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The Updated List of the Non-Native Freshwater Fishes in Slovenia with Note of their Potential Impact in Inland Waters

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Authors' Contribution
M Povz designed the study and prepared the manuscript. GJ and M Piria analyzed and interpreted the data and finalized the article.

Key words
Alien freshwater fish species, Introductions, Open waters, Slovenia.

ABSTRACT

In this paper up-to-date information on the known introductions of non-native fish species in Slovenia are summarized. Since the last report of list of alien fish species in Slovenia, two new introduced species has been recorded, which makes 22 new fish species in total. New species was introduced accidentally, by natural range expansion and by aquaculture. The date of introduction to Slovenia, the reason for the introduction, the mode of recent expansion, the degree of acclimatization and potential impacts on newly introduced non-native fishes were presented. Moreover, measures for the prevention of the uncontrolled restocking and further dispersal of alien freshwater fish species are proposed.

INTRODUCTION

Biological invasions are recognized as a major threat to biodiversity and their impacts in freshwater environments are a of particular concern (Dudgeon *et al*., 2006). Introductions of invasive freshwater alien species (IAS) are a significant and growing problem worldwide, including in Slovenia. The ichthyofauna of Slovenian inland waters is represented by 97 fish species, of which 28% are endemic (Povž *et al*., 2015). Recent data show that alien fish introductions are a major threat to the native ichthyofauna in the region (Mrakovčić *et al*., 2006), particulary to the endemic species (Ćaleta *et al*., 2015).

Until 2005, in Slovenia, 16 fish species was introduced and, between them, 10 have already established in the inland waters of Slovenia (Povž and Šumer, 2005). The introductions of non-native fish species in Slovenia were performed mainly by fishermen either intentionally (angling and aquaculture), or accidentally while restocking rivers with native species (Povž and Šumer, 2005) and many introductions in Slovenia have been carried out on a repeated basis (Povž and Ocvirk, 1988; Piria *et al*., 2016a).

Protection of ichthyofauna is provided under the National Freshwater Fisheries Act, Nature Conservation Act and related decrees, but they are practically ineffective (Povž and Šumer, 2005), and measures to effectively control the introductions or translocations of non-native fish species in Slovenia are still lacking even after the recent EC Regulation 1143/2014 on Invasive Alien Species (EU, 2014) came into force.

To date, there has been only two studies on history of introductions in Slovenia (Povž and Šumer, 2005; Povž, 2017), and since then, several new records of non-native fish species occured in inland waters of Slovenia (Simonović *et al*., 2017; Piria *et al*., 2018). Thus, the aim of this paper was to: (i) update the checklist of known introduced freshwater species in Slovenia, (ii) register and evaluate the vectors and pathways of the recent introductions, and (iii) determine the recent expansion of non-native fish species in Slovenia. Also, potential impact of the newly introduced fish species are discussed.

MATERIALS AND METHODS

Study area

Slovenia's dense river network (river density of 1.33 km^{-2}) is extensive (26989 km) and consists of 59 rivers, which are divided hydrologically into the Black Sea and the Adriatic drainages. The majority of the inland waters fall within the Black Sea drainage (16423 km^2 or 81%); while a smaller part (3850 km^2 or 19%) belongs to the Adriatic Sea Basin. The Black Sea drainage is dominated by the transboundary Sava River, with its tributaries the Kolpa/Kupa bordering with Croatia and with the transboundary

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of the Drava River (and its tributary Mura) also shared with Croatia. The Soča/Isonzo (139 km) flows through Slovenia draining into the Adriatic at the northeastern Italian coast. However, unlike the large Danube tributaries, most of the rivers of the Adriatic Sea basin form small and isolated catchments, most with a seasonal regime and often drying out during summer. Slovenia has several natural lakes, mostly in alpine region, either of glacial origin, such as the Triglav Lakes (Triglavska jezera), Lake Bled and Lake Bohinj, the deepest and largest permanent natural lake in Slovenia (3.13 km^2), or of karst origin, which are intermittent, such as Cerknica Lake or the numerous smaller polje (karst field) lakes in the Notranjska region.

Data collection and data processing

Historical data were derived from Povž and Šumer (2005) and Povž (2017) and recent data from various published and 'grey' literature sources (*e.g.* books and manuscripts). The degree of acclimatization, suspected impacts on native species and the pathways and vectors of introduction was assessed according to Copp *et al.* (2005), Povž and Šumer (2005) and Piria *et al.* (2018), respectively. The area of recent distribution was estimated as a proportion of the total area of Slovenia, which encompasses 266 squares (Kryštufek *et al*., 2001), using the UTM Grid zones of the world, converted to classes: (i) <1%, (ii) 1–5%, (iii) 6–20%, (iv) 21–50% and (v) >51% (Table I).

RESULTS AND DISCUSSION

By the end of 2017, a total of 97 fish species had been recorded in Slovenian inland waters, 75 indigenous and 22 introduced (Povž and Ocvirk, 1988; Šumer *et al*., 2003; Povž and Šumer, 2005; Piria *et al*., 2016a). At the end of 19th century, five alien fish species (*i.e.* nearly 30% of all registered releases) had been introduced (Table I) which represents the beginning of all future ichthyotransfers. The second wave of introductions occured between the 1960s and 1970s with four new species recorded (Povž, 1986; Povž and Šumer, 2005). However, the most intensive introductions occured in the last 45 years (from 1970s until 2015) when 11 new fish species were recorded in Slovenian inland waters (Table I). Furthermore, since the last reported list of alien fish species (Povž and Šumer, 2005; Povž, 2017), two new introduced species has been recorded (No 17-22, Table I).

Most of the introduced species originated from North America (ten species), followed by Asia (seven species), Europe (three species) and two from Africa (Table I) with 18 species now naturalized or acclimatized (Copp *et al*., 2005) in Slovenian inland waters. Self-sustaining populations in nature 11 of the introduced species have already established (Table I; Povž and Šumer 2005). European whitefish *Coregonus lavaretus* (Linnaeus, 1758), which drift in from Austria (Kristofič, 1992), are casuals without natural reproduction in Slovenia (Povž and Sket, 1990).

Coho salmon *Oncorhynchus kisutch* (Walbaum, 1792) disappeared within 10 years after introduction (Povž and Sket, 1990) due unknown reasons, and the North African catfish *Clarias gariepinus* (Burchell, 1822) disappeared within a year of introduction from Slovenian inland waters (Povž *et al*., 2015) due to cold water temperatures during winter (De Graaf *et al*., 1996). The North African catfish was first reported in two gravel pits of the River Mura basin (Slovenia) in 1997, having been introduced for angling purposes without legal consent. Although not naturalized, North African catfish is still present in Slovenia at fish farms located near Mura River system. This species is widely tolerant of extreme environmental conditions (Froese and Pauly, 2016), but it is unable to survive winter conditions in the Black Sea Basin of Slovenia where water temperatures are < 10°C. Nevertheless, a threat of establishment does still exist due to potential translocations to thermal spring habitats, as demonstrated by the adaptation of Nile tilapia *Oreochromis niloticus,* and to the Adriatic Sea basin in Slovenia (Piria *et al*., 2016a). This alien fish species could represent a significant risk for the local fish community and other aquatic animals (Muhammad *et al*., 2017). Nile tilapia was first introduced 2007 to the thermal spring Topla struga near Čatež town, tributary of the Sava River, from where the species is transported downstream by the river currents every summer (Povž *et al*., 2015), entering the downstream to the Sava River tributaries (D. Zanella, pers. Comm). Black carp *Mylopharyngodon piceus* (Richardson, 1845) was first introduced in 2004 near Slovenska Bistrica at an aquaculture facility and was thereafter occasionally released into surronding inland waters for sportfishing purposes (Povž, 2009). Similarly, Mississippi paddlefish *Polyodon spathula* (Walbaum, 1792) was for the first time introduced at an aquaculture facility near Rogaška Slatina in 2012 (Povž, 2012). Although there are no confirmed observations of the species in Slovenian inland waters yet, existence of naturalized populations of this species cannot be excluded.

One of the newest records of introduced species are those of bighead goby *Ponticola kessleri* (Günther, 1861) in 2015 (Simonović *et al*., 2017) in the Sava River near Čatež town. Recently, four of the Ponto-Caspian (P-C) gobies (Pisces, Gobiidae) have been reported in the Danube River Basin in Croatia (bordering country with Slovenia): bighead goby, monkey goby *Neogobius fluviatilis* (Pallas, 1814), round goby *Neogobius melanostomus* (Pallas, 1814), and racer goby *Babka gymnotrachelus* (Kessler, 1857) (Jakovlić *et al*., 2015).

Table I.- List of alien fish species recorded in Slovenia, origin, time of introduction, reasons of introduction, mode of expansion, the degree of acclimatization (U, unknown; F, failure; A, acclimatization of adults only; SA, satisfactory; VG, very good), impacts on native species (U, unknown; C, suspected competition with native fish for resources (*e.g.* habitat and food)), the mode of the current expansion (S, continuous stockings for sportfishing; E, escapes from fish farms and fish ponds; SR, uncontrolled self reproduction; DR, drift; U, unknown) and the area of recent distribution (No. TM grid zones), (i) <1%, (ii) 1–5%, (iii) 6–20%, (iv) 21–50% and (v) >51%. (*established self sustaining populations).

No	Fish species	Origin	Introduction		Mode of expansion after first introduction	Degree of acclimatization	Impacts on native species	Mode of the current expansion	No. TM Grid zones	References
			Time	Reason						
1	Brook trout *Salvelinus fontinalis* (Mitchill, 1815)*	North America	1892	Sportfishing	Release for sportfishing, self reproduction, escapment from aquaculture	VG	U	S	(iii) (32 squares)	Povž and Šumer, 2005
2	Lake charr *Salvelinus umbla* (Linnaeus, 1758)*	North America	1928, 1943, 1998	To fill vacant niche, sportfishing	Release for sportfishing, self reproduction, escapment from aquaculture	VG	U	SR	(i) (5 squares)	Povž and Šumer, 2005
3	Rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792)*	North America	1891	Sportfishing, aquaculture	Release for sportfishing, self reproduction, escapment from aquaculture	VG	C	S, E	(v) (153 squares)	Povž and Šumer, 2005
4	Coho salmon *Oncorhynchus kisutch* (Walbaum, 1792)	North America	1977	Sportfishing, aquaculture	Disappeared after 10 years of introduction	U	U	U	(i) (1 square - l. 1987)	Povž and Šumer, 2005
5	European whitefish *Coregonus lavaretus* (Linnaeus, 1758)	Europe	Every year	Indipendent downstream spreading	Drift	F	U	DR	(ii) (6 squares)	Povž and Šumer, 2005
6	Largemouth (black) bass *Micropterus salmoides* (Lacepède, 1802)*	North America	1892, 1993	Sportfishing	Release for sportfishing, self reproduction	SA	U	SR	(ii) (4 squares)	Povž and Šumer, 2005
7	Pumpkinseed *Lepomis gibbosus* (Linnaeus, 1758)*	North America	End of 19th century	Ornamental fish	Self reproduction, escapment from aquaculture	SA	U	SR	(iii) (39 squares)	Povž and Šumer, 2005
8	Brown bullhead *Ameiurus nebulosus* (LeSueur, 1819)*	North America	1935	Sportfishing, aquaculture	Release for sportfishing, self reproduction, escapment from aquaculture	SA	U	SR	(iii) (18 squares)	Povž and Šumer, 2005
9	Eastern mosquitofish *Gambusia holbrooki* (Girard, 1859)*	North America	1927	Mosquitos' control	Self reproduction	VG	U	SR	(i) (2 squares)	Povž and Šumer, 2005
10	Silver carp *Hypophthalamichthys molitrix* (Valenciennes, 1848)	Asia	1963	Aquaculture, sportfishing	Escapment from aquaculture, sportfishing	A	U	S	(iii) (20 squares)	Povž and Šumer, 2005

No	Fish species	Origin	Introduction		Mode of expansion after first introduction	Degree of acclimatization	Impacts on native species	Mode of the current expansion	No. TM Grid zones	References
			Time	Reason						
11	Bighead carp *Hypophthalmichthys nobilis* (Richardson, 1845)	Asia	1963	Aquaculture, sportfishing	Escapment from aquaculture, sportfishing	A	U	S	(ii) (12 squares)	Povž and Šumer, 2005
12	Grass carp *Ctenopharyngodon idella* (Valenciennes, 1844)	Asia	1963	Aquaculture, sportfishing, weed control	Escapment from aquaculture, sportfishing	A	U	S	(iii) (30 squares)	Povž and Šumer, 2005
13	Topmouth gudgeon *Pseudorasbora parva* (Temminck and Schlegel, 1846)*	Asia	1986	Accidental introduction	Self reproduction, escapment from aquaculture	VG	U	SR	(ii) (13 squares)	Povž and Šumer, 2005
14	Gibel carp *Carassius gibelio* (Bloch, 1782)*	Asia	1962	Accidental introduction, sportfishing	Release for sportfishing, self reproduction, escapment from aquaculture	VG	U	SR	(iii) (35 squares)	Povž and Šumer, 2005
15	Goldfish *Carassius auratus* (Linnaeus, 1758)*	Asia	In 19th century	Ornamental	From aquaria, self reproduction	VG	U	SR	(iii) (35 squares)	Povž and Šumer, 2005
16	North African catfish *Clarias gariepinus* (Burchell, 1822)	Africa	1997	Sportfishing	Release for sportfishing (disappeared?)	F	U	U	(i) (1 square)	Povž and Šumer, 2005
17	Black bullhead *Ameiurus melas* (Rafinesque, 1820)*	North America	Un-known	Sportfishing, aquaculture	Self reproduction	SA	U	SR	(iii) (19 squares)	M. Povž, pers obs
18	Nile tilapia *Oreochromis niloticus* (Linnaeus, 1758)*	Africa	2007	Aquaculture	Self reproduction in hot springs, escapment from aquaculture	U	U	SR	(i) (1 square)	Povž, 2007
19	Black carp *Mylopharyngodon piceus* (Richardson, 1845)	Asia	2004; 2012	Accidental introduction or sportfishing	Unknown	U	U	U	(i) (1 square)	Povž, 2009
20	Mississippi paddlefish *Polyodon spathula* (Walbaum, 1792)	North America	2012	Aquaculture	Not yet present in the rivers and lakes	U	U	U	(i) (1 square)	Povž, 2012
21	Siberian sturgeon *Acipenser baerii* (Brandt, 1869)	Europe	2015	Accidental introduction	Unknown	U	U	U	(ii) (13 squares)	M. Povž personal observations
22	Bighead goby *Ponticola kessleri* (Günther, 1861)	Europe	2015	Indipendent upstream expansion	Independent upstream expansion	U	U	SR	(i) (1 square)	Simonović *et al.*, 2017

Native distributions of P-C gobies were mostly confined to the lower reaches of the Danube River and to the littoral zone of the Black Sea (Vassilev *et al*., 2012), while the Djerdap Gorge represented the uppermost range boundary (Miller, 2003). Due their natural dispersal from the native habitat, and their presence near the Slovenain border, bighead goby occurence was not unexpected in Slovenian inland waters. Monkey goby, round goby and racer goby may occur in near future in the Sava, Mura or Drava Rivers in Slovenia.

The last record of Siberian sturgeon *Acipenser baerii* Brandt, 1869 in 2016 (M. Povž personal observation) represents typical example of insufficient knowledge and lack of expertise. Namely, Siberian sturgeon was released into the Sava and Mura Rivers in Slovenia, due to misidentification of the species with sterlet *Acipenser ruthenus* Linnaeus, 1758, native species for this region.

Still is not known time of introduction of black bullhead *Ameiurus melas* (Rafinesque, 1820) in Slovenia and its recent distribution. Recent research in Croatia has confirmed the dominant presence of black bullhead in both basins (Adriatic and Black Seas), while the brown bullhead *Ameiurus nebulosus* (LeSueur, 1819) is restricted only to the Neretva River and its tributaries (Piria *et al*., 2018). In Slovenia, most reports refer to brown bullhead (Povž and Šumer, 2005), thus indicating ambiguities and possible misidentifications of these two species.

The major motive for the introduction of non-native fishes to Slovene inland waters was sportfishing (Table I). Other reasons are referred to fill a perceived vacant niche, introduction an exotic angling element to the water body or scapee fish from fish farms and ponds. Escapee from fish farms frequently occuring and probably is responsible for the large number of rainbow trout *Oncorhynchus mykiss* (Walbaum, 1792), and brook trout *Salvelinus fontinalis* (Mitchill, 1815) present downstream of the fish farm outlets (M. Povž personal observation). In most cases, escapee fish have gone unreported.

Research of the impacts to native fish in Slovenian inland waters entirely missing. Competition of several introduced species with native fish species for habitat, food and other resources, due their wide distributions, elevated densities in some locations, or trophic role as obligate or facultative piscivores was only assumed for Slovenia according researches elsewhere (Povž and Šumer, 2005). Furthermore, the new finding of bighead goby in Slovenia was interpreted as a warning of the presence of an yet undefined stressor (Simonović *et al*., 2017). According to Jurajda *et al*. (2005) the introduction of bighead goby could affect the native fish community by competing with bullhead *Cottus gobio*, stone loach *Barbatula barbatula* and white-finned gudgeon *Romanogobio albipinnatus*. Also, their feeding activity can cause a decline in the abundance of native fish species (Piria *et al*., 2016b). Thus, research on impacts in inland waters of Slovenia are strongly recommended.

Assessment of the proportional area of recent distribution (Table I) revealed that eight species (lake charr *Salvelinus umbla* (Linnaeus, 1758), coho salmon, eastern mosquitofish *Gambusia holbrooki* Girard, 1859, North African catfish, bighead goby, Nile tilapia, paddlefish, black carp) cover <1% of the Slovenian territory. Six species (European whitefish, largemouth bass *Micropterus salmoides* (Lacepède, 1802), bighead carp *Hypophthalmichthys nobilis* (Richardson, 1845), topmouth gudgeon *Pseudorasbora parva* (Temminck and Schlegel, 1846) and goldfish *Carassius auratus* (Linnaeus, 1758) cover 1–5% of the area (*i.e.* four to 13 squares). Six species (brook trout, pumpkinseed *Lepomis gibbosus* (Linnaeus, 1758), brown bullhead and silver *Hypophthalamichthys molitrix* (Valenciennes, 1848), grass *Ctenopharyngodon idella* (Valenciennes, 1844) and gibel *Carassius gibelio* (Bloch, 1782) carps) inhabit 6–20% (*i.e.* 18–39 squares). Still the most abundant and most widely spread is rainbow trout, occupies more than 51% of Slovene territory (153 squares), which was considered as widely distributed species (Kryštufek *et al*., 2001).

Actions to remediate and/or control introduced fishes are lacking for Slovenia, thus it is neccesary to develop better conservation measures and management program and further uncontrolled restocking and dispersal of alien species should be suppressed. Suggestion for future aquatic studies and research are assessing and mitigating impacts of non-native species.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Brancelj, A. 1997. Rek, da dokler so ribe v vodi, je vse v redu, žal ne drži vedno in povsod: slovenska visokogorska jezera ogroža (tudi) naseljevanje rib. *Delo,* **39**: 10. [in Slovene]

Brancelj, A., Urbanc-Berčič, O., Krušnik, C., Kosi, G., Povž, M. and Dobravec, J., 1995. Življenje v vodah

Triglavskega narodnega parka [Freshwater fauna in Triglav National Park]. *Razprave in Raziskave*, **4**: 95-96. [in Slovene]

Copp, G.H., Bianco, P.G., Bogutskaya, N., Eros, T., Falka, I., Ferreira, M.T., Fox, M.G., Freyhof, J., Gozlan, R.E., Grabowska, J., Kovac, V., Moreno-Amich, R., Naseka, A.M., Penaz, M., Povž, M., Przybylski, M., Robillard, M., Russell, I.C., Stakenas, S., Šumer, S., Vila-Gispert, A. and Wiesner, C., 2005. To be, or not to be, a non-native freshwater fish? *J. appl. Ichthyol.,* **21**: 242-262. https://doi.org/10.1111/j.1439-0426.2005.00690.x

Ćaleta, M., Buj, I., Mrakovčić, M., Mustafić, P., Zanella, D., Marčić, Z., Duplić, A., Mihinjač, T. and Katavić, I., 2015. *Endemic fish of Croatia*. Croatian Environment Agency, Zagreb, pp. 116.

De Graaf, G. and Janssen, J., 1996. *Handbook on the artificial reproduction and pond rearing of the African catfish* Clarias gariepinus *in Sub-Saharan Africa Nefisco Foundation, Amsterdam, the Netherlands*. Fisheries Techical Paper 362, FAO, Rome, pp. 109.

Dudgeon, D., Arthington, A.H., Gessner, M.O., Kawabata, Z.I., Knowler, D.J., Lévêque, C., Naiman, R.J., Prieur-Richard, A.H., Soto, D., Stiassny, M.L.J. and Sullivan, C.A., 2006. Freshwater biodiversity: Importance, threats, status and conservation challenges. *Biol. Rev.,* **81**: 163-182. https://doi.org/10.1017/S1464793105006950

EU, 2014. Regulation (EU) No 1143/2014 of the European Parliament and of the Council of 22 October 2014 on the prevention and management of the introduction and spread of invasive alien species. *Offic. J. Europ. Union,* **57**: 35-55.

Froese, R. and Pauly, D. (eds.), 2016. *FishBase.* World Wide Web Electronic Publication. www.fishbase.org (version 10/2016).

Jakovlić, I., Piria, M., Šprem, N., Tomljanović, T., Matulić, D. and Treer, T., 2015. Distribution, abundance and condition of invasive Ponto-Caspian gobies *Ponticola kessleri* (Gunther, 1861), *Neogobius fluviatilis* (Pallas, 1814), and *Neogobius melanostomus* (Pallas, 1814) in the River Sava basin, Croatia. *J. appl. Ichthyol*., **31**: 888-894. https://doi.org/10.1111/jai.12803

Jurajda, P., Černý, J., Polačik, M., Valová, Z., Janáč, M., Blažek, R. and Ondračková, M., 2005. The recent distribution and abundance of non-native Neogobius fishes in the Slovak section of the River Danube. *J. appl. Ichthyol*., **21**: 319-323. https://doi.org/10.1111/j.1439-0426.2005.00688.x

Kristofič, T., 1992. Zanimiv ulov nove vrste ribe v Sloveniji [New fish species in Slovenia]. *Ribic*, **51**: 8. [in Slovene]

Kryštufek, B., Bedjanič, M., Brelih, S., Budihna, N., Gomboc, S., Grobelnik, V., Kotarac, M., Lesnik, A., Lipej, L., Marti, A., Poboljšaj, K., Povž, M., Rebeušek, F., Salamun, S., Tome, S., Trontelj, P. and Wraber, T., 2001. *Raziskava razširjenosti evropsko pomembnih vrst v Sloveniji [Distribution of European important freshwater fishes in Slovenia]*. Prirodoslovni muzej Slovenije [Report], pp. 364. [in Slovene]

Miller, P.J., 2003. Mugilidae, Atherinidae, Atherinopsidae, Blennidae, Odontobutidae, Gobiidae. In: *The freshwater fishes of Europe*, Vol. 8/1. AULA-Verlag GmbH, Wielbelsheim, pp. 404.

Mrakovčić, M., Brigić, A., Buj, I., Ćaleta, M., Mustafić, P. and Zanella, D., 2006. *Crvena knjiga slatkovodnih riba Hrvatske (Red book of freshwater fish of Croatia)*. Ministry of Culture, State Institute for Nature Protection, Republic of Croatia, Zagreb, pp. 253. [in Croatian with English Introduction]

Muhammad, H., Iqbal, Z. and Saleemi, S., 2017. Diversity and distribution of fish fauna of Indus River at Taunsa Barrage in Punjab, Pakistan. *Pakistan J. Zool.,* **49**: 155-161. http://dx.doi.org/10.17582/journal.pjz/2017.49.1.155.161

Munda, A., 1924. Ameriški ostriž (*Grystes salmoides* Gunth.) (Large mouth bass). *Lovec*, **9**: 93. [in Slovene]

Nico, L. and Fuller, P., 2005. *Hypophthalmichthys nobilis.* USGS Nonindigenous Aquatic Species Database, Gainesville, FL. Available from http://nas.er.usgs.gov/queries/FactSheet.asp

Piria, M., Povž, M., Vilizzi, L., Zanella, D., Simonović, P. and Copp, G., 2016a. Risk screening of non-native freshwater fishes in Croatia and Slovenia using the fish invasiveness screening kit. *Fish. Manage. Ecol*., **23**: 21-31. https://doi.org/10.1111/fme.12147

Piria, M., Jakšić, G., Jakovlić, I. and Treer, T., 2016b. Dietary habits of invasive Ponto-Caspian gobies in the Croatian part of the Danube River basin and their potential impact on benthic fish communities. *Sci. Total Environ*., **540**: 386-395. https://doi.org/10.1016/j.scitotenv.2015.05.125

Piria, M., Simonović, P., Nikolić,V., Ristovska, M., Apostolou, A., Adrović, A., Povž, M., Zanella, D., Mrdak, D., Milošević, D., Vardakas, L., Koutsikos, N., Kalogianni, E., Gregori, A., Kostov, V., Škrijelj, R., Korro, K., Bakiu, R., Tarkan, A.S. and Joy, M.K., 2018. Alien freshwater fish species in the Balkans – vectors and pathways of introduction. *Fish*

Fisheries, **19**: 138–169. https://doi.org/10.1111/faf.12242

Povž, M., 1986. Naseljevanje in preseljevanje sladkovodnih rib v Sloveniji. [Introductions and transplantations of freshwater fishes in Slovenia] Ljubljana. *Ribič*, **45**: 252-254. [in Slovene].

Povž, M. and Ocvirk, A., 1988. Freshwater fish introductions and transplantations in Slovenia. *Ichthyos,* **8**: 1-10.

Povž, M. and Sket, B., 1990. *Naše sladkovodne ribe [Freshwater fishes]*. Mladinska Knjiga, Ljubljana, pp. 367.

Povž, M., 2009. Black carp *Mylopharyngodon piceus* (Richardson, 1845). *Ribič,* **7-8**: 197-198. [in Slovene]

Povž, M., 2012. American paddlefish, *Polyodon spathula* (Walbaum, 1792). *Ribič,* **1-2**: 16-17. [in Slovene]

Povž, M. and Šumer, S., 2005. A brief review of non-native freshwater fishes in Slovenia. *J. appl. Ichthyol.*, **21**: 316-318. https://doi.org/10.1111/j.1439-0426.2005.00687.x

Povž, M., Gregori, A. and Gregori, M., 2015. *Freshwater fish and lampreys in Slovenia*. Zavod Umbra, Ljubljana, pp. 293. [in Slovene]

Povž, M., 2017. Non-native Freshwater Fishes in Slovenia. *Acta Zool. Bulg.*, **9**: 105-110.

Simončič, A., 1948. Jezerska zlatovčica v Bohinjskem jezeru [Arctic charr in the lake Bohinjsko jezero]. *Ribič*, **7**: 609 [in Slovene]

Simonović, P., Piria, M., Zuliani, T., Ilić, M., Marinković, N., Kračun-Kolarević, M. and Paunović, M., 2017. Characterization of sections of the Sava River based on fish community structure. *Sci. Total Environ.*, **574**: 264-271. https://doi.org/10.1016/j.scitotenv.2016.09.072

Šumer, S., Povž, M. and Seliškar, T., 2003. *Analiza bioloških obremenitev in vplivov na vode – pregled in posledice vnosov in preseljevanj sladkovodnih ribjih vrst in po Sloveniji in vpliv na oceno ekoloskega stanja vodnih teles v okviru direktive o vodah [Biological impacts on inland waters in Slovenia: non-native freshwater fishes in Slovenia]*. Report. EBRA d.o.o. Ljubljana, pp. 56. [in Slovene]

Vassilev, M., Apostolou, A., Velkov, B., Dobrev, D. and Zarev, V., 2012. *Atlas of the gobies (Gobiidae) in Bulgaria*. Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Science, Sofia, Bulgaria, pp. 112.

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Impact of Azomite Supplemented Diets on the Growth and Body Composition of Catfish (*Pangasius hypophthalmus*)

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Authors' Contribution
SSB, NK and UA planned the work. KJI, HA, SD and NK wrote the article. SB, SM, MN and SA studied growth, proximate and physicochemical parameters. SB, DHM and MSA analysed the data statistically.

Key words
Catfish (*Pangasius hypophthalmus*), Azomite, Growth, Body composition.

ABSTRACT

The study was conducted to evaluate the impact of azomite, a natural mineral of volcanic ash, on the growth performance and body composition of catfish (*Pangasius hypophthalmus*) for 90 days. Experimental fish (n=25) was stocked in six fibreglass aquariums (18L capacity) at 25 fish/aquaria. Azomite was added in the basal diet containing crude protein (CP 38.3%) with three graded levels 0% control, 0.5% T_1 and 1.0% T_2 and the fish was fed at 4% of wet body weight. After 90 days of study period, the increase in weight gain was obtained as 4.3g, 5.9g and 6.7 g in control, T_1 and T_2, respectively. The treatments with azomite supplementation showed significantly higher growth than control group ($P \leq 0.05$). The feed conversion ratio (FCR) was found significantly ($P \leq 0.05$) better (1.9) in T2 than (2.4) control and (2.2) T1. Proximate analysis showed that the fish fed azomite supplemented diet has a significant difference ($P \leq 0.05$) in fat, moisture, protein and ash contents while non-significant differences observed in fibre and phosphorus contents as compared to the control group. In conclusion the Pangasius fry showed best growth performance without any significant increase in biochemical nutrients profile with 1.0% inclusion of azomite in the diet.

INTRODUCTION

Catfish *Pangasius hypophthalmus* is Asian Catfish which is mainly produced in Vietnam and Thailand. It is considered major fish spp. in the Mekong River fishery and is the largest and most valued inland fisheries in the world. The Pangasius are prolific spawners and produce large numbers of larvae which are harvested easily from the flowing river. The establishment of capture-based aquaculture for this specie was begun in Vietnam and to some extent in Thailand and Cambodia (Nguyen, 2009). Thai *Pangasius hypophthalmus* becomes an auspicious species due to its omnivorous nature, rapid growth and good market. To achieve optimal growth potential Pangasius requires high protein diet (> 40%) (Ali *et al*., 2001). The operating cost mainly feed for cat fish is very high which accounts for more than 50% of the total production cost that is one of the major hindrance in its culture (Sehagal and Toor, 1991; De Silva and Davy, 1992). The nutritionists are working to reduce feed cost down so that suitable feeding strategies are made to improve the husbandry techniques by improving fish feed utilization. The studies revealed that an increase of feeding frequency has positive effect on the growth and production performance of catfish pangasiid and silver carp culture (Khan *et al*., 2009).

Azomite is considered a very useful natural mineral product found from Utha in USA and also abundantly used in Asia. It is a certified organic trace mineral booster and is used as supplement in livestock and aquatic animal feeds globally for over a decade. It is also used in the feed of poultry, shrimps, and tilapia as trace mineral mix for several years and declares to improve the feed quality, increase weight gain, feed conversion and likability. It has been reported that azomite supplemented diet improves growth, intestinal digestive enzymes activity, nutrient digestibility and serum non-specific immune function in tilapia (*Oreochromis niloticus aureus*) (Liu *et al*., 2009), grass carp (*Ctenopharyngodon idellus*) (Liu *et al*., 2011),

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white shrimp *(Litopenaeus vannamei)* (Tan *et al*., 2014), koi carp (*Ciprinus carpio*) (Jaleel *et al*., 2015). The trace minerals are necessary to incorporate in fish diets because they participate in biochemical processes required for normal fish growth and development (Hooge, 2008). Keeping in view the importance of azomite inclusion in experimental as well as commercial diets in animal and poultry feeds and its effects on the growth and survivability; the present study was therefore planned to test the impact of various levels of azomite concentration on the growth performance and body composition of catfish (*Pangasius hypophthalmus*) fry.

MATERIALS AND METHODS

Experimental setup

The experimental fish *Pangasius hypophthalmus* was imported from Thailand and kept in fiberglass aquariums in fish hatchery at Fisheries Research and Training Facilities, Department of Fisheries and Aquaculture, UVAS, Ravi Campus, Pattoki. The fish was fed with powdered diet having 38.3% crude protein three times a day at 2% fish body weight. After four weeks of acclimatization and quarantine measures, the experimental fish was distributed into three treatment groups (levels 0% control, 0.5% T_1 and 1.0% T_2) each having two replicates. *Pangasius hypophthalmus* having an average weight of 0.98 g was stocked at 25 fish/aquaria and the fish was fed at 4% of wet body weight. The morphometric characters *viz*. fish wet body weight (g) and total body length (mm) of each fish was measured and recorded at the time of stocking. Aerators were installed in each aquarium for oxygenation of water. The aquaria water was change 10% daily and completely on third day. The uneaten feed and feces were siphoned on daily basis.

Table I.- Feed formulation and feed ingredients.

Ingredients and CP composition	Percent inclusion	Crude protein (5)
Fish meal (50%)	30	15.0
Corn glutton (60%)	22	13.2
Rice polish (12%)	7	0.84
Soybean meal (43%)	10	4.3
Wheat bran (16.5%)	30	4.95
Vitamins premix and oil	1	0
Azomite	0%, 0.5%, 1%	
Total	100	38.3%

Feed formulation and feeding protocol

Three types of experimental feeds control, T_1 and T_2 were prepared using following feed ingredients: fish meal, maize glutton, rice polish, soybean meal, wheat bran, minerals mix, vitamins, cooking oil and azomite as a supplement in different ratios (0.5% and 1.0%) in feed designated as T_1 and T_2, respectively (Table I). The proximate composition of experimental feeds are given in Table II. Pelleted feeds were prepared on local pelleting machine which were then sun dried and broken down in to small crumbles and fed to fish at 4% of its wet body weight twice a day up to six days a week. Feed was adjusted after fortnightly sampling of fish and its growth increment.

Fish growth parameters

To measure the morphometric records, all the fish were caught on fortnightly basis using small nylon mesh hand net and glass beaker to avoid stress. After taking morphometric measurements fish was released back to their respective aquaria. Mortality of fish was also recorded if found. Growth parameters like net weight gain, feed conversion ratio (FCR) and specific growth rate (SGR) were also calculated for each tank.

$$\text{Net WG} = \text{Final BW (g)} - \text{Initial BW (g)}$$

$$\%\text{WG} = \frac{\text{Final BW (g)} - \text{Initial BW (g)}}{\text{Initial BW (g)}} \times 100$$

$$\text{FCR} = \frac{\text{Feed given}}{\text{WG}}$$

$$SGR\% = \frac{\ln(W1) - \ln(W2)}{T} \times 100$$

Where, WG is weight gain, BW is body weight, W1is initial weight, W2 is final weight and T is the number of days in the feeding trial.

Table II.- Proximate composition of fish feed.

Parameters	Control (0.0%)	T_1 (0.5%)	T_2 (1.0%)
Moisture (%)	7.3	8.0	7.9
Crude protein (%)	38.3	38.3	38.3
Crude fat (%)	8.7	8.5	8.4
Crude fiber (%)	0.85	0.7	0.7
Ash (%)	15.5	14.3	14.3

Proximate analysis

On termination of feeding trials, 7-8 fishes from each aquarium were taken for whole body proximate analysis. The fish sample and the formulated feed were subjected to proximate analysis to determine the dry matter, ash, crude protein, crude lipids and gross energy following (AOAC, 2003; Mehbood *et al*., 2017) in the Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki and Buffalo Research Institute (BRI), Pattoki using Near Infrared Spectrophotometry (NIR) method.

Physico-chemical parameters

Water quality parameters such as, dissolved oxygen (DO), pH, water temperature, total dissolved solids (TDS) and salinity were monitored and recorded on daily basis by using DO meter (YSI 55 Incorporated, Yellow Springs, Ohio, 4387, USA), pH meter (LT-Lutron pH-207 Taiwan) and TDS meter, respectively (APHA, 1998).

Statistical analysis

Data obtained was statistically analyzed using SAS 9.1 version through one-way ANOVA (Analysis of Variance Techniques). Means were compared by using Duncan's Multiple Range test.

Table III.- Fortnightly average growth of fish using various levels of azomite in artificial feed.

Parameter	Control (0%)	T1 (0.5%)	T2 (1.0%)
Initial wt. (g)	$0.7^c \pm 0.002^c$	0.9 ± 0.001^b	0.8 ± 0.008^a
Final wt. (g)	4.3 ± 0.002^c	5.9 ± 0.025^b	6.7 ± 0.019^a
Net wt.gain (g)	3.6 ± 0.028^c	5.0 ± 0.34^b	5.9 ± 0.23^a
Wt. gain (%)	514.28 ± 1.9^c	555.55 ± 3.89^b	737.5 ± 6.03^a
Initial length (mm)	53.3 ± 0.14^a	53.4 ± 0.57^a	53.1 ± 0.07^a
Final length (mm)	69.4 ± 0.14^c	82.4 ± 0.07^b	85.3 ± 0.07^a
Length gain (mm)	16.1 ± 0.28^c	28.9 ± 0.49^b	32.1 ± 0^a
Initial biomass	18.6 ± 0.07^c	21.9 ± 0.04^a	20.7 ± 0.21^b
Final biomass	90.7 ± 0.04^c	135.1 ± 0.59^b	155.2 ± 0.44^a
FCR	2.4 ± 0.01^a	2.2 ± 0^b	1.9 ± 0.007^c
SGR%	1.1 ± 0.01^a	1.1 ± 0.01^a	1.2 ± 0.01^b

*Figures having different super scripts are significantly different.

RESULTS

Growth

The growth parameters of fish under all the treatments are presented in Table III. Statistical analysis using one-way ANOVA revealed significant differences ($P \leq 0.05$) among the initial weight, final weight, net weight gain, and percent weight gain of fish in all the three treatment groups. The fish in T_2 exhibited significantly ($P \leq 0.05$) higher growth indices compared to T_1 and control, respectively (Table IV). Length increment also revealed same significant difference with higher increase in T_2 followed by T_1 and control, respectively (Table IV). Results regarding final biomass exhibited significantly higher increase in T_2 followed by T_1 and control. FCR and SGR% reveals significantly better values in T_2, T_1 and then control, respectively. SGR% in control and T_1 showed non-significant differences compared to T_2.

Proximate composition

The detail of proximate composition of *Pangasius hypophthalmus* fingerlings is given in Table IV. The fish was also analyzed for its proximate composition at the start and post-trial as well. The dry matter, ash content, crude protein and crude fat contents showed significant differences ($P \leq 0.05$) with control group but fiber content in the fish sample revealed non-significant differences.

Table IV.- Proximate composition pre and post-trial of *Pangasius hypophthalmus.*

Parameters	Pre-treatment	Control (0.0%)	T_1 (0.05%)	T_2 (1.0%)
Dry matter (%)	7.69 ± 0.37^b	9.55 ± 0.35^a	8.35 ± 0.21^b	8.15 ± 0.21^b
Crude protein (%)	58.15 ± 0.49^c	63.4 ± 0.14^a	60.2 ± 0.28^b	63.5 ± 0.28^a
Crude lipids (%)	8.2 ± 0.14^a	8.55 ± 0.21^a	7.7 ± 0.28^b	8.35 ± 0.35^a
Ash (%)	17.4 ± 0.14^c	18.55 ± 0.35^a	18.35 ± 0.35^a	17.65 ± 0.07^c
Crude fiber (%)	0.78 ± 0.09^a	0.95 ± 0.07^a	0.78 ± 0.04^a	0.75 ± 0.08^a

*Figures having different super scripts are significantly different.

Table V.- Physico-chemical parameters of aquariums water in different treatments.

Parameters	Control (0.0%)	T_1 (0.05%	T_2 (1.0%)
DO (mg/l)	4.96 ± 0.06^a	4.52 ± 0.39^a	4.79 ± 0.33^a
TDS (mg/l)	1252 ± 3.92^a	1262 ± 3.94^a	1273 ± 3.64^a
Salinity (ppt)	1.08 ± 0.007^a	1.12 ± 0.01^a	1.14 ± 0.03^a
Temp. (°C)	25.84 ± 0.09^a	25.91 ± 0.31^a	25.76 ± 0.16^a
pH	7.18 ± 0.04^c	7.50 ± 0.26^a	7.36 ± 0.01^b

Physico-chemical parameters

The physico-chemical parameters temperature, dissolved oxygen (DO), total dissolved solids (TDS), and salinity shows a non-significant difference in each treatment but pH shows a significant difference ($P \leq 0.05$) in each treatment and was in acceptable range throughout the study period (Table V).

DISCUSSION

The importance of trace minerals as essential ingredients in diets, although in small quantities, is also evident in fish. The results of current study based on the impact of adding different ratios of azomite *i.e.,* control (0%), T_1 (0.5%) and T_2 (1%) in the diet containing 38.3% crude protein on the growth, survival and body composition of *Pangasius hypophthalmus* fry. The statistical analysis of growth parameters revealed a significantly higher final

weight, net weight gain and percent weight gain in T_2 followed by T_1 and then control. The significantly higher fish biomass and FCR values were also observed in T_2 than T_1 and control group. Our results are comparable with the findings of Liu *et al*. (2009) who reported that addition of 0.25%, 0.50% and 0.75% azomite in the diet of *Oreochromis niloticus* increased significant growth and better FCR. Watanabe *et al*. (1997) described the role of trace elements in biological systems in several animals where macro and micro or trace minerals played an important role in the growth, survival, cellular and physiological levels of animals. Investigations of mineral contents in fish are comparatively difficult and complicated because both dietary mineral intake and waterborne mineral uptake have to be considered while calculating the mineral budgets. Liu *et al*. (2011) evaluated various levels of azomite on the growth and body composition of grass carp (*Ctenopharyngodon idella*) and found significant difference in growth and FCR with the addition of 0.2% azomite compared to control and non-significant changes in fish body composition. Tan *et al*. (2014) also supported our results who fed azomite at 2.0 and 4.0 g kg^{-1} in basal feed of shrimp and found significant higher growth compared to control group with 0g inclusion of azomite in the diet. Liu *et al*. (2011) reported that carps fed with azomite treatment showed higher growth and lower FCR than the control group with 0% azomite. Tan *et al*. (2014) also reported that the weight gain of shrimp increased by 14.0% ($P \leq 0.05$), while feed conversion ratio decreased by 0.11 and 0.09 ($P \leq 0.05$) in shrimp given feed containing 2.0 and 4.0 g kg-^{1} azomite.

Regarding proximate composition the results revealed non- significant differences among treated and control groups and is in line with the findings of Liu *et al*. (2009) who did not found any significant change in fish body composition.

The physico-chemical parameters were found within acceptable range accept temperature which showed fluctuations that might be due to winter season and addition of freshwater which was exchanged 10% on daily basis in the culture system. The growth and survival of fish is affected directly or indirectly by temperature (El-Sayed *et al*., 1996). Increase in temperature increases fish growth while decrease in temperature decreases growth (Hannibal *et al*., 2011; Shah *et al*., 2014). Other water quality parameters were recorded throughout the study period and were within the acceptable ranges for Pangas growth.

CONCLUSION

In conclusion supplementation of azomite at 1.0% in Pangasius fry diet can significantly increase growth without any significant alteration in the fish biochemical nutrient profile when fed with high protein 38.3% CP feed.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Ali, M.Z., Hossain, M.A. and Mazid, M.A., 2005. Effect of mixed feeding schedules with varying dietary protein levels on the growth of sutchi catfish, *Pangasius hypophthalmus* (Sauvage) with silver carp, *Hypophthalmichthys molitrix* (Valenciennes) in ponds. *Aquacult. Res.,* **36**: 627-634. https://doi.org/10.1111/j.1365-2109.2005.01262.x

AOAC, 2003. *Official methods of analysis,* 17th ed. Association of Official Analytical Chemists, Washington DC, USA.

APHA, 1998. *Standard methods for the examination of water and wastewater*, 20th ed. American Public Health Association, Washington DC, USA.

De Silva, S.S. and Davy, F.B., 1992. Fish nutrition research for semi-intensive culture systems in Asia. *Asian Fish. Sci.,* 5: 129-144.

El-Sayed, A.F.M., El-Ghobashy, A. and Al-Amoudi, M., 1996. Effects of pond depth and water temperature on the growth, mortality and body composition of Nile tilapia, *Oreochromis niloticus* (L.). *Aquacult. Res.,* **27**: 681-687. https://doi.org/10.1111/j.1365-2109.1996.tb01303.x

Hannibal, M.C., Amparo, L. F. and Aurelio, A. C., 2011. Effect of stocking density on growth performance, survival and production of silver pompano, *Trachinotus blochii* (Lacépède, 1801) in marine floating cages. *Asian Fish. Sci.*, **24**: 321-330.

Hooge, D., 2008. Natural minerals can benefit broiler diets. *Feedstuffs*, **80**: 24-26.

Jaleel, M.A., Musthafa, M.S., Ali, A.J., Muhammad, M.J., Kumar, M.S.A., Natarajan, G. and Thiagarajan, V., 2015. Studies on the growth performance and immune response of koi carp fingerlings (*Cyprinus carpio* koi) fed with azomite supplemented diet. *J.*

Biol. Nature, **4**: 160-169.

Khan, S., Hossain, M.S. and Haque, M.M., 2009. Effects of feeding schedule on growth, production and economics of pangasiid catfish (*Pangasius hypophthalmus*) and silver carp (*Hypophthalmichthys molitrix*) polyculture. *J. Bangladesh Agric. Univ.,* **7**: 175-181.

Liu, A.J., Leng, X.J., Li, X.Q., Wang, L.P., Luo, Y.X. and Zhu, R.J., 2009. Effects of Azomite on growth, intestinal structure and non-specific immunity of tilapia (*Oreochromis niloticus* × *O. aureus*). *Chinese J. Anim. Nutr.,* **21**: 1006-1011.

Liu, M.Z., Leng, X.J., Li, X.Q., Chang, X. and Chen, D.R., 2011. Effects of azomite on growth performance, intestinal digestive enzyme activities and serum nonspecific immune of grass carp (*Ctenopharyngodon idellus*). *J. Zhejiang Univ. (Agric. Life Sci.),* **37**: 312-318.

Mehboob, A., Khan, N., Atiq, U., Iqbal, K.J., Tayyab, R., Batool, S.S., Batool, H.S., Amjad, S. and Tanveer, M., 2017. Effect of fenugreek as a feed additive on the growth, body composition and apparent nutrients digestibility of striped catfish *Pangasius hypophthalmus* fry. *Pakistan J. Zool.,* **49**: 2037-2042.

Nguyen, T.T., 2009. Patterns of use and exchange of genetic resources of the striped catfish *Pangasianodon hypophthalmus* (Sauvage 1878). *Rev. Aquacult.,* **1**: 224-231. https://doi.org/10.1111/j.1753-5131.2009.01016.x

Sehagal, H.S. and Toor, H.S., 1991. Comparison of feeding strategies based on biomass and biomass-pond interactions. In: *Fish nutrition research in Asia* (ed. S.S. de Silva). Asian Fisheries Society, Manila, Philippines, pp. 181-192.

Shah, A.S., Malik, A., Kalhoro, H., Kalhoro, I.B., Wadhar, G.M. and Maher, G.M., 2014. Growth performance of exotic catfish Pangas, *Pangasius hypophthalmus*. *Sindh Univ. Res. J. (Sci. Ser.),* **46**: 205-208.

Tan, G.C., Li, X.Q., Leng, X.J., Su, X.G., Chen, L., Liu, B., Ma, F., Cai, X.Q. and Guo, T., 2014. Effect of supplemental azomite in diets on growth, immune function and diseases resistance of white shrimp (*Litopenaeus vannamei*). *Aquacult. Nutr.,* **20**: 324-331. https://doi.org/10.1111/anu.12081

Watanabe, T., Kiron, V. and Satoh, S., 1997. Trace minerals in fish nutrition. *Aquaculture*, **151**: 185-207. https://doi.org/10.1016/S0044-8486(96)01503-7

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Effect of Various Levels of Iron on Peroxidase Activity in the Fish, *Cirrhina mrigala*

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Authors' Contribution
MJ conceived the idea and supervised the findings. KS conducted the research. FL statistically analyzed the data. MA and KS wrote the manuscript.

Key words
Peroxidase, Organs, Iron, *Cirrhina mrigala*.

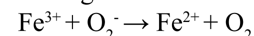
ABSTRACT

Present research work was conducted to evaluate the effect of various levels of iron on peroxidase activity in the fish, *Cirrhina mrigala*. For this purpose, four groups of *Cirrhina mrigala* (one-year old) were exposed to different treatments *viz*. 96 h LC_{50}, $2/3^{rd}$, $1/4^{th}$ and $1/5^{th}$ of LC_{50} concentrations of iron for 30 days in the glass aquaria with three replications for each treatment. Activity of peroxidase in the gills, liver, kidney and brain was assessed by measuring the conversion of guaiacol to tetraguaiacol, spectrophotometrically, at a wavelength of 470nm. The results reveal that peroxidase activity was increased significantly ($p<0.01$) in gills, liver, kidney and brain after exposure of iron as compared to the control group. Peroxidase activity of 0.697±0.005, 0.584±0.003, 0.456±0.002 and 0.287±0.004UmL^{-1} was recorded in the metal stressed fish gills, liver, kidney and brain, respectively while in control fish, the same for all the organs was observed as 0.119±0.003, 0.111±0.005, 0.107±0.005 and 0.024±0.005UmL^{-1}, respectively. Physico-chemical parameters *viz*. pH, dissolved oxygen, carbon dioxide, total ammonia, total hardness, calcium and magnesium of the test media were monitored daily. All the physico-chemical variables of the test media varied significantly at $p<0.01$. All these variables exerted significantly positive effect on peroxidase activity except dissolved oxygen in both the organs.

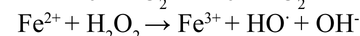
INTRODUCTION

Production of reactive oxygen species (ROS) is an inevitable phenomenon in all aerobically respiring organisms (Nishida, 2011). These reactive oxygen species (ROS) are produced naturally by mitochondrial respiration including superoxide, hydrogen peroxide and hydroxyl radicals. Reactive oxygen species (ROS) result in the oxidation of proteins, lipids and nucleic acids (Tripathi and Gaur, 2004). To minimize the hazardous impact of ROS on cells, there existed an antioxidant defense system in the animals (Geoffroy *et al*., 2004). This system comprises on the enzymes lipid peroxidase, superoxide dismutase, catalase, glutathione-S-transferase and glutathione peroxidase (Tripathi *et al*., 2006). Oxidative stress occurs as a consequence of the imbalance between reactive oxygen species (ROS) production and antioxidant enzymes (Nishida, 2011). Peroxidases are antioxidant enzymes that convert H_2O_2 into water (Pereira *et al*., 2000).

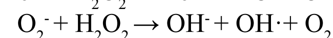
The fluctuating nature of the environment and human activities are continuously adding pollutants to the water bodies causing deleterious effects on the aquatic ecosystem (Ambreen and Javed, 2016). Metals are distinctive among various pollutants due to their non-biodegradable nature and having carcinogenic effects (Javed, 2012). Metallic ions can cause increase in the production of reactive oxygen species (Tripathi and Gaur, 2004). Among these metals, iron is a vital micro-nutrient necessary to perform different physiological processes in the living organisms. Highly toxic levels of iron can induce DNA, lipids and protein oxidation by increasing the production of ROS (Valko *et al*., 2005). Hydroxyl ion is produced by iron ion-dependent breakdown of hydrogen peroxide by means of Fenton reaction. In response to iron toxicity, production of ROS by Fenton reactions increases, leading towards oxidative stress (Sevcikova *et al*., 2011). The process of formation of ROS through Fenton reaction is as follows:



$$Fe^{3+} + O_2^{-} \rightarrow Fe^{2+} + O_2$$



$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^{\cdot} + OH^{-}$$



$$O_2^{-} + H_2O_2 \rightarrow OH^{-} + OH\cdot + O_2$$

Cirrhina mrigala is one of the most cultured fish species of Pakistan (Naz and Javed, 2013) as Indian major carps are fast-growing fish that attain marketable size of 0.8-1.0 kg in less than a year (Jhingran and Pullin, 1988). Therefore, fish gills, liver, kidney and brain are selected as organs to measure the effect of various levels of iron on peroxidase activity in the fish, *Cirrhina mrigala*.

MATERIALS AND METHODS

The proposed research work was performed in the laboratories of Fisheries Research Farms, Department of

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Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad. For this purpose, fingerlings of *Cirrhina mrigala* were brought to the laboratory and acclimatized to laboratory conditions prior to the experiments. After acclimatization, the one year old fingerlings of *Cirrhina mrigala*, having similar weights and lengths, were transferred to the glass aquaria of 50L water capacity. Chemically pure compound of iron chloride was dissolved in 1000ml deionized water and the metal stock solution was prepared. Four groups of fish (n=10) were exposed to sub-lethal concentrations *viz*. 96 h LC_{50}, 2/3rd, 1/4th and 1/5th of LC_{50} values of iron chloride as determined by Abdullah and Javed (2006). The experiment was conducted with three replications for each test concentration. Another group of fish regarded as "control" was kept in the metal free media. After 30 days of iron exposure, the fish were dissected and their gills, liver, kidney and brain tissues were isolated and stored at -4°C for enzyme assay. The physico-chemical parameters of water *viz*. pH, dissolved oxygen, carbon dioxide, total ammonia, total hardness, calcium and magnesium were monitored on daily basis by following the methods of APHA (1998).

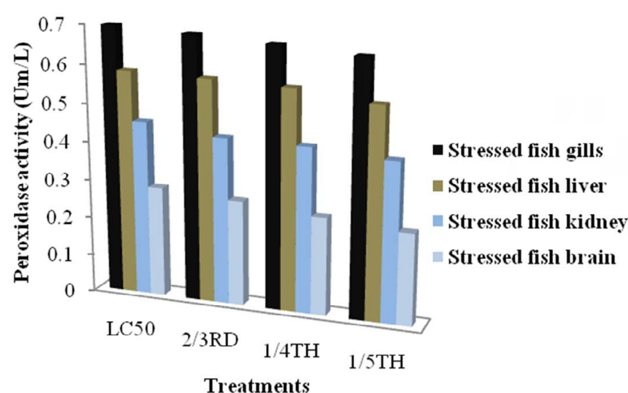

Fig. 1. Peroxidase activity of the fish organs (gills, liver, kidney and brain) at different treatments of iron.

For preparation of enzymes extract, the gills, liver, kidney and brain of the fish were rinsed with phosphate buffer of pH 6.5 (0.2M) and homogenized in cold buffer (1:4W/V) by using a blender. This was done for the removal of red blood cells from the tissues (gills, liver, kidney and brain). After homogenization, at 10,000 rpm, the organ homogenate was centrifuged for 15 min at 4°C. After centrifugation, the clear supernatant was preserved at -4°C for the enzyme assay. However, the residues were discarded.

The samples were subjected to enzyme assay as described by Civello *et al*. (1995) for the determination of peroxidase activity. The enzyme peroxidase activity was determined spectrophotometrically at a wavelength of 470nm by measuring the conversion of guaiacol to tetraguaiacol.

The buffer substrate solution was prepared by adding 750µL of Guaiacol to 0.2 M phosphate buffer pH 6.5 (47 ml) and mixed well. Later 300 µL H_2O_2 was added to buffer solution. The reaction mixture contained: Buffered substrate solution (300µL), enzymes extract (60µL) and the phosphate buffer was used as a blank.

A cuvette containing 3ml of blank solution was inserted into the Spectrophotometer and set it to zero at wave length of 470 nm. Then a cuvette containing buffered substrate was placed in the Spectrophotometer. The reaction was started by adding 0.06 ml enzyme extract. The reaction time was 3 min and after that absorbance was recorded.

$$\text{Activity (UmL}^{-1}) = \frac{\Delta A/3}{26.60 \times 60/3000}$$

Statistical analyses

Factorial experiments were performed, with three replications for each test concentration, to find out statistical differences among various sub-lethal concentrations of iron. The means were compared using LSD and the correlation and regression analyses were also performed to find out statistical relationships among different parameters under study.

RESULTS

After exposure to various sub-lethal concentrations of iron, peroxidase activity was analyzed in the gills, liver, kidney and brain of the fish, *Cirrhina mrigala*. In the gills of *Cirrhina mrigala,* the activity of peroxidase was found to be higher as 0.697±0.005UmL^{-1} in the 96 h LC_{50} treatment whereas it was significantly lower as 0.119±0.003UmL^{-1} in the control group (Fig. 1). In the liver of *Cirrhina mrigala,* peroxidase activity was significantly higher as 0.584±0.003UmL^{-1} during 96 h LC_{50} exposure while it was significantly lower as 0.111±0.005UmL^{-1} in control group (Fig. 2A). In the kidney of metal stressed *Cirrhina mrigala*, maximum activity of peroxidase was measured as 0.456±0.002UmL^{-1}at LC_{50} while it was significantly minimum (0.107±0.005UmL^{-1}) in control fish (Fig. 2B). In the brain of metal stressed *Cirrhina mrigala*, maximum activity of peroxidase was measured as 0.287±0.004UmL^{-1} at LC_{50} while it was significantly minimum (0.024±0.005UmL^{-1}) in control fish (Fig. 2C). Among the organs, peroxidase activity was in ascending order as gills> liver> kidney> brain. Peroxidase enzyme activity among the treatments followed the order, LC_{50}>

2/3rd> 1/4th> 1/5th> control (Fig. 2D). Increased peroxidase activity in the gills of iron exposed fish revealed that continuous contact of metal with the gills enhanced its absorption while the increase in the peroxidase activity in the liver of iron exposed fish revealed the fact that hepatocytes respond quickly to protect the tissues from oxidative damage caused by the enhanced production of ROS.

DISCUSSION

In the present study, after chronic exposure to iron the activity of peroxidase enzyme was significantly increased in the gills, liver, kidney and brain of the fish, *Cirrhina mrigala*, as compared to the control fish group (Mushtaq et al., 2017). In control group, the lower activity of peroxidase may be attributed to the minimum production of ROS. As peroxidase belongs to antioxidant enzymes family and causes the oxidation of a particular substrate at the expense of hydrogen peroxide (H_2O_2) it acts to reduce the harmful effects of ROS (Pereira *et al*., 2000). Therefore, activity of this enzyme was significantly increased in the metal stressed fish to overcome the severe effects of increasingly produced ROS on biomolecules. The present results are also in line with the findings of Rajeshkumar *et al*. (2013) who reported increased peroxidase activity (1.7±1.0UmL^{-1}) in the iron treated fish, *Chanos chanos* than that of control fish (0.7±0.1UmL^{-1}). It was also concluded that enhanced peroxidase activity in iron exposed fish helped in the removal of oxyradicals. Sevcikova *et al*. (2011) reported that sub-lethal exposure of iron resulted into increased peroxidase activity in the tissues of the fish, *Carassius auratus*. In relation to the iron exposed fish, the control fish showed minimum values of peroxidase activity during the whole experimental period. The present results are in parallel to the findings of Ercal *et al*. (2001). They concluded that iron caused significantly higher production of ROS that lead to the oxidative stress and the peroxidase activity in iron exposed fish (*Labeo rohita)* was significantly increased as compared to the un-exposed fish.

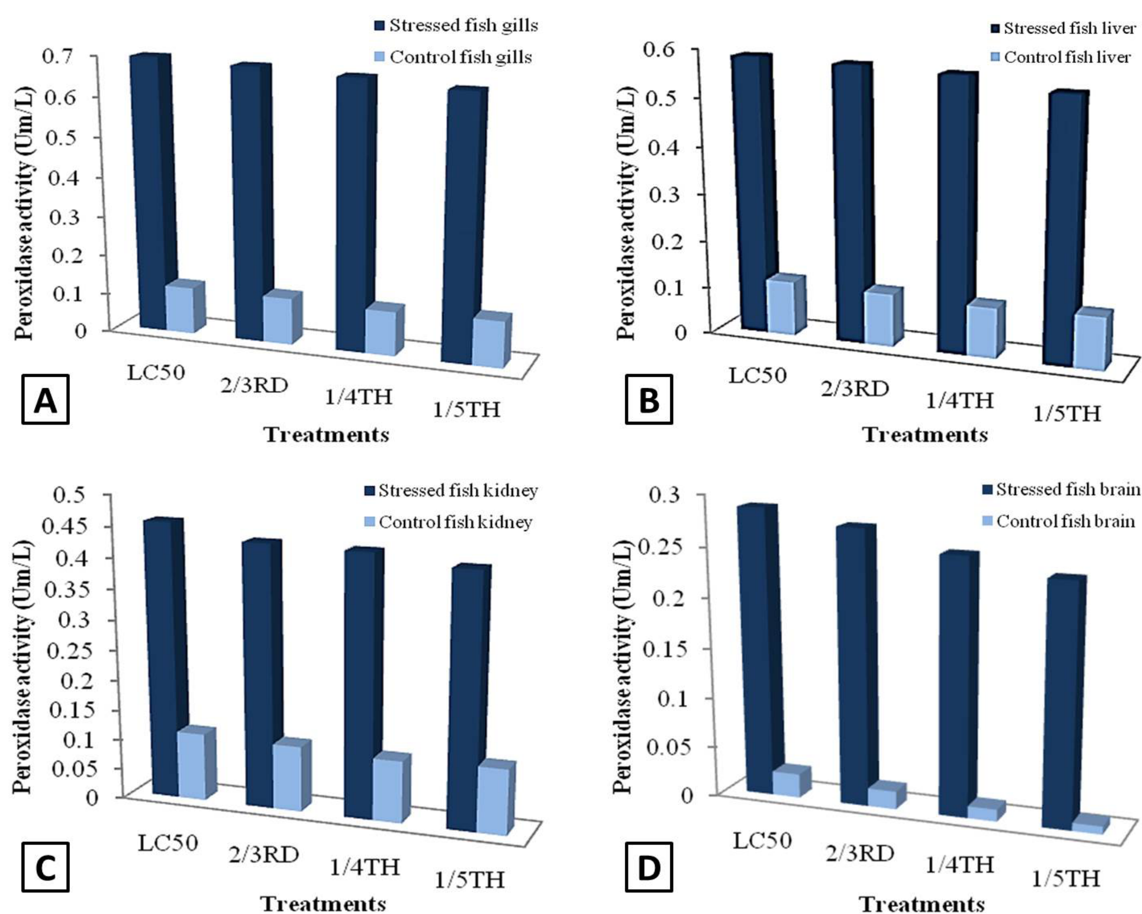

Fig. 2. Peroxidase activity (UmL^{-1}) in the gills (A), liver (B), kidney (C) and brain (D) of iron chloride treated and control fish groups.

The present results are also in conformity with the findings of Atli and Canli (2010), who observed that the enzyme activities in the cichlid fish (*Oreochromis niloticus*) were increased with increasing concentration (10µM and 20µM) of iron in test media, which was also reported by Ruas *et al*. (2008).

During the course of this experiment, peroxidase activity was found to be increased significantly ($p<0.05$) in the gills, liver, kidney and brain of the iron exposed fish, elevating the production of ROS at the cellular level and ultimately causing cell death. Peroxidase activity was higher in the gills in relation to other organs as gills directly encounter the external medium and it was higher in the liver than kidney because of the fact that liver is the main detoxification site and has higher affinity to accumulate the metal because of presence of metallothionins that are the metal binding proteins (Lushchak *et al*., 2005). Increased activity of peroxidase in the organs of metal stressed *Cirrhina mrigala* may be explained as a defensive mechanism against oxidative stress which was similar to the findings of Farombi *et al*. (2007) who observed 168% increase in the peroxidase activity of gills, 177% increase in the liver while 102% increase in the kidney. In contrast to our findings, Bagnyukova *et al*. (2006) reported lower peroxidase activity in the gills, liver, kidney and brain of Goldfish, *Carassius auratus*, when exposed to waterborne iron because of inactivation of peroxidase enzymes due to the oxidative stress. Fatima *et al*. (2000) also reported a decreased peroxidase activity in various tissues of the fish, *Carassius auratus*, resulting into inefficiency of fish organs to neutralize the harmful impacts of heavy metals.

Significant difference among peroxidase activity in the gills, liver, kidney and brain of control and metal stressed fish, *Cirrhina mrigala* was observed during present study. The physico-chemical parameters of the test media also influenced the toxicity of different metals to the fish. Therefore, during present experiment, water quality parameters *viz*. pH, dissolved oxygen, carbon dioxide, total ammonia, total hardness, calcium and magnesium were measured on daily basis. Results revealed that the concentration of total ammonia and carbon dioxide increased while dissolved oxygen decreased as a result of increasing iron concentration in the test media. These results are in parallel with the findings of Abdullah and Javed (2006) who also observed that ammonia and carbon dioxide increased significantly with an increase in metal concentration. Total hardness of water increased with the increasing concentrations of iron that showed significantly direct influence on the peroxidase activity because of elevation in the production of ROS. Javed and Mahmood (2001) found that in the treated media, total hardness increased with increasing concentrations of iron that is same as of current research results. During present studies, carbon dioxide, total ammonia and total hardness were increased with an increase in metal concentration that are similar to the findings of Correa et al. (2008). Sampaio *et al*. (2012) also found that peroxidase activity was increased with an increase in the carbon dioxide concentrations of water. Serafim *et al*. (2002) reported that heavy metals can induce oxidative stress due to alterations in the antioxidant enzyme activity as a result of increase in carbon dioxide and total ammonia concentrations.

CONCLUSION

Activity of peroxidase enzyme in metal stressed gills, liver, kidney and brain was reported as 0.697±0.005, 0.584±0.003, 0.456±0.002 and 0.287±0.004UmL^{-1}, respectively while in control group these values were 0.111±0.005UmL^{-1} (liver) and 0.107UmL^{-1} (kidney). Peroxidase activity was found to be metal concentration dependent as significantly higher activity was recorded in the 96 h LC_{50} exposed fish group while significantly lower activity was observed in control group. In all the treatments, peroxidase activity was significantly higher in the gills than that of other liver, kidney and brain. The physico-chemical variables *viz*. pH, carbon dioxide, total ammonia, total hardness, calcium and magnesium exhibited significantly positive dependence on peroxidase activity in both organs of fish. However, dissolved oxygen exhibited negative but significant regression on gills, liver, kidney and brain peroxidase activities. The partial regression co-efficients for all the physico-chemical variables were positive except for dissolved oxygen contents.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abdullah, S. and M. Javed. 2006. Studies on 96-hr LC_{50} and lethal toxicity of metals to the fish, *Cirrhina mrigala*. *Pakistan J. Agri. Sci.*, **43**: 180-185.

Ambreen, F. and Javed, M., 2016. Metal mixtures toxicity and bioaccumulation in *Tilapia nilotica* at 96-Hr LC50 exposure. *J. Biores. Manag.,* **3**: 14-21.

APHA, 1998. *Standard methods for the examination of water and wastewater*. 20th Ed. American Public Health Association, New York, pp. 1193.

Atli, G. and Canli, M., 2010. Response of antioxidant system of fresh water fish *Oreochromis niloticus* to acute and chronic metal (Cd, Cu, Cr, Zn, Fe) exposures. *Ecotoxicol. Environ. Safe*., **73**: 1884-1889. https://doi.org/10.1016/j.ecoenv.2010.09.005

Bagnyukova, T.V., Chahrak, O.I. and Lushchak, V.I., 2006. Coordinated response of goldfish antioxidant defenses to environmental stress. *Aquat. Toxicol*., **78**: 325-331. https://doi.org/10.1016/j.aquatox.2006.04.005

Civello, P.M., Arting, G.A., Chaves, A.R. and Ann, M.C., 1995. Peroxidase from strawberry and fruit by partial purification and determination of some properties. *J. Agric. Fd. Chem*., **43**: 2596-2601. https://doi.org/10.1021/jf00058a008

Correa, L.M., Kochhann, D., Becker, A.G., Pavanato, M.A., Llesuy, S.F., Loro, V.L., Raabe, A., Mesko, M.F., Flores, E.M.M., Dressler, V.L. and Baldisserotto, B., 2008. Biochemistry, cytogenetics and bioaccumulation in silver catfish (*Rhamdia quelen*) exposed to different thorium concentrations. *Aquat. Toxicol.,* **88**: 250–256.

Ercal, N., Gurer-Orhan, H. and Aykin-Burns, N., 2001. Toxic metals and oxidative stress part I: Mechanisms involved in induced oxidative damage. *Curr. Top. Med. Chem*., **1**: 529-539. https://doi.org/10.2174/1568026013394831

Farombi, E.O., Adelowo, O.A. and Ajimoko, Y.R., 2007. Biomarkers of oxidative stress and heavy metal levels as indicators of environmental pollution in African catfish (*Clarias gariepinus*) from Nigeria Ogun River. *Int. J. environ. Res. Publ. Hlth*., **4**: 158-165.

Fatima, M., Ahmed, I., Sayeed, I., Athat, M. and Raisuddin, S., 2000. Pollutant induced over activation of phagocytes is concomitantly associated with peroxidative damage in fish tissues. *Aquat. Toxicol*., **49**: 243-250. https://doi.org/10.1016/S0166-445X(99)00086-7

Geoffroy, L., Frankart, C. and Eullaffroy, P., 2004. Comparison of different physiological parameter responses in *Lemna minor* and *Scenedesmus obliquus* exposed to herbicide flumioxazin. *Environ. Pollut*., **131**: 475-480. https://doi.org/10.1016/j.envpol.2004.02.021

Javed, M., 2012. Growth responses of fish under chronic exposures of water-borne and dietary metals. *Int. J. Agric. Biol*., **14**: 281-285.

Javed, M. and Abdullah, S., 2006. Studies on acute and lethal toxicities of iron and nickel to the fish. *Pak. J. biol. Sci*., **9**: 330-335. https://doi.org/10.3923/pjbs.2006.330.335

Javed, M. and Mahmood, G., 2001. Metal toxicity of water in stretch of river Ravi from Shahdera to Baloki headworks. *Pak. J. agric. Sci*., **38**: 37-42.

Jhingran, V.G. and Pullin, R.S.V., 1988. A hatchery manual for the common, Chinese and Indian major carps. *ICLARM Stud. Rev*., **11**: 191.

Lushchak, O.V., Kubrak, O.I., Torous, I.M., Tetyana, Y., Kenneth, N., Storey, B. and Lushchak, V.I., 2005. Trivalent chromium induces oxidative stress in goldfish brain. *Chemosphere*, **75**: 56-62. https://doi.org/10.1016/j.chemosphere.2008.11.052

Mushtaq, T., Javed, M. and Abbas, S., 2017. Peroxidase activity in liver and kidney of Labeo rohita exposed to zinc chloride. *Pakistan J. Zool.,* **49**: 2335-2337.

Naz, S. and Javed, M., 2013. Evaluation of acute toxicity of metals mixture and bioaccumulation in freshwater fish. *Biosci. Methods*, **3**: 11-18. https://doi.org/10.5376/bm.2013.04.0003

Nishida, Y., 2011. The chemical process of oxidative stress by copper (II) and iron (III) ions in several neurodegenerative disorders. *Monatsh. Chem*., **142**: 375-384. https://doi.org/10.1007/s00706-010-0444-8

Pereira, L.F., Goodwin, P.H. and Erickson, L., 2000. Peroxidase activity during susceptible and resistant interactions between cassava (*Manihote sculenta*) and *Xanthomonas axonopodis* pv. *manihotis* and *Xanthomonas cassava*. *J. Phytopathol*., **148**: 575-577. https://doi.org/10.1046/j.1439-0434.2000.00548.x

Rajeshkumar, S., Mini, J. and Munuswamy, N., 2013. Impact of heavy metals on antioxidant activity in different tissue of milk fish, Chanos chanos. *Int. J. appl. Biol. Pharm. Technol*., **8**: 272-279.

Ruas, C.B.G., Carvalho, C.D., Arauijo, H.S.S., Espindola, E.L.G. and Feranandes, M.N., 2008. Oxidative stress biomarkers of exposure in the blood of cichild species from metal contaminated river. *Ecotoxical. Environ. Safe*., **71**: 86-93. https://doi.org/10.1016/j.ecoenv.2007.08.018

Sampaio, F.G., Boijink, C.L., Santos, L.R.B.D., Oba, E.T., Kalinin, A.L., Luiz, A.J.B. and Rantin, F.T., 2012. Antioxidant defenses and biochemical changes in the neotropical fish pacu, (*Piaractus mesopotamicus*) responses to single and combined copper and hypercarbia exposure. *Comp. Biochem. Physiol*., **156**: 178-186. https://doi.org/10.1016/j.cbpc.2012.07.002

Serafim, A., Company, R.M., Bebianno, M.J. and Lanjston, W.J., 2002. Effect of temperature and size on metallothien and synthesis in the gill of *Mytlus galloprovincialis* exposed to cadmium. *Mar. Environ. Res*., **54**: 361-365. https://doi.org/10.1016/S0141-1136(02)00121-6

Sevcikova, M., Modra, H., Slaninova, A. and Svobodova, Z., 2011. Metals as a cause of oxidative stress in fish. *Rev. Vet. Med*., **11**: 537-546. https://

doi.org/10.17221/4272-VETMED

Tripathi, B.N. and Gaur, J.P., 2004. Relationship between copper and zinc-induced oxidative stress and proline accumulation in *Scenedesmus* sp. *Planta*, **219**: 397-404. https://doi.org/10.1007/s00425-004-1237-2

Tripathi, B.N., Mehta, S.K., Amar, A. and Gaur, J.P., 2006. Oxidative stress in *Scenedesmus* sp. during short- and long-term exposure to Cu^{2+} and Zn^{2}. *Chemosphere*, **62**: 538-544. https://doi.org/10.1016/j.chemosphere.2005.06.031

Valko, M., Morris, H. and Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem*., **12**: 1161-1208. https://doi.org/10.2174/0929867053764635

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DNA Damage in Peripheral Erythrocytes of *Ctenopharyngodon idella* during Chronic Exposure to Pesticide Mixture

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Authors' Contribution
FA designed the experiment, collected and analyzed the data and wrote the manuscript. MJ reviewed the final version of manuscript. SA, SK, RI and MB helped in manuscript writing.

Key words
***Ctenopharyngodon idella*, Pesticide mixture, DNA damage, Sub-lethal exposure.**

ABSTRACT

Effluents from industries and runoff from agricultural fields contain highly toxic pesticides and their residues that persist in aquatic environment, posing a serious threat to aquatic fauna and flora. Therefore, laboratory tests were conducted to assess the DNA damage in peripheral blood erythrocytes of fish, *Ctenopharyngodon idella* (Grass carp) by using single cell gel electrophoresis (SCGE). Fish fingerlings were exposed to four sub-lethal concentrations of binary pesticide mixture (chlorpyrifos+endosulfan) *i.e.* 1/3rd, 1/4th, 1/5th and 1/6th of LC_{50} along with negative and positive control for the duration of 60-days. Fish were exposed to these concentrations, separately, in glass aquaria at constant temperature (30ºC), pH (7.75) and total hardness (225mgL^{-1}) of water. Peripheral erythrocytes were sampled after 60 day exposure and slides were prepared and examined under Epi-Fluorescence microscope for the estimation of DNA damage. DNA damage was examined by using three parameters *viz.* %age of damaged nuclei, genetic damage index (GDI) and cumulative tail length of comets (CTL). Pesticide mixture gave significantly ($p<0.05$) variable DNA damage in fish erythrocytes at various concentrations. Statistically significant results were observed at sub-lethal concentrations in-terms of %age of DNA damage, GDI and CTL (µm) of comets. Dose dependent DNA damage in terms of %age of damaged nuclei and GDI were observed, with highest damage at 1/3rd of LC_{50} as compared to control groups. Incidence of CTL was also higher (856.23±0.09µm) due to 1/3rd of LC_{50} while it was significantly lower due to negative control. This study confirmed that the SCGE is a useful tool for assessing the DNA integrity in fish and might be appropriate as a part of environmental monitoring programs for estimation of geno-toxicants in aquatic environment.

INTRODUCTION

Different classes of pesticides used against different species of pests, to increase crop yields even though these are highly toxic to other non-target species (Pandey *et al*., 2008; Ambreen and Javed, 2018). The aquatic environment is actually the last receptacle for pesticide residues (Boithias *et al*., 2011), finding their way to the food chain therefore; threatening the ecological balance and biodiversity (Asita and Makhalemele, 2008). Non-target organisms are exposed to different combinations of pesticides which may induce DNA damage in these organisms (Konen and Cavas, 2008). Exposure of aquatic organisms to pesticides could pose health hazards to humans through the food chain (Ambreen and Javed, 2015) and also linked with the induction of transmittable mutations leading to loss of biodiversity (Bickham *et al*., 2000; Jha, 2008). Health status of fish is a suitable reflection for the monitoring of aquatic pollutants because; fish is very sensitive to even minute changes in their surrounding environment (Boon *et al*., 2002).

Organochlorine and organophosphate are commonly detected pesticide classes in freshwater. Endosulfan belongs to class organochlorine is a very controversial chemical because of its high acute toxicity, harmful for the existence of life during its whole run from the time of spraying till its degradation (Liu *et al*., 2006). Its manufacturing and use is internationally banned by the Stockholm Convention in the year 2011. Unfortunately, it is still used in more than seventy countries, including Pakistan (Ahmed and Ahmad, 2006; Parbhu *et al*., 2009). Among organophosphate pesticides, chlorpyrifos is one of the most widely used pesticides. Chlorpyrifos have potential of high acute toxicity, it also elicit a number of other effects such as genotoxicity, developmental disorders, hepatic dysfunction, teratoginicity, neurotoxicity, irreversible inhibitor of cholinesterase and neurochemical changes (Gomes *et al*., 1999).

Several studies have focused on acute toxicity of individual pesticides but genotoxic effects of pesticides

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in the form of mixture are scant in the literature (Naz *et al*., 2012; Balistrieri and Mebane, 2014). Keeping in view the toxic nature, genotoxicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern. Integrity of DNA used as a sensitive biomarker for the monitoring of environmental pollutants (Lourenco *et al*., 2013). Pesticides induce strand breaks in DNA (single and double strand breaks) representing a constant threat to the genomic integrity (Nahas *et al*., 2017). Therefore, there exists need for rapid and sensitive assays that can be able to detect DNA damage (Mohanty *et al*., 2013). Among different techniques, the Single Cell Gel Electrophoresis (SCGE) can detect DNA damage at single cell level (Singh *et al*., 1988). Therefore, present study will endeavor to assess the dose dependent DNA damage in peripheral erythrocytes of *Ctenopharyngodon idella* under chronic exposure to binary pesticide mixture (chlorpyrifos+endosulfan).

MATERIALS AND METHODS

Experimental fish

Fingerlings of *Ctenopharyngodon idella* (180-day old) were purchased from local market and transported to Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Before start of experiment, fingerlings of *C. idella* having similar weight and lengths were acclimatized in cemented tanks for two weeks under laboratory conditions.

Pesticides

Pesticides *viz*. chlorpyrifos and endosulfan were dissolved, separately, in 95% analytical grade methanol (J.T Baker) as a carrier solvent to prepare the stock-I solutions (1g/100ml) while binary mixture of pesticides was prepared by further mixing of stock solutions.

Determination of sub-lethal concentrations

The 96 h LC_{50} value of chlorpyrifos+endosulfan mixture on *C. idella* was determined previously by Ambreen and Javed (2015). Based on this 96 h LC_{50} ($4.23\pm0.02\mu gL^{-1}$) value, four sub-lethal concentrations *viz*. 1/3rd ($1.41\mu gL^{-1}$), 1/4th ($1.06\mu gL^{-1}$), 1/5th ($0.85\mu gL^{-1}$) and 1/6th ($0.71\mu gL^{-1}$) of LC_{50} were calculated for these experiments.

Single cell gel electrophoresis (SCGE)

C. idella (n=6) were exposed, separately, to 1/3rd, 1/4th, 1/5th and 1/6th of LC_{50} in glass aquaria (70L capacity) along with negative and positive control. One group of healthy fish was kept in tap water, which was considered as "Negative control" (unstressed group) while 4% saline solution of cyclophosphamide (CP) was used as "Positive control". During 60 days of exposure, *C. idella* were fed daily small quantity of food. Water temperature (30ºC), pH (7.75) and hardness ($225mgL^{-1}$) were kept constant throughout the experimental duration. The exposure was continued for 60 days and peripheral blood slides were prepared on day 60th of exposure and subjected to SCGE. This experiment was conducted with three replications for each sub-lethal concentration. Peripheral blood erythrocytes were sampled from caudal vein of fish, transferred to eppendorf and treated with anticoagulant (heparin salt). SCGE was performed by following the protocols of Singh *et al*. (1988) as three layer procedure, followed by lysis, unwinding, electrophoresis, neutralization and staining. DNA was stained with ethidium bromide and slides were examined at 400X magnification by using the Epi-Fluorescence microscope (N-400M, American Scope; USA) equipped with light source of mercury and low lux digital (MD-800, American Scope; USA) camera. For each sub-lethal concentration/treatment, three slides and 50 cells per slide were randomly scored. Each image was classified according to the intensity of fluorescence in the comet tail and designated as following five categories (measured through TriTek CometScore™): Type-0, undamaged; Type-I, low level damage; Type-II, medium level damage; Type-III, high level damage and Type-IV, complete damage.

DNA damage (%)

The percentage of DNA damage was calculated as the mean percentage of cells with medium, high and complete damaged DNA by using following formula:

$$\text{DNA damage}(\%) = \text{Type} - \text{II} + \text{III} + \text{IV}$$

Genetic damage index (GDI)

From the arbitrary values assigned to the different categories (from Type-0 to Type-IV) a genetic damage index (GDI) was calculated for each subject by using following formula:

$$\text{GDI} = \frac{(\text{Type I}) + 2(\text{Type II}) + 3(\text{Type III}) + 4(\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

Cumulative tail lengths (CTL)

TriTek CometScore ™ software was used to calculate the comet tail length of damaged cells (Jose *et al*., 2011) and cumulative tail length (µm) was obtained by adding the tail length of all examined cells (n = 50/replicate).

Statistical analyses

Statistical analyses were performed through MSTATC computer software and results were expressed as Means±SD. Data means were compared for the statistical

differences by using Duncan Multiple Range test (DMR) by following Steel *et al*. (1996) and a value of $p<0.05$ was accepted as statistically significant.

RESULTS

Table I shows the variable proportion of Type-0 (undamaged nuclei), Type-I, II, III and IV (damaged nuclei), %age of damaged nuclei (Type-II+III+IV), GDI and CTL (µm) observed under sub-lethal concentrations of pesticide mixture in the peripheral blood erythrocytes of *C. idella* along with control groups after 60 days.

Undamaged and damaged nuclei (%)

Under all tested concentrations (1/3rd, 1/4th, 1/5th and 1/6th of LC_{50}), frequencies of undamaged nuclei (Type-0) were observed higher under negative control (96.67±2.31%) while same was lower due to 1/3rd of LC_{50} exposure. However, percentage of Type-I nuclei were significantly higher under 1/4th of LC_{50} (evident from mean value of 20.00±2.00%) while it was minimum as 2.67±1.15% in negative control. Regarding other damaged nuclei, Type-II damaged nuclei were maximum (32.00±3.46%) at 1/4th and Type-III nuclei were observed higher (36.67±3.06%) under 1/3rd of LC_{50} exposure as compared to control groups. However, Type-II nuclei under 1/3rd and 1/4th of LC_{50} exposure did not vary significantly at $p<0.05$. The Type-IV damaged nuclei under sub-lethal concentrations and control groups in peripheral erythrocytes of *C. idella* followed the sequence: positive control > 1/3rd > 1/4th ≥ 1/5th > 1/6th > negative control.

DNA damage (%)

The percentage of DNA damage (Type-II+Type-III+Type-IV) under negative control, positive control, 1/3rd, 1/4th, 1/5th and 1/6th of LC_{50} was significantly higher ($p<0.05$) due to 1/3rd of LC_{50}, followed by 1/4th, positive control, 1/5th, 1/6th of LC_{50} and negative control, representing dose dependent DNA damage.

GDI

C. idella also respond differently towards damage induction measured in terms of genetic damage indices (GDI). Maximum (2.32±0.03) GDI was observed at 1/3rd of LC_{50} mixture exposure while same was least in negative control samples.

CTL (µm)

Cumulative tail length of comets, induced due to various sub-lethal concentrations of mixture (chlorpyrifos+endosulafan), and control groups, ranged between the mean values of 3.38±0.06 to 856.23±0.09µm with statistically significant ($p<0.05$) differences.

DISCUSSION

Pesticides comprise an extensive range of synthetic organic compounds (Lazartigues *et al*., 2013) and these compounds are among more than 1000 active ingredients that are marketed as herbicides, insecticides and fungicides (Mostafalou and Abdollahi, 2013). During present study, the alkaline version of SCGE was applied to estimate the DNA damage in the peripheral blood erythrocytes of

Table I.- DNA damage in peripheral erythrocytes of *Ctenopharyngodon idella* after 60 day exposure to binary pesticide mixture.

Treatments/ Concentrations	Undamaged nuclei (%)	Damaged nuclei				Damaged nuclei (%)	GDI	CTL (µm)
	Type-0	Type-I	Type-II	Type-III	Type-IV			
Negative control (0.00)	96.67±2.31a	2.67±1.15e	0.67±1.15e	0.00±0.00e	0.00±0.00e	0.67±1.15f	0.04±0.03e	3.38±0.06 f
Positive control (CP: 20µgg^{-1})	25.33±1.15d	18.00±2.00bc	20.67±1.15d	16.67±4.16cd	19.33±5.03a	56.67±1.15c	1.87±0.06b	124.52±0.11e
1/3rd of LC_{50} (1.41µgL^{-1})	8.00±2.00f	12.67±1.15d	30.67±1.15ab	36.67±3.06a	12.00±2.00bc	79.33±1.15a	2.32±0.03a	856.23±0.09a
1/4th of LC_{50} (1.06µgL^{-1})	17.33±2.31e	20.00±2.00abc	32.00±3.46ab	20.00±2.00b	10.67±1.15c	62.67±1.15b	1.87±0.06b	566.20±0.10b
1/5th of LC_{50} (0.85µgL^{-1})	27.33±3.06cd	17.33±1.15c	30.00±2.00b	16.00±2.00d	9.33±1.15c	55.33±3.06d	1.63±0.08c	541.18±0.06c
1/6th of LC_{50} (0.71µgL^{-1})	38.00±2.00b	13.33±1.15d	26.67±4.16c	16.67±3.06cd	5.33±1.15d	48.67±2.31e	1.38±0.03d	408.36±0.05d

The means with similar letters in a single column for each variable are statistically non-significant at $p<0.05$.

C. idella exposed to sub-lethal concentrations of binary pesticide mixture and compared with negative and positive control. Pesticides may cause direct DNA damage due to action of parental compound or their metabolites or indirectly due to over-production of reactive oxygen species (Oliveira *et al*., 2009). During present study, dose dependent DNA damage was observed under sub-lethal exposures of chlorpyrifos, endosulfan mixture. Dose dependent DNA damage associated with pesticide exposure by using the SCGE in fish erythrocytes is well documented (Nwani *et al*., 2013; Pandey *et al*., 2011; Yong *et al*., 2011; Rani and Kumaraguru, 2013). However, previously discussed DNA damage was based on evaluation of acute exposure. Therefore, such approach fails to provide appropriate information regarding the long-term effects of pesticide burden on the genome. The main idea of the present study was to characterize DNA damage induced by prolonged exposure to mixture.

DNA damage was estimated by measuring the percentage of damaged nuclei, GDI and CTL. Statistically significant increase in DNA damage was observed in the erythrocytes of fish due to exposure of polluted water (Klobucar *et al*., 2010) while mixture of pesticides (chlorpyrifos+endosulfan+thiram) has been reported to cause significantly ($p<0.05$) higher DNA damage (Tope and Rogers, 2009). Altinok *et al*. (2012) also observed significantly higher DNA damage in terms of comet tail length, tail intensity, tail moment and tail migration in erythrocytes of *Oncorhynchus mykiss* exposed to different concentrations of carbosulfan for 60 days as compared to positive control.

Pesticides have higher ability to make variety of reactive oxygen species (H_2O_2, O^{2-} and OH^- and electrophilic free radical metabolites) that interact with nucleophilic sites of DNA, thereby cause strand breakage (Banudevi *et al*., 2006). Pesticides can form strong covalent bonds with DNA resulting in the formation of DNA adducts (Hartwell *et al*., 2000), modulate antioxidant defensive systems and cause oxidative damage in aquatic organisms (Monteiro *et al*., 2006). The DNA damage detected in the present study could have originated from DNA single strand breaks, DNA double strand breaks, DNA-DNA/DNA-protein cross linking or inhibition of the enzymes involved in DNA repair resulting from the interaction of pesticides or their metabolites with DNA (Guilherme *et al*., 2012).

The present study reveals that 1/3rd LC_{50} exposure of pesticide mixture to the fish caused significantly higher DNA damage while negative control exerted significantly least damage to the nuclei. Nwani *et al*. (2010) also observed dose and time dependent DNA damage in fish erythrocytes and gill cells under carbosulfan exposure by using SCGE. Exposure of fish to sub-lethal concentrations *viz*. 1/4th LC_{50}, 1/2nd LC_{50} and 3/4th of LC_{50} of carbosulfan gave significantly higher DNA damage ($p<0.01$) in erythrocytes and gill cells in terms of percentage of tail DNA than that of control group. Similarly, Ilyas (2015) observed dose-dependent response of major carps towards waterborne exposure of individual pesticides *viz*. endosulfan, bifenthrin and chlorpyrifos to induce DNA damage in their peripheral blood erythrocytes by employing SCGE. Naqvi *et al*. (2016) also evaluate genotoxic potential of different pesticides in the peripheral blood erythrocytes of fish by using micronuclei assay. Genotoxicity of pesticides in fish found to be in the order of cypermethrin > chlorpyrifos > malathion > lambda-cyhalothrin > buctril. Cavalcante *et al*. (2008) also reported significantly higher DNA damage in fish, *Prochilodus lineatus* exposed to $10 mgL^{-1}$ of roundup as compared to negative control.

Tested sub-lethal concentrations in the present study could be environmentally relevant concentrations, although repeated applications of the pesticides in most developing countries may be higher, suggesting the relevance of test concentrations. Pesticides in sub-lethal concentration present in water are too low to cause rapid death directly but may affect the functioning of organisms, disrupt normal behavior and reduce their survival (Susan *et al*., 2010). Present results are also in accordance with Ambreen and Javed (2016) who also reported concomitant increase in DNA damage with sub-lethal concentrations of mixture in erythrocytes of *Cyprinus carpio* as compared to control groups.

Similarly, significant increase in DNA damage under the exposure of pesticides mixture (metolachlor + isoproturon + chlorotoluron + atrazine + deethylatrazine) as compared to control group was observed by Polard *et al*. (2011). Dose and time dependent response of *Oreochromis niloticus* on DNA integrity was also observed by Rani and Kumaraguru (2013) due to endosulfan exposure. Altinok *et al*. (2012) also observed significantly higher DNA damage in terms of tail length, tail intensity, tail moment and tail migration in fish, exposed to various concentrations of carbosulfan for 60 days than that of control group. Exposure to different concentrations of chlorpyrifos induced higher DNA damage in fish as compared to controls, indicating genotoxic potential of this pesticide to aquatic organisms (Ali *et al*., 2008; Yong *et al*., 2011). Present results are also in accordance with findings of Pandey *et al*. (2011) who observed dose dependent DNA damage in fish (*Channa punctatus*) under exposure of organophosphate pesticide. DNA damage induced by mixture of pesticides recommended a serious health concern towards their possible danger to the survival of *C. idella* in their aquatic environment.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Ambreen, F. and Javed, M., 2018. Pesticide mixture induced DNA damage in peripheral blood erythrocytes of freshwater fish, *Oreochromis niloticus. Pakistan J. Zool.,* **50**: 339-346.

Ahmed, S. and Ahmad, M.S., 2006. *Effect of insecticides on the total number of soil bacteria under laboratory and field conditions*. Department of Agricultural Entomology, University of Agriculture, Faisalabad 38040, Pakistan, pp. 63-67.

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R. and Kushwaha, B., 2008. Genotoxicity assessment of acute exposure of chlorpyriphos to fresh water fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Chemosphere*, **71**: 1823-1831. https://doi.org/10.1016/j.chemosphere.2008.02.007

Altinok, I., Capkin, E. and Boran, H., 2012. Mutagenic, genotoxic and enzyme inhibitory effects of carbosulfan in rainbow trout *Oncorhynchus mykiss*. *Pestic. Biochem. Physiol*., **102**: 61-67. https://doi.org/10.1016/j.pestbp.2011.10.011

Ambreen, F. and Javed, M., 2015. Assessment of acute toxicity of pesticides mixtures for *Cyprinus carpio* and *Ctenopharyngodon idella*. *Pakistan J. Zool*., **47**: 133-139.

Ambreen, F. and Javed, M., 2016. Effects of 30 day sub-lethal exposure of cadmium and lead mixture on DNA damage in fish. *J Anim. Pl. Sci*., **26**: 674-679. https://doi.org/10.1016/j.scitotenv.2013.07.034

Asita, A.O. and Makhalemele, R., 2008. Genotoxicity of chlorpyrifos, alpha-thrin, efekto virikop and springbok to onion root tip cells. *Afri. J. Biotechnol*., **7**: 4244-4250.

Balistrieri, L.S. and Mebane, C.A., 2014. Predicting the toxicity of metal mixtures. *Sci. Total Environ*., **466-467**: 788-799.

Banudevi, S., Krishnamoorthy, G., Venkatataman, P., Vignesh, C., Aruldhas, M.M. and Arunakaran, J., 2006. Role of a tocopherol on antioxidant status in liver, lung and kidney of PCP exposed male albino rats. *Fd. Chem. Toxicol*., **44**: 2040-2046. https://doi.org/10.1016/j.fct.2006.07.017

Bickham, J.W., Sandhu, S., Hebert, P.D.N., Chikhi, L. and Athwal, R., 2000. Effects of chemical contaminants on genetic diversity in natural populations: Implications for biomonitoring and ecotoxicology. *Mutat. Res. Rev. Mutat. Res*., **463**: 33-51. https://doi.org/10.1016/S1383-5742(00)00004-1

Boithias, L., Sauvage, S., Taghavi, L., Merlina, G., Probst, J.L. and Sanchez-Perez, J.M., 2011. Occurrence of metolachlor and trifluralin losses in the save river agricultural catchment during floods. *J. Haz. Mat*., **196**: 210-219. https://doi.org/10.1016/j.jhazmat.2011.09.012

Boon, J.P., Lewis, W.E., Tjoen-A-Choy, M.R., Allchin, C.R., Law, R.J. and De Boer, J., 2002. Levels of polybrominated diphenyl ether (PBDE) flame retardants in animals representing different trophic levels of the North Sea food web. *Environ. Sci. Technol*., **36**: 4025-4032. https://doi.org/10.1021/es0158298

Cavalcante, D.G.S.M., Martinez, C.B.R. and Sofia, S.H., 2008. Genotoxic effects of roundup® on the fish *Prochilodus lineatus*. *Mutat. Res. Fund. Mol. Mechan. Mutag*., **655**: 41-46. https://doi.org/10.1016/j.mrgentox.2008.06.010

Gomes, J., Dawodu, A.H., Llyod, O., Revitt, D.M. and Anilal, S.V., 1999. Hepatic injury and disturbed amino acid metabolism in mice following prolonged exposure to organophosphorus pesticides. *Human Exp. Toxicol*., **18**: 33-37. https://doi.org/10.1191/096032799678839365

Guilherme, S., Santos, M.A., Barroso, C., Gaivao, I. and Pacheco, M., 2012. Differential genotoxicity of roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): Considerations on chemical interactions and DNA damaging mechanisms. *Ecotoxicology*, **21**: 1381-1390. https://doi.org/10.1007/s10646-012-0892-5

Hartwell, L.H., Hood, L., Goldberg, M.L., Reynolds, A.E., Silver, L.M. and Veres, R.C., 2000. *Genetics: From genes to genomes*. McGraw-Hill, New York.

Ilyas, R., 2015. *Effects of selected pesticides on growth and DNA damage in peripheral blood erythrocytes of fish*. Ph. D. thesis, Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan, pp. 1-329.

Jha, A.N., 2008. Ecotoxicological applications and significance of the comet assay. *Mutagenesis*, **23**: 207-221. https://doi.org/10.1093/mutage/gen014

Jose, S., Jayesh, P., Mohandas, A., Philip, R. and Singh, I.S.B., 2011. Application of primary haemocyte

culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. *Mar. environ. Res*., **71**: 169-177. https://doi.org/10.1016/j.marenvres.2010.12.008

Kapour, D. and Nagpure, N.S. (eds.), 2005. *Training on genotoxic assays in fishes*. National Bureau of Fish Genetic Resources, Dilknsha, Telibagh, India, pp. 68.

Klobucar, G.I.V., Stambuk, A., Pavlica, M., Peric, M.S., Hackenberger, B.K. and Hylland, K., 2010. Genotoxicity monitoring of freshwater environments using caged carp (*Cyprinus carpio*). *Ecotoxicology*, **19**: 77-84. https://doi.org/10.1007/s10646-009-0390-6

Konen, S. and Cavas, T., 2008. Genotoxicity testing of the herbicide trifluralin and its commercial formulation Treflan using the piscine micronucleus test. *Environ. Mol. Mutagen*., **49**: 434-438. https://doi.org/10.1002/em.20401

Lazartigues, A., Thomas, M., Banas, D., Brun-Bellut, J., Cren-Olive, C. and Feidt, C., 2013. Accumulation and half-lives of 13 pesticides in muscle tissue of freshwater fishes through food exposure. *Chemosphere*, **91**: 530-535. https://doi.org/10.1016/j.chemosphere.2012.12.032

Liu, Y., Zhang, Y., Liu, J. and Huang, D., 2006. The role of reactive oxygen species in the herbicide acetachlor-induced DNA damage on Bufo-raddei tadpole liver. *Aquat. Toxicol*., **78**: 21-26. https://doi.org/10.1016/j.aquatox.2006.01.016

Lourenco, J., Pereira, R., Goncalves, F. and Mendo, S., 2013. Metal bioaccumulation, genotoxicity and gene expression in the European wood mouse (*Apodemus sylvaticus*) inhabiting an abandoned uranium mining area. *Sci. Total Environ*., **443**: 673-680. https://doi.org/10.1016/j.scitotenv.2012.10.105

Mohanty, G., Mohanty, J., Garnayak, S.K., Rath, S.K. and Dutta, S.K., 2013. Use of comet assay in the study of DNA break in blood and gill cells of rohu (*Labeo rohita*) after an exposure to furadan, a carbamate pesticide. *Res. J. Biotechnol*., **8**: 83-89.

Monteiro, D.A., Almeida, J.A., Ratnin, F.T. and Kalinin, A.L., 2006. Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorous insecticide folisuper 600 (methyl parathion). *Comp. Biochem. Physiol. C: Toxicol. Pharmacol*., **143**: 141-149.

Mostafalou, S. and Abdollahi, M., 2013. Pesticides and human chronic diseases: Evidences, mechanisms, and perspectives. *Toxicol. appl. Pharmacol*., **268**: 157-177. https://doi.org/10.1016/j.taap.2013.01.025

Nahas, A.F., Abdel-Razek, M.A.S., Helmy, N.M., Mahmoud, S. and Ghazy, H.A., 2017. Impaired antioxidant gene expression by pesticide residues and its relation with other cellular biomarkers in Nile tilapia (*Oreochromis niloticus*) from Lake Burullus. *Ecotoxicol. environ. Safe*., **137**: 202-209. https://doi.org/10.1016/j.ecoenv.2016.12.006

Naqvi, G.Z., Shoaib, N. and Ali, A.M., 2016. Genotoxic potential of pesticides in the peripheral blood erythrocytes of fish (*Oreochromis mossambicus*). *Pakistan J. Zool*., **48**: 1643-1648.

Naz, S., Javed, M., Tahir, A. and Azmat, H., 2012. Impact of physico-chemical variables of test media on growth performance of metal stressed major carps. *Pakistan J. Zool*., **44**: 1291-1296.

Nwani, C.D., Nagpure, N.S., Kumar, R., Kushwaha, B. and Lakra, W.S., 2013. DNA damage and oxidative stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. *Environ. Toxicol. Pharmacol*., **36**: 539-547. https://doi.org/10.1016/j.etap.2013.06.001

Nwani, C.D., Lakra, W.S., Nagpure, N.S., Kumar, R., Kushwaha, B. and Srivastava, S.K., 2010. Mutagenic and genotoxic effects of carbosulfan in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Fd. Chem. Toxicol*., **48**: 202-208. https://doi.org/10.1016/j.fct.2009.09.041

Oliveira, M., Maria, V.L., Ahmad, I., Serafim, A., Bebianno, M.J., Pacheco, M. and Santos, M.A., 2009. Contamination assessment of a coastal lagoon (Ria de Averiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* – an integrated biomarker approach. *Environ. Pollut*., **157**: 959-967. https://doi.org/10.1016/j.envpol.2008.10.019

Pandey, A.K., Nagpure, N.S., Trivedi, S.P., Kumar, R. and Kushwaha, B., 2011. Profenofos induced DNA damage in freshwater fish, *Channa punctatus* (Bloch) using alkaline single cell gel electrophoresis. *Mutat. Res. Genet. Toxicol. Environ. Mutag*., **726**: 209-214. https://doi.org/10.1016/j.mrgentox.2011.09.011

Pandey, R.K., Singh, R.N. and Das, V.K., 2008. Effect of temperature on mortality and behavioural responses in freshwater catfish, *Heteropneustes fossilis* (Bloch) exposed to dimethoate. *Glob. J. environ. Res*., **2**: 126-132.

Parbhu, B., Rodgers, G. and Sullivan, J.E., 2009. Death in a toddler following endosulfan ingestion. Pediatric death caused by endosulfan

ingestion. *Clin. Toxicol*., **47**: 899-901. https://doi.org/10.3109/15563650903328879

Polard, T., Jean, S., Gauthier, L., Laplnche, C., Marlina, G., Sanchez-Perez, J.M. and Pinelli, E., 2011. Mutagenic impact on fish of runoff events in agriculture areas in south-west France. *Aquat. Toxicol*., **101**: 126-134. https://doi.org/10.1016/j.aquatox.2010.09.014

Rani, A.P.A. and Kumaraguru, A.K., 2013. DNA integrity as molecular biomarker of genotoxic effect of endosulfan in *Oreochromis mossambicus* (Peters). *Int. J. Eng. Sci. Invent*., **2**: 58-61.

Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L., 1988. A simple technique for the quantization of low levels of DNA damage in individual cells. *Exp. Cell Res.,* **175**: 184-191. https://doi.org/10.1016/0014-4827(88)90265-0

Steel, R.G.D., Torrie, J.H. and Dinkkey, D.A., 1996. *Principles and procedures of statistics,* 3rd ed. McGraw Hill Book Co., Singapore, pp. 627.

Susan, T.A., Sobha, K. and Tilak, K.S., 2010. A study on acute toxicity, oxygen consumption and behavioural changes in the three Major carps, *Labeo rohita* (Ham), *Catla catla* (Ham) and *Cirrhina mrigala* (Ham) exposed to Fenvalerate. *Biores. Bull*., **1**: 33-40.

Tope, A.M. and Rogers, P.F., 2009. Evaluation of protective effects of sulforaphane on DNA damage caused by exposure to low levels of pesticide mixture using comet assay. *J. environ. Sci. Hlth*., **44**: 657-662. https://doi.org/10.1080/03601230903163624

Yong, C., Guo, J., Xu, B. and Chen, Z., 2011. Genotoxicity of chlorpyrifos and cypermethrin to ICR mouse hepatocytes. *Toxicol. Mech. Methods*, **21**: 70-74. https://doi.org/10.3109/15376516.2010.529192

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Genotoxicity of Dietary Bifenthrin in Peripheral Blood Erythrocytes of Fresh Water Fish, *Cirrhina mrigala*

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Authors' Contribution
RI conceived the idea and performed the research. MJ helped in this study. SK helped in paper writing. FA helped in statistical analyses of data. MB and HA helped in the preparation of this manuscript.

Key words
Genotoxicity, Dietary bifenthrin, Fish, Comet assay.

ABSTRACT

Bifenthrin belongs to class pyrethroid, most commonly used pesticides throughout the world. Their extensive usage is a threat to the natural environments including aquatic ecosystems. Although bifenthrin are rapidly degraded in soil and plants, but they are extremely toxic to the fish. Keeping in view the high sensitivity of fish towards bifenthrin, the present study was conducted to evaluate the genotoxic effects of dietary bifenthrin in peripheral blood erythrocytes of freshwater fish, *Cirrhina mrigala* through comet assay. Fish were exposed to four sub-lethal concentrations *viz*. 10, 20, 33 and 50% LD_{50} of dietary bifenthrin along with positive and negative control groups, separately, under controlled laboratory conditions for 30 days. The DNA damage in terms of percentage of DNA damage, genetic damage index (GDI) and cumulative tail length of comets (µm) in peripheral erythrocytes of *Cirrhina mrigala* were determined. The exposure of dietary bifenthrin at 50% LD_{50} caused significantly higher damage to DNA followed by that of positive control, 33%, 20%, 10% LD_{50} and negative control. However, 33% and 20% LD_{50} exposures did not show any significant difference in percentage of DNA damaged cells. The GDI of *Cirrhina mrigala* showed significant differences due to exposure of various concentrations of bifenthrin. However, the patterns of DNA damage followed the order: 50% > positive control > 33% > 20% > 10% LD_{50} > negative control. The dietary exposure of 50% LD_{50} caused significantly higher cumulative tail length of comets followed by that of 33%, positive control, 20% , 10% LD_{50} and negative control with statistically significant differences at $p<0.05$. Significantly dose dependent increase in DNA damage was also observed during present investigation. This study also reveals that dietary bifenthrin is very toxic to the fish and comet assay can be used as useful tool for the determination of genotoxic effects of pesticides on fish.

INTRODUCTION

Aquatic pollution has become a severe and mounting crisis all over the world. The pollution of toxic chemicals exists in various countries, including Pakistan (Shoaib *et al*., 2011) that needs strict legislation to deal with this problem. Fish and other aquatic organisms have the tendency to accumulate pollutants directly from contaminated water and indirectly through the ingestion of contaminated food (Al-Ghanim, 2012). Genotoxic pollutants taint not only aquatic organisms but also the entire ecosystem and ultimately the humans through intake of contamination of food (Pandey *et al*., 2011).

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A basic contributor to the green revolution has been the expansion in the application of pesticides to control a wide diversity of insectivorous and herbaceous pests that would otherwise reduce the quantity and quality of food produced since the mid 1940s (Zhang *et al*., 2010; Bolognesi *et al*., 2011; Ambreen and Javed, 2018). Unfortunately, the benefits of chemistry have been decreased when heavily used pesticides were found in the aquatic habitats. Growth in industrial, agricultural and many other human activities, as a result of increasing population pressure, have resulted in poor environmental management that caused chemical contamination of important water bodies across the world (Ali and Kumar, 2012).

Bifenthrin is active insecticide belongs to pyrethroid group of insecticide and is used in agriculture and public health control programs (control of mosquitoes) that act as a contact stomach poison. It affects the central and

peripheral nervous system and cause synaptic discharge, depolarization and ultimately cause death and it also act as ATPase inhibitor in fish (Ponepal *et al*., 2010). Pyrethroid compounds are potentially toxic, they can damage the DNA directly and produce reactive oxygen species that can in turn damage the DNA (Cachot *et al*., 2006).

Keeping in view the toxic nature, genotoxicity of pesticides for non-target organisms and their influence on ecosystems are of worldwide concern. Integrity of DNA used as a sensitive biomarker for the monitoring of environmental pollutants (Lourenco *et al*., 2013). Pesticides induce strand breaks in DNA (single and double strand breaks) representing a constant threat to the genomic integrity (Nahas *et al*., 2017). Therefore, there exists need for rapid and sensitive assays that can be able to detect DNA damage (Mohanty *et al*., 2013). Among different techniques, comet assay can detect DNA damage at single cell level (Singh *et al*., 1988). Therefore, present study is the investigation of genotoxicity evaluation of dietary bifenthrin through comet assay in peripheral blood erythrocytes of fresh water fish, *Cirrhina mrigala.*

MATERIALS AND METHODS

Experimental fish

Fingerlings of *Cirrhina mrigala* (150-day old) were purchased from local market and transported to Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan. Before start of experiment, fingerlings of *Cirrhina mrigala* having similar weight and lengths were acclimatized in cemented tanks for two weeks under laboratory conditions.

Bifenthrin

Bifenthrin were dissolved, separately, in 95% analytical grade methanol (J.T. Baker) as a carrier solvent to prepare the stock-I solution (1g/100ml). Working stock solutions of pesticide were dissolved in one liter deionized water and mixed well with the feed ingredients prior to pelleting according to required sub-lethal concentrations. Pallets were prepared without heating by using 2 mm diameter module. After preparation, each diet was placed in small plastic bags and filled with nitrogen gas and vacuumed before refrigeration.

Determination of sub-lethal concentrations

The 96 h LD_{50} value of dietary bifenthrin on *Cirrhina mrigala* was determined during previous study of Ilyas (2015). Based on this 96 h LD_{50} (11.97±0.98 μgg^{-1}) value, four sub-lethal concentrations *viz*. 10% (1.20μgg^{-1}), 20% (2.39μgg^{-1}), 33% (3.95μgg^{-1}) and 50% (5.99μgg^{-1}) of LD_{50} were calculated for these experiments.

Comet assay

Cirrhina mrigala (n=10) was exposed, separately, to four dietary treatments *viz*. 10, 20, 33 and 50% LD_{50} of dietary bifenthrin for 30 days in glass aquaria. Each test dose was tested with three replications on fish. One group of fish (n=10) was used as negative control (un-stressed) while other as positive control (cyclophosphamide treated or CP). Fish was fed, separately, the diets containing sub-lethal concentrations (10, 20, 33 and 50% of LD_{50}) of dietary bifenthrin while the fish of both control groups were fed with pesticide free diet. Water temperature (30ºC), pH (7.5) and hardness (300mgL^{-1}) were kept constant throughout the experimental duration. The peripheral blood slides were prepared on day 30th of exposure and subjected to comet assay. This experiment was conducted with three replications for each sub-lethal concentration. Peripheral blood erythrocytes were sampled from caudal vein of fish, transferred to eppendorf and treated with anticoagulant (heparin salt). Comet assay was performed by following the protocols of Singh *et al*. (1988) as three layer procedure, followed by lysis, unwinding, electrophoresis, neutralization and staining. DNA was stained with ethidium bromide and slides were examined at 400X magnification by using the Epi-Fluorescence microscope (N-400M, American Scope; USA) equipped with light source of mercury and low lux digital (MD-800, American Scope; USA) camera. The DNA damage was quantified by visual classification of cells into five categories "comets" corresponding to the tail length, with three replicates (n = 50), undamaged: Type 0, low-level damage: Type I, medium-level damage: Type II, high-level damage: Type III and complete damage: Type IV. Cells with no DNA damage were intact nucleus without a tail, whereas cells with DNA damage showed comet like appearance. The length of DNA migration in the comet tail is an estimate of DNA damage. The cells with no head or dispersed head were regarded as apoptotic cells and not included in the analysis. The extent of DNA damage was expressed as the mean percentage of cells with medium, high and complete damaged DNA which were calculated as the sum of cells with damage Types II, III and IV. From the values assigned to the different categories (from Type 0 = 0 to Type IV = 4), %age of damaged cells, a genetic damage index {GDI: (TypeI+2×TypeII+ 3×Type III+4×Type IV) / (Type 0+Type I+ Type II+ Type III+ Type IV)} and cumulative tail length of comets (µm) were calculated for each subject. DNA damage was measured by using visual counting and cumulative tail length measured by adding the tail length of all examined cells (n=50 per replicate) through Tri Tek CometScore™ (Summerduck, USA) Software (Josea *et al*., 2011).

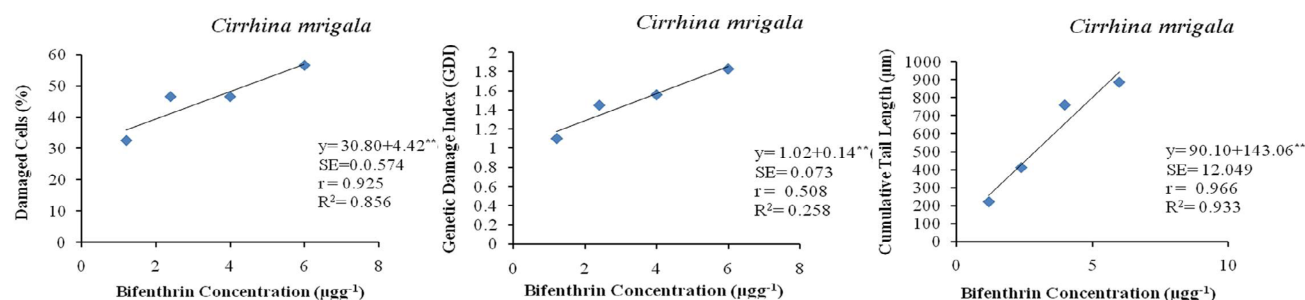

Fig. 1. Dependence of DNA damaged cells (%), genetic damage index (GDI) and tail length of comets (µm) on exposed dietary bifenthrin concentrations. **, significant at $p<0.01$; SE, standard error; r, correlation coefficient; R^2, coefficient of determination.

Table I.- Effect of dietary bifenthrin on DNA damage in the peripheral blood erythrocytes of *Cirrhina mrigala*.

Treatments	Exposure Concent.	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)				Damaged cells (%) (II+III+IV)	GDI	CTL (µm)
		Type 0	Type I	Type II	Type III	Type IV			
Negative control	0.00	92.67±1.15 a	3.33±3.06e	2.67±1.15e	0.67±1.15f	0.67±1.15f	4.00±3.46e	0.13±0.08f	4.59±2.87f
Positive control	CP (20 µgg^{-1})	36.00±2.00 e	14.00±2.00c	13.33±3.06d	16.00±4.00c	20.67±3.06a	50.00±2.00b	1.71±0.07b	486.94±9.21c
10% of LD_{50}	1.20 µgL^{-1}	50.67±3.06 b	16.67±5.03b	13.33±3.06d	10.67±1.15e	8.67±1.15e	32.67±2.31d	1.10±0.02e	224.64±9.12 e
20% of LD_{50}	2.39 µgL^{-1}	44.67±5.03 c	8.67±5.03 d	17.33±1.15b	15.33±1.15d	14.00±2.00d	46.67±1.15c	1.45±0.08d	414.15±10.35d
33% of LD_{50}	3.95 µgL^{-1}	36.67±3.06 d	16.67±1.15b	15.33±3.06c	16.67±3.06b	14.67±3.06c	46.67±3.06c	1.56±0.11c	761.38±10.49b
50% of LD_{50}	5.99 µgL^{-1}	23.33±3.06 f	20.00±2.00a	22.67±1.15a	18.00±2.00a	16.00±2.00b	56.67±3.06a	1.83±0.08a	887.17±14.46a

The means with similar letters in a single column for each variable are statistically non-significant at $p<0.05$.

Statistical analyses

Statistical analyses were performed through MSTATC computer software and results were expressed as Means±SD. Data means were compared for the statistical differences by using Duncan Multiple Range test (DMR) by following Steel *et al*. (1996) and a value of $p<0.05$ was accepted as statistically significant. Regression and correlation analyses were also performed to find-out relationships among various parameters under investigation.

RESULTS

Table I shows the variable proportion of Type-0 (undamaged nuclei), Type-I, II, III and IV (damaged nuclei), percentage of damaged nuclei (Type-II+III+IV), GDI and CTL (µm) observed under sub-lethal concentrations of dietary bifenthrin in the peripheral blood erythrocytes of *Cirrhina mrigala* along with control groups after 30 days.

Four concentrations of bifenthrin, negative and positive controls caused significantly variable damage to the DNA of peripheral erythrocytes of *Cirrhina mrigala*. The exposure of dietary bifenthrin at 50% LD_{50} caused significantly higher damage to DNA as 56.67±3.06%, followed by that of positive control, 33%, 20%, 10% LD_{50} and negative control with the mean values of 50.00±2.00, 46.67±3.06, 46.67±1.15, 32.67±2.31 and 4.00±3.46%, respectively. However, 33% and 20% LD_{50} exposures did not show any significant difference in percentage of DNA damaged cells. The GDI of *Cirrhina mrigala* showed significant differences due to exposure of various concentrations of bifenthrin. However, the patterns of DNA damage followed the order: 50% > positive control > 33% > 20% > 10% LD_{50} > negative control. The dietary exposure of 50% LD_{50} caused significantly higher cumulative tail length of comets as 887.17±14.46 µm in the peripheral erythrocytes, followed by that of 33%, positive control, 20%, 10% LD_{50} and negative control with statistically significant differences at $p<0.05$.

The data regarding frequency of DNA damage cells, GDI and cumulative tail length of comets induced due to various concentrations of dietary bifenthrin are presented in Table I. This data were statistically analyzed to see the relationships between frequency of DNA damage and dietary exposure of bifenthrin to the fish. There existed significantly positive relationship between exposure concentrations and frequency of DNA damaged cells, GDI and cumulative tail length of comets. DNA damage

in peripheral erythrocytes of fish increased linearly with the increase of pesticide concentration in the diet (Fig. 1).

DISCUSSION

Insecticides, fungicides and herbicides constitute major source of potential environmental hazards not only to fish, birds, mammals and other animals but also to humans when they become part of food chains (Abd-Alla *et al*., 2002). Various chemicals have been used as insecticides/fungicides/pesticides in public health programs, veterinary and agriculture. Pyrethroids are preferred above organophosphates, carbamates and organochlorines because of their high efficiency, low toxicity and easy biodegradability (Sharaf *et al*., 2010). For more than 30 years, pyrethroids are in use for home formulations and agricultural purposes, cover nearly one-fourth of the world market (Ahmad *et al*., 2012).

During present study, comet assay was applied to estimate the DNA damage in the peripheral blood erythrocytes of *Cirrhina mrigala* exposed to sub-lethal concentrations of dietary bifenthrin and compared with negative and positive control. Pesticides may cause direct DNA damage due to action of parental compound or their metabolites or indirectly due to over-production of reactive oxygen species (Oliveira *et al*., 2009). During present study, dose dependent DNA damage was observed under sub-lethal exposures of dietary bifenthrin. Dose dependent DNA damage associated with pesticide exposure by using comet assay in fish erythrocytes is well documented (Pandey *et al*., 2011; Yong *et al*., 2011; Rani and Kumaraguru, 2013). However, previously discussed DNA damage was based on evaluation of acute exposure. Therefore, such approach fails to provide appropriate information regarding the long-term effects of pesticide. The main idea of the present study was to characterize DNA damage induced by prolonged exposure of pesticide in diet.

Pesticides make variety of reactive oxygen species (ROS) which interact with nucleophilic sites of DNA, thereby cause strand breakage. Pesticides can form strong covalent bonds with DNA resulting in the formation of DNA adducts (Hartwell *et al*., 2000; Banudevi *et al*., 2006), and cause oxidative damage in aquatic organisms (Monteiro *et al*., 2006). The DNA damage detected in the present study could have originated from DNA single strand breaks, DNA double strand breaks, DNA-DNA/DNA-protein cross linking or inhibition of the enzymes involved in DNA repair resulting from the interaction of pesticide or their metabolites with DNA (Guilherme *et al*., 2012).

Similar study conducted by Ali *et al*. (2008, 2009) reported genotoxic effect of different concentrations of chlorpyrifos in freshwater fish, *Channa punctatus*, by using comet assay. The fish exhibited significantly higher DNA damage in their tissues than the control groups. A concentration and dose dependent increase in DNA damage were also observed. Kumar *et al*. (2010) also observed genotoxic effect of different concentrations of malathion in different tissues of freshwater fish, *Channa punctatus* by using comet assay. The DNA damage in kidney, lymphocytes and gills were significantly higher ($p<0.05$) in exposed groups as compared to the control. All the tissues showed significantly linear concentration dependent increase in DNA damage and non-linear decrease with time. The genotoxic and mutagenic effects of different concentrations (6.25, 12.50 and 25.00μgL^{-1}) of Atrazine on *Oreochromis niloticus* were investigated by Ventura *et al*. (2008) by using comet assay. Present results are also in accordance with findings of Pandey *et al*. (2011), who observed dose dependent DNA damage in fish (*Channa punctatus*) under exposure of organophosphate pesticide. DNA damage induced by dietary bifenthrin recommended a serious health concern towards their possible danger to the survival of *Cirrhina mrigala* in their aquatic environment.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abd-Alla, E.A.M., Nassar, A.M., Neamat-Allah, A.A. and Aly, S.E., 2002. Prevalence of pesticide residues in fish, cheese and human milk. *Assiut Vet. Med. J*., **47**: 110-124.

Ahmad, L., Khan, A., Khan, M.Z., Hussain, I., Mahmood, F., Sleemi, M.K., Lodhi, L.A. and Abdullah, I., 2012. Toxico-pathological effects of cypermethrin upon male reproductive system in rabbits. *Pestic. Biochem. Physiol*., **103**: 194-201. https://doi.org/10.1016/j.pestbp.2012.05.004

Al-Ghanim, K.A., 2012. Malathion toxicity in Nile tilapia, *Oreochromus niloticus*- A haemotological and biochemical study. *Afri. J. agric. Res*., **7**: 561-567.

Ali, D., Nagpure, N.S., Kumar, R., Kumar, S. and

Kushwaha, B., 2008. Genotoxicity assessment of acute exposure of chlorpyriphos to fresh water fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Chemosphere*, **71**: 1823-1831. https://doi.org/10.1016/j.chemosphere.2008.02.007

Ali, D., Nagpure, N.S., Kumar, S., Kumar, R., Kushwaha, B. and Lakraa, W.S., 2009. Assessment of genotoxic and mutagenic effects of chlorpyrifos in freshwater fish *Channa punctatus* (Bloch) using micronucleus assay and alkaline single-cell gel electrophoresis. *Fd. Chem. Toxicol*., **47**: 650-656. https://doi.org/10.1016/j.fct.2008.12.021

Ali, D. and Kumar, S., 2012. Study the effect of chlorpyrifos on acetylcholinesterase and hematological response in freshwater fish *Channa punctatus* (Bloch). *IIOAB J*., **3**: 12-18.

Ambreen, F. and Javed, M., 2018. Pesticide mixture induced DNA damage in peripheral blood erythrocytes of freshwater fish, *Oreochromis niloticus. Pakistan J. Zool.,* **50**: 339-346.

Banudevi, S., Krishnamoorthy, G., Venkatataman, P., Vignesh, C., Aruldhas, M.M. and Arunakaran, J., 2006. Role of a tocopherol on antioxidant status in liver, lung and kidney of PCP exposed male albino rats. *Fd. Chem. Toxicol*., **44**: 2040-2046. https://doi.org/10.1016/j.fct.2006.07.017

Bolognesi, C., Creus, A., Ostrosky-Wegman, P. and Marcos, R., 2011. Micronuclei and pesticides exposure. *Mutagenesis*, **26**: 19-26. https://doi.org/10.1093/mutage/geq070

Cachot, J., Geffard, O., Augagneur, S., Lacriox, S., Le Manach, K., Peluhet, L., Couteau, J., Denier, X., Devier, M.H., Pottier, D. and Budzinski, H., 2006. Evidence of genotoxicity related to high PAH content of sediments in the upper part of the Seine estury (Normandy, France). *Aquat. Toxicol*., **79**: 257-267. https://doi.org/10.1016/j.aquatox.2006.06.014

Guilherme, S., Santos, M.A., Barroso, C., Gaivao, I. and Pacheco, M., 2012. Differential genotoxicity of roundup® formulation and its constituents in blood cells of fish (*Anguilla anguilla*): Considerations on chemical interactions and DNA damaging mechanisms. *Ecotoxicology*, **21**: 1381-1390. https://doi.org/10.1007/s10646-012-0892-5

Hartwell, L.H., Hood, L., Goldberg, M.L., Reynolds, A.E., Silver, L.M. and Veres, R.C., 2000. *Genetics: From genes to genomes*. McGraw-Hill, New York.

Ilyas, R., 2015. *Effects of selected pesticides on growth and DNA damage in peripheral blood erythrocytes of fish*. Ph. D. thesis, Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan, pp. 1-329.

Josea, S., Jayesha, P., Mohandasa, A., Philipb, R. and Singha, I.S.B., 2011. Application of primary haemocyte culture of *Penaeus monodon* in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. *Mar. environ. Res*., **71**: 169-177. https://doi.org/10.1016/j.marenvres.2010.12.008

Kumar, A., Sharma, B. and Pandey, R.S., 2010. Toxicological assessment of pyrethroid insecticides with special reference to cypermethrin and λ-cyhalothrin in freshwater fishes. *Int. J. Biol. Med. Res*., **1**: 315- 325.

Lazartigues, A., Thomas, M., Banas, D., Brun-Bellut, J., Cren-Olive, C. and Feidt, C., 2013. Accumulation and half-lives of 13 pesticides in muscle tissue of freshwater fishes through food exposure. *Chemosphere*, **91**: 530-535. https://doi.org/10.1016/j.chemosphere.2012.12.032

Lourenco, J., Pereira, R., Goncalves, F. and Mendo, S., 2013. Metal bioaccumulation, genotoxicity and gene expression in the European wood mouse (*Apodemus sylvaticus*) inhabiting an abandoned uranium mining area. *Sci. Total Environ*., **443**: 673-680. https://doi.org/10.1016/j.scitotenv.2012.10.105

Mohanty, G., Mohanty, J., Garnayak, S.K., Rath, S.K. and Dutta, S.K., 2013. Use of comet assay in the study of DNA break in blood and gill cells of rohu (*Labeo rohita*) after an exposure to furadan, a carbamate pesticide. *Res. J. Biotechnol*., **8**: 83-89.

Monteiro, D.A., Almeida, J.A., Ratnin, F.T. and Kalinin, A.L., 2006. Oxidative stress biomarkers in the freshwater characid fish, *Brycon cephalus*, exposed to organophosphorous insecticide folisuper 600 (methyl parathion). *Comp. Biochem. Physiol. C: Toxicol. Pharmacol*., **143**: 141-149.

Mostafalou, S. and Abdollahi, M., 2013. Pesticides and human chronic diseases: Evidences, mechanisms, and perspectives. *Toxicol. appl. Pharmacol*., **268**: 157-177. https://doi.org/10.1016/j.taap.2013.01.025

Nahas, A.F., Abdel-Razek, M.A.S., Helmy, N.M., Mahmoud, S. and Ghazy, H.A., 2017. Impaired antioxidant gene expression by pesticide residues and its relation with other cellular biomarkers in Nile tilapia (*Oreochromis niloticus*) from Lake Burullus. *Ecotoxicol. environ. Safe*., **137**: 202-209. https://doi.org/10.1016/j.ecoenv.2016.12.006

Nwani, C.D., Nagpure, N.S., Kumar, R., Kushwaha, B. and Lakra, W.S., 2013. DNA damage and oxidative

stress modulatory effects of glyphosate-based herbicide in freshwater fish, *Channa punctatus*. *Environ. Toxicol. Pharmacol*., **36**: 539-547. https://doi.org/10.1016/j.etap.2013.06.001

Oliveira, M., Maria, V.L., Ahmad, I., Serafim, A., Bebianno, M.J., Pacheco, M. and Santos, M.A., 2009. Contamination assessment of a coastal lagoon (Ria de Averiro, Portugal) using defence and damage biochemical indicators in gill of *Liza aurata* – an integrated biomarker approach. *Environ. Pollut*., **157**: 959-967. https://doi.org/10.1016/j.envpol.2008.10.019

Pandey, A.K., Nagpure, N.S., Trivedi, S.P., Kumar, R. and Kushwaha, B., 2011. Profenofos induced DNA damage in freshwater fish, *Channa punctatus* (Bloch) using alkaline single cell gel electrophoresis. *Mutat. Res. Genet. Toxicol. environ. Mutag*., **726**: 209-214.

Ponepal, M.C., Paunescu, A., Draghici, O. and Marinescu, A.G., 2010. Research on the changes of some physiological parameters in several fish species under the action of the talstar insecticide. *Analele Univ. Oradea Fasc. Biol*., **17**: 175-179.

Rani, A.P.A. and Kumaraguru, A.K., 2013. DNA integrity as molecular biomarker of genotoxic effect of endosulfan in *Oreochromis mossambicus* (Peters). *Int. J. Eng. Sci. Invent.*, **2**: 58-61.

Sharaf, S., Khan, A., Khan, M.Z., Aslam, F., Saleemi, M.K. and Mahmood, F., 2010. Clinico-hematological and micronuclear changes induced by cypermethrin in broiler chicks: Their attenuation with vitamin E and selenium. *Exp. Toxicol. Pathol*., **62**: 333-341. https://doi.org/10.1016/j.etp.2009.05.002

Shoaib, N., Siddique, P.J.A., Ali, A., Burhan, Z. and Shafique, S., 2011. Toxicity of pesticides on photosynthesis of diatoms. *Pak. J. Bot*., **43**: 2067-2069.

Singh, N.P., McCoy, M.T., Tice, R.R. and Schneider, E.L., 1988. A simple technique for the quantization of low levels of DNA damage in individual cells. *Exp. Cell Res*., **175**: 184-191. https://doi.org/10.1016/0014-4827(88)90265-0

Steel, R.G.D., Torrie, J.H. and Dinkkey, D.A., 1996. *Principles and procedures of statistics*, 3rd ed. McGraw Hill Book Co., Singapore, pp. 627.

Ventura, B.C., Angelis, D.F. and Marin-Morales, M.A., 2008. Mutagenic and genotoxic effects of the atrazine herbicide in *Oreochromis niloticus* (Perciformes, Cichlidae) detected by the micronuclei test and the comet assay. *Pestic. Biochem. Physiol*., **90**: 42-51. https://doi.org/10.1016/j.pestbp.2007.07.009

Yong, C., Guo, J., Xu, B. and Chen, Z., 2011. Genotoxicity of chlorpyrifos and cypermethrin to ICR mouse hepatocytes. *Toxicol. Mech. Methods*, **21**: 70-74. https://doi.org/10.3109/15376516.2010.529192

Zhang, Z.Y., Yu, X.Y., Wang, D.L., Yan, H.J. and Liu, X.J., 2010. Acute toxicity to zebrafish of two organophosphates and four pyrethroids and their binary mixtures. *Pest Manage. Sci*., **66**: 84-89. https://doi.org/10.1002/ps.1834

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Cadmium Induced Oxidative Stress in Different Organs of *Labeo rohita* and *Catla catla*

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ABSTRACT

Untreated run off water from agricultural land and industrial effluents contain toxic pollutants, including heavy metals, which can accumulate in the aquatic organisms, especially the fish and cause oxidative stress. The present study was planned to measure the cadmium (Cd) induced oxidative stress in terms of antioxidant enzyme (peroxidase) activity in fish, *Labeo rohita* and *Catla catla.* 120-day old fingerlings of both fish species were exposed, separately, to 2/3rd and 1/3rd of their respective 96 h LC_{50} values of Cd in glass aquaria for 15 days at constant temperature (30°C), pH (8) and total hardness (250mgL^{-1}), along with a control in which fish remained unexposed to any metal concentration. After 15-day exposure, fish were sacrificed and their organs (liver, kidney, muscles and gills) separated for enzyme (peroxidase) assay. Results indicated that Cd exposure induced significant variations in the activity of peroxidase in the selected organs of *L. rohita* and *C. catla* as compared to control. Among fish species, *C. catla* was more sensitivity as compared to *L. rohita* as the variations in peroxidase activity were more pronounced in *C. catla* with the mean value of 0.268±0.103 UmL^{-1}. Peroxidase activity in the organs followed the order: liver > kidney > gills > muscles. However, maximum peroxidase activity was measured in 2/3rd of LC_{50} of Cd exposed fish followed by 1/3rd and control.

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Authors' Contribution
FL did the research work and wrote the paper. MJ conceived the idea and supervised the research.

Key words
Cadmium stress, Antioxidant enzyme, Peroxidase.

INTRODUCTION

A tremendous increase in the population, urbanization and industrialization has led to the degradation of environment, particularly the aquatic environment, which is the main receiving end for pollutants. Consequently, genetic disorders, physiological problems, diseases and ultimately mortality of various aquatic species may take place (Isani *et al.*, 2009). The metals such as copper, mercury, zinc, lead, chromium, iron, cobalt, manganese, arsenic and cadmium are potentially hazardous pollutants as they can cause toxicity in aquatic organisms (Javed *et al.*, 2016). An important pathway for metallic ions toxicity is through the production of reactive oxygen species (ROS) which include the singlet oxygen, superoxide anion, hydroxyl radical and hydrogen peroxide. Cellular redox balance favouring pro-oxidants production caused oxidative stress which can lead to macromolecules disruptions including cross-links in DNA, degradation of proteins and peroxidation of membrane fatty acids. These elevated ROS levels can also act in signal transduction I messenger pathways (The´venod, 2009).

According to Younis *et al.* (2013), cadmium (Cd) is one of the most toxic contaminant in aquatic environments causing toxicity at each level of the ecological station. Cd increases the production of ROS in tissues as well as inhibits the activity of some antioxidant enzymes (Zikic *et al.*, 2001). Water-borne Cd can be absorbed through the skin and gills and then distributed unevenly in different fish tissues. Unlike Fe and Cu, Cd is not a redox-active metal, but it could generate free radicals and ultimately oxidative stress, that may cause lipid peroxidation thus DNA damage (Cuypers *et al.*, 2010). Therefore, at biochemical levels, antioxidant systems have great potential to indicate the cellular responses to the toxic effects of Cd (Cirillo *et al.*, 2012).

Cd interferes with the structure and function of several molecules with marked endpoints such as uncontrolled cell proliferation and unprogrammed cell death. In general, Cd induces both protective as well as damaging signaling pathways, but the exact underlying mechanisms remain to be resolved. At the cellular level, a common mechanism in both Cd-induced damage and repair processes is oxidative stress. Peroxidases are broadly present in the microorganisms and animal tissues (Boeuf *et al.*, 2000). The amino acid (cysteine) and heme cofactor provides the active site for the enzyme peroxidase. When oxidative stress increases, peroxidase functions as first line of defense towards ROS (Kurutas *et al.*, 2009). Therefore, during present research work, effects of two different sub-lethal concentrations of Cd on peroxidase activity has been assessed on different organs of two fish species. The present research work will help in sustainable conservation of major carps, *Labeo rohita* and *Catla catla* in the natural aquatic bodies of Pakistan.

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Table I.- Peroxidase activity (UmL^{-1}) in the selected organs of *Labeo rohita* and *Catla catla* after sub-lethal exposure of cadmium.

Treatments	Fish species	Organs			
		Liver	Kidney	Muscles	Gills
Control	*Labeo rohita*	0.169±0.005 a	0.130±0.004 b	0.078±0.002 c	0.116±0.003 d
	Catla catla	0.193±0.004 a	0.182±0.003	0.097±0.002 c	0.142±0.002 d
1/3rd	*Labeo rohita*	0.263±0.003 a	0.221±0.005 b	0.190±0.003 c	0.218±0.007 d
	Catla catla	0.335±0.005 a	0.302±0.004 b	0.259±0.007 c	0.285±0.004 d
2/3rd	*Labeo rohita*	0.313±0.005 a	0.270±0.006 b	0.225±0.004 c	0.236±0.005 d
	Catla catla	0.389±0.005 a	0.356±0.005 b	0.329±0.009 c	0.349±0.009 d

MATERIALS AND METHODS

Fingerlings of *Catla catla* and *Labeo rohita* were kept in cemented tanks for acclimation for two weeks. After this acclimation period, the healthy group of 120-day old fish fingerlings of similar weights and lengths were selected for these experiments. These fingerlings of *L. rohita* and *C. catla* were exposed, separately, to 1/3rd and 2/3rd of their respective 96 h LC_{50} values of Cd determined by Yaqub and Javed (2012) in glass aquaria for 15 days at constant temperature (30°C), pH 8 and total hardness ($250mgL^{-1}$). The 96 h LC_{50} value of Cd for *L. rohita* and *C. catla* was 153.23±2.74 and 155.08±2.63 mgL^{-1}, respectively.

After 15-day exposure, metal stressed fish was sacrificed and their organs (liver, kidney, muscles and gills) separated for enzyme (peroxidase) assay. Each test was conducted with three replications for each concentration/ treatment and activity of antioxidant in the selected organs was compared with the control group. The activity of peroxidase was determined by measuring its ability to reduce the concentration of H_2O_2 at A470nm (Civello *et al*., 1995).

Extraction and essay of enzyme

The dissected organs were rinsed with phosphate buffer of pH 6.5 (0.2M) and homogenized in cold buffer (1:4W/V) using a blender. After homogenization, organ homogenate was centrifuged for 15 min at 10,000 rpm (×g) and 4ºC. After centrifugation process, clear supernatants were stored at -80ºC for enzyme assay while the residue was discarded.

Preparation of buffer substrate solution

Guaiacol (750µL) was added to 47 ml of 0.2M phosphate buffer pH 6.5 and mixed well on vortex agitator. After agitation, H_2O_2 (0.3mL) was added to buffer solution. Reaction mixture contain 300µL buffered substrate solution 60µL enzyme extract and phosphate buffer used as a blank. A cuvette containing 3mL of blank solution was inserted into the spectrophotometer and set it to zero at wavelength of 470nm. Then a cuvette containing buffered substrate solution was put into the spectrophotometer and initiation of reaction was occurred by adding 0.06mL of enzyme extract. The reaction time is 3minutes. Activity of peroxidase was measured by following the formula as:

$$\text{Activity (Unit/mL)} = \Delta A/3/26.60 \times 60/3000$$

Where, ΔA is Absorbance at 470nm, 26.60, extinction coefficient of tetraguaiacol.

RESULTS AND DISCUSSION

Heavy metals can persist in the aquatic environment for a long time and may bio-magnified due to their nature to bio-accumulate in the tissues of aquatic organisms. Ultimately, they can cause adverse effects in humans through the food chain (Luoma and Rainbow, 2008). Despite the presence of well-developed antioxidant enzymes defense system, severe oxidative damage may occur in aquatic organisms exposed to these metals, that increases the production of ROS and subsequent oxidative damage associated with metallic ions mediated mechanisms of toxicity in fish (Livingstone, 2001). According to Sudha and Lall (2013), metabolic adaptive strategies of fish affected by oxidative stress can be measured in the form of change in the activities of antioxidant enzymes. In aquatic environments, cadmium (Cd) is one of the most toxic metals that cause toxicity at each level of ecological station (Younis *et al*., 2013). Therefore, during present research work, Cd mediated oxidative stress in term of antioxidant enzyme (peroxidase) was assessed in the liver, kidney, muscles and gills of *Labeo rohita* and *Catla catla.* Present results indicated significant increase in the antioxidant enzyme (peroxidase) activity in the Cd stressed fish as compared to control (Table I). It has been reported by Dorta *et al.* (2003) that Cd increases the concentration of free Fe by replacing it in many proteins and ultimately enhances the cellular concentration of free redox active metals like Fe and Cu. These free redox metals increase the production of hydroxyl radicals through Fenton reaction and to overcome these free ions, peroxidase activity increased subsequently

in metals stressed fish (Raza *et al*., 2016). Among organs, maximum peroxidase activity was measured in the liver of both fish species followed by kidney, gills and muscles with the mean values of 0.277±0.085, 0.243±0.082, 0.224±0.087 and 0.196±0.096 UmL^{-1}, respectively. During present experiment, enhanced production of antioxidant enzyme in the liver is revealing important protective and adaptational responses against toxic stress caused by Cd which is also observed by Zhang *et al*. (2013) and Kumar and Yadav (2014). Bhanu and Deepak (2015) reported that increased activity of peroxidase in Cd stressed fish is due to the enhanced ability of cells to overcome the effects of ROS. These results are also supported by the findings of Mamidala (2012) and Soorya *et al*. (2013) who have reported that the induction of antioxidant compounds is a sensitive preliminary warning signal of oxidative stress. With increasing concentration of Cd from 1/3rd to 2/3rd of LC_{50}, peroxidase activity enhanced subsequently which showed concentration dependent increase in peroxidase activity. Least peroxidase activity was measured in the control fish groups, which may be attributed to the fact that in the absence of any external stressor, the level of ROS and antioxidant enzymes is parallel to each other which is a normal equilibrium because oxidative stress mainly occurs either due to increased production of ROS or decreased production of antioxidant enzymes which is also reported by Mushtaq *et al*. (2017). Among fish species, maximum peroxidase activity was measured in *C. catla* as compared to *L. rohita* with mean values of 0.268±0.103 and 0.202±0.071 UmL^{-1}, respectively. The results reported here and in other studies indicate that antioxidant enzyme responses are variable and transient for different fish species (Livingstone, 2001). The present results are concluded as Cd exposure caused significant increase in peroxidase activity in the organs of both fish species.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Bhanu, A.P. and Deepak, M., 2015. The effect of Cadmium on antioxidant enzymes in the liver of fresh water fish *Cyprius carpio* (Linn). *Biolife*, **3**: 50-53.

Boeuf, G.B., Legrand, B. and Rambour, S., 2000. Purification and characterization of a basic peroxidase from the medium of cell suspension culture of chicory. *Pl. Physiol. Biochem*., **38**: 217-224. https://doi.org/10.1016/S0981-9428(00)00731-2

Cirillo, T., Amodio, C.R., Fasano, E., Lucisano, A., Tafuri, S., Ferrante, M.C. and Isani, G., 2012. Cadmium accumulation and antioxidant responses in *Sparus aurata* exposed to waterborne cadmium. *Arch. environ. Contam. Toxicol*., **62**: 118-126. https://doi.org/10.1007/s00244-011-9676-9

Civello, P.M., Arting, G.A., Chaves, A.R. and Anan, M.C., 1995. Peroxidase from strawberry fruit by partial purification and determination of some properties. *J. Agric. Fd. Chem*., **43**: 2596-2601. https://doi.org/10.1021/jf00058a008

Cuypers, A., Plusquin, M., Remans, T., Jozefczak, M., Keunen, E., Gielen, H. and Nawrot, T., 2010. Cadmium stress: An oxidative challenge. *Biometals*, **23**: 927-940. https://doi.org/10.1007/s10534-010-9329-x

Dorta, D.J., Leite, S., Demarco, K.C. and Curti, C., 2003. A proposed sequence of events for cadmium-induced mitochondrial impairment. *J. Inorg. Biochem*., **97**: 251-257. https://doi.org/10.1016/S0162-0134(03)00314-3

Mamidala, E. and Lunavath, V., 2012. A study on human immunodeficiency virus-1 (HIV-1) protease inhibition by extracts of fresh water mussel. *Int. J. Med. Pharm. Res*., **2**: 38-42.

Hu, C.X., Li, D.H. and Liu, Y.D., 2013. Lipid peroxidation and antioxidant responses in zebrafish brain induced by Aphanizomenon flos-aquae DC-1 aphantoxins. *Aquat. Toxicol*., **144**: 250-256.

Isani, G., Andreani, G., Cocchioni, F., Fedeli, D., Carpene, E. and Falcioni, G., 2009. Cadmium accumulation and biochemical responses in *Sparus aurata* following sub-lethal Cd exposure. *Ecotoxicol. environ. Safe*., **72**: 224-230. https://doi.org/10.1016/j.ecoenv.2008.04.015

Javed, M., Abbas, S. and Latif, F., 2016. Acute toxicity of cadmium and its bio-accumulation in the carnivorous fish species *Channa marulius*, *Mystus seenghala* and *Wallago attu*. *Int. J. Agric. Biol*., **18**: 1169-1173. https://doi.org/10.17957/IJAB/15.0221

Kumar, V. and Yadav, U., 2014. Screening of antifungal activity of *Pleurotus ostreatus* and *Agaricus bisporus*. *Biolife*, **2**: 913-923.

Kurutas, E.B., Sahan, A. and Altun, T., 2009. Oxidative stress biomarkers in liver and gill tissues of spotted barb (*Capoeta barroisi Lortet*) living in the river Ceyhan, Adana, Turkey. *Turk. J. Biol*., **33**: 275-282.

Livingstone, D.R., 2001. Contaminant reactive oxygen species production and oxidative damage in aquatic organisms. *Mar. Pollut. Bull*., **42**: 656-666. https://doi.org/10.1016/S0025-326X(01)00060-1

Luoma, S.N. and Rainbow, P.S., 2008. *Sources and cycles*

of trace metals: Metal contamination in aquatic environments: Science and lateral management. Cambridge University Press, Cambridge, pp. 47-66.

Mushtaq, T., Javed, M., and Abbas, S., 2017. Peroxidase activity in liver and kidney of *Labeo rohita* exposed to zinc chloride. *Pakistan J. Zool.*, **49**: 2335-2337.

Raza, B., Javed, M., Ambreen, F. and Latif, F., 2016. Toxic effect of lead chloride on antioxidant enzyme in the liver and kidney of fish. *J. Bioresour. Manage*., **3**: 1.

Soorya, S.R., Devia, A., Binitha, R.N., Apirtha, B.V., Jayalakshmi, G. and Sunny, F., 2013. Quantitative changes in antioxidant enzyme activities, glutathione content and malondialdehyde in a freshwater fish *Anabus Testudialdehyde* (Bloch) exposed to sewage. *J. Aquat. Biol. Fish*., **1**: 68-76.

Sudha, S. and Lall, D., 2013. Biochemical alterations of antioxidant enzymes in fishes of Bisalpur reservoir. *Ind. J. Fund. appl. Life Sci*., **3**: 128-132.

The'venod, F., 2009. Cadmium and cellular signaling cascades: to be or not to be. *Toxicol. appl. Pharm*., **238**: 221-239.

Yaqub, S. and Javed, M., 2012. Acute toxicity of water-borne and dietary cadmium and cobalt for fish. *Int. J. Agric. Biol*., **14**: 276-280.

Younis, E.M., Abdel-Warith, A.A., Al-Asgah, N.A., Ebaid, H. and Mubarak, M., 2013. Histological changes in the liver and intestine of Nile tilapia, *Oreochromis niloticus* exposed to sub-lethal concentrations of cadmium. *Pakistan J. Zool*., **45**: 833-841.

Zhang, D.L., Hu, C.X., Li, D.H. and Liu, Y.D., 2013. Zebrafish locomotor capacity and brain acetylcholinesterase activity is altered by Aphanizomenon flos-aquae DC-1aphantoxins. *Aquat. Toxicol.,* **15**:138–139,

Zikic, R.V., Stajn, A.S., Pavlovic, S.Z., Ognjanovic, B.I. and Saicic, Z.S., 2001. Activities of superoxide dismutase and catalase in erythrocytes and plasma transaminases of goldfish (*Carassius auratus gibelio)* exposed to cadmium. *Physiol. Res*., **50**: 105-111.

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Effect of Phytase Supplementation in Formulated Feed for *Pangasius hypophthalmus* to Increase the Use of Cotton Seed meal by Replacing Fish Meal

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Authors' Contribution
TY, NK and FR planned the research. TY, KJI, SD, MHR and HA wrote the article. SD, TY, AS and GI studied growth, proximate and physicochemical parameters. IAQ and KMA analysed the data statically.

Key words
Catfish (*Pangasius hypophthalmus*), Growth, Body composition, Phytase supplementation.

