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RESEARCH ARTICLE

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Actions of 17β-estradiol on carbohydrate metabolism in liver, gills, and brain of gilthead sea bream *Sparus auratus* during acclimation to different salinities

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Abstract The influence of gonadal maturation on seawater adaptability at the metabolic level was assessed in the euryhaline teleost *Sparus auratus* by treatment of immature fish with 17ß-estradiol (E_2) followed by acclimation to different environmental salinities. Fish were injected with coconut oil alone (sham) or containing E_2 (10 µg g^{-1} body weight) and maintained in seawater (40‰). After 5 days, fish from oil- and E_2-implanted groups were sampled. At the same time, fish of both groups were transferred to brackish water (5‰, hypoosmotic test), seawater (40‰, transfer test), and hypersaline water (55‰, hyperosmotic test); 4 days after transfer (9 days post-implant) all groups were sampled. Data obtained from liver suggest that E_2 treatment produced effects comparable to those already reported in literature, including decreased glycogen levels, increased glycolytic potential, and decreased gluconeogenic potential. This, together with the fact that many changes displayed in shams among salinities disappeared in E_2-treated fish allow us to suggest that the response of liver carbohydrate metabolism to osmotic acclimation is exceeded by the response elicited by E_2 treatment. In gills, E_2 treatment produced increased lactate levels, decreased capacity for use of exogenous glucose, and decreased the potential of the pentose phosphate pathway. These findings suggest that the energy demand occurring in gills during osmotic acclimation should be increasingly fuelled by substrates other than exogenous glucose. Finally, data obtained in brain of E_2-treated fish suggest a lower necessity of exogenous glucose, increased lactate levels, and decreased glycolytic potential.

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Introduction

Gilthead sea bream (*Sparus auratus*) is a euryhaline teleost capable of adapting to extreme changes in environmental salinity (Chervinski 1984; Mancera et al. 1993a, 1993b). The osmoregulatory system of this species has been analyzed previously (Mancera et al. 1993a, 1993b, 1994, 1995). Recently, we have also examined, in this species, the influence of different hormones (prolactin, growth hormone, and cortisol) on osmoregulatory capacity (Mancera et al. 2002), the metabolic actions of cortisol (Láiz-Carrión et al. 2002, 2003), and the influence of acclimation to different salinities on energy metabolism of gills, kidney, liver, and brain (Sangiao-Alvarellos et al. 2003). Metabolic changes observed argue for a temporary reallocation of energy resources and increased energy demand in both osmoregulatory and non-osmoregulatory organs during osmotic acclimation. The increased energy demand at the osmoregulatory epithelia is predominantly supported by oxidation of glucose obtained from the circulation (Mommsen et al. 1985; Perry and Walsh 1989). Any alterations in the availability, mobilization, or oxidation of energy substrates could likely affect the osmotic acclimation process.

One process known to alter the availability of energy substrates is gonad maturation and reproduction

(Mommsen and Walsh 1988). The process of gonad development is energetically expensive and known to produce alterations in the energy metabolism of gonads and liver (Soengas et al. 1995). The administration of 17ß-estradiol (E_2) to immature fish is known to induce some of the physiological changes occurring in sexually maturing teleosts, including those at the metabolic level in the liver (Mommsen and Walsh 1988). Thus, E_2 treatment significantly alters the metabolic flux in the liver and the supply of metabolites from peripheral tissues needed for increased hepatic synthesis of the precursor protein vitellogenin (Korsgaard and Mommsen 1993; Woo et al. 1993). Increased levels of plasma vitellogenin have been addressed in immature gilthead sea bream after treatment with E_2 (Mosconi et al. 1998; Guerreiro et al. 2002).

The influence of sexual maturation on seawater adaptability has been well established in several species of teleosts (McCormick and Naiman 1985; Le François et al. 1997). In addition, treatment with E_2 alters the chloride cell density as well as the Na^+,K^+-ATPase activity in gills (Madsen and Korsgaard 1991; Madsen et al. 1997). Thus, considering that E_2 treatment induces high rates of protein synthesis, the ability of fish to cope with exposure to different environmental salinities will be compromised in a euryhaline teleost like gilthead sea bream and, therefore, the known metabolic responses observed in this species during osmotic acclimation (Sangiao-Alvarellos et al. 2003) could be altered by E_2 treatment. A similar approach was taken earlier by Vijayan et al. (2001), who showed that the carbohydrate metabolism of liver and gills in tilapia acclimated to 50% seawater was modified after 24 h of E_2 exposure. However, there are no studies in which: (1) a significant number of parameters related to carbohydrate metabolism were assessed; (2) information was provided regarding metabolic changes in tissues other than liver and gills, such as brain; (3) a wide range of different salinities was assessed; and (4) metabolic changes were evaluated in the time period of steady-state acclimation to salinity, which, in the species assessed, the gilthead sea bream, is known to be reached after 4 days of acclimation (Guzmán et al. 2004; Láiz-Carrión et al., unpublished data).

Therefore, to assess whether or not changes in carbohydrate metabolism observed during the chronic regulatory period of osmotic acclimation (Sangiao-Alvarellos et al. 2003) are modified by gonadal maturation, we treated fish with E_2 followed by subsequent acclimation to seawater (SW, 40‰), brackish water (BW, 5‰), and hypersaline water (HSW, 55‰). Under those experimental conditions, levels of several metabolites and the activities of key enzymes of the major pathways of carbohydrate metabolism (use of exogenous glucose, glycolysis, glycogenolysis, gluconeogenesis, and pentose phosphate) were assessed in osmoregulatory (gills) and non-osmoregulatory (brain and liver) organs of gilthead sea bream.

Materials and methods

Fish

Immature gilthead sea bream (*Sparus auratus* L., 60–70 g body weight) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cadiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories at the Faculty of Marine Sciences (Puerto Real, Cádiz). They were acclimated to SW in 300-l aquaria at a final density of 5 kg m^{-3} for, at least, 2 weeks in an open system (40‰ salinity, 1,000 mOsm/kg H_2O) before the experiments. During the experiments (June), fish were maintained under natural photoperiod and constant temperature (18°C). Fish were fed daily with 1% body weight commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). Proximate food analysis was: 48% crude protein, 6% carbohydrates, 25% crude fat, and 11.5% ash (20.2 MJ kg^{-1} of feed). They were fasted for 24 h before hormone injection and sampling. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the University of Cádiz (Spain) for the use of laboratory animals.

