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Time course of osmoregulatory and metabolic changes during osmotic acclimation in *Sparus auratus*

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Summary

Changes in different osmoregulatory and metabolic parameters over time were assessed in gills, kidney, liver and brain of gilthead sea bream *Sparus auratus* transferred either from seawater (SW, 38 p.p.t.) to hypersaline water (HSW, 55 p.p.t.) or from SW to low salinity water (LSW, 6 p.p.t.) for 14 days. Changes displayed by osmoregulatory parameters revealed two stages during hyperosmotic and hypo-osmotic acclimation: (i) an adaptive period during the first days of acclimation (1–3 days), with important changes in these parameters, and (ii) a chronic regulatory period (after 3 days of transfer) where osmotic parameters reached homeostasis. From a metabolic point of view, two clear phases can also be distinguished during acclimation to hyperosmotic or hypo-osmotic conditions. The first one coincides with the adaptive period and is characterized by enhanced levels of plasma metabolites (glucose, lactate, triglycerides and protein), and use of these metabolites by different tissues in processes directly or indirectly involved in osmoregulatory work. The second stage coincides with the chronic regulatory period observed for the osmoregulatory parameters and is metabolically characterized in HSW-transferred fish by lower energy expenditure and a readjustment of metabolic parameters to levels returning to normality, indicative of reduced osmoregulatory work in this stage. In LSW-transferred fish, major changes in the second stage include: (i) decreased glycolytic potential, capacity for exporting glucose and potential for amino acid catabolism in liver; (ii) enhanced use of exogenous glucose through glycolysis, pentose phosphate and glycogenesis in gills; (iii) increased glycolytic potential in kidney; and (iv) increased glycogenolytic potential and capacity for use of exogenous glucose in brain.

Key words: gilthead sea bream, *Sparus auratus*, osmoregulation, energy metabolism.

Introduction

The capacity of euryhaline teleosts to adapt to changes in environmental salinity depends on several factors including energy supply and demand, which in turn depend on the external salinity and the energy demand necessary for electrolyte shift between intracellular and extracellular spaces or the external medium. Successful salinity acclimation may require a metabolic reorganization to meet the increased energetic demands associated with the exposure to the new environmental salinity. Euryhaline fish showed several metabolic changes and spent large amounts of energy, particularly in osmoregulatory (gills, intestine and kidney) and metabolic (liver) organs, to compensate for these salinity changes. However, the energetics of these additive responses to salinity changes are not yet fully understood (Morgan and Iwama, 1991; Boeuf and Payan, 2001). The metabolic response of teleosts to different osmotic conditions undoubtedly includes both stress and osmoregulatory components, but the relative energetic demands of these processes cannot be discerned from whole animal oxygen consumption (Morgan and Iwama, 1991). Thus, not unexpectedly, alterations in intermediary metabolism related to osmoregulation are poorly studied in teleosts (Morgan et al., 1997; Nakano et al., 1998).

There are several studies addressing metabolic changes in euryhaline fish during acclimation to brackish water (BW), seawater (SW) or hypersaline water (HSW; Kelly and Woo, 1999a,b; Kelly et al., 1999; Nakano et al., 1997, 1998; Sangiao-Alvarellos et al., 2003; Soengas et al., 1995a,b; Vijayan et al., 1996; Woo and Murat, 1981). However, few studies have assessed what metabolic changes occur in osmoregulatory and non-osmoregulatory organs during acclimation to low salinity water (LSW; Kelly and Woo, 1999a,b; Kelly et al., 1999; Roche et al., 1989; Woo and Fung, 1981), and very few of those studies have assessed the time course of metabolic changes during acclimation to LSW, BW or HSW (Kelly and Woo, 1999a,b; Kelly et al., 1999).

Gilthead sea bream *Sparus auratus* is an euryhaline teleost

capable of living in different environmental salinities ranging from 5 to 60 p.p.t. (Chervinski, 1984). The osmoregulatory system of this species has been studied previously by analysing aspects related to changes in chloride cells, gill Na^+,K^+-ATPase activity and plasma parameters in fish adapted to different salinities or subjected to salinity transfer (Laiz-Carrión et al., 2005a,b; Mancera et al., 1993a), modifications in adenohypophyseal cells after acclimation to a hypo-osmotic environment (Mancera et al., 1993b, 1995), and the role of different hormones in the adaptation to hyperosmotic and hypo-osmotic environments (Guzmán et al., 2004; Laiz-Carrión et al., 2002, 2003; Mancera et al., 1994, 2002; Sangiao-Alvarellos et al., 2003, 2005b).

In a previous study using gilthead sea bream, we addressed modifications in osmoregulatory and metabolic parameters after a 14-day acclimation of this euryhaline species to brackish water (BW, 12 p.p.t.), seawater (SW, 38 p.p.t.) or high salinity water (HSW, 55 p.p.t.; Sangiao-Alvarellos et al., 2003). In addition, our group has studied the time course of changes in osmoregulatory parameters during transfer from seawater to different environmental salinities (5, 15, 38 and 60 p.p.t.; Laiz-Carrión et al., 2005a). In different euryhaline species (Holmes and Donaldson, 1969; Maetz, 1974), including gilthead sea bream (Laiz-Carrión et al., 2005a; Mancera et al., 1993a), osmoregulatory changes occurring during acclimation to different osmotic conditions normally exhibit two different stages: (i) an adaptive period, with changes in osmotic parameters, and (ii) a chronic regulatory period, where these parameters again reach homeostasis. However, there is no information about (i) what metabolic adjustments occur during the time course of acclimation to different salinities, and (ii) whether or not metabolic changes display a two-stage pattern similar to that exhibited by osmoregulatory parameters.

The purpose of the present study was therefore to assess the time course of the osmoregulatory and metabolic changes occurring during acclimation of *S. auratus* to hyperosmotic and hypo-osmotic environments. Thus, changes in different osmoregulatory parameters and levels of several metabolites and activities of key enzymes of the major pathways of energy metabolism (use of exogenous glucose, glycolysis, glycogenolysis, gluconeogenesis, pentose phosphate) were measured throughout a 14-day period in gills, kidney, liver and brain of *S. auratus* transferred to SW (38 p.p.t.), HSW (55 p.p.t.), and LSW (6 p.p.t.).

Materials and methods

Fish

Immature male gilthead sea bream *Sparus auratus* L. (150–200 g body mass) were provided by Planta de Cultivos Marinos (C.A.S.E.M., Universidad de Cádiz, Puerto Real, Cádiz, Spain) and transferred to the laboratory at Faculty of Marine Science (Puerto Real, Cádiz). They were acclimated to seawater (SW, 38 p.p.t. salinity, osmolality 1162 mOsm kg^{-1} H_2O) in 5000 l aquaria in an open system during an initial acclimation period (30 days). Fish were then transferred to a re-circulating system of experimental tanks (each 500 l volume; *N*=10–11 fish in each tank) containing SW for another 2-week acclimation period. During the experiments (May–June 2004), fish were maintained under natural photoperiod and constant temperature (18°C). Fish were fed daily with commercial dry pellets at 1% of body mass (Dibaq-Diprotg SA, Segovia, Spain). 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

In a first experiment (May 2004), we assessed changes due to hyperosmotic acclimation. Water in three tanks (*N*=32) was progressively replaced, within 1 h, by hypersaline water (HSW, 55 p.p.t., 1354 mOsm kg^{-1} H_2O), while water in the other three tanks (*N*=32) was replaced by SW. Salinity in the group denoted as HSW was obtained by mixing full SW with natural marine salt (Unionsal, Cádiz, Spain).

In a second experiment (June 2004), we assessed changes due to hypo-osmotic acclimation. Water in three tanks (*N*=32) was progressively replaced, within 1 h, by low salinity water (LSW, 6 p.p.t., 160 mOsm kg^{-1} H_2O), while water in the other three tanks (*N*=32) was replaced by SW. Salinity in the group denoted as LSW was obtained by mixing full SW with dechlorinated tapwater.

In both experiments fish remained in their specific salinity conditions for 2 weeks, during which the common water quality criteria were assessed with no major changes being observed. Average values for those parameters were 5 mg l^{-1} for oxygen, 0.3 mg l^{-1} for nitrite, 0.4 mg l^{-1} for nitrate, 0.4 mg l^{-1} for ammonia, and less than 0.1 mg l^{-1} for chlorine, calcium and hydrogen sulphide. The water salinity was checked every day and corrected as necessary. No mortality was observed during the experiments.

