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Growth performance of gilthead sea bream *Sparus aurata* in different osmotic conditions: Implications for osmoregulation and energy metabolism

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Abstract

The influence of three different environmental salinities (seawater, SW: 38 ppt salinity; brackish water, BW: 12 ppt; and low salinity water, LSW: 6 ppt salinity) on the growth, osmoregulation and metabolism of young gilthead sea bream (*Sparus aurata* L.) was studied over a period of 100 days. 480 inmature fish (20 g mean body weight) were randomly divided into six tanks of 2500 l (80 fish per tank) and maintained under three different salinities (38 ppt, 12 ppt and 6 ppt) in an open system. Every three weeks, 10 fish from each tank were anesthetized, weighed and lenghed. At the end of experiment, 10 fish from each tank were anesthetized, weighed and sampled for plasma, brain, gill and liver. Gill Na^+, K^+-ATPase activity, plasma osmolality, ions (sodium and chloride), glucose, lactate, protein and triglyceride, and hepatosomatic index were examined. In addition, levels of glycogen, lactate, ATP and activities of potential regulatory enzymes (hexokinase, pyruvate kinase, glycogen phosphorylase, and glucose 6-phosphate dehydrogenase) were assesed in liver, brain, and gill. BW-acclimated fish showed a better growth with respect to SW- or LSW-acclimated fish (12>38>6 ppt). The same relationship was observed for weight gain and specific growth rate. Osmoregulatory parameters in plasma (osmolality, Na^+ and Cl^- levels) were similar in SW- and BW-acclimated fish but significantly higher than those of LSW-acclimated fish. Gill Na^+, K^+-ATPase activity showed lower values in intermediate salinity (6>38>12 ppt). No changes were observed in metabolic parameters analyzed in plasma, whereas only minor changes were observed in metabolic parameters of liver, gills and brain that could be correlated with the higher growth rates observed in fish acclimated to BW, which do not allow us to attribute the best growth rate observed at 12 ppt to lower metabolic rates in that salinity.

Keywords: Gilthead sea bream; *Sparus aurata*; Growth; Osmoregulation; Energy metabolism

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1. Introduction

Euryhaline fish present the capacity to live in different environmental salinities. The osmolality of the internal milieu in marine teleost is equivalent to $10 \pm 2\%$ of the environmental salinity (Holmes and Donaldson, 1969; Maetz, 1974). At this environmental salinity (around 12 ppt) fish are almost isotonic with respect to the environment. Most researchers agree that salinities that differ from the internal osmotic concentration of the fish must impose energetic regulatory costs for active ion transport. There is less agreement concerning the magnitude of these costs and very little information on the related energetic and physiological consequences of life in different salinities (Eddy, 1982; Swanson, 1998; Boeuf and Payan, 2001). In this way, many studies support the hypothesis that the energetic cost of ion regulation is lowest in an isotonic environment, while others did not support this idea (Morgan and Iwama, 1991). The energy saved from osmoregulatory processes could be derived from other physiological processes, such as growth. Thus, a relationship between environmental salinity and growth has been reported in different euryhaline fish, but the optimal salinity to obtain the best growth depends on different factors such us species, temperature, dietary intake, sex, etc (Brett, 1979; Brett and Groves, 1979; Kirschner, 1995; Boeuf and Payan, 2001).

The gilthead sea bream (*Sparus aurata* L.) is a member of the Sparidae, and has a high commercial value (Basurco and Abellán, 1999). This species can live in environments of different salinities and temperatures: coastal waters, estuaries (brackish water) and lagoons (hypersaline water) (Arias, 1976; Suau and López, 1976). Several studies have focussed on the influence of environmental salinity on different aspects of sea bream biology such as limits of salinity tolerance for juveniles (Chervinski, 1984) and optimal salinity for larval development (Freddi et al., 1981; Tandler et al., 1995). Early studies showed that juvenile gilthead sea bream can grow in brackish water, under laboratory conditions, at least for two months (Chervinski and Chanin, 1985). In addition, Klaoudatos and Conides (1996) and Conides et al. (1997) showed that 28 ppt was the best salinity for growth in fry gilthead sea bream.

The previous studies of our group on the osmoregulatory system have indicated that this species is capable of facing extreme changes in environmental salinities and to adapt to environments of different salinity (Mancera et al., 1993). Also, the role of different hormones (prolactin, cortisol, growth hormone) for hypoosmotic or hyperosmotic adaptation has been studied (Mancera et al., 1994, 2002; Laiz-Carrión et al., 2003). In addition, the interrelationship between osmoregulation and metabolism has been analyzed in fish acclimated to different environmental salinities (12, 38 and 55 ppt) for two weeks (Sangiao-Alvarellos et al., 2003) addressing that hypoosmotic and hyperosmotic environments elicited an increased energy cost in this species. However, few studies have made simultaneous measurements on growth and energy metabolism of fish in relation to salinity. Therefore, our objective was to describe and compare the overall energy relationships, growth, and performance of fish acclimated to different salinities. To study those relationships we examined growth, metabolic rates, and ionic regulation in juvenile gilthead seabream acclimated over a period of 100 days to a range of salinities (36, 12 and 6 ppt), with one near isotonicity.