ABSTRACT

Present study was focused to observe the effect of phytase supplementation in formulated feeds on *Pangasius hypophthalmus* to increase the use of cotton seed meal by replacing fish meal. The trial was conducted in eight aquariums for 75 days. Each treatment was replicated twice and stocked 10 fish per aquarium with average weight (30g). Four iso-nitrogenous (45% CP) experimental diets were formulated by gradually replacement of fishmeal 0%, 10%, 20% and 30% with cotton seed meal (CSM) designated control, T1, T2 and T3, respectively. Statistically significant difference (P≤0.05) was observed in net weight gain of experimental fish among treatments. Higher net weight gain (32.55±0.77g) and highest length increase (76±0.35mm) was observed in T3 and lowest net weight gain and length increase was recorded in T1 (25.5±0.70g) and control (38±1.13 mm). The FCR values revealed non-significant differences (P ≥ 0.05) among treatments. Significantly higher SGR% was observed in T3 (1.0±0) compared to other treatments. Proximate analyses parameters were found significantly different (P ≤ 0.05) among treatments. It was concluded that 30% fish meal replaced with cotton seed meal (CSM) along with 750ftu^{-kg} phytase was found better combination for the growth and nutrient composition of *P. hypophthalmus*.

INTRODUCTION

Fish always preferred over red meat due to its higher protein contents, unsaturated fatty acid and small amount of cholesterol (Arts *et al*., 2001; Mozaffarian *et al*., 2003; Foran *et al*., 2005; Fawole *et al*., 2007). Catfish (Pangasius) is an important and fastest growing group of fish in the world aquaculture after tilapia and carps (Phan *et al*., 2009; Lakra and Singh, 2010). Among catfishes, *Pangasius hypophthalmus* is the most popular candidate fish due to its fast growth, easy culture, highly resistance to diseases, and fluctuation of environmental factors (Begum *et al*., 2012).

Aquaculture is a feed based industry and the preparation of balance fish feed depends on the availability of good quality feed ingredients. Worldwide fishmeal is used as a main component in aquaculture feed due to its palatability and high nutritional value (NRC, 2011). Fish meal (FM) is the major protein source in a special feed for fish because they are an excellent source of essential nutrients (Zhou *et al*., 2004). Due to high cost of fishmeal, increasing demand and shortage of supply, fish nutritionists making efforts to find out alternatives to substitute fish meal in fish feed (Pham *et al*., 2008; Lech and Reigh, 2012; Shapawi *et al*., 2013). Cottonseed meal (CM) due to its high protein content, availability and low cost has long been used in diets for both terrestrial animals as well as for fish (Barros *et al*., 2002; Pham *et al*., 2008; Colin-Negrete *et al*., 1996). It has been tested in several fish species such as rainbow trout, channel catfish, tilapia, largemouth bass and sunshine bass (Cheng and Hardy, 2002; Mabahinzireki

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et al., 2001; Rawles and Gatlin, 2000). Due to high crude protein level and economically viable, cotton seed meal is used in feeds. But due to some anti-nutritional factors especially phytic acid restricts its use for fish feeds. To address this issue phytase enzyme is used to hydrolyze phytic acid and enhance digestibility of cottonseed meal to increase fish growth (Baruah *et al*., 2004; Cao *et al*., 2007; Hussain *et al*., 2011). Keeping in view the importance of cotton seed meal, the present study was designed to study the effect of phytase supplementation in formulated feeds to increase the use of cotton seed meal by replacing fish meal and its impact on the growth of *Pangasius hypophthalmus*.

MATERIALS AND METHODS

Experiment site

The study was conducted at Research and Training Facilities, Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus Pattoki. The catfish (*Pangasius hypophthalmus*) fingerlings were collected from fish ponds situated at C-block Ravi Campus Pattoki.

Experimental setup

This trial was conducted in eight (8) aquariums for 75 days. Each treatment was replicated twice and stocked 10 fish per aquarium with average weight (30g). Four iso-nitrogenous (45%) crude protein (CP) experimental diets were formulated by gradually replacement of fishmeal 0%, 10%, 20% and 30% with cotton seed meal (CSM) and designated as control, T1, T2 and T3, respectively. The ingredients were finely grinded, well mixed in mechanical mixer and then molasses (600 ml) were added as binder before extrusion in local extruder to prepare desired size pellets (Table I).

Addition of phytase enzyme

Phytase solution was prepared by mixing 2g phytase with 1000 ml of distilled water and solution contained total 20,000 FTU of phytase. Feed pellets of 0 (control), T_1, T_2 and T_3 were sprayed with phytase solution at 0, 250, 500,750 FTU kg^{-1}, respectively and then dried feed was stored in air tight plastic bags and used subsequently (Table I).

Growth parameters

Fish morphometeric measurements (total body weight and length) were recorded at the time of initial stocking and then after fortnightly interval and fish were caught by using hand nets from aquarium. After taking measurements the fishes were transferred to their respective aquariums. The following parameters were used to evaluate the growth of fish:

$$\text{NWG} = \text{Final BW (g)} - \text{Initial BW (g)}$$

$$\text{SGR} = \frac{\text{lin}\left(\text{Final BW (g)}\right) - \text{lin}\left(\text{Initial BW (g)}\right)}{\text{Duration of exp.period (days)}} \times 100$$

$$\text{FCR} = \frac{\text{Feed intake (g)}}{\text{Weight gain (g)}}$$

Where, NWG is net weight gain, BW is body weight, SGR is specific growth rate and FCR is feed conversion ratio.

Proximate analysis fish

At the end of trial five (5) fish from each tank were caught for proximate analysis. The parameters like, dry matter, ash contents, % crude fat and crude protein of the feed and fish samples were carried out by following AOAC (2005).

Table I.- Inclusion level of different feed ingredients in experimental diet.

Ingredients	Proximate analyzed CP % of ingredients	Control		T1 (10% CSM)		T2 (20% CSM)		T3 (30% CSM)	
		Inclusion level	Contrib. of CP%	Inclusion level	Contrib. of CP%	Inclusion level	Contrib. of CP%	Inclusion level	Contrib. of CP%
Fish meal	62.5	60	37.5	50	31.25	42	26.25	36	22.5
Cotton seed meal	41.5	0	0	10	4.15	18	7.47	27	11.21
Maize gluten	29.5	14	4.13	26	7.67	36	10.62	36	10.62
Rice polish	12	14	1.68	2	0.24	2	0.24	2	0.24
Wheat bran	14	12	1.68	12	1.68	2	0.28	2	0.28
Total	-	100	44.99	100	44.99	100	44.86	100	44.84
Phytase level (FTU/kg)		0		250		500		750	

CSM, cotton seed meal.

Evaluation of the water quality parameters

Water quality parameters *viz*. temperature, dissolved oxygen (DO), pH, salinity, electrical conductivity (EC) and total dissolved solids (TDS) were measured on daily basis. All the parameters were determined by using digital meters (Mehboob *et al*., 2017).

Statistical analysis

The data obtained was analyzed by using SAS 9.1 version statistical software. The data on different variables was statistically analyzed by using Analysis of Variance (ANOVA) technique under Duncan's Multiple Range Test for comparison of means.

RESULTS

Fish growth studies

There was a non-significant difference observed in the initial weights of fish among treatments while in final weight a significant difference (P≤0.05) observed among treatments (Table II). Significantly (P≤0.05) highest final weight was observed in T3 (62.55±0.77 g) and lowest in T1 (55.0±0.70 g). Statistically significant difference (P≤0.05) was observed in the net weight gain among treatments and control. Highest net weight gain was observed in T3 (32.55±0.77 g) and lowest in T1 (25.5±0.70 g) (Table II).

The results regarding initial and final length revealed significant differences (P≤0.05) among treatments and control while non-significant variations were recorded in the increase in length among treatments and control groups. The highest increase in length was observed in T3 (76±0.35 mm) and lowest in control (38±1.13 mm) (Table II).

The FCR values for various treatments and control group were observed as control (2.20±0.14), T1 (2.15±0.07), T2 (2.2±0.14) and T3 (2.10±0) whereas the SGR% values recorded in control (0.90±0), T1 (0.85±0.07), T2 (0.9±0) and T3 (1±0), respectively. The statistical analysis of FCR values revealed non-significant differences (P ≥ 0.05) among treatments and control. The statistically higher SGR% value was recorded in T3 (1.0±0) compared to T1 (Table II).

Proximate composition

The fish were analyzed for its proximate composition at the start and at the end of the experiment. The results of proximate analysis revealed significant differences (P ≤ 0.05) among treatments. There was a significant differences observed for dry matter where T2 and T3 showed significantly higher dry matter percentage than pre-trial, control and T1. The crude protein contents showed similar pattern as dry matter. A non-significant difference was observed among all the treatments except pre-trial that showed significantly lower values than post-trial treatment groups (Table III). Crude fat contents were significantly higher in pre-treated group followed by T3 and control, respectively (Table III).

Table II.- Comparison of growth performance of *Pangasius hypophthalmus* among different treatments.

Parameters	Control	T1 (10% CSM)	T2 (20% CSM)	T3(30 % CSM)
Initial weight (g)	30.0±0.5[a]	30.5±0[a]	30±0[a]	30±0[a]
Final weight (g)	59.0±1.41[b]	55.0±0.70[c]	58.9±0.56[b]	62.5±0.77[a]
Net wt gain (g)	29.0±1.14[b]	25.5±0.70[c]	28.9±0.56[b]	32.5±0.77[a]
Initial length (mm)	150±0[d]	192±0[b]	214±0[a]	191±0[c]
Final length (mm)	188±1.13[b]	239.5±2.70[a]	2575.±1.60[a]	267.5±0.35[a]
Inc. in length (mm)	38±1.13[a]	46±2.96[a]	43±1.06[a]	76±0.35[a]
FCR	2.20±0.14[a]	2.15±0.07[a]	2.2±0.14[a]	2.10±0[a]
SGR%	0.90±0[a]	0.85±0.07[b]	0.9±0[ab]	1.0±0[a]

*Figures with same letters are not significantly different

Table III.- Proximate composition of experimental fish under various treatments.

Parameters	Pre treatment	Control	T1	T2	T3
Dry matter (%)	82.95±1.41[c]	98.00±0.71[a]	92.76±0.37[b]	93.49±0.69[b]	93.04±0.06[b]
Crude protein (%)	41.85±0.92[c]	45.21±0.30[b]	44.05±0.09[b]	49.23±0.33[a]	49.94±0.06[a]
Ash (%)	19.00±1.41[b]	24.25±0.35[a]	24.75±0.35[a]	25.75±0.35[a]	24.25±0.35[a]
Crude fat (%)	15.50±0.71[a]	9.70± 0.14[c]	13.20±0.28[b]	8.60± 0.28[d]	10.65±0.35[c]

*Figures with same letters are not significantly different.

Table IV.- Physico-chemical parameters in various treatments.

Parameters	Control	T1	T2	T3
DO (mg/l)	5.35±0.07[a]	5.35 ± 0.07[a]	5.35 ± 0.07[a]	5.35 ± 0.071[a]
Temp (°C)	21.65±0.07[a]	21.50 ±0.28[a]	21.80 ± 0.14[a]	21.45 ± 0.21[a]
Salinity(ppt)	1.00± 0[a]	1.00 ± 0[a]	1.00 ± 0[a]	1.00 ±0[a]
EC (µS/cm)	1704±1.41[a]	1704.50±3.54[a]	1703.50±2.12[a]	1706.00±1.41[a]
TDS(mg/l)	654± 1.41[a]	656.50± 0.71[a]	654.50±3.54[a]	655.50 ±0.71[a]
pH	8.00±0[a]	7.750±0.07[a]	7.90 ± 0.14[a]	7.80 ±0.14[a]

*Figures with same letters are not significantly different.

Physico-chemical parameters of water quality

The results obtained regarding water quality parameters during the study period are presented in Table IV. All the physico-chemical parameters showed statically non-significant differences among all treatments.

DISCUSSION

During present study significant differences ($P \leq 0.05$) were observed in the net weight gain among treatments and control. The highest net weight gain was observed in T3 (32.55±0.77) and lowest was in T1 (25.5±0.70). In T3 where cotton seed meal (CSM) was added upto 30% in the replacement of fish meal (FM) along with 750FTU-kg^{-1} phytase supplementation for *P. hypophthalmus* that enhanced the feed intake and significantly increased fish growth. The reason might be the addition of phytase that released phosphate from their phytin binders, thus making phosphorus availability to the fish and also increase the digestibility of protein as reported by Liebert and Partz (2007). Our results are also in line with Hussain *et al.* (2015), who reported that the phytase supplementation to cottonseed meal based diet at 750 FTU kg^{-1} level is optimum to release adequate chelated minerals for maximum growth performance. According to Sejin and Kyeongjun (2009), CSM could replace up to 30% FM for larger size of parrot fish (*Oplegnathus fasciatus*) and the supplementation of iron and phytase do not significantly affect the growth performance of fish. Similarly, Shah *et al.* (2016) reported that phytase supplementation in diet significantly ($p<0.05$) improve the growth of *L. rohita* either supplemented individually or mutually with citric acid. Debnath *et al.* (2005) testified different phytase levels in isonitrogenous diet (35.67% crude protein) on *P. pangasius* fingerlings and observed optimum growth performance with inclusion of 500 FTU kg^{-1}of phytase.

During present study, proximate composition of fish whole body revealed significant differences among different treatment and control groups. Similarly, Shah *et al.* (2016) also reported that phytase supplemented diet significantly ($p<0.05$) affect the proximate composition of *L. rohita* either supplemented individually and mutually with citric acid. Contradictory to our results, Yang (2011) reported non-significant difference in proximate composition of rainbow trout (*Oncorhynchus mykiss*) by the replacement of fish meal with phytase-treated SBM. Crude protein concentration was found significantly ($P \leq 0.05$) higher in T3 than T1, T2 and control during current study. Our results are also supported by Pham *et al.* (2008) who reported that supplementation of microbial phytase could improve the apparent digestibility of protein and phosphorus in juvenile olive flounder fed the CS-containing diets. According to Nwannaa *et al.* (2008), there were no significant differences in protein concentrations of the fish whole body but fish fed diet containing phytase had slightly more body protein than others.

Crude lipid contents were found significantly ($P \leq 0.05$) higher in control group as compare to other treatments. The results revealed that with the increased level of cotton seed meal along with phytase the protein contents also increased significantly. Crude lipid and ash contents followed opposite trend with respect to crude protein. Nwannaa *et al.* (2008) reported that lipid contents in all the fish showed non-significant differences but fish fed diets without phytase had the highest lipid concentration. The ash contents in fish body significantly increases with the phytase supplementation in diet than without phytase diet (Nwannaa *et al.*, 2008).

In conclusion phytase supplementation upto 750FTU-kg^{-1} can successfully replace 30% cotton seed meal (CSM) in the replacement of fish meal for *Pangaisus hopophthalmus* diets.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

AOAC, 2005. *Official method of analysis*, 15th edn. Association of Official Analytical Chemists, Washington, DC, USA.

Arts, M.T., Ackman, R.G. and Holub, B.J., 2001. Essential fatty acids in aquatic ecosystems: a crucial link between diet and human health and evolution. *Can. J. Fish. aquat. Sci.*, **58**: 122-137. https://doi.org/10.1139/f00-224

Barros, M.M., Lim, C. and Klesius, P.H., 2002. Effect of soybean meal replacement by cottonseed meal and iron supplementation on growth, immune response, and disease resistance of channel catfish (*Ictalurus punctatus*) to *Edwardsiella ictaluri* challenge. *Aquaculture,* **207**: 263-279. https://doi.org/10.1016/S0044-8486(01)00740-2

Baruah, K., Pal, A.K., Sahu, N.P. and Debnath, D., 2007. Microbial phytase supplementation in rohu, *Labeo rohita*, diet enhances growth performance and nutrient digestibility. *J. World aquat. Soc.,* **38**: 129-137.

Baruah, K., Sahu, N.P., Pal, A.K. and Debnath, D., 2004. Dietary phytase: An ideal approach for cost effective and low-polluting aqua-feed. *NAGA, World Fish Cent.,* **27**: 15-19.

Begum, M., Akhter, T. and Minar, M.H., 2012. Analysis of the proximate composition of domesticated pangus (*Pangasius hypophthalamus*) in laboratory condition. *J. environ. Sci. Nat. Resour.,* **5**: 69-74.

Cao, L., Weimin, W., Yanga, C., Yangb, Y., Dianac, J., Yakupitiyageb, A., Luoa, Z. and Lia, D., 2007. Application of microbial phytase in fish feed. *Enzyme Microb. Technol.*, **40**: 497-507. https://doi.org/10.1016/j.enzmictec.2007.01.007

Cheng, Z.J. and Hardy, R.W., 2002. Effect of microbial phytase on apparent nutrient digestibility of barley, canola meal, wheat and wheat middlings, measured *in vivo* using rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr.,* **8**: 271-277. https://doi.org/10.1046/j.1365-2095.2002.00219.x

Colin-Negrete, J., Kiesling, H.E., Ross, T.T. and Smith, J.F., 1996. Effect of whole cottonseed on Serum constituents, fragility of erythrocyte cells and reproduction of growing *Holstein heifers*. *J. Dairy Sci.,* **79**: 2016-2023. https://doi.org/10.3168/jds.S0022-0302(96)76574-8

Debnath, D., Pal, A.K., Narottam, P.S., Jain, K.K., Yengkokpam, S. and Mukherjee, S.C., 2005. Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. *Aquacult. Res*., **36**: 180-187. https://doi.org/10.1111/j.1365-2109.2004.01203.x

Fawole, O.O., Ogundiran, M.A., Ayandiran, T.A. and Olagunju, O.F. 2007. Mineral composition in some selected fresh water fishes in Nigeria. *J. Fd. Safe.*, **9**: 52-55.

Foran, J.A., Carpenter, D.O., Hamilton, M.C., Knuth, B.A. and Schwager, S.J., 2005. Risk- based consumption advice for farmed Atlantic and wild pacific salmon contaminated with dioxins and dioxinlike compounds. *Environ. Hlth. Persp.,* **33**: 552-556. https://doi.org/10.1289/ehp.7626

Hussain, S.M., Afzal, M., Rana, S.A., Javid, A. and Iqbal, M., 2011. Effect of phytase supplementation on growth performance and nutrient digestibility of *Labeo rohita* fingerlings fed on corn gluten meal based diets*. Int. J. Agric. Biol.,* **13**: 916-922.

Hussain, S.M., Ahmad, N., Jabeen, F., Javid, A., Aslam, N., Hussain, M., Ahmad, S., Arsalan, M.Z.H., Riaz, D. and shahzad, M.M., 2015. Effect of citric acid and phytase supplementation on nutrients digestibility of *Cirrhinus mrigala* fingerlings fed on corn gluten (30%) meal based diets. *Int. J. Biosci.,* **6**: 82-91. https://doi.org/10.12692/ijb/6.7.82-91

Lakra, W.S. and Singh, A.K., 2010. Risk analysis and sustainability of *Pangasianodon hypophthalmus* culture in India. *Aquacult. Asia Mag.*, **15**: 35-40.

Lech, P.L. and Reigh, R.C., 2012. Plant products affect growth and digestive efficiency of cultured Florida pompano (*Trachinotus carolinus*) fed compounded diets. *PLoS One*, **7**: 1-11. https://doi.org/10.1371/journal.pone.0034981

Liebert, F. and Partz, L., 2007. Different sources of microbial phytase in plant based low phosphorus diet for Nile tilapia may provide different effect on phytate degradation. *Aquaculture*, **267**: 292-299. https://doi.org/10.1016/j.aquaculture.2007.01.023

Mehboob, A., Khan, N., Atiq, U., Iqbal, K.J., Tayyab, R., Batool, S.S., Batool, H.S., Amjad, S. and Tanveer, M., 2017. Effect of fenugreek as a feed additive on the growth, body composition and apparent nutrients digestibility of striped catfish *Pangasius hypophthalmus* fry. *Pakistan J. Zool.,* **49**: 2037-2042. http://dx.doi.org/10.17582/journal.pjz/2017.49.6.2037.2042

Mabahinzireki, G.B., Dabrowski, K., Lee, K.J., El-Saidy, D. and Wisner, E.R., 2001. Growth, fed utilization and body composition of tilapia (*Oreochromis* sp.) fed with cottonseed meal-based diets in a

recirculating system. *Aquacult. Nutr.*, **7**: 189-200. https://doi.org/10.1046/j.1365-2095.2001.00172.x

Mozaffarian, M.D., Rozenn, N.L., Lewis, H.K., Gregory, L.B., Russell, P.T. and Davis, S.S., 2003. Cardiac benefits of fish consumption may depend on type of fish meal consumed. *Circulation,* **107**: 1372-1382. https://doi.org/10.1161/01.CIR.0000055315.79177.16

NRC, 2011. *Nutrient requirements of fish and shrimp*. National Academies Press, National Research Council, Washington, DC, USA, pp. 376.

Nwannaa, L.C., Oishib, C.A. and Pereira-Filhob, M., 2008. Use of phytase to improve the digestibility of alternative feed ingredients by Amazon tambaqui, *Colossoma macropomum*. *Sci. Asia,* **34**: 353-360. https://doi.org/10.2306/scienceasia1513-1874.2008.34.353

Pham, M.A., Lee, K.J., Dang, T.M., Lim, S.J., Ko, G.Y., Eo, J. and Oh, D.H., 2008. Improved apparent digestibility coefficient of protein and phosphorus by supplementation of microbial phytase in diets containing cottonseed and soybean meal for juvenile olive flounder (*Paralichthys olivaceus*). *Asian-Austral. J. Anim. Sci.*, **21**: 1367-1375. https://doi.org/10.5713/ajas.2008.80053

Phan, L.T., Bui, T.M., Nguyen, T.T.T., Gooley, G.J., Ingram, B.A. and Nguyen, H.V., 2009. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. *Aquaculture,* **296**: 227-236. https://doi.org/10.1016/j.aquaculture.2009.08.017

Rawles, S.D. and Gatlin, D.M., 2000. Nutrient digestibility of common feedstuffs in extruded diets for sunshine bass, *Morone chrysops* female × *M. sasatilis* male. *J. World Aquacult. Soc.,* **31**: 570-579. https://doi.org/10.1111/j.1749-7345.2000.tb00906.x

Sejin, L. and Kyeongjun, L., 2009. Partial replacement of fish meal by cottonseed meal and soybean meal with iron and phytase supplementation for parrot fish *Oplegnathus fasciatus*. *Aquaculture,* **290**: 283-289. https://doi.org/10.1016/j.aquaculture.2009.02.018

Shah, S.Z.H., Afzal, M., Akmal, A., Fatima, M. and Hussain, S.M., 2016. Effect of citric acid and phytase on growth performance and mineralization of *Labeo rohita* juveniles fed soybean meal based diet. *Int. J. Agric. Biol.,* **18**: 111-116. https://doi.org/10.17957/IJAB/15.0071

Shapawi, R., Ebi, I. and Yong, A., 2013. Soybean meal as a source of protein in formulated diets for tiger grouper, *Epinephelus fuscoguttatus* juvenile. Part I: Effects on growth, survival, feed utilization and body compositions. *Agric. Sci.,* **4**: 317-323. https://doi.org/10.4236/as.2013.47045

Yang, Y.H., Wang, Y.Y., Lu, Y. and Li, Q.Z., 2011. Effect of replacing fish meal with soybean meal on growth, feed utilization and nitrogen and phosphorus excretion on rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Int.,* **19**: 405-419. https://doi.org/10.1007/s10499-010-9359-y

Zhou, Q.C., Tan, B.P., Mai, K.S. and Liu, Y.J., 2004. Apparent digestibility of selected feed ingredients for juvenile cobia *Rachycentron canadum*. *Aquaculture,* **241**: 441-451. https://doi.org/10.1016/j.aquaculture.2004.08.044

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Changes in Glutathione S-Transferase Activity in Fish *Channa striata* Exposed to Different Aquatic Pollutants (Heavy Metals and Pesticides Mixture)

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Authors' Contribution
SA executed the experimental work. KA and HN helped in experimental work. WH statistically analyzed the data. AM helped in preparing the manuscript.

Key words
Heavy metals mixture, Pesticides mixture, Acute toxicity, Antioxidant enzyme.

ABSTRACT

The widespread use of chemicals like heavy metals and pesticides has resulted in the pollution of many aquatic habitats worldwide. Therefore, assessment of toxicity result from these chemicals is necessary to conserve the aquatic life in natural habitat. Present research was performed to calculate the 96-h LC_{50} and lethal concentrations of heavy metals (lead+nikel) and pesticides (endosulfan+deltamethrin) mixture for the fish, *Channa striata*. Acute toxic effects of metals and pesticides mixture on glutathione S-transferase (GST) activity, specific activity and total protein contents in gills, liver, kidney and muscle of fish was also evaluated. Fish fingerlings were collected from natural breeding sites and acclimatized to the laboratory conditions. Fingerlings were exposed to the different concentrations of metals (lead+nickel) and pesticides (endosulfan+deltamethrin) mixture to determine the 96-h LC_{50} and lethal concentrations. The 96-h LC_{50} and lethal concentration of lead+nikel mixture was calculated as 52.147±3.069 and105.446±8.382 mgL^{-1} while for endosulfan+deltamethrin mxture was as1.374±0.1007 and 2.957±0.278 μgL^{-1}, respectively. Lower value of pesticides mixture showed that this mixture was more toxic than metals mixture. The exposure of metals and pesticide mixture significantly increased the GST activity, specific activity and protein contents in gills, liver, kidney and muscle of experimental groups when compare with control.

INTRODUCTION

Environmental stressors and related risks have constantly a natural part of our society (Adeyemo *et al*., 2008). In different parts of the world the discharge of anthropogenic pollutants has resulted in ecotoxicological effects (Ramesh *et al*., 2009). Among contaminants, metals and pesticides are of great concern due to their stability (metals) and high toxicity (pesticides), these chemicals may have adverse effects on aquatic ecosystems (Forget *et al*., 1999). The main sources of pollution are advanced agriculture, industry, mining, motor traffic and household wastes. These contaminants can gather in fish and other aquatic organisms, also stick with the sediments and persist in water (Luoma and Rainbow, 2008). Environmental pollutants cause a severe effect on many aquatic organisms by disturbing genetic, behavioral, biochemical, and physiological parameters (Scott *et al*., 2003). Aquatic ecosystems are particularly susceptible to contact with toxic contaminants. In the aquatic environments, fish is most vulnerable to these essential contaminants and more susceptible to the metal contaminants than other organisms which live in aquatic environment (Alinnor, 2005). These toxicants have ability to produce reactive oxygen species through numerous mechanisms, such as interference of reactive intermediates with electron transport, inhibition of antioxidative enzymes, and reduction of antioxidants (Maran *et al*., 2009). Animals had endogenous enzymatic and non-enzymatic antioxidants defence system to convert the reactive oxygen species into harmless metabolites as well as to defend and repair normal cellular metabolism and functions (Bebe and Panemanga-lore, 2003). Glutathione-S-transferase (GST) enzymes can be used as biochemical marker for inorganic and organic (pesticides) contaminant yielding oxidative stress (Yang *et al*., 2001). Glutathione-S-transferases (GST) are a family of enzymes plays an important role in detoxification of many xenobitics (Thom *et al*., 2001) by catalyzing the conjugation of several xenobiotics with Glutathione (GSH) (Edwards *et al*., 2000). According to Edwards *et al*. (2000) these enzymes also protect the lipids from peroxidation by scavenging reactive oxygen

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species in the cells. The growing amount of pollutants in the environment for example heavy metals, detergents, organophosphorus and carbamic pesticides require quick and perceptive analytical techniques (Snejdarkova *et al.*, 2004). Therefore, assessment of antioxidant enzymes are provided useful information related to environmental stress before facing harmful effects in fish, and are significant parameters for evaluating water quality for the occurrence of contaminants (Geoffroy *et al.*, 2004). The present research work was designed to evaluate the changes in antioxidant enzyme (GST) activity in fish, *Channa striata* exposed to different aquatic pollutants (heavy metals and pesticides mixtures).

MATERIALS AND METHODS

Determination of LC_{50}

The acute toxicity tests in term of 96-h LC_{50} and lethal concentration were conducted in the wet laboratory at Fisheries Research Farms, University of Agriculture, Faisalabad, Pakistan.*Channa striata* fingerlings were acclimatized to the laboratory conditions in the cemented tanks for two weeks. During acclimatization fish were fed with commercial feed at 3% wet body weight. Technical grade $NiCl_2$ and $PbCl_2$ were separately dissolved in deionized water for stock solutions preparation while binary mixture of both metals was prepared by its further dilution on ions equivalence basis (1:1 ratio). Stock solutions of pesticides *viz*. endosulfan (END) and deltamethrin (DM) were made in methanol while pesticides mixture (END+DM) of required concentration were made by further dilutions in the deionized water. The acute toxicity tests for LC_{50} value were replicated three times. Glass aquaria of water capacity 70 liters was used having ten fish in each with three replicates for a period of 4 days. Different concentrations of heavy metals (lead+nickel) mixture (0, 7, 14, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84 and 91 mgL^{-1}) and pesticides (endosulfan+deltamethrin) mixture (0, 0.25, 0.5, 0.75, 1, 1.25, 1.5, 1.75, 2, 2.25 and 2.5 μgL^{-1}) were used for the determination of 96-h LC_{50} and lethal concentrations. During acute toxicity tests data recorded on fish mortality were analyzed by using Probit analysis method. Mortality was recorded after 12-h interval and dead fish were removed immediately.

Physico-chemical analysis of water

During acute toxicity tests, physico-chemical parameters were monitored on daily basis while water temperature, pH, total hardness and DO were maintained throughout the study period at 30°C, 7.5, 250 mgL^{-1} and 5ppm, respectively. However, total ammonia, hardness, calcium, magnesium and carbondioxide were measured by following the method of APHA (1998).

Glutathione-S-transferase (GST) activity

During acute toxicity test GST activity in liver, gills, kidney and muscles of fish *C. striata* exposed to metals and pesticides mixture was also evaluated. Along with this, control fish were kept in metal and pesticide free medium.

To isolate GST from the liver, gills, kidney and muscle of *C. striata*. All the dissected organs were weighted. For removal of RBCs, the dissected organs were rinsed with 50mM Tris HCl buffer of pH 7.4 and containing 0.2 M sucrose 4 times greater than the weight of organ *i.e.* 1:4 and homogenized for 15 min in cold buffer (1:4 w/v) using a pestle and morter. After the homogenization, organ homogenates were centrifuged for 15 min at 10,000 rpm and 4°C. After centrifugation process, clear supernatants were stored at 80°C for enzyme assay while residues were discarded.

Activity of GST was measured by following the method of Habig and Jakoby (1981) at 340nm against the reagent blank on spectrophotometer after interval of 1-min.

To estimate total protein content of samples Biuret method (Gornall *et al.*, 1994) was used.

Specific activity of an enzyme was measured by using the following formula:

$$\text{Specific activity} = \frac{\text{Activity of Sample}}{\text{Protein contents of Sample}}$$

Table I.- 96-h LC_{50} and lethal concentrations of metals/pesticides mixture for *Channa striata*.

Treatments	Mixture ratio	LC_{50} (μgL^{-1})	95% C.I. (LCL-UCL)	Lethal conc. (μgL^{-1})	95% C.I. (LCL-UCL)	Pearson Goodness of Fit Tests			Regression equation (Y=a+bx)
						χ^2	df	p-value	
Pb+Ni	1:1	52.14±3.07a	46.00-58.54	105.45±8.38a	92.35-127.80	4.30	12	0.997	Y= -2.27607+0.0436471**x (0.0062129)
END+DM	1:1	1.37±0.10ab	1.17-1.59	2.96±0.28b	2.53-3.73	0.68	9	0.988	Y= -2.01916+1.46917**x (0.233623)

Means with the same letters (small) in a single column are statistically similar at p<0.05. Pb, lead; Ni, nickel; END, endosulfan; DM, deltamethrin; C.I., confidence interval (μgL^{-1}); LCL, lower confidence limit (μgL^{-1}); UCL, upper confidence limit (μgL^{-1}); Lethal Conc., lethal concentrations (μgL^{-1}); χ^2, Chi-Square; df, degree of freedom; y, dependent variable; x, independent variable; value within bracket is the standard error; **, significant at p<0.01.

Statistical analyses

The experiment was performed with three replicates. The fish mortality data recorded during 96-h acute toxicity tests were analyzed through Probit Analysis method (Hamilton *et al*., 1977) at 95% confidence interval. Two-way ANOVA was used to compare variables among fish. A p-value less than 0.05 were considered as statistically significant.

RESULTS

LC_{50}

During acute toxicity test mortality data for *C. striata* exposed to heavy metals/pesticides mixture were analysed through Probit analysis method. Table I showed the acute toxicity data with mean 96-h LC_{50} and lethal concentration values, concentration of metals/pesticides in mixture, 95% lower and upper confidence interval limits with their calculated chi-square values. The Deviance Chi-Square values of metals and pesticides mixture for *C. striata* were calculated as 4.303 and 0.681, respectively. The p-value of metals and pesticides mixture for *C. striata* was computed as 0.997 and 0.988, respectively. For heavy metals (Pb+Ni) and pesticides (EDS+DM) mixture 96-h LC_{50} values were computed as 52.147±3.069 mgL^{-1} and 1.374±0.1007 μgL^{-1}, respectively. The 96-h lethal concentration values of metals (Pb+Ni) and pesticides (EDS+DM) mixture were computed as 105.446±8.382 mgL^{-1} and 2.957±0.278 μgL^{-1}, respectively. The lower 96-h LC_{50} and lethal concentration value of pesticides mixture showed that the pesticides mixture is more toxic than heavy metal mixture.

Table II.- Effect of heavy metals/pesticides mixture on GST activity (U/mL) in different organs of *C. striata*.

Organs	Control	Treatments	
		Pb+Ni	EDS+DM
Liver	40.53±0.35Ac	45.57±0.21Ab	48.67±1.66Aa
Kidney	26.83±0.31Cc	30.63±0.31Cb	38.57±0.35Ca
Gills	30.57±0.35Bc	35.70±0.36Bb	40.87±0.25Ba
Muscle	23.52±0.35Dc	28.77±0.15Db	29.87±0.35Da

Means with similar letters in a single row are statistically similar at $p<0.05$.

GST activity

In present study GST activity in different organs of fish, *C. striata* exposed to metals/pesticides mixture was measured. The GST activity was increased significantly in all selected organs of metals and pesticides mixture exposed fish as compared to control.

During 96-h exposure period, LC_{50} represents the concentration of both treatments at which 50% fish die while lethal concentration represents the concentration at which 100% fish population die.

Among all selected organs liver showed significantly maximum GST activity followed by the order: gills>kidney>muscle. Comparison between treatments showed that the pesticides mixture had more pronounced effect on GST activity in comparison of metals.

Total protein contents

Biuret method was used to estimate the total protein contents in all the selected organs of fish *C. striata* under control, metals and pesticides mixtures. Table III showed the total protein contents were increased in all observed organs of treated fish as compared to control. However, maximum total protein contents were observed in muscle of fish. Results showed that the protein contents were higher (1.24±0.14) in pesticides mixture exposed fish as compared to metals mixture (1.12±0.09).

The specific activity of GST was significantly accelerated in liver, gills, kidney and muscle of treated fish. The overall mean specific activity value was significantly maximum in liver of fish. Statistically higher specific activity of GST was showed by pesticides mixtures treated fish (Table IV).

Table III.- Effect of heavy metals/pesticides mixture on total protein contents (mg/mL) in different organs of *Channa striata*.

Organ	Control	Treatments	
		Pb+Ni	EDS+DM
Liver	1.12±0.02c	1.19±0.09b	1.20±0.14a
Kidney	1.09±0.03c	1.13±0.03b	1.17±0.03a
Gills	1.02±0.02c	1.09±0.02b	1.13±0.03a
Muscles	1.23±0.03c	1.40±0.02b	1.44±0.02a

Means with similar letters in a single row are statistically similar at $p<0.05$.

Table IV.- Effect of heavy metals/pesticides mixture on specific activity (U/mg) of GST in different organs of *Channa striata*.

Organs	Control	Treatments	
		Pb+Ni	EDS+DM
Liver	36.00±1.73c	38.29±1.73b	40.55±2.08a
Kidney	24.61±1.19c	27.11±1.12b	31.97±1.52a
Gills	29.97±1.96c	33.06±1.02b	36.17±1.71a
Muscles	19.12±0.95c	20.74±0.57b	20.74±0.16a

Means with similar letters in a single row are statistically similar at $p<0.05$.

DISCUSSION

Aquatic pollution by heavy metals is a major issue due to its harmful effects on organism. Like heavy metals (organic pollutants), inorganic compounds such as pesticides enter directly or indirectly into the water, are coming from rising agricultural and industrial activities that generate anthropogenic inputs (Baugartem and Niencheski, 1998). Intensive anthropogenic activities cause the accumulation of different xenobiotics whose final destination is predominantly the aquatic environment. Many environmental pollutants can amass in the organs of aquatic organisms and cause toxic effects that are associated to oxidative stress (Winston and Di-Giulio, 1991). Studies of the stress response related to oxidative stress in aquatic organisms have been considered as an important source of information that could be used as tools for assessing the quality of the environment (Valavanidis *et al*., 2006).

Therefore, in this work effect of heavy metals and pesticides mixture on GST activity in different organs of fish was studied. The results of present research work showed that exposure of heavy metals (Pb+Ni) and pesticides (EDS+DM) mixture increased the GST activity and specific activity. The highest GST activity and protein contents were observed in the organs of pesticides mixture treated fish as compared to metals mixture treated fish. Present results are supported by Costin *et al*. (2007) who observed the increased GST activity in gills of *Carassius auratus gibelio* exposed to deltamethrin for 7 days. The increase in antioxidant system may consider as an adaptation of organism against xenobiotics (Doyotte *et al*., 1997). Lamoureus and Rueness (1987) reported that the GST plays an important role in detoxification of many xenobiotic and protect the cells against toxicants, neutralizing them and rendering the product more water soluble. They act by rapidly metabolizing the pesticides to less toxic metabolites, or by quickly binding and very slowly turning over the pesticides (Kostaropoulos *et al*., 2001). The increased level of GST in tissue samples of fish from metals polluted sites. Whereas, the decreased levels of all enzymes were recorded in less polluted sites. According to Carvalho *et al*. (2012) GST activity was elevated in gills and liver of *O. niloticus* sampled from metals polluted Monjolinho River in comparison to reference site (non-polluted).

Stimulation of GSTs is known to show the existence of different xenobiotics like polycyclic aromatic hydrocarbons (PAHs) (Ahmad *et al*., 2005), pesticides (Printes *et al*., 2011) and $HgCl_2$ (Monteiro *et al*., 2010). Our results are also supported by Bouraoui *et al*. (2008) who reported the increased GST activity in liver of *Sparus aurata* exposed to $CdCl_2$. The increased level of GST in different organs after exposure to inducer depends upon the nature of inducer and type of tissue (Ahmad *et al*., 2005; Maria *et al*., 2009; Monteiro *et al*., 2010; Oliveira *et al*., 2010; Printes *et al*., 2011).

In present research work exposure of heavy metals (Pb+Ni) and pesticides (EDS+DM) mixture increased the protein contents in organs of fish. In a previous study increased level of protein and detoxifying enzyme was observed under stress condition (Sreejai and Jaya, 2010; Hossain *et al*., 2012). These finding are also supported by Padmini and Geetha (2007) who reported the increased level of oxidized protein in flounders captured from contaminated waters with xenobiotics. Liver showed the highest GST activity under exposure of metals and pesticides mixtures. In present research, the increased glutathione-S-transferase in fish organs may be due to its role in detoxification of toxicants. The role of the liver in antioxidant enzyme response as a result of its higher sensitivity to metals as compared to the gills, kidney and muscle has been elucidated by several investigations as the liver has to overcome the oxidative stress than the other organs because of the elevated antioxidant enzyme activities (Basha and Rani, 2003; Anushia *et al*., 2012). Liver of vertebrates exhibits a high metabolism and oxygen consumption and it is the key organ for the detoxification of xenobiotic. It is a predominantly rich source of GST (Nimmo, 1987).

CONCLUSION

The present research concluded that the metals/ pesticides mixture can induced the GST activity in various organs of fish *C. striata*. It was also concluded that the highest activity of GST in liver may be due its role in detoxification of toxicants. The GST activity can be used as a biomarker of aquatic pollution with toxic substances.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Adeyemo, O.K., Ajani, F., Adedeji, O.B. and Ajiboye, O.O., 2008. Acute toxicity and blood profile of adult *Clarias gariepinus* exposed to lead nitrate. *Int. J. Hematol*., **4**: 91-94.

Ahmad, I., Oliveira, M., Pacheco, M. and Santos, M.A., 2005. *Anguilla anguilla* L. oxidative stress biomarkers responses to copper exposure with or without bnaphthoflavone pre-exposure. *Chemosphere*, **61**: 267-275. https://doi.

org/10.1016/j.chemosphere.2005.01.069

Alinnor, I., 2005. Assessment of elemental contaminants in water and fish samples from Aba River. *Environ. Monit. Assess*., **102**: 15-25. https://doi.org/10.1007/s10661-005-1011-3

Anushia, C., Kumar, P.S. and Karthikeyan, P., 2012. Biochemical changes induced in *Tilapia mossambicus* during copper and cadmium acute intoxication. *Int. J. Inst. Pharm. Life Sci*., **2**: 38-52.

APHA, 1998. *Standard method for examination of water and waste water,* 20th ed. American Public Health Association, New York, pp. 1193.

Basha, P.S. and Rani, A.U., 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). *Ecotoxicol. environ. Safe*., **56**: 218-221. https://doi.org/10.1016/S0147-6513(03)00028-9

Baugarten, M.G.Z. and Niencheski, L.F., 1998. *Avaliacao da qualidade hidroquimica da area portuaria da cicade de rio grande RS Documentos Te´ cnicos 09*. Rio Grande, editora da FURG, pp. 5-66.

Bebe, F.N. and Panemangalore, M., 2003. Exposure to low doses of endosulfan and chlorpyrifos modifies endogenous antioxidants in tissue of rats. *J. environ. Sci. Hlth*., **38**: 349-363.

Bouraoui, Z., Banni, M., Ghedira, J., Clerandeau, H., Guerbej, H., Narbonne, J.F. and Bousseta, H., 2008. Acute effects of cadmium on liver phase I and II enzymes and metallothionein accumulation on sea bream *Sparus aurata*. *Fish Physiol. Biochem*., **34**: 201-207. https://doi.org/10.1007/s10695-007-9177-y

Carvalho, C.D.S., Bernusso, V.A., Selistre-de-Araujo, H.S., Espindola, E.L.G. and Fernandes, M.N., 2012. Biomarker responses as indication of contaminant effects in *Oreochromis niloticus*. *Chemosphere*, **89**: 60-69. https://doi.org/10.1016/j.chemosphere.2012.04.013

Costin, D., Staicu, A.C., Dinu, D., Huculeci, R., Costache, M. and Dinischiotu, A., 2007. Biochemical and histological effects of deltamethrin exposure on the gills of *carassius auratus gibelio* (*pisces cyprinidae*). *Lucrari Stiintifice: Zooteh. Biotehnol.,* **40**: 65-72.

Doyotte, A., Cossu, C., Jacquin, M.C., Babut, M. and Vasseur, P., 1997. Antioxidant enzymes, glutathione and lipid peroxidation as relevant biomarkers of experimental or field exposure in the gills and the digestive gland of the freshwater bivalve *Unio tumidus*. *Aquat. Toxicol*., **39**: 93-110. https://doi.org/10.1016/S0166-445X(97)00024-6

Edwards, R., Dixon-anal, D.P. and Walbot, V. 2000. Plant glutathione-S-transferase enzymes with multiple functions in sickness and in health. *Trends Pl. Sci*., **5**: 193-198. https://doi.org/10.1016/S1360-1385(00)01601-0

Forget, J., Pavillon, J., Beliaeff, B. and Bocquene, G., 1999. Joint action of pollutant combinations (pesticides and metals) on survival (LC_{50} values) and acetylcholinesterase activity of *Tigriopus brevicornis* (Copepoda, Harpacticoida). *Environ. Toxicol. Chem*., **18**: 912-918. https://doi.org/10.1002/etc.5620180514

Geoffroy, L., Frankart, C. and Eullaffroy, P., 2004. Comparison of different physiological parameter responsesin *Lemna minor* and *Scenedesmus obliquus* exposed to herbicide flumioxazin. *Environ. Poll*., **131**: 233-241. https://doi.org/10.1016/j.envpol.2004.02.021

Gornal, A.G., Bardwill, C.S. and David, M.M., 1994. Determination of serum proteins by means of biuret reaction. *J. biol. Chem*., **177**: 751-766.

Habig, W.H. and Jakoby, W.B., 1981. Assays for differentiation of glutathione-S-transferases. *Meth. Enzymol*., **77**: 398-405. https://doi.org/10.1016/S0076-6879(81)77053-8

Hamilton, M.A., Rusoo, R.C. and Thurstan, R.V., 1977. Trimmed spearman-karber method for estimation for medical lethal concentration in toxocity bioassay. *Environ. Sci. Toxicol*., **11**: 714-719.

Hossain, M.A., Piyatida, P., Da-Silva, J.A.T. and Fujita, M., 2012. Molecular mechanism of heavy metal toxicity and tolerance in plants: Central role of glutathione in detoxification of reactive oxygen species and methyl-glyoxal and in heavy metal chelation. *J. Bot*., **2012**: 872-875. https://doi.org/10.1155/2012/872875

Kostaropoulos, I., Papadopoulos, A.I., Metaxakis, A., Boukouvala, E. and Papadopoulou-Mourkidou, E., 2001. Glutathione S-transferase in the defence against pyrethroids in insects. Insect Biochem. *Mol. Biol.,* **31**: 313-339.

Kumar, N., Prabhu, P.A.J., Pal, A.K., Remya, S., Aklakur, M., Rana, R.S., Gupta, S., Raman, R.P. and Jadhao, S.B., 2011. Anti-oxidative and immuno-hematological status of Tilapia (*Oreochromis mossambicus*) during acute toxicity test of endosulfan. *Pestic. Biochem. Physiol*., **99**: 45-52. https://doi.org/10.1016/j.pestbp.2010.10.003

Lamoureus, G.L. and Rueness, D.G., 1987. Synergism of diazinon toxicity and inhibition of diazinon metabolism in the hose fly by Tridiphane: Inhibition of glutathione-S-transferase activity.

Pestic. Biochem. Physiol., **27**: 318-329. https://doi.org/10.1016/0048-3575(87)90061-7

Luoma, S.N. and Rainbow, P.S., 2008. *Sources and cycles of trace metals in metal contamination in aquatic environments: Science and lateral management*. Cambridge University Press, Cambridge, pp. 47-66.

Maran, E., Fernandez, M., Barbieri, P., Font, G. and Ruiz, M.J., 2009. Effects of four carbamate compounds on antioxidant parameters. *Ecotoxicol. environ. Safe*., **72**: 922-930. https://doi.org/10.1016/j.ecoenv.2008.01.018

Maria, V.L., Ahmad, I., Oliveira, M., Serafimb, A., Bebianno, M.J., Pacheco, M. and Santos, M.A., 2009. Wild juvenile *Dicentrarchus labrax* L. liver antioxidant and damage responses at Aveiro Lagoon, Portugal. *Ecotoxicol. environ. Safe*., **72**: 1861-1870. https://doi.org/10.1016/j.ecoenv.2009.06.001

Monteiro, D.A., Rantin, F.T. and Kalinin, A.L., 2010. Inorganic mercury exposure: Toxicological effects, oxidative stress biomarkers and bioaccumulation in the tropical freshwater fish matrinxa, *Brycon amazonicus* (Spix and Agassiz, 1829). *Ecotoxicology*, **19**: 105-123. https://doi.org/10.1007/s10646-009-0395-1

Nimmo, I.A., 1987. The glutathione-S-transferase of fish. *Fish Physiol. Biochem*., **3**: 163-172. https://doi.org/10.1007/BF02180277

Oliveira, M., Ahmad, I., Maria, V.L., Pacheco, M. and Santos, M.A., 2010. Antioxidant responses versus DNA damage and lipid peroxidation in golden grey mullet liver: A field study at Ria de Aveiro (Portugal). *Arch. Environ. Contam. Toxicol*., **59**: 454-463. https://doi.org/10.1007/s00244-010-9491-8

Padmini, E. and Geetha, B.V., 2007. A comparative seasonal pollution assessment study of Ennore estuary with respect to metal accumulation in the grey mullet, *Mugil cephalus*. *Oceanol. Hydrobiol. Stud*., **36**: 91-103. https://doi.org/10.2478/v10009-007-0031-4

Printes, L.B., Fernandes, M.N. and Espíndola, E.L.G., 2011. Laboratory measurements of biomarkers and individual performances in *Chironomus xanthus* to evaluate pesticide contamination of sediments in a river of southeastern Brazil. *Ecotoxicol. environ. Safe.,* **74**: 424-430. https://doi.org/10.1016/j.ecoenv.2010.10.033

Rajeshkumar, S., Mini, J. and Munuswamy, N., 2013. Effects of heavy metals on antioxidants and expression of HSP70 in different tissues of Milk fish (*Chanos chanos*) of Kaattuppalli Island, Chennai, India. *Ecotoxicol. environ. Safe.,* **98**: 8-18. https://doi.org/10.1016/j.ecoenv.2013.07.029

Ramesh, M., Saravanan, M. and Kavitha, C., 2009. Hormonal responses of the fish, *Cyprinus carpio*, to environmental lead exposure. *Afri. J. Biotechnol*., **8**: 4154-4158.

Scott, G.R., Sloman, K.A., Rouleau, C. and Wood, C.M., 2003. Cadmium disrupts behavioural and physiological responses to alarm substance in juvenile rainbow trout *Oncorhynchus mykiss*. *J. exp. Biol*., **206**: 1779-1790. https://doi.org/10.1242/jeb.00353

Snejdarkova, M., Svobodova, L., Evtugyn, G., Budnikov, H., Karyakin, A., Nikolelis, D.P. and Hianik, T., 2004. Acetylcholin esterase sensors based on gold electrodes modified with dendrimer and polyaniline: A comparative research. *Analyt. Chim. Acta*, **514**: 79-88. https://doi.org/10.1016/j.aca.2004.03.019

Sreejai, R. and Jaya, D.S., 2010. Studies on the changes in lipid peroxidation and antioxidants in fishes exposed to hydrogen sulfide. *Toxicol. Int*., **17**: 71-77. https://doi.org/10.4103/0971-6580.72674

Thom, R., Dixon, D.P., Edward, R., Cole, D.L. and Lapthorn, A.J., 2001. The structure of a zeta class glutathione-S-transferase from *Arabidopsis thaliana*: Characterization of a GST with novel active site tyrosine catabolism. *J. mol. Biol*., **308**: 949-962. https://doi.org/10.1006/jmbi.2001.4638

Valavanidis, A., Vlahogianni, T., Dassenakis, M. and Scoullos, M., 2006. Molecular biomarkers of oxidative stress in aquatic organisms in relation to toxic environmental pollutants. *Ecotoxicol. environ. Safe*., **64**: 178-189. https://doi.org/10.1016/j.ecoenv.2005.03.013

Winston, G.W. and Di-Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol*., **19**: 137-161. https://doi.org/10.1016/0166-445X(91)90033-6

Yang, Y., Cheng, J., Singhal, S.S., Saini, M., Pandya, U. and Awasthi, S., 2001. Role of glutathione-S-transferases in protection against lipid peroxidation. *J. biol. Chem*., **276**: 19220-19230. https://doi.org/10.1074/jbc.M100551200

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Effect of Feed with Varying Dietary Protein Levels on Growth, Survival and Rate of Cannibalism in Fry Rearing of *Channa marulius*

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Authors' Contribution
MHR designed the experiment. FA executed the experimental work. SA and KJI performed chemical tests. MA assisted in juvenile stoking. AH statistically analyzed the data. AJ and GA helped in preparing the manuscript.

Key words
***Channa marulius*, Dietary protein level, Fry, Growth, Survival, Cannibalism.**

ABSTRACT

Studies were spread over three trials (warmer and colder months). In first trial (June to September), net weight gain of fry was 94.18g, 102.66 and 115.46g in T_1 (30%CP), T_2 (40%CP) and control (50%CP), respectively. The specific growth rates (SGR) and feed conversion ratio (FCR) were uniform among all the dietary groups with slight variations which were statistically not detectable. The survival rate in T_2 (40%CP) and control (50%CP) was similar (75.5 and 71.25%) and significantly higher ($P<0.05$) than in T_1 (30%CP). The cannibalism rate in first phase of the experiment was significantly higher in T_1 (30%CP) while the natural loss was 7.5% higher in control (50%CP) followed by 5.0% in T_2 (30%CP) and 1.0% in T_2 (40%CP). In the second phase of the experiment (October to January), the net weight gain of fingerlings was 57.63g, 68.09 and 77.28g in T_1 (30%CP), T_2 (40%CP) and control (50%CP), respectively. The cannibalism rate in second phase of the experiment was 28.8, 24.4 and 16.8% in T_1, T_2 and control, respectively which was significantly higher in T_1 (30%CP) as compared to control (50%CP). Net weight gain and cannibalism (%) was significant among various dietary treatments ($p\leq0.05$) level while SGR (%), FCR, survival rate (%) and natural losses (%) remained non-significant. In the third phase of this experiment (February to May), the net weight gain of fingerlings was 107.6, 119.05 and 130.64g in T_1 (30%CP), T_2 (40%CP) and control (50%CP), respectively. The cannibalism was significantly higher in T_1 (30%CP) when compared to control (50%CP, the lowest). The natural loss recorded was 2.00, 2.66 and 2.0% in T_1, T_2 and control, respectively. Net weight gain, survival and cannibalism rate showed significant while specific growth rate, FCR and natural losses showed non-significant variation at $P\leq0.05$ levels in different treatments. The results of the present study showed that the dietary protein levels in the feed and sorting of *C. marulius* fry has a positive impact on the growth, survival and reduced the cannibalism in the earthen ponds.

INTRODUCTION

Murrels (Family Channidae or Ophiocephalidae) is recognized as its peculiar quality of air-breathing and predaceous feeding behavior (Qasim, 1996). It inhabits in freshwater swamps, slow moving water and fissures in the vicinity of riverbanks of all the tributaries of River Indus and other rivers of Pakistan, running through the warmer parts of the country. The fish have a therapeutic and recuperating quality and helps in quick wound healing in injury or debilitation after serious illness. Its flesh contains sufficient omega-3 polyunsaturated fatty acids to serve as precursor to biosynthesis of prostaglandins ultimately improving immune system of this fish and/or its consumer (Bowman and Rand, 1980; Mat-Jais *et al*., 1994; Baie and Sheikh, 2000a, b).

Like other animals in general and in fish in specific quality protein diet in adequate amounts improve fish palatability, enhances growth, survival and minimize the cannibalism in carnivorous fishes including *C. marulius* (Hafeez-ur-Rehman *et al*., 2017). Sometimes nutritionally completed diets may be rejected and in some cases

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formulated diets which is considered to be an unbalanced with definite nutrients have been accepted rapaciously in some of the species (Flickinger and Smektzer, 1983; Brandt *et al*., 1987; Lovshin and Rushing, 1989; Sloane, 1993; Kubitza, 1995; Hafeez-ur-Rehman *et al*., 2018).

The frequency of cannibalism is one of the major constraints for the culture of *C. marulius* in the earthen ponds. Cannibalism may take place in different age groups, various sizes, and expanded over between and within associated individuals or age classes, depending on the environmental condition and species (Smith and Reay, 1991). It is generally related to size differences, high stocking densities, insufficient quality feed and unfriendly living environment (Hecht and Pienaar, 1993). Unavailability of alternative food instead of natural in captivity and size heterogeneity are considered to be the main causes of cannibalism (Hecht and Appelbaum, 1988; Katavice *et al*., 1989). Hecht and Pienaar (1993) suggested that cannibalism can be reduced by reducing size differences, providing sufficient live food or alternative dietary protein to fish stock. Continuous sorting and stocking uniform sized fish during intensive culture is an effective toll (Jensen, 1990) to minimize cannibalism and early achievement of desired marketable size (Wallat *et al*., 2005). The objective of this study was to evaluate the effect of varying dietary protein levels on the growth, survival and cannibalism of *C. marulius* fry ultimately leading to improvement in management practices during fry rearing, the most critical stage of fish to fingerling (stockable size).

MATERIALS AND METHODS

Collection of C. marulius *fry and study site*

Fry was collected from Dhandh No. 3, Balloki Headworks (on the River Ravi, Pakistan) and transported and transferred to circular concrete tanks for acclimatization in Fish Hatchery, Department of Fisheries and Aquaculture, UVAS, Ravi Campus for 36 h. Fry was carefully taken out from the holding tanks and distributed in earthen ponds for further rearing.

Experimental design

The experiment was conducted in three treatments with two replicates in each. Feed in control group contained 50% protein, treatment T2 40% and T1 had 30% protein. A total 2400 *C. marulius* fry with an average weight of 1.58±0.30, 1.61±0.25, 1.58±0.5g each were equally randomly stocked @ 400 fry/pond (90'x70'x4') into 6 earthen ponds. Underground turbine water was supplied to these ponds when required. The study was continued for whole one growing season. Growing season was further divided into three phases/trials depending on season and feeding behavior. Each phase spanned 120 days. At the end of each phase all the fish were harvested, counted for determination of survival and cannibalism and then weighed for growth increments. Ponds were then dried, fish was graded and then restocked for the next growth trial.

Different feed ingredients including fish meal, soybean meal, maize gluten meal, rice polish, molasses, Vitamin and mineral premixes were procured from the local market, pulverized to suit mouth gape of fry and mixed in different ratios to achieve desired protein levels in different treatment groups (Table I). Three dietary treatments contained 50% (CP), 40% (CP) and 30% (CP) hereafter designated as control, treatment 2 and 1, respectively. The feed was offered to fish @ 10% of their body weight twice a day at 08:30 and 16:30 h by dust feeding method.

Table I.- Feed formulation of different crude protein levels for *C. marulius* fry.

Ingredients	T_1 (30% CP)		T_2 (40% CP)		Control (50%CP)	
	%age	CP	%age	CP	%age	CP
Fish meal	15	7.5	25	12.5	30	15
Soybean meal	20	8.4	20	8.4	20	8.4
Maize gluten	20	12	30	18	45	27
Rice polish	40	4.8	20	2.4	5	0.6
Molasses	4	-	4	-	4	-
Vitamins	1	-	1	-	1	-
Total	100	32.7	100	41.3	100	51.0

Fish survival, cannibalism and growth

A total of 30 fish were randomly netted out on fortnightly basis from each pond for morphometric measurements. At the end of the trial some fingerlings were preserved in polythene bags and refrigerated for nutritional evaluation. At the end of each feeding trial following growth indexes were calculated for each trial.

$$FCR = \frac{\text{Feed intake (g)}}{\text{Wet weight gain (g)}}$$

$$SGR\ \% = \frac{\ln(\text{Final wet BW}) - \ln(\text{Initial wet BW})}{\text{Number of days}} \times 100$$

$$NWG = \frac{\text{Final average weight (g)}}{\text{Initial average weight (g)}}$$

$$\text{Survival (\%)} = \frac{LC}{LS}$$

$$\text{Cannibalism (\%)} = 100 \times \frac{LS - M - LC}{LS}$$

Where, BW is body weight, NWG is net weight gain, LC is the number of fish caught at the termination of the experiment (individuals), LS is the number of fish stocked at the commencement of the experiment (individuals) and M is natural mortality (individuals).

Cannibalism (C) was calculated from the difference between the number of fish stocked at the initiation of the trial minus natural mortality (Szkudlarek and Zakes, 2007) and then from this figure subtraction of available stock which gave a reliable number of individual cannibalized.

Proximate analysis

Crude protein, crude fat, crude fiber, ash, nitrogen free extract and water contents of each experimental diet and flesh from individual treatment were determined before the trial and at the end of the trial respectively following standard analytical procedures (Takeuchi, 1988). Crude protein was determined by Kjeldahl method, crude fat by Soxhlet extraction using petroleum ether as solvent and ash was determined by incinerating sample in a muffle furnace at 600°C. For crude fiber sample was digested in boiling acid then in strong base. Moisture was determined by drying sample in an oven at 105°C to constant weight.

Water quality

Water temperature, dissolved oxygen (DO), pH, salinity, total dissolved solids (TDS), and electrical conductivity (EC), were monitored on daily basis between 9:00 and 10:00 h following the standard methods (APHA, 1998). Water temperature, dissolved oxygen (DO) was measured with the help of DO meter (YSI Model 55 Handheld Dissolved Oxygen and Temperature System, Ohio, 4387, USA), pH was monitored by pH meter (LT Lutron pH -207, Taiwan), and total dissolved solids (TDS), electrical conductivity (EC) and salinity by conductivity meter (Condi 330i WTW 82362 Weilheim, Germany).

RESULTS

In the first phase of the experiment (June to September), the maximum average weight gain was 15.0, 14.20 and 17.0, in T_1 (30%CP), T_2 (40%CP) and control (50%CP), respectively (Table II). The SGR in different treatments ranged from 0.564 to 0.590% day^{-1} and was statistically non-significant among treatments. The NWG of fry was 94.18g, 102.66 and 115.46g in T_1, T_2 and control, respectively. FCR was nearly similar 0.409, 0.411 and 0.416 in T_1, T_2 and control respectively. The survival rate in T_2 (40%CP) and control (50%CP) was similar (75.5 and 71.25%) and significantly higher ($P<0.05$) than in T_1 (30%CP). The cannibalism rate in first phase of the experiment was 30.75, 23.5 and 21.25% respectively which was significantly higher in T_1 (30%CP) while the natural loss was 7.5% higher in control (50%CP) followed by 5.0% in T_2 (30%CP) and 1.0% in T_2 (40%CP). The average length (cm) and length gain were also non-significant (Tables II, III, IV). There were slight variations in water quality parameters. The water temperature remained from 32.9±1.54 to 34.0±1.12, DO, 5.06±0.42 to 5.30±1.72, pH 7.53±0.15 to 7.62±0.14, salinity 0.86±0.07 to 0.90±0.07, TDS, 1805.42±20.07 to 1865.83±70.05, and EC, 2.09±0.08 to 2.15±0.12 (Table V).

Table II.- Fortnightly increase in average body weight of *C. marulius* fry under varying dietary protein levels during June-September.

No. of nettings	Fortnights	T_1 (30%CP)		T_2 (40%CP)		Control (50%CP)	
		Avg. BW (g)	WG (g)	Avg. BW (g)	WG (g)	Avg. BW (g)	WG (g)
Stocking	01-06-2010	0.83±0.02	-	0.84±0.02	-	0.82±0.03	-
1	15-06-2010	5.19±0.15	4.36±0.20	7.17±0.30	6.33±0.80	9.17±0.19	8.35±0.08
2	30-06-2010	16.27±2.20	10.08±0.80	15.66±1.33	8.49±0.85	20.35±1.24	12.00±0.29
3	15-07-2010	28.10±4.32	11.83±0.90	29.56±1.25	13.90±0.12	34.97±2.12	14.62±0.21
4	30-07-2010	40.10±3.55	12.00±2.12	43.85±2.17	14.29±1.13	50.47±0.20	15.50±1.22
5	15-08-2010	53.31±2.11	13.21±1.50	58.50±2.15	14.65±2.17	66.35±1.25	15.88±1.23
6	30-08-2010	66.41±3.13	13.10±2.12	73.80±2.12	15.30±2.17	82.35±2.24	16.00±2.10
7	15-09-2010	80.01±2.20	13.60±1.18	89.30±0.85	15.50±3.20	99.28±2.25	16.93±3.20
8	30-09-2010	95.01±2.15	15.00±1.20	103.5±0.90	14.20±1.21	116.28±2.32	17.00±3.05
ANOVA*							
Average body weight (g)		42.80±33.32^a		46.91±36.82^a		53.34±40.66^a	
Weight gain (g)		11.65±3.28^a		12.83±3.44^a		14.54±2.96^a	

Avg. BW, average body weight; WG, weight gain. *, Analysis of variance on fortnightly average body weight and weight gain (g) of *C. marulius* fry. Values represent means±SD of replicates.

Table III.- Fortnightly increase in body length of *C. marulius* fry under varying dietary protein levels during June-September.

No. of nettings	Fortnights	T_1 (30%CP)		T_2 (40%CP)		Control (50%CP)	
		Avg. BL (cm)	LG (cm)	Avg. BL (cm)	LG (cm)	Avg. BL (cm)	LG (cm)
Stocking	01-06-2010	4.6±0.01	-	4.7±0.01	-	4.5±0.01	-
1	15-06-2010	8.5±0.03	3.9±0.02	8.6±0.02	3.9±0.01	8.9±0.02	4.4±0.01
2	30-06-2010	12.8±0.01	4.3±0.04	12.1±0.03	3.5±0.01	12.9±0.01	4.0±0.04
3	15-07-2010	15.4±0.02	2.6±0.02	15.6±0.05	3.5±0.02	15.8±0.07	2.9±0.04
4	30-07-2010	17.5±0.03	2.1±0.03	17.5±0.02	1.9±0.03	18.2±0.04	2.4±0.03
5	15-08-2010	18.5±0.02	1.0±0.01	19.2±0.03	1.7±0.05	20.1±0.03	1.9±0.02
6	30-08-2010	20.1±0.04	1.6±0.04	21.0±0.07	1.8±0.03	21.3±0.06	1.2±0.03
7	15-09-2010	21.8±0.02	1.7±0.05	22.7±0.06	0.9±0.04	22.8±0.03	1.5±0.03
8	30-09-2010	23.3±0.02	1.2±0.02	23.0±0.03	0.3±0.01	24.0±0.02	1.2±0.04
ANOVA*							
Average body length (cm)		15.83±6.22[a]		16.04±6.41[a]		16.50±6.62[a]	
Length gain (cm)		2.30±1.22[a]		2.19±1.31[a]		2.44±1.24[a]	

Avg. BL, average body length; LG, length gain. *, Analysis of variance of average gain in length in *C. marulius* fry. Values represent means±SD of replicates.

Table IV.- Growth, survival and cannibalism in *C. marulius* fry under varying dietary protein levels during June-September.

Parameters	T_1 (30%CP)	T_2 (40%CP)	Control (50%CP)
No. of fish stocked	400	400	400
Initial Weight (g)	0.83±0.02	0.84±0.02	0.82±0.03
Final Weight (g)	95.01±1.45	103.5±1.23	116.28±1.23
Net weight gain (g)	94.18[b]	102.66[b]	115.46[a]
Weight gain (%)	11346.99[b]	122213[b]	14080.49[b]
SGR (%)	0.564± 0.07[a]	0.573± 0.003[a]	0.590± 0.056[a]
FCR	1.02±0.11[a]	1.01±0.25[a]	1.01±0.21[a]
Survival rate (%)	64.25±0.19[c]	75.5±1.44[a]	71.25±1.45[b]
Cannibalism rate (%)	30.75±0.62[a]	23.5±1.41[b]	21.25±0.1.06[b]
Natural losses (%)	5.0±1.548[a]	1.0±0.353[b]	7.5±1.33[a]

Data figures with different superscript letters across the rows are significantly different from each other (P≤0.05).

Table V.- Physico-chemical analysis of all the ponds under varying dietary protein levels during June-September.

Parameters	T1 (30%CP)	T2 (40%CP)	Control (50%CP)
Water temp. (°C)	34.09±1.12	33.70±1.25	32.95±1.54
DO (mg/l)	5.20±0.93	5.06±0.42	5.30±1.72
pH	7.61±0.13	7.62±0.14	7.53±0.15
Salinity (mg/l)	0.90±0.07	0.86±0.07	0.88±0.09
Total dissolved solid	1865.83± 70.05	1850.40± 25.95	1805.42± 20.07
Electrical conductivity	2.15±0.12	2.09±0.08	2.10±0.07

In the second phase of the experiment (October to January), the maximum average weight gain ranged from 5.30-12.40g, 6.15-13.65g and 6.70-15.68 in T_1 (30%CP), T_2 (40%CP) and control (50%CP), respectively. The maximum growth was recorded during 1st fortnight in October and minimum was in the 6th fortnights of December (Table VI). The SGR ranged from 1.549 to 1.766 %/day. The NWG of fingerlings was 57.63g, 68.09 and 77.28g in T_1, T_2 and control, respectively. FCR was 2.062, 1.829 and 1.674 in T_1, T_2 and control, respectively. The survival rate was 76.0% maximum in control (50%CP), while 68.8 in T_2 (40%CP) and 64.0% was observed in T_1 (30%CP). The cannibalism rate in second phase of the experiment was 28.8, 24.4 and 16.8% in T_1, T_2 and control, respectively which was significantly higher in T_1 (30%CP) as compared to control (50%CP). The natural loss was 7.2, 6.8 and 7.2% in T_1, T_2 and control, respectively (Tables VI, VII, VIII). NWG and cannibalism (%) was significant among various dietary treatments (p≤0.05) level while SGR (%), FCR, survival rate (%) and natural losses (%) remained non-significant. The ponds water quality parameters showed slight variations. The water temperature remained from 17.7±2.61 to 20.3±1.30, DO, 4.98±0.96 to 5.10±1.14, pH 7.42±0.12 to 7.45±0.21, salinity 0.87±0.08 to 0.90±0.05, TDS, 1502.00±82.72 to 1665.06±90.70, and EC 2.03±0.54 to 2.15±2.12±0.12 (Table IX).

In the third phase of this experiment (February to May), the maximum average weight gain was observed in the range of 10.53-15.05g, 11.25-16.60g and 12.55-17.66g in T_1 (30%CP), T_2 (40%CP) and control (50%CP), respectively. The maximum growth was recorded during 7th fortnight in May and minimum was in the 1st fortnight of February (Table X).

Table VI.- Fortnightly increase of average body weight *C. marulius* fry under varying dietary protein levels during the cold months (October-January).

No. of nettings	Fortnights	T_1 (30%CP)		T_2 (40%CP)		T_3 (50%CP)	
		Avg. BW (g)	WG (g)	Avg. BW (g)	WG (g)	Avg. BW (g)	WG (g)
Stocking	01-10-2010	100.20±1.22	-	100.50±2.13	-	101.25±1.89	-
1	15-10-2010	112.60±2.09	12.40±0.92	114.30±2.19	13.65±0.84	116.93±1.99	15.68±1.07
2	30-10-2010	121.35±1.79	8.75±0.65	125.50±3.14	11.20±0.88	128.68±4.23	11.75±1.02
3	15-11-2010	128.15±3.24	6.80±0.35	134.66±2.09	9.16±0.56	139.43±3.45	10.75±1.22
4	30-11-2010	133.95±2.08	5.80±0.45	141.46±3.24	6.80±0.67	146.53±3.24	7.10±0.78
5	15-12-2010	139.23±3.45	5.28±0.87	147.61±2.77	6.15±0.92	154.18±2.09	7.65±0.77
6	30-12-2010	144.53±2.00	5.30±0.42	153.06±2.65	5.45±0.23	160.88±3.04	6.70±0.66
7	15-01-2011	150.75±3.34	6.22±0.70	159.41±2.17	6.35±0.28	167.53±4.44	6.65±0.81
8	30-01-2011	157.83±3.22	7.08±0.21	168.59±3.76	9.18±1.02	178.53±4.35	11.00±1.09
ANOVA*							
Average body weight (g)		132.07±18.53[a]		138.34±21.96[a]		143.77±24.89[a]	
Weight gain (g)		7.20±2.39[a]		8.49±2.86[a]		9.66±3.21[a]	

Avg. BW, average body weight; WG, weight gain. *, Analysis of variance on fortnightly of average body weight and weight gain of *C. marulius* fry. Values represent means±SD of replicates.

Table VII.- Fortnightly increase in average length of *C. marulius* fry under varying dietary protein levels during October-January.

No. of nettings	Fortnights	T_1 (30%CP)		T_2 (40%CP)		Control (50%CP)	
		Avg. BL (cm)	LG (cm)	Avg. BL (cm)	LG (cm)	Avg. BL (cm)	LG (cm)
Stocking	01-10-2010	24.0±1.67	-	24.1±1.33	-	24.5±1.87	-
1	15-10-2010	25.0±1.08	1.0±0.02	25.4±1.46	1.3±0.03	25.8±1.92	1.3±0.08
2	30-10-2010	26.1±2.01	1.1±0.09	26.5±1.02	1.1±0.07	26.8±1.09	1.0±0.03
3	15-11-2010	26.7±1.04	0.6±0.02	26.9±2.02	0.4±0.03	27.0±1.06	0.2±0.01
4	30-11-2010	26.8±1.06	0.1±0.04	27.1±1.04	0.2±0.02	27.3±1.44	0.3±0.02
5	15-12-2010	27.0±1.02	0.2±0.01	27.4±2.01	0.3±0.03	28.1±1.45	0.8±0.08
6	30-12-2010	27.2±2.08	0.2±0.03	28.0±1.47	0.6±0.03	29.0±2.11	0.9±0.09
7	15-01-2011	28.0±1.02	0.8±0.06	28.8±2.09	0.8±0.02	29.8±1.92	0.8±0.19
8	30-01-2011	28.5±2.08	0.5±0.02	30.0±2.01	1.2±0.98	30.1±1.67	0.3±0.02
ANOVA*							
Average body length (cm)		26.59±1.40a		27.13±1.75a		27.60±1.85a	
Length gain (cm)		0.56±0.38a		0.74±0.43a		0.70±0.39a	

Avg. BL, average body length; LG, length gain. *, Analysis of variance of average length increments in *C. marulius* fry. Values represent means±SD of replicates.

The SGR ranged from 1.549 to 1.766 %/day. The NWG of fingerlings was 107.6, 119.05 and 130.64g in T_1, T_2 and control, respectively. FCR was 2.062, 1.829 and 1.674 in T_1, T_2 and control respectively. There was observed maximum survival rate (99.33%) in control group (50%CP), while it was the second highest (88.67) in T_2 (40%CP) and the lowest (84.46%) in T_1 (30%CP). The % cannibalism was 14.0 (the highest) 8.76 and 4.67% (the lowest) in T_1, T_2 and control, respectively. The cannibalism was significantly higher in T_1 (30%CP) when compared to control (50%CP, the lowest). The natural loss recorded was 2.00, 2.66 and 2.0% in T_1, T_2 and control, respectively (Tables X, XI, XII). NWG, survival and cannibalism rate showed significant while SGR, FCR and natural losses showed non-significant variation at P≤0.05 levels in different treatments. Like other phases water quality varied though variations were not so substantial. The water temperature was in the range of 27.7±2.61 to 28.6±1.30, DO, 5.02±0.95 to 5.50±1.12, pH 7.40±0.21 to 7.45±0.14, salinity 0.82±0.08 to 0.88±0.05, TDS, 1502.00±65.71 to 1654.06±92.60, and EC was 2.01±0.54 to 2.15±0.12 (Table XIII). The overall statistical analysis of growth data showed non-significant difference among all the three dietary groups (Table XIV).

Table VIII.- Growth, survival and cannibalism in *C. marulius* fry under varying dietary protein levels during October-January.

Parameters	T_1 (30%CP)	T_2 (40%CP)	Control (50%CP)
No. of fish stocked	250	250	250
Initial weight (g)	100.20±0.30	100.50±0.80	101.25±0.75
Final weight (g)	157.83±1.50	168.59±2.10	178.53±2.40
Net weight gain (g)	57.63±1.27[c]	68.09±2.82[b]	77.28±2.827[a]
Percent weight gain	57.51±1.85 [c]	67.75±2.52[b]	76.32±2.70 [a]
SGR (%)	1.766±0.28[a]	1.654±0.14[a]	1.549±0.282[a]
FCR	2.062±0.03[a]	1.829±0.12[a]	1.674±0.312[a]
Survival rate (%)	64.0±14.14[a]	68.8±1.41[a]	76.0±2.228[a]
Cannibalism (%)	28.8±2.82[a]	24.4±1.41[ab]	16.8±2.139[b]
Natural losses (%)	7.2±1.41[a]	6.8±1.27[a]	7.2±0.0.341[a]

DISCUSSION

Current findings have revealed that the initial stocking size of *C. marulius is* very important for its successful rearing and reduce cannibalism. When *C. marulius* exceeds 160 gram of body weight readily accepts artificial feed compared to smaller individuals which reduces predation due in part to low availability of food in the system (Tables III, VII, XI). The results are in line with the investigation of Zdzisaw (2012) who reported that early size of fish is very important for successful rearing of pike-perch fry on artificial feed. Zakeoe (1997) and Zakeoe and Demska-Zakeoe (1996) during their observation reported that the size variability affected the survival and successful rearing of fish and the variation in size ultimately enhanced cannibalism specific fish losses. Sanderson (1974) observed that the smallest size of *S. vitreum* fry fed on artificial feed would be almost 35mm while Nickum (1978) in his studies reported appropriate size range of 35 to 75 mm. Cheshire and Steele (1972), Nagel (1985), Nickum (1978) and Kuipers and Summerfelt (1994) concluded that initial body size of fish should be more than 50 mm for successful rearing. Longer rearing of fry to fingerlings in the earthen ponds increased fish cannibalism and mortality due to high stocking density, starvation and low protein level in the diet (Tables III, VII, XI). These results are in line with Nickum and Stickney (1993), who determined that the fish should be netted-out from the ponds at the size of 25-35 mm for grading.

Table IX.- Physico-chemical parameters observed in different ponds in varying dietary protein levels during October-January.

Parameters	T1 (30%CP)	T2 (40%CP)	Control (50%CP)
Water temp. (°C)	18.4±1.72	17.7±2.61	20.3±1.30
DO (mg/l)	5.10±1.14	5.05±0.62	4.98±0.96
pH	7.45±0.21	7.42±0.12	7.43±0.13
Salinity (mg/l)	0.90±0.05	0.89±0.06	0.87±0.08
Total dissolved solid	1502.00± 82.72	1625.93± 81.22	1665.06± 90.70
Electrical conductivity	2.12±0.12	2.03±0.54	2.10±0.22

Table X.- Fortnightly increase in average body weight gains in *C. marulius* fry under varying dietary protein levels during February-May.

No. of nettings	Fortnights	T_1 (30%CP)		T_2 (40%CP)		Control (50%CP)	
		Avg. BW (g)	WG (g)	Avg. BW (g)	WG (g)	Avg. BW (g)	WG (g)
Stocking	01-02-2011	160.50±2.89	-	162.30±2.46	-	162.35±2.89	-
1	15-02-2011	171.03±3.03	10.53±1.09	173.55±2.76	11.25±0.83	174.9±3.09	12.55±0.66
2	28-02-2011	182.36±2.76	11.33±0.77	187.13±3.15	13.58±1.02	189.95±3.00	15.05±0.79
3	15-03-2011	195.25±2.91	12.89±0.47	201.46±2.69	14.33±1.23	206.5±2.10	16.55±1.09
4	30-03-2011	209.4±3.15	14.15±0.58	216.69±3.06	15.23±1.03	223.1±2.09	16.60±0.68
5	15-04-2011	223.93±3.33	14.53±1.09	232.49±3.09	15.80±0.49	240.53±3.04	17.43±2.44
6	30-04-2011	238.43±3.01	14.50±082	248.92±3.66	16.43±1.07	257.98±3.14	17.45±1.11
7	15-05-2011	253.48±2.99	15.05±1.02	265.52±2.66	16.60±1.04	275.64±3.02	17.66±1.00
8	30-05-2011	268.23±3.90	14.75±3.80	281.35±2.70	15.83±0.80	292.99±1.09	17.35±3.40
ANOVA*							
Average body weight (g)		211.40±37.44[a]		218.82±41.44[a]		224.88±45.45[a]	
Weight gain (g)		13.47±1.71[b]		14.88±1.79a[b]		16.33±1.75[a]	

Avg. BW, average body weight; WG, weight gain. *, Analysis of variance in average body weight of *C. marulius* fry. Values represent means±SD of replicates.

Table XI.- Fortnightly increase of average body length *C. marulius* fry under varying dietary protein levels during the warm month (February-May).

No. of nettings	Fortnights	T_1 (30%CP)		T_2 (40%CP)		Control (50%CP)	
		Avg. BL (cm)	LG (cm)	Avg. BL (cm)	LG (cm)	Avg. BL (cm)	LG (cm)
Stocking	01-02-2011	28.4±1.022	-	28.5±1.221	-	28.5±1.291	-
1	15-02-2011	29.2±2.091	0.8±0.212	29.2±2.231	0.7±0.031	29.7±2.915	1.2±0.891
2	28-02-2011	30.5±1.939	1.3±0.671	30.2±1.861	1.0±0.061	30.3±2.343	0.6±0.121
3	15-03-2011	31.2±2.110	0.7±0.213	31.0±2.422	0.8±0.091	31.3±1.983	1.0±0.021
4	30-03-2011	31.8±3.002	0.6±0.032	32.0±1.572	1.0±0.091	32.5±2.901	1.2±0.451
5	15-04-2011	32.7±3.071	0.9±0.034	32.8±1.887	0.8±0.061	33.0±2.336	0.5±0.023
6	30-04-2011	32.9±2.771	0.2±0.021	33.6±1.990	0.8±0.341	34.0±3.002	1.0±0.212
7	15-05-2011	33.7±2.676	0.8±0.043	34.5±3.0953	0.9±0.045	35.0±4.011	1.0±0.064
8	30-05-2011	34.6±2.551	0.9±0.121	35.5±2.995	1.0±0.092	36.6±2.113	1.6±0.789
ANOVA*							
Average body length (cm)		31.67 ± 2.05a		31.92 ± 2.40a		32.32 ± 2.63a	
Length gain (cm)		0.78 ± 0.31a		0.86 ± 0.12a		1.01 ± 0.35a	

Avg. BL, average body length; LG, length gain. *, Analysis of variance of Fortnightly length gain in *C. marulius* fry. Values represent means±SD of replicates.

Table XII.- Growth, survival and cannibalism in *C. marulius* fry under varying dietary protein levels during February-May.

Parameters	T_1 (30%CP)	T_2 (40%CP)	Control (50%CP)
No. of fish stocked	150	150	150
Initial weight (g)	160.50	162.30	162.35
Final weight (g)	268.23	281.35	292.99
Net weight gain (g)	107.6±2.82[c]	119.05±2.78[b]	130.64±2.76[a]
Percent weight gain	67.04±0.12[c]	73.35±0.12[b]	80.46±0.13[a]
SGR (%)	1.766±0.28[a]	1.654±0.14[a]	1.549±0.02[a]
FCR	1.768±o.14[a]	1.654±0.02[a]	1.549±0.04[a]
Survival rate (%)	84.0±2.86[b]	88.67±0.27[ab]	93.33±0.33[a]
Cannibalism rate (%)	14.0±1.41[a]	8.67±0.16[b]	4.67±0.12[c]
Natural losses (%)	2.0±0.39[a]	2.66±0.12[a]	2.0±0.37[a]

Table XIII.- Physico-chemical parameters of ponds under varying dietary protein levels during February-May.

Parameters	T1 (30%CP)	T2 (40%CP)	Control (50%CP)
Water temp. (°C)	28.4±1.72	27.7±2.61	28.6±1.30
DO (mg/l)	5.50±1.12	5.03±0.65	5.02±0.95
pH	7.40±0.21	7.43±0.12	7.45±0.14
Salinity (mg/l)	0.88±0.05	0.85±0.06	0.82±0.08
Total dissolved solid	1502.00± 65.71	1621.97± 70.20	1654.06± 92.60
Electrical conductivity	2.15±0.12	2.01±0.54	2.12±0.22

Table XIV.- Average weight gains in *C. marulius* fry in different dietary treatments at different stocking densities.

Treatment	Stocking density of *C. marulius* fry		
	400 No.	250 No.	150 No.
T_1(30%CP)	42.803 ± 11.106[aa]	132.066 ± 6.178[ab]	211.401 ± 12.481[ac]
T_2(40%CP)	46.909 ± 12.273[aa]	138.343 ± 7.321[ab]	218.823 ± 13.813[ac]
Control (50%CP)	53.338 ± 13.553[aa]	143.771 ± 8.296[ab]	224.882 ± 15.150[ac]

Data figures with different superscript letters in columns are significantly different from each other at P<0.05 while data figures with similar superscript letters in rows are not significant at P>0.05.

The growth of *C. marulius* in all three phases of the experiment was non-significant despite offering feed with different protein levels. Nevertheless there were slight variations in growth when compared among different treatments. The lowest growth was observed in 30% crude protein containing diet. Variation within the group observed in current studies might be selective feeding in fish. This variability in feeding habit stippled fish size and provided ample opportunity to bigger fish to eat more. This drastic size variation resulted in heavy cannibalism. Similar results were found during the rearing of juvenile (initial body weight from 7.1 to 88.7 g) (Melard *et al*., 1995), of pikeperch (initial body weight 40 g) (Zakes *et al*., 2004), yellow perch *Perch flavescens* (Wallat *et al*., 2005) and turbot, *Scophthalmus maximus* (Sunde *et al*., 1998).

Sorting and grading have positive effect on European perch, *Perch fluviatilis* which resulted in increased body weight than those where sorting was not exercised (Melard *et al*.,1995).

The growth and intensity of cannibalism in *C. marulius* was non-significant during the pahse-1 (June-September) and phase-2 (October-January) of the trial. In the first phase the cannibalism was the maximum at initial stage of the fry while in the second phase though it continued but at low pace. On the onset of winter season (October to January) temperature decreased (10-15°C) which halted feed intake in fish and growth. The maximum survival rate was observed in 50% CP (control) due to sufficient protein level to meet the requirement of fish which might avert cannibalism. The cannibalism rate was almost double in 30% crude protein containing feed group. Kestemont *et al*. (2007) and Szkudlarek and Zakes (2007) had similar findings and reported that cannibalism play decisive role in rearing of fish larvae of pike perch which further conform our results. Baras and Jobling (2002) further stressed that during rearing of smaller fish cannibalism increases due to size variations.

To prevent the loss of fry diverse approaches have been proposed which comprise continuous food supply to the fry (Katavic *et al*., 1989) and control drastic size variation (Qin and Fast, 1996) at stocking and during rearing. Size variation proved to be more effective in predation than provision of continuous food to largemouth bass *Micropterus salmonides*. Cannibalism was closely monitored in all the three phases of the trial. During each phase fish was netted out and graded into uniform size groups and then restocked in earthen ponds separately. In the first phase, due to greater size variations cannibalism remained on the higher side than observed in remaining two phases. Bigger fish predated small fry during the first phase (June-September) and second phase (October-January) of experiment. These results are in line with Pantastico *et al*. (1988) who observed bigger tilapia eating the smaller ones (*Oreochromis niloticus*) in the hatchery (Fessehaye *et al*., 2004).

Maximum cannibalism was observed in T_1 (30%CP), then in T_2 (40%CP) and in Control (50% CP) the least. Pantastico *et al*. (1988) stated that the availability of natural food in the culture tank affected the survival of tilapia (*Oreochromis niloticus*) fry when stocked with fingerlings in aquarium. Francis and Bengtson (1999) investigated that the selection of diet reduces the cannibalism in summer flounder, *P. dentatus*, and both inert and live *Artemia* contributed differently in occurrence of cannibalism. The gradual decrease in protein levels from 54% (Control) to 40% and 30% proportionately increased cannibalism. The survival rate was maximum in 50% CP followed by 40%CP and the lowest was observed in 30%CP containing diet. These results are in line with Abdel-Tawwab *et al*. (2006) who observed maximum cannibalism in 25%CP containing feed group. Similarly, Francis and Bengtson (1999) reported that summer flounder (*P. dentatus)* showed non-significant difference in survival when fed on 50%CP and 55%CP diets. When more than required protein level was used, it significantly reduced cannibalism in tilapia fry (Ahmed *et al*., 2004). It is worth mentioning that provision of excess feed did not stop cannibalism in all the three phases of the current trial but elevated levels of protein did. Though insufficient food increases cannibalism and associated losses (Smith and Reay, 1991), but it is not universal phenomenon because larger rations in perch (Kestemont *et al*., 2003) could not prevent fish from cannibalistic behavior.

CONCLUSION

The results of the present experiment showed that maximum dietary protein levels in feed significantly increased growth and survival and decreased cannibalism. Cannibalism can be further minimized by stocking graded *C. marulius* fry till fingerling stage. Future research should focus on the intensive rearing of fry to further investigate the role of high protein diet in prevention of cannibalism and growth of fish at various stages of its development.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abdel-Tawwab, M., Hany, I.E. and Mohammad, H.A., 2006. Cannibalism in *Oreochromis niloticus* (Linnaeus): Effect of stocking density, feed quality and submerged macrophytes. *Indian J. Fish*., **53**: 245-251.

Ahmad, M.H., Abdel-Tawwab, M. and Khattab, Y.A.E., 2004. Effect of dietary protein levels on growth performance and protein utilization in Nile tilapia (*Oreochromis niloticus* L.) with different initial body weights. In: *The 6th International Symposium of Tilapia in Aquaculture* (eds. R. Bolivar, G. Mair and K. Fitzsimmons). Manila, Philippines, pp. 249-263.

Baie, S.H.J. and Sheik, K.A., 2000a. The wound healing properties of *Channa striatus* cetrimide cream-tensile strength measurement. *J. Ethnopharm.,* **71**: 93-100. https://doi.org/10.1016/S0378-8741(99)00184-1

Baie, S.H.J. and Sheik, K.A., 2000b. The wound

healing properties of *Channa striatus* cetrimide cream-wound contraction and glycosaminoglycan measurement. *J. Ethnopharm.,* **73**: 15-30. https://doi.org/10.1016/S0378-8741(00)00253-1

Bowman, W.C. and Rand, M.J., 1980. *Textbook of pharmacology,* 2nd ed. Willey-Blackwell, Oxford, London.

Brandt, T.M., Jones R.M. and Anderson, R.J., 1987. Evaluation of prepared feeds and attractants for largemouth bass fry. *Progr. Fish Cult.,* **49**: 198-203. https://doi.org/10.1577/1548-8640(1987)49<198:EOPFAA>2.0.CO;2

Baras, E. and Jobling, M., 2002. Dynamics of intracohort cannibalism in cultured fish. *Aquacult. Res.,* **33**: 461-479.

Cheshire, W.F. and Steele, K.L., 1972. Hatchery rearing of walleye using artificial food. *Progr. Fish Cult.*, **34**: 96-99. https://doi.org/10.1577/1548-8640(1972)34[96:HROWUA]2.0.CO;2

Fessehaye, Y., Rezk, M., Bovenhuis, H. and Komen, H., 2004. Size dependent cannibal-ism in Juvenile Nile tilapia (*Oreochromis niloticus*). In: *The 6th International Symposium of Tilapia in Aquaculture* (eds. R. Bolivar, G. Mair and K. Fitzsimmons). Manila, Philippines, pp. 230-238.

Flickinger, S.A. and Smeltzer, J.F., 1983. Training spotted bass to accept a prepared diet. *Progr. Fish Cult.,* **45**: 185-186. https://doi.org/10.1577/1548-8659(1983)45[185:TSBTAA]2.0.CO;2

Francis, A.W. and Bengtson, D.A., 1999. Partition of fish and diet selection as methods for the reduction of cannibalism in *Paralichthys denatus* larviculture. *J. World Aquacult. Soc.,* **30**: 302-310. https://doi.org/10.1111/j.1749-7345.1999.tb00680.x

Hafeez-ur-Rehman, M., Abbas, F., Abbas, G., Javid, A., Hussain, A., Andleeb, S., Iqbal, K.J. and Akmal, M., 2018. Effect of different dietary protein levels on growth and proximate composition of the eggs and broodstock of Giant Murrel, *Channa marulius* (Forsskal). *Pakistan J. Zool*., **50**: 595-602.

Hafeez-ur-Rehman, M., Abbas, F., Ashraf, M., Narejo, N.T., Iqbal, K.J., Abbas, G. and Andleeb, S., 2017. Effect of different dietary protein levels on egg development and its response to inducing agents during induced spawning of *Channa marulius*. *Pakistan J. Zool*., **49**: 337-343. https://doi.org/10.17582/journal.pjz/2017.49.1.337.343

Hecht, T. and Appelbaum, S., 1988. Observations on interspecific aggression and coeval sibling cannibalism by larval and juvenile *Clarias gariepinus* (Clariidae: Pisces) under controlled conditions. *J. Zool.,* **214**: 21-44. https://doi.org/10.1111/j.1469-7998.1988.tb04984.x

Hecht, T. and Pienaar, A., 1993. A review of cannibalism and its implications in fish larviculture. *J. World Aquacult. Soc.,* **24**: 246-261. https://doi.org/10.1111/j.1749-7345.1993.tb00014.x

Hecht, T. and Pienaar, A.G., 1991. Cannibalism: The hidden mortality factor in larviculture (abstract). In: *Larvi '91-Fish and Crustacean Larviculture Symposium* (eds. P. Lavens, P. Sorgeloos, E. Jaspers and F. Ollevier). European Aquaculture Society, Gent, Belgium, pp. 277.

Jensen, G.L., 1990. *Sorting and grading warm water fish.* Southern Region Aquaculture Center, SRAC Publication No. 391, pp. 1-8.

Katavic, I., Judgujakovic, J. and Glamuzina, B., 1989. Cannibalism as a factor affecting the survival of intensively cultured sea bass (*Dicentrarchus labrax*) fingerlings. *Aquaculture*, **77**: 135-143. https://doi.org/10.1016/0044-8486(89)90197-X

Kestemont, P., Jourdan, S., Houbart, M., Mélard,C., Paspa-tis, M., Fontaine, P., Cuvier, A., Kentouri, M. and Baras, E., 2003. Size heterogeneity, cannibalism and competition in cultured predatory fish larvae: biotic and abiotic influences. *Aquaculture*, **227**: 333-356. https://doi.org/10.1016/S0044-8486(03)00513-1

Kubitza, F., 1995. *Intensive culture of largemouth bass* Micropterus salmoides. *Production of advanced juveniles and food-size fish*. Ph.D. dissertation, Auburn University, Alabama, USA, pp. 145.

Kuipers, K.L. and Summerfelt, R.C., 1994. Converting pond-reared walleye fingerlings to formulated feeds: Effects of diet, temperature, and stocking density. *J. appl. Aquacult.*, **4**: 31-57. https://doi.org/10.1300/J028v04n02_04

Lovshin, L.L. and Rushing, J.H., 1989. Acceptance by largemouth bass fingerlings of pelleted feeds with a gustatory additive. *Progr. Fish Cult.,* **51**: 73-78. https://doi.org/10.1577/1548-8640(1989)051<0073:ABLBFO>2.3.CO;2

Mat-Jais, A.M., McCulloch, R. and Croft, K., 1994. Fatty acid and amino acid composition in Haruan as a potential role in wound healing. *Gen. Pharmacol.,* **25**: 947-950. https://doi.org/10.1016/0306-3623(94)90101-5

Melard, C., Baras, E. and Kestemont, P., 1995. Preliminary results of European perch (*Perca fluviatilis*) intensive farming trials: Effect of temperature and size grading on growth. *Bull. Franç. Pêcheet Piscicult.,* **336**: 19-27.

Nagel, T., 1985. Development of a domestic walleye broodstock. *Progr. Fish Cult*., **47**: 121. https://doi.

org/10.1577/1548-8640(1985)47<121:DOADWB>2.0.CO;2

Nickum, J.G., 1978. Intensive culture of walleyes: The state of the art. *Am. Fish. Soc. Spec. Publ.*, **11**: 187-194.

Nickum, J.G. and Stickney, R.R., 1993. Walleye. In: *Culture of non-salmonid freshwater fishes* (ed. R.R. Stickney). CRC Press, Ann Arbor, London, Tokyo, pp. 231-249.

Pantastico, J.B., Dangilan, M.M.A. and Egula, R., 1988. Cannibalism among different sizes of tilapia (*Oreochromis niloticus*) fry/fingerlings and the effect of natural food. In: *The Second International Symposium on Tilapia in Aquaculture* (eds. R.S.V. Pullin, T. Bhukaswan, K. Tonguthi and J.L. Maclean). ICLARM, Department of Fisheries, Bangkok, Thailand and Iclarm, Manila, Philippines, pp. 465-468.

Qasim, 1996. The growth of the freshwater murrel, *Ophiocephalus punctatus* Bloch. *Hydrogiolo*., **27**: 289-316.

Qin, J. and Fast, A.W., 1996. Size and feed dependent cannibalism with juvenile snakehead *Channa striatus. Aquaculture,* **144**: 313-320. https://doi.org/10.1016/0044-8486(96)01299-9

Sanderson, C.H., 1974. *Dry diet testing results* Pennsylvania Fish Commission. Linesville, Pennsylvania, Mimeographed Report, pp. 11.

Saoud, I.P., Davis, D., Roy, L.A. and Phelps, R.P., 2005. Evaluating the benefits of size sorting tilapia fry before stocking. *J. appl. Aquacult.,* **17**: 73-85. https://doi.org/10.1300/J028v17n04_05

Sloane, M.B., 1993. *Performance of training and grow-out diets for largemouth bass.* M.S. thesis, Auburn University, Auburn, Alabama, USA, pp. 66.

Smith, C. and Reay, P., 1991. Cannibalism in teleost fish. *Rev. Fish Biol. Fish.,* **1**: 41-64. https://doi.org/10.1007/BF00042661

Sunde, L.M., Imsland, A.K., Folkvord A. and Stefansson, S.O., 1998. Effects of size grading on growth and survival of juvenile turbot at two temperatures. *Aquacult. Int.,* **6**: 19-32. https://doi.org/10.1023/A:1009265602388

Szkudlarek, M., and Zakes, Z., 2007. Effect of stocking density on survival and growth performance of pikeperch, *Sander lucioperca* (L.), larvae under controlled conditions. *Aquacult. Int.,* **15**: 67-81. https://doi.org/10.1007/s10499-006-9069-7

Takeuchi T., 1988. Laboratory work-chemical evaluation of dietary nutrients. In: *Fish nutrition and mariculture* (ed. Watanabe T.). JICA Textbook the General Aquaculture Course, Tokyo, pp.179-233.

Wallat, G.K., Tiu, L.G., Wang, H.P., Rapp, D. and Leighfield, C., 2005. The effects of size grading on production efficiency and growth performance of yellow perch in earthen ponds. *N. Am. J. Aquacult*., **67**: 34-41. https://doi.org/10.1577/FA04-003.1

Zakeoe, Z., 1997. The effect of stock density on the survival, cannibalism and growth of summer fry of European pikeperch (*Stizostedion lucioperca* L.) fed with artificial food in controlled conditions. *Arch. Pol. Fish.*, **5**: 305-311.

Zakeoe, Z. and Demska-Zakeoe, K., 1996. Effect of diets on growth and reproductive development of juvenile pike perch, *Stizostedion lucioperca* (L.), reared under intensive culture conditions. *Aquacult. Res.,* **27**: 841-845. https://doi.org/10.1111/j.1365-2109.1996.tb01243.x

Zakes, Z. and Szczepkowski, M., 2004. Induction of out-of-season spawning of pikeperch, *Sander lucioperca* (L.). *Aquacult. Int.,* **12**: 11-18. https://doi.org/10.1023/B:AQUI.0000017183.40691.7d

Zdzisaw, Z., 2012. The effect of body size and water temperature on the results of intensive rearing of pike-perch, *Stizostedion lucioperca* (L.) fry under controlled conditions. *Arch. Pol. Fish.,* **20**: 165-172.

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Effect of Onion Powder as Feed Additive on the Growth, Hematology and Biochemical Profile of Grass Carp (*Ctenopharyngodon idella*)

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Authors' Contribution
MHR designed and supervised the study. IA performed the experimental work. FA performed chemical analyses. AH statistically analyzed the data. NA assisted in juvenile stoking. MA assisted in blood analyses. FR assisted in biochemical profiling of fish samples. HA assisted in organoleptic studies. GA and AJ assisted in preparation of manuscript.

Key words
Onion powder, Feed additive, Growth, Hematology, Grass carp.

ABSTRACT

This study was conducted to find out the dietary effect of *Allium cepa* (onion) as a feed additive on the growth, hematology, body composition and organoleptic profile of grass carp (*Ctenopharyngoden idella).* Three experimental diets (T1, T2, T3) containing 1%, 2% and 3% of onion powder and a control diet were fed to the fish in the aquariums. The total of 10±3 fish were stocked in each aquarium with 2 replicates. Fish fed with 1% and 2% gained significantly higher weight gain, specific growth ratio and feed conversion ratio than control group and T3. Increase in WBC and RBC in T1 as compare to other treatment groups exhibit positive health effect in grass carp. Fish flesh quality with respect to consumer's approval did not show any significant differences irrespective of diet composition among various dietary treatments. The body composition of grass carp result the moisture content ranged from 71 to 73.6%, crude protein (15 to 17%), ash content (2.15 to 2.7%) and dry matter (26.4 to 29%) with significant differences with respect to concentrations of onion powder.

INTRODUCTION

In Pakistan, new fish marketing practices aimed to introduce aquaculture products not only as a main source of protein but also ensure the nutrients quality and cost effectiveness. A complete and nutritionally balanced diet is compulsory which results best yield with maximum weight gain in shortest conceivable time (Bhosale *et al*., 2010). There is an increasing need to understand the roles of phyto-additives in aquaculture. To date, various herbs extracts and spices are reported to improve fish growth performance and immunity. Besides, feed additives that could enhance the immune systems of farmed fish species, facilitates ingestion rates, induce digestive secretions, high protein synthesis (Khalil *et al*.,, 2001; Citarasu, 2010), attains the desired flesh and skin pigmentation, as well as improving the organoleptic properties and direct bactericidal effect on gut microflora of the farmed fish product without having negative effects to fish species and the farming environment (Ajiboye *et al*., 2012).

Plants which belong to genus *Allium* have been used in food industry because it fights against the disease and improve immunity. Onion (*Allium cepa*) belongs to the *Liliaceae* family and used as antiseptic, antioxidant and food supplement due to its bioactivity. It is a rich source of a wide variety of beneficial nutrients such as trace macronutrients and micronutrients flavonoids and sulfur containing compounds that are used in artificial feed formulated by agricultural by products *e.g.* wheat bran, rice polish (Cho *et al*., 2010).

Grass carp is a very delicious and profitable fish species widely cultured throughout the world. It is herbivorous gain weight rapidly and can be grown together with other fish species (Fiertak *et al*., 2002). There is a little confirmation about the nutritive value and optimum feeding level of grass carp (Cui *et al*., 1992). The ideal production rate of fish requires research into fish nutrition and different feeding methods which result in better growth and reduced waste material which released in the water (Singh *et al*., 2005). The addition of food additives in the feed ingredient could results to improve fish performance, resistance against disease and higher values of flesh. Thus, the present study was aimed to determine the effect of onion on the growth, meat quality and composition of

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silver carp under different concentrations.

MATERIALS AND METHODS

Grass carp (7.8±5g) were obtained from Fish farm complex, UVAS, Ravi campus, Pattoki. Fish were divided into three treatments and one control group with 2 replicates (10fish/aquarium) and fed @ of 2% body weight for 60 days after conditioning. Onion was taken from market, cut and sun dried for complete dryness. Sun dried onion was crushed to powder with the help of grander and mixed in the diet at the levels of 0% (control), 1%, 2% and 3% dry food for the four experimental groups. The approximate chemical composition of formulated diet was determined by hit and trial method (Table I).

Table I.- Feed formulation for control and treatment group.

Ingredients	Control	1% onion powder	2% onion powder	3% onion powder
Fish meal	33%	33%	33%	33%
Cotton seed meal	25%	25%	25%	25%
Sunflower meal	18%	18%	18%	18%
Wheat bran	21%	20%	19%	18%
Onion	0%	1%	2%	3%
Fish oil	1%	1%	1%	1%
Molasses	1%	1%	1%	1%
Vitamin premix	1%	1%	1%	1%

Growth performance and biometric parameters

In order to analyze the growth catalogs of the grass carp juveniles, all fish from each aquarium were sampled weekly after 12 h of last feeding. At the end of the feeding trial, body weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) were calculated according to the formulas given by Bekcan *et al*. (2016):

$$WG(\%) = 100 \times \frac{\text{Final BW} - \text{Initial BW}}{\text{Initial BW}}$$

$$FCR = \frac{\text{Total feed given (g)}}{\text{Wight gain (g)}}$$

$$SGR = \frac{\ln(\text{Final wet BW}) - \ln(\text{Initial wet BW})}{\text{Number of periods}} \times 100$$

Where, WG is weight gain and BW is body weight.

Hematological assays

At the termination of the experiment, 5 fish were taken randomly from each tank and about 1ml of blood was drawn from the caudal vein in heparinized vacationer for blood cell investigations. The erythrocyte (RBC) and leukocyte (WBC) counts were determined using a Neubaeur hemocytometer (Blaxhall and Daisley, 1973). Hemoglobin levels (Hb) were obtained by the cyanomethemoglobin spectrophotometry method (Dorafshan *et al*., 2008). To estimate the differential leukocyte counts (lymphocytes, neutrophils, and eosinophils), blood smears were prepared, air-dried, fixed in methanol, and stained using Giemsa solution (Blaxhall and Daisley, 1973).

Organoleptic evaluation

At the end of trial, 5 fish were randomly collected from each treatment, degutted, well cleaned and uniformly filleted. One tablespoon iodized salt was sprinkled on each fillet and then steamed in Orient microwave oven at medium high temperature for 12 min. The samples were allowed to equilibrate with room temperature before serving to panel of judges for sensory evaluation (Khan *et al*., 2011). Sensory descriptors were defined for the parameters of odor, texture, flavor, whiteness, oiliness and overall acceptability.

Chemical analysis

The proximate analysis was done at the end of trial for dry matter, crude protein, and ash content according to AOAC (2010). Three samples of each trial were dried in oven at 60ºC and grind. Dry matter (DM) was determined by oven drying, ash contents by muffle furnace at 600°C and crude protein (CP) by Kjeldhal analysis using following formulas:

$$Ash(\%) = \frac{\text{Ash weight(g)}}{\text{Weight of dry sample(g)}} \times 100$$

$$\text{Crude protein } (\%) = \frac{\text{Sample} \times 0.875}{\text{Sample weight}}$$

Where, volume is the volume for titration of sample and 0.875 is the factor for protein.

Statistical analysis

The data was subjected to ANOVA for statistical significance among treatments. Mean values were compared to assess their intensity of significance among treatment groups by Duncan's multiple range test. The SAS (statistical Analysis Software) version 9.1 was used for all statistical analysis due to its wider application.

RESULTS

Growth parameters

The initial average body weight of grass carp in control, treatment 1, 2 and 3 were 7.85±2.7g, 7.78±2.2g, 7.64±2.4g and 9.34±1.7 g, respectively while the final body weight of grass carp were 9.7±0.95g, 11.13±0.3g, 10.28±0.48g and 12.25±0.74g for control, treatment 1, 2 and 3, respectively. The maximum weight gain was

Table II.- Average body weight (g) and net weight gain of grass carp on weekly basis (n=10).

No. of netting	Weakley	Control group		T1 (0.P 1%)		T2 (O.P 2%)		T4 (O.P3%)	
		Avg. BW (g)	NWG (g)	Avg. BW (g)	NWG (g)	Avg. BW (g)	NWG (g)	Avg. BW (g)	NWG (g)
Stocking	01-11-2015	7.85	-	7.78	-	7.64	-	9.30	-
1	09-11-2015	7.85±0.43	-	7.78±0.32	-	7.64±0.35	-	9.30±0.35	-
2	17-11-2015	8.05±0.38	0.2	8.13±0.35	0.4	7.99±0.25	0.35	9.83±0.31	0.53
3	25-12-2015	8.36±0.56	0.31	8.53±0.36	0.4	8.62±0.43	0.63	10.56±0.45	0.73
4	13-12-2015	8.6±0.66	0.24	8.59±0.45	0.06	8.85±0.49	0.23	10.71±0.65	0.15
5	11-12-2015	8.8±0.59	0.2	9.4±0.56	0.81	9.29±0.69	0.44	10.82±0.56	0.11
6	18-12-2015	9.0±0.54	0.3	9.80±0.59	0.5	9.49±0.68	0.20	11.23±0.76	0.42
7	28-12-2015	9.3±0.86	0.4	10.39±0.5	0.74	10.02±0.48	0.53	11.65±0.56	0.42
8	10-01-2016	9.7±0.95	o.4	11.13±0.3	0.91	10.28±0.48	0.26	12.25±0.74	0.6

Avg. BW, average body weight; NWG, net weight gain.

Table III.- Growth performance and feed utilization of grass carp fingerlings in different treatment groups (n=10).

Parameters	Control	T1 (1%)	T2 (2%)	T3 (3%)
Initial weight (g)	7.85±0.43	7.78±0.32	7.64±0.35	9.30±0.35
Final weight (g)	9.7±0.95	11.13±0.3	10.28±0.48	12.25±0.74
Net weight gain (g)	1.90±0.29^{d}	3.50±0.47^{a}	3.20±0.23^{b}	3.00 ±0.47^{c}
Percent weight gain	24.20±0.25^{d}	43.52±0.71^{a}	41.89 ±0.45^{b}	31.95 ±0.27^{c}
Feed conversion ratio (FCR)	5.42 ±0.007^{a}	3.13±0.42^{d}	3.31 ± 0.21^{c}	4.22±0.21^{b}
Specific growth rate (SGR)	0.36 ±0.007^{c}	0.59±0.14^{a}	0.46±0.14^{b}	0.46±0.14^{b}

observed in treatment 1 followed by treatment 2 and 3 (Table II). Table III indicates the values of net weight gain, FCR and SGR of experimental groups. Statistical analysis revealed that the control group had highest FCR and lowest SGR as compared to the diets containing onion powder. Lowest FCR and highest SGR were observed in T1 followed by T2 and T3. Water quality parameters were remaining within control range.

Hematology

Hematology of fish reveals that values of Hb and RBC showed significant differences in all experimental groups whereas; WBC and PLT vales were higher for T1 and T2 with respect to T3 and control. All other parameters *i.e.* neutrophils, lymphocytes, monocytes and eosinophils showed no significant differences between the experimental groups (Table IV).

Proximate analysis

Proximate analysis of experimental fish was given in Table V. Statistical analysis showed that feed with 1% onion powder produced fish with highest crude protein whereas, food with no onion gain lowest crude protein values. Dry mater did not showed significant differences among experimental groups however, moisture and ash values did not vary for control and T3 with respect to T1 and T2.

Table IV.- Hematological parameters of grass carp in different treatment groups.

Parameter	Control group	T1	T2	T3
Hb (g/dl)	7.6±0.2^{d}	8±0.1^{c}	8.8±0.3^{a}	8.5±0.1^{b}
RBC (x10^{6} µl)	0.88±0.2^{d}	1.25±0.1^{a}	1.10±0.1^{b}	0.92±0.1^{c}
WBC (10^{3} µl)	8±0.7^{b}	10.5±1.2^{a}	9.7±2.2^{a}	9±1.2^{b}
Thromb (10^{3} µl)	39±2.8^{b}	47±4.6^{a}	41±2.4^{a}	40±3.7^{b}
PCV (%)	30±1.0^{a}	35±2 .0^{a}	33±1.0^{a}	32±1.0^{a}
Neutophils (%)	5±4.0^{a}	8±2.0^{a}	7±2.0^{a}	6±2.0^{a}
Lymphosites (%)	69±1.0^{a}	72±3.0^{a}	71±4.0^{a}	70±6.0^{a}
Monocytes (%)	8±1.3^{a}	11±2.0^{a}	10±1.0^{a}	9±0.2^{a}
Eosinophils (%)	0.89±0.0^{a}	1.7±0.2^{a}	1±0.9^{a}	1±0.4^{a}

Table V.- Proximate analysis of fingerlings of grass carp (% on dry basis).

Ingredients (%)	Control group	T1 (OP-1%)	T2 (OP-2%)	T3 (OP-3%)
Crude protein	15±1.0^{c}	19.5±2.1^{a}	18.35±1.7^{a}	17±1.4^{b}
Dry matter	26.4±1.0^{a}	29±2.3^{a}	28.5±2.1^{a}	27.5±2.7^{a}
Moisture	73.6±1.0^{a}	71.00±0.1^{b}	71.5±0.2^{b}	72.5±2.3^{a}
Ash	2.7±0.3^{a}	2.15±0.9^{c}	2.31±0.5^{b}	2.56±0.1^{a}

Sensory evaluation

Sensory evaluation showed that odor, texture, flavor, whiteness, oiliness and overall acceptability of fish flesh showed significant differences (p>0.05) irrespective of diet composition among various dietary treatments (Table VI).

Table VI.- Organoleptic/sensory score of fish flesh against various treatments.

Character	T1	T2	T3	T4
Odor	6.41 ±0.26[a]	6.60±0.14[a]	6.67±0.46[a]	6.55±0.35[a]
Texture	6.55 0.21[a]	6.80±0.28[a]	6.65± 0.63[a]	7.06±0.36 [a]
Flavor	6.85 ±0.49[a]	6.70± 0.14[a]	7.20±0.42[a]	6.85±0.07[a]
Whiteness	6.45±0.21[a]	6.55 ± 0.21[a]	6.45±0.07[a]	6.45± 0.63[a]
Oiliness	6.55 ±0.21[a]	6.65±0.07[a]	6.45±0.07[a]	6.45±0.63[a]
Overall acceptability	6.56±0.04[a]	6.66 ±0.14[a]	6.68±0.11[a]	6.45 ±0.63[a]

DISCUSSION

The findings of present trial on Juvenile grass carp fed on onion showed that weight gain (WG), specific growth ratio (SGR) and feed conversion ratio (FCR) of fish fed with 1% and 2% onion was significantly higher than that of fish fed 3% and control diet. These results are in line with the Raza *et al*. (2015). They also use diets containing onion powder as 0 (control), 0.5 and 1% fed to beluga juvenile. At the end of the experiment, the highest weight gain and specific growth rate (SGR) was observed in group fed with 1% onion ($P<0.05$) with no significant difference ($P>0.05$) of weight gain in other treatment groups. Our results indicate that it has been reported that onion stimulates the digestive process, accelerating digestion and reducing food transit time in the gastrointestinal tract (Platel *et al*., 2001). Onion prebiotic activity is also being investigated by Benkeblia *et al*. (2006) and Sharma *et al*. (2006). They also find high soluble fiber content, like fructo-oligosaccharides which stimulate specific growth of microorganisms especially bifidobacteria and lactobacilli in the colon creating positive health effect just like studied by Ernst *et al*. (2000) and Binaii *et al*. (2014) and were in accordance with our studies.

Increase in growth performance and feed efficiency were recorded in juvenile beluga after feeding ginger (Kanani *et al*., 2014) and nettle (Binanii *et al*., 2014) that coincide with our studies. Studies of Cho *et al*. (2010) revealed that onion extract was one of the most effective dietary additives that improve weight gain of juvenile olive flounder. However, unlike this study, dietary inclusion of various 0, 0.5, 1, 2, 3 and 5% onion powder had no distinctive improvement on weight gain, specific growth rate and feed efficiency of the juvenile olive flounder. The effects of dietary additives on fish performance may vary depending on fish species, size, the dose of the additive, fish nutritional, physiological status, and or ambient culturing conditions.

Increase in white blood cells counts (WBC), erythrocyte count (RBC) and Hb level in experimental groups as compare to control group exhibiting positive health effect in grass carp. Fish feeding of onion diet demonstrates the immuno-stimulatory effects and anti-infection properties of onion which is similar to the work of Kanani *et al*. (2014) and Binaii *et al*. (2014) who obtained increased WBC after feeding juvenile beluga with ginger and nettle diets, respectively. The increase in WBC count and RBC count treated with 1% onion powder, also similar to the Raza *et al*. (2014). This is may be due to the quercetin found in onion that prevent fish from infection by triggering immune system. Results of Jian *et al*. (2004) were also in accordance with our results and support the phenomena that herbal plant could be act as immune-stimulants and increase WBC. However, in case of lymphocyte, neutrophil and monocyte in grass carp there was no significant differences which were closely related to the results of Kanani *et al*. (2014) and Binaii *et al*. (2014) in juvenile beluga.

Sensory evaluation of our experiment did not showed any significant effect in term of odor, texture, flavor, witness, oiliness and overall acceptability of grass carp fish flesh in all treatment groups. These results are similar in line with earlier researchers who reported similar sensory attributes when fish was fed on varying protein levels (Khan *et al*., 2011). In other studies, Khan *et al*. (2012) had similar findings when fingerlings of Indian major carps fed on plant based artificial feeds. But contrary to our study the sensory attributes like juiciness and tenderness scores of *Labeo rohita* differed significantly in treatments versus control. This difference could be due to the species difference. Findings of Tahir (2008) further support our non-significant differences result in taste and overall quality of the Indian major carps fed on different diets. From the present studies we can hypothesized that this type of fish, culture environment and type of feed offered have not significant effect on flesh quality of fish.

The chemical compositions of the whole body of grass carp at the end of the 8 week feeding trial are shown in Table V. These results were similar in line with Cho and Lee (2012) who reported that chemical compositions of the whole body of olive flounder at the end of the 8 week feeding trial was: moisture content ranged from 73.3 to 74.9%, crude protein content ranged from 17.4 to 18.6%, and ash content ranged from 3.5 to 3.8%. These measurements were affected by dietary concentrations of

onion powder. In oppose to this study inclusion of additives (*Opuntia ficus-indica*) did not affect the proximate composition of olive flounder (Park *et al*., 2003; Cho *et al*., 2010).

CONCLUSION

Results of this study demonstrated the onion powder as feed additive had a positive effect on growth performance; hematology and body composition of grass carp while sensory attributes remain same. Further, inclusion of 1% onion powder in feed was the best diet that improves growth and immunity of grass carp as compared to 2% and 3% diets.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Ajiboye, O.O., Yakubu, A.F. and Adams, T.E., 2012. A perspective on the ingestion and nutritional effects of feed additives in farmed fish species. *World J. Fish Mar. Sci*., **4**: 87-101.

AOAC, 2010. *Official method of analysis* (17th ed.) Association of official Analytical chmemist Washington D.C U.S.A.

Awad, E., Austin, D. and Lyndon, A.R., 2013. Effect of black cumin seed oil (*Nigella sativa*) and nettle extract (Quercetin) on enhancement of immunity in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Aquaculture,* **388-391**: 193-197. https://doi.org/10.1016/j.aquaculture.2013.01.008

Bekcan, S., Dogankaya, L. and Cakirogullari, G.C., 2006. Growth and body composition of European catfish (*Silurus glanis*) fed diet containing different percentages of protein. *Israeli J. Aquacult. Bamidgeh,* **58**: 137-142.

Benkeblia, N. and Shiomi, N., 2006. Hydrolysis kinetic parameters of DP 6, 7, 8 and 9e12 fructooligosaccharides (FOS) of onion bulb tissues. Effect of temperature and storage time, *J. Agric. Fd. Chem.,* **54**: 2587-2592. https://doi.org/10.1021/jf052848i

Bhosale, S.V., Bhilave, M.P. and Nadaf, S.B., 2010. Formulation of fish feed using ingredients from plant sources. *Res. J. Agric. Sci.,* **1**: 284-287.

Binaii, M., Ghiasi, M., Farabi, S.M.V., Pourgholam, R., Fazli, H., Safari, R., Alavi, S.E., Taghavi, M.J. and Bankehsaz, Z., 2014. Biochemical and hemato-immunological parameters in juvenile beluga (*Huso huso*) following the diet supplemented with nettle (*Urtica dioica*). *Fish Shellf. Immunol*., **36**: 46-51.

Blaxhall, P.C. and Daisley, K.W., 1973. Routine hematological methods for use with fish blood. *J. Fish Biol.,* **5**: 771-781. https://doi.org/10.1111/j.1095-8649.1973.tb04510.x

Cho, C.Y. and Kaushik, S.J., 1990. Nutritional energetics in fish: energy and protein utilization in rainbow trout (*Salmo gairdneri*). *World Rev. Nutr. Diet,* **61**: 132-172. https://doi.org/10.1159/000417529

Cho, H.C. and Lee, S.M., 2012. Onion powder in the diet of the Olive flounder (*Paralichthys olivaceus*) effects on the growth, body composition and lysozyme activity. *J. World Aquacult. Soc*., **43**: 30-38. https://doi.org/10.1111/j.1749-7345.2011.00489.x

Cho, S.H., Cho, Y.J., Lee, S. and Yoo, J., 2010. Effects of putative growth or health enhancing dietary additives on juvenile olive flounder, *Paralichthys olivaceus*, performance. *J. World Aquacult. Soc.,* **42**: 90-95. https://doi.org/10.1111/j.1749-7345.2010.00447.x

Citarasu, T., 2010. Herbal biomedicines: A new opportunity for aquaculture industry. *Aquacult. Int*., **18**: 403-414. https://doi.org/10.1007/s10499-009-9253-7

Cui, Y., Liu, X.F., Wang, S.M. and Chen, S.L., 1992. Growth and energy budget in young grass carp *Ctenopharyngodon idella* fed plant and animal diets. *J. Fish Biol.,* **41**: 231-238. https://doi.org/10.1111/j.1095-8649.1992.tb02653.x

Dorafshan, S., Kalbassi, M.R., Pourkazemi, M., Mojazi-Amiri, B. and Soltan-Karimi, S., 2008. Effects of triploidy on the Caspian salmon (*Salmo trutta*, caspius) haematology. *Fish Physiol. Biochem.,* **34**: 195-200. https://doi.org/10.1007/s10695-007-9176-z

Ernst, M. and Feldheim, W.J., 2000. Fructans in higher plants and in human nutrition. *Angew. Bot.,* **74**: 5-9.

Erondu, E.S., Bekibela, D. and Gbulubo, A.T., 2006. Optimum crude protein requirement of cat fish, *Chrysichthys nigrodigitatus*. *J. Fish Int.,* **1**: 1-2.

Fiertak, A. and Kilarski, W.M., 2002. Glyco-conjugates of the intestinal goblet cells of four cyprinids. *Cell. Mol. Life Sci.,* **59**: 1724-1733. https://doi.org/10.1007/PL00012500

Guillary, V. and Gasaway, R.D., 1978. Zoogeography of the grass carp in the United States. *Trans. Am. Fish. Soc*., **107**: 105-112. https://doi.org/10.1577/1548-8659(1978)107<105:ZOTGCI>2.0.CO;2

Jian, J. and Wu, Z., 2004. Influence of traditional Chinese medicine on non-specific immunity of

Jian carp (*Cyprinus carpio* var. jian). *Fish Shellfish Immunol*., **16**: 185-191.

Kanani, H.G., Nobahar, Z., Kakoolaki, S.H. and Jafarian, H., 2014. Effect of ginger and garlic-supplemented diet on growth performance, some hematological parameters and immune responses in juvenile *Huso huso*. *Fish Physiol. Biochem.,* **40**: 481-490. https://doi.org/10.1007/s10695-013-9859-6

Khalil, R.H., Nadia, B.M. and Soliman, M.K., 2001. Effects of biogen and levamisole HCl on the immune response of cultured *Oreochromis niloticus* to *Aeromonas hydrophila* vaccine. *Beni-Suef Vet. Med. J. Egypt*, **11**: 381-392.

Khan, M.A., Ahmad, I. and Abidi, S.F., 2004. Effect of ration size on growth, conversion efficiency and body composition of fingerling mrigal, *Cirrhinus mrigala* (Hamilton). *Aquacult. Nutr*., **10**: 47-53. https://doi.org/10.1046/j.1365-2095.2003.00279.x

Khan, N., Qureshi, N.A., Nasir, M., Rasool, F. and Iqbal, K.J., 2011. Effect of artificial diet and culture systems on sensory quality of fried fish flesh of Indian Major Carps. *Pakistan J. Zool.,* **43**: 1177-1182.

Khan, N., Qureshi, N.A., Nasir, M., Vandenberg, G.W., Mughal, M.S., Maqbool, A. Jabbar, M.A. and Zikria, N., 2012. Effect of artificial feed on sensory attributes of flesh of Indian major carps (*Labeo rohita, Catla catla* and *Cirrhinus mrigala)* fed in monoculture and polyculture systems. *Pak. Vet. J.,* **32**: 349-353.

Logambal, S.M. and Michael, R.D., 2000. Immunostimulatory effect of leaf of *Ocimum sanctum* in *oreochromis mossambicus* (peters). *Aquaculture,* **430**: 113-120.

Park, S.U., Kwon, M.G., Lee, Y.H., Shin, I.S. and Min, S.M., 2003. Effects of supplemental *Undaria*, obosan and wasabi in the experimental diets on growth, body composition, blood chemistry and non-specific immune response of juvenile flounder, *Paralichthys olivaceus*. *J. Aquacult.*, **16**: 210-215.

Pês, T.S., Saccol, E.M.H., Ourique, G.M., Londero, E.P., Gressler, L.T., Finamor, I.A., Rotili, D.A., Golombieski, J.I., Glanzner, W.G., Llesuy, S.F., Gonçalves, P.B.D., Radünz Neto, J., Baldisserotto, B. and Pavanato, M.A., 2015. Effect of diets enriched with rutin on blood parameters, oxidative biomarkers and pituitary hormone expression in silver catfish (*Rhamdia quelen*). *Fish Physiol. Biochem. Res. Int*., **25**: 289-307.

Platel, K. and Srinivasan, K., 2001. Studies on the influence of dietary spices on food transit time in experimental rats. *Nutr. Res.,* **21**: 1309-1314. https://doi.org/10.1016/S0271-5317(01)00331-1

Raza, Y. Iri, H., Khosh, B. and Razeghi, M., 2014. Effect of dietary supplementation of fructooligo saccharide (FOS) on growth performance, survival, lactobacillus bacterial population and hemato-immunological parameters of stellate sturgeon (*Acipenser stellatus*) juvenile. *Fish Shellfish Immunol.,* **35** : 1235-1239.

Raza, G., Ahma, R. and Ali, G., 2015. Effects of dietary onion (*Alliumcepa*) powder on growth, innate immune response and hemato-biochemical parameter of beluga (*Husohuso linnaeus*, 1754) juvenile. *Fish Shellfish Immunol*., **45**: 828-834. http://dx.doi.org/10.1111/anu.12219

Saccol, E.M.H., Uczay, J., Pês, T.S., Finamor, I.A., Ourique, G.M., Riffel, A.P.K., Schimidt, D., Caron, B.O., Heinzmann, B.M., Llesuy, S.F., Lazzari, R., Baldisserotto, B. and Pavanato, M.A., 2013. Addition of *Lippia alba* (Mill) N.E. Brown essential oil to the diet of the silver catfish: An analysis of growth, metabolic and blood parameters and the antioxidant response. *Aquaculture*, **416**: 244-254. https://doi.org/10.1016/j.aquaculture.2013.09.036

Shalaby, A.M., Khattab, Y.A. and Abdel-Rahman, A.M., 2006. Effects of garlic (*Allium sativum*) and chloramphenicol on growth performance, physiological parameters and survival of Nile tilapia (*Oreochromis niloticus*). *J. Venom. Anim. Toxins Trop. Dis*., **12**: 172-201.

Sharma, A.D., Kainth, S. and Gill, P.K., 2006. Inulinase production using garlic *(Allium sativum*) powder as a potential substrate in *Streptomyces* sp. *J. Fd. Engin.,* **77**: 486-491. https://doi.org/10.1016/j.jfoodeng.2005.06.072

Sheikh, B.A. and Sheikh, S.A., 2004. Aquaculture and integrated farming system. *Pak. J. Agric. agric. Engg. Vet. Sci.,* **20**: 52-58.

Sheunn, D.Y., Tain, S.L., Chyng, H.L. and Hung, K.P., 2003. Influence of dietary protein levels on growth performance, carcass composition and liver lipid classes of juvenile *Spinibarbus hollandi* (Oshima). *Aquacult. Res.,* **34**: 661-666. https://doi.org/10.1046/j.1365-2109.2003.00880.x

Singh, P.K., Gaur, S.R., Barik, P., Sulochana, S., Shukla, S. and Singh, S., 2005. Effect of protein levels on growth and digestibility in the Indian major carp, *Labeo rohita* (Hamilton) using slaughter house waste as the protein source. *Int. J. Agric. Biol.,* **7**: 939-941.

Tahir, M.Z.I., 2008. *Studies on partial replacement of fish meal with oil seed meals in the diet of major carps in semi-intensive culture system*. Ph.D.

Thesis. Department of Zoology, University of Agriculture, Faisalabad, Pakistan, pp. 174-178.
Zhai, S.W. and Liu, S.L., 2013. Effects of dietary quercetin on growth performance, serum lipids level and body composition of tilapia (*Oreochromis niloticus*). *Ital. J. Anim. Sci.,* **12**: 85. https://doi.org/10.4081/ijas.2013.e85

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Assessment of Copper Induced Genotoxicity in Peripheral Erythrocytes of *Cirrhina mrigala* by using Comet Assay and Micronucleus Test

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Authors' Contribution
KS designed and performed the experiments, collected and analyzed the data, and wrote manuscript. MJ reviewed the final manuscript. RI helped in data collection. FA, AA, AB and HA helped in writing manuscript.

Key words
***Cirrhina mrigala*, Copper, DNA damage, Peripheral erythrocytes, Comet assay, Micronucleus test.**

ABSTRACT

A wide variety of agricultural and industrial chemicals containing heavy metals are contaminating the aquatic ecosystem. Heavy metals are biologically non-degradable that can cause severe toxicity in aquatic animals through oxidative stress to various biological molecules. The present experiment was conducted to determine concentration dependent DNA damage in peripheral erythrocytes of 150-day old *Cirrhina mrigala* by using comet assay and micronucleus test under controlled laboratory conditions. Fish were exposed to four different sub-lethal concentrations *viz*. 17%, 25%, 33% and 50% of LC_{50} of copper, separately, for 30 days. Peripheral blood of chronically exposed fish was examined for damaged nuclei (%), genetic damage index (GDI), cumulative tail length (µm) micronuclei frequency (%) and frequency of other nuclear abnormalities. Chronic exposure of copper to *Cirrhina mrigala* induced DNA damage in the erythrocytes of fish that varied significantly ($p<0.05$) with exposure concentration. The DNA damage caused by copper was significantly higher than that recorded in negative control group. Values for percentage of damaged nuclei (78.00±2.00 %), GDI (2.46±0.04) and cumulative tail length of comets (238.00±0.39 µm) were significantly higher at 50% of copper LC_{50} exposure to the fish while this damage was significantly minimum at 17% of LC_{50}. Significantly higher micronuclei frequency of 35.05±0.88% was observed at 50% of LC_{50} of copper while it remained significantly least (12.21±0.54 %) due to 17% of LC_{50} concentration exposure. This study reveals that both comet assay and micronucleus test can be used as useful tools for the determination of genotoxic effects of metals on fish.

INTRODUCTION

Genotoxic compounds from industries and agriculture runoff represent major ecological threat because they may cause very unusual disorders in animals that could be transmitted to the progeny (Haldsrud and Krokje, 2009). Xenobiotics, especially heavy metals, are present in aquatic ecosystems of Pakistan (Rauf *et al*., 2009; Jabeen *et al*., 2012). Metals gained popularity because of their toxic effects and accumulation in aquatic organisms especially in fish (Javed, 2006; Mudgal *et al*., 2010). Heavy metals generate reactive oxygen species which may cause oxidative damage including oxidation of DNA, lipid peroxidation and enzyme inactivation (Sevcikova *et al*., 2011). In general, metallic ion genotoxicity appears to be associated with the formation of reactive oxygen species (Soto-Reyes *et al*., 2005). Oxidative damage to DNA due to reactive oxygen species, DNA strand breakage occurs which represents a major class of DNA damage under oxidative stress (Cadet *et al*., 1997; Shaukat *et al*., 2018).

A growing interest in the environmental genotoxicity studies has led to the development of several tests for detecting genotoxicants in aquatic media (Fenech *et al*., 2003). Both micronuclei and Comet assay have recently gained popularity over other assays in aquatic toxicity research due to their sensitivity for detecting DNA damage at single cell level (Bopp *et al*., 2008). Comet assay is used as one of the best approaches to study the genotoxic effects of pollutants on fish (Avishai *et al*., 2002). It is considered reliable, responsive and fast technique (Tice *et al*., 2000) for the detection of DNA single/double strand breakage and alkali-labile sites, induced in individual eukaryotic cells (Kim *et al*., 2002). This assay has been very affectively applied to blood erythrocytes of various fish species exposed to different genotoxicants (Matsumoto *et al*., 2006). Micronuclei test has been widely used to

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determine the clastogenic effects of a genotoxicants on fish erythrocytes. It is a standard assay to determine chromosomal aberrations during initial damage in S-phase (Obe *et al*., 2002).

The heavy discharge of untreated water into the rivers of Pakistan is badly affecting the aquatic ecosystem especially fresh water fisheries (Jabeen *et al*., 2012). Comparative toxicity studies are important to identify the toxic effects of metals on the fish as well as their tolerance limits to devise proper strategies for their conservation in the natural aquatic habitats. Thus, it is imperative to study the acute and chronic responses of fish towards metallic ions that could induce genotoxicity in their bodies. This study was conducted to determine the extent of DNA damage in fish peripheral erythrocytes of *Cirrhina mrigala* during sub-lethal chronic exposure of copper. This work will help in sustainable conservation of fresh water fisheries in Pakistan.

MATERIALS AND METHODS

The present experiment was conducted, to determine the extent of DNA damage in the peripheral erythrocytes of copper exposed *Cirrhina mrigala*, in the wet laboratory of Fisheries Research Farms, University of Agriculture, Faisalabad.

Procurement of fish and solution preparation

For this experiment fingerlings were purchased from Fish Seed Hatchery, Faisalabad. All the fish fingerlings were acclimatized to laboratory conditions for two weeks prior to experiments. Fingerlings were fed to satiation on feed (34% DP and 3.00Kcal/g DE) twice daily and also exposed to 12 h photoperiod using fluorescent light. After acclimatization, only healthy fingerlings were selected for the experiments. Analytical grade copper was selected to test their effects on *Cirrhina mrigala*. For the preparation of stock solutions, appropriate quantity of copper ($CuCl_2.6H_2O$:Merck) was poured into 1000 ml flask and flask was filled upto 1000 ml with deionized water. The stock solutions were diluted to make required concentrations of metallic ions.

Experimental conditions

The 96-h LC_{50} value of copper for *Cirrhina mrigala* determined in previous experiment (Kousar and Javed, 2012) was used to prepare four different sublethal concentrations (17%, 25%, 33% and 50% of 96-h LC_{50}) of copper. Fingerlings of *Cirrhina mrigala* were exposed to sub-lethal concentrations of copper in glass aquaria, separately, with three replications under controlled laboratory conditions at constant water pH (7.5), temperature (30^oC) and hardness (300mgL^{-1}). However, control fish of each species were kept unstressed in metal free clean tap water for comparison. The exposure concentrations of copper at four sublethal levels for *Cirrhina mrigala* are given in Table I.

Continuous air was supplied to all the aquaria with automatic air pump through capillary system. The fish under chronic metal's exposure were fed to-satiation twice a day (at 0900 and 1700 h) throughout the period of 30 days. During experiments the test media were replaced weekly and desired metallic ion concentrations were maintained throughout test duration of 30 days. At the end of 30-day exposure to various concentrations of copper, the DNA damage in peripheral erythrocytes of fish was evaluated by using Comet assay and Micronucleus test.

Table I.- The exposure concentrations of copper at four sublethal levels for *Cirrhina mrigala.*

Treatments	Sub-lethal concentrations of copper (mgL^{-1})	Exposure concentrations (mgL^{-1})
Copper	17% of 96 h LC_{50}	2.92
	25% of 96 h LC_{50}	4.29
	33% of 96 h LC_{50}	5.67
	50% of 96 h LC_{50}	8.59
-ve Control	Metal free tap water	--
+ve Control	Cyclophosphamide	20µgg^{-1} body weight

Comet assay (single cell gel electrophoresis)

Fish blood samples were processed for comet assay according to Singh *et al*. (1988) with minor modifications. After 30-day metallic ions exposure period, blood samples were collected from the caudal vein of fish (n = 5) for Comet assay through sterilized syringe. Utmost care was exercised to avoid the mixing of water and mucus into the blood samples. Heparin sodium salt was used for the stabilization of the blood samples. Blood sample was centrifuged at 1000rpm for 2 min in order to separate erythrocytes from the whole blood. Each blood sample, collected from the fish, was diluted with 1 ml of phosphate buffer saline (PBS). Slides were prepared by applying three layers of agarose gel. Out of which second layer contain blood sample to be tested. After solidification of the gel, slides were immersed in cold lysing solution and refrigerated at 4°C for 1 h. After lysis, the slides were placed in a comet assay tank (CSL-COM 20; Cleaver, UK) filled with fresh electrophoresis solution. The slides were left in the solution for 20 min to allow the unwinding. Electrophoresis was performed by using the same solution at 25V, 300mA for a period of 25 min. The slides were neutralized gently and DNA stained with

ethidium bromide. One hundred and fifty cells (50 per replicate) were scored and examined randomly under Epi-Fluorescence microscope (N-400M, American Scope; UK) equipped with light source of mercury short arc reflector lamp filters for ethidium bromide at 400 X magnification and low lux camera. The DNA damage was quantified by visual classification of cells into five categories "comets" corresponding to the tail length, undamaged: Type 0, low-level damage: Type I, medium-level damage: Type II, high-level damage: Type III and complete damage: Type IV.

The %age of DNA damage was calculated as the mean percentage of cells with medium, high and complete damaged DNA by using following formula:

$$\%\text{age of DNA Damage} = \text{Types} - \text{II} + \text{III} + \text{IV}$$

From the arbitrary values assigned to the different categories (from Type-0 to Type-IV) a genetic damage index (GDI) was calculated for each subject by using following formula:

$$\text{GDI} = \frac{(\text{Type I}) + 2(\text{Type II}) + 3(\text{Type III}) + 4(\text{Type IV})}{\text{Type 0} + \text{Type I} + \text{Type II} + \text{Type III} + \text{Type IV}}$$

TriTek CometScore ™ software was used to calculate the comet tail length of damaged cells (Costa *et al*., 2011; Jose *et al*., 2011) and cumulative tail length (µm) was obtained by adding the tail length of all examined cells (n = 50/replicate).

Micronucleus test

A drop of blood taken from the fish caudal vein was directly smeared on slides and stained with the 10% Giemsa solution. The nuclear abnormalities and frequency of micronuclei were scored under oil emersion (100 X) lens of binocular microscope. A total of 2,000 erythrocytes (1000/slide) with intact cellular and nuclear membranes were examined for each fish specimen. The frequency of micronuclei and other nuclear abnormalities, including bi-nucleated, dumble, blebbed, notched and de-shaped nuclei were evaluated (per 1,000 cells) by scoring them at a 1,000 X magnification by using binocular microscope. Micronuclei were scored through criteria devised by Fenech *et al*. (2003) by using the following formula:

$$\text{Micronucleus frequency (\%)} = \frac{\text{No. of cells with micronuclei}}{\text{Total No. of cells counted}} \times 100$$

Statistical analyses of data

Statistical analyses were performed through MSTATC computer software and results were expressed as Means ± SD. Data means were compared for the statistical differences by using Duncan Multiple Range test (DMR) by following Steel *et al*. (1996) and a value of $p<0.05$ was accepted as statistically significant.

RESULTS

During present experiment, DNA damage and nuclear abnormalities in peripheral erythrocytes of *Cirrhina mrigala* were observed through Comet Assay and Micronucleus Test (Fig. 1). Peripheral erythrocytes of *Cirrhina mrigala* showed significantly ($p<0.05$) higher proportions of damaged nuclei at 50% copper LC_{50} exposure, followed by that of 33%, 25%, positive control, 17% and negative control with the mean values of 78.00±2.00, 68.00±2.00, 58.00±0.00, 46.67±0.00, 20.67±3.15 and 0.00±0.00%, respectively. The values of GDI, for four sublethal copper concentrations, positive and negative controls, ranged between a minimum value of 0.04±0.02 while it was maximum (2.46±0.04) due to negative control and 50% copper LC_{50} exposure, respectively. However, increase in copper exposure concentration caused concomitant increase in GDI value for the peripheral erythrocytes of fish. Cumulative tail length of comets also showed significant variability due to exposure of six concentrations with significantly maximum cumulative tail length of 238.00±0.39µm at 50% LC_{50} exposure, followed by that of 33%, 25%, positive control, 17% LC_{50} and negative control (Table II).

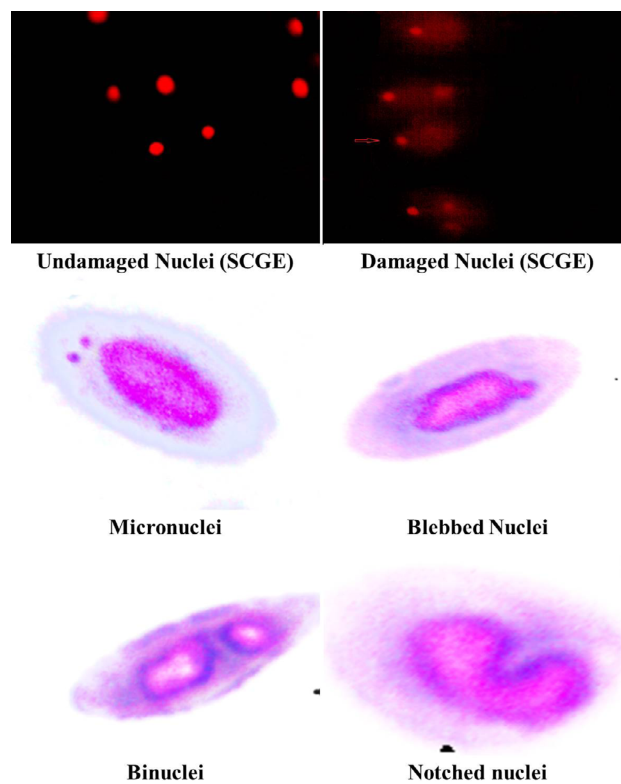

Fig. 1. DNA damage and nuclear abnormalities observed through Comet Assay and Micronucleus Test

Table II.- DNA damage in peripheral erythrocytes of *Cirrhina mrigala* exposed to copper.

Treatments	Exposure concentrations ($mg\ L^{-1}$)	Un-damaged nuclei (%)	Proportions of damaged nuclei (%)				% age of damaged cells (II+III+III)	GDI*	Cumulative tail length (µm)
		Type 0	Type I	Type II	Type III	Type IV			
-ve control	0.00	96.00±2.00a	4.00±2.00f	0.00±0.00f	0.00±0.00e	0.00±0.00f	0.00±0.00f	0.04±0.02f	3.66±0.26f
+ve control	CP (20 μgg^{-1})	28.00±0.00c	25.33±2.31b	26.67±1.15a	12.00±1.15c	8.00±2.00d	46.67±0.00d	1.47±0.06d	119.22±0.2d
17% of LC_{50}	2.92	34.67±1.15b	44.67±3.06a	12.00±2.00e	6.67±1.15d	2.00±0.00e	20.67±3.15e	0.97±1.43e	91.87±0.33e
25% of LC_{50}	4.29	24.00±2.00d	18.00±2.00d	19.33±1.15d	26.00±2.00b	12.67±1.15c	58.00±0.00c	1.85±0.02c	136.47±0.20c
33% of LC_{50}	5.67	12.00±0.00e	20.00±2.00c	20.67±1.15c	32.00±2.00a	15.33±1.15b	68.00±2.00b	2.19±0.06b	177.21±0.27b
50% of LC_{50}	8.59	8.67±1.15f	13.33±1.15e	24.00±2.00b	31.33±1.15a	22.67±1.15a	78.00±2.00a	2.46±0.04a	238.00±0.39a

The means with similar letters in a single column for each variable are statistically non-significant at $p<0.05$. *GDI, Genetic damage index = {Type I + 2(Type II) + 3(Type III) + 4(Type IV) / Type 0 + Type I + Type II + Type III + Type IV}; CP, Cyclophosphamide.

Table III.- Chronic exposure effects of copper on micronuclei frequency (mean±SD) and other nuclear abnormalities in the peripheral blood erythrocytes of *Cirrhina mrigala*.

	Negative control	Positive control	17% of LC_{50}	25% of LC_{50}	33% of LC_{50}	50% of LC_{50}
Micronuclei frequency (%)	0.24±0.12 f	13.57±0.33d	12.21±0.54 e	17.26±0.06 c	23.54±0.21 b	35.05±0.88 a
Other nuclear abnormalities (%)						
Binucleated cells	0.24±0.02 f	1.35±0.05 c	0.83±0.03 d	0.75±0.07 e	6.36±0.28 b	7.77±0.38 a
Cells with dumble shape nucleus	0.82±0.02 f	2.75±0.03 d	1.67±0.02 e	2.99±0.05 c	5.26±0.21 b	6.69±0.29 a
Cells with blebbed nucleus	0.14±0.04 f	1.96±0.06 e	3.19±0.07 c	2.19±0.12 d	4.88±0.08 b	6.78±0.17 a
Cells with notched nucleus	0.82±0.04 f	1.68±0.04 d	2.21±0.01 b	2.39±0.02 a	1.44±0.16 e	2.16±0.03 c
Deshaped cells	0.10±0.01 f	3.64±0.03 e	4.95±0.03 d	6.93±0.10 c	7.03±0.09 b	10.42±0.21 a
Total frequency of other nuclear abnormality	2.12±0.37 f	11.38±0.92e	12.84±1.58 d	15.26±2.32 c	24.98±2.17 b	33.82±2.98 a
Total number (±SD) of analyzed cells	2072±3.91	2145±2.77	2040±3.72	2005±3.27	2090±4.28	2034±3.72

Means with similar letters in a single row are statistically non-significant at $p<0.05$.

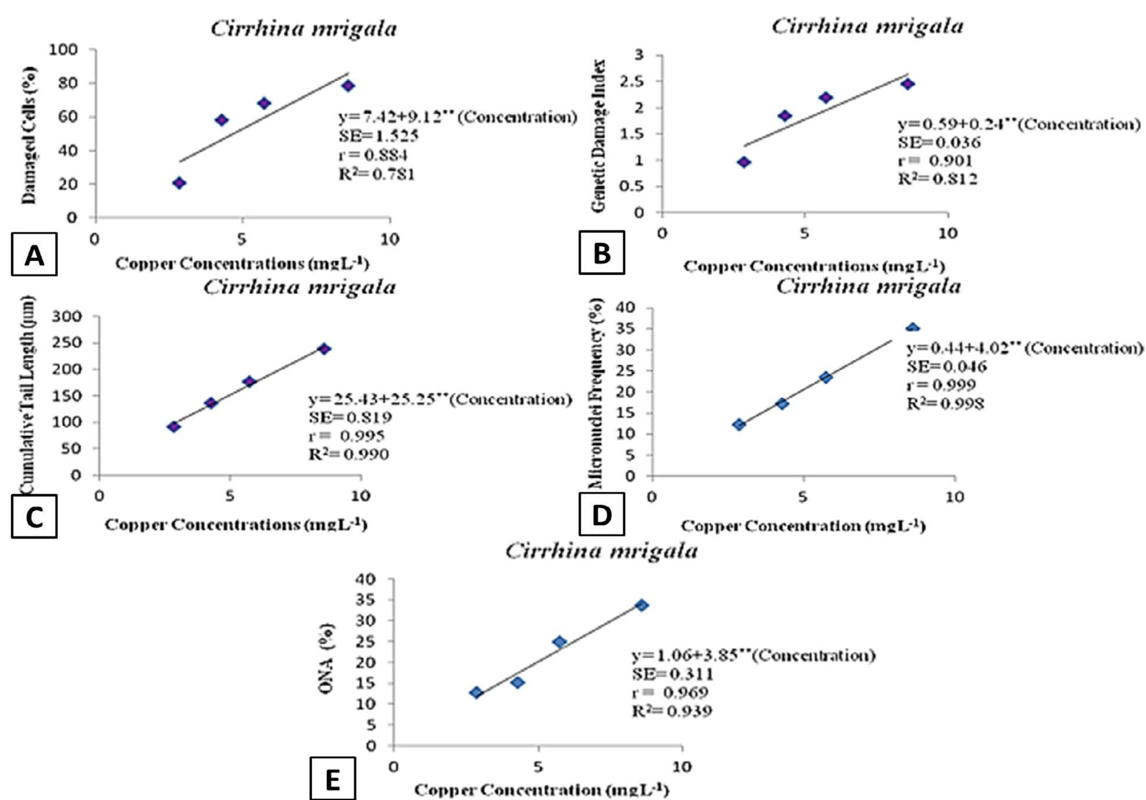

Fig. 2. Relationships between exposure concentrations of copper and DNA damage in peripheral erythrocytes of fish.

