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Influence of Cortisol on Osmoregulation and Energy Metabolism in Gilthead Seabream *Sparus aurata*

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ABSTRACT Gilthead seabream *Sparus aurata* were injected intraperitoneally with slow-release implants of coconut oil alone or containing cortisol (50 and 100 $\mu g.g^{-1}$ body weight), and sampled after two, five, and seven days to assess the simultaneous effects of cortisol on both osmoregulation and energy metabolism. Plasma cortisol levels increased in treated fish to 50–70 $ng.ml^{-1}$. An enhanced hypoosmoregulatory capacity of cortisol-implanted fish is suggested by the increase observed in gill Na^+, K^+-ATPase activity, and the decrease observed in plasma ion concentration (Na^+ and Cl^-) and osmolality. Cortisol also elicited metabolic changes in liver (increased gluconeogenic potential suggested by elevated FBPase activity, and decreased potential of glycolysis and pentose-phosphate shunt, suggested by the decreased activities of both PK and G6PDH) supporting changes in levels of plasma metabolites suitable for use in other tissues. Thus in this study, we demonstrate for the first time in fish that cortisol treatments elicit changes in the use of exogenous glucose in gills (decreased HK activity) and an increased glycolytic and glycogenic potential in brain (increased GPase, PK and PFK activities). *J. Exp. Zool. 298A:105–118, 2003.*
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INTRODUCTION

Cortisol directly and/or indirectly plays an important role in several aspects of fish physiology including intermediary metabolism, ionic and osmotic regulation, growth, stress, and immune function (Henderson and Garland, '80; McCormick, '95; Wendelaar Bonga, '97; Mommsen et al. '99).

The role of cortisol in fish in the regulation of substrate mobilization for energy purposes is poorly understood (Van der Boon et al., '91; Mommsen et al., '99). The role of cortisol in increasing plasma glucose concentration is believed to be due to activation of gluconeogenesis, since hepatic gluconeogenic and aminotransferase enzyme activities, and circulating glucose concentrations, are enhanced in cortisol-implanted fish (Mommsen et al., '99). Another reported consequence of cortisol administration in fish is the deposition of liver glycogen. However, this phenomenon is not consistently observed. Few studies have examined the effects of corticosteroids on tissue enzyme activities, a potentially more sensitive indicator of steroid-induced metabolic changes (Mommsen et al., '99).

The actions of cortisol on the energy metabolism of tissues other than liver, such as the gills (Pottinger et al., 2000), brain (Knoebl et al., '96), or kidney (Kloas et al., '98), in which receptors for cortisol have been described, have been less studied to date (Mommsen, '84; Mommsen et al., '99).

Cortisol also plays an important role in the adaptation to hyperosmotic environments. In this way, cortisol induces salinity tolerance, development and proliferation of gill chloride cells, gill Na^+, K^+ -ATPase activity, and expression of

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Na^+ K^+ -ATPase α-subunit (McCormick, '90, '95; Madsen et al., '95; Seidelin and Madsen, '97). In addition, in some species, this hormone can also promote adaptation to hypoosmotic environments (Perry, '97; Eckert et al., 2001; McCormick, 2001).

Osmotic adaptation of euryhaline fish is usually accompanied by increased oxygen consumption suggesting increased energetic demands for osmoregulation. Although the functions of osmoregulatory organs, such as the gills, kidneys, and intestines, have been extensively investigated, less attention has been paid to metabolic costs of osmoregulation in other tissues (Nakano et al., '97). Some of these metabolic changes may be related to the secondary effects of increased cortisol production on other metabolic processes (Morgan and Iwama, '96; Morgan et al., '97). In summary, cortisol is involved in teleost fish in the control not only of osmoregulatory capacity (McCormick, '95, 2001) but also of energy metabolism (Mommsen et al., '99). However, very little is known about the role of cortisol in the interrelationship between energy metabolism and osmoregulation in teleosts.

Gilthead seabream (*Sparus aurata*) is an euryhaline teleost capable of adapting to extreme changes in environmental salinity (Chervinsky, '84; Mancera et al., '93a). Different aspects of the osmoregulatory system in this species have been assessed previously (Mancera et al., '93a,b, '94, '95), including the role of different hormones in the adaptation to hyperosmotic and hypoosmotic environments (Mancera et al., 2002). However, there are no references regarding the interrelationship between osmoregulation and metabolism in this species. Therefore, the primary objective of the present study was to achieve cortisol levels similar to those observed in this euryhaline fish during osmotic adaptation to assess their effects on osmoregulatory and metabolic parameters simultaneously, in order to elucidate whether or not cortisol is involved in the energy repartitioning process associated with osmoregulation.

MATERIALS AND METHODS

Fish

Immature male gilthead seabream (*Sparus aurata* L., 100–150 g body weight) were provided by Planta de Cultivos Marinos (C.A.S.E.M., University of Cádiz, Puerto Real, Cádiz, Spain) and transferred to the laboratories at Faculty of Marine Science (Puerto Real, Cádiz). They were acclimated to seawater in 300 l aquaria for at least two weeks in an open system (40 p.p.t. salinity, 1000 $mOsm.kg^{-1}$ H_2O). During the experiments, fish were maintained under natural photoperiod (May) and constant temperature (18°C). Fish were fed daily with 1% body weight using commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain). They fasted for 24 h before hormone injection and throughout the experiment. 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 protocol

Three different groups (35 fish per group) of seawater acclimated seabream (100–150 g weight) were used. Fish were anaesthetized with 2–phenoxyethanol (0.5 $ml.l^{-1}$ water), weighed, and intraperitoneally implanted with slow-release coconut oil implants following procedures previously described (Soengas et al., '92). Thus, fish were injected with 5 $\mu l.g^{-1}$ body weight of coconut oil alone (controls) or containing different doses of cortisol (50 and 100 $\mu g.g^{-1}$ body weight) and placed back in seawater. Fish were sampled (n=7 per group and time) at two, five, and seven days after implant. Before implant, one group of fish (n=7) was sampled and served as control (untreated fish) as time 0 day. No mortality was observed during the experiment.

Sampling

Fish were anaesthetized with 2–phenoxyethanol (1 $ml.l^{-1}$ water), weighed, and sampled. 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. In order to assess gill Na^+,K^+-ATPase activity, and according to Mancera et al. (2002), three to five filaments coming from one branchial arch were cut just above the septum with fine point scissors and placed in 100 μl of ice-cold SEI buffer (150 $mmol.l^{-1}$ sucrose, 10 $mmol.l^{-1}$ EDTA, 50 $mmol.l^{-1}$ imidazole, pH 7.3) and frozen at −80°C. The liver, brain, and the remaining branchial arches were quickly removed (within a few seconds) from each fish, weighed, frozen on dry ice and stored at −80°C until assayed.

