JOURNAL OF EXPERIMENTAL ZOOLOGY 301A:828–836 (2004)

Osmoregulatory Action of 17β-Estradiol in the Gilthead Sea Bream *Sparus auratus*

JOSÉ MARÍA GUZMÁN[1], SUSANA SANGIAO-ALVARELLOS[2], RAÚL LAIZ-CARRIÓN[1], JESÚS M. MÍGUEZ[2], MARÍA DEL PILAR MARTÍN DEL RÍO[1], JOSÉ LUIS SOENGAS[2], AND JUAN MIGUEL MANCERA[1]*

[1]*Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Cádiz, Spain*

[2]*Laboratorio de Fisioloxía Animal, Facultade de Ciencias do Mar, Universidade de Vigo, 36200 Vigo, Spain*

ABSTRACT The osmoregulatory action of 17β-estradiol (E_2) was examined in the euryhaline teleost *Sparus auratas*. In a first set of experiments, fish were injected once with vegetable oil containing E_2 (1, 2 and 5 μg/g body weight), transferred 12h after injection from sea water (SW, 38 ppt salinity) to hypersaline water (HSW, 55 ppt) or to brackish water (BW, 5 ppt salinity) and sampled 12h later (i.e. 24 h post-injection). In a second experiment, fish were injected intraperitoneally with coconut oil alone or containing E_2 (10 μg/g body weight) and sampled after 5 days. In the same experiment, after 5 days of treatment, fish of each group were transferred to HSW, BW and SW and sampled 4 days later (9 days post-implant). Gill Na^+,K^+-ATPase activity, plasma E_2 levels, plasma osmolality, and plasma levels of ions (sodium and calcium), glucose, lactate, protein, triglyceride, and hepatosomatic index were examined. Transfer from SW to HSW produced no significant effects on any parameters assessed. E_2 treatment did not affect any parameter. Transfer from SW to BW resulted in a significant decrease in plasma osmolality and plasma sodium but did not affect gill Na^+,K^+-ATPase activity. A single dose of E_2 attenuated the decrease in these parameters after transfer from SW to BW, but was without effect on gill Na^+,K^+-ATPase activity. An implant of E_2 (10 μg/g body weight) for 5 days significantly increased plasma calcium, hepatosomatic index, plasma metabolic parameters, and gill Na^+,K^+-ATPase activity. In coconut oil-implanted (sham) fish, transfer from SW to HSW or BW during 4 days significantly elevated gill Na^+,K^+-ATPase. Gill Na^+,K^+-ATPase activity remained unaltered after transfer of E_2-treated fish to HSW or BW. However, in E_2-treated fish transferred from SW to SW (9 days in SW after E_2-implant), gill Na^+,K^+-ATPase activity decreased with respect to HSW- or BW-transferred fish. Shams transferred to HSW showed increased levels of lactate, protein, and trygliceride in plasma, while those transferred to BW only displayed increased trygliceride levels. E_2-treated fish transferred to HSW showed higher protein levels without any change in other plasmatic parameters, while those transferred to BW displayed elevated plasma glucose levels but decreased osmolality and protein levels. These results substantiate a chronic stimulatory action of E_2 on gill Na^+,K^+-ATPase activity in the euryhaline teleost *Sparus auratas*. *J. Exp. Zool. 301A:828–836, 2004.* © 2004 Wiley-Liss, Inc.

INTRODUCTION

The negative influence of sexual maturation on seawater adaptability has been well established in several salmonid species (McCormick and Naiman, '85; Lundqvist et al., '89; Stuarnes et al., '94; Le François et al., 2000). In addition, treatment with 17β-estradiol (E_2) reduces the chloride cell density, as well as Na^+,K^+-ATPase activity in gills of salmonids (Miwa and Specker, '86; Ikuta et al., '87; Madsen and Korsgaard, '89, '91; Madsen et al., '97), and reduced hepatic flavin-containing mono-

Grant support: This work was supported by grants BOS2001–4031-C02–01 (Ministerio de Ciencia y Tecnología, Spain) and PETRI PTR1995–0431-OP (Ministerio de Educación y Cultura, Spain) to J.M.M., and grants BOS2001–4031-C02–02 (Ministerio de Ciencia y Tecnología, Spain) and PGIDT01PXI30113PR and PGIDT02P-XI30105IF (Xunta de Galicia, Spain) to J.L.S. S.S-A. was recipient of a predoctoral fellowship from the Xunta de Galicia. R.L-C. was recipient of a MIT predoctoral fellowship from the Ministerio de Ciencia y Tecnología.

*Correspondence to: Juan Miguel Mancera, Departamento de Biología, Facultad de Ciencias del Mar y Ambientales, Universidad de Cádiz, Cádiz, 1150 Puerto Real, Spain E-mail: juanmiguel.mancera@uca.es

Received 8 December 2004; Accepted 11 May 2004

Published online in Wiley Interscience (www.interscience.wiley.com). DOI: 10.1002/jez.a.102

© 2004 WILEY-LISS, INC.

oxygenase activity in rainbow trout, which increased after seawater transfer and it has been related to salinity adaptation (Schlenk et al., '97).

In non-salmonids species, it has also been demonstrated this negative relationship between E_2 and hypoosmotic capacity. Thus, in *Oreochromis mossambicus*, E_2 treatment reduced this capacity through a decrease in branchial Na^+,K^+-ATPase activity (Vijayan et al., 2001). Recently, similar negative effects of E_2 treatment on gill Na^+,K^+-ATPase activity and hypoosmotic capacity have been reported in *Fundulus heteroclitus* (Mancera et al., 2003).