Sampling

For both experiments, on day 0, eight fish from one tank containing SW were sampled (time 0). In experiment 1, on days 1, 3, 7 and 14 after transfer, eight fish from each group (SW and HSW) were dipnetted from six tanks (three replicates per group), anaesthetized with 2-phenoxyethanol (1 ml l^{-1} water), weighed and sampled. A similar procedure was used in experiment 2 to obtain samples from fish in SW and LSW. Blood was obtained in ammonium-heparinized syringes from the caudal peduncle. Plasma samples were obtained after centrifugation of blood (30 s at 13 000 ***g***; Eppendorf 5415R, Eppendorf AG, Hamburg, Germany), and divided into two portions. One portion was immediately frozen on liquid nitrogen for assessment of plasma osmolality, cortisol and protein levels, whereas the other portion, for assessment of plasma metabolites, was deproteinized immediately (using 6% perchloric acid) and neutralized (using 1 mol l^{-1} potassium bicarbonate) before freezing on liquid nitrogen and storage at –80°C until further assay. In order to assess gill Na^+,K^+-

ATPase activity, 3–5 filaments coming from the second 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. Brain, liver, kidney, and the remaining branchial arches were removed within a few seconds from each fish, freeze-clamped in liquid nitrogen, and stored at –80°C until assay.

Analytical techniques

Plasma samples were centrifuged and supernatants used to assess metabolites. Glucose and lactate concentrations were measured using commercial kits from Spinreact (Barcelona, Spain) adapted to microplates. Plasma protein was measured using the bicinchoninic acid method with the BCA protein kit (Pierce, Rockford, IL, USA) for microplates, and bovine serum albumin as standard. Plasma triglyceride levels were determined enzymatically using a commercial kit from Spinreact (Spain) in microplates. Those 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, USA). Plasma osmolality was measured using a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, Norwood, VT, USA).

Gill and kidney Na^+,K^+-ATPase activity was determined using the micro assay method of McCormick (1993) adapted to *S. auratus* (Mancera et al., 2002), as previously described (Laiz-Carrión et al., 2003; Sangiao-Alvarellos et al., 2003).

Plasma cortisol levels were measured (in duplicate) by indirect enzyme immunoassay (ELISA) validated for gilthead sea bream (Tintos et al., 2005). The ELISA satisfied the criteria of specificity (testing cross-reactivity with other steroids), reproducibility (interassay coefficient of variation <6%), precision (intrassay coefficient of variation <4%) and accuracy (average recovery >98%).

Frozen brain, liver, gill and kidney samples were quickly minced on a chilled Petri dish to very small pieces that were mixed and (still frozen) divided into two homogeneous portions to assess enzyme activities and metabolite levels, respectively. The tissue to be used for assessment of metabolite levels was homogenized immediately by ultrasonic disruption in the cold with 7.5 vol. of ice-cooled 6% perchloric acid, and neutralized (using 1 mol l^{-1} potassium bicarbonate). The homogenate was centrifuged (2 min at 13 000 ***g***, Eppendorf 5415R), and the supernatant used for assays. Tissue lactate levels were determined spectrophotometrically using a commercial kit (Spinreact, Spain). 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 tissue used for assessment of enzyme activities was homogenized by ultrasonic disruption in the cold with 10 vol. of ice-cold stopping-buffer containing: 50 mmol l^{-1} imidazole-HCl (pH 7.5), 15 mmol l^{-1} 2-mercaptoethanol, 100 mmol l^{-1} KF, 5 mmol l^{-1} EDTA, 5 mmol l^{-1} EGTA, and a protease inhibitor cocktail (Sigma, P-2714). The homogenate was centrifuged (2 min at 13 000 ***g***, Eppendorf 5415R) and the supernatant used for assays. In those cases where non-cytosolic enzymes were assessed, appropriate centrifugations were carried out to obtain samples.

The activities of several enzymes representative of major pathways of carbohydrate metabolism (glycogen phosphorylase, GPase; 6-phosphofructo 1-kinase, PFK; pyruvate kinase, PK; fructose 1,6-bisphosphatase, FBPase; glutamate dehydrogenase, GDH; hexokinase, HK; glucose 6-phosphatase, G6Pase; glucose 6-phosphate dehydrogenase, G6PDH) were assessed. Reaction rates of enzymes were determined using a Unicam UV-2 spectrophotometer (Thermo Unicam, Waltham, MA, 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. No changes were found in tissue protein levels in any of the groups studied, and therefore enzyme activities are expressed in terms of mg^{-1} protein. Homogenate protein was assayed in triplicate as detailed by Bradford (1976), using bovine serum albumin (Sigma, St Louis, MO, 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 (Laiz-Carrión et al., 2002, 2003; Sangiao-Alvarellos et al., 2003, 2005b).

PFK activity was 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.

PK activity was determined at low (0.05 mmol l^{-1} for kidney, and 0.1 mmol l^{-1} for gills) 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 0.1 mmol l^{-1} fructose 1,6-bisphosphate, and 0.1 mmol l^{-1} PEP concentrations.

GPase *a* activity was measured with 2.5 mmol l^{-1} AMP and 10 mmol l^{-1} caffeine present, and total GPase activities were estimated in the presence of 2.5 mmol l^{-1} AMP but without caffeine. The ratio of GPase activities with and without caffeine ×100 represents the percentage of total GPase (*a*+*b*) in the active form (%GPase *a*). Recent studies in mammalian skeletal muscle (Rush and Spriet, 2001) report that caffeine inhibits GPase *a*. Therefore, we cannot discard an understimation of GPase *a* activity in the present study, which may also alter the %GPase *a*, and therefore the results provided regarding GPase activity must be interpreted with caution.

Statistics

Data were statistically analysed by a two-way analysis of variance (ANOVA) test in which treatment (SW and HSW transference in experiment 1; SW and LSW transference in experiment 2) and time (0, 1, 3, 7 and 14 days) were the main factors. Logarithmic transformations of the data were made when necessary to fulfil the conditions of the analysis of variance but data are shown in their decimal values for clarity. *Post-hoc* comparisons were made using a Tukey test, with the differences considered to be statistically significant at $P<0.05$.

Results

In all parameters assessed no changes were observed among the different sampling times for control fish transferred from SW to SW.

Plasma osmolality in fish transferred from SW to HSW and LSW significantly increased (up to 20%) and decreased (up to 15%), respectively (Fig. 1). Changes were especially apparent on the first days after transfer, followed by recovery later. Gill Na^+,K^+-ATPase activity increased significantly with time in HSW- and LSW-transferred groups, the increase being higher in fish in HSW (80%) than in LSW (60%). Kidney Na^+,K^+-ATPase activity was 70% higher in HSW- with respect to SW-transferred fish at day 1, but returned to initial values at day 3. In fish transferred to LSW this activity decreased by 20% after 7 days of transfer.

Plasma cortisol levels increased in fish transferred from SW to HSW and LSW on the first day of transfer, with levels being restored after 14 days of transfer (Fig. 2). This increase was higher in fish transferred to HSW (50-fold) than in those transferred to LSW (threefold). Plasma glucose levels increased dramatically (sixfold) on the first day of transfer from SW to HSW and returned to values similar to those in SW transferred fish at day 3. A lower increase (up to 20%) was noticed in fish transferred to LSW on days 1, 7 and 14. Plasma lactate levels increased 2.5-fold the first day after transfer from SW to HSW and remained at levels approximately 40% higher for the rest of the experiment; a lower increase (up to 20%) was noticed in fish transferred to LSW on days 1, 3 and 7 after transfer. Plasma protein concentration increased (up to 25%) in fish transferred from SW to HSW on days 3 and 7 after transfer whereas no changes were noticed on transfer to LSW. Plasma triglyceride levels increased from day 3 of transfer from SW to HSW onwards, reaching a 25% increase at the end of period, whereas a 200% increase was noticed on the first day for fish transferred to LSW that was restored to initial levels from day 7 onwards.

In liver, glycogen levels presented two stages in fish transferred from SW to HSW: a 50% decline on the first day followed by an increase (twofold) from day 7 of transfer onwards (Fig. 3). In fish transferred to LSW these levels were always lower (up to 70%) than in fish in SW. Glucose levels increased 60% after 1 day of transfer from SW to HSW, whereas in LSW a 35% increase occurred after 14 days. Lactate levels decreased (up to 40%) in fish transferred from SW to HSW from day 7 of the experiment onwards, whereas in LSW fish a 50% decrease was only observed after 1 day of transfer.

