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New findings in the searching of an optimal diet for the axolotl *Ambystoma mexicanum*: protein levels

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ABSTRACT

Objective: To determine the protein nutritional requirements in juvenile axolotls, for this purpose, four isocaloric diets (8 % lipids) with 30, 35, 30 and 45 % protein were prepared.

Design/methodology/approach: Six axolotls were used per test, during a period of 81 days. The diets were prepared using fishmeal as a protein source and fish oil as a lipid source. The feed was supplied every 48 hours with 4% of the weight of the biomass of organisms per experimental reservoir. Four biometries were performed throughout the experiment and growth parameters were determined: height, weight gained per day, specific growth rate, survival, Fulton's K, and protein efficiency rate. The digestibility of each of the diets was also determined.

Results: There were significant differences ($p > 0.05$) in the growth and survival of the axolotls, the diet with 45% protein showed the best growth results.

Limitations/Implications: No more protein levels could be tested, due to the number of organisms available for bioassays.

Findings/conclusions: Diets for *A. mexicanum* containing 45% protein level promote good development and survival. This allows for improved cultivation and management plans for the species.

Keywords: nutrition, digestibility, axolotl, amphibian, caudata.

INTRODUCTION

Aquatic animal husbandry requires a thorough understanding of their nutrition, which is relatively new compared to the nutrition of terrestrial farm animals. Aquatic species

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require protein, lipids, carbohydrates, vitamins, minerals and other food additives to meet the physiological needs for growth and reproduction just like terrestrial species. However, there are immense differences between the two since, due to the great diversity of farmed aquatic animals, their nutritional requirements can be radically different (Hertrampf & Piedad-Pascual, 2000).

The nutritional requirements of the different species are a function of several factors, such as: migratory habits, adaptations to the temperature of the environment, type of feeding, stage of life, sex, type of feeding in their natural environment. As well as the behaviors that are directly related to the collection, search and ingestion of nutrients (Gutiérrez-Espinosa *et al.*, 2019; Manopanta *et al.*, 2021; Puchades-Murgadas, 2021). A poorly structured diet can lead to infectious diseases, malnutrition, erratic swimming, and even death (Slight *et al.*, 2015). Most of the studies related to the nutrition of organisms are focused on mammals and birds and in the case of aquaculture to the most popular species with high commercial value. There are few studies that address rearing methods and the development of experimental diets that enhance the growth of amphibian (Bonnet *et al.*, 2002). For the particular case of the genus *Ambystoma*, there are no studies that address the development of inert diets for maintenance in captivity and some enthusiasts limit themselves to giving general recommendations (McWilliams, 2008) or establish feeding habits in free life or in captivity, based on organisms of their habitat (Chaparro-Herrera *et al.*, 2011). The possibility of having a specific diet for this species, considered in danger of extinction, is of priority importance to develop adequate culture and management plans.

MATERIALS AND METHODS

Organism obtention

The juveniles of the axolotl *A. mexicanum* were obtained by donation from the production center Axos-PIMVS, located in Tepic, Nayarit, México. They were transported individually, in plastic bags inside coolers (with coolant gel) to maintain an optimal temperature for transport to the Laboratory for Water Quality and Experimental Aquaculture (LACUIC) belonging to the University of Guadalajara in the city of Puerto Vallarta, Jalisco, México. An average initial size of 17.4±4.2 g and an initial length of 12.2±1.0 cm were recorded for the experimental axolotls. Once in the laboratory, the organisms were quarantined for their observation and monitoring, which consisted of individually separating the axolotls in half-liter plastic containers. As prophylactic treatment, a commercial medicament was administered (Azoo Diesase Treatment®) in a dose of 1 mL per 10 L of water over a period of 45 days. During quarantine and experimental development, a temperature of 19.0±1.2 °C was maintained in a room with a controlled environment. The axolotls were fed a commercial brand of trout diet (Silver cup®) with 55% protein and 12% lipids, with a particle size of 5.5 mm. Water exchanges were carried out with filtered water previously dechlorinated with sodium thiosulfate.

Conditions of the experimental units

Six organisms (measured and weighed) were placed per experimental unit (80 L tubs with a working volume of 25 L). Each treatment was tested in triplicate, with a total of 72 organisms. The bioassay lasted 81 days. During the same, four biometries were performed, one initial, one final and two intermediate. The temperature and water quality conditions were similar to those of acclimatization. The feed was supplied every 48 hours with 4% of the weight of the biomass of organisms per experimental reservoir. The non-ingested feed was removed and a 100% replacement of the water was conducted.