The pathway used by E_2 to affect the osmoregulatory system is not known. There are several in vivo and in vitro evidences about stimulatory effects of E_2 treatment on prolactin (PRL) production in teleosts (Nagahama et al., '75; Wigham et al., '77; Olivereau and Olivereau, '79; Olivereau et al., '86; Barry and Grau, '86). It is well established the negative effect of PRL on gill Na^+,K^+-ATPase activity (Hirano, '86; McCormick, '95; Manzon, 2002). In this way, it is possible that E_2 exerts a negative effect on the osmoregulatory system by increasing plasma PRL levels though a direct effect of E_2 on osmoregulatory organs cannot be discarded. Accordingly, E_2 receptors have been demonstrated in both intestine and kidney of salmonids (Persson et al., 2000) and gilthead sea bream (Socorro et al., 2000).

Gilthead sea bream (*Sparus auratus*) is an euryhaline teleost capable of adapting to extreme changes in environmental salinity. Our group has analysed the osmoregulatory system of this species, by studying responses of plasma parameters to abrupt changes in salinity (Mancera et al., '93) or the osmoregulatory and metabolic effects of treatment with cortisol (Mancera et al., '94; Laiz-Carrión et al., 2003). Recently, we have also analysed the influence of different hormones (PRL, growth hormone -GH-, and cortisol) on the osmoregulatory capacity of gilthead sea bream, showing a clear role of PRL for adaptation to hypoosmotic environments, and a possible dual role of cortisol for adaptation to hypo- and hyperosmotic environments (Mancera et al., 2002). However, as far as we are aware, there are no studies available regarding the osmoregulatory effects of E_2 in this species. The aim of the present study was, therefore, to establish effects of E_2 treatment on hypoosmoregulatory and hyperosmoregulatory capacity in *S. auratus*. The results are discussed in relation to the osmoregulatory role of this hormone in other teleosts.

MATERIAL AND METHODS

Fish

Immature male gilthead sea bream (*Sparus aurata* L., 40–60 g body weight) were provided by Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) and transferred to the web laboratories at Faculty of Marine Science (Puerto Real, Cádiz). They were acclimated to SW in 300 L aquaria for, at least, 2 weeks in an open system (38 ppt salinity, 1000 mOsm/kg H_2O) before the experiments. BW (5 ppt salinity, 130 mOsm/kg H_2O) was obtained by mixing SW with dechlorinated tap water in a recirculating system. HSW (55 ppt salinity, 1554 mOsm/kg H_2O) was obtained by mixing full SW with natural marine salts (Unionsal, Cádiz, Spain) in a recirculating system. In the Experiment 2 (see below) the system in the tanks containing SW was also recirculated to be comparable with that of BW and HSW fish. Once the systems became recirculated, the common water quality criteria (hardness, and the levels of oxygen, carbon dioxide, hydrogen sulphide, 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. During the experiments (May-June), fish were maintained under natural photoperiod and constant temperature (18°C), and fed daily with 1% body weight commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). 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.

Injection protocol

Fish were caught by netting, lightly anaesthetized with 2-phenoxyethanol (0.05% v/v; Sigma), weighed, injected intraperitoneally (5 μl g^{-1} body weight) with E_2 (Sigma E-8875) and returned to their tanks. Hormone for short-term treatment (Experiment 1) was suspended in vegetable oil (commercial sunflower oil) while for long-term treatment (Experiment 2) was suspended in coconut oil (Sigma C-1758). Hormone was injected intraperitoneally, and vehicle injections served as controls. No mortality was observed during the experiments.

Experimental design

Experiment 1: SW-adapted fish received a single injection of E_2 (1, 2 or 5 μg/g body weight) suspended in vegetable oil and were transferred to HSW 12h after the injection. Twelve h after the transfer (24h post-injection) the fish were sampled (see below). A similar protocol was also followed transferring fish from SW to BW.

Experiment 2: SW-adapted fish were implanted with slow-release coconut oil implants alone (sham) or containing E_2 (10 μg/g body weight). This dose has been previously used in this species by other authors who showed a chronic increase in E_2 levels during at least 2 weeks (Mosconi et al., '98; Guerreiro et al., 2002; Cavaco et al., 2003). In our study, 5 days after implant, 12 fish of coconut oil-implanted (sham) group and 12 fish of E_2-implanted group were sampled. At the same time, 12 fish of each group were transferred to HSW (hyperosmotic test), BW (hypoosmotic test) and SW (transfer test). Four days after transfer, all groups were sampled (see below).

Sampling

Fish were deeply anaesthetized with 2-phenoxy-ethanol (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 (1 min at 10,000 g) and were immediately frozen on dry ice and stored at −80°C until further assay. Gill tissue was dissected and placed in 100 μl of ice-cold SEI buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80°C. In the Experiment 2, gills and liver were weighed, frozen on dry ice and stored at −80°C until assayed.

Analytical techniques

Gill Na^+,K^+-ATPase activity was determined using the microassay method of McCormick ('93) adapted to gilthead seabream (Mancera et al., 2002). Gill tissue was defrozen and homogenized in 125 μl of SEI buffer with 0.1% deoxycholic acid, then centrifuged at 2000 g for 30 s. Duplicate 10 μl homogenate samples were added to 200 μl assay mixture with and without 0.5 $mmol.l^{-1}$ ouabain in 96-well microplates at 25°C and read at 340 nm for 10 min with intermittent mixing. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as μmol ADP mg^{-1} $protein.h^{-1}$. The protein content was determined using the Pierce BCA Protein kit (Pierce, Rockford, IL) with bovine albumin as standard. Both assays were run on a microplate reader as above.

Plasma glucose and lactate were measured using commercial kits from Sigma (Sigma #16–20UV and Sigma #735, respectively) adapted to microplates (Stein, '63; Iwama et al., '89). Plasma protein was measured using the bicinchoninic acid method (Smith et al., '85) with the BCA protein kit (Pierce) for microplates using bovine serum albumin as standard. Plasma triglycerides were determined enzymatically with a commercial kit (Sigma #334-UV; Bucolo and David, '73) in microplates. These assays were run on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments, Winooski, VT) using DeltaSoft3 software for Macintosh (BioMetallics, Inc., Princeton, NJ). Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT) and expressed as $mOsm.kg^{-1}$. Plasma Na^+ was measured using a atomic absorption spectrophotometer (Philips PU7000), and plasma Ca^{++} levels with a commercial kit (Spinreact, Madrid, Spain).