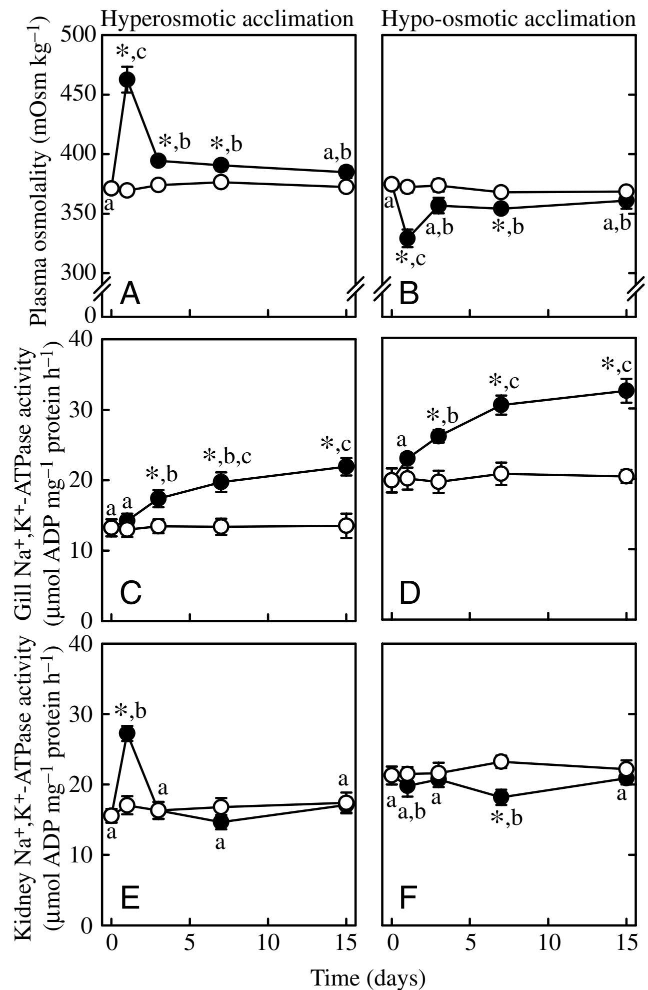

Fig. 1. Time course of changes in osmoregulatory parameters in plasma osmolality (A,B) and Na^+,K^+-ATPase activity in gills (C,D) and kidney (E,F) of gilthead sea bream acclimated to hyperosmotic (A,C,E) or hypo-osmotic (B,D,F) conditions. Fish were transferred for 14 days from seawater (SW) to (A,C,E) hypersaline water (HSW; closed circles) or (B,D,F) low salinity water (LSW; closed circles). Open circles indicate control fish transferred from SW to SW. Each value is the mean ± S.E.M. of N=8 fish per group and sampling time. *Significantly different ($P<0.05$) from SW group at the same sampling time. Different letters indicate significant differences ($P<0.05$) among sampling times (0, 1, 3, 7 and 14 days) in fish acclimated to HSW or LSW.

Liver GPase activity decreased threefold in fish transferred from SW to HSW on the first day, with activity recovered at day 7 (Fig. 4). In fish transferred to LSW a lower increase (up to 30%) was observed from day 7 onwards. Also, a decrease (up to 50%) was noticed for the %GPase in the active form (data not shown) on days 1 and 3 after transfer. PFK activity displayed no significant changes in fish transferred from SW to HSW whereas a small decline (20%) was noticed after 1 day in fish transferred to LSW. The activity ratio of PFK revealed

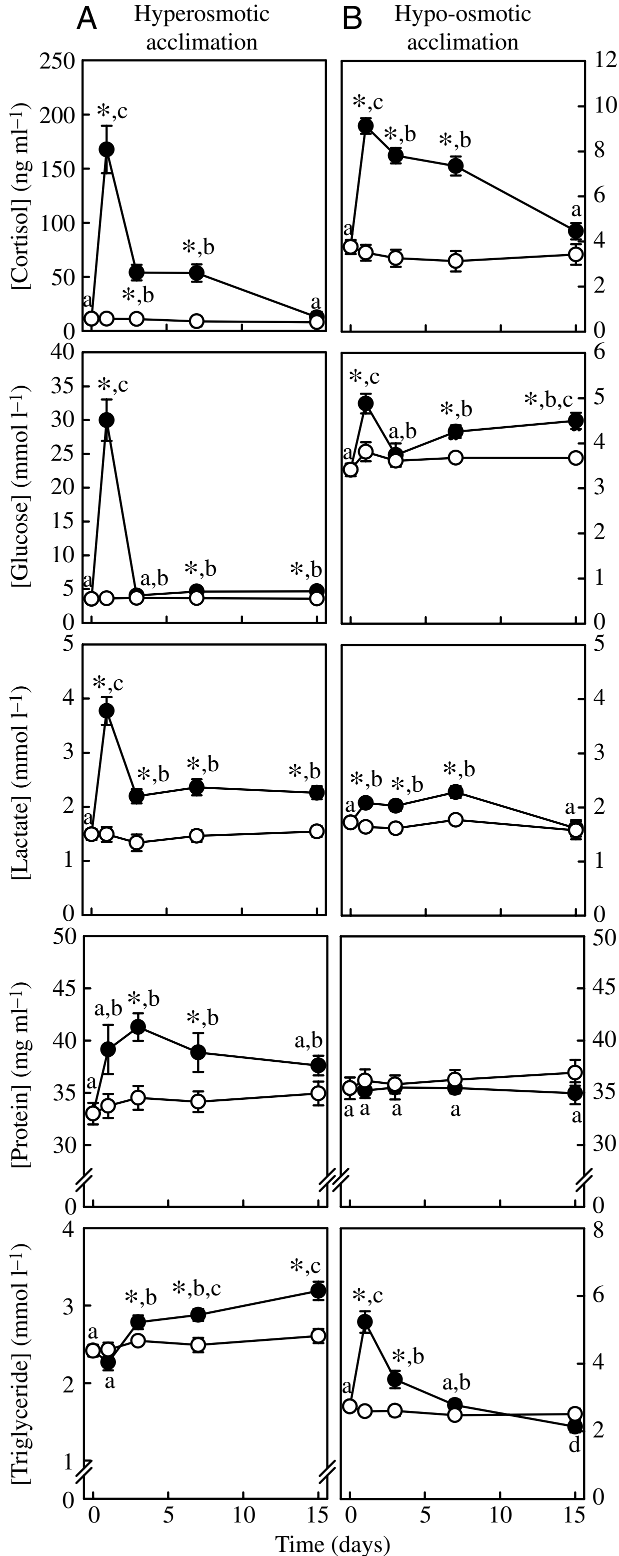

Fig. 2. Time course of changes in cortisol and metabolic parameters (glucose, lactate, protein and triglyceride levels) in plasma of gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

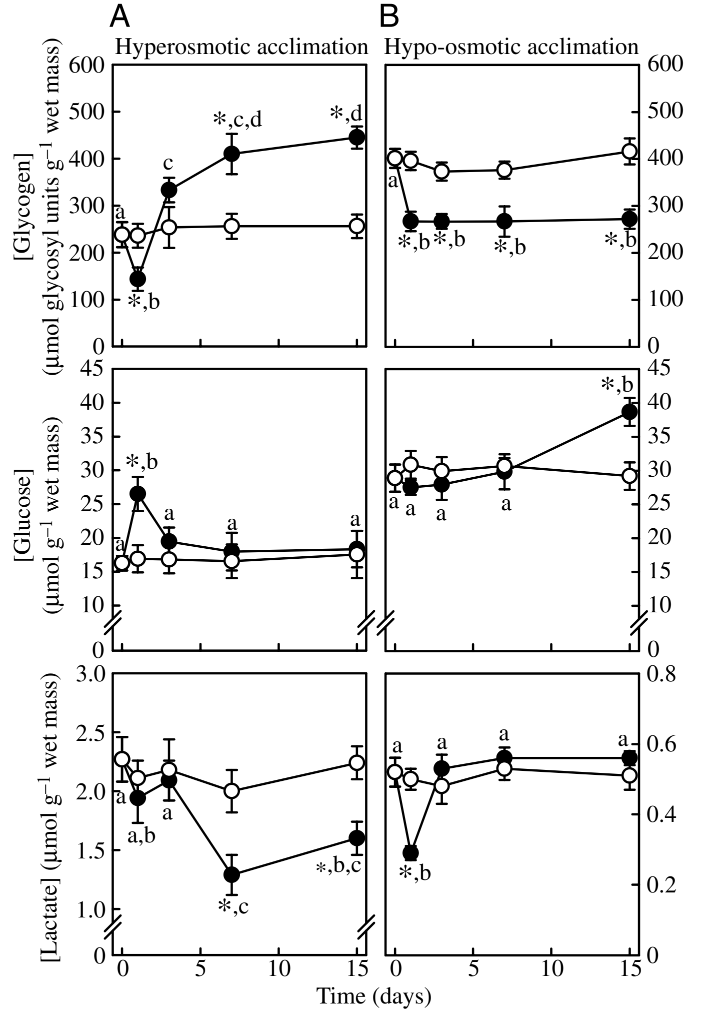

Fig. 3. Time course of changes in liver metabolite levels (glycogen, glucose and lactate) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

a 50% increase in fish transferred to HSW on day 3 whereas a low decline (25%) was noticed on day 14 for fish transferred to LSW. The cofactor activation ratio of PFK displayed a 50% increase on day 3 after transfer from SW to HSW whereas no changes were noticed for fish transferred to LSW (not shown). G6PDH activity continuously decreased (up to 30%) in fish transferred from SW to LSW whereas no changes were noticed in fish transferred to HSW. G6Pase activity displayed a 100% increase on the first day after transfer to HSW and a subsequent 50% increase was also observed after 14 days; in contrast, a threefold decrease was observed in activity of fish transferred to LSW from day 3 onwards. FBPase activity did not display significant changes (data not shown). Finally, GDH activity decreased approx 20% after 7 days of transfer to LSW but not presented changes in fish transferred to HSW (data not shown).