Analytical techniques

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, Rockford, USA) for microplates, with bovine 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, USA) using DeltaSoft3 software for Macintosh (BioMetallics, Inc. NJ). Plasma osmolality was measured with a vapor pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA) and expressed as $mOsm.kg^{-1}$. Plasma Na^+ was measured using a atomic absorption spectrophotometer, and plasma Cl^- levels with the Chloride Sigma kit (n° 461).

Plasma cortisol was determined by RIA in 15 μl plasma samples diluted in 300 μl phosphate buffer containing $0.5g.l^{-1}$ gelatine, pH 7.6 and denatured at 70°C for 30 min. The antiserum used for the assay was raised in rabbits against cortisol–3–(0-carboxymethyl)oxime-bovine serum albumin conjugate (Sigma-Aldrich). The cross-reactivity of this antiserum was determined in preliminary experiments with gilthead seabream giving values of 100% for cortisol, 54% for 11–desoxycortisol, 10% for cortisone, 16% for 17,21–dihydroxy–5β–pregnan-3,11,20–trione, 5% for 11b,17,21–trihydroxy–5β–pregnan–3,20–dione, 0.05% for 11–hydroxytestosterone, and $<0.001\%$ for testosterone. Binding of the antisera to thin layer chromatography fractionated plasma confirmed the specificity of the assay by revealing a single peak in the cortisol specific fraction.

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 thawed 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 25C 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, USA) with bovine albumin as standard. Both assays were run on a microplate reader as above.

After −80°C freezing, brain, liver, and gill samples were finely minced on a chilled Petri dish to very small pieces that were vigorously mixed and divided into two different (but relatively homogeneous) aliquots to assess enzyme activities and metabolite levels, respectively.

The aliquots of liver, brain, and gill used for the assessment of metabolite levels were homogenized immediately by ultrasonic disruption in the cold (Microson XL2007) with 10 vols of ice-cooled 6% perchloric acid, neutralized (using 1 $mol.l^{-1}$ potassium bicarbonate), centrifuged (2 min at 13,000 g, Eppendorf 5415R), and the supernatant used to assay tissue metabolites. Lactate levels were determined using the enzymatic method of Gutman and Wahlefeld ('74). Glycogen levels were assessed using the method of Keppler and Decker ('74). Glucose obtained after glycogen breakdown in brain and liver (after substracting free glucose levels), was determined with a glucose oxidase-peroxidase method (Biomérieux, Spain).

The aliquots of tissue used for the assessment of enzyme activities were homogenized by ultrasonic disruption in the cold (Microson XL2007) with 10 vols of ice-cold stopping-buffer containing 50 $mmol.l^{-1}$ imidazole-HCl (pH 7.5), 1 $mmol.l^{-1}$ 2–mercaptoethanol, 50 $mmol.l^{-1}$ NaF, 4 $mmol.l^{-1}$ EDTA, 250 $mmol.l^{-1}$ sucrose, and 0.5 $mmol.l^{-1}$ 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 used in enzyme assays.

Enzyme activities were determined using a Unicam UV6–220 spectrophotometer. 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. Protein was assayed in triplicate in homogenates as detailed by Bradford ('76), using bovine serum albumin (Sigma, USA) as standard. Enzymatic analyses were all carried out under optimal substrate concentrations determined in preliminary tests to render maximal activities. The specific conditions for enzyme assays were set up for use in gilthead seabream following original

methods previously described for salmonids (Soengas et al., '95, '96, '98), and were as follows:

Glycogen phosphorylase (*EC* 2.4.1.1.; GPase) was assayed in brain and liver using 50 mmol.l^{-1} phosphate buffer (pH 7.0), 27 mmol.l^{-1} $MgSO_4$, 19.5 mmol.l^{-1} EDTA, 0.5 mmol.l^{-1} NADP, 50 μmol.l^{-1} glucose 1,6–biphosphate, 2.5 mmol.l^{-1} AMP, excess phosphoglucomutase, excess glucose 6-phosphate dehydrogenase, and 5 mg.ml^{-1} glycogen (omitted for control). GPase *a* activity was measured with 10 mmol.l^{-1} caffeine present, and total GPase activities were estimated without caffeine. The ratio of GPase activities with and without caffeine multiplied by 100 represents the percentage of total GPase (*a*+*b*) in the active form (% GPase *a*).

6–Phosphofructo 1–kinase (*EC* 2.7.1.11.; PFK) was assessed in brain using 100 mmol.l^{-1} imidazole-HCl (pH 8.25), 5 mmol.l^{-1} $MgCl_2$, 50 mmol.l^{-1} KCl, 4 mmol.l^{-1} $SO_4(NH_4)_2$, 0.15 mmol.l^{-1} NADH, 1 mmol.l^{-1} ATP, excess aldolase, excess triose phosphate isomerase, and excess α-glycerol phosphate dehydrogenase. Activities were determined at low (0.1 mmol.l^{-1}) and high (5 mmol.l^{-1}) fructose 6–phosphate concentrations (omitted for controls). An activity ratio was calculated as the activity at low [fructose 6P]:high [fructose 6P]. Similarly, a fructose 2,6–bisphosphate (F 2,6–P_2) activation ratio was determined using 5 μmol.l^{-1} fructose 2,6–bisphosphate, and 0.1 mmol.l^{-1} fructose 6–phosphate concentrations.

Pyruvate kinase (*EC* 2.7.1.40.; PK) was assessed in liver and brain using 50 mmol.l^{-1} imidazole-HCl (pH 7.4), 10 mmol.l^{-1} $MgCl_2$, 100 mmol.l^{-1} KCl, 0.15 mmol.l^{-1} NADH, 0.5 mmol.l^{-1} ADP, and excess lactate dehydrogenase. Activities were determined at low (0.1 mmol.l^{-1}) and high (2.8 mmol.l^{-1}) phosphoenolpyruvate (PEP) concentrations (omitted for controls). An activity ratio was calculated as the activity at low [PEP]:high [PEP]. Similarly, a fructose 1,6–bisphosphate (F 1,6–P_2) activation ratio was determined using 1 (liver) and 0.01 (brain) mmol.l^{-1} fructose 1,6–bisphosphate, and 0.1 mmol.l^{-1} fructose 6-phosphate concentrations.

Fructose 1,6-bisphosphatase (*EC* 3.1.3.11.; FBPase) was assessed in liver using 85 mmol.l^{-1} imidazole-HCl (pH 7.7), 0.5 mmol.l^{-1} NADP, 5 mmol.l^{-1} $MgCl_2$, excess phosphoglucose isomerase, excess glucose 6–phosphate dehydrogenase, and 0.05 mmol.l^{-1} fructose 1,6–bisphosphate (omitted for control). Glucose–6–phosphate dehydrogenase (*EC* 1.1.1.49.; G6PDH) was determined in brain and liver using 78 mmol.l^{-1} imidazole-HCl (pH 7.7), 5 mmol.l^{-1} $MgCl_2$, 0.5 mmol.l^{-1} NADP, and 1 mmol.l^{-1} glucose 6-phosphate (omitted for control).