Experimental design

Fish were implanted with slow-release coconut oil implants alone (sham) or containing E_2 at a dose of 10 µg g^{-1} body weight. A homogeneous suspension of the hormone was prepared in coconut oil and injected intraperitoneally according to methods previously described (Láiz-Carrión et al. 2003). This dose has been previously used in this species by other authors who showed: (1) a chronic increase in E_2 levels during, at least, 2 weeks with levels similar to those observed in nature during sexual maturation (Mosconi et al. 1998; Guerreiro et al. 2002; Cavaco et al. 2003) and (2) increased levels of plasma vitellogenin similar again to those found in nature (Mosconi et al. 1998; Guerreiro et al 2002). Fish were caught by netting, lightly anaesthetized with 2-phenoxyethanol (0.05% v/v), weighed, injected intraperitoneally (5 µl g^{-1} body weight) with E_2 (Sigma Chemical), and returned to the tanks. Then, 5 days after implantation, 12 fish of the oil-implanted group (sham) and 12 fish of the E_2-implanted group were sampled. Moreover, seven uninjected fish were also sampled and served as basal values to assess the action of the vehicle.

After sampling, sham and treated fish were separately transferred to separate tanks containing BW, HSW, or SW. BW (5‰ salinity, 130 mOsm/kg H_2O) was obtained by mixing SW with dechlorinated tap water in a recirculated system. HSW (55‰ salinity, 1,554 mOsm/kg H_2O) was obtained by mixing full SW with natural marine salts (Unionsal, Cádiz, Spain) in a recirculated system. The two-tank systems (sham and treated)

containing SW were also recirculated to be comparable with those of BW and HSW fish. In the recirculated systems the common water quality criteria (hardness and the levels of oxygen, carbon dioxide, hydrogen sulfide, nitrite, nitrate, ammonia, calcium, chlorine, and suspended solids) were assessed, with no major changes being observed. Water salinity was checked every day and corrected when necessary. After 4 days of acclimatization to different salinities, 12 fish from each group (sham and E_2) were sampled from separate tanks at the three different salinities assessed. Seven uninjected fish were also sampled at the same time to assess the possible vehicle effect. No mortality was observed during the experiments.

Sampling

Fish were deeply anaesthetized with 2-phenoxyethanol (0.1% v/v) and weighed. Blood was obtained in ammonium-heparinized syringes from the caudal peduncle. Plasma samples were obtained after centrifugation of blood (10,000 *g* for 1 min) and were immediately frozen in liquid nitrogen and stored at −80°C. Brain, liver, and gills were weighed, freeze-clamped, and stored at −80°C until assayed.

Analytical techniques

Plasma glucose and lactate were measured using commercial kits from Sigma (Sigma no. 16-20UV and Sigma no. 735, respectively) adapted to microplates (Stein 1963; Iwama et al. 1989). Plasma protein was measured using the bicinchoninic acid method with the BCA protein kit (Pierce, Rockford, USA) for microplates and bovine serum albumin as standard. Plasma calcium levels were measured with a commercial kit (Spinreact, Spain).

Brain, liver, and gill samples were minced on a chilled Petri dish to very small pieces (approximately 1 mm) that, once formed, were mixed and divided into two homogeneous aliquots to assess enzyme activities and metabolite levels, respectively. The first aliquot of tissue used for the assessment of metabolite levels was homogenized immediately by ultrasonic disruption in the cold (Dr Hielscher UP200H), with 7.5 vol of ice-cooled 6% perchloric acid, and then neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged (2 min at 13,000 *g*, Eppendorf 5415R), and the supernatant was used for assays. Tissue lactate and adenosine triphosphate (ATP) levels were determined enzymatically using commercial kits (Spinreact, Spain, and Sigma Chemical, USA, for lactate and ATP, respectively). Liver protein levels were assessed following the method of Bradford (1976). Tissue glycogen levels were assessed using the method of Keppler and Decker (1974). Glucose obtained after glycogen breakdown (after subtracting free glucose levels) was determined enzymatically using a commercial kit (Biomérieux, Spain).

The second aliquot of tissue used for the assessment of enzyme activities was homogenized by ultrasonic disruption in the cold (Dr Hielscher UP200H), with 10 vol of ice-cold stopping-buffer containing (l^{-1}): 50 mmol imidazole-HCl (pH 7.5), 1 mmol 2-mercaptoethanol, 50 mmol NaF, 4 mmol EDTA, 250 mmol sucrose, and 0.5 mmol p-methyl-sulphonyl-fluoride (PMSF, added as dry crystals immediately before homogenization). The homogenate was centrifuged (2 min at 13,000 *g*, Eppendorf 5415R), and the supernatant was used for assays.

Enzyme activities were determined using a Unicam UV6-220 spectrophotometer (Thermo Unicam, Waltham, Mass., USA). Reaction rates of enzymes were determined by the increase or decrease in absorbance of NAD(P)H at 340 nm. The reactions were started by the addition of homogenates (0.05 ml), at a pre-established protein concentration, omitting the substrate in control cuvettes (final volume 1.35 ml) and allowing the reactions to proceed at 15°C for pre-established times. Homogenate protein was assayed in triplicate as detailed by Bradford (1976), using bovine serum albumin (Sigma, USA) as standard. Enzyme analyses were all carried out to achieve maximum rates in each tissue, as defined in preliminary tests. The specific conditions for enzyme assays were described previously (Láiz-Carrión et al. 2002, 2003; Sangiao-Alvarellos et al. 2003) after adaptation of methods described for salmonids (Soengas et al. 1996, 1998).

Statistical analyses

The differences between sham and E_2-treated fish 5 days after implantation were assessed with a Student's *t*-test (significance level $P < 0.05$). The differences observed between different groups assessed 5 days after implantation followed by subsequent transfer to different salinities for 4 days were analyzed using a two-way ANOVA with treatment (sham and E_2) and salinity (BW, SW, and HSW) as main factors. When significant differences were obtained from the ANOVA, multiple comparisons were carried out using the Student–Newman–Keuls' test. Significance level was set at $P < 0.05$.

Results

No differences were observed when comparing uninjected fish and fish injected with coconut oil alone (sham) in any parameter assessed after 5 or 9 days of treatment (data not shown).

Plasma calcium and protein, liver protein, and hepatosomatic index were significantly higher in E_2-treated fish compared with shams (Table 1). *P*-values resulting from the two-way ANOVA of the remaining parameters assessed are displayed in Table 2.