2. Materials and methods

2.1. Fish

Immature male gilthead sea bream (*Sparus aurata* L., 20 g mean body weight) were provided by ACUINOVA S.L. (San Fernando, Cádiz, Spain) in whose facilities the experiments were undertaken. The fish were acclimated and maintained during 100 days in three different environmental salinities (38, 12 and 6 ppt) (see below). During the experiments, fish were maintained under natural photoperiod (July–October) and temperature (18–27 °C). Daily maximum and minimum temperatures were recorded using a thermometer. The difference between maximum and minimum temperatures was always less that 1 °C. The variation in daily mean temperature of water during the experimental period is depicted in Fig. 1. Fish were fed once at day with 1% body weight using commercial dry pellets (D-5 Europa, Dibaq-Diprotg SA, Segovia, Spain) according to their size. The fish

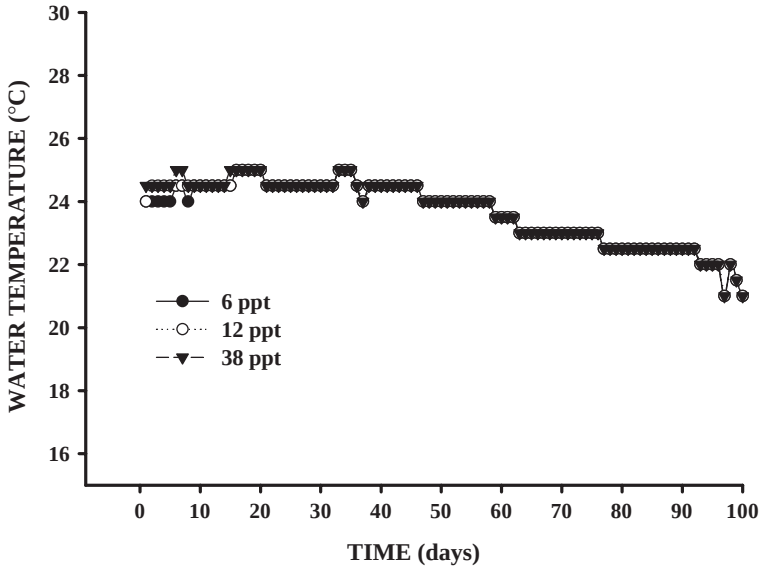

Fig. 1. Changes with time in daily mean temperature of water (°C) in different experimental salinities.

feed composition was: 48% crude protein, 6% carbohydrates, 25% crude fat, and 11.5% ash (20.2 megaJ/kg of feed). Fish were fasted for 24 h before each sample day. 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.

2.2. Experimental protocol

Fish were randomly divided into three different groups (80 fish per group), kept in six open system tanks of 2500 l each containing seawater (SW, 38 ppt and range 37–39 ppt). The experiment was carried out in duplicates. After an initial acclimation period (15 days) salinity was progressively changed to brackish water (BW, 12 ppt and range 11–13 ppt) or low saline water (LSW, 6 ppt and range 5–7 ppt) in open systems, whereas in the tanks with the SW groups the salinity remained the same. Salinity in the group denoted as BW and LSW was decreased gradually to 12 or 6 ppt over 2 salinity points per hour by mixing full SW with dechlorinated tap water. Fish from 3 groups remained in their specific conditions during 100 days during which the common water quality criteria (hardness, pH, levels of oxygen, carbon dioxide, hydrogen sulphide, nitrite, nitrate, ammonia, calcium, chlorine and suspended solids) were assessed with no major changes being observed. The water salinity was checked every day, and corrected when it was necessary. No mortality was observed during the experiment.

2.3. Sampling

At end of experiment, 10 fish from each tank (20 fish per salinity treatment) were capture and 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 in liquid nitrogen and stored 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, pH7.3) and frozen at −80 °C. From each fish, brain, liver, and the remaining branchial arches were removed in a few seconds, frozen in liquid nitrogen, and stored at −80 °C until assay.

2.4. Growth trial

At time 0 (start of experiment), 30, 52, 80 and 100 days (end of experiment), 10 fish from each tank (20 fish per salinity treatment) were captured by netting, slightly anaesthetized with 2-phenoxyethanol (0.5 ml l^{-1} water), weighed, measured and placed back into the respective tank. No mortality was observed during this manipulation. To evaluate the effects of environmental salinity on the performance of the fish several parameters were calculated:

- Fulton's condition factor (K) was calculated as $K = 100 \cdot W/L^3$, where W = fish weight (g) and L = total length (cm).
- Weight gain (%) = $100(W_f - W_i)/W_i$, where W_f = final body weight (g) and W_i = initial body weight (g).
- Hepatosomatic index (HSI) = (liver weight/total weight) · 100.
- Specific growth rate (SGR) was calculated as $SGR = 100(\ln W_f - \ln W_i)/T$, where W_f = final body weight (g), W_i = initial body weight (g) and T is number of days between each two weighing.

2.5. Analytical techniques

Plasma glucose and lactate were measured using commercial kits from Sigma (Sigma #16-20UV and Sigma #735, respectively) adapted to microplates (Stein, 1963; Iwama et al., 1989). Plasma protein was measured using the bicinchoninic acid method (Smith et al., 1985) with BCA protein kit (Pierce, Rockford, USA) for microplates, with bovine albumin as standard. Plasma triglyceride levels were determined enzymatically with a commercial kit from Sigma No #334-UV (Bucolo and David, 1973) 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). Plasma osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT, USA). Plasma Na^+ was measured using an atomic absorption spectrophotometer, and plasma Cl^- levels with the Chloride Sigma kit (no. 461–463).