The frequency of micronuclei in peripheral erythrocytes of *Cirrhina mrigala* was significantly higher as 35.05±0.88 % at 50% copper LC_{50}, followed by that of 23.54±0.21, 17.26±0.06, 13.57±0.33, 12.21±0.54 and 0.24±0.12% observed due to 33%, 25%, positive control, 17% LC_{50} and negative control treatments, respectively. Frequency of all other nuclear abnormalities, except cells with notched nuclei, was significantly higher at 50% LC_{50} exposure. However, positive control caused significantly lower frequency of other nuclear abnormalities as compared to 50% LC_{50} (Table III).

Relationships between exposure concentrations and DNA damage

All the four exposure concentrations of copper (17%, 25%, 33% and 50% of Cu LC_{50}) to *Cirrhina mrigala* were analyzed separately for their impacts on DNA damage determined in-terms of percentage of damaged cells (%), genetic damage index (GDI), cumulative tail length of comets (µm) and frequency of micronuclei and other nuclear abnormalities. Regression models computed for fish under copper exposure revealed linear (significantly positive) relationship between exposure concentration of copper and DNA damaged cells in peripheral erythrocytes of fish. The higher values of R^2 for the regression equation reveal high precision of this regression model (Fig. 2A). The genetic damage index and cumulative tail length of comets of peripheral erythrocytes in fish showed significantly positive correlation with the exposed concentration of copper also (Fig. 2B, C). The high values of correlation coefficient (r) for each regression equation show strong relationship between concentrations of exposure metal and genotoxic damage in fish. The micronuclei frequency and frequency of other nuclear abnormalities in peripheral erythrocytes of fish exhibited highly significant and direct relationships with the exposure concentration of copper. Significantly higher values of R^2 for each regression equation reveal high reliability of these regression models (Fig. 2D, E).

DISCUSSION

Contamination of heavy metals poses a serious threat to the aquatic organisms especially fish due to their ability to induce oxidative stress through production of reactive oxygen species that leads to oxidation of bio-molecules

like DNA (Sevcikova *et al*., 2011). Metals may change physiological processes and biochemical parameters of blood and tissues (Basa and Rani, 2003; Saravanan and Mohamed, 2003). In contaminated waters, the toxicity of metallic ions is severe because they form complexes with sulfhydryl groups in proteins and small bio-molecules (Foulkes, 2000) resulting into increased vulnerability of aquatic animals (Liao *et al*., 2004). Deb and Santra (1997) observed marked variability in specific toxicity and accumulation of copper and zinc for different fish species. However, toxic effects of metals were strongly influenced by the responses of tested fish to the amount of metals entering into the fish body as well as the rate of their retention and excretion as observed during present investigation (Sobha *et al*., 2007). Due to industrial advancement, a variety of toxic chemicals, including metals, are released into the aquatic environments of Pakistan (Jabeen *et al*., 2012) which not only disturb the physico-chemical properties of the water bodies but also influence the aquatic food chain to cause physiological and cytogenetic alterations in the aquatic animals (Barbosa *et al*., 2009). Metals can also act through redox cycle to induce ROS which possibly cause DNA strand breakage (Ventura-Lima *et al*., 2009).

Comet assay has gained popularity in eco-toxicological studies and successfully employed to detect DNA damage in aquatic organisms (Frenzilli *et al*., 2009) especially in fish (Kosmehl *et al*., 2008; Pereira *et al*., 2009). This assay allows examination of whole genome at any step of the cell cycle, rather than just during mitosis (Sunjog *et al*., 2012). Metals produce ROS either through redox cycling or by impairing the antioxidant defense system of animals (Stohs and Bagchi, 1995). In addition to that Fenton reactions are also important for the generation of ROS (Valko *et al*., 2005). Single strand breakage occurred either due to the direct attack of ROS on the deoxyribose or its indirect effects on intermediate steps of excision repair pathway leading to disruption of repair process of oxidized DNA bases. This all ultimately ends up in double strand breakage during the process of replication (Hedge *et al*., 2008). Generally, the DNA damage resulting from metal toxicity is broadly used as biomarker of biological effects of toxicants (van der Oost *et al*., 2003). With increasing DNA damage in the cell nucleus, more broken DNA fragments migrate towards the tail region of the comet resulting in an increased amount of fluorescence in the tail region, as well as enlargement of tail length (Mitchelmore and Chipman, 1998) as observed during present investigation. Comet tail length has been appeared as a basic parameter in quantifying DNA damage in fish peripheral erythrocytes.

Micronucleus test has been commonly used for the estimation of biological impacts of water pollutants on genotoxic damage in fish (Ergene *et al*., 2007). Micronuclei are cytoplasmic chromatin containing bodies developed from broken section of chromosome or from the chromosomes that could not be incorporated into daughter nuclei (Fagr *et al*., 2008). Various metallic ions act as valuable genotoxins at particular concentrations just because of their ability to bind to thiol groups and induce instability in the spindle formation in the cells (Patra *et al*., 2004).

Jiraungkoorskul *et al*. (2007) exposed three fish species *viz*. *Oreochromis niloticus*, *Poronotus triacanthus* and *Puntius altus* to 25% of 96-h LC_{50} of lead, copper and cadmium and reported significant increase in micronuclei frequency and other nuclear abnormalities (notched, blebbed, lobed and binucleated cells) in fish erythrocytes after 48-h exposure. The overall ability of metals to cause DNA damage followed the order: lead > cadmium > copper. Previous literature indicated a relationship between genotoxicity response variability and metabolic and pharmacokinetic factors in animals (Al-Sabti and Metcalfe, 1995). In general, nuclear abnormalities like notched, blebbed, lobed and binucleated cells can be considered as good indicators of genotoxic damage and therefore, they may complement micronuclei scoring in routine genotoxicity surveys.

The DNA damage, determined in-terms of percentage of damaged cells (%), GDI, cumulative tail length (µm), micronuclei frequency (%) and frequency of other nuclear abnormalities (%) showed significantly direct relationships with the metallic ion concentrations. Ergene *et al*. (2007) reported significant linear relationship between metal concentration (Cu, Cd, Ni, Pb) and frequency of micronuclei and other nuclear abnormalities in erythrocytes of mullet and catfish. Summak *et al*. (2010) also reported significantly positive correlation (r=0.980) between metal concentrations and frequency of nuclear abnormalities in *Oreochromis niloticus* also. Kousar and Javed (2014) reported arsenic concentration dependent increase in DNA damage in peripheral erythrocytes of major carps. Similarly, Ambreen and Javed (2016) also reported increase in DNA damage (%age of damaged cells, GDI, cumulative tail length of comets) in erythrocytes of *Cyprinus carpio* with gradual increase in concentration of Cd+Pb mixture.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Al-Sabti, K. and Metcalfe, C.D., 1995. Fish micronuclei for assessing genotoxicity in water. *Mutat. Res*., **343**: 121-135. https://doi.org/10.1016/0165-1218(95)90078-0

Ambreen, F. and Javed, M., 2016. Effect of 30 day sublethal exposure of cadmium and lead mixture in DNA damage in fish. *J. Anim. Pl. Sci*., **26**: 674-679.

Avishai, N., Rabinowitz, C., Moiseeva, E. and Rinkevich, B., 2002. Genotoxicity of the Kishon River, Israel: The application of an in vitro cellular assay. *Mutat. Res*., **518**: 21-37. https://doi.org/10.1016/S1383-5718(02)00069-4

Barbosa, J.S., Cabral, T.M., Ferreira, D.N., Agnez-Lima, L.F. and de-Medeiros, S.R., 2009. Genotoxicity assessment in aquatic environment impacted by the presence of heavy metals. *Ecotoxicol. Environ. Safe*., **73**: 320-325. https://doi.org/10.1016/j.ecoenv.2009.10.008

Basa, S. and Rani, P.S., 2003. A cadmium induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). *Ecotoxicol. Environ. Safe*., **56**: 218-221. https://doi.org/10.1016/S0147-6513(03)00028-9

Bopp, S.K., Abicht, H.K. and Knauer, K., 2008. Copper-induced oxidative stress in rainbow trout gill cells. *Aquat. Toxicol*., **86**: 197-204.

Cadet, J., Berger, M., Douki, T. and Ravanat, J.L., 1997. Oxidative damage to DNA: Formation, measurement and biological significance. *Rev. Physiol. Biochem. Pharmacol*., **131**: 1-87. https://doi.org/10.1007/3-540-61992-5_5

Costa, P.M., Neuparth, T.S., Caeiro, S., Lobo, J., Martins, M., Ferreira, A.M., Caetano, M., Vale, C., DelValls, T.A. and Costa, M.H., 2011. Assessment of the genotoxic potential of contaminated estuarine sediments in fish peripheral blood: Laboratory versus in situ studies. *Environ. Res*., **111**: 25-36. https://doi.org/10.1016/j.envres.2010.09.011

Deb, C. and Santra, S.C., 1997. Bioaccumulation of metals in fishes. An *in vivo* experimental study of a sewage fed ecosystem. *Environmentalist*, **17**: 27-33. https://doi.org/10.1023/A:1018579312038

Ergene, S., Cavas, T., Celik, A., Koleli, N., Kaya, F. and Karahan, A., 2007. Monitoring of nuclear abnormalities in peripheral erythrocytes of three fish species from the Goksu Delta (Turkey): Genotoxic damage in relation to water pollution. *Ecotoxicology*, **16**: 385-391. https://doi.org/10.1007/s10646-007-0142-4

Fagr, A., El-Shehawi, A.M. and Seehy, M.A., 2008. Micronucleus test in fish genome: A sensitive monitor for aquatic pollution. *Afri. J. Biotechnol*., **7**: 606-612.

Fenech, M., Chang, W.P., Kirsch-Volders, M., Holland, N., Bonassi, S. and Zeiger, E., 2003. HUMN project: Detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte culture. *Mutat. Res*., **534**: 65-75. https://doi.org/10.1016/S1383-5718(02)00249-8

Foulkes, E.C., 2000. Transport of toxic heavy metals across cell membranes. *Exp. Biol. Med*., **223**: 234-240. https://doi.org/10.1046/j.1525-1373.2000.22334.x

Frenzilli, G., Nigro, M. and Lyons, B.P., 2009. The comet assay for the evaluation of genotoxic impact in aquatic environments. *Mutat. Res. Rev. Mutat. Res*., **681**: 80-92. https://doi.org/10.1016/j.mrrev.2008.03.001

Haldsrud, R. and Krokje, A., 2009. Induction of DNA double-strand breaks in the H4IIE cell line exposed to environmentally relevant concentrations of copper, cadmium, and zinc, singly and in combinations. *J. Toxicol. environ. Hlth*., **7**: 155-163. https://doi.org/10.1080/15287390802538964

Hedge, M.L., Hazra, T.K. and Mitra, S., 2008. Early steps in the DNA base excision/single strand interruption repair pathway in mammalian cells. *Cell Res*., **18**: 27-47. https://doi.org/10.1038/cr.2008.8

Jabeen, G., Javed, M. and Azmat, H., 2012. Assessment of heavy metals in the fish collected from the river Ravi, Pakistan. *Pak. Vet. J*., **32**: 107-111.

Javed, M., 2006. Studies on growth responses of fish during chronic exposure of nickel and managanese. *Pak. J. biol. Sci*., **99**: 318-322.

Jiraungkoorskul, W., Sahaphong, S. and Kangwanrangsan, N., 2007. Toxicity of copper in butterfish (*Poronotus triacanthus*): Tissues accumulation and ultra-structural changes. *Environ. Toxicol*., **22**: 92-100. https://doi.org/10.1002/tox.20238

Jose, S., Jayesh, P., Mohandas, A., Philip, R. and Singh, I.S.B., 2011. Application of primary haemocyte culture of Penaeus monodon in the assessment of cytotoxicity and genotoxicity of heavy metals and pesticides. *Mar. Environ. Res*., **71**: 169-177. https://doi.org/10.1016/j.marenvres.2010.12.008

Kim, B., Park, J.J., Edlaer, L., Fournier, D., Haase, W., Sautter-Bihl, M., Gotzes, F. and Thielmann,

H.W., 2002. New measurement of DNA repairs in the single-cell gel electrophoresis (comet) assay. *Environ. Mol. Mutagen*., **40**: 50-56. https://doi.org/10.1002/em.10090

Kousar, S. and Javed, M., 2014. Assessment of DNA damage in peripheral blood erythrocytes of fish exposed to arsenic under laboratory conditions. *Int. J. Curr. Microbiol. appl. Sci*., **11**: 877-888.

Kosmehl, T., Hallare, A.V., Braunbeck, T. and Hollert, H., 2008. DNA damage induced by genotoxicants in zebrafish (*Danio rerio*) embryos after contact exposure to freeze-dried sediment and sediment extracts from Laguna Lake (The Philippines) as measured by the comet assay. *Mutat. Res. Gen. Toxicol. Environ. Mutagen.,* **650**: 1-14.

Liao, C.M., Tsai, J.W., Ling, M.P., Liang, H.M., Chou, H.Y. and Yang, P.T., 2004. Organ-specific toxicokinetics and dose response of arsenic in tilapia *Oreochromiss mosambicus*. *Arch. environ. Contam. Toxicol*., **47**: 502-510. https://doi.org/10.1007/s00244-004-3105-2

Matsumoto, S.T., Mantovani, M.S., Malaguttii, M.I.A., Dias, A.U., Fonseca, I.C. and Marin-Morales, M.A., 2006. Genotoxicity and mutagenicity of water contaminated with tannery effluents, as evaluated by the micronucleus test and comet assay using the fish *Oreochromis niloticus* and chromosome aberrations in onion root-tips. *Genet. Mol. Biol*., **29**: 148-158. https://doi.org/10.1590/S1415-47572006000100028

Mitchelmore, J.K. and Chipman, C.L., 1998. DNA strand breakage in aquatic organisms and the potential value of the comet assay in environmental monitoring. *Mutat. Res*., **399**: 135-147. https://doi.org/10.1016/S0027-5107(97)00252-2

Mudgal, V., Madaan, N., Mudgal, A., Singh, R.B. and Mishra, S., 2010. Effects of metals on human health. *Open Nutraceutical. J.* **3**: 94-99.

Obe, G., Pfeiffer, P., Savage, J., Johannes, C., Goedecke, W., Jeppesen, P., Natarajan, A., Martinez-López, A., Folle, G. and Drets, M., 2002. Chromosomal aberrations: Formation, identification and distribution. *Mutat. Res*., **504**: 17-36. https://doi.org/10.1016/S0027-5107(02)00076-3

Patra, M., Bhowmik, N., Bandopadhyay, B. and Sharma, A., 2004. Comparison of mercury, lead and arsenic with respect to genotoxic effects on plant systems and the development of genetic tolerance. *Environ. Exp. Bot*., **52**: 199-223. https://doi.org/10.1016/j.envexpbot.2004.02.009

Pereira, C.S.A., Guilherme, S.I.A.G., Barroso, C.M.M., Verschaeve, L., Pacheco, M.G. and Mendo, S.A.L.V., 2009. Evaluation of DNA damage induced by environmental exposure to mercury in *Liza aurata* using the comet assay. *Arch. environ. Contamin. Toxicol*., **58**: 112-122. https://doi.org/10.1007/s00244-009-9330-y

Rauf, A., Javed, M., Ubaidullah, M. and Abdullah, S., 2009. Assessment of heavy metals in sediments of river Ravi, Pakistan. *Int. J. Agric. Biol*., **11**: 197-200.

Saravanan, T.S. and Mohamed, A.M., 2003. Freshwater fishes as indicators of Kaveri River pollution. *J. environ. Biol*., **24**: 381-389.

Sevcikova, M., Modra, H., Slaninova, A. and Svobodova, Z., 2011. Metals as a cause of oxidative stress in fish: A review. *Vet. Med*., **56**: 537-546. https://doi.org/10.17221/4272-VETMED

Singh, N.P., Mccoy, M.T., Ticer, R.R. and Schneder, E.L., 1988. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.,* **175**: 184-191.

Shaukat, N., Javed, M., Ambreen, F. and Latif, F., 2018. Oxidative stress biomarker in assessing the lead induced toxicity in commercially important fish, *Labeo rohita. Pakistan J. Zool.,* **50**: 735-741.

Sobha, K., Poornima, A., Harini, P. and Veeraiah, K., 2007. A study on biochemical changes in fresh water fish, *Catla catla* (Hamilton) exposed to the heavy metals toxicant cadmium chloride. *J. Sci. Engin. Technol*., **1**: 1-11.

Soto-Reyes, E., Razo, L.M.D., Valverde, M. and Rojas, E., 2005. Role of the alkali-labile sites, reactive oxygen species and antioxidants in DNA damage induced by methylated metabolites of inorganic arsenic. *BioMetals*, **18**: 493-506. https://doi.org/10.1007/s10534-005-0858-7

Steel, R.G.D., Torrie, J.H. and Dinkkey, D.A., 1996. *Principles and procedures of statistics*, 3rd ed. McGraw Hill Book Co., Singapore.

Stohs, S.J. and Bagchi, D., 1995. Oxidative mechanisms in the toxicity of metals ions. *Free Radical Biol. Med*., **2**: 321-336. https://doi.org/10.1016/0891-5849(94)00159-H

Summak, S., Aydemir, N.C., Vatan, O., Yilmaz, D., Zorlu, T. and Bilaloglu, R., 2010. Evaluation of genotoxicity from Nilufer stream (Bursa/Turkey) water using piscine micronucleus test. *Fd. Chem. Toxicol*., **48**: 2443-2447. https://doi.org/10.1016/j.fct.2010.06.007

Sunjog, K., Gacic, Z., Kolarevic, S., Visnjic-Jeftic, Z., Jaric, I., Knezevic-Vukcevic, J., Vukovic-Gacic, B. and Lenhardt, M., 2012. Heavy metal accumulation and the genotoxicity in barbel

(*Barbus barbus*) as indicators of the Danube river pollution. *Scient. World J*., **2012**: 351074. https://doi.org/10.1100/2012/351074

Tice, R.R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., Miyamae, Y., Rojas, E., Ryu, J.C. and Sasaki, Y.F., 2000. Single cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen*., **35**: 206-221. https://doi.org/10.1002/(SICI)1098-2280(2000)35:3<206::AID-EM8>3.0.CO;2-J

Valko, M., Morris, H. and Cronin, M.T.D., 2005. Metals, toxicity and oxidative stress. *Curr. Med. Chem*., **12**: 1161-1208. https://doi.org/10.2174/0929867053764635

van der Oost, R., Beyer, J. and Vermeulen, N.P.E., 2003. Fish bioaccumulation and biomarkers in environmental risk assessment: A review. *Environ. Toxicol. Pharmacol*., **13**: 57-149. https://doi.org/10.1016/S1382-6689(02)00126-6

Ventura-lima, J., Castro, M.R., Acosta, D., Fattorini, D., Regoli, F., Carvalho, L.M., Bohrer, D., Geracitano, L.A., Barros, D.M., Marins, L.F., Silva, R.S., Bonan, C.D., Bogo, M.R. and Monserrat, J.M., 2009. Effects of arsenic (As) exposure on the antioxidant status of gills of the zebrafish *Danio rerio* (Cyprinidae). *Comp. Biochem. Physiol*., **149**: 538-543. https://doi.org/10.1016/j.cbpc.2008.12.003

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Heavy Metals (Cadmium and Lead) Induced Oxidative Stress in *Channa marulius* and *Wallago attu* during Acute Toxicity Experiments

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Authors' Contribution
MB executed and designed the experiments and collected and analyzed the data. SA supervised the work. SK and FA performed statistical analysis. MB, RI and KTM wrote the manuscript. MUI and MF reviewed the manuscript.

Key words
Oxidative stress, Antioxidant enzyme activity, Heavy metals, Pollution.

ABSTRACT

In recent decades, as a result of rapid urbanization and industrialization there has been a great variation in the human environment. Heavy metals are important inducers of oxidative stress in aquatic animals, promoting formation of reactive oxygen species (ROS). The present study was planned to assess the metals induced oxidative stress in *Channa marulius* and *Wallago attu*. After the determination of LC_{50} 96-h and lethal values of cadmium (75.70±1.29 and 166.03±3.58 mg L^{-1}), lead (53.42±0.94 and 128.79±2.68 mg L^{-1}) to *Channa marulius* and (32.96±0.51 and 77.54±2.91 mg L^{-1}), (25.08±0.93 and 71.67±2.00 mg L^{-1}) to *Wallago attu* respectively, the fish organs like liver, kidney and gills were used to estimate the antioxidant enzymes assay (SOD) from the metal treated fishes. Highest overall mean activity of superoxide dismutase was observed in *Channa marulius* liver (258.278 U/ml) followed by kidney (245.571 U/ml) and gills (198.029 U/ml). However, superoxide dismutase activity in *Wallago attu* kidney was minimum as 84.201 U/ml. The activity of superoxide dismutase in lead exposed *Channa marulius* and *Wallago attu* were noted maximum in liver *i.e.* 25.261 and 55.141 U/ml and minimum in gills *i.e.* 18.745 and 39.513 U/ml, respectively. Activity of SOD increased with increasing metallic ion concentrations in the test mediums for both fish species. However, superoxide dismutase activity was least in all organs of control fish. The result demonstrates that alteration in the antioxidant enzymes reflects the presence of heavy metals which may cause oxidative stress in *Channa marulius* and *Wallago attu*. The study therefore, provides a rational use of biomarkers of oxidative stress in biomonitoring of aquatic pollution.

INTRODUCTION

Heavy metals pollution in aquatic ecosystem is a global problem due to persistence and continuous accumulation of these polluants in aquatic environment (Hyun *et al*., 2006). It is impossible to biologically degrade heavy metals because these metals store in the organs system of aquatic organisms, become deleterious and consequently pass to other living organisms including human, who consume aquatic species as food (Ashraf, 2005). In freshwater ecosystem, fish is the most powerful indicator of pollution (Rashed, 2001).

A pollutant can enter the fish through food, skin, gills, non-food particles and oral consumption of water. The intake of heavy metals accumulates them in fish organs at different levels (Rao and Padmaja, 2000; Vinodhini and Narayanan, 2008). After absorption, metals are transported by the blood stream to other parts of the body and liver for transformation, detoxification and storage. Therefore, it is imperative to determine the concentrations of heavy metals in commercial fish species in order to evaluate the possible risk associated with fish consumption (Cid *et al*., 2001). Acute toxicity bioassays (LC_{50} and lethal concentration) are used to assess the toxicity of physiologically active heavy metals and to evaluate the potential of various fish species against metal toxicity (Munshi *et al*., 2005; Shukla *et al*., 2007; Abdullah, 2007).

To know the mechanistic characteristics of heavy metals as toxicant in aquatic ecosystem, fish may serve as a good model (Prabakaran *et al*., 2007). Enzyme plays an important role in body metabolism but heavy metals induce alterations in normal metabolic reactions (Suresh *et al*., 2013). Destruction of antioxidant balance leads to potential organ damage, which is called as oxidative stress (Sies, 1991). Oxidative damage results in increased reactive oxygen species and impaired antioxidant defense mechanism (Di-Giulio and Meyer, 2008). The major organs involved in antioxidant defense systems of fish are kidney and liver (Atli and Canli, 2008).

Riverine system of Pakistan is polluted by large quantities of heavy metals, which are adversely affecting the indigenous fish fauna of Pakistan. The *Channa marulius* and *Wallago attu* are fishes of high economic value and are threatened species in Pakistan due to heavy load of

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metals (Khan *et al*., 2011). As heavy metals are increasing oxidative stress day by day, the study of antioxidant enzyme like superoxide dismutase in *Channa marulius* and *Wallago attu* is of increased concern. Therefore, the current study was carried out to assess the oxidative stress and tolerance limits of *Channa marulius* and *Wallago attu* induced by lead and cadmium in laboratory conditions.

MATERIALS AND METHODS

The experimental fish

The fingerlings of *Channa marulius* and *Wallago attu* were collected from their natural breeding grounds (Head Chanawa and Head Khanki). The fingerlings of both fish species were transported to the laboratory and placed in cemented tanks of 1000 liters water capacity. The fish were acclimatized in the laboratory for 15 days with 12h Light and 12h Dark period. During this period, the fingerlings were fed with diet, containing 40% crude protein.

Chemicals

The pure chloride compounds of cadmium and lead were used as metal toxicant. Desired concentrations of Cd and Pb (Merk) were prepared by dissolving an appropriate volume of stock solution (APHA, 2005).

Metals acute toxicity bioassays

The 96-h LC_{50} and lethal concentrations were determined in static bioassay system. Metal's toxicity concentration for each fish species were started from 0 and increased as 0.05 and 5 mg L^{-1} (as total concentration) for low and high metals concentrations, respectively. The fish were not fed during acute toxicity trials. In each aquarium the concentration of metal was increased gradually in order to avoid the fish from stress. Continuous air was supplied to all the test and control mediums with an air pump through capillary network. Fish mortality data obtained against each concentration of metals during 96 h test duration was recorded.

Physico-chemical parameters

The acute toxicity tests were performed at constant water temperature (28°C), pH (7.5) and total hardness (150 mg/L) in static bioassay systems. The acute toxicity bioassay procedure, based on standard method of APHA (2005) was conducted to determine 96-h LC_{50} and lethal concentrations of Cd and Pb for each fish species.

Enzyme assays

The fish used in the acute (96-h LC_{50}) and lethal test trails were weighed and removed from the media. All the fish were dissected and samples of liver, kidney and gills were taken and washed with phosphate buffer (pH 6.5) to remove RBCs. The samples were kept at -80°C for the further enzyme assays.

Enzyme extract preparation

Organ samples were weighed and homogenized in phosphate buffer (0.2M, pH 6.5) with a ratio of 1:4. The tissue homogenates were then centrifuged at 10,000 rpm for 15 min at 4°C. The clear supernatant was preserved and used for further enzyme analysis.

Table I.- Metals acute toxicity (96-h LC_{50} and lethal concentrations) for fish, *Channa marulius* and *Wallago attu* calculated by probit analysis.

Metals	Fish species	Replications	96-h LC_{50} (mg L^{-1})	95 % C.I. (mg L^{-1})	Lethal concentrations (mg L^{-1})	95 % C.I. (mg L^{-1})
Cadmium	*C. marulius*	R1	74.33	67.26-81.15	162.66	146.35-187.19
		R2	75.34	68.27-82.22	164.43	147.86-189.38
		R3	77.43	70.15-84.59	170.99	153.18-198.11
		Means±SD	75.70±1.29		166.03±3.58	
	W. attu	R1	33.67	29.41-57.84	78.98	68.71-95.95
		R2	32.68	28.74-36.79	73.48	64.31-88.41
		R3	32.51	28.03-37.04	80.15	69.32-98.39
		Means±SD	32.96±0.51		77.54±2.91	
Lead	*C. marulius*	R1	54.76	48.45-61.00	132.41	117.07-156.07
		R2	52.76	46.59-58.85	127.95	113.33-150.38
		R3	52.77	46.72-58.75	126.01	111.87-147.55
		Means±SD	53.42±0.94		128.79±2.68	
	W. attu	R1	24.81	20.46-29.10	71.38	60.48-90.64
		R2	26.34	21.95-30.80	74.26	62.85-94.36
		R3	24.10	19.79-28.26	69.38	58.86-87.80
		Means±SD	25.08±0.93		71.67±2.00	

Superoxide dismutase (SOD U/L)

The activity of superoxide dismutase was determined by measuring its ability to inhibit the photo-reduction of nitro-blue tetrazolium (NBT) by superoxide (Giannopolitis and Ries, 1977). At 560 nm wave length 1 ml blank (non-illuminated) solution was inserted into the spectrophotometer and reading was noted after 1 minute. A set of eight cuvettes were used to place in the light box. In each cuvette 1 ml of buffer solution was added, to which 0.05 ml enzyme extract and 0.016 ml riboflavin at timed intervals was added. All the cuvettes were incubated in the light box (internally mounted by a 30 W fluorescent bulb to provide the uniform light intensity) for 12 min. After incubation each cuvette was transferred to spectrophotometer, where 0.067 ml of EDTA/NaCN solution and 0.033 ml NBT solution was added to illuminated reaction mixture. The absorbance of the samples was measured at 560 nm.

Statistical analyses

The experiment was performed in triplicates and the data of acute toxicity were analyzed by applying probit analysis (Finney, 1971). The data of enzymes activities were subjected to statistical analysis by using MSTATC (Steel *et al*., 1997).

RESULTS

96-h LC_{50} and lethal concentrations

The experimental fish *viz*. *Channa marulius* and *Wallago attu* separately, were tested to determine their 96-h LC_{50} and lethal concentrations for lead and cadmium. Both the fish species were exposed to different concentrations of these metals. Figure 1 shows the graphical presentation of fish mortality during 96-h LC_{50} and lethal concentrations of lead and cadmium for *Channa marulius* and *Wallago attu*.

The mean 96-h LC_{50} values calculated for *Channa marulius* (75.70±1.29 and 53.42±0.94 mg L^{-1}) and *Wallago attu* (32.96±0.51 and 25.08±0.93 mg L^{-1}) for cadmium and lead, respectively, showed that *Channa marulius* was significantly less sensitive than *Wallago attu* (Table I). While the comparison between metals lead become more toxic than the cadmium. The exposure of metals (Cd and Pb)) to *C. marulius* showed least sensitivity (166.03±3.58 and 128.79±2.68 mg L^{-1}) while *W. attu* (77.54±2.91 and 71.67±2.00 mg L^{-1}) showed significantly highest sensitivity in terms of lethal exposure.

Lead showed significantly direct relationship between 96-h LC_{50} and lethal concentrations for *Channa marulius* and *Wallago attu*. While the cadmium showed statistically direct relationship between 96-h LC_{50} and lethal concentrations for *Channa marulius*. The high value of R^2 (Co-efficient of determination) computed for 96-h LC_{50} and lethal concentrations relationships showed high reliability of these models (Table II).

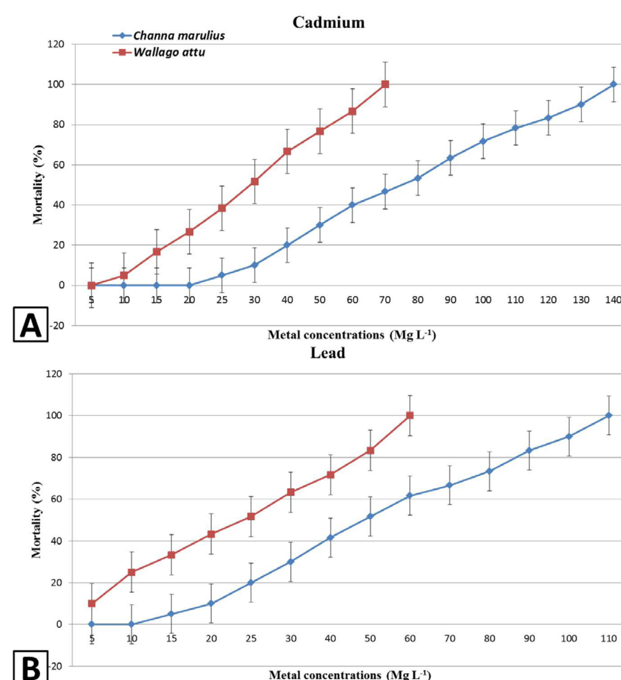

Fig. 1. The percentage mortality of *Channa marulius* and *Wallago attu* at different cadmium (A) and lead (B) concentrations during 96-h acute toxicity tests.

Table II.- The computed relationships between LC_{50} and lethal concentrations of the test mediums.

Metals/ Treatments	Fish species	Lethal concentration y	LC_{50} x	Regression equation (y = a + bx)	r	R²
Cadmium	*C. marulius*	166.03	75.70	y = -42.21 + 2.751** x (0.340)	0.992	0.985
	W. attu	77.54	32.96	y = 37.22 + 1.223 x (5.558)	0.215	0.046
Lead	*C. marulius*	128.79	53.42	y = -19.08 + 2.768** x (0.868)	0.954	0.910
	W. attu	71.67	25.08	y = 18.12 + 2.135** x (0.215)	0.995	0.990

y, dependent variable; x, Independent variable; r , correlation coefficient; R², coefficient of determination. **, significant at P<0.01; *, significant at P<0.05. Values within brackets are the standard errors.

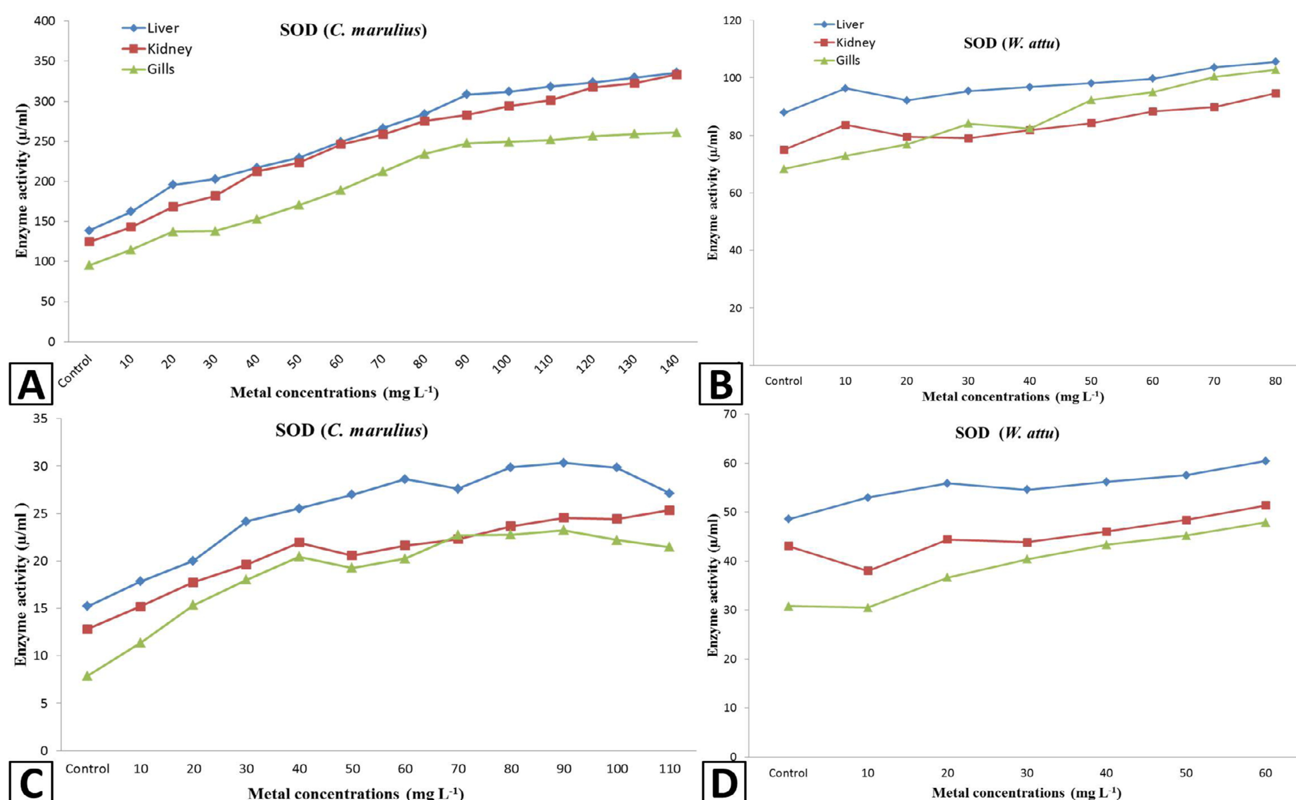

Fig. 2. SOD activity of cadmium (A, B) and lead (C, D) exposed *Channa marulius* and *Wallago attu*.

Antioxidant enzyme activity

During acute exposure the activity of antioxidant enzymes like superoxide dismutase was also studied in the test and control mediums. The activities of the enzyme were determined in fish organs *viz*. kidney, gills and liver.

In cadmium exposed fish the highest mean activity of superoxide dismutase was observed in *Channa marulius* liver (258.278 U/ml) followed by that of kidney (245.571 U/ml) and gills (198.029 U/ml). However, *Wallago attu* kidney showed minimum activity of superoxide dismutase as 84.201 U/ml. Figure 2 showed increased superoxide dismutase activity with increased cadmium and lead concentrations in the test medium. The activity of superoxide dismutase in lead exposed *Channa marulius* and *Wallago attu* were noted maximum in liver *i.e.* 25.261 and 55.141 U/ml and minimum in gills *i.e.* 18.745 and 39.513 U/ml, respectively. Overall SOD activity increased with increasing metallic ion (Cd, Pb) concentrations in the test mediums for both fish species, which is an indicator of stress conditions of fish.

DISCUSSION

The measurement of 96-h LC_{50} values is an important parameter to check the susceptibility of fish to pollutions. The sensitivity of fish varies from species to species and metals to metals. The metal that is toxic at low level concentration to one individual may be non-toxic or less toxic to other animal at similar concentrations (Shah and Altindu, 2005). The LC_{50} values for the same toxicant differ from individual to individual due to the mode of action and responses of the animals (King, 1992). Sanjay *et al*. (2006) exposed *C. marulius* to Cr (VI) for 96 h. They observed that toxicity of Cr to fish is dose and time dependent *i.e.,* with the increase in metal concentration and time, the mortality was also increased. Similar trend was also noted that toxicity of metals increased with increasing/decreasing concentration of these metals. Tiwari *et al*. (2011) studied the toxicity of cadmium for freshwater teleost, *Channa punctata* (Bloch) at 24, 48, 72 and 96-h exposure durations. The LC_{50} values with 95% confidence level were estimated by SPSS as 26.88 (21.69-71.68), 18.76 (17.13-20.81), 16.70 (14.77-17.96) and 14.95 (13.13-15.88) mgL^{-1} for dissolved metal concentrations, respectively. Dhanalakshmi and Chitra (2014) examined the chromium exposed *Cirrhina mrigala* for 24, 48, 72 and 96-h, and the consequential LC_{50} values were calculated using Finney's Probit analysis. Rajkumar *et al*. (2011) studied the fingerlings of *Mugil cephalus* was

exposed to acute toxicity test under static renewal bioassay to cadmium, copper, lead and zinc. The fingerlings were sensitive to copper followed by cadmium and lead. Jasim Aldoghachi (2016) tested the juvenile hybrid tilapia (*Oreochromis* sp.) with different concentration of heavy metals under varying exposure time to examine acute toxicity effect on their survival rate and bioaccumulation in fish tissues. Copper (Cu), cadmium (Cd) and zinc (Zn) was used at rates 0.0, 0.50, 1.0, 3.0 and 5.0 mg L^{-1}. The medial lethal concentration of Cu, Cd and Zn (96-h LC_{50}) was determined to be 0.45, 0.7 and 2.1 mgL^{-1}, respectively, in a Probit transformed concentration - response curves. Mortality of fish was significantly higher with higher concentration of toxic metals. The observed fish toxicity by heavy metals was in the following order: Cu > Cd > Zn.

During present investigation superoxide dismutase activity in fish increased by increasing metalic ion concentrations in the test medium. Liver is the organ that showed maximum superoxide dismutase activity against cadmium and lead in both fish species. Velma and Tchounwou (2010) isolated the kidney and liver of chromium stressed fish to study the antioxidant enzyme activity. Increased activity in superoxide dismutase was noted at all concentrations of chromium as compared to control fish, *Carassius auratus*. Roberts and Oris (2004) reported that rainbow trout liver is more sensitive because it showed increase in the activity of superoxide dismutase by increasing metallic ion contents and exposure time. The gills and kidney were the organs that showed non-significant increase in the activity of superoxide dismutase. Cadmium accumulation in fish organs also affects the activity of SOD in catfish *(Clarias gariepinus)*. An increase in SOD activity in fish with increasing metal concentration was noted by Asagba *et al*. (2008) and Vieria *et al*. (2009). Chromium-induced oxidative stress in kidney and liver of gold fish was studied by Lushchak *et al*. (2009). Four different chromium concentrations (1, 2.5, 5 or 10 mgL^{-1}) and a control medium was used. The results indicate the higher SOD activity in liver than kidney. The activity of superoxide dismutase decreased with increasing medium concentration. However, this decrease was sharper in kidney as compared to liver.

CONCLUSION

The metals (lead and cadmium) 96-h LC_{50} and lethal responses by both, *C. marulius* and *W. attu* showed statistically significant differences. Cadmium was significantly more toxic than chromium. Between the two fish species *W. attu* was found significantly more sensitive in terms of 96-h LC_{50} and lethal responses. Overall the SOD activity increased with increasing metallic ion (Cd, Pb) concentrations in the test mediums for both fish species that indicate stress conditions in fish.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

APHA, 2005. *Standard methods for the examination of water and wastewater*, 21st ed. American Public Health Association, New York.

Abdullah, S., 2007. *Studies on acute toxicity of metals to the fish* viz. Catla catla, Labeo rohita *and* Cirrhina mrigala. Ph.D. thesis, Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan, pp. 1-191.

Asagba, S.O., Eriyamremu, G.E. and Igberaese, M.E., 2008. Bioaccumulation of cadmium and its biochemical effect on selected tissues of the catfish *(Clarias gariepinus). Fish Physiol. Biochem*., **34**: 61-69. https://doi.org/10.1007/s10695-007-9147-4

Ashraf, W., 2005. Accumulation of heavy metals in kidney and heart tissues of *Epinephelus microdon* fish from the Arabian Gulf. *Environ. Monit. Assess*., **101**: 311-316. https://doi.org/10.1007/s10661-005-0298-4

Atli, G. and Canli, M., 2008. Enzymatic response to metal exposures in a freshwater fish *Oreochromis niloticus*. *Comp. Biochem. Physiol*., **145**: 282-287.

Cid, B.P., Boia, C., Pombo, L. and Rebelo, E., 2001. Determination of trace metals in fish species of the Ria de Aveiro (Portugal) by electro thermal atomic absorption spectrometry. *Fd. Chem*., **75**: 93-100. https://doi.org/10.1016/S0308-8146(01)00184-4

Dhanalakshmi, B. and Chitra, G., 2014. Biochemical changes in the fish *Cirrhinus mrigala* (Hamilton) after acute and chronic exposure of heavy metal. *Int. J. Curr. Res*., **6**: 4362-4368.

Di Giulio, R.T. and Meyer, J.N., 2008. Reactive oxygen species and oxidative stress. In: *The toxicology of fishes* (eds. R.T. Di Giulio and D.E. Hinton). CRC Press, Taylor and Francis Group, pp. 273-324. https://doi.org/10.1201/9780203647295.ch6

Finney, D.J., 1971. *Probit analysis*, 3rd ed. Cambridge University Press, London, pp. 318.

Giannopolitis, C.N. and Ries, S.K., 1977. Superoxide dismutase. I. Occurrence in higher plants. *J. Pl. Physiol*., **59**: 309-314. https://doi.org/10.1104/pp.59.2.309

Hyun, S., Lee, T. Lee, C.H. and Park, Y.H., 2006. The effects of metal distribution and anthropogenic effluents on the benthic environment of Gwangyang

Bay, Korea. *Mar. Poll. Bull*., **52**: 113-120. https://doi.org/10.1016/j.marpolbul.2005.10.011

Jasim-Aldoghachi, M.A., Motior-Rahman, M., Yusoff, I. and Sofian-Azirunl, M., 2016. Acute toxicity and bioaccumulation of heavy metals in red tilapia fish. *J. Anim. Pl. Sci*., **26**: 507-513.

Khan, A.M., Ali, Z., Shelly, S.Y., Ahmad, Z. and Mirza, M.R., 2011. Aliens: A catastrophe for native fresh water fish diversity in Pakistan. *J. Anim. Pl. Sci*., **21**: 435-440.

King, S.G., 1992. Some effects of DDT on the guppy and brown trout. *Spec. Scient. Res. U.S. Fish. Wildl. Surv.,* **399**: 1-22.

Lushchak, O.V., Kubraka, O.I., Lozinskya, O.V., Storey, J.M., Storey, K.B. and Lushchak, V.I., 2009. Chromium (III) induces oxidative stress in goldfish liver and kidney. *Aquat. Toxicol*., **93**: 45-52. https://doi.org/10.1016/j.aquatox.2009.03.007

Munshi, A.B., Jamil, K. and Shirin, K., 2005. Acute toxicity of copper and cadmium and their mixture to Tilapia *(Oreochromis mossambicus). J. Chem. Soc. Pak*., **27**: 268-270.

Prabakaran, M., Binuramesh, C., Steinhagen, D. and Michael, R.D., 2007. Immune response in the tilapia, *Oreochromis mossambicus* on exposure to tannery effluent. *Ecotoxicol. environ. Safe*., **68**: 372-378. https://doi.org/10.1016/j.ecoenv.2006.11.016

Rajkumar, J.S.I., John-Milton, M.C. and Ambrose, T., 2011. Acute toxicity of water borne Cd, Cu, Pb and Zn to *Mugil cephalus* fingerlings. *Int. J. Chem. Sci*., **9**: 477-480.

Rao, L.M. and Padmaja, G., 2000. Bioaccumulation of heavy metals in *M. cyprinoids* from the harbour waters of Visakhapatnam. *Bull. Pure appl. Sci*., **19**: 77-85.

Rashed, M.N., 2001. Monitoring of environmental heavy metals in fish from Nasser Lake. *Environ. Int. J*., **27**: 27-33. https://doi.org/10.1016/S0160-4120(01)00050-2

Roberts, A.P. and Oris, J., 2004. Multiple biomarker response in rainbow trout during exposure to hexavalent chromium. *Comp. Biochem. Physiol*., **138**: 221-228. https://doi.org/10.1016/j.cca.2004.08.006

Sanjay, P., Ravindra, K., Shilpi, S., Nagpure, N.S., Satish, K., Srivastava, S. and Verma, M.S., 2006. Acute toxicity of chromium exposed *Channa marulius. Indian J. environ. Protec.,* **38**: 118-121.

Shah, S.L. and Altindu, A., 2005. Effects of heavy metals accumulation in the 96-h LC_{50} values in *Tench tinca tinca* L., 1758. *Turk. J. Vet. Anim. Sci*., **29**: 139-144.

Shukla, V., Dhankhar, M., Prakash, J. and Sastry, K.V., 2007. Bioaccumulation of Zn, Cu and Cd in *Channa punctatus. J. environ. Biol*., **28**: 395-397.

Sies, H. (ed.), 1991. Oxidative stress: introduction. In: *Oxidative stress: Oxidants and antioxidants*. Academic Press, London, pp. 15-22.

Steel, R.G.D., Torrie, J.H. and Dinkkey, D.A., 1997. *Principles and procedures of statistics: A biometrical approach*, 2nd ed. McGraw Hill Book Co., Singapore.

Suresh, V., Senthil-Kumar, N., Thangam, R., Rajkumar, M., Anbazhagan, C., Rengasamy, R., Gunasekaran, P., Kannan, S. and Palani, P., 2013. Separation, purification and preliminary characterization of sulfated polysaccharides from *Sargassum plagiophyllum* and its in vitro anticancer and antioxidant activity. *Process Biochem*., **48**: 364-373. https://doi.org/10.1016/j.procbio.2012.12.014

Tiwari, M., Nagpure, N.S., Saksena, D.N., Kumar, R., Singh, S.P., Kushwaha, B. and Lakra, W.S., 2011. Evaluation of acute toxicity levels and ethological responses under heavy metal cadmium exposure in freshwater teleost, *Channa punctata* (Bloch*). Int. J. aquat. Sci*., **2**: 36-47.

Velma, V. and Tchounwou, P.B., 2010. Chromium-induced biochemical, genotoxic and histopathologic effects in liver and kidney of goldfish, *carassius auratus*. *Mutat. Res*., **698**: 43-51. https://doi.org/10.1016/j.mrgentox.2010.03.014

Vieria, L.R., Gravato, C., Soares, A.M.V.M., Morgado, F. and Guilhermino, L., 2009. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: Linking biomarkers to behavior. *Chemosphere*, **76**: 1416-1427. https://doi.org/10.1016/j.chemosphere.2009.06.005

Vinodhini, R. and Narayanan, M., 2008. Bioaccumulation of heavy metals in organs of fresh water fish, *Cyprinus carpio* (common carp). *Int. J. environ. Sci. Technol*., **5**: 179-182. https://doi.org/10.1007/BF03326011

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Determination of Toxic Heavy Metal Load (Mn, Cu and Cd) in *Labeo rohita* (Rohu) Collected from Wild and Local Fish Farms

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Authors' Contribution
AY supervised and planned the research. KMA did data collection and analysis. MSM co-supervised the research. MA helped in the analytical facilities. NM performed experiments.

Key words
Bioaccumulation, Heavy metal, Pollution, *Labeo rohita.*

ABSTRACT

The present study was conducted to determine heavy metal (zinc, lead, manganese, copper and cadmium) concentrations in muscle tissues of *Labeo rohita* inhabiting local water bodies as well as those being raised at farms. Sampling was performed to collect *Labeo rohita* from two different stations along river Ravi, *viz*. Lahore Siphona (upstream) and Balloki Headworks (downstream). Additionally, samples were also obtained from govt. operated as well as private fish farms located in Lahore District, Pakistan. The fish were dissected, their muscles were digested, filtered, and finally analyzed for concentrations of heavy metal ions, such as Cd, Cu and Zn. Significant variation of heavy metal ions were found between two sites along river Ravi as well as govt. operated and private fish farms. The order of accumulation of heavy metals in muscle tissues of *Labeo rohita* was Mn> Cu> Cd. Elevated mean concentrations of these heavy metals were determined at Head Balloki which were as Mn (0.44±0.045), Cu (0.31±0.081) and Cd (0.24±0.057) mg/kg; whereas, the lowest levels were found in the samples collected from private fish farms, the values being as Mn (0.22±0.014), Cu (0.082±0.002), and Cd (0.019±0.0008) mg/kg; it was also observed that these values were within permissible limits as laid down by WHO/FAO. The highest level of heavy metals in water samples collected from river Ravi indicates that the bioaccumulation of heavy metals may affect the aquatic life of fresh water and industries should not be allowed pouring their effluent directly into the river Ravi in their vicinity.

INTRODUCTION

Indiscriminate addition of pollutants in the natural water bodies, such as river, streams etc. from anthropogenic sources has resulted in the degradation of environmental health of aquatic ecosystems (Eisler, 2010). So much so, the influx of these pollutants into living system, such as fish and other organisms has worsened the situation. These pollutants are not only harmful for fish itself but also for other animals which consume fish meat. Human population may be directly affected by this alarming scenario (Mendil *et al*., 2005). Fishes are one of the most important bio-indicators of toxic heavy metal pollution (Alinnor and Obiji, 2010). Currently, there is dire need to monitor the situation and to assess the risk (Dural *et al*., 2007).

Fish is considered at apex level of food chain within aquatic system because of which a large amount of heavy metals may accumulate in their soft and hard tissues (Mansour and Sidky, 2002). So the result of pollution of trace metals is dangerous for human health by consuming this contaminated fish muscles. As a result, the monitoring of trace metal pollution within aquatic ecosystem including water, sediments and biota is very essential.

Heavy metals (such as Cd, Cu, Mn, *etc*.) in elevated concentration are added to the freshwater bodies through domestic sewage as well as industrial effluents dumping on routinely basis which may ultimately may affect the health of water bodies (Vineeta *et al*., 2007). The measure of the addition of these pollutants into the fauna and flora would be bio-concentration factor and the extent of bio-magnification by which organisms are generally affected. In a number of recently conducted studies, the elevated levels of thee trace metals have been reported and even more alarmingly, in a number of cases, these values have been beyond permissible limits as prescribed by authentic bodies, such as WHO, US-EPA, *etc*. (Csuros and Csaba, 2002; Vineeta *et al*., 2007).

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Fish consumption by human population is on the rise mainly due to its nutritional value and it production as well as consumption will mount to 98.6 million in 2020 (Retnam and Zakaria, 2010). On the other hand, risk associated with the heavy toxicity accumulated in fish fauna is also on the rise. Recently, Ita-Ita disease has been reported in Japan which was caused due to intake of cadmium contaminated seafood; it was also reported that digging in mines in the proximate area was the ultimate cause of the same (Chen and Chen, 2001; Dural *et al*., 2007).

In the current scenario, there is urgent need to assess the risk of influx of heavy metals, such as manganese, cadmium, and chromium within the fish fauna. This information will help to understand the route and risk assessment concerning heavy metal pollution. Also, it would help us design future strategies to mitigate the risk.

MATERIALS AND METHODS

Samples of *Labeo rohita* were collected from wild as well fish farms during the months from November to April and were transported to the laboratory. Identification of fishes was done with the help of identification key based on morphological characters (Mirza *et al*., 2013). For this purpose selected sites are: (i) Two sites along river Ravi: one was upstream, Siphon bridge (site 1) and the other was downstream, Balloki headworks (site 2). (ii) Three Government operated fish arms: locate in Lahore district: Manga Mandi Fish Hatcheries, Govt. Model Fish Farm and Rahkbakanth Army Fish Farm, were designated as sites 3, 4 and 5, respectively and (iii) Three private farms: Sunder Fish Farm, Mansha Fish Farm and Varioline Intercool (Kahna Kacha) were designated as sites 6, 7 and 8, respectively were placed in group 3.

Additionally, water samples from each site were also collected in glass in acidified vials (10% HNO_3). At the spot tests of various physico-chemical parameters, such as temperature and pH of water were recorded.

Muscle tissues (100 mg) form each fish were obtained through dissection, washed with deionized water and were subjected to wet digestion method (APHA, 2005; Bano and Afzal, 2017). For this, muscle samples (2g) of each sample were chopped with sterilized stainless steel knife to achieve homogeneity. Then it was subjected to 10 ml of HNO_3 and H_2O_2 (1:1 ratio) treatment and heated on hot plate to about 140°C and refluxed for 10 to 15 min. Sample were cooled and placed back onto hot plate, added 35% H_2O_2 in 1ml portions and again the solution was cooled until effervescence stops. The digested samples were syringe filtered by using 0.2µm nylon filter (Nalgene) and were sent to PCSIR, Lahore for the element analysis by using atomic absorption spectrometer.

Bioconcentration factors (BCF) of Mn, Cd and Cu were also calculated by using the following expression.

$$BCF = Tc/Wc$$

Where, Tc is concentration of heavy metals in tissue and Wc is concentration of heavy metals in water.

Statistical analysis of the data was performed by using statistical program named as SPSS Software, whereas, various tests were performed, such as uni-variate analysis of variance (One-Way ANOVA), T-Test and Post Hoc Test.

RESULTS

Water parameters

Physicochemical characteristics of water of all the studied sites are given Table I. Highest mean temperature was found at site 7 and lowest of the same was at site 4 (Table I). pH of all the sites was similar which was slightly alkaline (Table I). No significant variation of both these parameters was found.

Table I.- Physico-chemical characteristics of water at all studied sites.

Sites	Temp. (ºC) (Mean ± SD)	pH (Mean ± SD)
Site 1	30.5±1.50	8.30±0.1
Site 2	30±1.00	8.35±0.05
Site 3	28.5±1.50	8.40±0.1
Site 4	28±1.00	8.25±0.05
Site 5	27.5±1.50	8.45±0.05
Site 6	28.5±2.5	8.20±0.1
Site 7	31.5±1.5	8.30±0.1
Site 8	29±3.0	8.40±0.1

Concentrations of various Mn, Cd, and Cu were recorded as given in Figures 1 and 2.

Metal concentrations in Labeo rohita *muscles*

Mean average concentration of manganese (Mn) ion with SEM at various studied sites such as site 1, 2, 3, 4, 5, 6, 7 and 8 were measured as 0.22 ± 0.014, 0.14 ± 0.008, 0.12 ± 0.004, 0.11 ± 0.008, 0.08 ± 0.008, 0.07 ± 0.012, 0.07 ± 0.008 and 0.05 ± 0.008 mg/L, respectively (Fig. 1A).

Mean average concentration of copper (Cu) with SEM at various studied sites such as site 1, 2, 3, 4, 5, 6, 7 and 8 were measured as 0.082 ± 0.002, 0.044 ± 0.003, 0.046 ± 0.002, 0.036 ± 0.001, 0.036 ± 0.001, 0.034 ± 0.001, 0.027 ± 0.0 and 0.018 ± 0.0 mg/L, respectively (Fig. 1B).

Mean average value of cadmium (Cd) at various sites such as site 1, 2, 3, 4, 5, 6, 7 and 8 were measured as 0.019 ± 0.0008, 0.015 ± 0.0004, 0.015 ± 0.0008, 0.013 ± 0.0008, 0.011 ± 0.0008, 0.013 ± 0.0008, 0.002 ± 0.0008 and 0.002 ± 0.0008 mg/L, respectively (Fig. 1C).

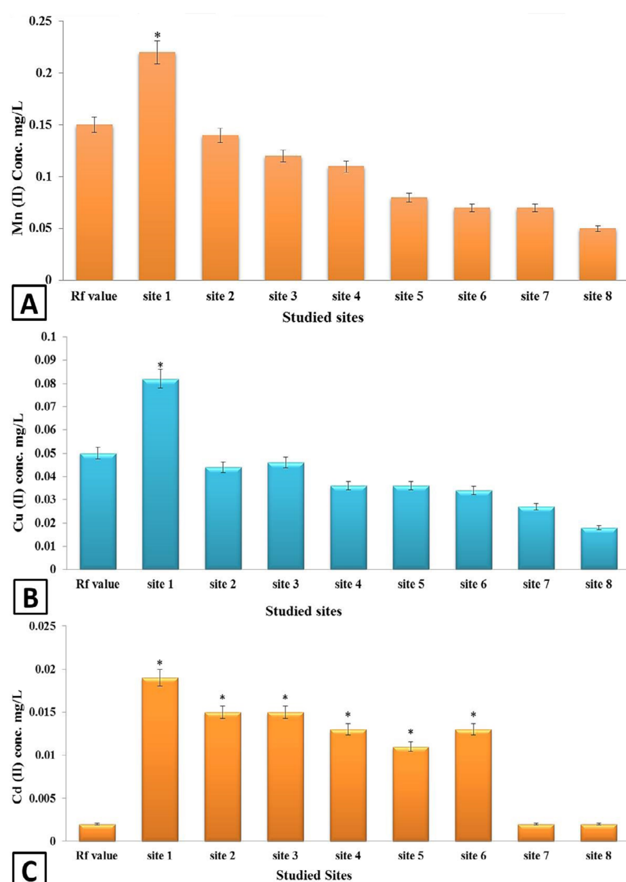

Fig. 1. Water analysis showing concentrations (mg/L) of Mn (Reference value = 0.15 mg/L (US EPA) (**A**), Cu (Reference value = 0.05 mg/L (US EPA) (**B**) and Cd (Reference value = 0.002 mg/L (US EPA) (**C**). *These values exceed permissible limits as prescribed by US EPA.

DISCUSSION

Head Balloki reservoir is very important for commercial fishing but receives 168 cusecs of effluents from nearly 200 industries and 3136 cusecs of domestic waste water from Lahore City as reported by Tabinda *et al.* (2013) without any treatment.

In 1993, National Environmental Quality Standards (NEQS) were approved by Government of Pakistan for permissible limits of pollutants in municipal and industrial effluents which were enacted in the year 2000; dilution of industrial effluents by 1:10 was recommended before discharge in water bodies. However, implementation has not been strict resulting in the continuously deteriorating the environmental health of local water bodies and ultimately damaging not only inhabiting fauna, such as fish but also the human population consuming these fish as food (Widianarko *et al*., 2000).

Manganese (Mn) concentration in water

The value of Mn (II) recorded in current study is beyond the permissible limits of US-EPA as 0.15 mg/L. However, the value of Mn (II) in water samples of river Jehlam was ranged 0.002 - 0.158 mg/L less than the current study (Khan *et al*., 2004). The reason of this fact might be due to less discharge of effluents into the river Jehlam.

Water pollution affects the aquatic flora and fauna due to discharge of effluents into the Laan River China by disturbing ecological and toxicological parameters (Wang and Tang, 1998). Mn (II) toxicity in bed sediments of river Ravi at Balloki Head works was measured as 14.19 µg/g (Javed and Mahmood, 2001). Which may be another reason for increased level of Mn metal content at River Ravi. Rest of the studied metal were within permissible limits.

Table II.- One way analysis of variance (ANOVA) for different metals concentration (mg/Kg) in muscle tissues of group 1, 2 and 3.

Sites		n	M	SD	SEM	F	P
Mn	Wild	2	0.34	0.15	0.11	9.562	0.020*
	Govt.	3	0.12	0.04	0.02		
	Private	3	0.05	0.01	0.01		
Cu	Wild	2	0.23	0.11	0.08	8.306	0.026*
	Govt.	3	0.07	0.02	0.01		
	Private	3	0.04	0.01	0.01		
Cd	Wild	2	0.17	0.10	0.07	5.782	0.050
	Govt.	3	0.06	0.02	0.01		
	Private	3	0.03	0.01	0.01		

*, significantly high value at $P \leq 0.05$.

Mn (II) concentration in fish muscle

Mn (II) concentration in muscle tissues at upstream (Shiphon Bridge) was found significantly (Table II) less than the downstream (Head Balloki) because pollutants are naturally carried downstream and after has been introduced into the river do not further diluted. The present study revealed that heavy discharge of domestic sewage and industrial waste has badly affected the quality of river water. Logical reason behind this fact are three major tributaries like Farrukhabad, Taj Company and Hudiara Nullas discharge large amount of industrial waste into the river and increases metal toxicity level (Javed, 2005).

Mn (II) metal content in present investigation was found similar in the range of 0.27-1.50 mg/Kg recorded in a study (Andreji, 2006). In a previous study Mn (II) bioconcentration in muscles of seabass and red seabream

was measured in the range of 0.04-0.11µg/g and 0.02-0.10 µg/g which are close to the values calculated in Government operated and private fish farms in present study. In another study mean value of Mn (II) ranged 11.7-72.9 µg/g in fish species found in Takot lakes in Turkey (Mendil *et al*., 2005). In a similar investigation accumulation level of Mn (II) metal content in cultured fish of local farms (*O. niloticus* and *S. aurata*) was ranged 0.37- 0.8 µg/g (Muzyed, 2011) greater than the values calculated in Government operated fish farm (Site 4). This difference may be due to species specific metal accumulation.

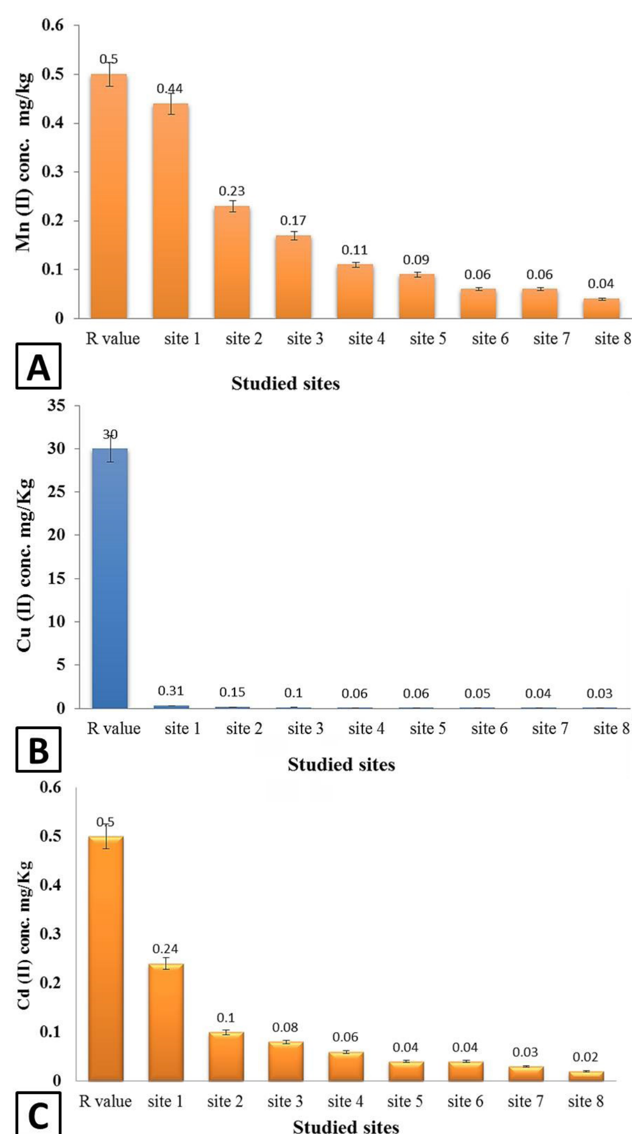

Fig. 2. Concentration of Mn (II) (**A**), Cu (II) (**B**) and Cd (II) (mg/Kg) (**C**) in muscle tissues collected from different sites.

By comparison, the bioaccumulation of Mn (II) in muscle tissues of marine fish of USA was found as 0.15-0.70µg/g (Burger and Gochfeld, 2005), and about 0.08-2.78µg/g in mullet of Turky (Tepe *et al*., 2008; Turkmen *et al*., 2009). Manganese level in muscle tissues of fish found in sea of china was measured as 0.26-43µg/g (Asante *et al*., 2008), and in Southeast Asia as 0.05-0.810µg/g (Agusa *et al*., 2007). Amount of Mn (II) in frozen fish samples imported from Thailand and Malaysia was 0.35µg/g and 0.94µg/g, respectively. A concentration of Mn in canned fish imported from Greenford, England was whit in the range of 0.00-100µg/g.

Copper (Cu) concentration in water

Copper (Cu) concentration in water samples at all the selected sites was significantly low than the USEPA recommended values of 0.05 mg/L. In an investigation at same studied site concentration of Cu (II) content ranged (0.172 - 0.173 mg/L) was almost close to the present value (Tabinda *et al*., 2013).

In this study Cu (II) toxicity level was less at Lahore Siphon as compared to the Balloki Headworks it has been found that effluents discharge from tributaries are responsible for the toxic effect (Fig. 2C). Effluents discharged from industries and domestic wastes from nine tributaries are responsible for the increase of impurity of river Ravi, which is responsible for increased toxicity of Cu (II) in water and bioaccumulation in biota (Rauf and Javed, 2007). Mean concentration of Cu (II) in river Jehlum was ranged 1.21 - 2.24 mg/L comparatively greater than our calculated value. Low level of Cu (II) metal content is found in water samples collected from private fish farms (Sites 6, 7 and 8). Low Cu (II) content at private fish Farms might be due to irrigation of fields with ground water.

Copper (Cu) concentration muscle in fish muscle

In present study significantly low level of Cu (II) has been found in muscle tissues of *L. rohita* than the WHO/FAO (1989) recommended values. Comparatively less amount of Cu (II) is bioaccumulated with regard to other selected metals. In this investigation the order of accumulation at studied sites (group 2, Government operated fish farms) was Mn > Cu > Cd. The Accumulation level was significantly different at $P \leq 0.05$.

Eisler (2010) reported that in most of the organisms Cu (II) is being regulated properly and usually less bioaccumulation of it occurs in fresh water food chains.

Comparatively high level of Cu (II) is found in muscle tissues from Head Balloki. One possible reason behind this is the presence of large amount of Cu in sediments at collection site. Cyprinus fishes are benthic, burrowing species and bioaccumulation of copper depends

on its bioavailability from sediments (Luoma, 1983). Accumulation of Cu (II) in bed sediments of River Ravi at Balloki Headworks was found to be 46.28 µg/g (Rauf *et al*., 2009) might be cause of high accumulation level of Cu (II) at Balloki Headworks as compared to samples from fish farms.

Plankton in river Ravi has affinity to bioaccumulate Cu (II) ions in greater amount as compared to its concentration in water (Rauf *et al*., 2009) one plausible reason for increased BCF in muscle tissues.

Cu (II) accumulation in muscle tissues of fish from China was below the permissible levels given by management department of China (Tepe *et al*., 2008) similar to our investigation. It has been invested that less Cu (II) is present in natural water bodies because copper minerals are relatively insoluble in natural water provided that no polluted effluents are being discharged into river water.

Pesticides sprays extensively used for agriculture purpose contain copper compounds which is responsible of Cu (II) to be available in surface water and ground water which might be the reason of minor accumulation of Cu (II) in cultured fish samples. It has also been found that Cu (II) toxicity depend on physico-chemical properties of water specially in water having low alkalinity (Taha, 2004) and pH value at the studied site supports this argument, might be responsible for bioaccumulation of this metal in muscle tissues.

Statistical analysis showed that less amount of Cu (II) has been measured in muscle tissues of Government operated fish farms and private fish farms as compared to the wild samples. Cu levels within caged seabass and red sea bream muscles were 0.06-0.16 µg/g and 0.11-0.22 µg/g, respectively. Thus accumulation level of Cu (II) in studied samples was confirmatory with the calculated values of Onsanit (2010). The concentration of Cu (II) in cultured seabass (*D. labrax*) ranged 0.25-1.0 µg/g (Fernandes *et al*., 2008) which is greater than the values found in our present study. This might be due to different physico-chemical properties of water and use of different analytical instrument.

In a similar investigation accumulation level of Cu (II) in cultured fish of local farms (*O. niloticus* and *S. aurata*) was ranged 0.25-0.9 mg/Kg (Muzyed, 2011), greater the values calculated in Government operated fish farm. Previously it has been reported that in Pakistan frozen fish imported from Thailand and Malaysia contain 5.23 and 0.01 µg/g of copper, respectively. However, Cu (II) content in frozen fish collected from Pakistan was 0.320 µg/g lower than USEPA allowable limit (120 µg/g). In our study the values of bioaccumulation factor (BCF) ranged 1.4 - 3.7 higher than the values (0.93) previously calculated in muscle tissues of fish samples from Laizhou Bay (Liu *et al*., 2014).

Cadmium (Cd) concentration in water

Cadmium (Cd) concentration in water samples at Head Balloki Reservoir (downstream) was significantly high as compared to Siphon Bridge (upstream). Increased value at Siphon Bridge may due to inclusion of more untreated waste from different tributaries into river water.

In this study statistical analysis showed lowest level of Cd (II) metallic content in water samples of Government operated and private fish farms. One plausible reason behind this fact might be the culturing of fishes in ground water not polluted with untreated industrial waste. Cd (II) is rarely found in natural water (Hem, 1989) one supportive statement to the present investigation. The concentration of Cd in drinking and irrigation water is thought to be dangerous if it exceeds 0.01mg/L (Taha, 2004).

Cadmium (Cd) concentration in fish muscle

Amount of Cd (II) in sediments of river Ravi at Siphon was found in the range of 0.99-3.17 µg/g responsible for less bioconcentration of Cd (II) at Lahore Siphon.

The concentration of cadmium in sediments serves as secondary source of pollution to water in which these are present (Rauf *et al*., 2009) responsible for accumulation of Cd (II) in muscle tissues of fish. Cd (II) and Pb (II) don't have any biological function considered potentially toxic even these are present in trace concentrations in the body (Robert, 1991).

The highest value of Cd (II) measured was significantly low than permissible values that was suggested by FAO and WHO (FAO, 1989) indicates that fish can be used by human community.

Bioaccumulation of trace amount of Cd (II) in Government operated fish farms and private fish farms as compared to the wild fish samples is supported by the work of Ali and Abdel-Sater (2005) who found that cultured fishes may get heavy metals through artificial diet and surrounding water.

In a similar investigation accumulation level of Cd (II) in cultured fish of local farms (*O. niloticus* and *S. aurata*) was ND- 0.09 µg/g similar to the values calculated in Government operated fish farm (Muzyed, 2011). Recently, highest bioconcentration factor (BCF) values (12) were found at head Balloki (site 1) similar to the values previously measured in frog species ranged 3-10 (Ololade *et al*., 2011) and comparatively less than that calculated by Taiwo *et al*. (2014).

In the present study accumulation level at private fish farms was significantly low and the order of accumulation was as Mn> Cu> Cd. Similar ranking order of studied

heavy metals such as Mn> Cu> Cd were found in muscle tissues of reverine fish species polluted with industrial effluents (Damodharan and Reddy, 2013). Among all the selected heavy metals it has been found that high level of Mn as comparison to other heavy metals is present in muscle tissues of fish (Fig. 2A). Similarly, the order of accumulation of selected heavy metals at Balloki Headworks was in the order of Mn >Cu > Cd (Sites 7 and 8 (group 3-Private fish farms)). Accumulation order was significantly different at $P \leq 0.05$.

CONCLUSION AND RECOMMENDATIONS

In present study this has been concluded that concentration of heavy metals in muscle tissues differed significantly among two sites on river Ravi, Government operated fish farms and private fish farms of Lahore district. Overall heavy metal accumulation was in maximum in wild fish samples, followed by Government operated, and private fish farms (wild > Government operated fish farms> private fish farms); amongst wild fish, specimens of Baloki showed elevated values than the Siphon. Low level of heavy metals' accumulation was found in muscle tissues of *L. rohita* collected from private fish farms. The order of accumulation of heavy metals in muscle tissues of *L. rohita* was Mn> Cu> Cd in the specimens collected from all the sources. The present investigation will enable us to establish food safety in terms of source of fish meat. This study will help us to understand which source is safer in terms of fish meat for consumption.

In the light of the current study, following recommendations may be laid: (i) The effluents discharge from industrial units into water bodies, such as river Ravi as well as its tributaries must be banned as far as these effluents are not treated to remove toxic metal ion to significant extent. (ii) Regular monitoring of the water quality of river Ravi carried out by competent and concerned authorities, such Punjab Fisheries Department, Environmental Protection Department and Irrigation Department, etc. and it must be ensured the pollutants must not exceed permissible limits of National Environmental Quality Standards (NEQS). (iii) Waste water treatment plants may be constructed at larger scale dealing with the effluent of many industries lying adjacent to each other along river Ravi and its tributaries. (iv) Bio-remedial measures may be employed at large scale which would be helpful in large extent to control the alarming situations. (v) Necessary improvement at Government operated fish farms may be brought upon to improve the quality of fish meat in terms of trace metal ion contents. (vi) For consumer of fish meat, it is recommended to use farm fish instead of wild.

Statement of conflict of interest
Authors have declared no conflict of interest.

REFERENCES

Agusa, T., Kunito, T., Sudaryanto, A., Monirith, I., Kan-Atireklap, S., Iwata, H., Ismail, A., Sanguansin, J., Muchtar, M., Tana, T.S. and Tanabe, S., 2007. Exposure assessment for trace elements from consumption of marine fish in Southeast Asia. *Environ. Pollut.,* **145**: 766-777. https://doi.org/10.1016/j.envpol.2006.04.034

Alinnor, I.J. and Obiji, I.A., 2010. Assessment of trace metal composition in fish samples from Nworie River. *Pak. J. Nutr*., **9**: 81-85. https://doi.org/10.3923/pjn.2010.81.85

Andreji, J., Stranai, I., Massanyi, P. and Valent, M., 2006. Accumulation of some metals in muscles of five fish species from lower Nitra River. *J. environ. Sci. Hlth.,* **41**: 2607-2622.

Ali, H.M. and Abdel-Satar, M.A., 2005. Studies of some heavy metals in water, sediment, fish and fish diet in some fish farms in El-Fayoum Province, Egypt. *Egypt. J. aquat. Res*., **31**: 261-273.

APHA, 2005. *Standard methods for the examination of water and wastewater*, 21st ed. American Public Health Association, New York, pp. 258-259.

Asante, K.A., Agusa, T., Mochizuki, H., Ramu, K., Inoue, S., Kubodera, T., Takahashi, S., Subramanian, A. and Tanabe, S., 2008. Trace elements and stable isotope (δ^{13}C and δ^{15}N) in shallow and deep-water organisms from the East China Sea. *Environ. Pollut.,* **156**: 862-873. https://doi.org/10.1016/j.envpol.2008.05.020

Bano, N. and Afzal, M., 2017. Combined effect of acidification and phytase supplementation on calcium and phosphorus digestibility and body composition of rohu (*Labeo rohita*). *Pakistan J. Zool*., **49**: 2093-2101. http//:dx.org./10.17582/journal.pjz.2017.49:2093-2101

Burger, J. and Gochfeld, M., 2005. Heavy metals in commercial fish in New Jersey. *Environ. Res.,* **99**: 403-412.

Chen, C.Y. and Chen, H.M., 2001. Heavy metal concentrations in nine species in fishes caught in coastal waters off Ann- Ping, S.W Taiwan*. J. Fd. Drug Analy.,* **9**: 107-114.

Csuros, M. and Csaba, C., 2002. Atomic absorption spectrometry. *Environ. Samp. Analy. Metals,* **20**: 121-133. https://doi.org/10.1201/9781420032345.ch8

Damodharan, U. and Reddy, M.V., 2013. Heavy metal

bioaccumulation in edible fish species from an industrially polluted river and human health risk assessment. *Arch. Pol. Fish.,* **21**: 19-27. https://doi.org/10.2478/aopf-2013-0003

Dural, M., Goksu, M.Z.L. and Ozak, A.A., 2007. Investigation of heavy metal levels in economically important fish species captured from the Tuzla Lagoon. *Fd. Chem.,* **102**: 415-421. https://doi.org/10.1016/j.foodchem.2006.03.001

Eisler, R., 2010. *Handbook of chemical risk assessment: Health hazards to humans, plants and animals*, Vol. 1. CRC Press, Florida, United States.

FAO/WHO, 1989. *Evaluation of certain food additives and the contaminants mercury, lead and cadmium*. WHO Technical Report Series No. 505.

Fernandes, D., Zanuy, S., Bebianno, M.J. and Porte, C., 2008. Chemical and biological tools to assess pollution exposure in cultured fish. *Environ. Pollut*., **152**: 138-146. https://doi.org/10.1016/j.envpol.2007.05.012

Javed, M. and Mahmood, G., 2001. Metal toxicity of water in a stretch of river Ravi from Shahdera to Baloki headworks. *Pak. J. Agric. Sci.,* **38**: 37-42.

Hem, D.J., 1989. *Study and interpretation of the chemical characterization of natural water*. U.S. Geological Survey, Water Supply Paper 2254, 3rd ed. pp. 263.

Javed, M., 2005. Heavy metal contamination of freshwater fish and bed sediments in the river Ravi stretch and related tributaries. *Pak. J. biol. Sci.* **8**: 1337-1341. https://doi.org/10.3923/pjbs.2005.1337.1341

Khan, M.J., Sarwar, S. and Khattak, R.A., 2004. Evaluation of river Jehlum water for heavy metals (Zn, Cu, Fe, Mn, Ni, Cd, Pb, and Cr) and its stability for irrigation and drinking purposes at district Muzaffarabad (AK). *J. Chem. Soc. Pak.,* **26**: 436-442.

Liu, J., Cao, L., Huang, W., Zhang, C. and Dou, S., 2014. Zinc and copper bioaccumulation in fish from Laizhou Bay, the Bohai Sea. *Chinese J. Oceanol. Limnol.,* **32**: 491-502. https://doi.org/10.1007/s00343-014-3032-7

Luoma, S.N., 1983. Bioavailability of trace metals to aquatic organisms: A review. *Sci. Total Environ.,* **28**: 1-22. https://doi.org/10.1016/S0048-9697(83)80004-7

Mansour, S.A. and Sidky, M.M., 2002. Heavy metals contaminating water and fish from Fayoum Governate, Egypt. *Ecotoxicol. Stud.,* **78**: 15-22.

Mendil, D., Uluozlu, O.D., Hasdemir, E., Tuzen, M., Sari, H. and Suicmez, M., 2005. Determination of trace metals levels in seven fish species in lakes in Tokat, Turkey. *Fd. Chem*., **90**: 175-179. https://doi.org/10.1016/j.foodchem.2004.03.039

Mirza, Z.S., Mirza, M.R., Nadeem, M.S. and Sulehria, A.Q.K., 2013. Revised checklist of fishes of Mangla Reservoir. *Biologia,* **5**: 1-6.

Muzyed, S.K., 2011. *Heavy metals concentration in commercially available fishes in Gaza strip markets*. M.Sc. thesis, The Islamic University of Gaza, Palestine, pp. 41-87.

Onsanit, S., 2010. Trace elements in two marine fish cultured in fish cages in Fujian province, China. *Environ. Pollut*., **5**: 1334-1342. https://doi.org/10.1016/j.envpol.2010.01.012

Ololade, I.A., Lajide, L., Olumekun, V.O., Ololade, O.O. and Ejelonu, B.C., 2011. Influence of diffuse and chronic metal pollution in water and sediments on edible seafoods within Ondo oil-polluted coastal region, Nigeria. *J. environ. Sci. Hlth.,* **46**: 898-908. https://doi.org/10.1080/10934529.2011.580208

Rauf, A. and Javed, M., 2007. Copper toxicity to water and plankton in the river Ravi, Pakistan. *Int. J. Agric. Biol.,* **9**: 771-774.

Rauf, A., Javed, M. and Ubaidullah, M., 2009. Heavy metal level in three major carps (*Catla catla, Labeo rohita, Cirrhinus mrigala*) from the river Ravi, Pakistan. *Pak. Vet. J.,* **29**: 24-26.

Retnam, A. and Zakaria, M., 2010. *Hydrocarbons and heavy metals pollutants in aquaculture*. Proceedings of Postgraduate Qolloquium Semester 1 2009/2010, pp. 40-46.

Robert, G., 1991. *Toxic effects of metals, Casarett and Doull's toxicology*. Pergamon Press, pp. 662-672.

Tabinda, A.B. Bashir, S., Yasar, A. and Hussain, M., 2013. Metals concentration in the riverine water, sediments and fishes from river Ravi at Balloki headworks. *J. Anim. Pl. Sci.,* **23**: 76-84.

Tabinda, A.B., Bashir, S., Yasar, B. and Munir, S., 2013. Heavy metals concentrations in water, sediment and fish in River Sutlej at Sulemanki Headworks. *Pakistan J. Zool.,* **45**: 1663-1668.

Taha, A.A., 2004. Pollution sources and related environmental impacts in the new communities, Southeast Nile Delta, Egypt. *Emirate J. Engin. Res.,* **19**: 44.

Taiwo, I.E., Henry, A.N., Imbufe, A.P. and Adetoro, O.O., 2014. Heavy metal bioaccumulation and biomarkers of oxidative stress in the wild African tiger frog, Hoplobatrachus occipitalis. *Afri. J. environ. Sci. Tech.*, **8**: 6-15.

Tepe, Y., Turkmen, M. and Turkmen, A., 2008.

Assessment of heavy metals in two commercial fish species of four Turkish seas. *Environ. Monit. Assess.,* **146**: 277-284. https://doi.org/10.1007/s10661-007-0079-3

Turkmen, M., Turkmen, A., Tepe, Y., Tore, Y. and Ates, A., 2009. Determination of metals in fish species from Aegean and Mediterranean seas. *Fd. Chem.,* **113**: 233-237. https://doi.org/10.1016/j.foodchem.2008.06.071

Vineeta, S., Dhankhar, M., Prakash, J. and Sastry, K.V., 2007. Bioaccumulation of Zn, Cu, and Cd in *Channa punctatus*. *J. environ. Biol.,* **28**: 395-397.

Wang, Z.J. and Tang, H.X., 1998. The chemical toxicological and ecological studies in assessing the heavy metal pollution in Lean river, China. *Water Res.,* **32**: 510-518. https://doi.org/10.1016/S0043-1354(97)00229-7

Widianarko, B., Van Gestel, C.A.M., Verweij, R.A. and Van Straalen, N.M., 2000. Associations between trace metals in sediment, water, and guppy, *Poecilia reticulate* (Peters), from urban streams of Semarang, Indonesia. *Ecotoxicol. environ. Safe.,* **46**: 101-107. https://doi.org/10.1006/eesa.1999.1879

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Evaluation of Cd (II) and Cr (VI) Accumulation in Thaila Fish *Gibelion catla* (Formerly *Catla catla*) Collected from Industrially Polluted Water

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Authors' Contribution
AY supervised and planned the research. KMA did field collection and data analysis. SB performed experimental work. MK helped in the experiment and NM helped in data analysis.

Key words
Heavy metal pollution, River Ravi, *Gibelion catla*, Bioaccumulation.

ABSTRACT

The present study was designed to evaluate bioaccumulation of Cd and Cr in some tissues like muscle, liver and gills of Thaila Fish (*Gibelion catla*) inhabiting river Ravi. Three sites were selected for the collection of fish and water samples, Balloki headworks (S1), Shahdara Bridge (S2) and Lahore Siphon (S3). Samples of *G. catla* were also collected from ponds of Manawan Fisheries Research and Training Institute (FR&TI), Lahore and used as control. After capturing the fish were anesthesized, sacrificed and the organs were obtained. The cadmium and chromium concentration in these fish organs collected from various sites was measured through inductively coupled plasma optical emission spectrometry (ICP-OES). The same was used to measure water samples. Variation of concentrations of Cd (II) ions was non-significant among different organs whereas that of Cr (II) was significant (≤ 0.05). Also, significantly higher concentrations of Cd (II) and Cr (II) were observed in liver, muscle and gills as compare to control samples. In liver of fish samples, bio-concentration factors (BCF) of Cd (II) were calculated as 10.7, 3.6 and 6.4 at site S1, S2, and S3, respectively and those of Cr (VI) as 1.4, 1.0, and 1.4, respectively; In muscles, BCF values for Cd (II) were 8.8, 7.6 and 9.0 and for Cr (VI) it was 1.1, 0.8, and 1.1, respectively; In gills, values for Cd (II) were 10.8, 7.6, 9.0 and for Cr (VI) it was 1.1, 0.8, and 1.0, respectively. These findings for heavy metal bioaccumulation indicate that heavy metals persist throughout the river stretch. The results also suggest that this fish acts as a biomarker of exposure for heavy metals and can be used to report heavy metal pollution.

INTRODUCTION

Natural waters are being polluted by heavy metals from numerous anthropogenic as well as geological sources. It is an increasing global issue and many publications have described the heavy metals contamination in aquatic ecosystems. The bodies of aquatic animals accumulate large amounts of heavy metals under a variety of environmental conditions. Since heavy metals accumulate in different organs of fish, there is a need to evaluate heavy metal concentrations in water and fish organs to determine health risks to humans who consume fish as food (Yilmaz *et al*., 2007).

Due to the rapid increase in industrial and agricultural activities over the past few years the wastes from point and non-point sources are indiscriminately dumped into the surrounding water ecosystems and as a result heavy metals progressively accumulate in fish organs through aquatic food chain (Abdel-Baki *et al*., 2013). Heavy metal bioaccumulation in fish is mainly dependent on fish species, heavy metal type and also on tissues of fish (Qiao-Qiao *et al*., 2007). Fishes in aquatic ecosystems are able to accumulate heavy metals in their tissues in greater concentrations than water, micro flora and sediments in their environment. As the levels of heavy metals increase in water, the accumulation rate of heavy metals in fish body tissues also increase. Iron, zinc and copper are considered essential for fish for their biological and enzymatic activities if present in low concentration. However, other metals, such as cadmium, mercury and lead can be highly toxic if they are present in minute quantity because they do not have any essential role in fish body. Nevertheless, even essential metals can also become very toxic if present in high concentration in fish tissues (Yaduma and Humprey, 2009).

Fish, as food is consumed by humans all over the world and there is a threat to human life due to consumption of toxic fish. Many mutagenic, cytotoxic

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and carcinogenic effects in fish body are caused by these heavy metals (Al-Kahtani, 2009). Imbalances of aquatic ecosystem are caused by heavy metals and they also disrupt the aquatic food chain. Accumulation of heavy metals varies depending upon route of metal uptake, fish species and type of heavy metal which is accumulated by fish (Alyahya *et al*., 2011). These accumulations result in changes in biochemical metabolism, histopathological changes, serum biochemical changes and other induce stresses if they are present in any part of the fish body (Shivakumar *et al*., 2014).

Fishes are used as bio-indicators in aquatic environments for the determination of heavy metals. Metal deposition in different organ of fish is caused by temperature of water. Gills of fishes in acidic conditions usually absorb divalent ions of heavy metals (Bat *et al*., 2012). Cadmium is usually accumulated in liver and kidneys under high temperatures. This accumulation probably results from high metabolic rate under high temperatures which promote high rate of metal uptake and binding. Acidified lakes have high concentration of cadmium and lead but not zinc. Uptake of heavy metals across the gills is also affected by hardness of water (Akan *et al*., 2012).

The heavy metals are deposited, incorporated or assimilated in water, sediments and even in the body of aquatic animals because these metals cannot be degraded and it is the main cause of water pollution. These metals can be biomagnified within the food chain and when assimilated by humans they become a part of human body resulting in health risks (Wuana and Okieimen, 2011).

This study was planned to evaluate heavy metals (Cd-II, Cr-VI) in the water bodies and accumulation of these metals in various tissues of *Gibelion catla* inhibiting polluted water of river Ravi.

MATERIALS AND METHODS

Study area

The current study was conducted at three sites of river Ravi which originate from India than moving along Indo-Pak border it enters in Pakistan and passes through Lahore. (Tabinda *et al*., 2013). The three sites chosen along the river are S1 (Balloki headworks), S2 (Shahdara Bridge), S3 (Lahore Siphon).

Collection of samples

Samples of *G. catla* were collected from designated sites. The control samples were obtained from Government operated Fish Farm, Manawan Fisheries Complex, Lahore. Fish were collected by using dip nets and were kept in polythene bags in ice cooler and transported to the Research Laboratory at the Department of Zoology, GC Universsity, Lahore. Fish were identified with the help of identification key based on morphological characters (Mirza, 2003) and weight of each fish sample was recorded.

Water samples were collected and preserved by adding concentrated HCl 2ml/ liter of samples at the time of collection. These samples were wrapped in aluminum foil and stored in -4°C freezer in laboratory.

Physico-chemical parameters, such as temperature and pH of water were also measured at the time of collection.

Heavy metal analysis

Fish samples were prepared by taking muscles, gills and liver after dissection. These organs undergo wet difestion as described by Enamorado-Baez *et al*. (2013). Briefly, the tissues were weighed and 2g was taken, chopped and homogenized. HNO_3 (10 ml) was taken and mixed with fish solid sample and allow to heat on hot plate at 90 to 95°C and then refluxed for 10 to 15 min. Upon cooling 3ml of 30% hydrogen peroxide (H_2O_2) and 2ml of deionized water was added. Sample were placed again onto the hot plate and allowed to heat until the effervescence stops. Again 1 ml of 30% H_2O_2 was added and warmed until the effervescence stop and cooled at room temperature. By using the syringe filter, Nalgene 0.25 nm the digested samples of fish tissues were filtered. Filtrate was added to a new glass vials and stored at room temperature until analytical procedures could be done.

For digestion of water samples Ozturk *et al*. (2009) method was used. A beaker was filled with 100 ml of water and 3 ml of concentrated HNO_3 and allowed to heat on hot plate slowly until the volume was decreased to 5ml. The beaker was allowed to cool after removing from the hot plate and 3ml of conc. HNO_3 was added and again it was placed on hot plate. With the continuous addition of acid, samples were heated until the sample solution was clear and light in color. The sample solution was evaporated until the volume reached to 3ml. Upon cooling of sample 10ml of HCl was added to sample and heated for 15 min. The sample solution was filtered by using filtration apparatus to remove the suspended particles. Final volume of sample was adjusted to 100ml at the end with deionized water. Filtrate was added to a new glass vials and stored until analytical procedures could be done.

Determination of metal ions

The filtrates were analyzed for Cd (II) and Cr (VI) by using "inductively coupled plasma optical emission spectrometry (ICP-OES, Perkin Elmer Optima 7000DV)".

Determination of bio concentration factor

Relative concentration of cadmium and chromium among body organs was determined by Bio concentration factor (BCF).

$$BCF = Tc/Wc$$

Where, Tc is heavy metal concentration in fish tissues and Wc is heavy metal concentration in water.

Statistical analysis

By using statistical software (SPSS) for social sciences, statistical analysis of the data was performed. Correlation analysis was done to examine the interrelationships between the investigated metal concentrations. T-test was performed for the determination of significance between the values.

RESULTS

Water analysis

Temperature and pH at all the selected sites were determined while collecting the water samples. The values for temperature and pH are given in Table I.

Table I.- Mean values of water temperature and pH at all studied sites (n=3).

Sites	Temp. (°C) Mean±SD	pH Mean±SD
S1	33 ±2	9.6±0.2
S2	30±2	8.4±0.3
S3	30±4	8.4±0.2
Control	28±3	7.9±0.2

Cadmium and chromium concentration in water samples

Mean concentrations of divalent cadmium (Cd II) and hexavalent chromium (Cr VI) at sites 1, 2, 3, and control were measured and compared with the WHO and NEQS, Pakistan provided standard concentrations as shown in Table II.

Lowest concentration of Cd (II) was observed in control samples whereas highest concentration of Cd (II) was observed at site 1 which is downstream of the river. In case of Cr (VI) lowest concentration was observed in control samples whereas highest concentration was at site 2 which is downstream of the river.

Table II.- Concentrations (Mean ± SD) of heavy metals (mg/L) in water collected from different sampling sites.

Sampling sites	Cd (II) Mean ± S.D	Cr (VI) Mean ± S.D
Site 1	0.03±0.008	0.42 ± 0.26
Site 2	0.03±0.004	0.50 ± 0.32
Site 3	0.02±0.005	0.39 ± 0.12
Control	0.01±0.002	0.05 ± 0.02
NEQS	0.01 mg/L	1 mg/L
WHO	0.72 ug/L	11 ug/L

*NEQS, permissible standard values for Pakistan.

Analysis of fish tissues

Liver

Cd (II) and Cr (VI) concentration in liver tissue of *G. catla* collected from study sites S1, S2, S3, and control were measured. The highest concentration of Cd (II) and Cr (VI) was observed in the liver of fish collected from site 1 (Balloki headworks) as shown in Table III.

Muscle

Cd (II) and Cr (VI) concentration in muscle tissue of *G. catla* collected from study sites S1, S2, S3, and control were measured. The highest concentration of Cd(II) and Cr(VI) was observed at S1 (Balloki Headworks) as shown in Table III.

Gills

Cd (II) and Cr (VI) concentration in gills of *G. catla* collected from study sites S1, S2, S3, and control were measured. The highest concentration of Cd(II) and Cr (VI) was observed in fish collected from S1 (Balloki headworks) as shown in Table III.

Table III.- Mean concentrations (Mean ± SD) of heavy metals in mg/kg in liver tissues of *G. catla.*

Sampling sites	Liver		Muscle		Gills	
	Cd	Cr	Cd	Cr	Cd	Cr
Site 1	0.29 ±0.006	0.62 ±0.05	0.24 ±0.05	0.47±0.02	0.29±0.02	0.46±0.04
Site 2	0.09±0.004	0.50±0.03	0.19±0.04	0.40 ±0.05	0.19±0.04	0.44±0.03
Site 3	0.15±0.006	0.56±0.02	0.21±0.006	0.43±0.04	0.21±0.006	0.39±0.009
Site 4	0.01 ± 0.002	0.19 ± 0.05	0.02 ± 0.03	0.18±0.02	0.02±0.002	0.18 ± 0.001
Rf value*	0.10 mg/kg	0.05 mg/kg	0.10 mg/kg	0.05 mg/kg	0.10 mg/kg	0.05 mg/kg
EPA (Pakistan)	0.05 mg/kg	0.01 mg/kg	0.05 mg/kg	0.01 mg/kg	0.05 mg/kg	0.01 mg/kg

*, WHO/FAO (1989).

Bio-concentration of heavy metals

The bio-concentration of Cd(II) and Cr(VI) was calculated by dividing the heavy metal concentration in fish tissues divided by heavy metal concentration in respective water sample. The bio-concentration values in all three tissues are shown in Table IV.

Table IV.- Bio-concentration factor of Cd (II) and Cr (VI) in *G. catla* tissues.

	Cadmium (Cd II)			Chromium (Cr VI)		
	Liver	Muscle	Gills	Liver	Muscle	Gills
Site 1	10.7	8.8	10.8	1.4	1.1	1.1
Site 2	3.6	7.6	7.6	1.0	0.8	0.8
Site 3	6.4	9.0	9.0	1.4	1.1	1.0
Control	1.1	2.2	1.9	3.7	3.6	3.6

DISCUSSION

The area of river Ravi used in this study is stretched from Lahore Siphon to Shahdara Bridge and Balloki head-works. The main sources of pollution in river Ravi are industrial, urban and agricultural wastes. The discharge from electroplating workshops, paper and pulp industries, scientific and medicine laboratories, steel factories as well as municipal sewage and surface runoff contribute to the contamination of this water body (Rauf *et al*., 2009).

The data concerning concentrations of Cd (II) and Cr (VI) in water at different studied sites revealed that the amounts of the two toxic metals were significantly higheras compared to control. Significantly elevated concentration of Cd (II), from the samples collected along river Ravi clearly indicates the pollution burden rendered upon these sites. This situation is quite alarming because of the persistent elevated level of Cd (II) ions beyond permissible limits set by World Health Organization (WHO) as well as Environmental Protection Department, Pakistan (EPD). The previous studies for the evaluation of Cd (II) at these areas showed the similar trend (Yaqub, 2003; Tabinda *et al*., 2013). On the other hand, values in the current study are slightly elevated as compared to the previous reports of the same area (Khan *et al*., 2003). This might be due to fact that water bodies, located along Ravi may have overburdened by the set-up of increasingly developing industrial zones along the tributaries of this river.

A comparison of chromium (Cr VI) concentration showed that water from Head Balloki, Shahdara Bridge and Lahore Siphon contained higher concentrations of Cr (VI) and is beyond acceptable limits set by World Health Organization (WHO) as well as Environmental Protection Department, Pakistan (EPD).

The concentrations of Cr (VI), at studied areas were much lower than previously reported studies of the same area (Yaqub, 2003; Khan *et al*., 2003).

The concentrations of Cr (VI) in water were in accordance with a study by Qadir *et al*. (1997) but are much higher than another study at the same area (Kashif *et al*., 2009). Higher concentration of heavy metals can be attributable to the industries along the sides of this river (Rauf *et al*., 2009).

As aquatic ecosystem is being polluted by untreated chemical discharges from industrial, domestic and agricultural wastes, the adverse effects are being produced on populations of aquatic fauna and thereby becoming an important environmental problem. Pollutants tend to bio-accumulate in the aquatic food web and can become a threat to human health due to the consumption of intoxicated organisms such as fish (Benson *et al*., 2007). Fish are often at the top of aquatic food chain and had greater tendency to accumulate heavy metals from river water (Gitet *et al*., 2016).

Cd (II) concentration in Fish muscles showed its bioavailability in water bodies which could cause carcinogenic effects in aquatic biota and human health (US EPA). Presence of Cd (II) is highly toxic which could cause anomalies such as developmental reduction and slow growth rate as well as lowest concentration of Cd (II) could cause skeletal ossification (Rodríguez *et al*., 2016). Bioaccumulation of Cd (II) ions in control samples (MFRTI pomd) showed that Fish from the farms may get some amount of heavy metals through surrounding water and artificial diet. Levels of Cd (II) were observed high among edible fishes with mean value of 0.48 ppm averaging about 0.45 ppm in most liked species of Pakistan *G. catla* and *Labeo rohita* (Hussain *et al*., 2016).

Active metabolite organs, such as gill and liver sensitively accumulate large amount of metals as compared to the muscles. Different tissues have different tendencies to accumulate metals because these tissues have different functions as well as different metabolic roles of metals in organs (Hussain *et al*., 2016).

Cr (VI) is more concentrated in liver tissues of all fish which is beyond the permissible limits set by World Health Organization (WHO) as well as Environmental Protection Department, Pakistan (EPD). Toxicity of Cr (VI) was statistically significant among all studied sites.

Liver is the main organ for the detoxification and it is an active site for pathological responses induced by contamination. Gills may be direct in contact with water and toxins and it perform the function of respiration in fish bodies reported by Uzairu *et al*. (2009).

Usually fish in aquatic ecosystems absorb metals through drinking of water and ingestion of contaminated

food. These metals bio-accumulate in the tissues of fish and in turn are transferred to humans through consumption of fish and fish products (Gitet *et al*., 2016).

Metal concentration in fish tissues is due to its contact with sediments and water (Rauf *et al*., 2009). *Labeo rohita* and *G. catla* had less metal concentration as compare to the other species because *G. catla* is upper water column dweller and *Labeo rohita* is mid column water dweller (Tabinda *et al*., 2013).

Cr (III) and Cr (VI) compounds have ability to bind with the soil and very persistent in water and sediments. Cr (VI) compounds are very toxic and can be potential carcinogens while Cr (III) is an essential nutrient. High level of Cr (VI) cause irritation to the lining of nose, nose ulcers and breathing problems in human (Benson *et al*., 2017).

This study revealed that bioaccumulation of heavy metals in fish farms was significantly and Cr (VI) can bio-accumulated more than Cd (II).

CONCLUSION AND RECOMMENDATIONS

The present study revealed that concentration of heavy metals, cadmium and chromium in liver, muscle and gills of fish differed significantly between fish samples collected from River Ravi and those collected from private fish farms of Manawan Fisheries Complex, Lahore. Low levels of Cd (II) heavy metal accumulation was observed in muscles of *G. catla* collected from River Ravi. The descending order of accumulation of Cd (II) in various body parts of examined fish was gills > liver > muscles. High levels of Cr (VI) concentration were noted in liver of *C. catla* collected from River Ravi. The descending order of accumulation of Cr (VI) in the fish was liver > muscles > gills. Presence of heavy metals in tissues of fish collected from River Ravi was most likely due to point and non-point source industrial and domestic discharges into the River Ravi.

On the basis of present findings, the following recommendations are proposed: (i) Prior to discharge of domestic and industrial effluents into the link canals sewage, they should be treated properly before finally falling into the barrages of the respective cities. (ii) There should be proper systematic pollution checking system for daily pollution loads at all the head works especially at downstream locations from the headworks. (iii) Trees should be planted on the boundaries of the water systems to prevent direct entry of agricultural wastes into the waterways. (iv) Serious efforts should be made to reduce anthropogenic discharges in the head works as gradually increasing water pollution levels will greatly influence area. (v) Seasonal variations of physico-chemical parameters of river water quality must be considered while establishing water quality parameters for pollution reduction and proper management of the aquatic ecosystems.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abdel-Baki, A.S., Dkhil, M.A. and Al-Quraishy, S., 2013. Bioaccumulation of some heavy metals in tilapia fish relevant to their concentration in water and sediment of Wadi Hanifah, Saudi Arabia. *Afri. J. Biotechnol*., **10**: 2541-2547.

Akan, J.C., Mohmoud, S., Yilkala, B.S. and Ogugbuaja, V.O., 2012. Bioaccumulation of some heavy metals in fish samples from River Benue in Vinikilang, Adamawa State, Nigeria. *Am. J. Analyt. Chem*., **3**: 727-736. https://doi.org/10.4236/ajac.2012.311097

Al-Kahtani, M.A., 2009. Accumulation of heavy metals in tilapia fish (*Oreochromis niloticus*) from Al-Khadoud Spring, Al-Hassa, Saudi Arabia. *Am. J. appl. Sci*., **6**: 2024-2029. https://doi.org/10.3844/ajassp.2009.2024.2029

Alyahya, H., El-Gendy, A.H., Al Farraj, S. and El-Hendeny, M., 2011. Evaluation of heavy metal pollution in the Arabian Gulf using the calm *Meretrix meretrix* Linnaeus, 1758. *Water Air Soil Pollut*., **214**: 499-507. https://doi.org/10.1007/s11270-010-0441-x

Bat, L., Sezgin, M., Ustun, F. and Sahin, F., 2012. Heavy metal concentration in ten species of fishes caught in Sinop coastal waters of Black Sea, Turkey. *Turk. J. Fish. aquat. Sci*., **12**: 371-376.

Benson, N.U., Essien, J.P., Williams, A.B. and Bassey, D.E., 2007. Mercury accumulation in fishes from tropical aquatic ecosystems in Niger Delta, Nigeria. *Curr. Sci*., **92**: 781-785.

Benson, R., Conerly, O.D., Sander, W., Batt, A.L., Boone, J.S., Furlong, E.T. and Simmons, J.E., 2017. Human health screening and public health significance of contaminants of emerging concern detected in public water supplies. *Sci. Total Environ*., **579**: 1643-1648. https://doi.org/10.1016/j.scitotenv.2016.03.146

Enamorado-Baez, S.M., Abril, J.M. and Gomez-Guzman, J.M., 2013. Determination of 25 trace element concentrations in biological reference materials by ICP-MS following different microwave assisted acid digestion methods based on scaling masses of digested samples. *ISRN Analyt. Chem*., **2013**: Article ID 85171. https://doi.org/10.1155/2013/851713

Gitet, H., Hilawie, M., Muuz, M., Weldegebriel, Y., Gebremichael, D. and Gebremedhin, D., 2016. Bioaccumulation of heavy metals in crop plants grown near Almeda Textile Factory, Adwa, Ethiopia. *Environ. Monitor. Assess*., **188**: 500. https://doi.org/10.1007/s10661-016-5511-0

Hussain, B., Sultana, T., Sultana, S., AlGhanim, K.A. and Mahboob, S., 2016. Study on effect of pollution on genotoxic damage in *Cirrhinus mrigala* and *Catla catla* from river Chenab. *Environ. Sci. Pollut. Res. Int*., **23**: 14589-14599. https://doi.org/10.1007/s11356-016-6645-x

Kashif, S.R., Akram, M., Yaseen, M. and Ali, S., 2009. Studies on heavy metals status and their uptake by vegetables in adjoining areas of Hudiara drain in Lahore. *Soil Environ*., **28**: 7-12.

Khan, M., Khan, H.N. and Aslam, H., 2003. Hudiara drain-a case of trans-boundary water pollution between India and Pakistan. *Pakistan J. biol. Sci*., **6**: 167-175. https://doi.org/10.3923/pjbs.2003.167.175

Mirza, M.R., 2003. Checklist of freshwater fishes of Pakistan. *Pakistan J. Zool. Suppl. Ser*., **3**: 1-30.

Ozturk, M., Ozozen, G., Minareci, O. and Minareci, E., 2009. Determination of heavy metals in fish, water and sediments of Avsar Dam Lake in Turkey. *Iranian J. environ. Hlth. Sci. Engin*., **6**: 73-80.

Qadir, M., Qureshi, R.H. and Ahmad, N. 1997. Nutrient availability in a calcareous saline-sodic soil during vegetative bioremediation. *Arid Soil Res., Rehab.,* **11**: 343-352. https://doi.org/10.1080/15324989709381487

Qadir, A. and Malik, R.N., 2011. Heavy metals in eight edible fish species from two polluted tributaries (Aik and Palkhu) of the River Chenab, Pakistan. *Biol. Trace Elem. Res*., **143**: 1524-1540. https://doi.org/10.1007/s12011-011-9011-3

Qiao-Qiao, C., Guang-wei, Z. and Langdon, A., 2007. Bioaccumulation of heavy metals in fishes from Taihu Lake, China. *J. environ. Sci*., **19**: 1500-1504. https://doi.org/10.1016/S1001-0742(07)60244-7

Rauf, A., Javed, M. and Ubaidullah, M., 2009. Heavy metal levels in three major carps (*Catla catla*, *Labeo rohita* and *Cirrhina mrigala*) from the river Ravi, Pakistan. *Kidney*, **2**: 4-43.

Rauf, A., Javed, M. and Ubaidullah, M., 2009. Heavy metal levels in three major carps (*Catla catla, Labeo rohita* and *Cirrhina mrigala*) from the river Ravi, Pakistan. *Pakistan Vet. J*., **29**: 24-26.

Rodríguez, J. and Mandalunis, P.M., 2016. Effect of cadmium on bone tissue in growing animals. *Exp. Toxicol. Pathol*., **68**: 391-397. https://doi.org/10.1016/j.etp.2016.06.001

Shivakumar, C.K., Thippeswamy, B., Tejaswikumar, M.V. and Prsahanthakumar, S.M., 2014. Bioaccumulation of heavy metals and its effects on organs of edible fishes located in Bhandra River, Karnatka. *Int. J. Res. Fish. Aquacult*., **4**: 90-98.

Tabinda, A. B., Bashir, S., Yasar, A. and Hussain, M., 2013. Metal concentration in riverine water, sediments and fishes from River Ravi at Balloki Headworks. *J. Anim. Pl. Sci*., **23**: 76-84.

Uzairu, A., Harrison, G.F.S., Balarabe, M.L. and Nnaji, J.C., 2009. Concentration levels of trace metals in fish and sediments from Kubanni River, northern Nigeria. *Bull. Chem. Soc. Ethipoia*, **23**: 9-17. https://doi.org/10.4314/bcse.v23i1.21293

Wuana, R.A. and Okieimen, F.E., 2011. Heavy metals in contaminated soils: A review of sources, chemistry, risks and best available strategies for remediation. *ISRN Ecol*., **2011**: Article ID 402647. https://doi.org/10.5402/2011/402647

Yaduma, J.B. and Humprey, M.M., 2009. Accumulation of some heavy metals on *Clarias anguillaris* and *Heterotis niloticus* from Lake Geriyo Yola Nigeria. *Nature Sci*., **7**: 40-43.

Yaqub, A., 2003. *Determination of heavy metal/metalloid load (As, Cd, Cr, Cu, Fe, Ni, Zn) in water of Hudiara Drain, adjoin soils and some crops irrigated with Hudiara Drain*. M. Phil. thesis, Department of Zoology, GC University, Lahore, Pakistan.

Yilmaz, F., Ozdemir, N., Demirak, A. and Tuna, A.L., 2007. Heavy metal levels in two fish species *Leuciscus cephalus* and *Lepomis gibbosus*. *Fd. Chem*., **100**: 830-835. https://doi.org/10.1016/j.foodchem.2005.09.020

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ATPase 6/8 Gene Assisted Variability among the Populations of *Labeo rohita* and its Global Genealogy Relationship with Cyprinids

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Authors' Contribution
FR supervised the study and wrote the article. SSH conducted the research. NK collected the data. SK helped in DNA extraction and data collection. TH analyzed the data. HA helped in arranging data and statistical analysis. KMA helped in writing and proof-reading of the article.

Key words
***Labeo rohita*, ATPase 6/8, Phylogeography, Genealogy, Cyprinids.**

ABSTRACT

The study on evaluation of potential of the whole ATPase 6/8 region of the mitochondrial DNA as a molecular marker was conducted for determination of phylogeographic analysis of *Labeo rohita*. For detection of variations among the different weight groups from the same age class of this species samples were collected from the fish farming area in Punjab-Pakistan and its phylogeographic relationship on the basis of ATPase 6/8 gene studies conducted on other cyprinids of the world and registered with NCBI was analyzed. The ATPase6/8 region was more variable but gave the wide distribution of *Labeo rohita,* the overall levels of sequence divergence were low. Levels of haplotype diversity varied widely among countries with Chinese and India *spp.* showing the greatest diversity whereas Japanese *Labeo* had undetectable nucleotide variation. Chinese and Japanese carp strains were the most divergent and their relationships did not support the evolution of independent Asian and European lineages and current taxonomic treatments. The results revealed that 878 bp of ATPase 6/8 region could be a promising marker for determining variations at inter-population as well as intra-population levels in *Labeo rohita*. These results would facilitate conservation and management of this important species.

INTRODUCTION

Labeo rohita is the important member of the carp family *Cyprinidae.* In Pakistan and other surrounding countries, this fish is famous due its fast growth rate with local names; rohu, dumbra *etc*. *L. rohita* occupies almost all the natural reservoirs *viz*. rivers, canals and freshwater lakes of the South and South-East Asiatic regions. In Pakistan, Bangladesh, India, Nepal and other Asiatic parts, *L. rohita* is considered as a delicacy due to its taste and fast growing rate with its rare omnivorous nature in the niche. The breeding behavior of this species is that it does not breed in stagnant water bodies, widely adopted methods of its breeding is induced breeding by induction of hormonal injection in the manmade hatcheries (Froese, 2006). Luhariya *et al*. (2014) sequenced ATPase 6/8 gene of mitochondrial DNA in *Labeo rohita* samples collected from nine rivers belonging to four river basins; Indus, Ganges, Brahmaputra and Mahanadi. The analysis after their study discovered 44-haplotypes with high haplotype diversity (0.694) and low nucleotide diversity (0.001). The within population variation was larger (83.44%) than among population differences (16.56%). The mean FST value (0.166; $P < 0.05$) for overall populations revealed moderate level of genetic structuring in the wild *L. rohita* populations. The haplotype network presented a single clade for wild *L. rohita* population, from different rivers. Negative values for Fu's index (*FS*), mismatch distribution analysis indicated period of expansion in *L. rohita* population. The time after recent expansion was estimated for each population, between 0.042 to 0.167. The analysis of data demonstrated that ATPase6/8 gene polymorphism is a potential marker to understand genetic population structure of wild *L. rohita* existing in different rivers. The study identified population substructure in wild *L. rohita* with common ancestral origin.

For sustainable aquaculture practices, the studies on genetic variation within and between the species and populations of the cultureable fishes for maintenance and improvements in the genetic makeup are need of the time. The level of variation within and between the species is helpful to ascertain the taxonomic/systematic components and to identify distinction of a species. Genetic variability studies at the level of population execute the minute differences studies for speciation

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purposes for identification of new differentiated genetic groups from sub species and phylogenetic analysis of the present status of a population with its wild relatives. The genetic variability within population is extremely useful to gather the information on individual identity, breeding outline, degree of kinship and conflicts of genetic differences among them (Schierwater *et al*., 1994). Inter- and intraspecific mitochondrial DNA (mtDNA) variation is a very useful marker to study the evolution of animals, especially in taxonomy, systematics, ecology and population biology. The mtDNA analyses based on partial nucleotide sequences usually assume that most of the variations conform to a neutral model of molecular evolution, but some experimental studies and human mitogenome data have suggested that selective forces might act upon mtDNA (Hirayama *et al*., 2010). Liu and Cordes (2004) stated that molecular genetic markers have become valuable tools in population genetics, conservation biology and evolutionary studies. These markers have been used to estimate effective population size (Kuhner *et al*., 1998), historical bottlenecks and sex specific gene flow. However, selection of appropriate markers that is useful to determine genetic variation is prerequisite for any population genetic study. Mitochondrial DNA is widely used to determine the variation at interspecific and intraspecific levels. There is a need of specific knowledge of intraspecific genetic variations to plan rehabilitation and management strategies for populations of the species which are declining in their natural habitat. The ATPase8 and ATPase6 regions of mitochondrial DNA have been successfully analyzed for both phylogeny as well as phylogeography in many fish species (Chow and Ushiama, 2004; Dammannagoda *et al*., 2008; Vergara-Chen *et al*., 2009). Gjedrem and Baranski (2009) demonstrated that knowledge of genetic parameters of the species cultured is vital for sustainability of the production system. More emphasis is being given on genetic improvement of aquaculture species, following the success story of salmon and tilapia selective breeding programs. However, information on genetic relationships and diversity of these species at the molecular level is not yet available. Genetic diversity is the existence of variants (alleles) of individual genes due to the change of the DNA sequence. The alleles of a particular gene may occur at different frequencies in different groups of individuals interbred (population) and genetic variation of a particular species because it is distributed both within populations and between populations (difference in occurrence and frequency between populations). Latest technological developments in the field of genetics have shown great potential for their application in fish conservation. Genetic variation can be directly assessed through controlled genetic markers. Use of more than one marker can help enlarge the scope of utilization data. Information on the genetic structure of cultivable fish species are useful to optimize the identification of populations, improvement of the population, improvement programs, performance management and sustainable conservation of genetic diversity (Garcia and Benzie, 1995). Rohu, Catla, Mrigal and Kalbasu have been previously classified on the basis of their morphological characteristics. These four species do not intersect, but produce fertile hybrids in any combination (Jhingran, 1991). These species may have a common ancestral origin (Khanna, 1988). In Pakistan there is no baseline data for genetic identification of the brood stock, so the aim the present study is to provide a substitute mitochondrial DNA polymorphism analysis to launch appropriateness for the genetic stock identification program for *Labeo rohita* amongst the locally available stocks. This study will also provide smaller input for the demographic data and phylogeny analysis of the locally available stocks of *L. rohita.*

MATERIALS AND METHODS

Sample collection

Samples for three weight groups having different size with same age group for the determination of intraspecific variation and recording morphometeric data were collected from the fish farm of the Department of Fisheries and Aquaculture, UVAS, Ravi Campus, Pattoki.

Amplification of ATPase 6/8 gene

Genomic DNA extraction

Total genomic DNA isolation was carried out from the stored fish samples using the procedure described by Lopera-Barrero *et al*. (2008). This procedure is based on the protocol given by Aljanabi and Martinez (1997) which was modified by the use of NaCl. In this procedure lysis buffer was used which carried 50mM tris which was taken from a stock of 1 M pH: 8 tris buffer, 50mM EDTA taken from a stock of 0.5 M pH: 8 , 100mM NaCl taken from a stock of 5 M NaCl and 1% SDS. From this lysis buffer working lysis buffer was prepared by adding 7µl of 200µgmL^{-1} proteinase K. Stock solution of the proteinase K was prepared by preparing the buffer of 100mM Tris-base, 50mM EDTA, 500mM NaCl and then Proteinase K was added and dissolved at 200µgmL^{-1}. DNA was visualized on 1% agarose gel.

Table I.- Primers sequence.

Primer name	5'-3' Sequence
ATPase6/8-Fish	F: AAAGCRTTRGCCTTTTAAGC
	R: GTTAGTGGTCAKGGGCTTGGRTC

PCR

Specific primers for Mitochondrial ATPase 6/8 genes were used from Thai *et al*. (2004) (Table I). With the help of the primers, polymerase chain reactions were devised. Each reaction was performed in 0.2 ml PCR tube and 25 µL reaction mixtures. To prepare this 25 µL reaction mix 2.5 µL 10x PCR buffer, 2 µL 1.6 mM MgCl2 2µL 10 nM primer, 2 µL 2.5 mM dNTPs, 0.3 µL 5 units/µL Taq polymerase enzyme and 11.2 µL sterilized deionized double distilled water was mixed. In each reaction a negative control was also run using sterilized water as template.

PCR reaction was carried out in thermal cycler (Bio-Rad) USA. For ATPase6/8 primer was amplified using the following conditions: one cycle of 5 min denaturation at 95°C and then 35 cycles of 1 min at 94°C, 45 sec at 55°C for annealing and 1 min at 72°C for extension and finally 10 min final extension time was given at 72°C. Then the machine was allowed to hold the reaction contents at 4°C. PCR product was visualized on 1.5% agarose gel.

DNA sequencing

Double stranded PCR product was purified using elution method from low melting Agarose gel. The purified PCR Product was used in setting up sequencing reaction with same set of primers using Mega Bace ET Terminator Dye kit. The sequencing reaction was performed for 30 cycles of: 95°C for 10 sec; 50°C for 20 sec; 60°C for 2 min. PCR products were precipitated using ethanol and ammonium acetate and were dissolved in Mega Bace Loading Buffer. The DNA sequencing was carried out on an automated DNA sequencer, ABI 3100 Genetic Analyzer (Prism) using manufacturer's recommendations.

Analysis of DNA sequences

The analysis of ATPase6 and ATPase8 gene of *Labeo rohita* was done using the bioinformatics software. The Codon Code Aligner software was used for the alignment and to find single nucleotide polymorphisms (SNPs) in the sequences. The phylogenetic analysis of Pakistani *L. rohita* sequences was done using already reported sequences of *Labeo rohita* and some other related fish species

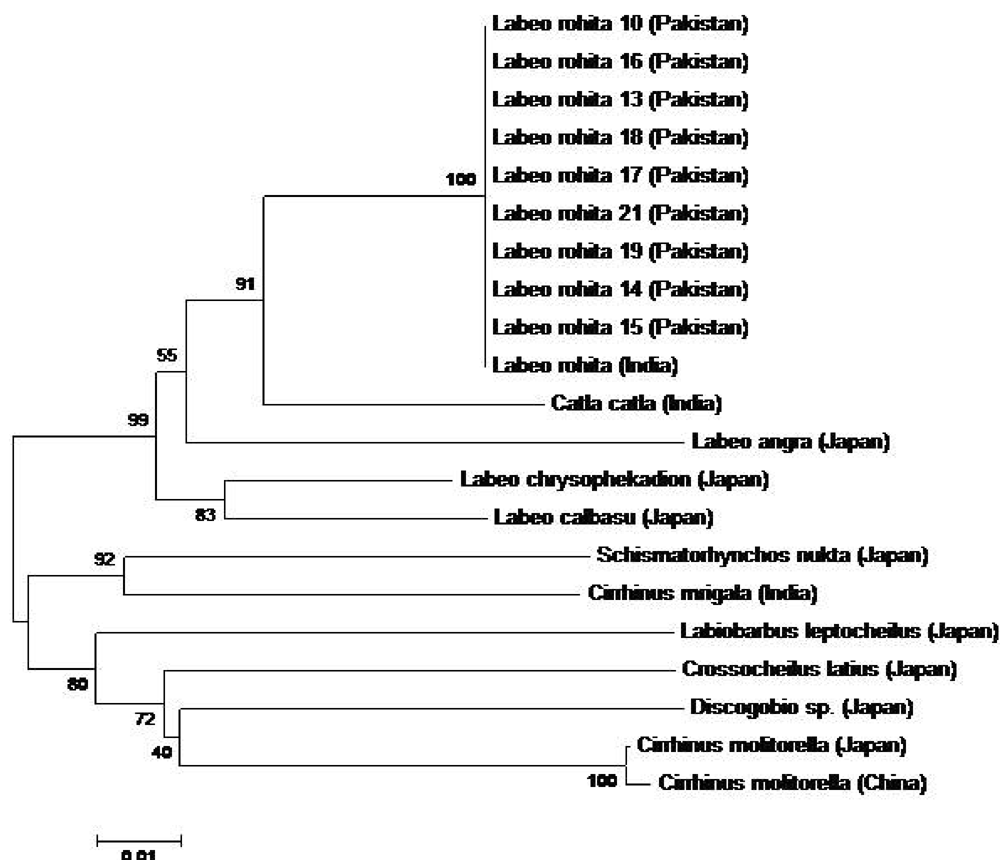

Fig. 1. The NJ phylogenetic tree (rectangular view) based on ATPase6/8 gene sequences. The NJ Phylogenetic tree (Rectangular view) based on ATPase6 and ATPase8 gene sequences of *Labeo rohita* from Pakistan with reported sequences of different fish species on GenBank (NCBI) using MEGA 6.1.

from different parts of the world available on GenBank (NCBI). MEGA 6.1 software was used for construction of phylogenetic tree using Neighbor Joining method with 1000 bootstrap value. The DNA sequences were aligned using CLUSTAL W (Thompson *et al*., 1994) and sequence composition was estimated using software MEGA 6.1 (Gupta *et al*., 2013). The phylogenetic trees were constructed by CLUSTAL OMEGA for analysis of ATPase6/8 gene variation and the sequences differences among the cyprinids of Pakistan and from the different parts of the world taken from NCBI GenBank.

RESULTS AND DISCUSSION

The neighbor joining tree was constructed to depict genetic relatedness between the samples using software MEGA 4.1 (Tamura *et al*., 2007) and molecular clock was calibrated using nucleotide substitution rate of 1.3% per million years (My) as suggested for ATPase 6 and 8 genes in animals.