Table 1 *Sparus auratus*. Changes in plasma calcium and protein, and liver protein, and hepatosomatic index in SW-acclimated gilthead sea bream after 5 or 9 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (E_2, 10 μg g^{-1} body weight). Values are the means (±SEM, $n=12$ fish per group). *Asterisk* indicates significantly different ($P<0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P<0.05$) among days within each treatment (sham and E_2)

Parameter	Treatment	Days after treatment	
		5 days	9 days
Plasma calcium (μmol ml^{-1})	Sham	2.61 ± 0.21^{a}	2.60 ± 0.25^{a}
	E_2	$8.16 \pm 0.21^{*,a}$	$11.2 \pm 0.10^{*,b}$
Plasma protein (mg ml^{-1})	Sham	34.9 ± 1.10^{a}	34.5 ± 1.41^{a}
	E_2	$56.3 \pm 1.09^{*,a}$	$94.5 \pm 1.11^{*,b}$
Liver protein (mg g^{-1} wet wt)	Sham	30.9 ± 1.78^{a}	31.7 ± 2.03^{a}
	E_2	$51.7 \pm 1.38^{*,a}$	$84.6 \pm 5.79^{*,b}$
Hepatosomatic index (%)	Sham	1.24 ± 0.05^{a}	1.37 ± 0.04^{a}
	E_2	$2.61 \pm 0.08^{*,a}$	$2.92 \pm 0.08^{*,b}$

After 5 days, plasma glucose levels (Fig. 1A) were significantly higher in E_2-treated fish than in shams, whereas, after 9 days, E_2-treated fish acclimated to BW and SW displayed higher levels than shams. Values of plasma glucose of shams were higher in HSW- than those of BW- and SW-acclimated fish, whereas values of treated fish were higher in BW- than those of SW- and HSW-acclimated fish. After 5 days, plasma lactate levels (Fig. 1B) in E_2-treated fish were higher than those of shams, whereas, after 9 days, E_2-treated fish displayed higher levels than shams in all salinities assessed, and levels increased in shams in parallel with the increase in salinity.

After 5 days, liver glycogen levels (Fig. 2A) decreased in E_2-treated fish compared with shams. In fish sampled after 9 days, E_2-treated fish displayed lower glycogen levels in liver than those of shams at all salinities assessed, whereas levels of shams were higher in SW- than those in BW- or HSW-acclimated fish. After 5 days, liver lactate levels (Fig. 2B) increased in E_2-treated fish compared with shams, whereas, after 9 days, levels of treated fish were higher than those of shams at all salinities assessed. Liver lactate levels of shams were lower in SW- than in BW-acclimated fish, whereas, in treated fish, levels in SW- were lower than those of BW- or HSW-acclimated fish. Liver ATP levels (Fig. 2C) in E_2-treated fish acclimated to both BW and HSW were significantly lower than their respective shams.

Liver enzyme activities are displayed in Table 3. After 5 days, glycogen phosphorylase (GPase) activity increased in livers of E_2-treated fish compared with shams, whereas activity in E_2-treated fish sampled at different salinities was higher than that of shams in both SW and HSW. The percentage of GPase activity in the active form measured in livers of E_2-treated fish acclimated to BW and HSW was significantly higher than that of shams. After 5 days, the optimal activity of pyruvate kinase (PK) was higher in livers of E_2-treated

Table 2 *Sparus auratus*. *P*-values from two-way ANOVAs of parameters measured in plasma, liver, gills, and brain of gilthead seabream after 5 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (sham) or containing 17ß-estradiol (E_2, 10 μg g^{-1} body weight), followed by subsequent transfer to different salinities for 4 days. Treatment (sham and E_2) and salinity (*BW* brackish water; *SW* seawater; *HSW* hypersaline water) are the main factors (*n.s.* not significant) (enzyme abbreviations: *ATP* adenosine triphosphate; *GPase* glycogen phosphorylase; *PK* pyruvate kinase; *FBPase* fructose 1,6-bisphosphatase; *G6PDH* glucose 6-phosphate dehydrogenase; *HK* hexokinase)

Tissue	Parameter	Treatment	Salinity	Treatment×salinity
Plasma	Glucose levels	<0.001	0.037	n.s.
	Lactate levels	<0.001	0.031	n.s.
Liver	Glycogen levels	<0.001	<0.001	n.s.
	Lactate levels	0.009	<0.001	n.s.
	ATP levels	0.044	n.s.	n.s.
	GPase activity (total)	0.041	n.s.	n.s.
	GPase *a* activity (%)	0.036	n.s.	n.s.
	PK activity (optimal)	0.001	0.006	n.s.
	PK activity (activity ratio)	<0.001	0.003	n.s.
	PK activity (activation ratio)	<0.001	0.002	n.s.
	FBPase activity	<0.001	n.s.	n.s.
	G6PDH activity	<0.001	n.s.	0.005
Gills	Glycogen levels	n.s.	n.s.	n.s.
	Lactate levels	<0.001	<0.001	n.s.
	ATP levels	n.s.	0.008	n.s.
	GPase activity (total)	n.s.	0.014	n.s.
	GPase *a* activity (%)	n.s.	0.035	n.s.
	PK activity (optimal)	0.023	n.s.	n.s.
	PK activity (activity ratio)	n.s.	0.004	n.s.
	PK activity (activation ratio)	n.s.	n.s.	n.s.
	HK activity	0.021	<0.001	n.s.
	G6PDH activity	0.026	0.047	n.s.
Brain	Glycogen levels	n.s.	0.046	n.s.
	Lactate levels	0.011	<0.001	n.s.
	ATP levels	<0.001	n.s.	n.s.
	GPase activity (total)	0.034	0.026	<0.001
	GPase *a* activity (%)	n.s.	n.s.	0.033
	PK activity (optimal)	<0.001	0.001	n.s.
	PFK activity (activity ratio)	n.s.	n.s.	n.s.
	PFK activity (activation ratio)	n.s.	n.s.	n.s.
	HK activity	n.s.	0.002	n.s.
	G6PDH activity	n.s.	0.044	n.s.