Experimentation

Four diets were prepared with one level of lipids (8%) and four levels of protein (30, 35, 40 and 45%), represented as follows: P30/L8, P35/L8, P40/L8 y P45/L8, with fishmeal as a source of protein and fish oil as a source of fat (Table 1). The ingredients were weighed on a microbalance (Nimbo NBL® (d=0.0001 g)) and mixed in a food processor (Kitchen Aid®) for a period of 15 min until the desired consistency was achieved; The mixture was pelleted in a food mill with a 5.0 mm sieve and allowed to dry in an oven (Novatech®) at 65 °C for 24 h. The feed was cooled and was packed in plastic bags at −4 °C for its conservation and later use.

Proximal analysis

Proximal analysis of the experimental diets were performed according to the protocols established by the A.O.A.C (1995).

Table 1. Formulation and proximal composition of the experimental diets for juvenile axolotls *A. mexicanum*.

INGREDIENTS (g 100 g^{-1})	Experimental treatments			
	P30/L8	**P35/L8**	**P40/L8**	**P45/L8**
Fish meal	36.4	44.0	51.6	59.1
Corn meal	16.0	16.0	16.0	16.0
Fish oil	4.9	4.3	3.7	3.1
Corn starch	33.8	26.8	19.9	12.9
Grenetin	5.0	5.0	5.0	5.0
Vitamins and minerals	3.0	3.0	3.0	3.0
Vitamin C	0.5	0.5	0.5	0.5
Sodium benzoate	0.2	0.2	0.2	0.2
Alpha tocopherol	0.01	0.01	0.01	0.01
Proximal composition (% basis in dry matter)				
Total protein (%)	30.9±0.1	34.9±0.9	40.7±0.1	45.1±0.9
Total lipid (%)	7.6±0.1	7.4±0.8	7.9±0.2	7.6±0.3
Total ashes (%)	11.1±0.1	13.4±0.4	14.2±0.5	15.2±0.4
Nitrogen Free Extracts (ELN)	50.2	44.1	37.0	32.0
Diet digestibility (%)	nd	87.3±0.8	84.0±0.3	77.6±0.1

Values are expressed as mean and standard deviation, nd=The collected feces were not sufficient for this treatment.

Diet digestibility

To determine the digestibility of the diets, feces were collected (using a siphon) for 30 days. Once the feces were collected, they were kept in plastic containers and frozen at −20 °C until subsequent analysis. The determination of the apparent digestibility coefficient (ADC) of the nutrients of the fedd was carried out with the determination of acid-insoluble ashes of the food and feces, with the method proposed by Tejeda-de Hernández (1992) modified by Montaño-Vargas *et al*. (2002).

The acid insoluble ashes (CIA) were determined with the following formula:

$$CIA(\%)=((Ash\ weight\ g-melting\ pot\ weight\ g)/Dry\ sample\ g)\times 100$$

The apparent digestibility (DA) was determined with the formula:

$$DA(\%)=100-((100\times\%\ ash\ in\ food)/\%\ ash\ in\ stool)$$

Biological indices

After 81 days of experimentation, the following growth rates were determined:

Survival was calculated using the formula:

$$SE(t_i)=SP(t_i)\times SP(t_2)\times SP(t_1)$$

where $SP(t_i)$=mean population survival in the interval (t_i-1, t_i).

The specific growth rate was calculated with the formula:

$$(SGR\ \%\ weight\ increase\ per\ day)=[(\ln W_f-\ln W_i)/t]\times 100$$

where W_f=final weight (g); W_i=initial weight (g); t=time (days).

$$Size\ Heterogeneity=CV_{wf}/CV_{wi}$$

where: W_f=final weight; W_i=initial weight.

$$Food\ Conversion\ Factor\ (FCA)=M_i\ (g)/Gp\ (g)$$

where: M_i (g): ingested food; Gp (g): weight gain.

$$Condition\ factor\ (K)=(W/L^3)$$

where: w=wet body weight g; L=length cm.

Statistical analysis

The response variables were final weight, final length, total weight gain, weight gained, weight gained per day, specific growth rate, and survival. To determine the statistical differences among treatments, an analysis of variance was used (ANOVA). In the case of significant differences among the treatments ($p<0.05$) in some variables, a post-hoc Tukey test was used. The tests were carried out with the statistical program Statistica 6.1.