Statistics

In Experiment 1 differences among groups were assessed by one-way ANOVA. In Experiment 2, the differences between sham and E_2-treated fish after 5 days of implantation were assessed with a Student t test (significance level $P<0.05$). The differences observed between different groups assessed after 5 days of implantation followed by subsequent transfer to different salinities for 4 days were analyzed using a two-way ANOVA with treatment (sham and 17β-estradiol) and salinity (BW, SW, and HSW) as independent variables. 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

Transfer of *S. auratas* from SW to HSW for 12 h did not significantly change any osmoregulatory parameter assessed. E_2 treatment previous to transfer had not effect on gill Na^+,K^+-ATPase activity and plasma osmolality, and the remaining parameters evaluated in plasma (Table 1). Only a significant increase was observed in plasma calcium levels in fish treated with the higher dose of E_2.

TABLE 1. Effects of a single dose of 17β-estradiol (E_2) on gill Na^+,K^+-ATPase activity, several parameters in plasma, and hepatosomatic index (HSI) following transfer from SW to HSW. Fish were kept in SW 12h following treatment and then transferred to HSW for 12h

	SW	E_2 0 μg/g	E_2 1 μg/g	E_2 2 μg/g	E_2 5 μg/g
Gill Na^+,K^+-ATPase (μmol ADP/mg prot./h)	12.12 ± 0.49^{a}	12.90 ± 0.92^{a}	11.58 ± 0.57^{a}	12.01 ± 0.79^{a}	10.86 ± 0.60^{a}
Osmolality (mOsm/kg)	347.1 ± 7.8^{a}	351.1 ± 3.8^{a}	378.3 ± 6.5^{a}	374.8 ± 8.0^{a}	363.3 ± 10^{a}
Na^+ (mM)	168.8 ± 3.8^{a}	167.2 ± 4.4^{a}	165.0 ± 5.3^{a}	162.3 ± 3.7^{a}	159.7 ± 5.7^{a}
Ca^{++} (mM)	2.11 ± 0.09^{a}	$2.27 \pm 0.06^{a,b}$	$2.26 \pm 0.11^{a,b}$	$2.44 \pm 0.08^{a,b}$	2.54 ± 0.09^{b}
Glucose (mM)	3.91 ± 0.19^{a}	4.42 ± 0.22^{a}	4.41 ± 0.15^{a}	4.31 ± 0.16^{a}	4.84 ± 0.12^{a}
Lactate (mM)	$1.01 \pm 0.08^{a,b}$	$1.11 \pm 0.07^{a,b}$	0.99 ± 0.07^{a}	$1.24 \pm 0.14^{a,b}$	1.39 ± 0.11^{b}
Protein (mg/ml)	36.4 ± 1.3^{a}	37.1 ± 0.8^{a}	39.4 ± 1.0^{a}	40.4 ± 0.8^{a}	38.6 ± 0.9^{a}
Triglycerides (mM)	1.55 ± 0.05^{a}	$2.00 \pm 0.09^{a,b}$	2.37 ± 0.21^{b}	$1.99 \pm 0.14^{a,b}$	$2.19 \pm 0.09^{a,b}$
HSI (%)	1.38 ± 0.02^{a}	1.23 ± 0.02^{a}	1.37 ± 0.06^{a}	1.28 ± 0.02^{a}	1.32 ± 0.04^{a}

Values are the mean ± SEM (n=7–8).
[a,b]Different letters indicate significant differences among groups ($P < 0.05$, one-way ANOVA).

TABLE 2. Effects of a single dose of 17β-estradiol (E_2) on gill Na^+,K^+-ATPase activity, several parameters in plasma, and hepatosomatic index (HSI) following transfer from SW to BW. Fish were kept in SW 12h following treatment and then transferred to BW for 12h

	SW	E_2 0 μg/g	E_2 1 μg/g	E_2 2 μg/g	E_2 5 μg/g
Gill Na^+,K^+-ATPase (μmol ADP/mg prot./h)	11.83 ± 1.06^{a}	11.53 ± 1.01^{b}	11.35 ± 0.44^{a}	11.25 ± 1.05^{a}	11.18 ± 0.63^{a}
Osmolality (mOsm/kg)	349.5 ± 5^{a}	291.8 ± 3.9^{b}	311.2 ± 4.7^{b}	310.2 ± 6.1^{b}	302.3 ± 4.6^{b}
Na^+ (mM)	166.8 ± 4.8^{a}	124.4 ± 2.4^{b}	133.6 ± 2.2^{b}	134.8 ± 4.8^{b}	125.2 ± 2.3^{b}
Ca^{++} (mM)	2.15 ± 0.11^{a}	1.70 ± 0.09^{a}	$1.80 \pm 0.06^{a,b}$	$1.82 \pm 0.07^{a,b}$	$1.98 \pm 0.07^{a,b}$
Glucose (mM)	4.01 ± 0.18^{a}	3.64 ± 0.22^{a}	3.79 ± 0.15^{a}	4.45 ± 0.16^{b}	3.68 ± 0.12^{a}
Lactate (mM)	1.16 ± 0.05^{a}	1.19 ± 0.07^{a}	1.12 ± 0.09^{a}	1.45 ± 0.07^{b}	1.15 ± 0.05^{a}
Proteins (mg/ml)	36.9 ± 0.8^{a}	36.2 ± 1.0^{a}	37.3 ± 0.6^{a}	37.3 ± 0.8^{a}	37.8 ± 1.1^{a}
Triglycerides (mM)	1.75 ± 0.05^{a}	1.76 ± 0.09^{a}	2.34 ± 0.21^{b}	2.48 ± 0.14^{b}	2.49 ± 0.09^{b}
HSI (%)	1.36 ± 0.04^{a}	1.37 ± 0.06^{a}	1.37 ± 0.04^{a}	1.34 ± 0.06^{a}	1.32 ± 0.02^{a}

Values are the mean ± SEM (n=7–8).
[a,b]Different letters indicate significant differences among groups ($P < 0.05$, one-way ANOVA).