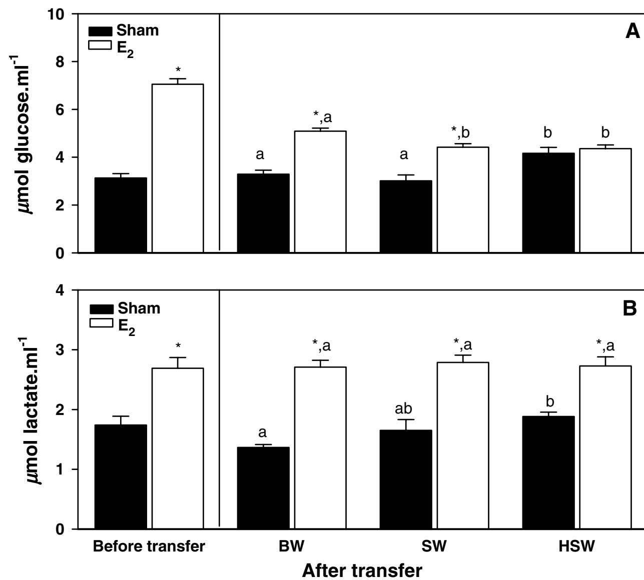

Fig. 1A, B *Sparus auratus*. Changes in the levels of glucose (**A**), and lactate (**B**) in plasma of SW-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (*E_2*, 10 μg g^{-1} body weight), followed by 4 days of acclimation to BW (5‰), SW (40‰), or HSW (55‰). Values are the means (±SEM, $n=12$ fish per group). *Asterisk* indicates significantly different ($P<0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P<0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

fish than in shams, whereas, after 9 days, enzyme activity was higher in E_2-treated fish than in shams at all salinities assessed, and the activity of shams in SW- was higher than those of BW- or HSW-acclimated fish. The activity ratio of PK in livers of E_2-treated fish was higher than that of shams at all salinities assessed. The same activity ratio in livers of treated fish was higher in HSW- than that of SW-acclimated fish. The fructose 1,6-bis-phosphatase (FBPase) activation ratio of PK in livers of E_2-treated fish was higher than that of shams at all salinities assessed, whereas, after 5 days, the ratio of E_2-treated fish was higher in HSW- than those of BW- and SW-acclimated fish. FBPase activity was significantly lower in livers of E_2-treated fish acclimated to SW than in shams, and the activity of E_2-treated fish was lower than that of shams in fish acclimated to all salinities assessed. After 5 days, liver glucose 6-phosphate dehydrogenase (G6PDH) activity was lower in E_2-treated fish than in shams, and the activity of E_2-treated fish was lower than that of shams at all the salinities assessed.

Gill lactate levels (Fig. 3B) increased after 5 days in E_2-treated fish compared with shams. After 9 days, lactate levels in gills of treated fish were higher than those of shams in BW- and SW-acclimated fish, whereas levels in shams were higher in HSW- than those of BW- and SW-acclimated fish. ATP levels in gills of E_2-treated fish were lower in SW- and HSW- than those of BW-acclimated fish.

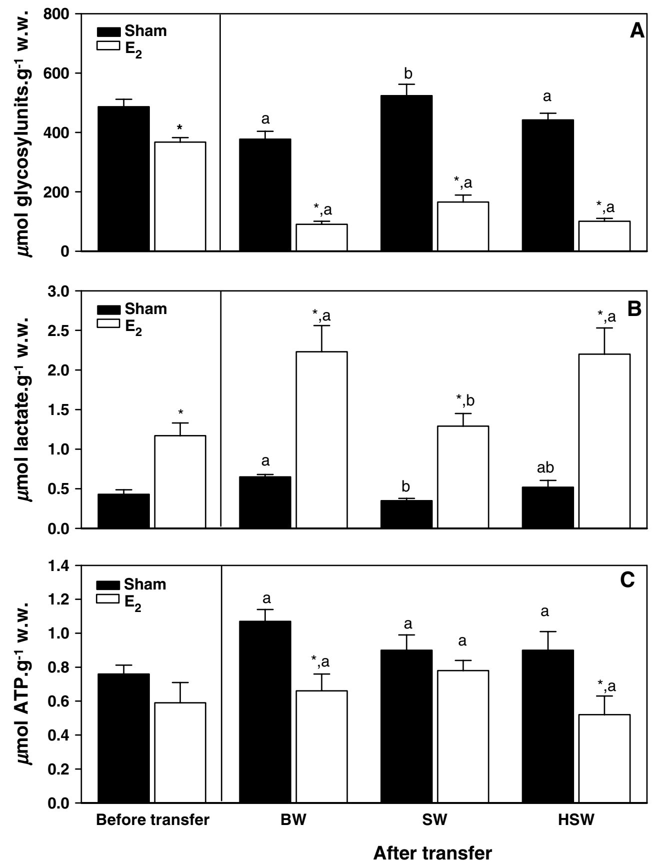

Fig. 2A–C *Sparus auratus*. Changes in the levels of glycogen (**A**), lactate (**B**) and ATP (**C**) in liver of SW-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (*E_2*, 10 μg g^{-1} body weight), followed by 4 days of acclimation to BW (5‰), SW (40‰), or HSW (55‰). Values are the means (±SEM, $n=12$ fish per group). *Asterisk* indicates significantly different ($P<0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P<0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

The activities of gill enzymes are displayed in Table 4. GPase activity in gills of E_2-treated fish decreased in parallel with the increase in salinity. When considering the percent GPase *a* of gills, shams displayed higher activities in SW- and HSW- than those of BW-acclimated fish, and the same differences were observed when comparing E_2-treated fish acclimated to different salinities. The optimal activity of PK in gills of E_2-treated fish was lower than that of shams in SW- and HSW-acclimated fish. The activity ratio of PK in gills of shams was lower in SW- than that of HSW-acclimated fish, whereas no significant differences were observed for the cofactor activation ratio of PK in gills. Hexokinase (HK) activity in gills of E_2-treated fish was lower than that of shams in HSW-acclimated fish, and a significant increase was observed in shams in parallel with increased salinity. G6PDH activity was lower in gills of E_2-treated fish than in shams after 5 days of treatment, whereas, after 9 days, the activity of E_2-treated fish was lower than that

Table 3 *Sparus auratus*. Changes in enzyme activities (abbreviations, see Table 1) in liver of seawater-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (*E_2*, 10 μg g^{-1} body weight), followed by 4 days of acclimation to brackish water (*BW*, 5‰), seawater (*SW*, 40‰), or hypersaline water (*HSW*, 55‰). Values are the means (± SEM, $n = 12$ fish per group). *Asterisk* indicates significantly different ($P < 0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P < 0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