Hexokinase (*EC* 2.7.1.1.; HK) was assayed in liver, brain, and gills with 50 mmol.l^{-1} imidazole-HCl (pH 8), 5 mmol.l^{-1} $MgCl_2$, 0.15 mmol.l^{-1} NADP, 1 mmol.l^{-1} ATP, excess glucose 6–phosphate dehydrogenase, and 5 mmol.l^{-1} glucose (omitted for control).

Statistics

All data were statistically analysed by a two-way ANOVA test with treatment (0, 50, and 100 μg.g^{-1}) and time (two, five, and seven days) as the main factors. Logarithmic transformations of the data were made when necessary to fulfil the conditions of the analysis of variance. Post-hoc comparisons were made using a Tukey HSD test for unequal N, considering the differences statistically significant at $p < 0.05$.

RESULTS

No differences were observed between untreated fish (time 0 days) and those fish implanted with coconut oil alone (control) either for plasma cortisol levels (Fig. 1) or for any other parameter assessed (data not shown).

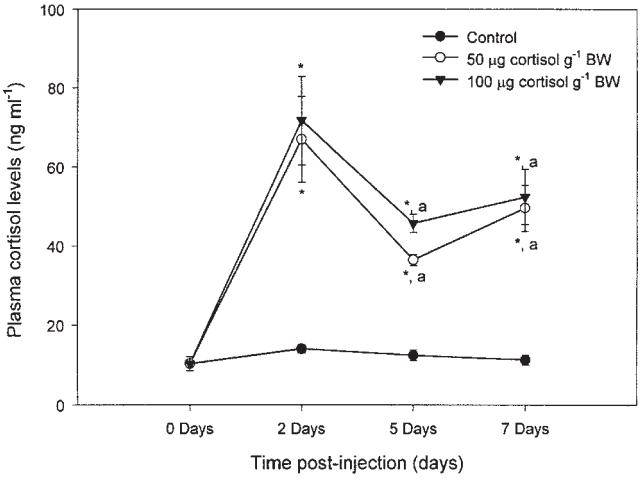

Fig. 1. Changes in the levels of cortisol in plasma of gilthead seabream after 2, 5, and 7 days of intraperitoneal implantation of 5 μl.g^{-1} body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 μg.g^{-1} body weight). Each value is the mean ± S.E.M. of n=7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). a, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 days.

Plasma cortisol levels increased in cortisol-treated fish compared to controls throughout the experiment, though significant differences were not observed between the two doses used (Fig. 1). Also, values at five and seven days for fish treated with both doses of cortisol were significantly lower than those observed in treated fish at two days (Fig. 1).

Cortisol treatment clearly increased gill Na^+,K^+-ATPase activity over five days post-implant. However, by seven days post-implant values of gill Na^+,K^+-ATPase activity were similar to controls (Fig. 2A). The ATPase activity of treated fish after seven days of treatment with 50 and 100 $\mu g.g^{-1}$ was significantly lower than the activity observed in fish treated with the same doses after two days (Fig. 2A). Cortisol treatment also decreased gill HK activity compared with controls (Fig. 2B).

Consistent with the increased gill Na^+,K^+-ATPase activity, plasma levels of Na^+ (Fig. 3B) and Cl– (Fig. 3C) decreased two days post-implant in cortisol-treated fish. However, plasma osmolality did not show any significant change after two days of treatment (Fig. 3A). In addition, in cortisol-treated fish, plasma osmolality decreased at day seven compared with controls, without any change in plasma Na^+ and Cl^- levels (Fig. 3). As for the days effect, plasma osmolality of fish treated with 50 $\mu g.g^{-1}$ cortisol at seven days was significantly lower than levels measured in fish treated with the same dose of cortisol at two and five days (Fig. 3A). Plasma sodium levels of fish treated with cortisol at five and seven days were significantly higher than those observed in treated fish at two days (Fig. 3B), whereas for plasma chloride levels fish treated with the dose of 100 $\mu g.g^{-1}$ levels were higher at five days than those observed in fish treated with the same dose at two days. (Fig. 3C).

When addressing the effects of cortisol treatment on the levels of plasma metabolites, a significant increase was observed in both glucose and triglyceride levels of treated fish compared with controls (Fig. 4A, B). Several days' effects were also noticed for both parameters since levels of glucose in fish treated with 50 $\mu g.g^{-1}$ cortisol at five days were significantly lower than those observed in fish treated with the same dose at two and seven days (Fig. 4A). Plasma triglyceride levels of fish treated at five days with both doses of cortisol were significantly lower than those measured in fish treated with the same doses at two days; a similar decrease was noticed at seven days but only for fish treated with the dose of 100 $\mu g.g^{-1}$ (Fig. 4B). As for lactate levels, an increase in treated fish compared with controls was apparent only at day five with the dose of 50 $\mu g.g^{-1}$ whereas on day two a decrease was recorded (Fig. 4C). Lactate levels were also higher in fish treated with both doses of cortisol after five and seven days of treatment than those treated with the same doses after two days (Fig. 4C).

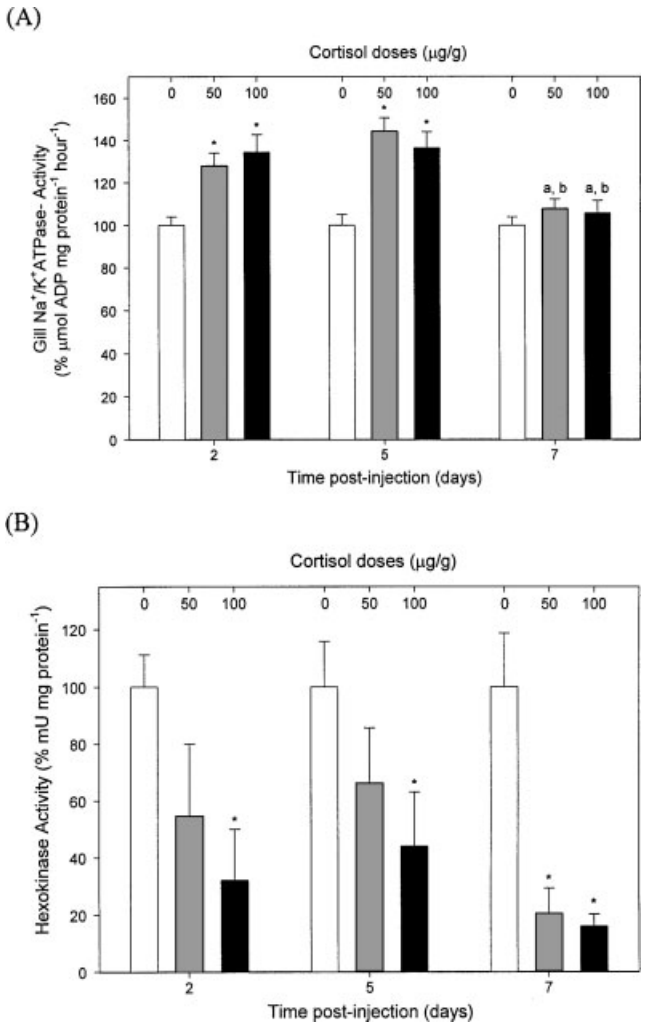

Fig. 2. Changes in the activity of Na^+,K^+, ATPase (A) and hexokinase (B) in gills of gilthead seabream after 2, 5, and 7 days of intraperitoneal implantation of 5 $\mu l.g^{-1}$ body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 $\mu g.g^{-1}$ body weight). Results are shown as percent of control (control = 100%; Average value of controls: 10.73 ± 0.17 µmol $ADP.mg^{-1}$ $protein.h^{-1}$, and 1405 ± 173 $mU.mg^{-1}$ protein, for activities of Na^+, K^+, ATPase, and HK, respectively). Each value is the mean $\pm$ S.E.M. of n=7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). a, b, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 and 5 days, respectively.