Gill glycogen levels displayed a 100% increase on the first

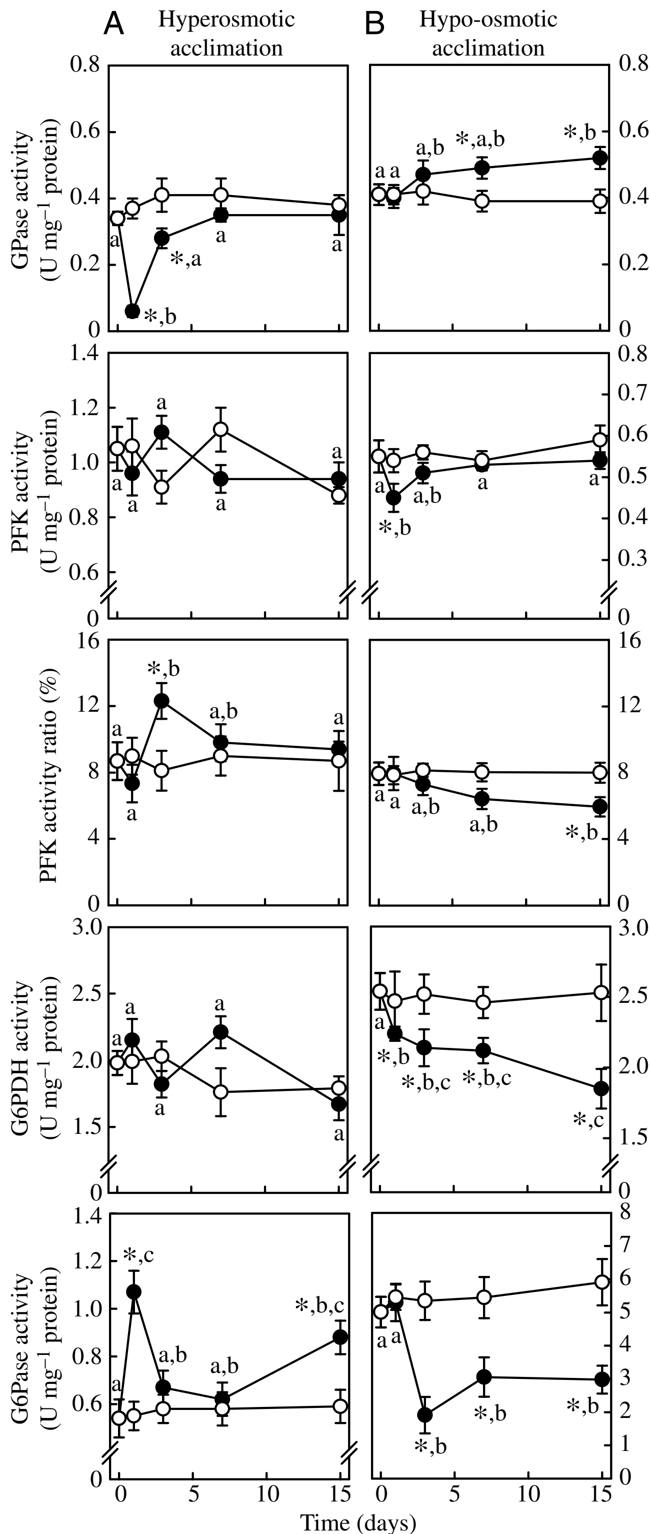

Fig. 4. Time course of changes in liver enzyme activities (glycogen phosphorylase, GPase; 6-phosphofructo 1-kinase, PFK; glucose 6-phosphate dehydrogenase, G6PDH; and glucose 6-phosphatase, G6Pase) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

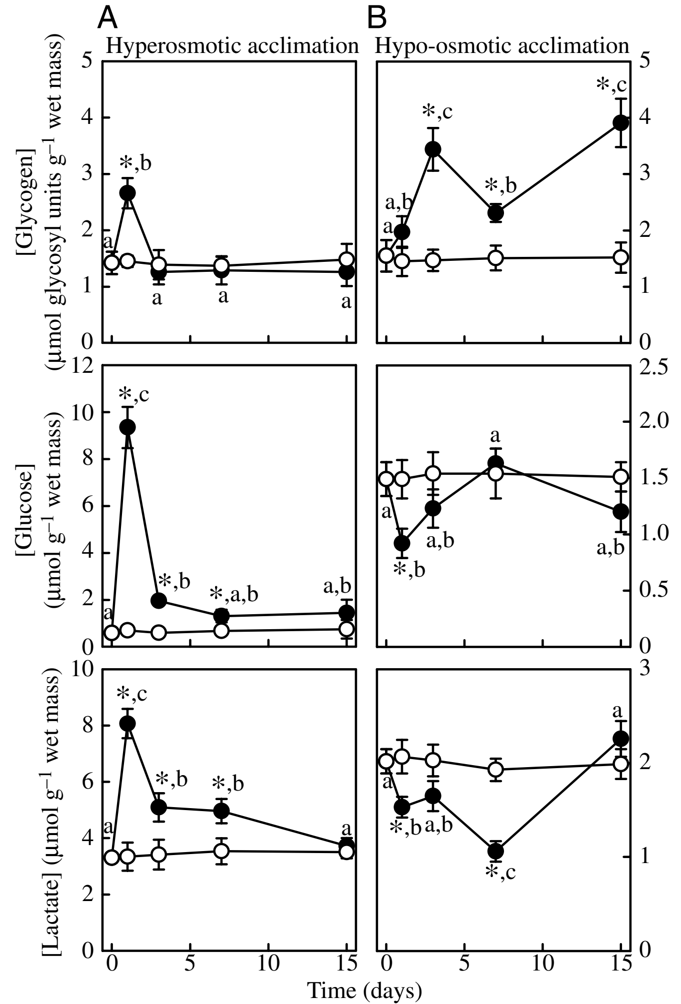

Fig. 5. Time course of changes in gill metabolite levels (glycogen, glucose, and lactate) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

day in fish transferred from SW to HSW but returned to similar values as in SW fish at day 3; a higher increase (up to 250%) was observed in levels of fish transferred to LSW from day 7 of transfer onwards (Fig. 5). Free glucose levels increased tenfold in gills of fish transferred from SW to HSW on the first day after transfer, with levels being restored on day 14; in contrast the only significant change noticed in fish transferred to LSW was a 50% decline on the first day after transfer. Lactate levels displayed a pattern similar to that of glucose, thus in HSW-transferred fish a threefold increase was observed after 7 days that recovered on day 14 whereas in LSW-transferred fish a 50% decrease was observed between 1 and 7 days after transfer.

Gill GPase activity did not present any significant changes (data not shown) whereas the percentage of enzyme in the active form (%GPase *a*) increased 50% on the first day after transfer from SW to HSW in contrast to a threefold increase