Parameter	Treatment	Before transfer	After transfer		
			BW-acclimated	SW-acclimated	HSW-acclimated
GPase activity					
Total activity (U mg^{-1}protein)	Sham	0.29 ± 0.02	0.31 ± 0.04[a]	0.31 ± 0.02[a]	0.26 ± 0.03[a]
	E_2	0.41 ± 0.03*	0.32 ± 0.02[a]	0.42 ± 0.02*[,a]	0.39 ± 0.02*[,a]
GPase *a* (%)	Sham	41.3 ± 4.51	34.3 ± 1.72[a]	35.8 ± 1.67[a]	33.6 ± 1.84[a]
	E_2	39.6 ± 1.13	43.3 ± 1.47*[,a]	37.1 ± 1.31[a]	41.9 ± 1.41*[,a]
PK activity					
Optimal activity (U mg^{-1}protein)	Sham	0.35 ± 0.02	0.27 ± 0.02[a]	0.36 ± 0.01[b]	0.28 ± 0.02[a]
	E_2	0.45 ± 0.03*	0.42 ± 0.02*[,a]	0.48 ± 0.03*[,a]	0.41 ± 0.02*[,a]
Activity ratio	Sham	0.31 ± 0.07	0.24 ± 0.02[a]	0.21 ± 0.03[a]	0.25 ± 0.02[a]
	E_2	0.25 ± 0.04	0.36 ± 0.02*[,ab]	0.30 ± 0.03*[,a]	0.46 ± 0.03*[,b]
Fructose 1,6-P_2 activation ratio	Sham	0.34 ± 0.03	0.27 ± 0.03[a]	0.23 ± 0.02[a]	0.31 ± 0.04[a]
	E_2	0.36 ± 0.06	0.56 ± 0.04*[,a]	0.54 ± 0.03*[,a]	0.79 ± 0.05*[,b]
FBPase activity					
Optimal activity (U mg^{-1}protein)	Sham	0.49 ± 0.03	0.47 ± 0.03[a]	0.46 ± 0.04[a]	0.49 ± 0.01[a]
	E_2	0.30 ± 0.02*	0.21 ± 0.03*[,a]	0.22 ± 0.02*[,a]	0.16 ± 0.01*[,a]
G6PDH activity					
Optimal activity (U mg^{-1}protein)	Sham	1.19 ± 0.09	1.26 ± 0.09[a]	1.11 ± 0.07[a]	1.34 ± 0.07[a]
	E_2	0.73 ± 0.05*	0.65 ± 0.04*[,a]	0.68 ± 0.03*[,a]	0.53 ± 0.05*[,a]

of shams in SW- and HSW-acclimated fish, and the activity in treated fish decreased significantly in parallel with the increase in salinity.

Brain glycogen levels of E_2-treated fish were lower than those of shams in SW and HSW, and levels in shams were higher in HSW- than those of BW- and SW-acclimated fish. Brain lactate levels (Fig. 4B) were higher in E_2-treated fish than those of shams in BW and SW, and levels of shams increased in parallel with the increase in salinity. Brain ATP levels of E_2-treated fish were higher than those of shams at all salinities assessed.

Enzyme activities in brain are reported in Table 5. The activity of GPase in brains of E_2-treated fish was higher than that of shams in SW-acclimated fish, but lower than that of shams in HSW-acclimated fish, whereas the activity of shams was lower in SW- than that of HSW-acclimated fish, and the activity of treated fish was higher in SW- than that of HSW-acclimated fish. The optimal activity of 6-phosphofructo 1-kinase (PFK) in brains of E_2-treated fish acclimated to SW and HSW was lower than their respective shams, whereas the activity observed in E_2-treated fish decreased in parallel with the increase in salinity. HK activity in brains of shams was lower in SW- than that of BW- and HSW-acclimated fish. Finally, G6PDH activity in brains of E_2-treated fish was higher in BW- than that of SW- and HSW-acclimated fish.

Discussion

The use of coconut oil as a slow-release implant is an effective and practical method to produce a stable increase in the level of E_2 in the blood for several weeks in different species of teleosts (Flett and Leatherland 1989), including gilthead sea bream (*Sparus auratus*) (Mosconi et al. 1998; Guerreiro et al. 2002; Cavaco et al. 2003). Moreover, the dose used in the present study was similar to that previously used by other authors to assess changes due to E_2 action (Washburn et al. 1992; Korsgaard and Mommsen 1993; Bjerkeng et al. 1999; Vijayan et al. 2001). E_2 treatment is known to increase plasma levels of vitellogenin and calcium, as well as hepatosomatic index (HSI) in teleost fish (Mommsen and Walsh 1988). In the present experiments, the significant increases in plasma calcium and protein, liver protein, and hepatosomatic index in E_2-treated fish compared with shams and uninjected fish suggest that treatment was effective in elevating plasma E_2 levels.

Previous studies in fish have shown that glycogen levels and activities of the enzymes involved in carbohydrate metabolism may be affected by the increased protein synthesis and energy expenditure elicited by E_2 treatment (Peter and Oomen 1989; Olin et al. 1992). The results obtained in the present study when comparing sham and E_2-treated fish after 5 days are in good agreement with that general model. Thus, glycogen levels in liver of E_2-treated fish decreased when compared with shams in a way similar to that already described in several fish species (Petersen et al. 1983; Haux and Norberg 1985; Olin et al. 1992; Sehgal and Goswani 2001). In addition, the increase observed in plasma glucose levels of E_2-treated fish is in agreement with similar increases recorded in rainbow trout (Washburn et al. 1992; Korsgaard and Mommsen 1993), red sea bream (Woo et al. 1993), and tilapia (Sunny et al. 2002). Plasma lactate levels displayed a marked increase in E_2-treated fish, which has not been observed in any