Glycogen levels in liver and brain displayed a similar trend in cortisol-treated fish, i.e., an increase compared with controls (Fig. 5). Levels of glycogen in liver were also significantly higher after seven days of treatment in fish treated with the two doses of cortisol compared with those fish sampled after two days of treatment; a similar increase was noticed after five days but only for fish treated with 50 $\mu g.g^{-1}$ (Fig. 5A). In contrast, lactate levels displayed no significant changes in either liver or brain when comparing cortisol-treated fish and controls (data not shown).

As for enzyme activities in liver (Table 1), a decrease in the activity of the glycogenolytic enzyme GPase of cortisol-treated fish compared with controls was observed when considering the percentage of the enzyme in the active form, whereas no significant differences were observed for total activity. The percent GPase *a* displayed in fish treated with 100 $\mu g.g^{-1}$ showed a decrease after five and seven days when compared with fish treated with the same dose at day two; a similar decline was observed in fish treated with 50 $\mu g.g^{-1}$ after five days. This was significantly lower than those measured after two and seven days. A decline in activity of cortisol-treated fish compared to controls was also observed in the activity of the glycolytic enzyme PK, considering the optimal activity or the activity ratio and fructose 1,6–bisphosphate activation ratio of the enzyme. More decreases in activity in cortisol-treated fish compared to controls were observed when considering G6PDH and HK enzymes. Also, for G6PDH activity values of fish treated with 100 $\mu g.g^{-1}$ after five and seven days were significantly higher than those fish treated with the same dose after two days. A significant increase in activity in cortisol-treated fish compared with controls was also noticed for liver FBPase.

Brain enzyme activities are presented on Table 2. No significant differences were noted in GPase activity when comparing the optimal activity of treated and control fish, whereas in the

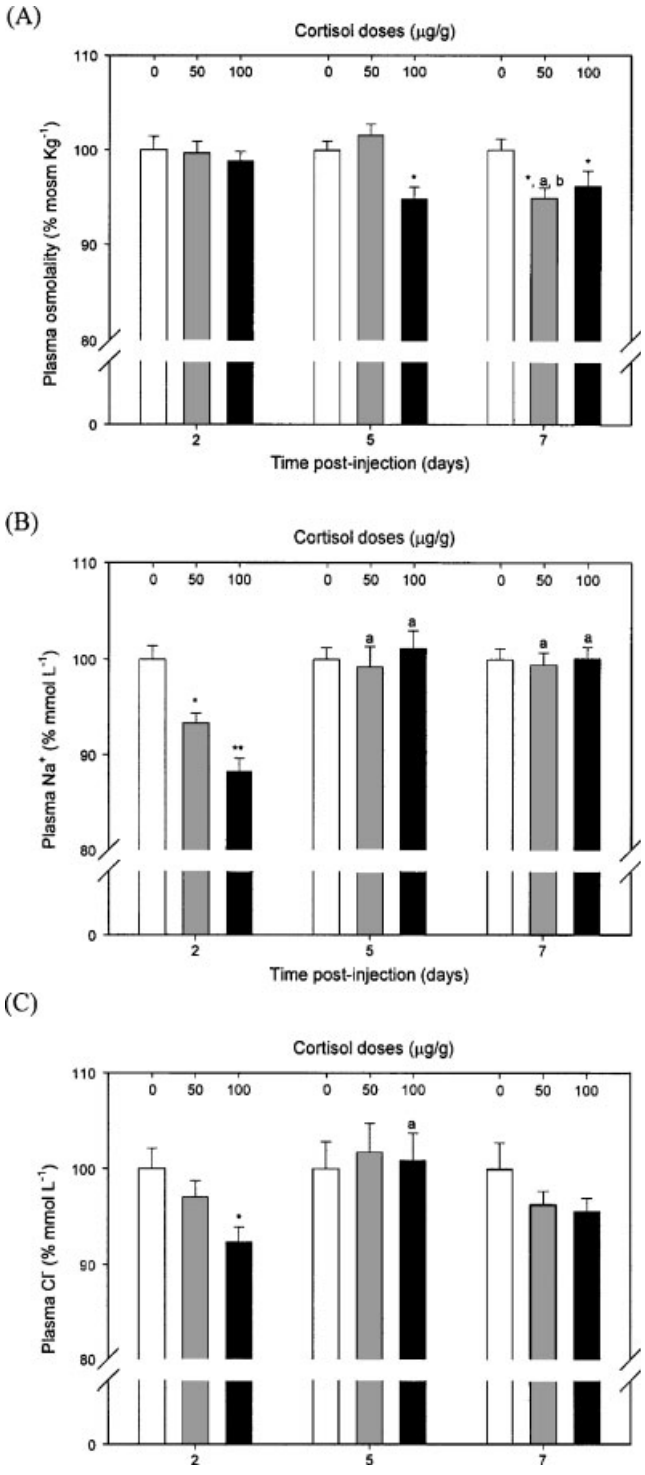

Fig. 3. Changes in osmolality (A), sodium levels (B) and chloride levels (C) in plasma of gilthead seabream after 2, 5, and 7 days of intraperitoneal implantation of 5 $\mu l.g^{-1}$ body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 $\mu g.g^{-1}$ body weight). Results are shown as percent of control (control=100%; Average value of controls: 358.8 ± 5.18 $mOsm.kg^{-1}$, 175.3 ± 1.91 $mmol.l^{-1}$, and 157.7 ± 4.21 $mmol.l^{-1}$ for plasma osmolality and plasma levels of sodium and chloride, respectively). Each value is the mean $\pm$ S.E.M. of n=7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). **, significantly different ($P < 0.05$) from control fish and fish implanted with 50 μg $cortisol.g^{-1}$ body weight. a, b, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 and 5 days, respectively.

percent of GPase *a,* a significant increase was noticed in cortisol-treated fish compared to controls. The percent of GPase *a* was also lower in fish treated with 50 $\mu g.g^{-1}$ at seven days than that observed in fish treated with the same dose at two and five days. The glycolytic potential of brain tissue was assessed using PFK and PK activities. PFK activity was not significantly different when comparing treated and control fish in its optimal activity. However, the activity ratio and the fructose 2,6–bisphosphate activation ratio were significantly higher in cortisol-treated fish than in controls. For both ratios, values of fish treated with both doses at seven days were significantly higher than those fish treated with the same doses after two and five days. A similar cortisol effect, though only in its optimal activity was also observed for PK activity that displayed higher values in cortisol-treated fish than in controls. Values of PK activity of fish treated with both doses of cortisol at five and seven days were also significantly lower than those observed in treated fish at two days. In contrast to those changes described above, no cortisol or days effects were observed in HK and G6PDH activities.