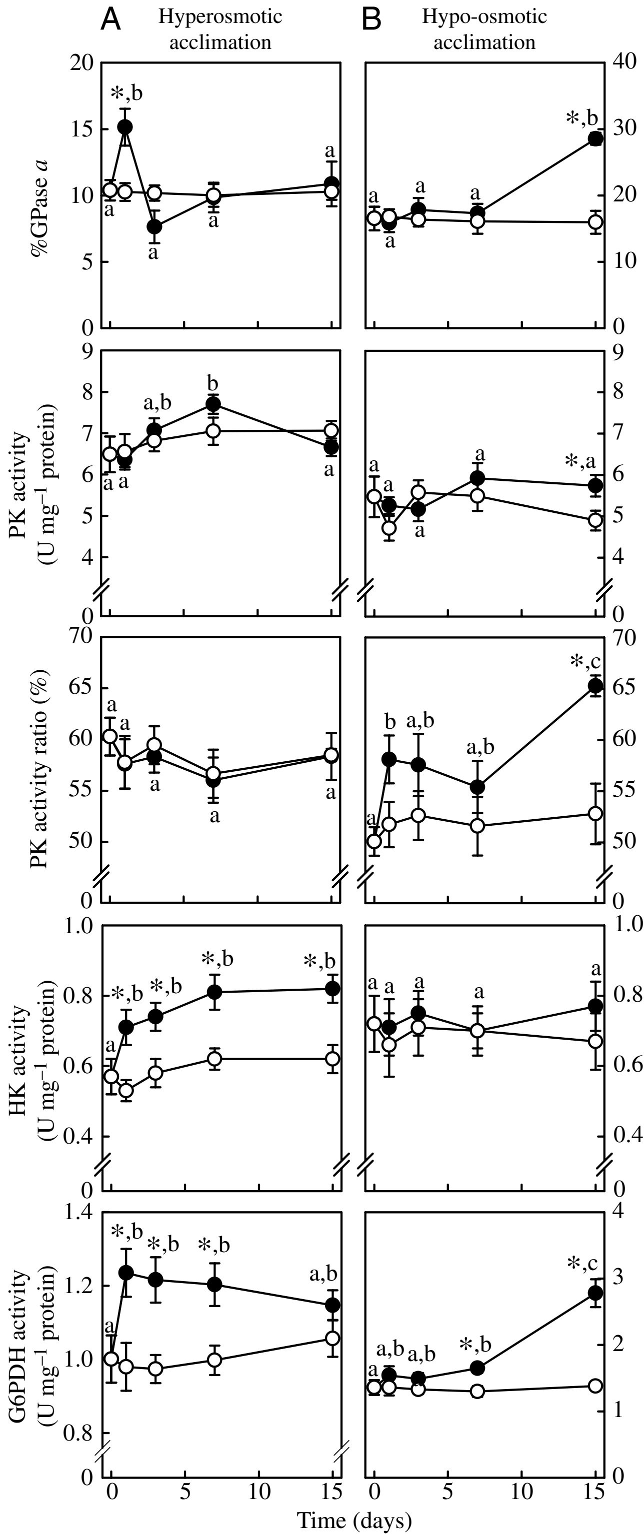

Fig. 6. Time course of changes in gill enzyme activities (glycogen phosphorylase, GPase; pyruvate kinase, PK; hexokinase, HK; and glucose 6-phosphate dehydrogenase, G6PDH) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

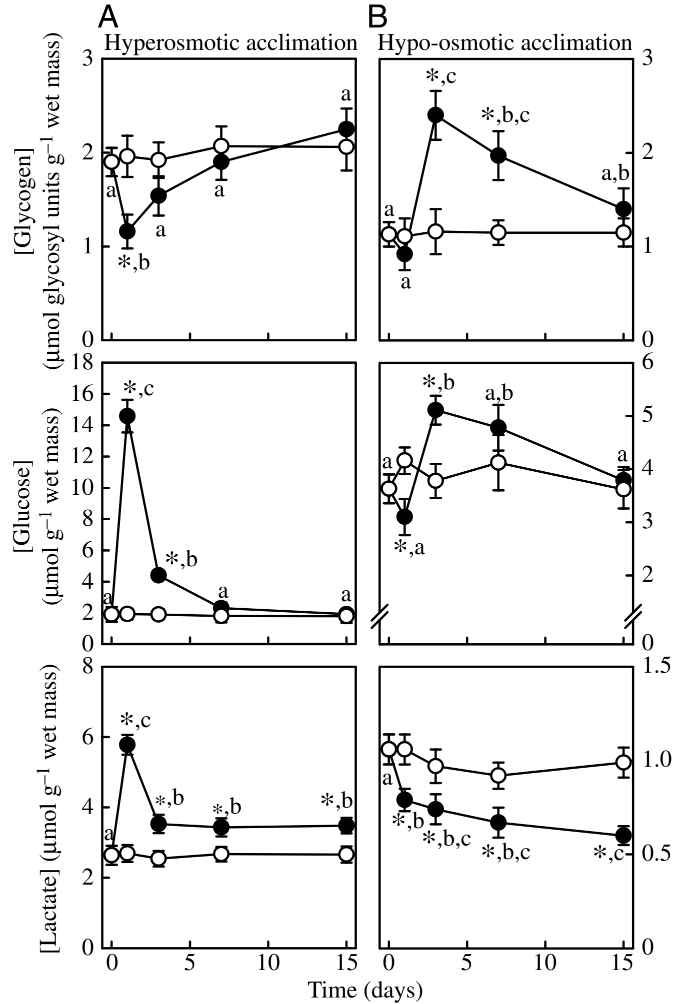

Fig. 7. Time course of changes in kidney metabolite levels (glycogen, glucose and lactate) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

in fish transferred to LSW on day 14 (Fig. 6). PK activity, either total activity or the activity ratio of the enzyme only, increased in fish transferred from SW to LSW after 14 days (a similar increase in the cofactor activation ratio of the enzyme was also observed in fish transferred to LSW after 14 days, data not shown). HK activity increased all along the transfer period in fish transferred from SW to HSW, reaching a 40% increase; in contrast, no changes were noticed in LSW. Finally, G6PDH activity was stimulated approx. 20% in the time period between 1 and 7 days of transfer from SW to HSW, whereas a higher increase (up to 100%) was observed after 7 and 14 days of transfer to LSW.

Kidney glycogen levels decreased 100% in fish transferred from SW to HSW on day 1, with levels being recovered from that time onwards; in contrast, a 200% increase was noticed after 3 and 7 days of transfer to LSW (Fig. 7). Free glucose levels in fish transferred from SW to HSW displayed an

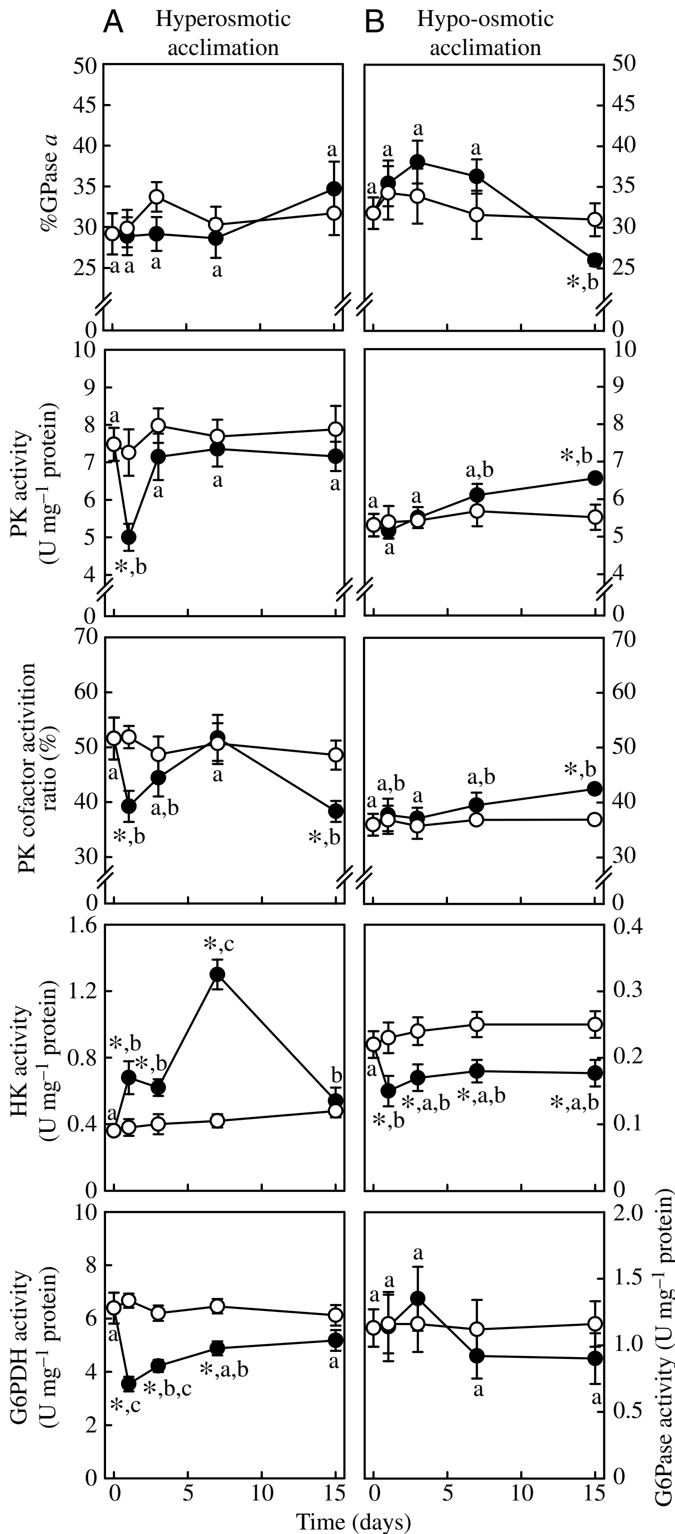

Fig. 8. Time course of changes in kidney enzyme activities (glycogen phosphorylase, GPase; pyruvate kinase, PK; hexokinase, HK; and glucose 6-phosphate dehydrogenase, G6PDH) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