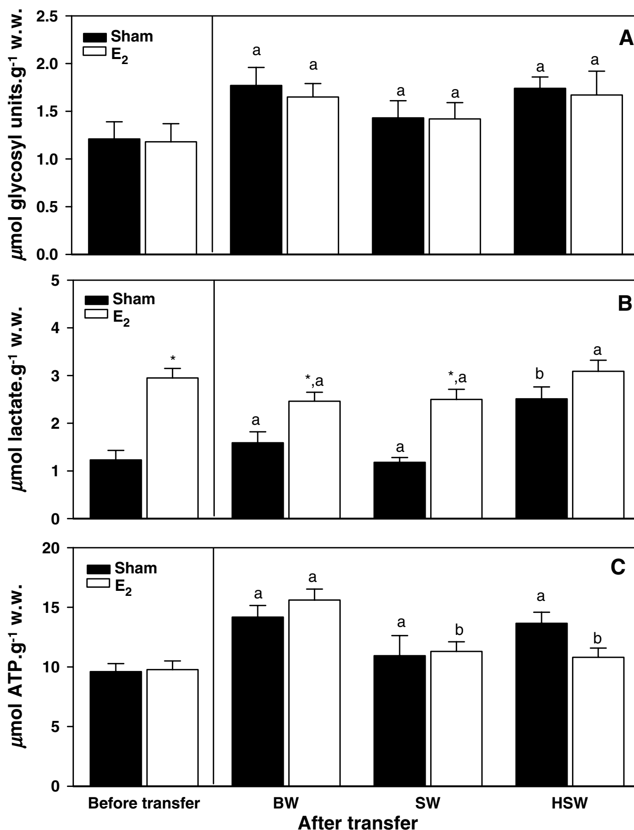

Fig. 3A–C *Sparus auratus*. Changes in the levels of glycogen (**A**), lactate (**B**) and ATP (**C**) in gills of SW-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 µl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (*E_2*, 10 µg g^{-1} body weight), followed by 4 days of acclimation to BW (5‰), SW (40‰), or HSW (55‰). Values are the means (±SEM, $n=12$ fish per group). *Asterisk* indicates significantly different ($P<0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P<0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

preceding study, and could be the result of increased muscle glycolysis considering that the liver lactate load was very low.

The remaining parameters assessed in liver pointed to changes in the potential of several carbohydrate-metabolizing pathways. Thus, an increased potential of liver glycogenolysis is apparent in E_2-treated fish, as judged by the increase observed in GPase activity, which is similar to that already observed in other studies after E_2 treatment (Peter and Oomen 1989; Woo et al. 1993), and during gonadal development (Soengas et al. 1995). An enhanced glycolytic capacity in liver is also suggested from data observed in E_2-treated fish, including increased PK activity, decreased FBPase activity, and increased lactate levels. This higher glycolytic capacity in liver of gilthead sea bream agrees well with results reported previously in other E_2-treated fish, such as: (1) the increased oxygen consumption in eel hepatocytes (Peyon et al. 1998), (2) the increased activities of liver glycolytic enzymes in several fish species (Sand et al. 1980; Petersen et al. 1983; Korsgaard and Mommsen 1993; Woo et al. 1993; Sehgal and Goswani 2001), and (3) the decreased gluconeogenic capacity of rainbow trout liver (Korsgaard and Mommsen 1993). In addition, the capacity of the pentose phosphate pathway appears to be reduced in E_2-treated fish, as judged by the decrease observed in G6PDH activity, which is similar to that described previously (Sand et al. 1980).

The second and major part of the study dealt with the effect of E_2 on the metabolic changes occurring in gilthead sea bream during acclimation to different environmental salinities, to assess whether or not metabolic changes associated with osmotic acclimation (Sangiao-Alvarellos et al. 2003) are modified by the reorganization of energy resources imposed by E_2 treatment. In the fish used in the present experiment, the osmoregulatory parameters of sham fish acclimated to different salinities, gill Na^+/K^+-ATPase activities, and plasma osmolality and ion levels (Guzmán et al. 2004), and these levels were similar to those previously reported for the same species (Mancera et al. 2002; Sangiao-Alvarellos et al. 2003). Moreover, E_2 treatment produced in the fish used in the present experiment an increase in Na^+/K^+-ATPase activity in gills and osmolality in plasma compared with shams (Guzmán et al. 2004). These osmoregulatory changes suggest that E_2 treatment further enhances the energy demands for osmoregulation. Furthermore, E_2 treatment induced increased levels of glucose in plasma compared with shams, which is in contrast with the lack of changes observed by Vijayan et al. (2001) during acclimation of E_2-treated tilapia to 50% SW. However, it should be remembered that their study was conducted with a lower osmotic challenge (50% SW) and over a shorter time period (24 h). The source of these increased levels of glucose could be the mobilization of liver glycogen to satisfy the increased energy demand of other tissues. The increase in plasma lactate levels observed in sham fish in parallel with the increase in environmental salinity was changed by higher levels in E_2-treated fish compared with shams reaching similar levels in all salinities assessed.

The fall in liver glycogen levels observed after 5 days in E_2-treated fish acclimated to SW is further enhanced in treated fish after 9 days, when levels of E_2-treated fish are lower than those of shams and equal in all salinities assessed. Changes observed in glycogen levels agree with those displayed by GPase activity (higher in treated fish and not different among the salinities assessed). It seems that E_2 treatment increases the necessity of glycogen mobilization from the liver independent of the salinity at which the fish were acclimated.

Liver glycolytic potential increased after 9 days in E_2-treated fish compared with shams. This increased potential was higher (as judged by activity ratio and activation ratio of PK) in HSW- than in BW- and SW-acclimated fish. This observation coincides with changes reported in liver lactate levels that not only increased in E_2-treated fish (at levels higher than those observed after 5 days), but also that the increase was remarkably

Table 4 *Sparus auratus*. Changes in enzyme activities (abbreviations, see Table 1) in gills of SW-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (*E_2*, 10 μg g^{-1} body weight), followed by 4 days of acclimation to BW (5‰), SW (40‰), or HSW (55‰). Values are the means (± SEM, $n=12$ fish per group). *Asterisk* indicates significantly different ($P<0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P<0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

Parameter	Treatment	Before transfer	After transfer		
			BW-acclimated	SW-acclimated	HSW-acclimated
GPase activity					
Total activity (U mg^{-1}protein)	Sham	0.12 ± 0.01	0.10 ± 0.01^{a}	0.11 ± 0.02^{a}	0.08 ± 0.01^{a}
	E_2	0.13 ± 0.02	0.10 ± 0.01^{a}	0.09 ± 0.01^{ab}	0.07 ± 0.01^{b}
GPase *a* (%)	Sham	15.4 ± 1.51	8.32 ± 2.20^{a}	19.2 ± 3.58^{b}	22.4 ± 3.07^{b}
	E_2	10.4 ± 2.11	7.02 ± 2.74^{a}	15.60 ± 2.77^{b}	18.2 ± 2.64^{b}
PK activity					
Optimal activity (U mg^{-1}protein)	Sham	4.67 ± 0.33	3.98 ± 0.16^{a}	4.39 ± 0.31^{a}	4.48 ± 0.24^{a}
	E_2	4.37 ± 0.55	3.57 ± 0.22^{a}	3.31 ± 0.13^{*,a}	3.22 ± 0.17^{*,a}
Activity ratio	Sham	0.50 ± 0.02	0.51 ± 0.02^{ab}	0.47 ± 0.02^{a}	0.56 ± 0.02^{b}
	E_2	0.49 ± 0.03	0.48 ± 0.03^{a}	0.48 ± 0.03^{a}	0.54 ± 0.02^{a}
Fructose 1,6-P_2 activation ratio	Sham	0.93 ± 0.03	0.92 ± 0.03^{a}	0.98 ± 0.02^{a}	0.99 ± 0.04^{a}
	E_2	0.94 ± 0.02	0.96 ± 0.02^{a}	0.99 ± 0.04^{a}	0.99 ± 0.02^{a}
HK activity					
Optimal activity (U mg^{-1}protein)	Sham	0.74 ± 0.03	0.66 ± 0.03^{a}	0.80 ± 0.05^{ab}	1.02 ± 0.04^{b}
	E_2	0.66 ± 0.04	0.71 ± 0.05^{a}	0.64 ± 0.05^{a}	0.69 ± 0.06^{*,a}
G6PDH activity					
Optimal activity (U mg^{-1}protein)	Sham	1.55 ± 0.08	1.53 ± 0.11^{a}	1.58 ± 0.09^{a}	1.57 ± 0.10^{a}
	E_2	1.22 ± 0.04*	1.48 ± 0.05^{a}	1.26 ± 0.05^{*,ab}	1.13 ± 0.04^{*,b}