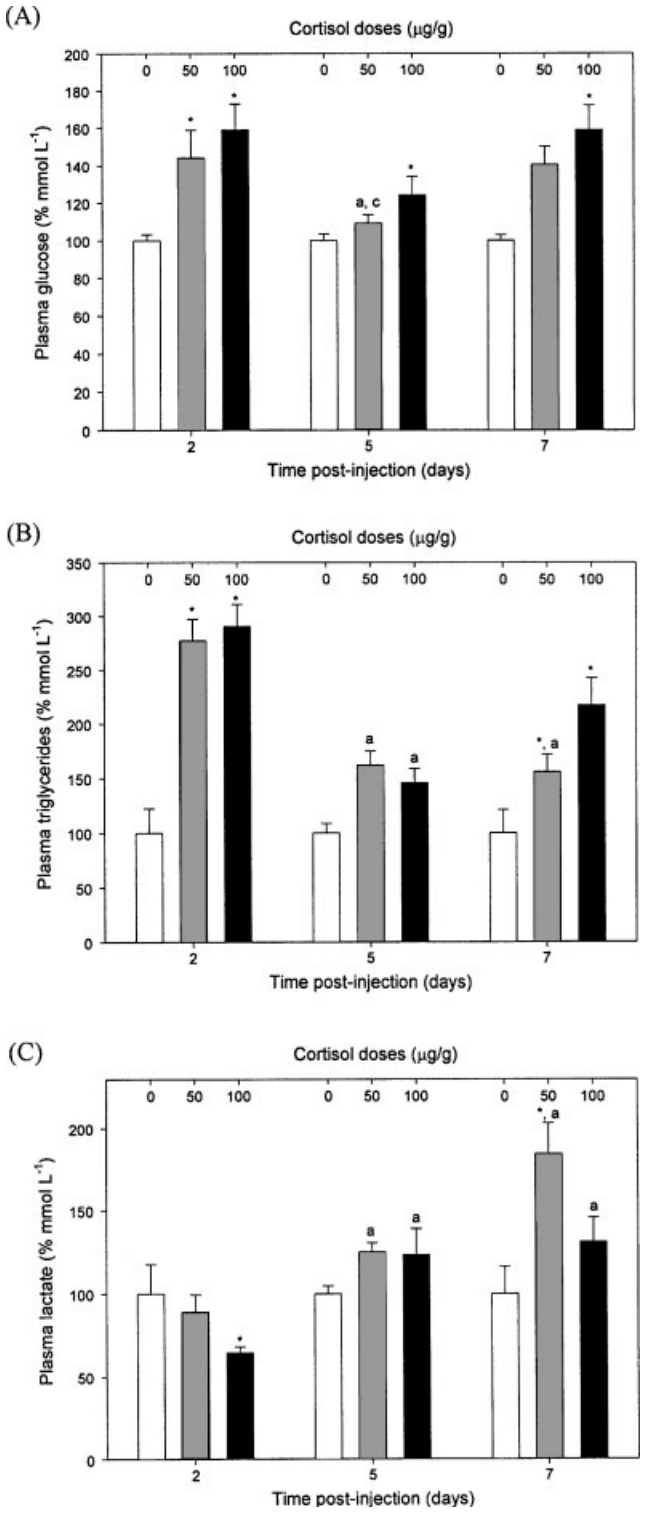

Fig. 4. Changes in the levels of glucose (A), triglycerides (B) and lactate (C) in plasma of gilthead seabream after 2, 5, and 7 days of intraperitoneal implantation of 5 $\mu l.g^{-1}$ body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 $\mu g.g^{-1}$ body weight). Results are shown as percent of control (control = 100%; Average value ($mmol.l^{-1}$) of controls: 3.81 ± 0.18, 2.61 ± 0.46, and 1.54 ± 0.17, for levels of glucose, triglycerides and lactate, respectively). Each value is the mean $\pm$ S.E.M. of n=7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). a, c, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 and 7 days, respectively.

DISCUSSION

The use of coconut oil as a slow-release implant has been shown to be an effective and practical method to continuously raise plasma cortisol levels in different species of teleosts (see review by Gamperl et al., '94). Thus, slow-release implants have been used successfully to evaluate the chronic effects of cortisol in the physiology of several species such as killifish (Leach and Taylor, '82), Atlantic salmon (Vijayan and Leatherland, '89), brook trout (Vijayan et al., '91), sea raven (Vijayan et al., '96a), tilapia (Vijayan and Pereira, '97) and rainbow trout (Soengas et al., 1992; Dugan and Moon, '98).

Thus, the cortisol implant procedure employed in the present study proved effective in producing a chronic elevation in plasma cortisol titres similar to 1) those previously obtained in other fish

