JOURNAL OF EXPERIMENTAL ZOOLOGY 303A:563–576 (2005)

Branchial Osmoregulatory Response to Salinity in the Gilthead Sea Bream, *Sparus auratus*

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ABSTRACT The branchial osmoregulatory response of gilthead sea bream (*Sparus auratus* L.) to short-term (2–192 hr) and long-term (2 weeks) exposure to different environmental salinities (5‰, 15‰, 25‰, 38‰ and 60‰) was investigated. A ''U-shaped'' relationship was observed between environmental salinity and gill Na^+,K^+-ATPase activity in both long- and short-term exposure to altered salinity, with the increase in activity occurring between 24 and 96 hr after the onset of exposure. Plasma osmolality and plasma ions (sodium, chloride, calcium and potassium) showed a tendency to increase in parallel with salinity. These variables only differed significantly ($P < 0.05$) in fish adapted to 60‰ salinity with respect to fish adapted to full-strength sea-water (SW). Plasma glucose remained unchanged whereas plasma lactate was elevated at 5‰ and 60‰. Muscle water content (MWC) was significantly lower in fish adapted to 60‰. Chloride cells (CC) were only present on the surface of the gill filaments and absent from the secondary lamellae. CC distribution was not altered by external salinity. However, the number and size of CC were significantly increased at salinity extremes (5‰ and 60‰), whereas fish exposed to intermediate salinities (15‰ and 25‰) had fewer and smaller cells. Furthermore, the CC of fish exposed to diluted SW became rounder whereas they were more elongated in fish in full-strength and hypersaline SW. This is consistent with previous reports indicating the existence of two CC types in euryhaline fish. At likely environmental salinities, gilthead sea bream show minor changes in plasma variables and the effective regulation of gill Na^+,K^+-ATPase. However, at very low salinities both haemodilution and up-regulation of gill Na^+,K^+-ATPase predict a poor adaptation most likely related to deficiency or absence of specific components of the CC important for ion uptake. *J. Exp. Zool. 303A:563–576*, 2005.

INTRODUCTION

Euryhaline teleosts have the ability to adapt to different environmental salinities while maintaining essentially constant their internal milieu by the activation of several osmoregulatory mechanisms, namely in the branchial and renal epithelia. In this adaptive process, two consecutive phases occur: an initial period characterized by changing osmotic variables, followed by a chronic regulatory period, when these variables reach a new homeostasis (Holmes and Donaldson, '69; Maetz, '74).

The modulation of Na^+,K^+-ATPase pump activity of chloride cells (CC) in the branchial epithelia is essential for acclimation to a new environmental salinity (Epstein et al., '80; Zadunaisky, '84; McCormick, '95; Marshall, 2002). The alterations in gill Na^+,K^+-ATPase activity in relation to environmental salinity are diverse, but two typical situations seem to prevail: (i) a direct relationship, characteristic of anadromous species, in which higher salinities induce higher values of gill Na^+, K^+-ATPase activity (McCormick, '95) and (ii) a

Grant sponsors: This work was supported by grants BOS2001-4031-C02-01 (Ministerio de Ciencia y Tecnología, Spain) and PTR95-0431-OP (DGES, Ministerio de Educación y Ciencia, Madrid, Spain) to J.M.M. Travelling between labs was partially funded by grants DGES HP1999-0098 and HP2001-0061 (Ministerio de Ciencia y Tecnología) from integrated actions to J.M.M. and CRUP, Portugal, to J.F. R.L.-C. was in receipt of a MIT-F2 pre-doctoral fellowship from Ministerio de Ciencia y Tecnología, Spain. P.M.G. was in receipt of fellowship PRAXIS/BD/9207/96 from Fundação para a Ciência e Tecnologia (Portugal) and visited the University of Cadiz in the frame of the EU Socrates Exchange Programme.

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Received 7 October 2004; Accepted 21 February 2005
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jez.a.183.