RESULTS AND DISCUSSION

Originally, a duration of 65 days had been established for the development of the bioassays; however, at the end of this period, it was observed that there was an overlap in the final weights in the P35/L8 and P30/L8 diets. Therefore, even though the effect of the diets with higher percentages was already evident at 65 days, it was decided to extend the feeding period by two weeks, with the intention of observing if there was any significant variation. At 81 days, a trend was observed that showed that diets with low percentages of protein can negatively affect the proper development of axolotls.

The results obtained in the present study, show that with a level of 45% protein, the best growths, specific growth rate and survival of *A. mexicanum* were achieved. Figure 1 shows the growth of juvenile axolotls during 81 days of experimentation using four levels of protein and the same level of lipids. Regarding the weight gained after 81 days of experimentation, the treatments showed statistically significant differences ($p<0.05$). The organisms fed with the P45/L8 and P40/L8 treatments obtained a higher average weight of 39.7±11.2 and 34.4±12.1 g, respectively. Comparing the results obtained in this study with others previously published with *Ambystoma mexicanum* is practically impossible. Although there are works that address the problem of the nutrition of the species, most only provide general information without proposing a specific formulated diet based on ad hoc scientific studies. Much of the information published on the feeding

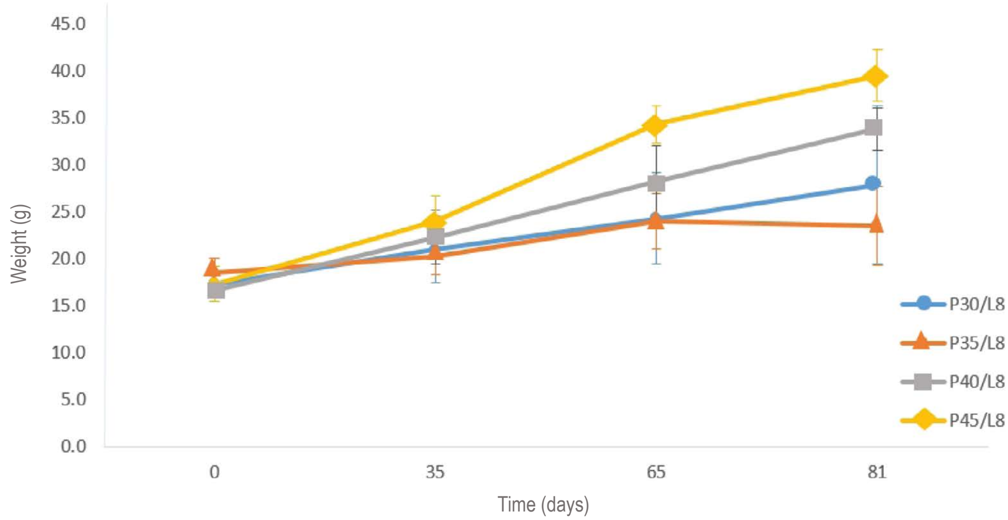

Figure 1. Weight of juvenile axolotls *A. mexicanum* during a period of 81 days of experimentation, fed with four different diets of P30/L8, P35/L8, P40/8 and P45/L8 % (protein/lipids).

of *Ambystoma* is aimed at establishing the diet of animals in the wild or under laboratory conditions, trying to emulate natural conditions, always with consistent diets of living organisms typical of their habitat (Sarma *et al.*, 2017), the behavior and predation preferences of such organisms (Chaparro-Herrera *et al.*, 2011, 2013). García *et al.* (2003), mention the feeding of this species will depend on the stage of development and the size of the organism, feeds mainly on living organisms such as Daphnia, Artemia, algae (Spirulina) and some small insects, even fish fillet and chicken meat. McWilliams (2008) comments that it is advisable to give some live food adding calcium carbonate in the diet to increase the level of this mineral in the body and thus have a balanced diet. Likewise, Wildy *et al.*, (1998) and McWilliams (2008) establish that the mixed diets tested in *Ambystoma* are less effective compared to solid diets, made up of a single high-quality product. Recently, Ocaranza-Joya *et al.* (2021) evaluated the attractability and palatability of various oils in *A. mexicanum*, to determine if they were feasible to be used as attractant additives for species-specific diets. The authors found that krill and chicken oil were effective in promoting the feeding of the tested axolotls. Among the few works that evaluate the performance of diets on the growth of Caudata, specifically *A. mexicanum*, the one by Slight *et al.* (2015), who studied diets based on i) bloodworm, ii) Daphnia and iii) mixed diet of both organisms (alternating their offering to axolotls). The highest growth was achieved with the single bloodworm diet and the lowest with Daphnia, intermediate growths resulted with the mixed diet. Also, Carmen *et al.* (2015) studied the effect of diets, in which they include three probiotics isolated from the same gastrointestinal tract of *A. mexicanum* and added to their diet. It was determined whether these had a beneficial effect on their growth and survival. The vehicle used for the dosage of the probiotics was live *Artemia franciscana* metanauplii. Probiotics were shown to improve axolotl development and survival. The two studies mentioned above are a sample that, despite its great importance as a model organism, species at risk and even ornamental, there are no studies aimed at evaluating specific formulated foods for this or another species of *Ambystoma*.