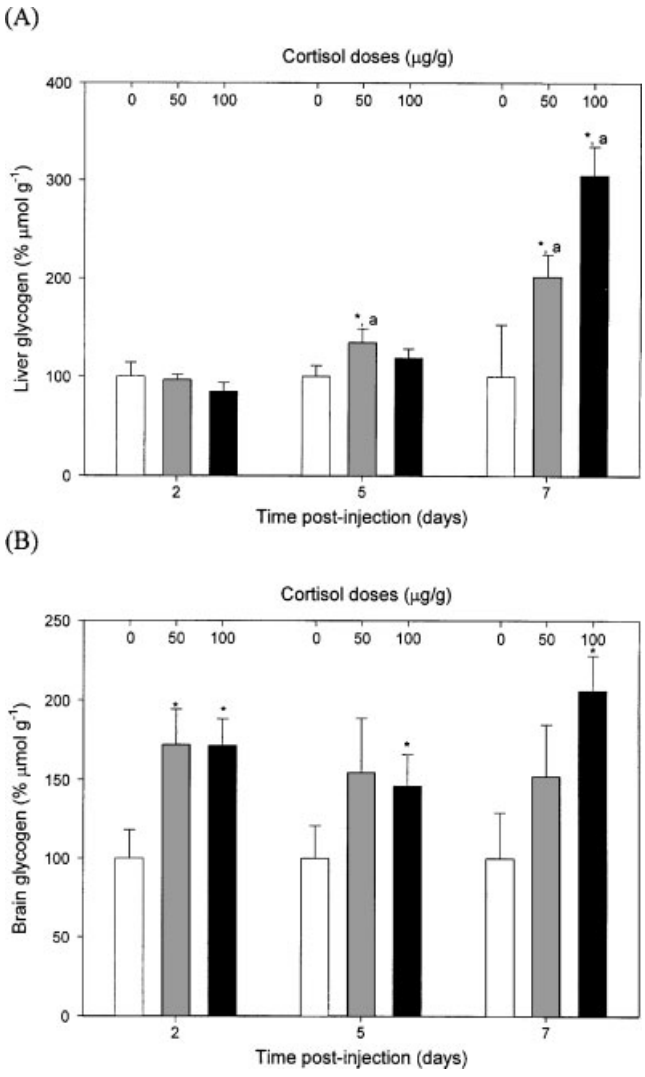

Fig. 5. Changes in the levels of glycogen in liver (A) and brain (B) of gilthead seabream after 2, 5, and 7 days of intraperitoneal implantation of 5 $\mu l.g^{-1}$ body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 $\mu g.g^{-1}$ body weight). Results are shown as percent of control (control = 100%, Average value (μmol glycosyl units.g^{-1} wet weight) of controls: 215.8 ± 34.7, and 0.17 ± 0.03, for liver and brain, respectively). Each value is the mean $\pm$ S.E.M. of n=7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). a, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 days.

species (see above), 2) those experienced during osmotic adaptation in several species of euryhaline fish (Morgan and Iwama, '96), 3) those of fish subjected to a substantial chronic stress (Andersen et al., '91), and 4) those reported in gilthead seabream 24h after abrupt salinity changes (Mancera et al., '93a, '94) and during the first days (1–3 days) of confinement under high density (Arends et al., '99). It is interesting to note the lack of differences in plasma cortisol levels between the two experimental groups that received the different cortisol doses used (50 and 100$\mu g.g^{-1}$ body weight), which can be due to a possible increased clearance of cortisol in those fish injected with the higher dose used.

Gill Na^+,K^+-ATPase activity increased in cortisol-treated fish with respect to controls, which agrees with the stimulatory role of cortisol reported previously in other teleosts (see McCormick, 1995, 2001). Thus, seawater-adapted gilthead seabream treated with cortisol (4 injections of 50$\mu g.g^{-1}$ body weight every other day during one week) also showed increased gill Na^+,K^+-ATPase activity versus controls (Mancera et al., 2002). In the present study plasma ion levels (Na^+ and Cl^-) decreased 2 days post-implant, when gill Na^+,K^+ATPase activity was highest, in contrast to that observed for plasma osmolality which did not show any change. This discrepancy between plasma osmolality and ion levels can be attributable to the increased values of glucose and triglycerides observed at two days post-implant thus resulting in increased osmolality. In addition, the decrease observed at day 7, without any change in plasma Na^+ and Cl^- levels, could be attributed to a reduction in plasma levels of other substances that increase plasma osmolality (ions and/or metabolites). Our results demonstrated that cortisol improve gill Na^+,K^+-ATPase activity in seawater-adapted gilthead seabream. However, this increase was only observed during five days post-implant. At seven days post-implant values of gill Na^+, K^+-ATPase activity were similar to controls even with cortisol levels remaining higher. A similar situation has been reported in seawater-acclimated specimens of the sparid silver seabream (*Sparus sarba*) treated daily during one week with 4$\mu g.g^{-1}$ cortisol, which showed increased cortisol levels but not gill Na^+,K^+-ATPase activity (Deane et al., 2000). This could be due to a loss in sensitivity of gill chloride cells to exogenous cortisol. In this way, it has been demonstrated that a downregulation of corticosteroids receptors occurs in coho salmon gills due to cortisol treatment (Maule and Schreck, '91; Shrimpton and Randall, '94), and that the responsiveness of gill Na^+,K^+-ATPase to cortisol is related to gill corticosteroid receptor concentration (Shrimpton and McCormick, '99). An exhaustion of gill chloride cells as a consequence of high cortisol levels during one week could be also responsible for the data obtained. However, this seems

*TABLE 1. Changes in the activities of potential regulatory enzymes (Glycogen phosphorylase, GPase; Pyruvate kinase, PK; Fructose 1,6-bisphosphatase, FBPase; Hexokinase, HK; glucose 6-phosphate dehydrogenase, G6PDH) assayed in liver of gilthead seabream after 2, 5 and 7 days of intraperitoneal implantation of 5 $\mu l.g^{-1}$ body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 $\mu g.g^{-1}$ body weight)**

Parameter	Days	Treatment		
		Control	Cortisol 50 $\mu g.g^{-1}$	Cortisol 100 $\mu g.g^{-1}$
GPase activity				
Total activity	2	100 ± 27.90	66.08 ± 9.97	106.20 ± 32.05
($mU.mg^{-1}$ protein)	5	100 ± 17.35	102.47 ± 18.52	145.62 ± 32.31
	7	100 ± 25.09	124.38 ± 20.65	133.56 ± 8.82
% GPase *a*	2	100 ± 9.57	99.77 ± 4.85	143.69 ± 1.83
	5	100 ± 15.74	24.57 ± 5.34*,a,c	58.80 ± 4.45*,a
	7	100 ± 16.65	112.01 ± 19.25	52.11 ± 5.07*,a
PK activity				
Optimal activity	2	100 ± 9.60	73.01 ± 5.31*	70.28 ± 7.34*
($mU.mg^{-1}$ protein)	5	100 ± 5.07	92.98 ± 4.26	117.78 ± 5.43*
	7	100 ± 7.09	98.52 ± 6.42*	69.93 ± 8.10*
Activity ratio	2	100 ± 3.77	101.71 ± 2.52	88.42 ± 6.99
	5	100 ± 4.19	96.21 ± 2.13	75.72 ± 6.39*
	7	100 ± 12.57	93.16 ± 3.47	70.81 ± 9.28*
Fructose 1,6-P_2	2	100 ± 5.78	101.72 ± 7.69	88.37 ± 11.31
activation ratio	5	100 ± 5.21	78.01 ± 4.04*	52.08 ± 9.26**
	7	100 ± 33.44	121.65 ± 45.55	82.40 ± 13.70
FBPase activity				
Optimal activity	2	100 ± 6.10	88.91 ± 7.41	91.36 ± 8.02
($mU.mg^{-1}$ protein)	5	100 ± 4.20	118.32 ± 2.99*	117.08 ± 3.28*
	7	100 ± 6.99	119.63 ± 4.06*	121.44 ± 6.99*
HK activity				
Optimal activity	2	100 ± 11.15	54.68 ± 15.29*	32.17 ± 11.76*
($mU.mg^{-1}$ protein)	5	100 ± 10.72	66.12 ± 19.47	44.01 ± 19.08*
	7	100 ± 11.68	20.59 ± 8.89*	15.97 ± 4.32*
G6PDH				
Optimal activity	2	100 ± 5.39	71.95 ± 2.67*	36.38 ± 3.85*
($mU.mg^{-1}$ protein)	5	100 ± 5.67	92.61 ± 7.34	83.05 ± 4.31*,a
	7	100 ± 15.14	129.87 ± 10.01	133.60 ± 10.49a