Phylogenetic analysis of mitochondrial ATPase6 and ATPase8 genes

The Neighbor Joining phylogenetic tree was constructed using MEGA 6.1 software based on finally selected 878 bp fragment of APTPase6 and ATPase8 genes in all the fish samples from Pakistan. We constructed the trees using 1000 bootstrap value. All the ATPase6/8 sequences from Pakistani *Labeo rohita* and same region sequences (given below) from other parts of the word (from NCBI) were used for the construction of phylogenetic trees.

```
CAATTAAACCCCGGCCCCTGATTCGCAATTTTAGTATTCTCTTGATTAATTTTCCTAA
CCATCATTCCAACTAAAATCCTAAACCATATTTCACCAAATGAACCAACCCCAGTAA
GTGCTGAAAAACACAAAACTGAATCCTGAGATTGACCATGATAACAAGCTTCTTCG
ACCAATTCGCAAGCCCATCATACCTGGGAATCCCCCTAATCGCAATCGCAATTGCAC
TACCATGAGTTCTCTATCCAACCCCATCATCCCGATGAATCAATAACCGACTTATTA
CAATCCAAGGATGGTTCATTAACCGATTTACAAACCAACTGCTACTCCCCCTAAATG
TAGGAGGCCACAAATGAGCACTTTTACTAGCCTCACTAATAATTTTCCTAATTACTA
TTAATATATTAGGCCTACTTCCATACACCTTCACACCTACAACACAACTATCACTCA
ACATAGGATTTGCCGTACCACTGTGACTCGCCACAGTAATTATTGGAATACGTAACC
AACCTACAGTTGCCCTGGGACACCTTCTCCCAGAAGGAACACCCATTCCTCTAATCC
CAGTACTTATTATTATCGAAACAATCAGCCTACTTATCCGACCACTAGCCCTAGGAG
TCCGACTTACAGCAAACTTGACCGCAGGTCACCTACTAATCCAACTAATCGCTACAG
CCGTATTTGTCCTCTTACCAATAATGCCTACAGTAGCAATCCTAACTGCTACAGTACT
CTTCCTGTTAACACTACTAGAAGTCGCAGTAGCAATAATTCAAGCCTATGTATTTGT
ACTTCTCCTAAGCCTTTACTTGCAAGAAAACGTCTAATGGCCCACCAAGCACATGCC
TATCATATAGTTGACCCAAGCC
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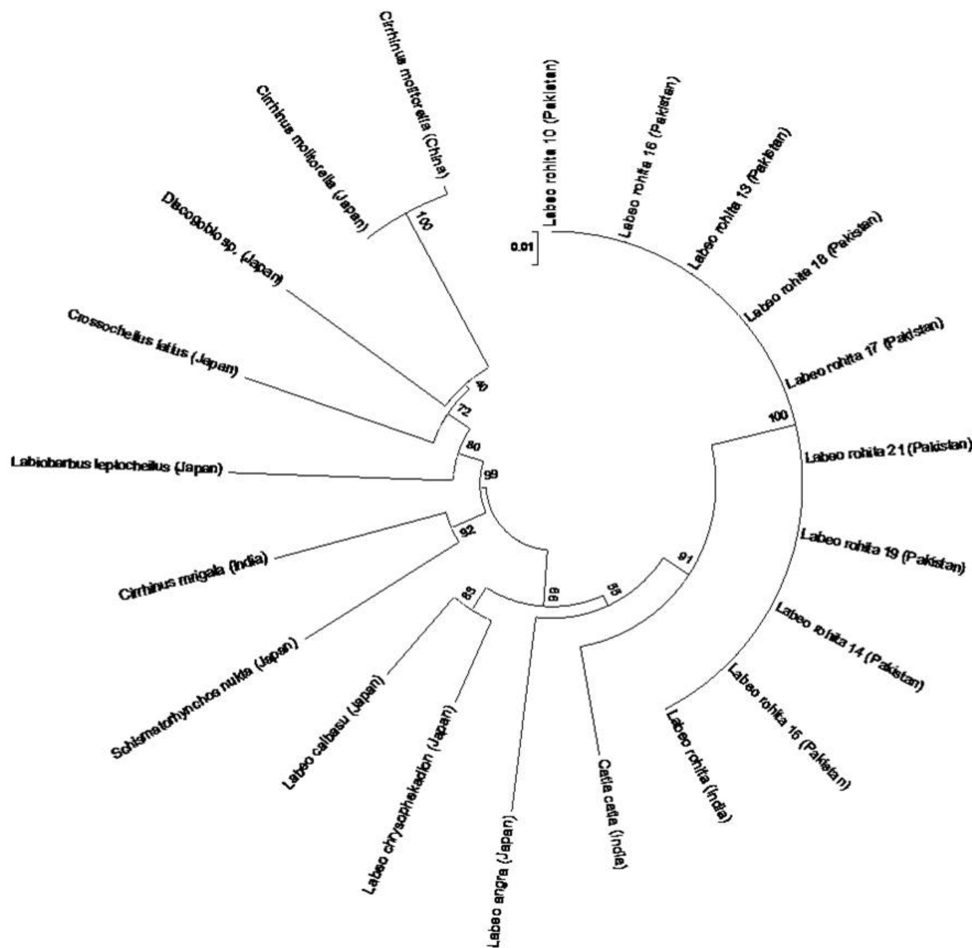

Fig. 2. The NJ Phylogentic Tree (circular view) based on ATPase6/8 gene sequences of *Labeo rohita.*

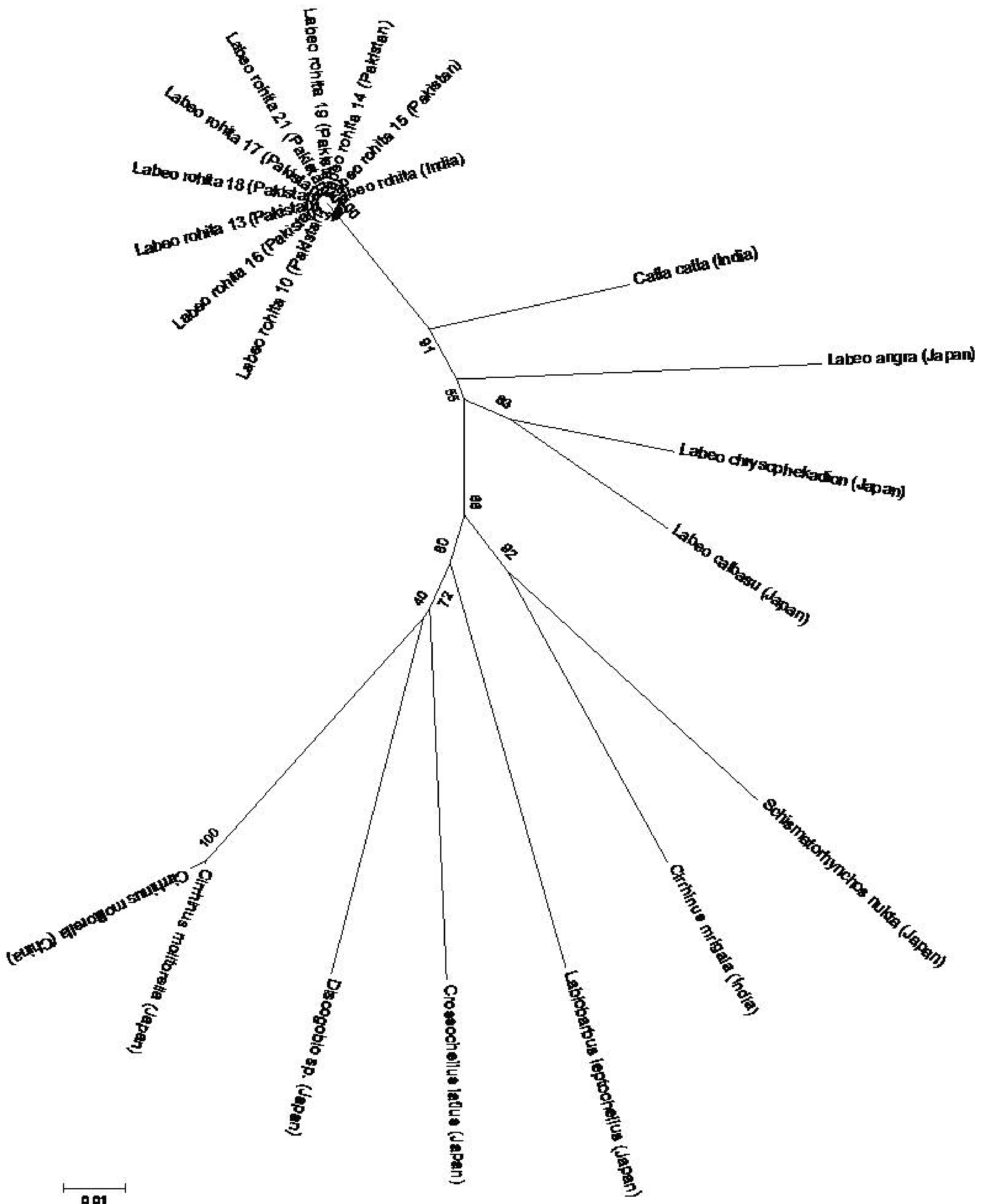

Fig. 3. The NJ Phylogenetic tree (radiational view) based on ATPase6 and ATPase8 gene sequences of *Labeo rohita* from Pakistan with reported sequences of different fish species on GenBank (NCBI) using MEGA 6.1.

NJ Phylogenetic tree (Figs. 1-3) based on ATPase6 and ATPase8 gene sequences of *Labeo rohita* from Pakistan with reported sequences of different fish species on GenBank (NCBI) using MEGA 6.1. A phylogenetic tree using a representative ATPase6/8 sequence (878 bp) from Pakistani *Labeo rohita* and sequences from different fish species from GenBank NCBI was constructed to see the biological position of Pakistani isolates. Pakistani *Labeo rohita* and same region sequences from other parts of the word (from NCBI) were used for the construction of these phylogenetic trees. The phylogenetic tree constructed for ATPase 6 and ATPase 8 in comparison with same region gene for the cyprinids of Asia, showed that *L. rohita* samples collected for our study and were designated as samples from Pakistan in the tree were clustered in the same class and showed no variability amongst the collected sample while they were found 9% variable from the cyprinids (*Catla*) of India and 45%, 1%, 17%, 20%, different from *Labeo* genus from Japan and 60% variation was recorded from the cyprinids (*Cirrhinus*) from Japan and China in rectangular view of the tree and almost similar trends of variation were found from the circular and radiational view.

Phylogentic tree amongst the sequences of ATPase6/8 (878 bp) region were also constructed in comparison with sequences found from the same region for cyprinids of the other Asiatic regions from GenBank NCBI to have an idea about phylogeny of Pakistani isolates. Very interesting results that *L. rohita* samples collected for our study and were designated as samples from Pakistan in the tree were clustered in the same class with *Labeo rohita* samples from india which indicated that the phylogenetic history of *Labeo* genus from both the countries is evolved from the same ancestors in the past and also showed somewhat very minute variation (1%) from the same genus found from NCBI for the region from Japan while showed a huge variation (about 50%) from the other cyprinids (*Cirrhinus*) from Japan and China in the rectangular view of the tree and almost similar trends of variation were found from the circular and radiational view. This study confirmed the results of the study conducted by the Luhariya *et al*. (2014) on samples collected from different four rivers and concluded after the analysis that population substructure in wild *L. rohita* showed common ancestral origin. These results of this study are also related to the work done by Vera *et al*. (2013) during their studies on identification and conservation of remnants genetic resources of brown trout in relict populations from Mediterranean streams. In their studies they concluded that neighbor joining population tree based on Nei's standard genetic distances and the branches indicate the number of times a clade on the original tree is present in the trees estimated from 1000 replicates of their studies. These results for mitochondrial diversity are in accordance with the results concluded by the Martinez *et al*. (2007), Cortey *et al*. (2009), Baric *et al*. (2010), Sanz *et al*. (2009) and Vera *et al*. (2013) on the Mediterranean species.

CONCLUSION

This paper is a study on the variation amongst the different populations of Labeo rohita on the basis of ATPase 6/8 gene. The study evaluates the potential of complete ATPase 6/8 region of mitochondrial DNA as a marker region to determine the phylogeography of *Labeo rohita* from fish farm of region Punjab, Pakistan. ATPase6/8 (878 bp) region was used to investigate genetic variation within *Labeo rohita* and develop a global genealogy of genus *Labeo* strains. The results revealed that 878 bp of ATPase 6/8 region could be a promising marker for determining variations at inter-population as well as intra-population levels in *Labeo rohita*. And the results would facilitate conservation and management of this important species.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Aljanabi, M. and Martinez, I., 1997. Universal and rapid salt-extraction of high quality genomic DNA for PCR based techniques. *Nucl. Acids Res*., **25**: 4692-4693. https://doi.org/10.1093/nar/25.22.4692

Baric, S., Riedl, A., Meraner, A., Medgyesy, N., Lackner, R., Pelster, B. and Dalla-Via, J., 2010. Alpine headwater streams asreservoirs of remnant populations of the Danubian clade brown trout. *Freshw. Biol*., **55**: 866-880. https://doi.org/10.1111/j.1365-2427.2009.02318.x

Cortey, M., Vera, M., Pla, C. and Garcia-Marin, J.L., 2009. Northern and southern expansions of Atlantic brown trout (*Salmo trutta*) populations during the Pleistocene. *Biol. J. Linn. Soc*., **97**: 904-917. https://doi.org/10.1111/j.1095-8312.2009.01220.x

Chow, S. and Ushiama, H., 2004. Global population structure of albacore (*Thunnus alalunga*) inferred by RFLP analysis of the mitochondrial ATPase gene. *Mar. Biol*., **123**: 39-45. https://doi.org/10.1007/BF00350321

Dammannagoda, S.T., Hurwood, D.A. and Mather, P.B., 2008. Evidence for fine geographical scale heterogeneity in gene frequencies in yellowfin tuna (*Thunnus albacares*) from the north Indian Ocean around Sri Lanka. *Fish. Res*., **90**: 147-157. https://

doi.org/10.1016/j.fishres.2007.10.006

Froese, R., 2006. Cube law, condition factor and weight–length relationships: History, meta-analysis and recommendations. *J. appl. Ichthyol*., **22**: 241-253. https://doi.org/10.1111/j.1439-0426.2006.00805.x

Garcia, D.K. and Benzie, J.A.H., 1995. RAPD markers of potential use in penaeid prawn (*Penaeus monodon*) breeding programs. *Aquaculture*, **130**: 137-144. https://doi.org/10.1016/0044-8486(94)00311-B

Gjedrem, T. and Baranski, M., 2009. *Selective breeding in aquaculture: An introduction*, 1st ed. Springer Hedenberg, pp. 221. https://doi.org/10.1007/978-90-481-2773-3

Gupta, A., Lal, K.K., Mohindra, V., Singh, R.K., Punia, P., Dwivedi, A.K., Gupta, B.K., Luhariya, R.K., Masih, P., Mishra, R.M. and Jena, J.K., 2013. Genetic divergence in natural populations of bronze feather back, *Notopterus notopterus* (Osteoglossiformes: Notopteridae) from five Indian rivers, analyzed through mtDNA ATPase6/8 regions. *Meta Gene*, **1**: 50-57. https://doi.org/10.1016/j.mgene.2013.10.007

Hirayama, M., Mukai, T., Miya, M., Murata, Y., Sekiya, Y., Yamashita, T., Nishida, M., Watabe, S., Oda, S. and Mitani, H., 2010. Intraspecific variation in the mitochondrial genome among local populations of Medaka Oryzias latipes. *Gene*, **457**: 13-24. https://doi.org/10.1016/j.gene.2010.02.012

Jhingran, V.G., 1991. Procurement of stocking material. In: *Fish and fisheries of India* (ed. V.G. Jhingran). Hindustan Publishing, Delhi, India, pp. 379-380.

Khanna, S.S., 1988. *An introduction to fishes*, 3rd ed. Indian Universities Press, Allahabad, India.

Kuhner, M.K., Yamato, J. and Felsenstein, J., 1998. Maximum likelihood estimation of population growth rates based on the coalescent. *Genetics*, **149**: 429-434.

Liu, Z.J. and Cordes, J.F., 2004. DNA marker technologies and their applications in aquaculture genetics. *Aquaculture*, **238**: 1-37. https://doi.org/10.1016/j.aquaculture.2004.05.027

Lopera-Barrero, N.M., Povh, J.A., Ribeiro, R.P., Gomes, P.C., Jacometo, C.B. and Lopes, T.D.S., 2008. Comparison of DNA extraction protocols of fish fin and larvae samples: Modified salt (NaCl) extraction. *Cienc. Investig. Agrar*., **35**: 65-74.

Luhariya, R.K., Lal, K.K., Singh, R.K., Mohindra, V., Gupta, A., Masih, P., Dwivedi, A.K., Das, R., Chauhan, U.K. and Jena, J.K., 2014. Genealogy and phylogeography of Cyprinid fish *Labeo rohita*(Hamilton, 1822) inferred from ATPase 6 and 8 mitochondrial DNA gene analysis. *Curr. Zool*., **60**: 460-471. https://doi.org/10.1093/czoolo/60.4.460

Martinez, P., Bouza, C., Castro, J., Hermida, M., Pardo, B.G. and Sanchez, L., 2007. Analysis of a secondary contact between divergent lineages of brown trout *Salmo trutta* L. fromDuero basin using microsatellites and mtDNA RFLPs. *J. Fish Biol*., **71**: 195-213. https://doi.org/10.1111/j.1095-8649.2007.01551.x

Sanz, N., Araguas, R.M., Fernández, R., Vera, M. and García-Marín, J.L., 2009. Efficiency of markers and methods for detecting hybrids and introgression in stocked populations. *Conserv. Genet.,* **10**: 225-236.

Schierwater, B., Streit, B., Wagner, G.P. and Desalle, R., 1994. *Molecular ecology and evolution: Approaches and applications*. Birkhauser Verlag, Basel, Switzerland, pp. 495-508. https://doi.org/10.1007/978-3-0348-7527-1_28

Tamura, K., Dudley, J., Nei, M. and Kumar, S., 2007. MEGA 4: Molecular evolutionary genetics analysis (MEGA) software, version 4.0. *Mol. Biol. Evolut*., **241**: 1596-1599. https://doi.org/10.1093/molbev/msm092

Thai, B.T., Burridge, C.P., Pham, T.A. and Austin, C.M., 2004. Using mitochondrial nucleotide sequences to investigate diversity and genealogical relationships within common carp (*Cyprinus carpio* L.). *Anim. Genet*., **36**: 23-28. https://doi.org/10.1111/j.1365-2052.2004.01215.x

Thompson, J.D., Higgins, D.G. and Gibson, T.J., 1994. CLUSTAL W improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucl. Acids Res*., **22**: 4673-4680. https://doi.org/10.1093/nar/22.22.4673

Vergara-Chen, C., Aguirre, W.E., Gonzalez-Wanguemert, M. and Bermingham, E., 2009. A mitochondrial DNA based phylogeny of weakfish species of the Cynoscion group (Pisces: Sciaenidae). *Mol. Phylogen. Evolut*., **53**: 602-607. https://doi.org/10.1016/j.ympev.2009.06.013

Vera, M., Garcia-Marin, J.L., Martinez, P., Araguas, R.M. and Bouza, C., 2013. Identification and conservation of remnant genetic resources of brown trout in relict populations from Western Mediterranean streams. *Hydrobiologia,* **707**: 29-45. https://doi.org/10.1007/s10750-012-1402-8

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Effect of Ascorbic Acid on Survival and Bacterial Contents in the Gut Contents of *Oreochromis niloticus*

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Authors' Contribution
FR supervised the study and wrote the manuscript. SP helped in data collection. NA conducted research. NK helped in microbial work. KMA helped in writing and proofreading the manuscript.

Key words
Ascorbic acid, Diet, Bacteria, Survival and *Oreochromis niloticus*.

ABSTRACT

Studies were conducted to evaluate the dietary impact of Ascorbic acid on the survival and bacterial load in the gut contents of *Oreochromis niloticus*. Fish stocked in the earthen ponds which were to be marketed for human consumption was taken and the *Vit* C as a source of ascorbic acid was added in the standard feed for evaluation. There were three tanks in replicates selected as control, treatment 1 (T_1) with 3% addition and treatment 2 (T_2) with 5% addition of ascorbic acid, respectively. After a ninety day trial results 100% survival was observed in all the replicates were analyzed by statistical software SAS and analysis of variance showed significant differences among the treatments. The highest microbial load was observed in control (1.12E±07 to 1.67E±06) while in the treated tanks it was observed as 1.03E±05 to 1.23E±06 in T_1 and 7.10E±05 to 9.8E±05 in T_2.

INTRODUCTION

Tilapia fish (*Oreochromis niloticus)* have some features, such as, the ability to persist in poor water quality such as in saline water and quick growth. Tilapia aquaculture production has been doubled between 1986 and 1992. Tilapia offer commercial and home-grown protein sources because of their superior culture facilities. More common species in brackish water ponds are mullets, tilapia and catfish (FAO, 2006). In homeotherms, specific dietary deficiency is known to modulate the immune system. Such specific nutritional factors include vitamins, proteins, lipids, and minerals. Similarly in fish, variations in diet often affect immune-competence as reflected by disease resistance. It is well known that the deficiency of some micronutrients produces pathological signs and immune-depression. Vitamins and minerals are now included in farmed fish food to promote optimal growth and health. Dietary supplementation of L-ascorbic acid (AA) is a prerequisite for normal cell function and the development of farmed fish. Vitamin C functions as a general water-soluble redox reagent, a cofactor in collagen synthesis, a regulator of steroid synthesis, growth activator in wound healing, a modulator of the hexose monophosphate shunt and an activator of hepatic microsomal hydroxylases (Panush and Delafuente, 1985).

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Ascorbic acid/Vit C (AA) has influences on the production and feeding efficiency of fish. It has been used for its impacts on growth and feeding parameters, carcass composition and survival rate of *Cyprinus carpio*. The 5 different kinds of partially refined feeds of *Cyprinus carpio* fry with supply of AA in terms of Vit C in ratio of 0/Kg, 400/Kg, 800/Kg, 1200/Kg and 200/Kg were designed. At the end of the experiment, growth and feeding parameters, carcass composition and survival rate of fries were evaluated. The average net weight gains were 6.82±0.09, 7.38±0.03, 8.20±0.03, 8.07±0.09 and 8.12±0.08 g/90 days, respectively, for fish fed diets 0, 400, 800, 1200 and 2000 mg vitamin C/kg. The fish fed diets containing less than 800 mg supplemental vitamin C/kg had significantly reduced weight gain, feed efficiency and other nutritional indices compared to those fed diets supplemented with vitamin C at 800-2000 mg/kg. In vitamin C treatments the specific growth rate, food conversation efficiency were increased significantly and highest SGR was observed in treatment 4. There were no significant differences in survival rate observed between the treatments (Faramarzi, 2012). The ascorbic acid has pronounced impacts on the immunity of fish. Teleost and Cyprinid lack the enzyme for endogenous synthesis of AA, an essential micronutrient for fish. The study on the impacts of high ratio of ascorbic acid in form of Vit C in included in the supplementary feeds of *Labeo rohita* and its influence on production, feed intake and after effects and immune-modulation. This species was divided into 4-groups and were fed with feed without AA as control and with supplementation

of AA @ 500 mg/ kg as Experiment-1, 1000 mg/kg as experiment-2 and 1500 mg/kg in experiment-3 for the interval of sixty days. The parameters under observation were for growth of fish, serology of fish, hematology of fish and various unspecific parameters for the immunology of the fish. The experimental groups *i.e.* the fish having supplementation of AA in feed expressed high levels of specific growth rate upto 1000 mg/kg in comparison with the fish grown without supplementation of AA. Various parameters for the hematology and serology including the unspecific immunology limits were inclined by addition of AA. Amongst the unspecific parameters of immunology, bustle for phagocytic process and respiratory burst activity (NBT cells) were considerably improved by growing quantity of AA addition. Greater ration of AA considerably promoted the immune response for *Aeromonas hydrophila* contagion in contrast to fish fed with diet having zero levels of AA. These findings promoted the idea of aquaculturists about the valuable impacts of AA on the production and enhancement in immune response of the *Labeo rohita* (Tewary and Patra, 2008). Similarly many other scientists studied the impacts of AA/Vit C on the growth immunology and other beneficial impacts of supplementation of vitamins on boosting the other enzymes activities in fish. The total aerobic bacteria and enterobacteria load in African catfish (*Clarias gariepinus*) and Nile Tilapia (*Oreochromis niloticus*) randomly sampled from different aquatic environments in Ibadan, Southwest Nigeria was studies and found considerably high levels of entero-bacterial count in the stomach of caught tilapia (Emikpe *et al*., 2011). Mai *et al*. (2010) evaluated the *in vivo* activities of inulin and AA experimentally via *O. niloticus* that were distributed into 3 equal groups. Their results suggest that vit C @ 500 mg for one month could be a potential, less expensive and promising dietary supplementation than inulin that would positively affect growth, hematology, innate immunity, and resistance of *O. niloticus* in aquaculture. The aim of the current study is to evaluate the potential effect (beneficial or harmful) of AA (5mg/100g) on the immunity of tilapia taken from hatchery of University of Veterinary and Animal Sciences, Ravi campus, Pattoki. It is on the target to find either AA reduce or enhance the bacterial content in gut of tilapia (*O. niloticus*). Striped catfish *Pangasius hypophthalmus* was fed with fenugreek as feed additive to evaluate its survival, growth and body composition. Fenugreek as an additive was added in experimental feed @ of 0.5% in T1 and 1.0% in T2 while control T0 was without fenugreek supplementation. Fish was fed for six days a week at the rate of 4% of its body weight twice a day. Results showed significant ($P<0.05$) higher growth performance among the three treatment groups. Highest weight gain (5.07±0.72g), increase in total length (24.25±4.31 mm) was observed in T2. Better feed conversion ratio (FCR) 1.8 and specific growth rate 1.4 were observed in T1 (Mehboob *et al*., 2017).

MATERIALS AND METHODS

Location

The present study was conducted at Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki. The experiment was conducted in the fish hatchery using cemented tanks.

Experimental protocol

Tilapia (*Oreochromis niloticus*) was procured from fish ponds at UVAS Ravi Campus, Pattoki. Two month trial on *O. niloticus* (30) was done from 27-10-2014 to 27-12-2014 to find out the dietary effect of different concentration of vitamin C (AA).
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Feeding trial

Thirty (30) samples of tilapia were collected from a pond of varying sizes from 11 to 23kg and were placed in three different tanks situated in university hatchery. Each Tank was with 10 fishes. There were two treatment and one control group. Control group was fed with normal diet (mentioned in Table I) but was devoid of AA and indicated as treatment 1 (control) (T1) whereas the treatment groups were fed with feed recipes given in Table I. Treatment 2 (T2) tank was given feed with 3% AA and treatment 3 (T3) with 5% AA.

Table I.- Ingredients for treatment 1 (control), treatment 2 (3 % AA) and Treatment 3 (5 % AA).

Ingredients	T1	T2	T3
Fish meal	25%	25%	25%
Soybean meal	25%	25%	25%
Maize glutton	25%	25%	25%
Rice polish	20%	20%	20%
Mollases	3%	0%	0%
Vitamins	1%	1%	0%
Ascorbic acid	0	3%	5%

Feed preparation

Feed ingredients were collected from university and the following formula was used during the course of this experiment.

Ingredients

Fish meal, soybean meal, maize glutton, rice polish,

mollases, vitamins and AA.

Fishes were fed seven days a week with their formulated diets for about 60-days. Fish were fed two times a day, 8:30 am in morning and 4:30 pm in evening. Fishes were fed at the rate of 2% of their body weight.

Ration

All three tank fishes were fed with 2% of their body weight feed per day throughout the trial.

Sampling for microbiological work

From each tank, samples were collected carefully weighed and length measured to check the growth. Fishes were collected in polythene bag and sent to the Microbiological Lab of Department of Fisheries of University of Veterinary and Animal Sciences, Ravi Campus, Pattoki after two months of trial.

Collection of samples for microbial work

Samples were collected aseptically in clear and sterilized polythene bags and were immediately transported to laboratory.

Sample preparation

Gut of the fish was separated by using a sharp scissors and forceps and immediately transferred to 9% normal saline solution in flask and shaken well for release of bacteria to solution. Serial dilution was used for isolation and culturing of bacteria. The eppendorf tubes were filled with 900 µl 9% sterilized normal saline solution, 100 µl solution from each flask was added to 900 µl normal saline in eppendorf tubes. This was the 1st dilution. Further dilutions were made likewise. This suspension (10 µl) was spread on LB medium and incubated at 37°C for 24 and 48 h.

Microbial count

For the estimation of viable counts, pour plate method was used. 1 ml from each of the appropriate dilutions was transferred aseptically to sterile triplicate petri plates by means of sterile pipette. Then to each plate transferred 15-20 ml Tryptic soya agar sterilized, melted and cooled to about 45°C was poured. After solidification the plates were inverted to prevent condensation of moisture on the agar surface. The dilutions were spread thoroughly with the glass rod to distribute the microbial cells uniformly on the solidified plates. After incubation the colonies were counted. Average number of colonies in triplicates petri-plates of a suitable dilution was multiplied by the dilution factor and was reported as total viable count per ml of sample.

Total viable count=
Avg. No. of colonies x Dilution factor

lokiTotal of thirty samples of *Oreochromis niloticus* were collected from treated tanks. Ten samples from Treatment 1, T1 (control), Ten from Treatment 2, T2 (3% ascorbic acid) Tank, and Ten from Treatment 3, T3 (5% ascorbic acid) tank and were subjected for microbiological examination (Fig. 1). The samples were analyzed for total plate count.

Statistical analysis

The data obtained was analyzed by using Minitab Computer Software. The data on different variables was statistically analyzed by using Analysis of Variance (ANOVA).

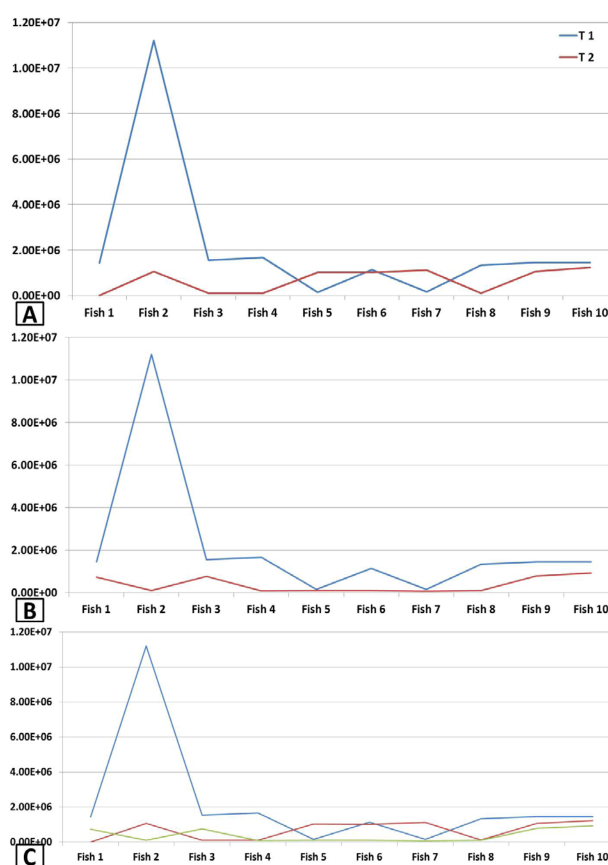

Fig. 1. Showing comparison between T1 and T2 (A), T1 and T3 (B) and T1, T2 and T3 (C).

RESULTS

Comparison of microbial counts

Microbial count in gut of T1 tank samples was highest, which ranged between 1.12E+07 to 1.67E+06. The value is significant (Tables II, III). Microbial count in gut of T2 samples were high and ranged 1.03E+05 to 1.23E+06. The values are significant (Table II). Microbial

Table II.- CFU and body weight with relation to Treatment 1 (control), Treatment 2 (3 % ascorbic acid) and Treatment 3 (5 % ascorbic acid).

Sample	T1		T2		T3	
	CFU	Weight	CFU	Weight	CFU	Weight
Fish sample 1	1.44E+06	19.83g	1.08E+03	23.61g	7.30E+06	6.7g
Fish sample 2	1.12E+07	24.91g	1.06E+06	19.21g	9.60E+05	14.38g
Fish sample 3	1.55E+06	16.06g	1.03E+05	13.21g	7.60E+06	16.96g
Fish sample 4	1.67E+06	21.91g	1.05E+05	22.92g	8.77E+05	12.91g
Fish sample 5	1.55E+05	17.72g	1.03E+06	17.99g	9.80E+05	25.63g
Fish sample 6	1.14E+06	11.29g	1.02E+06	19.53g	9.60E+05	17.01g
Fish sample 7	1.56E+05	9.75g	1.12E+06	18.81g	7.10E+05	7.57g
Fish sample 8	1.34E+06	9.30g	1.14E+05	7.29g	9.80E+05	9.36g
Fish sample 9	1.45E+06	10.35g	1.06E+06	10.91g	7.80E+06	9.58g
Fish sample 10	1.46E+06	10.21g	1.23E+06	11.56g	9.20E+06	10.53g

Table III.- Analysis of variance for T1 and T2, and T1 and T3.

Groups	Count	Sum		Average		Variance
For T1 and T2						
Column 1	30	64683000		2156100		9.68E+12
Column 2	30	20529243		684308.1		2.55E+11
For T1 and T3						
Column 1	30	64683000		2156100		9.68E+12
Column 2	30	11210000		373666.7		1.26E+11
			ANOVA			
Source of variation	**SS**	**Df**	**MS**	**F**	**P-value**	**F crit**
For T1 and T2						
Between groups	3.25E+13	1	3.25E+13	6.538817	0.013193	4.006873
Within groups	2.88E+14	58	4.97E+12			
Total	3.21E+14	59				
For T1 and T3						
Between groups	4.77E+13	1	4.77E+13	9.716647	0.00284	4.006873
Within groups	2.84E+14	58	4.9E+12			
Total	3.32E+14	59				

Values are significant at $P<0.05$.

count in gut of T3 samples was lowest, which ranged between 7.10E+05 to 9.80E+05. The values are significant (Tables II, III). The analysis of variance test applied on Excel version 2010.

The analysis test of T1, T2 (Table III) shows that results are significant as $p< 0.05$. P value is 0.013. Results are significant.

Values are: DF for between groups is 1 and within group is 58, total is 59. MS for between groups is 3.25E+13 and within groups is 4.97E+12. F is 6.538817 and F-crit

is 4.006873. P Value is 0.013193. The value is significant (Table III). The analysis test of T1, T3 (Table III) shows that results are significant as $p < 0.05$. P value is 0.013. Results are significant.

Values are: DF for between groups is 1 and within group is 58, total is 59. MS for between groups is 3.25E+13 and within groups is 4.97E+12. F is 6.538817 and F-crit is 4.006873. P Value is 0.013193. The value is significant (Table III).

DISCUSSION

Among diet components, antioxidant vitamins, especially vitamin C have been described as affecting the vertebrate immune system (Panush and Delafuente, 1985). Present study focuses the effect of vitamin C on the immunity and survival of *O. niloticus*. Experiment revealed increasing minute concentration of AA (vitamin C) in fish diet enhanced the immunity of *O. niloticus*. Experimental group fed on 5% AA (T3) had low microbial content which ranged between 7.10E+05 to 9.80E+05 (Tables I, II). The effects of vitamin C absence have been described in several fish species and deficiencies have been correlated to reduced growth rate, skeletal deformation, capillary fragility, slow wound repair, and depression of the immune system specimens being more susceptible to bacterial diseases (Lovell, 1973; Lim and Lovell, 1978). Present study investigated control group with normal diet show less signs of immunity had high microbial content ranged 1.12E+07 to 1.67E+06 (Table I). The experimental group fed with 3% AA (T2) ranged microbial content in between T1 and T2 as 1.03E+05 to 1.23E+06 (Tables I, II). AA is also known to be beneficial for immune responses in fish. A number of studies reported the improved immune responses and disease resistance in many fish species by feeding a higher level of dietary AA than required for growth (Ortuno *et al*., 2003). The results of the present study indicate that high concentration of AA prevented the occurrence of pathological signs of AA deficiency and resulted in improved growth and immunity compared with the control, AA free diet T1 or 3% containing diet T2.

Increasing AA concentration in T1 increased considerably the immunity of fish by reducing overall microbial population but, too much increase may harm the fish. The quantitative requirements for dietary vitamin C have been determined for several fish species, and the recommended values ranged from 20 to 50 mg AA Kg^{-1} diet. The requirement of vitamin C varies, to some degree, with fish species, size, diet and experimental conditions. Paper has demonstrated a requirement for 125mg AA per 100g diet (Soliman *et al*., 2008). Our study revealed that by adding AA to a fish diet at the rate of 5mg per 100g of fish feed would not only enhance the fish immunity but also help it grower saver and grow well. Therefore it is recommended for farmers to add AA in fish feed at given rate. A prebiotic is a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth or the activity of one or a limited number of bacteria in the gut (Ringo *et al*., 2010). Increased levels of vitamin C (mega doses) in the diet have been shown to enhance disease resistance against the bacterial pathogens *Edwardsiella ictaluri* and *E. tarda* of catfish held in aquaria and of salmonids. Specific antibodies against *E. ictaluri* and serum complement activity were suppressed in channel catfish fed a vitamin C-free diet while in aquaria, and antibody titers and complement activity were significantly higher when fish were fed doses of vitamin C that were much higher than the normal requirement (Durve *et al*., 1982). Halver *et al*. (1969) studied that vitamin C plays an important role in growth and immunity of fish. Most fishes are unable to synthesize AA due to the lack of L-gulonolactone oxidase that is responsible for synthesis of vitamin C. Therefore, an exogenous source of vitamin C is required in fish diet. Inadequate supply of dietary vitamin C usually results in a number of deficiency symptoms such as spinal deformation, impaired collagen formation, internal hemorrhaging, retarded growth and depressed immunity. The quantitative requirements on vitamin C have been determined for several species and the recommended values varied by various studies. A number of studies reported the improved immune responses and disease resistance in many fish species by feeding a higher level of dietary AA than required for growth. Increased immunity was demonstrated by the increased immunological parameters, as lysozyme, complement activities, phagocytic activity and respiratory burst (Navarre *et al*., 1989). Fish take a large number of bacteria into their gut from water sediment and food. It has been well known that both fresh and brackish water fishes can harbor human pathogenic bacteria particularly the coliform group (Sugita *et al*., 1988). Experimental fish for present study was obtained from earthen ponds of University contained fresh water. Vitamin C intake for two months decreased the previous microbial diversity of fish. In conclusion, the present study provides further evidence to support the hypothesis that dietary administration of 5% AA enhances immunity of *Oreochromis nioltcus* by reducing gut microbial content.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Durve, V.S. and Lovell, R.T., 1982. Vitamin C and

disease resistance in channel catfish (*Ictalurus punctatus*). *Can. J. Fish. aquat. Sci*., **39**: 948. https://doi.org/10.1139/f82-129

Emikpe, B.O., Adebisi, T. and Adedeji, O.B., 2011. Bacteria load on the skin and stomach of *Clarias gariepinus* and *Oreochromis niloticus* from Ibadan, South West Nigeria: Public health implications. *J. Microbiol. Biotech. Res*., **1**: 52-59.

FAO, 2006. *State of world aquaculture*. Fisheries Technical Paper No. 500, FAO, Rome.

Faramarzi, M., 2012. Effect of dietary vitamin C on growth and feeding parameters, carcass composition and survival rate of common carp (*Cyprinus carpio*). *Glob. Vet*., **8**: 507-510.

Halver, J.E., Ashley, L.M. and Smith, R.E., 1969. Ascorbic acid requirements of coho salmon and rainbow trout. *Trans. Am. Fish. Soc.,* **98**: 762-772. https://doi.org/10.1577/1548-8659(1969)98[762:AAROCS]2.0.CO;2

Lovell, R.T., 1973. Essentiality of vitamin C in feeds for intensively fed caged channel catfish. *J. Nutr.,* **103**: 134-138. https://doi.org/10.1093/jn/103.1.134

Lim, C. and Lovell, R.T., 1978. Pathology of the vitamin C deficiency syndrome in channel catfish (*Ictalurus punctatus*). *J. Nutr*., **108**: 1137-1146. https://doi.org/10.1093/jn/108.7.1137

Mai, D.I., Mohamed, F., Salah, M. and Abdelaty, A.M., 2010. Effect of dietary supplementation of inulin and vitamin C on the growth, hematology, innate immunity, and resistance of Nile tilapia (*Oreochromis niloticus*). *Fish Shellf. Immunol*., **29**: 241-246.

Mehboob, A., Khan, N., Atiq, U., Iqbal, K.J., Tayyab, R., Batool, S.S., Batool, H.S., Amjad, S. and Tanveer, M., 2017. Effect of fenugreek as a feed additive on the growth, body, composition and apparent nutrients digestibility of striped catfsh *Pangasius hypophthalmus* fry. *Pakistan J. Zool.,* **49**: 2037-2042. http://dx.doi.org/10.17582/journal.pjz/2017.49.6.2037.2042

Navarre, O. and Halver, J., 1989. Disease resistance and humoral antibody production in rainbow trout fed high levels of vitamin C. *Aquaculture*, **79**: 207-221. https://doi.org/10.1016/0044-8486(89)90462-6

Ortuno, J., Estcban, M.A. and Meseguer, J., 2003. The effect of dietary intake of vitamins C and E on the stress response of gilthead seabream (*Sparus aurata* L.). *Fish Shellf. Immunol*., **14**: 145-156. https://doi.org/10.1006/fsim.2002.0428

Panush, R.S. and Delafuente, J.C., 1985. Vitamins and immunocompetence. *World Rev. Nutr. Diet.,* **45**: 97-123. https://doi.org/10.1159/000410265

Ringo, E. and Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: A review. *Aquaculture*, **160**: 177-203. https://doi.org/10.1016/S0044-8486(97)00299-8

Soliman, A.K., Jauncey, K. and Roberts, R.J., 2008. Water-soluble vitamin requirements of tilapia: Ascorbic acid (vitamin C) requirement of Nile tilapia, *Oreochromis niloticus* (L.). *Aquacult. Res.,* **25**: 269 ¨C 278. https://doi.org/10.1111/j.1365-2109.1994.tb00690.x

Sugita, H., Tsunohara, M., Ohkoshi, T. and Deguchi, Y., 1988. The establishment of an intestinal microflora in developing goldfish (*Carassius auratus*) of culture ponds. *Microb. Ecol.,* **15**: 333. https://doi.org/10.1007/BF02012646

Teway, A. and Patra, B.C., 2008. Use of vitamin C as an immunostimulant. Effect on growth, nutritional quality, and immune response of Labeo rohita (Ham.). *Fish Physiol. Biochem*., **34**: 251-259. https://doi.org/10.1007/s10695-007-9184-z

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Studies on Growth Performance of *Cyprinus carpio* through Supplementary Feed in Monoculture Production System

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Authors' Contribution
SP conducted the research. FR helped in lab work and wrote the manuscript. IA supervised the research. IZ and AB helped in feed formulation and data collection. NK helped in arranging data and statistical analysis. MSA helped in writing the article.

Key words
Supplementary feed, *Cyprinus carpio*, Mono-culture and Manuring.

ABSTRACT

This project was launched to study the growth performance of *Cyprinus carpio* through supplementary feed in monoculture production system. Three earthen ponds were selected and manured with cow-dung at the rate of 0.10g N/100g of wet fish body weight daily and each pond was stocked with 50 common carps. A standard diet was formulated, containing 30% crude protein by using different feed ingredients *i.e.* corn gluten (30% C.P.), sun flower meal (35% C.P.) and rice polish (10% C.P.), equal by weight. These ponds were supplemented with two feeding levels, *i.e.* 2% of body weight (T_1) and 4% of body weight (T_2) while one of the ponds was kept as control. The amount of feed was increased fortnightly according to the measurement of fresh fish body weight. The morphometric characteristics of the fish *i.e.* body weight and total length were measured and recorded on fortnightly basis. Fortnightly water samples from fish ponds were also collected and analyzed for physical and chemical characteristics.

INTRODUCTION

Supplementary feeding plays an important role in semi-intensive and intensive fish culture. It offers the best means of fish production within shortest possible time in the ponds. It is molding of low value foodstuffs into a quality protein (Devaraj and Krishna, 1981). Supplemental feeding with an artificial feed is necessary to obtain increased production in ponds (Sinha *et al.*, 1980). Artificial feed increases the carrying capacity of the culture systems and can augment fish production by many fold (Hepher, 1975; Devaraj *et al.*, 1986). Various feed ingredients of both plant and animal origin, with different contents of crude protein have been used in semi-intensive and intensive culture systems. With the expansion and trend towards conversion of extensive aquaculture to intensive and semi-intensive ones, the need for specialized feeds designed for particular production situation is increasing. To date, nutritionists and feed manufacturers have concentrated their efforts on determining that which of the wide variety of the feedstuff may be used to produce a cost-efficient fish feed formulation (Alam *et al.*, 1996). Pond fertilization is one of the key factors in increasing the maximum carrying capacity. Fertilization as a means of increasing fish production is well accepted (Alam *et al.*, 1996; Heckling, 1971; Lin and Chen, 1967). The natural productivity of a pond can be greatly enhanced by the use of fertilizers. Fertilization with organic manures and inorganic fertilizers is also recommended for fish food production in rearing and stocking ponds. The most commonly used organic manure is cow dung (Wolney, 1967). It stimulates the growth of natural food by providing some essential elements, which found to be deficient in pond. When the natural feed forms the main source of nutrition, supplemental feeding with artificial feed is necessary to enhance production in ponds (New and Fedoruk, 2003). Indian major carps, common carp (*Cyprinus carpio*), and Chinese carps show best nitrogen incorporation efficiency under artificial feed (Mahboob *et al.*, 1995). It is pertinent to use supplementary feed along with chemical fertilizers and organic manure to get maximum yield of fish from confined water bodies within the shortest possible time (Mahboob and Sheri, 1997). Therefore, for commercial culture of these fishes in ponds, with the formulation of low-cost balanced diet from locally available agro-industry by-products is required.

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At present, no formulated aquafeed is available in Pakistan. This situation is discouraging and has been adversely affecting the growth of aquaculture industry in the country. Considerable work has been done to evaluate the effect of artificial feed on growth, feed conversion, feed intake, body composition, and nutrient retention *etc*., in different fish species (Singh and Srivastava, 1984; Chiu *et al*., 1987; Hossain and Jauncey, 1989; Webster *et al*., 1992a, b; Sarker *et al*., 2000; Jabeen *et al*., 2004; Singh *et al*., 2006; El-Ebiary, 2005; Mondal *et al*., 2007; Latif *et al*., 2008; Abid and Ahmed, 2009; Siddiqui and Khan, 2009). Most of these studies were carried out on a laboratory scale, where growth of fish was based only on artificial feed. The artificial supplementary feed increases the growth of Indian major carps and common carp in intensive system.

Minor modifications of different inputs into semi-intensive systems can bring about major changes in terms of growth, reproductive performance and overall productivity of the system (de Silva and Davy, 1992; Veerina *et al*., 1993; Bjerkan, 1996; Tacon and de Silva, 1997; Miaje *et al*., 1999). Since, feed and fertilizers represent about 60-80% of the total cost of aquaculture production, understanding feed management strategies and their implementation is of major importance.

So the present experiment has been planned to study "Studies on growth performance of *C. carpio* through supplementary feed in monoculture production system".

MATERIALS AND METHODS

The experiment was conducted in three earthen fishponds, each with dimensions 25 x 8 x 1.5 m (length x width x depth) at Fisheries Research Farms, University of Agriculture, Faisalabad. Before stocking, the earthen ponds were disinfected and pH was stabilized by liming with CaO (Sinha *et al*., 1980). Essential precautionary measures were taken to screen the water inlets to avoid the entry of intruders into or exit of fish out of ponds. After one week of these steps, each pond was watered up to level of 1.5 meter. This water level was maintained throughout the experimental period. Fertilization of all the ponds was done with cowdung at the rate of 0.10g N/100 g of wet fish body weight daily. Common carp (*C. carpio*) was stocked at the rate of 50 in each pond. At the time of stocking, the growth parameters such as body weight and total length of fish were measured and recorded as initial data. A standard diet was formulated, containing 30% crude protein by using corn gluten (30% CP), sunflower meal (35%) and rice polish (10%), in equal amount. The stocked fish was fed at two different levels, *i.e.* 2% of body weight (T_1) and 4% of body weight (T_2), while one of the ponds was kept as control. After every fortnight, the cultured fish stock was captured randomly with nylon drag net and their wet body weight and total length were measured and recorded and then released back into their respective ponds. Following parameters of physico-chemical properties of water were analyzed in the ponds for the entire study period after every fortnight; air temperature, water temperature, pH, light penetration, dissolved oxygen, total alkalinity, carbonates, bicarbonates, total hardness, calcium, magnesium, total solids, total dissolved solids and planktonic biomass. The data thus obtained was subjected to statistical analysis (Hora and Pillay, 1962). The comparison of mean values of various parameters was computed by using the Analysis of Variance (ANOVA) and Duncan's Multiple Range Test with repeated sampling.

Table I.- Analysis of variance on increase in body weight (g) and total length (cm) of *Cyprinus carpio*.

S.O.V.	D.F.	S.S.	M.S.	F. Value
Increase in body weight (g)				
Fortnights	10	18823.250	1882.325	13.8637**
Ponds	2	3763.223	1881.612	13.8584**
Error	20	2715.475	135.774	
Total	32	25301.948		
Increase in total length (cm)				
Fortnights	10	29.096	2.910	8.7774**
Ponds	2	2.157	1.078	3.2535^{NS}
Error	20	6.630	0.331	
Total	32	37.882		

**, highly significant; *, significant; NS, non-significant.

RESULTS AND DISCUSSION

The initial and final average body weights of *C. carpio* in control pond were 19.50 and 205.70g and in pond$_2$ (T_1) were 19.00 and 385.33g while in pond$_3$ (T_2) remained as 19.50 and 490.05g. In all the ponds the maximum gain in body weights by *C. carpio* were during the 6th fortnight as 54.80, 98.93 and 84.65g in control, T_1 and T_2, respectively. While the minimum gain in average body weights were during the 12th fortnight as 1.77, 5.50 and 3.00g in control, T_1 and T_2, respectively. The graphs between the fortnights and increase in body weight of *C. carpio* are the clear indication of these results. Analysis of variance shows that there was a highly significant difference in the increase in body weight of *C. carpio* during each fortnight and also among the ponds (Table I). The comparison of mean values indicates the variation in body weight of *C. carpio* during the fortnights and among the ponds (Table II).

Table II.- Comparison of mean values of increase in body weight (g) and total length of *Cyprinus carpio.*

Ponds	Original order	Ranked order
Control pond	16.93 B	42.78 A
Treated pond 1 (T1)	33.32 A	33.32 A
Treated pond 2 (T2)	42.78 A	16.93 B
Increase in average body weight (g)		
Fortnight 1	13.47 CD	79.46 A
Fortnight 2	20.37 CD	68.17 A
Fortnight 3	28.72 BC	42.89 B
Fortnight 4	42.80 B	42.80 B
Fortnight 5	79.46 A	28.72 BC
Fortnight 6	68.17 A	25.38 BCD
Fortnight 7	42.89 B	20.37 CD
Fortnight 8	25.38 BCD	13.47 CD
Fortnight 9	9.94 CD	9.94 CD
Fortnight 10	6.45 D	6.45 D
Fortnight 11	3.42 D	3.42 D
Increase in average total length		
Fortnight 1	2.00 BCDE	4.30 A
Fortnight 2	1.933CDEF	3.03 B
Fortnight 3	3.03 B	2.67 BC
Fortnight 4	2.67 BC	2.57 BCD
Fortnight 5	4.30 A	2.00 BCDE
Fortnight 6	2.57 BCD	1.93 CDEF
Fortnight 7	1.77 CDEF	1.77 CDEF
Fortnight 8	1.53 DEF	1.60 CDEF
Fortnight 9	1.60 CDEF	1.53 DEF
Fortnight 10	0.87 F	1.00 EF
Fortnight 11	1.00 EF	0.87 F

The initial and final total length of *C. carpio* recorded was 10.5 and 29.8cm in control, while 10.0 and 35.0cm in T_1 and 10.5 and 36.0cm in T_2. The maximum total lengths gained by C. carpio were 3.5, 5.3 and 4.1cm during the 6th fortnight in control pond, T_1 and T_2, respectively. Similarly, the minimum increase in total length of *C. carpio* was 1.0cm in control and 0.5cm in T_2 during the 12th fortnight also 0.5cm in T_1, during the 11th fortnight. The graphs between fortnights and increase in length confirm these results. Analysis of variance indicates that increase in total length of fish was highly significant in each fortnight while the increase in total length of fish was non-significant among ponds (Table I). The comparison of mean values indicates the variation in total length of *C. carpio* during the fortnights and among the ponds (Table II).

After six months of rearing, cultured fish species was harvested from three ponds. Survival rate for *C. carpio* was found to be 100% throughout the experimental period. The net gain in average body weight of *C. carpio* in control, T_1 and T_2 ponds, was 186.20, 366.33 and 470.55g, respectively. The gross fish yield for *C. carpio* per pond per year was recorded, it was found to be 20.57, 38.53 and 49.005kg in control, T_1 and T_2 ponds, respectively. The gross fish production for *C. carpio* per acre per year was calculated to be 416.22, 779.69 and 991.59kg in control, T_1 and T_2 ponds, respectively. The net production per pond per year was calculated, it was found to be 18.62, 36.63 and 47.05kg in control, T_1 and T_2 ponds, respectively. The net production per acre per year was found to be 376.77, 741.25 and 952.13kg in control, T_1 and T_2 ponds, respectively. The net production values per hectare per year were 941.92, 1853.13 and 2380.37kg in control, T_1 and T_2 ponds, respectively for *C. carpio*, while gross fish production for *C. carpio* was calculated to be 1040.55 for control, 1949.23 for T_1 and 2478.98kg for T_2 per hectare per year (Table III).

Table III.- Total fish production.

	Cyprinus carpio		
	Control	**T_1**	**T_2**
No. of fish stocked	50	50	50
Survival rate	100%	100%	100%
Initial average weight (g)	19.50	19.00	19.50
Final average weight (g)	205.70	385.33	490.05
Gain average weight (g)	186.20	366.33	470.55
Initial average total length (cm)	10.5	10.0	10.5
Final average total length (cm)	29.8	35.0	36.0
Gain average total length (cm)	19.3	25.0	25.5
Gross fish prod./pond/6 months(g)	10285.00	19266.50	24502.50
Gross fish prod./pond/year (kg)	20.57	38.53	49.005
Gross fish prod./acre/year (kg)	416.22	779.69	991.59
Gross fish prod./ha/year (kg)	1040.55	1949.23	2478.98
Net fish prod./pond/6 months(g)	9310.00	18316.50	23527.50
Net fish prod./pond/year (kg)	18.62	36.63	47.05
Net fish prod./acre/year (kg)	376.77	741.25	952.13
Net fish prod./ha/year (kg)	941.92	1853.13	2380.34
Gross fish prod./ha/year	1040.55	1949.23	2478.98
Net fish prod./ha/year	941.92	1853.13	2380.34

In the present study it is clear that T_2 supplied at the rate of 4% wet fish body weight daily gave good results as compared to the control and T_1. Same results were postulated by Omer and Nour (1986) and Abid and Ahmed (2009). The analysis of variance of this experiment showed the growth of *C. carpio* in terms of weight and length were highly significantly different during fortnights and weight were also highly significant among ponds while the length was non-significantly differet among ponds. Such conclusions were also made by Gosh *et al.* (1984) while studying the effect of artificial feed (rice

bran) and also by Janjua (1996) during his studies on the response of *Catla catla* to different levels of supplementary feed (rice polish). Gross and net production results of this study showed the same results as conclusions were made by Janjua (1996), during their studies on effect of supplementary feed ingredients on growth performance of major carps supplied at the rate of 4% wet fish body weight daily and also by Abbas *et al*. (2004) during their studies on effect of inorganic fertilizers and organic manure and their combination on the growth performance of major carps.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abbas, K., Ahmed, I., Rehman, M H. and Kanwal, S., 2004. Effect of rice bran and maize gluten on growth performance of major carps in fertilized ponds. *Indus J. Pl. Sci*., **3**: 391-395.

Abid, M. and Ahmed, M.S., 2009. Efficacy of feeding frequency on growth and survival of *Labeo rohita* (Ham.) fingerlings under intensive rearing. *J. Anim. Pl. Sci*., **19**: 111-113.

Alam, A.K., Maughan, O.E. and Matter, W.J., 1996. Growth response of indigenous and exotic carp species to different protein sources in pelleted feeds. *Aquacul. Res*., **27**: 673-679. https://doi.org/10.1111/j.1365-2109.1996.tb01302.x

Bjerkan, T., 1996. *Strategies of supplementary feeding in semi-intensive carp farming systems in Bangladesh*. M. Sc. thesis, Zoologisk Institutt, Norges Teknisk-naturvitenskapelige Universitet, Dragvoll, Norway, pp. 64.

Chiu, Y.N., Sumagaysay, N.S. and Sashillo, M.A.S., 1987. Effect of feeding frequency and feeding rate on the growth of milkfish, *Chanus chanus*, juveniles. *Asian Fish. Sci*., **1**: 27-31.

de Silva, S.S. and Davy, F.B., 1992. Fish nutrition research for semi-intensive culture systems in Asia. *Asian Fish. Sci*., **5**: 129-144.

Devaraj, K.J., Krishna, D.S., 1981. Utilization of duck weeds and waste cabbage leaves in the formulation of fish feed. *Mysore J. agric. Sci*., **15**: 132-135.

Devaraj, K.V., Keshavappa, G.Y. and Manissery, J.K., 1986. Growth of grass carp, *Ctenopharyngodon idella* (val.), fed on two terrestrial fodder plants. *Aquacul. Res*., **17**: 123-128. https://doi.org/10.1111/j.1365-2109.1986.tb00093.x

El-Ebiary, S.H., 2005. Use of soybean meal and /or corn gluten meal as partial substitutes for fish meal in Nile tilapia (*Oreochromis niloticus*) fingerlings. *Egypt. J. Aquacul. Res*., **31**: 432-442.

Gosh, S.K., Mandal, B.K. and Barthapur, D.N., 1984. Effects of feeding rated on production of common carp and water quality in paddy-cum fish culture. *Aquaculture*, **40**: 97-101. https://doi.org/10.1016/0044-8486(84)90347-8

Heckling, C.F., 1971. *Fish culture*, 2nd ed. Faber & Faber, London.

Hepher, B., 1975. Supplementary feeding in fish culture. In: *Proc. 9th Int. Congr. Nutr*. 3, Karger, Baselm, Maxico, pp. 183-198.

Hora, S.L. and Pillay, T.V.R., 1962. Handbook of fish culture in the Indo-Pacific region. *FAO Fish Biol*., **14**: 204-208.

Hossain, M.A. and Jauncey, K., 1989. Nutritional evaluation of some Bangladeshi oilseed meals as partial substitutes for fish meal in the diet of common carp, *Cyprinus carpio* L. *Aquacul. Res.*, **20**: 255-268. https://doi.org/10.1111/j.1365-2109.1989.tb00351.x

Jabeen, S., Salim, M. and Akhtar, P., 2004. Feed conversion ratio of major carp *Cirrhinus mrigala* fingerlings fed on cotton seed meal, fish meal and barley. *Pak. Vet. J*., **24**: 42-45.

Janjua, I.M., 1996. *Effect of supplementary feed and fertilizer on survival and growth rate of fingerlings of* Labeo rohita. M. Phil. thesis, Dept. Zool. and Fish., Univ. Agri., Faisalabad, Pakistan, pp. 120.

Latif, K.A., Alam, M.T., Sayeed, M.A., Hussain, M.A., Sultana, S. and Hossain, M.A., 2008. Comparative study on the effects of low cost oil seed cakes and fish meal as dietary protein sources for *Labeo rohita* (Hamilton) fingerling. *Univ. J. Zool. Rajshahi Univ*., **27**: 25-30.

Lin, S.Y. and Chen, T.P., 1967. Increase of production in fresh-water fish ponds by the use of inorganic fertilizers. *FAO Rep*., **44**: 210-225.

Mahboob, S., Sheri, A.N. and Javed, M., 1995. Influence of fertilizers and artificial feed on nitrogen incorporation efficiency of fish. *Pakistan J. Zool*., **27**: 349-351.

Mahboob, S. and Sheri, A.N., 1997. Growth performance of major, common and some Chinese carps under composite culture system with special reference to pond fertilization. *J. Aquacult. Trop*., **12**: 201-207.

Miaje, M.A.H., Hossain, M.A., Hasan, M.R. and Siddique, M.A.L., 1999. Effect of form of supplemental feed on the growth and production of Indian major carps and silver barb in polyculture. *Bangladesh J. Aquacult*., **21**: 41-46.

Mondal, K., Kaviraj, A., Mukhopadhyay, P.K., Datta, M.

and Sengupta, C., 2007. Evaluation of fermented fish offal in formulated diet of the Indian major carp, Rohu, *Labeo rohita* (Hamilton). *Acta Icthyol. Piscat*., **37**: 99-105. https://doi.org/10.3750/AIP2007.37.2.06

New, M. and Fedoruk, M., 2003. *Report of CIFRI on the Regional Workshop on Aquaculture Planning in Asia, Bangkok, Thailand*, pp. 154.

Omer, E.A. and Nour, A.M., 1986. Effect of different protein sources and levels on growth and feed utilization of common carp (*Cyprinus carpio*) fingerlings. *Egypt. J. Anim. Prod*., **26**: 185-196.

Rehman, M.H., Ahmed, I., Abbas, K. and Tabassum, S., 2005. Effect of inorganic fertilizer, organic manure and their combination on the growth performance of major carps. *Indus J. biol. Sci*., **2**: 196-201.

Sarker, P.K., Chakraborty, S.C., Islam, M.N. and Chowdhary, B.B.P., 2000. Effects of animal and plant protein diets on growth of Indian major carp (*Labeo rohita* Ham.) fingerlings. *Bangladesh J. Fish. Res*., **4**: 113-120.

Siddiqui, T.Q. and Khan, M.A., 2009. Effects of dietary protein levels on growth, feed utilization, protein retention efficiency and body composition of young *Heteropneustes fossilis* (Bloch). *Fish Physiol. Biochem*., **3**: 479-488. https://doi.org/10.1007/s10695-008-9273-7

Singh, K.P. and Srivastava, S.K., 1984. Effect of feeding frequency on the growth, consumption and gross conversion efficiency in the siluroid catfish, *Heteropneustes fossilis* (Bloch), *Bamidgeh*, **36**: 80-91.

Singh, P.K., Gaur, S.R. and Chari, M.S., 2006. Effcet of varying protein levels on the growth of Indian major carp Rohu, *Labeo rohita* (Hamilton). *Int. J. zool. Res*., **2**: 186-191.

Sinha, V.R.P., Khan, H.A., Chakraborty, D.P. and Gupta, M.V., 1980. Preliminary observation on nitrogen balance of some ponds under composte fish culture. *Aquacul. Hung*., **2**: 105-116.

Steel, R.G.D., Torrie, J.H. and Dinkkey, D.A., 1996. *Principles and procedure of statistics*, 2nd ed. McGraw Hill Book Co., Singapore.

Tacon, A.G.J. and de Silva, S.S., 1997. Feed preparation and feed management strategies within semi-intensive fish farming systems in the tropics. *Aquaculture*, **151**: 379-404. https://doi.org/10.1016/S0044-8486(96)01494-9

Tacon, A.G.J., 1997. Feeding tomorrow's fish: Keys for sustainability. In: *Proceedings of feeding tomorrow's fish* (eds. A. Tacon and B. Basurco). Zaragoza, Spain, 24-26 June 1996, pp. 11-33.

Veerina, S.S., Nandeesha, M.C. and Rao, K.G., 1993. *Status and technology of Indian major carp farming in Andhra Pradesh, India, Mangalore, India*. Asian Fisheries Society, Indian Branch, pp. 52.

Webster, C.D., Tidwell, J.H. and Yancey, D.H., 1992a. Effect of protein level and feeding frequency on growth and body composition of cage-reared channel catfish. *Progr. Fish-Cultur*., **54**: 92-96. https://doi.org/10.1577/1548-8640(1992)054<0092:EOPLAF>2.3.CO;2

Webster, C.D., Tidwell, J.H. and Yancey, D.H., 1992b. Effects of feeding diets containing 34 and 38% protein at two feeding frequencies on growth and body composition of channel catfish. *J. appl. Aquacul*., **1**: 67-80. https://doi.org/10.1300/J028v01n03_06

Wolney, P., 1967. Fertilization of warm water fish ponds in Europe. *FAO Fish. Rep*., **44**: 64-81.

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Effect of Various Protein Feeds on the Growth, Body Composition, Hematology and Endogenous Enzymes of Catfish (*Pangasius hypophthalmus*)

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Authors' Contribution
NK and MA designed the study. UA and NK conducted the study. HA and FR analyzed data. MSM ans AM help in writing the article. MT and KJI supervised enzymes study.

Key words
Artificial feed, Growth, Body composition, Digestive enzymes, Hematology, *Pangasius hypophthalmus*.

ABSTRACT

The study was conducted to evaluate the effect of various concentrations of crude protein on the growth, body composition, hematology, and endogenous enzymes of catfish, *Pangasius hypophthalmus*. The trial was carried out in HAPAS (10 x 10 x 5 feet) for 90-days. Fish was fed at 3% of wet body weight twice daily with the experimental feeds having crude protein (CP) levels 44% (T_1), 35% (T_2), and 40% (T_3), each having three replicates. Fish fed on 40% protein feed (T_3) showed significantly ($P<0.05$) higher growth (1378.57±53.20g) compared to T_2 and T_1. The percent weight gain was also highest in T_3 (718.81±43.27) followed by T_2 (69.83±10.83) and T_1 (61.69±1.41). The FCR of fish in T_3 was significantly lower (2.51±0.095) than T_1 (4.00±0.06) and T_2 (3.75±0.42). Proximate analysis values remained uniform among treatments. Concentration of amylase was significantly higher ($P\leq 0.05$) in T_1 and T_2 than T_3 while phytase and lipase enzymes showed non-significant differences among all the treatments. Values of Alanine aminotranspherase (ALT) and Aspartate aminotranspherase (AST) in blood of *P. hypophthalmus* increased where 35%-40% protein was offered. The overall performance of fish fed 40% CP feed remained significantly better with no adverse effects on nutritional value of fish.

INTRODUCTION

Catfish culture has become very popular across the globe due to its fast growth rate and higher market demand. Catfish is an important and fastest growing group of fish in the world aquaculture after carps and tilapia (Phan *et al*., 2009; Lakra and Singh, 2010). That is why World-wide catfish production has increased by six folds during the last decade (FAO, 2009). Among the most popular catfish species, *Pangasius hypophthalmus* and *Pangaisus pangasius* are promising fish species for prospective and progressive fish aquaculturists due to their fast growth, easiness of culture, high resistance to disease, and wide range of environmental factors (Begum *et al*., 2012a). It has been introduced widely outside of its native range and has established population in Myanmar and Indonesia.

The culture of *Pangasius* spp. is increased in South Asia and South East Asian region due to its size and taste. Among Asian countries, Vietnam is the leading country producing about 1.4 million tons of *Pangasius* annually and contributed more than 50% of its total aquacultural output that elicited the development of a fish processing sector and becomes a source of livelihoods to more than 150,000 individuals (Wilkinson, 2008).

Good nutrition dictates the success of aquaculture entrepreneur. Formulation of good quality feed requires complete knowledge of fish species and its biology. Pangasius also accepts artificial feed with varying levels at different life stages. Among catfish, nutritional aspects of Pangasiid has been least explored when compared with Clariids and Ictalurids. Protein requirements of *Pangasius hypophthalmus,* vary from 16 g kg^{-1} day^{-1} to 25 g kg^{-1} day-

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[1] (Pathmasothy and Lin, 1988). Similarly, Hung (1999) described protein requirement of 16 and 17 g kg^{-1} day^{-1} for *Pangasius hypophthalmus* and *Pangasius bocourti,* respectively. The cause of this variation may be due to the varying levels of feed supply, quality as well as quantity of given protein sources or energy to protein ratio in the experimental diets (Wilson and Moreau, 1996). Keeping in view the future scope of these species, an attempt was made to introduce Catfish *Pangasius hypophthalmus* in Pakistan and started work on feed formulation to promote its culture in more beneficial and convenient way. The key objective was to observe its response to local feed on growth, nutritional status, hematology along with enzyme secretion and role ending with some observations on blood feed interactions.

MATERIALS AND METHODS

The study was carried out at Research and Training Facilities, Department of Fisheries and Aquaculture, UVAS, Ravi Campus, Pattoki. The trial was conducted in HAPAS installed in one of the earthen ponds (0.04 acre) filled with tube well water with desired aeration through paddle wheel aerator. The pond was upgraded with the provision of plastic sheet on the top for successful overwintering.

Procurement of fish

Fish weighing 350 g on average, was procured from one of the progressive fish farmer near Head Baloki, Pattoki, District Kasur who imported this fish from Thailand and nourished it in his fish culture facility for 4 months. Fish was transported in oxygen permeated cans and safely transferred to above mentioned facility for further trials.

Experimental design and setup

Trial was based on complete randomized design (CRD) statistical experiment design. Three feeds with different crude protein levels (44%, 35%, and 40%) were purchased from Oryza Organics Feed Mill near Raiwind, Lahore. These three different feeds were hereby designated as T_1, T_2 and T_3. Each treatment has three replicate HAPPAs with 7 fish in each which was reared for further 3 months. Fish was offered 3% feed of its wet biomass during the course of the trial twice a day in two equal installments.

Fish growth studies

Morphometric measurements of fish such as wet body weight and total body length was recorded on the day of stocking and then at fortnight intervals for examination of fish and re-adjustment of diet plan. Net weight gain, percentage weight gain, specific growth rate (SGR) and feed conversion ratio (FCR) were calculated using following formulae:

Net weight gain = final body weight (g) - initial body weight (g)

% body weight gain = body weight gain× 100 ÷ initial body weight

SGR%= lin (final mean body weight (g) – lin (initial mean body weight (g) ×100 ÷ duration of the experimental period (days)

FCR= feed intake (g) /weight gain (g)

Table I.- Ingredient composition and proximate analysis of experimental feeds.

Ingredients composition (%)	T_1 (44%)	T_2 (35%)	T_3 (40%)
Fish meal (52%)	15	10	35
Soybean meal	10	25	25
Wheat bran	14	20	10
Wheat flour	-	-	4
Rice bran	-	15	6
Rice polish	10	-	-
Cotton seed meal	-	8	-
Maize gluten meal (60%)	50	19	16
Fish oil	-	1.5	1.5
DCP	-	1	0.5
Lysine	-	0.3	0.4
Methionine	-	0.1	0.8
Seldox	-	0.1	0.1
Kimera mould	-	0.1	0.1
Whex powder	-	-	0.5
Vit. C	-	-	0.1
Vit. premix	1	-	-
Total	100	100	100
Proximate composition of prepared feed			
Moisture (%)	8.60± 0.42[a]	8.45 ± 0.49[a]	8.45 ± 0.35[a]
Crude protein (%)	44 ±0.28[a]	35 ±0.55[ab]	40 ± 0.70[a]
Crude fat (%)	7.5 ± 0.42[a]	7.5 ±0.35[a]	7.70 ± 0.28[a]
Ash (%)	18.60±0.42[a]	19.24± 0.33[a]	18.95±0.70[a]
Fiber contents (%)	0.75 ±0.70[a]	0.85 ± 0.70[a]	0.80 ±0.14[a]

Water quality parameters

Dissolved oxygen, temperature, salinity, total

dissolved solid, pH and Secchi disc visibility were measured on daily basis.

Proximate analysis of feed and fish

Proximate analysis of the experimental feeds and fish was determined by following the technique Near Infrared Reflectance Spectroscopy (NIRS 5000 model, Foss Tecator, Sweden) (Martinez *et al*., 2003) (Table I).

Sample preparation for detection of enzymes

After three months feeding trial three fish samples were collected randomly from each treatment, degutted, removed the whole intestine, and then homogenized in chilled Tris-HCl. The homogenate was centrifuged at 6000×g at 4°C for 15 min. The supernatant was removed and stored at -4°C for enzyme estimation.

Phytase activity

Phytase concentration was measured by using a modified ferrous sulphate-molybdenum blue assay. Enzyme solution (25µl) was incubated with 475 µl of 5mM sodium phytase in 50Mm Tris HCL buffer (pH 7) at 37 °C for 15 min. Addition of 500 µl (10% trichloro acetic acid) terminated enzyme reaction. Addition of 1000 µl of freshly prepared color reagent containing 1% ammonium molybdate, 3.2 % sulfuric acid and 7.2% of ferrous sulphate, released phosphorus which was then measured (Sabir *et al*., 2017).

Amylase activity

Amylase activity assay was measured by using DNS method. A tube was taken and 1 mL of supernatant of fish sample was taken in this tube and 2 mL of starch phosphate buffer was added in it and was incubated at 37ºC for 30 min. After incubation, at 37°C, 3 mL of DNS solution was added and incubated at boiling temperature for 5-10 min until color of DNS changes to reddish brown. Placed the tube to cool and 4 mL of H_2O was added to make up the volume to 10 mL standard ranging from 1 to 100 mM. Optical density (O.D.) of samples and glucose solutions were measured at 540 nm.

Lipase activity

A sample was taken in a test tube and then added 0.5 mL olive oil, 0.5 mL 50 mM phosphate buffer such as disodium hydrogen phosphate 50 mM 200 mL and sodium dihydrogen phosphate 100 mM 50 mL in the sample by adding 10 mM $CaCl_2$ and made 50µl of sample. Sample was incubated at 30°C for 30 min at 150 rpm and then reaction was stopped by adding 20 mL of ethanol. Amount of fatty acids was determined by 50 mM of KOH at pH 10.

Heamatological studies

Blood samples were collected from caudal vein and then by puncturing heart by syringe from each fish taken from each HAPA; blood samples were collected in vials containing EDTA as an anticoagulant for heamatological analysis. For biochemical estimation, blood was stored in vials without EDTA. The blood was diluted 1:200 in modified Natt-Herrick's solution; red blood cells (RBCs $\times 10^{12}$ cells/L), and white blood cells (WBCs $\times 10^{9}$ cells / L) were counted manually using a Neubauer heamocytometer. Haemoglobin (Hb) was determined using the cyanmethemoglobin technique. Differential blood cell counts were accomplished on blood films fixed with absolute methanol and stained with modified Wright-Giemsa stain. A total of 600 white blood cells (WBCs) per slide were counted, recorded, and described according to Ellis (1976) and Ainsworth (1992). Samples without EDTA were centrifuged at 10,000 g for 5 min in a microhaematocrit centrifuge at room temperature (Morris and Davey, 1996) to determine the activities of Aspartate aminotransferase (AST) and Alanine aminotransferase (ALT) by using commercial kits (DIALAB GmbH, Wiener Neudorf, Austria).

Statistical analysis

The data was analyzed by using SAS 9.1 version statistical software. The data on different variables was statistically analyzed by using Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test.

RESULTS

There was a significant difference observed between initial weights in T_3 and T_2 whereas non-significant difference was found between the T_2 and T_1 (Table II). Similar relationship was repeated in the final weight gain of all the three treatments. T_3 exhibited the highest weight gain *i.e.* T_3 (1378.57g±53.20) whereas non-significant difference was observed between the weight gain of T_2 and T_1, respectively. The percent weight gain was also highest in T_3 (718.81±43.27) compared to T_2 (69.83±10.83) and T_1 (61.69±1.41), respectively. Non-significant differences were observed among the initial length, final length and increase in length of all the treatments under consideration.

The FCR values for three treatments were observed as T_1 (4.00±0.06) T2 (3.75±0.42) and T_3 (2.51±0.095) where T_3 was found significantly lower than T_1 and T_2, respectively. The SGR (%) values were observed as T_1 (0.22±0.005) T2 (0.25±0.03) and T_3 (0.34±0.015) with T_3 was found significantly higher compared to T_1 and T_2 (Table II). Body composition of fish remained uniform among treatments (Table II).

Table II.- Growth performance and proximate composition of *Pangasius hypophthalmus* under different dietary treatments.

Parameters	T_1(44%CP)	T_2(35%CP)	T_3 (40%CP)
Initial weight(g)	703.56 ± 27.6^{a}	685.23 ± 42.5^{a}	659.75 ± 29.6^{a}
Final weight(g)	1137.38 ± 34.9^{b}	1160.95 ± 6.4^{b}	1378.57 ± 53.2^{a}
Weight gain(g)	433.81 ± 7.6^{b}	475.71 ± 39.9^{b}	718.81 ± 43.3^{a}
Weight gain (%)	61.69 ± 1.4^{b}	69.83 ± 10.8^{b}	109.09 ± 8.04^{a}
Initial length (cm)	43.11 ± 0.5^{a}	42.89 ± 1.7^{a}	42.58 ± 3.3^{a}
Final length (cm)	47.16 ± 0.8^{a}	46.89 ± 0.5^{a}	48.25 ± 1.2^{a}
Increase in length (cm)	4.11 ± 1.3^{a}	3.92 ± 2.21^{a}	5.67 ± 2.83^{a}
FCR	4.00 ± 0.06^{a}	3.75 ± 0.42^{a}	2.51 ± 0.09^{b}
SGR%	0.22 ± 0.005^{b}	0.25 ± 0.03^{b}	0.34 ± 0.01^{a}
Proximate composition			
Moisture (%)	10.9 ± 0.40^{b}	11.5 ± 0.2^{b}	11.3 ± 0.1^{b}
Crude protein (%)	59 ± 1.65^{a}	59.7 ± 1.5^{a}	59.9 ± 3.0^{a}
Crude fat (%)	8.23 ± 0.11^{a}	8.4 ± 0.2^{a}	8.7 ± 0.4^{a}
Crude fiber (%)	$0.86\pm0.05_{a}$	0.83 ± 0.1^{a}	0.9 ± 3.3^{a}
Ash (%)	17.6 ± 0.57^{a}	17.1 ± 0.6^{a}	17.4 ± 1.7^{a}

Values with different superscripts are significantly different from each other ($P<0.05$).

Table III.- Endogenous enzymes concentration under three treatments.

Enzymes	T_1	T_2	T_3
Amylase	0.21^{a}	0.20^{a}	0.11^{b}
Phytase	7.27^{a}	6.46^{a}	5.96^{a}
Lipase	1.06^{a}	1.03^{a}	1.0^{a}

*Values with different superscripts are significantly different from each other ($P<0.05$).

Endogenous enzymes

Level of endogenous enzymes such as amylase were found significantly higher in T_1 and T_2 compared to T_3 while phytase and lipase enzymes showed non-significant differences among all the treatments with slightly higher numerical values in T_1 followed by T_2 and T_3, respectively (Table III).

Heamatological parameters

Heamatological parameters were also significantly affected by CP level. Results revealed that heamatological values for all parameters such as RBCs, WBCs, Hb level, monocytes and neutrophil in experimental fish were the highest in 40% CP level feed and the lowest at 35% CP level however, there were no significant differences between T_1 and T_3 (Table IV). Level of ALT and AST were found significantly different from each other in all dietary treatments. Their maximum activity was found in 40% CP level feed than 35% and lowest for 44% CP level (Table IV).

Table IV.- Heamatological parameters and liver function tests (LFTs) of *Pangasius hypophthalmus* fed three different dietary crude protein diets.

Hematological parameters	T_1	T_2	T_3
RBCs	1.50 ± 0.06^{b}	1.47 ± 0.015^{b}	1.93 ± 0.045^{a}
WBCs	5.76 ± 0.15^{b}	2.80 ± 0.86^{c}	6.86 ± 0.450^{a}
Hb level (g/dl)	6.30 ± 0.10^{a}	5.60 ± 0.17^{b}	6.52 ± 0.105^{a}
Neutrophils (%)	14.0 ± 1.00^{b}	12.0 ± 1.00^{b}	16.66 ± 1.52^{a}
Lymphocytes (%)	85.33 ± 2.51^{a}	89.66 ± 2.08^{a}	91.33 ± 4.72^{a}
Monocytes (%)	3.00 ± 1.00^{ab}	1.33 ± 0.57^{b}	4.00 ± 1.00^{a}
Liver functioning blood parameters			
ALT	17.66 ± 0.57^{c}	26.0 ± 2.00^{b}	29.0 ± 1.00^{a}
AST	21.66 ± 0.57^{b}	24.33 ± 1.52^{b}	32.0 ± 2.64^{a}

*Values with different superscripts are significantly different from each other ($P<0.05$).

Physico-chemical relationship with growth

Table V indicates the co-relational studies between the length-weight increments as well as the water quality parameters under consideration. Dissolved oxygen has a highly negative co-relation with the weight and length gain of the fish under study. Temperature narrates interesting relationship. It has negative co-relation (-0.29) with weight whereas length shows a positive co-relation. Total dissolved solids (TDS) have a positive relation with length and weight in the Pangasius pond. Similarly, pH also shows a positive relation with the length and weight of the given fish whereas salinity has similar relation with the temperature. It has a negative relation with the weight and positive relation with the length.

In Table V, T_1 showed positive relation between DO and weight but T_2 and T_3 showed negative relation between DO and weight. Weight and temperature showed positive relation between T_1, T_2 and T_3.Weight and TDS showed negative relation in T_1 but T_2 and T_3 both showed positive relation between weight and TDS. Weight and salinity showed negative relation with both T_1 and T_2 but they showed positive relation with T_3.Weight and pH showed negative relation in T_1, T_2 and T_3.

Table V.- Relationship of physico-chemical parameters with length-weight co-relation, fish weight gain and total length.

Parameters			Weight		Length
Dissolved oxygen (DO)			-0.28		-0.66
Temperature			-0.29		0.01
Total dissolved solids (TDS)			0.29		0.34
Salinity			-0.03		0.20
pH			0.1 1		0.19
Treatments	**Wt and DO**	**Wt and temp**	**Wt and TDS**	**Wt and sal**	**Wt and pH**
T1	0.38	0.12	-0.87	-0.99	-0.99
T2	-0.27	0.14	0.76	-0.53	-0.66
T3	-0.95	0.9	0.59	0.54	-0.45
T	L and DO	L and temp	L and TDS	L and salinity	L and pH
T1	-0.72	0.97	0.14	-0.24	-0.24
T2	-0.9	0.83	0.02	-0.98	-1
T3	-0.73	0.82	-0.98	0.99	0.53

Wt., Weight gain; DO, dissolved oxygen; Temp., temperature; Sal., salinity.

The length and DO showed highly negative relation among T_1, T_2 and T_3. Length and temperature showed highly positive relation in all treatments. Length and TDS showed positive relation in T_1 and T_2 but positive with T_3. Length and salinity showed positive relation with T_3 but showed highly negative relation between T_1 and T_2. Length and pH showed highly negative relation between $T_1$and T_2 but in T_3 both showed positive relation (Table V).

DISCUSSION

In the current study, *Pangasius* was cultured first time in Pakistan to study the effect of locally prepared feed on growth, body composition, and hematology when fed on diets with various protein concentrations. *Pangasius* is an omnivorous fish which shows faster growth rates when given a reasonably balanced diet. Fish showed an impressive growth with 40% CP feed in current trial. Sayeed *et al*. (2008) reported that final weight and specific growth rate of Thai pangus increased with increasing protein level in feed which agrees with ours results though protein range varied from 26 to 40% CP diet means comparatively higher protein levels are inevitable for better growth and production. Abdel-Tawwab *et al*. (2010) found similar results and reported highest growth of fry with 45% protein diets and the lowest with 25% protein diet ($P<0.05$). The fish used for current trial showed optimum growth efficiency at 40% whereas the survival rate of each treatment group remained unaffected at all protein levels. The reason for comparatively better values of FCR at 40% CP might be due better composition of diets with higher fish meal and higher feed intake. Similar was the case with the SGR values as it was directly related to growth and then FCR for the treatment under consideration. Whatever the situation is the pond water quality parameters have significant role in enhancing growth and feed intake.

The results obtained by El-Dahhar *et al*. (2000) revealed similar pattern that with increasing dietary crude protein final body weight (FBW) increased proportionately and significantly ($p \leq 0.05$). The fish fed at 20 or 24% protein level showed significantly better mean final body weight (FBW) in comparison to that fed 12% protein. With increase in protein to 20% the feed conversion ratio (FCR) was improved.

Temperature has a direct effect on the feed consumption in fish especially in *Pangasius*. When the temperature is up to or more than 30ºC, the fish feeds voraciously and this ultimately leads towards a recorded growth increments but the case becomes vice versa when it drops to a significant level and leads stunted or minimal growth. Similar case prevailed in the present study under consideration as well.

Non-significant differences were observed among proximate composition of fish among various treatment groups. Numerically higher values were recorded in T_3 which was fed 40% CP diet that played significant role in growth enhancement compared to T_1 and T_2.

Similarly, the enzyme production was directly related

with the level of protein, time duration and ratio of feeding in an organism. In our study, where T_3 showed higher growth performance and better feed acceptance also results in higher amylase enzyme secretion in the experimental fish. Several exogenous and endogenous factors are responsible for affecting protein retention efficiency in fish (Halver and Hardy, 2002). Several studies revealed that modification in the digestive enzymes activity is due to changes in feed ingredients, food manipulations, starvation, feeding time and protein concentration (Hakim *et al*., 2006). It has been revealed by some studies that herbivorous fishes might compensate their low protein availability by increasing their enzymatic activity. Some herbivorous fishes exhibit trypsin activities similar to or even higher than carnivorous species, to maximize protein digestive efficiency (Chan *et al*., 2004).

Heamatological results in this study were also significantly (P<0.05) affected due to CP level. RBCs and WBCs were positively influenced by CP level up to 40% and on further increase in dietary protein their number started to decline at 44% CP level. According to Akinwande *et al*. (2005) the increase in protein level in the diet of *Heterobroanchus longifilisan* increase the values of the hematological indexes. Further he observed maximum RBC, WBC, PCV and Hb in fingerlings fed 40% crude protein followed by 45% crude protein. During present study, Hb level was positively influenced by dietary protein level with no significant differences (P<0.05) between 44% and 40% CP however, the value obtained at 40% CP was slightly greater than 44% CP and lowest at 35% CP with significant differences between 40% and 44% CP feed. In the differential leucocyte count neutrophils, lymphocyte and monocyte percentage was observed non-significantly (P<0.05) different from 35% and 44% CP diets, however, at 35% CP their percentage was minimum (12.0 ± 1.00). Maximum percentage (16.66±1.52) of neutrophils was observed in fish fed 40% diet (T_3) and was significantly different from the other two treatments. Abou-Daoud *et al*. (2014) reported that number of neutrophil in respective fish blood was considerably less which was fed 35% and 50% CP level in diet than in all other treatments (P <0.05). Furthermore, they stated that variation of CP levels did not significantly (P <0.05) affect the numbers of thrombocytes, eosinophil, lymphocytes, monocytes and basophils.

Liver functioning of fish can be judged by the level of these two enzymes alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the blood. In the current study level of ALT and AST in blood of *P. hypophthalmus* increased with 35%-40% diets however, further increase in CP level up to 44% affected their level negatively and lowest value was observed in this treatment. ALT revealed significant differences (P<0.05) from each other in all dietary treatments however, AST level in T_1 and T_2 was found non-significantly different. Gallagher (1999) observed similar results and reported that liver alanine aminotransferase activities increased significantly on higher dietary protein and lipid concentration, which indicated that dietary protein is transformed for non-protein activities. They hypothesized from these results that excess dietary protein is consumed for energy generation. Melo *et al*. (2012) observed similar results that by increasing the protein content in the diet increase the hepatic activity of ALT and AST. The increasing protein concentration in the diets resulted increase gain in weight. The increased hepatic activity of protein metabolizing enzymes in the fish fed higher protein level diets indicated efficient utilization of dietary amino acids that trigger growth and act as a substrate for gluconeogenesis. Ayyat *et al*. (2011) reported that the fish fed high protein diet significantly increased the blood total protein, albumin and ALT. According to Abdel-Tawwab (2012) the actions of physiological variables, comprising aspartate aminotransferase (AST), alanine aminotransferase (ALT) and total protein were significantly altered by the varying levels of dietary protein.

CONCLUSION

The overall performance of the fish fed with three different levels of crude protein commercial diets showed better growth in T_3 (40% CP) with decreased enzyme secretions. Heamatological parameters varied significantly (P<0.05) due to CP level but these diets have no adverse effects on the fish body composition. Nevertheless, fish was found very sensitive to temperature and its feed intake and growth performance was directly affected by temperature variations.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abdel-Tawwab, M., 2012. Effects of dietary protein

levels and rearing density on growth performance and stress response of Nile tilapia, *Oreochromis niloticus* (L.). *Int. aquat. Res.*, **4**: 1-13. https://doi.org/10.1186/2008-6970-4-3

Abdel-Tawwab, M., Ahmad, M.H., Khattab Y.A.E. and Shalaby, A.M.E., 2010. Effect of dietary protein level, initial body weight, and their interaction on the growth, feed utilization, and physiological alterations of Nile tilapia, *Oreochromis niloticus* (L.). *Aquaculture*, **298**: 267-274. https://doi.org/10.1016/j.aquaculture.2009.10.027

Abou-Daoud, Y., Ghanawi, J., Farran, M., Davis, D.A. and Saoud, I.P., 2014. Effect of dietary protein level on growth performance and blood parameters of marbled spinefoot *Siganus rivulatus*. *J. appl. Aquacult.,* **26**: 103-118. https://doi.org/10.1080/10454438.2014.901077

Akinwande, A.A., Moody, F.O., Sogbesan, O.A., Ugwumba, A.A.A. and Ovie, S.O., 2005. Heamatological response of *Heterobranchus longifilis* fed varying dietary protein levels. In: *19th Annual Conference of the Fisheries Society of Nigeria (FISON), 29 Nov - 03 Dec 2004, Ilorin, Nigeria*, pp. 715-718.

Ainsworth, A.J., 1992. Fish granulocytes: Morphology, distribution, and function. In: *Annual review of fish diseases* (eds. M. Faisal and F.M. Hetrick). Pergamon Press, New York, pp. 123-148. https://doi.org/10.1016/0959-8030(92)90060-B

Ayyat, M.S., El-Marakby, H.I. and Sharaf, S.M., 2011. Effect of dietary protein level, stocking density, and dietary pantothenic acid supplementation rate on performance and blood components of Nile tilapia, *Oreochromis niloticus. J. appl. Aquacult.,* **23**: 122-135. https://doi.org/10.1080/10454438.2011.581572

Begum, M., Akhter, T. and Minar, M.H., 2012a. Analysis of the proximate composition of domesticated pangus (*Pangasius hypophthalamus*) in laboratory condition. *J. environ. Sci. Nat. Resour.,* **5**: 69-74.

Chan, A.S., Horn, M.H., Dickson, K.A. and Gawlicka, A., 2004. Digestive enzyme activities in carnivores and herbivores: Comparison among four closely related prickle back fishes (Teleostei: Stichaeidae) from California rocky intertidal habitat. *J. Fish Biol.,* **65**: 848-858. https://doi.org/10.1111/j.0022-1112.2004.00495.x

El-Dahhar, A.A., Zewel, H.S. and El-Tawil, N.E., 2000. Effect of protein and energy levels in commercial diets on growth performance of juvenile Nile tilapia (*Oreochromis niloticus*). *Egyptian J. aquat. Biol. Fish.,* **4**: 267-285.

Ellis, A.E., 1976. Leucocytes and related cells in the plaice *Pleuronectus platessa*. *J. Fish Biol.,* **8**: 143-156. https://doi.org/10.1111/j.1095-8649.1976.tb03928.x

FAO, 2009. *FISHSTAT Plus*, ver 2.30. Food and Agriculture Organisation, Rome, Italy.

Gallagher, M.L., 1999. Growth responses, tissue composition, and liver enzyme changes in juvenile sunshine bass, *Morone chrysops* × *M. saxatilis*, associated with dietary protein and lipid level. *J. appl. Aquacult.,* **9**: 41-51. https://doi.org/10.1300/J028v09n04_05

Hakim, Y., Uni, Z., Hulata, G. and Harpaz, S., 2006. Relationship between intestinal brush border enzymatic activity and growth rate in tilapias fed diets containing 30 or 48% protein. *Aquaculture,* **257**: 420-428. https://doi.org/10.1016/j.aquaculture.2006.02.034

Halver, J.E. and Hardy, D.M., 2002. *Fish nutrition*, 3rd Ed. Academic Press, California, USA.

Hung, L.T., 1999. *Contribution to the study of larval rearing and juvenile nutrition of two Me'kong catfish,* Pangasius bocourti *(Wild, 1880) and* Pangasius hypophtalmus *(Wild, 1878)*. PhD thesis, Institut National Agronomique Paris-Grignon, Paris, France.

Lakra, W.S. and Singh, A.K., 2010. Risk analysis and sustainability of *Pangasianodon hypophthalmus* culture in India. *Aquacult. Asia Mag*., **15**: 35-40.

Martinez, S., Navalon, J.L., Ortiz, A., Montalvo, G., Ferrero, J.L. and Gonzalez, A., 2003. *The use of NIRS to predict chemical composition of ungrounded poultry feeds*. 14th Eurpean Symposium Poultry Nutrition August 2003, Lillehammer, Norway, pp. 91-92.

Melo, J.F.B., Lundstedt, L.M., Moraes, G. and Inoue, L.A.K.A., 2012. Effect of different concentrations of protein on the digestive system of juvenile silver catfish. *Arq. Bras. Med. Vet. Zootec.,* **64**: 450-457. https://doi.org/10.1590/S0102-09352012000200027

Morris, M.W. and Davey, F.R., 1996. Basic examination of blood. In: *Clinical diagnosis and management by laboratory methods* (ed. J.B. Henry) 19th ed. Saunders, New York, pp. 1278.

Pathmasothy, S. and Lin, L.T., 1988. The response of *Pangasius sutchi* (Fowler) fingerlings fed an isocaloric diets with variable protein levels. *Malaysian Agric. J.,* **54**: 81-90.

Phan, L.T., Bui, T.M., Nguyen, T.T.T., Gooley, G.J.,

Ingram, B.A. and Nguyen, H.V., 2009. Current status of farming practices of striped catfish, *Pangasianodon hypophthalmus* in the Mekong Delta, Vietnam. *Aquaculture,* **296**: 227-236. https://doi.org/10.1016/j.aquaculture.2009.08.017

Sabir, F., Tayyab, M., Muneer, B., Hashmi, A.S., Awan, A.R., Rashid, N., Wasim, M. and Firyal, S., 2017. Characterization of recombinant thermostable phytase from *Thermotoga naphthophila*: A step for the fulfilment of domestic requirement of phytase in Pakistan. *Pakistan J. Zool.,* **49**: 1945-1951.

Sayeed, M.A.B., Hossain, G.S., Mistry, S.K. and Huq, K.A., 2008. Growth performance of Pangus (*Pangasius hypothalamus*) in polyculture system using different supplementary feeds. *Univ. J. Zool. Rajshahi Univ.,* **27**: 59-62.

Wilson, R.P. and Moreau, Y., 1996. Nutrient requirements of catfishes (*Siluroidei*). *Aquat. Living Resour.,* **9**: 103-111. https://doi.org/10.1051/alr:1996045

Wilkinson, S., 2008. Better management practices for Vietnamese catfish. *Aquacult. Asia Mag.,* **13**: 7-11.

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Growth Response, Antioxidant Status and Fatty Acid Profile of *Cirrhinus mrigala* Fingerlings Fed on Practical Diets after Treated with Vitamin E Supplementation

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Authors' Contribution
MI performed the experiment. MA supervised the research and provided the research facilities. AY helped in write-up. KMA helped in the data analysis and compilation of results. SZHS and MF plannedand conducted the experiment.

Key words
Vitamin E, Growth, TBARS, Antioxidant enzymes, Fatty acid profile, *C. mrigala*.

ABSTRACT

An 8-week feeding trial was conducted to investigate the effect of dietary intake of vitamin E (a-tocopherol acetate) for enhancing productivity of *Cirrhinus mrigala* fingerlings. Fish fingerlings were kept in V shaped tanks with stocking density of 15 fish/tank (ini. avg. wt. 2.78±g per individual). Six diets were formulated by supplementing vitamin E at the level of 0, 25, 50, 75, 100 and 125 mg kg^{-1} and each treatment was applied to triplicates. At the end of feeding experiment, improved growth performance was measured in groups received vitamin E supplemented diets as compared to control. A significant ($p<0.05$) increase in weight gain (9.76 g) was showed by the group fed on diet supplemented with 100 mg kg^{-1}. Similarly, better feed intake (FI) and feed conversion ratio (FCR) was recorded at 100 mg kg^{-1} level of supplementation while, maximum survival rate (SR) % was observed at 50 and 75 mg kg^{-1} diets. Consistently, significant ($p<0.05$) decline was observed in hepatic TRABS contents with increased vitamin E supplementation. Antioxidant enzymes *i.e.* catalase (CAT), superoxide dismutase (SOD) and peroxidase (POX) displayed significantly ($p<0.05$) decreased activities in gills of fish provided 100 mg kg^{-1} vitamin E supplemented diet. Higher PUFAs (n-3, n-6) values were examined in muscles of fish fed on increased supplementation levels. Based on Quadratic regression analysis of WG % 106.58 mg kg^{-1} vitamin E was the optimum level for the supplementation of practical diet for *C. mrigala* fingerlings. In conclusion, as a proficient antioxidant, vitamin E has potential to reduce oxidative stress in *C. mrigala* fingerlings.

INTRODUCTION

Vitamins are complex organic compounds used efficiently as feed additives in animal nutrition to ensure animal health by regulating the normal physiological functions such as growth, development and reproduction. Vitamin E (α-tocopherol) is a lipophilic compound and functions as a chain breaking antioxidant, positively influences the immune system, reduces the mortality and consequently improves the growth performance (Shiau and Hsu, 2002). It is also a principle component in various biochemical processes like prevention of lipid oxidation in fish tissues (Zhong *et al*., 2007).

Vitamin E possesses greatest potency as a cofactor for several biochemical processes in enzyme activation and plays considerable role for the maintenance of vertebrate fitness and consequently prevents muscular dystrophy and hepatic necrosis. Appropriate cellular functioning needs adequate vitamin E supplementation. Inadequate vitamin E in diet can have significant negative consequences for proper immune system development as well as proper immune system functioning. In teleosts, erythrocyte fragility, decline in growth, anemia and high mortality rates are considered main complications caused by hypovitaminosis E (Halver, 2002). Previous studies revealed potential benefits of vitamin E as an immunostimulant showed resistance against several infectious diseases and significant decreased mortalities were observed in chicks due to *E. coli* (Heinzerling *et al*., 1974) and turkeys (Julseth, 1974) fed on vitamin E supplemented diet.

Dietary vitamin E requirements vary from species to

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species and depend upon dietary lipid requirements and fatty acid profile in fish. In previous studies requirements of dietary vitamin E has been reported for many commercially important fish species such as for channel cat fish (Murai and Andrews, 1974), 30-50 mg/kg, 200-300 mg/kg (Watanabe *et al*., 1977) for common carp, 120 mg/kg (Hamre and Lie, 1995), for Atlantic salmon, 28 mg/kg (Kocabas and Gatlin, 1999) for hybrid striped bass, 40-44 mg/kg (Shiau and Shiau, 2001), for juvenile hybrid tilapia and 131.9 mg/kg (Sau *et al*., 2004) for rohu.

High production rate of reactive oxygen species (ROS) and decreased antioxidant defense mechanism at cellular level mainly contributing in oxidative damage that is the main reason of deterioration of biological molecules such as proteins, lipoproteins, enzymes, DNA and fatty acids present within the cell membranes and stored in various body tissues like liver and muscles. The defense mechanism of vitamin E as antioxidant is a sequential process contained three steps; initiation, propagation and termination by scavenging free radicals produced by ROS and proved an effective defensive tool for essential biological membranes and intracellular components (Paul *et al*., 2004).

Lipid peroxidation produced a principle end product called malondialdehyde (MDA); is determined by TBARS test (Shahidi and Hong, 1991). High concentration of TBARS reflects high oxidative stress in liver and muscles tissues. Dietary inclusion of vitamin E significantly reduced the concentration of hepatic TBARS in (Zhou *et al*., 2013) juvenile cobia, *Rachycentron canadum*.

Status of liver antioxidant defense enzymes like liver glutathione (GSH), catalase (CAT) and superoxide dismutase (SOD) is considered as a biomarker of oxidative damage (Miller *et al*., 1993; Reed, 1990). In juvenile sea bream, turbot and halibut increase in hepatic enzyme activities were recorded when fed on un-supplemented vitamin E diet as compared to supplemented diet (Tocher *et al*., 2002). Vitamin E content and muscle fatty acids profile directly correlates with α-tocopherol content in diet. Lower contents of α-tocopherol, unsaturated fatty acids, polyunsaturated fatty acids and PUFA/saturated fatty acids ratio were recorded in Korean rock fish when fed on diet contained low vitamin E (Bai and Lee, 1998). The findings of several authors strengthen this fact that the incorporation of α-tocopherol in diet showed significant positive responses for growth improvements. Findings reported by Bae *et al*. (2013) suggested that inclusion of α-tocopherol in diet showed significant effect for eel fry growth. Similarly, Zhou *et al*. (2013) reported that incorporation of vitamin E in diet positively influences the growth performance in juvenile cobia.

Cirrhinus mrigala also known as mrigal is one of the well-known and commercially important Indian carp widely cultured and consumed in Asian subcontinent particularly in Northern India, Berma, Bangladesh and Pakistan. Mrigal efficiently cultured in pond system as well as in paddy fields under mono and polyculture system (Khan *et al*., 2004). Available information was not adequate about basic vitamin E requirement of *Cirrhinus mrigala*. Investigation about dietary requirement of vitamin E of mori, *Cirrhinus mrigala* was the main concern of the present research work.

MATERIALS AND METHODS

The present research was executed to study the dietary vitamin E requirements of mori, *Cirrhinus mrigala* fingerlings. The experiment was carried out in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.

Fish and experimental conditions

Cirrhinus mrigala fingerlings were obtained from Government Fish Hatchery, Faisalabad and fingerlings were allowed to acclimatized to experimental condition for two weeks in V-shaped tanks (UA system) and during this period fingerling were fed once a day to apparent satiation on the experimental diet without vitamin E. Before performing the experiment fingerlings were given a prophylactic dip in 5g/L NaCl solution (Rowland and Ingram, 1991). The feeding experiment was started with an initial average weight of 2.78±0.01 g. Dissolved oxygen in water media was monitored by using digital meter (HANNA, model HI 9147). Similarly, water temperature and pH was estimated by using AMPROBE pH meter (model WT-80). Aeration was provided through capillary system in all the tanks during experimental period.

Feed ingredients and formulation of experimental diet

Dietary feed ingredients were procured from local market and analyzed chemically by following AOAC (1995) before the preparation of experimental diets. The composition of experimental diet is given in Table I. The feed ingredients were grounded and sieved to require particle size before the formulation of Vitamin E based diet. The samples of experimental diets were homogenized using a motor and pestle and analyzed by standard methods AOAC (1995): moisture was determined by oven-drying at 105°C for 12 h; crude protein (N x 6.25) by micro Kjeldahl apparatus; crude fat, by petroleum ether extraction method through Soxtec HT2 1045 system; crude fiber, as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH; ash, by ignition at 650°C for 12 h in electric furnace (Eyela-TMF 3100) to constant

weight; carbohydrates; by the subtracting the values of crude protein (%), crude fat (%), crude fibre (%).

Six experimental diets D1, D2, D3, D4, D5 and D6 were formulated by supplementing vitamin E in the form of α-tocopherol acetate at the level of 0, 25, 50, 75, 100 and 125 mg/kg diet, respectively. Fish were given respective diet at the rate of 2% of live wet weight. Two replicates were allotted for each experimental diet and each tank was stocked with 15 fingerlings.

Table I.- Ingredient composition (%) of experimental diet.

Ingredient	%
Sunflower	40
Fishmeal	20
Rice polish	18
Wheat flour	10
Fish oil	6
Mineral mixture*	3
Vitamin premix without vitamin E**	3

*Each kg of mineral mixture contains; Ca (calcium), 155 gm; P (phosphorous), 135gm; Mg (magnesium), 55gm; Na (sodium), 45gm; Zn (zinc), 3000 mg; Mn (manganese), 2000 mg; Fe (iron), 1000 mg; Cu (copper), 600 mg; Co (cobalt), 40 mg; I (iodine), 40mg; Se (selenium), 3mg. **Each kg of Vitamin premix contains; Vitamin A (retinoic acid), 5.0 mg; Vitamin B1 (thiamine), 0.5 mg; Vitamin B2 (riboflavin), 3.0 mg; Vitamin B3 (niacin), 5.0 mg; Vitamin B6 (pyridoxine), 1.0 mg; Vitamin B7 (biotin), 0.05 mg; Vitamin B9 (folic acid), 0.18 mg; Vitamin B12 (cobalamin), 0.002 mg; Vitamin C (ascorbic acid), 5.0 mg; Vitamin D3 (cholecalciferol), 0.002 mg; Cellulose, 815.26 mg; Choline, 100 mg.

Feeding procedure and collection of uneaten diet

Fish were given respective diet at the rate of 2% of its live wet weight. Duplicates tanks were allotted for each experimental diet and each tank was stocked with 15 fingerlings. After the feeding session of three hours, the uneaten diet was collected from each tank with help of collection tube by opening the valve I and valve II subsequently of the tanks. The tanks were washed completely to remove the particles of diets and refilled with water. After that the collected diets were dried and stored for the calculation of feed conversion ratio (FCR). The feeding experiment lasted for 60 days.

Growth study and vitamin E requirement determination

Growth performance was evaluated after every week by taking gross weight of fish from each treatment. Growth performance and feed utilization rates were evaluated in terms of weight gain (WG), weight gain %, feed conversion ratio (FCR), specific growth rate (SGR) and survival rate %.

Determination of TBARS, antioxidant enzyme activities and fatty acid profile

At the termination of feeding experiment, fish from each replicate were collected, euthanized by overdose of clove oil and sacrificed. Liver, gills and muscles were extracted and stored at -20°C for determination of TBARS, antioxidant enzymes activities and fatty acid profile, respectively.

TBARS assay

The measurement of thiobarbituric acid reactive substances (TBARS) in fish liver was specifically assayed by following Gatta *et al*. (2000). Each sample (1 g) was homogenized in a solution of KCl 11.5 g/L and 3 ml of 80 Mm Tris-maleate, pH 7.4 with homogenizer. After addition of 1 ml of 2 mM ascorbic acid, samples were incubated at 37°C for 30 min to induce lipid peroxidation. Colorimetric reaction was obtained by adding 5 ml of 0.7 M HCl and 5 ml of 0.05 M thiobarbituric acid (TBA) to the sample and boiling the tubes for 25 min. Then, before final reading, samples were refrigerated and centrifuged at 495 x g for 5 min with the addition of 5 ml of 200 g/L trichloroacetic acid. TBA values, expressed as l g malondialdehyde equivalents mg liver tissue, were determined photometrically at 530 nm. A standard solution of malondialdehyde was used to obtain a calibration curve and absorbance values were correlated with this curve in order to calculate the amount of MDA in fish liver.

Antioxidant enzymes assays

Gills were homogenized phosphate buffer (pH 7.4) for 15 min in pestle and mortar, pass through the muslin cloth and filtered through Whatman Filter Paper No. 1. The filtrate was centrifuged at 10,000 rpm for 15 min. Supernatants were used for enzyme activities.

The activity of superoxide dismutase (SOD) in fish gills was determined by measuring its ability to inhibit the reduction of nitroblue tetrazole (NBT) by superoxide following the method of Giannopolitis and Ries (1997). Catalase (CAT) activity was determined by measuring its ability to decompose hydrogen peroxide concentration at 240 nm following the method of Chance and Meahly (1955). Peroxidase activity was calculated by measuring its ability to reduce the concentration of hydrogen peroxide at 470 nm (Civello *et al*., 1995).

Determination of fatty acid profile

The extracted fat from muscles (as described earlier) was used for the determination of fatty acid profile. Fatty acid profile in fish muscles was determined following by IUPAC (1987) standard method. Fatty acid profile in muscles was determined from the fatty acid methyl esters (FAME) derivatives of the transesterified lipid.

Table II.- Influence of dietary vitamin E intake on growth performance, FI, FCR and SR% of *Cirrhinus mrigala* fingerlings.

Diet	Vitamin E level (mg/kg)	Initial weight (g)	Final weight (g)	AWG (g)	Weight gain (%)	SGR	FI (g)	FCR	SR (%)
D1	0	2.37	8.03[e]	5.65[e]	237.89[e]	2.03[e]	10.94[a]	1.94[a]	95.83[a]
D2	25	2.36	8.32[d]	5.95[d]	251.79[d]	2.10[d]	9.78[b]	1.64[b]	95.83[a]
D3	50	2.38	8.85[c]	6.47[c]	271.85[c]	2.19[c]	8.32[c]	1.29[c]	100.00[a]
D4	75	2.40	9.73[a]	7.32[a]	305.21[a]	2.33[a]	7.40[d]	1.01[a]	100.00[a]
D5	100	2.40	9.76[a]	7.35[a]	305.61[a]	2.33[a]	7.12[d]	0.97[a]	95.83[a]
D6	125	2.40	9.42[b]	7.01[b]	292.29[b]	2.28[b]	8.02[c]	1.14[d]	87.50[a]

AWG, absolute weight gain; FCR, feed conversion ratio; FI, feed intake; SGR, specific growth rate, SR, survival rate.

FAMEs were prepared by using methanol. A sample of 200 to 300 mg of fat was taken in flask and reflux with 15 ml of 0.5 N KOH solution for 3-5 min. When the solution became hot, 15 ml of ammonium-chloride-methanol-H_2SO_4 mix was added and refluxed for 15 min. Swirled to mix, and refluxed for 3 min. Cooled and added light petroleum ether and shaked. Then the ether layer was separated and evaporated the ether under vacuum. The residues were dissolved in 3-10 ml of petroleum ether for determination of fatty acid profile by gas chromatography. Fatty acid methyl esters were analyzed by gas chromatography (GC) (SHIMADZU, model GC-17A FID) and identified by comparing their relative and absolute retention time with those of authentic known standards. Data was subjected to One-way analysis of variance (Steel *et al*., 1996).

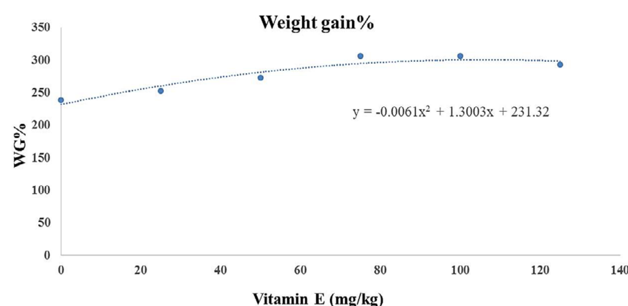

Fig. 1. Graphical representation of dietary vitamin E requirement of weight gain % for *Cirrhinus mrigala* fingerlings on the basis of quadratic regression analysis.

RESULTS

The data regarding the growth performance, feed intake, FCR and survival rate (%) of *Cirrhinus mrigala* fingerlings fed with graded levels of vitamin E supplemented diet is summarized in Table II. According to the results, significant (p<0.05) increase in final weight (g), AWG (g), WG % and SGR of *Cirrhinus mrigala* fingerlings was observed in groups fed with vitamin E supplemented diet when compared with group fed on un-supplemented (control) diet. All the above discussed parameters of growth showed their best in supplemented groups up to the certain level (100 mg/kg) of vitamin E, after which growth was started decreasing. Figure 1 showed the dietary vitamin E requirement of *Cirrhinus mrigala* fingerlings. Quadratic regression analysis on the basis of weight gain % showed that 106.58 mg/kg vitamin E was the optimum level for supplementation in the practical diets of the *Cirrhinus mrigala* fingerlings.

Table III.- Influence of dietary vitamin E intake on TBARS contents in liver and antioxidant enzymes activities in gills of *Cirrhinus mrigala* fingerlings.

Diet	Vitamin E level (mg/kg)	TBARS (mg/g)	SOD (U/mg protein)	CAT (U/mg protein)	POX (mU/mg protein)
D1	0	3.58[a]	4.68[d]	31.36[d]	81.79[d]
D2	25	3.45[ab]	4.73[c]	31.81[c]	83.38[c]
D3	50	3.35[bc]	4.78[b]	32.31[b]	85.02[b]
D4	75	3.20[c]	4.83[a]	32.90[a]	87.16[a]
D5	100	3.04[d]	4.84[a]	32.92[a]	87.44[a]
D6	125	2.86[e]	4.75[bc]	32.12[bc]	82.79[cd]

CAT, catalase; POX, peroxidase; TBARS, thiobarbituric acid reactive substances; SOD, superoxide dismutase.

Improved responses for FI (g) and FCR were recorded in supplemental groups as compare to the control group in *Cirrhinus mrigala* fingerlings. Significant (p<0.05) differences were recorded for FI (g) and FCR at 100 mg/kg of vitamin E. Non-significant (p>0.05) effect of vitamin E supplementation was observed on survival rate (%) of *Cirrhinus mrigala* fingerlings. Table III shows the data of TBARS contents in liver and antioxidant enzyme activities in gills of *Cirrhinus mrigala* fingerlings fed with graded levels of vitamin E supplementation. Inverse relationship was observed in TBARS contents and vitamin E supplementation in diet. Significant (p<0.05) differences

were recorded in TBARS contents between the groups fed on vitamin E supplemented and unsupplemented diet. Lowest TBARS contents was recorded in group fed on diet with maximum supplementation (125 mg/kg of vitamin E), while group fed with control diet showed maximum TBARS contents in liver of *Cirrhinus mrigala* fingerlings. Values for TBARS contents at different dietary vitamin E levels were significantly ($p<0.05$) different among treatments. Significant ($p<0.05$) variations were evident in the values of SOD activity in gills of *Cirrhinus mrigala* fingerlings among control group and the treatment. Increased SOD activity was observed with increased dietary vitamin E supplementation in diet up to a certain level, above which a decrease in enzyme activity was observed. Maximum SOD activity was given in group fed on diet supplemented with 100 mg/kg of vitamin E, while minimum value was observed in control group. Similar trend was followed by the activities of CAT and POX enzyme. CAT and POX activities were significantly ($p<0.05$) higher in gills of *Cirrhinus mrigala* fingerlings with increased vitamin E supplementation in diet up to a certain level. Among all the treatments, highest CAT and POX activities were recorded in group received diet supplemented with 100 mg/kg of vitamin E, while lowest values of these enzymes were observed in control group. Table IV is showing that dietary vitamin E supplementation had showed significant ($p<0.05$) effect on fatty acids profile *i.e.* increased values of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), n-7 and n-9 fatty acids were recorded in control group, while unsaturated fatty acids (USFAs), n-3 and n-6 fatty acids showed their improved ratios by feeding vitamin E diet. Fish received diet contained 25 mg/kg of vitamin E had showed significant improvement in values of polyunsaturated fatty acids (PUFAs), n-3, n-6 and n-3/n-6 when compared with control group.

DISCUSSION

Vitamin E is a collective term used for a category of lipophilic molecules contained four tocopherols and four tocotrienols. Among all of these molecules, α-tocopherol is functionally active for the protection of biological membranes against lipid oxidation and also has other, more specific biochemical functions. Similar to other vertebrates, in fish, α-tocopherol also indirectly contributes in the modulation of growth by controlling the damage in tissues during stress conditions (Tocher *et al.*, 2002). Inadequate vitamin E in diet can have significant negative consequences for proper immune system development as well as proper immune system functioning. In teleosts, reduced growth, poor survival rate, hemolysis, anemia and erythrocyte malformation are considered the main consequences of hypovitaminnosis E (Halver, 2002).

Table IV.- Influence of dietary vitamin E intake on fatty acid profile in muscles of *Cirrhinus mrigala* fingerlings.

Fatty acid	Vitamin E level (mg/kg)					
	0	25	50	75	100	125
14:0n-0	5.80[a]	3.23[c]	3.32[bc]	3.33[bc]	3.30[bc]	3.37[b]
16:0n-0	12.30[a]	8.42[b]	8.48[b]	8.50[b]	8.46[b]	8.49[b]
18:0n-0	4.09[a]	1.86[b]	1.81[b]	1.75[b]	1.81[b]	1.79[b]
16:1n-7	14.52[a]	8.67[b]	8.72[b]	8.77[b]	8.69[b]	8.68[b]
18:1n-7	12.28[a]	8.96[b]	8.93[b]	9.01[b]	8.98[b]	9.03[b]
18:1n-9	18.85[a]	13.22[b]	13.20[b]	13.17[b]	13.16[b]	13.21[b]
18:2n-6	2.69[b]	5.32[a]	5.30[a]	5.29[a]	5.31[a]	5.31[a]
20:4n-6	1.84[b]	3.03[a]	3.13[a]	3.09[a]	3.09[a]	3.06[a]
18:3n-3	3.90[b]	7.40[a]	7.40[a]	7.40[a]	7.40[a]	7.40[a]
20:5n-3	8.59[b]	14.46[a]	14.36[a]	14.39[a]	14.43[a]	14.36[a]
22:5n-3	11.01[b]	17.36[a]	17.32[a]	17.25[a]	17.28[a]	17.27[a]
22:6n-3	4.14[b]	8.05[a]	8.10[a]	8.06[a]	8.08[a]	8.04[a]
Total SFAs	22.19[a]	13.51[b]	13.60[b]	13.58[b]	13.57[b]	13.64[b]
Total MUFAs	45.65[a]	30.85[b]	30.85[b]	30.95[b]	30.83[b]	30.92[b]
n-3	27.65[b]	47.30[a]	47.14[a]	47.10[a]	47.21[a]	47.08[a]
n-6	4.52[b]	8.34[a]	8.42[a]	8.37[a]	8.39[a]	8.36[a]
n-9	18.85[a]	13.22[b]	13.20[b]	13.17[b]	13.16[b]	13.21[b]
ARA/EPA	0.21[a]	0.21[a]	0.22[a]	0.21[a]	0.21[a]	0.21[a]
EPA/DHA	2.08[a]	1.80[b]	1.77[b]	17.90[b]	1.79[b]	1.79[b]
n-3/n-6	1.47[a]	3.58[b]	3.57[b]	3.58[b]	3.59[b]	3.56[b]
Monoenes/ polyenes	0.89[b]	0.45[a]	0.45[a]	0.45[a]	0.45[a]	0.45[a]

ARA, arachidonic acid; EPA, ecosapentaenoic acid; DHA, docosahexaenoic acid; MUFAs, monounsaturated fatty acids; SFAs, saturated fatty acids.

The present research work was aimed to determine the dietary vitamin E requirement of mori, *Cirrhinus mrigala* fingerlings. Growth performance, TBARS content, antioxidant status and fatty acid profiles were the main concerning areas of present research work.

In the present study, results clearly demonstrated that the dietary intake of vitamin E significantly improved the growth performance in *Cirrhinus mrigala* fingerlings. A significant increase in growth responses was recorded in fish fed at the supplementation level of 100 mg/kg of vitamin E and beyond this level of supplementation a decrease in growth performance was recorded. It is believed that α-tocopherol acetate is a powerful antioxidant, actively contributes as disease resistant, modulates the immune system and considered essential dietary nutrient for the maintenance of normal growth in fish (Hamre, 2011). Findings of present research work are in agreement with the previous report that the dietary supplementation of vitamin E showed significant influence on the growth in

young-of-the-year beluga (Amlashi *et al*., 2012). Kocabas and Gatlin III (1999) suggested that, the dietary vitamin E supplementation leads to significant increase in growth responses of hybrid striped bass. In another experiment, increased growth response was reported with increase in dietary α-tocopherol in juvenile grass carp (Li *et al*., 2014), but it tends to decrease with high supplementation level. Results of another trial conducted by Abdel-Hameid *et al*. (2012) on fingerlings of *Channa punctatus* are in accordance with our findings that fish receiving high level of α-tocopherol supplementation (>140 mg/kg) showed significant reduction in weight gain. In another experiment, poor growth and feed utilization efficiencies were reported by Kiron *et al*. (2004) by incorporating 1000 mg/kg of vitamin E for rainbow. At high dietary levels of vitamin E growth depression were resulted may be due to the formation of vitamin E radicals which accumulate in cell and cause imbalance, consequently leads towards pro-oxidant effects (Hamre *et al*., 1997). In contrast, some studies reported that dietary vitamin E supplementation showed no significant effects on growth responses in some fish species like channel catfish (Gaylord *et al*., 1998), gilthead seabream (Montero *et al*., 1999), Nile tilapia (Lim *et al*., 2010) and rainbow trout (Kiron *et al*., 2004). Several factors are responsible for the confliction in results like fish age, fish species, dietary lipid level and lipid type, vitamin E source, interaction of vitamin E with other nutrients present in diet and experimental regimes (Norouzitallab *et al*., 2009; Jaramillo *et al*., 2009). Present study showed non-significant effect of vitamin E supplementation on survival of fish. Similar non-significant effect was also reported by Paul *et al*. (2004) for *Cirrhinus mrigala* fry.

In the present study, the dietary vitamin E requirement of *Cirrhinus mrigala* fingerlings for enhancing weight gain % on the basis of quadratic regression analysis was 106.58 mg/kg of vitamin E. Similarly, Sau *et al*. (2004) and Paul *et al*. (2004) determine that the dietary vitamin E requirement of *Labeo rohita* is 131.91 mg/kg vitamin E and 99 mg/kg vitamin E for *Cirrhinus mrigala* fingerlings on the basis of broken line regression analysis.

Quantification of malondialdehyde (MDA) concentration by TBARS test is considered one of the important parameter for assessment of lipid peroxidation. Previous studies indicated that α-tocopherol level and concentration of MDA showed an inverse relationship with one another. The results of present study suggested that increased dietary vitamin E content (125 mg/kg) significantly decreased liver TBARS concentration in *Cirrhinus mrigala* fingerlings. Results reported by the Gatta *et al*. (2000) indicated that lowest amount of TBARS appeared in muscles of sea bass fed on diet contained vitamin E at high level. Similarly, findings of Lin and Shiau (2005) are in accordance with our results that the values of hepatic TBARS were showed their peak value in fish fed on vitamin E un-supplemented diet and gradually decreased with increased Vitamin E in grouper, *Epinephelus malabaricus*. Low concentrations of vitamin E supplementation in diet might be responsible for increased peroxidation of fatty acids mainly due to the formation of oxidative radicals in tissues on large amount (Tocher *et al*., 2002).

The SOD, CAT and POX activities in gills of *Cirrhinus mrigala* fingerlings were found significantly increased with the high dietary vitamin E concentrations in present study. Higher SOD activity was showed in the gills of fish fed with diet containing 100 mg/kg of vitamin E beyond that a decrease in activity was observed. Similar trend in activities of CAT and POX was observed in the gills of *Cirrhinus mrigala* fingerlings. Similar conclusion drawn by the studies of Mourente *et al*. (2000) and Tocher *et al*. (2002) conducted with juvenile marine fish that the level of dietary α-tocopherol had significant effects on the activities of the liver antioxidant enzymes. The reason behind this increased hepatic enzyme activity might be to make the fish able to counteract the pro-oxidant effect caused by utilization of α-tocopherol (Li *et al*., 2014).

Fish fed the vitamin E deficient (control) diet showed increased values of saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs), EPA/DHA and monoenes/polyenes. Fish received diet contained 25 mg/kg of vitamin E showed significant improvement in values of unsaturated fatty acids (USFAs), n-3, n-6 fatty acids, n-3/n-6 when compared with control group in the muscles of *Cirrhinus mrigala* fingerlings, beyond this level of supplementation no further increase was observed. From the results of present experiment, it is concluded that low level (25 mg/kg vitamin E) of dietary vitamin E supplementation is enough to protect the unsaturated fatty acids from deterioration in the muscles of *Cirrhinus mrigala* fingerlings and supplementation beyond this is of no use. Presence of α-tocopherol in the interior hydrophobic part of the biological membrane with phytyl chain (Quinn, 2004), protects PUFAs by donating its own hydrogen atom to the lipid peroxyl radical and breaks the chain of reactions involved in the lipid peroxidation. Similar to our results, Agradi *et al*. (1993) reported that sturgeon (*Acipenser naccarii*) fed on vitamin E supplemented diet showed highest percentage of total n-3 PUFAs in muscle and liver tissues. In another research work, Bai and Lee (1998) concluded that unsaturated fatty acids and polyunsaturated fatty acid ratios in fish liver and muscles fed vitamin E un-supplemented diet were lower than those of fish fed the diet supplemented with 120 mg/kg of vitamin E in juvenile Korean rockfish.

In conclusion, dietary vitamin E supplementation was proved efficient for the improvements in growth, antioxidant status and fatty acid profile. Vitamin E showed great capacity to work as an effective tool to reduce oxidative damage in muscles, liver and gills of *Cirrhinus mrigala* fingerlings

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Abdel-Hameid, N.A.H., Abidi, S.F. and Khan M.A., 2012. Dietary vitamin E requirement for maximizing the growth, conversion efficiency, biochemical composition and haematological status of fingerling *Channa punctatus*. *Aquacult. Res*., **43**: 226-238. https://doi.org/10.1111/j.1365-2109.2011.02819.x

Agradi, E., Abrami, G., Serrini, G., Mckenzie, D., Bolis, C. and Bronzi, P., 1993. The role of dietary N-3 fatty acid and vitamin E supplements in growth of sturgeon (*Acipenser naccarii*). *Comp. Biochem. Physiol*., **105**: 187-195. https://doi.org/10.1016/0300-9629(93)90194-9

Amlashi, A.S., Falahatkar, B. and Sharifi, D.S., 2012. Dietary vitamin E requirements and growth performance of young-of-the-year beluga. *Huso huso* (L.) (Chondrostei: Acipenseridae). *Arch. Pol. Fish*., **20**: 299-306. https://doi.org/10.2478/v10086-012-0034-y

AOAC, 1995. *Official methods of analysis*, 15th ed. Association of Official Analytical Chemist, Washington, D.C., USA, pp. 1094.

Bai, S.C. and Lee, K.J., 1998. Different levels of dietary DL-α-tocopheryl acetate affect the vitamin E status of juvenile Korean rockfish, *Sebastes schlegeli*. *Aquaculture*, **161**: 405-415. https://doi.org/10.1016/S0044-8486(97)00288-3

Bae, J.Y., Park, G.H., Yoo, K.Y., Lee, J.Y., Kim, D.J. and Bai, S.C., 2013. Evaluation of optimum dietary vitamin E requirements using DL-α-tocopheryl acetate in the juvenile eel, *Anguilla japonica*. *J. Appl. Icthol.,* **29**: 213-217. https://doi. 10.1111/jai.12001

Chance, B. and Maehly, A.C., 1955. Assay of catalase and peroxidases. *Methods Enzymol*., **2**: 764-775. https://doi.org/10.1016/S0076-6879(55)02300-8

Civello, P.M., Marting, G.A., Chaves, A.R. and Anan, M.C., 1995. Peroxidase from strawberry fruit by partial purification and determination of some properties. *J. Agric. Fd. Chem*., **43**: 2596-2601. https://doi.org/10.1021/jf00058a008

Gatta, P.P., Pirini, M., Testi, S., Vignola, G. and Monetti, P.G., 2000. The influence of different levels of dietary vitamin E on sea bass, *Dicentrarchus labrax* flesh quality. *Aquacult. Nutr*., **6**: 47-52. https://doi.org/10.1046/j.1365-2095.2000.00127.x

Gaylord, T.G., Rawles, S.D. and Gatlin III, D.M., 1998. Re-evaluation of vitamin E supplementation of practical diets for channel catfish, *Ictalurus punctatus*, production. *Aquacult. Nutr*., **4**: 109-114. https://doi.org/10.1046/j.1365-2095.1998.00054.x

Giannopolitis, C.N. and Ries S.K., 1997. Superoxide dismutase I occurrence in higher plant. *Pl. Physiol*., **59**: 309-314. https://doi.org/10.1104/pp.59.2.309

Halver, J.E., 2002. The vitamins. In: Fish nutrition (eds. J.E. Halver and R.W. Hardy). Academic Press, San Diego, CA, pp. 61-141.

Hamre, K. and Lie, O., 1995. Minimum requirement of vitamin E for Atlantic salmon (*Salmo salar* L.) at first feeding. *Aquacult. Res*., **26**: 175-184. https://doi.org/10.1111/j.1365-2109.1995.tb00900.x

Heinzerling, R., Tengerdy, R.P., Wick, L.L. and Lueker, D.C., 1974. Vitamin E protects mice against Diplococus pneumonia type I infection. *Infect. Immun*., **10**: 1292-1295.

Hamre, K., 2011. Metabolism, interactions, requirements and functions of vitamin E in fish. *Aquacult. Nutr*., **17**: 98-115. https://doi.org/10.1111/j.1365-2095.2010.00806.x

Hamre, K., Waagbo, R., Berge, R.K. and Lie, O., 1997. Vitamin C and E interact in juvenile Atlantic salmon (*Salmo salar* L.). *Free Radic. Biol. Med*., **22**: 137-149. https://doi.org/10.1016/S0891-5849(96)00281-X

IUPAC, 1987. *Standard methods for the analysis of oils, fats and derivatives*, 7th rev. (eds. C. Paquot and A. Hautfenne). International Union of Pure and Applied Chemistry, Blackwell Scientific, London.

Jaramillo, J.R., Peng, L.I. and Gatlin III, D.M., 2009. Selenium nutrition of hybrid striped bass (*Morone chrysops* x *M. saxatilis*) bioavailability, toxicity and interaction with vitamin E. *Aquacult. Nutr*., **15**: 160-165. https://doi.org/10.1111/j.1365-2095.2008.00579.x

Julseth, D.R., 1974. *Evaluation of vitamin E and disease stress in turkey performance*. M.S. thesis, Colorado State University, Colorado, pp. 51.

Khan, M.A., Jafri, A.K. and Chadha, N.K., 2004. Growth and body composition of rohu, *Labeo rohita* (Hamilton), fed compound diet. Winter feeding and rearing to marketable size. *J. appl. Ichthyol*., **20**: 265-270. https://doi.org/10.1111/

j.1439-0426.2004.00550.x

Kiron, V., Puangkaew, J., Ishizaka, K., Satoh, S. and Watanabe, T., 2004. Antioxidant status and nonspecific immune responses in rainbow trout (*Oncorhynchus mykiss*) fed two levels of vitamin E along with three lipid sources. *Aquaculture*, **234**: 361-379. https://doi.org/10.1016/j.aquaculture.2003.11.026

Kocabas, A.M. and Gatlin III, D.M., 1999. Dietary vitamin E requirement of hybrid striped bass (*Morone chrysops* female x *M. saxatilis* male). *Aquacult. Nutr.*, **5**: 3-7. https://doi.org/10.1046/j.1365-2095.1999.00074.x

Li, J., Lang, X.F., Tan, Q., Yuan, X., Liu, L., Zhou, Y. and Li, B., 2014. Effects of vitamin E on growth performance and antioxidant status in juvenile grass carp *Ctenopharyngodon idellus*. *Aquaculture*, **430**: 21-27. https://doi.org/10.1016/j.aquaculture.2014.03.019

Lim, C., Yildirim-Aksoy, M., Welker, T. and Klesius, P.H., 2010. Growth performance, immune response, and resistance to *Streptococcus iniae* of Nile Tilapia, *Oreochromis niloticus*, Fed diets containing various levels of vitamins C and E. *J. World Aquacult. Soc.*, **41**: 35-48. https://doi.org/10.1111/j.1749-7345.2009.00311.x

Lin, Y.H. and Shiau, S.Y., 2005. Dietary vitamin E requirement of grouper, *Epinephelus malabaricus*, at two lipid levels and their effects on immune responses. *Aquaculture*, **248**: 235-244. https://doi.org/10.1016/j.aquaculture.2005.04.020

Miller, J.K., Brzezinska-Slebodzinska, E. and Madsen, F.C., 1993. Oxidative stress, antioxidants, and animal function. *J. Dairy Sci.*, **76**: 2812-2823. https://doi.org/10.3168/jds.S0022-0302(93)77620-1

Montero, D., Marrero, M., Izquierdo, M.S., Robaina, L., Vergara, J.M. and Tort, T., 1999. Effect of vitamin E and C dietary supplementation on some immune parameters of gilthead seabream (*Sparus aurata*) juveniles subjected to crowding stress. *Aquaculture*, **171**: 269-278. https://doi.org/10.1016/S0044-8486(98)00387-1

Murai, T. and Andrews, J.W., 1974. Interactions of dietary a-tocopherol, oxidized menhaden oil and ethoxyquin on channel catfish (*Ictalurus punctatus*). *J. Nutr.*, **104**: 1416-1431. https://doi.org/10.1093/jn/104.11.1416

Mourente, G., Daiz-Salvago, E., Tocher, D.R. and Bell, G.J., 2000. Effect of dietary polyunsaturated fatty acid/vitamin E (PUFA/tocopherol) ratio on antioxidant defence mechanisms of juvenile gilthead sea bream (*Sparus aurata* L. Osteichthyes Sparidae). *Fish Physiol. Biochem.*, **23**: 337-351. https://doi.org/10.1023/A:1011128510062

Norouzitallab, P., Farhangi, M., Babapour, M., Rahimi, R., Sinha, A.K. and Baruah, K., 2009. Comparing the efficacy of dietary α-tocopherol with that of DL-α-tocopheryl acetate, both either alone or in combination with ascorbic acid, on growth and stress resistance of angelfish, *Pterophylum scalare*, juveniles. *Aquacult. Int.*, **17**: 207-216. https://doi.org/10.1007/s10499-008-9192-8

Paul, B.N., Sarkar, S. and Mohanty, S.N., 2004. Dietary vitamin E requirement of mrigal, *Cirrhinus mrigala* fry. *Aquaculture*, **242**: 529-536. https://doi.org/10.1016/j.aquaculture.2004.08.037

Quinn, P.J., 2004. Is the distribution of a-tocopherol in membranes consistent with its putative functions? *Biochemistry (Moscow)*, **69**: 58-66. https://doi.org/10.1023/B:BIRY.0000016352.88061.02

Reed, D.J., 1990. Glutathione: toxicological implications. *Annu. Rev. Pharmacol. Toxicol.*, **30**: 603-631. https://doi.org/10.1146/annurev.pa.30.040190.003131

Rowland, S.J. and Ingram, B.A., 1991. *Diseases of Australian native fishes*. Fisheries Bulletin 4 NSW Fisheries, Sydney, NSW, Australia.

Sau, S.K., Paul, B.N., Mohanta, K.N. and Mohanty, S.N., 2004. Dietary vitamin E requirement, fish performance and carcass composition of rohu, *Labeo rohita* fry. *Aquaculture*, **240**: 359-368. https://doi.org/10.1016/j.aquaculture.2004.02.008

Shahidi, F. and Hong, C., 1991. Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *J. Fd. Biochem.*, **15**: 97-105. https://doi.org/10.1111/j.1745-4514.1991.tb00147.x

Shiau, S.Y. and Hsu, C.Y., 2002. Vitamin E sparing effect by dietary vitamin C in juvenile hybrid tilapia, *Oreochromis niloticus* x *O. aureus*. *Aquaculture*, **210**: 335-342. https://doi.org/10.1016/S0044-8486(01)00853-5

Shiau, S.Y. and Shiau, L.F., 2001. Reevaluation of the vitamin E requirements of juvenile tilapia (*Oreochromis niloticus* x *O. aureus*). *Anim. Sci.*, **72**: 529-534. https://doi.org/10.1017/S135772980005205X

Steel, R.G.D., Tories, J.H. and Dickey, D.A., 1996. *Principles and procedures of statistics*, 3rd ed. McGraw Hill International Book Co. Inc., New York, USA, pp. 336-352.

Tocher, D.R., Mourente, G., Van der Eecken, A., Evjemo, J.O., Diaz, E., Bell, J.G., Geurden, I., Lavens, P. and Olsen, Y., 2002. Effects of dietary vitamin E on antioxidant defense mechanisms of

juvenile turbot (*Scophthalmus maximus* L.), halibut (*Hippoglossus hippoglossus* L.) and sea bream (*Sparus aurata* L.). *Aquacult. Nutr*., **8**: 195-207. https://doi.org/10.1046/j.1365-2095.2002.00205.x

Watanabe, T., Takeuchi, T., Matsui, M., Ogino, C. and Kawabata, T., 1977. Effect of α-tocopherol deficiency on carp. VII. The relationship between dietary levels of linoleate and α-tocopherol requirement. *Bull. Jpn. Soc. Sci. Fish*., **43**: 935-946. https://doi.org/10.2331/suisan.43.935

Zhong, Y.S., Lall, P. and Shahidi, F., 2007. Effects of oxidized dietary oil and vitamin e supplementation on lipid profile and oxidation of muscle and liver of juvenile Atlantic cod (*Gadus morhua*). *J. Agric. Fd. Chem*., **55**: 6379-6386. https://doi.org/10.1021/jf070124w

Zhou, Q.C., Wang, L.G., Wang, H.L., Wang, T., Elmada, C.Z. and Xie, F.J., 2013. Dietary vitamin E could improve growth performance, lipid peroxidation and non-specific immune responses for juvenile cobia (*Rachycentron canadum*). *Aquacult. Nutr*., **19**: 421-429. https://doi.org/10.1111/j.1365-2095.2012.00977.x

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Intraspecific Variation in Thermal Tolerance among Three Hatchery Reared Populations of *Labeo rohita* Acclimated to Different Temperatures

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Authors' Contribution
AS conducted experiments, collected and analyzed the data and wrote the manuscript. AZ planned and supervised this research and reviewed the manuscript. MA assisted in research work, statistical analysis and interpretation of results. SA helped in acquisition of data and preparation of manuscript.

Key words
Major carp, Thermal tolerance, Hatchery reared populations, Intraspecific variation.

ABSTRACT

The ability of species and populations to respond to increase or decrease in temperature depend on their genetic make-up and adaptive potential. *Labeo rohita* is a prized warm water species that has been widely stocked across Pakistan. We studied the upper and lower thermal tolerance in three captive populations of a single species from three different ancestral environments, *i.e.* Charbanda Fish Hatchery District Mardan (MDN-s), Rawal Town Fish Seed Hatchery, Islamabad (IBD-s) and Manawa Fish Hatchery, District Lahore (LHR-s) at four acclimation temperature (22, 26, 30 and 34°C),. A thermal tolerance polygon over the range of 22-34°C showed a calculated area of 418°C^2 for IBD-s and LHR-s, while 417.5°C^2 for MDN-s. Results indicate that East origin *L. rohita* (LHR-s) have better potential to cope warmer conditions due to tolerance of Upper thermal limit (45 ± 0.25°C) and North East origin have better ability to tolerate Minimum thermal limits (10 ± 0.25°C), while MDN-s has moderate thermal tolerance. Overall results suggest consideration of the thermal tolerance range for successful culture of *L. rohita* in different regions.

INTRODUCTION

In all vertebrates, with every degree rise in temperature relate to increase in metabolism unless stable temperature is gained. At the thermal optimum, every system of organism performs efficiently (Ficke *et al.*, 2007). Every species have a particular range of temperature and beyond that limits, behaviour, growth, reproduction, migration and physiology (Hirayama *et al.*, 2004; Zhou *et al.*, 2009; Akhtar *et al.*, 2011; Wilson *et al.*, 2014) or overall performance of the organism is adversely affected (Ficke *et al.*, 2007). For instance, warm water fish show higher mortality in winter season, as the temperature fall below 13°C (beyond lower thermal tolerable limits) and adversely affect their activity, feed intake and growth (Ibarz *et al.*, 2003; Hurst, 2007).

Organisms have tendency to exceed their tolerance range according to climatic condition (Chown *et al.*, 2010; Hofmann and Todgham, 2010), hence indicating the genetically controlled flexibility (Seebacher and Franklin, 2012). The ability to adjust physiological performance to prevailing environmental conditions (acclimation) may allow individuals to maximize fitness in changing environmental condition (Seebacher *et al.*, 2012). However, if such adaptation did not occur, then organism's loss their performance (Ficke *et al.*, 2007; Schulte *et al.*, 2011) or disappearance of populations may occur due to migration towards better habitats (Visser, 2008). Generally, ectoderms in nature timely alter their habitat (Anderson *et al.*, 2007; Cadena and Tattersall, 2009), however, when would not have any other option then extinction of particular species is started.

Currently, comparative methods are in practice for evaluating evolutionary changes in thermal tolerance of organism with respect to habitat use or geographical variation in climate as a result of regional, global warming (Feder *et al.*, 2000; Angilletta *et al.*, 2002; Rivadeneira and Fernández, 2005; Pörtner and Knust, 2007; Yanik and Aslan, 2018). Under different environmental conditions, different strains of the same species from various geographical regions might respond differently (Imsland *et al.*, 2003), because of the confounded effects of many factors, including gene environment interaction, other than habitat uniqueness (Pearson *et al.*, 2002). Many studies demonstrated variation in thermal tolerance within species (McDermid *et al.*, 2012; Stitt *et al.*, 2014) and among species (Chatterjee *et al.*, 2004; Das *et al.*, 2004; Vinagre *et al.*, 2013).

The impact of climate changes on thermal tolerance

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and life of the organism has widely been recognized (Ficke *et al*., 2007; Bozinovic *et al*., 2011). The upper and lower temperature tolerance of fish in the laboratory could be quantified by adopting a critical thermal methodology (CTM) (Cowles and Bogert, 1944) that involve exposure of fish to a continuous and constant decrease or increase in temperature until a sub-lethal or near-lethal endpoint *i.e.* disorganization of locomotive activity of fish (Beitinger *et al*., 2000) is attained. Furthermore, the acclimation response ratio (ARR) *i.e.,* magnitude of thermal acclimation reflects the dependence of ARR on the geographic zone of the organisms, as fish inhabiting subtropical and tropical regions have high values in comparison to warm and cold region fish (Diaz *et al*., 2002; Re *et al*., 2005). It further emphasize on the knowledge regarding thermal tolerance (CTMax and CTMin) of fish being exposed to spatial or temporal, temperature extremes during winter or dry season.

L. rohita is cultured almost throughout Pakistan from Islamabad, Punjab, Sindh and even in Baluchistan and Khyber Pakhtunkhwa (KPK). Throughout the year, climatic condition of these areas shows variability with respect to rainfall, drought and in higher and lower temperature limit. Hence, fish under culture in these areas are in the influence of these fluctuations and show various adaptations to overcome these climatic challenges. Successful culture of any species depends upon the availability of fish seed. In Pakistan, most of fish seed is provided to the fish farmer from Punjab province and to a lesser extent by the KPK. In the present study, three government fish Hatcheries, Charbanda Fish Hatchery (KPK), Rawal Town Fish Seed Hatchery (Islamabad) and Manawa Fish Hatchery (Punjab) differ on the basis geographical location, climatic condition, management and husbandry practices were selected for the evaluation of thermal tolerance of their stock of *L. rohita* with the assumption that will help in the successful culture of *L. rohita* by the selection and suggestion of seed on the basis of thermal tolerance with respect their particular area.

MATERIALS AND METHODS

Maintenance and acclimation trials

Fingerling of *Labeo rohita,* average body weight and length 10.44± 0.24 g, 9.11±0.24 cm, 11.88±0.17g, 10.75±0.17 cm and 12.26± 0.11g, 10.96 ± 0.10 cm were collected from Charbanda Fish Hatchery, Mardan (33°40'55.28"N, 73° 6'57.88"E), Manawa Fish Hatchery, Lahore (31°35'23.50"N, 74°29'0.83"E) and Rawal Town Fish Seed Hatchery, Islamabad (34°15'41.96"N, 72° 3'26.69" E), respectively and considered as Mardan strain (MDN-s), Lahore strain(LHR-s) and Islamabad strain (IBD-s). These hatcheries mostly provide fish seed throughout Pakistan for commercial and for re-stocking purposes. Fingerlings were transported in aerated plastic bags to the Fisheries and Aquaculture Research Station, Quaid-i-Azam University. After tempering, they were shifted to separate circular tanks, to avoid mixing of strains and acclimatized at 26±1ºC for one week before starting the experiment. Fish were offered prepared 35% protein basal diet twice a day at ad libitum, while undigested feed and fecal material were siphoned on a daily basis. Then fingerling of each hatchery were tagged on the dorsal side with three different water proof colored materials and tag specimen were shifted to different aquaria with thermostat heater (4 fish/aquarium and 8 fish/treatment *i.e.* 4 for CTMax and 4 for CTMin, from each population). Experiment was conducted in replicates of two, so total of 48 specimens per temperature treatment; 12 per aquarium (MDN-s, 4; LHR-s, 4; IBD-s, 4) were used for one treatment. An experimental/environmental effect was avoided by providing thermal treatment to all three strains in the same aquarium. The water temperature of each aquarium was decreased or increased at a rate of 1°C/day from 26±1°C temperature to reach the experimental temperatures *i.e.* 22, 26, 30, and 34°C. After reaching the chosen treatment, fish were acclimated for a period of 30 days before starting the trial. The mentioned acclimation temperature was created on the basis of previous investigation on *C. carpio* and *L. rohita* (Chatterjee *et al*., 2004; Das *et al*., 2004), and yellowtail catfish (Debnath *et al*., 2006). During trial, dissolved oxygen (DO) and acclimatized temperature were checked on a daily basis. Daily water was exchanged (20-30%) in order to maintain quality of water. Before starting the thermal tolerance experiment, fish were starved for 24 h.

Thermal tolerance estimation

Critical thermal methodology (CTM) was adopted to assess thermal tolerance. Further strain based thermal tolerance was performed by using Mora and Ospina (2001) method. The method has been previously evaluated as effective because CTM does not involve death as the experimental end point, thus showing significance for estimating the thermal tolerances of endangered or threatened fish species (Bennett and Beitinger, 1997). To evaluate thermal tolerance CTMax and CTMin test was conducted by gradual increment and reduction of water temperature, *i.e.,* at the rate of 0.3°C per minutes of each aquarium set at acclimated temperature until fish showed loss of equilibrium (LOE). Lowest and highest temperature points at which LOE was observed was considered as critical thermal minima (CTMin), and critical thermal maxima (CTMax), respectively (Beitinger *et al*., 2000). Aeration was well maintained throughout the experiment,

and a digital thermometer set on each aquarium was used to determine time to time temperature fluctuations. Fish were adjusted in temperature gradient aquariums, respectively.

The polygon of thermal tolerance was created by drawing acclimatization temperatures on the X-axis and the CTMax and CTMin mean values on the Y-axis. The thermal tolerance zone range was calculated from polygon and expressed as 2°C. The area of thermal tolerance for all three populations was designed from the graph (Fig. 1).

Statistical evaluation

Thermal tolerance results are presented in the text and tables as mean±S.E. and experimental data of each strain was tested using two-way analysis of variance (ANOVA) followed by LSD comparison test by using statistical package program SPSS (version 20). Simple regression analysis was performed using Microsoft Office Excel spread sheet.

RESULTS

Analysis of variance revealed significant intraspecies difference in upper thermal tolerance among strains ($F_{2, 48}$ = 103.705, P =0.000) and among higher acclimation temperature ($F_{3, 48}$ = 391.788, P = 0.000). Similarly CTMin also showed significant difference among three strains ($F_{2, 48}$= 108.250, P = 0.000) and among lower acclimation temperature ($F_{3, 48}$ = 585.407, P = 0.000). CTMax and CTMin of all strains increased significantly with increasing acclimation temperatures (P<0.05) (Table I). At higher acclimation temperature, all strains had higher CTMax and CTMin values while at lower temperature had lower values. Furthermore, with 0.3°C / min heating and cooling rate at 22, 26, 30 and 34°C acclimation temperatures, CTMax and CTMin of all strains ranged within 36.13 ± 0.44°C to 45 ± 0.25°C and 10 ± 0.25 °C to 19.63 ± 0.38°C, respectively. Moreover, in all strains, acclimation temperatures and thermal tolerance level (CTM) showed strong relationship , LHR-s: CTmax = 27.71+ 0.515 × Acclimation temperature; P= 0.007, r2 = 0.98 and CTMin = -0.1563 + 0.57 × Acclimation temperature, P= 0.001, r2 = 0.99).MDN-s: CTmax = 26.40 + 0.51 × Acclimation temperature; *P* = 0.001, r2 = 0.99 and CTMin = -1.52 + 0.58 × Acclimation temperature, P= 0.001, r2 = 0.99). IBD-s: CTmax = 25.15 + 0.51 × Acclimation temperature; P= 0.007, r2 = 0.98 and CTMin = -2.9 + 0.58 × Acclimation temperature, P= 0.001, r2 = 0.99).

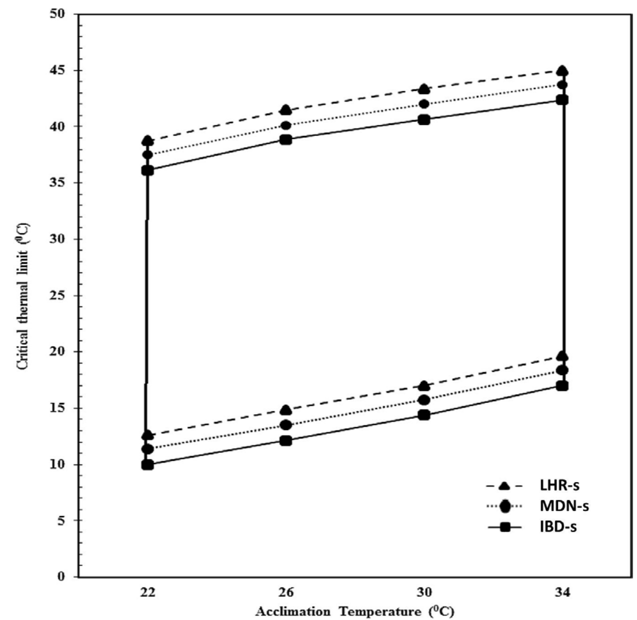

Fig. 1. Thermal tolerance polygon (C^2) of LHR-s, MDN-s and IBD-s created by using CTM data. It shows thermal tolerance zone of *L. rohita* strains acclimated to four different temperatures (22, 26, 30 and 34°C). Thermal tolerance polygon 418°C^2 for IBD-s and LHR-s and 417.5°C^2 for MDN-s.

Table I.- Critical thermal maxima (CT_{Max}) and critical thermal minima (CT_{Min}) of three strain of *Labeo rohita* acclimated at four different temperatures.

Acclimation temperature	CT_{Max}			CT_{Min}		
	LHR-s	MDN-s	IBD-s	LHR-s	MDN-s	IBD-s
22	38.75 ± 0.5^{aD}	37.5 ± 0.5^{abC}	36.13 ± 0.63^{bD}	12.63 ± 0.38^{aD}	10.38 ± 0.13^{abD}	10± 0.25^{bD}
26	41.5 ± 0.5^{aC}	40.25 ± 0.88^{abB}	38.5 ± 0.13^{bC}	14.88 ± 0.63^{aC}	13.5 ± 0.25^{abC}	12.13 ± 0.13^{bC}
30	43.38 ± 0.13^{aB}	42± 0.25^{bAB}	40.63 ± 0.38^{cB}	17 ± 0.25^{aB}	15.75 ± 0.25^{bB}	14.38 ± 0.13^{cB}
34	45 ± 0.25^{aA}	43.75 ± 0.25^{bA}	42.38 ± 0.13^{cA}	19.63 ± 0.38^{aA}	18.38 ± 0.13^{bA}	17 ± 0.25^{cA}

Values are expressed as mean ± SEM (n=4). Means sharing similar letters (a, b, ab, c) within the row are not significantly different from each other (P >0.05) amongst different strains, while Different superscripts (A, B, AB, C and D) in the same column indicate significant difference amongst different acclimation temperatures in each strain. (ANOVA followed by LSD test). MDN-s, Mardan strain; IBD-s, Islamabad strain; LHR-s, Lahore strain.

The regression slope for CTMax and CTMin of LHR-s revealed that for every 1 °C increase in the acclimation temperature, the CTMax and CTMin increased by 0.51 and 0.58 °C, respectively. Similarly, IBD-s and MDN-s followed the same trend for regression slope. These results also showed that, in LHR-s, acclimation temperature variation had a greater effect on their tolerance to high temperatures as compared to other two strains. But IBD-s showed better tolerance to cold temperature as compared to other groups. Thermal tolerance zone was calculated as 418°C^2 for IBD-s and LHR-s, while 417.5°C^2 for MDN-s by plotting thermal tolerance polygon (Fig. 1).

DISCUSSION

Selection of fast growing and thermal tolerant fish stock is always prerequisites for the sustainable development of fish culture. The significant variation in thermal tolerance among different strains (*i.e.* MDN-s, IBD-s and LHR-s) of *L. rohita* at four acclimation temperatures (22, 26, 30 and 34°C (Table I; Fig. 2) indicates the presence of Intra-specific-variation. The experimental condition for all strains was similar; hence the differences in performance are likely attributable to genetic factor (Nakajima *et al*., 2009) or environmental factors (Imsland *et al*., 2003). It appears that different strain had historically different thermal regimes, environmental condition (Vecerek *et al*., 2002; Graczyk *et al*., 2003), seasonal pattern (Jawad *et al*., 2004) and temperature exposure (Cho *et al*., 2015). Thermal tolerance difference among strains was also observed by Nakajima *et al.* (2009) in guppy *Poecilia reticulata* and suggested the sex-linked inheritance of thermal tolerance gene or genes, while according to Imsland and Jonassen (2001) thermal effect resulted from the interaction of both extrinsic (food supply, salinity and oxygen) and intrinsic factors (strain, age, sex *etc*.)