Gill Na^+, K^+-ATPase activity was determined using the micro assay method of McCormick (1993) adapted to *S. aurata* (Mancera et al., 2002; Laiz-Carrión et al., 2003). Liver, brain, and gill samples were minced on a chilled Petri dish to very small pieces that, once formed, were mixed and divided into two homogeneous aliquots to assess enzyme activities and metabolite levels, respectively. The tissue used for the assessment of metabolite levels was homogenized immediately by ultrasonic disruption in cold (Dr Hielscher UP200H) with 7.5 volumes 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 and ATP levels were determined spectrophotometrically using commercial kits (Sigma diagnostics, USA). 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 the assessment of enzyme activities was homogenized by ultrasonic disruption in cold (Dr Hielscher UP200H) with 10 volumes 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 homogenisation). The homogenate was centrifuged (2 min at 13,000 g, Eppendorf 5415R) and the supernatant used for assays.

Enzyme activities were determined using a Unicam UV6-220 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. Homogenate protein was assayed in triplicate as detailed by Bradford (1976), using bovine serum albumin (Sigma, USA) as standard. Enzyme analyses were all carried out to achieve maximum rates in each tissue, as defined in preliminary tests. The specific conditions for enzyme assays were described previously (Laiz-Carrión et al., 2002, 2003; Sangiao-Alvarellos et al., 2003, 2005).

2.6. Statistics

Data were statistically analysed by a one-way ANOVA test in which treatment (38, 12 and 6 ppt) was the main factor and treated as a nominal independent variable. Growth trial data were statistically analysed by a two-way ANOVA test in which treatment (38, 12 and 6 ppt) and time (0, 30, 52, 80 and 100 days) were the main factors and treated as nominal independent variables. 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 $P<0.05$. Since no significant differences were found between experimental tanks for each salinity and sampling time (*t*-test) we pooled data for each salinity.

3. Results

The temperatures recorded in tanks used in experiment did not significantly change, as can be observed in Fig. 1, when comparing temperatures registered in replicate tanks within salinities and comparing temperatures registered among salinities.

The effect of salinity on growth of juvenile gilthead sea bream acclimated to three different salinities over a period of 100 days is represented in Fig. 2. In addition, the effect of salinity on several growth performance indexes is summarized in Table 1. In the present study, after 3 months BW-acclimated fish showed a better growth than SW- or LSW-acclimated fish (12 ppt>38 ppt>6 ppt). The same relationship was observed for weight gain and specific growth rate (SGR).

Osmoregulatory plasmatic parameters were similar in SW- and BW-acclimated fish, while LSW-acclimated fish presented significantly lower levels with respect to the other two groups (Table 2). Gill Na^+, K^+-ATPase activity showed the lowest values in intermediate salinity (12 ppt) showing LSW-acclimated fish higher activity than SW-acclimated fish. The relationship between gill Na^+, K^+-ATPase activity and environmental salinity presented a "U-shape" curve.

Metabolic parameters in plasma (glucose, lactate, triglyceride and protein) did not show significant changes in fish acclimated to different environmental salinities (Table 3). Metabolic parameters assessed in gills are displayed on Table 4. ATP levels were lower in LSW- than in SW- and BW-acclimated fish. The optimal activity of PK was higher in BW- than in BW- and LSW-acclimated fish, whereas the activity ratio of

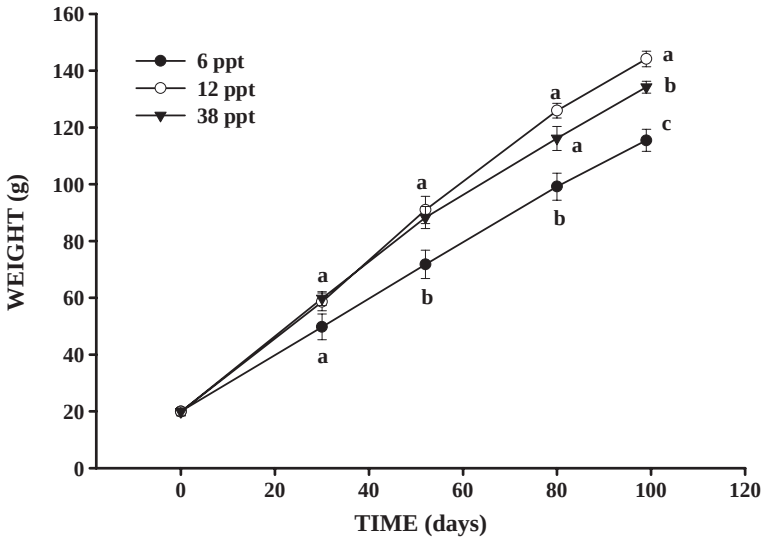

Fig. 2. Change in weight over 100 days of juvenile of gilthead sea bream adapted to three different salinities. Different letters indicate significant differences among groups at each time ($P<0.05$). In each salinity weight was significantly different ($P<0.05$) among sampling times (symbols not shown).