Hypoosmotic transfer for 12 h significantly decreased plasma osmolality and plasma ions (sodium and calcium) but did not affect gill Na^+,K^+-ATPase activity (Table 2). E_2 treatment previous to transfer was without effect on gill Na^+,K^+-ATPase activity, or in glucose, lactate, and protein levels in plasma while increased plasma levels of triglycerides (Table 2).

The implant of E_2 (10 μg/g body weight in coconut oil, Experiment 2) for 5 days significantly increased plasma levels of calcium and metabolic parameters, hepatosomatic index, and gill Na^+,K^+-ATPase activity (Fig. 1, Table 3). In sham fish, transfer from SW to HSW during 4 days significantly increased gill Na^+,K^+-ATPase activity but was without effect on plasma osmolality and plasma sodium. On the other hand, transfer of sham fish to BW induced an increase in gill Na^+,K^+-ATPase activity. Gill Na^+,K^+-ATPase activity remained unaltered after transfer of E_2-treated fish to HSW or BW. However, in treated fish transferred from SW to SW (9 days in SW after E_2-implant), gill Na^+,K^+-ATPase activity decreased with respect to HSW- or BW-transferred fish (Table 3). Plasma osmolality decreased significantly in E_2-treated fish under hyposmotic change, whereas no changes were observed after transfer to HSW. As for plasma metabolic parameters, sham fish transferred to BW for 4 days displayed increased triglyceride levels. However, transfer to HSW increased significantly plasma levels of lactate, protein and triglyceride. E_2-treated fish transferred to HSW showed higher protein levels compared with shams without any change in other plasmatic parameters. Finally, transfer of treated fish to BW increased plasma glucose levels while decreased protein levels compared with shams.

DISCUSSION

In this study, we aimed to assess the osmoregulatory action of E_2 on the hyperosmotic and hypoosmotic capacity of gilthead sea bream using

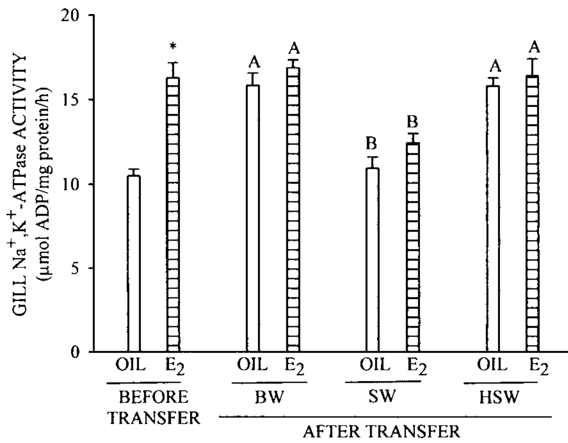

Fig. 1. Effect of a implant of coconut oil alone (sham) or containing E_2 (10 μg/g body weight) on gill Na^+,K^+-ATPase activity of gilthead sea bream after 5 days in SW followed by transfer to, HSW (hyperosmotic test), BW (hypoosmotic test) and SW (transfer test) during 4 days. Values are the mean ± SEM (n=7–8). *, 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 estradiol).

two types of E_2 treatment: short-term treatment with a single injection of different doses of E_2, and long-term treatment using a slow-release implant. In addition, two sampling times were selected for checking osmoregulatory action of E_2: 12h after transfer for short-term experiments, and 4 days for long term experiment. Thus, in the experiments involving short term treatment (Experiment 1 with sampling time at 12h post-transfer) we aimed to check the influence of E_2 on the adaptative period after transfer, while in the long-term treatment (Experiment 2 with sampling time at 4 days post-transfer) we aimed to test the influence of E_2 on the chronic regulatory period after transfer.

The method for delivery and the dose of E_2 used have been demonstrated to produce a stable increase in the levels of E_2 in the blood in different species of teleosts including gilthead seabream (Mosconi et al., '98; Guerreiro et al., 2002; Cavaco et al., 2003). Moreover, E_2 treatment is known to increase plasma levels of vitellogenin and calcium, and hepatosomatic index in teleost fish (Mommsen and Walsh, '88). In the present experiments, plasma calcium and protein, and hepatosomatic index were significantly higher in E_2-treated fish compared with controls, which is a fair indicator of

TABLE 3. Effect of a implant of coconut oil alone (sham) or containing E_2 (10 μg/g body weight) on several parameters in plasma, and hepatosomatic index (HSI) of gilthead sea bream after 5 days in SW followed by transfer to HSW (hyperosmotic test), BW (hypoosmotic test) and SW (transfer test) for 4 days