*Results are shown as % of control values (control = 100%; Average value ($mU.mg^{-1}$ protein) of controls: 49.6 ± 4.07, 815 ± 97.9, 691 ± 53.1, 55.1 ± 7.13, and 1790 ± 156, for activities of GPase, PK, FBPase, HK, and G6PDH, respectively). Each value is the mean ± S.E.M. of n = 7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). **, significantly different ($P < 0.05$) from control fish and fish implanted with 50 μg cortisol.g^{-1} body weight. a, c, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 and 7 days, respectively.

unlikely since we found an increase in gill Na^+, K^+-ATPase activity only at three days after transfer from seawater to hyperosmotic environments, and the maintenance of these higher values in the new environment lasted at least two more weeks (Guerreiro et al., unpublished observations).

Several metabolic changes were observed in the livers of cortisol-treated gilthead seabream, including an enhancement of the gluconeogenic potential (as suggested by changes observed in FBPase activity), and a decrease in glycolytic potential (suggested by the decrease observed in PK activity, and the absence of changes in lactate levels). Changes in enzyme activity are similar to those previously observed in other teleosts (Vijayan et al., '91; Vijayan and Pereira, '97) and agree with the role of cortisol as an inductor of hepatic gluconeogenesis as suggested earlier in other fish species (Mommsen et al., '99). Moreover, cortisol-treated fish showed increased liver glycogen levels, which is further supported by the decrease observed in GPase activity. This increase in glycogen levels after cortisol treatment coincides

*TABLE 2. Changes in the activities of potential regulatory enzymes (Glycogen phosphorylase, GPase; 6-Phosphofructo 1-kinase, PFK; Pyruvate kinase, PK; Hexokinase, HK; glucose 6-phosphate dehydrogenase, G6PDH) assayed in brain of gilthead seabream after 2, 5 and 7 days of intraperitoneal implantation of 5 μl.g^{-1} body weight of coconut oil alone (control) or containing different doses of cortisol (50 and 100 μg.g^{-1} body weight)**

Parameter	Days	Treatment		
		Control	Cortisol 50 μg.g^{-1}	Cortisol 100 μg.g^{-1}
GPase activity				
Total activity	2	100 ± 3.08	94.57 ± 5.12	98.82 ± 5.76
(mU.mg^{-1} protein)	5	100 ± 7.25	92.66 ± 3.27	87.42 ± 6.89
	7	100 ± 9.21	84.37 ± 5.99	91.23 ± 6.63
% GPase *a*	2	100 ± 6.83	133.92 ± 4.95	139.60 ± 7.05
	5	100 ± 3.54	126.37 ± 2.78	135.90 ± 5.73*
	7	100 ± 1.85	83.57 ± 2.30^{a,b}	124.84 ± 1.61*
PFK activity				
Optimal activity	2	100 ± 4.28	99.00 ± 6.66	97.59 ± 4.20
(mU.mg^{-1} protein)	5	100 ± 3.63	95.94 ± 5.21	98.12 ± 6.55
	7	100 ± 10.40	98.09 ± 4.86	94.19 ± 11.21
Activity ratio	2	100 ± 12.48	103.14 ± 16.51	99.88 ± 12.86
	5	100 ± 6.81	114.99 ± 14.86	134.26 ± 9.89*
	7	100 ± 17.90	202.25 ± 43.72*^{,a,b}	245.68 ± 15.32**^{,a,b}
Fructose 2,6-P_2	2	100 ± 10.57	86.46 ± 14.42	92.68 ± 7.18
activation ratio	5	100 ± 5.02	116.39 ± 17.72	141.66 ± 10.87*
	7	100 ± 13.36	197.34 ± 20.57*^{,a,b}	284.79 ± 34.87*^{,a,b}
PK activity				
Optimal activity	2	100 ± 4.08	162.07 ± 15.59*	175.43 ± 13.36*
(mU.mg^{-1} protein)	5	100 ± 5.73	100.42 ± 7.00^{a}	104.89 ± 3.09^{a}
	7	100 ± 4.61	92.43 ± 4.36^{a}	118.22 ± 3.45*^{,a}
Activity ratio	2	100 ± 5.78	96.78 ± 7.55	72.93 ± 4.06*
	5	100 ± 4.41	90.80 ± 2.76	98.32 ± 7.11
	7	100 ± 8.31	118.52 ± 5.35	99.36 ± 3.66^{a}
Fructose 1,6-P_2	2	100 ± 4.77	105.90 ± 7.44	111.68 ± 9.76
activation ratio	5	100 ± 10.71	106.49 ± 9.08	83.92 ± 5.45
	7	100 ± 2.21	117.77 ± 3.80	112.09 ± 2.35
HK activity				
Optimal activity	2	100 ± 4.99	103.74 ± 5.03	113.52 ± 5.67
(mU.mg^{-1} protein)	5	100 ± 4.41	98.57 ± 4.98	97.18 ± 3.49
	7	100 ± 6.24	98.16 ± 2.57	87.37 ± 7.24
G6PDH				
Optimal activity	2	100 ± 6.39	91.74 ± 7.76	99.11 ± 3.33
(mU.mg^{-1} protein)	5	100 ± 5.62	105.38 ± 4.15	103.21 ± 4.73
	7	100 ± 3.88	100.73 ± 4.95	93.68 ± 4.67

*Results are shown as % of control (control = 100%; Average value (mU.mg^{-1} protein) of controls: 294 ± 16.9, 2865 ± 201, 12286 ± 751, 733 ± 19.4, and 431 ± 22.5, for activities of GPase, PFK, PK, HK, and G6PDH, respectively). Each value is the mean ± S.E.M. of n = 7 fish per group in each sampling time. *, significantly different ($P < 0.05$) from fish implanted with coconut oil alone (control). **, significantly different ($P < 0.05$) from control fish and fish implanted with 50 μg cortisol.g^{-1} body weight. a, b, significantly different ($P < 0.05$) from fish implanted with the same dose of cortisol at 2 and 5 days, respectively.

with data obtained from several species such as killifish (Leach and Taylor, '82), rainbow trout (De la Higuera and Cardenas, '86), and brook trout (Vijayan and Leatherland, '92), and is accompanied by changes in the activities of both GSase and GPase (see Mommsen et al., '99) but not with others, reinforcing the concept that the effects of cortisol on glycogen metabolism are variable and species-specific (Van der Boon et al., '91; Mommsen et al., '99).