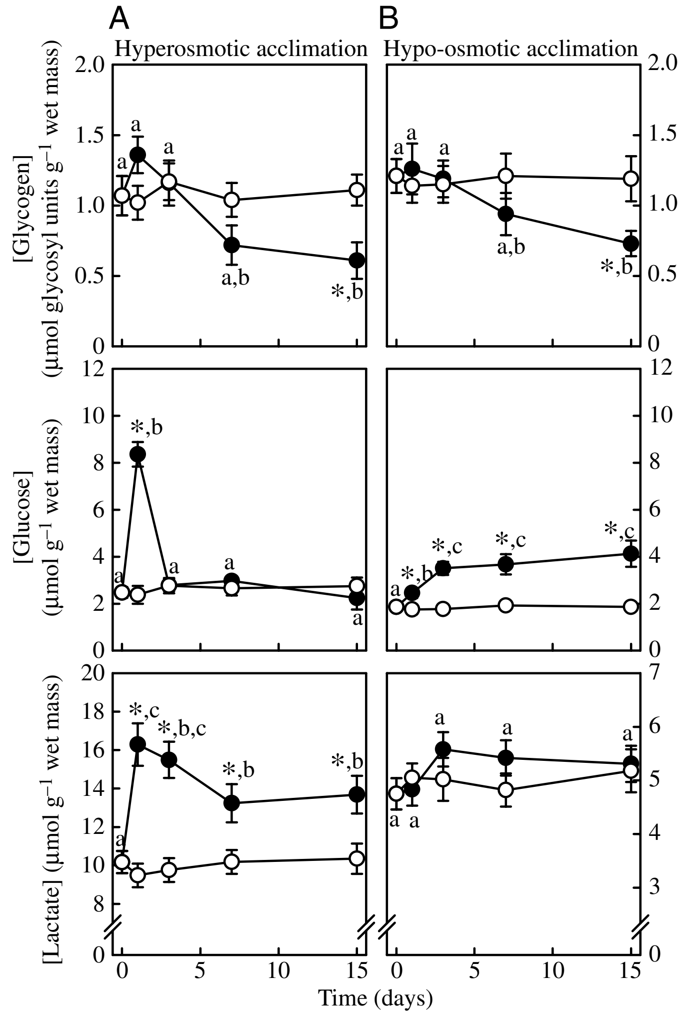

Fig. 9. Time course of changes in brain metabolite levels (glycogen, glucose, and lactate) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1. Note the different *y*-axis scales in A and B.

eightfold increase on day 1 after transfer with levels being restored from day 7 onwards; in contrast a small decrease on day 1 followed by a 20% increase on day 3 was noticed in fish transferred from SW to LSW. Lactate levels presented a 2.5-fold increase on the first day after transfer from SW to HSW with levels raised by approx. 80% for the remaining time period; in contrast a continuous decline up to 50% was observed in fish transferred to LSW.

Kidney GPase activity did not show significant changes (data not shown) whereas the percentage of the enzyme in the active form (GPase *a*) showed a 20% decrease after 14 days of transfer from SW to LSW (Fig. 8). PK activity presented a 40% decrease on day first after transfer to HSW whereas a 20% increase was observed after 14 days of transfer to LSW. No changes were observed for the activity ratio of PK (data not shown), but for the cofactor activation ratio of the enzyme, a

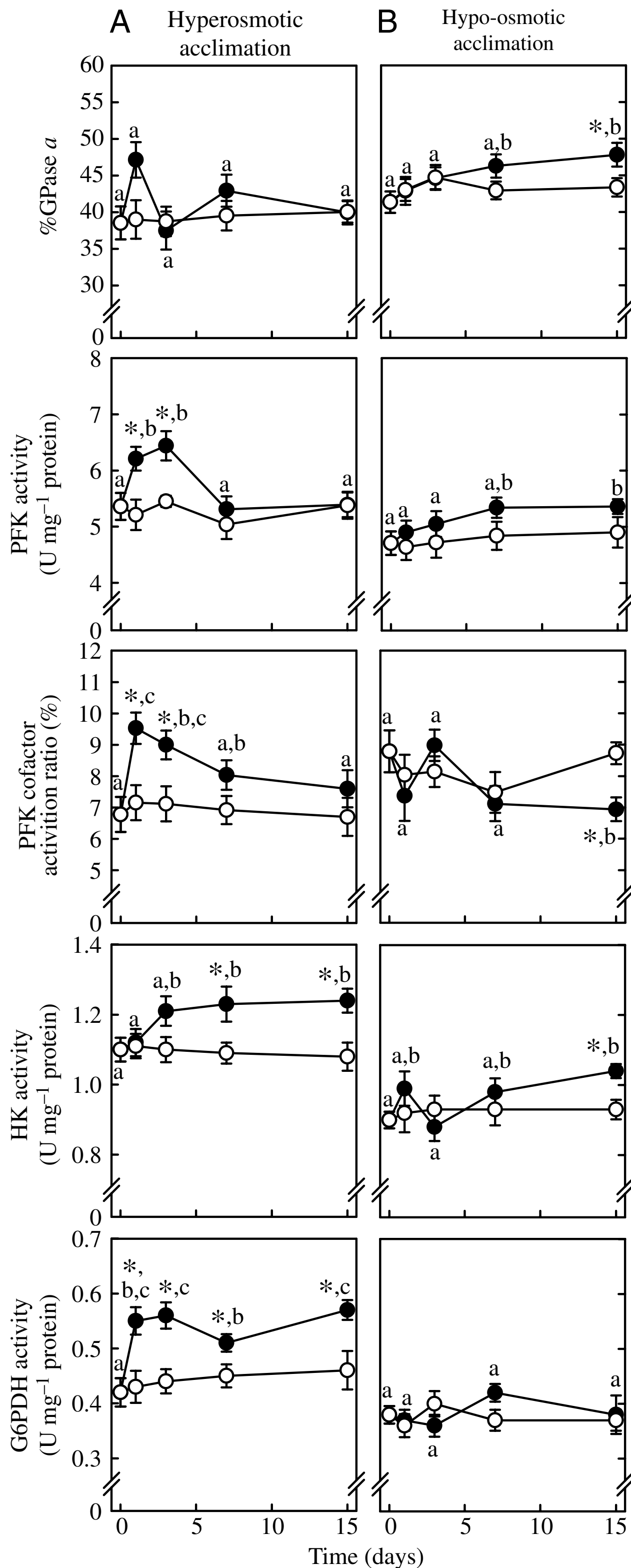

Fig. 10. Time course of changes in brain enzyme activities (glycogen phosphorylase, GPase; 6-phosphofructo 1-kinase, PFK; hexokinase, HK; and glucose 6-phosphate dehydrogenase, G6PDH) in gilthead sea bream acclimated to hyperosmotic (A) or hypo-osmotic (B) conditions. Fish were transferred for 14 days from seawater (SW) to (A) hypersaline water (HSW; closed circles) or (B) low salinity water (LSW; closed circles). Further details as in legend of Fig. 1.

20% decrease on days 1 and 14 were observed in fish transferred to HSW, and a 15% increase was noticed after 14 days of transfer to LSW. HK activity increased in fish transferred to HSW from days 1 to 7 after transfer, with activity observed on day 7 being 2.5-fold higher than in SW; in contrast a 40% decrease from day 1 after transfer onwards was observed for fish in LSW. G6PDH activity was only assessed in fish transferred from SW to HSW, displaying a 50% decrease on day 1 that was recovered at day 14. G6Pase activity was only assessed in fish transferred to LSW without showing any significant change.

Brain glycogen levels displayed a similar trend in fish transferred from SW to HSW or LSW: decreased levels from day 7 of transfer onwards, resulting in a 30% decrease after 14 days (Fig. 9). Free glucose levels increased fivefold after 1 day of transfer to HSW whereas a continuous increase was observed in fish transferred to LSW from day 1 to day 14 of transfer, reaching a 50% increase at the end. Lactate levels increased (up to 60%) in fish transferred to HSW from day 1 of transfer onwards whereas no changes were observed for fish in LSW.

Finally, brain GPase activity did not show any changes (data not shown) whereas the percentage of the enzyme in the active form displayed a 15% increase after 14 days of transfer from SW to LSW (Fig. 10). PFK activity increased approx. 20% on days 1 and 3 after transfer to HSW whereas no changes were noticed in fish in LSW. The activity ratio of PFK did not display any major changes (data not shown) whereas the cofactor activation ratio of PFK increased by up to 40% on days 1 and 3 after transfer to HSW. HK activity increased in fish transferred to HSW from day 7 of transfer onwards, reaching a 15% increase after 14 days; a similar increase was also observed in fish transferred to LSW but only after 14 days. Finally, G6PDH activity increased (up to 40%) in fish transferred to HSW from day 1 of transfer onwards whereas no changes were observed in fish transferred to LSW.