	Before transfer		After transfer					
	SW		BW		SW		HSW	
	Sham	E_2	Sham	E_2	Sham	E_2	Sham	E_2
Osmolality (mOsm/kg)	373.1 ± 5.1	386.2 ± 5.5	356.1 ± 6.2^a	360.4 ± 5.5^a	368.3 ± 6.2^a	382.9 ± 5.5^b	370.2 ± 5.8^a	387.2 ± 5.1^{b,1}
N^+ (mM)	166.2 ± 4.6	154.5 ± 4.3	166.6 ± 4.3^a	156.1 ± 3.7^a	175.7 ± 4.1^a	161.2 ± 3.7^{a,1}	169.0 ± 4.1^a	158.1 ± 3.7^a
Ca^{++} (mM)	2.61 ± 0.21	8.16 ± 0.21^1	2.55 ± 0.20^a	11.58 ± 0.1^{a,b,1}	2.60 ± 0.25^a	11.18 ± 0.1^{a,1}	2.97 ± 0.17^a	12.06 ± 0.1^{b,1}
Glucose (mM)	3.73 ± 0.19	7.05 ± 0.16^1	3.49 ± 0.17^a	5.08 ± 0.16^{a,1}	3.30 ± 0.19^a	4.41 ± 0.16^{b,1}	3.94 ± 0.18^a	4.56 ± 0.17^{a,b,1}
Lactate (mM)	1.74 ± 0.14	2.69 ± 0.12^1	1.33 ± 0.14^a	2.72 ± 0.12^{a,1}	1.65 ± 0.14^a	2.78 ± 0.12^{a,1}	2.24 ± 0.12^b	2.72 ± 0.12^{a,1}
Protein (mg/ml)	34.9 ± 1.1	56.3 ± 1.1^1	33.9 ± 1.1^a	87.8 ± 1.1^{a,1}	34.5 ± 1.4^a	94.5 ± 1.1^{b,1}	40.4 ± 1.1^b	99.3 ± 1.1^{c,1}
Triglycerides (mM)	2.06 ± 0.13	16.68 ± 0.68^1	3.30 ± 0.19^a	10.64 ± 0.92^{a,1}	2.15 ± 0.20^b	13.51 ± 1.21^{a,1}	3.48 ± 0.24^a	12.84 ± 1.45^{a,1}
HSI (%)	1.24 ± 0.05	2.61 ± 0.08^1	1.17 ± 0.04^a	2.97 ± 0.13^{a,1}	1.37 ± 0.04^b	2.92 ± 0.08^{a,1}	1.23 ± 0.04^{a,b}	3.03 ± 0.06^{a,1}

Values are the mean ± SEM (n=7–8). ^{1}significantly different ($P < 0.05$) from fish implanted with coconut oil alone (sham) under the same experimental conditions. ^{a,b,c}Different letters indicate significant differences ($P < 0.05$) among groups (BW, SW, and HSW) within each treatment (sham and estradiol).

an effective E_2 treatment in the present experiments.

It is well known that in euryhaline fish, the abrupt transfer to extreme salinities (such as those in our experimental design: from SW-38 ppt- to HSW-55ppt- and from SW-38 ppt- to BW-5ppt-) results in changes in plasma osmotic parameters, with the subsequent activation of the osmoregulatory system trying to recover the original values. Two phases have been described in that process such as: i) an adaptative period involving changes in osmotic parameters, and ii) a chronic regulatory period, where these parameters reach again homeostasis (Holmes and Donaldson, '69; Maetz, '74). Our group have demonstrated previously that gilthead sea bream presented a short adaptative period of 24–48h during hypoosmotic transfer (from SW to BW) (Mancera et al., '93; Mancera et al., 2002). The results of the present experiment agree with that model showing plasma hypoosmolality, and a reduction in plasma ion levels after hypoosmotic transfer. However, hyperosmotic transfer (from SW to HSW) did not induce any change in plasma osmolality or plasma ion levels, either suggesting that the adaptative period is shorter or that fish present a higher resistance to changes in the internal milieu under this situation. On the other hand, we have found previously (in the chronic regulatory period) an increase in gill Na^+,K^+-ATPase activity only 3 days after hyperosmotic or hypoosmotic transfer (Laiz-Carrión, Guerreiro, Fuentes and Mancera, unpublished data). The results of Experiment 2 confirmed these previous data.

There are no data available regarding the dynamics of gill Na^+,K^+-ATPase activity in gilthead sea bream under hyperosmotic and hyposmotic transfer. In teleosts, it has been reported a rapid non-genomic activation of gill Na^+,K^+-ATPase pump involving phosphorylation and/or membrane insertion of the protein, while the slow activation is associated with genomic changes, and involve an increase in the biogenesis of chloride cells, as well as in the synthesis of new molecules (Fosket et al., '81; Hwang et al., '89; Uchida and Kaneko, '96; Mancera and McCormick, 2000; Tipsmark and Madsen, 2001). In the present experiments, changes in gill Na^+,K^+-ATPase activity were only noticed at 3 days, but not at 24h, suggesting a genomic nature for changes due to an enhanced production of new molecules of Na^+,K^+-ATPase, which is associated with chloride cell differentiation/synthesis in this species upon hyperosmotic and hypoosmotic transfer. Short-term treatment of *S. auratus* with a single injection of different doses of E_2 did not affect gill Na^+,K^+-ATPase activity after hyperosmotic or hypoosmotic transfer. These results, together with results from long-term treatment with E_2 provide further support for a genomic action of E_2 on enzymatic activity.

In salmonids, long-term E_2 treatment is known to reduce chloride cell density, as well as gill Na^+,K^+-ATPase activity (Miwa and Specker, '86; Ikuta et al., '87; Madsen and Korsgaard, '89, '91; Madsen et al., '97). In non-salmonid fish, a negative effect of E_2 on gill Na^+,K^+-ATPase activity has been also demonstrated in *O. mossambicus* (Vijayan et al., 2001), and *F. heteroclitus* (Mancera et al., 2003). In addition, Vijayan and colleagues (2001) suggested that the reason for decreased branchial Na^+,K^+-ATPase activity and hypoosmoregulatory capacity after E_2 treatment in *O. mossambicus* was the reduced metabolic capacity observed in liver and gills. In the present study, E_2 treatment for 5 days clearly induced an increase in gill Na^+,K^+-ATPase activity. To our knowledge, this is the first report about a stimulatory action of this hormone on gill Na^+,K^+-ATPase activity. E_2 implant also increased plasma osmolality, which could be attributable to the increased levels of plasma metabolites observed in plasma of these fish (glucose, lactate, and protein).