Table 1
Total length, initial and final body weight, weight gain, specific growth rate (SGR), hepatosomatic index (HSI) and condition factor (K) in gilthead sea bream cultured in SW (38 ppt), BW (12 ppt) or LSW (6 ppt) over a period of 100 days

Parameter	Groups		
	SW	BW	LSW
Final total length (cm)	18.9 ± 0.2 a	19.0 ± 0.3 a	17.7 ± 0.4 b
Initial body weight (g)	19.8 ± 0.5 a	19.7 ± 0.5 a	20.0 ± 0.4 a
Final body weight (g)	134.2 ± 2.1 a	144.1 ± 2.7 b	120.5 ± 3.9 c
Weight gain (%)	576 ± 11 a	632 ± 14 b	502 ± 19 c
SGR (g/day)	1.911 ± 0.016 a	1.990 ± 0.019 b	1.791 ± 0.032 c
HIS (%)	1.4 ± 0.1 a	1.1 ± 0.1 a	1.3 ± 0.1 a
K	1.99 ± 0.04 a	2.10 ± 0.09 a	2.17 ± 0.20 a

Each value is the mean ± S.E.M. of $n = 15$–20 fish per group. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).

the same enzyme was higher in LSW- than in SW- and BW-acclimated fish. G6PDH activity was higher in BW- and LSW-acclimated fish than in SW-acclimated fish. The remaining metabolic parameters assessed in gills (levels of glycogen and lactate, and the activities of GPase and HK) did not display significant differences among groups.

The results obtained when assessing metabolic parameters in liver are displayed in Table 5. ATP levels were lower in BW- and LSW-acclimated fish than in SW-acclimated fish. The activity of GPase was also lower in BW- than in SW- and LSW-acclimated fish. The remaining parameters (levels of glycogen and lactate, and the activities of PK, G6PDH and FBPase) did not display significant differences when comparing the groups assessed.

Table 2
Osmolality, sodium and chloride levels in plasma, and gill Na^+, K^+-ATPase activity in gilthead sea bream cultured in SW (38 ppt), BW (12 ppt) or LSW (6 ppt) over a period of 100 days

Parameter	Groups		
	SW	BW	LSW
Plasma osmolality (mosm kg^{-1})	377 ± 11 a	370 ± 4 a	348 ± 7 b
Plasma sodium (mmol l^{-1})	182 ± 3 a	177 ± 3 a	169 ± 2 b
Plasma chloride (mmol l^{-1})	151 ± 3	149 ± 2 a	144 ± 3 a
Gill Na^+, K^+-ATPase activity (μmol ADP mg $protein^{-1}$ h^{-1})	15.87 ± 0.39 a	12.35 ± 0.72 b	26.89 ± 1.21 c

Each value is the mean ± S.E.M. of $n = 15$–20 fish per group. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).

Table 3
Levels of glucose, lactate, triglyceride and protein in plasma of gilthead sea bream cultured in SW (38 ppt), BW (12 ppt) or LSW (6 ppt) over a period of 100 days

Parameter	Groups		
	SW	BW	LSW
Glucose (mmol l^{-1})	4.13 ± 0.19 a	4.31 ± 0.42 a	4.21 ± 0.21 a
Lactate (mmol l^{-1})	2.55 ± 0.19 a	2.39 ± 0.27 a	2.01 ± 0.22 a
Triglyceride (mmol l^{-1})	2.76 ± 0.42 a	3.10 ± 0.46 a	2.38 ± 0.35 a
Protein (mg ml^{-1})	42.29 ± 1.10 a	39.97 ± 1.55 a	41.75 ± 1.4.5 a

Each value is the mean ± S.E.M. of $n = 15$–20 fish per group. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).

Finally, the results obtained in metabolic parameters of brain are displayed on Table 6. ATP levels were higher in BW- and LSW-acclimated fish than in SW-acclimated fish. PFK activity decreased in BW-acclimated fish compared with SW-acclimated fish. The percentage of GPase in the active form significantly increased in BW- and LSW-acclimated fish compared with SW-acclimated fish. The remaining metabolic parameters assessed (levels of glycogen and lactate, and the activities of HK and G6PDH) did not show significant differences when comparing groups.

4. Discussion

Fish growth is affected by environmental and endogenous factors, such as salinity, temperature, quality of water, age, sex, etc (Brett, 1979; Brett and Groves, 1979; Boeuf and Payan, 2001). The effect of salinity seems to vary among the different species studied, which show different and specific optimal salinities for growth (*Mugil cephalus*: De Silva and Perera, 1976; *Salmo gairdneri*: McKay and Gjede,

Table 4
Levels of glycogen, lactate, ATP and activities of potential regulatory enzymes (hexokinase, HK; pyruvate kinase, PK; glycogen phosphorylase, GPase; glucose 6-phosphate dehydrogenase, G6PDH) in gills of gilthead sea bream cultured in SW (38 ppt), BW (12 ppt) or LSW (6 ppt) over a period of 100 days