Feeding with live feed is the usual way in which these animals are kept in captivity, with the implications that this entails in technical resources to maintain support cultures, increased space for facilities and additional financial requirements. In an attempt to address the lack of information on amphibian nutrition, Ferrie *et al.* (2014) mention that the nutritional requirements of the Anura order have been the most studied and that for species whose requirements are unknown, those published for "related species" are used, taking as a reference those of the National Research Council (NRC). In this way, theoretical diets formulated for the various species of amphibians that are poorly understood could be designed. The choice of "related species" may be based on environment, life stage, metabolism or feeding habits in wildlife. These authors suggest that for amphibians in general, the model species that can be used to establish, in a preliminary way, the nutritional requirements are cats and dogs (carnivore and omnivore, respectively), fish (omnivores and carnivores), poultry (requirements for egg production and uric acid excretion) and rats (basic omnivore model). The same authors comment that integrating all the NRC recommendations into a single set of nutrient recommendations for amphibians can provide valuable guidance for offering adequate diets for amphibians; however, they also mention that it is a challenge due to the differences between species. Undoubtedly, trying to establish a diet based on theoretical data can be economical in time, maintenance of organisms and development of growth bioassays with different diets, with the possibility of obtaining favorable results. Unfortunately, the substitution of nutritional experimentation for a set of theoretical data obviates, as the authors of the aforementioned study well mention, the extreme differences that may exist between species, especially in feeding behavior and specific nutritional requirements for growth and reproduction. Even so, they propose a protein percentage of 44% for amphibians in general, which coincides with what was found in the present study. McWilliams (2018) also mentions that for aquatic salamanders, as insectivores, a natural percentage of protein would be between 30 and 60%.

Regarding the development of nutritional research in amphibians, anurans have had a better performance in the production of scientific publications, probably due to their commercial importance as food. Schiesari *et al.* (2009) and Ruibal & Laufer (2012) mention that the diet of amphibians is based on algae and detritus due to their oral disc, but it has been verified by stomach analysis that their feeding range can include organisms with a higher level in the trophic chains. The bullfrog (*Lithobates catesbeianus*), consumes on average between 25 and 35% of the protein in the diet, however, few are the species in which research has been developed to determine their nutritional needs. Carmona-Osalde *et al.* (1996) evaluated the nutritional requirements in bullfrog tadpoles, where they used isocaloric diets (6.0% lipids) and four protein levels (30, 35, 40 and 45%). They evaluated the effect of different protein levels on the metamorphosis and growth of the tadpoles. Obtaining as a result that a 45% protein level was statistically different from the other treatments ($p<0.05$) reflected in better metamorphosis rates, better weight gain and growth of the animals. Result that coincides with the most effective protein percentage found in the present study. Olvera-Novoa *et al.* (2007) evaluated the optimal level of protein in growth for bullfrog juveniles, with different protein levels (20, 28, 34, 42, 50 and 58%), using fish meal as the main protein source. They evaluated survival, growth and feed efficiency after 60 days of experimentation. It was observed that the highest growth was reached with the 58% protein diet, although there was no significant difference between the diets that contained 42, 50% protein, all treatments showed a survival of approximately 83% except for the treatments that contained 20 and 28% protein. They conclude that diets with protein levels between 20 and 34% affected the growth of the bullfrog significantly ($p<0.05$) and that levels greater than 42% showed a better development of the organisms. This coincides with the results of the present study, since after 81 days of experimentation the organisms fed the P45/L8 diet presented the highest weight gain, the best specific growth rate and survival ($p<0.05$).