After 5 days, E_2 treatment increased hepatosomatic index and plasma calcium, and protein levels suggesting an increase in vitellogenin production, as demonstrated previously in this species under similar hormonal treatment (Mosconi et al., '98; Guerreiro et al., 2002; Cavaco et al., 2003). In addition, this treatment also increased plasma levels of glucose, lactate and triglycerides. The results obtained for glucose agree with those previously found in several teleost species after E_2 treatment (Korsgaard and Mommsen, '93; Woo et al., '93; Sunny et al., 2002), whereas no previous studies have dealt with changes in plasma levels of lactate and triglycerides. In a previous paper (Sangiao-Alvarellos et al., 2003) we have suggested that lactate and tryglicerides could be used as fuel in gill cells during acclimation of *S. aurata* to different environmental salinities, which could support the higher gill Na^+,K^+-ATPase activity observed in the hyperosmotic environment. In addition, we have also demonstrated (Laiz-Carrión et al., 2003) that cortisol administration to gilthead sea bream induces a simultaneous increase

in both Na^+,K^+-ATPase activity in gills, and lactate and triglyceride levels in plasma. Our results after E_2 treatment suggest that this hormone increased levels of plasma metabolites to support the enhanced osmoregulatory work of gill epithelia. Moreover, considering the important metabolic changes elicited by 17β-estradiol on liver carbohydrate metabolism (Mommsen and Walsh, '88; Korsgaard and Mommsen, '93), the increased levels of plasma metabolites could be also the consequence of enhanced hepatic production to satisfy the liver production of vitellogenin and its uptake and further accumulation in gonads. The presence of E_2 receptors has been demonstrated in both intestine and kidney, but not in chloride cells of fish (Persson et al., 2000; Socorro et al., 2000). However, considering the stimulatory effect of E_2 on gill Na^+,K^+-ATPase activity, the existence of receptors in those cells could be a reasonable hypothesis.

In addition to the possible interaction of E_2 with gill receptors, E_2 treatment can also affect indirectly the parameters evaluated in the present study by an influence on other endocrine systems. In several teleosts, it has been demonstrated that E_2 treatment increased plasma PRL levels (Nagahama et al., '75; Wigham et al., '77; Olivereau and Olivereau, '86; Barry and Grau, '86; Poh et al., '97; for other references see Brinca et al., 2003). Moreover, it is known that in several teleosts, including *S. auratas*, PRL treatment decrease gill Na^+,K^+-ATPase activity (Manzon, 2000; Mancera et al., 2002). In gilthead sea bream the presence of PRL secretion due to E_2 treatment is depending on season since E_2 treatment for 7 days increased in vitro PRL release in winter while lowered PRL release in spring (Brinca et al., 2003). Since we have carried out the present E_2-implant experiments during spring (May-June), a decrease in the basal secretion of PRL with lower PRL plasma levels could be expected. Thus, E_2 treatment could reduce circulating PRL levels and therefore produce a decrease in the inhibitory effect of this hormone on gill Na^+,K^+-ATPase activity.

Considering that E_2 treatment is known to elevate GH production in tilapia (Poh et al., '97), a similar situation could be expected in the present experiments. However, considering that GH treatment has no clear effects on hypoosmotic capacity in gilthead sea bream (Mancera et al., 2002) an effect of E_2 mediated by GH do not seem apparent. It is also well known that cortisol stimulates gill Na^+,K^+-ATPase activity in teleosts, including gilthead sea bream (McCormick, '95; Mancera et al., 2002, Laiz-Carrión et al., 2003). We have no data about cortisol levels in E_2-treated gilthead sea bream, but considering that E_2 treatment in tilapia does not affect cortisol production (Vijayan et al., 2001), we may hypothesize a similar situation in our study, thus excluding a stimulation of gill Na^+,K^+-ATPase activity by cortisol.

After transfer from SW to BW or HSW, sham fish increased gill Na^+,K^+-ATPase activity with respect to fish transferred to SW. These data agree with the typical ''U-shaped'' relation between gill Na^+,K^+-ATPase and environmental salinity showed previously in some euryhaline species (see Jensen et al., '98), including gilthead sea bream (Laiz-Carrión, Guerreiro, Fuentes and Mancera, unpublished data). However, E_2-treated gilthead sea bream transferred to BW and HSW showed similar gill Na^+,K^+-ATPase activity with respect to values observed before transfer. The non existence of an increase in this activity associated to hypo- or hyperosmotic transfer in E_2-treated fish could suggest the inability of chloride cells to enhance Na^+,K^+-ATPase under a new stimuli (salinity transfer after E_2 treatment).

It is interesting to remark that SW-adapted E_2-implanted fish presented higher gill Na^+,K^+-ATPase activity at 5 days post-implant, but showed a decrease in this activity 9 days post-implant. A loss in sensitivity of gill chloride cells to exogenous E_2 or an exhaustion of gill chloride cells as a consequence of high E_2 levels during nine days could be also responsible for the data obtained. The presence of E_2 receptors in the chloride cells of fish has not been demonstrated yet (Persson et al., 2000; Socorro et al., 2000). If those receptors were present, a downregulation by high hormonal levels, similarly to that demonstrated previously for corticosteroids receptors (Maule and Schreck, '91; Shrimpton and Randall, '94), could help to explain the results of the present study. However, E_2-implanted fish under hyperosmotic or hypoosmotic transfer maintained higher gill Na^+,K^+-ATPase activity at least for 4 more days indicating that chloride cells were able to maintain this enzyme activity. These results also suggest that, in addition to hormones, other factors (like changes in salinity) are also necessary for the activation of chloride cells (Foskett et al., '81; Zadunaisky et al., '95; Mancera and McCormick, '98).

In conclusion, our results showed a clear stimulatory effect of E_2 on gill Na^+,K^+-ATPase activity after long- but not after short-term

treatments. To our knowledge, this is the first report about this stimulatory effect of E_2 in teleosts. Previously, it has been reported that E_2 treatment decreases gill Na^+,K^+-ATPase activity and osmoregulatory capacity in salmonids (Miwa and Specker, '86; Ikuta et al., '87; Madsen and Korsgaard, '89, '91; Madsen et al., '97), *O. mossambicus* (Vijayan et al., 2001) and *F. heteroclitus* (Mancera et al., 2003). Differences among species as well as in the history of the fish and in their environment may be at the basis for such discrepancies.