Parameter	Groups		
	SW	BW	LSW
Glycogen levels (μmol glycosyl U g^{-1} wet weight)	0.39 ± 0.05 a	0.75 ± 0.08 b	0.45 ± 0.09 a
Lactate levels (μmol g^{-1} wet weight)	2.26 ± 0.26 a	2.86 ± 0.33 a	1.78 ± 0.21 a
ATP levels (μmol g^{-1} wet weight)	1.35 ± 0.48 a	1.93 ± 0.50 a	1.63 ± 0.49 b
HK activity (U mg^{-1} protein)	0.75 ± 0.06 a	0.80 ± 0.09 a	0.92 ± 0.07 a
PK activity			
Optimal activity (U mg^{-1} protein)	2.06 ± 0.11 a	2.76 ± 0.14 b	2.33 ± 0.07 a
Activity ratio	0.46 ± 0.01 a	0.49 ± 0.02 a	0.55 ± 0.02 b
Fructose 1,6-P_2 activation ratio	0.99 ± 0.03 a	0.94 ± 0.03 a	1.00 ± 0.03 a
GPase activity			
Total activity (U mg^{-1} protein)	0.09 ± 0.01 a	0.10 ± 0.01 a	0.09 ± 0.01 a
% GPase *a*	9.89 ± 2.31 a	5.96 ± 2.54 a	6.34 ± 3.25 a
G6PDH activity (U mg^{-1} protein)	1.14 ± 0.06 a	1.57 ± 0.06 b	1.60 ± 0.10 b

Each value is the mean ± S.E.M. of $n = 15$–20 fish per group. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).
One unit of enzyme activity is defined for HK as that which utilizes 1 μmol glucose min^{-1}, for PK as that which utilizes 1 μmol phosphoenolpyruvate min^{-1}, for GPase as that which produces 1 μmol NADPH min^{-1}, and for G6PDH as that which utilizes 1 μmol glucose 6-phosphate min^{-1}; % GPase *a*, percentage of total glycogen phosphorylase (a+b) in the active form (a).
The activity ratio of pyruvate kinase is defined as activity at low (0.1 mmol l^{-1})/high (2.8 mmol l^{-1}) substrate (fructose 6P) concentration. Similarly, a fructose 1,6-bisphosphate activation ratio was determined using 0.01 mmol l^{-1} fructose 1,6-bisphosphate concentrations, and 0.1 mmol l^{-1} phosphoenolpyruvate concentrations.

Table 5
Levels of glycogen, lactate, ATP and activities of potential regulatory enzymes (pyruvate kinase, PK; glycogen phosphorylase, GPase; glucose 6-phosphate dehydrogenase, G6PDH; fructose 1,6-bisphosphatase, FBPase) in liver of gilthead sea bream cultured in SW (38 ppt), BW (12 ppt) or LSW (6 ppt) over a period of 100 days

Parameter	Groups		
	SW	BW	LSW
Glycogen levels (μmol glycosyl U g^{-1} wet weight)	460.4 ± 20.3 a	426.4 ± 14.8 a	394.5 ± 28.4 a
Lactate levels (μmol g^{-1} wet weight)	1.44 ± 0.25 a	0.96 ± 0.13 a	1.36 ± 0.33 a
ATP levels (μmol g^{-1} wet weight)	1.02 ± 0.16 a	0.59 ± 0.06 b	0.53 ± 0.08 b
PK activity			
Optimal activity (U mg^{-1} protein)	0.36 ± 0.01 a	0.46 ± 0.04 a	0.34 ± 0.04 a
Activity ratio	0.25 ± 0.09 a	0.29 ± 0.02 a	0.29 ± 0.06 a
Fructose 1,6-P_2 activation ratio	0.13 ± 0.03 a	0.20 ± 0.02 a	0.28 ± 0.06 a
GPase activity			
Total activity (U mg^{-1} protein)	0.15 ± 0.02 a	0.08 ± 0.01 b	0.16 ± 0.02 a
% GPase *a*	48.7 ± 6.76 a	46.6 ± 4.96 a	46.1 ± 2.54 a
G6PDH activity (U mg^{-1} protein)	1.30 ± 0.06 a	1.39 ± 0.10 a	1.47 ± 0.06 a
FBPase activity (U mg^{-1} protein)	0.49 ± 0.04 a	0.54 ± 0.02 a	0.52 ± 0.03 a

Each value is the mean ± S.E.M. of $n = 15$–20 fish per group. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).
One unit of enzyme activity is defined for PK as that which utilizes 1 μmol phosphoenolpyruvate min^{-1}, for GPase as that which produces 1 μmol NADPH min^{-1}, for G6PDH as that which utilizes 1 μmol glucose 6-phosphate min^{-1}, and for FBPase as that which utilizes 1 μmol fructose 1,6-bisphosphate min^{-1}; % GPase *a*, percentage of total glycogen phosphorylase (a + b) in the active form (a).
The activity ratio of pyruvate kinase is defined as activity at low (0.1 mmol l^{-1})/high (2.8 mmol l^{-1}) substrate (fructose 6P) concentration. Similarly, a fructose 1,6-bisphosphate activation ratio was determined using 1 mmol l^{-1} fructose 1,6-bisphosphate concentrations, and 0.1 mmol l^{-1} phosphoenolpyruvate concentrations.