higher in the two extreme salinities assessed (BW and HSW) compared with E_2-treated fish acclimated to SW. The effect of energy mobilization in the two extreme salinities can also be observed for ATP levels that decreased in E_2-treated fish compared with shams only in HSW- and BW-acclimated fish.

Changes observed in liver FBPase and G6PDH activities have in common that E_2 treatment produced a marked decrease in enzyme activity after only 9 days, and that no differences were observed among treated fish acclimated to the different salinities assessed. These changes agree with those reported by Vijayan et al. (2001) in PEPCK (phosphoenolpyruvate-carboxykinase) and G6PDH activities when acclimating E_2-treated tilapia to 50% SW.

E_2 treatment is known to activate vitellogenin synthesis in fish liver and subsequent energy demand (Mommsen and Walsh 1988). Data obtained in the present experiments also demonstrate increased levels of protein in both liver and plasma of E_2-treated fish compared with shams. As protein synthesis accounts for a significant portion of O_2 consumption and nearly 80% of hepatocyte demand, a repartitioning of the energy budget is likely with E_2 stimulation (Vijayan et al. 2001). Accordingly, marked changes in enzyme activities were noted with E_2 treatment in liver. Thus, data obtained from liver suggest that E_2 treatment produced in those fish acclimated to extreme salinities (BW, HSW) changes in metabolic parameters similar to the changes in fish acclimated to medium (SW) salinity, with the exception of lactate and PK activity. Considering that most changes displayed in shams among salinities disappeared in E_2-treated fish, we suggest that the carbohydrate resources of liver in E_2-treated fish are used in a higher portion endogenously, probably through the increased synthesis of vitellogenin.

The increase in the potential for use of exogenous glucose in gills in parallel with the increase in environmental salinity in sham fish disappeared in E_2-treated fish, which evidenced the same enzyme activity among the different salinities assessed. It seems that the necessity of exogenous glucose for gills diminished in the presence of E_2, perhaps reflecting a higher use of other fuels under such circumstances. On the other hand, the lower use of exogenous glucose in E_2-treated fish compared with shams would match with the decrease observed in the activity of G6PDH in E_2-treated fish among the different salinities assessed.

The differences in lactate levels observed in gills of sham fish acclimated to different salinities disappeared in treated fish. The increased lactate production could suggest an enhanced glycolytic potential in gills of E_2-treated fish. However, gill PK activity did not show any significant increase and actually showed a decrease compared with shams in SW- and HSW-acclimated fish. A similar decrease in the glycolytic capacity of gills in E_2-treated fish during the acclimation of tilapia to 50% SW has previously been reported (Vijayan et al. 2001). Thus, the source of increased lactate levels does not appear to be a higher production through glycolysis, and is probably related to increased use of exogenous lactate. Considering the ability of gills to use lactate as fuel (Mommsen 1984; Perry and Walsh 1989), the latter seems a reasonable hypothesis. The enhanced use of alternative fuels would support the enhanced osmoregulatory work in gilthead sea bream elicited by the E_2

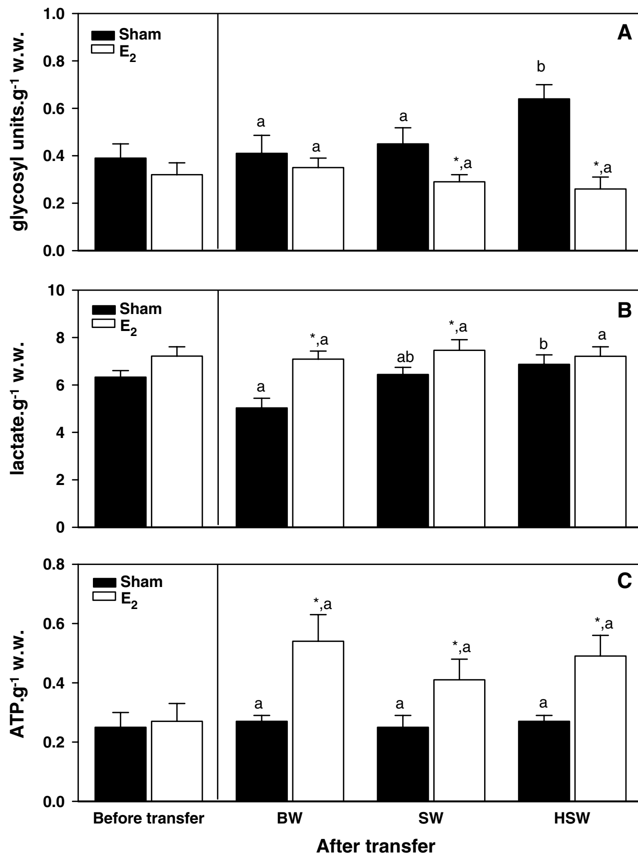

Fig. 4A–C *Sparus auratus*. Changes in the levels of glycogen (**A**), lactate (**B**) and ATP (**C**) in brain of SW-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 µl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (E_2, 10 µg g^{-1} body weight), followed by 4 days of acclimation to BW (5‰), SW (40‰), or HSW (55‰). Values are the means (±SEM, $n=12$ fish per group). *Asterisk* indicates significantly different ($P<0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P<0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

treatment (Guzmán et al. 2004), which could not be sustained by exogenous glucose alone.