The increasing trend of CTMax and CTMin in MDN-s, IBD-s and LHR-s with a rise in acclimation temperatures showed the conformity with the earlier investigation on early fingerlings of *Cyprinus carpio* (Chatterjee *et al*., 2004), advance fingerlings of Indian Major Carps (Das *et al*., 2004) and yellowtail catfish, *P. pangasius* (Debnath *et al*., 2006). However, some difference in CTMax and CTMin values from previous investigation may be due to difference in geographical temperature gradient (Herrera *et al*., 1998), acclimation temperature (Das *et al*., 2004), thermal tolerance history, species, age and size of fish. However, involvement of the sex related genetic factor cannot be ignored in explaining the results. Our present results support the strain-specific thermal tolerance in fish (McDermid *et al*., 2012).

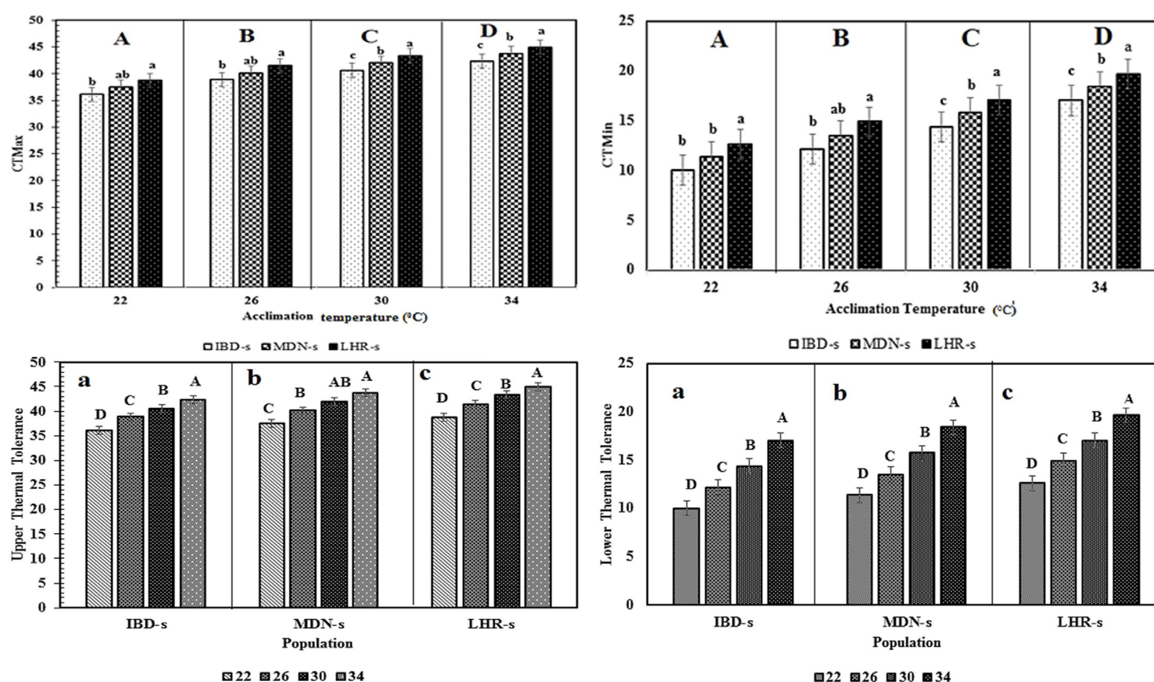

Fig. 2. Values are expressed in means SEM. Upper thermal tolerance or CTMax (left) and lower thermal tolerance or CTMin (right) of three strains of Rohu acclimated to four temperatures. Significant differences between strains are indicated by different lowercase letters, and significant differences between acclimation temperatures are indicated by different uppercase letters above the bars.

Thermal tolerance polygons provide comprehensive information about fish ecology and distribution strategy of fish for temperature tolerance (Dulger *et al*., 2012). It is also an important comparative index of eurythermicity between species (Eme and Bennett, 2009). In the present study thermal tolerance polygon with four fixed temperatures in IBD-s, and LHR-s were similar, 418°C^2 as compared to MDN-s (417.5°C^2). However, the observed thermal polygons of three strain *L. rohita* were higher than (273.5°C^2) reported by Das *et al*. (2005) for the same species. The variability in result might be related to differences in acclimation temperature as the area of thermal-tolerance polygon is reliant on acclimation temperatures used in the experiment (Chatterjee *et al*., 2004). To compare our findings, no parallel report on *L. rohita* of this region is available. Furthermore, the strong effect of acclimation temperature on tolerance of high temperature compared to low temperature indicated the better adaptability of *L.rohita* towards upper temperature than lower temperature.

The effect of acclimation temperature on temperature tolerances is reflected mathematically by the magnitude of the slopes relating these two variables. All strains showed 0.55-point increase for each 1°C increased in acclimation temperature, while 0.59 decreased for each 1°C decreased in acclimation temperature. The former represents a gain in heat tolerance and the latter, a loss of cold tolerance. It appears that changes in acclimation temperature had a stronger effect on tolerance of low temperature than high temperatures.

For an experiment, the tested strains were collected from three different hatcheries geographically located in different regions and are in practice of using limited broodstock with minor replacement. Therefore, strong effects of increase in temperature on thermal tolerance of testing strains support the concept that both genetic and physical parameters (Portner, 2002) are influencing the physiological response of *L. rohita* to temperature fluctuation. LHR-s fingerlings were collected from Manawa Hatchery where temperature exceed to 48°C, therefore, this strain showed more upper temperature-tolerance and better adaptability to warmer conditions. However, IBD-s (North east strain), suggesting some degree of ancestral local adaptation for temperature tolerance, where the temperature reaches to 38°C in summer while in winter decrease up to -3.9°C. Due to moderate temperature Mardan-s showed the moderate result. Reduced tolerance either of CTmax or CTMin in each strain likely resulted from their historical bottleneck and subsequent inbreeding (Balon, 1995). Intraspecific variations in thermal performance were also observed by many other scientists (Haugen and Vøllestad, 2000; Myrick and Cech, 2004; Kavanagh *et al*., 2010; Eliason *et al*., 2011). Overall results indicate that IBD-s have less potential to adapt to predicted temperature increases and LHR-s to the predicted temperature decrease. Based on the present study LHR-s strain is likely the best candidate for the warmer climate of Pakistan, but IBD-s is best for colder area of this country.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Akhtar, M., Pal, A., Sahu, N., Alexander, C. and Meena, D., 2011. Dietary pyridoxine enhances thermal tolerance of *Labeo rohita* (Hamilton) fingerlings reared under endosulfan stress. *J. Therm. Biol*., **36**: 84-88. https://doi.org/10.1016/j.jtherbio.2010.10.003

Anderson, J.L., Albergotti, L., Proulx, S., Peden, C., Huey, R.B. and Phillips, P.C., 2007. Thermal preference of *Caenorhabditis elegans*: a null model and empirical tests. *J. exp. Biol*., **210**: 3107-3116. https://doi.org/10.1242/jeb.007351

Angilletta, M.J., Niewiarowski, P.H. and Navas, C.A., 2002. The evolution of thermal physiology in ectotherms. *J. Therm. Biol*., **27**: 249-268. https://doi.org/10.1016/S0306-4565(01)00094-8

Balon, E.K., 1995. Threatened fishes of the world: Aurora form of the brook charr, *Salvelinus fontinalis* (Mitchill, 1814) (*Salmonidae*). *Environ. Biol. Fish*., **43**: 107-108. https://doi.org/10.1007/BF00001823

Beitinger, T.L., Bennett, W.A. and McCauley, R.W., 2000. Temperature tolerances of North American freshwater fishes exposed to dynamic changes in temperature. *Environ. Biol. Fish*., **58**: 237-275. https://doi.org/10.1023/A:1007618927527

Bennett, W.A. and Beitinger, T.L., 1997. Temperature tolerance of the sheepshead minnow, *Cyprinodon variegatus*. *Copeia*, **1997**: 77-87. https://doi.org/10.2307/1447842

Bozinovic, F., Calosi, P. and Spicer, J.I., 2011. Physiological correlates of geographic range in animals. *Annu. Rev. Ecol. Evol. Syst*., **42**: 155-179. https://doi.org/10.1146/annurev-ecolsys-102710-145055

Cadena, V. and Tattersall, G.J., 2009. The effect of thermal quality on the thermo regulatory behaviour of the bearded dragon *Pogona vitticeps*: Influences

of methodological assessment. *Physiol. Biochem. Zool.*, **82**: 203-217. https://doi.org/10.1086/597483

Chatterjee, N., Pal, A., Manush, S., Das, T. and Mukherjee, S., 2004. Thermal tolerance and oxygen consumption of *Labeo rohita* and *Cyprinus carpio* early fingerlings acclimated to three different temperatures. *J. Therm. Biol.*, **29**: 265-270. https://doi.org/10.1016/j.jtherbio.2004.05.001

Cho, H.C., Kim, J.E., Kim, H.B. and Baek, H.J., 2015. Effects of water temperature change on the haematological responses and plasma cortisol levels in growing of red spotted grouper, *Epinephelus akaara*. *Dev. Reprod.*, **19**: 19-24. https://doi.org/10.12717/DR.2015.19.1.019

Chown, S.L., Hoffmann, A.A., Kristensen, T.N., Angilletta, M.J., Stenseth, N.C. and Pertoldi, C., 2010. Adapting to climate change: a perspective from evolutionary physiology. *Clim. Res.*, **43**2: 3-15. https://doi.org/10.3354/cr00879

Cowles, R.B. and Bogert, C.M., 1944. A preliminary study of the thermal requirement of desert reptiles. *Am. Mus. Nat. Hist. Bull.*, **83**: 259-296.

Das, T., Pal, A., Chakraborty, S., Manush, S., Chatterjee, N. and Mukherjee, S., 2004. Thermal tolerance and oxygen consumption of Indian Major Carps acclimated to four temperatures. *J. Therm. Biol.*, **29**: 157-163. https://doi.org/10.1016/j.jtherbio.2004.02.001

Das, T., Pal, A., Chakraborty, S., Manush, S., Sahu, N. and Mukherjee, S., 2005. Thermal tolerance, growth and oxygen consumption of *Labeo rohita* fry (Hamilton, 1822) acclimated to four temperatures. *J. Therm. Biol.*, **30**: 378-383. https://doi.org/10.1016/j.jtherbio.2005.03.001

Debnath, D., Pal, A., Sahu, N., Baruah, K., Yengkokpam, S., Das, T. and Manush, S., 2006. Thermal tolerance and metabolic activity of yellowtail catfish *Pangasius pangasius* (Hamilton) advanced fingerlings with emphasis on their culture potential. *Aquaculture*, **258**: 606-610. https://doi.org/10.1016/j.aquaculture.2006.04.037

Díaz, F., Sierra, E., Re, A.D. and Rodríguez, L., 2002. Behavioural thermoregulation and critical thermal limits of *Macrobrachium acanthurus* (Wiegman). *J. Therm. Biol.*, **27**: 423-428. https://doi.org/10.1016/S0306-4565(02)00011-6

Dülger, N., Kumlu, M., Türkmen, S., Ölçülü, A., Eroldoğan, O.T., Yılmaz, H.A. and Öçal, N., 2012. Thermal tolerance of European Sea Bass (*Dicentrarchus labrax*) juvenile acclimated to three temperature levels. *J. Therm. Biol.*, **37**: 79-82. https://doi.org/10.1016/j.jtherbio.2011.11.003

Eliason, E.J., Clark, T.D., Hague, M.J., Hanson, L.M., Gallagher, Z.S., Jeffries, K.M., Gale, M.K., Patterson, D.A., Hinch, S.G. and Farrell. A.P., 2011. Differences in thermal tolerance among sockeye salmon populations. *Science*, **332**: 109-112. https://doi.org/10.1126/science.1199158

Eme, J. and Bennett, W.A., 2009. Critical thermal tolerance polygons of tropical marine fishes from Sulawesi, Indonesia. *J. Therm. Biol.*, **34**: 220-225. https://doi.org/10.1016/j.jtherbio.2009.02.005

Feder, M.E., Bennett, A.F. and Huey, R.B., 2000. Evolutionary physiology. *Annu. Rev. Ecol. Evol. Syst.*, **31**: 315-341. https://doi.org/10.1146/annurev.ecolsys.31.1.315

Ficke, A.D., Myrick, C.A. and Hansen, L.J., 2007. Potential impacts of global climate change on freshwater fisheries. *Rev. Fish Biol. Fish.*, **17**: 581-613. https://doi.org/10.1007/s11160-007-9059-5

Graczyk, T., Conn, D., Marcogliese, D., Graczyk, H. and de Lafontaine, Y., 2003. Accumulation of human waterborne parasites by zebra mussels (*Dreissena polymorpha*) and Asian freshwater clams (*Corbicula fluminea*). *Parasitol. Res.*, **89**: 107-112. https://doi.org/10.1007/s00436-002-0729-x

Haugen, T.O. and Vøllestad, L.A., 2000. Population differences in early life history traits in grayling. *J. Evol. Biol.*, **13**: 897-905. https://doi.org/10.1046/j.1420-9101.2000.00242.x

Herrera, F.D., Uribe, E.S., Ramirez, L.F.B. and Mora, A.G., 1998. Critical thermal maxima and minima of *Macrobrachium rosenbergii* (Decapoda: *Palaemonidae*). *J. Therm. Biol.*, **23**: 381-385. https://doi.org/10.1016/S0306-4565(98)00029-1

Hirayama, M., Kobiyama, A., Kinoshita, S. and Watabe, S., 2004. The occurrence of two types of hemopexin-like protein in medaka and differences in their affinity to heme. *J. exp. Biol.*, **207**: 1387-1398. https://doi.org/10.1242/jeb.00897

Hofmann, G.E. and Todgham, A.E., 2010. Living in the now: Physiological mechanisms to tolerate a rapidly changing environment. *Annu. Rev. Physiol.*, **72**: 127-145. https://doi.org/10.1146/annurev-physiol-021909-135900

Hurst, T., 2007. Causes and consequences of winter mortality in fishes. *J. Fish Biol.*, **71**: 315-345. https://doi.org/10.1111/j.1095-8649.2007.01596.x

Ibarz, A., Fernández-Borràs, J., Blasco, J., Gallardo, M.

and Sánchez, J., 2003. Oxygen consumption and feeding rates of gilthead sea bream (*Sparus aurata*) reveal lack of acclimation to cold. *Fish Physiol. Biochem*., **29**: 313-321. https://doi.org/10.1007/s10695-004-3321-8

Imsland, A.K. and Jonassen, T.M., 2001. Regulation of growth in turbot (*Scophthalmus maximus Rafinesque*) and Atlantic halibut (*Hippoglossus hippoglossus* L.): Aspects of environment× genotype interactions. *Rev. Fish Biol. Fish*., **11**: 71-90. https://doi.org/10.1023/A:1014240430779

Imsland, A., Foss, A., Conceiçao, L.E., Dinis, M.T., Delbare, D., Schram, E. and White, P., 2003. A review of the culture potential of *Solea solea* and *S. senegalensis*. *Rev. Fish Biol. Fish*., **13**: 379-408. https://doi.org/10.1007/s11160-004-1632-6

Jawad, L., Al-Mukhtar, M. and Ahmed, H., 2004. The relationship between haematocrit and some biological parameters of the Indian shad, *Tenualosa ilisha* (Family *Clupeidae*). *Anim. Biodivers. Conserv*., **27**: 47-52.

Kavanagh, K.D., Haugen, T.O., Gregersen, F., Jernvall, J. and Vøllestad, L.A., 2010. Contemporary temperature-driven divergence in a Nordic freshwater fish under conditions commonly thought to hinder adaptation. *BMC Evol. Biol*., **10**: 350. https://doi.org/10.1186/1471-2148-10-350

McDermid, J.L., Fischer, F.A., Al-Shamlih, M., Sloan, W.N., Jones, N.E. and Wilson, C.C., 2012. Variation in acute thermal tolerance within and among hatchery strains of brook trout. *Trans. Am. Fish. Soc*., **141**: 1230-1235. https://doi.org/10.1080/00028487.2012.688917

Mora, C. and Ospina, A., 2001. Tolerance to high temperatures and potential impact of sea warming on reef fishes of Gorgona Island (tropical eastern Pacific). *Mar. Biol. Res*., **139**: 765-769. https://doi.org/10.1007/s002270100626

Myrick, C.A. and Cech J.J., 2004. Temperature effects on juvenile anadromous salmonids in California's Central Valley: What don't we know? *Rev. Fish Biol. Fish*., **14**: 113-123. https://doi.org/10.1007/s11160-004-2739-5

Nakajima, K., Yoshie-Stark, Y. and Ogushi, M., 2009. Comparison of ACE inhibitory and DPPH radical scavenging activities of fish muscle hydrolysates. *Fd. Chem*., **114**: 844-851. https://doi.org/10.1016/j.foodchem.2008.10.083

Pearson, D., Shine, R. and Williams, A., 2002. Geographic variation in sexual size dimorphism within a single snake species (*Morelia spilota*, Pythonidae). *Oecologia*, **131**: 418-426. https://doi.org/10.1007/s00442-002-0917-5

Pörtner, H.O. and Knust, R., 2007. Climate change affects marine fishes through the oxygen limitation of thermal tolerance. *Science*, **315**: 95-97. https://doi.org/10.1126/science.1135471

Pörtner, H.O., 2002. Climate variations and the physiological basis of temperature dependent biogeography: systemic to molecular hierarchy of thermal tolerance in animals. *Comp. Biochem. Physiol. A: Mol. Integr. Physiol*., **132**: 739-761. https://doi.org/10.1016/S1095-6433(02)00045-4

Re, A.D., Diaz, F., Sierra, E., Rodriguez, J. and Perez, E., 2005. Effect of salinity and temperature, on thermal tolerance of brown shrimp *Farfantepenaeus aztecus* (Ives) (Crustace: Penaeidae). *J. Therm. Biol*., **30**: 618-622. https://doi.org/10.1016/j.jtherbio.2005.09.004

Rivadeneira, M.M. and Fernández, M., 2005. Shifts in southern endpoints of distribution in rocky intertidal species along the south-eastern Pacific coast. *J. Biogeogr*., **32**: 203-209. https://doi.org/10.1111/j.1365-2699.2004.01133.x

Schulte, P.M., Healy, T.M. and Fangue, N.A., 2011. Thermal performance curves, phenotypic plasticity, and the time scales of temperature exposure. *Integr. Comp. Biol*., **51**: 691-702. https://doi.org/10.1093/icb/icr097

Seebacher, F. and Franklin, C.E., 2012. Determining environmental causes of biological effects: the need for a mechanistic physiological dimension in conservation biology. *Proc. biol. Sci*., **367**: 1607-1614. https://doi.org/10.1098/rstb.2012.0036

Seebacher, F., Holmes, S., Roosen, N.J., Nouvian, M., Wilson, R.S. and Ward, A.J., 2012. Capacity for thermal acclimation differs between populations and phylogenetic lineages within a species. *Funct. Ecol*., **26**: 1418-1428. https://doi.org/10.1111/j.1365-2435.2012.02052.x

Stitt, B.C., Burness, G., Burgomaster, K.A., Currie, S., McDermid, J.L. and Wilson, C.C., 2014. Intraspecific variation in thermal tolerance and acclimation capacity in brook trout (*Salvelinus fontinalis*): Physiological implications for climate change. *Physiol. Biochem. Zool*., **87**: 15-29. https://doi.org/10.1086/675259

Vecerek, V., Strakova, E., Suchy, P. and Voslarova, E., 2002. Influence of high environmental temperature on production and haematological and biochemical

indexes in broiler chickens. *Czech J. Anim. Sci*., **47**: 176-182.

Visser, M.E., 2008. Keeping up with a warming world; assessing the rate of adaptation to climate change. *Proc. biol. Sci*., **275**: 649-659. https://doi.org/10.1098/rspb.2007.0997

Vinagre, C., Dias, M., Roma, J., Silva, A., Madeira, D. and Diniz, M.S., 2013. Critical thermal maxima of common rocky intertidal fish and shrimps- A preliminary assessment. *J. Sea Res*., **81**: 10-12. https://doi.org/10.1016/j.seares.2013.03.011

Wilson, T.E., Klabunde, R.E. and Monahan, K.D., 2014. Using thermal stress to model aspects of disease states. *J. Therm. Biol*., **43**: 24-32. https://doi.org/10.1016/j.jtherbio.2014.03.003

Yanik, T. and Aslan, I., 2018. Impact of global warming on aquatic animals. *Pakistan J. Zool.,* **50**: 353-363.

Zhou, X., Li, M., Abbas, K. and Wang, W., 2009. Comparison of haematology and serum biochemistry of cultured and wild Dojo loach *Misgurnus anguillicaudatus*. *Fish Physiol. Biochem*., **35**: 435-441. https://doi.org/10.1007/s10695-008-9268-4

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Effect of Fat Supplementation from Different Sources on Growth and Digestibility of Fatty Acid Profile in *Labeo rohita* Fingerlings

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Authors' Contribution
SZHS conceived and designed the study and wrote the article. RI performed the experiment and collected the data. MF helped in collection and analysis of data and writing of manuscript. MA supervised the study.

Key words
Fish oil, Sunflower oil, Corn oil, Meat quality, Feed intake

ABSTRACT

Present research work was designed to determine the effect of fat supplementation from different sources on growth, digestibility of fatty acid profile and meat quality of *Labeo rohita* fingerlings. Diets were supplemented with three different fat sources. First diet served as control diet having no oil, second diet was added with fish oil, third diet was supplemented by sunflower oil and forth diet had corn oil as fat source at the level of 9%. Chromic oxide as inert marker was added at 1% concentration in experimental diets for fatty acid profile digestibility estimation. Water quality parameters *i.e.* temperature, dissolved oxygen and pH were monitored throughout the feeding trial. At the end of the trail, 10 fingerlings were collected and sacrificed to analyze the growth, digestibility of fatty acid profile and meat quality of *Labeo rohita* fingerlings. Data was subjected to one way analysis of variance (ANOVA) under completely randomized design. In *Labeo rohita* fingerlings, FCR and FI were improved and proximate composition of muscles was not affected in response to different fat sources. Digestibility of saturated and monounsaturated fatty acids elevated by supplementing sunflower oil as fat source. In conclusion, supplementation of fat from different sources effected growth, digestibility of fatty acid profile and muscles proximate composition of rohu.

INTRODUCTION

Half of the population of the world is relying on aquaculture for food; so, the main prerequisites on which the aquaculture industry is developing and flourishing include the diets that are cost effective and show a positive effect on the growth and health status of fish, so that it would be a healthy product for consumers (Hunter and Roberts, 2000). Main requirements include fish meal and fish oil in aquafeed. Because fish meal is a source of proteins and fish oil provides energy in the form of lipids and lipids are readily available and highly unsaturated essential fatty acids (Sargent and Tacon, 1999), but fish meal and fish oil have become scarce because of their extensive use and they are expensive sources of energy (Barlow and Pike, 2001).

Because of the scarcity of fish oil, the use of fish oil in aquafeed is no more an economical source of energy in the form of lipids. So it is a prerequisite of developing aquaculture to replace the fish oil with different sources of oils that are nutritionally valuable and effective (Tacon, 2004; Pike, 2005). The fish oil is substituted with many sources of oils mainly with oils having plant based origins.

Plant oils have many advantages over fish oil including cost effectiveness, higher and easily availability and improved economic value. Plant oils have high production by 100 times and are low in price than fish oil (Bimbo, 1990). Most common plant or vegetable oils that are used in compound fish feed include soybean, palm oil, linseed oil, rapeseed, sunflower and olive oil. Some vegetable oils proved to be more effective alternatives of fish oil in salmonids and freshwater fish. These vegetable oils include soybean and linseed oil (Bell *et al*., 2001; Rosenlund, 2001; Caballero *et al*., 2002). Soybean oil and rapeseed oils are rich in poly unsaturated fatty acid PUFA especially in linoleic acid and oleic acid but poorer in n-3, are considered as the best alternative sources of fish oil for salmonids and marine fish species (Caballero *et al*., 2002; Izquierdo *et al*., 2005). But in European Sea Bass (*Dicentrarchus labrax*), when fish oil was replaced with 60% rapeseed oil, it showed reduction in the growth of sea bass (Montero *et al*., 2005).

For gilthead sea bream (*Sparus aurata*) growth, soybean oil is considered as better lipid source of plant origin (E1-Kerdawy and Salama, 1997; Wassef *et al*.,

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2009). Moreover, the diet having palm oil showed the same and equal level of growth and FCR to that fish oil in atlantic salmon and rainbow trout (Caballero *et al*., 2002; Rosenlund, 2001; Torstensen *et al*., 2000). Olive oil can also be used as a partial alternate of fish oil in the feed of European sea bass (Mourente and Bell, 2005), Atlantic salmon (*Salmo salar)* (Torstensen *et al*., 2004) and rainbow trout (*Onchorhynchus mykiss*) (Caballero *et al*., 2002). This alteration of fish oil with olive oil manifested excellent results and showed the same growth rate to that fish that were fed on 100% fish oil in their diet. When the total fish oil was replaced by vegetable oils including palm oil and sunflower oils, they did not exhibit any change in growth, feed efficiency and fatty acid composition of the Atlantic salmon (Torstensen *et al*., 2000).

Fish oil can also be substituted with corn oil. Fish oil was substituted with corn oil in different proportions to check the potential of corn oil in marine species Malabar grouper (*Epinepheus malabaricus*) (Lin and Shiau, 2007). Results showed that when the FO was substituted with higher proportions of corn oil, it didn't show any significant growth in these species. But the low level substitution was even more effective than that of higher substitution level.

The supplementation of fish oil with vegetable oils can alter the fatty acid profile and even may cause the severe results for fillet quality (Martinez-Liorens *et al*., 2007). The main changes observed were the reductions in the n-3 highly poly unsaturated fatty acids especially EPA (Izquierdo*et al*., 2005; Montero *et al*., 2005).

Rohu (*Labeo rohita*) is an important fish species and is being cultured at high level in the world especially in subcontinent to meet the food requirements because it has very delicious taste to eat and also have economic value (Khan *et al*., 2004). The culture of rohu is almost 35% of the total culture of Indian major carps (FAO, 2001).

The aim of study is to investigate the effect of fat supplementation from different sources on growth, digestibility of fatty acid profile and meat quality of *Labeo rohita* fingerlings.

MATERIALS AND METHODS

The present research work was carried out to study the efficacy of citric acid and phytase in soybean meal based diet to improve trace mineral retention in rohu (*Labeo rohita*) juveniles. The experiment was conducted in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.

Fish and experimental conditions

Labeo rohita fingerlings were obtained from Government Fish Seed Hatchery, Faisalabad and fingerlings were distributed in V-shaped tanks (UA system) where the fish were acclimated to experimental conditions in laboratory for two weeks. During this period, feed was given to fingerlings to apparent satiation with the basal diet once a day. Before performing the experiment, fish were dipped in 5 g/L NaCl to prevent the fingerlings from fungal infections (Rowland and Ingram, 1991). Optimum temperature and pH were monitored during this period. The tanks were aerated to maintain the oxygen level for proper growth of fingerlings.

Feed ingredients and experimental diets

The feed ingredients were purchased from a commercial feed mill and before the formulation of the experimental diet, dietary ingredients were analyzed for chemical composition following (AOAC, 1995). The composition of experimental diets is shown in the Table I. The feed ingredients were converted into powder form before the incorporation of plant oil based diet. The proximate composition and fatty acid profile of experimental diets is given in Table II and III, respectively.

Table I.- Ingredient composition (%) of basal diet.

Ingredients	Diet 1	Diet 2	Diet 3	Diet 4
Fish meal	18	18	18	18
Corn gluten meal	20	20	20	20
Soybean meal	25	25	25	25
Rice polish	12	12	12	12
Wheat flour	20	11	11	11
Mineral mixture*	1	1	1	1
Vitamins premix**	1	1	1	1
Ascorbic acid	2	2	2	2
Chromic oxide	1	1	1	1
Fish oil	-	9	-	-
Sunflower oil	-	-	9	-
Corn oil	-	-	-	9
Total	100	100	100	100

*Each kg of mineral mixture contains; Ca (calcium), 155 gm; P (phosphorous), 135gm; Mg (magnesium), 55gm; Na (sodium), 45gm; Zn (zinc), 3000 mg; Mn (manganese), 2000 mg; Fe (iron), 1000 mg; Cu (copper), 600 mg; Co (cobalt), 40 mg; I (iodine), 40mg; Se (selenium), 3mg. **Each Kg of vitamin premix contains; Vit. A, 15 M.I.U.; Vit. B1, 5000 mg; Vit. K3, 4000 mg; Vit. B12, 9000 mcg; Vit. D3, 3 M.I.U.; Vit. E, 6000 IU; Vit. B6, 4000 mg; Vit. C, 15000 mg; Nicotinic acid, 25000 mg; Vit. B2, 6000 mg; Folic acid, 750 mg; Calcium pantothenate, 10000 mg.

Feeding procedure and collection of uneaten diet

Feed was given to *Labeo rohita* fingerlings at 2% of live wet body weight. For each experimental diet, duplicate tanks were assigned and ten fish were stocked in each tank. After the feeding session of three hours, valves

of the tanks were opened to drain out the uneaten diet from each tank. The diet particles were removed by washing the tanks thoroughly and filled with water again (Habib *et al*., 2018). After that feed conversion ratio (FCR) was calculated by drying and storing the experimental diets. Fatty acids digestibility was measured by the collection of fecal material throughout the feeding trial. The feeding trial lasted for two months.

Table II.- Chemical composition (%) of experimental diets (dry basis).

Fat sources	Diet	Moisture	Crude protein	Crude fat
No oil	D1	91.055	32.1905	10.7345
Fish oil	D2	90.695	32.595	11.3755
Sunflower oil	D3	90.94	31.919	11.329
Corn oil	D4	91.71	32.4115	10.9145

Table III.- Fatty acid composition of experimental diets (dry basis).

Fat sources	Control (D1)	Fish oil (D2)	Sunflower oil (D3)	Corn oil (D4)
14:0 n-0	12.685	6.6	3.05	6.29
16:0 n-0	27.85	20.795	8.005	34.62
18:0 n-0	9.48	6.38	15.98	14.675
16:1 n-7	6.71	7.35	2.02	1.915
18:1 n-7	ND	2.1	1.29	0.375
18:1 n-9	8.9	18.1	41.64	3.82
18:2 n-6	14.16	8.2	8.635	28.055
20:4 n-6	3.155	1.075	0	3.815
18:3 n-3	1.63	2.555	0.62	0
20:5 n-3	7.33	10.295	7.34	0
22:5 n-3	ND	1.155	0.79	0.975
22:6 n-3	8.1	15.395	10.63	5.46
Saturated	50.015	33.775	27.035	55.585
Monounsaturated	15.61	27.55	44.95	6.11
n-3	17.06	29.4	19.38	6.435
n-6	17.315	9.275	8.635	31.87
n-9	8.9	18.1	41.64	3.82
ARA/EPA	0.43	0.10	ND	ND
EPA/DHA	0.90	0.66	0.69	ND
n-3/n-6	0.98	3.17	2.24	0.20
Monoenes/ polyenes	0.36	0.48	0.64	0.14

Fish oil with different sources of oils were added to formulate four experimental diets. Diets containing different oils *i.e.* canola oil, fish oil and sunflower oil was given to *Labeo rohita* fingerlings. First diet contained no oil, designated as control diet, second diet had 9% fish oil, third diet contained 9% sunflower and forth diet supplemented with 9% corn oil as fat source. Three group of replicates were assigned for each experimental diet and 25 fingerlings were distributed to each tank. The experiment was completed in two months.

Proximate analysis of muscles

The standard methods of AOAC (1995) were used to analyze the proximate composition of muscles; moisture was determined by oven-drying at 105°C for 12 h, crude protein (N x 6.25) by micro Kjeldahl apparatus, crude fat by petroleum ether extraction method through Soxtec HT2 1045 system, crude fiber as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH, ash by ignition at 650°C (Eyela-TMF 3100) to constant weight, carbohydrates; by the subtracting the values of crude protein (%), crude fat (%), crude fibre (%) from dry matter.

Study of growth performance

After every week, growth performance was checked by taking gross weight of fish from each treatment. Growth and feed performance and feed were estimated in terms of absolute weight gain (WG), weight gain (%), feed conversion ratio (FCR), specific growth rate (SGR) and survival rate (%).

$$\text{Absolute weight gain (g)} = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{Weight gain \%} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{SGR} = \ln \frac{\text{Initial weight (g)} - \text{Final weight (g)}}{\text{Experimental duration in days}} \times 100$$

$$\text{Survival \%} = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

$$\text{FCR} = \frac{\text{Total dry feed intake (g)}}{\text{Wet weight gain (g)}}$$

Determination of fatty acid profile

Fatty acid profile was determined by taking above extracted fat (as described earlier). IUPAC (1987) standard method was used to estimate the Fatty acid profile in fish feces. Fatty acid profile in feces was determined from the fatty acid methyl esters (FAME) derivatives of the trans-esterified lipid. Methanol was used to prepare FAMEs.

Statistical analysis

Growth performance was measured to determine the optimum level of fatty acid requirements. One-way

analysis of variance (ANOVA) was applied on statistical analysis of data (Steel *et al.*, 1996). Student-Newman-Kuels Test was used to compare the differences among means and considered significant at $p<0.05$ (Snedecor and Cochran, 1991).

RESULTS

The effect of different fat sources on growth performance of Nile tilapia is given in Table IV. Supplementation of different fat sources exhibited significant effects on feed intake and feed conversion ratio in *Labeo rohita* fingerlings.

The effect of different fat sources on proximate composition (%) of experimental diets is given in Table V. Supplementing fat from different sources, crude fat, crude proteins and crude ash contents were not affected significantly while moisture contents showed significant effect in *Labeo rohita* fingerlings.

Table IV.- Effect of different fat sources on growth performance of Nile tilapia.

Fat sources	Control (D1)	Fish oil (D2)	Sunflower oil (D3)	Corn oil (D4)	PSE	P-value
Final weight (g)	10.46	14.16	12.71	11.95	0.65	0.0651ns
Absolute weight gain	7.21	10.9	9.47	8.7	0.65	0.0664ns
Weight gain (%)	222.35	334.5	292.38	267.8	20.4	0.0709ns
SGR	1.95	2.44	2.27	2.16	0.08	0.0600ns
FI (g)	14.73a	12.07b	12.64b	12.36b	0.17	0.0014**
FCR	2.04a	1.11b	1.35b	1.42b	0.1	0.0116*
Survival rate (%)	97.5	100	95	100	2.79	0.5841ns

Table V.- Effect of different fat sources on proximate composition (%) of experimental diets.

Fat sources	Control (D1)	Fish oil (D2)	Sunflower oil (D3)	Corn oil (D4)	PSE	P-value
Dry matter	77.04a	75.60b	75.59b	75.56b	0.17	0.102
Crude protein	17.6	17.75	17.69	17.72	0.1	0.7583ns
Crude fat	3.79b	4.38a	4.56a	4.56a	0.11	0.267ns
Crude ash	1.43	1.36	1.5	1.36	0.02	0.0527ns

Table VI.- Effect of different fat sources on fatty acid profile of Nile tilapia.

Fat sources	Control (D1)	Fish oil (D2)	Sunflower oil (D3)	Corn oil (D4)	PSE	P-value
14:0 n-0	92.89	93.54	93.08	93.06	0.4	0.7096ns
16:0 n-0	85.94b	86.68b	90.25a	85.1b	0.45	0.0048**
18:0 n-0	81.09	81.16	84.08	83.11	0.53	0.0388*
16:1 n-7	89.05b	88.55b	92.6a	93.15b	0.37	0.0019**
18:1 n-7	ND	98.69a	98.54a	95.48b	0.18	0.0020**
18:1 n-9	96.23b	98.19a	98.5a	96.44b	0.36	0.0238*
18:2 n-6	97.09	97.75	97.08	98.3	0.39	0.2241ns
20:4 n-6	97.55	97.1	ND	96.87	0.46	0.6260ns
18:3 n-3	98.58	97.87	99.24	ND	0.31	0.1229ns
20:5 n-3	98.03	99.14	98.44	ND	0.29	0.1612ns
22:5 n-3	ND	96.89	97.12	97.56	0.37	0.5155ns
22:6 n-3	97.98	98.66	98.08	98.18	0.42	0.7016ns
Saturated	86.64b	87.13b	89.13a	87.09b	0.28	0.0122*
Monounsaturated	92.64c	95.14b	96.55a	95.02b	0.17	0.0005***
n-3	98.2	98.14	98.22	97.87	0.16	0.4968ns
n-6	97.32	97.42	97.08	97.58	0.31	0.7348ns
n-9	96.23b	98.19a	98.5a	96.44b	0.36	0.0238
ARA/EPA	0.99	0.97	ND	ND	0.006	0.2532ns
EPA/DHA	1	1	1	ND	0.005	0.8484ns
n-3/n-6	1.02	0.99	0.99	1.01	0.004	0.0382*
Monoenes/polyenes	0.31c	0.32a	0.32b	0.32b	0	0.0016**

The effect of different fat sources on fatty acid profile of Nile tilapia is given in Table VI. Fat supplementation from different sources showed significantly increasing effect on digestibility of saturated, unsaturated and polyunsaturated fatty acids.

DISCUSSION

The current experiment was executed to study the effect of different sources of fat supplementation on *Labeo rohita* fingerlings. The main areas of concern about research were proximate composition, growth and fatty acids digestibility.

The result of present study demonstrated that fat supplementation from different sources did not affect the growth of *Labeo rohita* fingerlings. Specifically, significant ($P<0.05$) effect was observed on feed intake and feed conversion ratio while non-significant effect was observed on final weight, weight gain, absolute weight gain, significant growth rate and survival rate of *Labeo rohita*. In contrast to present study, Cho and Kaushik (1990) observed significantly increasing trend in feed conversion ratio and feed intake by giving sunflower oil and soybean oil as different sources of fat in trout. Similar to our work, Bell *et al*. (2001) also observed decreased feed intake and feed conversion ratio while a non-significant effect on final weight, absolute weight and significant growth rate when Atlantic salmon was fed with soybean oil and sunflower oil. Growth and survival of European sea bass (Mourente *et al*., 2005) was not affected on supplementation of linseed oil, rapseed oil and olive oil.

The present study exhibited that supplementation of fat from different sources had non-significant effect on muscle proximate composition of *Labeo rohita* fingerlings. Specifically, significant ($P<0.05$) effect on moisture contents while non-significant effect on crude fat, crude protein and crude ash contents was recorded. In contrast to our study, Bell *et al*. (2001) reported that vegetable based oils supplementation showed significant ($P<0.05$) trends in moisture, crude fat, crude protein and crude ash contents of *Salmo salar*. Fountoulaki *et al*. (2009) also observed that supplementation of fat from different sources affected the muscles proximate non-significantly in gilthead sea bream.

In the present study results manifested non-significant variations in the digestibility of fatty acid profile in *Labeo rohita* fingerlings by supplementing fat from different sources. However, 16:1 n-7, 18:1 n-7, 18:1 n-9, saturated, monounsaturated fatty acids, n-9, n-3/n-6 and monoenes/polyenes were affected significantly. Mourente *et al*. (2005) reported contrary results to our study when European sea bass showed a significant increase of decosahexanoic acid, eicosapentanoic acid, linolenic acid, linoleic acid and oleic acid in muscles on fat supplementation from different sources. Moreover, Bell *et al*. (2001) observed significant effects on digestibility of decosahexanoic acid, eicosapentanoic acid and n-3/n-6 in Atlantic salmon by giving vegetable based fat sources. Similar to our work, Piedecausa *et al*. (2006) also reported non-significant effects on DHA, EPA and ARA in sharpsnout seabream by giving vegetable based fat sources.

CONCLUSION

Thus it is concluded, growth and muscle proximate composition were not affected significantly ($P>0.05$) in *Labeo rohita* fingerlings fed with fat from different sources also results showed non-significant variations in digestibility of fatty acid profile in *Labeo rohita* fingerlings by supplementation of fats from different sources.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Anwar, F., Hussain, A.I., Ashraf, M., Jamil, A. and Iqbal, S., 2006. Effect of salinity on yield and quality of *Moringa oleifera* seed oil. *Grasas Yaceites,* **57**: 349-401. https://doi.org/10.3989/gya.2006.v57.i4.65

AOA, 1995. *Official methods of analysis*, 15th Ed. Association of Official Analytical Chemist, Washington, DC, USA, pp. 1094.

Barlow, S.M. and Pike, I.H., 2001. *Sustainability of fish meal and oil supply*. Paper presented at the Scottish Norwegian Marine Fish Farming Conference, Sustainable Future for Marine Fish Farming, University of Stirling, Stirling, Scotland, June 14–15, 2001.

Barrington, K., Chopin, T. and Robinson, S., 2009. Integrated multi-trophic aquaculture (IMTA) in marine temperate waters. In: *Integrated mariculture: A global review* (ed. D. Soto). FAO Fisheries and Aquaculture, Rome, Italy, pp. 7-46.

Bell, J.G., McEvoy, J., Tocher, D.R., McGhee, F., Campbell, P.J. and Sargent, J.R., 2001. Replacement of fish oil with rapeseed oil in diets of Atlantic salmon (*Salmo salar*) affects tissue lipid composition and hepatocyte fatty acid metabolism. *J. Nutr.,* **131**: 1535-1543. https://doi.org/10.1093/jn/131.5.1535

Bimbo, A.P., 1990. Production of fish oil. In: *Fish oils nutrition* (ed. M.E. Stansby). Van Nostrand Reinhold, New York, pp. 141-180.

Bligh, E.G. and Dyer, W.J., 1959. A rapid method of total fat extraction and purification. *Can. J. Biochem. Physiol.,* **37**: 911-917. https://doi.org/10.1139/y59-099

Caballero, M.J., Obach, A., Rosenlund, G., Montero, D., Gisvold, M. and Izquierdo, M.S., 2002. Impact of different dietary lipid sources on growth, lipid digestibility, tissue fatty acid composition and histology of rainbow trout (*Oncorhynchus mykiss)*. *Aquaculture,* **214**: 253-271. https://doi.org/10.1016/S0044-8486(01)00852-3

Cho, C.Y. and Kaushik, S.J., 1990. Nutritional energetics in fish: Energy and protein utilization in rainbow trout (*Salmo gairdneri*). *World Rev. Nutr. Diet,* **61**: 132-172. https://doi.org/10.1159/000417529

El-Kerdawy, A. and Salama, A., 1997. Effect of dietary lipid sources on the growth and fatty acid composition of gilthead bream (*Sparus aurata*) feeding tomorrow's fish. *Cah. Options Mediterr.,* **22**: 235-241.

FAO, 2001. *Fishery statistics*. Food and Agriculture Organization, Rome, Italy, pp. 92.

Fountoulaki, E., Vasilaki, A., Hurtado, R., Grigorakis, K., Karacostas, I., Nengas, I., Rigos, G., Kotzamanis, Y., Venou, B. and Alexis, M.N., 2009. Fish oil substitution by vegetable oils in commercial diets for gilthead sea bream (*Sparus aurata* L.); effects on growth performance, flesh quality and fillet fatty acid profile: recovery of fatty acid profiles by a fish oil finishing diet under fluctuating water temperatures. *Aquaculture,* 289: 317-326. https://doi.org/10.1016/j.aquaculture.2009.01.023

Goldburg R.J., Elliot, M.S. and Naylor, R.L., 2001. *Marine aquaculture in the United States: Environmental impacts and policy options*. Pew Oceans Commission, Arlington.

Habib, R.Z., Afzal, M., Shah, S.Z.H., Fatima, M., Bilal, M. and Hussain, S.M. 2018. Potential of phytase and citric acid treated canola meal based diet to enhance the minerals digestibility in *Labeo rohita* fingerlings. *Pakistan J. Zool.,* **50**: 2045-2050. http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2045.2050

Hunter, B.J. and Roberts, D.C.K., 2000. Potential impact of the fat composition of farmed fish on human health. *Nutr. Res.,* **20**: 1047-1058. https://doi.org/10.1016/S0271-5317(00)00181-0

IUPAC, 1987. *Standard methods for the analysis of oils, fats and derivatives*, 7th rev (eds. C. Paquot and A. Hautfenne). International Union of Pure and Applied Chemistry, Blackwell Scientific, London.

Izquierdo, M.S., Montero, D., Robaina, L., Caballero, M.J., Rosenlund, G. and Ginés, R., 2005. Alterations in fillet fatty acid profile and flesh quality in gilthead seabream (*Sparus aurata*) fed vegetable oils for a long term period. Recovery of fatty acid profiles by fish oil feeding. *Aquaculture,* **250**: 431-444. https://doi.org/10.1016/j.aquaculture.2004.12.001

Khan, M.A., Jafri, A.K. and Chadha, N.K., 2004. Growth and body composition of rohu (*Labeo rohita)* (Hamilton), fed compound diet: winter feeding and rearing to marketable size. *J. appl. Ichthyol.,* **20**: 265-270. https://doi.org/10.1111/j.1439-0426.2004.00550.x

Lee, B.L., New, A.L. and Ong, C.N., 2003. Simultaneous determination of tocotrienols, tocopherol, retinol and major carotenoids in human plasma. *Clin. Chem.,* **49**: 2056-2066. https://doi.org/10.1373/clinchem.2003.022681

Lin, Y.H., and Shiau, S.Y., 2007. The effects of dietary selenium on the oxidative stress of grouper, *Epinephelus malabaricus*, fed high copper. *Aquaculture,* 267: 38-43. https://doi.org/10.1016/j.aquaculture.2006.12.015

Martinez-Llorens, S., Tomas, A., Andris, V., Morino, V., Torres, M.P. and Cerda, J.M., 2007. Effects of dietary soybean oil concentration on growth, nutrient utilization and muscle fatty acid composition of gilthead sea bream (*Sparus aurata* L.). *Aquacult. Res.,* **38**: 76-81. https://doi.org/10.1111/j.1365-2109.2006.01636.x

Montero, D., Robaina, L., Caballero, M.J., Ginés, R. and Izquierdo, M.S., 2005. Growth, feed utilization and flesh quality of European sea bass (*Dicentrarchus labrax*) fed diets containing vegetable oils: A time-course study on the effect of are-feeding period with a 100% fish oil diet. *Aquaculture,* **248**: 121-134. https://doi.org/10.1016/j.aquaculture.2005.03.003

Mourente, G. and Bell, J.G., 2006. Partial replacement of dietary fish oil with blends of vegetable oils (rapeseed, linseed and palm oils) in diets for European sea bass (*Dicentrarchus labrax* L.) over a long term growth study: effects on muscle and liver fatty acid composition and effectiveness of a fish oil finishing diet. *Comp. Biochem. Physiol.,* **145**: 389-399. https://doi.org/10.1016/j.cbpb.2006.08.012

Mourente, G., Good, J.E. and Bell, J.G., 2005. Partial substitution of fish oil with rapeseed, linseed and olive oils in diets for European sea bass

(*Dicentrarchus labrax* L.): Effects on flesh fatty acid composition, plasma prostaglandins E2 and E2a immune function and effectiveness of a fish oil finishing diet. *Aquacult. Nutr.,* **11**: 25-40. https://doi.org/10.1111/j.1365-2095.2004.00320.x

NRC, 1993. *Nutrient requirements of fish*. National Academy Press, National Research Council, Washington DC, USA, pp. 114.

Piedecausa, M.A., Mazon, M.J., Garcia, B.G. and Hernandez, M.D., 2006. Effects of total replacement of fish oil by vegetable oils in the diets of sharp snout seabream (*Diplodus puntazzo*). *Aquaculture,* **263**: 211-219. https://doi.org/10.1016/j.aquaculture.2006.09.039

Pike, I., 2005. Eco-efficiency in aquaculture: global catch of wild fish used in aquaculture. *Int. Aquafeed*, **8**: 38-40.

Robbins, K.R., Norton, H.W. and Baker, D.H., 1979. Estimation of nutrient requirement from growth data. *J. Nutr.,* **109**: 1710-1714. https://doi.org/10.1093/jn/109.10.1710

Rosenlund, G., 2001. Effect of alternative lipid sources on long-term growth performance and quality of Atlantic salmon (*Salmo salar* L.). *Aquacult. Res.,* **32**: 323-328. https://doi.org/10.1046/j.1355-557x.2001.00025.x

Rowland, S.J. and Ingram, B.A., 1991. Diseases of Australian native fishes. In: *Fish. Bull*. 4-NSW Fisheries, Sydney, NSW, Australia.

Sargent, J.R. and Tacon, A.G.J., 1999. Development of farmed fish: a nutritionally necessary alternative to meat. *Proc. Nutr. Soc.,* **58**: 377-383. https://doi.org/10.1017/S0029665199001366

Snedecor, G.W. and Conhran, W.G., 1991. *Statistical methods*, 8th ed. Iowa State University Press, Amsterdam, USA, pp. 503.

Steel, R.G.D., Torrie, J.H. and Dickey, D.A., 1996. *Principles and procedures of statistics*, 3rd ed. McGraw Hill International Book Co. Inc., New York, USA, pp. 336-352.

Tacon, A.G.J., 2004. Use of fish meal and fish oil in aquaculture. *Aquacult. Resour. Cult. Dev.,* **1**: 3-14.

Torstensen, B.E., Frøyland, L., Ørnsrud, R. and Lie, O., 2004. Tailoring of a cardio-protective muscle fatty acid composition of Atlantic salmon *(Salmo salar)* fed vegetable oils. *Fd. Chem.,* **87**: 567-580. https://doi.org/10.1016/j.foodchem.2004.01.009

Torstensen, B.E., Øyvind, L. and Frøyland, L., 2000. Lipid metabolism and tissue composition in Atlantic salmon *(Salmo salar)* effects of capelin oil, palm oil, and oleic acid-enriched sunflower oil as dietary lipid sources. *Lipids,* **35**: 653-664. https://doi.org/10.1007/s11745-000-0570-6

Wassef, E.A., Saleh, N.E. and El-Hady, H.A.E., 2009. Vegetable oil blend as alternative lipid resources in diets for gilthead seabream (*Sparus aurata)*. *Aquacult. Int.,* **17**: 421-435. https://doi.org/10.1007/s10499-008-9213-7

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Evaluation of Zinc Bioavailability from Organic and Inorganic Sources in Practical Diet for *Labeo rohita* Juveniles

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Authors' Contribution
SZHS conceived and designed the study and wrote the article. UA performed the experiment and collected the data. MF helped in collection and analysis of data and writing of manuscript. MA supervised the study.

Key words
Zn-gluconate, Inorganic Zn, Growth, Proximate composition, TBARS, Alkaline Phosphatase

ABSTRACT

Present study was designed to evaluate the Zinc (Zn) bioavailability from organic (Zn-gluconate) and inorganic sources ($ZnCl_2$, ZnO and $ZnSO_4$) in practical diet of *Labeo rohita* juveniles. Eight experimental diets were formulated by supplementing the basal diet with two different levels of Zn (25 mg kg^{-1} and 50 mg kg^{-1}) from both sources (organic and inorganic). Three replicates were allocated to each experimental diet and each tank was stocked with 18 juveniles. The water quality parameters including pH, temperature and dissolved oxygen (DO) were monitored and kept constant throughout the feeding experiment. The feeding experiment was lasted for 3 months. Results showed that growth performance was not affected by different Zn sources. Moreover, muscle proximate composition also remain unaffected by the quality of dietary Zn. Supplementation of Zn from different sources significantly ($p<0.05$) affected the Zn contents in bones and scales of juviniles. Maximum Zn deposition in these tissues was recorded in fish fed with diet containing Zn-gluconate at the level of 50 mg/kg. Thiobarbituric acid reactive substances (TBARS) contents in kidney and spleen were remain unaffected by different Zn sources. However, alkaline phosphatase (ALP) activity in kidneys was significantly higher in fish group fed Zn-gluconate (50 mg kg^{-1} Zn) compared to other Zn sources. In conclusion, Zn from organic source showed higher bioavailability compared to other inorganic sources in practical diets of *Labeo rohita* juveniles.

INTRODUCTION

Animals require Zn as an essential micronutrient in their biochemical processes. It has been reported that the excessive or limited amount of Zn has adverse effect on growth, biochemical reactions and skeleton of fish (Watanabe *et al*., 1997). The deficiency of Zn has detrimental consequence on red blood cells production in bone marrow (Hughes *et al*., 2006) and decreases the production of B and T lymphocytes (Shils *et al*., 1997; Haddad *et al*., 2008). Moreover, its deficiency causes the limited growth rate, low content of Zn in serum, psychological disorder like anorexia and poor storage of Zn and calcium in the bones of channel catfish (National Research Council, 1993).

In fresh and seawater, normal levels of Zn are found to be limited to fulfill the demand of Zn in growing aquatic species (Spry *et al*., 1988; Willis and Sunda, 1984). Hence, Zn is considered as an essential micronutrient in fish diet (Lall, 1989; NRC, 1993; Wei *et al*., 1999).

The nutritional requirement of Zn in rainbow trout (*Oncorhynchus mykiss*) is 15-30 mg kg^{-1} diet (Ogino and Yang, 1978) and in Atlantic salmon (*Salmo salar*) is 37-57 mg kg^{-1} diet (Maage and Julshamn, 1993; Maage *et al*., 1993). In channel catfish, the requirement of Zn is estimated as 20 mg kg^{-1} in purified diets (Gatlin and Wilson, 1983).

Fishmeal and plant protein sources are being used in fish feed as protein sources. Tricalcium phosphate and phytate are two main antinutritional factors present in fishmeal and plant protein sources (Apines *et al*., 2003), that reduces the bioavailability of mineral (Hardy and Shearer, 1985; Richardson *et al*., 1985; Satoh *et al*., 1987a, b, c; Lovell, 1989; Francis *et al*., 2001). It was found that increasing level of phytic acid significantly decreased the level of Zn in vertebral column (Satoh *et al*., 1989). Thus, diets with the rising level of inhibitors increases the demands of fish for minerals. Chemical nature of supplemental Zn source is one of the factors that affect the bioavailability of Zn (Solomons, 1993) along with diet and protein source.

Chelates and other complexes are less sensitive to the mineral inhibitors (phytate and tricalcium phosphate) and compete with these inhibitors present in diet thus, increases

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the availability of mineral to the animals in experimental diets in contrast to inorganic forms of Zn (Ashmead, 1992; Garcia-Aranda *et al*., 1983). As compare to the inorganic salts, lower molecular weights and structural stability of chelates and complexes increase the absorbance of Zn in the intestine of animals (Ashmead, 1992; Davis and Gatlin, 1996). Hence, diet formulation should be carried out with the supplementation of Zn from those sources which have higher rate of bioavailability.

Organic minerals are good source of micro minerals, because they enhance the transport of trace elements in the intestinal mucosa by inhibiting the formation of insoluble complexes (such as with tricalcium phosphate and phytate) in the gastro-intestinal tract (Ashmead, 1993). In animal's intestine, amino-acid-chelated Zn has higher rate of absorption than inorganic Zn source which include Zn oxide, Zn chloride, Zn sulfate and Zn carbonate (Ashmead, 1992).

In channel catfish, relative bioavailability of ZnMet increases as compare to $ZnSO_4$ by 500% based on the concentration of Zn in bones (Paripatananont and Lovell, 1995). Bioavailability of Zn from organic chelate such as Zn methionine (ZnMet) was higher than that of inorganic salt like Zn sulfate ($ZnSO_4$) (Wedekind *et al*., 1992). Moreover, higher bioavailability of Zn from organic sources compare to inorganic sources was also observed in channel catfish (Paripatananont and Lovell, 1995), *Haliotis discus hannai* (abalone) (Tan and Mai, 2001), rainbow trout (Apines *et al*., 2001) and in the terrestrial vertebrates (Ma *et al*., 2014).

The aim of this study was to evaluate the Zn bioavailability from organic and inorganic sources in practical diets for *Labeo rohita* juveniles.

MATERIALS AND MATHODS

The present research work was carried out to evaluate the bioavailability of Zn from organic (Zn-gluconate) and inorganic ($ZnCl_2$, ZnO and $ZnSo_4$) sources in practical diet for *Labeo rohita* juveniles. The research was carried out in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.

Feed ingredients and experimental diets

Eight experimental diets were formulated by supplementating organic and inorganic sources of Zn in the basal practical diet at the levels of (25 mg/kg and 50 mg/kg). Prior to the formation practical diet, feed contituents were purchased from feed mill and their chemical composition was evaluated following (AOAC, 1995). A cereal oppressive machine (FFC-45, JIMO, China) was used for sieving (0.05 mm) and grinding of feed ingredients. Mixing of feed ingredients like chromic oxide, vitamin premix, fish oil and Zn free mineral mixture was done in an electrical mixture. The components of Zn free mineral mixture is presented in Table I. Dough was prepared by adition of 150 ml of distilled water in 1 kg diet. Floating pellets (3 mm) were formed by hand pelletizer. Approximately 10% moisture is maintained in pellets by evaporating water contents in moist pellets with an electrical fan. Pellets were ground, sieved to suitable sizes and stored at -20°C in self-sealing plastic bags. Proximate composition of experimental is shown in Table I.

Fish husbandry

Labeo rohita juveniles were purchased from Govt. Fish Seed Hatchery, Faisalabad. Fish were treated with 5 g/L NaCl to avoid fungal infections and to obtain juveniles free from ectoparasites upon arrival in laboratory. Cemented tanks (1000 L) were used to acclimatize the fish for two weeks. During acclamation, fish were fed on basal diet once a day to apparent satiation (Allan and Rowland, 1992). At the beginning of experiment, eighteen fish were kept in V-shaped tanks. Fish were fed twice a day to apparent satiation, 6 days in a week for 3 months. Uneaten diet was removed out of the tank by opening of valves after feeding period of three hours (Habib *et al*., 2018).

Table I.- Proximate energy and zinc composition of experimental diets.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$	
Zinc level (mg/kg)	25	50	25	50	25	50	25	50
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8
Dry matter (%)	90.97	90.36	90.72	92.51	90.51	90.61	90.11	89.8
Crude protein (%)	32.81	32.45	32.45	33.18	33.54	32.45	33.18	33.18
Crude fat (%)	6.33	6.28	6.36	6.34	6.37	6.47	6.4	6.51
Ash (%)	10.24	11.77	10.69	11.95	10.68	12.41	10.43	12.14
Gross energy (kcal/kg)	4104.4	4135	4153.2	4139.2	4120	4136.4	4107.3	4091.6
Zinc (ug/g)	62.21	92.07	64.06	92.32	62.08	91.2	63.57	90.95

The data are mean of three replicates.s

Particles of diet were removed out by complete washing of tanks and fish were refilled in experimental tanks. Water quality variables specifically dissolved oxygen (DO), temperature and pH were observed by the usage of DO meter (Model 970), hermomete and Jenway pH meter (Model 3510) respectively throughout the experiment. Capillary system was used to provide aeration to the tanks round the clock.

Sample collection

Fish were sedated by using MS-22 and slayed at the end of 90 days feeding experiment. For whole body proximate analysis, four fish were crushed and oven dried at at 60°C. Moreover, bones, kidney, spleen and scales, heart, skin, liver, eyes, intestine and muscle sample were obtained by sacrificing ten more fishes. These samples were seprately pooled and refrigrated at -20°C.

Proximate analysis of whole body

Whole body samples were homogenized with the help of pestle and morter and different chemical analysis *viz*. ash, crude fat, crude protein and moisture contents were determined by following AOAC (1995) methods. Samples were oven dried at 105°C for 12 h to estimate the moisture contents in muscle tissues. Crude protein was determined by Kjeldahl apparatus, crude fat by soxtec HT2 1045 system following ether extraction method (Bligh and Dyer, 1959). Ash was determined by heating at 65°C for 12 h in an electric furnace (Eyela-TMF 3100) until constant weight was obtained.

Absorption of Zn

After 2 h intervals, feces were collected from each tank through fecal collection pipe. Nutrients leaching was reduced by avoiding the break down of thin fecal strings. Fecal matter was oven dried at 60ºC, finally ground and stored for analysis of Zn contents.

Zn absorption was measured by following formula:

$$\text{Absorption (\%)} = 100 - 100 \times \frac{(\text{\% marker in diet} \times \text{\% nutrient in feces})}{(\text{\% marker in feces} \times \text{\% nutrient in diet})}$$

Growth performance

Growth (as weight) was measured at the initiation and on weekly basis and at the end of feeding trial. Weight gain, weight gain%, Feed conversion ratio (FCR) and Specific growth rate (SGR) were calculated by standards formulae:

$$\text{Weight gain} = \text{Final weight gain (g)} - \text{Initial weight gain (g)}$$

$$\text{Weight gain \%} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}}$$

$$\text{SGR} = \frac{\text{In [Initial weight (g)} - \text{Final weight (g)]}}{\text{No. of days}} \times 100$$

Mineral analysis

After acid digestion, Zn contents in heart, kidney, spleen, eyes, whole body, muscles, skin bones and scales, liver, intestine and feces were determined through atomic absorption spectrophotometer. Wet digestion of these samples was carried out by using nitric acid and perchloric acid in ratio 3:1. Dilution of digested sample was done up to the applicable volume.

Retention of Zn was checked by the formula:

$$\text{Retention (\%)} = \frac{\text{Final nutrient content} - \text{Initial nutrient content}}{\text{Nutrient intake}} \times 100$$

Excretion of Zn was checked by the formula:

$$\text{Excretion} \left(\frac{\text{kg}}{\text{t}}\text{production}\right) = \frac{[\text{FCR} \times \text{Nutrient in diet (kg)} - \text{Nutrient retained in fish (kg)}]}{\text{Production (kg)}} \times 1000$$

Where, FCR is feed conversion ratio.

TBARS assay

The thiobarbituric acid reactive substances (TBARS) in liver, intestine, kidney, muscles, spleen and heart tissues were specifically analysed by following Gatta *et al*. (2000).

Alkaline phosphatase activity

The 1g sample of liver, intestine, spleen, kidney, heart and muscles was homogenized in ice-cold 0.25M sucrose solution, homogenates were kept frozen overnight followed by centrifugation at 5000 ×g for 15 min in a cooling centrifuge (5°C), supernatant collected and stored at -20 °C until analyzed for alkaline phosphatase enzyme (Yakubu *et al*., 2005). The ALP activity was determined by following Vitro Scient (ISO 13485) kit method. The reaction mixture having 20 µL enzyme extract and 1000 µL working solution was mixed and OD was measured at the wavelength of 405 nm. Same procedure was used to prepare a reagent blank (having no enzyme extract).

The activity of alkaline phosphatase enzyme was determined by using following formula:

$$\frac{U}{L} = \frac{\Delta A/\text{min} \times TV \times 1000}{\Sigma \times \text{SV} \times \text{LP}}$$

Statistical analysis

Finally, the data was subjected to two-way analysis of variance for statistical analysis (Steel *et al*., 1996). The differences among means were compared by Tukey`s Honestly Significant Difference Test and considered significant at p<0.05 (Snedecor and Conhran, 1991).

RESULTS AND DISCUSSION

Chelation increases the bioavailability of Zn to

channel catfish (Paripatananont and Lovell, 1995).

In the present study, Zn supplementation, either through different sources or at different levels, did not affect the growth performance of fish. The chemical form of element seems to have no influence on the growth performance of fish (Table II). The non-significant effect of Zn sources and Zn levels on growth performance may owe to the fact that Zn is a micro mineral and is required in a very small quantity, which in the present study, was provided by the feed ingredients. In accordance with our results, supplementation of chelated Zn showed non-significant effect on growth performance and feed efficiency of channel catfish (*Ictalurus punctatus*) (Li and Robinson, 1996), rainbow trout (Apines *et al*., 2001), Atlantic salmon (Maage *et al*., 2001), Nile tilapia (Sa *et al*., 2005) and turbot (*Scophthalmus maximus*) (Ma *et al*., 2014). Contrary to these reports, Shimei *et al*. (2011) observed enhanced growth and feed performance of Nile tilapia by supplementation of organic trace elements. Moreover, enhanced growth performance was also observed in red sea bream, when fed with chelated mineral compared to sulfate salts (Sarker *et al*., 2005). A similar increase in growth performance was observed in rainbow trout, *Oncorhynchus mykiss* (Satoh *et al*., 2001) and Japanese sea bass, *Lateolabrax japonicas* (Wang *et al*., 2015) by feeding the diets supplemented with chelated minerals. The discrepancies in the reported results might had occurred due to the differences in the nature of fish feed as Zn supplementation in practical diets showed no effect on growth performance, while, it improved the growth performance of fish in purified diets.

In the current experiment, organically chelated Zn showed non-significant effect on body proximate composition of *L. rohita,* either from different Zn sources or at its different levels except ash contents which were higher from organic source (Table III). Higher ash contents in fish body may attribute to higher bioavailability of Zn from organic source. In line with our study, no significant difference was observed in proximate composition of turbot, *Scophthalmus maximus* (Ma *et al*., 2014) and cobia, *Rachycentron canadum* (Qiao *et al*., 2013) by feeding diets supplemented with chelated minerals and inorganic mineral salts. However, in contrast to our study Zn supplementation from sulfate source showed higher whole body crude lipid content in turbot compared to that fed on Zn-methionine (Ma *et al*., 2014).

Table II.- Effect of Zinc sources and Zinc levels on growth of *L. rohita* fingerlings.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$		PSE	P-value		
Zinc level (mg/kg)	25	50	25	50	25	50	25	50		Zn source	Zn level	Zn source × Zn level
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8				
Initial weight (g)	3.46	3.52	3.52	3.61	3.67	3.59	3.58	3.63				
Final weight (g)	14.16	14.25	13.9	14.39	14.51	14.24	14.3	14.41	0.26	$p > 0.05$	$p > 0.05$	$p > 0.05$
Absolute weight gain	10.69	10.73	10.38	10.78	10.84	10.64	10.72	10.78	0.19	$p > 0.05$	$p > 0.05$	$p > 0.05$
Weight gain %	309.01	304.98	295.07	298.64	295.69	296.46	300.01	297.17	5.68	$p > 0.05$	$p > 0.05$	$p > 0.05$
SGR	2.35	2.33	2.29	2.3	2.29	2.3	2.31	2.3	0.02	$p > 0.05$	$p > 0.05$	$p > 0.05$
FI (g)	13.03	12.9	12.17	12.23	13.22	12	12.4	12.17	0.35	$p > 0.05$	$p > 0.05$	$p > 0.05$
FCR	1.22	1.2	1.17	1.14	1.22	1.13	1.16	1.13	0.03	$p > 0.05$	$p > 0.05$	$p > 0.05$
Survival rate %	98.15	100	96.3	100	96.3	100	96.3	94.44	1.73	$p > 0.05$	$p > 0.05$	$p > 0.05$

The data are mean of three replicates. Mean values sharing different superscript letters within a row are significantly different (p<0.05). PSE, pooled standard error = √MSE/n (where MSE is mean-squared error).

Table III.- Effect of Zinc sources and Zinc levels on body proximate composition of *L. rohita* fingerlings.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$		PSE	P-value		
Zinc level (mg/kg)	25	50	25	50	25	50	25	50		Zn source	Zn level	Zn source × Zn level
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8				
Moisture (%)	71.95	71.92	72.13	71.91	72.09	71.88	72.13	71.52	0.23	$p > 0.05$	$p > 0.05$	$p > 0.05$
Crude protein (%)	20.42	19.32	19.69	20.05	19.69	20.05	19.69	20.42	0.58	$p > 0.05$	$p > 0.05$	$p > 0.05$
Crude fat (%)	5.53	5.67	5.59	5.48	5.75	5.8	5.73	5.63	0.08	$p > 0.05$	$p > 0.05$	$p > 0.05$
Ash (%)	2.66^b	2.87^a	2.65^b	2.86^a	2.47^c	2.65^b	2.46^c	2.67^b	0.01	$p < 0.05$	$p < 0.05$	$p > 0.05$

For abbreviations and statistical data, see Table II.

Table IV.- Effect of Zinc sources and Zinc levels on Zn (µg/g) in heart, kidney, spleen, eyes, whole body, muscles, skin, bones, liver, scales, intestine and feces of *L. rohita* fingerlings.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$		PSE	P-value		
Zinc level (mg/kg)	25	50	25	50	25	50	25	50		Zn source	Zn level	Zn source × Zn level
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8				
Heart	28.28^{c}	30.92^{b}	27.76^{cd}	32.09^{a}	25.06^{e}	30.89^{b}	27.26^{d}	30.73^{b}	0.18	$p < 0.05$	$p < 0.05$	$p < 0.05$
Kidney	21.62^{e}	27.17^{a}	21.5^{e}	27.04^{a}	23.57^{d}	26.15^{b}	21.47^{e}	24.66^{c}	0.15	$p < 0.05$	$p < 0.05$	$p < 0.05$
Spleen	28.9^{b}	31.85^{a}	29^{b}	32.06^{a}	28.87^{b}	31.72^{a}	28.72^{b}	31.66^{a}	0.11	$p < 0.05$	$p < 0.05$	$p > 0.05$
Eyes	36.86^{c}	42.81^{a}	37.11^{c}	42.63^{a}	35.94^{d}	40.21^{b}	37.14^{c}	42.41^{a}	0.16	$p < 0.05$	$p < 0.05$	$p < 0.05$
Whole body	64.62^{d}	79.18^{a}	61.83^{e}	73.02^{b}	61.65^{e}	68.46^{c}	58.71^{f}	68.49^{c}	0.47	$p < 0.05$	$p < 0.05$	$p < 0.05$
Muscles	17.72^{c}	19.55^{ab}	18.56^{abc}	19.89^{a}	18^{bc}	18.9^{abc}	18.99^{abc}	19.58^{ab}	0.38	$p > 0.05$	$p < 0.05$	$p > 0.05$
Skin	210.25^{c}	232.16^{a}	205.05^{d}	230.67^{a}	186.96^{f}	221.38^{b}	200.19^{e}	230.95^{a}	1.15	$p < 0.05$	$p < 0.05$	$p < 0.05$
Bones	128.13^{e}	152.42^{a}	133.67^{d}	145.54^{b}	127.14^{e}	138.51^{c}	127.14^{e}	144.73^{b}	0.67	$p < 0.05$	$p < 0.05$	$p < 0.05$
Liver	15.58^{c}	18.93^{b}	15.8^{c}	21.65^{a}	11.4^{d}	18.9^{b}	11.28^{d}	18.46^{b}	0.27	$p < 0.05$	$p < 0.05$	$p < 0.05$
Scales	90.02^{e}	96.96^{a}	89.71^{e}	94.76^{b}	89.41^{e}	92.13^{d}	89.34^{e}	94.18^{c}	0.19	$p < 0.05$	$p < 0.05$	$p < 0.05$
Intestine	335.72^{f}	394.36^{c}	339.56^{e}	397.58^{b}	348.11^{d}	404.03^{a}	348.73^{d}	403.72^{a}	0.27	$p < 0.05$	$p < 0.05$	$p < 0.05$

For abbreviations and statistical data, see Table II.

Growth performance alone is not considered as a good indicator of mineral status of fish, to determine the mineral bioavailability, analysis of tissue mineral contents should also be performed (Baker, 1986; Cowey, 1992). The nutritional value of a di*et al*so does not depend upon the mineral contents it contain but on the bioavailability of those mineral contents to the animals (Paripatananont and Lovell, 1997). Results of the present study showed a significant increase in Zn content of heart, kidney, spleen, eyes, whole body, skin, bone, liver and scales in response to Zn supplementation from organic source compared to in organic sources (Table IV). However, the intestinal Zn contents were higher in fish fed Zn from inorganic source than fish fed organically chelated Zn. A significant increase in organ Zn contents by feeding Zn gluconate, compared to inorganic mineral sources, indicates an increased Zn bioavailability from organic source. The higher bioavailability of trace element from organic source is thought to be due to the chelation of element, which renders it making insoluble complexes in the gastrointestinal tract of the animal and improves the transportation of the element through intestinal mucosa (Ashmead, 1992). Moreover, Ashmead (1992) also suggested that these elements remain intact until they reach the site, where they are needed.

In correspondence to the present results, chelated mineral supplementation, to rainbow trout diets, resulted in higher Cu and Zn whole body content compared to sulfate supplemented group (Apines *et al*., 2003). Similarly, in another study, higher mineral deposition was observed in liver and bones of rainbow trout fed chelated mineral compared to those fed inorganic mineral sources (Apines *et al*., 2004). Furthermore, supplementation of trace elements from organic sources, as compared to inorganic sources, resulted in higher carcass mineral content in red sea bream (Sarker *et al*., 2005). Higher mineral deposition was reported, by Satoh *et al*. (2001), in whole body and bones of rainbow trout fed amino acid chelated mineral than other sources.

Contradictory to our results, non-significant effect of chelated Zn supplementation was reported in catfish (Li and Robinson, 1996), Atlantic salmon (Maage *et al*., 2001), rainbow trout (Apines *et al*., 2001) and Nile tilapia (Sa *et al*., 2005). Interestingly, lower performances of organic Zn sources, as compared Zn-sulfate, have also been reported in whole body and different tissues of fish (Sa *et al*., 2005; Rider *et al*., 2010).

Absorption of elements is greatly influenced by the source from which they are ingested (Apines *et al*., 2001). Significant differences were observed in Zn digestibility and retention in present study, when *L. rohita* were fed with different Zn sources and at different Zn levels (Table V). The enhanced absorption of chelated Zn may attribute to its stability which keep the molecule intact throughout the absorption process (Ashmead, 1992). In line with our results, Apines *et al*. (2001) observed significantly enhanced Zn retention in rainbow trout fed with amino acid chelated Zn compared to glass embedded Zn and ZnSO4. Moreover, Satoh *et al*. (2001) reported significantly increased mineral retention in the whole body of rainbow trout due to chelated mineral supplementation rather than inorganic mineral sources (Satoh *et al*., 2001). A Similar increase in absorption and retention of Zn, Mn and Cu was recorded in rainbow trout fed amino acid chelated elements compared to their counter sulfate sources (Apines *et al*., 2003, 2004). However, Zn absorption remained unaffected

in Nile tilapia (Sa *et al*., 2005) and rainbow trout (Rider *et al*., 2010) fed with either organic or inorganic Zn sources.

The activity of metalloenzymes can serve as a tool to evaluate the mineral bioavailability because they use mineral as their cofactor. Dietary Zn is known to affect the ALP activity as it serve as a cofactor for this enzyme (Roth and Kirchgessner, 1980). In the present study, Zn gluconate supplementation resulted in the maximum ALP activity in fish compared to inorganic sources (Table VI). Lower level of ALP activity in fish fed inorganic Zn sources may be attributed to Zn deficiency which causes structural changes in enzyme, leading to decreased ALP activity in tissues (Reinhold *et al*., 1969). A series of experiments conducted on rainbow trout revealed significantly increased ALP activity when fish were fed with amino acid chelated trace elements than those fed on inorganic sources (Apines *et al*., 2001, 2003, 2004). Similarly, Shimei *et al*. (2011) also observed higher ALP activity in tilapia fed with amino acid chelated trace elements compared to the groups supplemented with inorganic element sources (Shimei *et al*., 2011).

Table V.- Effect of zinc sources and zinc levels on zinc digestibility (%) and zinc retention (%) in *L. rohita* fingerlings.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$		PSE	P-value		
Zinc level (mg/kg)	25	50	25	50	25	50	25	50		Zn source	Zn level	Zn source × Zn level
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8				
Zinc digestibility	53.13^{a}	47.94^{b}	37.6^{c}	36.08^{c}	36.66^{c}	29.67^{d}	31.25^{d}	29.93^{d}	0.58	$p < 0.05$	$p < 0.05$	$p < 0.05$
Zinc retention	86.75^{a}	76.97^{bcd}	82.62^{ab}	73.64^{cd}	81.7^{abc}	69.09^{d}	78.92^{abc}	69.25^{d}	2.01	$p < 0.05$	$p < 0.05$	$p > 0.05$

For abbreviations and statistical data, see Table II.

Table VI.- Effect of zinc sources and zinc levels on ALP activity in liver, intestine, kidney, muscle, spleen and heart of *L. rohita* fingerlings.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$		PSE	P-value		
Zinc level (mg/kg)	25	50	25	50	25	50	25	50		Zn source	Zn level	Zn source × Zn level
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8				
Liver (μ/mg)	191.93^{b}	198.18^{a}	188.17^{d}	195.44^{b}	176.34^{f}	182.24^{d}	181.08^{e}	188.87^{c}	0.41	$p < 0.05$	$p < 0.05$	$p > 0.05$
Intestine (μ/mg)	54.94^{c}	59.98^{a}	49.48^{e}	50.84^{b}	45.52^{g}	48.88^{f}	46.08^{d}	52.56^{b}	0.48	$p < 0.05$	$p < 0.05$	$p < 0.05$
Kidney (μ/mg)	76.01^{c}	80.6^{a}	67.45^{d}	75.75^{b}	59.13^{f}	65.28^{e}	70.79^{e}	75.8^{d}	0.42	$p < 0.05$	$p < 0.05$	$p > 0.05$
Muscle (μ/mg)	143.09^{c}	150.2^{a}	137.53^{d}	145.68^{b}	129.93^{f}	135.49^{d}	140.1^{e}	145.35^{d}	0.38	$p < 0.05$	$p < 0.05$	$p < 0.05$
Spleen (μ/mg)	27.1^{b}	33.82^{a}	24.26^{de}	29.27^{d}	20.02^{f}	25.04^{e}	22.15^{f}	24.64^{c}	0.53	$p < 0.05$	$p < 0.05$	$p < 0.05$
Heart (μ/mg)	171.08^{b}	177.2^{a}	164.56^{d}	170.72^{b}	158.51^{f}	164.03^{e}	162.34^{c}	168.59^{b}	0.31	$p < 0.05$	$p < 0.05$	$p < 0.05$

For abbreviations and statistical data, see Table II.

Table VII.- Effect of zinc sources and zinc levels on TBARS in liver, intestine, kidney, muscle, spleen and heart of *L. rohita* fingerlings.

Zinc source	Zinc gluconate		$ZnSO_4$		ZnO		$ZnCl_2$		PSE	P-value		
Zinc level (mg/kg)	25	50	25	50	25	50	25	50		Zn source	Zn level	Zn source × Zn level
Experimental diets	D1	D2	D3	D4	D5	D6	D7	D8				
Liver (mg/g)	2.34^{c}	2.32^{c}	2.65^{b}	2.74^{b}	3.05^{a}	3.05^{a}	2.74^{b}	2.72^{b}	0.04	$p < 0.05$	$p > 0.05$	$p > 0.05$
Intestine (mg/g)	0.98^{g}	0.96^{g}	1.22^{f}	1.29^{e}	1.85^{b}	1.92^{a}	1.56^{d}	1.65^{c}	0.02	$p < 0.05$	$p < 0.05$	$p > 0.05$
Kidney (mg/g)	1.31^{c}	1.34^{c}	1.71^{b}	1.65^{b}	2^{a}	1.96^{a}	1.98^{a}	2^{a}	0.04	$p < 0.05$	$p > 0.05$	$p > 0.05$
Muscle (mg/g)	1.38^{b}	1.4^{b}	1.4^{b}	1.4^{b}	1.69^{a}	1.67^{a}	1.74^{a}	1.71^{a}	0.03	$p < 0.05$	$p > 0.05$	$p > 0.05$
Spleen (mg/g)	0.71^{c}	0.73^{c}	1.07^{b}	1.14^{b}	1.43^{a}	1.47^{a}	1.18^{b}	1.07^{b}	0.03	$p < 0.05$	$p > 0.05$	$p > 0.05$
Heart (mg/g)	0.49^{a}	0.51^{a}	1.92^{a}	0.51^{a}	0.98^{a}	1.05^{a}	0.71^{a}	0.73^{a}	0.49	$p > 0.05$	$p > 0.05$	$p > 0.05$

For abbreviations and statistical data, see Table II.

Thiobarbituric acid reactive substances (TBARS) are produced as a result of lipid peroxidation which increase the oxidative stress in animal. The activities of antioxidant enzymes, such as Cu-Zn superoxide dismutase (SOD), reduces the oxidative stress and in turn may lower the production of TBARS. In the current study, significantly reduced TBARS level was observed in fish fed with organic Zn compared to other inorganic sources (Table VII). This positive response may be attributed to the increased activity of Zn dependent antioxidant enzymes (particularly Cu-Zn SOD) as higher availability of Zn from organic source might have enhanced their activity. Significantly increased activities of Cu-Zn SOD and total SOD (T-SOD) was observed in tilapia fed with organic trace elements (including Cu and Zn) compared to those fed on inorganic mineral sources (Shimei *et al*., 2011). Moreover, increased production and activity of glutathione peroxidase was observed in turbot juveniles fed organic Zn than Zn-sulfate (Ma *et al*., 2014).

CONCLUSION

In conclusion, present study evidenced that organic Zn improved the tissue Zn contents, retention of Zn; ALP enzyme activity and TBARS status in different body tissues of *L. rohita* juveniles compared to the inorganic Zn. However, both Zn sources and levels have non-significant effect on whole body proximate except for the ash contents which were greater from organic source; growth performance and absorption of Zn in the fish. So, organic Zn improved the Zn bioavailability compared to the inorganic Zn in the practical diet of *L. rohita* juveniles

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Allan, G.L. and Rowland, S.J., 1992. Development of an experimental diet for silver perch (*Bidyanus bidyanus*). *Aust. Aquacult.,* **6**: 39-40.

AOAC, 1995. *Official methods of analysis*, 15th Ed. Association of Official Analytical Chemists, Washington, DC, USA, pp. 1094.

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T., Nasu, N. and Fujita, S., 2001. Bioavailability of amino acids chelated and glass embedded Zn to rainbow trout, *Oncorhynchus mykiss*, fingerlings. *Aquacult. Nutr*., **7**: 221-228. https://doi.org/10.1046/j.1365-2095.2001.00178.x

Apines, M.J.S., Satoha, S., Kirona, V., Watanabea, T. and Aoki, T., 2003. Availability of supplemental amino acid-chelated trace elements in diets containing tricalcium phosphate and phytate to rainbow trout, *Oncorhynchus mykiss. Aquaculture,* **225**: 431-444. https://doi.org/10.1016/S0044-8486(03)00307-7

Apines-Amar, M.J.S., Satoh, S., Caipang, C.M.A., Kiron, V., Watanabe, T. and Aoki, T., 2004. Amino acid-chelate: A better source of Zn, Mn and Cu for rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, **240**: 345-358. https://doi.org/10.1016/j.aquaculture.2004.01.032

Ashmead, H.D., 1992. *The roles of amino acids chelates in animal nutrition*. Noyes Publications, NJ, pp. 479.

Ashmead, H.D., 1993. *Comparative intestinal absorption and subsequent metabolism of metal amino acid chelates and inorganic metal salts. The roles of amino acid chelates in animal nutrition*. Noyes, New Jersey, pp. 47-75.

Baker, D.H., 1986. Problems and pitfalls in animal experiments designed to establish dietary requirement for essential nutrients. *J. Nutr.,* **116**: 2239-2249. https://doi.org/10.1093/jn/116.12.2339

Bligh, E.G. and Dyer, W.J., 1959. A rapid method of total fat extraction and purification. *Can. J. Biochem. Physiol.,* **37**: 911-917. https://doi.org/10.1139/y59-099

Cowey, C.B., 1992. Nutrition: Estimating requirements of rainbow trout. *Aquaculture,* **100**: 177-189. https://doi.org/10.1016/0044-8486(92)90370-Z

Davis, D.A. and Gatlin III, D.M., 1996. Dietary mineral requirements of fish and marine crustaceans. *Rev. Fish. Sci.,* **4**: 75-99. https://doi.org/10.1080/10641269609388579

Francis, G., Makkar, H.P.S. and Becker, K., 2001. Anti-nutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture,* **199**: 197-227. https://doi.org/10.1016/S0044-8486(01)00526-9

Garcia-Aranda, J.A., Wapnir, R.A. and Lifshitz, F., 1983. *In vivo* intestinal absorption of manganese in the rat. *J. Nutr.,* **113**: 2601-2607. https://doi.org/10.1093/jn/113.12.2601

Gatlin III, D.M. and Wilson, R.P., 1983. Dietary Zn requirement of fingerling channel catfish. *J. Nutr.,* **113**: 630-635. https://doi.org/10.1093/jn/113.3.630

Gatta, P.P., Pirini, M., Test, S., Vignola, G. and Monetti,

P.G., 2000. The influence of different levels of dietary vitamin E on sea bass *Dicentrarchus labrax* flesh quality. *Aquacult. Nutr.,* **6**: 47-52. https://doi.org/10.1046/j.1365-2095.2000.00127.x

Habib, R.Z., Afzal, M., Shah, S.Z.H., Fatima, M., Bilal, M. and Hussain, S.M. 2018. Potential of phytase and citric acid treated canola meal based diet to enhance the minerals digestibility in *Labeo rohita* fingerlings. *Pakistan J. Zool.,* **50**: 2045-2050. http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2045.2050

Haddad, A.S., Subbiah, V. and Lichtin, A.E., 2008. Hypocupremia and bone marrow failure. *Haematologica*, **93**: 12121. https://doi.org/10.3324/haematol.12121

Hardy, R.W. and Shearer, K.D., 1985. Effect of dietary calcium phosphate and Zn supplementation on whole body Zn concentration of rainbow trout (*Salmo gairdneri*). *Can. J. Fish. aquat. Sci*., **42**: 181-184. https://doi.org/10.1139/f85-022

Hughes, S., Diet, M.N. and Samman, S., 2006. The effect of Zn supplementation in humans on plasma lipids, antioxidant status and thrombogenesis*. J. Am. Coll. Nutr*., **25**: 285-291. https://doi.org/10.1080/07315724.2006.10719537

Lall, S.P., 1989. The minerals. In: *Fish nutrition* (ed. J.E. Halver). Academic Press, San Diego, CA, pp. 19-257.

Li, M.H. and Robinson, E.H., 1996. Comparison of chelated zinc and zinc sulfate as zinc sources for growth and bone mineralization of channel catfish (*Ictalurus punctatus*) fed practical diets. *Aquaculture*, **146**: 237-243. https://doi.org/10.1016/S0044-8486(96)01388-9

Lovell, R.T., 1989. *Nutrition and feeding of fish*. Van Nostrand Reinhold, New York, USA. https://doi.org/10.1007/978-1-4757-1174-5

Ma, R., Hou, H.K., Mai, A.S., Bharadwaj, F.J. and Zhang, W., 2014. Comparative study on the bioavailability of chelated or inorganic Zn in diets containing tri-calcium phosphate and phytate to turbot (*Scophthalmus maximus*). *Aquaculture*, **420-421**: 187-192. https://doi.org/10.1016/j.aquaculture.2013.11.003

Maage, A. and Julshamn, K., 1993. Assessment of Zn status in juvenile Atlantic salmon (*Salmo salar*) by measurement of whole body and tissue levels of Zn. *Aquaculture,* **117**: 179-191. https://doi.org/10.1016/0044-8486(93)90134-K

Maage, A., Julshamn, K. and Berge, G.E., 2001. Zinc gluconate and zinc sulphate as dietary zinc sources for Atlantic salmon. *Aquacult. Nutr.,* **7**: 183-187. https://doi.org/10.1046/j.1365-2095.2001.00170.x

Maage, A., Lorentzen, M., Bjùrnevik, M. and Julshamn, K., 1993. Zn requirement for Atlantic salmon *Salmo salar* fry during start feeding. *Fish Nutr. Prac.,* **61**: 873-882.

NRC, 1993. *Nutrient requirements of fish*. National Academy Press, National Research Council, Washington, DC, pp. 114.

Ogino, C. and Yang, G.Y., 1978. Requirement of rainbow trout for dietary Zn. *Bull. Japan. Soc. Sci. Fish*., **44**: 1015-1018. https://doi.org/10.2331/suisan.44.1015

Paripatananont, T. and Love1, R.T., 1995. Chelated Zn reduces the dietary Zn requirement of channel catfish, *Ictalurus punctatus*. *Aquaculture,* **133**: 73-82. https://doi.org/10.1016/0044-8486(94)00404-C

Paripatananont, T. and Lovell, R.T., 1997. Comparative net absorption of chelated and inorganic trace minerals in channel catfish *Ictalurus punctatus* diets. *J. World Aquacult. Soc.,* **28**: 62-67. https://doi.org/10.1111/j.1749-7345.1997.tb00962.x

Qiao, Y.G., Tan, B.P., Mai, K.S., Ai, Q.H., Zhang, W.B. and Xu, W., 2013. Evaluation of iron methionine and iron sulphate as dietary iron sources for juvenile cobia (*Rachycentron canadum). Aquacult. Nutr.,* **19**: 721-730. https://doi.org/10.1111/anu.12019

Reinhold, J.G. and Kfoury, G.A., 1969. Zinc-dependent enzymes in zinc-depleted rats; intestinal alkaline phosphatase. *Am. J. clin. Nutr*., **22**: 1250-1263. https://doi.org/10.1093/ajcn/22.9.1250

Richardson, N.L., Higgs, D.A., Beames, R.M. and Bride, J.R.M., 1985. Influence of dietary calcium, phosphorus, Zn and sodium phytate level on cataract incidence, growth and histopathology in juvenile Chinook salmon (*Oncorhynchus tshawytscha*). *J. Nutr.,* **115**: 553-567. https://doi.org/10.1093/jn/115.5.553

Rider, S.A., Davies, S.J., Jha, A.N., Clough, R. and Sweetman, J.W., 2010. Bioavailability of co-supplemented organic and inorganic zinc and selenium sources in a white fishmeal-based rainbow trout (*Oncorhynchus mykiss*) diet. *J. Anim. Physiol. Anim. Nutr*., **94**: 99-110. https://doi.org/10.1111/j.1439-0396.2008.00888.x

Roth, H.P. and Kirchgessner, M., 1980. Zn metalloenzyme activities. Changes and biochemical aspects in Zn deficiency. *World Rev. Nutr. Diet,* **34**: 144-160. https://doi.org/10.1159/000403310

Sa, M.V.D.C.E., Pezzato, L.E., Barros, M.M. and Padilha, P.D.M., 2005. Relative bioavailability of zinc in supplemental inorganic and organic sources for Nile tilapia *Oreochromis niloticus*

fingerlings. *Aquacult. Nutr.,* **11**: 273-281. https://doi.org/10.1111/j.1365-2095.2005.00352.x

Sarker, S.A., Satoh, S. and Kiron, V., 2005. Supplementation of citric acid and amino acid-chelated trace element to develop environment-friendly feed for red sea bream, *Pagrus major*. *Aquaculture,* **248**: 3-11. https://doi.org/10.1016/j.aquaculture.2005.04.012

Satoh, S., Izume, K., Takeuchi, K. and Watanabe, T., 1987a. Availability to rainbow trout of Zn contained in various types of fish meals. *Nippon Suisan Gakkaishi*, **53**: 1861-1866. https://doi.org/10.2331/suisan.53.1861

Satoh, S., Tabata, K., Izume, K., Takeuchi, K. and Watanabe, T., 1987b. Effect of dietary tri-calcium phosphate on availability of Zn to rainbow trout. *Nippon Suisan Gakkaishi,* **53**: 1199-1205. https://doi.org/10.2331/suisan.53.1199

Satoh, S., Takeuchi, K. and Watanabe, T., 1987c. Availability to rainbow trout of Zn in white fish meal and of various Zn compounds. *Nippon Suisan Gakkaishi*, **53**: 595-599. https://doi.org/10.2331/suisan.53.595

Satoh, S., Apines, M.J., Tsukioka, T., Kiron, V., Watanabe, T. and Fujita, S., 2001. Bioavailability of amino acid-chelated and glass-embedded manganese to rainbow trout, *Oncorhynchus mykiss* (Walbaum) fingerlings. *Aquacult. Res*., **2**: 18-25. https://doi.org/10.1046/j.1355-557x.2001.00002.x

Satoh, S., Poe, W.E. and Wilson, R.P., 1989. Effect of supplemental phytate and/or tricalcium phosphate on weightgain, feed efficiency and Zn content in vertebrae of channel catfish. *Aquaculture,* **80**: 155-161. https://doi.org/10.1016/0044-8486(89)90281-0

Shils, M.E., Ross, M.L. and Witshi, J.C., 1997. Specific dietary component. In: *Modern nutrition in health and disease* (eds. M.E. Shils, J.A. Olson, M. Shike and A.C. Ross), 8th ed., Vol. 1. Williams and Wilkins, USA, pp. 156-238.

Shimei, L., Yu, P., Li, L., Xia, M., Ruorui, Z. and Shuhong, M., 2011. Effects of different sources of iron, zinc, manganese and copper on growth, metabolism and non-specific immunity of tilapia (*Oreochromis niloticus*). *Chinese J. Anim. Nutr.,* **5**:

Snedecor, G.W. and Conhran, W.G., 1991. *Statistical methods*. Iowa State University Press, Ames., USA.

Solomons, N.W., 1993. Zn. In: *Physiology: Encyclopaedia of food science, food technology and nutrition* (eds. R. Macrae, R.K. Robinson and M.J. Sadler). Academic Press, London, pp. 4984-4987.

Spry, D.J., Hodson, P.V. and Wood, C.M., 1988. Relative contributions of dietary and waterborne Zn in the rainbow trout, *Salmo gairdneri*. *Can. J. Fish. aquat. Sci.,* **45**: 32-41. https://doi.org/10.1139/f88-005

Steel, R.G.D., Torrie, J.H. and Dickey, D.A., 1996. *Principles and procedures of statistics*, 3rd Ed. McGraw Hill International Book Co. Inc., New York, USA.

Tan, B. and Mai, K., 2001. Zn methionine and Zn sulfate as sources of dietary Zn for juvenile abalone, Haliotis discus hannai Ino. *Aquaculture,* **192**: 67-84. https://doi.org/10.1016/S0044-8486(00)00435-X

Wang, L.M., Wang, J., Bharadwaj, A.S., Xue, M., Qin, Y.C., Wu, X.F. and Han, F., 2015. Effects of dietary copper sources on growth, tissue copper accumulation and physiological responses of Japanese sea bass (*Lateolabrax japonicas)* fed semi-purified or practical diets. *Aquacult. Res*., **46**: 1619-1627. https://doi.org/10.1111/are.12322

Watanabe, T., Kiron, V. and Satoh, S., 1997. Trace minerals in fish nutrition. *Aquaculture*, **151**: 185-207. https://doi.org/10.1016/S0044-8486(96)01503-7

Wedekind, K.J., Hortin, A.E. and Baker, H.D., 1992. Methodology for assessing Zn bioavailability: efficacy estimates for Zn-methionine, Zn sulfate, and Zn oxide. *J. Anim. Sci*., **70**: 178-187. https://doi.org/10.2527/1992.701178x

Wei, W., Li, A. and Li, D., 1999. Effect of dietary supplemented Zn on the growth and some biochemical parameters of juvenile flounder, *Paralichthys oliĺaceus*. *J. Ocean Univ. Qingdao,* **18**: 60-66.

Willis, J.N. and Sunda, W.G., 1984. Relative contributions of food and water in the accumulation of Zn by two species of marine fish. *Mar. Biol.,* **80**: 273-279. https://doi.org/10.1007/BF00392822

Yakubu, M.T., Adebayo, O.J., Egwim, E.C. and Owoyele, V.B., 2005. Increased liver alkaline phosphatase and aminotransferase activities following administration of ethanolic extract of *Khaya senegalensis* stem bark to rats. *Biokemistri,* **17**: 27-32. https://doi.org/10.4314/biokem.v17i1.32585

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Supplementation of Citric Acid, Phytase and Organic Trace Elements Affects Excretion of Nitrogen and Phosphorus in *Labeo rohita* Fingerlings

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Authors' Contribution
SZHS designed the study, performed the experiment and wrote the manuscript. MF and MB helped in data collection and analysis of data and writing of the manuscript. MA supervised the experiment. M Arshad critically reviewed the draft.

Key words
Rohu, Nutrient excretion, Amino acid chelated mineral, Body mineralization.

ABSTRACT

The present study with *Labeo rohita* fingerlings (3.27±0.04 g) was designed to investigate the effect of citric acid (CA), phytase (PHY) and organically chelated trace elements (TEs) supplementation on nitrogen and phosphorus excretion. The three supplements *i.e.* CA (0 and 3%), PHY (0 and 1000 FTU/kg) and TEs {(inorganic (0.15%) and organic (0.1% and 0.05%)}, were supplemented in factorial arrangement (2×2 ×3) resulting in the formulation of 12 treatments. Fish were fed in triplicates to apparent satiation twice a day, 6 days a week for a period of 90 days. Results showed higher deposition of Cu, Mn, Fe and Zn in whole body by feeding organic TEs compared to their traditional sulfate salts. Deposition of major mineral was also significantly influenced by the supplementation of organically chelated TEs. Similarly, dietary addition of CA and PHY enhanced the deposition of all observed mineral in whole body of fish. Citric acid and PHY interacted positively to enhance the whole body mineralization. Interestingly, all the three additives and their all kind of interactions increased the DM, CP and ash content while decreased ($p<0.05$) the EE level in fish body. Inclusion of CA and PHY in the diets showed significant increase in the retention of N and P in the fingerlings. Their retentions were also affected by the source of TEs. Trace elements supplementation from organic chelates showed higher retention of these elements (N and P) compared to inorganic TEs. Furthermore, all the main as well as interaction effects reduced the P and N excretions in the waste water. In conclusion, supplementation of CA, PHY and organically chelated TEs alone as well as in combination, in SBM based diet, improved the nutrient utilization in fingerlings leading to reduced loading to the aquatic environment.

INTRODUCTION

Aquatic water bodies are basic component of our environment which provide food to living organisms (Milenkovic *et al*., 2005). In aquaculture, the undigested nutrients excreted in the feces of living animals limits the life in an aquatic ecosystem. High load of undigested fecal nitrogen and phosphorus cause eutrophication in water bodies (Lewis and Wurtsbaugh, 2008) which deteriorate the water quality (Sobolewski, 2016). Efforts are being made to lower nutrient leaching into the environment as quality of water determines the health of a population (Bian *et al*., 2016).

Fishmeal is being used as a major protein source in aquafeeds (Hardy, 1995) as it has high protein content, balanced amino acid profile, utmost digestibility and presence of relatively less anti-nutritional factors. However, its short supply and high price influence the researchers to find other sustainable protein sources (Lunger *et al*., 2007). Interest has been developing in the use of plant proteins including soybean meal (SBM), sunflower meal (SFM) and canola meal (CM) as alternative to fishmeal in aquafeeds (Cain and Garling, 1995). These plant proteins are of low cost and have high protein contents (Cheng and Hardy, 2004). However, these plant based protein meals contain phytate which is an anti-nutritional factor. It is the

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main storage form of P in plants and nearly 60-80% of total P in plants meals is found bound to it (Cao *et al*., 2007). This phytate-P is not available to agastric or mono gastric fish species (Hussain *et al*., 2011). Phosphorus deficiency induces skeletal deformities in fish (Lall and Lewis, 2007) as well as undigested phytate-P can contribute to eutrophication of water bodies (Baruah *et al*., 2007b). Moreover, phytate reduces the digestibility of many important nutrients like protein, fat and mineral by making insoluble complexes with them (Nwanna *et al*., 2008).

The use of organic acids to hydrolyse the phytate is one of the modern approaches being used in fish nutrition now a day. Citric acid (CA) and its salt complexes have been widely used for the acidification of diet because of its unique flavour and high buffering capacity (Hossain *et al*., 2007). It dephosphorylates the phytate to release the bound P and other nutrients (Zyla *et al*., 1995). Sugiura *et al*. (2001) reported that addition of CA in fish diet improved the P and other mineral utilization. Khajepour and Hosseini (2011) reported that the addition of CA improved P and Ca contents in Beluga (*Huso huso*) fed SBM based diets. Other studies have also reported improved fish growth by addition of organic acid in fish feed (Hossain *et al*., 2007; Ng *et al*., 2009).

A more precise approach to hydrolyze the phytate is the supplementation of phytase (PHY) in the feed. PHY is an enzyme which hydrolyses the phytate to release the bound P and other chelated nutrients for absorption (Kumar *et al*., 2012). Agastric or monogastric fish species lack this enzyme, however, its supplementation in feed resulted in enhanced bioavailability of plant P and other nutrients in fish (Cao *et al*., 2007). Significant improvements in growth as well as feed efficiency in tra catfish by PHY addition in feed has been reported recently (Hung *et al*., 2015). Also, increased availability of nutrients including mineral (Cu, Ca, Mg, Fe and Zn) in rainbow trout has been reported by adding PHY in the diet (Sugiura *et al*., 2001).

Negative influence of phytate on mineral availability can also be reduced by supplementing the chelated mineral instead of mineral from in-organic sources. It has been shown that organically chelated mineral have the capability to compete with the dietary mineral inhibitor like phytate resulting in improved mineral availability to the animal. Recently, interest in the use of amino acid chelated trace elements (TE) has increased because of their higher availability compared to inorganic sources (Ashmead, 1992). Supplementation of methionine chelated Zn improved the growth and bone Zn deposition in chicks compared to inorganic Zn sources ($ZnSO_4$ and ZnO) (Wedekind *et al*., 1992).

Table I.- Composition (%) of the experimental diets.

Ingredients	Test Diets											
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Soybean meal	44	44	44	44	44	44	44	44	44	44	44	44
Fishmeal	5	5	5	5	5	5	5	5	5	5	5	5
Corn-gluten 60%	9	9	9	9	9	9	9	9	9	9	9	9
Rice polish	17	17	17	17	17	17	17	17	17	17	17	17
Wheat flour	20	20	20	20	20	20	20	20	20	20	20	20
Fish oil	3	3	3	3	3	3	3	3	3	3	3	3
Vitamin premix[a]	1	1	1	1	1	1	1	1	1	1	1	1
Chromic oxide	1	1	1	1	1	1	1	1	1	1	1	1
Total	100	100	100	100	100	100	100	100	100	100	100	100
Addition of supplements*												
CA (%)	0	0	0	0	0	0	3	3	3	3	3	3
PHY (FTU/kg)	0	0	0	1000	1000	1000	0	0	0	1000	1000	1000
Macro mineral mixture [b]	2	2	2	2	2	2	2	2	2	2	2	2
TEs mixture												
From inorganic source (%)[c]	0.15	0	0	0.15	0	0	0.15	0	0	0.15	0	0
From organic source (%)[d]	0	0.1	0.05	0	0.1	0.05	0	0.1	0.05	0	0.1	0.05

[a]Each kg of vitamin premix contains Vitamin A, 15 M.I.U.; Vitamin D_3, 3 M.I.U.; Vitamin B_1, 5000 mg; Vitamin E, 6000 IU; Vitamin B_2, 6000 mg; Vitamin K_3, 4000 mg ; Vitamin B_6, 4000 mg; Folic acid, 750 mg; Vitamin B_{12}, 9000 mg; Calcium pantothenate, 10000 mg; Vitamin C, 15000 mg; Nicotinic acid, 25000 mg. [b]Macro mineral mixture (mg/g): $CaCO_3$ 316, KH_2PO_4 479, $MgSO_4.7H_2O$ 153, NaCl 51, $CoCl.6H_2O$ 0.0816, Ammonium molybdate 0.061, $AlCl_3.6H_2O$ 0.255. [c]TEs mixture from inorganic source (mg/g): $ZnSO_4.7H_2O$ 121.33, $CuSO_4.5H_2O$ 210.67, $MnSO_4.5H_2O$ 116.67, $FeSO_4.H_2O$, 100.67, $SeCl_2$ 0.56, KI 1.58. [d]TEs mixture from organic source (mg/g): Zn (as methionine hydroxy analogue chelate) 40, Cu (as methionine hydroxy analogue chelate) 80, Mn (as methionine hydroxy analogue chelate) 40, Fe (as iron glycerin chelate) 50, Se as selenium yeast 0.3, I as KI 1.2. Note: 0.15% inclusion of TEs mixture from inorganic source shares the same amount of TEs as from 0.1% organic TEs mixture.

Paripatananont and Lovell (1995) also reported improved Zn methionine availability than $ZnSO_4$ in channel catfish. However, many discrepancies have been reported in the availability of chelated mineral complexes. Studies with swine showed non-significant differences in the availability of Zn form organic and inorganic sources (Swinkels *et al*., 1996).

The aim of present study was to investigate the effect of CA, PHY and organic TE on proximate composition, mineral deposition and excretion of phosphorus (P) and nitrogen (N) in *L. rohita* juveniles.

MATERIALS AND METHODS

The present experiment was conducted in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad, Pakistan.

Experimental diets and design

The three selected supplements *i.e.* CA (0, and 3%) (OmniPur®, Merck Millipore), PHY (0 and 1000 FTU/kg) (Phyzyme® XP 10000 FTU/g; Danisco Animal Nutrition, Fin-65101 Vaasa, Finland) and TEs {(inorganic (0.15%; Merck Millipore) and organic (0.1% and 0.05%; (MINTREX®, Poultry TMO Plus, NOVUS Int. USA))}, were supplemented in factorial arrangement (2 × 2 × 3) (Table I). The experimental layout was as followed; T1 contained 0.15% inorganic TEs, 0% CA and 0 FTU/kg PHY; T2 contained 0.1% organic TEs, 0% CA and 0 FTU/kg PHY; T3 contained 0.05% organic TEs, 0% CA and 0 FTU/kg PHY; T4 as T1 plus 1000 FTU/kg PHY; T5 as T2 plus 1000 FTU/kg PHY; T6 as T3 plus 1000 FTU/kg PHY; T7 as T1 plus 3% CA, T8 as T2 plus 3% CA; T9 as T3 plus 3% CA; T10 as T1 plus 3% CA and 1000 FTU/kg PHY; T11 as T2 plus 3% CA and 1000 FTU/kg PHY and T12 as T3 plus 3% CA and 1000 FTU/kg PHY.

Feed ingredients were procured from local poultry feed market and ground to 0.05 mm particle size in cereal grinding machine (FFC-45, JIMO, China). Ingredients were analyzed chemically before the formulation of experimental diet (AOAC, 1995) and mixed in an electric mixer. Required moisture for pelleting was provided by adding 15% water into above mixture and was processed through hand pelletizer with 3 mm die for pellets formation. Citric acid and TEs were added in their relevant diets during mixing while PHY was top sprayed on the prepared pellets. For PHY spray, its required amount was dissolved in 50 mL of distilled water and sprayed on 1 kg of the finished diet. Other experimental diets were sprayed with same amount of distilled water to maintain an equal level of moisture. The diets were stored at -20°C throughout the feeding trial. All the three experiments were run in factorial arrangement under completely randomized design with three replicates per treatment. Each feeding trial last for 90 days. Proximate and mineral compositions of SBM based diet is given in Table II.

Table II.- Proximate and mineral composition of the soybean meal based test diets.

Ingredients	Test Diets											
	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12
Proximate composition												
DM (%)	96.44	96.74	96.65	96.46	96.27	96.64	96.6	96.5	96.38	96.59	96.76	96.52
CP (%)	29.84	30.04	30.67	30.56	30.14	29.82	28.57	30.33	28.99	29.28	29.73	29.9
EE (%)	9.77	9.49	9.77	9.58	9.71	9.7	9.75	9.7	9.95	9.4	10.04	9.73
GE (kcal/g)	4.15	4.12	4.13	4.18	4.11	4.18	4.16	4.18	4.18	4.12	4.13	4.14
Mineral composition												
P (mg/g)	15.99	16.12	15.83	16.01	15.64	16.08	15.73	15.96	16.12	15.8	15.52	15.78
Ca (mg/g)	17.88	17.69	17.39	18.18	17.41	17.75	17.72	17.6	17.69	17.96	17.91	18.09
Mg (mg/g)	5.37	4.6	4.63	4.66	4.82	4.57	4.58	4.57	5.06	5.55	5	5.1
Na (mg/g)	7.58	7.41	8.29	8.13	7.58	7.41	7.75	7.9	8.16	8.3	7.88	7.38
K (mg/g)	10.56	10.51	10.34	10.59	10.81	10.59	11.03	10.61	10.68	10.82	10.91	11.11
Cu (µg/g)	145.7	145.6	105.5	141.1	147.3	105.4	145.9	144.3	107.6	139.2	145.9	107.3
Zn (µg/g)	74.55	74.56	53.57	74.56	74.54	54.5	73.86	74.57	52.6	73.76	74.53	54.38
Mn (µg/g)	84.56	84.55	63.51	84.52	84.58	64.53	84.55	84.42	64.62	84.43	85.42	62.66
Fe (µg/g)	164.28	162.66	139.95	162.92	163.52	138.24	162.35	164.32	138.55	163.29	165.39	139.32

Experimental fish, feeding and feces collection

Labeo rohita fingerlings were obtained from Government Fish Seed Hatchery, Faisalabad and stored into cemented tanks (1000 L) for acclimation to laboratory conditions for two weeks before initiation of feeding trial. During this period, fingerlings were fed with basal diet once a day. After acclimation, 720 fish were shifted into 36 experimental tanks (70 L) such that every tank contained 20 fish while another 15 fish were randomly selected and used for the initial whole body proximate and mineral analysis. Average initial weight of fingerlings was 3.27±0.04 g. Fish were fed the experimental diets to satiation, 6 days a week. The daily ration was divided into two, and fed to the fish at 08:00 and 16:00 h. The fish were weighed every 2 weeks and their ration was adjusted accordingly. After the feeding session of 3 h, fish from each experimental tank were moved to separate clean water, the experimental tanks were washed completely to remove the particles of diet, refilled with fresh water and fish were shifted back to experimental tanks. Uneaten diet was collected and dried to determine feed intake. Round the clock aeration was provided through capillary system to all the experimental tanks. During experimental period the dissolved oxygen and pH were monitored constant throughout the experimental duration with the help of HANNA DO meter (model HI 9147) and AMPROBE pH meter (model WT-80), which were regulated between 5.8-7.3 mg/L and 7.4-8.6, respectively. The water temperature ranged from 24.9-28.7°C throughout the trial.

Proximate and mineral composition

Proximate composition of experimental diets and feces were determined by following standard methods of AOAC (1995). Crude protein (N×6.25) contents of samples were determined by micro Kjeldahl apparatus after acid digestion, EE by following petroleum ether extraction method through soxtec HT2 1045 system, DM contents by oven drying at 105°C up to a constant weight. Samples were ashed via incineration at 550°C in electric furnace (Eyela-TMF 3100) until stable weight. Gross energy of diet and feces was determined using adiabatic oxygen bomb calorimeter (Parr Instrument Co., Moline, USA).

For mineral estimation, the samples of test diets and feces were digested in boiling nitric acid and perchloric acid mixture (3:1). After appropriate dilution, Ca, Mg, Fe, Cu, Mn and Zn contents were estimated by atomic absorption spectrophotometer (Hitachi Polarized Zeeman AAS, Z-8200, Japan). While P contents were analyzed colorimetrically on UV/VIS spectrophotometer (U-2001, Hitachi) at 720 nm wavelength. Na and K were analyzed by using flamephotometer (Jenway PFP-7, UK) (AOAC, 1995). Chromic oxide contents were estimated after oxidation with molybdate reagent (Divakaran *et al*., 2002) using spectrophotometer at 370 nm absorbance.

Whole body proximate and mineral composition

At the end of each trial, 5 fish from each experimental tank were chemically analyzed for whole body proximate and mineral composition. After 24 h starvation, fish were anesthetized using MS-222 (Sigma-Aldrich) and sacrificed. Whole body samples of fish were taken, sealed in self-sealing plastic bags and stored at -20°C until further analyses. Proximate and mineral analyses were performed as described above for dietary analysis.

Retention and excretion

The values obtained from proximate and mineral analyses of whole body samples and diets were used for calculating P and N retention in the whole body following standard procedures and according to methods described by Satoh *et al*. (1987, 1996). Retention (%) of P and N was obtained using the formula presented by Watanabe (1988) while excretion (kg/t fish produced) was calculated based on the intake and retention in the whole body, on weight gain basis as described by Parveen (1999).

$$\text{Retention (\%)} = \left(\frac{\text{Final nutrient content (g)} - \text{Initial nutrient content (g)}}{\text{Nutrient intake (g)}}\right) \times 100$$

$$\text{Excretion}\left(\frac{\text{kg}}{\text{t}}\text{production}\right) = \left(\frac{\text{FCR} \times \text{Nutrient in diet (kg)} - \text{Nutrient retained in fish (kg)}}{\text{Production (kg)}}\right) \times 1000$$

Statistical analysis

Results were analyzed using three-way analysis of variance (ANOVA) and considered significant at $p<0.05$. When significant differences occurred, Student-Newman-Keuls test was used for comparison of means. CoStat computer package (Version 6.303, PMB 320, Monterey, CA, 93940 USA) was used for statistical analyses.

RESULTS

Whole body proximate composition

Effect of dietary CA, PHY and chelated TEs supplementation on whole body proximate composition is given in Table III. Interestingly, all the three additives and their all kind of interactions increased ($p<0.05$) the DM, CP and ash content while decreased ($p<0.05$) the EE level in fish body.

Major mineral deposition in whole body

Main and interaction effects of CA, PHY and TEs to improve the major mineral contents in whole body of *L.*

rohita fingerlings are given in Table IV. Citric acid and PHY supplementations improved the whole body major mineral deposition in the fingerlings fed SBM based diet. Higher major mineral deposition was also recorded in organic TEs supplemented diets compared to inorganic TEs. Moreover, P and Na were not affected by the level of these chelated mineral while Ca, Mg and K depositions showed dose dependent response. Citric acid with TEs and PHY with TEs interacted positively to improve the major mineral deposition. Highest degree interaction (CA×PHY×TEs) was also found significant (p<0.05) to increase the Ca and Mg deposition.

Table III.- Effect of CA, PHY and organic TEs on whole body proximate composition of *L. rohita* fingerlings fed soybean meal based diet.

CA (%)	0						3						PSE*
PHY (FTU/kg)	0			1000			0			1000			
TEs	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	
Test diets	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	
DM (g/kg)	234.66^{i}	236.20^{g}	235.92^{h}	236.75^{f}	238.57^{d}	237.53^{e}	236.88^{f}	238.64^{d}	237.59^{e}	239.49^{c}	240.31^{a}	239.90^{b}	0.056
CP (g/kg)	178.22^{l}	180.80^{j}	180.32^{k}	182.17^{h}	184.91^{d}	184.60^{e}	181.84^{i}	184.52^{f}	184.23^{g}	185.90^{c}	188.20^{a}	187.73^{b}	0.025
EE (g/kg)	56.72^{a}	55.11^{c}	55.69^{b}	54.77^{d}	52.91^{g}	53.17^{f}	53.57^{e}	51.51^{j}	52.02^{h}	51.58^{i}	49.27^{l}	49.82^{k}	0.021
Ash (g/kg)	41.66^{l}	42.71^{j}	42.17^{k}	42.85^{i}	44.48^{f}	43.93^{g}	43.31^{h}	45.30^{c}	44.71^{e}	44.89^{d}	49.01^{a}	47.79^{b}	0.023

ANOVA

Parameter	CA	PHY	TEs	CA×PHY	CA×TEs	PHY×TEs	CA× PHY ×TEs
DM	S	S	S	S	S	S	S
CP	S	S	S	S	S	S	S
EE	S	S	S	S	S	S	S
Ash	S	S	S	S	S	S	S

Data are means of three replications. Mean values bearing different small superscripts under each parameter vary significantly (p<0.05). *PSE = pooled SE = √MSE/n (where MSE is mean-squared error). TEs1 = 0.15% inorganic TEs; TEs2 = 0.1% organic TEs; TEs3 = 0.05% organic TEs.

Table IV.- Effect of CA, PHY and organic TEs on whole body macro mineral of *L. rohita* fingerlings fed soybean meal based diet.

CA (%)	0						3						PSE*
PHY (FTU/kg)	0			1000			0			1000			
TEs	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	
Test diets	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	
P (mg/g)	9.88^{j}	10.67^{i}	10.49^{i}	11.30^{h}	12.58^{ef}	12.07^{fg}	11.87^{g}	12.89^{de}	13.74^{bc}	13.34^{cd}	14.48^{a}	14.18^{ab}	0.19
Ca (mg/g)	13.29^{l}	14.06^{j}	13.94^{k}	15.00^{h}	15.97^{d}	15.66^{f}	14.77^{i}	15.79^{e}	15.49^{g}	16.63^{c}	17.09^{a}	16.82^{b}	0.019
Mg (mg/g)	1.22^{l}	1.37^{j}	1.34^{k}	1.46^{i}	1.62^{e}	1.60^{f}	1.52^{h}	1.67^{c}	1.65^{d}	1.54^{g}	1.80^{a}	1.73^{b}	0.004
Na (mg/g)	4.37^{i}	4.53^{h}	4.49^{h}	4.81^{f}	5.07^{d}	4.96^{e}	4.67^{g}	4.96^{e}	4.85^{f}	5.15^{c}	5.61^{a}	5.40^{b}	0.018
K (mg/g)	6.40^{l}	6.61^{j}	6.53^{k}	6.75^{i}	7.11^{f}	7.03^{g}	6.81^{h}	7.38^{c}	7.26^{d}	7.17^{e}	7.88^{a}	7.72^{b}	0.016

ANOVA

Parameter	CA	PHY	TEs	CA×PHY	CA×TEs	PHY×TEs	CA×PHY×TEs
P	S	S	S	S	S	S	NS
Ca	S	S	S	S	S	S	S
Mg	S	S	S	S	S	S	S
Na	S	S	S	S	S	S	NS
K	S	S	S	NS	S	S	NS

For abbreviations and statistical details, see Table III.

Table V.- Effect of CA, PHY and organic TEs on whole body TEs of *L. rohita* fingerlings fed soybean meal based diet.

CA (%)	0						3						PSE*
PHY (FTU/kg)	0			1000			0			1000			
TEs	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	
Test diets	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	
Cu (µg/g)	6.19^{l}	6.58^{j}	6.43^{k}	6.78^{i}	7.48^{f}	7.31^{g}	6.91^{h}	7.80^{c}	7.61^{e}	7.72^{d}	8.72^{a}	8.54^{b}	0.025
Zn (µg/g)	71.57^{j}	75.21^{hi}	74.46^{i}	76.36^{h}	84.39^{e}	83.23^{f}	82.02^{g}	89.70^{c}	87.08^{d}	88.13^{d}	95.33^{a}	92.28^{b}	0.393
Mn (µg/g)	24.73^{l}	27.97^{j}	26.05^{k}	30.44^{i}	34.67^{e}	32.67^{g}	31.22^{h}	35.12^{d}	33.69^{f}	37.88^{c}	40.08^{a}	38.61^{b}	0.151
Fe (µg/g)	125.31^{k}	136.00^{i}	133.34^{j}	139.43^{h}	155.23^{e}	152.36^{f}	142.86^{g}	159.66^{c}	157.59^{d}	159.68^{c}	170.54^{a}	168.98^{b}	0.273

ANOVA							
Parameter	**CA**	**PHY**	**TEs**	**CA×PHY**	**CA×TEs**	**PHY×TEs**	**CA×PHY×TEs**
Cu	S	S	S	S	S	S	S
Zn	S	S	S	S	S	S	S
Mn	S	S	S	S	S	NS	S
Fe	S	S	S	S	S	NS	S

For abbreviations and statistical details, see Table III.

Table VI.- Effect of CA, PHY and organic TEs on N and P retention (%) and excretion (kg/t production) of *L. rohita* fingerlings fed soybean meal based diet.

CA (%)	0						3						PSE*
PHY (FTU/kg)	0			1000			0			1000			
TEs	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	TEs1	TEs2	TEs3	
Test diets	T1	T2	T3	T4	T5	T6	T7	T8	T9	T10	T11	T12	
N retention	52.25^{e}	62.44^{c}	60.15^{d}	62.55^{c}	63.34^{bc}	63.59^{bc}	66.16^{a}	62.41^{c}	65.57^{ab}	65.48^{ab}	64.78^{abc}	64.2	0.58
N excretion	191.95^{a}	107.65^{cd}	120.78^{b}	106.37^{cd}	107.80^{cd}	107.21^{cd}	91.89^{e}	112.77^{bc}	97.37^{de}	98.99^{de}	104.56^{cd}	107.43^{cd}	2.9
P retention	46.50^{f}	62.44^{e}	61.35^{e}	68.01^{d}	79.09^{c}	72.67^{d}	73.18^{d}	79.37^{c}	85.19^{b}	83.73^{bc}	93.61^{a}	89.79^{a}	1.5
P excretion	11.20^{a}	5.79^{b}	6.05^{b}	4.70^{c}	3.13^{de}	4.33^{c}	3.95^{cd}	3.23^{de}	2.24^{ef}	2.46^{ef}	0.98^{g}	1.63^{fg}	0.29

ANOVA							
Parameter	**CA**	**PHY**	**TEs**	**CA×PHY**	**CA×TEs**	**PHY×TEs**	**CA×PHY×TEs**
N retention	S	S	S	S	S	S	S
N excretion	S	S	S	S	S	S	S
P retention	S	S	S	S	S	S	NS
P excretion	S	S	S	S	S	S	S

For abbreviations and statistical details, see Table III.

TEs deposition in whole body

Effect of CA, PHY and TE on whole body TEs is given in Table V. Main effect data of CA, PHY and organic TEs supplementations showed significant ($p<0.05$) increment in the TEs contents in the whole body of *L. rohita* juveniles. Also, Cu, Mn and Fe deposition showed dose dependent response to the chelated mineral supplementation. Moreover, PHY and TEs interacted positively to enhance the Cu and Zn deposition in fish body while Mn and Fe deposition did not respond to their interaction. Nevertheless, CA×PHY, CA×TEs and CA×PHY×TEs interactions were significant in enhancing the trace element deposition in the fingerlings.

N and P retention and excretion

Retention and excretion of N and P are presented in Table VI. Phytase supplementation positively affected the N and P retentions. Also, acidified diet (CA supplemented diet) showed significant increase in the retention of N and P in fingerlings fed SBM based diet. Their retentions were also affected by the source of TEs. Trace elements supplementation from organic source showed higher retention of these elements (N and P) compared to in-organic TEs. Moreover, all the 2nd order interactions (CA×PHY, CA×TEs, PHY×TEs) showed positive influence on N and P retention while the highest degree interaction (CA×PHY×TEs) did not affect the P

retention in fingerlings. Furthermore, all the main as well as interaction effects significantly reduced the P and N excretions in the waste water.

DISCUSSION

Dietary CA supplementation increased the body DM, CP and ash contents while decreased the EE contents in fingerlings fed SBM based diet. Lowering of gut pH due to dietary acidification may cause the conversion of pepsinogen into pepsin at higher rate (Park *et al*., 2009) and may also result in higher pepsin activity (Kirchgessner and Roth, 1982). These factors may have contributed to higher protein digestibility observed in the present study. In agreement to our results, Khajepour and Hosseini (2012) reported that 2% and 3% CA supplemented diets increased the protein while decreased the lipid contents in the muscles of beluga. Studies carried out by Hossain *et al*. (2007) in red sea bream and Sarker *et al*. (2012) in yellow tail observed higher body ash content with CA and maleic acid supplementations, respectively. However, in contrast to our results, most of the proximate parameters remained non-responsive to the supplementation of organic acids across many fish species (Sarker *et al*., 2012; Zhu *et al*., 2015).

Mineral deposition in whole body were increased under CA supplementation in the present study. Hossain *et al*. (2007) suggested that organic acids improve the mineral bioavailability by protecting the dietary mineral against the inhibitory actions of anti-nutritional factors present in the diet. In line with our results, higher whole body mineral contents were also observed in rainbow trout fed organic acid added diets (Pandey and Satoh, 2008). Similar positive results were observed by other investigators (Hossain *et al*., 2007; Khajepour and Hosseini, 2012; Vielma *et al*., 1999).

P and N retention efficiencies are considered as important parameters for the evaluation of feed qualit (Pandey and Satoh, 2008). In the current study, higher P and N retention and thereby, their lower excretion was observed in *L. rohita* juveniles when fed with CA treated diets. Higher retention and lower excretion of these elements will not only be beneficial to reduce their use in the feed resulting in the formulation of cost effective feed but will also be helpful for the development of environment friendly feed as excessive release of these elements cause eutrophication in natural water bodies. Similar to our study, higher retention and lower excretion of P has been observed in red sea bream (Hossain *et al*., 2007) fed with CA supplemented diets. Likewise, Pandey and Satoh (2008) in rainbow trout and Hossain *et al*. (2007) in red sea bream observed that CA supplementation enhanced the N retention.

Supplementation of PHY enhanced the DM, CP and crude ash while reduced the EE (crude lipid) in the body of rohu juveniles. Studies by Liu *et al*. (2014) in grass carp and Cao *et al*. (2007) in Nile tilapia reported that dietary PHY addition caused a reduction in body lipids. However, in the present study, hydrolysis of phytate by PHY might release the sufficient P. Sufficient levels of dietary P showed a similar decrease in body lipid content in other studies (Rodehutscord, 1996; Roy and Lall, 2003). Therefore, higher P availability was the main cause of reduced lipid level in the whole body. P deficiency probably causes the inhibition of beta-oxidation of fatty acids (Schafer *et al*., 1995).

In lieu with the results of proximate composition, supplementation of PHY also resulted in the fish body compared to non-supplemented group. Present results showed effectiveness of PHY in decomposing the phytate and subsequent release of bound elements. These findings are in consistence with those observed for rainbow trout (Sugiura *et al*., 2001), *Pangasius pangasius* (Debnath *et al*., 2005b), *L. rohita* (Baruah *et al*., 2007b) and yellow catfish (Zhu *et al*., 2014; Cheng *et al*., 2015).

The results of the present study showed lower P and N loading while their higher retention has been observed by feeding the fish with PHY treated diet. Morales *et al*. (2015) observed 50% reduction in the excretion of P from rainbow trout fed PHY containing diet. Japanese flounder having PHY treated SBM based diet exhibited similar reduction in P load to the natural water bodies (Sarker *et al*., 2006). Similar to excretion studies, several studies have reported positive impact of PHY addition on P retention in different fish species including catfish (Debnath *et al*., 2005b), tilapia (Tudkaew *et al*., 2008; Tahoun *et al*., 2009), rainbow trout (Cheng and Hardy, 2004) and Korean rockfish (Yoo *et al*., 2005). This increased P retention may likely be explained on the basis of higher P digestibility caused by PHY addition in the diet resulting in its lower excretion into the aquatic environment (Habib *et al*., 2018). Similar to P, higher N retention in response to PHY supplementation has also been observed in many fish species (Hung *et al*., 2015; Tahoun *et al*., 2009; Debnath *et al*., 2005b; Phromkunthong and Gabaudan, 2006; Vandenberg *et al*., 2012).

Supplementation of organic TEs, in the present study, resulted in higher contents of macro and micro mineral in whole body of juveniles in comparison to their traditional sulfate groups. Ligands in the chelates protect the element even after absorption and facilitate the transport and metabolism of these elements (Ashmead,

1992). In correspondence to present results, Apines *et al*. (2004) reported higher contents of Cu and Zn in whole body of rainbow trout which was fed with chelated mineral compared to sulfate supplemented groups. Furthermore, results from the present study were in general agreement with reports on crucian carp (Shao *et al*., 2010), red sea bream (Sarker *et al*., 2005) and rainbow trout (Satoh *et al*., 2001).

Organic TEs supplementation in SBM based diets resulted in higher P and N retention and their lower loading in water bodies. Spears (1989) pointed out that mineral from different sources are metabolized differently after absorption. Ashmead (1992) suggested that chelation may facilitate the post-absorption transport and thus metabolism of absorbed element. Our results agree with previous observations by Apines *et al*. (2003, 2004) and Satoh *et al*. (2001) who observed higher retention of TEs in chelate supplemented groups than their comparable inorganic sources in rainbow trout.

Supplementation of CA in a PHY treated diets, in the present study, resulted in an interaction in improving the whole body DM, CP, EE, ash and mineral deposition. These findings support the hypothesis that addition of organic acid in the diet gives the favourable environment to PHY for optimum performance. These results agree with those reported by Baruah *et al*. (2005, 2007a, b) in a series of experiments who evaluated the combined effect of CA (3%) with PHY (500 IU) in a SBM based diet. They found a synergism between these supplements to enhance the mineral content in whole body, bones and plasma of *L. rohita* juveniles. Moreover, they observed a synergistic response on ash content only while other whole body proximate parameters remained non-responsive to the combined supplementation of CA and PHY. By contrast, Morales *et al*. (2015) observed non-significant interaction between sodium diformate and PHY for whole body DM, CP, P, Ca, Mg and Zn contents in rainbow trout. Similarly, whole body proximate and mineral analysis and vertebral mineral analysis showed no synergistic response in catfish when given simultaneous supplementations of CA and PHY (Zhu *et al*., 2014). Similar observations were also recorded in pig (Radcliffe *et al*., 1998) and laying hens (Nezhad *et al*., 2008) where adding CA in the diet does not affect the PHY efficacy.

Under the experimental conditions of the present study, a significant interaction between PHY and CA was also recorded to enhance the N and P retention in the fingerlings. Better retention of these elements as a consequence of simultaneous supplementation of CA and PHY resulted in reduced excretion of these elements. In contrast to our observations, Morales *et al*. (2015) did not observe such a positive interaction between sodium diformate and PHY for N and P retention and loading in rainbow trout. These differences could be explained considering the differences in diet composition, experimental conditions and gastric pH of fish.

Concerning the interaction behavior of CA and PHY with TE (CA×TE; PHY×TE) and highest degree interaction (CA×PHY×TE), variations have been recorded for the observed parameters. In the present study, CA×TEs and PHY×TEs interactions showed additional affects to improve mineral deposition in whole body of fish compared to their individual affects. Similar affect has also been observed with highest degree interaction (CA×PHY×TEs). However, these interactions were not significant for all the observed parameters. This showed the positive role of CA and PHY in improving the bioavailability of chelated TEs. Further research is needed to confirm this fact. In the present study, higher retention while lower excretion of N and P were recorded in fingerlings in response to 2^{nd} (CA×TEs and PHY×TEs) and highest order interactions (CA×PHY×TEs) with little variations in all the three experimental diets. By contrast, Sarker *et al*. (2007) did not report any significant interaction between CA and organic TEs to influence these parameters.

Positive interaction between CA and TE can be attributed to ligand like properties of CA. Citric acid chelates the Ca and other mineral and prevent the formation of precipitates on the intestinal brush border. This phenomenon also helps in avoiding the co-precipitation of mineral on intestinal membrane and provides more space to the chelated TEs for absorption. Positive interaction of PHY with TE can be attributed to phytate hydrolyzing capability of PHY. Presence of PHY damages the epithelial cells and reduces their absorptive capacity. Phytate-nutrient complexes also precipitate on the intestinal brush border membrane. So, by hydrolyzing the phytate with PHY supplementation, these chelated TEs will be more absorbed.

CONCLUSION

In conclusion, addition of CA, PHY and organically chelated TEs enhanced the bioavailability of major nutrients and various mineral from plant sources which lead to higher body mineralization. Bioavailability of organic TEs seems to be superior to that of traditional inorganic salts. Moreover, supplementation of these additives caused a reduction of N and P excretion in the fingerlings which showed the potential of these additives to reduce the environmental pollution from aquaculture waste, which is the serious problem attached with culture

of aquatic species now-a-days.

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Statement of conflict of interest

The authors declare no conflict of interests regarding the publication of this article

REFERENCES

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T. and Aoki, T., 2001. Availability of supplemental amino acid chelated trace elements in diets containing tricalcium phosphate and phytate to rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, **225**: 431-444. https://doi.org/10.1016/S0044-8486(03)00307-7

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T., Nasu, N. and Fujita, S., 2001. Bioavailability of amino acids chelated and glass embedded zinc to rainbow trout, *Oncorhynchus mykiss*, fingerlings. *Aquacult. Nutr*., **7**: 221-228. https://doi.org/10.1046/j.1365-2095.2001.00178.x

Apines, M.J., Satoh, S., Kiron, V., Watanabe, T. and Fujita, S., 2003b. Bioavailability and tissue distribution of amino acids chelated trace elements in rainbow trout, *Oncorhynchus mykiss*. *Fish. Sci*., **69**: 722-730. https://doi.org/10.1046/j.1444-2906.2003.00679.x

Apines, M.J., Satoh, S., Caipang, C.M.A., Kiron, V., Watanabe, T. and Aoki, T., 2004. Amino acid-chelate: A better source of Zn, Mn and Cu for rainbow trout, *Oncorhynchus mykiss*. *Aquaculture*, **240**: 345-358. https://doi.org/10.1016/j.aquaculture.2004.01.032

Ashmead, H.D., 1992. *The roles of amino acid chelates in animal nutrition*. Noyes Publications, New Jersey, pp. 419.

AOAC, 1995. *Official methods of analysis*, 16th ed. Association of Official Analytical Chemists, Arlington, Virginia, USA.

Baruah, K., Pal, A.K., Sahu, N.P., Jain, K.K., Mukherjee, S.C. and Debnath, D., 2005. Dietary protein level, microbial phytase, citric acid and their interactions on bone mineralization of *Labeo rohita* (Hamilton) juveniles. *Aquacult. Res.,* **36**: 803-812. https://doi.org/10.1111/j.1365-2109.2005.01290.x

Baruah, K., Sahu, N.P., Pal, A.K., Jain, K.K., Debnath, D. and Mukherjee, S.C., 2007a. Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. *Aquacult. Res*., **38**: 109-120. https://doi.org/10.1111/j.1365-2109.2006.01624.x

Baruah, K., Sahu, N.P., Pal, A.K., Debnath, D. and Yengkokpam, S., 2007b. Interactions of dietary microbial phytase, citric acid and crude protein level on mineral utilization by rohu, *Labeo rohita* (Hamilton), juveniles. *J. World Aqucult. Soc*., **38**: 238-249. https://doi.org/10.1111/j.1749-7345.2007.00092.x

Bian, J., Liu, C., Zhang, Z., Wang R. and Gao, Y., 2016. Hydro-geochemical characteristics and health risk evaluation of nitrate in groundwater. *Pol. J. environ. Stud*., **25**: 521. https://doi.org/10.15244/pjoes/61113

Cain, K.D. and Garling, D.L., 1995. Pretreatment of soybean meal with phytase for salmonid diets to reduce phosphorus concentrations in hatchery effluents. *J. Progr. Fish-Cultur*., **57**: 114-119. https://doi.org/10.1577/1548-8640(1995)057<0114:POSMWP>2.3.CO;2

Cao, l., Wang, W., Yang, C., Yang, Y., Diana, J., Yakupitiyage, A., Luo, Z. and Li, D., 2007. Application of microbial phytase in fish feed. *Enzyme Microb. Technol*., **40**: 497-507.

Cheng, N., Chen, P., Lei, W., Feng, M. and Wang, C., 2015. The sparing effect of phytase in plant-protein-based diets with decreasing supplementation of dietary NaH_2PO_4 for juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquacult. Res*., **47**: 3952-3963. https://doi.org/10.1111/are.12845

Cheng, Z.J. and Hardy, R.W., 2004. Effects of microbial phytase supplementation and dosage on apparent digestibility coefficients of nutrients and dry matter in soybean product based diets for rainbow trout *Oncorhynchus mykiss*. *J. World Aquacult. Soc*., **35**: 1-15. https://doi.org/10.1111/j.1749-7345.2004.tb01054.x

Debnath, D., Pal, A.K. and Sahu, N.P., 2005a. Effect of dietary microbial phytase supplementation on growth and nutrient digestibility of *Pangasius pangasius* (Hamilton) fingerlings. *Aquacult. Res*., **36**: 180-187. https://doi.org/10.1111/j.1365-2109.2004.01203.x

Debnath, D., Sahu, N.P., Pal, A.K., Jain, K.K., Yengkokpam, S. and Mukherjee, S.C., 2005b. Mineral status of *Pangasius pangasius* (Hamilton) fingerlings in relation to supplemental phytase: absorption, whole-body and bone mineral contents. *Aquacult. Res*., **36**: 326-335. https://doi.org/10.1111/j.1365-2109.2004.01204.x

Divakaran, S., Leonard, G.O. and Ian, P.F., 2002. Note on the methods for determination of chromic oxide in shrimp feeds. *J. Agric. Fd. Chem*., **50**: 464-467. https://doi.org/10.1021/jf011112s

Habib, R.Z., Afzal, M., Shah, S.Z.H., Fatima, M., Bilal, M. and Hussain, S.M. 2018. Potential of phytase and citric acid treated canola meal based diet to enhance the minerals digestibility in *Labeo rohita* fingerlings. *Pakistan J. Zool.,* **50**: 2045-2050. http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2045.2050

Hardy, R.W., 1995. Current issues in salmonid nutrition. In: *Nutrition and utilization technology in aquaculture* (eds. C.E. Lim and D.J. Sessa). AOCS Press, Champaign, IL, USA, pp. 26-35.

Hossain, M.A., Pandey, A. and Satoh, S., 2007. Effects of organic acids on growth and phosphor usutilization in red sea bream *Pagrus major. Fish. Sci*., **73**: 1309-1317.

Hung, L.T., Thanh, N.T., Pham, M.A. and Browdy, C.L., 2015. A comparison of the effect of dietary fungal phytase and dicalcium phosphate supplementation on growth performances, feed and phosphorus utilization of tra catfish juveniles (*Pangasianodon hypophthalmus* Sauvage, 1878). *Aquacult. Nutr*., **21**: 10-17. https://doi.org/10.1111/anu.12134

Hussain, S.M., Afzal, M., Rana, S.A., Javid, A. and Hussain, M., 2011. Impact of phytase supplementation on nutrient digestibility for *Labeo rohita* fingerlings fed on sunflower meal based diets. *Pak. J. Life Soc. Sci*., **9**: 85-90.

Khajepour, F. and Hosseini, S.A., 2011. Effect of dietary citric acid supplementation and partial replacement of dietary fish meal with soybean meal on calcium and phosphorus of muscle, scute and serum of Beluga, *Huso huso*. *Afr. J. Biotechnol*., **10**: 14652-14655. https://doi.org/10.5897/AJB11.1312

Khajepour, F. and Hosseini, S.A., 2012. Citric acid improves growth performance and phosphorus digestibilityin Beluga (*Huso huso*) fed diets where soybean meal partly replaced fish meal. *Anim. Feed Sci. Technol*., **171**: 68-73. https://doi.org/10.1016/j.anifeedsci.2011.10.001

Kirchgessner, M. and Roth, F.X., 1982. Fumaric acid as a feed additive in pig nutrition. *Pig News Inf*., **3**: 259-264.

Kumar, V., Sinha, A.K., Makkar, H.P.S., Boeck, D.D. and Becker, K., 2012. Phytate and phytase in fish nutrition. *J. Anim. Physiol. Anim. Nutr*., **96**: 335-364. https://doi.org/10.1111/j.1439-0396.2011.01169.x

Lall, S.P. and Lewis, L.M., 2007. Role of nutrients in skeletal metabolism and pathology in fish an overview. *Aquaculture*, **267**: 3-19. https://doi.org/10.1016/j.aquaculture.2007.02.053

Lall, S.P., 1991. Digestibility, metabolism and excretion of dietary phosphorus in fish. In: *Nutritional strategies and aquaculture waste* (eds. C.B. Cowey and C.Y. Cho). Proceedings of the First International Symposium on Nutritional Strategies in Management of Aquaculture Waste. University of Guelph, Guelph, pp. 21-35.

Lewis, Jr. W.M. and Wurtsbaugh, W.A., 2008. Control of lacustrine phytoplankton by nutrients: erosion of the phosphorus paradigm. *Int. Rev. Hydrobiol*., **93**: 446. https://doi.org/10.1002/iroh.200811065

Liu, L., Zhou, Y., Wu, J., Zhang, W., Abbas, K., Fang, L.X. and Luo, Y., 2014. Supplemental graded levels of neutral phytase using pretreatment and spraying methods in the diet of grass carp, *Ctenopharyngodon idellus*. *Aquacult. Res*., **45**: 1932-1941. https://doi.org/10.1111/are.12145

Lunger, A.N., Mclean, E. and Craig, S.R., 2007. The effects of organic protein supplementation upon growth, feed conversion and texture quality parameters in juvenile cobia (*Rachycentron canadum*). *Aquaculture*, **264**: 342-352. https://doi.org/10.1016/j.aquaculture.2006.12.012

Milenkovic, N., Damjanovic, M. and Ristic, M., 2005. Study of heavy metal pollution in sediments from the iron gate (Danuae river), Serbia and Montenegro. *Pol. J. environ. Stud*., **14**: 781-787.

Morales, G.A., Denstadli, V., Collins, S.A., Mydland, L.T., Moyano, F.J. and Overland, M., 2015. Phytase and sodium diformate supplementation in a plant-based diet improves protein and mineral utilization in rainbow trout (*Oncorhynchus mykiss*). *Aquacult. Nutr.,* **22**: 1301-1311. https://doi.org/10.1111/anu.12340

Nezhad, Y.E., Sis, M.N., Gholshani, A.A., Saedi, Y. and Aminvakili, R., 2008. The effects of combination of citric acid and microbial phytase on the concentration of some minerals of serum and parameters of mineralization of tibia in commercial laying hens. *Asian J. Anim. Vet. Adv*., **3**: 375-380. https://doi.org/10.3923/ajava.2008.375.380

Ng, W.K., Koh, C.B., Sudesh, K. and Zahrah, A.S., 2009. Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, *Oreochromis* sp., and subsequent survival during a challenge test with *Streptococcus agalactiae*. *Aquacult. Res*., **40**: 1490-1500. https://doi.org/10.1111/j.1365-2109.2009.02249.x

Nwanna, L.C., Kolahsa, M., Eisenreich, R. and Schwarz,

F.J., 2008. Pre-treatment of dietary plant feed stuffs with phytase and its effect on growth and mineral concentration in common carp (*Cyprinus carpio*). *J. Anim. Physiol. Anim. Nutr*., **92**: 677-682. https://doi.org/10.1111/j.1439-0396.2007.00764.x

Pandey, A. and Satoh, S., 2008. Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. *Fish. Sci.*, **74**: 867-874. https://doi.org/10.1111/j.1444-2906.2008.01601.x

Paripatananont, T. and Lovell, R.T., 1995. Chelated zinc reduces the dietary zinc requirement of channel catfish, *Ictalurus punctatus*. *Aquaculture*, **133**: 73-82. https://doi.org/10.1016/0044-8486(94)00404-C

Park, K.W., Rhee, A.R., Um, J.S. and Paik, I.K., 2009. Effect of dietary available phosphorus and organic acids on the performance and egg quality of laying hens. *J. appl. Poult. Res*., **18**: 598-604. https://doi.org/10.3382/japr.2009-00043

Parveen, J., 1999. *Reduction of environmental phosphorus loading from carp culture*. Master's thesis, Tokyo University of Fisheries, Tokyo.

Phromkunthong, W. and Gabaudan, J., 2006. Use of microbial phytase to replace inorganic phosphorus in sex-reversed red tilapia: 1 dose response. *Songklanakarin J. Sci. Technol*., **28**: 731-743.

Radcliffe, J.S., Zhang, Z. and Kornegay, E.T., 1998.The effects of microbial phytase, citric acid, and their interaction in a corn-soybean meal-based diet for weanling pigs. *J. Anim. Sci*., **76**: 1880-1886. https://doi.org/10.2527/1998.7671880x

Rodehutscord, M., 1996. Response of rainbow trout (*Oncorhynchus mykiss*) growing from 50 to 200 g to supplements of dibasic sodium phosphate in a purified diet. *J. Nutr*., **126**: 324-331. https://doi.org/10.1093/jn/126.1.324

Roy, P.K. and Lall, S.P., 2003. Dietary phosphorus requirement of juvenile haddock (*Melanogrammus aeglefinus*, L.). *Aquaculture*, **221**: 451-468. https://doi.org/10.1016/S0044-8486(03)00065-6

Sarker, S.A., Satoh, S. and Kiron, V., 2005. Supplementation of citric acid and amino acid-chelated trace element to develop environment-friendly feed for red sea bream, *Pagrus major*. *Aquaculture*, **248**: 3-11. https://doi.org/10.1016/j.aquaculture.2005.04.012

Sarker, P.K., Fukada, H., Hosokawa, H. and Masumoto, T., 2006. Effects of phytase with inorganic phosphorus supplement diet on nutrient availability of Japanese flounder (*Paralichthys olivaceus*). *Aquacult. Sci*., **54**: 391-398.

Sarker, A.S.A., Satoh, S. and Kiron, V., 2007. Inclusion of citric acid and/or amino acid-chelated trace elements in alternate plant protein source diets affects growth and excretion of nitrogen and phosphorus in red sea bream *Pagrus major*. *Aquaculture*, **262**: 436-443. https://doi.org/10.1016/j.aquaculture.2006.10.007

Sarker, M.S.A., Satoh, S., Kamata, K., Haga,Y. and Yamamoto, Y., 2012. Partial replacement of fish meal with plant protein sources using organic acids to practical diets for juvenile yellowtail, *Seriola quinqueradiata*. *Aquacult. Nutr*., **18**: 81-89. https://doi.org/10.1111/j.1365-2095.2011.00880.x

Satoh, S., Takeuchi, T. and Watanabe, T., 1987. Availability to rainbow trout of zinc in white fish meal and of various zinc compounds. *Nippon Suisan Gakk*., **53**: 595-599. https://doi.org/10.2331/suisan.53.595

Satoh, S., Apines, M.J., Tsukioka, T., Kiron, V., Watanabe, T. and Fujita, S., 2001. Bioavailability of amino acid-chelated and glass-embedded manganese to rainbow trout, *Oncorhynchus mykiss* (Walbaum), fingerlings. *Aquacult. Res*., **32**: 18-25. https://doi.org/10.1046/j.1355-557x.2001.00002.x

Satoh, S., Porn-Ngam, N.K., Takeuchi, T. and Watanabe, T., 1996. Influence of dietary phosphorus levels on growth and mineral availability in rainbow trout. *Fish. Sci*., **62**: 483-487. https://doi.org/10.2331/fishsci.62.483

Schafer, A., Koppe, W.M., Meyer-Burgdorff, K.H. and Gunther, K.D., 1995. Effects of a microbial phytase on the utilization of native phosphorus by carp in a diet based on soybean meal. *Water Sci. Technol*., **31**: 149-155. https://doi.org/10.2166/wst.1995.0372

Shao, X., Liu, W., Xu, W., Lu, K., Xia, W. and Jiang, Y., 2010. Effects of dietary copper sources and levels on performance, copper status, plasma antioxidant activities and relative copper bioavailability in *Carassius auratus gibelio*. *Aquaculture*, **308**: 60-65. https://doi.org/10.1016/j.aquaculture.2010.07.021

Sobolewski, W., 2016. Effect of agricultural land use on the water quality of Polish lakes: A regional approach. *Pol. J. environ. Stud*., **25**: 2705-2710. https://doi.org/10.15244/pjoes/63654

Spears, J.W., 1989. Zinc methionine for ruminants: Relative bioavailability of zinc in lambs and effects of growth and performance of growing heifers. *J. Anim. Sci*., **67**: 835-843. https://doi.org/10.2527/jas1989.673835x

Sugiura, S.H., Gabaudan J., Dong, F.M. and Hardy, R.W., 2001. Dietary microbial phytase supplementation and the utilization of phosphorus, trace minerals and protein by rainbow trout [*Oncorhynchus mykiss* (Walbaum)] fed soybean meal-based diets. *Aquacult. Res*., **32**: 583-592. https://doi.

org/10.1046/j.1365-2109.2001.00581.x

Swinkels, J.W., Kornegay, E.T., Zhou, W., Lindemann, M.D., Webb, K.E. and Verstegen, M.W., 1996. Effectiveness of a zinc amino acid chelate and zinc sulfate in restoring serum and soft tissue zinc concentrations when fed to zinc-depleted pigs. *J. Anim. Sci*., **74**: 2420-2430. https://doi.org/10.2527/1996.74102420x

Tahoun, A.M., Abo-State, H.A. and Hammouda, Y.A., 2009. Effect of adding commercial phytase to DDGS based diets on the performance and feed utilization of Nile tilapia (*Oreochromis niloticus*) fingerlings. *Am. Eurasian J. Agric. Environ. Sci*., **5**: 550-555.

Tudkaew, J. Gabaudan, J. and Phromkunthong, W., 2008. The supplementation of phytase RONOZYME® on the growth and the utilisation of phosphorus by sex-reversed red tilapia (*Oreochromis niloticus* L.). *Songklanakarin J. Sci. Technol*., **30**: 17-24.

Vandenberg, G.W., Scott, S.L. and Noue, J.D.L., 2012. Factors affecting nutrient digestibility in rainbow trout (*Oncorynchus mykiss*) fed a plant protein based diet supplemented with microbial phytase. *Aquacult. Nutr*., **18**: 369-379. https://doi.org/10.1111/j.1365-2095.2011.00901.x

Vielma, J., Rouhonen, K. and Lall, S.P., 1999. Supplemental citric acid and particle size of fish-bone meal influence the availability of minerals in rainbow trout *Onchorhynchus mykiss* (Walbaum). *Aquacult. Nutr*., **5**: 65-71. https://doi.org/10.1046/j.1365-2095.1999.00092.x

Watanabe, T., 1988. *Fish nutrition and mariculture. The general aquaculture course*. Kanagawa International Fisheries Training Centre, Japan International Corporation Agency (JICA), Tokyo.

Wedekind, K.J., Hortin, A.E. and Baker, D.H., 1992. Methodology for assessing zinc bioavailability: Efficacy estimates for zinc-methionine, zinc sulfate, and zinc oxide. *J. Anim. Sci*., **70**: 178-187. https://doi.org/10.2527/1992.701178x

Yoo, G.Y., Wang, X., Choi, S., Han, K., Kang, J.C. and Bai, S.C., 2005. Dietary microbial phytase increased the phosphorus digestibility in juvenile Korean rockfish *Sebastes schlegeli* fed diets containing soybean meal. *Aquaculture*, **243**: 315-322. https://doi.org/10.1016/j.aquaculture.2004.10.025

Zhu, Y., Qiu, X., Ding Q., Duan, M. and Wang, C., 2014. Combined effects of dietary phytase and organic acid on growth and phosphorus utilization of juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture*, **430**: 1-8. https://doi.org/10.1016/j.aquaculture.2014.03.023

Zhu, Y., Ding Q., Chan, J., Chen, P., Wang, C., 2015. The effects of concurrent supplementation of dietary phytase, citric acid and vitamin D_3 on growth and mineral utilization in juvenile yellow catfish *Pelteobagrus fulvidraco*. *Aquaculture*, **436**: 143-150. https://doi.org/10.1016/j.aquaculture.2014.11.006

Zyla, K., Ledoux, D.R., Garcia, A. and Veum, T.L., 1995. An *in vitro* procedure for studying enzymatic phosphorylation of phytate in maize-soybean feeds for turkey poults. *Br. J. Nutr*., **74**: 3-17. https://doi.org/10.1079/BJN19950102

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Dietary Requirement of Myo-Inositol for Nile Tilapia (*Oreochromis niloticus*) Fingerlings Fed Practical Diet

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Authors' Contribution
SZHS generated the idea, designed the study and wrote the manuscript. RM performed the experiment and collected the data. MF helped in collection and analysis of data and writing of the manuscript. MA supervised the experiment.

Key words
Growth, Oxidative stress, TBARS, Antioxidant enzyme activities, Fatty acid Profile

ABSTRACT

Present research work was designed to determine the dietary requirement of *myo*-inositol for Nile tilapia (*Oreochromis niloticus*) fingerlings fed practical diet. Five semi-purified diets namely D1, D2, D3, D4 and D5 were formulated by supplementing *myo*-inositol at the graded levels of 0, 100, 200, 400 and 800 mg/kg diet, respectively. Duplicate tanks were allotted for each experimental diet and each tank was stocked with 15 fingerlings. Water quality parameters i.e. temperature, dissolved oxygen and pH were monitored throughout the feeding trial. Growth and survival rate were observed every week during the whole experimental period. At the end of the trial, 10 fingerlings were sacrificed from each replicate to analyze the thiobarbituric acid reactive substances (TBARS), anti-oxidant enzymes and fatty acid composition of gills tissues. Data was subjected to one-way analysis of variance (ANOVA) under completely randomized design (CRD). The results indicated significantly improved growth in Nile tilapia fingerlings in response to *myo*-inositol supplementation. Based on weight gain (%), the quadratic regression analysis indicated that Nile tilapia fingerlings required 457.78 mg/kg *myo*-inositol for normal growth. The activities of antioxidant enzymes (SOD, CAT, GPx) and TBARS content were also enhanced in fishes fed *myo*-inositol added diets than the control group. Also, a significant increase in palmitic acid (16:0 n-0), arachidonic acid (20:4 n-6) and ARA/EPA ratio was recorded in gills tissue in response to *myo*-inositol supplementation. Inclusion of *myo*-inositol significantly decreased the hepatosomatic index while it did not affect the viscerosomatic index in Nile tilapia fingerlings. In conclusion, addition of *myo*-inositol in the diets improved the fish growth and activities of antioxidant enzymes in gills of Nile tilapia fingerlings.

INTRODUCTION

Myo-inositol (MI) is a six-fold alcohol (polyol) of cyclohexane and a very active isomer of inositol in living tissues as a part of cell membrane. In past, it was considered as a vitamin that is necessary for growth and is frequently added in aquatic feeds (Jiang *et al*., 2010). The reason behind this concept was that some fish and shellfish synthesize insufficient amounts of MI in their bodies to compensate their metabolic requirements and thus they need an external source of this vitamin in their diets (Aoe and Masuda, 1967; Shiau and Su, 2004; Jiang *et al*., 2009).

Now concept about MI as a vital vitamin has been changed and is regarded as a vitamin like nutrient that is frequently included in feeds of aquatic organisms (Shiau and Su, 2005). The change in concept about MI as an essential vitamin is due to its de novo synthesis which takes place by cyclization of Glucose-6-Phosphate with the help of an enzyme inositol-1-phosphate synthase (Combs, 1992). Brain and liver are the sites of its synthesis in channel catfish (Burtle and Lovell, 1989), sea bass (Boonyaratpalin and Wanakowat, 1993), and sunshine bass (Deng *et al*., 2002). Microbial flora of intestine is also responsible for its synthesis in common carp (Aoe and Masuda, 1967) some fishes and other vertebrates. However, in some fishes amounts of MI produced by body do not meet body's metabolic demands (Shiau and Su, 2005; Lee *et al*., 2009).