Table 6
Levels of glycogen, lactate, ATP and activities of potential regulatory enzymes (hexokinase, HK; 6-phosphofructo 1-kinase, PFK; glycogen phosphorylase, GPase; glucose 6-phosphate dehydrogenase, G6PDH) in brain of gilthead sea bream cultured in SW (38 ppt), BW (12 ppt) or LSW (6 ppt) over a period of 100 days

Parameter	Groups		
	SW	BW	LSW
Glycogen levels (μmol glycosyl U g^{-1} wet weight)	0.23 ± 0.01 a	0.20 ± 0.03 a	0.24 ± 0.03 a
Lactate levels (μmol g^{-1} wet weight)	4.33 ± 0.30 a	4.54 ± 0.43 a	3.96 ± 0.36 a
ATP levels (μmol g^{-1} wet weight)	0.04 ± 0.003 a	0.07 ± 0.009 b	0.06 ± 0.006 b
HK activity (U mg^{-1} protein)	0.71 ± 0.03 a	0.66 ± 0.03 a	0.64 ± 0.03 a
PFK activity			
Optimal activity (U mg^{-1} protein)	4.06 ± 0.13 a	4.57 ± 0.11 b	4.12 ± 0.09 a
Activity ratio	0.06 ± 0.005 a	0.06 ± 0.005 a	0.06 ± 0.006 a
Fructose 2,6-P_2 activation ratio	0.18 ± 0.009 a	0.20 ± 0.01 a	0.20 ± 0.01 a
GPase activity			
Total activity (U mg^{-1} protein)	0.21 ± 0.01 a	0.20 ± 0.01 a	0.23 ± 0.01 a
% GPase *a*	42.6 ± 1.79 a	52.4 ± 2.38 b	45.4 ± 2.21 a
G6PDH activity (U mg^{-1} protein)	0.23 ± 0.01 a	0.22 ± 0.01 a	0.22 ± 0.0005 a

Each value is the mean ± S.E.M. of $n = 15-20$ fish per group. Different letters indicate significant differences among groups (one way ANOVA, $P < 0.05$).
One unit of enzyme activity is defined for HK as that which utilizes 1 μmol glucose min^{-1}, for PK as that which utilizes 1 μmol phosphoenolpyruvate min^{-1}, for GPase as that which produces 1 μmol NADPH min^{-1}, and for G6PDH as that which utilizes 1 μmol glucose 6-phosphate min^{-1}; % GPase *a*, percentage of total glycogen phosphorylase (a + b) in the active form (a).
The activity ratio of 6-phosfofructo 1-kinase is defined as activity at low (0.1 mmol l^{-1})/high (2.8 mmol l^{-1}) substrate (fructose 6P) concentration. Similarly, a fructose 1,6-bisphosphate activation ratio was determined using 5 μmol l^{-1} fructose 2,6-bisphosphate concentrations, and 0.1 mmol l^{-1} fructose 6-phopsphate concentrations.

1985; *Oreochromis niloticus*: De Silva and Perera, 1985; Woo et al., 1997; *Dicentrachus labrax*: Dendrinos and Thorpe, 1985; Zanuy and Carrillo, 1985; Barnabé and Guissi, 1993; *Sparus sarba*: Woo and Kelly, 1995; *Chanos chanos*: Swanson, 1996; Alava, 1998; *Acanthopagrus butcheri*: Partridge and Jenkins, 2002; *Paralichthys orbignyanus*: Sampaio and Bianchini, 2002). This effect of salinity on growth rate may be mediated by the energy cost of osmotic and ionic regulation that limits the energy supply for growth (see Eddy, 1982; Boeuf and Payan, 2001). However, other possibilities related to the influence of environmental salinity on food intake (see Le Bail and Boeuf, 1997), metabolic reorganization (Sangiao-Alvarellos et al., 2003) or stimulation of osmoregulatory hormones related to growth (McCormick, 2001) should be considered.

In the present study, we have analyzed the influence of three different environmental salinities (6, 12 and 38 ppt) on the growth of juvenile gilthead sea bream over a period of 100 days. Growth of *Sparus auratus* was better in intermediate salinity (12 ppt) when compared with extreme salinities (12>38>6 ppt) (Fig. 2). The absence of changes in water temperature among salinities (Fig. 1) further supports that changes observed in parameters can be attributed to the different salinity of the environment. At this environmental salinity (12 ppt) fish is almost isotonic with respect to the environment and the energetic cost of osmoregulation would be lower than in other higher (38 ppt) or lower (6 ppt) salinities, saving energy for other physiological processes (such as growth) (Brett, 1979; Brett and Groves, 1979; Kirschner, 1995; Boeuf and Payan, 2001). The higher growth observed in gilthead sea bream acclimated to 12 ppt with respect to 6 ppt or 38 ppt agrees with this hypothesis. In other sparids the best growth is also observed between 12–15 ppt (golden-line sea bream *Sparus sarba*:Woo and Kelly, 1995; black sea bream *Mylio macrocephalus*: Kelly et al., 1999). Our results also agree with those observed in other species such as the grey mullet *Mugil cephalus* (De Silva and Perera, 1976), different species of tilapia (*Oreochromis mossambicus*: De Silva and Perera, 1985; *O. niloticus*: Woo et al., 1997), cod *Gadus morhua* (Lambert et al., 1994) and turbot *Scopthalmus maximus* (Gaumet et al., 1995; Imsland et al., 2001) where the best growth occurred at 10–15 ppt environmental

salinity. However, in other studies this stimulatory effect of intermediate salinity on growth was not demonstrated (Shaw et al., 1975; McCormick et al., 1989; for more references see Boeuf and Payan, 2001).