In brain, no significant differences in any parameter assessed were recorded between sham and E_2-treated fish after 5 days. After 9 days, brain glycogen levels significantly decreased in E_2-treated fish compared with shams in SW- and HSW-acclimated fish, which is reflected by changes in GPase activity. The glycolytic potential appears to be reduced in E_2-treated fish compared with shams, as judged by changes displayed by PK activity. The increased lactate levels in the brains of shams in parallel with increased salinity disappeared in treated fish, in which levels were higher than those of shams, but not different among the salinities assessed. Again, considering the fall observed in PK activity, in a way similar to gills, the increased lactate levels must be the result of increased uptake into the brain or decreased rates of lactate utilization. Considering that lactate is as a good fuel as glucose for teleost brain (Soengas et al. 1998), an increased use of lactate could be possible. The enhanced lactate levels, together with the decrease in PK activity of E_2-treated fish compared with shams, would help to explain the significant increase observed in brain ATP levels of E_2-treated fish in all salinities assessed compared with shams, indicating a general decrease in ATP consumption in brain.

The pathway used by E_2 to affect energy metabolism is not known. In gilthead sea bream, the presence of estrogen receptors (ER) has been demonstrated in liver, brain, kidney, or intestine (Socorro et al. 2000). However, many steroids are able to exert effects via membrane-bound receptors and second messenger pathways (Borsky 2000; Sunny and Oomen 2001). On the other hand, a possible effect of E_2 through other endocrine systems cannot be disregarded. This is of particular importance for results obtained in gills, since no signal for ER in gills was apparent in the only study performed to date in gilthead sea bream (Socorro et al. 2000). Possible candidates for the indirect action of E_2 would be those hormones whose levels are known to be altered by E_2 treatment, like prolactin (Barry and Grau 1986; Brinca et al. 2003) or growth hormone (Poh et al. 1997), and are known to produce changes in fish energy metabolism (Sheridan 1986; Leung et al. 1991). Cortisol does not appear to be involved in the indirect action of E_2, since no significant changes in plasma cortisol levels have been observed when comparing sham and E_2-treated gilthead sea bream under similar experimental conditions (Láiz-Carrión et al., unpublished data).

In conclusion, we submitted fish to E_2 treatment (thus simulating the increased energy demand occurring during gonadal maturation) and then acclimated to different salinities as a method of determining the competition with the energy demands arising from osmoregulatory work. E_2 treatment increases the intermediary metabolic capacity of liver, indicating an overall repartitioning of energy, which is further sustained after acclimation to different salinities. Energy metabolism in the brain and gills is not affected by E_2 treatment under normal conditions. However, when E_2-treated fish are acclimated to different salinities, this species showed a decrease in the capacity for use of exogenous glucose through glycolysis in the gills and brain, possibly reflecting the higher metabolic necessities of the liver for glucose and suggesting the use of alternative fuels in the gills and brain to deal with the necessities imposed by osmoregulatory work. On the whole, at least part of the tissue-specific reorganization of energy metabolism in the liver, gills, and brain of gilthead sea bream observed during osmotic acclimation seems altered under E_2 treatment, reflecting a possible influence of sexual maturation on seawater adaptability at the metabolic level.

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Table 5 *Sparus auratus*. Changes in enzyme activities (*PFK* 6-phosphofructo 1-kinase; other abbreviations, see Table1) in brain of SW-acclimated gilthead sea bream after 5 days of intraperitoneal implantation of 5 μl g^{-1} body weight of coconut oil alone (*sham*) or containing 17ß-estradiol (E_2, 10 μg g^{-1} body weight), followed by 4 days of acclimation to BW (5‰), SW (40‰), or HSW (55‰). Values are the means (± SEM, $n = 12$ fish per group). *Asterisk* indicates significantly different ($P < 0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. *Different letters* indicate significant differences ($P < 0.05$) among groups (BW, SW, and HSW) within each treatment (sham and E_2)

Parameter	Treatment	Before transfer	After transfer		
			BW-acclimated	SW-acclimated	HSW-acclimated
GPase activity					
Total activity (U mg^{-1}protein)	Sham	0.49 ± 0.06	0.58 ± 0.03^{ab}	0.51 ± 0.03^{a}	0.72 ± 0.04^{b}
	E_2	0.48 ± 0.03	0.65 ± 0.04^{ab}	0.75 ± 0.04^{*,a}	0.55 ± 0.03^{*,b}
GPase *a* (%)	Sham	49.2 ± 1.30	47.8 ± 1.91^{a}	48.1 ± 3.10^{a}	54.1 ± 1.74^{a}
	E_2	50.7 ± 0.92	52.0 ± 1.93^{a}	52.3 ± 1.45^{a}	49.1 ± 2.45^{a}
PFK activity					
Optimal activity (U mg^{-1}protein)	Sham	9.45 ± 0.06	9.46 ± 0.46^{a}	9.67 ± 0.36^{a}	9.81 ± 0.29^{a}
	E_2	9.14 ± 0.40	8.58 ± 0.30^{a}	7.80 ± 0.31^{*,ab}	7.30 ± 0.18^{*,b}
Activity ratio	Sham	0.16 ± 0.03	0.13 ± 0.01^{a}	0.18 ± 0.02^{a}	0.16 ± 0.02^{a}
	E_2	0.14 ± 0.02	0.10 ± 0.01^{a}	0.15 ± 0.02^{a}	0.14 ± 0.02^{a}
Fructose 2,6-P_2 activation ratio	Sham	0.66 ± 0.11	0.70 ± 0.07^{a}	0.99 ± 0.13^{a}	0.84 ± 0.10^{a}
	E_2	0.69 ± 0.09	0.84 ± 0.09^{a}	0.93 ± 0.16^{a}	0.66 ± 0.10^{a}
HK activity					
Optimal activity (U mg^{-1}protein)	Sham	0.62 ± 0.02	0.79 ± 0.03^{a}	0.58 ± 0.03^{b}	0.76 ± 0.04^{a}
	E_2	0.68 ± 0.07	0.83 ± 0.04^{a}	0.76 ± 0.08^{a}	0.83 ± 0.05^{a}
G6PDH activity					
Optimal activity (U mg^{-1}protein)	Sham	0.40 ± 0.03	0.41 ± 0.03^{a}	0.33 ± 0.04^{a}	0.38 ± 0.02^{a}
	E_2	0.40 ± 0.02	0.45 ± 0.02^{a}	0.38 ± 0.02^{b}	0.37 ± 0.01^{b}

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