It is a part of phospholipid molecule phosphatidylinositol which is a component of cell membrane and also involves in signal transduction pathway known as phosphoinositide system regulated by hormones, neurotransmitters or growth factors (Aukema and Holub, 1994). In eukaryotic cells, it acts as a structural component of secondary messenger such as phosphatidylinositol (Shiau and Su, 2004) and as a regulator of lipid transport

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metabolism in liver and blood (Shiau and Su, 2003; Wen *et al*., 2007; Diao *et al*., 2010). It improves structure and function of intestinal tissues in fish by increasing its length, weight and enzymatic activity (Jiang *et al*., 2009) and facilitate growth of liver and bone marrow cells, transport of cholesterol and RNA synthesis (Shiau and Su, 2003). Control of cytosolic calcium and relation with secondary messenger indicates its role in central nervous system (Hughes and Michell, 1993; Colodny, 1998; Irvine, 2002). It improves activity of enzymes of enteroyctes in jian carp including catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (Jiang *et al*., 2013) and also acts as an anti-oxidant by improving anti-oxidant enzyme activity and growth in jian carp (Jiang *et al*., 2009b, c, 2010). Oxidative damage to gill by Cu can also be prevented by myo-inositol (Jiang *et al*., 2011).

Its deficiency in some fish species is identified by symptoms including poor growth and appetite, anemia, skin lesion, darkening of skin, edema, delayed gastric emptying, enlarged stomach and lesser activity of enzymes like cholinesterase and aminotransferase (NRC, 2011).

It is considered essential for rainbow trout (McLaren *et al*., 1947; Kitamura *et al*., 1967), chinook salmon (Halver, 1953), common carp (Aoe and Masuda, 1967), red sea bream (Yone *et al*., 1971), Japanese eel (Arai *et al*., 1972), Japanese parrot fish (Ikeda *et al*., 1988) and yellowtail (Hosokawa, 1989). On the other hand, MI in diet is not required by Atlantic salmon, sunshine bass and Nile tilapia for their growth (Waagbø *et al*., 1998; Deng *et al*., 2002; Peres *et al*., 2004).

Dietary requirement for myo-inositol ranges from 400 mg kg^{-1} diet, 250-500, 3400, 440, 550-900, 300, 166, 518 and 617 in hybrid tilapia, *Oreochromis niloticus*, *O. aureus* (Shiau and Su, 2005), *Penaeus monodon* (Shiau and Su, 2004), *Oncorhynchus mykiss* (Kitamura *et al*., 1967), *Cyprinus carpio* L. (Aoe and Masuda, 1967), *Chrysophrys major* (Yone *et al*., 1971), *Salmo salar* L. (Waagbø *et al*., 1998), *Cyprinus carpio* (Jiang *et al*., 2009a), *Ctenopharyngodon idella* (Wen *et al*., 2007) and *Paralichthys olivaceus* (Lee *et al*. 2009). It has not been clarified yet, whether myo-inositol is essential or not for Nile tilapia in practical diet.

MATERIALS AND METHODS

The present research was executed to study the dietary requirement of myo-inositol for Nile tilapia, *Oreochromis niloticus* fingerlings fed practical diet. The experiment was carried out in the Fish Nutrition Laboratory, Department of Zoology, Wildlife and Fisheries, University of Agriculture, Faisalabad.

Fish and experimental conditions

O. niloticus fingerlings were procured from Fisheries Research Farms, University of Agriculture, Faisalabad and acclimatized to experimental conditions for two weeks in V-shaped tanks (UA system) and basal diet was given to fingerlings once a day to apparent satiation. Before the initiation of experiment, fingerlings were dipped in 5g/L NaCl solution to remove fungal infections (Rowland and Ingram, 1991). Dissolved oxygen, pH and water temperature were regulated and tanks were fully aerated through capillary system during the experimental period.

Feed ingredients and experimental diets

Dietary feed ingredients were obtained from local market and chemical composition was analyzed by following AOAC (1995) before the formulation of experimental diets. The composition of basal diet is given in Table I. The feed ingredients were ground to powder form before the preparation of myo-inositol based diets. Five experimental diets D1, D2, D3, D4 and D5 were formulated by supplementing myo-inositol at the levels of 0, 100, 200, 400 and 800 mg/kg diet, respectively and diets were stored at -20 ºC. The proximate composition and fatty acid profile of experimental diets is given in Table II and III, respectively.

Table I.- Composition (%) of basal diet.

Ingredients	Percentage	Ingredients	Percentage
Fishmeal	30	Fish Oil	7
Sunflower meal	20	Minerals mixture*	1
Corn gluten meal	15	Vitamins premix**	1
Wheat flour	14.9	Ascorbic acid	1
Rice Polish	10	Choline Chloride	0.1

*Each kg of mineral mixture containss: Zn (zinc), 3000 mg; Co (cobalt), 40 mg; Cu (copper), 600 mg; I (iodine), 40mg; Fe (iron), 1000 mg; Se (selenium), 3mg; Mn (manganese), 2000 mg. **Each Kg of Vitamin premix contains: Vitamin A, 15 M.I.U.; Vitamin B12, 9000mg; Vitamin B1, 5000mg; Vitamin C, 15000mg; Vitamin B2, 6000mg; Vitamin D3, 3 M.I.U.; Vitamin B6, 4000 mg; Vitamin K3, 4000mg; Folic acid, 750mg; Nicotinic acid, 25000mg; Calcium pantothenate, 10000mg.

Table II.- Proximate composition of experimental diets.

Myo-inositol (mg/kg)	Diet	Dry matter (%)	Crude protein (%)	Crude fat (%)
0	D1	90.54	30.60	8.87
100	D2	91.04	31.22	9.00
200	D3	90.98	30.93	8.99
400	D4	91.13	31.07	9.03
800	D5	90.99	31.07	8.46

Table III.- Fatty acid profile of experimental diets (% of total fatty acid detected).

Fatty acid	D1	D2	D3	D4	D5
14:0 n-0	4.11	4.17	4.22	4.16	4.26
16:0 n-0	16.43	16.49	16.52	16.47	16.42
18:0 n-0	3.77	3.79	3.74	3.76	3.80
16:1 n-7	12.57	12.55	12.57	12.6	12.59
18:1 n-7	5.16	5.18	5.17	5.14	5.11
18:1 n-9	17.68	17.72	17.74	17.71	17.66
18:2 n-6	9.51	9.54	9.49	9.53	9.53
20:4 n-6	0.94	0.98	0.95	0.98	0.96
18:3 n-3	5.37	5.33	5.37	5.30	5.35
20:5 n-3	10.29	10.11	10.17	10.21	10.25
22:5 n-3	3.26	3.20	3.13	3.16	3.12
22:6 n-3	10.91	10.94	10.93	10.98	10.95
Total	100	100	100	100	100
Saturated	24.31	24.45	24.48	24.39	24.48
Monounsaturated	35.41	35.45	35.48	35.45	35.36
n-3	29.83	29.58	29.60	29.65	29.67
n-6	10.45	0.98	10.44	10.51	10.49
n-9	17.72	17.74	17.74	17.71	17.66
ARA/EPA	0.368	0.369	0.093	0.095	0.093
EPA/DHA	0.94317	0.924	0.930	0.929	0.936
n-3/n-6	1.683	1.667	1.668	1.674	1.680
Monoenes/Polyenes	0.610	0.733	0.614	0.612	0.611

Feeding procedure and collection of uneaten diet

Fish were given experimental diets at 2% of their live wet weight and 15 fingerlings were placed in each tank. After three hours of feeding session, the uneaten diet particles were collected by opening the valve I and valve II subsequently of the tanks (Habib *et al*., 2018). After removing particles, tanks were washed completely and refilled with water. The collected diets were dried and stored to calculate feed conversion ratio (FCR). The feeding experiment was run for a period of 2 months.

At the termination of feeding experiment, four fish from each replicate were collected, euthanized by overdose of clove oil and sacrificed. Samples were extracted and stored at -20°C for different analysis.

Proximate analysis of diets

The standard methods of AOAC (1995) were followed to analyze the moisture (by oven-drying at 105°C for 12 h), crude fat (by petroleum ether extraction method hrough Soxtec HT2 1045 system), crude protein ((N x 6.25) by micro Kjeldahl apparatus), crude fiber (as loss on ignition of dried lipid-free residues after digestion with 1.25% H_2SO_4 and 1.25% NaOH), and crude ash (by ignition at 650°C (Eyela-TMF 3100) to constant weight) contents of experimental diets. Carbohydrates were calculated by subtracting the values of crude protein (%), crude fat (%), crude fiber (%) from dry matter.

Fatty acid profile

The extracted fat was used for the estimation of gills fatty acid profile following IUPAC (1987) standard method.

Growth study

Growth performance was evaluated after every week by taking gross weight of fish from each treatment. Growth and feed performance and feed were evaluated in terms of absolute weight gain (WG), weight gain %, feed conversion ratio (FCR), specific growth rate (SGR) and survival rate (%) using the following formula:

$$\text{Absolute WG (g)} = \text{Final weight (g)} - \text{Initial weight (g)}$$

$$\text{Weight gain \%} = \frac{\text{Final weight} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{SGR} = \frac{\ln[\text{Initial weight (g)} - \text{Final weight (g)}]}{\text{Experimental duration in days}} \times 100$$

$$\text{Survival rate \%} = \frac{\text{Final number of fish}}{\text{Initial number of fish}} \times 100$$

$$\text{FCR} = \frac{\text{Total dry feed intake (g)}}{\text{Wet weight gain (g)}}$$

At the end of the experiment, three fishes were selected randomly from each tank and their body, viscera and gills weights were recorded individually to measure the hepatosomatic and viscerosomatic index.

$$\text{Hepatosomatic index} = \frac{\text{Weight of liver}}{\text{Total body weight}} \times 100$$

$$\text{Viscerosomatic index} = \frac{\text{Weight of Viscera}}{\text{Total body weight}} \times 100$$

TBARS assay

The measurement of thiobarbituric acid reactive substances (TBARS) in fish gills was specifically assayed by following Gatta *et al*. (2000).

Antioxidant enzymes determination

A homogenate was prepared by homogenizing gills samples in phosphate buffer solution in a tissue homogenizer (Wisd HG-15D, DAIHAN Scientific). The homogenate was filtered and then filtrate was centrifuged at 10, 000 rotates per minutes for 15 min.. The supernatant collected and frozen in sample vials for the determination of superoxide dismutase, catalase and peroxidase enzyme activity. Superoxide dismutase activity was determined following the method of Giannopolitis and Ries (1977). Catalase (CAT) activity was determined by measuring its

ability to decompose hydrogen peroxide concentration at 240 nm following the method of Chance and Mehaly (1977). Peroxidase activity was calculated by measuring its ability to reduce the concentration of hydrogen peroxide at 470 nm (Civello *et al*., 1995).

Statistical analysis

The optimum level of dietary myo-inositol requirements was determined with reference to growth performance. Statistical analysis of data was done by applying one-way analysis of variance (ANOVA) (Steel *et al*., 1996). The differences among means were compared by Student Newman-Keuls Test and considered significant at $p<0.05$ (Sneadecor and Cochran, 1991).

RESULTS

Effect of myo-inositol supplemented diets on growth performance of Nile tilapia is given in Table IV. Significantly improved growth was observed in Nile tilapia fingerlings in response to myo-inositol supplementation. Based on weight gain (%), the quadratic regression analysis indicated that Nile tilapia required 457.78 mg/kg myo-inositol for normal growth (Fig. 1).

The effect of antioxidant enzyme activity in gills of Nile tilapia fed myo-inositol supplemented diet is given in Table V. The activities of antioxidant enzymes (SOD, CAT, GPx) were enhanced in fishes fed myo-inositol added diets than the control group.

The effect of myo-inositol supplemented diet on fatty acid profile of Nile tilapia is represented in Table VI. A significant increase in palmitic acid (16:0 n-0), arachidonic acid (20:4 n-6) and ARA/EPA ratio was observed in response to myo-inositol supplementation in gills tissue.

The effect of hepatosomatic index, Viscerasomatic index and TBARS contents in gills of Nile tilapia fed myo-inositol supplemented diet is given in Table VII. Inclusion of myo-inositol significantly decreased hepatosomatic index while it did not affect the viscerosomatic index in Nile tilapia fingerlings. Fishes fed basal diet had significantly lower gills TBARS compared with those fed myo-inositol supplemented diets and showed an increasing trend with increasing myo-inositol level.

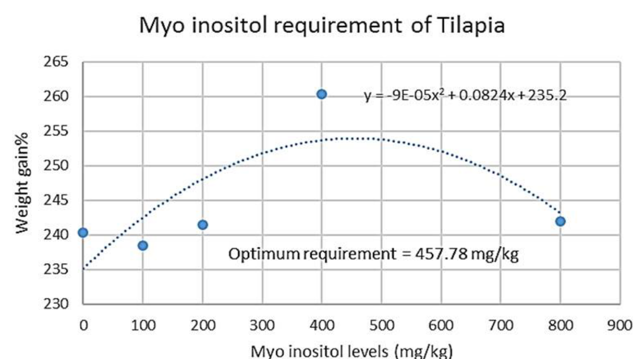

Fig. 1. Quadratic regression analysis showing the dietary requirement of myo-inositol for Nile tilapia.

Table IV.- Effect of myo-inositol supplemented diets on growth performance of Nile tilapia.

Myo-inositol (mg/kg)	0	100	200	400	800	PSE	P-value
Diets	**D1**	**D2**	**D3**	**D4**	**D5**		
Final weight (g)	21.69b	21.64b	21.79b	22.99a	21.87b	0.102	0.0011 **
Absolute weight gain	15.32b	15.25b	15.41b	16.61a	15.47b	0.102	0.102
Weight gain %	240.31b	238.47b	241.53b	260.34a	241.98b	1.752	0.0015 **
SGR	2.04b	2.03b	2.04b	2.13a	2.04b	0.008	0.0015 **
FI (g)	27.30a	26.20a	26.10a	23.97b	25.80a	0.408	0.0177 *
FCR	1.78a	1.71a	1.69a	1.44b	1.66a	0.034	0.0062 **
Survival rate (%)	87.5	91.66	91.66	91.66	87.5	2.635	0.5983 ns

Table V.- Effect of antioxidant enzyme activity in gills of Nile tilapia fed myo-inositol supplemented diet.

Myo-inositol (mg/kg)	0	100	200	400	800	PSE	P-value
Diets	**D1**	**D2**	**D3**	**D4**	**D5**		
Superoxide dismutase (U/mg protein)	5.25[b]	4.97[b]	5.89[b]	6.22[b]	8.25[a]	0.352	0.0069 **
Catalase (U/mg protein)	36.00[d]	39.91[c]	44.40[b]	45.72[b]	50.26[a]	0.942	0.0008 ***
Glutathione peroxidase (mU/g protein)	75.02[c]	77.64[b]	79.19[b]	83.92[a]	85.81[a]	0.532	0.0001 ***

Table VI.- Effect of myo-inositol supplemented diet on fatty acid profile of Nile tilapia.

Myo-inositol (mg/kg)	0	100	200	400	800	PSE	P-value
Diets	D1	D2	D3	D4	D5		
14:0 n-0	5.93	5.89	5.56	5.85	5.77	0.17	0.594
16:0 n-0	17.57^{ab}	17.61^{ab}	17.77^{a}	17.29^{b}	17.34^{b}	0.074	0.0269 *
18:0 n-0	6.17	6.4	6.14	6.3	6.41	0.118	0.4509 ns
16:1 n-7	13.34	13.37	13.26	13.21	13.4	0.224	0.9667 ns
18:1 n-7	4.88	4.96	5.15	5.51	4.9	0.127	0.0704 ns
18:1 n-9	15.21	15.13	15.31	15.19	15.24	0.179	0.9650 ns
18:2 n-6	7.29	7.08	7.13	7.31	6.98	0.142	0.4972 ns
20:4 n-6	0.65^{ab}	0.53^{b}	0.77^{a}	0.81^{a}	0.55^{b}	0.044	0.0193 *
18:3 n-3	4.06	4.21	3.98	3.91	4.21	0.059	0.0506 ns
20:5 n-3	11.3	11.3	11.16	11.18	11.46	0.178	0.7714 ns
22:5 n-3	2.45	2.38	2.51	2.5	2.34	0.057	0.2936 ns
22:6 n-3	11.12	11.1	11.22	10.9	11.37	0.098	0.1263 ns
Saturated	29.68	29.9	29.48	29.45	29.53	0.257	0.7175 ns
Monounsaturated	33.44	33.47	33.73	33.92	33.55	0.293	0.7569 ns
n-3	28.93	29.01	28.89	28.5	29.38	0.190	0.1471 ns
n-6	7.94	7.61	7.9	8.13	7.53	0.117	0.0674 ns
n-9	15.21	15.13	15.31	15.19	15.24	0.179	0.9650 ns
ARA/EPA	0.05^{ab}	0.04^{b}	0.06^{ab}	0.07^{a}	0.04^{b}	0.004	0.0242 *
EPA/DHA	1.01	1.01	0.99	1.02	1	0.02	0.8391 ns
n-3/n-6	1.9	1.91	1.88	1.87	1.92	0.006	0.4069 ns
Monoenes/Polyenes	0.64	0.64	0.64	0.65	0.64	0.002	0.7758 ns

Table VII.- Hepatosomatic index, Viscerasomatic index and Gills TBARS of Nile tilapia fed myo-inositol supplemented diet.

Myo-inositol (mg/kg)	0	100	200	400	800	PSE	P-value
Diets	D1	D2	D3	D4	D5		
Viscerosomatic index	2.25	2.24	2.22	2.13	2.18	0.026	0.0960 ns
Hepatosomatic index	0.99^{a}	0.88^{b}	0.90^{b}	0.87^{b}	0.89^{b}	0.015	0.0115*
Gills TBARS (mg/g protein)	1.62^{c}	1.68^{c}	2.05^{b}	2.39^{a}	2.49^{a}	0.056	0.0003***

DISCUSSION

Growth performance is always considered as an important indicator for nutritional evaluation of a diet. In the present study, significantly higher final weight, absolute weight gain, weight gain and specific growth rate clearly confirmed that maximal growth of Nile tilapia fingerlings necessarily require the addition of myo-inositol in diet. Similar results were obtained in different studies on hybrid tilapia (Shiau and Su, 2005), olive flounder (Lee *et al*., 2009), jian carp (Jiang *et al*., 2009) and juvenile barramundi (Diao *et al*., 2010) that showed a requirement for external source for optimal growth and to prevent clinical deficiency symptoms. In contrast to our results, studies of Deng *et al*. (2002) and Peres *et al*. (2004) showed that addition of MI in experimental diets did not show any significant effect on juvenile sunshine bass and Nile tilapia, respectively. In above mentioned species *de novo* synthesis of myo-inositol was found sufficient for normal growth (Deng *et al*., 2002; Peres *et al*., 2004). In present study, the quadratic regression analysis of weight gain (%) indicated that Nile tilapia fingerlings required

457.78 mg/kg myo-inositol for normal growth which is close to the value recorded in hybrid tilapia fed purified diet who required 400 mg/kg myo-inositol for optimal growth (Shiau and Su, 2005). The requirement of myo-inositol for Nile tilapia fingerlings recorded in our study was higher than reported for parrot fish (94.3 mg/kg, Khosravi*et al*., 2015), gibel carp (165.3 mg/kg, Gong *et al*., 2014), grass carp (166 mg/kg, Wen *et al*., 2007) and Atlantic salmon (300 mg/kg, Waagbø *et al*., 1998) but lower than reported for juvenile barramundi (507 mg/kg, Diao *et al*., 2010), jian carp (518 mg/kg, Jiang *et al*., 2009a) and olive flounder (617 mg/kg, Lee *et al*., 2009). In various aquatic species, the requirement of dietary myo-inositol differs and depends upon fish species (Shiau and Su, 2005; Jiang *et al*., 2009), physiological and nutritional stress, growth stage (Kukiss and Mookerjea, 1978; Lee *et al*., 2009; NRC, 2011) experimental conditions and diet composition. Myo-inositol requirement is also affected by different dietary sources of lipids and carbohydrates (Chu and Geyer, 1983; Kukiss and Mookerjea, 1978).

Body indices comprising of hepatosomatic index (HSI) and viscerosomatic index (VSI) also influenced by supplementation of myo-inositol. In current study, significantly lower hepatosomatic index was observed while viscerosomatic index did not influence significantly in fishes fed myo-inositol added diets. Gong *et al*. (2014) found significantly lower HSI in juvenile gibel carp which is in accordance to our results. In contrast to our results, Deng *et al*. (2002) reported that supplementation of myo-inositol did not influence HSI significantly among all dietary treatments.

Myo-inositol possesses lipotropic properties and helps in proper fat metabolism by increasing fat export from liver (Halver, 1989). In the present study, inclusion of myo-inositol remained unsuccessful in affecting gill fatty acid composition except for palmitic acid (16:0 n-0), arachidonic acid (20:4 n-6) and ARA/EPA. The reason behind this may be the synthesis of phospholipids by using the myo-inositol in fish gills. Similar results were obtained in previous study conducted on parrot fish that showed non-significant differences in fatty acid composition in liver tissue in response to myo-inositol supplementation (Khosravi *et al*., 2015). Lee *et al*. (2009) found a significant increase in polyunsaturated fatty acids in liver of olive flounder which was in accordance to our results. The possible reason suggested in their study for higher PUFA content was an increment in the synthesis of phospholipids due to addition of myo-inositol in diets.

Thiobarbituric reactive substances (TBARS) are one of the most important indicators of oxidative changes in lipids (Shahidi and Hong, 1991). In our study, significantly higher levels of TBARS were obtained in fishes fed different dietary levels of myo-inositol. To our knowledge, there is no study indicating the effect of supplementation of myo-inositol on TBARS level in any fish species.

Increase in antioxidative capacity of cell increases its viability and integrity (Chen *et al*., 2009; Jiang *et al*., 2009a) and also can limit the detrimental effects of lipid peroxidation (Cabrini *et al*., 1998). Superoxide dismutase (SOD) is the first and only endogenous antioxidant enzyme that respond to oxidative stress by removing toxic superoxide anions and oxygen radicals (Winston and Di-Giulio, 1991; Reddi *et al*., 2009) while catalase (CAT) provides a defense against the toxic hydroxal radicals (David *et al*., 2008). According to our results, the activities of SOD, CAT and GPx significantly increased by addition of myo-inositol in gills tissues. Myo-inositol possess the chelating character which enhance the ability of scavenging of hydroxyl radical in gills, which indicated that myo-inositol may prevent the free radical generation and oxidative damage of tissue (Santoro *et al*., 2007; Jiang *et al*., 2010b). Our results were in accordance to the studies conducted by Jiang *et al*. (2010, 2013) who reported increased antioxidant activity in muscles and intestine of jian carp, respectively. Jiang *et al*. (2009b) reported similar increase in activities of antioxidant enzymes in jian carp body. Based on the above mentioned results, we can conclude that myo-inositol supplementation can increase antioxidant activity of SOD, CAT and GPx but the mechanism which is responsible for it is still unknown.

CONCLUSION

In conclusion, supplementation of myo-inositol significantly enhanced growth, and antioxidant enzymes (SOD, CAT, GPx) activities in gills of Nile tilapia. Also a significant increase in palmitic acid (16:0 n-0), arachidonic acid (20:4 n-6) and ARA/EPA ratio was observed in response to myo-inositol supplementation in gills tissue. However, inclusion of myo-inositol significantly decreased hepatosomatic index while it did not affect viscerosomatic index in Nile tilapia fingerlings.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

AOAC, 1995. *Official methods of analysis*, 15th ed., Association of Official Analytical Chemist, Washington, DC, USA, pp. 1094.

Aoe, H. and Masuda, L., 1967. Water-soluble vitamin requirements of carp-II. Requirements for para-aminobenzoic acid and inositol. *Bull. Japanese Soc.*

Sci. Fish., **33**: 674-685. https://doi.org/10.2331/suisan.33.674
Arai, S., Nose, T. and Hashimoto, Y., 1972. Qualitative requirements of young eels, *Anguilla japonica*, for water-soluble vitamins and their deficiency symptoms. *Bull. Freshw. Res. Lab. Tokyo,* **22**: 69-83.
Aukema, H.M. and Holub, B.J., 1994. Inositol and pyrroloquinolinequinone. In: *Modern nutrition in health and disease* (eds. M.E. Shils, J.A. Olson and M. Shike). 8th edn., Lea and Febiger, Philadelphia, pp. 466-472.
Boonyaratpalin, M. and Wanakowat, J., 1993. Effect of thiamin, riboflavin, pantothenic acid and inositol on growth, feed efficiency and mortality of juvenile sea bass. In: *Fish nutrition in practice* (eds. S.J. Kanshik and P. Luquet). INRA, Cedex, France, pp. 819-828.
Burtle, G.J. and Lovell, R.T., 1989. Lack of response of channel catfish (*Ictalurus punctatus*) to dietary myo-inositol. *Canadian J. Fish. aquat. Sci.,* **46**: 218-222. https://doi.org/10.1139/f89-030
Cabrini, L., Bergami, R., Fiorentini, D., Marchetti, M., Landi, L. and Tolomelli, B., 1998. Vitamin B6 deficiency affects antioxidant defenses in rat liver and heart. *Biochem. Mol. Biol. Int.,* **46**: 689-697.
Chance, B. and Mehaly, A.C., 1977. Assay of catalase and peroxidases. *Methods Enzymol.,* **2**: 764-775. https://doi.org/10.1016/S0076-6879(55)02300-8
Chen, J., Zhou, X.Q., Feng, L., Liu, Y. and Jiang, J., 2009. Effects of glutamine on hydrogen peroxide-induced oxidative damage in intestinal epithelial cells of jian carp (*Cyprinus carpio* var. Jian). *Aquaculture,* **288**: 285-289. https://doi.org/10.1016/j.aquaculture.2008.10.053
Chu, S.H.W. and Geyer, R.P., 1983. Tissue content and metabolism of myo-inositol in normal and lipodystrophic gerbils. *J. Nutr.,* **113**: 293-303. https://doi.org/10.1093/jn/113.2.293
Civello, P.M., Marting, G.A., Chaves, A.R. and Anan, M.C., 1995. Peroxidase from strawberry fruit by partial purification and determination of some properties. *J. Agric. Fd. Chem.,* **43**: 2596-2601. https://doi.org/10.1021/jf00058a008
Colodny, L., 1998. Inositol clinical applications for exogenous use. *Altern. Med. Rev.,* **3**: 432-447.
Combs, G.F. 1992. Quasi-vitamins. In: *The vitamins: Fundamental aspects in nutrition and health* (ed. G.F. Combs). Academic Press, San Diego, CA, USA, pp. 411-419.
David, M., Munaswamy, V., Halappa, R. and Marigoudar, S.R., 2008. Impact of sodium cyanide on catalase activity in the freshwater exotic carp, *Cyprinus carpio* (Linnaeus). *Pest. Biochem. Physiol.,* **92**: 15-18. https://doi.org/10.1016/j.pestbp.2008.03.013
Deng, D.F., Hemre, G.I. and Wilson, R.P., 2002. Juvenile sunshine bass (*Morone chrysops* female × *Morone saxatilis* male) do not require dietary myo-inositol. *Aquaculture,* **213**: 387-393. https://doi.org/10.1016/S0044-8486(02)00119-9
Diao, S.Q., Huang, Z., Chen, S.S., Niu, J., Li, Z.J., Ding, X. and Lin, H.Z., 2010. Effect of dietary inositol on growth, feed utilization and blood biochemical parameters for juvenile barramundi (*Lates calcarifer* Bloch). *Am. J. Agric. Biol. Sci.,* **5**: 370-375. https://doi.org/10.3844/ajabssp.2010.370.375
Gatta, P.P., Pirini, M., Testi, S., Vignola, G. and Monetti, P.G., 2000. The influence of different levels of dietary vitamin E on sea bass *Dicentrarchus labrux* flesh quality. *Aquacult. Nutr.,* **6**: 47-52. https://doi.org/10.1046/j.1365-2095.2000.00127.x
Giannopolitis, C.N. and Ries, S.K., 1977. Superoxide dismutase occurrence in higher plants. *Pl. Physiol.,* **59**: 309-314. https://doi.org/10.1104/pp.59.2.309
Gong, W., Lei, W., Zhu, X., Yang, Y., Han, D. and Xie, S., 2014. Dietary myo-inositol requirement for juvenile gibel carp (*Carassius auratus gibelio*). *Aquacult. Nutr.,* **20**: 514-519. https://doi.org/10.1111/anu.12104
Habib, R.Z., Afzal, M., Shah, S.Z.H., Fatima, M., Bilal, M. and Hussain, S.M. 2018. Potential of phytase and citric acid treated canola meal based diet to enhance the minerals digestibility in *Labeo rohita* fingerlings. *Pakistan J. Zool.,* **50**: 2045-2050. http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2045.2050
Halver, J.E., 1953. Fish diseases and nutrition. *Trans. Am. Fish. Soc.,* **83**: 254-261. https://doi.org/10.1577/1548-8659(1953)83[254:FDAN]2.0.CO;2
Halver, J.E., 1989. The vitamins, In: Fish nutrition (ed. J.E. Halver), 2nd edn. Academic Press, San Diego, CA, USA, pp. 31-109.
Hosokawa, H., 1989. *The vitamin requirements of fingerling yellowtail*, Seriola quinqueradiata. Ph. D. dissertation, Kochi University, Japan.
Hughes, P.J. and Michell, R.H., 1993. Novel inositol containing phospholipids and phosphates: Their synthesis and possible new roles in cellular signaling. *Curr. Opin. Neurobiol.,* **3**: 383-400. https://doi.org/10.1016/0959-4388(93)90132-I
Ikeda, S., Ishibashi, Y., Murata, O., Nasu, T. and Harada, T., 1988. Qualitative requirements of the

Japanese parrot fish for water-soluble vitamins. *Bull. Japanese Soc. Sci. Fish.,* **54**: 2029-2035. https://doi.org/10.2331/suisan.54.2029

IUPAC, 1987. *Standard methods for the analysis of oils, fats and derivatives*, 7th rev. (eds. C. Paquot and A. Hautfenne). International Union of Pure and Applied Chemistry Blackwell Scientific, London.

Irvine, R.F., 2002. *Nuclear lipid signaling*. Science's STKE: Signal Transduction Knowledge Environment 2002, re13. https://doi.org/10.1126/stke.2002.150.re13

Jiang, W.D., Zhou, X.Q., Feng, L., Liu, Y. and Jiang, J., 2009a. Growth, digestive capacity and intestinal microflora of juvenile jian carp (*Cyprinus carpio* var. Jian) fed graded levels of dietary inositol. *Aquacult. Res.,* **40**: 955-962. https://doi.org/10.1111/j.1365-2109.2009.02191.x

Jiang, W.D., Feng, L., Liu, Y., Jiang, J. and Zhou, X.Q., 2009b. Myo-inositol prevents oxidative damage, inhibits oxygen radical generation, and increases antioxidant enzyme activities of juvenile jian carp (*Cyprinus carpio* var. Jian). *Aquacult. Res.,* **40**: 1770-1776. https://doi.org/10.1111/j.1365-2109.2009.02283.x

Jiang, W.D., Feng, L., Liu, Y., Jiang, J., Hu, K., Li, S.H. and Zhou, X.Q., 2010a. Lipid peroxidation, protein oxidant and antioxidant status of muscle, intestine and hepatopancreas for juvenile jian carp (*Cyprinus carpio* var. Jian) fed graded levels of myo-inositol. *Fd. Chem.,* **120**: 692-697. https://doi.org/10.1016/j.foodchem.2009.10.062

Jiang, W.D., Feng, L., Liu, Y., Jiang, J., Hu, K., Li, S.H. and Zhou, X.Q., 2010b. Effects of graded levels of dietary myo-inositol on nonspecific immune and specific immune parameters in juvenile jian carp (*Cyprinus carpio* var. Jian). *Aquacult. Res.,* **41**: 1413-1420.

Jiang, W., Wu, P., Kuang, S., Liu, Y., Jiang, J., Hu, K., Li, S., Tang, L., Feng, L. and Zhou, X.Q., 2011. Myo-inositol prevents copper-induced oxidative damage and changes in antioxidant capacity in various organs and the enterocytes of juvenile jian carp (*Cyprinus carpio* var. Jian). *Aquat. Toxicol.,* **105**: 543-551. https://doi.org/10.1016/j.aquatox.2011.08.012

Jiang, W.D., Kuang, S.Y., Liu, Y., Jiang, J., Hu, K., Li, S.H., Tang, L., Feng, L. and Zhou, X.Q., 2013. Effects of myo-inositol on proliferation, differentiation, oxidative status and antioxidant capacity of carp enterocytes in primary culture. *Aquacult. Nutr.,* **19**: 45-53. https://doi.org/10.1111/j.1365-2095.2011.00934.x

Khosravi, S., Lim, S.J., Rahimnejad, S., Kim, S.S., Lee, B.J., Kim, K.W., Han, H.S. and Lee, K.J., 2015. Dietary myo-inositol requirement of parrot fish, *Oplegnathus fasciatus*. *Aquaculture,* **436**: 1-7. https://doi.org/10.1016/j.aquaculture.2014.10.034

Kitamura, S., Suwa, T., Ohara, S. and Nakagawa, K., 1967. Studies on vitamin requirements of rainbow trout: 2. The deficiency symptoms of fourteen kinds of vitamins. *Bull. Japanese Soc. Sci. Fish.,* **33**: 1120-1125. https://doi.org/10.2331/suisan.33.1120

Kukiss, A. and Mookerjea, S., 1978. Inositol. *Nutr. Rev.,* **36**: 233-238.

Lee, B.J., Lee, K.J., Lim, S.J. and Lee, S.M., 2009. Dietary myo-inositol requirement for Olive flounder, *Paralichthys olivaceus* (Temminchet Schlegel). *Aquacult. Res.,* **40**: 83-90. https://doi.org/10.1111/j.1365-2109.2008.02067.x

McLaren, B., Keller, A.E., O'Donnel, D.J. and Elvehjem, C.A., 1947. The nutrition of rainbow trout: I. Studies of vitamin requirements. *Arch. Biochem. Biophys.,* **15**: 169-178.

NRC, 2011. *Nutrient requirements of fish*. National Academy Press, National Research Council, Washington, DC, USA.

Peres, H., Lim, C. and Klesius, P.H., 2004. Growth, chemical composition and resistance to *Streptococcus iniae* challenge of juvenile Nile tilapia (*Oreochromis niloticus*) fed graded levels of dietary inositol. *Aquaculture,* **235**: 423-432. https://doi.org/10.1016/j.aquaculture.2003.09.021

Reddi, A.R., Jensen, L.T., Naranuntarat, A., Rosenfeld, L., Leung, E., Shah, R. and Culotta, V.C., 2009. The overlapping roles of manganese and Cu/Zn SOD in oxidative stress protection. *Free Radic. Biol. Med.,* **46**: 154-162. https://doi.org/10.1016/j.freeradbiomed.2008.09.032

Rowland, S.J. and Ingram, B.A., 1991. Diseases of Australian native fishes. In: *Fisheries Bulletin*, 4 NSW Fisheries, Sydney, NSW, Australia.

Santoro, M., Caffaratti, E., Peregrin, J.M.S., Korecz, L., Rockenbauer, A. and Luis, F.S., 2007. Signorella kinetics and mechanism of the chromic oxidation of myo-inositol. *Polyhedron*, **26**: 169-177. https://doi.org/10.1016/j.poly.2006.08.003

Shahidi, F. and Hong, C., 1991. Evaluation of malonaldehyde as a marker of oxidative rancidity in meat products. *J. Fd. Biochem.,* **15**: 97-105. https://doi.org/10.1111/j.1745-4514.1991.tb00147.x

Shiau, S.Y. and Su, L.W., 2003. Ferric citrate is half as effective as sulfate in meeting the iron requirement of juvenile tilapia, *Oreochromis niloticus* × *O. aureus*. *J. Nutr.,* **133**: 483-488. https://doi.

org/10.1093/jn/133.2.483

Shiau, S.Y. and Su, S.L., 2004. Dietary inositol requirement for juvenile grass shrimp, *Penaeus monodon*. *Aquaculture,* **241**: 1-8. https://doi.org/10.1016/j.aquaculture.2004.01.013

Shiau, S.Y. and Su, S.L., 2005. Juvenile tilapia (*Oreochromis niloticus* × *Oreochromis aureus*) requires dietary myo-inositol for maximal growth. *Aquaculture,* **243**: 273-277. https://doi.org/10.1016/j.aquaculture.2004.10.002

Sneadecor, G.W. and Cochran, W.G., 1991. *Statistical method*, 8th ed. Iowa State University Press, Ames., USA, pp. 503.

Steel, R.G.D., Tories, J.H. and Dickey, D.A., 1996. *Principles and procedures of statistics*, 3rd ed. McGraw Hill International Book Co. Inc., New York, USA, pp. 336-352.

Waagbø, R., Sandnes, K. and Lie, Ø., 1998. Effects of inositol supplementation on growth, chemical composition and blood chemistry in Atlantic salmon, *Salmo salar* L., fry. *Aquacult. Nutr.,* **4**: 53-59. https://doi.org/10.1046/j.1365-2095.1998.00043.x

Wen, H., Zhao, Z.Y., Jiang, M., Liu, A.L., Wu, F. and Liu, W., 2007. Dietary myo-inositol requirement for grass carp, *Ctenopharyngodon idella* fingerling. *J. Fish. Sci. China,* **14**: 794-800.

Winston, G.W. and Di-Giulio, R.T., 1991. Prooxidant and antioxidant mechanisms in aquatic organisms. *Aquat. Toxicol.,* **19**: 137-161. https://doi.org/10.1016/0166-445X(91)90033-6

Yone, Y., Furuichi, M. and Shitanda, K., 1971. Vitamin requirements of the red sea bream: 1. Relationship between inositol requirements and glucose levels in diet. *Bull. Japanese Soc. Sci. Fish.,* **37**: 149-155. https://doi.org/10.2331/suisan.37.149

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Dietary Effect of *Allium sativum* as a Prebiotic on the Growth and Immunity of *Oreochromis niloticus*

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Authors' Contribution
FR supervised the study and wrote the manuscript. SP co-supervised the research. ZH conducted research. IZ and AB helped in feed formulation and data collection. NK helped in arranging data and statistical analysis. KMA helped in writing and proofreading the manuscript.

Key words
***Allium sativum*, Growth, *Oreochromis niloticus*, Immunological parameters.**

ABSTRACT

The present study was focused to find the dietary effect of *Allium sativum* (garlic) as a prebiotic on the growth and immunity of *Oreochromis niloticus* (tilapia). A total of 20 fishes were stocked in each aquarium. These aquariums were named as treatment 1, treatment 2 and treatment 3. *A. sativum*, a prebiotic given to the fish in the form of powder in varying concentrations. Treatment 1 fish was fed with normal diet but was devoid of *A. sativum*. Treatment 2 was given feed with 3% *A. sativum* and treatment 3 with 6% *A. sativum*. Fish from treatment 3 gained significantly higher weight and increased length than treatment 1 and treatment 2. Water quality parameters such as temperature, pH, DO, salinity and TDS were daily checked which were in suitable range. Microbial content of skin was counted through plate colony method. Tryptic soya agar (TSA), nutrient agar and Eosine methylene blue (EMB) were used to study the growth of bacterial colonies. After results it was cleared that total microbial load was less in treatment 3 samples as compared to treatment 2 and treatment1 samples. Treatment 2 had high microbial content than treatment 3 but less than treatment 1, which was fed with normal control diet. It can be concluded that microbial content reduced in fish supplemented with 6% *A. sativum* feed, at the rate of 2mg/100g. The reduction in microbial content indicates enhanced immunity. At the end of two month trial slides of selected bacterial colonies were prepared, and examined under the microscope to study the presence of Gram positive and Gram negative bacteria in treatment 1, treatment 2 and treatment 3 under the effect of *A. sativum*.

INTRODUCTION

Different types of antibiotics are usually used to stimulate growth and health in trout, carp and *Oreochromis niloticus* (tilapia) (Shalaby *et al*., 2006). Some antibiotics are used to avert fish from diseases but the use of antimicrobials has many disadvantages in the form of pathogens defiance and the problem of drug resistance in treated fish which also causes the pollution of environment (FAO, 2006). Therefore immunostimulants seems to be an irresistible alternative to control fish diseases (Shalaby *et al*., 2006). So, the use of immunostimulants in fish farming has become essential to increase the activity and defense against diseases in fish (Raa, 1996). Therefore *A. sativum* as an immunostimulants can be used for the control of pathogens, especially bacteria and fungi. Moreover, *A. sativum* has also proven effective against gram positive or gram negative bacteria (Adetumbi *et al*., 1986). *A. sativum* is known as imperative medicinal plant due to its antiprotozoal, antibacterial, antifungal properties (Corzo-Martinez *et al*., 2007). Gupta *et al*. (2008) studied that in aquaculture operations *A. sativum* promotes growth, stimulates appetite, enhances the immune system and strengthens the control of pathogens, especially bacteria and fungi. Many reports have documented that *A. sativum* can effectively eliminate principal pathogenic bacteria in freshwater fish. *A. sativum* consists of allicin which increases the process of digestion and enhance the use of energy which results in body weight gain (Khalil *et al*., 2001). *A. sativum* is also used to promote growth in *O. niloticus* (Shalaby *et al*., 2006). Therefore, this study is aimed and focused to determine the effects of *A. sativum* on growth and immunological parameters of *O. niloticus*. Furthermore total bacterial count and bacterial population was also examined under the effect of *A. sativum* on *O. niloticus*.

MATERIALS AND METHODS

Location

The present study was conducted at Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki. The experiment was conducted in the fish hatchery using glass aquariums.

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Experimental protocol

Two month trial on *O. niloticus* was done to find out the dietary effects of *A. sativum* on growth and immunological parameters of *O. niloticus*.

Feeding trial

Twenty (20) samples of tilapia were collected from a pond of varying sizes from 5 to 10g and were placed in three different aquariums situated in fish hatchery. Each aquarium was with 20 fishes. There were two treatment and one control group. Control group was fed with normal diet (mentioned in Table I) but as devoid of *A. sativum* and was indicated as Treatment 1 (control) T1 whereas the treatment groups were fed with feed recipes given in Table I. Treatment 2 (T2) tank was given feed with 3% *A. sativum* and treatment 3 (T3) with 6% *A. sativum*.

Table I.- Feed chart.

Ingredients	Treatment 1	Treatment 2	Treatment 3
Fish meal	25 %	25%	25%
Soybean meal	25%	25%	25%
Maize glutton	25%	255	25%
Rice polish	20%	20%	20%
Molasses	3%	0	0
Vitamins	1%	1%	1%
Garlic	0	3%	6%

Feed preparation

The feed ingredients fish meal, soybean meal, maize Glutton, rice Polish, mollases, vitamins and *A. sativum* (garlic) were used for preparation of feed. Fish were fed six days a week with their formulated diets for about two months. Fish was fed two times a day, 8:30 am in morning and 4:30 pm in evening. Fish were fed at the rate of 2% of their body weight.

From each aquarium, samples were collected carefully. Weight and length measured to check the growth. Fishes collected in polythene bag and sent to the microbiological Lab after two months of trial.

Microbiological analysis

Each media was poured in petri plates according to the organs of fish; each petri-plate contained 20ml of prepared media. Then allowed them to become cool and solidify. After that skin of fish was separated by using a sharp scissor and forceps at sterilized and cleaned laminar air flame, so that there were less chances of contamination.

The separated organ was immediately transferred to 9% normal saline solution in flask and was shacked well with the help of electric shaker (vortex mixer) to allow the bacteria to be shifted to solution. Serial dilution was used for isolation and culturing of bacteria. The eppendroff tubes were filled with 900 µl, 9% normal saline solution (sterilized in autoclave) which has capacity of 2 ml. Transferred 100 µl solution from each flask which has solution of flesh, to 900 µl normal saline solutions of eppendroff tubes. This was acted as the 1st dilution, then took 100 µl solutions from that dilution and added into 900 µl of next tube. That became 100 times diluted and acted as second dilution. The bacterial colonies were cultured by pouring and spreading 10 µl of the suspension from corresponding dilution on the surface of the relevant solidified media by micropipette. The inoculated plates were then kept in incubator at 37^{o}C. Colonies of bacteria were counted after 24 and 48 h of incubation.

Microbial count

For the estimation of viable counts, pour plate method was used. 1 ml from each of the appropriate dilutions was transferred aseptically to sterile triplicate petri plates by means of sterile pipette. Then to each plate transferred 15-20 ml Tryptic soya agar sterilized, melted and cooled to about 45^{o}C was poured. After solidification the plates were inverted to prevent condensation of moisture on the agar surface. The dilutions were spread thoroughly with the glass rod to distribute the microbial cells uniformly on the solidified plates. After incubation the colonies were counted. Same method was used by using Nutrient agar and Eosin methylene blue (EMB) for microbial count.

Average number of colonies in triplicates petri-plates of a suitable dilution was multiplied by the dilution factor and was reported as total viable count per ml of sample.

Total Viable Count =
Average No. of colonies x Dilution factor

Gram staining

At the end of two month trial slides of selected bacterial colonies were prepared, stained and examined under the microscope.

Gram staining is the most important differential staining used in Bacteriology. Following Gram staining method; a drop of sample was placed on clean glass slide and smear was prepared. The smear was air dried and then heat fixed. The smear was stained with crystal violet solution for one minute. Then washed with distilled water and flooded with iodine solution for one minute. After washing with distilled water, the smear was flooded with 95% ethyl alcohol for decolorization purpose. The smear was washed with distilled water and counter-stained with safranin for 30 sec.

The smear was washed with distilled water, air dried and observed under microscope with oil immersion lens.

Then checked that either bacterium was gram-positive or gram-negative. Bacterial population of treatment 1, 2 and 3 is given in Table V.

Statistical analysis

The data obtained was analyzed by using Excel software. The data on different variables was statistically analyzed by using Analysis of Variance (ANOVA).

RESULTS

Growth parameters

At the beginning of the experimental trial in aquariums sampling was done and the initial average body weight of fish for *O. niloticus* in treatment 1 was 9.7g, in treatment 2 the initial body weight was 5.74 g and in treatment 3 the initial body weight was 13.14g. The final body weight for *O. niloticus* in treatment 1 for Treatment 2 was 10.12 g, in treatment A the final body weight was 6.12g and in treatment 3 the final body weight was 14.54g. The maximum weight gain was observed in Treatment 3 under the influence of *A. sativum*. There is also a variation in length of treated fishes of all aquariums as maximum average length gain for treatment 3 was 10.55cm and for treatment 1 and treatment 2 the average maximum length gain was 8.48cm and 8.8cm, respectively (Table II).

Table II.- Variations in growth parameters (Mean±SD) in *Oreochromis niloticus* between treatment 1, treatment 2 and treatment 3.

Parameters	Treatment 1	Treatment 2	Treatment 3
Initial weight	9.74±0.52[b]	5.74±0.52[c]	13.14±2.13[a]
Final weight	10.12±0.54[b]	6.12±0.54[c]	14.54±1.83[a]
Net weight	0.38±0.13[b]	0.38±0.13[b]	1.50±0.57[a]
Total weight gain	7.60±2.60[b]	7.60±2.60[b]	26.0±10.48[a]
Weight gain (%)	3.84±1.37[b]	6.66±2.47[b]	12.10±6.03[a]
Initial length	8.28±0.25[b]	8.16±0.96[b]	9.42±1.11[a]
Final length	8.48±0.27[b]	8.80±1.13[b]	10.18±1.27[a]
Net length	0.20±0.10[b]	0.64±0.28[a]	0.76±0.24[a]
Total length increase	4.0±2.0[b]	12.8±5.76[a]	15.20±4.81[a]
Length gain (%)	2.37±1.17[b]	7.74±3.14[a]	8.35±2.78[a]

Different letter in the same row are significant ($p<0.05$).

Water quality parameters

Temperature, DO, pH, TDS and salinity of treatment 1, treatment 2 and treatment 3 were not significantly different (Table III).

Microbial count

Total of forty five samples of *O. niloticus* were collected from treated aquariums. Fifteen samples from Treatment 1, T1 (control group), fifteen from Treatment 2, T2 (3% garlic) aquarium, and fifteen from Treatment 3, T3 (6% garlic) aquarium and were subjected for microbiological examination. The samples were analyzed for total plate count.

Table III.- Average water quality parameters of the treatments.

Parameters	Treatment 1	Treatment 2	Treatment 3
Temp. (ºC)	29.1±1.43	29.4±1.25	29.8±0.45
DO (MgL[1])	5.59±0.33	5.39±0.27	5.52±0.23
pH	8.69±0.31	8.9±0.37	8.67±0.29
TDS (MgL[1])	944.76±332.83	949±337.29	928±357.67
Salinity	1±0	1±0	1±0

Table IV.- Summary statistics.

Treatment	Samples	Mean±SD
1	15	12267866.6±739806.39[a]
2	15	6599533.33±4436561.75[b]
3	15	64533.33±7936.05[c]

Values are significant at the level of $p<0.05$ and the values with different letters are significant.

Table V.- Microbial species identified during present study.

Treatment	Name of species	Gram
1, 2, 3	*Bacillus subtilis*	+ve
1, 2, 3	*Micrococcus*	+ve
1, 2, 3	*Lactobacillus*	+ve
1, 2, 3	*Flavobacterium*	-ve
2, 3	*Staphylococcus*	+ve
2, 3	*Coryneforms*	+ve
1	*Aeromonas hydrophila*	-ve
1	*P. fluorescence*	-ve

Microbial count in skin of treatment 1 samples was highest, which ranged 12,267,866.6±739,806.39. The value was significant (Table IV). Microbial count in skin of treatment 2 samples was high but less than Treatment 1 and ranged 6,599,533.33±4,436,561.7. The value was significant (Table IV). Microbial count in skin of Treatment 3 samples was lowest, which ranged 64,533.33±7,936.05. The remained significant (Table IV).

Bacterial identification

Bacterial population in treatment 1, treatment 2 and treatment 3 (Tables IV, V).

DISCUSSION

The immense use of different chemical compounds and antibiotics has resulted in resistant pathogens and drug remnants in treated fish. Drug remnants not only pollute the environment, but also affect human consumers badly. In contrast, *A. sativum* as a well-known natural antibiotic and as an immunostimulant causes no environmental pollution and physical side effects. Due to its antioxidant, antimicrobial, and antihypertensive properties it becomes effective for the treatment of many diseases in animals and humans. *A. sativum* assists in growth, promotes appetite, strengthen immune system, and enhance the control of pathogens, especially fungi and bacteria in aquaculture applications. These effects of *A. sativum* are due to the presence of various organosulfur compounds, including allicin (Augusti *et al*., 1974). Higher levels of allicin are advantageous for medicinal applications (Huchette *et al*., 2005). Allicin has a powerful garlic flavor with a strong rousing effect on olfaction in most aquatic animals, including *Ctenopharyngodon idellus* (grass carp), *Pelodiscus sinensis* (Chinese softshell turtule), *Cyprinus carpio* (common carp), *Carassius auratus* (gold fish) and *O. niloticus*. Allicin can also promote immune competence, prevent and kill various pathogenic bacteria, moderate the secretion of various enzymes to refine digestion and nutrient absorption and raise gastrointestinal motility. Therefore *A. sativum* is strongly advised with dose optimization in aquaculture applications. Zeng *et al*. (1996) stated that when 50 mg/kg synthesized allicin was added to the feed of *O. niloticus*, the survival rate and weight gain increased by more than 2–3% after 45 days than in the control group. Present study focuses on the effect of *A. sativum* on the growth and immunity of *Orechromis niloticus* (tilapia). Experimental group fed on 6% *A. sativum* (garlic) (treatment B) had maximum weight and length with averages 12.10±6.033 and 8.35±2.786, respectively. Present study investigated that treatment 1 with normal diet show less signs of weight gain and length increase with averages 3.84±1.37 and 2.37±1.177, respectively. The experimental group fed with 3% garlic (treatment 2) ranged weight and length in between treatment 1 and treatment 3 as 6.66±2.471 and 7.74±3.143, respectively. In conclusion, the present study documented that by increasing minute concentration of *A. sativum* in fish diet it can enhanced the growth of *O. niloticus*. *O. niloticus* is considered the hardiest fish being cultured and it can tolerate water quality conditions and physical handling. *O. niloticus* can withstand a wider range of environmental conditions-including factors such as salinity, temperature, dissolved oxygen (DO), pH, salinity and total dissolved salts (TDS). However, the equipment should be of good quality that can examine the minimum basic water quality parameters of DO, temperature, pH, TDS and salinity. The results of key physicochemical parameters of water quality of this study were found within the acceptable range for survival of *O. niloticus* and the values of temperature, DO, TDS, pH and salinity of water are non-significant. The results of physicochemical parameters of water quality are according to the study of Roos (2000). Experimental group fed on 6% garlic (T3) had low microbial content which ranged 64533.33±7936.05 (Table IV). *A. sativum* have been reported to control pathogens, defy stress, advantageous for fish health (Ress *et al*., 1993) and increase the immunity (Adetumbi *et al*, 1986) as well as *A. sativum* (garlic) positively affects the survival rate of fish. The results of the present study indicate that high concentration of *A. sativum* inhibit the occurrence of pathological signs of *A. sativum* deficiency and resulted in improved growth and immunity compared with the control, *A. sativum* (garlic) free diet T1 or 5% containing diet T2.

Increasing *A. sativum* concentration in T1 enhanced considerably the immunity of fish by reducing overall microbial population but, too much increase in concentration of *A. sativum* is not suitable for fish. The present study revealed that by adding *A. sativum* to a fish diet at the rate of 6mg per 100g of fish feed would not only enhance the fish immunity but it also help to improve growth. Therefore it is recommended for fish farmers to add *A. sativum* in fish feed at given rate. After staining, it was observed that some bacteria were Gram-Positive while some were Gram-Negative. Microorganisms are considered to present in the intestines of live and newly caught fish and on all the outer surfaces including gills and skin of fish. Treatment 1 contains bacteria which are named *Aeromonas hydrophila* and *Pseudomonas fluorescens* and these two types of bacteria are absent in treatment 2 and treatment 3. *A. hydrophila* associated with the occurrence of different diseases in aquaculture. *Pseudomonas fluorescens* also considered as an expedient pathogen and it is mostly found in *O. niloticus*, gold fish, carp, sea bream and rainbow trout. Choudhury *et al*. (1991) described that *A. sativum* was considered very effective against two tested harmful bacteria including *A. hydrophila* and *P. fluorescens*.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Augusti, K.T., Narayanan, A., Pillai, L.S., Ebrahim, R.S., Sivadasan, R., Sindhu, K.R., Subha, I., Abdeen, S. and Nair, S., 2001. Beneficial effects of

garlic (*Allium sativum* Linn) on rats fed with diets containing cholesterol and either of the oil seeds, coconuts or groundnuts. *Indian J. exp. Biol*., **39**: 660-667.

Adetumbi, M., Javora, G.T. and Lau, B.H., 1986. *Allium sativum* (garlic) inhibits lipid synthesis by *Candida albicans*. *J. Antimicrob. Agents Chemother*., **30**: 499-501. https://doi.org/10.1128/AAC.30.3.499

Choudhury, D., Pal, A.K., Sahu, N.P., Kumar, S. and Das, S.S., 2005. Dietary yeast RNA supplementation reduces mortality by *Aeromonas hydrophila* in rohu (*Labeo rohita* L.) juveniles. *J. Fish Shellf. Immunol*., **19**: 281-291.

Food and Agriculture Organization (FAO), 2006. Country Profile. http://www.fao.org/countryprofiles/index/en/

Gupta, A.D., Das, S.N., Dhundasi, S.A. and Das, K.K., 2008. Effect of garlic (*Allium sativum*) on heavy metal (Nickel II and Chromium VI) induced alteration of serum lipid profile in male albino rats. *Int. J. environ. Res. Publ. Hlth*., **5**: 147-151.

Huchette, O., Kahane, R. and Bellamy, C., 2005. Influence of environ and genetic factors on the allicin content of garlic bulbs. *Acta Horticul*., **688**: 93-99. https://doi.org/10.17660/ActaHortic.2005.688.9

Khalil, R.H., Nadia, B.M. and Soliman, M.K., 2001. Effects of Biogen and Levamisol Hcl on the immune response of cultured *Oreochromis niloticus* to *Aeromonas hydrophila. Vaccine*, **2**: 381-392.

Corzo-Martinez, M., Corzo, N. and Villamiel, M., 2007. Biological properties of onions and garlic. *Trends Fd. Sci. Technol*., **18**: 609-625. https://doi.org/10.1016/j.tifs.2007.07.011

Raa, J., 1996. The use of immune stimulatory substances in fish and shellfish farming. *Rev. Fish Sci*., **4**: 229-288. https://doi.org/10.1080/10641269609388587

Ress, L.P., Minney, S.F., Plummer-Slatter, J.H. and Skyrme, D.A., 1993. A quantitative assessment of the antimicrobial activity of garlic (*Allium sativum*). *J. Microbiol. Biotechnol*., **9**: 303-307. https://doi.org/10.1007/BF00383068

Ross, L.G., 2000. Environmental physiology and energetic. In: *Tilapias: Biology and exploitation* (eds. M.C.M. Beveridge and B.J. McAndrew). Kluwer Academic Publishers, UK, pp. 89-128. https://doi.org/10.1007/978-94-011-4008-9_4

Shalaby, A.M., Khattab, Y. and Abdel-Rahman, A.M., 2006. Effects of garlic, *Allium sativum* and chloramphenicol on growth performance, physiological parameters and survival of Nile tilapia, *Oreochromis niloticus*. *J. Venom. Anim. Toxins Trop. Dis*., **12**: 172-201.

Zeng, H., Ren, Z.L. and Guo, Q., 1996. Application of allicin in tilapia feed. *China Feed*, **21**: 29-30.

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Acute Toxicity of Aluminium to *Channa marulius*, *Mystus seenghala* and *Wallago attu*

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ABSTRACT

In freshwater ecosystems of Pakistan, metallic ion pollutants that are released continuously from both natural as well as anthropogenic sources cause serious hazards to the carnivorous fish fauna. Therefore, acute toxicity tests of aluminium, in terms of 96 h LC_{50} and lethal concentrations were conducted under laboratory conditions to evaluate the sensitivity of three carnivorous fish species *viz*., *Channa marulius*, *Mystus seenghala* and *Wallago attu*. The fingerlings of 150mm total length were first acclimatized to the laboratory conditions for ten days and then shifted to the glass aquaria for toxicity experiments. During the whole acute toxicity trials, fish mortality and physico-chemical variables of water *viz*. temperature, pH, dissolved oxygen, carbondioxide, total hardness, total ammonia, electrical conductivity, calcium, magnesium, sodium and potassium were determined at 12 h intervals. Fish mortality data were analyzed through Probit analysis method with 95% confidence interval to estimate 96 h LC_{50} and lethal concentrations of aluminium for each fish species. Among the three fish species, *M. seenghala* showed significantly ($p<0.01$) higher sensitivity towards aluminium, followed by that of *W. attu* and *C. marulius*. However, the lethal concentrations of aluminium for the three fish species *viz*., *C. marulius*, *M. seenghala* and *W. attu* were computed as 193.85±8.35, 105.93±6.67 and 123.54±5.77mgL^{-1}, respectively. The 96 h LC_{50} revealed statistically significant and positive relationship with lethal concentration of aluminium determined for the three fish species. Both LC_{50} and lethal concentrations showed positively significant ($p<0.05$) correlation with dissolved oxygen contents of the test media while their relationship with total ammonia was found negative but non-significant. Calcium and sodium exhibited significantly direct correlation while magnesium exhibited inverse relationship, with both LC_{50} and lethal concentrations of the test media.

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Authors' Contribution
SA conducted experiments, collected and statistically analysed the data. MJ assisted in the write-up of article.

Key words
96 h LC_{50}, Lethal concentration, Aluminium, Carnivorous fish, Physico-chemistry.

INTRODUCTION

Anthropogenic activities have resulted into increased discharge of various concentrations of metals into the aquatic environments. The trace metals although essential for the normal metabolic processes, their abnormally higher concentrations pose toxic effects on the aquatic organisms (Goldoni *et al*., 2006). The heavy metals cause toxicity to the living organisms as they change behavioural, biochemical, physiological and genetic parameters (Scott and Sloman, 2004; Kumar *et al*., 2007; Odo *et al*., 2017). Among the aquatic organisms, fish are considered as the most significant biomonitors for the estimation of heavy metal's pollution level (Alinnor, 2005), as they offer specific advantages in describing natural characteristics of the aquatic ecosystems and in assessing changes to the habitats (Lamas *et al*., 2007). In addition, the fish are present at the top of aquatic food chain and may bioconcentrate metals and pass them to the human beings through food causing acute or chronic effects (Al-Yousuf *et al*., 2000).

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Aluminium is one of the most frequent elements present in the biosphere and serves as an important factor in the toxicity of acidified water to the freshwater fish. Acid precipitation has led towards increasing concentrations of various trace elements, including aluminium in lakes and rivers which may cause fish mortality.

The acute toxicity test is used to determine concentration of the test material or level of an agent that produce deleterious effect on a group of test organisms, during short-term exposure *i.e.* 96 h under controlled conditions and to assess the impact of toxic chemical on the biology of aquatic organisms (Rani *et al*., 2011). Estimation of 96 h LC_{50} and lethal concentrations serve as reliable parameters to measure the susceptibility and survival potential of organisms to particular toxic substances such as heavy metals (Claude, 2005).

Aquatic contamination with heavy metals may adversely affect the immune system of fish (Nawaz *et al*., 2018) leading to decreased production, increased susceptibility to diseases and ultimately mortality. Studies from both field and laboratory works demonstrated that some environmental factors such as water temperature, oxygen concentration, hardness, pH, alkalinity, salinity and dissolved organic carbon may influence and play

significant roles toward metal's toxicity to the fish (Benaduce *et al*., 2008; Has-Schon *et al*., 2008; Ebrahimi and Taherianfard, 2011). The alterations in various physico-chemical parameters may serve as important bioindicators against metallic ions pollution. This in turn may help in proper management of aquatic pollution as well as saving the aquatic fauna from damage, disturbance in food chain, biomagnification and extinction.

Over the last few decades, there has been found a gradual decline in the density of commercial carnivorous fish species of Pakistan *viz*. *Channa marulius*, *Mystus seenghala* and *Wallago attu* due to the metallic ions pollution. Therefore, in order to evaluate the harmful effects of aluminium on the fish and to build strategies for sustainable conservation of these fish species in freshwater environs of Pakistan, the acute toxicity trials were conducted.

MATERIALS AND METHODS

Acute toxicity tests

The fish species *viz*., *C. marulius*, *M. seenghala* and *W. attu* were obtained from Head Qadirabad and brought to the wet laboratory for acclimatization in cemented tanks for ten days. The fish were fed with pelleted feed having 40% digestible protein and 3.50 $Kcalg^{-1}$ energy, thrice a day. However, fish were not fed during whole acute toxicity trials. After acclimatization, the healthy fish of 150mm total length, for each group, were selected for acute toxicity trials. Chemically pure compound of aluminium ($AlCl_3.6H_2O$) was dissolved, separately, in 1000 mL deionized water and stock solution prepared for the metal.

The tolerance limits of three fish species for aluminium were determined in terms of 96 h LC_{50} and lethal concentrations. The toxicity tests were conducted in glass aquaria of 50 litre water capacity at constant water temperature, pH and total hardness of 28°C, 8 and 250 mgL^{-1}, respectively. The three fish species *viz*., *C. marulius*, *M. seenghala* and *W. attu* having average wet weights of 16.11±0.82, 12.06±0.43 and 17.59±0.67 g, respectively, were exposed separately to aluminium. The ten fish of each species were placed in aquarium separately, with three replications for each test dose. The concentration of exposed metal (aluminium) was started from zero with an increment of 0.01 and 0.1 mgL^{-1} for low and high concentrations, respectively. The metal concentration in each aquarium was increased gradually and 50% test concentration maintained within 3 h and full toxicant concentration in 6 h. Each exposure concentration was tested with three replications, separately, for each fish species. During acute toxicity trials, the observations on fish mortality were made after every two hours. The metal exposure concentrations were started from zero up to that concentration at which 50% (LC_{50}) and 100% mortality of fish (lethal concentration) occurred during 96 h duration. The dead fish was immediately removed from the test media and mortality data were recorded.

Physico-chemistry of the test media

During the whole experimental duration, the physico-chemical variables of the test media *viz*., temperature, pH, dissolved oxygen, carbondioxide, total hardness, total ammonia, electrical conductivity, calcium, magnesium, sodium and potassium were analyzed on 12 hourly basis by following the methods of APHA (1998). Constant aeration of the aquarium water was done with an air pump fitted with capillary system.

Statistical analyses

The acute toxicity of aluminium was computed by using Probit analysis method (Hamilton *et al*., 1977). Mean values of 96 h LC_{50} and lethal concentrations of the metal for three fish species were obtained at 95% confidence intervals. The means were compared for statistical differences by employing Tukey's/Student Newman-Keul test (Steel *et al*., 1996). Correlation and Regression analyses were also performed to find-out statistical relationships among various parameters under study.

RESULTS AND DISCUSSION

Table I shows the 96 h LC_{50} and lethal concentrations of aluminium for the three carnivorous fish species with 95% confidence interval limits. It was found that *M. seenghala* showed significantly ($p<0.01$) higher sensitivity towards aluminium with the mean 96 h LC_{50} value of 62.34±3.08mgL^{-1}, followed by that of *W. attu* and *C. marulius* for which the same was computed as 86.97±2.72 and 138.20±3.46mgL^{-1}, respectively. Higher LC_{50} values are less toxic because greater concentrations are required to produce 50% mortality in test organisms (Hedayati *et al*., 2010). However, the lethal concentrations of aluminium for the three fish species *viz. C. marulius*, *M. seenghala* and *W. attu* were calculated as 193.85±8.35, 105.93±6.67 and 123.54±5.77mgL^{-1}, respectively. Kaushal and Mishra (2013) found that the differences in 96 h LC_{50} and lethal concentration values between *Channa punctatus* and other fish species were due to the property of metal to induce changes in the physiology and survival of aquatic organisms during acute exposure. Such changes differ from species to species, metal to metal and from one experimental condition to another.

Table I.- Calculated 96 h LC_{50} and lethal concentrations (±SE) of aluminium for the three fish species.

Fish species	Mean 96 h LC_{50} (mgL^{-1})	95% confidence intervals (mgL^{-1})	Mean lethal concentrations (mgL^{-1})	95% confidence intervals (mgL^{-1})
Channa marulius	138.20±3.46 a	130.73 - 144.91	193.85±8.35 a	180.84 - 216.33
Mystus seenghala	62.34±3.08 c	55.30 - 68.06	105.93±6.67 c	95.66 - 124.36
Wallago attu	86.97±2.72 b	80.87 - 92.12	123.54±5.77 b	114.62 - 139.44

Means with similar letters in a single column are statistically similar at $p<0.05$.

Table II.- Mean values (±SD) of physico-chemical characteristics of test media observed during 96 h acute toxicity trials of aluminium for the three fish species.

Fish species	Temp. (°C)	pH	DO (mgL^{-1})	CO_2 (mgL^{-1})	Total hardness (mgL^{-1})	Total ammonia (mgL^{-1})	EC (mgL^{-1})	Ca (mgL^{-1})	Mg (mgL^{-1})	Na (mgL^{-1})	K (mgL^{-1})
Channa marulius	28.01± 0.02	7.99± 0.02	5.14± 0.75	1.41± 0.20	250.00± 0.01	1.25± 0.35	2.62± 0.43	23.97± 1.38	47.52± 0.86	271.30± 8.81	10.68± 1.58
Mystus seenghala	28.00± 0.02	8.01± 0.02	4.91± 0.14	1.30± 0.14	250.00± 0.01	1.31± 0.14	2.42± 0.10	23.50± 1.44	47.81± 0.90	281.69± 9.71	10.62± 1.70
Wallago attu	28.01± 0.01	8.00± 0.01	5.25± 0.28	1.34± 0.18	250.00± 0.01	1.22± 0.34	2.98± 0.32	24.44± 1.64	47.22± 1.03	271.82± 11.90	10.95± 1.50

Mg, magnesium; DO, dissolved oxygen; EC, electrical conductivity; Na, sodium; CO_2, carbon dioxide; Ca, calcium; K, potassium.

Table III.- Correlation coefficients among acute toxicity of aluminium for fish and physico-chemical variables of the test media.

	LC_{50}	Lethal	DO	CO_2	T. Amm.	EC	Ca	Mg	Na
Lethal	0.99626								
DO	0.50622	0.49068							
CO_2	-0.23024	-0.24468	0.03228						
T. Amm.	-0.26167	-0.25445	-0.38837	0.55861					
EC	0.36291	0.32248	0.19194	-0.05530	0.12095				
Ca	0.55992	0.53704	0.59600	-0.26175	-0.28312	0.27676			
Mg	-0.55730	-0.53445	-0.59910	0.26341	0.28600	-0.27673	-0.99903		
Na	0.61526	0.65172	0.31288	-0.30281	0.00644	0.12089	0.41510	-0.41317	
K	-0.29763	-0.31479	-0.24617	-0.14998	-0.38222	-0.11212	-0.32997	0.32812	-0.35430

Critical Value (2 tail 0.05±0.38009). Conc., concentration (mgL^{-1}); T. Amm., total ammonia (mgL^{-1}); Mg, magnesium (mgL^{-1}); DO, dissolved oxygen (mgL^{-1}); EC, electrical conductivity ($mScm^{-1}$); Na, sodium (mgL^{-1}); CO_2, carbon dioxide (mgL^{-1}); Ca, calcium (mgL^{-1}); K, potassium (mgL^{-1}).

The exact causes of fish mortality due to the heavy metal poisoning are variable and depend mainly on the duration and concentration of exposure. The results of the present investigation are parallel to the findings of Javed *et al*. (2016) who reported that *Channa marulius* was more tolerant to the metallic ion (Cd) toxicity as compared to *Mystus seenghala* and *Wallago attu*. During acute toxicity trials of Al for the three fish species, it was observed that with an increase in concentration, the mortality rate was also increased. Johnson and Radhakrishnan (2015) found that susceptibility of African walking catfish (*Clarias batrachus*) to the toxic impacts of chromium was concentration dependent, as mortality increased with an increase in metal exposure concentration.

All the determined physico-chemical parameters of the test media (Table III) were correlated with both 96 h LC_{50} as well as lethal concentrations of Al in order to evaluate the impact of metal's toxicity on the water quality parameters and vice versa. Table III shows the correlation coefficients of 96 h LC_{50} and lethal concentration of aluminium with dissolved oxygen, carbon dioxide, total ammonia, electrical conductivity, calcium, magnesium, sodium and potassium. The 96 h LC_{50} showed positive and strong relationship with lethal concentration of Al, determined for the three fish species. Both LC_{50} and lethal concentrations exhibited significantly ($p<0.05$) positive

correlation with dissolved oxygen of the aluminium exposed media while their relationship with total ammonia contents was found negative but non-significant. Calcium and sodium revealed significantly direct correlation with LC_{50} and lethal concentrations while magnesium exhibited inverse relationship with both LC_{50} as well as lethal concentrations of the test media. Azmat *et al*. (2016) reported that the average sodium contents of the test media significantly increased due to the exposure of *Labeo rohita* to the metal. During present investigation, the correlation coefficient between total ammonia and dissolved oxygen appeared negatively significant. However, the total ammonia contents of aluminium test media showed significantly positive correlation with the carbon dioxide. This may be due to the fact that exposure of metals to the fish caused stressful conditions for the fish to respire more frequently and hence resulted into significant decrease in the dissolved oxygen contents of the test media along with an enhanced ammonia excretion. Significantly inverse correlation was observed between calcium and magnesium contents of the aluminium exposed test media during acute toxicity trials for the three fish species. Shafiq *et al*. (2012) also reported that there existed negatively significant correlation between calcium and magnesium contents of the nickel exposed test media.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Alinnor, I.J., 2005. Assessment of elemental contaminants in water and fish samples from Aba River. *Environ. Monit. Assess*., **102**: 15-25. https://doi.org/10.1007/s10661-005-1011-3

Al-Yousuf, M.H., El-Shahawi, M.S. and Al-Ghais, S.M., 2000. Trace metals in liver, skin and muscle of *Lethrinus lentjan* fish species in relation to body length and sex. *Sci. Total Environ*., **256**: 87-94. https://doi.org/10.1016/S0048-9697(99)00363-0

APHA, 1998. *Standard methods for the examination of water and wastewater*, 20th ed. New York, pp. 1193.

Azmat, H., Javed, M., Husssain, S.M., Javid, A. and Jabeen, G., 2016. Impacts of physico-chemical parameters on fish grown under heavy metal stress. *Pakistan J. Zool*., **48**: 795-807.

Benaduce, A.P.S., Kochhann, D., Flores, E.M.M., Dressler, V.L. and Baldisserotto, B., 2008. Toxicity of cadmium for silver catfish *Rhamdia quelen* (Heptapteridae) embryos and larvae at different alkalinities. *Arch. environ. Contam. Toxicol*., **54**: 274-282. https://doi.org/10.1007/s00244-007-9024-2

Claude, E.B., 2005. *Toxicity can result from contamination of culture systems by pesticides, heavy metals, or industrial chemicals. Sustainable aquaculture practices*. Global Aquaculture Alliance.

Ebrahimi, M. and Taherianfard, M., 2011. The effects of heavy metals exposure on reproductive systems of cyprinid fish from Kor River. *Iran. J. Fish. Sci*., **10**: 13-26.

Goldoni, M., Caglieri, A., Poli, D., Vettori, M.V., Corradi, M., Apostoli, P. and Mutti, A., 2006. Determination of hexavalent chromium in exhaled breath condensate and environmental air among chrome plating workers. *Anal. Chim. Acta*, **562**: 229-235. https://doi.org/10.1016/j.aca.2006.01.065

Hamilton, M.A., Russo, R.C. and Thurston, R.V., 1977. Trimmed Spearman-Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Tech*., **11**: 714-719. https://doi.org/10.1021/es60130a004

Has-Schon, E., Bogut, I., Kralik, G., Bogut, S., Horvatic, J. and Cacic, M., 2008. Heavy metal concentration in fish tissues inhabiting waters of “BuskoBlato” reservoar (Bosnia and Herzegovina). *Environ. Monit. Assess*., **144**: 15-22. https://doi.org/10.1007/s10661-007-9627-0

Hedayati, A., Safahieh, A., Savar, A. and Ghofleh, M.J., 2010. Detection of mercury chloride acute toxicity in Yellow fin sea bream (*Acanthopagrus latus*). *World J. Fish Mar. Sci*., **2**: 86-92.

Javed, M., Abbas, S. and Latif, F., 2016. Acute toxicity of cadmium and its bio-accumulation in the carnivorous fish species *Channa marulius*, *Mystus seenghala* and *Wallago attu*. *Int. J. Agric. Biol.,* 18: 1169-1173. https://doi.org/10.17957/IJAB/15.0221

Johnson, C. and Radhakrishnan, M.V., 2015. Estimation of acute toxicity of chromium to the freshwater catfish *Clarias batrachus* (Linn.). *Int. J. Res. environ. Sci*., **1**: 30-37.

Kaushal, B.T. and Mishra, A., 2013. Investigation of acute toxicity of cadmium on snakehead fish *Channa punctatus*- A comparative toxicity analysis on median lethal concentration. *Int. J. Adv. biol. Res*., **3**: 289-294.

Kumar, P., Prasad, Y., Patra, A.K. and Swarup, D., 2007.

Levels of cadmium and lead in tissues of freshwater fish (*Clarias batrachus* L.) and chicken in Western UP (India). *Bull. Environ. Contam. Toxicol*., **79**: 396-400. https://doi.org/10.1007/s00128-007-9263-y

Lamas, S., Fernandez, J.A., Aboal, J.R. and Carballeira, A., 2007. Testing the use of juvenile *Salmo trutta* L. as biomonitors of heavy metal pollution in freshwater. *Chemosphere*, **67**: 221-228. https://doi.org/10.1016/j.chemosphere.2006.10.040

Rani, M.J., John, M.M.C., Uthiralingam, M. and Azhaguraj, R., 2011. Acute toxicity of mercury and chromium to *Clarias batrachus* (Linn). *Biores. Bull*., **1**: 368-372.

Nawaz, R., Javed, M., Abbas, S. and Kousar, S., 2018. Effect of iron chloride on peroxidase activity in kidney and liver of *Labeo rohita. Pakistan J. Zool.,* **50**: 1-4. http://dx.doi.org/10.17582/journal.pjz/2018.50.1.sc4

Odo, G.E., Agwu, E.J., Ossai, N.I., Ezea, C.O., Madu, J. and Eneje, V., 2017. Effects of aluminium phosphide on the behaviour, haematology, oxidative stress biomarkers and biochemistry of African catfish (*Clarias gariepinus*) juvenile. *Pakistan J. Zool.,* **49**: 433-444. http://dx.doi.org/10.17582/journal.pjz/2017.49.2.433.444

Scott, G.R. and Sloman, K.A., 2004. The effects of environmental pollutants on complex fish behaviour: Integrative behavioural and physiological indicators of toxicity. *Aquat. Toxicol*., **68**: 369-392. https://doi.org/10.1016/j.aquatox.2004.03.016

Shafiq, A., Abdullah, S., Javed, M., Hayat, S. and Batool, M., 2012. Effect of combined exposure of water-borne and dietary nickel on the growth performance of *Cirrhina mrigala*. *J. Anim. Pl. Sci*., **22**: 425-430.

Steel, R.G.D., Torrie, J.H. and Dinkkey, D.A., 1996. *Principles and procedures of statistics,* 3rd ed. McGraw Hill Book Co., Singapore, pp. 666.

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Assessment of Fish Diversity of Downstream Indus River, Sindh, Pakistan

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Authors' Contribution
MS conducted the research work and collected data. MYL designed th project and guided to collect the data. PKL and ARK analyzed the data. NTN wrote the manuscritp and all other authors helped him.

Key words
Assessment, Downstream, Fish diversity, Indus River, Sindh.

ABSTRACT

In the present study fishery sources were investigated quantitatively by studying the ichthyofaunal biodiversity of Downstream Indus River, Sindh Pakistan. A fortnightly sampling was conducted from eight stations during March to October 2015. A total 92 fish species were recorded. Out of which 77 species were fresh water and 15 were identified as anadromous. Based on their edible values, 52 fishes were edible and 40 were found as trash fishes. In edible fishes 11 species were export quality, 24 standard quality (high value) and 17 were local quality (low value). The greater fish biodiversity was found in Railo Miyan (n=81) followed by Branch Morrie (n= 66) and lowest in Wasi Malook Shah (n= 32). Overall, *Cirrihinus mrigala* was in abundance (45%) followed by *Labeo rohita* (38%) and *Catla catla* was found rare (17%). While in the trash fishes *Labeo gonius* was found in abundant quantity. All of the recoded 92 fishes belonged to 11 orders and 30 families. The order Siluriformes found to be dominant with 29 fish species followed by Cypriniformes (27 species) and Perciformes (13 species). Out of 30 families, the family Cyprinidae recorded 24 fish species (19%) followed by Bagridae 13 fish species (14%) and Siluridae 7 species (8%) and Clupeidae and Channidae 5 species (5%) each.

INTRODUCTION

The Indus River is one of the longest important River in Asia. It flows through Pakistan and merges into the Arabian Sea near the port city of Karachi in Sindh. Ichthyofaunal diversity refers the spatial and temporal patterns of diversity, distribution and species composition of freshwater fishes are useful to examine factors influencing the structure of the fish community (Galactos *et al*., 2004). Mostly it provides essential ecological services as provision of water for drinking, fisheries, food production, cultural purposes and transportation (Balian *et al*., 2008). However, with the increased recognition of the importance of the indigenous aquatic biodiversity and inherent ecological processes (Leal *et al*., 2005). Further, physical and chemical changes culminate in new environmental conditions that can result in permanent alterations of biological communities (Karr, 1981; Li *et al*., 1987). Freshwater fish fauna is considered as highly diverse and representative of all the warm water fish fauna of Pakistan in the Indus plain (Rafique, 2000). Human activities such as modification of the environment, harvesting and culture and effects of modernization have contributed to the pollution of water bodies which causes species reduction because of damaging their habitats. Indus River needs serious attention in its management and conservation of fishery resources. And also, due to flood the quantity of silt concentration is increases in the different water bodies of River which block the water routes and destroys the spawning ground of fish species.

MATERIALS AND METHODS

The fishes were collected from the Downstream of River Indus during March to October 2015. Eight major landing areas (From Jamshoro to Thatta) were selected and monthly collection of fish species was done. These eight landing area includes RailoMiyan (RM), Karokho (Kk), Khanpur (Kp), Mullakatiyar (Mk), Wasi Malook Shah (WMS), Branch morie (BM), Sujawal (Sj) and Jangseer (JS). There was about 50km average distance between all sites.

Sample collection and preservation

The samples were collected by the help of various crafts and gears. Following four type of crafts were utilized to collect the samples, Hora, Dhonda, Sail boat (6mLOA) and large sail boat (15mLOA). While, various gears were used to collect the fish samples that include hook and line, barricades, pot net, dip net, drag net, trawl net, seine nets, cast nets and gill nets. The caught fish were immediately put in the icebox and brought to the lab, washed with

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distilled water and preserved with 5% formalin (Glass jars) for further analysis.

Fish identification

Fish identified with their morphological characteristics, Morphometric measurements with the help of Manuals (Day, 1980; Talwar and Jhingran, 1991) and Websites (http://www.fishbase.org.com; http://www.tns.org.com; http://www.iucnredlist.org).

RESULTS

The present study revealed the occurrence of 92 species of fish (Table I). Out of which 77 species were identified as freshwater and 15 identified as migratory fishes. All of these species belonging to 11 orders and 30 families from eight sites of Indus River downstream. The larger fish biomass collection from the RM where 9 orders, 21 families with 81 species was recorded, in which maximum species were found in order Siluriformes 29 species followed by the order Cypriniformes 28 species, in this site the most dominant specie was *Rita rita* belonging to family Bagridae and sub-dominant species is *Wallago attu* belonging to the family Siluridae. The list of fish species recorded from the Kk here 7 orders, 14 families with 44 species, here order Siluriformes is dominant in which 14 species are present towards the order Cypriniformes having 9 species. In this site the specie *Cirrhinus mrigala* is dominant followed by *Labeo rohita* belonging to the same family Cyprinidae. The distribution of fish species is quite variable at Kp in which 6 orders, 15 families and 38 species was collected, here order Siluriformes having 16 species compared with the order Cypriniformes having 12 species. On this site the *Rita rita* is in good quantity which belongs to family Bagridae while the *Labeo gonius* is dominant which is belong to the family Cyprinidae. Fish fauna at the Mk in which 8 orders, 17 families and 40 species were taken by the fishermen, in which both orders Cypriniformes and Siluriformes are in abundance having 14 species each of them followed by the order Perciformes had 4 species. From this site the specie *Cirrihinus mrigala* is dominant which belongs to the family Cyprinidae rather than the specie *Rita rita* is in recessive manner which belongs to the family Bagridae competed by the specie *Channa oriental* which belongs to the family Channidae. The lowest collection of fish species in all the sites of downstream of Indus River from WMS just 7 orders, 13 families and 32 species was found, the prominent order is Cypriniformes 13 species followed by the order Siluriformes 9 species. In which the specie *Separata seenghala* is in abundant quantity belongs to the family Bagridae while the specie *Labeo rohita* is in minimum quantity belongs to the family Cyprinidae. Considerable relative abundance of the species was also observed at BM here 8 orders, 17 families and 66 species was caught by fisherman, wiz the first site where the dominance of order Cypriniformes with 22 species followed by the order Siluriformes having 19 species. In which the most abundant fish species is *Labeo rohita* belongs to the Cyprinidae family followed by the specie *Wallago attu* belongs to the family Siluridae. The collection of fish species at the Sj are 9 orders, 14 families and 46 species was found in which the order Siluriformes is on high level 13 species followed by the order Cypiriniformes 12 species. On this site a highly commercial fish species is in abundancy name *Tenualosa ilisha* belongs to the family Clupeidae rather than the native species *Cirrihinus mrigala* belongs to the family Cyprinidae. And the last JS which is near to the delta region, in this station 8 orders, 20 families and 48 species was collected. Here mostly estuary water species are found in which order Siluriformes observed in maximum quantity including 17 species while order Perciformes in minimum quantity having 10 species. In which the abundant specie is *Liza aurata* and *Chelon subviridis* both belongs to the family Mugilidae followed by the specie *Rita macracanthus* belongs to the family Bagridae. A great collection of migratory fishes was also recorded during collection those includes 15 species.

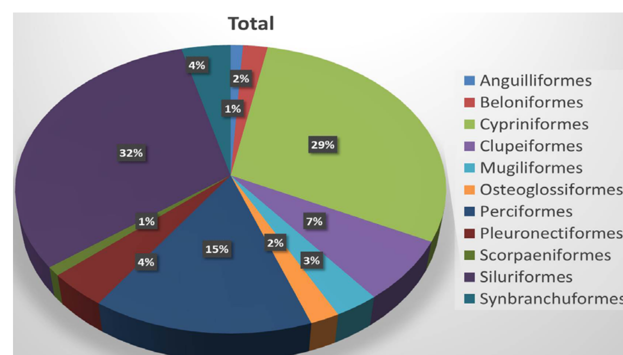

Fig. 1. The order dominance.

The distribution of fish species density, abundance and distribution is quite variable because of geographical and hydrological conditions which was collected from 8 landing areas are shown in Table I. Although, 92 species were recorded, overall, the order Siluriformes was maximum quantity with 29 or 31 fish species followed by order Cypriniformes 27 or 29 species and Perciformes 13 or 15 species. In all of them family Cyprinidae was observed as the dominant family with 24 fish species (26%) followed by Bagridae 13 fish species (14%) and Siluridae 7 species (8 %), as shown in Figure 1. However, the Genus

Mystus is in abundant quantity in which the collection of species are five the names are *M. bleekre, M. cavasius, M. gulio, B. halepinus, M. seengtee* and *M. vittatus* followed by the family Cyprinidae the dominant Genus is *Labeo* in which four species was found *L. bata, L. calbasu, L. gonius* and *L. rohita*, while in family Siluridae there isn't abundancy of any genus. When collection of all sites were compared, highly edible fish quantity is *Cirrihinus mrigala* followed by the *Labeo rohita* and in rare quantity specie is *Catla catla* similarly changes occurs in distribution and abundance of fish is quite natural in natural waters.

Table I.- The systematic list of downstream of Indus River species collection.

S. No	Species	Local name	St-1	St-2	St-3	St-4	St-5	St-6	St-7	St-8
Order: Anguilliformes										
Family: Anguillidae										
1	*Anguilla anguilla*	Baam	√	√	×	√	×	√	√	×
Order: Beloniformes										
Family: Belonidae										
2	*Strongylura strongylura*	Kaang	√	×	√	×	×	×	√	×
3	*Xenentodon cancila*	Kaang	√	×	×	×	×	√	√	×
Order: Cypriniformes										
Family: Botiidae										
4	*Botia almorhae*	Rani machli 1	√	×	×	√	×	×	×	×
Family: Cyprinidae										
5	*Bangana ariza*	Rahu minnow	√	×	×	×	√	×	×	×
6	*Barbonymus gonionotus*	Popro (daro)	√	×	√	×	√	√	√	×
7	*Barilius tilio*	Krail	√	×	×	×	×	√	×	×
8	*B. barila*	Krail	×	×	×	×	×	√	×	×
9	*Catla catla*	Theela	√	√	√	√	√	√	√	×
10	*Chagunius chagunius*	Kather	√	×	√	×	×	×	×	√
11	*Cirrhinus reba*	Sohni	√	√	√	×	√	√	√	×
12	*C. mirigala*	Morakhi	√	√	√	√	√	√	√	×
13	*Crossocheilus crossocheilus*	Kather	√	×	×	×	×	×	×	×
14	*Ctenopharyngodon idella*	Grass carp	√	√	√	√	√	√	√	×
15	*Cyprinus carpio*	Gulfam	√	√	√	√	×	√	√	×
16	*Hypophthalmichthys nobilis*	Big Head Carp	√	×	×	×	×	×	√	√
17	*H. molitirx*	Silver Carp	√	√	×	√	√	√	×	×
18	*Labeo calbasu*	Dahi	√	√	√	√	√	√	×	×
19	*L. gonius*	Ganier	√	×	√	√	×	√	√	×
20	*L. bata*	Rahu minnow/Seriyo	√	√	√	√	√	√	×	×
21	*L. rohita*	Kororo	√	√	√	√	√	√	√	×
22	*Osteobrama cotio*	Chelori	√	×	×	√	×	√	√	√
23	*Pethia phutunio*	Popery 3	√	×	×	×	×	×	×	×
24	*P. ticto*	Chitti Popery	√	√	×	√	√	√	×	×
25	*Puntius sophore*	Popery 1	√	√	×	×	×	√	×	×
26	*P. chola*	Popery 2	√	×	×	×	×	√	×	√
27	*P. terio*	Ticto Popery	√	×	×	√	×	√	×	×
28	*Systomus sarana*	Popero	√	×	×	×	×	√	√	×
29	*Salmophasia bacaila*	Chandi/Othiyar	√	√	√	√	√	√	√	×
Family: Cobitidae (Loach)										
30	*Botia birdi*	Rani machli 2	√	×	×	×	√	√	×	×

S. No	Species	Local name	St-1	St-2	St-3	St-4	St-5	St-6	St-7	St-8
Order: Clupeiformes										
Family: Clupeidae										
31	*Anodontostoma chacunda*	Palla (black spot)	×	×	×	×	×	×	√	√
32	*Dayella malabarica*	Kather	√	×	×	√	√	×	×	×
33	*Gudusia chapra*	Palri	√	√	√	√	√	√	√	√
34	*Hilsa kelee*	GoberPalla	×	×	×	×	×	√	√	√
35	*Tenualosa ilisha*	Palla	√	√	√	√	√	√	√	√
Family: Pristigasteridae										
36	*Ilisha megaloptera*	Palla (big eye)	×	×	×	×	×	×	×	√
Order: Mugiliformes										
Family: Mugilidae										
37	*Chelon subviridis*	Chodi	×	×	×	√	√	√	√	√
38	*Liza aurata*	Phaar 1	×	×	×	×	×	√	×	√
39	*Mugil cephalus*	Phaar 2	√	×	×	×	×	×	√	√
Order: Osteoglossiformes										
Family: Notopteridae										
40	*Notopterus chitala*	Gandan	√	√	√	√	√	√	√	√
41	*N. notopterus*	Bati	√	√	×	×	√	√	×	×
Order: Perciformes										
Family: Ambassidae										
42	*Parambassis ranga*	Glass Fish (Hajam)	√	×	√	√	√	√	√	×
Family: Channidae										
43	*Channa gachua*	Mukur	√	×	×	×	×	√	√	×
44	*C. marulius*	Chitti mundi	√	√	×	√	×	√	√	√
45	*C. orientalis*	Mundo	√	×	×	√	√	√	√	√
46	*C. punctata*	Shakur	√	×	√	×	×	√	×	√
47	*C. striata*	Mundi	√	√	×	×	×	×	×	×
Family: Cichlidae										
48	*Oreochromis mossambicus*	Black dayo	√	×	√	×	×	√	×	×
49	*O. niloticus*	Irani dayo	√	×	×	×	×	√	×	×
Family: Ambassidae										
50	*Chanda nama*	Glass Fish (Kangii)	√	√	×	×	×	√	√	√
Family: Latidae										
51	*Lates calcarifer*	Dangri	√	√	√	√	×	√	√	√
Family: Gobiidae										
52	*Glossogobius giuris*	Gup guloo	√	√	√	√	×	×	×	√
53	*G. platycephalus*	AndhGuloo	√	×	×	×	√	√	×	√
Family: Totobas										
54	*Nibea coibor*	Muska/Seeiyer	×	×	×	×	×	√	×	√
Family: Sciaenidae										
55	*Aplodinotus grunniens*	Muska 2	×	×	×	×	×	×	√	√
Family: Sphyraenidae										
56	*Sphyraena barracuda*	Chela	×	×	×	×	×	×	×	√
Order: Pleuronectiformes										
Family: Soliedae										
57	*Brachirus orientalis*	Phareen	×	×	×	×	×	√	√	√
Family: Cynoglossidae										
58	*Cynoglossus lingua*	Phareen 2	×	×	×	×	×	√	×	√

S. No	Species	Local name	St-1	St-2	St-3	St-4	St-5	St-6	St-7	St-8
Order: Scorpaeniformes **Family: Platycephalidae**										
59	*Platycephalus indicus*	Khokar	×	×	×	×	×	√	√	√
Order: Siluriformes **Family: Ailiidae**										
60	*Clupisoma garua*	Chali	√	√	√	×	×	√	√	√
61	*Bagrus halepensis*	Mulreet 2	√	×	×	×	×	×	×	×
Family: Bagridae										
62	*Batasio batasio*	Rehra 1	√	×	×	×	×	√	×	×
63	*B. flavus*	Rehra 2	√	×	×	√	×	×	√	×
64	*Mystus bleekre*	Tengra 1	√	√	√	√	×	√	√	√
65	*M. vittatus*	Mulreet 1	√	√	√	√	√	×	√	√
66	*M. cavasius*	Mulreet 3	√	×	√	×	×	√	×	√
67	*M. gulio*	Tengra 3	√	×	×	√	×	×	√	×
68	*Ria rita*	Khagga	√	√	√	√	√	√	√	√
69	*R. kuturnee*	Sindhi Khaggi	√	√	×	×	×	√	×	√
70	*R. macracanthus*	Desi khagga	√	×	×	×	×	×	×	√
71	*Sperata aor*	Singhara	√	×	×	×	×	√	×	√
72	*S. seenghala*	Singhari	√	√	√	√	√	√	√	√
73	*Mystus seengtee*	Tengra 2	√	×	√	×	×	×	×	√
Family: Bagridae (S. catfish)										
74	*Bagarius bagarius*	Fogikhagga	√	√	×	√	√	√	√	√
Family: Sisoridae										
75	*Bagarius yarrelli*	Terbela	√	√	√	×	×	√	×	√
76	*Gagata cenia*	Kal'lo	√	√	×	×	×	×	×	×
77	*Eutropiichthys vacha*	Badshah	√	√	√	√	×	√	√	√
Family: Schilbeidae										
78	*Silonia childreni*	chalri	√	√	√	×	×	×	×	×
79	*Clarias batrachus*	Moor mangro	√	×	×	√	√	√	×	√
Family: Clariidae										
80	*Heteropneustes fossilus*	Singhi	√	√	√	√	×	√	√	√
Family: Heteropneustidae										
81	*Heteropneustes fossilus*	Loarh	√	√	√	×	√	×	×	×
82	*Belodontichthys macrochir*	Jhumro/ Rhinghi	√	×	√	×	×	√	×	√
Family: Siluridae										
83	*Wallago attu*	Jerko	√	√	√	√	√	√	√	√
84	*Wallagonia leeri*	Jerki	√	×	×	√	×	√	×	×
85	*Ompok siluroides*	Phabun	√	√	×	√	√	√	×	×
86	*O. pabo*	Pabu'n	√	√	×	×	×	√	√	×
87	*O. bimaculatus*	Pabo	√	√	√	√	×	×	×	×
88	*Pinniwallago kanpurensis*	Dhingri / Dhumro	√	√	√	×	√	√	√	×

S. No	Species	Local name	St-1	St-2	St-3	St-4	St-5	St-6	St-7	St-8
Order: Synbranchuformes										
Family: Mastacembelidae										
89	*Mactacembelus armatus*	Bhu'an	√	√	×	×	×	√	√	√
90	*Macrognathus aculeatus*	Behanri	√	√	×	×	×	×	√	√
91	*M. pancalus*	Otho	√	√	√	×	√	√	×	×
92	*M. siamensis*	Gooj	√	√	×	√	×	√	×	×

St-1, RM (Railo Miyan); St-2, Kk (Karokho); St-3, Kp (Khanpur); St-4, Mk (Mullakatiyar); St-5, WMS (Wasi Malook Shah); St-6, BM (Branch Morie); St-7, Sj (Sujawal); St-8, Js (Jangseer).

Human activities increase the pace of this change by applying catch pressure on certain species and stressing others by polluting the water. During the visits, discussion with people living around the Indus River and fishermen, it had been revealed that the Indus River had a large variety of fish in the past, the major reasons for fish population decline are illegal fishing as using prohibited nets, catch of small size fish, catch during breeding season and while, pollution due to industrial waste flow towards the River without treatment, household wastes, agricultural runoff, and irregular flow of water from upstream are the major factor affecting downstream biodiversity.

DISCUSSION

Pakistan is endowed with rich fishery potential. Located in the Northern part of the Arabian Sea. The diversity of any aquatic body had therefore been correlated with changes in flow regimes, which created new environments, providing opportunity to the species found in nearby water bodies to establish there (Mirza *et al*., 2011). Positive correlations between biomass production and species abundance have been recorded by various earlier workers (Nikolosky, 1978). Globally, in the recent years it has been reported that freshwater fish species could greatly change their present-day distribution in response to climate change (Mohseni *et al*., 2003; Chu *et al*., 2005) and has now become a serious threat to the freshwater diversity (Habit *et al*., 2006). The rich fish diversity in the lower stretch may be attributed to the significant contributions of larger numbers of tributaries and presence of protected area. The results of the present study showed that Ichthyofauna of the Downstream of Indus River was gradually need proper attention because it is going to be decreasing considerably. In previous report 180 fish species had been identified throughout the River and its tributaries (Mirza, 2003). The Indus River is lifeline of the people nearby villages mostly for various domestic activities like fishing for livelihood and food is common practice of the local community. The fish fauna of the Indus River and its tributaries had always been of interest. The damming of rivers and streams is often implicated as a cause for fish population decline and local extinction of freshwater fish (Christopher *et al*., 2001).

Approximately not much information available in the literature regarding the fish fauna in downstream of Indus River. The recent recorded fish species are 92 which almost half of previous recorders. It is possible that there might be changes occur in upstream which also effects on the downstream. In downstream of Indus River due to the sequential and significant decrease in the richness of fish species were observed due to the shortage of food availability, excessive fishing and scarcity of water in downstream. The fish productivity in Rivers is declining.

CONCLUSION

Present investigation suggests that we should take conservation measures and regular water flow towards the downstream. In further, government must take serious action against illegal fishing, control the industrial waste and the siltation at downstream. Hence, we can ensure the existence and growth of fish in order to improve the fish fauna of downstream. Likewise, it will play a great rule on the socio-economic conditions of fish farmers; those are directly dependant for their livelihood on the fisheries. If we could not control the water flow, towards downstream, to keep the water quality in normal range for growth and survival of fish then we could not uplift of the socio-economic conditions of the local people. Otherwise, poverty will increase and aquatic protein requirement will reach on high level. Hence, necessary measurements should be taken to ensure the conservation of species that will be increase the fisheries yield.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Ambasht, R.S., Srivastava, A.K. and Ambasht, N.K., 1994. Conserving the bio diversity of India: An

ecological approach. *Indian Forester*, **120**: 791-798.

Balian, E.V., Segers, H., Leveque, C. and Martens, K., 2008. An introduction to the fresh water animal diversity assessment: An overview of the results. *Hydrobiologia.,* **595**: 627-637.

Christopher, A.T., Knouft, J.H. and Hiland, T.M., 2001. Consequences of stream impoundment on fish communities in a small North American drainage. *Regul. Rivers Res. Manage*., **17**: 687-698. https://doi.org/10.1002/rrr.629

Chu, C., Mandrak, N.E. and Minns, C.K., 2005. Potential impacts of climate change on the distributions of several common and rare freshwater fishes in Canada. *Divers. Distrib*., **11**: 299-310. https://doi.org/10.1111/j.1366-9516.2005.00153.x

Day, F., 1880. *On the fishes of Afghanistan.* Proc. Zool. Soc. London, pp. 224-232.

Ehrlich, P.R. and Wilson, E.O., 1991. Biodiversity studies science and policy. *Science*, **253**: 758-762. https://doi.org/10.1126/science.253.5021.758

Galactos, K., Barriga-Salazar, R. and Stewart, D.J., 2004. Seasonal and habitat influences on fish communities within the lower Yasuni River basin of the Edu Amazon. *Environ. Biol. Fishes*, **71**: 33-51. https://doi.org/10.1023/B:EBFI.0000043156.69324.94

Habit, E., Belk, M.C., Tuckfield, R.C. and Parra, O., 2006. Response of the fish community to human induced changes in the Biobio River in Chile. *Freshw. Biol*, **51**: 1-11. https://doi.org/10.1111/j.1365-2427.2005.01461.x

Karr, J.R., 1981. Assessment of biotic integrity using fish communities. *Fisheries*, **6**: 21-27. https://doi.org/10.1577/1548-8446(1981)006<0021:AOBIUF>2.0.CO;2

Leal, I.R., Silva, J.M.C., Tabarelli, M. and Lacher, Jr. T.E., 2005. Changing the course of biodiversity conservation in the Caatinga of Northeastern Brazil. *Conserv. Biol*., **19**: 701-706. https://doi.org/10.1111/j.1523-1739.2005.00703.x

Li, S., Weimen, L. and Changdie, P., 1987. A genetic study of the growth performance of silver carp from Chiangjiang and Zhujiang rivers. *Aquaculture*, **65**: 93-104. https://doi.org/10.1016/0044-8486(87)90254-7

Mirza, M.R., 2003. Checklist of freshwater fishes of Pakistan. *Pakistan J. Zool. Suppl. Ser*., **3**: 1-30.

Mirza, M.R., 2006. Revised ichtyogeography of Pakistan and adjoining areas. *Biologia*, **2**: 105-115.

Mirza, Z.S., Mirza, M.R., Mirza, M.A. and Sulehria, A.Q.K., 2011. Ichtyofaunal diversity of River Jhelum, Pakistan. *Biologia.*, **57**: 23-32.

Mohseni, O., Stefan, H.G. and Eaton, J.G., 2003. Global warming and potential changes in fish habitat in U.S. streams. *Climat. Change*, **59**: 389-409.

Nikolosky, G.V., 1978. *The ecology of fishes*. T F H Publications, USA, pp. 352.

Rafique, M., 2000. Fish diversity and distribution in Indus River and its drainage system. *Pakistan J. Zool*., **32**: 321-332.

Rafique, M., Akhtar, S. and Niazi, M.H.K., 2003. Fish fauna of Jinnah Barrage and adjoining areas. *Pakistan J. Zool*., **35**: 95-98.

Talwar, P.K. and Jhingran, A., 1991. *Inland fishes of India and adjacent countries*. Oxford and IBH Publishing Co. Pvt. Ltd., New Delhi, pp. 1158.

Wilson, E.O., 1988. The current state of biological diversity. In: *Biodiversity* (ed. E.O. Wilson). National Academy Press, Washington, USA.

to be continued.....

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Effect of Processed Fish Waste on Growth Rate and Digestive Enzymes Activities in *Cyprinus carpio*

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Authors' Contribution
MSH, HA, AJ, S Abbas and S Ashraf designed the project and wrote the manuscript. MA and SI helped in lab work. UA contributed in lab work as well as in discussing the project. KJI and MB helped in data analysis.

Key words
Fish silage, Fatty acid profile, Protease, Amylase, Lipases.

ABSTRACT

Enzymes play an important role in digestion process and growth rate of fish. Enzyme activity is strongly affected by diet and ingredients used for feed formulation. So, present study was conducted to analyze effect of processed fish body viscera on digestive enzymes in *Cyprinus Carpio*. Fish body viscera were collected from local fish processing units and treated by formic acid and its fatty acid profile was also determined. Lipids were extracted from this processed waste and fatty acid profile was determined by gas liquid chromatography (GLC). Fatty acid profile showed that this processed body viscera contain large amount of unsaturated fatty acids and relative pattern of different fatty acids is mono un saturated fatty acid (MUFA) > saturated fatty acids (SFA) > poly unsaturated fatty acids (PUFA). Proximate analysis of this treated viscera showed that it can adequate amount of essential nutrients. So further, three treatment diets were prepared by incorporation of different percentages of each silage 100% silage (T_1), 75% (T_2) silage and 50% (T_3) while in fourth treatment diet, control diet (T_4) fish body viscera was replaced by fish meal. Treatment diets were offered to *Cyprinids carpio,* fingerlings having body weight 15±4.55g, at the rate of four percent body weight twice a day. Fortnightly growth paprameters like, weight gain, length gain, feed conversion ratio and mortality rate as recorded. After completion of three month feeding trial, three fish were randomly dissected, their intestines were excised out, and enzymes *i.e.* protease, amylase and lipase activity was determined. The statistical analysis showed that enzyme activity of these enzymes showed that there was non-significant variation in protease activity while amylase and lipase differ significantly amonfg all treatment diets. So it was be concluded that waste fish body viscera can be used safely to replace costly fish meal in fish diet. Moreover, protease enzyme activity vary according to protein concentration in diet.

INTRODUCTION

After degutting a large amount of fish waste is produced in form of scale, skin, fins and body viscera. Fish is degutted because its digestive system contains a large amount of enzymes which cause petrification. Improper disposing of this waste *i.e.* buried off inland or wasted in sea, causes environmental pollution (Nagai and Suzuki, 2000). Proper utilization of this body viscera not only reduce expenditures of disposing off but it may also use to generate revenue. Despite some valuable nutrients, this wasted body viscera also contains a high amount of valuable substances like collagen, gelatin and many enzymes can also be extracted from this waste (Tidwell and Allan, 2001). Another possible alternative method to get rid of, this waste material is to convert it into animal feed after proper processing as a valuable protein source (Krogdahl *et al*., 2005).

Among already used ingredients fish meal is a major ingredient but due to its high cost and scarcity, efforts are turning to search its suitable alternate (Lapie and Bigueras, 1992). As this waste contain a large amount of macro nutrients like protein and fats. Protein is a major components in aqua diets so this waste can be used in feed

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formulation after proper processing (Haga *et al*., 2003). An alternate which can successfully replace fish meal is this processed fish body viscera treated with formic acid or processed through fermentation. This processed waste is also called fish silage (Geron *et al*., 2007).

Both types of prepared fish silages either acid or fermented silage, are rich with essential nutrients like proteins and fats (Chakrabarti and Sharma, 2005; Bolasina *et al*., 2006). Lipids present in fish silage are usually categorized as saturated, unsaturated, mono unsaturated and poly unsaturated fatty acids. These fatty acids play an important and dynamic role in fish growth (Nettleton, 1991; Klor *et al*., 1997; Jabeen and Chaudhry, 2011). So fatty acid profile analysis is an essential tool to estimate quantity and quality of fatty acids. Because fats contains double amount of energy as compared to other non-protein energy sources. Utilization of fats spare protein for other constructive purposes in fish body (Lee and Putnam, 1973). Different researchers have already successfully used prepared silage in diet of different fish species e.g. Atlantic salmon, *Salmo salar* (Parrish *et al*., 1991), for rainbow trout, *Oncorhynchus mykiss* (Aksnes *et al*., 2006), for tilapia, *Oreochromis niloticus* (Fagbenro *et al*., 1994) and for Silver carp larvae, *Cyprinus carpio* (Carvalho *et al*., 1998).

Composition of this processed silage depends upon quality of body viscera used for silage preparation and its digestibility depends upon enzymes concentration in fish digestive tract (Stone, 2003; Seenappa and Devaraj, 1995). These digestive enzymes work in collaboration with other beneficial bacteria and micro-organisms already present in fish digestive system and help in digestion process (Garcia-Carren and Haard, 1993; Furne *et al*., 2008). Digestion process mainly depends upon feed ingredients type and concentration of digestive enzymes (Alexis, 1990). So study of enzymes activity may help in feed formulation and ingredient selection and feed management (Fernandez *et al*., 2011). It has already been proved that herbivore fish have more concentration of carbohydrate digestive enzymes while carnivorous fishes contains high concentration of protease enzymes (Hidalgo *et al*., 1999).

In south Asia and especially in Indian sub-continent *Cyprinus carpio* is most preferred and most cultured fish (Misra and Samantaray, 2004). So this fish species was selected as experimental fish in this project.

Keeping in view all this scenario present research project was designed to analyse fatty acid profile of fish acid silage and to check its possible effects on growth rate and enzyme activity in above said fish.

MATERIALS AND METHODS

Fish body viscera were collected from fish processing plants located, University of Veterinary and Animal Sciences, Lahore. Collected body viscera were washed with distills water to remove debris and minced with electric mincer. Minced body viscera were treated with further processed following Jialin and Lied (2001). Minced fish body viscera were mixed up with 80% concentrated Formic acid @ 3% v/w. Methoxyquine, 2ml/kg was added as anti-oxidant to preserve fatty acids. Whole mixture was thoroughly mixed and homogenize. Mixture was stored at ambient temperature covered with lid. Twice a day, mixture was stirred on daily basis and pH was maintained at 3.5. Lipids were removed weekly from upper surface of silage. At the end of thirty days storage period dark brown pasty type material with strong fishy smell was produced. This mixture was sun dried and grinded by feed grinder electric machine. The powdered silage was stored at dry place till further processing.

Sample analysis for fatty acid analysis

Bligh and Dyer (1959) method were followed for lipids extraction from prepared fish sialge. Fatty acid analysis was performed by following Kiessling *et al*. (2001). Boron trifluoride-methanol complex was used for esterifications. The fatty acid methyl esters were used as standers for fatty acid comparisons. Reagent 2, 7-dichlorofluorescein was used for colored spots of lipid under ultra violet light; λ 366 nm.

Table I.- Percentages of various ingredients and their protein level used in four diets.

Ingredients	100% silage		75% silage		50% silage		Control	
	Weight (g)	CP (%)	Weight (g)	CP (%)	Weight (g)	CP (%)	Weight (g)	CP (%)
Fish meal	-	-	-	-	-	-	32	14.8
Fish silage	100	31.18	75	21.5	50	15.15	-	
Soy bean meal	-	-	19	9.4	30	14.13	29	11.63
Rice bran	-	-	8	0.9	20	2.33	39	5.1
Total	100	31.18	100	31.8	100	31.51	100	31.53

Gas-liquid chromatography (GLC) was performed in Pakistan Counsel of Scientific and Industrial Research (PCSIR), Lahore. Fatty acid methyl esters (FAME) were separated identified and quantified using a GLC. Helium gas was used as a carrier agent. Methyl esters were identified by their comparison with chromatograms of standards. Peaks were identified by their retention time. Beside fatty acid analysis proximate analysis of above prepared silage was performed by following AOAC (2001) to check the nutritional quality of silage. When it was found having appropriate nutritional values it was used in fish feed for further estimation.

Diet preparation

Four is nitrogenous diets were prepared incorporation of this silage with other three conventional feed ingredients *i.e.* rice bran and soya bean meal at different ratiosviz 100%, 75%, 50%. These diets were termed as T_1, T_2, T_3 while fourth control diet was prepared by inclusion of fish meal instead of fish silageand termed as T_0. All ingredients were well mixed and grinded (Table I).

Feeding trial

The *Cyprinus carpio* fingerlings with an average weight of 13.25±1.23 g were kept in glass aquaria dimensions (3′×2′×2′) having water retention capacity 150 liters water. The one third of aquaria was filled with fresh water and water quality parameters *i.e.* dissolved oxygen level, temperature, pH was kept within optimum range. The fingerlings were fed twice a day @ 4% of their body weight. The feed was offered in powder form. Fifteen fingerlings were kept in each aquarium and experiment was run in triplicate. Growth parameters like weight gain, length gain, FCR, SGR and mortality was calculated fortnightly. The experiment continued for 92 days from 15 May 2015 to 15 August 2015.

Enzyme activity

After completion of feeding trial three fish were randomly selected and sacrificed from each aquaria. Fish intestine was separated and washed with cool distilled water. Excised out Intestine were homogenized by electrical homogenizer and centrifuged @20,000rpm for 10 min at 0°C. Then supernatant was removed and used as enzyme assay for further processing.

Protease enzyme activity was determined by following Kunitz (1947). Already prepared supernatant 5ml phosphate buffer 2 ml having pH 7.5 and reaction mixture containing 1% casein 2ml (as substrate) were mixed and incubated at 35°C for 20 min in oven. After 20 min incubation, 5% TCA 5ml was added in mixture to stop reaction. Then optical density of sample was checked by photometer at λ 280nm. Tyrosine curve was used as standard and activity was expressed as mole of tyrosine released/min/mg protein 37°C.

Cherry and Crandall (1932) was followed to determine lipase activity. Phosphate buffer solution (pH 7.5) 5 ml, tissue homogenate1 ml, distilled water 5 ml, and olive oil emulsion 3.5 ml were mixed for preparation of reaction mixture. After mixing the mixture was incubated at 24°C for 24 h in electric oven. After incubation this mixture was titrated with 0.05 N NaOH until appearance of pink color. Amount of NaOH consumed was calculated to measure amount of fatty acids released. Enzyme source without emulsion and buffer was used as standard.

For amylase activity Sangeetha *et al*. (2010) were followed. Amylase starch solution, prepared in phosphate buffer (pH 7.5) 5 ml, was used as substrate. Already prepared reaction mixture consisting of enzyme supernatant 5 ml, starch solution 5ml and distilled water 3.5 ml were mixed well and incubated at 35°C for 30 min in electric oven. After 30 min, 5ml of 5% dinitrosalicylic acid (DNS) solution was added in mixture to stop reaction. Mixture was diluted by addition of 20mL distilled water and its optical density was recorded by photometer at ƛ540 nm. Standard maltose curve was used for comparison of amylase activity. The amylase activity was expressed as mole of maltose released from starch/min/mg protein at 35°C.

RESULTS AND DISCUSSIONS

As diets were iso nitrogenous having 30% protein level and were prepared by mixing Soya bean meal and rice bran along with prepared fish silage. These diets were prepared by keeping in view diet formulation method prescribed by Govoni *et al*. (1986) who stated that many factors like price, availability of ingredients used in raw material, anti-nutritional factors present in raw material and palatability of mixtures must be considered while formulating fish feed. Soybean meal along with other ingredients such as rice bran, corn flour, milk powder can be successfully used for fish feed formulation. The similar procedure has been described by Aksnes *et al*. (2006).

Fatty acid analysis

The fat contents obtained were observed as 5.12±1.17 g/100g of sample in our prepared fish acid silage. These results are similar to the results as described by Wassef *et al*. (2002a) and seabass (Sakr, 2004). They found that lipid concentration may vary from 14.84, 12.20 and 14.29 g/100 g lipid contents, respectively in different fish silage samples. Similar findings has been described by Kiessling *et al*. (1991) and Kaushik *et al*. (2006) who also concluded that the final composition and nutritive value of silage

varies according to season of fish harvesting, and even if same type of fish is used for silage preparation it vary according to part of fish used for silage preparation.

Table II.- Fatty acid analysis of pure fish acid silage.

S No.	FA*	Acid fish silage			
		I	II	III	Average
1	$C_{12:0}$	0.08	0.07	0.08	0.076±0.005
2	$C_{14:0}$	13.17	13.26	12.77	13.06±0.02
3	$C_{16:0}$	18.33	17.26	18.35	17.980.62
4	$C_{16:1}$	10.82	11.99	10.63	11.14±0.73
5	$C_{18:0}$	3.51	3.44	3.45	3.46±0.03
6	$C_{18:1}$	30.88	29.16	29.57	29.87±0.89
7	$C_{18:2}$	16.49	17.28	17.41	17.06±0.49
8	$C_{18:3}$	1.61	1.83	1.16	1.53±0.34
9	$C_{20:0}$	0.69	0.17	0.04	0.3±0.34
10	$C_{20:1}$	0.92	0.98	0.86	0.92±0.06
11	TSFA	35.78	34.2	34.69	34.89±0.80
12	MUFA	42.62	42.13	40.2	41.65±1.27
13	PUFA	18.1	19.11	18.57	18.59±0.50
14	TFA	ND	ND	ND	ND

TSFA, total saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

The fatty acid analysis results are presented in Table II. Results revealed that saturated fatty acids (SFA), mono un-saturated fatty acids (MUFA), and poly unsaturated fatty acids (PUFA) were presented in our prepared silage in large quantity. These values were recorded as 34.89±0.80, 41.65±1.27, 18.59±0.50 for TSFA, MUFA and PUFA, respectively. The results indicates these fatty acids are present in relative pattern of MUFA>TSFA>PUFA. Our results are in line with those of Arruda *et al*. (2004) and Jabeen and Chaudhry (2011). They reported similar pattern of fatty acids analysis *e.g.* SFA>MUFA>PUFA while analyzing profile of carp fish species collected from Indus river. However, Din *et al*. (2004) stated that relative pattern of fatty acid may be reversed in some cases, and amount of MUFA may exceed greater than PUFA as shown in carp cases (Stancheva and Merdzhanova, 2011). Differences in pattern of fatty acids can be justified by Tocher (2003) and Nurasyikin and Jumat (2006) who reported that fatty acid profile of processed fish body viscera vary according to type and concentration of different fatty acid present in fish body viscera. According to Ozogul and Ozogul (2007) and Kalyoncu *et al*. (2010), quality and quantity of unsaturated fatty acids strongly vary between wild and farmed fish species due to variation in their diet as wild fish feed a variety of natural ingredients as compared to farmed fish. According to Sargent *et al*. (1999) eicosapentaenoic acid (EPA 20:5n-3), docosahexaenoic acid (DHA 22:6n-3) and probably, arachidonic acid (AA, 20: 4n-6) are considered essential in diet for normal growth and survival of aquatic organisms. However this ratio may vary according to species type and their life stage. This variation is primarily due to their ability to convert fatty acids from one form to an other (Martino and Portz, 2006).

Enzyme activity

Enzyme activity of digestive enzymes *i.e.* protease, amylase and lipase is presented in Table III. According to our results protease enzyme showed non-significant variation in activity among all treatment diets T_1, T_2, T_3 and T_0 prepared by different inclusion level of processed fish body viscera. Different researchers have shown different results for protease activity. Lopez-Lopez *et al*. (2005) reported that there is no strong correlation between protease activity and dietary crude protein. According to Le Moullac *et al*. (1994) and Krogdahl *et al*. (1999) the quantity of protease and amylase enzyme fluctuate with variation in concentration of carbohydrate and protein in fed diets. However, if concentration of these components increases beyond limits, concentration of amylase and protease start to decrease (Cara *et al*., 2003). In our experiment all treatment diets were iso-nitrogenous so there was no significant difference in protease activity.

Table III.- Enzyme activity of different enzymes protease, amylase, lipase in digestive tract of *Cyprinus carpio* fingerlings fed on different treatment diets.

Enzymes	Fish acid silage			Control
	T_1	T_2	T_3	T_0
Protease	14.25±0.97^{ab}	13.61±0.52^{ab}	15.85±1.72^{a}	12.87±1.52^{b}
Amylase	8.44±1.13^{ab}	9.35±0.49^{a}	8.25±0.07^{ab}	11.88±0.68^{ab}
Lipase	1.31±0.39^{a}	1.66±0.51^{a}	0.94±0.24^{a}	1.33±1.01^{a}

Enzyme activities are expressed as: Protease, micromol of tyrosine released/min/g protein; Amylase, micromol of maltose released/min/g protein; Lipase, units/mg protein. Different superscripts in the same column signify statistical differences ($p<0.05$).

Amylase enzyme showed significant variation among treatment diets T_1, T_2, T_3, T_0 in our experiment. This significant difference can be justified by comparison with findings of Sabapathi and Teo (1993) and Hidalgo *et al*. (1999) who found lower amylase activity in Carnivorous fish and higher one in Omnivorous fish because in diet of carnovourous fish there is low concentration of carbohydrates as compared to omnivorous fish. As concentration of processed body viscera vary among all treatment diets so there is variation in carbohydrate concentration among all treatment diets as a result amylase activity showed variation in our results.

Table IV.- Weight gain, length gain, FCR, SGR and survival rate of *Cyprinus carpio* fingerlings.

Treatments	Total weight gain	Total length gain	FCR	SGR	Survival (%)
T_1	37.31±6.17a	13.02±1.43a	2.48±1.08b	1.29a	100
T_2	33.70±5.21a	12.89±1.61b	3.36±1.53a	0.99b	100
T_3	31.18±9.34a	11.68±1.56b	3.51±1.92a	0.87b	100
T_0	38.17±6.94a	12.60±1.29ab	2.53±1.37b	1.19a	100

Values are Mean±SE. Means with the same latter in the same column are not statistically significantly different ($P<0.05$).

Our results about amylase activity can also be justified by Cahu *et al.* (1999) and Lopez-Lopez *et al.* (2005) who reported non-corelation among amylase and dietary carbohydrates contents in diet in *Homarus americanus* and *Cherax quadricarinatus*, respectively. Different researchers (Sabapathi and Teo 1993; Kurokawa *et al*., 2000; Kurokawa and Suzuki, 2002) have proved that fish feeding habit, type of carbohydrate present in feed (NRC, 1993), temperature of environment and season of fish harvesting (Kuźmina *et al*., 1996) can influence the digestive enzyme activity in fish. So our results may vary to some extent from previous ones.

Lipase enzyme also showed significant difference in all treatments. T_1 treatment of both types showed greater Lipase enzyme activity as compared to other treatments. This variation in results can be justified by comparison with findings of Ma *et al*. (2005), Lundstedt *et al*. (2004) and Chakrabarti *et al*. (1995) who reported same observations. They reported that lipase activity varies according to amount of lipid contents in diets and this activity is more prominent in carnivorous fishes. As for as our results are concerned there is variation in concentration of fish silage among all treatment diets and with variation in silage concentration lipid contents also vary so there is concentration of lipase enzyme activity among all treatment diets and same findings has been described by Klomklao *et al*. (2006).

Growth rate

Grwoth data indicates that maximum weight gain was in T_1 and T_0 *i.e.* 39.31±6.71 and 38.17±6.94g, respectively. Statistical analysis showed that T_1 and T_0 have significant difference from T_2 and T_3. This difference can be justified by Alwan *et al.* (1993) and Gildberg (2001) who stated that other nutrients, other than protein also play critical role in growth of fish. As diets proximate analysis results is presented in Table IV, which indicates that although all treatment are iso nitrogenous but other nutrients concentration vary in all treatment diets so results about weight gain may vary in all treatment diets.

FCR was also showed non-significant difference among T_1 and T_0 while these two treatments showed significant difference from T_2 and T_3. Best FCR was observed in T_1 which is pure silage and contain maximum protein. Our results are in line with Jeena *et al.* (1998) who reported that FCR value decrease as quality of diet improves. Kumar *et al.* (2005) also gave same findings that, well balance diet have maximum protein and it gave best FCR. In our result as T_1 is purely processed body viscera and T_0 also contain fish meal which also contain good nutrition quality so both treatment diets gave good result.

CONCLUSIONS

It is concluded that fish acid silage and fermented fish silage, both contain high concentrations of monounsaturated fatty acids which are beneficial for fish growth. The activity of protease, amylase and lipase changes with change in protein, carbohydrate and lipid concentration in diet.

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Statement of conflict of interest

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REFERENCES

Aksnes, A., Hope, B., Jönsson, E., Björnsson, B.T. and Albrektsen, S., 2006. Size fractionated fish hydrolysate as feed ingredient for rainbow trout (*Oncorhynchus mykiss*) fed high plant protein diets. I. Growth, growth regulation and feed utilization. *Aquaculture*, **261**: 305-317. https://doi.org/10.1016/j.aquaculture.2006.07.026

Alawan, S.R., Buckley, D.J. and Connor, T.P., 1993. Silage from fish waste: Chemical and microbial aspects. *Ir. J. Fd. Sci*., **32**: 75-81.

Alexis, M.N., 1990. Comparative evaluation of

soybean meal and carob seed germ meal as dietary ingredients for rainbow trout fingerlings. *Aquat. Living Resour*., **3**: 235-241. https://doi.org/10.1051/alr:1990024

AOAC, 2001. *Official methods of analysis*. Association of Official Analytical Chemists, Arlington, VA, pp. 1141.

Arruda, S.F., Siqueira, E.M. and Souza, E.M., 2004. Malanga (*Xanthosoma sagittifolium*) and purslane (*Portulaca oleracea*) leaves reduce oxidative stress in vitamin A-deficient rats. *Annls. Nutr. Metab*., **48**: 288-295. https://doi.org/10.1159/000081075

Berge, G.M. and Storebakken, T., 1996. Fish protein hydrolyzate in starter diets for Atlantic salmon (*Salmo salar*) fry. *Aquaculture*, **145**: 205-212. https://doi.org/10.1016/S0044-8486(96)01355-5

Bligh, E.G. and Dyer, W.J., 1959. A lipid method of total lipid extraction and purification. *Can. J. Biochem*., **37**: 911-917.

Bolasina, S., Perez, A. and Yamashita, Y., 2006. Digestive enzymes activity during ontogenetic development and effect of starvation in Japanese flounder (*Paralichthys olivaceus*). *Aquaculture*, **252**: 503-515. https://doi.org/10.1016/j.aquaculture.2005.07.015

Cahu, C.L., Zambonino-Infante, J.L., Quazuguel, P. and Le-Gall, M.M., 1999. Protein hydrolysate vs fish meal in compound diets for 10-day-old sea bass *Dicentrarchus labrax* larvae. *Aquaculture*, **171**: 109-119. https://doi.org/10.1016/S0044-8486(98)00428-1

Cara, J.B., Moyano, F.J., Cardenas, S., Fernandez-Diaz, C. and Yufera, M., 2003. Assessment of digestive enzyme activities during larval development of white bream. *J. Fish Biol*., **63**: 48-58. https://doi.org/10.1046/j.1095-8649.2003.00120.x

Carvalho, E.D., Flavia-Batista Da Silva, V., Fujihara, C.Y., Henry, R. and Foresti, F., 1998a. Diversity of fish species in the River Paranapanema - Jurumirim Reservoir transition region (São Paulo, Brazil). *Ital. J. Zool*., **65**: 325-330. https://doi.org/10.1080/11250009809386841

Chakrabarti, I., Gani, M.D.A., Chaki, K.K., Sur, R. and Misra, K.K., 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. *Comp. Biochem. Physiol. A*, **112**: 167-177. https://doi.org/10.1016/0300-9629(95)00072-F

Chakrabarti, R. and Sharma, J.G., 2005. Digestive physiology of fish larvae during ontogenic development: A brief overview. *Indian J. Anim. Sci*., **75**: 1337-1347.

Cherry, I.S. and Crandall, L.A., 1932. The specificity of pancreatic lipase: Its appearance in the blood after pancreatic injury. *Am. J. Physiol*., **100**: 266-274. https://doi.org/10.1152/ajplegacy.1932.100.2.266

Din, J.N., Newby, D.E. and Flapan, A.D., 2004. Omega 3 fatty acids and cardiovascular disease fishing for a natural treatment. *Br. med. J*., **328**: 30-35. https://doi.org/10.1136/bmj.328.7430.30

Fagbenro, O., Jauncey, K. and Haylor, G., 1994. Nutritive value of diets containing dried lactic acid fermented fish silage and soybean meal for juvenile *Oreochromis niloticus* and *Clarias gariepinus*. *Aquat. Living Resour*., **7**: 79-85. https://doi.org/10.1051/alr:1994010

Fernandez, I., Moyano, F.J., Diaz, M. and Martınez, T., 2011. Characterization ofamylase activity in five species of Mediterranean sparid fishes (Sparidae, Teleostei). *J. exp. Mar. Biol. Ecol*., **262**: 1-12. https://doi.org/10.1016/S0022-0981(01)00228-3

Furne, M., Garcia-Gallego, M., Hidalgo, M.C., Morales, A.E., Domezain, A., Domezain, J. and Sanz, A., 2008. Effect of starvation and refeeding on digestive enzyme activities in sturgeon (*Acipenser naccarii*) and trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. A: Mol. Integr. Physiol*., **149**: 420-425. https://doi.org/10.1016/j.cbpa.2008.02.002

Garcia-Carren˜o, F.L., Navarrete del Toro, A. and Ezquerra, J.M., 1997. Digestive shrimp proteases for evaluation of protein digestibility *in vitro*: I. Effect of protease inhibitors in protein ingredients. *J. Mar. Biotechnol*., **5**: 36-40.

Geron, L.J.V., Zeoula, L.M., Vidotti, R.M., Matsushita, M., Kazama, R., Neto, S.F.C. and Fereli, F., 2007. Chemical characterization, dry matter and crude protein ruminal degradability and *in vitro* intestinal digestion of acid and fermented silage from tilapia filleting residue. *Anim. Feed Sci. Technol*., **136**: 226-239. https://doi.org/10.1016/j.anifeedsci.2006.09.006

Gildberg, A., 2001. Utilisation of male Arctic caplin and Atlantic code intestine for fish sausce production. *Bioresour. Technol*., **76**: 119-123. https://doi.org/10.1016/S0960-8524(00)00095-X

Gobran, A.S., 2000. *Development of diets for Nile and blue tilapia (*Oreochromis niloticus *and* O. aureus*) culture in Egypt*. Ph. D. thesis, Faculty of Science, Menoufiya University, Menofia Governorate, Egypt, pp. 166.

Gokoglu, N., Yerlikaya, P. and Cengiz, E., 2004. Effects of cooking methods on the proximate composition and mineral contents of rainbow trout (*Oncorhynchus mykiss*). *Fd. Chem*., **84**: 19-22.

https://doi.org/10.1016/S0308-8146(03)00161-4

Govoni, J.J., Boehlert, G.W. and Watanabe, Y., 1986. The physiology of digestion in fish body. *Environ. Biol. Fish*., **16**: 59-77. https://doi.org/10.1007/BF00005160

Haga, Y., Suzuki, T., Kagechika, H. and Takeuchi, T., 2003. A retinoic acid receptor-selective agonist causes jaw deformity in the Japanese flounder, *Paralichthys olivaceus*. *Aquaculture*, **221**: 381-392. https://doi.org/10.1016/S0044-8486(03)00076-0

Hidalgo, M.C., Urea, E. and Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits: Proteolytic and amylase activities. *Aquaculture*, **170**: 267-283. https://doi.org/10.1016/S0044-8486(98)00413-X

Jabeen, F. and Chaudhry, A., 2011. Chemical compositions and fatty acid profiles of three freshwater fish species. *Fd. Chem*., **125**: 991-996. https://doi.org/10.1016/j.foodchem.2010.09.103

Jena, J.K., Aravidakshan, P.K., Suresh, C., Muduli, H.K., Ayyappan, S. and Chandra, S., 1998. Comparative evaluation of growth and survival of Indian major carps and exotic carps in raising fingerlings. *J. Aquacult. Trop*., **13**: 143-149.

Jialin, W. and Lied, E., 2001. Two types of fish silage and its use in fish feed. *Mar. Fish. Res*., **22**: 80-86.

Kalyoncu, L., Yaman, Y. and Aktumsek, A., 2010. Seasonal changes on total fatty acid composition of carp (*Cyprinus carpio* L.) in İvriz Dam Lake, Turkey. *Afr. J. Biotechnol*., **9**: 3896-3900.

Kaushik, S.J., Corraze, G., Radunz-Neto, J., Larroquet, L. and Dumas, J., 2006. Fatty acid profilesof wild brown trout and Atlantic salmon juveniles in the Nivelle basin. *J. Fish Biol*., **68**: 1376-1387. https://doi.org/10.1111/j.0022-1112.2006.01005.x

Kiessling, A., Aasgaard, T., Storebakken, T., Johansson, L. and Kiessling, K.H., 1991. Changes in the structure and function of the muscle of rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age. *Aquaculture*, **93**: 373-383. https://doi.org/10.1016/0044-8486(91)90225-V

Kiessling, A., Pickova, J., Johansson, L., Asgard, T., Store-Bakken, T. and Kiessling, K.H., 2001. Changes in fatty acid composition in muscle and adipose tissue of farmed rainbow trout (*Oncorhynchus mykiss*) in relation to ration and age. *Fd. Chem*., **73** : 271-284. https://doi.org/10.1016/S0308-8146(00)00297-1

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K., 2006. Purification and characterization of trypsin from the spleen of tongol tuna (*Thunnus tonggol*). *J. Agric. Fd. Chem*., **54**: 5617-5622. https://doi.org/10.1021/jf060699d

Klor, H., Hauenschild, H., Holbachi, I., Kreshtchmer, H. and Stroh, S., 1997. Nutrition and cardiovascular disease. *Eur. J. med. Res*., **2**: 243-257.

Krogdahl, A., Hemre, G.I. and Mommsen, T.P., 2005. Carbohydrates in fish nutrition: Digestion and absorption in postlarval stages. *Aquacult. Nutr*., **11**: 103-122. https://doi.org/10.1111/j.1365-2095.2004.00327.x

Kumar, S., Srivastva, A. and Chakrabarti, R., 2005. Study of digestive proteinases and proteinase inhibitors of *Daphnia carinata*. *Aquaculture*, **243**: 367-372. https://doi.org/10.1016/j.aquaculture.2004.10.011

Kunitz, M., 1947. Isolation of a crystalline protein compound of trypsin and of soybean trypsin-inhibitor. *J. Gen. Physiol*., **30**: 311-320. https://doi.org/10.1085/jgp.30.4.311

Kurokawa, T., Suzuki, T. and Andoh, T., 2000. Development of cholecystokinin and pancreatic polypeptide endocrine systems during the larval stage of Japanese flounder, *Paralichthys olivaceus*. *Gen. Comp. Endocrinol*., **120**: 8-16. https://doi.org/10.1006/gcen.2000.7512

Kurokawa, T. and Suzuki, T., 2002. Development of neuropeptide Y-related peptides in the digestive organs during the larval stage of Japanese flounder, *Paralichthys olivaceus*. *Gen. Comp. Endocrinol*., **126**: 30-38. https://doi.org/10.1006/gcen.2001.7774

Kuźmina, V.V., Golovanova, I.L. and Izvekova, G.I., 1996. Influence of temperature and season on some characteristics of intestinal mucosa carbohydrates in six freshwater fishes. *Comp. Biochem. Physiol*., **113**: 255-260. https://doi.org/10.1016/0305-0491(95)02019-5

Lapie, L.P. and Bigueras-Benitez, C.M., 1992. Feeding studies on tilapia *Oreochromis* sp. using fish silage. In: *Proceedings of the IPFC workshop on fish silage* (ed. Ž.D. James). FAO Fisheries Report No. 470, FAO, Rome, pp. 165-177.

Le-Moullac, G., van Wormhoudt, A. and AQUACOP, 1994. Adaptation of digestive enzymes to dietary protein, carbohydrate and fiber levels and influence of protein and carbohydrate quality in *Penaeus vannamei* larvae (Crustacea, Decapoda). *Aquat. Living Resour*., **7**: 203-210. https://doi.org/10.1051/alr:1994022

Lee, D.J. and Putnam, G.B., 1973. The response of rainbow trout to varying protein/energy ratios in a test diet. *J. Nutr*., **103**: 916-912. https://doi.org/10.1093/jn/103.6.916

Lopez-Lopez, S., Nolasco, H., Villarreal-Colmenares, H. and Civera-Cerecedo, R., 2005. Digestive enzyme response to supplemental ingredients in practical diets for juvenile freshwater crayfish *Cheraxquadri carinatus*. *Aquacult. Nutr*., **11**: 79-85. https://doi.org/10.1111/j.1365-2095.2004.00305.x

Lundstedt, L.M., BibianoMelo, J.F. and Moraes, G., 2004. Digestive enzymes and metabolic profile of *Pseudoplatysto macorruscans* (Teleostei: Siluriformes) in response to diet composition. *Comp. Biochem. Physiol. B*, **137**: 331-339. https://doi.org/10.1016/j.cbpc.2003.12.003

Ma, H., Cahu, C., Zambonino, J., Yu, H., Duan, Q., Le-Gall, M.M. and Mai, K., 2005. Activities of selected digestive enzymes during larval development of large yellow croaker (*Pseudosciaena crocea*). *Aquaculture*, **245**: 239-248. https://doi.org/10.1016/j.aquaculture.2004.11.032

Martino, R.C. and Portz, L., 2006. Estratégias paradesenvolvimento de raçõesparapeixescarnívoross de águadoce: Fontes de proteína e lipídios. In: *Tópicose speciaisembiologiaaquática e aqüicultura* (eds. J.E.P. Cyrino and E.C. Urbinati). Sociedade Brasileira de Aqüicultura e Biologia Aquática, Jaboticabal, pp. 125-138.

Misra, K. and Samantaray, K., 2004. Interacting effects of dietary lipid level and temperature on growth, body composition and fatty acid profile of rohu (*Labeo rohita*). *Aquacult. Nutr*., **10**: 359-369. https://doi.org/10.1111/j.1365-2095.2004.00311.x

Nagai, T. and Suzuki, N., 2000. Isolation of collagen from fish waste material – skin, bone and fins. *Fd. Chem*., **68**: 277-281. https://doi.org/10.1016/S0308-8146(99)00188-0

Nettleton, J.A., 1991. n-3 Fatty acids: Comparison of plant and seafood sources in human nutrition. *J. Am. Diet. Assoc*., **91**: 331-337.

NRC, 1993. *Nutrient requirements of fish*. National Academy Press, Washington, pp. 114.

Nurasyikin, A.R. aand Jumat, S., 2006. Physicochemical characteristics of Terubok, Tenualosa toil fish oil. *Malaysian J. Analyt. Sci.,* **10**: 115-120.

Ozogul, Y. and Ozogul, F., 2007. Fatty acid profiles and fat content of commercially important seawater and freshwater fish species of Turkey: A comparative study. *Fd. Chem*., **100**: 1634-1638. https://doi.org/10.1016/j.foodchem.2005.11.047

Parrish, C.C., Li, H., Indrasena, W.M. and Ackman, R.G., 1991. Silage feeds in Atlantic salmon farming: Flavour volatiles and lipid composition, feeding trials, and taste panels. *Bull. Aquacult. Assoc. Canada,* **91**: 75-84.

Sabapathi, U. and Teo, L.H., 1993. A quantitative study of some digestive enzymes in rabbitfish, *Siganu scanaliculatus* and the sea bass, *Lates calcarifer*. *J. Fish Biol*., **42**: 595-602. https://doi.org/10.1111/j.1095-8649.1993.tb00362.x

Sakr, E.M. 2004. *Feeding gilthead bream Sparus aurata and seabass* Dicentrarchus labrax *macroalgae supplemented diets*. Ph. D. thesis, Faculty of Science, Alexandria University, Alexandria Governorate, Egypt.

Sangeetha, R., Geetha, A. and Arulpandi, I., 2010. Concomitant production of protease and lipase by *Bacillus licheniformis* VSG1: Production, purification and characterization. *Braz. J. Microbiol*., **41**: 179-185. https://doi.org/10.1590/S1517-83822010000100026

Sargent, J.R., McEvoy, L., Estevez, A., Bell, J.G., Bell, M.V., Henderson, J.R. and Tocher, D.R., 1999. Lipid nutrition of marine fish during early development: Current status and future directions. *Aquaculture*, **179**: 217-229. https://doi.org/10.1016/S0044-8486(99)00191-X

Seenappa, D. and Devaraj, K.V., 1995. Effect of different levels of protein, fat and carbohydrate on growth, feed utilization and body carcass composition of fingerlings in *Catla catla* (Ham.). *Aquaculture*, **129**: 243-249. https://doi.org/10.1016/0044-8486(94)00273-Q

Stancheva, M. and Merdzhanova, A., 2011. Fatty acid composition of common carp, rainbow trout and grey mullet fish species. *Agric. Sci. Technol*., **3**: 285-289.

Stone, D.A.J., 2003. Dietary carbohydrate utilization by fish. *Rev. Fish. Sci*., **11**: 337-369. https://doi.org/10.1080/10641260390260884

Tidwell, J.H. and Allan, G.L., 2001. Fish as food: Aquaculture's contribution. *EMBO Rep.*, **2**: 958-963. https://doi.org/10.1093/embo-reports/kve236

Tocher, D., 2003. Metabolism and functions of lipid and fatty acid in teleost fish. *Rev. Fish. Sci*., **11**: 107-184. https://doi.org/10.1080/713610925

Wassef, E.A., El-Masry, M.H., Eissa, M.A. and Mikhail, F.R., 2001a. Evaluation of five supplementary feeds for grey mullet Mugilcephalus fry. *Egypt J. Nutr. Feeds*, **4**: 731-741.

Wassef, E.A., El-Masry, M.H. and Mickhail, F.R., 2001b. Growth enhancement and muscle structure of stripped mullet, *Mugil cephalus* L. fingerlings by feeding algal meal-based diets. *Aquacult. Res*., **32**(Suppl-1): 315-322. https://doi.org/10.1046/j.1355-557x.2001.00043.x

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Quality Assessment of Marine Shellfisheries: Dietary Exposure to Metal Contaminants in Seafood

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Key words
Shellfish, Heavy metals, Nutritional value, Contamination, Seafood safety.

ABSTRACT

This study was conducted to assess the concentration of heavy metals (Zn, Cu, Cd, Pb and Cr) and biochemical analysis (carbohydrate, lipid moisture, total protein) in edible muscle of commercially important shellfish *Portunus reticulatus, P. segnis, P. sanguinolentus, Scylla olivaceae, Penaeus monodon* and *P. indicus*). The concentration of Cu, Pb and Cd showed significant difference ($p>0.05$) among species except Zn. The bioaccumulation of metals both essential and toxic in marine animals, through natural and anthropogenic source is an actual food safety issue. The studied species are commonly found in the area and have significant commercial value. Additionally, it is long lived, thus suitable as bio-indicator of the environmental monitoring. In order to ensure the seafood safety, risk managers may implement measures to reduce human exposure to contaminants via seafood consumption. The biochemical results of the present work clearly indicate that there are differences in the content, carbohydrate, lipid and protein and protein structure of the muscles in different species of shellfish. It was concluded that seafood specially shellfish are the main source of essential nutrient, but more effective controls should be focused on metal pollution for the quality and safety of the fish and fisheries items. However, the current research provides a conceptual framework for addressing these issues systematically.

INTRODUCTION

Chemical pollution in the marine environment is common due to the widespread use of organic chemicals and metals worldwide, and the tendency for long-range transport and persistence of many of thèse compounds (Finlayson *et al*., 2016; Bano *et al*., 2017). Levels of pollutants in aquatic ecosystems are steadily increasing because of anthropogenic activities, including mining, chemical processing, fossil fuel burning, industrial waste discharges, incineration, *etc*. (Ghedira *et al*., 2016).

Heavy metals are natural trace components of the marine environment, but they constitute one of the most hazardous substances that could be accumulated in biota. According to Munoz-Olivas and Camara (2001) heavy metals are classified as: potentially toxic (*e.g.* aluminum, arsenic, cadmium, lead, mercury), probably essential (*e.g.* nickel, vanadium, cobalt) and essential (*e.g.* copper, zinc, selenium).

The global food system faces significant stressors in population growth, limited land and water resources, rising demand for animal products, overreliance on fossil fuels, and a changing climate (Fry *et al*., 2016). In addition, seafood production has changed substantially over the last few decades. Half of seafood consumed globally currently comes from aquaculture, or farmed seafood, which is increasing at a faster rate than any other animal production sector (UNFAO, 2014).

Seafood is good source of protein having high biological values. Seafood such as Finfish, shellfish and other aquatic organisms suitable for human food and feed, are of worldwide importance and are excellent sources of high quality proteins, superior to those in meat and poultry. Especially, crustacean species reflects the highly rich composition of protein, calcium, vitamins and various extractable compounds. On this basis, decapod crustacean represents an important economic source and seafood consumption is linked with improvements in health conditions including cardiovascular disease, arthritis, and cancer (Kamal *et al*., 2015).

The nutritional benefits of seafood are mainly due to the content of high-quality protein (fish provide 17% of the total animal protein and 6% of all protein consumed by humans), and other essential nutrients. The quality of seafood tissue is function of their body compositions and energy values, important for that vary among different species. Determination of proximate composition as protein contents, carbohydrates, lipids, moisture contents and ash percentage is often necessary to ensure that fish tissues have a good nutrition quality and that they meet the requirements of food regulations and commercial specifications (WHO/FAO, 2011). The aim of this study

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to assess the nutritional quality and metals (Zn, Cu, Pb, Cd and Cr) contamination in edible tissues of shellfish speciesand also to evaluate the contamination status of metals in commercially important seafood species found along the Karachi coast.

MATERIALS AND METHODS

Sample collection and preparation

The shellfish samples including *Scylla olivacea, Portunus reticulatus, P. segnis, P. sanguinolentus, Penaeus monodon* and *P. indicus* (crabs and shrimp) were randomly obtained (ten individuals were taken randomly of each species) from local fish harbours in Karachi duringthe March to June 2014. These species were selected due to their economic value and human consumption. After collection, the samples were immediately transported to laboratory in ice and freeze until analysis. In the laboratory, crabs and shrimps were sorted, identified and sexed. Measurements of the different body parts (carapace width (CW), carapace length (CL) and chela length (Chl.L) were recorded. The specimen was then blotted dry and body weight was measured using an analytical balance then soft tissues in shell were scraped out with a sterile scalper. The edible tissues were oven dried at 70°C for 24 h and ground into powder for the determination for heavy metals and proximate composition.

Proximate analysis

For the nutritional quality assessment, proximate composition of each studied shellfish was analyzed. The Protein content was estimated according to the method described by Lowry *et al*. (1951). Lipid was estimated by following Folch *et al*. (1956) and carbohydrate content was estimated by the procedure of Dubois *et al*. (1956). The moisture content was determined by drying the sample in a hot air oven at 75°C until constant weight was obtained. The percentage difference due to loss of water was then calculated.

Measurements of heavy metal concentration

Approximately 2 g of dry tissues samples was placed in a beaker with 10ml freshly prepared concentrated HNO_3 and H_2O_2. The mixture was reacted at 100°C on a water bath to allow the sample to be digested by solution in the fume hood. After acid digestion, the beaker was carefully removed from the water bath and the contents were left to cool, also allowing the acid to evaporate. After evaporation of the acid, the digested samples were filtered and made up with 25ml of 1% HNO_3 solution. Zn, Cu, Pb, Cd and Cr were analyzed by an Atomic absorption spectrometry AAS air acetyl atomic absorption spectrometer (Perkin Elmer (USA), model A Analyst 700). Triplicate determinations for each heavy metal were carried out. The concentration of metal was determined from calibration curves of the standard element.

Dietary exposure assessment

Dietary exposure was estimated based on the consumption data and calculated by the following equation:

$$EDI = \frac{C_{metals} \times W_{shellfish}}{B_w}$$

Where, C is the concentration of heavy metals in shellfish (mg Kg^{-1}), $W_{shellfish}$ is the daily consumption of shellfish seafood and B_w is the body weight of an adult.

Statistical analysis

Statistical analysis was carried out using Microsoft Excel, Minitab 17 and SPSS 18 Software. All the analyses were done in triplicate andthe means were compared using Analysis of Variance (ANOVA). ANOVA was carried out for species wise data comparison. The level of significance was fixed at $p<0.05$.