Determining the digestibility of nutrients in diets provides the first indication of their nutritional value and is considered the first step in their quality assessment (Rahman *et al.*, 2016). In this study, an apparent digestibility was obtained in a range of 77.6 to 87.3%, and the best digestibility was presented by the 35P/8L diet with 87.3±0.8 %,

(Table 1) this coincides with that reported by Vásquez-Torres *et al.* (2013), where they mention that a good apparent digestibility in fish ranges from 76.6 to 84.0%, having fish meal as a protein base in food, which contains a high level of essential amino acids and essential fatty acids for animals, so it is considered an essential product and is classified as a high impact raw material in the development of balanced diets (Coronel-Rodríguez, 2015).

Another very important response variable is the condition factor (K) since this parameter provides information on the strategy that the organism has to grow, its nutritional status and the feasibility of reproduction when it reaches adulthood, describing the relationship length-weight and the well-being during nutrition studies (Cifuentes *et al.*, 2012). A K close to or greater than 1.0% reflects that the organism is in a zero or low state of stress, which indicates that the organisms are kept in suitable conditions for their development. In the present study, all the treatments presented a K greater than 0.94%; being the 45P/8L treatment the one that presented a K greater than 1.04%. These results coincide with those found in other freshwater organisms typical of cold water such as rainbow trout, fed with 46% protein in a growth ration, reporting a K of 1.13% (Morales & Quirós, 2007). Regarding the feed conversion factor and the protein efficiency rate, the best results were obtained by the 35P/8L diet with 2.8±0.3 and 45P/8L with 4.7±0.9 g (Table 2).

Table 2. Biological indices of juvenile axolotls *A. mexicanum* fed four experimental diets with different protein levels 30, 35, 40, 45% and 8% in: weight, length, survival, weight gained, weight gained per day, condition Index (Fulton K), specific growth rate (TCE), specific growth factor (FCE), protein efficiency rate (TEP) during and Food Conversion Factor (FCA) in 81 days.

Biological indices	Experimental treatments			
	P30/L8	P35/L8	P40/L8	P45/L8
Starting weight (g)	17.3 ± 3.5^{a}	18.5 ± 5.0^{a}	16.55 ± 4.7^{a}	17.2 ± 3.6^{a}
Final weight (g)	27.9 ± 7.2^{b}	24.8 ± 9.1^{b}	34.4 ± 12.1^{ab}	39.7 ± 11.2^{a}
Final weight (cm)	12.3 ± 1.1^{a}	12.5 ± 1.0^{a}	12.0 ± 1.1^{a}	12.1 ± 0.9^{a}
Final length (cm)	14.3 ± 1.1^{a}	13.8 ± 1.5^{a}	15.0 ± 1.7^{a}	15.6 ± 1.5^{a}
Total weight gain (%)	61.3 ± 4.4^{b}	35.3 ± 19.8^{b}	102.5 ± 40.1^{ab}	120.1 ± 23.7^{a}
Gained weight (g)	10.6 ± 1.0^{b}	6.5 ± 3.7^{b}	17.2 ± 7.3^{ab}	22.2 ± 3.6^{a}
Weight gained per day (g/day)	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.2 ± 0.1^{ab}	0.2 ± 0.0^{a}
Specific growth rate	0.2 ± 0.0^{b}	0.1 ± 0.1^{b}	0.3 ± 0.1^{ab}	0.4 ± 0.1^{a}
Survival (%)	83.3 ± 0.0^{a}	88.8 ± 9.6^{a}	77.7 ± 9.6^{a}	88.8 ± 9.6^{a}
Weight gained per day (g/day)	0.1 ± 0.0^{b}	0.1 ± 0.0^{b}	0.2 ± 0.1^{ab}	0.2 ± 0.0^{a}
FCE (%)	0.08	0.11	0.09	1.51
TCE (%)	0.15±0.0	0.29±0.0	0.23±0.1	0.37±0. 0
Fulton K (%)	0.95±0.05	0.94±0.10	0.98±0.11	1.04±0.15
TEP (%)	4.6±0.4	3.7±0.3	4.0±1.3	4.7±0.9
FCA (%)	2.6±0.0	2.8±o.3	2.6±0.3	2.0±0.3

Means with different superscripts within a row are significantly different (one-way ANOVA, $p<0.05$).

CONCLUSION

According to the results obtained in this study, it is recommended to include a level of 45% protein in the diets of *Ambystoma mexicanum* juveniles. These results contribute to the formulation of specific diets for the optimal development and nutrition of the axolotl in captivity conditions, which allows to expand the knowledge about the species to improve management and cultivation plans.

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