Discussion

Osmoregulatory parameters

Two different phases have been described in euryhaline fish after transfer between water of different salinities: an adaptive period and a chronic regulatory period (Holmes and Donaldson, 1969; Maetz, 1974). In our experiments, the time course of plasma osmolality in *S. auratus* transferred from SW to HSW or LSW also suggests the existence of two different phases in salinity acclimation, the first describing a sharp increase (or decrease) in osmolality, and a second several days later, when osmolality recovered to levels similar to those in SW-acclimated fish. These results confirmed the good euryhalinity of this species and agree with previous data obtained by our group (Guzmán et al., 2004; Laiz-Carrión et al., 2003, 2005a; Mancera et al., 1993a, 2002; Sangiao-Alvarellos et al., 2003, 2005b).

Gill Na^+,K^+-ATPase activity is related to the capacity of this osmoregulatory organ for extrusion of excess ions in a hyperosmotic environment (Marshall, 2002; McCormick, 1995, 2001) and for ion intake in a hypo-osmotic environment

(Deane and Woo, 2004; Jensen et al., 1998; Lin et al., 2004). In the present study, the time course of changes in gill Na^+,K^+-ATPase activity after hyperosmotic transfer agrees with that previously reported for this species (Laiz-Carrión et al., 2005a) and other euryhaline species (Deane and Woo, 2004; Jensen et al., 1998) as well as with the physiological role of this ion pump in euryhaline teleosts (Jensen et al., 1998; Marshall, 2002; McCormick, 1995, 2001). In addition, these results reinforce the U-shaped model for the relationship between gill Na^+,K^+-ATPase activity and environmental salinity observed for several several euryhaline teleosts (Deane and Woo, 2004; Jensen et al., 1998), including gilthead sea bream (Guzmán et al., 2004; Laiz-Carrión et al., 2005a). Our results agree with this model and also suggest a role for this enzyme in ion intake in hypo-osmotic environments (Marshall, 2002; McCormick, 2001). Since we have no indication whether or not sea bream is able to actively absorb ions in hypo-osmotic environments, it is entirely feasible that this species maintains ion balance for 14 days by decreasing ion efflux. However, specimens of gilthead sea bream maintained for 3 months in LSW also showed high gill Na^+,K^+-ATPase activity compared to fish maintained in brackish water (Laiz-Carrión et al., 2005b), suggesting that sea bream actively absorb ions in hypo-osmotic environments.

In euryhaline fish, kidney Na^+,K^+-ATPase activity also presents changes in response to variation in environmental salinity (Kelly and Woo, 1999a,b; Kelly et al., 1999; Lasserre, 1971; Venturini et al., 1992). To our knowledge, this is the first report on the time course of kidney Na^+,K^+-ATPase activity in gilthead sea bream after transfer from SW to HSW or LSW. The increase in activity seen on the first day of acclimation to HSW could be attributed to a reduction in urine production and/or to increased ion transport in the kidney. Since plasma osmolality also increased during acclimation to HSW, fish needed to eliminate the excess ions. However, no changes occurred in gill Na^+,K^+-ATPase activity during the adaptive period, so it is possible that the excess ions could be driven by rapid activation of kidney Na^+,K^+-ATPase activity, which returned to basal activity once the activity in gills was stimulated during the regulatory period. So, as shown in Fig. 1, at day 1, plasma osmolality and kidney Na^+,K^+-ATPase activity are high whereas gill Na^+,K^+-ATPase activity was normal. However, at day 3 an increase was noticed in gill Na^+,K^+-ATPase whereas renal Na^+,K^+-ATPase activity and plasma osmolality had almost recovered, suggesting that the heightened level of gill Na^+,K^+-ATPase activity may be responsible for correcting the osmotic disturbance.

Plasma cortisol and metabolites

Cortisol is considered to be an important hormone for acclimation to hyperosmotic and also hypo-osmotic environments (McCormick, 1995, 2001). In gilthead sea bream, a role for cortisol in osmotic acclimation (Laiz-Carrión et al., 2005a; Mancera et al., 1993a,b, 2002) and regulation of energy metabolism (Laiz-Carrión et al., 2002, 2003) has been proposed. The changes in plasma cortisol observed in the present study (i.e. increase in the first days of exposure to changed salinities followed by a subsequent return to basal levels) agree with similar cortisol pulses observed previously in several fish species, including gilthead sea bream (Laiz-Carrión et al., 2005a), after transfer to HSW (Marshall et al., 1999; Morgan et al., 1997; Richards et al., 2004; Scott et al., 2004) or LSW (Roche et al., 1989). Thus, it seems that the transient increase in plasma cortisol levels during the adaptive period of acclimation would be necessary for the enhancement of gill Na^+,K^+-ATPase activity and adaptation to the new salinity (McCormick, 1995, 2001). Since cortisol presents an important metabolic role in teleosts (Mommsen et al., 1999), including gilthead sea bream (Laiz-Carrión et al., 2002, 2003), this hormone could also assist to the osmoregulatory process by providing energy substrates for ion regulation. The higher increase in plasma cortisol levels in fish transferred to HSW than LSW also suggests more metabolic changes in HSW than in LSW (see below).

Plasma glucose levels in fish acclimated to HSW increased 1 day after salinity exposure followed by a slow decline over the following days, which is different from those reports in the literature following acclimation from freshwater (FW) to SW in rainbow trout (Soengas et al., 1993), from FW to brackish water in carp (De Boeck et al., 2000) and from SW to HSW in black sea bream (Kelly et al., 1999). In contrast, tilapia transferred from FW to SW showed a two-stage behaviour comparable to that described in the present study (Nakano et al., 1998). On the other hand, fish transferred to LSW displayed a peak of glucose on day 1 followed by elevated levels up to the end of experiment. This results agree with those previously reported for *S. auratus* under similar conditions of salinity transfer (Mancera et al., 1993a) and for the European sea bass (Roche et al., 1989).

The high values of plasma lactate levels in HSW-acclimated gilthead sea bream agree with data obtained after acclimation of this species (Sangiao-Alvarellos et al., 2003; Guzmán et al., 2004) and tilapia (Vijayan et al., 1996) to HSW. Since lactate can be used in tissues like gills, kidney and brain to supply their energy requirements (Mommsen, 1984; Mommsen et al., 1985; Soengas et al., 1998), the increase in plasma lactate levels observed in fish acclimated to LSW or HSW suggests that this metabolite becomes more important during osmotic acclimation, presumably reflecting its metabolic use in those organs.

Plasma triglyceride levels increased in the first days of acclimation to LSW and at the end of acclimation period in fish transferred to HSW, suggesting a role of this metabolite as a fuel for tissues during osmotic acclimation. The role of triglycerides in this process has not yet been addressed, but may be related to a metabolic reallocation of energy resources once carbohydrate stores have been mobilized. To our knowledge, there are no comparable studies in the literature, but this result may be in agreement with the enhanced production of plasma triglycerides already reported in Atlantic salmon during smoltification (Nordgarden et al., 2002).

Altogether, changes observed in plasma metabolite levels

suggested an increased availability of fuels, especially during the first days of transference (adaptive period), which can be used for an enhanced energy requirement in different tissues of the fish during HSW or LSW acclimation. However, the possibility that a change in plasma volume may account for some of the changes in plasma metabolites (like triglycerides) cannot be dismissed. The availability of fuels is very different in magnitude between HSW and LSW conditions. In fact, the increases in HSW were much more higher than those in LSW for all metabolites, especially glucose and lactate during the first stage, which strongly suggests an increased energy demand in HSW compared with LSW. Considering the increased plasma levels of cortisol during the adaptative period (especially in HSW), this hormone may be responsible of at least part of the metabolic changes observed (Laiz-Carrión et al., 2002, 2003).

Liver energy metabolism

Liver glycogen levels declined the first day after transfer to HSW and throughout the experiment in LSW. The mobilization of liver glycogen would provide glycosyl units ready to be used to fuel endogenous pathways such as glycolysis or to be exported to other tissues. However, considering the changes displayed in HSW by GPase activity, the later rise in glycogen and fall in lactate levels suggest resynthesis of glycogen from lactate.

An increase in the activity ratio and cofactor activation ratio of liver PFK was noticed after 3 days of transfer to HSW. In time course studies reported in the literature, increases were also noticed in liver glycolytic potential of rainbow trout (Soengas et al., 1993) and tilapia (Nakano et al., 1998) during the first days of acclimation from FW to SW. In contrast, in gilthead sea bream transferred to LSW we observed a decrease in this potential from approximately day 7 of experiment onwards. These changes suggest that a higher glucose use was taking place in liver of HSW-transferred fish during the first stage of acclimation, whereas in LSW-transferred fish a reduced use was apparent, especially from day 7 of acclimation onwards.