Table I.- Morphometric measurement (Mean±SD) of the shellfish (crabs and shrimps) found along Karachi Coast.

Species	Weight	C.L	C.W	Chl. L
S. olivaceae	116.89 ± 33.92	6.14± 0.56	8.88 ± 0.77	5.89± 0.96
P. reticultus	129.60± 22.63	12.29± 0.54	5.60± 0.313	7.54± 1.13
P. sagnis	178.1± 61.2	13.69± 0.86	6.31± 0.94	10.88± 1.36
P. sanguinolentus	157.2± 45.4	12.85± 0.55	5.73± 0.313	7.77± 0.72
P. monodon	26.00± 9.90	3.69 ± 0.44	-	-
P. indicus	34.13± 4.90	4.38± 0.47	-	-

CL, carapace length; CW, carapace width; Ch.L., Chela length.

RESULTS AND DISCUSSION

The mean weight, carapace length, carapace width and chela length of the shellfish crabs and shrimp's species used in this study presented (Table I), showed that the individual with highest weight (178.1±61.2), Chela length (10.88±1.36) and carapace length (13.69±0.86) was observed in *P. segnis*.

In different shellfish, a relatively large variations in metal concentration was observed, even within the same species. The median and ranges of examined heavy metal concentration in the crabs and shrimp meat listed in Table II.

Table II.- Heavy metal concentration in shellfish species analyzed (mg kg $^{-1}$).

Metal	*S. olivaceae*		*P. reticulates*		*P. sagnis*		*P. sanguinolentus*		*P. monodon*		*P. indicus*	
	Min-Max	M	Min-Max	Med	Min-Max	Med	Min-Max	Med	Min-Max	Med	Min-Max	Med
Cu	2.1-34.50	8.85	0.0-4.87	1.55	0.0-8.37	1.30	0.0-6.25	2.51	1.8-28.65	5.76	0.50-4.42	1.87
Zn	14.35-50.83	34.83	12.05-24.80	21.76	17.60-35.55	29.45	32.7-77.08	37.38	26.1-15.2	34.2	0.0-29.8	34.2
Pb	1.2-8.97	4.26	2.20-8.95	5.47	1.45-2.57	1.85	0.0 -3.4	2.42	-	-	-	-
Cd	0.22-1.10	0.46	0.62-0.825	0.70	1.05-2.65	1.16	0.0-3.57	1.67	-	-	-	-
Cr	0.1- 21.35	0.0	-	-	-	-	0.0-21.4	0.0	0.3-11.48	7.16	0.0-18.2	14.45

In current study, *P. sanguniolentus* had the highest Zn level (median 37.38 mg kg $^{-1}$) followed by *S. olivacea* (median 34.83 mg kg $^{-1}$) and *P. monodon* (median 34.2 mg kg $^{-1}$) and *P. indicus* (median 34.2 mg kg $^{-1}$) whereas the *P. reticulatus* presented the lower Zn level (median 21.76 mg kg $^{-1}$). Therefore, no significant difference was observed among species.Although zinc is an essential element, but at high concentrations, it can be toxic to shellfish, cause mortality, growth retardation and reproductive impairment. Zinc is capable of interacting with other elements and producing antagonistic, additive or synergistic effects.

The *S. olivacea* showed the highest Cu accumulation (median 8.85 mg kg-1) followed by the *P. Monodan* (median 5.76 mg kg $^{-1}$) and *P. sanguinolentus* (median 2.51 mg kg $^{-1}$), respectively. Significant difference $p<0.05$ was observed among species. Cu is an important metals and required in several enzymes and it is essential for the synthesis of haemoglobin but can cause harmful effect at higher concentration (McCluggage, 1991).

Lead is non-essential and toxic metal to human's health, pose the deleterious effects on the reproductive, hemopoietic, nervous systems and the urinary tract. The highest level of Pb was observed in *P. reticulatus* (median 5.475 mg kg $^{-1}$), and lowest in *P. segnis* (median 1.850 mg kg $^{-1}$), whereas in shrimps the concentration of Pb was below the detection limit. The maximum concentration of Cr was found in *S. olivacea* 21.35mg kg $^{-1}$ as showen in Table II. Cr is an essential trace element in humans, fish and shellfish but in excess, it could have a detrimental effect on shellfish and other wildlife (Akan *et al*., 2009).

Cadmium is highly toxic and ecotoxic metal. The occupational levels of Cd exposure prove to be a risk factor for chronic lung disease and testicular degeneration. Cadmium could originate from water, sediments and food and may accumulate in the human body as may induce kidney dysfunction, skeletal damage and reproductive deficiency (Stancheva *et al*., 2013). The high concentrations of Cd were obtained for analyzed crabs species (Table II) as shrimps did not showed Cd accumulation in edible tissues and are below the detection limits. The low or below the detection limit, concentration of Cd might be due to its low tendency of bioaccumulation or it's good ability to excretion from the body (Abdel Rahman *et al*., 2016).

Heavy metal content in shellfish species were compared with the maximum limits set out by the FAO (2012). In investigated shellfish species the concentration of Zn and Cu were below the limits but the Cd, Pb and Cd were present the high amounts.

Evaluation of the dietary exposure

To evaluate the dietary intake values the average concentration of each investigated heavy metals in each species was calculated and then multiplied by consumption rate (Santos *et al*., 2004). Table III showed the estimated dietary intake through the heavy metals Cu, Cd, Pb, Zn and Cr for an adult population including 75 %. The exposure of the Cd though the consumption of shellfish species appears to be relatively low, whereas, Zn the highest dietary intake values p75 4.37 mg kg $^{-1}$ body weight day $^{-1}$ and UB 4.96 mg kg $^{-1}$ body weight day $^{-1}$, respectively (Table III). Following these result it can be concluded that it is unlikely that the intake these heavy metals through shellfish would involve any risk for the average consumer.

Table III.- Estimated daily intake for adult people by consumption of shellfish species.

Metals	Dietary intake (ug kg $^{-1}$ bw day $^{-1}$)				
	Mean±SD	LB	UB	P_{50}	P_{75}
Cu	0.42±0.33	0.06	0.77	0.23	0.75
Zn	3.59±1.30	2.22	4.96	3.63	4.73
Pb	0.21±0.21	0.01	0.43	0.16	0.44
Cd	0.06±0.06	0.002	0.13	0.05	0.13
Cr	0.41±0.47	0.08	0.91	0.33	0.76

SD, standard deviation; P, percentile; LB, lower bound; UB, upper bound.
[a]In parentheses confidence interval 95%.

Proximate chemical composition of shellfish

The proximate composition in most seafood item is primarily based on water, proteins, and lipids. In fish meat these constituents make up about 98% of the total mass, and the other minor constituents include carbohydrates,

vitamins and minerals (WHO/FAO, 2011). The mean values of protein, carbohydrate, lipid and moisture contents are presented in Table IV.

The biochemical composition of seafood such as fish and shellfish, generally varies due to their geographical locations, stages and sizes of maturity. It is known that seafood or edible shellfish tissue contains 60–84% water, 15–24% protein and 0.1–22% lipids. The proportions of the constituents are species-specific and the main variations in proximate composition between species occurs in moisture and lipids content (WHO/FAO, 2011; Boran *et al*., 2011). Table IV shows that the biochemical composition of shellfish varies between species to species. The level of moisture were higher in edible tissues of shrimps (27.503 ± 1.228 and 24.611±1.760, respectively) for *P. indicus* and *P. monodon* than in the edible tissues of crabs as showed in Table IV. These results shows that the water is the main constitute of the shellfish. Water is required for the normal functioning of many biological molecules. It is present in two forms in the tissues, bound to the proteins and in the free form. These forms have well defined biological roles (Stancheva *et al*., 2013).

Table IV.- Proximate composition of different shellfish species found along the Karachi coast.

Species	Protein	Carbohydrate	Moisture	Lipid
S. olivaceae	18.84± 3.84	2.29 ± 0.06	19.38 ± 2.06	6.28 ± 4.05
P. reticulatus	24.46 ± 15.14	2.32 ± 0.04	23.26 ± 2.43	8.61 ± 0.65
P. sagnis	24.88± 22.96	2.19± 0.12	18.07 ± 1.47	7.18 ± 2.68
P. sanguinolentus	10.74 ± 10.0	1.58 ± 1.0	16.11 ± 3.49	0.76 ± 1.25
P. monodon	29.30 ± 6.56	4.54 ± 0.09	24.61 ± 1.76	6.18 ± 6.67
P. indicus	28.92 ± 6.71	4.32 ± 0.31	27.51 ± 1.23	9.16 ± 12.61

Quantitatively, protein is the second major component in edible muscle of shellfish. The protein content tends to vary much less widely from species to species (FAO, 2010). The protein content were high in the edible tissues of shrimps (29.30 ±6.56 and 28.92 ± 6.71) in *P. monodon* and *P. indicus* as compare to the crabs species. These results showed that these species can be used as an animal protein source (Njinkoue *et al*., 2016). Protein is the most important constituent in seafood from the nutritional point of view. However,compared to finfish species, shell fish falls under lower protein category, the range being 5-14% (Balachandran, 2001).

Carbohydrate in fishery products contain no dietary fiber but they contain glucose, fructose, sucrose and monosaccharides and disaccharides (Okuzumi and Fujii, 2000). The carbohydrate values were low in crab's species (1.58 ± 1.0) in *P. sanguinolentus* and high in shrimp's species (4.54 ± 0.09) in *P. monodon*. Carbohydrates make up only a minor proportion of total proximate composition.

Lipids act as a major food reserve along with protein and are subject to periodic variations influenced via environmental variables like temperature. Lipids are highly efficient as sources of energy and they contain more than twice the energy of carbohydrates and proteins (Baklouti *et al*., 2013). The lipid content was high in shrimp species (9.16 ± 12.60) for *P. monodon*. Lipids are hydrophobic in nature and thus require a special vehicle for their transport through the aqueous hemolymph. For this purpose, in crustaceans, as in other animals, the lipids get associated with proteins, forming lipoproteins. Two different lipoproteins were isolated from crustacean hemolymph, in addition to these, lipoprotein was present, which seemed to be vitellogenin (Komatsu *et al*., 1993; Fatima *et al*., 2013).

Table V.- Species comparisons of heavy metal concentrations, biochemical parameters and morphometric characters of some shellfish collected from Karachi coastal waters by one-way ANOVA.

Metals	Source	df	MS	F	P
Zinc (Zn)	Species	5	2048	1.56	0.18
Copper (Cu)	Species	5	191.78	7.21	0.000*
Lead (Pb)	Species	5	60.77	22.34	0.000*
Cadmium (Cd)	Species	5	5.11	14.53	0.000*
Chromium (Cr)	Species	5	284.93	10.41	0.000*
Protein	Species	5	509.9	3.57	0.007**
Carbohydrate	Species	5	15.79	82.42	0.000*
Lipid	Species	5	90.92	2.57	0.035***
Moisture	Species	5	195.45	40.92	0.000*
Weight	Species	5	43312	34.35	0.000*
Carapace length	Species	5	203.59	126.25	0.000*
Carapace width	Species	4	145.34	302.59	0.000*
Chela length	Species	4	186.27	106.79	0.000*

Level of significance, 0.001*, 0.01** and 0.05***. df, degrees' freedom; MS, mean squares; F, F values; P, significance.

Statistical analysis of ANOVA showed significant differences among heavy metal concentration, proximate composition and morphometric parameters in different shellfish (shrimps and crabs) studied. There are significant difference between heavy metal in species except the Zn which showed no significant difference.the biochemical and morphometric parameters showed highly significant

difference among studies species as presented in Table V.

CONCLUSION

Marine shellfish such as crabs and shrimps are an important food source for human consumption and a major constituent of the aquatic system, thus evaluation of the heavy metal content and biochemical composition of shellfish species is particularly important for the safety and nutritional point of view of edible species of shellfish seafood. Therefore, studies on the presence of metal accumulation in shellfish will contribution of the new data on their dietary exposure to the consumers or in human for safety risk in coastal waters of Karachi coast. Based on the analyses of shellfish samples, the concentrations of Zn and Cu were well within limits, therefore the Cd, Pb and Cr were exceeding the permissible limits set by FAO. Both studied crabs and shrimp species were characterized by a good nutritional quality, as mainly characterized by higher protein contents and lower lipid levels in shellfish. According to presented results of this study, we can give a positive evaluation of the nutritional quality, dietary assessment and the safety of shellfish species found in Karachi waters for consumers.

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Statement of conflict of interest

The author(s) declare(s) that there is no conflict of interests regarding the publication of this article.

REFERENCES

Abdel Rahman, S.T., Gohar, M.E., Ghannam, H.E. and Abdo, M.H., 2016. Chemical composition and heavy metal contents of *Oreochromis niloticus* from the main irrigated canals (rayahs) o Nile delta. *Egypt. J. Fish. Res*., **42**: 23-31.

Akan, J.C., Abdul Rahman, F.I., Sodipo, O.A. and Akan, D.U., 2009. Bioaccumulation of some heavy metals of six fresh water fishes caught from Lake Chad in Doron Buhari, Maiduguri, Borno, State, Nigeria. *J. appl. Sci. environ. Sanit*., **4**: 103-114.

Baklouti, S., Derbali, A.K., Dhieb, K., Kammoun, W. and Jarboui, O., 2013. Proximate composition and its seasonality of the Mediterranean green crab, *Carcinus aestuarii* Nardo, 1847 (Brachyura, Portunidae), in Southern Tunisian Waters (Central Mediterranean). *J. mar. Biol*., **2013**: Article ID 989467. https://doi.org/10.1155/2013/989467

Balachandran, K.K., 2001. *Post-harvest technology of fish and fish products*. Daya Publishing House, Delhi, pp. 77-79.

Bano, Z., Abdullah, S., Ahmad, W., Zia, M.A. and Hassan, W., 2017. Assessment of heavy metals and antioxidant enzyme in different organs of fish from farm, hatchery and Indus River of Pakistan *Pakistan J. Zool.,* **49**: 2227-2233.

Boran, G. and Karacam, H., 2011. Seasonal changes in proximate composition of some fish species from the Black Sea. *Turk. J. Fish. aquat. Sci*., **11**: 1-5.

Dubois, M., Gills, K.A., Hamitton, J.K., Rebers, P.A. and Smith, F., 1956. Colorimetric method for determination of sugars and related substances. *Analy. Chem*., **28**: 350-356. https://doi.org/10.1021/ac60111a017

FAO, 2010. *Nutrition elements of fish*. http://www.fao.org/fishery/topic/12319/en (Accessed on 26/8/12010).

FAO, 2012. *Fishery and aquaculture statistics*. http://www.fao.org/fishery/statistics/en (Accessed 2 May 2014)

Fatima, H., Ayub, Z., Ali, S.A. and Siddiqui, G., 2013. Biochemical composition of the hemolymph, hepatopancreas, ovary, and muscle during ovarian maturation in the penaeid shrimps *Fenneropenaeus merguiensis* and *F. penicillatus* (Crustacea: Decapoda). *Turk. J. Zool*., **37**: 334-347.

Finlayson, K.A., Leusch, D.F.L. and van de Merwe, J.P., 2016. The current state and future directions of marine turtle toxicology research. *Environ. Int*., **94**: 113-123. https://doi.org/10.1016/j.envint.2016.05.013

Folch, J., Loes, M. and Stanley, G.H.S., 1957. A simple method for the isolation and purification of total lipids from anuimal tissues. *J. biol. Chem*., **226**: 496-509.

Fry, P.J., Love, D.C., MacDonald, G.K., West, P.C., Engstrom, P.M., Nachman, K.E. and Lawrence, R.S., 2016. Environmental health impacts of feeding crops to farmed fish. *Environ. Int*., **91**: 201-214. https://doi.org/10.1016/j.envint.2016.02.022

Ghedira, J., Chicano-Gálvez, E., Fernández-Cisnal, R., Jebali, J., Banni, M., Chouba, L., Boussetta, H., López-Barea, J. and Alhama, J., 2016. Using environmental proteomics to assess pollutant response of *Carcinus maenas* along the Tunisian coast. *Sci. Total Environ*., **541**: 109-118. https://doi.

org/10.1016/j.scitotenv.2015.09.032

Kamal, T., Tanoli, M.A.K., Mumtaz, M., Ali, N. and Ayub, S., 2015. Bioconcentration potential studies of heavy metals in *Fenneropenaeus penicillatus* (Jaira or red tail shrimp) along the Littoral States of Karachi City. *J. Basic appl. Sci*., **11**: 611-618. https://doi.org/10.6000/1927-5129.2015.11.82

Komatsu, M., Ando, S. and Teshima, S.I., 1993. Comparison of hemolymph lipoproteins from four species of Crustacea. *J. exp. Zool*., **266**: 257-265. https://doi.org/10.1002/jez.1402660403

Lowry, O.H., Rosenberg, N.J., Farr, A.L. and Randall, R.F., 1951. Protein measurement witht the folin phenol reagent. *J. biol. Chem*., **193**: 265-275.

McCluggage, D., 1991. *Heavy metal poisoning*. NCS Magazine, The Bird Hospital, CO, USA.

Munoz-Olivas, R. and Camara, C., 2001. Speciation related to human health. In: *Trace element speciation for environment, food and health* (eds. L. Ebdon, L. Pitts, R. Cornelis, H. Crews, O.F.X. Donard and P. Quevauviller). Royal Society of Chemistry, Cambridge, pp. 331-353.

Njinkoue, J.M., Gouado, I., Tchoumbougnang, F., Yanga-Ngueguim, J.H., Ndinteh, D.T., Fomogne-Fodjo, C.Y. and Schweigert, F.J., 2016. Proximate composition, mineral content and fatty acid profile of two marine fishes from Cameroonian coast: *Pseudotolithus typus* (Bleeker, 1863) and *Pseudotolithus elongatus* (Bowdich, 1825). *Nutr. Fd. Sci. J*., **4**: 27-31. https://doi.org/10.1016/j.nfs.2016.07.002

Okuzumi, M. and Fujii, T., 2000. *Nutritional and functional properties of squid and cuttlefish*. Japan National Cooperative Association of Squid Processors, Tokyo, Japan.

Santos, E.E., Lauria, D.C. and Porto da Silveira, C.L., 2004. Assessment of daily intake of trace elements due to consumption of foodstuffs by adult inhabitants of Rio de Janeiro city. *Sci. Total Environ*., **327**: 69-79. https://doi.org/10.1016/j.scitotenv.2004.01.016

Stancheva, M., Merdzhanova, A., Petrova, E. and Petrova, D., 2013. Heavy metals and proximate composition of Black Sea Sprat (*Sprattus Sprattus*) and Goby *(Neogobius Melanostomus*). *Bulgar. J. agric. Sci*., **19**(Suppl. 1): 35-41.

UNFAO, 2014. *The state of food insecurity in the world 2014*. *Strengthening the enabling environment for food security and nutrition*. Food and Agriculture Organization of the United Nations, Rome.

WHO/FAO, 2011. *Report of the joint FAO/WHO expert consultation on the risks and benefi ts of fish consumption*. Food and Agriculture Organization of the United Nations, Geneva, World Health Organization, Rome, pp. 50.

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Evaluation of Protein (Nutritional Property) in some Shrimp Species Found in Coastal Waters of Pakistan by Three Different Methods

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Authors' Contribution
NS proposed the concept of the study and helped in manuscript writing. SHN carried out the study, analysed the data and did laboratory work.

Key words
Shrimps, Quantitative, Protein, Spectroscopy.

ABSTRACT

Shrimps are an extremely important source of protein, yet very low in fat and calories make them a very healthy choice of food. From Pakistan an average commercial size for export of shrimps are considered as Jaira that individual sizes ranges from 16 cm to 25 cm with an average weight from 30 g to 100 g. Estimation of the total protein content in any sample is a perilous step in an assessment of the food quality and the accurate estimation of protein can be a value added character in nutritional perspective. The present study investigates protein contents variations in five different species of shrimps by using multiple methods (Lowry, Bradford and Biuret method) through quantitative technique *i.e.* Molecular UV-Vis absorption spectroscopy. For protein quantification, the absorbance was measured at a wavelength of 660 nm for Lowry method, 595 nm for Bradford and 540 nm for Biuret assay method accordingly. The protein content estimated in species of genus *Fenneropenaeus* ranged, 19-67.8% in the Lowry estimation while 17.20-57% in Bradford and 8.81-50.5% via Biuret estimated, whereas in genus *Metapenaeus* protein estimated between the range of 10.1-73% in Lowry, while 11-6.9% in Bradford and 11.4-7% via Biuret, in genus *Parapenaeopsus* the highest protein content was estimated by Lowry method 55.7% and lowest value by 12% via Biuret. The result of our present study implies that the average nutritive values (proteins) of shrimp can help in overcoming daily protein dietary values. Hence, increasing the consumption of it might be helpful to achieve the nutritional requirement of protein.

INTRODUCTION

In the world, the millions of people have been suffering from malnutrition and protein deficiency especially in the developing countries. Protein deficiency may minimize to some extent by making available fish and shellfish items, which are available to local communities. Fish and shellfish are known to be highly nutritious and an important component of the diet and an excellent source of protein as well as other essential nutrients for human diets (Fawole *et al.*, 2007). These items fundamentally composed of water, lipid, and protein, which create the nutritional value, functional aspects and sensory characteristics of the flesh. It also contains vitamins and minerals, which plays an important role in post-mortem biochemical changes (Gokoglu and Yerlikaya, 2015). The Decapod crustaceans are one, among the better-investigated animal group mainly due to their ubiquity in aquatic ecosystems and their importance as human food. Aquatic food (fish and shellfish) products represent a significant market niche that includes a wide number of species of commercial interest such as shrimps, crabs, lobsters. These edible crustaceans constitute one of the major sources of amino acids, peptides, proteins, and other useful nutrient nutritious food for human beings. There is much studies encouraging crustacean consumption (Marques *et al.*, 2008; Bugel *et al.*, 2001; Kucukgulmez *et al.*, 2006; Prasuna *et al.*, 2015; Ravichandran *et al.*, 2009; Wardiatno and Mashar, 2010; Wardiatno *et al.*, 2012).

Among the shell fisheries, the shrimp is an extremely good source of proteins, very low in fats and calories, thus widely accepted as a healthy food choice for consumers and make them commercially important seafood product. The edible muscles of shrimp consist of highly unsaturated fatty acids (FAs), such as eicosapentaenoic (C20: 5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, which are essential in the human diet (King *et al.*, 1990; Dinçer and Aydin, 2014). Shrimp is an important product in the international fisheries trade and there is an indication of an increase in worldwide consumption. Shrimps fulfill more than 30% of the worldwide food demand. For many centuries, shrimp have been considered as a good source of food and have been harvested by extractive fishing or farmed in aquaculture. The important edible varieties of Shrimps belong to the family Penaeidae and abundantly distributed in the seas, backwaters, estuaries, lakes and freshwaters (Fatima *et al.*, 2013).

The nutritive values of crustaceans depend upon their

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biochemical composition, such as protein, amino acids, lipids, fatty acids, carbohydrates, vitamins and minerals. Among the seafood, shrimp and shrimps contribute about 20-22% by volume of the world seafood market (FAO, 2014). Many studies examined the proximate composition of different species of shrimp in various parts of the world (Nisa and Sultana, 2010; Fatima *et al*., 2013; Ravichandran *et al*., 2009; Hala, 2014). Studies have shown that shrimp meat contains low calories and low fat than chicken (Approx. 1 calorie/1g). Due to their nutritional nature, apart from the supply of good quality proteins, lipids, they also contain several dietary minerals such as calcium, iron, *etc*., which are essential and play an important beneficial role in the maintenance of physiological and biochemical activities in human beings. The DRI (Dietary Reference Intake) is 0.8 grams of protein per kilogram of body weight, or 0.36 grams per pound. This quantity is 56 grams per day for the average sedentary man and 46 grams per day for the average sedentary woman. According to this standard, shrimp meal reveals enrich with protein and meet the daily intake requirements of human consumption and shrimps considered as the most popular species as it is a part of almost every nation's traditional meal rich in protein and minerals.

From Pakistan, the twenty-five species of penaeid shrimps have been recorded and among them only 12 species are commercially exploited from the coastal waters of Pakistan (Kazmi, 2003; Nisa and Sultana, 2010). The present study aimed to estimate the total proteins in shrimp species through the three common methods (Lowry, Biuret and Bradford) as well as to compare the precision of the protein estimation of these methods.

Fig. 1. Map showing the coastline of Sind: A, Karachi City; B, Karachi Beaches; C, Karachi Fish Harbor.

MATERIALS AND METHODS

Sample collection

Shrimps were collected from commercial catch of three commercial harbors (Karachi Fish Harbor, Korangi Ibrahim Hyderi Harbor and Sonmiani Harbor during November 2015 to July 2016 and over 90 percent of the Pakistan's fish and seafood catch gathered by fishing boats and exports to different countries (Fig. 1). The shrimps placed in ice in an insulated ice bag and transported to the laboratory within two hours for sample preparation and analysis. Total 77 shrimp individuals were analyzed in this study (Table I). The morphometric analysis was done for each specimen through standard ruler and/or vernier caliper (cm). The total length (TL) was measured from the tip of the rostrum to tip of the telson and carapace length (CL) was measured from the tip of rostrum to postero-dorsal limit of carapace. The weight was measured using standard digital electronic weighing scale to weigh the shrimp's samples to two decimal places in g. The protein contents were analyzed in triplicate for each specimen by three different methods *i.e.* Biuret, Bradford and Lowry.

Table I.- Percent species composition of shrimps.

Species name	Male	Female	Total
Fenneropenaeus indicus	7	7	14
Fenneropenaues merguensis	7	7	14
Metapenaues monoceros	10	8	18
Metapenaues affinis	6	8	14
Parapeneopsis stylifera	9	8	17
Total	39	38	77

Identification and preservation

The shrimp species initially identified with the help of differences in upper and lower rostral teeth, carapace sutures, distal part of third maxilliped and sex was determined by the presence of a petasma on the first pair of pleopods in males and absence in female penaeid prawns (Pérez and Kensley, 1997) by using available taxonomic keys (Tirmizi , 1973). The works of Hall (1962) followed for identification of these sexual characters. After sorting and identification of samples, they were preserved freeze dry at -20 °C.

Protein quantification

The all shrimp samples were firstly de-shell and then muscles were oven dried at 75°C for 24 h. Dried samples were grounded and powdered. The total protein estimation was done by three different methods (Lowry, Bradford and Biuret). Analytical grade chemicals were used in this study and reagents were made manually in the laboratory. All the samples analyzed in replicates to avoid any ambiguity in the results.

According to the methodology of Lowry *et al.* (1951), 100 mg of the dried sample was taken in a test tube; after an addition of 10 ml of distilled water sample allowed to standing for 24 h at 4 °C. Followed by 10 ml b-mercaptethanol then 0.5 ml aliquot was taken along with 4.5 ml distill water and 0.5 ml NaOH was added, 6.9 gs approximately ammonium sulphate was added for ppt., 0.5 ml phosphate buffer, 5 ml of freshly prepared alkaline copper tartrate reagent was added, followed by 0.5 ml of IN Folin-Ciocalteu phenol reagent. The contents mixed thoroughly and allowed to stand for 30 mins for color development. The absorbance read at 660 nm against a reagent blank.

According to the methodology of Gornall *et al.* (1949) 100 mg of the dried sample was taken in a test tube, 10 ml of distilled water was added and allowed to stand for 24 h at 4 °C. followed by 10 ml b-mercaptethanol then 0.5 ml aliquot was taken along with 4.5 ml distilled water and 0.5 ml NaOH was added, then 6.9 g ammonium sulfate was added for ppt, 0.5ml phosphate buffer, 5 ml of freshly prepared alkaline copper tartrate reagent was added. The contents then mixed thoroughly and allowed to stand for 30 min for colour development. Then the absorbance read at 540 NM against a reagent blank. According to the methodology of Bradford (1976), a 100 mg of the dried sample taken in a test tube, 10 ml of distilled water added and allowed to stand for 24 h at 4 °C, followed by 10 ml b-mercapt ethanol, then 0.5 ml aliquot taken along with 4.5 ml distill water and 0.5 ml NaOH was added. The contents mixed thoroughly and allowed to stand for 5 min for color development. The absorbance read at 595 nm against a reagent blank.

RESULTS AND DISCUSSION

In this study, the three common assays were used to determine the concentrations of protein in tissues of five shrimp through the spectrophotometer (life science UV/VIS spectrophotometer) as well as the obtained results from different methods were further compared, the sensitivity of the three methods was listed in Table II. The five species of shrimps were identified includes *Fenneropenaeus indicus*, *F. merguensis*, *Metapenaues monocerose*, *M. affinis* and *Parapeneopsis stylifera* (Table I). The knowledge of proximate composition mainly protein contents of fishery species is essential to estimate the quality of the raw material and for its maximum utilization, which has fundamental importance in the application of different technological processes in preservation, processing and product development (Mridha *et al*., 2005). The proximate

composition of the shrimps, crustaceans and other aquatic organisms has found tobe varied due to the seasonal factors, climatic factors, geographic factors, habitat, developmental stage, sex, sexual maturation (Pillay and Nair, 1973; Nisa and Sultana, 2010).

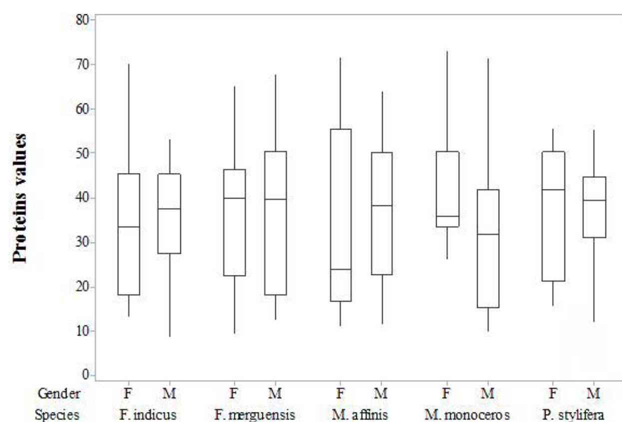

Fig. 2. The total proteins levels in genders and shrimp.

The concentration of total protein in the edible muscles of analyzing shrimp species summarized in Table III. No significant differences (p >0.05) were observed in protein quantity among the different shrimp species as well as between the genders of each shrimp species (Fig. 2). Whereas, the significant differences observed in protein contents, among the three methods in all shrimp species, except in *M. monocerose*. Figure 3 shows the variations in protein concentrations in shrimp species through the three different methods. Methods for the determination of protein concentration based on the quantity and nature of the protein to be analyzed, the presence of interfering substances and sensitivity requirements. The proteins levels in *F. indicus* was observed significantly higher in Lowry (43.05 ± 14.19) followed by Bradford (36.72 ± 12.55) and Biuret (24.85±13.33) methods. The total proteins in *F. merguensis* was observed significantly higher in Lowry (47.04 ± 10.76) and Bradford (38.79 ± 14.06) methods as compared to the Biuret method (24.08 ± 13.28). Estimated protein in *M. affinis* was observed significantly higher in Lowry (50.75 ± 18.26) as compared to Bradford (30.49 ± 18.76) and Biuret method (22.87 ± 8.93). However, in *P. stylifera* the protein contents were significantly higher in Lowry (44.83 ± 6.73) and Bradford (40.0 ± 10.86) methods as compared to the Biuret method (26.79 ± 12.63).

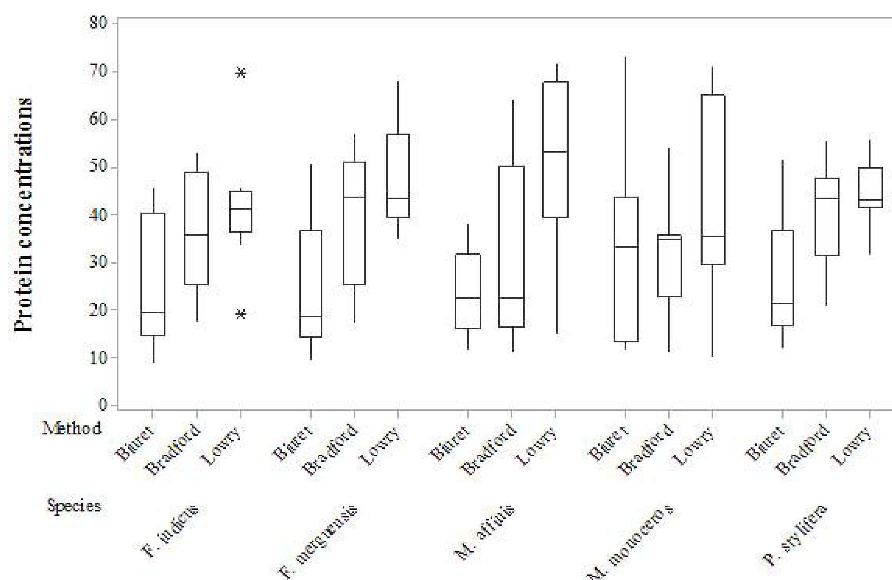

Fig. 3. The comparison of total proteins through three methods in five species of shrimps collected from Pakistan coast.

Table II.- Overview of methods for protein quantification.

Method	Sensitivity	Accuracy	Interference
Lowry	0−0.1 mg	Partially dependent on amino acid composition	Acids, EDTA, DTT, phenol, $(NH_4)_2SO_4$
Biuret	0−1 mg	High, no depend on amino acid composition	Amino-group $[(NH_4)_2SO_4]$
Bradford	0−0.01 mg	Dependent on amino acid composition	Detergents (soap, SDS, Triton X-100)

Table III.- Summary of protein concentrations determine through Lowry, Bradford and Biuret in shrimp species.

Species name	Lowry method		Bradford method		Biuret method	
	Male	Female	Male	Female	Male	Female
F. indicus	40.30 ± 3.53	46.90 ± 22.44	41.85 ±9.39	29.54 ± 13.80	23.50 ± 12.43	26.75 ± 15.77
	36.13–45. 38	19.00–69.84	29.00–53.00	17.76–51.80	8.81–45.00	13.36–45.40
F. merguensis	48.59 ±12.21	45.48 ± 9.79	37.94 ±15.92	39.63 ± 13.14	24.89 ±15.84	23.27 ± 11.37
	35.78–67.80	35.00–64.85	17.20–57.00	19.32–53.08	12.56–50.50	9.56–41.50
M. monoceros	46.75 ±24.20	38.03 ±12.75	27.72 ±11.12	38.42 ±7.64	22.59 ±14.48	45.94 ± 15.21
	10.1–71.3	26.04–57.3	11.00–41.00	34.79–54	11.52–49	33.05–73.00
M. affinis	44.89 ± 9.03	55.14 ± 22.58	46.43 ± 18.41	18.53 ± 5.51	19.93 ± 4.78	25.07 ± 10.89
	33.84–61.2	14.8–71.4	15.2–63.97	11.00–27.84	11.44–23.5	12.72–38.00
P. stylifera	42.89 ± 6.91	47.56 ± 6.08	41.31 ± 8.47	38.17 ± 14.47	25.18 ± 11.52	29.04 ± 15.11
	31.55–53.88	41.33–55.70	31.00–55.28	21.00–51.55	12.00–45.50	15.69–51.00

Table IV.- Some previous studies as conducted for the protein estimation in *Fenneropenaeus indicus*.

Species	Lowry*	Region	Reference
Fenneropenaeus indicus	7.49±0.072c	India	Karuppasamy et al., 2014
Indian White Prawn	35.13±1.39	India	Banu et al., 2016
	42.88±1.11	Egypt	Abdel salam, 2013
	41.3±0.3	India	Ravichandran, 2009

*No recoerd for Bradford and Biuret was found.

Our results clearly showed the usefulness of Lowry's method as compared to the other two methods for proteins estimation in the tissues of shrimps. As the variety of compounds interfere with the Lowry procedure includes some amino acid derivatives, certain buffers, drugs, lipids, sugars, salts, nucleic acids and sulphydryl reagents ammonium ions, Zwitter ionic buffers, non-ionic buffers and thiol compounds (Dunn, 1992; Price, 1996). The using of Folin-Ciocalteu reagent makes the Lowry assay nearly 100 times more sensitive than other methods (Martina and Vojtech, 2015). The principle of the Lowry method to determine protein concentrations based on the reactivity of the peptide nitrogen with the copper ions under alkaline environment and the subsequent reduction of the Folin-Ciocalteay phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic acids (Dunn, 1992). Table IV showed the comparison of protein levels estimated by different researchers from other parts of the world and most of the authors followed the Lowry's method to estimate the proteins. Table IV shows that in the highest amount of proteins were observed in the current study as compared to the other studies. Anggun (2013) also found the highest sensitivity of protein through the Lowry method in albumen. The Lowry method, sensitive to minimum concentrations of protein, Dunn (1992) suggested concentrations ranging from 0.10-2 mg of protein per ml while Price (1996) suggests concentrations of 0.005-0.10 mg of protein per ml.

The Bradford assay was founded the less sensitive to Lowry but showed high sensitivity towards the Biuret but this assay have some disadvantages for instance, it is sensitive to interference by many other compounds (basic conditions and detergents-SDS). However, there are detergent-compatible Bradford reagents. The Bradford assay depends on the sequence of the protein. If the protein does not contain enough number of arginine and/or aromatic residues, then the dye will not bind to the protein as efficiently, resulting in an underestimation of the protein concentration. The Biuret method indicated the lowest content of proteins in most of the shrimp species, which was probably due to the low sensitivity of this method. This study is also comparable with the work of Janairo *et al*. (2011), who tested the sensitivity of Biuret method. The Biuret assay is not much good for protein concentrations below 5 mg/ml.

CONCLUSION

In this study, Protein content of some shrimp species showed that they belong to a high-proteinaceous category. The recorded data of this study revealed that the Protein found the major content in the muscles no significant differences observed in the muscle protein of male and female in all studied species of the shrimps. Protein identified as the major content in the muscles of both the sexes. The high protein value observed in edible muscles

of *M. affinis,* hence it transform more dietary protein into tissue protein. The most reliable technique was Lowry because of its sensitivity, consistency and less variation in the result, but as it is time taking so Bradford sensitivity could come next as it has also better sensitivity, while Biuret sensitivity is quite low and detects 5 times lesser concentrations than the other two techniques in most of the samples.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Anggun, M., 2013. Determining the concentration of protein on egg albumin through Lowry method. http://www.scribd.com

Bano, Z., Abdullah, S., Ahmad, W., Zia, M.A. and Hassan, W., 2017. Assessment of heavy metals and antioxidant enzyme in different organs of fish from farm, hatchery and Indus River of Pakistan. *Pakistan J. Zool.,* **49**: 2227-2233.

Banu, S.K.S., Hareesh, K. and Srinivasulu-Reddy, M., 2016. Evaluation of nutritional status of penaeid prawns through proximate composition studies. *Int. J. Fish. aquat. Stud.*, **4**: 13-19.

Bradford, M.M., 1976. Rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analy. Biochem*., **72**: 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Bugel, S.H., Sandstrom, B. and Larsen, E.H., 2001. Absorption and retention of selenium from shrimps in man. *J. Trace Elem. Med. Biol*., **14**: 198-204. https://doi.org/10.1016/S0946-672X(01)80002-X

Cheng, C.S. and Chen, L., 1990. Growth and relationships among body length and tail weight of *Penaeus monodon* from culture environment in Taiwan. *Aquaculture*, **9**: 253-263. https://doi.org/10.1016/0044-8486(90)90192-P

Dincer, M.T. and Aydin, I., 2014. Proximate composition and mineral, and fatty acid profiles of male and female jinga shrimps (Metapenaeus attm,s. H M1ine Edwards, 1837). *Turk. J. Vet. Anim. Sci.,* **38:** 445-451.

Dunn, M.J., 1992. Protein determination of total protein concentration. In: *Protein purification methods* (eds. Harris, E.L.V., Angal S.), Oxford, IRL Press.

Faiz, M., Zhang, Z.F., Shao, M.Y. and Shafi, M., 2017. cDNA cloning and expression of Cyclophilin A (LvCypA) in white leg shrimp, *Litopenaeus vannamei. Pakistan J. Zool.,* **49**: 935-941.

FAO, 2012. Fishery and aquaculture statistics. www.fao.org/fishery/statistics/en (Accessed 2 May 2014)

FAO, 2014. *World aquaculture and fisheries statistics*. FAO Publication, Rome, Italy.

Fatima, H., Ayub, Z., Ali, S.A. and Siddiqui, G., 2013 Biochemical composition of the hemolymph, hepatopancreas, ovary, and muscle during ovarian maturation in the penaeid shrimps *Fenneropenaeus merguiensis* and *F. penicillatus* (Crustacea: Decapoda). *Turk. J. Zool*., **37**: 334-347.

Fawole, O.O., Ogundiran, M.A., Ayandiran, T.A. and Olagunju, O.F., 2007. Proximate and mineral composition in some selected fresh water fishes in Nigeria. *Internet J. Fd. Safe*., **9**: 52-57.

Gokoglu, N. and Yerlikaya, P., 2015. *Chemical composition of fish in seafood chilling, refrigeration and freezing: Science and technology*. John Wiley and Sons, Ltd., Chichester, UK. https://doi.org/10.1002/9781118512210

Gornall, A.G., Bardawill, C.S. and David, M.M., 1949. Determination of serum proteins by means of the biuret reaction. *J. biol. Chem*., **177**: 751.

Hala, A.A.S., 2014. Protein and electrophoretic analysis of edible muscle of commercially important crustaceans and mollusks species from Egyptian and Saudi Arabia costs Abdel-Salam Department of Zoology, Faculty of Science, Cairo University, Egypt. *Anim. Vet. Sci*., **2**: 109-117.

Hall, D.N.F. 1962. Observations on the taxonomy and biology of some Indo-West Pacific Penaeidae (*Crustecea decapoda*). *Fish. Publ. Col. Office London,* **17:** 1-229.

Janairo, G., Marianne-Linley, S.M., Yap, L., Llanos-Lazaro, N. and Robles, J. 2011. Determination of the sensitivity range of Biuret Test for undergraduate biochemistry experiments. *J. ScI. Tech.,* **5**: 77-83.

Karuppasamy, P.K., Sri Sakthi Priyadarshini, R., Ramamoorthy, N., Sujatha, R., Ganga, S., Jayalakshmi, T. and Santhanam, P., 2014. Comparison of proximate, amino and fatty acid composition of *Penaeus monodon* (Fabricius, 1798), *Fenneropenaeus indicus* (H. Milne Edwards, 1837) and *Aristeus virilis* (Bate, 1881) of Nagapattinam landing centre, Tamil Nadu. *J. Mar. Biol. Assoc. India*, **55**: 39-42.

Kazmi, Q.B., 2003. *Marine fauna Pakistan*, Ser. 1. *Shrimps,* pp. 1-16.

King, I.M.T., Marian T.C., Dorsett C., Ostrander J.G. and Monsen E.R. 1990. Shellfish: Proximate composition, minerals, fatty acids, and sterols. *J. Am. Diet. Assoc.,* **90**: 677-685.

Kucukgulmez, A.M., Celik, Y., Ersoy, Y.B. and Cikrikci, M., 2006. Proximate composition and mineral contents of the blue crab *Callinectes sapidus* breast meat, claw meat and hepatopancreas. *J. Fd. Sci. Technol*., **41**: 1023-1026.

Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., 1951. Protein measurement with the Folin Phenol Reagent. *J. biol. Chem*., **193**: 265-275.

Marques-Barrento, S.A., Teixeira, B., Vaz-Pires, P., Carvalho, M.L. and Nunes, M.L., 2008. Essential elements and contaminants in edible tissues of European and American lobsters. *Fd. Chem*., **111**: 862-867. https://doi.org/10.1016/j.foodchem.2008.04.063

Martina, V. and Vojtech, K.A., 2015. Comparison of Biuret, Lowry and Bradford methods for measuring the egg's proteins. *Mendel Net Conf. Proc.,* 394-398.

Mridha, M.A., Lipi, S.Y., Narejo, N.T., Uddin, M.S., Kabir, M.S. and Karim, M., 2005. Determination of biochemical composition of *Cirrhinus reba* (Hamilton, 1822) from Jessore, Bangladesh. *J. Sci. Technol. Univ. Peshawar*, **29**: 1-5.

Nisa, K.U. and Sultana, R., 2010. Variation in the proximate composition of shrimp, Fenneropenaeus penicillatus at different stages of maturity. *Pak. J. Biochem. Mol. Biol*., **43**: 135-139.

Pillay, K.K. and Nair, N.B., 1973. Observations on the biochemical changes in gonads and other organs of *Uca annulipes*, *Portunus pelagicus* and *Metapenaeus affinis* (Decapoda: Crustacea) during the reproductive cycle. *Mar. Biol.,* **18**: 167–198.

Prasuna, D.D.V., Hareesh, K. and Srinivasulu-Reddy, M., 2015. Studies on the proximate composition of tropical freshwater shrimp *Macrobrachium rosenbergii*. *Int. J. Fish. aquat. Stud*., **3**: 329-336.

Price, N.C., 1996. *Proteins*. Labfax, Oxford, Academic Press.

Pérez-Farfante, I. and Kensley, B., 1997. Penaeoid and Sergestoid shrimps and prawns of the world. Key and diagnoses for the families and genera. *Mém. Mus. Natl. Hist. Nat. Paris*, **175**: 1-233.

Trung, T.S. and Pham, P.D.T. 2012. Bioactive compounds from by-products of shrimp processing industry in Vietnam. *Food Drug Anal.,* **20**:194–197.

Ravichandran, S., Ramesh Kumar, G. and Prince, A.R., 2009. Biochemical composition of shell and flesh of the Indian white shrimp *Penaeus indicus*. *Am. Euras. J. scient. Res*., **4**: 191-194.

Wardiatno, Y., Santoso, J. and Mashar, A., 2012. Biochemical composition in two populations of the mantis shrimp, *Harpiosquilla raphidea* (Fabricius 1798) (Stomatopoda, Crustacea). *Maret*, **17**: 49-58.

Wardiatno, Y. and Mashar, A., 2010. Biological information on the mantis shrimp, *Harpiosquilla raphidea* (Fabricius 1798) (Stomatopoda, Crustacea) in Indonesia with highlight of its reproductive aspects. *J. Trop. Biol. Conserv*., **7**: 65-71.

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Comparative Effect of Similar Feed and Feeding Regimes on the Growth Performance, Proximate Composition and Economic Profitability of Indian Major Carps

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Authors' Contribution
NAQ and GWV planned the research. NK conducted the experiments and studied growth, proximate and PKS analyzed the data statically. NK, A Mustafa, KJI, MAJ, SD and A Maqbool wrote the article.

Key words
Indian major carps, Growth, Artificial feed, Body composition, Economic analysis.

ABSTRACT

Indian major carps (*Catla catla*, *Cirrhinus mrigala*, and *Labeo rohita*) fingerlings were evaluated for growth, body composition, and economic profitability under a similar feeding regime in monoculture system. The diet containing 42% protein was provided at 4% of fish wet body weight. When different species were compared, *C. catla* (1341.58) and *L. rohita* (1762.51) significantly ($P \leq 0.05$) grew faster than *Cirrhinus mrigala* (976.17). Feed conversion ratio (FCR) values of three species *C. catla, Cirrhinus mrigala* and *L. rohita* ranged from 1.63, 1.56, and 1.43, respectively. *C. catla* and *L. rohita* exhibited significantly higher gross nitrogen retention efficiency (GNRE %) at 10.4 and 9.3 compared to *Cirrhinus mrigala* at 6.5. The gross energy retention efficiency, proximate body composition, and mineral composition were non-significant among the species. In conclusion, the growth performance and economic anaylisis indicated that *C. catla* and *L. rohita* showed better performance compared to *Cirrhinus mrigala* fingerlings, when fed an artificial feed.

INTRODUCTION

Worldwide, attention toward healthy diet is increasing because now-a days, peoples have started to know that food choice may have significant importance in health (Franz and Nowak, 2010; Kaimakoudi *et al*., 2013). Fish is rich source of vital fatty acids especially omega-3 polyunsaturated fatty acids which are essential during pregnancy and early childhood for normal growth, mental development and heart functioning (FAO, 2003). Fish products provide admirable protein due to their balanced amino acid profile and protein digestibility that ranges from 85-90% (Rudolf, 1971; Astawan, 2004).

Fish is also rich in minerals (especially iodine, selenium, calcium and phosphorous etc) and fat soluble vitamins (A, D and E) and water soluble vitamins (B complex) (Choo and Williams, 2003; Sandhu, 2005; Razvi, 2006; Salim, 2006; Yildrim *et al*., 2008; Naeem and Salam, 2010). So, fish can provide an essential source of nutrients, especially for those people whose foods are deficient of these nutritious components (World Aquaculture, 2010).

Almost all over the world, fisheries sector is playing important role in betterment of socio-economic status of fishermen community (Chiarini *et al*., 2009). Pakistan has total fish ponds area around 60,470 hectares and 1050 km long coastline which provides job opportunities to about one million people directly or indirectly (Akhtar, 2001). In natural resources fish production is decreasing due to over fishing and pollutants (Hassan, 1996). Pakistan has been blessed with very rich aquatic resources. However, aquaculture in Pakistan, contributes only about 1% of the national GDP (FAO, 2003). Aquaculture is the fastest

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rising animal food production area and now provides more than wild-caught and beef to the human beings (FAO, 2016; USDA, 2017). It is the only alternate that can compensate these deficiencies and cater the emerging demands of quality protein. Indian major carps, such as *Catla catla*, *Labeo rohita*, and *Cirrhinus mrigala* have central position among the fish fauna of South Asia and are prime cultureable fish species in pond culture system both in public and private sectors since longtime (Khan *et al*., 2004; Hussain *et al*., 2011). Approximately 80% of carps and 65% tilapia are cultured throughout the world (Naylor *et al*., 2000). These carps are basically herbivorous in nature (Khan *et al*., 2011). A number of studies so far have been carried out at laboratory scale in flow-through systems or glass aquaria on growth, body composition, feed utilization, feeding frequency, and protein retention efficiency of various fish species (Sarker *et al*., 2000; Singh *et al*., 2006; Mondal *et al*., 2007; Latif *et al*., 2008; Abid and Ahmed, 2009; Siddiqui and Khan, 2009; Khan and Abidi, 2010), where growth is mainly dependent on artificial feed. Several researchers also worked on polyculture (major carps) and composite culture (major carps and Chinese carp) of these carp in semi-intensive culture system (Ahmed *et al*., 2005; Yaqoob *et al*., 2010; Naeem and Ishtiaq, 2011; Khan *et al*., 2012, 2018; Abbas *et al*., 2014; Mamun and Mahmud, 2014). But information regarding monoculture and its impact on the comparative growth rate and body composition of fingerlings of Indian major carps in monoculture system is limited. The present study is therefore, focused on the growth performance and body composition of Indian major carps (*L. rohita*, *C. catla* and *Cirrhinus mrigala*) and economic viability in monoculture system.

Table I.- Formulation and ingredient composition of experimental diet.

Ingredients	Composition (g/Kg)
Fish meal	200
Soybean meal	300
Maize gluten	240
Wheat bran	50
Rice polish	30
Maize grains	80
Molasses	80
Vitamins	10
Mineral mixtures	10
Total	1000

MATERIALS AND METHODOLOGY

Experimental site and design

The study was carried out in the Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Lahore, Ravi Campus, Pattoki, Pakistan. The experiment was conducted in nine earthen ponds having an area of 0.03 ha (59m× 30.5m× 1.8m) each and replicated three time for 90 days following complete randomized design (CRD).

Pond preparation

The ponds were filled with tube well water to a depth of 1.5 m. This water level was retained up to studied period (90 days) with regular additions of tube well water to compensate seepage and evaporation losses. One week prior to stocking of fish, ponds were fertilized with cattle manure at 90 kg/pond (3 ton/ ha) (Jena and Das, 2006; Sahu *et al*., 2007) and poultry manure at 45 kg (50% of cattle manure), 2.5 kg single super phosphate, and 1.25 kg urea/ pond for the production of planktonic life and followed by bi-weekly application of this quantity of fertilizer throughout the study period. Fertilizers are used in carp ponds to increase the primary productivity, which is considered staple natural food of carp.

Fingerlings and stocking

The fingerlings of three experimental species were procured from Punjab Government Fisheries Department Chenawan Fish Hatchery, District Gujranwala, Pakistan. Fish with an average weight of 20-30g size were randomly stocked at 80 fish/pond (0.03ha) (240 fishes/species). All the fish were weighed at the time of stocking and data was recorded for final comparison purposes.

Feed ingredients and preparation of feed

The ingredients were finely ground separately, steam cooked at 140°C, and passed through a 5 mm extruder die to prepare pellets at National Feed Mill, Sheikhupura, Pakistan. The dry pellets were mashed and reduced in size (2 mm) to meet the fish's requirement. The mash was then bagged and transported to experimental site and stored at room temperature 23-26°C until fed. The artificial supplementary feed was formulated using 20% fish meal and diet contained 42% protein level (Table II), based on several studies regarding the recommended ranges for protein level of Indian major carps 30-45% (De Silva and Gunasekera, 1991). The fish were fed the supplementary diet twice a day (9:00 and 16:30) at 4% body weight (Abid and Ahmed, 2009). Most of the studies decribed that fry to fingerlings are fed 4-5% fish wet body weight and when the fish grown to adult it may be reduced to 3%.

Table II.- Proximate composition of experimental diet and ingredients for fingerlings of Indian major carps (% dry matter basis).

Parameters	Diet	Fish meal	Soybean meal	Maize gluten	Wheat bran	Rice polish	Maize grains
Dry matter	92.07	91.68	92.56	91.59	89.9	90.85	86.93
Protein	42.34	47.89	43.09	58.92	21.5	15.3	12.03
Lipid	5.52	8.97	0.63	2.81	3.17	12.53	2.63
Ash	14.08	31.99	9.8	3.3	6.6	15.6	2.49
Crude fiber	3.89	2.77	11.64	1.07	11.79	12.75	3.3
Nitrogen free extract	26.24	0.06	27.4	25.49	46.84	34.67	66.48
Gross energy (MJ/g)	19.33	17.35	18.56	22.59	18.34	20.03	17.81
Mineral composition (%)							
Ca	1.7	5.72	1.78	0.86	1.32	1.79	0.13
Mg	0.22	0.34	0.36	0.08	0.51	0.98	0.17
K	1.13	0.6	1.11	0.02	1.2	1.36	0.56
P	0.75	1.54	0.45	0.06	0.95	1.73	0.4

Fish growth studies

At the end of each 2-week period, a random sample of 20 fish was taken from the bulk harvest from each pond, by a drag net and weighed to the nearest g and measured to the nearest mm. The fish were anesthetized with few drops of clove oil in bucket having half full of clean water. This practice continued up to the end of feeding trial. Data were recorded and fish were put back into ponds. At the end of experiment five sampled fish were randomly collected from each pond for bio-chemical analysis. Other growth parameters including condition factor (K), net weight gain (NWG), percent gain in weight, specific growth rate (SGR %), feed conversion ratio (FCR), protein efficiency ratio (PER), protein utilization (PU), gross nitrogen retention efficiency (GNRE%), and gross energy retention efficiency (GERE%) were calculated according to the following formulae:

Condition factor (Carlander, 1970):

$$K= W \times 10^5 / L^3$$

Net Weight Gain (NWG) = Mean final weight (g) - Mean initial weight (g)

Percent Weight Gain (PWG) = Final weight(g)-Initial weight(g) x 100/Initial weight(g)

SGR % = ln (Final wet body weight) - ln (Initial wet body weight) x 100 / Number of days (Hopkins, 1992)

Feed Conversion Ratio (FCR) = Feed intake (g) / Wet weight gain (g)

Protein Efficiency Ratio (PER) = Wet weight gain of fish (g) / protein intake (g)

Protein Utilization (PU) = protein content(g) of fish at the end of experiment - protein content (g) of fish at the start of experiment / dry protein fed (g)

Gross Nitrogen Retention Efficiency (GNRE):

$$GNRE\ \% = [[(FBW \times N\ content_{final}) - (IBW \times N\ content_{initial})] \times 100/ GNI]$$

Gross Energy Retention Efficiency (GERE):

$$GERE\ \% = [[(FBW \times E\ Content_{final}) - (IBW \times E\ content_{initial})] \times 100/ GEI]$$

Where, FBW is final body weight, IBW is initial body weight, GNI is gross nitrogen intake and GEI is gross energy intake.

Chemical analysis

The feed ingredients and feed were analyzed for moisture, crude protein, crude lipid, ash, amino acids, minerals, gross energy, and crude fiber whereas, nitrogen free extract (NFE) was estimated by subtraction. Proximate analysis of fish was determined at the start and end of experiment following AOAC (2003). Briefly, the samples were dried in a vacuum oven (Model: 524 Precision Scientific, USA) at 105°C for 18 h to determine the dry matter. Crude protein was determined by Kjeltec Auto Analyzer Tecator 1030 (FOSS, Hoganas, Sweden) by digesting the sample at high temperature (415 °C) in concentrated sulphuric acid (15 ml) in the presence of potassium sulphate and copper sulphate. Crude lipid was determined by extraction through Soxtec System (Model: HT 1043 Extraction Unit Tecator, Hoganas, Sweden) in diethyl ether (solvent). The ash content was determined by incinerating approximately 1 g of sample in a mufflefurnace (Thermolyne, Dubuque, Iowa, USA) at 550°C overnight. Crude fiber content of feed and feed ingredients was determined by digesting dry sample in 1.25% (Ankom 200/220, Model: A200, Macedon, NY,

USA). Gross energy was determined by bomb calorimeter (Parr 6300 Calorimeter, Moline, IL, USA) using benzoic acid as a standard. Calcium, magnesium and potassium were determined by atomic absorption spectrophotometer (Model: Z-8100 Polarized Zeeman Atomic Absorption Spectrophotometry, Hitachi, Ltd, Tokyo Japan) by Flame Atomization Method after digesting the samples, while phosphorus was determined by Spectrophotometer at 400 nm wavelength (Spectronic 1201 and 1001-plus, Milton Roy, Ivyland Road, USA).

Physico-chemical parameters

Water temperature, dissolved oxygen (DO), pH, salinity, total dissolved solids (TDS), electrical conductivity, and pond productivity (Secchi disc visibility) were monitored on a daily basis, while nitrates on a bi-weekly basis between 9.00 and 10.00 a.m. following the standard methods APHA (1998).

Economic analysis

The ingredients were purchased from local market, Lahore as per whole sale price. Organic fertilizer was procured from Livestock farm, Pattoki, District Kasur and inorganic fertilizers from Pattoki as per market rates. The cost of grinding of ingredients, transportation, and preparation was also included, which was than calculated finally per kg of prepared feed.

Statistical analyses

The obtained data was analyzed through statistical software SAS 9.1 and Analysis of Variance (ANOVA) was applied to compare the means.

RESULTS

During present study, net weight gain were compared among the three species and found significantly higher ($P \leq 0.05$) weight in *C. catla* and *L. rohita* than *Cirrhinus mrigala* (Table III). Feed conversion ratio (FCR), protein efficiency ratio (PER) and protein utilization (PU) were found non-significant ($P > 0.05$) among the species (Table III). Minor variation regarding these parameters among species was observed. Regarding gross nitrogen retention efficiency (GNRE %) *C. catla* (10.4) showed significantly higher ($P \leq 0.05$) nitrogen retention efficiency followed by *L. rohita* (9.3) and *Cirrhinus mrigala* (6.5) (Table III).

The whole body proximate composition among the three species was observed non-significant under the artificial diet 42% protein (Table IV). However, an increasing trend of protein and lipid deposition was observed with the increase in body weight, while ash contents remained the same.The percentage of macro minerals (Ca, Mg, K and P) minor variations were observed among the species (Table IV).

Table III.- Growth performance of three species of Indian major carps fingerlings fed 42% protein diet for 90 days in monoculture system.

Parameters	*Catla catla*	*Cirrhinus mrigala*	*Labeo rohita*	SEM	P value
Initial wt.(g)	32.46	29.78	28.3	3.53	0.109
Final wt.(g)	458.3	317.96	402.81	7.98	0
Net wt. gain(g)	425.83[a]	288.18[b]	374.34[a]	9.5	0.004
IL (mm)[1]	140.6	148.3	126.3	6	0.106
FL(mm)[2]	312.2	311.2	305	1.85	0.015
GL(mm)[3]	171.5[a]	161.9[a]	178.7[a]	5.99	0.258
P WG[4]	1341.58	976.17	1762.51	234	0.314
SGR%[5]	2.95	2.6	3.03	0.15	0.107
K[6]	1.59[a]	1.11[b]	1.42[a]	0.03	0.013
FCR[7]	1.63	1.56	1.43	0.07	0.234
PER[8]	1.4	1.4	1.6	0.63	0.228
PU[9]	0.7	0.7	0.9	0.08	0.224
GNRE%[10]	10.4[a]	6.5[b]	9.3[a]	0.44	0.002
GERE%[11]	68.3	71	76.9	2.79	0.165

SEM, standard error of mean; [1]IL, initial length; [2]FL, final length; [3]GN, gain in length; [4]PWG, percent weight gain; [5]SGR%, specific growth rate; [6]K, condition factor; [7]FCR, feed conversion ratio; [8]PER, protein efficiency ratio; [9]PU, protein utilization; [10]GNRE%, gross nitrogen retention efficiency; [11]GERE%, gross energy retention efficiency. *Figures in the same row with different superscripts are significantly ($P \leq 0.05$) different.

Table IV.- Whole body proximate composition of three fish species fingerlings fed 42% protein diet in monoculture system post treatment (% wet weight basis).

Parameters	*Catla catla*	*Cirrhinus mrigala*	*Labeo rohita*	SEM	ANOVA P values
Dry matter (%)	22.08	26.69	28.39	1.64	0.733
Protein (%)	12.9	14.09	16.44	1.02	0.929
Lipids (%)	4.82	6.96	6.52	0.42	0.144
Ash (%)	3.56	4.75	4.49	0.22	0.793
Gross energy (MJ/g)	5.00	6.17	6.53	0.4	0.593
Mineral composition					
Ca (%)	0.93	0.84	1.05	0.05	0.472
Mg (%)	0.02	0.05	0.03	0.01	0.117
K (%)	0.09	0.18	0.15	0.02	0.232
P (%)	0.49	0.55	0.55	0.03	0.413

SEM, standard error mean. *Means are of three replicates and the values in each represent means of two determinations.

Table V.- Total fish production (fingerlings), feed and fertilizer costs of three fish species under monoculture system in ponds.

Parameters	*Catla catla*	*Cirrhinus mrigala*	*Labeo rohita*
No. of fish stocked/pond	80	80	80
Survival rate (%)	100	100	100
Initial average body weight (g)	32.46	29.78	28.3
Final average body weight (g)	458.3	317.96	402.81
Increase in average body weight(g)	425.83	288.18	374.34
Gross fish production/pond/90days(kg)	36.66	25.43	32.22
Gross fish production/pond/year(kg)	148.67	103.13	130.67
Gross fish production/ha/year(kg)	4955.66	3437.66	4355.66
Net fish production /pond/90days (kg)	34.07	23.05	29.95
Net fish production /pond/year (kg)	138.17	93.48	121.46
Net fish production/ha/year(kg)	4605.66	3116	4048.66
Total Fertilizer used/pond/90days(kg)	971.25	971.25	971.25
Total Feed used/pond/90days(kg)	57.75	32.29	44.64
Fertilizer cost/pond/90days@Rs.1.45/kg	1408.31	1408.31	1408.31
Feed cost/pond/90days@Rs.37.43/kg	2156.38	1205.7	1666.85
Cost fertilizer /pond/year	5711.47	5711.47	5711.47
Cost feed /pond/year	8745.31	4889.81	6760
Cost of fertilizer/ha/year	190382.65	190382.65	190382.65
Cost of feed/ha/year	291510.63	162993.94	225334.45
Total cost of feed and fertilizer/ha/year	481893.28	353376.59	415717.1

The values of key physico-chemical parameters such as, water temperature ranged from 32.6-33.0°C, Dissolved oxygen (DO) 5.45- 5.77 mg/L, pH 7.4-7.7, light penetration 18.2-29.3cm, respectively. All these values were in acceptable range for the growth and culture of Indian major carps.

Total cost of feed and fertilizer of three species varied from Rs. 481893.28 (4589.45USD) for *C. catla*, Rs. 353376.59 (3365.49USD) for *Cirrhinus mrigala* and Rs. 415717.1 (3959.21USD)/ ha/ year for *L. rohita* (Table V). Total income in Pakistani rupees (Rs.) of three species *C. catla*, *Cirrhinus mrigala* and *L. rohita*, varied from Rs. 991133, 687533, and 871133/ ha/ year, respectively (Table V). Net profit of each species was Rs. 509240 (4849.90USD), 334156.74 (3182.44USD), and 455416 (4337.29USD), for *C. catla, Cirrhinus mrigala* and *L. rohita*, respectively. Based on the above mentioned figures, the economic analysis of the current study indicated that *C. catla* and *L. rohita* net profit is higher when compared to *Cirrhinus mrigala*.

DISCUSSION

In present study, net weight gain were compared among the three species and found significantly higher ($P \leq 0.05$) weight in *C. catla* and *L. rohita* than *Cirrhinus mrigala.* These differences in growth response among three species may be due to their feeding habits (ecological feeding niche) as *C. catla* is surface feeder; *L. rohita* is column feeder and *Cirrhinus mrigala* is bottom feeder. Same results were obtained by Sophin and Preston (2001), who concluded that the degree of response was highest for *H. molitrix*, followed by *C. catla* and *L. rohita* and least for *Cirrhinus mrigala*. Similarly, Abbas *et al*. (2014) found highest growth in *C. catla*, followed by *L. rohita* and *C. carpio* when cultured under semi-intensive system. Javed *et al*. (1992) and Tahir (2008), also found highest growth in *C. catla,* followed by *L. rohita* and *Cirrhinus mrigala* in polyculture system under different treatments. Differences in weight gain among species during current study are in line with the findings of Garg *et al*. (2002) for mrigal fingerlings, which remained significantly low in comparison to *L. rohita* (rohu) when fed on similar diets.

During present study, non-significant difference were observed in FCR, PER, PU and GERE% among the major carp. Khan *et al*. (2012) also supported our study and found significant difference in FCR, PER, PU and GERE% when they fed major carps similar feed. Low variability in energy retention efficiency was observed among species in present study, where *L. rohita* showed higher values followed by *Cirrhinus mrigala* and *C. catla*. Differences in nitrogen retention efficiency among species was also observed during the current study where values of GNRE% were significantly higher in *C. catla* followed by *L. rohita* than *Cirrhinus mrigala* which might be due to their voluntary feed intake, weight gain, composition, digestion, absorption, transport, metabolism and genetic variation (Javed *et al*., 1992; Medale, 1993). Mahboob *et al*. (1995) reported higher amount of the overall nitrogen incorporation efficiency of 28.25% under composite culture of Indian major carps (*C. catla, Cirrhinus mrigala* and *L. rohita*) with Chinese carps (*Hypophthylmicthys molitrix, Ctenopharyngodon idella*) and common carp (*Cyprinus carpio*) reared with artificial feed (35% protein) and fertilizer additions.

The proximate composition in the current study were observed non significant among species. However, an increasing trend of protein and lipid deposition was observed with the increase in body weight, while ash contents remained the same. Similar finding was reported by Khan *et al*. (2012), when they studied body composition of major carps (*C. catla, L. rohita* and *Cirrhinus mrigala*) under mono and polyculture system at similar feeding regime. While Naeem and Salam (2010) and Naeem and Ishtiaq (2011) studied proximate of *Aristichthys nobilis* and *Mystus bleekeri* and reported that the nutrients of fish body vary due to variation in species, body size and condition factor. Jirsak *et al*. (1984) suggested the intensity of feeding frequency and formulation increased fat and protein contents in carps significantly that may depend upon their feeding habits. Genetic factors and feeding regimes exercise significant change in some structural and flesh quality parameters of different fish species (Ashraf *et al*., 2011). The percentage of macro minerals (Ca, Mg, K and P) observed non-significant difference among species at similar feeding regime. Similar findings were also reported by Khan *et al*. (2012), when they studied mineral composition of major carps under mono or polyculture semi-intensive system at similar feeding regime. Kirchgessner and Schwarz (1986) studied mineral contents in *Cyprinus carpio* L. and found increased in mineral (Ca and P) contents in the carp carcass with increasing crude protein contents in the feed and with increasing energy supply the Ca and P values decreased.

The economic analysis of the current study indicated that *C. catla* and *L. rohita* as the net profit is higher when compared to *Cirrhinus mrigala* among three species of Indian major carps in semi-intensive monoculture system.

CONCLUSION

It has been concluded that fingerlings production performance of the three fish species of Indian major carps in terms of economic viability and growth indices revealed that *C. catla* and *L. rohita* are better candidates for monoculture production system than *Cirrhinus mrigala* with 42% CP artificial diet.

Statement of conflict of interest

We have not any conflict of interest to declare.

REFERENCES

Abbas, S., Ashraf, M. and Ahmed, I., 2014. Effect of fertilization and supplementary feeding on growth performamnce of *Labeo rohita, Catla catla* and *Cyprinus carpio. J. Anim. Pl. Sci.,* **24**: 142-148.

Abid, M. and Ahmed, M.S., 2009. Growth response of *Labeo rohita* fingerlings fed with different feeding regimes under intensive rearing. *J. Anim. Pl. Sci.*, **19**: 45-49.

Ahmed, I., Abbas, K. and Hafeez-ur-Rehman, M., 2005. Growth response of major carps in semi-intensive ponds supplemented with rice polishing. *Pakistan Vet. J.,* **25**: 59-62.

Akhtar, N., 2001. *Strategic planning for inland fisheries and aquaculture sector in Pakistan*. In: Proceedings of National Seminar on Strategic Planning for Fisheries and Aquaculture to Face the Challenges of New Millennium. Karachi, Pakistan.

AOAC, 2003. *Official methods of analysis,* 17th ed. Association of Official Analytical Chemist, Washington, DC, US.

APHA, 1998. *Standard methods for the examination of water and wastewater*, 20th ed. American Public Health Association, The American Water Works Association and the Water Environment Federation, pp. 1220.

Ashraf, M., Zafar, A., Rauf, A., Mehboob, S. and Qureshi, N.A., 2011. Nutritional values of wild and cultivated silver carp (*Hypophthalmichthys molitrix*) and grass carp (*Ctenopharyngodon idella*). *Int. J. Agric. Biol.,* **13**: 210-214.

Astawan, M., 2004. *Ikan yang sedap dan bergizi*. Penerbit Tiga Serangkai. Solo.

Carlander, D., 1970. *Handbook of freshwater fishery biology*, Vol. 1. The Iowa State University Press, Iowa, USA.

Chiarini, E., Tyler, K., Farber, J.M., Pagotto, F. and Destro M.T., 2009. Processing, products and food safety, *Listeria monocytogenes* in two different poultry facilities: Manual and automatic evisceration. *Poult. Sci.,* **88**: 791-797. https://doi.org/10.3382/ps.2008-00396

Choo, P.S. and Williams, M.J., 2003. Fisheries production in Asia: Its role in food security and nutrition. *World Fish Centre Quart.,* **26**: 11-16.

de Silva, S.S. and Gunasekera, R.M., 1991. An evaluation of the growth of Indian and Chinese major carps in relation to the dietary protein content. *Aquaculture,* **92**: 237-241. https://doi.org/10.1016/0044-8486(91)90025-3

Dhawan, A. and Kaur, S., 2002. Pig dung as pond manure: Effect on water quality, pond productivity and growth of carps in polyculture system. *The ICLARM Quart.,* **25**: 11-14.

FAO, 2003. *Review of the state of world aquaculture*. FAO Fisheries Circular No. 886, Rev. Rome, Italy.

FAO, 2003. *The role of aquaculture in improving food security and nutrition.* Committee on World Food Security, Twenty-Ninth Session, 12-16 May 2003. Rome, pp. 14.

FAO, 2016. *The state of world fisheries and aquaculture*. Rome, Italy. Available at: www.fao.org/3/a-i5555e.pdf (Accessed: 9 November 2017).

Franz, A. and Nowak, B., 2010. *Functional food consumption in Germany: A lifestyle segmentation study*. Discussion Papers Number, 1003.

Garg, S.K., Kalla, A. and Bhatnagar, A., 2002. Evaluation of raw and hydrothermically processed leguminous seeds as supplementary feed for the growth of two Indian major carp species. *Aquacult. Res.,* **33**: 151-163. https://doi.org/10.1046/j.1365-2109.2002.00642.x

Hassan, M., 1996. *Influence of pond fertilization with broiler droppings on the growth performance and meat quality of major carps*. Ph D thesis, Department of Zoology and Fisheries, University of Agriculture, Faisalabad, Pakistan, pp. 15.

Hopkins, K.D., 1992. Reporting fish growth: A review of the basics. *J. World Aquacul. Soc.,* **23**: 173-179. https://doi.org/10.1111/j.1749-7345.1992.tb00766.x

Javed, M., Hassan, M. and Sial, M.B., 1992. Fish pond fertilization. iv. Effect of cow-dung on the growth performance of major carps. *Pak. J. Agric. Sci.,* **29**: 111-115.

Jena, J.K., Ayyapan, S., Aravindakshan, P.K. and Muduli, H.K., 2001. Comparative evaluation of growth, survival and production of carp species at different stocking densities under polyculture. *Indian J. Fish.,* **48**: 17-25.

Jena, J.K. and Das, P.C., 2006. Carp culture. In: *Handbook of fisheries and aquaculture* (eds. S.A. Verm, A.T. Kumar and S. Pardhan). Indian Council of Agricultural Research, New Delhi, India, pp. 265-282.

Jirsak, J., Spurny, P. and Janesov, L., 1984. Effect of different feeding frequency on body growth, feed conversion and nutrient retention in carp fry. *Zivvcisna Skola Vyroba*, **29**: 983-989.

Kaimakoudi, E., Polymeros, K., Schinaraki, M.G. and Batzios, C., 2013. Consumers' attitudes towards fisheries products. *Proc. Technol.,* **8**: 90-96. https://doi.org/10.1016/j.protcy.2013.11.013

Khan, M.A. and Abidi, S.F., 2010. Optimum ration level for better growth, conversion efficiencies and body composition of fingerling *Heteropneustes fossilis* (Baloch). *Aquacult. Int.,* **18**: 175-188. https://doi.org/10.1007/s10499-008-9234-2

Khan, M.A., Ahmed, I. and Abidi, S.F., 2004. Effect of ration size on growth, conversion efficiency and body composition of fingerling mrigal, *Cirrhinus mrigala* (Hamilton). *Aquacult. Nutr.,* **10**: 47-53. https://doi.org/10.1046/j.1365-2095.2003.00279.x

Khan, M.R.I., Parvez, M.T., Talukder, M.G.S., Hossain, M.A. and Karim, M.S., 2018. Production and economics of carp polyculture in ponds stocked with wild and hatchery produced seeds. *J. Fish.,*

6: 541-548. https://doi.org/10.17017/jfish.v6i1.2018.306

Khan, N., Ashraf, M, Qureshi, N.A. and Sarker, P.K., Vandenberg, D.W. and Rasool, F., 2012. Effect of similar feeding regime on growth and body composition of Indian major carps (*Catla catla*, *Cirrhinus mrigala* and *Labeo rohita*) under mono and polyculture. *Afr. J. Biotechnol.,* **11**: 10280-10290.

Khan, N., Qureshi, N.A., Nasir, M., Rasool, F. and Iqbal, K.J., 2011. Effect of artificial diet and culture systems on sensory quality of fried fish flesh of Indian major carps. *Pakistan J. Zool.,* **43**: 1177-1182.

Kirchgessner, M. and Schwarz, F.J., 1986. Mineral content (major and trace elements) of carp (*Cyprinus carpio* L.) fed with different protein and energy supplies. *Aquaculture,* **54**: 3-9. https://doi.org/10.1016/0044-8486(86)90248-6

Latif, K.A., Alam, M.T., Sayeed, M.A., Hussain, M.A., Sultana, S. and Hossain, M.A., 2008. Comparative study on the effects of low cost oil seed cakes and fish meal as dietary protein sources for *Labeo rohita* (Hamilton) fingerling. *Univ. J. Zool. Rajshahi Uni.,* **27**: 25-30.

Mahboob, S., Sheri, A.N. and Javed, M., 1995. Influence of fertilizers and artificial feed on nitrogen incorporation efficiency of fish. *Pakistan J. Zool.,* **27**: 349-351.

Mamun, A.A. and Mahmud, A.I., 2014. Study on growth performance and production of juvenile Indian major carp (*Catla catla*, *Labeo rohita* and *Cirrhinus cirrhosus*) and their hybrids. *J. Fish. aquat. Sci.,* **9**: 92-108.

Medale, F., 1993. Relation between growth and utilization of energy substrates in three rainbow trout strains. In: *Proceedings of the 4th International Symposium on Fish Nutrition and Feeding. Fish Nutrition in Practice* (eds. S.J. Kaushik and P. Luquet). Les Colloques 61. INRA, Biarritz, France, pp. 37-48.

Mondal, K., Kaviraj, A., Mukhopadhyay, P.K., Datta, M. and Sengupta, C., 2007. Evaluation of fermented fish offal in formulated diet of the Indian major carp, Rohu, *Labeo rohita* (Hamilton). *Acta Icthyol. Piscat.,* **37**: 99-105. https://doi.org/10.3750/AIP2007.37.2.06

Naeem, M. and Salam, A., 2010. Proximate composition of fresh water bighead carp, *Aristichthys nobilis*, in relation to body size and condition factor from Islamabad, Pakistan. *Afr. J. Biotechnol.,* **9**: 8687-8692.

Naeem, M. and Ishtiaq, A., 2011. Proximate composition of *Mystus bleekeri* in relation to body size and condition factor from Nala Daik, Sialkot, Pakistan. *Afr. J. Biotechnol.,* **10**: 10765-10763.

Nandeesha, M.C., Gangadhara, B., Manisser, J.K. and Venkataraman, L.V., 2001. Growth performance of two Indian major carps catla (*Catla catla*) and rohu (*Labeo rohita*) fed diets containing different levels of *Spirulina platensis*. *Biores. Tech.,* **80**: 117-120. https://doi.org/10.1016/S0960-8524(01)00085-2

Naylor, R.L., Goldburg, R.J., Primavera, J.H., Beveridge, M.C.M., Clay, J., Folke, C., Lubchenco, J., Mooney, H. and Troell, M., 2000. Effect of aquaculture on world fish supplies. *Nature,* **405**: 1017-1024. https://doi.org/10.1038/35016500

Razvi, M., 2006. *Lahore-absolutely fishy: Nutritional value*. The Review, Dawn, pp. 12-13.

Rudolf, K., 1971. *Fish inspection and quality*. Fishing News Books Ltd., London, UK.

Sahu, P.K., Jena, J.K., Das, P.C., Mondal, S. and Das, R., 2007. Production performance of *Labeo calbasu* (Hamilton), *Labeo rohita* (Hamilton) and *Cirrhinus mrigala* (Hamilton) with provision of fertilizers, feed and periphytic substrate as varied inputs. *Aquaculture,* **262**: 333-339. https://doi.org/10.1016/j.aquaculture.2006.11.016

Salim, M. and Sheri, A.N., 1999. Influence of protein sources, levels of protein and levels of feeding on growth of rohu (*Labeo rohita*) fingerlings under intensive system. *Pak. J. scient. Res.,* **51**: 85-89.

Salim, M., 2006. Role of fish as food to human nutrition. International Conference on Solving Problems of Freshwater Fish Farming in Pakistan, November 27-28, 2006. UVAS, Lahore, pp. 20.

Sandhu, G.S., 2005. *A textbook of fish and fisheries*. Dominant Publishers and Distributors, New Delhi, India, pp. 39-40.

Sarker, P.K., Chakraborty, S.C., Islam, M.N. and Chowdhary, B.B.P., 2000. Effects of animal and plant protein diets on growth of Indian major carp (*Labeo rohita* Ham.) fingerlings. *Bangladesh J. Fish. Res.,* **4**: 113-120.

SAS, 2009. *Statistical Software for Windows*, version 9.2 (TSIM0). SAS Institute, Cary, NC, USA.

Satpathy, B.B., Mukherjee, D. and Ray, A.K., 2003. Effect of dietary protein and lipid levels on growth, feed conversion and body composition in rohu, *Labeo rohita* (Ham.), fingerlings. *Aquacul. Nutr.,* **9**: 17-24. https://doi.org/10.1046/j.1365-2095.2003.00223.x

Seenappa, D. and Devaraj, K.V., 1995. Effect of different levels of protein, fat and carbohydrate on growth,

feed utilization and body carcass composition of fingerlings in *Catla catla* (Ham.). *Aquaculture,* **129**: 243-249. https://doi.org/10.1016/0044-8486(94)00273-Q

Sehgal, H.S. and Sehgal, G.K., 2002. Aquacultural and socio-economic aspects of processing carps into some value-added products. *Biores. Tech.,* **82**: 291-293. https://doi.org/10.1016/S0960-8524(01)00182-1

Siddiqui, T.Q. and Khan, M.A., 2009. Effects of dietary protein levels on growth, feed utilization, protein retention efficiency and body composition of young *Heteropneustes fossilis* (Bloch). *Fish Physiol. Biochem.,* **35**: 479-488. https://doi.org/10.1007/s10695-008-9273-7

Singh, P.K., Gaur, S.R. and Chari, M.S., 2006. Effect of varying protein levels on the growth of Indian major carp Rohu, *Labeo rohita* (Hamilton). *Int. J. zool. Res.,* **2**: 186 -191.

Tahir, M.Z.I., 2008. *Studies on partial replacement of fish meal with oil seed meals in the diet of major carps in semi-intensive culture system.* Ph.D. thesis. Department of Zoology, University of Agriculture, Faisalabad, Pakistan, pp. 174-178.

Sophin, P. and Preston, T.R., 2005. *Effect of processing pig manure in a bio-digester as fertilizer input for ponds growing fish in polyculture*. Livestock Research for Rural Development. Volume 13, Article #60. http://www.lrrd.org/lrrd13/6/pich136.htm

USDA, 2017. *Livestock and poultry: World markets and trade*. United States Department of Agriculture. Available at: https://apps.fas.usda.gov/psdonline/circulars/livestock_poultry.pdf (Accessed: 9 November 2017)

World Aquaculture, 2010. Contribution to food security. In: *FAO Fisheries and Aquaculture Technical Paper*, **500/1**: 120.

Yaqoob, M., Ali, M.R. and Mehmood, S., 2010. Comparison of growth performance of major and Chinese carps fed on floating and sinking pelleted supplementary feeds in ponds. *Pakistan J. Zool.,* **42**: 765-769.

Yildrim, O., 2008. Aquafeed industry in Turkey: Its aquafeed projections towards the year 2015. *Turk. J. Fish. aquat. Sci.,* **8**: 93-98.

Zeitler, M.H., Kirchgessner, M. and Schwarz, F.J., 1984. Effects of different protein and energy supply on carcass composition of carp (*Cyprinus carpio* L.). *Aquaculture,* **36**: 37- 48. https://doi.org/10.1016/0044-8486(84)90052-8

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Comparison of Traditional (Rice Polish) and Commercial Aquafeed on the Growth and Body Composition of Indian and Chinese Carps in Composite Culture System

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Authors' Contribution
NK designed the experiment. AK, SD, SN, SP, SB, HW and TY executed the experimental work. NK, FR, HA, KMA and KJI analyzed the data and wrote the article.

Key words
Chinese carps, Indian major carps, Rice polish, Commercial aqufeed, Composite culture.

ABSTRACT

This study was carried out to compare traditional (rice polish) and commercial aqua feed effects on different carps species at fish farms of Irshadullah Chattha in Alipur Chattha, district Gujranwala. Out of 4400 fingerlings, 800 *Labeo rohita*, 100 *Catla catla,* 100 *Cirrhinus mrigala* and 100 *Hypophthalmichthys molitrix* respectively stocked/acre in treatments T1 and T2 with two replicates each. Fish were fed with commercial feed (CP 30%) and traditional feed (rice polish) in T1 and T2, respectively. Overall growth performance of different fish species showed significant ($P<0.05$) differences among T1 and T2. FCR and SGR values of different fish species showed non-significant ($P<0.05$) differences among treatments. Average percentage of crude protein in *L. rohita* and *H. molitrix* remained non-significant ($P<0.05$) while *C. mrigala and C. catla* showed significant differences ($P<0.05$) between the treatments. Crude fat was significantly ($P<0.05$) different while, dry matter remained non-significant between the treatments. Whereas, ash contents of *L. rohita* showed non-significant ($P<0.05$) differences amongst other species. On other hand, *C. mrigala, C. catla* and *H. molitrix* showed significant ($P<0.05$) differences. Water quality parameters were recorded within optimum range. In conclusion commercial aqua feed showed significantly higher growth of carps than traditional rice polish.

INTRODUCTION

Fish has highest quality, digestible animal protein for human and an excellent protein substitute to red meat. Fish flesh is basically a source of all essential amino acid, minerals and vitamins, and unsaturated fats, especially omega-3 fatty acids (Sandhu, 2005; Razvi, 2006).

Aquaculture in Asia mainly consists of semi-intensive freshwater, earthen pond culture systems, in which fertilizers are used to enhance the natural productivity and fish is provided with supplementary feeds. With the passage of time, rapid growth has been observed in fish culture in South Asian Subcontinent, however, only 10% of its potentials have been exploited (De Silva *et al*., 1986). The sustenance of any composite culture system depends upon specific interactions among fish species (Sahu *et al*., 2007). Productivity can be increased by suitable selection of suitable fish species (Rehman *et al*., 2006). It is conventional to introduce exotic species with indigenous species to improve yield and to occupy all the ecological niches of pond. Therefore, to increase per unit production, polyculture concept has widely been accepted (Hossain *et al*., 2005).

Commercially manufactured fish diets are either floating or sinking feeds that could produce satisfactory growth, but feed preference depends on fish species. Most fish species prefer floating pellets because the supplementary feed in sinking pelleted form goes waste as it sinks in bottom and fish is unable to consume it (Albert and Tacon, 1990). Both floating and sinking feeds produce satisfactory but it is advantageous to offer floating feed that could be consumed from surface or column of water however, floating feeds are more expensive. Feeding requirements of fish can be observed directly by the farmer and adjust feeding rates accordingly. Rice bran or rice polish is one of the most commonly used agriculture by-

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product in fish culture because it is rich in proteins and carbohydrates with low level of fat and fiber. This has significantly positive effect on fish growth but less than maize gluten due to nutritive value (Ahmed *et al*., 2005; Tekchandani *et al*., 1999). The current study was planned to compare their effects on growth performance and body composition of cultured carps species in composite fish culture system.

MATERIALS AND METHODS

The experiment was carried out at Irshadullah Chattha fish farm in Alipur Chattha, district Gujranwala. Out of 4400 fingerlings, 800 *Labeo rohita*, 100 *Catla catla, Cirrhinus mrigala* and *Hypophthalmichthys molitrix* each respectively were stocked/acre in both T1 and T2 treatments in replicates. The study was conducted in semi-intensive composite culture system and experimental ponds were filled with tube well water upto 5 feet. Fish were fed with rice polish as traditional feed (T1) and pelleted feed of Oryza Organics was used as commercial feed having 30% CP (T2). Fish was fed @ 2% body weight twice a day for three months. Organic and inorganic manures (urea and DAP) were added to increase the natural feed in ponds.

At the time of stocking, body measurements (initial weight and length) of fish were measured individually. During the course of experiment, fish were sampled on monthly basis for total body weight and body length to assess the feeds effect. Other growth parameters like net weight gain, feed conversion ratio (FCR), and specific growth rate (SGR) were calculated according to the following formulae:

$$\text{NWG} = \text{Final body wt. (g)} - \text{Initial body wt. (g)}$$

$$\text{FCR} = \frac{\text{Feed intake (g)}}{\text{Wet wt. gain (g)}}$$

$$\text{SGR} = \frac{\ln(\text{Initial wt.}) - \ln(\text{Final wt.})}{\text{No. of days}} \times 100$$

Proximate analysis

The formulated feeds, feed ingredients and fish samples were analyzed for proximate analysis (dry matter, ash, crude protein (CP), crude fat (CF) and gross energy) in Fish Nutrition Laboratory, Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki according to AOAC (2006).

Water quality parameters

Water quality parameters of water were measured on fortnightly basis. The temperature and dissolved oxygen of the water were recorded with YSI -55/25 FT (dissolved oxygen meter). Total dissolved solids (TDS) and electrical conductivity (EC) were measured by conductivity meter (WTW Cond 330i). pH was measured by pH meter (Lutron pH-207). The salinity of water was measured by hand refractor meter (ATAGO, ES-421).

Statistical analysis

Results thus obtained were statistically analyzed using Minitab version 1.5 and analysis of variance ANOVA techniques.

RESULTS

The overall growth performance of different fish species showed significant differences between T1 and T2. However, better growth performance was observed in T1 as compared to T2 which may be due to better availability of protein or feed that could fulfill their body requirement. Maximum average body weight gain (870.90±204.05g) was observed in *C. catla* and minimum (537.30±72.60g) in *L. rohita* in T2. Maximum average body length increase (41.00±3.24cm) was observed in *Cirrhinus mrigala* and minimum (33.35±1.63cm) was observed in *L. rohita* of T2 treatment.

The average value of FCR of different fish species revealed non-significant differences ($P<0.05$) between the treatments. Different fish species among treatments had better FCR (1.86) in T1 and poor (3.39) in *C. catla* in T2. Similarly, the average value of SGR% of different fish species showed non-significant ($P<0.05$) differences between the treatments. Among the different fish species and treatments the better SGR% (1.62) was found in T1 of *H. molitrix* and same was poor (1.05) in *C. catla* in T1.

The average percentage of crude protein (CP) of *L. rohita* and *H. molitrix* showed non-significant ($P<0.05$) difference between treatments and *Cirrhinus mrigala and C. catla* showed significant difference between the treatments. Among different fish species and also between the treatments, maximum CP (28.66) was recorded in *L. rohita* in T1 and minimum (17.62) was recorded in *Cirrhinus mrigala* in T2. Crude fat (CF) showed significant ($P<0.05$) difference between treatments. Maximum (5.46) CF was recorded in *C. catla* among different fish species and also between the treatments and minimum (1.30) was recorded in *Cirrhinus mrigala.* Dry matter remained non-significant ($P<0.05$) between the treatments. Maximum (22.90) dry matter was recorded in *L. rohita* in T1 and minimum (19.45) was in *Cirrhinus mrigala* in T1. Ash contents of *L. rohita* showed non-significant difference between the treatments while *Cirrhinus mrigala, C. catla* and *H. molitrix* showed significant difference between the

treatments. Maximum (2.85) ash contents were recorded in *L. rohita* in T1 and minimum (1.67) was recorded in *Cirrhinus mrigala* in T2 among different fish species and also between the treatments.

Water quality parameters (temperature, dissolved oxygen (DO), pH, alkalinity, phosphate, nitrate and hardness) were recorded within the optimum range.

DISCUSSION

The goal of aquaculture is to get higher production in terms of fish growth in addition to qualitative meat. During present study, overall growth performance of different fish species showed significant ($P<0.05$) differences between T1 and T2 and better growth performance was observed in T1 as compared to T2 which may be due to the better availability of protein or feed which could fulfill their body requirement. Maximum average body weight gain (870.90±204.05g) was observed in *C. catla* in T1 and minimum (537.30±72.60g) in *L. rohita* was in T2. Maximum average body length increase (41.00±3.24cm) was observed in *Cirrhinus mrigala* in T1 and minimum (33.35±1.63cm) in *L. rohita* was in T2. These results match with the results of Ashraf *et al*. (2008) and Abid and Ahmed (2009a, b) who compared artificially composed diets and naturally composed diets on growth of fingerlings of *L. rohita*. Similarly, Ahmed *et al*. (2012) observed significant ($P<0.05$) difference in average wet body weight and gross fish weight, *L. rohita* when fed with commercial diets and traditional diet (rice bran).

Feed conversion ratio is the preeminent factor to measure the rate of feed accepted by fish and ultimate fish growth performance (Inayat and Salim, 2005). The average value of FCR of different fish species showed non-significant ($P<0.05$) difference between the treatments. Among different fish species and also between the treatments better FCR (1.86) was in T1 and same was poor (3.39) in *C. catla* in T2. Similarly, Ahmed *et al*. (2012) observed significant ($P<0.05$) difference in FCR of *L. rohita* when fed with commercial diets than traditional diet (rice bran). Iqbal *et al*. (2015) recorded significant differences in FCR when *L. rohita* fed with different feed ingredients. Specific growth rate (SGR) of different fish species showed non-significant ($P<0.05$) difference among traditional and commercial diets. Among the different fish species and also between the treatments, better SGR (1.62) was in *H. molitrix* in T1 and same was poor (1.05) in *C. catla* in T1. Similar minor variation of SGR values in carps were also reported by Azim *et al*. (2001) and Sahu *et al*. (2007). Abid and Ahmed (2009a) also recorded non-significant ($P<0.05$) differences in SGR between treatments in which feed with different crude protein level was given to fish.

Average percentage of crude protein (CP) of *L. rohita* and *H. molitrix* showed non-significant ($P<0.05$) difference, and *Cirrhinus mrigala and C. catla* showed significant difference between the treatments. Crude fat (CF) showed significant ($P<0.05$) difference while dry matter remained non-significant ($P<0.05$) between the treatments. Ash contents of *L. rohita* showed non-significant difference between treatments while *Cirrhinus mrigala, C. catla* and *H. molitrix* showed significant difference between treatments. Khan *et al*. (2012) studied that when major carp were fed with different crude protein levels artificial feed, they showed non-significant differences in body composition which indicated that protein deposition in fish does not reveal the diet protein. Similarly, Hasan *et al*. (2005) could not find difference in body composition when common carp fed with plant based diet compared to the fishmeal based diet.

Water quality parameters (temperature, pH and DO) have significant effect on the growth of fish (Ali *et al*., 2000; Ahmad *et al*., 2008; Noor *et al*., 2010). Water quality parameters were recorded with minor variation between the treatments in the present study and these values were in accordance with the optimum range of cyprinid (Ali *et al*., 2000; Abid and Ahmed, 2009a, b).

CONCLUSION

Growth performance and proximate analysis of fish on the commercial feed showed better results than traditional feed (rice polish) which might be due to the availability of best combination of essential nutrients and formulation. These finding suggested that commercial feed should be used for better fish growth and per acre fish production.

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REFERENCES

Abbas, S., Ahmed, I., Salim, M. and Rehman, K., 2010a. Comparative effects of fertilization and supplementary feed on growth performance of three fish species. *Int. J. Agric. Biol*., **12**: 153-168.

Abbas, S., Ahmed, I., Salim, M. and Rehman, K.,

2010b. Comparative effects of fertilization and supplementary feed on growth performance of three fish species. *Int. J. Agric. Biol*., **12**: 276-280.

Abbas, S., Ashraf, M. and Ahmed, I., 2014. Effect of fertilization and supplementary feeding on growth performance of *Labeo rohita*, *Catla catla* and *cyprinus carpio*. *Int. J. Agric. Biol*., **24**: 142-148.

Abdel-Hakim, N.F., El-Gendymo, G.A.H. and Elkomy, H.M., 2014. *Influence of artificial feeding and fertilizer types on growth and productivity of four fish species stocked in polyculture in earthen ponds*. In: 4th Conference of Central Laboratory for Aquaculture Research, pp. 133-152.

Abid, M. and Ahmed, M.S., 2009a. Growth response of *Labeo rohita* fingerlings fed with different feeding regimes under intensive rearing. *J. Anim. Pl. Sci*., **19**: 45-49.

Abid, M. and Ahmed, M.S., 2009b. Efficacy of feeding frequency on growth and survival of *Labeo rohita* (Ham.) fingerlings under intensive rearing. *J. Anim. Pl. Sci*., **19**: 111-119.

Ahmed, I., Abbas, K. and Rehman, M.H., 2005. Growth performance of major carp in semi intensive ponds supplemented with rice polishing. *Pak. Vet. J.,* **25**: 59-62.

Ahmad, N., Ahmed, I., Saleem, M. and Ashraf, M., 2008. Effect of different levels of mineral phosphorus with and without nitrogen on the dry weight of planktonic biomass, increase in fish production and biomass conversion efficiencies. *Pak. J. Agric. Sci*., **45**: 122-128.

Ahmed, M.S., Shafiq, K. and Kiani, M.S., 2012. Growth performance of major carp, *Labeo rohita* fingerlings on commercial feeds. *J. Anim. Pl. Sci.,* **22**: 93-96.

Albert, G.J. and Tacon, C., 1990. *Standard methods for the nutrition and feeding of farmed fish and shrimp*. Argent Laboratories Press, Redmond, WA.

Ali, M., Salam, A., Azeem, A., Shafiq, M. and Khan, B.A., 2000. Studies on the effect of seasonal variations on physical and chemical characteristics of mixed water from River Ravi and Chenab at union site in Pakistan. *J. Res. Sci.*, **11**: 11-17.

AOAC, 2006. *Official methods and analysis*. Method Number 1125, 17th Edition. Association of Official Analytical Chemists.

Ashraf, M., Ayub, M. and Rauf, A., 2008. Effect of different feed ingredients and low temperature on diet acceptability, growth and survival of Mrigal, *Cirrhinus mrigala* fingerlings. *Pakistan J. Zool*., **40**: 83-90.

Azim, M.E., Sahib, M.A., van Dam, A.A., Beveridge, M.C.M. and Verdegem, M.C.J., 2001. Optimization of stocking ratios of two Indian major carps, *Labeo rohita* (Ham.) and *Catla catla* (Ham.) in a periphyton based aquaculture system. *Aquaculture*, **203**: 33-49. https://doi.org/10.1016/S0044-8486(01)00602-0

Azim, M.E., Wahab, M.A., van Dam, A.A., Beveridge, M.C.M., Milstein, A. and Verdegem, M.C.J., 2001. Optimization of fertilization rate for maximizing periphyton production on artificial substrates and their implications for periphyton based aquaculture. *Aquacult. Res*., **32**: 749-760. https://doi.org/10.1046/j.1365-2109.2001.00613.x

Bakhtiyar, Y., Langer, S., Karlopia, S.K. and Ahmed, I., 2011. Growth, survival and proximate body composition of *Labeo rohita* larvae fed artificial food and natural food organisms under laboratory condition. *Int. J. Fish. Aquacul.,* **3**: 114-117.

Britz, P.J., Hecht, T. and Mangold, S., 1997. Effect of water temperature on growth, feed consumption and nutritional indices of Haliotusmidae fed on formulated diet. *Aquaculture*, **152**: 191-203. https://doi.org/10.1016/S0044-8486(97)00002-1

Burel, C., Ruyet, P.L., Gaumet, F., Roux, A.L., Sever, A. and Boeuf, D., 1996. Effects of temperature on growth and metabolism in juvenile turbot. *J. Fish Biol*., **49**: 678-692. https://doi.org/10.1111/j.1095-8649.1996.tb00064.x

Chakrabarti, N.M., 1998. *Biology, culture and production of Indian major carps*. Fisheries Scientist in Indian Council of Agricultural Research, pp. 175.

Chakrabarty, D., Das, S.K. and Das, M.K., 2008. Low cost fish fed for aquarium fish: A test case using earthworms. *Adv. Environ. Biol*., **1**: 96-100.

Chatta, A.M., Khan, A.M., Khan, M.N. and Ayub, M., 2015. A study on growth performance and survival of Indus golden Mahseer (*Tor macrolepis*) with Indian major carps in semi-intensive polyculture system. *J. Anim. Pl. Sci.,* **25**: 561-566.

Chughtai, M.I., Mahmood, K. and Awan, A.R., 2015. Growth performance of carp species fed on salt-tolerant roughages and formulated feed in brackish water under polyculture system. *Pakistan J. Zool*., **47**: 775-781.

De Silva, S.S. and Anderson, T.A., 1995. *Fish nutrition in aquaculture*. Chapman and Hall Publications, London, UK, pp. 36-37.

De Silva, S.S., Gunasekera, R.M. and Keembiyahetty, C., 1986. Optimum ratio and feeding frequency in *Oreochromis niloticus* young. In: *Conference of the Asian Fisheries Society*, May 26-31, 1986 (eds. J.L. Maclean, L.B. Dizon and Hosillos, L.V.). Manila, Philippines, pp. 727-732.

Diana, J.S., Lin, C.K. and Jaiyen, K., 1994. Supplemental feeding of tilapia in fertilized ponds. *J. World Aquacul. Sci*., **25**: 497-506. https://doi.org/10.1111/j.1749-7345.1994.tb00818.x

Hasan, M.R., Macintosh, D.J. and Jauncey, K., 2005. Evaluation of some plant ingredients as dietary protein sources for common carp (*Cyprinus carpio* L.) fry. *Aquaculture,* **151**:55-70.

Hossain, M.A., Sultana, N., Hossain, M.G. and Hussain, M.G., 2005. Optimum dietary protein requirement of Gulsha *Mystuscavasius* (Hamilton) fingerlings. *J. Aquacul*., **20**: 237-233.

Inayat, L. and Salim, M., 2005. Feed conversion ratio of major carp, *Cirrhinus mrigala* fingerlings fed on soybean meal, maize and maize gluten. *Pak. Vet. J.,* **25**: 13-16.

Iqbal, K.J., Ashraf, M., Javid, A., Qureshi, N.A., Khan, N., Azmat, H., Altaf, M., Majeed, H., Haider, M.S., Irfan, W. and Zahoor, M.Y., 2015. Effect of different feed ingredients on growth, sensory attributes and body composition of *Labeo rohita. J. Anim. Pl. Sci.,* **25**(3 Supp. 2): 514-518.

Jena, J.K., Ayyappan, S., Aravindakshan, P.K. and Muduli, H.K., 2001. Comparative evaluation of growth, survival and production of carp species at different stocking densities under polyculture. *Ind. J. Fish.,* **48**: 17-25.

Khan, N., Ashraf, M., Qureshi, N.A., Sarker, P.K., Vandenberg, G.W. and Rasool, F., 2012. Effect of similar feeding regime on growth and body composition of Indian major carps (*Catla catla, Cirrhinus mrigala, Labeo rohita*) under mono and polyculture. *Afr. J. Biotechnol.,* **11**: 10280-10290.

Lakshmanan, M.A.V., Sukumaran, K.K., Murty, D.S., Chakraborty, D.P. and Philipose, M.T., 1971. Preliminary observations on intensive fish farming in freshwater ponds by the composite culture of Indian and exotic species. *Inland Fish. Sci. India,* **2**: 1-21.

Noor, E.L., Deen, A.I.E. and Mona, S.Z., 2010. Impact of climatic changes (oxygen and temperature) on growth and survival rate of Nile tilapia (*Oreochromis niloticus*). *Report and Opinion,* **2**: 192-195.

Rahman, M.A., Hossain, M.K., Azad, M.A.K., Kamruzzzaman, M., Rashid, H.A., Rahman, M.M. and Hasan, K.M.M., 2006. Culture potential of Thai Sharpunti, *Barbodesgonionotus* (Bleeker) with major carps in seasonal ponds. *Pak. J. biol. Sci.,* **9**: 1891-1897. https://doi.org/10.3923/pjbs.2006.1891.1897

Rahman, M.M., Verdegem, M.C.J., Nagelkerke, L.A.J., Wahab, M.A., Milstein, A. and Verreth, J.A.J., 2006. Growth, production and food preference of rohu (*Labeo rohita*) (H.) in monoculture and in polyculture with common carp *(Cyprinus carpio*) (L.) under fed and non-fed ponds. *Aquaculture,* **257**: 359-372. https://doi.org/10.1016/j.aquaculture.2006.03.020

Razvi, M., 2006. *Lahore-absolutely fishy: Nutritional value*. The Review, Dawn, pp. 12-13.

Sahu, P.K., Jena, J.K., Das, P.C., Mondal, S. and Das, R., 2007. Production performance of *Labeo calbasu* (Hamilton) in polyculture with three Indian major carps *Catla catla* (Hamilton), *Labeo rohita* (Hamilton) and *Cirrhinus mrigala* (Hamilton) with provision of fertilizers, feed and periphytic substrate as varied inputs. *Aquaculture*, **262**: 333-339. https://doi.org/10.1016/j.aquaculture.2006.11.016

Sana-Ullah, Hasan, Z., Zuberi, A., Younus, N. and Rauf, S., 2014. Comparative study on body composition of two Chinese carps, common carp (*Cyprinus carpio*) and silver carp (*Hypophthalmichthys molitrix*). *Glob. Vet*., **13**: 867-876.

Sandhu, G.S., 2005. *A textbook of fish and fisheries dominant publishers and distributors*. New Delhi, pp. 39-40.

Tekchandani, H.K., Dias, F.F. and Mehta, D., 1999. Maize wet milling co-products as feed additives: Perspectives and opportunities. *J. Sci. Indust. Res.,* **58**: 83-88.

Watanabe, T., Verakunpiriya, V., Watanabe, K., Kiron, V. and Satoh, S., 1997. Feeding in rainbow trout with non-fish meal diets. *J. Fish. Sci.,* **63**: 258-266. https://doi.org/10.2331/fishsci.63.258

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Moringa Meal as an Alternate Protein Source for *Labeo rohita* Fingerlings: Effects on Growth and Body Composition

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Authors' Contribution
MI conducted the experiment. NK supervised the experiment. CJS, SAMM, FR and KJI helped in experiment design, finalized the experiment and writing the paper. SD, DM, AH and AA helped in smaple collection and analysis. HA and MF helped in paper write up.

Key words
***Moringa oleifera*, Rohu, Liver and intestine histology, Whole body proximate composition.**

ABSTRACT

The present study was designed to assess the effects of moringa meal as an alternate protein source on the growth, body composition, liver and gut health of *Labeo rohita* fingerlings. Study was carried out in 10 glass aquariums of 60 L water capacity, having randomly stocked 20 fingerlings (average biomass =18.62g) per aquarium for three months. Four experimental diets having 30% crude proteins (CP) designated as whole plant meal (T_1), root meal (T_2), stem meal (T_3) and leaf meal (T_4) of *Moringa oleifera*. Control group was without moringa meal (T_0) contained 30% CP only. Fish were fed twice a day at the rate of 3% of their wet body weight. Significantly highest ($p<0.05$) weight gain was observed in T_1 (111.6±24.56g) and lowest in T_4 (43.6±8.20g). Similarly, maximum % age weight gain was recorded in T_1 (35.17±7.09%) and lowest in T_2 (20.18±3.7%) and T_4 (20.68±1.65%). Feed conversion ratio (FCR) showed statistically non-significant differences among treatments.

INTRODUCTION

Fisheries industry is playing imperative role to meet the nutritional requirements of growing human population and its production is rising around 10% annually (FAO, 1997). Fish meal is widely used as best protein source in fish feed. However, high price and unsteady supply have diverted the attention of fish culturist to search for alternate sources of protein for fish. In intensive aquaculture, protein is the most vital part of fish meal, representing over half of total feed costs (Nwanna *et al.*, 2008). In aquaculture, many plants and their parts are used in fish feed for stimulating appetite, promoting growth, boosting immunity, curing diseases, preventing infection and reducing stress to grow healthy fish (Shalaby, 2004). Significant accentuation was given on the utilization of plant proteins including soybean, groundnut, cottonseed and rapeseed meal (Sadiku and Jauncey, 1995).

Moringa oleifera commonly known as 'Sohanjna' of family Moringaceae is considered to be highly nutritious plant. The different parts (leaves, stem and roots) of *M. oleifera* contained rich amount of ascorbic acid, carotene, other amino acids and nutrient components are used as vegetables for human consumption (Makkar and Becker, 1996). Moringa has the potential to be used in fish feed formulations due to its local availability and relatively good nutritional value (Anwar *et al.*, 2007).

Studies has shown the extensive use of *M. oleifera* leaves meal as protein alternate in the diet of *Labeo rohita* and *Clarias gariepinus* fingerlings up to inclusion level of 10% (Bello *et al.*, 2013; Arsalan *et al.*, 2016; Ezekiel *et al.*, 2016; Mehdi *et al.*, 2016). In addition, *M. oleifera* seed meal has also been successfully used as protein source in the diet of Nile tilapia (*Oreochromis niloticus*) (Hashem *et al.*, 2017). Therefore, the current study was conducted to assess the effects of different parts of *M. oleifera* (whole plant, root, stem, leaves) on growth and body composition of *Labeo rohita* to maximize the cost effective production of fish.

METHODOLOGY

Study area, experimental design and fish species

The current study was conducted at Fish Hatchery of the Department of Fisheries and Aquaculture, University of Veterinary and Animal Sciences, Ravi Campus, Pattoki,

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Punjab, Pakistan. The *Labeo rohita* fingerlings having average body weight of 18.62 g were used as experimental fish species and captured from fish ponds at Ravi Campus, Pattoki. Ten aquariums of 60 L water capacity were used for the experimental trial and 20 fish were stocked randomly in each aquarium. Before the initiation of feeding trial, net body weight (g) and total body length (cm) of *L. rohita* fingerlings was carefully measured and recorded.

Plant collection, fish feed formulation and processing

Fish feed formulation and its further processing was carried out according to procedure described by AOAC (2006). The different parts of *M. oleifera* (whole plant, root, stem and leaves) were collected, washed properly and dried under shade at room temperature. All dried collected parts of moringa plant were ground separately into coarse fine powder using electrical grinder and then packed for feed formulation. Five different types of experimental diets designated as T_0, T_1, T_2, T_3, T_4, having 30% crude protein (CP) were formulated using locally available feed ingredients (Table I). The fish were fed twice a day at 9:00 AM and 4:00 PM at feeding rate of 3% of the fish wet body weight. Re-adjustment of feed allowance was carried out after every fortnight (Habib *et al*., 2018).

Measurement of growth parameters of fish

Initial body weight of *L. rohita* fingerlings was measured before stocking. At the end of feeding experiment, all growth parameters such as final weight, net weight gain, weight gain%, specific growth rate (SGR %) and feed conversion ratio (FCR) were calculated according to the formulae:

$$\text{NWG} = \text{Avg. final wt. (g)} - \text{Avg. initial wt. (g)}$$

$$\text{Weight Gain(\%)} = \frac{\text{Final weigt} - \text{Initial weight}}{\text{Initial weight}} \times 100$$

$$\text{SGR} = \frac{\ln \text{Final body wt.} - \ln \text{Initial body wt.}}{\text{Total No. of days}} \times 100$$

$$\text{FCR} = \frac{\text{Feed given (g)}}{\text{Wet weight gain (g)}}$$

Proximate analysis

The proximate body composition of *L. rohita* including crude protein, dry matter, moisture, ash and fat contents of 5 fish from each replicate were determined at the end of experiment. The proximate analysis of fish diet was also performed. Proximate analysis was performed following the procedures established by AOAC (2006).

Table I.- Fish feed formulation using different parts of *M. oleifera.*

Ingredients	CP (%)	Inclusion (%)	Experimental feed CP (%)
Control (T_0)			
Fish meal	52.72	18	9.5
Guar meal	27.9	10	2.8
Soybean meal	43.09	16	6.9
Rice polish	15.1	23	3.5
Wheat bran meal	16.4	16	2.6
Sun flower plant meal	30.23	16	4.8
Vitamins	-	1	-
Total		100	30.1
Whole plant moringa meal (T_1)			
Fish meal	52.72	18	9.5
Guar meal	27.9	10	2.8
Soybean meal	43.09	14	6
Whole plant Moringa meal	18	14	2.7
Rice polish	15.1	14	2.1
Wheat bran meal	16.4	14	2.3
Sun flower plant meal	30.23	15	2.6
Vitamins	-	1	--
Total		100	30
Moringa root meal (T_2)			
Fish meal	52.72	18	9.5
Guar meal	27.9	20	5.6
Soyabean meal	43.09	16	6.9
Moringa root meal	15.5	16	2
Rice polish	15.1	11	1.7
Wheat bran meal	16.4	10	1.6
Sunflower plant meal	30.23	10	3.2
Vitamins	-	1	--
Total		100	30.5
Moringa stem meal (T_3)			
Fish meal	52.72	18	9.5
Guar meal	27.9	10	2.8
Soya bean meal	43.09	20	8.6
Moringa Stem meal	14.43	20	2.9
Rice polish	15.1	10	1.5
Wheat meal	16.4	11	1.8
Sun flower meal	30.23	10	3.2
Vitamins	-	1	--
Total		100	30.3
Moringa leaves meal (T_4)			
Fish meal	52.72	18	9.5
Guar meal	27.9	10	2.8
Soya bean meal	43.09	14	8
Moringa leaf meal	21.65	15	3.2
Rice polish	15.1	14	2.1
Wheat bran meal	16.4	14	2.3
Sun plant meal	30.23	14	4.2
Vitamins	-	1	--
Total		100	30.1

Physico-chemical parameters of water

Temperature, pH, salinity, total dissolved solids (TDS) and dissolved oxygen (DO) were recorded as the physico-chemical parameters of water in the fish aquarium on daily basis using multi meter and dissolved oxygen meter. The amount of nitrates was measured using HANNA-Nitrate-Test-Kit HI3874 on fortnightly basis.

Statistical analysis

The obtained data of body composition, growth and physico-chemical parameters was analyzed by applying one-way analysis of variance (ANOVA) and significant differences among all treatments were compared using Duncan's multiple range test (DMR) using SAS version 9.1. Significance was tested at 5% level ($p < 0.05$).

RESULTS

Statistically significant ($P \leq 0.05$) association in growth parameters were recorded at every fortnight sampling in all treatment groups is presented in Table II. The highest weight gain was observed in T_1 (111.6±24.56g) under the influence of artificial feed in the mixed form of *M. oliefera* whole plant meal. The total %age weight gain for Rohu was higher in T_1 (35.17±7.09%) and lowest was observed in T_2 (20.18±3.78%). The FCR values showed non-significant differences and significant association was recorded in SGR% values of all treatment groups.

Table II.- Growth performance of *Labeo rohita* under various moringa meal diets.

Treatment	Initial weight (g)	Final weight (g)	Gain in weight (g)	%weight gain (g)	FCR	SGR %
T_0	339.1 ± 1.34[a]	420.1 ± 16.46[a]	80.97 ± 17.80[ab]	23.89 ± 5.35[ab]	2.38 ± 0.43[a]	0.28 ± 0.06[ab]
T_1	316.5 ± 6.36[a]	428.4 ± 30.50[a]	111.6 ± 24.56[a]	35.17 ± 7.09[a]	1.75 ± 0.26[a]	0.41 ± 0.07[a]
T_2	341.1 ± 12.72[a]	409.6 ± 2.39[a]	68.60 ± 10.33[b]	20.18 ± 3.78[b]	2.72 ± 0.42[a]	0.24 ± 0.03[a]
T_3	336.6 ± 14.99[a]	407.8 ± 1.62[a]	71.22 ± 13.36[ab]	21.27 ± 4.92[b]	2.62 ± 0.50[a]	0.25 ± 0.04[b]
T_4	335.0 ± 7.1[a]	404.2 ± 2.90[a]	43.6 ± 8.20[c]	20.68 ± 1.65[b]	2.63 ± 0.17[a]	0.25 ± 0.01[b]

T_0, control; T_1, whole plant meal; T_2, root meal; T_3, stem meal; T_4, leaf meal.

The significant parameters for determining body composition of *L. rohita* were crude protein, dry matter, fat, moisture and ash contents (Table III). The maximum value of crude protein contents was recorded in T_4 (66.1±0.77%) and lowest in T_2 (57.4±0.67%). T4 showed maximum value of fat contents (14.8±0.28%) and lowest fat contents were observed in T_2 (4.90±0.14%). The maximum and minimum value of ash contents was (14.9±0.14%) and (10.5±0.70%) reported in T_3 and T_4 respectively. Again T4 exhibited the higher amount of moisture (10.7±1.06%). However, high proportion of dry matter was recorded in T_1 (96.75±1.06%) and lower in T_4 (88.9±0.57%).

Table III.- Proximate analysis of *Labeo rohita* under various moringa meal diets.

Parameters	Control	T1	T2	T3	T4
Dry matter %)	94.1 ± 0.84[b]	96.75 ± 1.06[a]	96.0 ± 0.70[ab]	94.5 ± 0.70[b]	88.9 ± 0.57[c]
Moisture (%)	5.90 ± 0.84[b]	2.75 ± 0.34[d]	3.60 ± 0.14[dc]	4.75 ± 0.35b[c]	10.7 ± 1.06[a]
Ash (%)	12.4 ± 0.60[b]	12.2 ± 0.35[b]	10.7 ± 0.42[c]	14.9 ± 0.14[a]	10.5 ± 0.70[c]
Crude fat (%)	5.70 ± 0.05[d]	6.75 ± 0.35[c]	4.90 ± 0.14[e]	7.70 ± 0.42[b]	14.8 ± 0.28[a]
Crude protein (%)	59.4 ± 0.79[c]	64.8 ± 1.14[ab]	57.4 ± 0.67[c]	62.9 ± 0.63[b]	66.1 ± 0.77[a]

T_0, control; T_1, whole plant meal; T_2, root meal; T_3, stem meal; T_4, leaf meal.

Table IV.- Physico-chemical parameters of water in various treatments of moringa meal diets.

Parameter	Control	T1	T2	T3	T4
pH (mg/l)	8.41 ± 0.03[a]	8.33 ± 0.08[a]	8.39 ± 0.05[a]	8.39 ± 0.04[a]	8.41 ± 0.03[a]
EC (mScm^{-1})	2178.04 ± 23.6[a]	2175 ± 28.3[a]	2177.1 ± 27.80[a]	2169.3 ± 24.7[a]	2178.4 ± 23.6[a]
Salinity (mg/l)	1.0 ± 0.00[a]	1.0 ± 0.00[a]	1.0 ± 0.00[a]	1.0 ± 0.00[a]	1.0 ± 0.00[a]
DO (mg/l)	5.42 ± 0.02[a]	5.43 ± 0.03[a]	5.42 ± 0.02[a]	5.42 ± 0.02[a]	5.42 ± 0.02[a]
Temp. (ºC)	19.9 ± 4.87[a]	19.9 ± 4.85[a]	19.82 ± 4.95[a]	19.96 ± 4.88[a]	19.9 ± 4.87[a]
TDS (mg/l)	1242.9 ± 15.3[a]	1241.8 ± 9.9[a]	1247.1 ± 4.98[a]	1245.8 ± 6.73[a]	1242.9 ± 15.3[a]

T_0, control; T_1, whole plant meal; T_2, root meal; T_3, stem meal; T_4, leaf meal.

Physico-chemical parameters were recorded on daily basis throughout the experimental period of 12 weeks and the mean values are presented in Table IV. Temperature remained constant in all test groups. Mean values of pH were recorded for T_0, T_1, T_2, T_3 and T_4 as 8.41±0.03, 8.33±0.08, 8.39±0.05, 8.39±0.04, 8.41±0.03, respectively. TDS values were showing non-significant difference for T_0, T_1, T_2, T_3 and T_4 treatments with the mean values of 1242.9±15.3, 1241.8±9.9, 1247.1±4.98, 1245.8±6.73 and 1242.9±15.3 (mg/l), respectively. Salinity remains

non-significant during whole experimental period with the constant value of 1.00±0.00 mg/l in all treatments. Electrical conductivity and DO values were observed non-significant in all treatments.

DISCUSSION

Fish is the basic source of high-quality protein globally about one billion people gets their animal protein from fish due to its healthier and high nutritional characteristics (FAO, 2000). However nowadays, major issues that confronting fish culturist are elevated price and shortage of fish feed. The elevated rate and variable superiority with the uncertain accessibility of fish meal have created the need to search for alternate protein sources. In recent times, fish culturist progressively deviating their careful consideration towards moringa (*Moringa oleifera*) meal to increase fish growth and production within the shortest possible time.

Previously *M. oleifera* leaves meal has been extensively applied as best alternate protein source in the diet of *Labeo rohita* and *Clarias gariepinus* fingerlings up to addition level of 10% (Bello *et al*., 2013; Ezekiel *et al*., 2016; Arsalan *et al*., 2016; Mehdi *et al*., 2016). Further, Yuangsoi (2012) studied the effect of *M. oleifera* leaves meal (MOLM) on the growth performance and digestibility of experimental fish (fancy carp). Yuangsoi (2012) demonstrated that *M. oleifera* leaves meal could be replaced not up to 200 gram per kg of plant protein in soybean to increase fish production. Moringa leaf meal results of current study are in line with those of Yuangsoi, (2012), Bello *et al*. (2013), Ezekiel *et al*. (2016), Arsalan *et al*. (2016) and Mehdi *et al*. (2016).

Furthermore, the effect and nutritional value of leaves of *Leucaena leucocephala* in the fish diet of the fingerings of *Clarias gariepinus* at inclusion level of 0%, 5%, 10%, 15% and 20% for two months was demonstrated by Tiamiyu (2015). Results showed that 20% of Leucaena leaf meal gave better results of weight gain, specific growth rate and feed conversion ratio. In contrast, moringa leaf meal revealed its best results at inclusion level of 10% in all studies that have been conducted so far for evaluating its effect on different fingerlings growth.

In addition, *M. oleifera* seed meal (MSM) has also been studied by Hashem *et al*. (2017) as plant-based protein source in the feed of Nile tilapia (*Oreochromis niloticus*) at the graded level of 0, 4, 8 and 12% for 83 days. Hashem *et al*. (2017) found excellent growth of Nile tilapia at 8 and 12 % MSM inclusion level. Current study results of Moringa root meal at inclusion level of 16% showed best impact on *L. rohita* growth exhibiting agreement with the results of Hashem *et al*. (2017). However, Moringa root meal of present study was found less effective as compared to moringa meal of whole plant and stem meal.

Feed conversion ratio (FCR) is the most excellent parameter to determine the acceptability of diet and its performance on fish growth (Mukherjee *et al*., 2011). Further, Ebrahim (2007) described that feed digestibility play imperative role in decreasing feed conversion ratio by efficient feed consumption. Feed conversion ratio in current research exhibited non-significant difference among the treatments and better FCR was recorded in T_1 (1.75±0.26). Significant difference was observed in SGR% in all treatments with highest SGR% in T_1 (0.41±0.07). The higher FCR value is an indication of some anti-nutrients presence in the *Moringa meal* (Richter *et al*., 2003). While low FCR value exhibit efficient fish feed utilization. It is a remarkable sign for the use of moringa meal in fish industry.

In present study, highest value of crude protein (66.1±0.77), moisture (10.7±1.06) and fat content (14.8±0.28) was recorded in moringa leaf meal treatment (T_4) at inclusion level of 15%. While lowest value for crude protein and fat contents was found in moringa root meal treatment (T_2) at 16% addition level. Similarly Mehdi *et al*. (2016) also observed the higher proportion of moisture content (10.12 %) and crude protein (63.98 %) by applying moringa leaf meal in the diet of *L. rohita*. Present study is showing agreement with the results of Olaniyi *et al*. (2013), Madalla *et al*. (2013) and Mehdi *et al*. (2016). However current findings are showing contradiction with that of Arsalan *et al*. (2016). They reported maximum value of crude protein (64%) and fat content at 10% addition level of MOL meal and lowest in fish feeding 40% of MOL meal. Arsalan *et al*. (2016) results are supporting the study of Thiam *et al*. (2015) and Ganzon-Naret (2014) in sea bass.

The corresponding proportion of moisture contents in control T_0, T_1, T_2, T_3 and T_4 was 5.90±0.84%, 2.75±0.34%, 3.60±0.14%, 4.75±0.35% and 10.7±1.06%. Present findings are supporting the observations of Madalla *et al*. (2013). They reported that moisture and ash contents of fish increases with the rise of MOL diet. Similarly, Olaniyi *et al*. (2013) described lowest value of moisture contents in *Clarias gariepinus* feeding 10% inclusion of moringa diet. However, present findings contradict with that of Arsalan *et al*. (2016) findings, who recorded higher proportion of moisture content in 40% MOL diet inclusion. Present findings of Moringa root meal at inclusion level of 16% exhibiting parallel line with the findings of Hashem *et al*. (2017). They reported highest crude protein (63.00%) and ash contents (10.12%) at maximum graded level of 12%. Similarly in present findings, peak value of crude protein and ash content recorded in moringa whole plant meal

treatment were 64.8% and 12.2%, respectively.

The physico-chemical parameters showed non-significant deviation and were in favorable range throughout the experimental period as reported from the study of Amisah *et al*. (2009) and Kumar *et al*. (2011). There was observed 100% survival in all treatment groups indicating favorable condition of aquarium water. Hlophe and Moyo (2014) also observed 100% survival rate by replacing fish meal with moringa meal and kikuyu grass in Tilapia.

Histological analysis of experimental *L. rohita* of present study treated with whole plant meal (T1) exhibited increase in enterocyte height and number of mucous cells and change in the size of a nucleus effecting liver metabolism leading to picnosis, kariolysis of the nucleus is in line with results of Raskovic *et al*. (2011). Slight damage in the periphery of intestinal wall and vaculation in few hepatocytes of *L. rohita* was observed feeding on 16% inclusion level of moringa root meal (T_2) showing agreement with the results of Markovic *et al*. (2012). However, decreased fish growth and severe cellular swellings in the liver of *Oreochrimis mossambicus* was observed with the increase of moringa meal as reported by Rapatsa, (2014). While Nwankpa (2017) findings exhibited contradiction with other ones. He found no change in liver of *Oreochromis niloticus* feeding at inclusion levels of 5, 10 and 15% of Moringa leaf meal. The severe damages in liver of fish might be due to hepato-protective factor absence in various plants but inherent in *M. Oleifera* leaves as it has been evaluated by Fakurazi *et al*. (2008).

CONCLUSION

In current study, four fish diets (whole plant, root, stem and leaf meals) that were applied for evaluating their effect on the body composition, growth and histological parameters of *Labeo rohita*, among these whole plant moringa meal revealed highest effectiveness, followed by the stem meal, root and leaf meal. The remarkable point of present research is that whole plant and stem meal of *Moringa oleifera* have not been tested so far for evaluating their protein source for fish meal of *Labeo rohita*. Another significant point is that whole plant moringa meal in current findings advocated its strong potential as excellent protein source for fish meal of *Labeo rohita*.

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Statement of conflict of interest

The authors declare that there is no conflict of interest regarding publication of this article

REFERENCES

Amisah, S., Oteng, M.A. and Ofori, J.K., 2009. Growth performance of the African catfish, *Clarias gariepinus*, fed varying inclusion levels of *Leucaena leucocephala* leaf meal. *J. appl. Sci. environ. Manage*., **13**: 21-26.

Anwar, F., Latif, S., Ashraf, M. and Gilani, A.H., 2007. *Moringa oleifera*: A food plant with multiple medicinal uses. *Phytother. Res*., **21**: 17-25. https://doi.org/10.1002/ptr.2023

AOAC, 2006. *Official methods and analysis, Method Number 1125*, 17th Edition. Association of Official Analytical Chemists.

Arsalan, M.Z.H., Hussain, S.M., Asrar, M., Anwar, H., Rehan, M.M.H., Shahzad, M.M., Riaz, D., Ahmad, N. and Wahab, N., 2016. Effects of *Moringa oleifera* leaf meal (MOLM) based diets on carcass composition and hematology of *Labeo rohita* fingerlings. *J. Biodiv. environ. Sci*., **9**: 214-223.

Bello, O.N. and Nzeh, G.C., 2013. Effects of varying levels of *Moringa oleifera* leaf meal diet on growth performance, hematological indices and biochemical enzymes of African catfish *Clarias gariepinus* (Burchell 1822). *Elixir Aquacult.*, **57A**: 14459-14466.

Ebrahim, M.S., Zeinhom, M., Abou, M. and Seif, R., 2007. Response of Nile tilapia (*Oreochromis niloticus)* fingerlings diets containing Azolla meal as a source of protein. *J. Arab. Aquacul. Soc*., **2**: 54-69.

Ezekiel, O.A., Fidelis, B.A. and Grace, N.U., 2016. Effect of partial replacement of fishmeal with *Moringa oleifera* leaf meal on the haematology, carcass composition and growth perfomance of *Clarias gariepinus* (Burchell 1822) fingerlings. *Int. J. Fish. aquat. Stud*., **4**: 307-311.

Fakurazi, S., Hairuszah, I. and Nanthini, U., 2008. *Moringa oleifera* Lam prevents acetaminophen induced liver injury through restoration of glutathione level. *Fd. Chem. Technol*., **46**: 2611-2615. https://doi.org/10.1016/j.fct.2008.04.018

FAO, 1997. Review of the state of world aquaculture. *FAO Fisheries Circular No. 886, Rev. 1*. Food and Agriculture Organization of the United Nations,

Rome, Italy.

FAO. 2000. *Fishery statistics (Aquaculture production)*. Food and Agriculture Organization of the United Nations, Rome, 90(2): 22-131.

Fasakin, E.A., Balogun, A.M. and Fasuru, B.E., 1999. Use of duckweed, *Spirodela pohyorhiza* L. Schieiden, as a protein feed stuff in practical diets for tilapia, *Oreochromis niloticus* L. *Aquacul. Res.*, **30**: 313-318. https://doi.org/10.1046/j.1365-2109.1999.00318.x

Ganzon-Naret, E.S., 2014. Utilization of *Moringa oleifera* leaf meals as plant protein sources at different inclusion levels in fish meal based diets fed to *Lates calcarifer*. *ABAH Bioflux*, **6**: 158-166.

Habib, R.Z., Afzal, M., Shah, S.Z.H., Fatima, M., Bilal, M. and Hussain, S.M. 2018. Potential of phytase and citric acid treated canola meal based diet to enhance the minerals digestibility in Labeo rohita fingerlings. *Pakistan J. Zool.*, **50**: 2045-2050. http://dx.doi.org/10.17582/journal.pjz/2018.50.6.2045.2050

Hashem, H., El-Rahman, A., Hanan, A., Abo-State, Ali, S.M., El-Nadi, Abozaid, H., Mamdouh, I., El-Fetoh, M.A. and Abdall, M., 2017. Growth performance, feed utilization and body composition of Nile tilapia (*Oreochromis niloticus*) fingerlings fed moringa (*Moringa oleifera* Lam.) seed meal. *J. Fish. aquat. Sci.*, **12**: 36-41.

Hlophe, S.N. and Moyo, N.A.G., 2014. A comparative study on the use of *Pennisetum clandestinum* and *Moringa oleifera*as protein sources in the diet of the herbivorous *Tilapia rendalli*. *Aquacul. Int.*, **22**: 1245-1262. https://doi.org/10.1007/s10499-013-9744-4

Humason, G.L., 1979. *Animal tissue techniques*, 4th edn. Freeman, San Francisco.

Kumar, V., Makkar, H.P.S. and Becker, K., 2011. Detoxified *Jatropha curcas* kernel meal as a dietary protein source: Growth performance, nutrient utilization and digestive enzymes in common carp (*Cyprinus carpio* L.) fingerlings. *Aquacul. Nutr.*, **17**: 313-326. https://doi.org/10.1111/j.1365-2095.2010.00777.x

Madalla, N., Agbo, N.W. and Jauncey, K., 2013. Evaluation of aqueous extracted moringa leaf meal as a protein source for Nile tilapia juveniles protein source for *Nile tilapia* juveniles. *Tanzania J. agric. Sci.*, **12**: 53-64.

Makkar, H.P.S. and Becker, K., 1996. Nutritional value and anti-nutritional components of whole and ethanol extracted *Moringa oleifera* leaves. *Anim. Feed Sci. Technol.*, **63**: 211-228. https://doi.org/10.1016/S0377-8401(96)01023-1

Markovic, Z., Poleksic, V., Lakic, N., Zivic, I., Dulic, Z., Stankovic, M., Spasic, M., Raskovic, B. and Sorensen, M., 2012. Evaluation of growth and histology of liver and intestine in juvenile carp (*Cyprinus carpio* L.) fed extruded diets with or without fish meal. *Turkish J. Fish. aquat. Sci.*, **12**: 301-308.

Mehdi, H., Khan, N., Iqbal, K.H., Rasool, F., Chaudhry, M.H. and Khan, K.M., 2016. Effect of *Moringa oleifera* meal on the growth, body composition and nutrient digestibility of *Labeo rohita*. *Int. J. Biosci.*, **8**: 11-17. https://doi.org/10.12692/ijb/8.4.11-17

Mukherjee, S., Parial, D., Khatoon, N., Chaudhuri, A., Senroy, S., Homechaudhuri, S. and Pal, R., 2011. Effect of formulated algal diet on growth performance of *Labeorohita* Hamilton. *J. Algal Biomass Utln.*, **2**: 1-9.

Nwankpa, K., 2017. The histological evaluation of the effects of *Moringa oleifera* leaf meal inclusions on the liver and ovaries of female *Oreochromis niloticus*. *Int. J. Fish. aquat. Res.*, **2**: 22-26.

Nwanna, L.C., Falaye, A.E. and Sotolu, A.O., 2008. *Water hyacinth* Eichhhomia crassipes *(Mart solms): A sustainable protein source for fish feed in Nigeria*. Food health and environmental issues in developing countries: The Nigeria situation (eds. O.C. Adeboye, K.A. Taiwo and A.A. Fatufe). Alexander Von Humboldt Foudation, Bonn, Germany, pp. 187-194.

Olaniyi, C.O., Ajani, N.O. and Adetomi, M.N., 2013. Growth performance and nutrient utilization of *Clarias gariepinus* fed *Moringa oleifera* leaf meal. *J. Nat. Sci. Res.*, **3**: 99-104.

Rapatsa, M.M. and Moyo, N., 2014. Effect of *Moringa oleifera* on the histology, haematology and growth of *Oreochromis mossambicus* in aquadams fertilised with chicken manure in South Africa. *Afr. J. aquacul. Sci.*, **39**: 295-303. https://doi.org/10.2989/16085914.2014.958977

Raskovic, B.S., Stankovic, M.B., Markovic, Z.Z. and Poleksic, V.D., 2011. Histological methods in the assessment of different feed effects on liver and intestine of fish. *J. agric. Sci.*, **56**: 87-100.

Richter, N., Siddhuraju, P. and Becker, K., 2003. Evaluation of nutritional quality of Moringa (*Moringa oleifera* Lam.) leaves as alternative protein source for tilapia (*Oreochromis niloticus* L.). *Aquaculture*, **217**: 599-611. https://doi.org/10.1016/S0044-8486(02)00497-0

Sadiku, S.O.E. and Jauncey, K., 1995a. Soybean flour–poultry meat meal blends as dietary protein source

in practical diets of *Oreochromis niloticus* and *Clarias gariepinus*. *Asian Fish. Sci*., **8**: 159-168.

Shalaby, S.M.M., 2004. Response of Nile tilapia, *Oreochromis niloticus*, fingerlings to diets supplemented with different levels of fenugreek seeds (Hulba). *J. Agric. Mansoura Univ*., **29**: 2231-2242.

Thiam, S., Fall, J., Loum, A., Sagne, M. and Diouf, M., 2015. Use of effective microorganisms (Em) in Tilapia diets: Effects of growth performance and carcass composition. *Int. J. Curr. Microbiol. appl. Sci*., **4**: 536-549.

Tiamiyu, L.O., Okomoda, V.T. and Aende, A., 2015. Growth performance of *Oreochromis niloticus* fingerlings fed *Moringa oleifera* leaf as replacement for soybean meal. *Turkish J. Fish. aquat. Sci*., **2**: 61-66.

Weliange, W.S., Amarasinghe, U.S., Moreau J. and Villanueva, M.C., 2006. Diet feeding periodicity, daily ration and relative food consumption in fish populations in three reservoirs of Sri Lanka. *Aquat. Liv. Resour*., **19**: 229-237. https://doi.org/10.1051/alr:2006023

Yuangsoi, B. and Masumoto, T., 2012. Replacing moringa leaf (*Moringa oleifera*) partially by protein replacement in soybean meal of fancy carp (*Cyprinus carpio*). *Song J. Sci. Technol*., **34**: 479-485.

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Studies on the Activity of Digestive Enzymes in *Labeo rohita* Fingerlings Fed with Formulated Feeds

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Authors' Contribution
NI conducted the research. SA supervised the research. KA helped in lab work and wrote the manuscript. AM helped in feed formulation and data collection. WH arranged data and did statistical analysis. HN and SA helped in writing up and proofreading the manuscript.

Key words
***Labeo rohita*, Formulated feed, Digestive enzymes.**

ABSTRACT

The activity of digestive enzymes (Amylase and Lipase) is affected by the animal's nutrition. Amylase catalyzes the hydrolysis of carbohydrates while lipase catalyzes the hydrolysis of ester bonds. The purpose of present study was to analyze the activity of above mentioned digestive enzymes in *Labeo rohita* under intensive culture system. Ten fish were stocked in each aquarium and fed with formulated feed containing 30 % (control), 40 and 50 % crude protein (CP) @ 3% of wet biomass twice a day for 60 days. Fish wet weight, fork and total length was measured and recorded on weekly basis. The activities of amylase and lipase were determined by using standard methods at the end of the experimental trial. Results indicated the highest weight, fork and total length increment in *L. rohita* fed with diet containing 50 % CP as compared to that in 40 and 30 % CP fed fish. The highest activity of amylase and lipase was recorded in fish fed with diet containing 30 % CP. As compared to that recorded in fish fed with 40 and 50 % CP containing diet.

INTRODUCTION

Aquaculture's development is firmly connected to nutritional improvement and progress of fish farming practices, a dare for upcoming improvement in culture of aquatic organisms. High quality fish production greatly depends on the magnification of appropriate feeding procedures to fulfill the dietary necessities of the growing species. Through the years, as the aquaculture industry is growing the requirement of particular feeds is also growing to make the species and production system better (Ismat *et al*., 2013).

Pakistan has massive fishery holdings but current amount of production is deficient to fulfill the needs of distressingly enhancing public of the country. In Southeast subcontinent even after years of fast growth in fish production, only ten percent of its potentials have been exploited (Bhatta, 2001). World fish culture has developed excessively during the last years becoming financially crucial corporation (Subasinghe *et al*., 2009).

Fish is nutrient rich and most accepted food worldwide it is edible, appetizing and having great nutritional values. The major nutrients present in fish are vitamins, calcium, phosphorus and unsaturated fat. These nutrients are offered to fish by feed either naturally or artificially and permit the fish to develop more rapidly for the improvement of fitness in individuals (Ayanda, 2003). An unlimited number of researches are now focused towards the expansion of artificial feed for cultured classes (Lopez-Vasquez *et al*., 2009). Formulated feed is important input in aquaculture, and their value depends on the capability of the cultured fish to assimilate and thus use the feeds for growth and development (Jauncey *et al*., 2007).

So, before considering any aquaculture nominee, the ability of digesting artificial diets is investigated (Kolkovski, 2001). To satisfy the protein requirements of the fish meal or to make an economical feed, materials from different plant and animal bases are used. Fish nutritionist pays greater attention for low cost artificial diets from alternate protein sources *i.e.* plants and animals because of the establishment of inter-relationship amongst the nutritional energy requirements, the importance of protein nutrition and the growth of the fish (Das *et al*., 1991).

Feeding habit of fish reveal the pattern of digestive enzyme in fish. Several mechanical and chemical processes start on feed after taken by fish. The capability of fish to digest a diet rest on the existence of proper gastric enzymes, which facilitate the specific degradation pathways controlling both physical and chemical nature of foods (Caruso *et al*., 2009). Fish digestive enzymes highlighting the mechanisms and greatest use of nutrients are of absolute importance as a background for the optimization of fish feeding processes (Suarez *et al*., 1995).

The knowledge and study of gastrointestinal enzymes

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involves in the breakdown process of food is a much needed step to understand about the digestion mechanism itself and how animals acclimatize to variations in dietary situation (Sunde *et al*., 2004). Digestive enzymes actions of fish are linked with intrinsic feeding pattern and di*et al*ignment (Ray, 1988). There are some other factors also present which influences the digestive enzyme activity, these are age, pH, temperature and anatomic-physiological characteristics (Kuzmina, 1996). Commonly, carnivorous fish exhibit complex protease activity whereas carbohydrases are extra active in omnivorous and herbivorous fishes (Ugolev and Kuzmina, 1994). The current research was designed to study the growth performance and digestive enzymes activities in fingerlings of *Labeo rohita* fed on formulated feeds.

MATERIALS AND METHODS

Labeo rohita fingerlings were purchased from the Government Fish Seed Hatchery, Faisalabad. Prior to the experiment, the fish were kept in laboratory condition for two weeks in 500 liter cemented tanks for acclimatization. During this time the fish fingerlings were fed with reference diet twice a day. Ten fish were stocked in each aquarium for a total period of 60 days. The experimental setup was divided in three treatments based on the different protein levels in feed. The experiment was conducted in 3 replications for each treatment. Fish were fed with diet containing 30, 40 and 50% CP (crude protein) @ 3% of their wet biomass twice a day.

The physico-chemical parameters *viz*., water temperature, pH, dissolved oxygen and total hardness were recorded after 24 h by following the method of APHA (1998). Before the experiment, fish were treated with pinch of $KMnO_4$. At the end of experimental period fish were randomly collected from each treatment. Fish wet weight, fork and total length were measured and recorded on weekly basis. Afterwards, the fish were dissected and organs (liver and intestine) were separated to measure the activity of experimental enzymes. The liver and intestine was stored at 1°C for extraction of enzymes.

Enzyme extraction and quantitative estimation

The stored samples were used for the extraction of amylases and lipases. The extracted samples of fish was collected and homogenized with chilled 50 mM tris HCl (0.788 g of tris HCl was weighed and dissolved in 100 mL of distilled water) in mortar and pestle. Homogenate was centrifuged at 6000 rpm (4°C) for 15 min; supernatant was used for quantitative estimation of enzymes.

For amylase assay starch solution (1%) was used as substrate in amylase assay as reported by Bernfeld (1955), for which 1 ml sample and 1 ml starch substrate (1%) was incubated for 2 min at room temperature. After incubation, 1 ml DNS reagent was added in test tube and placed in boiling water bath for 5 min, to stop the reaction. Absorbance was checked against blank at 540 nm wavelength.

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A \text{ enzyme} - \Delta A \text{ blank} \times \text{standard factor}}{\text{Time of incubation} \times \text{dilution factor}}$$

Standard factor calculated for amylase was 6.5.

For lipase assay 3.5 ml phosphate buffer, 1 ml sample and 0.5 ml olive oil were added in a glass flask. The reaction mixture was stirred for 30 min at 37°C in water bath and then 1 ml of acetic acid was added and titrated against NaOH till the pH of solution became 10 (Borlongan, 1990).

$$\text{Enzyme activity (U/ml)} = \frac{\text{Vol. of NaOH} \times \text{Molarity of NaOH} \times 1000 \times 2 \times \text{df}}{\text{Vol. of sample used}}$$

Statistical analysis

Data were analyzed by the analysis of variance at the 0.01 significance level. The Minitab 17 version was used for all the statistical analyses.

RESULTS AND DISCUSSION

In current study, the growth performance and digestive enzymes activity in *L. rohita* fed with diet containing different levels of protein was assessed. Comparison of the growth parameters among fish fed on formulated feeds revealed significant differences at $p<0.05$. The results of the growth parameters of current study indicated significant effect on weight gain in all the treatment groups. Higher wet weight, fork and total length increments were noted in fish fed with feed containing 50% CP than the fish fed with 30 and 40% CP (Table I).

Table I.- Growth comparison of *Labeo rohita* fed on different formulated feeds.

Growth parameters/ treatments	Avg. wet weight (g)		Avg. fork length (mm)		Avg. total length (mm)	
	Initial	Final	Initial	Final	Initial	Final
30 % CP	2.16±0.05	13.50±0.03	50.63±0.30	77.40±0.30	62.46±0.25	87.60±0.30
40 % CP	2.12±0.03	16.30±0.02	50.40±0.20	80.60±0.10	62.66±0.25	91.33±0.25
50 % CP	2.15±0.07	20.12±0.05	50.50±0.26	85.29±0.31	60.19±0.30	95.56±0.32

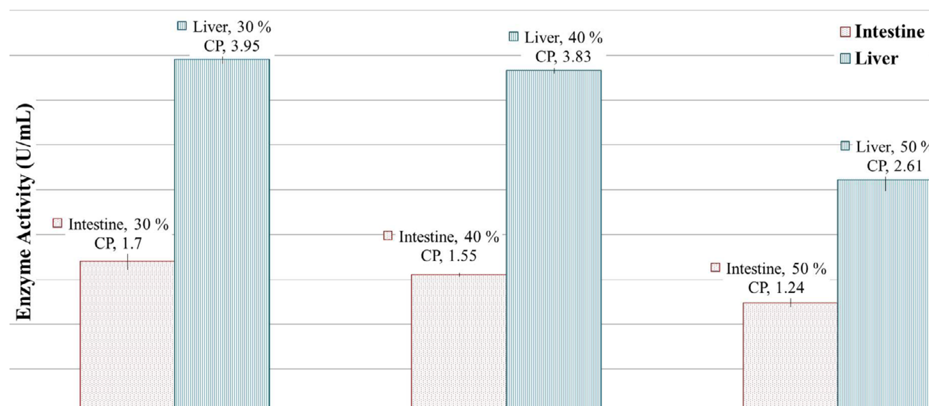

Fig. 1. Activity of amylase in different organs of *Labeo rohita* fed with different formulated feeds.

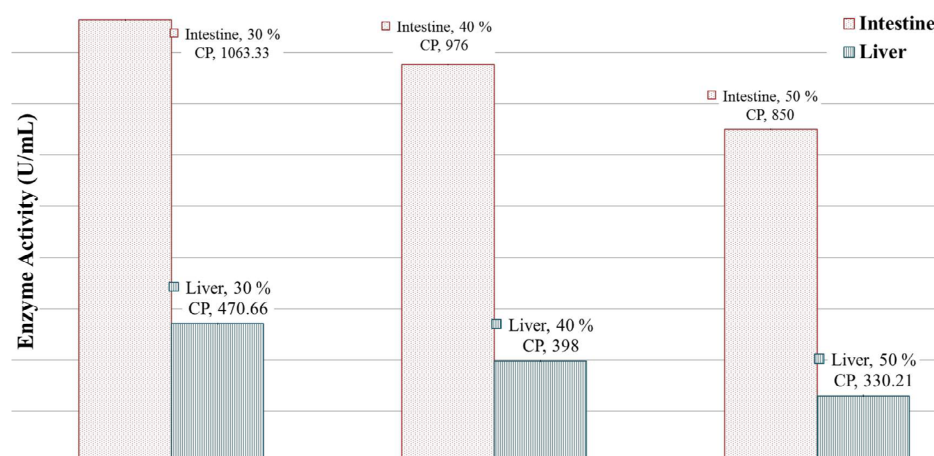

Fig. 2. Activity of lipase in different organs of *Labeo rohita* fed with different formulated feeds.

Kim *et al*. (2016) reported that fish fed with diet containing higher percentage of the crude protein (CP) exhibited significantly higher growth rate than those fed with the diet having lower percentage of CP. Maximum growth was noted in fish fed with 50% CP followed by 40 and 30%. Similarly, Hossain *et al*. (2010) described that the optimal dietary protein level for *Pampus argenteus*, was 49%. Protein requirement of some other fish species such as *Paralichthys olivaceus* was 46.4% (Kim *et al*., 2002) while for *Epinephelus malabaricus* it was 47.8% (Chen and Tsai, 1994). Mostly, with the increase in dietary protein level the growth performance of fish also increased (NRC, 1993).

Mannivnan and Sravnan (2012) formulated four different kinds of fish feed with different protein levels. *L. rohita* were fed with feed for 60 days to evaluate the growth performance. Results showed that fish fed with 40% feed had better growth as compared to the fish fed with other feeds, which supported my results completely. Abid and Ahmed (2009) also reported significantly higher weight gain in 45% protein fed fish fingerlings as compared those fed with other feeds.

In this study, fish fed with 30% CP exhibited higher amylase level as compared to the fish fed with 40 and 50% CP. In treated fish the activity of amylase was statistically different at $p<0.05$. The comparison of amylase activity between the organs showed that the liver had more amylase activity which was almost double than its activity in intestine (Fig. 1).

Fish fed with feed containing 30% CP showed more lipase activity as compared to the fish fed with 40 and 50% CP containing feed. The activity of lipase in treated fish was statistically different at $p<0.05$. The comparison between the organs showed that the intestine had more lipase activity than the liver (Fig. 2).

Similar results were reported by Klahan *et al*. (2009). They also found that amylase activity was significantly higher in the liver than other organs ($P<0.01$). Borlongan

(1990) examined the distribution pattern of digestive lipases along the digestive tract. Results of his study showed that major parts of lipase activity were the intestine, pancreas and pyloric caeca than the liver. These findings also support our results, that lipase activity is almost double in the intestine as compared to the liver. Caruso *et al*. (2009) studied the effect of protein rich and protein deficient feed on the sea bream and it was concluded that the secretion of gut enzymes reflected the type of diet on which fish feed. The activity of amylase and lipase enzymes was more in fish fed on diets containing low protein while diets rich in protein caused higher quantity of protease enzyme in fish. It can be concluded that fish may adjust their metabolic functions to the nutritional substrates through a regulation in enzyme production in order to increase the consumption of certain feed ingredients.

Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

APHA, 1998. *Standard methods for the examination of water and waste water*, 20th ed. American Public Health Association, New York, pp. 1193-1197.