ACKNOWLEDGMENTS

The authors are grateful to Planta de Cultivos Marinos (CASEM, Universidad de Cádiz, Puerto Real, Cádiz, Spain) for providing experimental fish.

LITERATURE CITED

Barry TP, Grau G. 1986. Estradiol-17β and thyrotropin-releasing hormone stimulate prolactin release from the pituitary gland of a teleost fish in vitro. Gen Comp Endocrinol 62:306–314.

Brinca L, Fuentes J, Power DM. 2003. The regulatory action of estrogen and vasoactive intestinal peptide on prolactin secretion in sea bream (*Sparus aurata*, L.). Gen Comp Endocrinol 131:117–125.

Bucolo G, David H. 1973. Quantitative determination of serum triglycerides by the use of enzymes. Clin Chem 19:476.

Cavaco JEB, Santos CRA, Ingleton PM, Canario VM, Power DM. 2003. Quantification of prolactin (PRL) and PRL receptor messenger RNA in gilthead seabream (*Sparus aurata*) after treatment with estradiol-17β. Biol Reproduct 68:588–594.

Foskett JK, Lodgson CD, Turner T, Machen TE, Bern HA. 1981. Diferentiation of the chloride extrusion mechanisms during seawater adaptation of a telesot fish, the cichlid *Sarotherodon mossambicus*. J Exp Biol 93:209–224.

Guerreiro PM, Fuentes J, Canario AVM, Power DM. 2002. Calcium balance in sea bream (*Sparus aurata*): the effect of oestradiol-17β. J Endocrinol 173:377–385.

Hirano T. 1986. The spectrum of prolactin action in teleosts. In: Ralph CL, editor. Comparative endocrinology: developments and directions. New York City, NY: Alan R. Liss. p 53–74.

Holmes WN, Donaldson EM. 1969. The body compartments and the distribution of electrolytes. In: Hoar WS, Randall DJ, editors. Fish Physiology, Vol. 1. San Diego, CA: Academic Press. p 1–89.

Hwang PP, Sun CM, Wu SM. 1989. Changes of plasma osmolality, chloride concentration and gill Na-K-ATPases activity in tilapia *Oreochromis mossambicus* during seawater acclimation. Mar Biol 100:295–299.

Ikuta K, Aida K, Okumoto N, Hanyu I. 1987. Effects of sex steroids on the smoltification of masu salmon, *Oncorhynchus masou*. Gen Comp Endocrinol 65:99–110.

Iwama GK, McGeer JC, Pawluk MP. 1989. The effects of five fish anesthetics on acid-base balance, hematocrit, blood gases, cortisol and adrenaline in rainbow trout. Can J Fish Aquat Sci 67:2065–2073.

Jensen MK, Madsen SS, Kristiansen K. 1998. Osmoregulation and salinity effects on the expression and activity of Na^+,K^+-ATPase in the gills of european sea bass, Dicentrarchus labrax (L.). J Exp Zool 282:290–300.

Korsgaard B, Mommsen TP. 1993. Gluconeogenesis in hepatocytes of immature rainbow trout (*Oncorhynchus mykiss*): Control by estradiol. Gen Comp Endocrinol 89:17–27.

Laiz-Carrión R, Martín del Río MP, Miguez JM, Mancera JM, Soengas JL. 2003. Influence of cortisol on osmoregulation and energy metabolism in gilthead sea bream *Sparus aurata*. J Exp Zool 298A:105–118.

Le François N, Blier P. 2000. Branchial Na^+K^+ATPase activity in brook charr (*Salvelinus fontinalis*): Effect of gonadal development in hypo- and hyperosmotic environments. J Exp Zool 286:647–655.

Lundqvist H, Borg B, Berglund I. 1989. Androgens impair seawater adaptability in smolting Baltic salmon (*Salmo salar*). Can J Zool 67:1733–1736.

Madsen SS, Korsgaard B. 1989. Time-course effects of repetitive oestradiol-17β and thyroxine injections on the natural spring smolting of atlantic salmon, Salmo salar L. J Fish Biol 35:119–128.

Madsen SS, Korsgaard B. 1991. Opposite effects of 17β-estradiol and combined growth hormone-cortisol treatment on hypo-osmoregulatory performance in sea trout presmolts, *Salmo trutta*. Gen Comp Endocrinol 83:276–282.

Madsen SS, Mathiesen AB, Korsgaard B. 1997. Effects of 17β-estradiol and 4-nonylphenol on smoltification and vitellogenesis in Atlantic salmon (*Salmo salar*). Fish Physiol Biochem 17:303–312.

Maetz J. 1974. Aspects of adaptation to hypo-osmotic and hyper-osmotic environments. In: Malins DC, Sargent JR, editors. Biochemical and Biophysical Perspectives in Marine Biology. New York City, NY: Academic Press. p 1–167.

Mancera JM, McCormick SD. 2000. Rapid activation of gill Na^+,K^+-ATPase in the euryhaline teleost *Fundulus heteroclitus*. J Exp Zool 287:263–274.

Mancera JM, Pérez-Fígares JM, Fernández-Llebrez P. 1993. Osmoregulatory responses to abrupt salinity changes in the euryhaline gilthead sea bream (*Sparus aurata*). Comp Biochem Physiol 106A:245–250.

Mancera JM, Pérez-Fígares JM, Fernández-Llebrez P. 1994. Effect of cortisol on brackish water adaptation in the euryhaline gilthead sea bream (*Sparus aurata* L.). Comp Biochem Physiol 107A:397–402.

Mancera JM, Laiz-Carrión R, Martín del Río MP. 2002. Osmoregulatory action of PRL, GH and cortisol in the gilthead seabream (*Sparus aurata* L.). Gen Comp Endocrinol 129:95–103.

Mancera JM, Smolenaars M, Laiz-Carrión R, Martín del Río MP, Wendelaar Bonga SE, Flik G. 2003. 17β-estradiol affects osmoregulation in *Fundulus heteroclitus*. Comp Biochem Physiol. in press.