Gilthead sea bream is a teleost capable of adapting to environments of different salinities (Chervinski, 1984; Mancera et al., 1993). Klaoudatos and Conides (1996) analyzed the influence of salinity (8, 18, 28 and 38 ppt) on growth of fry gilthead sea bream (1.9 initial body weight) reporting high mortality rates in fish acclimated to low salinity (8 ppt), and observing that the optimal salinity for growth was 28 ppt. However, in our experiment, we have not observed any kind of mortality in the LSW-acclimated fish (20 g initial body weigth), and our results showed that the best salinity for growth was 12 ppt. Differences in body size and the range of salinities tested would explain these discrepancies between both experiments. Further studies, using salinities between 12 and 38 ppt, could be necessary to determine the optimal salinity for growth in juvenile gilthead sea bream. Thus, according to the results of Klaoudatos and Conides (1996), it will be interesting to test environmental salinities of 18 and 28 ppt.

The osmoregulatory system of gilthead sea bream acclimated to different environmental salinities has been previoulsy analyzed by our group (Mancera et al., 1993, 1994, 2002; Sangiao-Alvarellos et al., 2003). The results obtained in the present experiment agree with those previously reported for this species. Thus, there are no significant differences in osmoregulatory parameters in plasma between SW-and BW-acclimated fish indicating that gilthead sea bream did not experience osmoregulatory difficulties. However, LSW-acclimated fish presented lower osmolality and ion levels suggesting that difficulties arise to maintain homeostasis in this low salinity. Similar findings have been observed in other euryhaline fish (Nordlie, 1985; Sampaio and Bianchini, 2002). It has been suggested that this response could be a symptom of a bad adaptation to this low salinity (Franklin et al., 1992) indicating that fish are subjected to a stressing salinity condition (Morgan and Iwama, 1998). In this way, the negative effect of stress conditions on growth is well known (Pickering, 1993; Wendelaar Bonga, 1997). However, plasma glucose levels, a classic marker of stress, did not show any increase in LSW-acclimated fish with respect to SW- or BW-acclimated fish. The pattern of gill Na^+, K^+-ATPase showed a "U-shape" with the lower activity being observed in fish acclimated to intermediate salinity. This result agrees with those observed previously for gilthead sea bream (Laiz-Carrión, Guerreiro, Fuentes, and Mancera, unpublished data) and other teleosts (see Jensen et al., 1998). However, in other teleosts gill Na^+, K^+-ATPase presented a direct relationship with respect to environmental salinity (see MCormick, 1995; Jensen et al., 1998; Marshall, 2002). Gill Na^+, K^+-ATPase activity is related to the processes of ion excretion and ion uptake across the gills (Perry, 1997; McCormick, 2001; Marshall, 2002), which are energy demanding. In this way, any reduction in gill Na^+, K^+-ATPase activity could save energy for other processes (see Boeuf and Payan, 2001).

In the present study, after 3 months, growth was 7% and 19% higher in BW- than in SW- and LSW-acclimated fish, respectively. In terms of the influence of salinity on the growing capacities numerous studies are available, and many fish species have been tested, and almost always the level of salinity influences growth. Most of the results are also dependent on the water temperature, but in any case, salinity clearly influences growth in fish (Brett, 1979; Brett and Groves, 1979; Sumpter, 1992; Boeuf and Payan, 2001). As for the growth rates, we have observed that the SGR was higher in fish acclimated to intermediate salinity (12>38>6 ppt.). Thus, our results agree with those previously described by other authors in marine species such as cod *G. morhua* or turbot *S. maximu*s, where growth rate was significantly higher in intermediate salinities around 12–19 ppt (Lambert et al., 1994; Gaumet et al., 1995; Dutil et al., 1997; Imsland et al., 2001). In our study, we do not have information about the exact amount of food eaten by fish in each salinity. It is possible that the better growth at intermediate salinity could be due only to a stimulation of food intake by environmental salinity as it has been demonstrated in other teleosts (see Le Bail and Boeuf, 1997; Boeuf and Payan, 2001). Some studies on the effects of salinity on growth and metabolic rates appear to support the hypothesis that the energy costs of osmoregulation are lowest in isotonic medium and that the energy savings are sufficient to result in increased growth relative to that seen in both hypo and hyperosmotic media (Arnesen et al., 1993;

Swanson, 1998; Boeuf and Payan, 2001). However, other authors (Morgan and Iwama, 1991) said that metabolic rates were inversely correlated with growth rates. Variations in growth rate bring about changes in the biochemical composition of fish tissues. Thus, theoretically, the higher growth rates observed in BW-acclimated fish should lead to changes in the energy metabolism of several tissues. The results obtained in plasma displayed no major differences when comparing metabolite levels of fish grown in the different salinities assessed. Accordingly, the higher growth rates in BW-acclimated fish did not appear to affect plasma levels of metabolites. The lower glucose levels observed in plasma of the same species after 14 days of acclimation to BW compared with SW (Sangiao-Alvarellos et al., 2003) disappeared in the present study performed after 100 days of acclimation, which can be attributed to the different sampling times used, possibly reflecting a new steady state for energy metabolism due to long-term acclimation to salinity.