Abid, M. and Ahmed, M.S., 2009. Growth response of *Labeo rohita* fingerlings fed with different feeding regimes under intensive rearing. *J. Anim. Pl. Sci*., **19**: 45-49.

Ayanda, J.O., 2003. Feeding practices and management of fish in farming system. In: *Proceedings of the National Workshop on Fish Feed Development and Feeding Practices in Aquaculture* (ed. A.A. Eyo). Organized by Fisheries Society of Nigeria (FISON) and National Institute for Freshwater Fisheries Research (NIFFR), New Bussa.

Bernfeld, P., 1955. Amylase α and β. *Method. Enzymol.,* **1**: 149-158. http://dx.doi.org/10.1016/0076-6879(55)01021-5

Bhatta, R., 2001. Production, accessibility and consumption patterns of aquaculture products in India. In: *Production, accessibility, marketing and consumption patterns of freshwater aquaculture products in Asia: A gross-country comparison*. FAO Fisheries Circular No. 973, Rome, FAO, pp. 275.

Borlongan, I.G., 1990. Studies on the digestive lipases of milkfish, *Chanos chanos*. *Aquaculture*, **89**: 315-325. https://doi.org/10.1016/0044-8486(90)90135-A

Caruso, G., Denaro, M.G. and Genovese, L., 2009. Digestive enzymes in some teleost species of interest for Mediterranean aquaculture. *Open Fish. Sci. J*., **2**: 74-86. https://doi.org/10.2174/1874401X00902010074

Chen, H.Y. and Tsai, J.C., 1994. Optimum dietary protein level for the growth of juvenile grouper, *Epinephelus malabaricus*, fed semi purified diets. *Aquaculture*, **119**: 265-271. https://doi.org/10.1016/0044-8486(94)90181-3

Das, K.M., Mohanty, S.N. and Sarkar, S., 1991. Optimum dietary protein to energy ratio for *Labeo rohita* fingerlings. In: *Proceedings of the Fourth Asian Fisheries Society*. Special Publication, pp. 205.

Hossain, M.A., Almatar, S.M. and James, C.M., 2010. Optimum dietary protein level for juvenile silver pomfret, *Pampus argenteus* (Euphrasen). *J. World Aquacult. Soc*., **41**: 710-720. https://doi.org/10.1111/j.1749-7345.2010.00413.x

Ismat, N., Ashraf, M., Naeem, M. and Hafeez, M., 2013. Effect of different feed ingredients on growth and level of intestinal enzyme secretions in juvenile *Labeo rohita, Catla catla, Cirrhinus mrigala* and *Hypophthalmichthys molitrix*. *Int. J. Aquacult*., **3**: 85-91.

Jauncey, K., Sorensen, P.L. and Areola, F., 2007. *A short handbook–Catfish feed for Nigeria. Materials storage, handling, stock and quality control, ingredients, feed formulation, feed use (feed husbandry) and technical aspects of feed manufacture*. CDE, Brussels.

Kim, K.W., Wang, X.J. and Bai, S.C., 2002. Optimum dietary protein level for maximum growth of juvenile olive flounder, *Paralichthys olivaceus*. *Aquacult. Res*., **33**: 673-679. https://doi.org/10.1046/j.1365-2109.2002.00704.x

Kim, K.W., Zaman, M.M., Kim, K.D., Han, H.S., Yun, H., Lee, S. and Bai, S.C., 2016. Effects of dietary protein levels on growth performance and body composition of juvenile parrot fish, *Oplegnathus fasciatus*. *Int. aquat. Res*., **8**: 239-245.

Klahan, R., Areechon, N., Yoonpundh, R. and Engkagul, A., 2009. Characterization and activity of digestive enzymes in different sizes of Nile tilapia (*Oreochromis niloticus L.*). *J. Nat. Sci*., **43**: 143-153.

Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles-implications and applications to formulated diets. *Aquaculture*, **200**: 181-201. https://doi.org/10.1016/S0044-8486(01)00700-1

Kuzmina, V., 1996. Influence of age on digestive enzyme activity in some freshwater teleosts. *Aquaculture*, **148**: 25-37. https://doi.org/10.1016/S0044-8486(96)01370-1

Lopez-Vasquez, K., Castro-Perez, C.A. and Val, A.L., 2009. Digestive enzymes of eight Amazonian teleosts with different feeding habits. *J. Fish Biol*., **74**: 1620-1628. https://doi.org/10.1111/j.1095-8649.2009.02196.x

Manivannan, S. and Saravanan, T.S., 2012. Impact of formulated protein diets on growth of the Indian major carp, *Labeo rohita* (Hamilton). *Fish. Aquacult. J*., **57**: 1-7.

NRC, 1993. *Nutrient requirements of fish*. National Research Council, National Academy Press, Washington.

Ray, A.K., 1988. On the digestive enzymes in three Indian freshwater perches in relation to food and feeding habits. *J. Fish. Soc. London*, **20**: 1-5.

Suarez, M.D., Hidalgo, M.C., Galego, M.G., Sanz, A. and Higuera, M., 1995. Influence of the relative proportions of the energy yielding nutrients on the liver intermediary metabolism of the European eel. *Comp. Biochem. Physiol*., **111**: 421-428. https://doi.org/10.1016/0300-9629(95)00035-6

Subasinghe, R., Soto, D. and Jia, J., 2009. Global aquaculture and its role in sustainable development. *Rev. Aquacult*., **1**: 2-9. https://doi.org/10.1111/j.1753-5131.2008.01002.x

Sunde, J., Eiane, S.A., Rustad, A., Jensen, H.B., Opstvedt, J., Nygard, E., Venturini, G. and Rungruangsak-Torrissen, K., 2004. Effect of fish feed processing conditions on digestive protease activities, free amino acid pools, feed conversion efficiency and growth in Atlantic salmon (*Salmo salar* L.). *Aquacult. Nutr*., **10**: 261-277. https://doi.org/10.1111/j.1365-2095.2004.00300.x

Ugolev, A.M. and Kuzmina, V.V., 1994. Fish enterocyte hydrolases: Nutrition adaptations. *Comp. Biochem. Physiol*., **107**: 187-193. https://doi.org/10.1016/0300-9629(94)90292-5

Wang, C., Xie, S., Zheng, K., Zhu, X., Lie, W., Yang, Y. and Liu, J., 2005. Effects of live food and formulated diets on survival, growth and protein content of first-feeding larvae of *Plelteobagrus fulvidraco*. *Appl. Ichthyol*., **21**: 210-214. https://doi.org/10.1111/j.1439-0426.2005.00620.x

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Assessment of the Inhibitory Effect of Different Diets and Plant Ingredients on the Digestive Progress of Tilapia (*Oreochromis niloticus*, Linnaeus, 1758)

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Authors' Contribution
AT and EP performed the lab work. AT wrote the article. MHD provided technical assistance in experimentation. FO, DK and GWV checked and analysed the data. GWV conceived the projet and codrafted the manuscript.

Key words
***Oreochromis niloticus*, Feed, Protease, Inhibitors, By-products.**

ABSTRACT

The inhibitory effects of plant ingredients on the digestive proteases of tilapia (*Oreochromis niloticus*) were evaluated. Ninety monosex male tilapia juveniles (mean weight 17.3 ± 0.2 g, mean length 9.6 ± 0.1 cm) were reared in nine tanks in a recirculating water system. Three diets were tested in triplicate: (1) *Rcongo*, a feed formulated from local ingredients from the Democratic Republic of the Congo, 2) *Rcanada*, formulated with the same ingredients as *Rcongo* but acquired in Canada and 3) *Rcommercial*, commercial feed. Three diets and five agricultural by-products (soybean meal, wheat bran, rice bran, corn grain, and brewers grains) were used to assess in vitro protease inhibition. Proteolytic activity was higher in the first four intestinal segments than in the terminal segment. The presence of protease inhibitors in the diets and plant ingredients was highlighted. The negative effects of plant inhibitors on digestive proteases were quantified with non-significant differences between diets. Soybean meal had the greatest inhibiting activity, followed by wheat and rice bran and brewers' spent grains. However, given the lack of significant differences in the effects of diets on enzyme activities and protease inhibition (limitations of the *in vitro* method) the use of other methods such as the Zymogram (SDS-PAGE) appears to be a more sensitive biochemical tool to characterize protease sensitivity to inhibitors.

INTRODUCTION

Nile tilapia (*Oreochromis niloticus*, Linnaeus, 1758) aquaculture is a promising opportunity in response to food insecurity challenges in developing countries. This species has been identified as a priority for development in the Democratic Republic of the Congo (DR Congo) and a number of sub-Saharan African countries. Several authors have reported that Nile tilapia easily adapts to very diverse conditions and that its flesh is highly appreciated by consumers. Also, its superior performance (rapid growth, prolificacy, disease resistance, *etc.*) has previously been reported (El-Sayed, 2006; Pouomogne and Pemsl, 2008; Lazard, 2009; Meyer, 2013; Ahmad and Zulqurnain, 2018).

However, intensive aquaculture production in developing countries is confronted with several limitations due to the difficulty of accessing commercial feed of adequate nutritional value (CNPMT, 2010; FAO, 2010, 2012). The use of local and readily-available ingredients, mostly consisting of agricultural by-products, could improve availability and reduce production costs. Unfortunately, their low quality, mainly that of plant-based by-products, limits their use in aquaculture because of their negative impact on fish growth (Moyano *et al*., 1999; El-Sayed, 2006; Drew *et al*., 2007).

Many authors have previously reported the low quality of plant ingredients, as well as their reduced digestibility and the presence of antinutritional compounds (Moyano *et al*., 1999; El-Sayed *et al*., 2000; Francis *et al*., 2001; El-Sayed, 2006; Drew *et al*., 2007). Several antinutritional compounds are identified in plant-sourced proteins, including protease inhibitors, phytates, saponins, antigenic proteins *etc*. (Médale and Kaushik, 2009; Médale *et al*., 2013; Burel and Médale, 2014). Protease inhibitors are proteins that reduce enzyme-catalyzed reactions and act in physiological processes, such as regulation of coagulation, fibrinolysis, complement activation and the inflammatory response in mammals (Whitaker, 1994; Voet and Voet,

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1995). These compounds affect the digestive capacity and growth of fish fed diets formulated with plant-based ingredients. El-Sayed (1999) reported a significant decrease in growth and feed efficiency in Nile tilapia due to a poor balance of amino acids and the presence of protease inhibitors in diets. Many studies reported that the heat treatment of plant proteins inactivates protease inhibitors, however other antinutritional factors are heat stable (Alarcón *et al*., 1999, 2001; El-Sayed *et al*., 2000; Azaza *et al*., 2006). It has also been demonstrated that the proper combination of different ingredients in the formulation of diets and essential amino acid supplementation improve growth and feed conversion (Moyano *et al*., 1992; El-Sayed, 1999; Médale and Kaushik, 2009).

Furthermore, it is documented that the nutritional value of a diet is not simply based on its chemical composition. It is also determined by the physiological capacity of fish to digest it, and to absorb the proteins and other nutrients contained therein, according to their eating habits. During digestion, proteins are hydrolyzed into peptides and free amino acids and are absorbed by specific intestinal membrane proteins. Protein hydrolysis in the digestive tract is carried out by proteolytic enzymes (Verri *et al*., 2011; Santos *et al*., 2013), this the link between the activity level of fish digestive proteases and the presence of antinutritional factors in the ingredients used in fish feed, particularly protease inhibitors, is of importance.

Intestinal proteases are classified into four major groups: acid protease (gastrin, pepsin) serine (trypsin and chymotrypsin), cysteine (cathepsin), and metalloproteases (aminopeptidases) (Whitaker, 1994; Voet and Voet, 1995; Chong *et al*., 2002). They are abundant in carnivorous fish such as trout. However, tilapia is an omnivorous and planktivorous species characterized with lower protease and higher amylase activity (Hidalgo *et al*., 1999; Moyano *et al*., 1999; El-Sayed, 2006). Protein digestion in fish stomach is achieved through the action of pepsin, secreted as inactive pepsinogen and activated by gastric acid secretion. When chyme arrives in the intestine, several pancreatic proteases and amylases (*e.g*., trypsin, α-amylase) are secreted and involved in protein and starch hydrolysis (Alarcón *et al*., 1999; Chan *et al*., 2004).

To date, the majority of the research on fish sensitivity to protease inhibitors has been focused on the use of soybean meal (Moyano *et al*., 1999; El-Sayed *et al*., 2000; Drew *et al*., 2007). This study was therefore conducted to assess the inhibitory effects of three different diets and five different plant ingredients (soybean meal, wheat bran, rice bran, milled corn and brewers grain) on digestive proteases in tilapia.

MATERIALS AND METHODS

Fish

This experiment was conducted at the Laboratoire de Recherche des Sciences Aquatiques (LARSA) at Université Laval (Québec, Canada). It was carried out in accordance with the requirements of the Animal Protection Committee of Université Laval. Ninety monosex male tilapia juveniles (*Oreochromis niloticus*; mean weight 17.3 ± 0.2 g; mean length 9.6 ± 0.1 cm) were obtained from Sand Plains Aquaculture (Ontario, Canada) and reared in nine acrylic basins supplied by closed water recirculation, for four weeks. Upon arrival of the fish, a two-week acclimation period immediately began. All fish were weighed, measured on total (LT) and standard (LS) lengths and transferred to 9 rectangular aquariums, each containing 10 litres of water. Each aquarium was stocked with 10 juveniles, giving a density of 17 g/L. During the acclimation period, the fish were fed to satiety with commercial feed (Corey Optimum, 2mm dia., Fredericton, NB Canada). The physicochemical parameters of water were regularly verified and maintained within tolerable limits for tilapia *O. niloticus* (El-Sayed, 2006; Ross, 2000). Temperature (26 ± 0.5°C), dissolved oxygen (9.2 ± 1.6 mg/L), and pH (7.1 ± 0.4) were continuously monitored. Ammonia (NH4 ≤ 0.01 mg/L), nitrites (NO2 ≤ 0.01 mg/L), and dissolved CO_2 (≤ 0.01 mg/L) were measured weekly using a Hach® Spectrophotometer; conductivity (117.8 ± 1.2 µs/cm), alkalinity (9.2 ± 0.1 mg/L $CaCO_3$), and water hardness (424 mg/L $CaCO_3$) were also verified weekly. The photoperiod (14h: 10 h light: dark) was maintained with the help of incandescent lamps. A water flow of 1 L/min, corresponding to 6 tank water renewals per hour, was verified and adjusted daily.

Feed and diets

Diet composition and nutrient content are presented in Table I. Three experimental diets were tested in triplicate: 1) Rcongo, a feed formulated from local ingredients from the Democratic Republic of the Congo, 2) Rcanada, a feed formulated with the same ingredients as the first one, but acquired in Canada and 3) Rcommercial, a fishmeal-based commercial feed (Corey Optimum 2 mm). An indigestible silicon dioxide marker (Sipernat™50® Evonik, Piscataway, NJ) was added to all three feeds (1% w: w). These diets were formulated according to the methods and recommendations regarding the nutritional needs of Nile tilapia and the apparent digestibility of the ingredients for the species (NRC, 1993, 2011; El-Sayed, 2006; FAO, 2015). One of the three feeds was fed in three different randomly selected aquariums. The fish were fed twice daily between 8:00 a.m. and 4:00 p.m. In order to avoid any waste or loss of feed, the diet (4% of the body

weight/day) was distributed in small meals that lasted one hour, after faeces collection. The amount of ingested and uneaten feed was recorded daily in every aquarium.

Table I. Biochemical composition and analysis of experimental diets in Nile tilapia *Oreochromis niloticus*[1]

Ingredients and production costs	Experimental diets		
	Rcongo	Rcanada	Rcommercial
Wheat bran (%)	46,7	46,7	-
Rice bran (%)	5	5	-
Corn (%)	5	5	-
Brewers grain (%)	5	5	-
Soybean meal (%)	23,3	23,3	-
Blood meal (%)	10	10	-
Bone meal (%)	2	2	-
African palm oil (%)	2	2	-
Sipernat™50 marker (%)	1	1	1
Feed cost USD/kg diet	0,38	0,50	1
Production costs USD/kg fish	1,45	1,81	2,28
Biochemical analysis			
Dry matter (%)	89,6	89,1	90,4
Crude protein (%)	32,2	32,0	51,5
Lipid (%)	5,0	6,3	18,3
Gross energy, Mj/kg (kcal/kg)	19,7 (4541)	19,9 (4541)	23 (5497)
Phosphorus (%)	1,0	1,0	1,4
Crude fiber (%)	9,7	9,3	1,2
Ash (%)	9,0	7,5	11,7

[1]Values are means of three fish in triplicate.

To prepare the feed, every dry ingredient was finely ground (appx. 120 µm) using a crusher (Foss CT 193 Cyclotech™, Sweden), then weighed and homogeneously mixed. Palm oil and Sipernat™50® were mixed together before being added to the dry ingredient meal. Distilled water was added to 45% in the final mixture to form a homogeneous dough. The commercial feed, which had been previously prepared, was crushed for 7 seconds at 2500 RPM for 1 h 30 min with a blender (Retsch®, Düsseldorf, Germany) in order to be able to integrate the Sipernat. Using a extrusion machine with a helical screw, the dough was pressed through the 1.9 mm mesh of matrix # 9. The knife attached to the output of the granular produced 2 mm long granules. The resulting granules were the spheronized to standardize the particle size, according to the Tilapia nutritional requirements guidelines (FAO, 2015). The feed produced was then dried overnight at room temperature (25°C) under the fume hood, sieved and stored at -20°C in sealed plastic bags until used.

Determination of protease activity in fish enzyme extracts

Prior to the start of the feeding study and one day prior to final sampling, the fish were subjected to a 24 h fast. Following harvest, fish were immediately euthanized by 5 min bath in a concentrated tricaine methanesulfonate solution (250 mg of MS-222 + 500 mg sodium bicarbonate/liter of water), according to the protocol described by Popovic *et al*. (2012). Euthanized fish were counted, individually weighed and their total and standard lengths were measured. They were then dissected by groups of 4 randomly sampled subjects in each aquarium. The digestive tract was carefully removed, stripped of visceral fat and weighed. The intestine was isolated from other digestive organs, its length was measured and then cut into 5 distinct segments, as described by Smith *et al*. (2000). The five intestinal segments were sectioned from the cranial extremity, after the stomach, to the caudal extremity in the following order: 1) hepatic loop (HL), 2) proximal major coil (PMC), 3) gastric loop (GL), 4) distal major coil (DMC), 5) terminal segment (TS). Each intestinal segment was weighed and separately homogenized in distilled water (1:10 ratio), using a homogenizer (Turrax® or Tissumizer®, Cologne, Germany). The supernatant was collected after centrifugation (16000 RPM x 30 min at 4°C) and stored at -20°C until used as the aliquot for enzyme extraction. Finally, the soluble proteins of the enzyme extracts were determined by using the Bradford (1976) method, using bovine serum albumin as a standard. It should be noted that the original batch sampling, consisting solely of juvenile tilapia, the entire intestine was collected for protease analysis.

The activity of the intestine's alkaline proteases was measured by the Walter (1984) method, which uses 500 µl of casein (0.5%) as substrate in a 50 mM Tris-HCl pH 9 buffer. The whole was mixed with 20 µl of enzyme extract in 500 µl of buffer solution (50 mM Tris-HCl + 10 mM $CaCl_2$ pH 7.5) and incubated at room temperature (25°C) for 0, 5, 10, 15 and 30 min. The reaction was interrupted by the adding 500 µl of trichloroacetic acid (TCA) 20% and centrifuged (12000 RPM x 5 min), before submitting the supernatant to the spectrophotometer at 280 nm absorbance. For each enzyme sample, two sets of Eppendorf microtubes (test and blank) were analyzed in triplicate. However, the sequential addition of casein substrate (0.5%) and trichloroacetic acid (TCA, 20%) in the control sample was done before adding enzyme extract into the mixture.

Table II.- Effect of diets on the concentration of soluble proteins, and volumetric and specific activities of alkaline proteases in fish according to the diets used.

Parameter	Experimental diets			SEM	p-value	
	Rcongo	Rcanada	Rcommercial		Diet	Segments
Soluble protein (mg/ml)	0.21	0.22	0.21	0.003	0.179	<0.001
Volumetric activity (U/ml)	94.2^{a}	106.4^{b}	89.6^{a}	4.7	0.001	<0.001
Specific activity (U/mg)	449.4^{ab}	493.2^{a}	419.3^{b}	23.8	0.006	<0.001

The values correspond to the mean of the observations in the intestinal segments. Values associated to different letters are significantly different according to Tukey's test (P <0.05). SEM, mean standard error.

Evaluation of digestive protease inhibition by plant extracts

The plant solutions used in this experiment were prepared from three diets and five vegetable ingredients considered as agricultural by-products valued in animal feed (soybean meal, wheat bran, rice bran, spent corn grain and brewers' spent grains; Table I) and known to be very rich in protease inhibitors by the aforementioned authors (El-Sayed, 1999, 2006). Dry ingredients were finely ground (120 µm) with a blender (Moulinex®, France). The resulting powder (250 mg) was homogenized using a manual homogenizing kit (Potter-ELV® and Wheaton®, USA) in 10 ml of buffer solution (50 mM Tris-HCl pH 7.5) and centrifuged (2000 RPM for 10 min at room temperature) for a final concentration of 25 mg/ml. The supernatant containing the plant proteins was collected and stored at -20°C for the evaluation of inhibitory effects on proteases.

The inhibitory effect of plant solutions on fish digestive proteases was evaluated according to the protocol of Moyano *et al*. (1999), which was adapted from the method presented by García-Carreño *et al*. (1996), and based on the measurement of residual protease activity after the incubation of enzyme extracts with the solutions containing inhibitors. A solution containing 10 µl of enzyme extract and different volumes of inhibitor extracts was incubated at room temperature (25°C) for 60 min in 500 µl of buffer solution (50 mM Tris-HCl + 10 mm $CaCl_2$ pH 7.5). Residual enzyme activity was evaluated by means of a second incubation of the same mixture for 30 min in 500 µl of casein 0.5% + buffer 50 mM Tris-HCl pH 9. The reaction was interrupted by the adding 500 µl of trichloroacetic acid (TCA) 20% and centrifuged (12000 RPM x 5 min), before submitting the supernatant to the spectrophotometer at 280 nm absorbance. For each plant ingredient analyzed, two sets of Eppendorf microtubes (test and control) containing the mixture were tested in duplicate. The first series of microtubes (test) allowed to evaluate the state of enzyme extracts incubated in with varying volumes of plant extracts, while the second series was used as a control of the enzyme activity in the presence of variable volumes of buffer 50 mM Tris-HCl pH 7.5 rather than plant extracts. Each microtube series was simultaneously tested with its specific control, whose reaction was stopped before the addition of casein 0.5%, and immediately followed by a 15 min incubation period at 4°C (on ice). Absorbance values were obtained from protein hydrolysis and the enzymatic reaction in the control tubes. Protease inhibition, expressed as a percentage, was estimated as the difference in proteolytic activity between the two series of tubes. Finally, the response curves (Fig. 2) were constructed based on the ratio increase observed during the incubation of the plant solutions with the predetermined quantities of enzyme extracts (9.4 to 300 µg of inhibitor/protease activity unit).

Statistical analysis

The software R*i*389 (version 3.3.1) was used for the statistical analysis of data. The measured parameters were subjected to an analysis of variance (one-way and two-way ANOVA). In the case of significant differences ($P < 0.05$), the results were subjected to Tukey's multiple comparison test ($P < 0.05$) to determine if the treatment means were different, and to determine factor effects.

Table III.- Effect of diets on the inhibition of digestive proteases (%) in intestines and/or intestinal segments.

Protease sources*	Inhibitor sources (Experimental diets)			SEM	P-value
	Rcongo	Rcanada	Rcommercial		
Initial pool	44.3^{a}	38.0^{bc}	34.1^{bc}	1.6	P<0.001
HL	42.3^{ab}	40.5^{a}	45.0^{b}	0.8	P=0.030
PMC	40.3^{a}	44.9^{b}	52.7^{c}	1.9	P<0.001
GL	46.4	44.1	38.7	1.8	P=0.200
DMC	37.7^{a}	34.3^{b}	40.5^{c}	0.9	P<0.001
Segment pool	41.7	41.0	44.3	0.6	P=0.060

*Protease sources represent different intestinal segments for adult tilapia and entire intestine for the initial group. The values correspond to the mean of the observations in the intestinal segments: HL, hepatic loop; PMC, proximal major coil; GL, gastric loop; DMC, distal major coil. Values associated to different letters are significantly different according to Tukey's test ($p < 0.05$). SEM, mean standard error.

RESULTS

Soluble proteins and protease activities in enzyme extracts

The results of the present experiment on the concentration of soluble proteins and the activity of alkaline digestive protease activity are shown in Table II and Figure 1, respectively. Experimental diets did not have a significant influence on soluble protein between fish samples (*P = 0.179*), but significant differences were found within intestinal segment types (*P <0.001*).

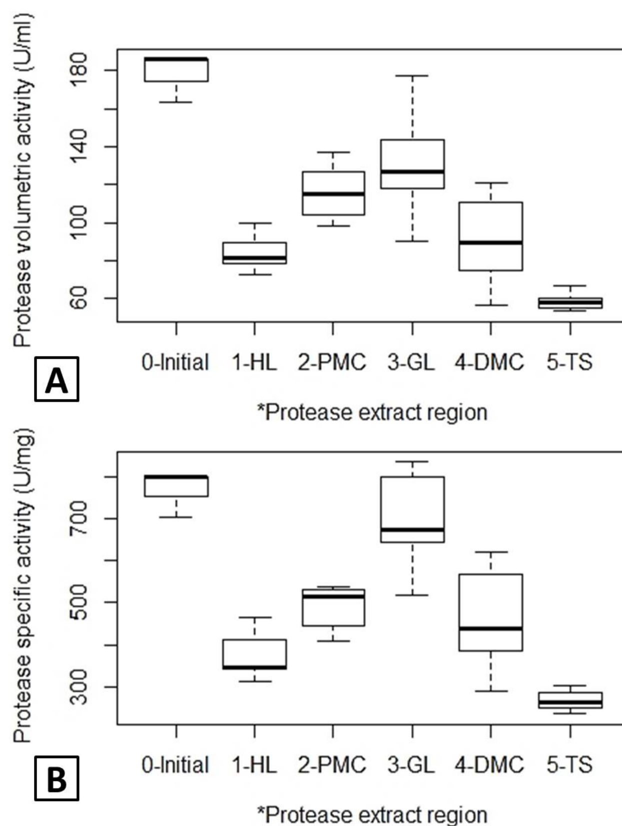

Fig. 1. Influence of intestinal segment on volumetric (U/ml) (A) and specific (U/mg) (B) activities of alkaline proteases in tilapia. *Logical order of the five intestinal segments of tilapia immediately after the stomach: 1-HL, hepatic loop; 2-PMC, proximal major coil; 3-GL, gastric loop; 4-DMC, distal major coil; 5-TS, terminal segment. Tilapia intestines from initial group.

As illustrated in Figure 1, intestinal segments had a significant effect on the specific activity of the proteases (*p <0.001*). This activity was higher in juvenile tilapias of the initial group (766.9 ± 55.1 U/mg) compared to adults, in which the activity was greater in the first four segments and lower in the fifth (*i.e.,* 370.7 ± 49.5 U/mg for HL, 489.6 ± 48.8 U/mg for PMC, 686.7 ± 111.9 U/mg for GL, 454.8 ± 115.1 U/mg for DMC and 268.0 ± 23.5 U/mg for TS, respectively). The effect of diets on specific protease activity was detected in fish (*p = 0.006*), showing a significant difference between the *Rcommercial* and Rcanada diets (*p = 0.004*), while no difference was observed between fish that were fed Rcongo and those that were fed Rcommercial (*p = 0.342*) and Rcanada (*P=0.114*).

Effects of diets on the inhibition of digestive proteases in fish

The results of intestinal extract incubation with diet-based solutions are included in Table III. These results show that the diets used did not lead to a significant difference in protease inhibition in all intestinal segments of adult tilapia, with the exception of the three HL segments (*p = 0.030*), PMC (*p < 0.001*) and DMC (*p <0.001*), where the Rcommercial diet resulted in higher inhibition compared to Rcongo and Rcanada. The significant effect of diets on protease inhibition was not found when the segments were pooled (*p = 0.06*). However, for tilapia of the initial group, the Rcongo diet resulted in higher protease inhibition, *i.e.,* 44.3 ± 2.2% vs. 38.0 ± 1.7% for Rcanada and 34.1 ± 0.8% for Rcommercial.

Effects of added ingredients on the inhibition of digestive proteases

The results of the incubation of fish intestinal extracts with the selected feed ingredient solutions are included in Figure 2. For all analyzed segments, high percentages of protease activity inhibition were observed when intestinal extracts were incubated with soybean meal solutions (*p <0.0001*), *i.e.,* 44.3 ± 1.0% for HL, 65.8 ± 5.0% for PMC, 64.2 ± 3.2% for GL, 56.3 ± 1.8% for DMC, and 66.9 ± 8.8% for young tilapias of the initial group. Depending on the intestinal segments, the protease inhibition caused by the soybean meal was 23 to 80% higher than the other ingredients. A protease inhibition greater than 20% with small amounts of plant solutions (37.5 µg inhibitor/U activity) was observed only for the enzymes from the GL segment and the initial group (Fig. 2). Along with soybean meal, wheat and rice bran also induced a significant antinutritional effect on digestive protease activity, followed by brewer's spent grains who generated less inhibition, and finally by corn whose inhibition did not exceed 20% in all segments.

The three enzyme sources, which are represented by three different diets, as well as the various ingredients used in the present study (Table IV) resulted in a significant effect on digestive protease inhibition (*p <0.001*). For certain ingredients, variations in protease response to inhibition

contained in the intestinal segments were observed: the soybean meal, for example, resulted in higher protease inhibition percentages in fish fed with the ingredients from the Congo compared with those from Canada, *i.e.,* 44.3 ± 1.0 to 65.8 ± 5.0% inhibition vs. 43.1 ± 2.1 to 48.9 ± 2.4%, respectively. For rice bran, the inverse situation was observed, *i.e.,* 45.2 ± 0.6 to 56.2 ± 1.1% protease inhibition for fish that consumed the ingredients of Canadian origin, compared to 24.2 ± 1.6 to 43.4 ± 0.6% for those who consumed ingredients of Congolese origin. In other words, tilapia were more sensitive to protease inhibitors in soybean meal from the Congo than Canada, and vice versa for rice bran. On the other hand, corn generated less inhibition than any other ingredient ($p < 0.0001$), *i.e.* from 10.1 ± 1.4 to 23.8 ± 2.1% protease inhibition.

Assessment of protease sensitivity to inhibitors according to fish age

Three digestive enzyme extracts were incubated *in vitro* with plant solutions of three corresponding diets to

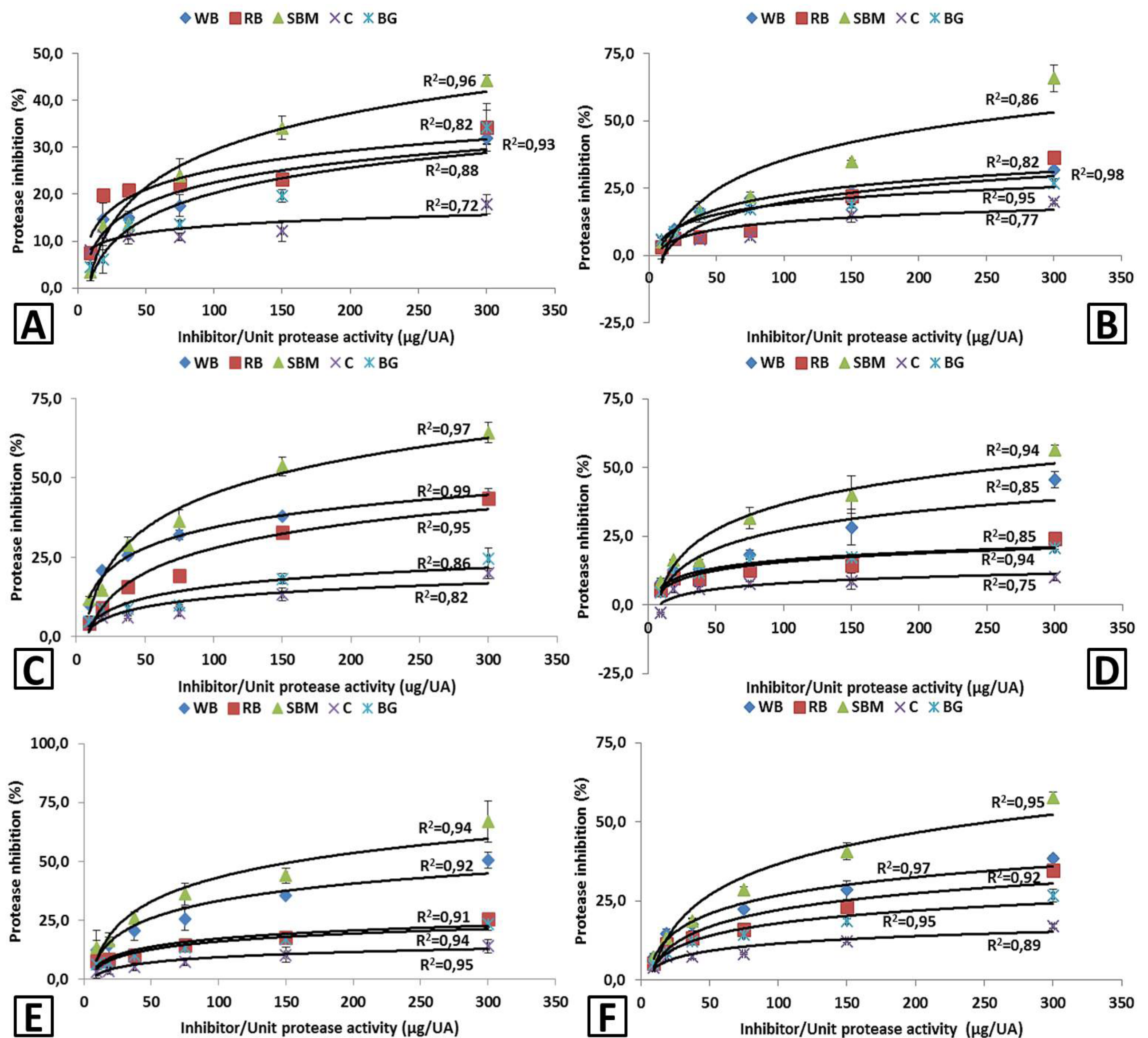

Fig. 2. Effect of the ingredients on the inhibition of digestive proteases (%) in intestines and/or intestinal segments logical order of the five intestinal segments of tilapia immediately posterior to the stomach: A, hepatic loop (HL); B, proximal major coil (PMC); C, gastric loop (GL); D, distal major coil (DMC); E, initial group; F, pooled segment.

Table IV.- Interaction between enzyme sources and inhibitors on protease inhibition (%) in tilapia.

Enzyme source (E)	Individual and pooled intestinal segments				
Inhibitors (I)	**HL**	**PMC**	**GL**	**DMC**	**Pooled**
Enzymes Rcongo	**32.5[A]**	**36.1[B]**	**39.3[B]**	**31.4[A]**	**34.8[B]**
Wheat bran	32.0[d]	31.6[cd]	44.1[c]	45.5[de]	38.3[c]
Wheat rice	34.3[d]	36.2[d]	43.4[c]	24.2[b]	34.5[c]
Soybean meal	44.3[e]	65.8[e]	64.2[d]	56.3[f]	57.7[f]
Corn	17.9[ab]	19.8[ab]	20.0[ab]	10.1[a]	16.9[a]
Brewers grain	34.2[d]	26.9[bc]	24.7[b]	20.6[ab]	26.6[b]
Enzymes Rcanada	**32.0[A]**	**32.2[A]**	**31.6[A]**	**37.5[B]**	**33.3[A]**
Wheat bran	34.5[d]	27.0[bc]	29.9[b]	46.0[de]	34.3[c]
Wheat rice	45.2[e]	56.2[f]	50.9[c]	54.5[f]	51.7[e]
Soybean meal	43.1[e]	40.9[d]	43.0[c]	48.9[e]	44.0[d]
Corn	14.1[a]	15.3[a]	11.3[a]	13.2[a]	13.5[a]
Brewers grain	23.0[bc]	21.8[b]	23.0[b]	25.1[b]	23.2[b]
Enzymes Rcommercial	**42.5[B]**	**33.5[A]**	**37.2[B]**	**38.8[B]**	**38.0[C]**
Wheat bran	44.7[e]	40.1[d]	47.3[c]	42.6[d]	43.7[d]
Wheat rice	55.8[f]	25.2[bc]	32.3[b]	30.3[c]	35.9[c]
Soybean meal	53.3[f]	65.8[e]	68.7[d]	77.1[g]	66.3[g]
Corn	23.8[bc]	22.2[b]	20.9[b]	16.0[a]	20.7[ab]
Brewers grain	35.0[d]	14.2[a]	16.7[ab]	28.0[bc]	23.5[b]
SEM	1.8	2.5	2.6	2.8	2.2
p value (E)	<0.001	<0.001	<0.001	<0.001	<0.001
p value (I)	<0.001	<0.001	<0.001	<0.001	<0.001
E x I	<0.001	<0.001	<0.001	<0.001	<0.001

The values correspond to the mean of the observations in the intestinal segments. Values associated to different letters (A, a) are significantly different according to Tukey's test ($P < 0.05$). SEM, mean standard error.

assess the sensitivity of tilapia to protease inhibitors by age (Fig. 3). Despite a much higher enzyme activity for juvenile extracts from the original group compared to adults ($p < 0.001$), *i.e.,* 766.9 ± 55.1 U/mg vs. 449.4 ± 150.6 U/mg for Rcongo, 493.2 ± 188.0 U/mg for Rcanada and 419.3 ± 138.4 U/mg for Rcommercial, these were relatively more sensitive to protease inhibitors in the diet ($P < 0.02$). The observed inhibition percentages were 44.3 ± 2.1% for the initial group vs. 41.7 ± 4.9% for adults fed Rcongo, 41.0 ± 4.4% for those fed Rcanada, and 44.3 ± 5.8% for those fed Rcommercial.

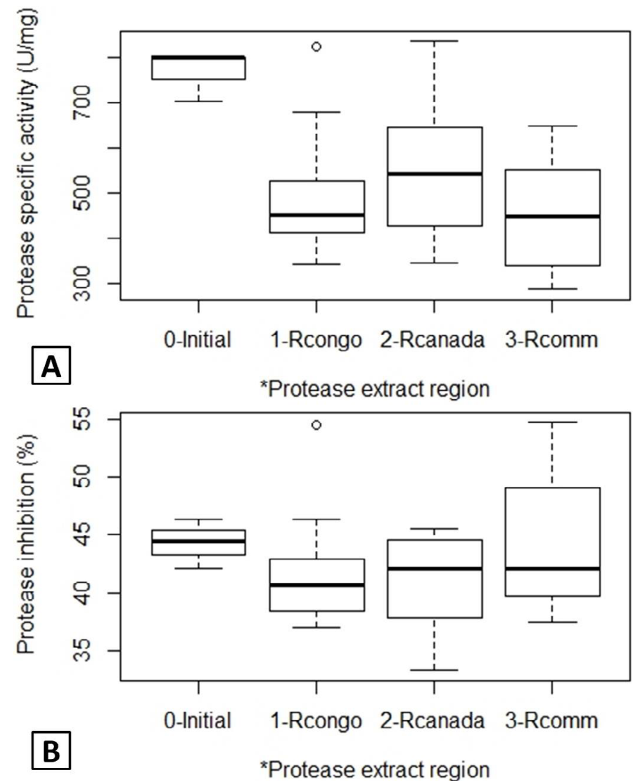

Fig. 3. Enzymatic activity (U/mg) (A) et sensibilité de tilapias aux inhibiteurs végétaux des protéases digestives (%) (B). *Sources d'extraits d'enzymes collectés au temps initial et chez tilapias adultes nourris avec les 3 rations utilisées.

DISCUSSION

Digestive protease activity

With regards to the results of this experiment (Table II; Fig. 1), a variation in the responses of protease activity was observed in association with the diet ($p=0.006$), even though the Rcongo test-diet did not lead to a statistically different activity from Rcanada and Rcommercial. This effect on diets corroborates previous works that reported that the activity of digestive enzymes may vary with fish eating habits, feed composition and nutritional value of diets (Moyano *et al*., 1999; El-Sayed *et al*., 2000; Santos *et al*., 2013). Kohla *et al*. (1992) recorded an improvement in trypsin activity due to increased dietary consumption in *O. mossambicus* and *Colossoma macropomum* tilapias. Different responses of alkaline protease activity were detected by Moyano *et al*. (1999) in three fish species (tilapia, sea bream, and sole), as a consequence of a variation in diets and their nutritional value. The inspection of the digestive tracts of different fish species

by Kolkovski (2001) allowed to identify digestive enzymes that are associated with the dietary metabolism (digestion, absorption and assimilation) of proteins, lipids and glycogen.

However, despite differences in dietary crude protein levels of Rcongo (32.2%) and Rcommercial (51.5%) diets, the presented results (Table II) also showed that the level of protease activity was not significantly different between fish that were fed these two diets. This seems to be in disagreement with the aforementioned researchers who support the concept of an intimate relationship between enzyme activity and fish feeding, resulting in adaptation. For example, a study published in NRC (1993) reported relatively high proteolytic activity in the intestine of tilapias after the administration of protein-rich diets. Indeed, the increase in soluble protein or protease activity in tilapia has been reported as a means of compensating for their inhibition and avoiding the decrease in digestibility of dietary proteins. This increase depends on a change in pancreatic secretion, which varies according to the diet (El-Sayed *et al*., 2000). A similar observation was made in the experiment of Papoutsoglou and Lyndon (2006), who observed an increase in chymotrypsin activity in fish of *Anarhichas minor* species, as a means of adapting to low levels of dietary protein.

Many divergent opinions were also issued in regards to the physiological responses of fish according to their species. Santos *et al*. (2013) stressed the existence of divergent results regarding the relationship between fish enzyme activity and feed composition. For example, by measuring 6 different enzyme activity levels in 11 teleost species, Chakrabarti *et al*. (1995) attributed the observed similarities in enzyme activities to the lacustrine habitat of fish, as these authors did not find a relationship between enzyme activity and dietary habits. According to the work of Haard *et al*. (1996), the physiological adaptation of fish oriented to compensate for protease inhibition by overproducing enzymes was valid in salmonids. However, Anderson *et al*. (1991) reported the difficulty of tilapia to compensate for protease inhibition, hence the consequences leading to decreased digestibility of plant proteins. By comparing herbivorous and carnivorous fish, Chan *et al*. (2004) confirmed the hypothesis that digestive enzyme activity is strongly correlated with fish phylogeny rather than the diet because they did not observe a strong correlation between diet and the profile of digestive enzymes.

Since the effect of the studied diets was not significantly proportional to their crude protein content (Tables I, II), it can be assumed that changes in protease activity, such as the one observed in this study, may be related to *in vitro* testing and sample manipulation. This could also be valid for soluble protein concentrations of the enzyme extracts, which were not significantly different in spite of different crude protein contents of the studied diets. Several authors suggested that the different methods used by various researchers for assessment of enzyme activity in fish further limit possible comparisons (Hidalgo *et al*., 1999; Chan *et al*., 2004). Depending on the fish species and type of tissue analyzed, it was suggested that the level of enzyme activity may vary with ambient temperature during enzyme incubation (Hidalgo *et al*., 1999; Kolkovski, 2001). Other authors also demonstrated variations in enzyme activity due to manipulations during sampling and preparation of enzyme extracts (Alarcón *et al*., 1998; Hidalgo *et al*., 1999; Chong *et al*., 2002).

When considering the age of fish, analysis of Figure 1 shows that enzyme extracts from juveniles of the initial group showed higher protease activity, compared to the adults (represented by five intestinal segments; HL, PMC, GL, DMC, and TS, respectively). This could be justified by the fact that for most species, young fish (larvae and juveniles) are generally exposed to greater protein needs (30–50%) for their growth. They are particularly fond of tiny animals and zooplankton for their nutrition, especially when they are in the wild (El-Sayed, 2006; Hlophe and Moyo, 2013). This nutritional preference could thus predispose them to secrete more proteolytic enzymes in their digestive tract, compared to adults of the same species. These results are supported by a report published in NRC (1993) which recorded relatively higher proteolytic activities in young tilapia compared to adults. On the other hand, the inverse situation was reported by Kolkovski (2001), which observed that enzyme activities were relatively lower in larvae than adults of different species, after feeding.

The present results (Figure 1) nevertheless showed that the first four intestinal segments distinguished themselves from the fifth by their higher proteolytic activity. These observations are consistent with several previous studies conducted on *O. niloticus* tilapia. Tengjaroenkul *et al*. (2000) identified two peptidases (leucine-aminopeptidase and dipeptidyl aminopeptidase IV) on the enterocytes' microvilli of the first four intestinal segments of tilapia, with the strongest activities in the first three intestinal segments. This arrangement is close to that observed in the present experiment, even though the strongest proteolytic activities were recorded in the first four segments rather than the first three, with a very sharp emphasis on the third segment. However, these authors (Tengjaroenkul *et al*., 2000) stressed the importance of these particular intestinal segments because of their content in peptidases capable of peptide hydrolysis, in particular peptides in which the N-terminal position contains the amino acid proline

(dipeptidyl aminopeptidase IV) and all the common amino acids except proline (leucine aminopeptidase). In the light of these results and referring to other research on the morphology and physiology of the intestinal tract of Nile tilapia, it can be considered that although the digestive system of this species is relatively simple and non-specialized, the first four segments, representing more than 90% of the total length of the intestine, have played an important role in the degradation of peptide (Smith *et al*., 2000; Tengjaroenkul *et al*., 2000; El-Sayed 2006). This is consistent with previous research that showed that even though omnivorous fish are not as well equipped as carnivores in terms of digestive proteases that effectively digest feed proteins (Chakrabarti *et al*., 1995; El-Sayed, 2006; NRC, 2011; Chaudhuri *et al*., 2012; Hlophe and Moyo, 2013), they may nevertheless possess a proteolytic potential allowing them to use hardly digestible plant proteins (Kuz'mina, 1990; Kuz'mina and Kuz'mina, 1990; Hidalgo *et al*., 1999). However, the lower enzymatic reaction recorded in the fifth and last segment (Fig. 1) would probably be due to its low participation in the hydrolysis of peptides, due to the resorption of the proteolytic and protein enzymes by the intestinal mucosa and the regressive variations of the pH involved in the distal third of the gastrointestinal tract (Hofer and Schiemer, 1981; Hofer *et al*., 1982; Hidalgo *et al*., 1999; Smith *et al*., 2000; Tengjaroenkul *et al*., 2000; El-Sayed, 2006).

Inhibition of digestive proteases

Considering the results hereby presented, we have observed, same as other authors in previous work (Moyano *et al*., 1999; El-Sayed *et al*., 2000), the existence of protease inhibitors in vegetable diets and ingredients. Their negative effects on digestive proteases and variations in the physiological responses of fish were clear (Tables III, IV; Fig. 2). This is consistent with authors who reported that protease inhibitors are particularly abundant in legume seeds, but also in cereals and agricultural by-products, and a thermal treatment is incapable of completely eliminating the inhibitory effects, especially for oilseeds such as soybeans (Liener, 1989; Alarcón *et al*., 1999, 2001; Francis *et al*., 2001; Drew *et al*., 2007).

Of all five ingredients analyzed in this study, soybean meal had the greatest inhibiting action (Fig. 2; Table IV). Research on the high percentage of protease inhibition in fish from plant-sourced feeds, including soybeans, has been documented by several authors. *In vitro* incubation of treated and untreated soybean meal inhibitors in the presence of tilapia digestive proteases allowed El-Sayed *et al*. (2000) to obtain high inhibition percentages (60.9 ± 0.3% to 80.1 ± 0.5%), which are close to the values obtained in this study. In their experiment on the characterization of intestinal trypsin in tilapia, Zhou *et al*. (2013) detected a strong inhibition of intestinal protease activity, which was induced by both the inhibitors contained in soybeans and different metal ions. According to El-Sayed *et al*. (2000), tilapia is more sensitive to protease inhibitors of soybean meal, with an *in-vitro* level of protease inhibition above 60%.

In addition to tilapia, the inhibitory effect of soybeans and other plant feeds have been evaluated several times in both salmonids and other carnivorous fish. The experience of Moyano *et al*. (1999) revealed that the digestive proteases of tilapia and sea bream were more sensitive to protease inhibitors present in soybean meal, resulting in a very high inhibition percentage (40%), even with very small solution quantities (62.5 mg/unit of activity). Despite the pretreatment of seeds, Alarcón *et al*. (1999) observed the persistence of alkaline protease inhibition in sea bream *Sparus aurata* after having administered a soybean meal-based diet, *i.e.* 42.6 ± 6.7% for raw meal and 39.9 ± 3.0% for extracted meal. In their experience on salmonids, Krogdahl *et al*. (1994) observed a high sensitivity of fish to trypsin inhibitors contained in soy-based diets. Aside from soybean meal, protease inhibition percentages induced by other analyzed plant ingredients (wheat bran, rice bran, brewers grain) were highlighted in this study. Although the sensitivity of digestive proteases to agricultural by-products has not been the subject of many evaluations, their antinutritional effect has been documented by several authors (El-Sayed, 2006; Médale and Kaushik, 2009; Médale *et al*., 2013; Burel and Médale, 2014). For example, the experiment of Nobah *et al*. (2014) on hybrid tilapia showed that rice and wheat bran diets did not induce the best growth rates, when compared to T4GF commercial feed. Bamba *et al*. (2008) did not record a better growth of Nile tilapia fed with wheat and rice bran compared to those that were fed corn bran. However, among the few assessments of the impact of these ingredients on digestive proteases inhibition is the study of Moyano *et al*. (1999), who simulated wheat bran consumption and observed a proportional increase of the inhibitory effect up to 40% in sea bream. On the other hand, sole was highly susceptible to wheat bran with approximately 60% inhibition of protease.

Referring to similar research, the results of this study (Fig. 3) confirmed the hypothesis that omnivorous fish, in this case tilapia, would be more sensitive to protease inhibitors than carnivorous fish (*e.g.,* trout). For example, by incubating intestinal extracts of three different fish species with soybean meal solutions, Moyano *et al*. (1999) found *O. niloticus* showed a high sensitivity (> 60%) to the digestive protease inhibitors present in this ingredient, whereas *Solea senegalensis* (African sole) was highly

resistant (< 30%). These authors made the same statement with corn germ meal, by registering over 20% inhibition for tilapia, and low inhibition (< 20%) was observed for sole and sea bream *Sparus aurata*, two carnivorous species.

It should be recalled that the two test diets' formulation Rcongo and Rcanada was based on a massive incorporation of the plant ingredients. Thus, the fact that there was no significant difference between the effect of Rcongo and Rcommercial on protease inhibition (Table III), would negate the hypothesis that high quantities of plant ingredients in diets would increase inhibitor concentration (El-Sayed, 1999, 2006; Drew *et al*., 2007; Médale *et al*., 2013; Burel and Médale, 2014). However, the high inhibition observed with the *Rcommercial* diet could be due to its components, which remain unknown for most commercial diets. Other authors have also observed high protease inhibition in fish fed commercial feed. Mitchell *et al*. (1993) cited by Alarcón *et al*. (1999) recorded protease inhibition induced by soy-based commercial diet in salmonids ranging from 10 to 86%. El-Sayed *et al*. (2000) also observed significant protease inhibition (37%) after incubating tilapia enzyme extracts with plant solutions of the fish meal-based control diet.

The percentages of protease inhibition recorded with individual ingredients were generally superior to those of the diets (Fig. 2; Tables III, IV). This situation could be justified by the physiological adaptation of the fish's digestive enzymes to the diets used, rather than to the crude ingredients (Haard *et al*., 1996; Santos *et al*., 2013). Moreover, according to the literature, the negative effects related to the use of ingredients containing protease inhibitors could be associated to several factors, such as the type of ingredient, the amount of ingredient incorporated, the duration of the experiment and the sensitivity of the fish species to antinutritional factors (Moyano *et al*., 1999). Two types of inhibitors responsible for negative effects were identified in the plant ingredients: a Kunitz thermolabile inhibitor (20000–25000 moles) with several trypsin-related disulfide bridges, and a thermostable Bowman-Birk inhibitor (6000–10000 moles) containing a high proportion of disulfide bonds capable of inactivating trypsin and chymotrypsin (Kunitz, 1947; El-Sayed *et al*., 2000).

Nevertheless, alternatives have been identified to counteract inhibitory effects of plant ingredients and to improve their digestibility, such as pretreatment and supplementation with exogenous proteases. For example, the experiment presented in Hlophe-Ginindza *et al*. (2016) on *O. Mossambicus* revealed that fish subjected to Natuzyme50® protease supplemented diets showed better growth performance and higher protease activity, compared to the control group. Although many of the aforementioned authors are unanimous that the pretreatment of plant ingredients does not effectively reduce their inhibitory effects on digestive proteases, Alarcón *et al*. (2001) observed a different trend. After studying two snapper species (*Lutjanus argentiventris* and *L. novemfasciatus*), they observed that a pretreatment at very high temperatures was effective in reducing the inhibitory capacity of plant ingredients on alkaline proteases, but that an acid treatment had no effect.

CONCLUSION

This study was conducted to assess the inhibitory effects of plant ingredients on fish digestive proteases. The sources of inhibitors used included three experimental diets and five different agricultural by-products (soybean meal, wheat bran, rice bran, spent corn grain, brewers grains). The results showed that tilapia was more sensitive to protease inhibitors when compared to similar studies. The first four intestinal segments distinguished themselves from the fifth by their higher proteolytic activities. The existence of protease inhibitors in diets and plant ingredients was demonstrated; of all plant ingredients, soybean meal had the greatest inhibiting activity, followed by wheat and rice bran, and brewers' spent grains. Corn grain had the lowest level of protease inhibition (about 20%) than all other studied ingredients. However, *in vitro* methods show certain limitations due to variations in the extracts' responses, and the lack of significant differences between the effects of diets on enzyme activity and protease inhibition. Thus, as many researchers have suggested, the use of other methods such as the zymogram electrophoresis technique (SDS-page) appears to be a more sensitive biochemical tool to detect and thoroughly characterize protease sensitivity to inhibitors (Alarcón *et al*., 1999, 2001; Moyano *et al*., 1999; Santos *et al*, 2013). Finally, supplementation of diets with exogenous protease or further processing to reduce or remove antiproteases should be considered to improve the feeding value of plant-based byproducts.

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Statement of conflict of interest

Authors have declared no conflict of interest.

REFERENCES

Ahmad, N. and Zulqurnain, 2018. Growth performance of two GIFT strains of Nile tilapia *Oreochromis niloticus* reared under semi-intensive culture. *Pakistan J. Zool.,* **50**: 2301-2305.

Alarcón, F.J., Díaz, M., Moyano, F.J. and Abell, E., 1998. Characterization and functional properties of digestive proteases in two sparids; gilthead seabream (*Sparus aurata*) and common dentex (*Dentex dentex*). *Fish Physiol. Biochem*., **19**: 257-267. https://doi.org/10.1023/A:1007717708491

Alarcón, F.J., Moyano, F.J. and Díaz, L.M., 1999. Effect of inhibitors present in protein sources on digestive proteases of juvenile sea bream (*Sparus aurata*). *Aquat. Living Resour*., **12**: 233-238. https://doi.org/10.1016/S0990-7440(00)86633-4

Alarcón, F.J., García-Carreño, F.L. and Navarrete del Toro, M.A., 2001. Effect of plant protease inhibitors on digestive proteases in two fish species, *Lutjanus argentiventris* and *L. novemfasciatus*. *Fish Physiol. Biochem*., **24**: 179-189. https://doi.org/10.1023/A:1014079919461

Anderson, J., Capper, B.S. and Bromage, N.R., 1991. Measurement and prediction of digestible energy values in feedstuffs for the herbivorous fish tilapia (*Oreochromis niloticus* Linn.). *Brit. J. Nutrit*., **66**: 37-48. https://doi.org/10.1079/BJN19910007

Azaza, M.S., Mensi, F., Toko, I.I., Dhraief, M.N., Abdelmouleh, A., Brini, B. and Kraïem, M., 2006. Effets de l'incorporation de la farine de tomate dans l'alimentation du tilapia du Nil (*Oreochromis niloticus*, L., 1758) en élevage dans les eaux géothermales du Sud Tunisien. *Bull. Inst. Natn. Scien. Tech*., **33**: 47-58.

Bamba, Y., Ouattara, A., Da Costa, K.S. and Gourène, G., 2008. Production de *Oreochromis niloticus* avec des aliments à base de sous-produits agricoles. *Sci. Nat*., **5**: 89-99.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem*., **72**: 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Burel, C. and Médale, F., 2014. Quid de l'utilisation des protéines d'origine végétale en aquaculture? *OCL*, **21**: D406. https://doi.org/10.1051/ocl/2014013

Chakrabarti, I., Gani, M.A., Chaki, K.K., Sur, R. and Misra, K.K., 1995. Digestive enzymes in 11 freshwater teleost fish species in relation to food habit and niche segregation. *Comp. Biochem. Physiol. A*, **112**: 167-177. https://doi.org/10.1016/0300-9629(95)00072-F

Chan, A.S., Horn, M.H., Dickson, K.A. and Gawlicka, A., 2004. Digestive enzyme activities in carnivores and herbivores: Comparisons among four closely related prickleback fishes (Teleostei: Stichaeidae) from a California rocky intertidal habitat. *J. Fish Biol*., **65**: 848-858. https://doi.org/10.1111/j.0022-1112.2004.00495.x

Chaudhuri, A., Mukherjee, S. and Homechaudhuri, S., 2012. Diet composition and digestive enzymes activity in carnivorous fishes inhabiting mudflats of Indian Sundarban estuaries. *Turk. J. Fish. aquat. Sci*., **12**: 265-275.

Chong, A.S., Hashim, R., Chow-Yang, L. and Ali, A.B., 2002. Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). *Aquaculture*, **203**: 321-333. https://doi.org/10.1016/S0044-8486(01)00630-5

CNPMT, 2010. *Cadre National des Priorités à Moyen Terme - RD Congo*. Ministère de l'Agriculture, Pêche et Elevage – FAO. Kinshasa, RD, Congo, pp. 56.

Drew, M.D., Borgeson, T.L. and Thiessen, D.L., 2007. A review of processing of feed ingredients to enhance diet digestibility in finfish. *Anim. Feed Sci. Tech*., **138**: 118-136. https://doi.org/10.1016/j.anifeedsci.2007.06.019

El-Sayed, A.F.M., 1999. Alternative dietary protein sources for farmed tilapia, *Oreochromis* spp. *Aquaculture*, **179**: 149-168. https://doi.org/10.1016/S0044-8486(99)00159-3

El-Sayed, A.F.M., Nmartinez, I. and Moyano, F.J., 2000. Assessment of the effect of plant inhibitors on digestive proteases of Nile tilapia using in vitro assays. *Aquacult. Int*., **8**: 403-415. https://doi.org/10.1023/A:1009262407725

El-Sayed, A.F.M., 2006. *Tilapia culture*. Cab International Publishing, London, UK, pp. 294. https://doi.org/10.1079/9780851990149.0000

FAO, 2010. *Globefish*. Food and Agricultural Organization of the United Nations, Rome, Italy.

FAO, 2012. *La situation mondiale des pêches et de l'aquaculture*. Food and Agricultural Organization of the United Nations, Rome, Italy.

FAO, 2015. *Système d'information sur les ressources alimentaires et d'engrais en aquaculture. Nile*

tilapia - Nutritional requirements. Food and Agricultural Organization of the United Nations, Rome, Italy.

Francis, G., Makkar, H.P. and Becker, K., 2001. Antinutritional factors present in plant-derived alternate fish feed ingredients and their effects in fish. *Aquaculture*, **199**: 197-227. https://doi.org/10.1016/S0044-8486(01)00526-9

García-Carreño, F.L., Navarrete del Toro, M.A., Díaz-López, M., Hernandez-Cortes, M.P. and Ezquerra, J.M., 1996. Proteinase inhibition of fish muscle enzymes using legume seed extracts. *J. Fd. Protect*., **59**: 312-318. https://doi.org/10.4315/0362-028X-59.3.312

Haard, N.F., Dimes, L.E., Arndt, R.E. and Dong, F.M., 1996. Estimation of protein digestibility-IV. Digestive proteinases from the pyloric caeca of coho salmon (*Oncorhynchus kisutch*) fed diets containing soybean meal. *Comp. Biochem. Physiol. B*, **115**: 533-540. https://doi.org/10.1016/S0305-0491(96)00189-7

Hidalgo, M.C., Urea, E. and Sanz, A., 1999. Comparative study of digestive enzymes in fish with different nutritional habits. Proteolytic and amylase activities. *Aquaculture*, **170**: 267-283. https://doi.org/10.1016/S0044-8486(98)00413-X

Hlophe-Ginindza, S.N., Moyo, N.A.G., Ngambi, J.W. and Ncube, I., 2016. The effect of exogenous enzyme supplementation on growth performance and digestive enzyme activities in *Oreochromis mossambicus* fed kikuyu-based diets. *Aquacult. Res.,* **47**: 3777-3787

Hlophe, S.N. and Moyo, N.A.G., 2013. The aquaculture potential of Tilapia rendalli in relation to its feeding habits and digestive capabilities. *Phys. Chem. Earth Pt. A/B/C*, **66**: 33-37.

Hofer, R. and Schiemer, F., 1981. Proteolytic activity in the digestive tract of several species of fish with different feeding habits. *Oecologia*, **48**: 342-345. https://doi.org/10.1007/BF00346492

Hofer, R., Dalla Via, G., Troppmair, J. and Giussani, G., 1982. *Differe proteolytic activity in the digestive tract of several species of fish with different feeding habits nces in digestive enzymes between cyprinid and non-cyprinid fish*. Memorie dell'Istituto Italiano di Idrobiologia, Dr. Marco de Marchi, Verbania Pallanza.

Kimou, N.B., Koumi, R.A., Koffi, M.K., Atsé, C.B., Ouattara, I.N. and Kouamé, P.L., 2016. Utilisation des sous-produits agroalimentaires dans l'alimentation des poissons d'élevage en Côte d'Ivoire. *Cah. Agricult*., **25**: 25006. https://doi.org/10.1051/cagri/2016012

Kohla, U., Saint-Paul, U., Friebe, J., Wernicke, D., Bravm, E. and Gropp, J., 1992. Growth, digestive enzyme activities and hepatic glycogen levels in juvenile *Colossoma macropomum* Cuvier from South America during feeding, starvation and refeeding. *Aquacult. Res*., **23**: 189-208. https://doi.org/10.1111/j.1365-2109.1992.tb00610.x

Kolkovski, S., 2001. Digestive enzymes in fish larvae and juveniles-implications and applications to formulated diets. *Aquaculture*, **200**: 181-201. https://doi.org/10.1016/S0044-8486(01)00700-1

Krogdahl, Å., Lea, T.B. and Olli, J.J., 1994. Soybean proteinase inhibitors affect intestinal trypsin activities and amino acid digestibilities in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. A*, **107**: 215-219. https://doi.org/10.1016/0300-9629(94)90296-8

Kunitz, M., 1947. Crystalline soybean trypsin inhibitor. *J. Gen. Physiol*., **30**: 291-310. https://doi.org/10.1085/jgp.30.4.291

Kuz'mina, V.V., 1990. Temperature influence on the total level of proteolytic activity in the digestive tract of some species of freshwater fishes. *J. Ichthyol.*, **30**: 97-109.

Kuz'mina, V.V. and Kuz'mina, Y.G., 1990. Level of total proteolytic activity in some species of fish from the Volga basin. *J. Ichthyol*., **30** : 25-35.

Lazard, J., 2009. La pisciculture des tilapias. *Cah. Agricult*., **18**: 174-182.

Liener, I.E., 1989. Antinutritional factors in legume seeds: State of the art. In: *International workshop on antinutritional factors (ANF) in legume seeds*. Wageningen, Netherlands, 23-25 Nov 1988.

Médale, F. and Kaushik, S., 2009. Les sources protéiques dans les aliments pour les poissons d'élevage. *Cah. Agricult*., **18**: 103-111.

Médale, F., Le Boucher, R., Dupont-Nivet, M., Quillet, E., Aubin, J. and Panserat, S., 2013. Des aliments à base de végétaux pour les poissons d'élevage. *INRA Prod. Anim*., **26**: 303-315.

Meyer, C., 2013. *Dictionnaire des sciences animales*. CIRAD, Montpellier, France.

Mitchell, A.I., Dawson, A. and Houlihan, D.F., 1993. Trypsin inhibitors in commercial fish food. In: *Fish nutrition in practice* (eds. S.J. Kaushik and P. Luquet). Colloques de l'INRA n°61, Biarritz, France, pp. 219-222.

Moyano, F.J., Cardenete, G. and De la Higuera, M., 1992. Nutritive value of diets containing a high percentage of vegetable proteins for trout, *Oncorhynchus mykiss*. *Aquat. Living Resour*., **5**:

23-29. https://doi.org/10.1051/alr:1992004

Moyano, F.J., Martinez, D.I., Diaz, L.M. and Alarcón, F.J., 1999. Inhibition of digestive proteases by vegetable meals in three fish species; seabream (*Sparus aurata*), tilapia (*Oreochromis niloticus*) and African sole (*Solea senegalensis*). *Comp. Biochem. Physiol. B*, **122**: 327-332. https://doi.org/10.1016/S0305-0491(99)00024-3

Nobah, C.S.K., Affourmou, A. and Alla, Y.L., 2014. Formulation d'aliments à base de sous-produits locaux et effets sur la croissance des tilapias hybrides [Tilapia zilii (mâle) x Tilapia guineensis (femelle)] en cages flottantes installées sur le lac de Barrage d'Ayame I (Côte d'Ivoire). *Sci. Vie Terre Agron*., **2**: 85-92. http://publication.lecames.org/index.php/svt/article/download/113/170

NRC, 1993. *Nutrient requirements of warm water, fishes and shellfishes: nutrient requirements of domestic animals*. National Academy Press, National Research Council, Washington, DC, pp. 114.

NRC, 2011. *Nutrient requirements of fish and shrimp*. Animal Nutrition Series, National Academy Press, National Research Council, Washington DC, pp. 346.

Popovic, N.T., Strunjak-Perovic, I., Coz-Rakovac, R., Barisic, J., Jadan, M., Persin Berakovic, A. and Sauerborn Klobucar, R., 2012. Tricaine methane-sulfonate (MS-222) application in fish anaesthesia. *J. appl. Ichthyol*., **28**: 553-564. https://doi.org/10.1111/j.1439-0426.2012.01950.x

Potempa, J., Korzus, E. and Travis, J., 1994. The serpin superfamily of proteinase inhibitors: structure, function, and regulation. *J. biol. Chem*., **269**: 15957-15960.

Pouomogne, V. and Pemsl, D.E., 2008. *Development and status of freshwater aquaculture in Cameroon: Reducing poverty and hanger by improving fisheries and aquaculture*. World Fish Center, Penang, Malaysia, pp. 72.

Papoutsoglou, E.S. and Lyndon, A.R., 2006. Digestive enzymes of *Anarhichas minor* and the effect of diet composition on their performance. *J. Fish Biol*., **69**: 446-460. https://doi.org/10.1111/j.1095-8649.2006.01108.x

Ross, L.G., 2000. Environmental physiology and energetics. In: *Tilapias: Biology and exploitation* (eds. M.C.M. Beveridge and B.J. Mcandrew). Kluwer Academic Publishers, Dordrecht, pp. 89-128. https://doi.org/10.1007/978-94-011-4008-9_4

Santos, J.F., Castro, P.F., Leal, A.L.G., de Freitas Júnior, A.C.V., Lemos, D., Carvalho, L.B. and Bezerra, R.S., 2013. Digestive enzyme activity in juvenile Nile tilapia (*Oreochromis niloticus*, L) submitted to different dietary levels of shrimp protein hydrolysate. *Aquacult. Int*., **21**: 563-577. https://doi.org/10.1007/s10499-012-9589-2

Smith, B.J., Smith, S.A., Tengjaroenkul, B. and Lawrence, T.A., 2000. Gross morphology and topography of the adult intestinal tract of the tilapian fish, *Oreochromis niloticus* L. *Cells Tissues Organs*, **166**: 294-303. https://doi.org/10.1159/000016743

Tengjaroenkul, B., Smith, B.J., Caceci, T. and Smith, S.A., 2000. Distribution of intestinal enzyme activities along the intestinal tract of cultured Nile tilapia, *Oreochromis niloticus* L. *Aquaculture*, **182**: 317-327. https://doi.org/10.1016/S0044-8486(99)00270-7

Voet, D. and Voet, J.G., 1995. *Biochemistry*, Second edition. John Wiley & Sons Inc., New York.

Verri, T., Terova, G., Dabrowski, K. and Saroglia, M., 2011. Peptide transport and animal growth: the fish paradigm. *Biol. Lett*., **7**: 597-600. https://doi.org/10.1098/rsbl.2010.1164

Walter, H.E., 1984. Proteinases: Methods with hemoglobin, casein and azocoll as substrates. *Met. Enz. Anal*., **5**: 270-277.

Whitaker, J.R., 1994. *Principles of enzymology for the food sciences*, 2nd ed. Marcel Dekker, New York.

Zhou, A., Yin, F., Zhao, L., Gong, C., Benjakul, S., Liu, X. and Cao, Y., 2013. Purification and characterization of trypsin from the intestine of genetically improved Nile tilapia (*Oreochromis niloticus*). *J. Aquat. Fd. Prod. Tech*., **22**: 421-433. https://doi.org/10.1080/10498850.2012.658528

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Biochemical Profile of Healthy and *Lernaea* Infected Major and Chinese Exotic Carps

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MHR, Designed the experiment and helped in conduction of experimental trial; FA, executed the experimental work; SA, assisted in the juvenile stocking; KJI, assisted in statistical analysis.

Key words
Biochemical profile, mineral composition, Indian and Chinese carps, *Cyprinus carpio, Catla catla*

ABSTRACT

Changes in the body composition of the host fish in case of disease is an important and informative indicator of type of parasite and level of its intensity. Several anabolic and catabolic changes take place in fish body to counteract this invasion. In present study proximate composition (% of dry matter, ash, protein, fat contents) and mineral composition of Indian and Chinese carps affected with *Lernaea* were compared with those of healthy, unaffected animals. There were differences in the biochemical profiles of whole body tissues between healthy and lernaeid fishes cultured under semi intensive conditions for three months. In healthy experimental fishes there were significant differences between body constituents expect ash contents. In diseased species there was considerable increase in moisture, and ash contents with proportionate decrease in dry matter, protein and fat. Mineral composition was compared between control and treated fishes indicated that all minerals tested varied significantly ($P<0.05$). Variations were quite evident within as well as between groups however; differences were more prominent between *Catla catla* (heavily infected) and *Cyprinus carpio* (with no infection) for Na, K, Ca, Fe, Zn and P. There was decrease in mineral contents in fishes from control group infected with *Lernaea* from that of treated healthy fishes. *Cyprinus carpio* was totally free of *Lernaea* attack hence composition remained the same both in control and treated group.

INTRODUCTION

In Pakistan carp fish farming is on rise in both public and private sector due to its adaptation to the current environment and ease of its culture. Presently *C. catla*, *L. rohita, C. mrigala* (Indian major carps) *C. idellus*, *H. moiltrix* and *C. carpio* (Chinese carps) are successfully cultured in 7829 fish farms covering an area of 45650 acres throughout the country (Punjab Fisheries Department, 2010). They are well accepted among consumers due to their nutritional value presumably having anti-cancerous effects, minimizing risk of heart ailments and consequently prolonging life expectancy (Jhingran and Pullin, 1985; Kulikove, 1978). In addition to its superiority in nutritional value it is also more efficient in converting food to body tissues than other farm animals.

Previously wild waters have been the main sources of food fish supply. From the last several years industrial revolution, drought and indiscriminate fishing has heavily damaged existing fish fauna. People gradually moved from extensive open water fish catches to more controlled facilities with better output. This intensification with better production incentives introduced diseases and other ailments in culture waters due to interactions and close orientations. Parasitic disease, among others remained prominent in this type of culture which not only resulted in heavy losses but still carries major share inflicting heavy losses to this resource (Kabata, 1985).

L. cyprinacea Linnaeus (1758), commonly called the anchor worm is one of the most studied parasites of freshwater fishes. It is widely distributed in Africa, Asia, North America and Europe (Kabata, 1979; Lester and Hayward, 2006; Post, 1987; Roberts, 1989) and recently has been reported in native fishes from Brazil (DeMagalhaes, 2006) and Western Australia (Hassan *et al*., 2008). *L. cyprinacea* is more prevalent in still and slow moving water than in fast flowing streams (Hoffman, 1976). Due to its external attachment this parasite can have serious pathogenic effects on the skin and fins of the fish and young and feeble fish are always more at risk of its attack. Heavy infestation can result in significant crop losses. *L. cyprinacea* is the only species of the genus that is known to have worldwide distribution because it lacks the host specificity shown by most other members of the genus (Tidd, 1934; Uzmann and Rayner, 1958). In current fish culture practices *C. idellus*, *H. molitrix*, *C. catla* and

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L. fimbriatus appeared more susceptible to *Lernaea* attack than *C. carpio* and *L. calbasu* where *L. cyprinacea L. cyprinacea* is apparently totally absent irrespective of to the type of fish culture and species combinations except *L. rohita* which demonstrated comparatively more resistance in monoculture than it experienced in polyculture system. Even when challenged with much higher copepodid intensity, it failed to produce any infectious symptoms (Hemaprasanth *et al*., 2011). Economic losses due to ectoparasite infestation are not restricted to direct damage to fish bodily functions and growth but also disfigure its physical appearance rendering it unsuitable for consumption and for sale if this happens in ornamental fish culture (Piasecki *et al*., 2004).

Immediate after attack parasite, tries to get hold of metabolic products of the host for utilization of its own benefits. Fish respond to this stimulus by manipulating and changing its nutrient contents and further dietary intakes. So changes in the body composition of the host fish in this case is an important and informative indicator of type of parasite and level of its intensity. Different macro and micronutrients play a pivotal role; some are mobilized at the place of infection while others are involved in strengthening body immunity. Among all of them, protein, fats, vitamins and minerals immediately reacts to foreign particles in variable forms, so their accurate assessment can give an idea about the level of infection and status of fish. Therefore, accurate information on the level and nature of fats, protein, vitamins and minerals and when and how they vary in relation to body changes is important for maintaining fish health, growth, reproduction and its suitability for consumers. This data also helps in selecting the most appropriate species for environment linked culture and fitness for human consumption (Dempson et. al., 2004).

Investigations concerning the change of fish bio-chemical composition in relation to their biology and physiology with respect to sex, age, nutrition and environmental factors have been carried out by many workers (Hadjinikolova and Tzekov, 1990; Ottolenghi *et al*., 1995; Hadjinikolova *et al*., 2000; Olivera–Novoa *et al*., 2002; Hadjinikolova and Zaikov, 2006; Hadjinikolova, 2008).

A number of studies on the *Lernaea* infestation, prevalence, intensity and susceptibility have been undertaken and many are on their way (Tasawar and Naseem, 1999; Tasawar *et al*., 2007a; Tasawar *et al*., 2007b; Tasawar *et al*., 2009; Piasecki, 2004; Kir, 2007) on different fish species at their different developmental stages and sexual differences. However, relationship between nutrient composition, parasitic infestations and determination of its health status and which nutrients or their level resist attack of parasite and how and when different remedial measures can be considered what remedial measures, still remains unexplored. Present study was therefore, planned to investigate what type of changes parasites induces in body of fish and if fish body deters its attack what is something special in one species and absent in others which results in selective attack of this parasite.

MATERIALS AND METHODS

Experimental site

The experiment was managed in four earthen rearing ponds (59m × 30.5m × 1.8m) located at Fisheries and Aquaculture Department of University of Veterinary and Animal Sciences, Ravi Campus Pattoki. Locally culturable 6 fish species viz. *C. idellus*, *H. moiltrix*, *L. rohita*, *C. mrigala*, *C. catla* and *C. carpio* were used as experimental animals.

Experimental design

In this trial two-group simple randomized design (Kothari, 2004) was used in which group without any treatment served as "control" and the second group tremed as "treatment" treated with DDVP. Each group randomly received two equal sized ponds. All the ponds were randomly stocked with 6 locally culturable fish species with standard combination ratios (Table I). Fishes in all the ponds received isonitrogenous and isocaloric diets with 40% crude protein and composed of fish meal, guar meal, soybean meal, cotton seed meal, corn gluten and canola meal. Feed was offered to fish @ 4% their wet biomass daily twice a day at 9.00 and 16.00 hours. At the outset of trial all the fish species were weighed, measured and comprehensively examined for presence of *L. cyprinacea* and general health condition. Fishes from both groups were dealt uniformly except administration of regular applications of DDVP (@ 0.25 ppm) in treatment group while there was no any medication in control group. Physico-chemical parameters of pond water were also recorded during the study period.

Proximate analysis

Duplicate samples of healthy and infected fishes of each species were collected from the experimental ponds. Parasites were removed and weight and length of fishes were recorded. Proximate compositions of diets were determined according to the Association of Official Analytical Chemists (AOAC, 2010). Moisture and dry matter test of healthy and infected fish samples were carried out in convection oven at 60 °C till constant weight. Ash determination was carried out in muffle furnace at 550 °C for 5-6 hours. Crude protein in all the samples was subjected to Kjeldahl method and crude fat was calculated according to the Bligh and Dyer (1959) method using Soxhlet apparatus.

Table I.- Morphometric measurements of experimental fish species at the initiation of trial.

Fish Species Stocked	Total No. of Fish	Pond No.	Type of fish group	Weight Range (g)	Avg. Weight (g)	Length Range (cm)	Avg. Length (cm)
L. rohita	450 Fishes/pond	1	treated	285-1040	662.5	26.7-5.2	35.9
C. mrigala	and fed @ 4% body	2	treated	400-1010	705	25.2-45.4	35.3
C. idella	weight twice a day						
H. molitrix	(*L. rohita: C. mrigal: C.*	3	control	280-1530	805	28.1– 50.1	39.1
C. catla	*idellus: H. molitrix: C.*						
C. carpio	*catla: C. carpio*)						
	140: 80: 80: 60: 40: 50 = 450	4	control	535-1260	898	26.7– 47.5	37.1

Mineral analysis

Mineral contents of samples were determined by atomic absorption spectrometry and flame photometry according to the methods of AOAC (2010). Wet digested samples were subjected to flame photometers for the determination of Na and K whereas, phosphorus, iron, zinc, calcium, cupper, and magnesium contents were determined by Atomic Absorption Spectrophotometer (Hitachi model 170-10).

Table II.- Feed formula used during the course of studies.

Ingredients	% Crude protein of ingredient	% Contribution in feed formula	% Protein contribution
Fish meal	50	20	10.0
Soybean meal	45	30	13.5
Maize gluten	60	24	13.7
Wheat bran	14	5.0	0.7
Rice polish	12	3.0	0.36
Maize grains	9.8	8.0	0.78
Molasses	3	8.0	0.24
Mineral mixtures	-	1.0	-
Vitamins	-	1.0	-
Total		100	39.28

Statistical analysis

One way Analysis of variance (Factorial ANOVA) followed by Duncan Multiple Range test was used to evaluate the statistical significance of differences among the species using SAS software version 9.1, however, factorial ANOVA was applied for the comparison of treated and controlled fish parameters. Differences between the means of different parameters were considered significant at $p<0.05$. Pearson correlation was done between Physico-chemical parameters.

RESULTS

Bio-chemical analysis

Proximate analysis of healthy (non infected) fish revealed that there was significant difference between body constituents of experimental species except ash contents. Dry mater was the lowest in *C. idella* (16.33±0.07%) followed by *C. catla* (17.07±0.8%) while it was the highest in *C. mrigala* (23.39±4.34%). Highest value for crude protein was observed in *L. rohita* (26.00±4.24%) which differed significantly from rest of the species in treated group. Crude fat significantly varied in *C. catla* and *C. mrigala* (7.30±0.28 and 10.55±0.92%) whereas values of crude fat remained the same in remaining species (Table III).

In control (infected fishes) group there was considerable increase in moisture, and ash contents with proportionate decrease in dry matter, protein and fat (Table IV). The various chemical constituents varied significantly from that of treated group (Table V). *C. carpio* was totally free of *Lernaea* attack hence composition remained the same both in control and treated group.

Mineral analysis

When mineral composition was compared between control and treated fishes, all minerals tested varied significantly ($P<0.05$). Variations were quite evident within as well as between groups. Differences were more prominent between *C. catla* (heavily infected) and *C. carpio* (with no infection) for Na, K, Ca, Fe, Zn and P (Table VI). Similar differences were also observed among other species too (Tables VI and VII). There was decrease in mineral contents in fishes from control group infected with *Lernaea* from that of treated healthy fishes (Table VII). *C. car*pio was free of such ailments during these studies.

Water quality parameters remained within acceptable ranges and were uniform in ponds from both groups (Table IX).

Table III.- Proximate analysis of fishes from treated group.

Parameters	Dry (%)	Moisture (%)	C. Fat (%)	C. Protein (%)	Ash (%)
C. catla	17.07±0.8^{b}	73.75±0.63^{b}	7.30±0.28^{b}	12.65±0.49^{b}	5.47±2.44^{a}
C. mrigala	23.39±4.34^{a}	66.75±2.33^{c}	10.55±0.92^{a}	13.13±0.00^{b}	3.28±1.24^{a}
L. rohita	24.17±1.48^{a}	77.74±1.22^{ab}	8.1±1.41^{ab}	26.00±4.24^{a}	5.80±0.85^{a}
C. idella	16.33±0.07^{ab}	78.75±0.63^{a}	9.50±2.26^{ab}	13.16±0.05^{b}	3.80±0.28^{a}
H. molitrix	18.89±1.26^{b}	69.15±1.34^{c}	7.55±0.64^{ab}	15.00±1.41^{b}	5.28±0.45^{a}
C. carpio	23.75±2.88^{a}	76.35±2.88^{ab}	9.35±0.77^{ab}	14.00±1.41^{b}	3.1±0.14^{a}

Figures with different superscript letters are significantly different from each other at $p<0.05$.

Table IV.- Proximate analysis of fishes from control group with *L. cyprinacea* infestation.

Species	Dry matter (%)	Moisture (%)	C. fat (%)	C. protein (%)	Ash (%)
C. catla	11.7±0.28^{b}	82.9±0.82^{a}	5.35±0.21^{b}	7.75±0.78^{c}	12.85±0.49^{a}
C. mrigala	20.80±3.25^{a}	76.61±4.34^{ab}	6.9±0.99^{a}	9.95±0.35^{b}	8.80±0.42^{b}
L. rohita	19.95±0.35^{a}	79.20±1.41^{ab}	5.9±0.28^{ab}	23.10±1.4^{a}	8.20±0.99^{b}
C. idella	13.90±0.57^{b}	83.67±0.08^{a}	5.10±0.57^{b}	8.75±0.35^{bc}	9.80±0.85^{b}
H. molitrix	13.75±0.78^{b}	81.10±1.26^{ab}	5.00±0.14^{b}	9.70±0.28^{bc}	13.95±1.06^{a}
C. carpio	23.75±2.88^{a}	76.35±2.88^{ab}	9.35±0.77^{ab}	14.00±1.41^{b}	3.1±0.14^{a}

Table V.- Comparison of macronutrients in fishes from treated and control group.

Species	Treatment	Dry matter %	Moisture %	Fat %	Protein %	Ash %
C. catla	Treated	17.07±0.8^{Ba}	73.75±0.63^{Ba}	7.30±0.28^{Ba}	12.65±0.49^{Ba}	5.47±2.44^{Aa}
	Control	11.7±0.28^{Bb}	82.9±0.82^{Ab}	5.35±0.21^{Bb}	7.75±0.78^{Cb}	12.85±0.49^{Ab}
C. mrigala	Treated	23.39±4.34^{Aa}	66.75±2.33^{Ca}	10.55±0.92^{Aa}	13.13±0.0^{Ba}	3.28±1.24^{Aa}
	Control	20.80±3.25^{Ab}	76.61±4.34^{ABb}	6.9±0.99^{Ab}	9.95±0.35^{Bb}	8.80±0.42$^{B b}$
L. rohita	Treated	24.17±1.48^{Aa}	77.74±1.22^{ABa}	8.1±1.41^{ABa}	26.00±4.24^{Aa}	5.80±0.85^{Aa}
	Control	19.95±0.35^{Ab}	79.20±1.41^{ABb}	5.9±0.28$^{AB b}$	23.10±1.4^{Ab}	8.20±0.99^{Bb}
C. idella	Treated	16.33±0.07^{ABa}	78.75±0.63^{Aa}	9.50±2.26^{ABa}	13.16±0.05$^{B a}$	3.80±0.28^{Aa}
	Control	13.90±0.57^{Bb}	83.67±0.08^{Ab}	5.10±0.57^{Bb}	8.75±0.35^{BCb}	9.80±0.85^{Bb}
H. molitrix	Treated	18.89±1.26^{Ba}	69.15±1.34^{Ca}	7.55±0.64^{ABa}	15.00±1.41^{Ba}	5.28±0.45^{Aa}
	Control	13.75±0.78^{Bb}	81.10±1.26^{ABb}	5.00±0.14^{Bb}	9.70±0.28^{BCb}	13.95±1.06^{Ab}
C. carpio	Treated + control	23.75±2.88^{Aa}	76.35±2.88A^{B}	9.35±0.77^{ABa}	14.00±1.41^{Ba}	3.1±0.14^{Aa}

Note: Figures with different superscript letters are significantly different from each other at $p<0.05$.

DISCUSSION

Proximate analysis

Protein, fats, carbohydrates, vitamins and minerals are the main constituents of fish feed as well as of body. After consumption these nutrients are processed and then finally become part of the body. Fish body is hence a true representative of fish feed. Changes in feed or environmental perturbations changes fish behavior and body processes which ultimately jeopardize nutrient balance of the body and its composition. Nutrient level in muscle and blood tissue is therefore, an important indicator of its functional normality and health status. This issue was comprehensively addressed in current studies and it was investigated how whole fish bio-chemical profile differs in healthy and infected fish and how this state of affairs can be controlled to obviate heavy production and economic losses.

Table VI.- Mineral contents of fishes from treated group.

Minerals	*C. catla*	*C. mrigala*	*L. rohita*	*C. idella*	*H.molitrix*	*C. carpio*
Na	37.96± 0.56^{a}	25.48±0.66^{cd}	34.2±0.42^{b}	24.58±0..86^{d}	34.61±1.32^{b}	27.09±0.34^{c}
K	48.12±0.53^{a}	34.22± 0.45^{d}	44.41±0.61^{b}	41.02± 0.96^{c}	31.85±0.91^{e}	45.30±0.28^{b}
Ca	16.18± 0.40^{a}	13.70±0.00^{c}	15.8± 0.61^{ab}	15.28±0.54^{abc}	16.85±1.06^{a}	14.20±0.98^{c}
Mg	2.82± 0.06^{c}	3.07± 0.01^{b}	2.97± 0.04^{b}	3.00±0.02^{b}	3.05± 0.08^{b}	3.20± 0.007^{a}
Fe	1.45± 0.01^{a}	1.42±0.04^{a}	1.28± 0.07^{b}	1.04±0.01^{c}	1.22± 0.02^{b}	1.04±0.00^{c}
Zn	1.34± 0.06^{a}	0.49± 0.04^{d}	1.01± 0.02^{b}	0.70± 0.10^{c}	0.52± 0.02^{d}	0.91±0.05^{b}
Cu	0.011± 0.00^{bc}	0.05± 0.001^{a}	0.02±0.00^{b}	0.000±0.00^{c}	0.01± 0.01^{bc}	0.005±0.006^{bc}
P	0.79± 0.03^{a}	0.41± 0.007^{c}	0.45±0.04^{c}	0.41± 0.01^{c}	0.66± 0.00^{b}	0.07± 0.008^{d}

Note: Figures with different superscript letters are significantly different from each other at p<0.05.

Table VII.- Mineral contents of fishes from control group infected with *L. cyprinacea*.

Minerals	*C. catla*	*C. mrigala*	*L. rohita*	*C. idella*	*H. molitrix*	*C. carpio*
Na	29.60±0.87^{a}	22.20±0.56^{c}	22.98±0.30^{b}	20.11±0.01^{d}	21.81±0.54^{c}	27.09±0.34^{c}
K	37.88±0.45^{b}	27.12±1.52^{c}	41.82±2.29^{a}	27.75±0.63^{c}	25.50±0.20^{d}	45.30±0.28^{b}
Ca	11.07±0.18^{c}	11.98±0.44^{b}	14.81±0.69^{a}	13.85±0.91^{b}	10.10±0.00^{d}	14.20±0.98^{c}
Mg	1.00±0.00^{b}	2.45±0.21^{a}	1.31±0.01^{b}	2.75±0.21^{a}	1.20±0.000^{b}	3.20± 0.007^{a}
Fe	0.99±0.00^{c}	1.44±0.00^{a}	1.32±0.00^{b}	1.00±0.00^{c}	0.99±0.00^{c}	1.04±0.00^{c}
Zn	0.78±0.12^{b}	0.46±0.05^{c}	1.01±0.01^{a}	0.44±0.03^{c}	0.26± 0.04^{d}	0.91±0.05^{b}
Cu	0.002±0.001^{bc}	0.001±0.00^{b}	0.003±0.001^{a}	0.00±0.00^{c}	0.001±0.00^{b}	0.005±0.006^{bc}
P	0.81±0.08^{a}	0.40±0.03^{c}	0.50±0.02^{b}	0.45±0.006^{c}	0.52± 0.001^{b}	0.07± 0.008^{d}

Note: Figures with different superscript letters are significantly different from each other at p<0.05.

Table VIII. Comparison of minerals (ppm) concentrations in treated and control group.

Species	Health status	Na (ppm)	K (ppm)	Ca(ppm)	Mg (ppm)	Fe (ppm)	Zn (ppm)	Cu (ppm)	P (ppm)
C. catla	Treated	37.95 0.55^{Aa}	48.12 0.53^{Aa}	16.18 0.40^{Aa}	2.82 0.06$^{C a}$	1.45 0.01^{Aa}	1.34 0.06^{Aa}	0.01 0.001^{BCa}	0.79 0.03^{Aa}
	Control	29.60 0.87^{Ab}	37.88 0.45^{Bb}	11.07 0.18$^{C b}$	1.00 0.00^{Bb}	0.99 0.007^{Cb}	0.78 0.12^{Bb}	0.002 0.001$^{BC b}$	0.81 0.08$^{A b}$
C. mrigala	Treated	25.48 0.66^{Da}	34.22 0.45^{Da}	13.70 0.00^{Ca}	3.07 0.01^{Ba}	1.42 0.04^{Aa}	0.49 0.04^{Da}	0.05 0.001^{Aa}	0.41 0.007^{Ca}
	Control	22.20 0.56$^{C b}$	27.12 1.52$^{C b}$	11.98 0.44$^{B b}$	2.45 0.21^{Ab}	1.44 0.007$^{A b}$	0.46 0.05^{Cb}	0.001 0.00^{Bb}	0.40 0.03^{Cb}
L. rohita	Treated	34.25 0.42$^{B a}$	44.41 0.61^{Ba}	15.83 0.61A^{Bab}	2.97 0.04^{Bb}	1.28 0.07^{Bb}	1.01 0.02^{Bb}	0.02 0.00^{Bb}	0.45 0.04^{Cc}
	Control	22.98 0.30^{Bb}	41.82 2.29^{Ab}	14.81 0.69^{Aa}	1.31 0.01^{Bc}	1.32 0.00^{Bb}	1.01 0.01^{Aa}	0.003 0.001^{Ab}	0.50 0.02^{Bbc}
C. idella	Treated	24.58 0.86$^{D a}$	41.02 0.96^{Ca}	15.28 0.54A^{BCa}	3.00 0.02^{Ba}	1.04 0.01^{Ca}	0.70 0.10^{Ca}	0.00 0.00$^{C a}$	0.41 0.01^{Cc}
	Control	20.11 0.01^{Db}	27.75 0.63^{Cb}	13.85 0.91^{Ba}	2.75 0.21^{Aa}	1.00 0.00^{Cc}	0.44 0.03^{Cc}	0.00 0.00^{Ca}	0.45 0.006^{Cbc}
H. molitrix	Treated	34.61 1.32^{Ba}	31.85 0.91$^{E a}$	16.85 1.06^{Aa}	3.05 0.08^{Bb}	1.22 0.02^{Bb}	0.52 0.02^{Dd}	0.01 0.01^{BCbc}	0.66 0.005^{Bb}
	Control	21.81 0.54^{Cb}	25.50 0.20^{Db}	10.10 0.00^{Dc}	1.20 0.00^{Bc}	0.99 0.007^{Cc}	0.26 0.04^{Dd}	0.001 0.000^{Bb}	0.52 0.001^{Bb}
C. carpio	Treated control	27.09 0.34$^{C a}$	45.30 0.28^{Ba}	14.20 0.98$^{C a}$	3.20 0.007^{Aa}	1.04 0.00^{Cc}	0.91 0.05^{Bb}	0.005 0.006^{BCbc}	0.07 0.008^{Dd}

Figures with different superscript letters are significantly different from each other at p<0.05.

Moisture, dry matter, ash, crude fat and protein contents of healthy fish were very close to the previous findings (Zelepuchin, 2007; Hadjinikolova, 2008; Ali *et al*., 2005; Natarajan and Sreenivasan, 1961; Salam and Janjua, 1992; Salam and Mahmood, 1993; Salam and Davies, 1994; Salam, 1983; Kalita *et al*., 2008) and were significantly higher than infected fishes except *C. carpio* which did not show any difference whether normal or infected at the termination of experiment. It is very obvious from our studies that there is drastic reduction of nutrient levels in infected fish which might be due to several reasons, prevention of fish from feeding, mobilization of nutrients to the site of infection and development and strengthening of immune system to defend against attack. Our findings were in accordance with the conclusions of Dörücü (2000) who observed similar trend of nutrient decrease in prawn *(C. lavaretus)* infected by *D. bothrium* species (Cestoda). Findings of Love (1980), Medford and Mackay (1978) corroborate our studies.

Table IX.- Mean values for water quality parameters during the course of study.

Parameters	Mean±Stdv.	Mini-mum	Maxi-mum
DO (ppm)	5.9±0.8	3.5	6.7
pH	8.3±0.4	7	8.9
TDS (ppm)	1421.0±337.5	805	1813
Salinity (ppt)	0.8±0.1	0.8	1.1
Temp.(°C)	31.6±1.9	27.5	35.5
EC (µS/cm)	2.1±0.1	1.9	2.5
PO_4^{-2} (ppm)	0.5±0.5	0	1
NO_3 (µg/L)	0.7±0.2	0.2	1
Cl^{-1} (ppm)	311.4±43.8	220	437
Secchi Disk Visibility (cm)	25.6±9.4	11	28
Zooplanktons (mL^{-1})	664.7±389.2	156	1875
Phytoplankton (mL^{-1})	1291.0±807.0	156	1375

Meakins (1974), Tierney (1991), Milinski (1984), Pennycuick (1971), Walkey and Meakins (1970) reported that parasitic worms of fish endeavor for energy sources with their respective host and cause severe impairment to their body parts. Meakins (1974) reported that infected stickleback female's gonadosomatic index depressed around half of the healthy ones may be due to the competition of parasite for energy with sticklebacks. Tierney (1991) further investigated that infected fish eat less than that of healthy ones irrespective of season and have great effect on the composition of fish as observed in our studies but also on their gonadal development, may be due to the parasite competition for nutrient reserves.

Like other nutrients fats also have direct relationship with percentage of water present in the body and fluctuate with changes in environment as well as in body (Sinclair and Duncan, 1972). These relationships exist in various fish species (Brett *et al*., 1969; Iles and Wood, 1965; Salam *et al*., 1993) and were also present in our studies in both healthy and infected species. Ash contents and moisture increased in infected fish while protein, fats and dry matter of fish body thus reducing its flesh quality. Our findings contradicts to those of Floreto *et al*. (2000) who while working with healthy and shell-diseased lobsters reported that protein, lipid and ash levels in muscles were not significantly different between the two groups but quite significant differences were present when we compared protein and fats between two groups. Protein and fat contents, however, decreased in hepatopancreas and hemolymph of diseased lobster which favorably supports our findings that disease has significant bearing on the nutrient level too.

Changes in liver nutrient levels (Floreto *et al*., 2000) may be due partly its function as nutrient synthesis and reservoir (Factor, 1995; Torreblanca *et al*., 1993) but unlike them we observed all these nutrients in whole body with quite tangible differences between both healthy and diseased fish. Leung *et al*. (1990) was of the view that animal uses them during poor feeding and/or when a disease ensues. In a similar study on American lobster larvae, Anger *et al*. (1985) reported that loss of lipid-storing capacity of the R-cells of the hepato-pancreas was induced by starvation and the larvae eventually died even if feeding was successfully resumed. Direct or indirect consequences of disease may have caused similar damage to the hepato-pancreas of affected fish. Also, if reduction in the hepato-somatic index were due to reduced feed intake, then there would have been a corresponding reduction in the protein content. Prince (1997) noted lower levels of serum protein and lower numbers of circulating hemocytes in the hemolymph of affected lobsters and postulated that blood cells are destroyed through phagocytosis and lysis to ward off the infection.

Mineral analysis

In fish, minerals perform important roles in osmoregulation, intermediary metabolism, and in formation of the skeleton and scales (Lall, 1981). Mineral requirements of fish are difficult to measure because many minerals are required in only trace amounts which fish can absorb through feed consumption and normal respiration. It is also very difficult to obtain mineral-free feed ingredients

for experimental diets. Most practical diets provide the major mineral requirements through fish meal which is also a major source of protein. However, diets which rely heavily on plant protein sources must be supplemented with carefully balanced mineral premixes. The minerals required in finfish diets include calcium, zinc, manganese, cobalt, selenium, iodine and fluorine. The functions of some of these have been described in detail by NRC, 1977 and 1981. The potential for toxicity of minerals must also be carefully assessed since fish are very sensitive to excess amounts of minerals which cause toxicity, weakens fish immune system and ultimately invite variety of infections that may lead to parasitic diseases.

Paterson *et al*. (1981) demonstrated that an imbalance of dietary minerals in certain diets predisposes Atlantic salmon to bacterial kidney disease under specific environmental conditions. They fed Atlantic salmon on a diet containing high levels of iodine (4.5 mg/kg feed) and fluorine (4.5 mg/kg feed) and observed a low incidence of symptomatic bacterial kidney disease than the fish fed with low levels.

Kirchgessner and Schwarz (1986) studied the effect of varied protein and energy supply on the retention of major elements (Ca, P, K, Na, Mg) and trace elements (Fe, Cu, Zn, Mn). They observed that composition of nutrients (crude protein, crude fat, crude ash) determine mineral (Ca, P) contents in carp carcass. With increasing energy supply the Ca and P values diminished. On average, 1 kg of carcass contained 6.1 g Ca, 5.0 g P, 2.1 g K, 0.85 g Na, 0.25 g Mg, 20 mg Fe, 1.1 mg Cu, 63 mg Zn and 0.70 mg Mn. Pirestani *et al*. (2009) reported comparatively higher mineral values than ours which might be due to different source of fishes i.e. Caspian sea. Gökoğlu (2002) reported similar values for Na (60 mg g^{-1}) in both fresh and marine water fish. Özyurt (2009) also observed Na contents of pike perch (66.51 mg/100 g) and common carp (61.25 mg/100 g) in the same ranges. Na content of European catfish (130.10 mg/kg) was significantly higher than the others ($P < 0.05$), but had similarities with blue whiting (136 mg/100 g) (Martínez-Valverde *et al*., 2000).

In general, K level is usually higher than sodium in both *C. catla* and *C. carpio*. K levels were also lower than the findings of Otitologbon *et al*. (1997) for fresh water fish species. Magnesium is needed for bone, protein, and fatty acid formation, formation of new cells, B vitamin activation, muscle relaxation, blood clot formation, and energy metabolism. Aquatic food products, like other animal products, are poor sources of magnesium (Lall, 1995). The magnesium content of freshwater fish species was reported as 18–36 mg/100g by Otitologbon *et al*. (1997) and Lall (1981) reported 18-36 mg 100 g^{-1} and 16-113 mg 100 g^{-1} respectively. Copper contents remained between 0.08 and 0.13 mg 100 g^{-1} that were comparatively lower than previous studies. The high intakes of Cu could cause health problems such as liver and kidney damage. Food and Agricultural Organization permits Cu for human consumption up to 0.3 mg/100 g in fish and fishery products (FAO, 1983). Cu contents of experimental fishes were found to be lower than the legal limit of FAO (1983) that further decreased in the diseased condition that makes the fish deficient in certain minerals.

Fish requires Zn in low concentrations if it exceeds required limits then it causes toxicity. Özyurt (2009) observed Zn contents of 1.25-1.32 mg g^{-1} in pike perch, common carp and European catfish which were comparatively higher than ours. According to Turkish Food Codex (2002/63), Anonymous suggests permitted Zn levels for fish are 5 mg 100 g^{-1}. It is well known that that species, size, age, sex, developmental stage food source, environment (water chemistry, salinity, temperature, and contaminants), and method of food processing (Lall and Parazo, 1995; Otitologbon *et al*., 1997) influence level of minerals and requirements in fish. Diseased fish not only disturbs the nutrient balance in fish body but also make it unfits for human consumption both nutritionally and pathologically. Normal values for mineral analysis were also in accordance of Hussain et al. (2018) in the tilapia. All values for tilapia were within the normal rage given by NRC (1981).

These studies have shown a slightly lower level of protein (12.65±0.49%) and fat (7.30±0.28%) in *C. catla* than rest of the species. The protein was the highest (26.00±4.24%) in *L. rohita* while the fats were the highest (10.55±0.92%) in *C. mrigala* and *C. carpio* the second highest. Similarly looking at mineral profile there is not much difference so it is hard to say that level and type of nutrients are solely responsible for *Lernaea* attack. Pathogenicity is a complex of so many factors, which encompass environmental, biological, and physiological factors so still lot remains to be explored before suggesting any concrete recommendation that which factor is more active and critical in inviting and attracting this parasite.

REFERENCES

Anger, K., Storch, V., Anger, V. and Capuzzo, J.M., 1985. Effects of starvation on moult cycle and hepato-pancreas of stage 1 lobster *Homarus americanus* larvae. *Helgol. Meeresuntersuch.,* **38**: 107–116.

Anonymous, 2002. *Regulation of setting maximum levels for certain contaminants in foodstuffs*. Official Gazette, Iss: 24908.

AOAC., 2010. *Official methods of analysis of the*

association of official's analytical chemists. 17th edn.Association of official analytical chemists, Arlington, Virginia.

Bligh, E.G. and Dyer, W.J., 1959. A rapid method for total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**: 911-917.

Brett, J.R., Shelbousne, J.E. and Shoop, C.T., 1969. Growth rate and body composition of fingerling Socheye salmon *Oncorhynchus mesha* in relation to temperature and ration size. *J. Fish Res.,* **26**: 2364-2394. https://doi.org/10.1139/f69-230

Dempson, J.B., Schwarz, C.J., Shears, M. and Furey, G., 2004. Comparative proximate body composition of Atlantic salmon with emphasis on Parr from fluvial and lacustrine habitats. *J. Fish Biol.,* **64**: 1257-1271. https://doi.org/10.1111/j.0022-1112.2004.00389.x

DeMagalhães, A.L.B., 2006. First record of lernaeosis in a native fish species from a natural environment in Minas Gerais state, Brazil. *Pan-Am. J. aquat. Sci.,* **1**: 8-10

Dörücü, M., 2000. Changes in the protein and lipid content of muscle, liver and ovaries in relation to *Diphyllo bothrium* species (Cestoda) infection in Powan (*Coregonus lavaretus*) from Loch Lomond, Scotland. *Turk. J. Zool.,* **24**: 211–218.

Factor, J.R., 1995. The digestive system. In: *The biology of the lobster*, (Ed. Factor, J.R.) Academic Press, San Diego, CA, USA, pp. 395–440. https://doi.org/10.1016/B978-012247570-2/50037-2

FAO., 1983. Compilation of legal limits for hazardous substances in fish and fishery products*.* FAO, 1983, *Fisher. Circul.,* **464**: 5-100.

Floreto, E.A.T., Deanna, L., Prince, P., Brown, B.R. and Bayer, C., 2000. The biochemical profiles of shell-diseased American lobsters*, Homarus americanus* Milne Edwards. *Aquaculture*, **188**: 247–262. https://doi.org/10.1016/S0044-8486(00)00350-1

Gökoğlu, N., 2002. *Su Ürünleri İşleme Teknolojisi*. Su Vakfı Yayınları, İstanbul.

Hadjinikolova, L. and Tshekov, A., 1990. Chemical composition of carp (*Cyprinus carpio* L.), from different breeding strains. *J. Anim. Sci.,* **27**: 75-79.

Hadjinikolova, L. and Zaikov, A., 2006. Investigations on the chemical composition of pike (*Esox lusius* L.). *Bulg. J. agric. Sci.,* **12**: 337-342.

Hadjinikolova, L., 2008. Investigations on the chemical composition of carp (*Cyprinus carpio L.*), bighead carp (*Aristichthys nobilis* Rich.) and pike (*Esox lusiusL.*) during different stages of individual growth. *Bulg. J. agric. Sci.,* **14**: 121-126.

Hadjinikolova, L., Atanasova, R. and Hubenova-Siderova, T., 2000. Comparative immuno-biochemical characteristic of stocking material of carp (*Cyprinus carpio* L.), big-head carp (*Aristichthys nobilis* Rich.), grass carp (*Ctenopharyngodon idella* Val.) and Tench (*Tinca tinca* L.) raised in polyculture. *Bulg. J. agric. Sci.,* **6**: 675-679.

Hassan, M., Beatty, S.J., Morgan, D.I., Doupe, R.G. and Lymbery, A.J., 2008. An introduced parasite, *Lernaea cyprinacea* L., found on native freshwater fishes in South West of Western Australia. *J. R. Soc. West. Aust.,* **91**: 149-153.

Hemaprasanth, S.R., Raghavendra, A., Sridhar, N., Raghunath, M.R. and Eknath, A.E., 2011. Comparative susceptibility of carp fingerlings to *Lernaea cyprinacea* infection. *Vet. Parasitol.,* **178**: 156-62. https://doi.org/10.1016/j.vetpar.2010.12.029

Hoffman, G., 1967. *Parasites of North American freshwater fishes*. University of California Press, Berkeley and Los Angeles.

Hoffman, G.L., 1976. *Parasites of fresh water fishes. IV. Miscellaneous. The anchor parasite* (Lernaea elegans) *and related species*. FDL 46, Dept. Interior, U.S. Fish Wildl. Serv., Wash. pp.8.

Hussain, M.S, Nosheen, A. Javid, A., Liaquat, S., Shahzad, M.M., Arsalan, M.Z. and Khalid, M.A. 2018. Efficacy of probiotics supplementation on mineral digestibility, haematological parameters and carcass composition of *Oreochromis niloticus* fingerlings fed canola meal based diets. *Pakistan J. Zool.,* **50**: 1825-1834. http://dx.doi.org/10.17582/journal.pjz/2018.50.5.1825.1834

Iles, I.D. and Wood, R.J., 1965. The fat/water relationship in North Sea herring, *Clupea harengus* and its possible significance. *J. Mar. Biol. Assoc.,* **45**: 353-366. https://doi.org/10.1017/S0025315400054886

Jhingran, V.G. and Pullin, R.S.V., 1985. *A hatchery manual for the common, Chinese and India major carps.* Asian Development Bank, Manila, Philippines. pp. 16-17.

Jonsson, N. and Jonsson, B., 1998. Body composition and energy allocation in life history stages of brown trout. *J. Fish Biol.,* **53**: 1313. https://doi.org/10.1111/j.1095-8649.1998.tb00250.x

Kabata, Z., 1985. *Parasites and diseases of fish cultured in tropics*. Taylor and Francis, London. pp. 310.

Kabata, Z., 1979. *Parasitic copepoda of British fishes.* Ry Society, London, pp. 468.

Kalita, P., Mukhopadhyay, P.K. and Mukherjee, A.K., 2008. Supplementation of four non-conventional aquatic weeds to the basal diet of *C. catla* and *C. mrigala* fingerlings: Effect on growth, protein utilization and body composition of fish. *Acta Icthy.*

Piscatoria, **38**: 21-27. https://doi.org/10.3750/AIP2008.38.1.03

Khalil, I.A. and Manan, F., 1990. *Text book of chemistry I. analytical biochemistry.* 2^{nd}Edn. Taj Kutab Khana, Peshawar.

Kir, I., 2007. The effects of parasites on the growth of the crucian carp (*Carassius carassius L.* 1758) inhabiting the Kovada Lake. *Turk. Parazitol. Derg.,* **31**: 162-166.

Kirchgessner, M. and Schwarz, F.J., 1986. Mineral content (major and trace elements) of carp (*Cyprinus carpio*) fed with different protein and energy supplies. *Aquaculture,* **54**: 3–9. https://doi.org/10.1016/0044-8486(86)90248-6

Kothari, C.R., 2004. *Research methodology: Methods and techniques* (second Revised Edition). New Age International Publishers India, pp. 401.

Kulikove, D.I., 1978. *Production of meal, oil and protein, vitamin preparation in the fishing industry.* Amerind Publishing Co., New York, USA.

Lall, S.P. and Parazo, M.P., 1995. Vitamins in fish and shellfish. In: *Fish and fishery products: Composition, nutritive properties and stability* (ed. A. Ruither), CAB Int., UK. pp. 157-186.

Lall, S.P., 1995. Macro and trace elements in fish and shellfish. In: *Fish and fishery products: Composition, nutritive properties and stability* (ed. A. Ruither), CAB International, UK. pp. 187-214.

Lall, S.P., 1981. Minerals -A review, In: *Biological aspects of aquaculture nutrition.* Proc. World Conf. Aquacult. Int. Trade Show, Sept. 20-23, Venice, Italy.

Lester, R.J.G. and Hayward, C.J., 2006. Phylum arthropoda. In: *Fish diseases and disorders: Protozoan and Metazoan Infections*. (ed. P.T.K. Woo), CAB International, Oxfordshire, pp. 466-565. https://doi.org/10.1079/9780851990156.0466

Leung, K.M., Chen, H.L. and Chu, K.H., 1990. *Effects of starvation on the biochemical composition and digestive enzyme activities in the hepato-pancreas of the shrimp Meta penaeusensis* (eds. R. Hirano and I. Hanyu). Procceeding of the Second Asian Fish Forum, Tokyo, Japan, 17–22 April 1989, pp. 445–448.

Linnaeus, C., 1758. *Systema naturæ per regna tria naturæ, secundum classes, ordines, genera, species, cum characteribus, differentiis, synonymis, locis*. 10th ed. Stockholm: Laurentius Salvius., pp. 1–824.

Love, R.M., 1980. *The chemical biology of fishes.* Academic Press, II, London, UK.

Ali, M., Iqbal, F., Salam, A., Sial, F. and Athar, M., 2005. Comparative study of body composition of four fish species in relation to pond depth. *Int. J. Environ. Sci. Tech.*, **2**: 359-364. https://doi.org/10.1007/BF03325897

Martínez-Valverde, I., Periago, M.J., Santaella, M. and Ros, G., 2000. The content and nutritional significance of minerals on fish flesh in the presence and absence of bone. *Fd. Chem.,* **71**: 503–509. https://doi.org/10.1016/S0308-8146(00)00197-7

Meakins, R.H., 1974. A qualitative approach to the effects of the plerocercoid of *Schistocephalus solidus* on the ovarian maturation of the three-spined stickleback, *Gasterosteus aculeatus*. *Para.,* **44**: 73-79. https://doi.org/10.1007/BF00328833

Medford, B.A. and Mackay, W.C., 1978. Protein and lipid contents of gonads, liver and muscle of Northern pike (*Esox lucius*) in relation to gonad growth. *J. Fish. Res. Bd. Canada*, **35**: 213-219. https://doi.org/10.1139/f78-035

Milinski, M., 1984. Parasites determine a predator's optimal feeding strategy. *Behav. Ecol. Sociobiol.,* **15**: 35-37. https://doi.org/10.1007/BF00310212

Natarajan, M.V. and Sreenivasan, A., 1961. Proximate and mineral composition of freshwater fishes. *Ind. J. Fish.,* **8**: 422- 429.

Nutrition Research Council, 1977. *Nutrient requirements of warm water fishes.* Natl. Acad. Sci., Washington, DC. pp. 78.

Nutrition Research Council, 1981. *Nutrient requirements of cold water fishes.* Natl. Acad. Sci., Washington, DC. p. 63.

Olivera-Novoa, M.A., Olivera-Castillo, L. and Martinez-Palacios, C.A., 2002. Sunflower seed meal as a protein source in diets for *Tilapia rendalli* (Boulanger, 1896) fingerlings. *Aqua. Res.,* **33**: 223-229. https://doi.org/10.1046/j.1365-2109.2002.00666.x

Otitologbon, S.A., Agbaji, E.B., Peters, O.A. and Oniye, S.J., 1997. Proximate and mineral composition of three Nigerian freshwater fishes. *J. Sci. Fd. Agric.,* **75**: 312-314. https://doi.org/10.1002/(SICI)1097-0010(199711)75:3<312::AID-JSFA865>3.0.CO;2-U

Ottolenghi, C., Puviani, A.C., Ricci, D., Brighenti, L. and Morsiani, E., 1995. The effect of high temperature on blood glucose level in two teleost fish (*Ictalurus melas* and *Ictalurus puncta*tus). *Comp. Biochem. Physiol. A.,* **11**: 229-235. https://doi.org/10.1016/0300-9629(95)00009-V

Özyurt, G., Abdurrahman, P. and GülBringen, L., 2009. Vitamin and mineral content of pike perch (*Sander lucioperca*), common carp (*Cyprinus carpio*), and European catfish (*Silurus glanis*). *Turk. J. Vet. Anim. Sci.,* **33**: 351-356.

Paterson, W.D., Lall, S.P. and Desautels, D., 1981. Studies on bacterial kidney disease in Atlantic salmon (*Salmo salar*) in Canada. *Fish Pathol.,* **15**: 283-292. https://doi.org/10.3147/jsfp.15.283

Pennycuick, L., 1971. Frequency distribution of a parasite in a population of three-spined sticklebacks, *Gasterosteus aculeatus* L., with particular reference to the negative binomial distribution. *Parasitolog.,* **63**: 389-406. https://doi.org/10.1017/S0031182000079920

Punjab Fisheries Department (PFD), 2010. *Annual report.* 2 Sanda Road Lahore.

Piasecki, W., Goodwin, A.E., Eiras, J.C. and Nowak, B.F., 2004. Importance of copepoda in freshwater aquaculture. *Zool. Stud.,* **43**: 193-205.

Pirestani, S., Ali, S.M., Barzegar, M. and Seyfabadi, S.J., 2009. Chemical compositions and minerals of some commercially important fish species from the South Caspian Sea. *Int. Fd. Res. J.,* **16**: 39-44.

Post, G., 1987. *Textbook of fish health.* T.F.H. Publ. Inc., pp. 288.

Prince, D.L., 1997. *Studies on the etiology and pathogenesis of shell disease in the American lobster, Homarus americanus*. PhD thesis. University of Maine, ME, USA.

Roberts, R.J., 1989. *Fish pathology*. Bailliere Tindal, London, pp. 467.

Salam, A., 1983. *Body composition and the thermal energetics of food consumption and growth in the Northern Pike* (Esox Lucius L.). Ph.D. thesis. Dept. Zool. Univ. Notts. Nottingham.

Salam, A. and Davies, P.M.C., 1994. Body composition of Northern pike (*Esox Lucius* L.) in relation to body size and condition factor. *Fish Res.,* **19**: 193-204. https://doi.org/10.1016/0165-7836(94)90038-8

Salam, A. and Janjua, M.Y., 1992. Morphometric studies in relation to body size of *Labeo rohita*: A cultureable major carp Multan. *J. Res. Sci.,* **3**: 59-63.

Salam, A. and Mahmood, J.A., 1993. Weight length and condition factor relationship of a fresh water under yearling wild *C. catla* (Hamilton) from river Chenab (Multan). *Pakistan J. Zool.,* **25**: 127-130.

Salam, A., Ali, M., Chatta, A.M. and Zaman, Q.U., 1993. Growth performance of cultured exotic silver carp (*Hypophthalmicthys molitrix*) under semi- intensive farming: An ecological approach. *Acta Sci.,* **3**: 89-100.

Sinclair, A.R.E. and Duncan, P., 1972. Indices of condition in tropical ruminants. East Africa. *Africa. J. Ecol.,* **10**: 143-149.

Tasawar, Z., Zafar, S., Lashari, M.H. and Hayat, C.S., 2009. The prevalence of lernaeid ectoparasites in grass carp (*Ctenopharyngodon idella*). *Pakistan Vet. J.,* **29**: 95-96.

Tasawar, Z. and Naseem, R., 1999. Observation on lernaeid parasites of *Ctenopharyngodon idella*. *Acta Sci.,* **9**: 33-38.

Tasawar, Z., Umer, K., and Hayat, C.S., 2007a. Observations on lernaeid parasites of *Catla catla* from a fish hatchery, Muzaffargarh, Pakistan. *Pak. Vet. J.,* **27**: 17-19.

Tasawar, Z., Hanif, M., Lashari, M.H. and Hayat, C.S., 2007b. The prevalence of Lernaeid ectoparasites in Mori (*Cirrhinus mrigala*) fish. *Pak. Vet. J.,* **27**: 175-178.

Tidd, W.M., 1934. Recent infestations of goldfish and carp by the "anchor parasite," *L. carassii*. *Trans. Am. Fish. Soc.,* **64**: 176-180. https://doi.org/10.1577/1548-8659(1934)64[176:RIOGAC]2.0.CO;2

Tierney, J.F., 1991. *Studies on the life history of* Schistocephalus solidus*: Field observation and laboratory experiments.* PhD thesis, University of Glasgow, Glasgow.

Torreblanca, A., Ramo, J. and Diaz-Mayans, J., 1993. Responses of marine organisms to pollutants. Part 2. Effect of sublethal exposure to mercury in the biochemical composition of the hepato-pancreas in *Procambarus clarkia* during the recovery after starvation. *Mar. Environ. Res.,* **35**: 73–77. https://doi.org/10.1016/0141-1136(93)90016-S

Uzmann, J.R., and Rayner, H.G., 1958. Record of the parasitic copepod *Lernaea* in Oregon and Washington fishes. *J. Parasitol.,* **44**: 452-453. https://doi.org/10.2307/3274337

Walkey, M. and Meakins, R.H., 1970. An attempt to balance the energy budget of a host-parasite system. *J. Fish Biol.,* **2**: 261-262. https://doi.org/10.1111/j.1095-8649.1970.tb03294.x

Zelepuchin, V., 2007. *Endogenic various quality concepts in the formation of biological resources of carp fish*. PhD thesis. Academic Press, London, UK.

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Ichthyofaunal Diversity, Physico-Chemical and Health Status of Fishes Inhabiting the River Ravi near Balloki Headworks, Pakistan

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Authors' Contribution
MI conducted the experiments. MHR supervised and executed the experiments. FA assisted in identification of disease samples. NK programmed chemical analysis of water quality parameters. AH analysed the data statistically. MA collected and preserved the fish samples. AJ assisted in manuscript writing.

Key words
Ichthyofaunal diversity, Physico-chemical, Health status, River Ravi

ABSTRACT

Present study was conducted to study the ichthyofaunal diversity and health status of fish inhabiting balloki headworks. To study the morphological parameters, a large number of specimens were captured that belonged to 19 species from different commercial fish landing sites in the year of 2015. The results showed that the species wise order of catch from different landing sites were *Labeo rohita* > *Cirrhinus mirigala* > *Labeo calbasu* > *Oreochromis mossambicus* > *Wallago attu* > *Sperata sarwari* > *Cirrhinus reba* > *Notopterus notopterus* > *Catla catla* > *Labeo boga* > *Cyprinus carpio* > *Chitala chitala* > *Ctenopharyngodon idella* > *Hypophthalmichthys molitrix* > *Mastacembelus armatus* > *Mastacembelus pancalus* > *Channa marulius* > *Channa punctata*. Approximately all the above species were present in all selected sites but *Cyprinus carpio* showed highest catch with the total number of 229 and *Chanan marulius* showed lowest catch with total number of 05 in river Ravi at balloki headworks. In river Ravi during the study period based on winter and summer months, water temperatures ranged between 11-16 and 18-34 (C°), dissolved oxygen (DO) 8.9-9.5 and 7.1-8.0 (mg/l), pH 6.2-8.0 and 6.5-8.0, electrical conductivity (EC) 801-808 and 815-832 (S/m), turbidity 5.0-7.4 and 5.7-60 (FTU), total dissolved solids (TDS) 433-441 and 441-447 (mg/l) respectively. pH and water temperature were the same at all sites. The skin, gills, fins, kidney, liver and gall bladder were all infested with parasites. The study revealed the existence of different species of parasites including: protozoa, monogenean, trematodes and five species of crustaceans. Lernaea on *Catla catla* was observed as the most prevalent species for the parasites whereas, *Aoricthys aor* showed the lowest infestation.

INTRODUCTION

The Punjab Province has a large irrigation system-based on diversion from five main rivers i.e. the Indus, the Jhelum, the Chenab, the Ravi and Sutlej. Punjab irrigation system is accompanied by a set-up of drainage system. The drains were originally constructed to meet farming drainage, counter the problem of water logging and to collect the spare irrigation water and flood water.

The rich diversity of fishes of river Ravi has been rapidly decreasing due to habitation destruction, excess of flood, harmful means of fishing, pollution, extensive use of pesticides and insecticides and introduction of exotic species etc. Taking into consideration the importance of fish and regular monitoring of water bodies for fish diversity, present survey was conducted in some of the lotic water bodies inhabiting the area of Balloki headworks, so that immediate steps regarding conservation may be taken.

Ever increasing anthropogenic activities are severely affecting and demeaning river habitats. This habitat degradation combined with changes in natural flows of rivers is changing the patterns of sharing of the fish fauna in these water bodies. Ganasan and Hughes (1998) found that fish assemblages are particularly useful indicators of ecosystem health especially when they involve high valued species. Therefore, the information on diversity indicates as a very useful tool for differentiating habitats, diagnosing the worldly changes in the ecosystem, and formulating the saving strategies (Costa and Schulz, 2010; Dale and Beyeler, 2001; Lin and Caramaschi, 2005; Sarkar *et al.*, 2010).

River Ravi is one of the tributary rivers of River Indus irrigating the plains of province of Punjab in Pakistan. Only few reports on Ichthyofauna of the Ravi River are available

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(Mirza and Ahmad, 1987; Mirza *et al*., 2006). These reports provided checklists of the fish fauna found but lacked information on any index of faunal diversity. There was an urgent need to determine the current Ichthyofaunal diversity of the river so that it could be incorporated in the development of conservation strategies. Balloki headworks (Latitude: 31° 13’ 10 N, Longitude: 73° 51’ 35 E) on river Ravi is located at a distance of about 42 miles from Lahore in the south-west direction. This barrage is very important for commercial fishing but receives 168 cusecs of effluents from nearly 200 industries and 3136 cusecs of home waste water from Lahore city as reported by Atlas (2002) without any treatment.

Water quality deals with the physical, chemical and natural characteristics in relation to all other hydrological properties. Any characteristic of water that effects the survival, reproduction, development and creation of aquaculture species, influences management decisions, causes environmental impacts or reduces product quality and safety can be considered a water quality up-and-down. Other factors being the same, aquaculture species will be healthier, production will be more, environmental impacts will be less and quality better in culture systems with “good” water quality than in those with “poor” water quality (Lin and Caramaschi, 2005).

The present study was, therefore, carried out to evaluate the fish species diversity and spatial variation in diversity and composition of fish assemblages in river Ravi at balloki headworks.

MATERIALS AND METHODS

Experimental sites

Head balloki is situated at river Ravi. Length of River Ravi in Pakistan is 422 miles. Catchment Area of river Ravi is 15,741 miles2. The Ravi is the smallest of the five main eastern tributaries of the Indus. It rises in the basin of Bangahal and drains the southern slopes of the Dhanladhar. The river leaves the Himalayas at Baseeli in India (Fig. 1).

Fish sampling

Fish species were collected from different the landing sites and used to measure total length (cm), standard length (cm) and body weight (gm) from the commercial fish catch of during the research period (Sep. 2014-May, 2015). The fishes were preserved in 10%formalin solution injections of 20”Formalin were also given in the gut region to the large sized fishes. Parameters like species identification (Mirza, 2003), Shannon-Weaver index and Simpson index were taken and fish samples stored in jars (Shannon and weaver, 1963).

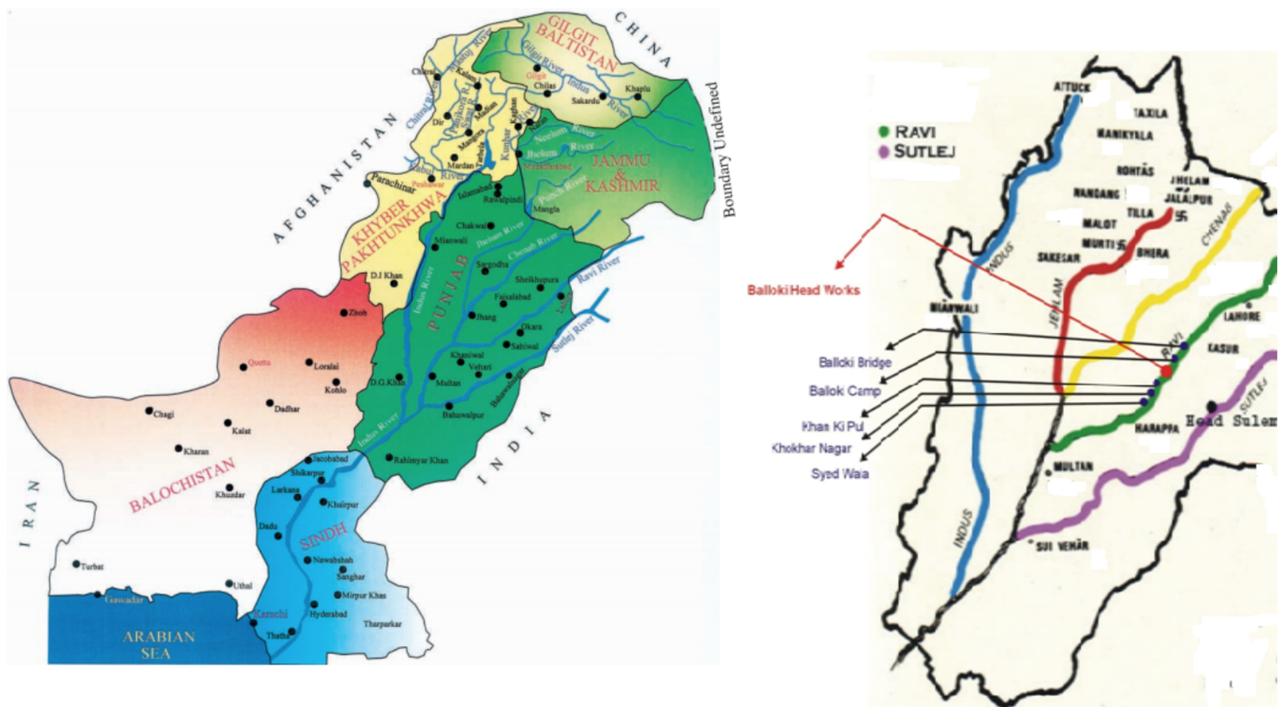

Fig 1: Balloki headworks fish landing sites.1, Balloki bridge landing site; 2, balloki camp landing site; 3, khanki pul landing site; 4, khokhar nagar landing site; 5, syedwala landing site.

Physico-chemical analysis of the water

Water quality parameters were measured through the whole period of the experiment based on the winter and summer months. Water temperatures, dissolved oxygen (DO), pH, Electrical Conductivity (EC), turbidity, total dissolved solids (TDS) were all measured thorough YSI multiparameter.

Water samples for analysis of ammonium ions were collected in 500 ml plastic bottle. The sampling started on 21st September 2014 and continued up to March 22, 2015. At the time of sampling, the air and water temperatures were recorded by using alcohalic bulb and digital thermometers. Light penetration was recorded with the help of Secchi's disk. Dissolved oxygen was determined by using an oxygen meter. pH and conductivity were determined by using digital pH and conductivity meter (Model WTW-pH 720). While all other parameters were determined by the methods as described by Boyd and Tucker (1998).

Examination of fish health

Parasitological examination was performed according to Robert (1978). Skin scrapping of fresh fish samples for external parasites was carried out. The sticky substance obtained from the skin and fins were separately washed into disinfected petri dish using physiological saline. The gills were extracted and also washed into sterile petri dish as with the mucus and viewed under the microscope at x 100 magnification. Wet squash preparations were made of internal organs like kidney, liver and intestine. The squash preparations were fixed for 60 sec in absolute methanol. Slides were then stained with parasitologic iodine microscopic studies. Bacteria were also stained according to Robert, 1978.

RESULTS

The fish diversity survey was conducted on head Balloki Headworks on River Ravi from September, 2014 to April, 2015. Total 1703 fish specimens were collected from landing sites of Balloki, river Ravi belonged to 19 species, 14 genera, 8 families and 7 orders (Table I). *Cyprinus carpio* showed the highest catch with a total number of 229 (13.5 ± 4.3 %) specimens followed by *Wallago attu* (225,13.2 ± 1.8 %) and *Sperata sarwari* (214, 12.6 ± 3.6 %). *Channa marulius* showed the lowest catch of only 5 (0.29 ± 0.3 %). It was observed that large number of fishes were present during winter (October to December) i.e., in the months of low water level than summer. The results showed that highest catch of fishes was recorded in the month of December whereas the lowest catch of fishes was recorded in the month of May (Table II).

Table I.- List of fish species found in the River Ravi.

Sr. No.	Scientific Names	Local Name
1	*Gudusia chapra*	Palli
2	*Notopterus notopterus*	Pari
3	*Labeo calbasu*	Di,Kalbansu,Kalu
4	*Labeo gonius*	Seereha
5	*Labeo rohita*	Rohu,Dambra
6	*Labeo dero*	Challi
7	*Cirrhinus mrigala*	Mrigal,Mori
8	*Cirrhinus reba*	Reba,Sunni
9	*Gibelion catla*	Thaila
10	*Ctenopharyngodon idella*	China rohu
11	*Amblypharyngodon mola*	Mola,Chilwa
12	*Aspidoparia morar*	Common chilwa
13	*Systomus sarana*	Jundoori
14	*Puntius chola*	Chola barb
15	*Puntius ticto*	Rittatus,Popra
16	*Puntius sophore*	Sophore popra
17	*Puntius conchonius*	Rosy barb
18	*Crossocheilus diplocheilus*	Dogra
19	*Garra gotyla*	Pather chat
20	*Osteobrama cotio*	Palero
21	*Chela cachius*	Bidda
22	*Salmophasia bacaila*	Small chal
23	*Salmophasia punjabiensis*	Punjabi chal
24	*Securicula gora*	Big chal
25	*Esomus danricus*	Soomara
26	*Barilius modestus*	Lahori chilwa
27	*Barilius vagra*	Lahori chilwa
28	*Mystus cavasius*	Tingara
29	*Mystus bleekeri*	Bleekri tingara
30	*Mystus vittatus*	Keenger fish
31	*Rita rita*	Tirkanda
32	*Heteropneustes fossilis*	Singhi
33	*Ompok bimaculatus*	Pallu,Pafta
34	*Ailia punctata*	Pootals
35	*Clupisoma garua*	Bachwa
36	*Eutropiichthys vacha*	Jhalli
37	*Pseudeutropius atheri-noides*	Pootlas
38	*Xenentodon cancila*	Cowa tokia, Kan

39	*Channa punctate*	Daula,Guddu
40	*Sicamugil cascasia*	Gachkigura
41	*Chanda nama*	Sheesha
42	*Chanda ranga*	Ranga sheesha
43	*Chanda baculius*	Baculius sheesha
44	*Glossogobius giuris*	Guloo
45	*Colisa fasciata*	Chidu, Kangee, Fider
46	*Colisa lalia*	Choti kangee
47	*Oreochromis aureus* Steindachner	Golden tilapia

Month wise relative percentage of fishes is shown in table. Highest percentage of fishes (18%) was found in the month of December, 2014. Lowest percentage of fishes (1.5) was found in May, 2015. The contribution of some exotic fishes, *Hypophthalmichthys molitrix, Ctenopharyngodon idella* and *Oreochromis mossambicus* has also been shown during the present period of studies, which confirmed the overflow of water from river Ravi during the flooding season. Numerically the most abundant order was Cypriniformes represented by 47.37% of the total individuals followed by Osteoglossiformes, Siluriformes, Channiformes, Mastacembeliformes was represented by 10.53% and Biloniformes and Perciformes were represented by 5.26% respectively. The rank abundance curve of fish species was plotted to display the relative species abundance of the fish species collected (Fig. 1). The species rank abundance plot showed a greater proportion of intermediate abundant species.

In order to show the sampling similarities in different species, the data was subjected to Multivariate Cluster Analysis for preparation of Dendrogram using Euclidian distance. The results showed that at Eucladian distance of almost 16, all the fish species were arranged in five different groups on the basis of their abundance and similarities. *Labeo boga, Notopterus notopterus, Ctenopharyngodon idella* and *Hypophthalmichthys molitrix* formed the first group with less number of fishes. *Xenentodon cancila, Mastacembelus armatus* and *Mastacembelus puncalus* formed second group in this particular river. *Labeo rohita, Wallago attu* and *Cirrhinus mrigala* formed the last cluster with highest number of fishes in the present studies.

Table II.- Different fish species of balloki headworks and the adjoining areas of the river Ravi.

Species	AU	S	O	N	D	J	F	M	APR	MA	Total	% age	MEAN	STDV	VAR	SEM
Chitala chitala	0	3	7	18	20	15	17	3	0	0	83	5	8.3	8.27	68	2.6
Cyprinus carpio	25	24	30	35	45	33	17	10	7	3	229	13.5	22.9	13.5	183	4.3
Labeo calbaso	15	21	21	20	25	21	16	12	10	1	162	9.51	16.2	7.07	50	2.2
Labeo boga	10	8	4	5	7	6	3	7	0	0	50	2.94	5	3.3	11	1
Catla catla	10	8	6	7	10	9	6	4	2	0	62	3.64	6.2	3.36	11	1.1
Sperata sarwari	15	18	25	30	43	30	21	20	9	3	214	12.6	21.4	11.4	130	3.6
Cirrhinus reba	15	11	17	15	17	15	3	2	0	0	95	5.58	9.5	7.34	54	2.3
Labeo rohita	6	3	5	4	7	6	4	3	3	1	42	2.47	4.2	1.81	3.3	0.6
Channa striata	11	7	15	20	25	17	10	8	5	5	123	7.22	12.3	6.75	46	2.1
Wallago attu	20	23	25	28	30	27	23	20	19	10	225	13.2	22.5	5.72	33	1.8
Channa marulius	0	1	0	0	3	0	1	0	0	0	5	0.29	0.5	0.97	0.9	0.3
Xenentodon cancila	10	13	8	9	10	8	7	6	3	1	75	4.4	7.5	3.5	12	1.1
Channa punctuata	15	15	10	6	15	3	3	2	2	1	72	4.23	7.2	5.96	36	1.9
Mastacembelus armatus	0	2	3	5	5	0	1	1	0	0	17	1	1.7	2	4	0.6
Mastacembelus pancalus	0	0	0	1	5	3	2	3	0	0	14	0.82	1.4	1.78	3.2	0.6
Total	195	188	241	222	309	206	145	108	63	26	1703	100	170.3			
Percentage	11	11	14	13	18	12	9	6.3	4	1.5	100					

S, September; O, October; N, November; D, December; J, January; F, February; M, March; Ap, April; Ma, May; STDV, Standard Deviation; VAR, Variance; SEM, Standard Error of Mean.

Table III.- Relative % Representation of fish orders.

No.	No. of Orders	No. of families	No. of genera	No. of species	Relative % representation of order
1	Osteoglossiformes	1	1	2	10.53
2	Cypriniformes	1	6	9	47.37
3	Siluriformes	2	2	2	10.53
4	Beloniformes	1	1	1	5.26
5	Channiformes	1	1	2	10.53
6	Perciformes	1	1	1	5.26
7	Mastacembeliformes	1	1	2	10.53
Total		8	14	19	100

Table IV.- Seasonal fluctuations of diversity indices, species richness and species evenness of fish species in the present studies.

Months	Shannon- Weaver Index (H)	Simpson Index of Dominance (D)	Simpson Index of Diversity (1-D)	Species Richness (SR)	Species Evenness (E)
August, 2014	2.52534	0.08865	18.9113	2.4653	0.95691
September, 2014	2.57466	0.08725	18.9127	3.0555	0.90874
October, 2014	2.54227	0.10081	18.8991	3.0925	0.87956
November, 2014	2.58898	0.091	18.909	3.1362	0.89572
December, 2014	2.67581	0.08306	18.9169	3.1413	0.90876
January, 2015	2.48265	0.10001	18.8999	3.0058	0.87626
February, 2015	2.48652	0.10146	18.8985	3.4206	0.86027
March, 2015	2.36139	0.11649	18.8835	2.799	0.89478
April, 2015	2.03085	0.16402	18.8359	2.1722	0.88198
May, 2015	1.80945	0.21893	18.781	2.4554	0.82351

The value of Shannon-Weaver index (2.67581) and Simpson index of diversity (18.9169) were highest in December and lowest in May (1.80945 and 18.781). Similarly, Simpson index of dominance (D) was highest in May (0.21893) and lowest in December (0.08306). The species richness (SR) was highest in February (3.4206) and lowest in April (2.1722) and species evenness (E) was highest in August (0.95691) and lowest in May (0.82351) (Tables III, IV).

The total discharge of water (Cusecs) in river Ravi at balloki headworks was measured by Flood and Drainage division, Punjab Irrigation Department, Lahore. The highest discharge was in August (45370 Cusecs) whereas the lowest discharge was in the month of January (6614) in the river Ravi at balloki headworks. The fish diversity was rich and consisting of different number of species which was used as a food source as well as for the economical importance.

Monthly variations in physico-chemical parameters

A statistical summary of different water parameters recorded during the whole year shown in table V. Maximum air and water temperature were recorded in May (37.78 ºC and 23 ºC) and minimum in January (16.60 ºC and 11 ºC). Both the temperatures started to increase from February, reached at peak during summer (April and May) and then dropped suddenly in August, mainly due to rain fall and mixing of incoming cold water of river with hot flood plain stagnant water.

pH was maximum in May (8.2) and minimum in March (6.2). pH started increasing steadily from January to May due to increase in temperature until May and dropped from Sep. 2014 to Jan, 2015. The increase in pH in warm months may be due to the increase of $CaCO_3$ in stagnant waters and increased amount of nitrates, phosphates and ultimately eutrophication in summer. DO is a very important indicator of a water body's ability to support

aquatic life. In present studies maximum concentration of dissolved oxygen was present from November (8.0 mg/l) to February (7.68 mg/l) being maximum in December ((9.5 mg/l), while minimum amount of Oxygen was observed in summer, being lowest in May (7.1 mg/l). Oxygen concentration started increasing from Oct, 2014.

Electrical conductivity was highest in May (832 µs/cm)) and lowest in January (801µs/cm). Electrical conductivity increased with increase in temperature. In warm month's evaporation in water bodies resulted in decrease in total quantity of water, causing increase in electrical conductivity. Similar trend was observed in case of total dissolved solids. It is seen that a linear relationship existed between TDS and EC.

Turbidity was found highest in May (60) and lowest was from September to April, being minimum in February (5FTU). Transparency values were recorded maximum in December (142 cm) and minimum in May (27 cm). Transparency and turbidity were inversely proportional to each other. The physico-chemical characteristics of water analyzed during the study period are given in Table V.

Diseases of fishes

The study reveals the existence of different species of parasites including: species of protozoa, monogenean, trematodes and five species of crustaceans (Table VI). Most common parasite was *Lernaea* on C*atla catla* whereas, *Aoricthys aor* showed lowest infestation. Highest parasitic load was recorded at balloki bridge on river Ravi. Class Monogenea is a group of parasitic worms commonly found on fishes. These parasites feed on mucus and epithelial cells of the skin and gills and sometimes on the blood. These parasites found in captive and wild fishes and this is found in Ravi and caused excessive mortalities.

Table V.- Physical-chemical parameters of ravi river from August 2014 to May 2015.

Season	Air Temp.	Landing site	Water temp.	Dissolved oxygen	pH	Salinity	Electrical conductivity (EC)	TDS	Turbidity (FTU)	Visibility (cm)	Total Hardness	Total Alkalinity (mg/l)	Chlorides (mg/l)
Winter months	23.72	1	16c	9.5	8	0.12	808ms/cm	438mg/l	7.4	84.36	136	111	23
	18.28	2	13	8.9	7	0.098	805	435	7.2	83	133	110	25
	16.92	3	11	9	6.2	0.1	802	433	5.2	142	130	111	26
	16.60	4	12	9.1	7.5	0.1	806	440	6.5	108	140	108	35
	29.26	5	13	9	7.1	0.097	801	441	5	100	144	107	27
	31.36	6	14	9.42	8	0.1	808	436	4.80	105	146	114	29
		Mini	11	8.9	6.2	0.097	801	433	5	83	130	107	25
		Maxim	16	9.5	8	0.1	808	441	7.4	142	146	114	35
		Mean	13.17	9.15	7.30	0.10	804.40	437.00	6.02	103.73	138.1	110.17	27.50
		STDEV	1.85	0.25	0.71	0.01	2.87	3.34	1.10	22.81	6.37	2.63	4.29
		S.E.	0.70	0.10	0.28	0.0035	1.29	1.50	0.47	8.76	2.56	1.01	1.71
Summer months	40.32	1	18	7.2	7.2	0.11	815	444	5.7	92	150	113	27
	32.65	2	19	7.1	6.7	0.14	817	445	28	95	152	117	30
	35.34	3	22	8	7	0.14	822	445	22	58	156	118	28
	37.78	4	23	7.3	8.2	0.11	832	442	60	27	146	109.25	24
	29.03	5	22	7.7	6.6	0.13	820	441	29	42	118	98	21
	29.75	6	20	7.2	6.5	0.12	823	447	11	90	126	111	22
		Mini	18	7.1	6.5	0.11	815	441	5.7	27	118	98	21
		Maxim	34	8.0	8.0	0.14	832	447	60	95	156	118	30
		Mean	20.67	7.42	7.03	0.12	821.5	444	25.95	67.33	141.33	111.04	25.33
		STDEV	1.99	0.36	0.63	0.01	6.41	2.29	20.51	28.76	15.64	7.71	3.60
		S.E.	0.80	0.14	0.25	0.01	2.43	0.89	7.79	11.89	6.34	2.95	1.45

Table VI.- Fish health status of the of the landing sites of the River Ravi.

Sr. No.	Landing site	Fish family	Fish	Monogenea and other parasites	Monogenea Family
1	Khokhar nagar landing site	Siluridae	*Wallago attu*	Bychowskella pripathu	Daclylogyridae
				Thaparoclidus gomtius	Daclylogyridae
				Bychowskyella wallagonia	Daclylogyridae
				Chauhanellus indicus	Daclylogyridae
		Cyprinadae	*Ctenotharyngdon idella*	-	-
		Cyprinadae	*Labeo calbsu*	Dactlylogyrus labei	Daclylogyridae
				Dactlylogyrus vicinus	Daclylogyridae
				Paradacly logyrus catalius	Daclylogyridae
		Cyprinadae	*Catla catla*	-	-
		Cyprinadae	*Labeo rohita*	Dactylogyrus batae	Dactylogyrus
				Dactylogyrus glossogobii	Dactylogyrus
2	Khan ki pull landing site	Cyprinadae	*Ctenotharyngdon idella*	Lernaea oryzophila	-
		Cyprinadae	*Labeo calbsu*	Dactlylogyrus labei	Daclylogyridae
		Cichlids	*Oreochromis niloticus*	Learnea	-
3	Balloki bridge landing site	Cichlids	*Cattla cattla*	Ichthyobodo, Apiosoma, Lernaea	-
		Bagridae	*Labeo rohita*	Clonorchis spp.	-
4	Balloki camp landing site	Osphrano-midae	*Chana stritor*	-	-
5	Sayedwala landing site	Bagridae	*Labeo rohita*	Clonorchis spp.	-

D. minutus and *Gyrodactylus elegans* were most common monogenea parasites of the study areas. *Learnea is* found on the fins, skin and gills in *Catla catla* and *Hypophthalmichthys molitrix. Clonorchis spp.* identified in the intestine of *Labeo rohita* and *Cirrhinus mrigala* while protozoan cysts were found in the intestine of *Aoricthys aor*. Parasites were more prevalent in fish of 8 to 14 cm total length size range.

DISCUSSION

The ichthyofaunal diversity study on river Ravi was conducted from Sep. 2014 to Apr. 2015. In this study 19 species of 14 different genera, 8 families and 7 orders were recorded from the river Ravi. The *Cyprinus carpio* was found to be the most dominant fish in river Ravi. The *Cyprinidae* was found to be the most dominant family which includes many species.

In Pakistan about 30 species of which the following economically important species *Labeo rohita*,, *Noemacheilus choprai*, *Noemacheilus Gibelion catla*, *Cirrhinus mrigala*, *Cirrhinus reba*, *Noemacheilus naziri* and *Channa striata*, *Channa marulius*, *Sperata sarwari*, *Wallago attu, Rita rita, Bagarius bagarius, Tenualosa ilisha, Notopterus notopterus* become rare in River Ravi.

Freshwater environments, unlike the marine ones, are subjected to variations in the environmental factors such as temperature, dissolved oxygen, light penetration, turbidity, density, etc. These factors are responsible for division of organisms in different freshwater habitats according to their adaptations, which allow them to survive in that specific habitat (Jaffries and Mills, 1990). The spreading of a fish, therefore, depends entirely on its facility to accommodate itself to a variety of physical conditions and degree of vitality by which it is enable to survive under more or less sudden changes (Rehage and Loftus, 2007). This is not the first study on seasonal variations in physical and chemical parameters of River Ravi but it is first study at balloki headworks. Temperature fluctuations, both diurnal and seasonal are more evident in freshwater habitats.

Flowing waters however, lack wide fluctuations in temperature hence directly related to each other (Odum, 1971). Photoperiod and temperature were maximum in

warmer months (Sep. 2014 and May, 2015). Dissolved oxygen also showed negative relationship with temperature and photoperiod. The minimum value of turbidity (5 FTU) was observed in Feb. 2015 and highest value (60 mg/L) was recorded in the May, 2015 on the onset of floods and rains. Salam and Rizvi (1999) and Ali *et al*. (2003, 2005) reached the same results while working on River Chenab and Rachna Doaab, respectively. Density of river varies at different sites and different times. These differences may be due to variations in temperature and salt concentration of water. Akhtar and Nawaz (2012) also found fluctuations in water density with increase or decrease in total dissolved solids. The change in density due to temperature fluctuations is more important however, specific gravity and density are related with each other.

The pH of water is important because many biological activities can occur only within a narrow range. Thus, any variation beyond acceptable range could be fatal to a particular organism. Akhtar and Nawaz (2012) reported favorable range of pH 6.5-9.0 at daybreak, are suitable for fish production. pH range in the present study was 6.2-8.2 touching the upper limit of favorable range in River Ravi, which indicates that water is suitable for fish production.

Dissolved oxygen showed maximum values in winter season as it is inversely proportion to water temperature (Ali, 1999). Similar type of results was observed in present study as dissolved oxygen decreased with increase in temperature. Dissolved oxygen also had an inverse relationship with photoperiod. When the photoperiod was long, the dissolve oxygen value was low and when photoperiod was short, dissolved oxygen value was high. Ali *et al*. (2000) and Zeb *et al*. (2011) also arrived at the same conclusion. Several factors influence the conductivity including temperature, ionic mobility and ionic valencies. In turn, conductivity provides a rapid mean of obtaining approximate knowledge of total dissolved solids concentration and salinity of water sample (Odum, 1971). Zeb *et al*. (2011) reported that total hardness acts as limiting factor for alkalinity. Calcareous water with alkalinity more than 50 ppm is most productive, zero- 20ppm for low production, 20-40 ppm for medium production and 40-90 ppm for higher production.

Examination of some specimens showed that it was infected with three opportunistic fungal genera, *Aspergillus* sp., *Mucor* sp. and *Rhizopus* sp. The gills, abdomen and caudal fin were the most affected areas by the fungi. *Aspergillus* sp. was the prevalent fungus isolated from koi followed by *Mucor* sp. and *Rhizopus* sp. *Aspergillus* spp. and *Mucor* sp. grew well on three culture media. Posterior part of both the fishes had significantly higher infection than anterior part.

Aspergillo mycosis is principally a disease of tilapia (*Oreochromis* sp.) caused by *Aspergillus sp*. as reported by Olufemi (1985) and Woo and Bruno (2014). These fungal species are infectious through contamination of fish feed (Saleem *et al*., 2012). Moreover, the fungal load increase significantly during storage and is more definite at high moisture levels in ground and tree nuts (Zeb *et al*., 2012). The factors which enhance the threat of fungal infection through feeds include environmental temperature (27°C), humidity level greater than 62% and moisture level in the feed above 14%. Poor water quality is one of the most important factors favoring the growth of fungus (Iqbal *et al*., 2012b). The infection on sensitive area like gills and eyes of fish become fatal, as the growth of fungal hyphae in eyes may cause partial or complete blindness. In such condition the treatment and cure is impossible and ultimately the fishes die (Srivastava, 2009; Iqbal *et al*., 2012a).

The results of the present study were also comparable with studies conducted on the parsitic fauna by Rohde *et al*. (1995), Ho and Kim (1997), Roberts (2004), Siddique *et al*. (2009), Medeiros and Maltchik (2002), Iqbal and Saleemi (2013) and Abbas *et al*. (2014). The most common parasite present in river water body were *Learnea* and *D. minutus*. The differences in species may be due to different climatic conditions, parasite host specificity and different host examined. The absence of *L. lophiara, L. arcuata* in *Ctenopharyngodon idella* in the present study was difficult to explain.

REFERENCES

Abbas, F., Ashraf, M., Hafeez-ur-Rehman, M., Iqbal, K. J., Abbas, S. and Javed, A., 2014. *Lernaea* susceptibility, infestation and its treatment in indigenous major and exotic chinese carps under polyculture system. *Pakistan J. Zool*., **46**: 1215-1222.

Akhtar, S. and Nawaz, M.M., 2012. Impact of water quality on aquatic life in River Ravi, Pakistan. *Nature Environ. Pollut. Technol.,* **11**: 219-224.

Ali, M., Salam, A., Iram, S., Bokhari, T.Z. and Qureshi, K.A., 2005. Studies on monthly variations in biological and physico-chemical parameters of brackish water fish pond, Muzaffargarh, *Pakistan. J. Res. Sci.,* **16**: 27-38.

Ali, M., Salam, A., Jamshaid, S. and Zahra, T., 2003. Studies on biodiversity in relation to seasonal variations in water of river Indus at Ghazi Ghatt, Punjab, Pakistan. *Pak. J. biol. Sci.,* **6**: 1840-1844. https://doi.org/10.3923/pjbs.2003.1840.1844

Atlas-Danmark, 2002. www.atlas-spareparts.com

Boyd, C.E. and Tucker, C.S., 1998. *Pond Aquaculture water quality management*. 1st Edn., Boston, Kluwer Academic, London, ISBN-10: 0412071819, pp: 700.

Claude, E.B. and Tucker, C.S., 1998. *Pond aquaculture water quality management*. Chapman and Hall Aquaculture Series, Springer Science and Business Media.

Costa, P.F. and Schulz, U.H., 2010. The fish community as an indicator of biotic integrity of the streams of Sinus River basin, Brazil. *Brazilian J. biol.,* **70**:1195-1205.

Dale, V.H. and Beyeler, S.C., 2001. Challenges in the development anduse of ecological indicators. *Ecol. Indicat.,* **1**: 3–10. https://doi.org/10.1016/S1470-160X(01)00003-6

Ganasan, V. and Hughes, R.M., 1998. Application of an index of biological integrity (IBI) to fish assemblages of the rivers Klan and Kshipra (Madhya Pradesh), India. *Freshw. Biol.,* **40**: 367-383. https://doi.org/10.1046/j.1365-2427.1998.00347.x

Ho, J.S. and Kim, I.H., 1997. Lernaeid copepod (Cyclopoida) parasites on freshwater fishes of Thailand*. J. nat. Hist.,* **31**: 69 – 84. https://doi.org/10.1080/00222939700770061

Iqbal, Z. and Saleemi, S., 2013. Isolation of pathogenic fungi from a freshwater commercial fish, *Catla catla* (Hamliton). *Sci. Int. (Lahore)*, **25**:851-855.

Iqbal, Z., Iffat. R. and Khalid, P., 2012a. Treatment of *Lernaeasis* in carps with thunder: An organophasphate. *Pakistan J. Zool.,* **44**: 415-420.

Iqbal, Z., Shafqat, A. and Haroon, F., 2012b. *Lernaea* diversity and infection in Indian and Chinese carps under Semi-intensive culture conditions in Lahore, Punjab. *J. Anim. Pl. Sci.,* **22**: 923-926.

Jeffries, M.J. and Mills, D.H., 1990. *Freshwater ecology: Principles and applications*. Belhaven Press, London, New York.

Lin, D.S.C. and Caramaschi, É.P., 2005. Responses of the fish community to the flood pulse and siltation in a floodplain lake of the Trombetas River, *Brazil. Hydrobiol.,* **545**:75-91. https://doi.org/10.1007/s10750-005-2186-x

Mediros, E.S.F. and Maltchik, A.D.L., 1999. The effects of hydrological disturbance on the intensity of infestation of *Lernaea cyprinacea* in an intermittent stream fish community. *J. Arid Environ.,* **43**: 351-356. https://doi.org/10.1006/jare.1999.0545

Mirza, M.R. and Ahmad, I., 1986. Fishes of river Jhelum in Sargodha district. *Biologia*, **33**: 253-263.

Mirza, M.R., 2003. Checklist of fresh water fishes of Pakistan. *Pakistan. J. Zool,* Supl. Ser., **9**: 31-30.

Odum, E.P., 1971. *Fundamentals in ecology*, 3rd Edition, W. B. Saunders Co., Philadelphia.

Olufemi, B.E., 1985. The Aspergilli as pathogens of cultured fishes. In: *Recent advances in aquaculture*, pp. 193-218. https://doi.org/10.1007/978-1-4684-8736-7_5

Rehage, J.S. and Loftus, W.F., 2007. Seasonal fish community variation in head water mangrove creeks in the south western Everglades: An examination of their role as dry down refuges. *Bull. mar. Sci.,* **80**: 625-645.

Roberts, R.J., 2004. *Fish pathology*, 3rd edition. Elsevier Limited, UK.

Rohde, K., Hayward, C., Heap, M. and Gosper, D., 1994. A tropical assemblage of ectoparasites: Gill and head parasites of *Lethrinus miniatus* (Teleostei, Lethrinidae). *Intl. J. Parasitol.*, **24**: 1031–1053. https://doi.org/10.1016/0020-7519(94)90169-4

Salam, A. and Rizvi, M.S., 1999. *Studies on biodiversity and water quality parameters of river Chenab, Muzaffargarh*. Semi. Aquat. Biol. Pakistan, Karachi.

Saleem, M.J., Hanan, A., Nisa, A.U. and Qasir, T.A., 2012. Occurrence of Aflatoxins in maize seed under different conditions. *Int. J. agric. Biol.,* **14**: 473–476.

Sarkar, U.K., Dubey, V.K. and Jena, J.K., 2013. Freshwater fish biodiversity of India: Pattern, utilization, importance, threats and challenges. *Rev. Fish Biol. Fish.* 10.1007/s11160- 013-9306-x

pattern, utilization, importance, threats and challenges. Rev Fish Biol Fish. https://doi.org/10.1007/s11160-013-9306-x

Siddique, M.M.R., Basher, M.A., Hussain, M.A. and Kibria, A.S.M., 2009. Fungal disease of freshwater fishes in natore district of Bangladesh. *J. Bangla. Agric. Univ.,* **7**: 157–162

Srivastava, R.C., 2009. *Fish mycopathology*. Today and Tomorrow's Printers and Publishers, New Dehli, pp. 103.

Woo, P.T.K. and Bruno, B.W.G., 2014. *Diseases and disorders of finfish in cage culture*, 2nd Edition, CABI.

Zeb, B.S., Malik, A.H., Waseem, A. and Mahmood, Q., 2011. Water quality assessment of Siran river, Pakistan. *Int. J. Phys. Sci.*, **6**: 7789 -7798.