The decrease observed in liver G6PDH activity of cortisol-treated fish point to a decreased

capacity of the pentose phosphate shunt, which does not agree with data from the literature in which no changes (Andersen et al., '91) or increases (Vijayan et al., '91) have been observed. However, this decreased enzyme activity suggests a fall in the potential of the pentose-phosphate shunt, thus providing less of the reducing power usually associated with lipogenesis. A reduced lipogenic capacity agrees with the usually observed increased lipolytic capacity of cortisol-treated fish (Van der Boon et al., '91; Mommsen et al., '99).

The increased production of glucose in liver through gluconeogenesis may be associated with its possible use in other tissues in which energy demand increases, in a way similar to that observed in liver during seawater adaptation (Hanke, '91; Soengas et al., '95). Accordingly, the plasma of cortisol-treated gilthead seabream clearly showed increased glucose and triglyceride levels when compared with controls. An increase in plasma glucose levels has been commonly observed in cortisol-treated fish (Van der Boon et al., '91; Mommsen et al., '99). The increase in plasma triglyceride levels has not been observed in any comparable study (Mommsen et al., '99). Cortisol stimulates total lipid depletion from the liver and dark muscle in coho salmon parr, and also stimulates depletion of triglycerides in the liver through increased triglyceride-lipase activity (Sheridan, '86), in accordance our data. Plasma protein levels displayed no changes in cortisol-treated fish in agreement with similar studies performed in rainbow trout (Andersen et al., '91) and brook trout (Vijayan et al., '91), whereas a small increase was recorded for plasma lactate levels similar to that described in rainbow trout by Dugan and Moon ('98). Altogether, these changes in plasma metabolite levels allow us to suggest that cortisol, either acting directly on the liver or acting indirectly through increased energy demand in other tissues, induced liver mobilization of substrates.

The energy requirement of fish gills is thought to be maintained by oxidation of glucose and lactate obtained from the circulation (Mommsen, '84; Perry and Walsh, '89; Soengas et al., '95). As the liver is the main site of glucose and lactate production in fish, it is likely that liver metabolism is enhanced during osmotic adaptation, thereby providing energy substrates for gill metabolism. Changes in plasma concentration of several hormones, including cortisol, have been associated with the process of ion regulation and, consequently with seawater acclimation in fish (Mayer-Gostan, '87; McCormick, '95, 2001). In addition, these hormones have been shown to play a role in the mobilization of energy substrates in fish (Mommsen et al., '99). Consequently, some of the effects of cortisol on osmotic acclimation may be mediated indirectly by providing substrates for gill metabolism and maintaining gill Na^+, K^+, ATPase activity in gills and other osmoregulatory organs. Thus, metabolic changes occurring during osmotic adaptation (Vijayan et al., '96b) are quite similar to those usually attributed to cortisol action (Mommsen et al., '99), and also similar to those described in the present experiment.

Unfortunately, the lack of tissue mass did not allow us to assess a significant number of metabolic parameters in gills of gilthead seabream under the present experimental conditions. The only parameter assessed, HK activity, clearly displayed decreased activity in cortisol-treated fish, suggesting that the increased glucose levels available in plasma at the same time may not be connected with an increased HK activity in this tissue of the seabream, in contrast to studies addressing a parallel increase in plasma glucose and gill HK activity of rainbow trout during adaptation to seawater (Soengas et al., '95). Therefore, the suspected increased energy demand of the gills (as suggested by increased Na^+,K^+ATPase activity) should be fuelled by other substrates either exogenous (lactate, triglycerides) or endogenous (glycogen). Further experiments are necessary to elucidate such questions in *Sparus aurata*. In this way, the study of temporal changes in liver and gill carbohydrate and lipid metabolism associated with transfer to hyperosmotic environment (where high gill Na^+,K^+-ATPase activity is expected) would be useful.

In theory, brain tissue should not be affected by the osmoregulatory changes, elicited by cortisol, occurring in gilthead seabream and, therefore, changes within this tissue should be exclusively attributed to a direct cortisol action, though a flow of metabolites from liver to brain cannot be discounted. However, the non-existence of changes in brain HK activity of cortisol-treated fish do not allow us to hypothesize that a significant flow of glucose, the main fuel for the brain in teleosts (Soengas et al., '98), occurs from liver to brain. The stress response induced by raised cortisol levels in plasma should be similar to that naturally occurring in several physiological processes, such as starvation, hypoxia, and toxic disruption, which generally lead to an enhance-

ment of the energy demands of the brain (Soengas and Aldegunde, 2002).

There is limited data on cortisol's effects on brain energy metabolism of fish to compare with our data (see review by Soengas and Aldegunde, 2002). Only Lynshiang and Gupta (2000) demonstrated an increased oxygen consumption in brains of cortisol-treated *Clarias batrachus*. Data obtained in the present experiment demonstrate, for the first time, any effects of cortisol in pathways of energy metabolism in the fish brain. Accordingly, a clear enhancement of glycolytic capacity (as judged by increased activity of both PFK and PK), together with increased glycogen levels, were observed in the brain of cortisol-treated sea bream. The increased glycogenic capacity resulting in increased glycogen levels has been previously observed in other tissues, such as liver and white muscle (Van der Boon et al., '91; Mommsen et al., '99), but this is the first time in which similar results were reported in any fish brain. The absence of changes in GPase activity do not appear to correlate well with the increased glycogen levels of cortisol-treated fish, but it must be stressed that glycogen levels are dependent on the balance between GPase and GSase activities. Increased GSase activity could be a reasonable hypothesis in brains of cortisol-treated fish. The increased glycolytic potential in cortisol-treated fish is quite surprising considering the role of cortisol as a metabolic depressor of glycolysis in other tissues (i.e., liver) (Mommsen et al., '99), but these results are comparable to those observed in our laboratory in rainbow trout brain after intracerebroventricular treatment with norepinephrine (Sangiao-Alvarellos et al., 2003), suggesting a possible interaction between both hormones. Reid et al. ('92) demonstrated that raised levels of plasma cortisol resulted in a significant increase in the number of hepatocyte surface adrenoceptors in rainbow trout. If a similar action occurred in the brain, changes observed in metabolic parameters of that tissue could also be attributable to an indirect action of cortisol through catecholamine receptors.

Therefore, in the present experiments, a significant activation of energy demand in the brains of cortisol-treated fish is observed in agreement with the enhanced oxygen consumption already reported by Lynshiang and Gupta (2000), and reflects an adaptive role for cortisol during stress. As a whole, brain tissue in cortisol-treated fish appears to enhance the use of those pathways involved in ATP production, whereas other synthetic pathways appear to be reduced. The role of brain glycogen, and what fuels are increasingly being used in the brain instead of exogenous glucose, still remain to be elucidated.

In summary, increased cortisol levels in plasma of gilthead seabream elicited not only an increased hypoosmoregulatory capacity but also several metabolic changes in liver, brain, and gills. These metabolic changes can be related to the energy repartitioning process occurring in nature during osmotic adaptation, and also suggest the existence of tissue-specific metabolic responses due to cortisol treatment. This will deserve future research.

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