The liver is the main site of ammoniogenesis, fatty acid synthesis, and gluconeogenesis and because of all these functions the liver is necessarily involved in the direct regulation of fish growth (Peragón et al., 1998). Cods with a higher growth rate are known to increase their capacity for hepatic gluconeogenesis from blood lactate (Couture et al., 1998). In the present study, no differences were observed in liver FBPase activity of sea bream acclimated to different salinities in spite of the different growth rates registered among fish not supporting the idea of enhanced liver gluconeogenesis in BW-acclimated fish. The glycolytic potential of gilthead seabream in liver also did not display significant differences among salinities. This is in contrast to expected considering that fish with higher growth rates have also been reported to follow the growth in mass in fish kept in lab including aerobic and glycolytic capacities of muscle, liver, and intestine (Couture et al., 1998). However, considering that after 14 days of acclimation glycolytic potential of gilthead sea bream liver was lower in BW- than in SW-acclimated fish (Sangiao-Alvarellos et al., 2003) it seems that an increase has occurred with time in this potential resulting that after 100 days levels were comparable to those of SW-acclimated fish in agreement with similar increases already reported in literature (Nakano et al., 1997; Kelly et al., 1999). The remaining parameters assessed in liver do not support the existence of major changes in energy metabolism of this tissue that could be comparable with different growth rates among fish either for glycogenolytic potential, or the capacity of the pentose phosphate pathway. The only significant difference registered was the lower levels of ATP noticed in livers of BW- and LSW-acclimated fish compared with those of SW-acclimated fish. Since ATP levels result from the equilibrium of several biochemical processes is difficult to elucidate one responsible for such a decrease. However, when this species was studied after 14 days of acclimation the situation was the converse, i.e. higher ATP levels in fish acclimated to BW compared with those in SW (Sangiao-Alvarellos et al., 2003) suggesting a reduced ATP consumption in liver with time. Considering the lack of changes in the main metabolites and enzyme activities of carbohydrate metabolism that drop may suggest that a lower ATP production occurs in livers of fish displaying the higher growth rates, i.e. those acclimated to BW.

In gills, the lower ATPase activity displayed by BW-acclimated fish may suggest a lower energy cost for osmoregulation at that salinity. This hypothesis agrees with results obtained for glycogen levels that increased in BW-acclimated fish compared with those in the other two salinities reflecting that a higher portion of energy is accumulated in the form of glycogen in this tissue. Moreover, the increased ATP levels observed in this tissue in BW-acclimated fish also reinforce the idea of a reduced ATP use in that salinity in gills in agreement with the general model of reduced energy consumption under isotonic salinity (Boeuf and Payan, 2001). The higher glycolytic capacity displayed by BW-acclimated fish indicate this pathway as responsible of the enhanced ATP production and agree with the higher glycolytic capacity already observed in fish with higher growth rates (Couture et al., 1998). However, it should be also considered that after 14 days of acclimation in the same species, fish in BW displayed a lower not higher glycolytic potential suggesting that glycolytic capacity of gill tissue performs differently under long-term acclimation to salinity. In this way, Morgan and Iwama (1999) also suggested different energy requirements of gill tissue in cutthroat trout when comparing short- and long-term periods of acclimation to salinity.

We included brain in this study since this tissue, contrary to muscle and liver, is not involved in accumulating energy reserves for the whole body (Soengas and Aldegunde, 2002), and hence has a high potential to show growth-related variations in composition that are independent of the energetic status of the fish. In another study performed in cod protein content of the brain did not change with growth rate, and the activities of indicators of aerobic capacity (citrate synthase and cytochrome C-oxidase) increased in the brain of fish with increasing growth in length but not with increasing growth in mass (Couture et al., 1998). In the present study fish displaying the higher growth rates also possessed higher glycolytic and glycogenolytic capacities in brain. A similar higher potential in BW for both pathways was noticed in fish acclimated for 14 days compared with those in SW (Sangiao-Alvarellos et al., 2003). Considering that, in contrast to brain, most of the differences observed after 14 days of acclimation disappeared in liver and gills. This is suggesting that brain effectively behaves in a way different to that shown by liver and gills not reflecting a lower energy demand in fish acclimated to 12 ppt. This enhanced the energy requirement of brain in fish acclimated to BW suggest that certain brain functions become more activated under those salinity conditions.

The remarkable low amount of metabolic changes observed in the tissues assessed should be taken with caution considering that the present study was a long-term one, and the important metabolic changes associated with osmotic acclimation occur in the first weeks of acclimation (Sangiao-Alvarellos et al., 2003). On the other hand, the different growth rates of fish could be reflected in the energy metabolism of other tissues like white muscle or in the metabolization of other fuels like lipids not considered in the present study.

In conclusion, our results indicate a better growth over a period of 100 days of juvenile gilthead sea bream (20 g initial body weight) acclimated to 12 ppt with respect to those acclimated to 6 or 38 ppt. This result could be attributed following our initial hypothesis to a lower standard metabolic rate at intermediate salinity. However since very few metabolic changes were observed when comparing gilthead sea bream displaying the higher growth rates (BW) with the others, our hypothesis is difficult to sustain and other possibilities should be considered. These include i) a stimulation of food intake by environmental salinity as it has been demonstrated in other teleosts (see Le Bail and Boeuf, 1997); ii) a metabolic reorganization at intermediate salinity implying a modified use of alternative fuels saving energy that could be invested in promoting growth (Sangiao-Alvarellos et al., 2003), and iii) a stimulation of different osmoregulatory hormones (growth hormone, cortisol, etc), which are also related to growth in teleosts (McCormick, 2001) including gilthead sea bream (Mancera et al., 1994, 2002). Future studies are necessary to elucidate these possibilities in the gilthead sea bream *Sparus aurata*.

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