Manzon LA. 2002. The role of prolactin in fish osmoregulation: A review. Gen Comp Endocrinol 125:291–310.

Maule AG, Schreck CB. 1991. Stress and cortisol treatment changed affinity and number of glucocorticoid receptors in leukocytes and gill of coho salmon. Gen Comp Endocrinol 84:83–93.

McCormick SD. 1993. Methods for nonlethal gill biopsy and measurement of Na^+,K^+-ATPase activity. Can J Fish Aquat Sci 50:656–658.
McCormick SD. 1995. Hormonal control of gill Na^+,K^+-ATPase and chloride ell function. In: Wood CM, Shuttleworth TJ, editors. Fish Physiology. Vol. 14. New York City, NY: Academic Press. p 285–315.
McCormick SD, Naiman RJ. 1985. Hypoosmoregulation in an anadromous teleost: influence of sex and maturation. J Exp Zool 234:193–198.
Miwa S, Inui Y. 1986. Inhibitory effects of 17α-methyltestosterone and estradiol-17β on smoltification of sterilized amago salmon (*Oncorhynchus rhodurus*). Aquaculture 53:21–39.
Mommsen TP, Walsh PJ. 1988. Vitellogenesis and oocyte assembly. In: Hoar WS, Randall DJ, editors. Fish Physiology. Vol 11. New York City, NY: Academic Press. p. 347–406.
Mosconi G, Carnevali O, Carletta R, Nabissi M, Polzonetti-Magni AM. 1998. Gilthead seabream (*Sparus aurata*) vitellogenin: purification, partial characterization, and validation of an enzyme-linked immunosorbent assay (ELISA). Gen Comp Endocrinol 110:252–261.
Nagahama Y, Nishioka RS, Bern HA, Gunther R. 1975. Control of prolactin secretion in teleosts, with special reference to *Gillichthys mirabilis* and *Tilapia mossambica*. Gen Comp Endocrinol 25:166–188.
Olivereau M, Olivereau J. 1979. Effect of estradiol-17β on the cytology of the liver, gonads and pituitary, and on plasma electrolytes in the female freshwater eel. Cell Tissue Res 199:431–454.
Olivereau M, Dubourg P, Chambolle P, Olivereau J. 1986. Effects of estradiol and mammalian LHRH on the ultrastructure of the pars distalis of the eel. Cell Tissue Res 246:425–437.
Persson P, Shrimpton JM, McCormick SD, Björnsson BT. 2000. The presence of high-affinity, low-capacity estradiol-17beta binding in rainbow trout scale indicates a possible endocrine route for the regulation of scale resorption. Gen Comp Endocrinol 120:35–43.
Poh LH, Munro AD, Tan, CH. 1997. The effects of oestradiol on the prolactin and growth hormone content of the pituitary of the tilapia, *Oreochromis mossambicus*, with observations on the incidence of black males. Zool Sci 14:979–986.
Sangiao-Alvarellos S, Laiz-Carrión R, Guzmán JM, Martín del Río MP, Miguez JM, Soengas JL. 2003. Acclimation of *Sparus aurata* to various salinities alters energy metabolism of osmoregulatory and nonosmoregulatory organs. Am J Physiol 285:R897–R907.
Schlenk D, El-Alfy A, Buhler DR. 1997. Down regulation of hepatic flavin-containing monooxygenase activity by 17β-estradiol in rainbow trout (*Oncorhynchus mykiss*). Comp Biochem Physiol 118C:199–202.
Shrimpton JM, Randall DJ. 1994. Downregulation of corticosteroid receptors in gill of coho salmon due to stress and cortisol treatment. Am J Physiol 267:R432–R438.
Smith OK, Krohon RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC. 1985. Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85.
Socorro S, Power DM, Olsson PE, Canario AV. 2000. Two estrogen receptors expressed in the teleost fish, *Sparus aurata*: cDNA cloning, characterization and tissue distribution. J Endocrinol 166:293–306.
Staurnes M, Sigholt T, Gulseth OA, Elliassen R. 1994. Effects of maturation on seawater tolerance of anadromous Artic charr. Trans Amer Fish Soc 123:402–407.
Stein MW. 1963. D-Glucose, determination with hexokinase and glucose-6-phosphate dehydrogenase. In: Bergmeyer HU, editor. Methods of Enzymatic Analysis. New York City, NY: Academic Press. p. 117.
Sunny F, Annamma J, Oomen OV. 2002. Sex steroids regulate intermediary metabolism in *Oreochromis mossambicus*. Endocrine Res 28:175–188.
Tipsmark CK, Madsen SS. 2001. Rapid modulation of Na^+,K^+-ATPase activity in osmoregulatory tissue of a salmonid fish. J Exp Biol 204:701–709.
Uchida K., Kaneko T. 1996. Enhanced chloride cell turnover in the gill of chum salmon fry in seawater. Zool Sci 13: 655–660.
Vijayan MM, Takemura A, Mommsen TP. 2001. Estradiol impairs hyposmoregulatory capacity in the euryhaline tilapia, *Oreochromis mossambicus*. Am J Physiol. 281:R1161–R1168.
Wigham T, Nishioka RS, Bern HA. 1977. Factors affecting in vitro activity of prolactin cells in the euryhaline teleost Sarotherodon mossambicus (*Tilapia mossambica*). Gen Comp Endocrinol 32:120–131.
Woo NYS, Chung ASB, Ng TB. 1993. Influence of oral administration of estradiol-17β and testosterone on growth, digestion, food conversion and metabolism in the underyearling red sea bream, *Chrysophrys major*. Fish Physiol Biochem 10:377–387.
Zadunaisky JA, Cardona S, Au L, Roberts DM, Lowenstein B, Crague EJ, Spring KR. 1995. Chloride transport activation by plasma osmolality during rapid adaptation to high salinity of *Fundulus heteroclitus*. J Membr Biol 143: 207–217.