Changes displayed by G6Pase activity suggested an increased capacity of liver for exporting glucose on days 1 and 14 in HSW and from day 3 of experiment onwards in LSW. The mobilization of liver glycosyl units from glycogen stores in HSW-transferred fish can be related to the sharp increase of liver and plasma glucose levels at the same time. Thus, liver of gilthead sea bream may have a higher capacity to export glucose to plasma when fish are transferred to HSW, whereas their capacity is lower when transfer is to LSW, especially in the first stages of acclimation.

Modifications observed in liver energy metabolism in gilthead sea bream reinforce the model of a two-stage metabolic response during osmotic acclimation. Interestingly, some of the changes described for both stages behave in a converse way when comparing fish acclimated to HSW and LSW, such as during the first stage for glucose (increase in HSW, decrease in LSW) or G6Pase activity (increase in HSW, decrease in LSW).

Gill energy metabolism

Changes in gill HK activity suggest that an increased use of exogenous glucose occurs in gills during acclimation to HSW but not to LSW. These changes in HK activity are reflected in changes in tissue glucose levels that increased in HSW-acclimated fish and decreased in LSW-acclimated fish. Thus, at least part of the increased glucose in HSW-transferred fish is apparently used to store as glycogen in the first days of acclimation. However in LSW-transferred fish, the increase in glycogen levels from day 3 onwards suggests synthesis from metabolites other than glucose (maybe lactate), since tissue glucose levels actually decrease.

It is accepted that gill tissue is able to oxidize lactate (Mommsen, 1984). In this way the rise in plasma lactate levels and decrease in gill lactate levels observed in LSW-acclimated fish could suggest (Le François et al., 2004) an enhancement of the use of exogenous lactate by gills through LDH working in the oxidative direction, covering the initial stage of LSW acclimation (adaptive period). Considering that the energy demand of gills produced by Na^+,K^+-ATPase activity displayed a U-shape in relation to environmental salinity, this would also match with an increased use of other fuels, namely lactate, in extreme salinities (hyper and hypo-osmotic environments). However, no changes were observed in PK activity in HSW- and SW-acclimated fish, and an increase was noticed in gill lactate levels during the adaptive period of acclimation. Therefore the hypothetical increased use of lactate should be considered only under LSW and not HSW acclimation.

The enhanced capacity of the pentose phosphate pathway after transfer to HSW and LSW, based on changes in G6PDH activity, is interesting, suggesting the enhanced need for reducing power in gills of fish transferred to extreme salinities, which may be related to an increased necessity for them to synthesize lipids.

The changes observed in Na^+,K^+-ATPase activity in gills are therefore accompanied by two metabolic stages: (i) a first stage, characterized by an important accumulation of glycogen, glucose and lactate in HSW and decreased levels of lactate and glucose in LSW, and (ii) a second stage, where glucose is progressively being used through the pentose phosphate shunt and levels return to normality after 14 days in HSW, whereas an enhanced use of glucose through glycolysis, pentose phosphate and glycogenesis occurs on subsequent days of acclimation in LSW.

Kidney energy metabolism

In kidney, an increase in lactate levels was observed in HSW-transferred fish, especially in the adaptive period when a return to basal levels of kidney Na^+,K^+-ATPase activity was observed, suggesting an increased importance for this metabolite in kidney of HSW-acclimated fish. Changes observed in the time course of glycogen and glucose during acclimation to HSW resemble those measured after acclimation of rainbow trout from FW to SW (Soengas et al., 1994). These changes may suggest that an enhanced production of glucose occurs in kidney (at least part coming

directly from glycogen stores) during the first days of HSW acclimation. However, considering that no changes were apparent for GPase activity and no G6Pase activity was measured, this must be interpreted with caution. Changes in kidney HK activity in HSW-acclimated fish suggest that another important part of the increased glucose levels noticed in this tissue during the regulatory period could come directly from the blood stream.

The situation in LSW-transferred fish was almost the converse since: (i) glucose levels declined at day 1 of transference and increased on day 3, then returning to levels similar to those of SW-acclimated fish; (ii) HK activity showed a continuous decline from day 1 onwards, suggesting a decreased potential in kidney for using exogenous glucose during the first days; and (iii) a decrease in kidney lactate levels occurred from day 1 onwards, which may suggest that lactate is increasingly used as a fuel for kidney. In fact, lactate metabolization may be so high that some of the lactate molecules could be used through gluconeogenesis to increase glycogen synthesis (Blasco et al., 2001), which could match with the increased glycogen levels observed in kidney of LSW-transferred fish on days 4 and 7. Another converse response was noticed for glycolytic capacity, which decreased in the first days of acclimation to HSW and increased at the end of the acclimation period in LSW.

Considering that kidney Na^+,K^+-ATPase activity sharply increased on the first day of transfer to HSW, the extent of changes in metabolite levels observed at the same time suggest the existence of increased energy demand of this tissue during the first days of acclimation. This energy demand appears to be reduced on the following days. In HSW-acclimated fish, increased excretion of ions by the kidney could be necessary, and thus our metabolic results would suggest an increased use of different metabolites to fuel the increased osmoregulatory work of the kidney during the first days of HSW acclimation. On the other hand, acclimation of gilthead sea bream to LSW should produce increased osmoregulatory work in kidney because of production of a more dilute urine and increased ion reabsorption. Changes observed in metabolic parameters assessed in LSW-transferred fish may also lend support to a lower activation of kidney metabolism during the adaptive period of hypo-osmotic compared with hyperosmotic acclimation.

Brain energy metabolism

Brain glycogen, which constitutes the major energy store of fish brain (Soengas and Aldegunde, 2002) was mobilized during acclimation to HSW and LSW, and this could be related to a stress effect of salinity on brain metabolism that led this tissue to activate processes involved indirectly in the osmoregulatory work. These changes are probably elicited by hormones known to produce metabolic changes in fish brain (Laiz-Carrión et al., 2002, 2003; Sangiao-Alvarellos et al., 2004, 2005a). Since plasma cortisol levels increased dramatically during transfer of gilthead sea bream to HSW and LSW, this hormone is probably the main responsive of changes described. The important increase observed in HK activity in HSW- and LSW-acclimated fish also suggests that at least part of the increased glucose within the brain is coming directly from the blood stream. An increased energy demand in brain during the first days of acclimation to HSW is also suggested by increased glycolytic capacity, which can be related to the increase observed in Na^+,K^+-ATPase and creatine kinase activities in brain of tilapia transferred from FW to SW (Weng et al., 2002). Another interesting finding was the increase in lactate levels in HSW-acclimated fish, which indicate that the increased use of carbohydrates is higher than the energy demand of the brain, resulting in an accumulation of lactate. The possibility that brain glucose and lactate levels are at least partly due to blood within the brain cannot be excluded.

Altogether, changes observed in brain energy metabolism again revealed the existence of two stages in metabolic changes occurring during hyper/hypo-osmotic acclimation: a first one of reduced glycogen mobilization and use of exogenous glucose, followed by a second period of increased mobilization of glycogen. The enhanced availability of glucose during this second stage would help to explain the sharp increase in brain free glucose levels also observed in LSW-acclimated fish. The increased availability of glucose within brain in the first stage is not apparently used through glycolysis or the pentose phosphate pathway in LSW-transferred fish since no important changes were noticed in the activity of selected enzymes from those pathways, whereas an increased use through glycolysis is apparent in HSW-transferred fish at that stage. Interestingly, changes in metabolic parameters in brain are in most cases irrespective of the direction of changes in salinity, which can be attributed to the action of cortisol (Laiz-Carrión et al., 2002, 2003).

Conclusions

In summary, the time course of acclimation to HSW and LSW in *S. auratus* displayed an adaptive and a regulatory period similar to those previously described for this species when subjected to equivalent transfers (Laiz-Carrión et al., 2005a; Mancera et al., 1993a). In addition, these periods are characterized by a tissue-specific reorganization of energy metabolism in two stages. The first stage involves an increase in the availability and use of fuels in different organs where an enhanced energy requirement of different osmoregulatory (gills and kidney) and non-osmoregulatory (liver and brain) tissues involved directly or indirectly on osmoregulatory work is observed. In the second stage, osmoregulatory parameters reached homeostasis, and most metabolic parameters returned to normality. When comparing changes in metabolic parameters between HSW and LSW-transferred fish, several interesting findings arise such as: (i) several metabolic parameters (i.e. liver glucose or G6Pase activity) in liver, gills and kidney, displayed converse responses that are not apparent in brain, and (ii) the magnitude of changes (as well as the two stages) is less important in LSW acclimation than in HSW acclimation, suggesting that acclimation of euryhaline marine

species like gilthead sea bream to LSW is less expensive in terms of energy than acclimation to HSW.

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