U-shaped relationship, described for some euryhaline teleosts (Towle et al., '77; Gallis et al., '79; Jensen et al., '98), in which lower values of gill Na^+,K^+-ATPase activity occur at intermediate salinities and higher values at low and high salinities. Changes in gill Na^+,K^+-ATPase activity are observed 2–3 days after transfer from a hypoosmotic to hyperosmotic environment in euryhaline teleosts (*Anguilla rostrata*: Forrest et al., '73; *Dormitator maculatus*: Evans and Mallery, '75; *Fundulus heteroclitus*: Jacob and Taylor, '83; *Dicentrarchus labrax*: Jensen et al., '98). In anadromous species (*Oncorhynchus kisutch*: Boeuf et al., '78; *Salvelinus fontinalis*: McCormick and Naiman, '85; *Salmo gairdneri*: Madsen and Naamansen, '89; *Salmo salar*: Berge et al., '95), activation of gill Na^+,K^+-ATPase takes place 3–7 days after transfer to seawater (SW). The delay in gill Na^+,K^+-ATPase activation in response to osmotic challenge is proposed to reflect changing gene expression. Thus modifications in environmental salinity alter not only the activity of Na^+,K^+-ATPase but also transcript expression and protein synthesis (Lee et al., 2000; Seidelin et al., 2000; Tipsmark et al., 2002). However, in addition to this slow activation, a rapid, non-genomic activation of gill Na^+,K^+-ATPase pump has been reported, involving phosphorylation and/or membrane insertion of the protein (Hwang et al., '89; Uchida and Kaneko, '96; Mancera and McCormick, 2000; Tipsmark and Madsen, 2001).

Changes in Na^+,K^+-ATPase pump activity during salinity adaptation in most fish are paralleled by alteration in the number and size of branchial CC, the site at which most of the branchial ionic regulation takes place (Perry, '97; Marshall and Bryson, '98; Marshall, 2002). Slow salinity adaptation usually involves the biogenesis or reshaping of existing CC, which undergo important changes in fish exposed to salinity variations (Pisam and Rambourg, '91; Sakamoto et al., 2001; Varsamos et al., 2002; Wilson and Laurent, 2002).

The gilthead sea bream (*Sparus auratus* L.) is a marine teleost living in coastal waters, capable of adapting to considerable changes in environmental salinity (Chervinski, '84; Mancera et al., '93a). Previous studies with this species showed that a decrease in environmental salinity (from 38‰ to 7‰) activates the prolactin, growth hormone and corticotrophic cells in the adenohypophysis (Mancera et al., '93b, '95) and that the transfer from seawater (SW) to brackish water (BW) leads to transitory blood hypomineralization (Mancera et al., '93a). Additionally, it was observed that prolactin and cortisol increase the Na^+,K^+-ATPase activity and blood osmolality in BW exposed gilthead sea bream, thus improving its hypoosmoregulatory capacity (Mancera et al., '94, 2002; Laiz-Carrión et al., 2003). The distribution, density and morphology of CC in response to the salinity challenge remain unknown in the marine gilthead sea bream. Most studies have focused on SW adaptation of fish previously kept in freshwater (FW) (Marshall et al., '99; Wong and Chan, '99; Lee et al., 2000; Tipsmark et al., 2002), and the response to acclimation to hypo- and hyperosmotic environments has received little attention.

Therefore, the aim of the present study was to describe and analyse the alterations and the compensatory mechanisms occurring in the branchial osmoregulatory system of juvenile gilthead sea bream exposed and acclimated to a wide range of environmental salinities, and to determine whether the adaptation in this species is an immediate phenomenon or a slow, long-lasting gradual process.

MATERIALS AND METHODS

Experimental protocol

Immature gilthead sea bream (40–60 g body weight) were provided by a commercial fish farm (CUPIMAR SA, San Fernando, Cádiz, Spain). Fish were transferred to the wet laboratories at the Faculty of Marine Sciences (Puerto Real, Cádiz), where they were acclimated for 30 days to full SW (38‰, 1162 mOsm kg^{-1} H_2O) in 300-l tanks in an open system. After this period, fish were used for experiments to assess how they adapted to altered salinities. The experimental salinities were achieved either by mixing full-strength SW with dechlorinated tap water or by mixing full-strength SW with natural marine salt (Instant Ocean,

TABLE 1. Osmolality and ionic compositon of the water at different salinities used in the experiments

	Salinity				
	5‰	15‰	25‰	38‰	60‰
Osmolality (mOsm)	130	366	613	1162	1494
Na^+ (mmol l^{-1})	55	185	304	468	734
Cl^- (mmol l^{-1})	71	210	353	534	821
Ca^{2+} (mmol l^{-1})	1.8	4.3	7.3	11.1	16.9
K^+ (mmol l^{-1})	1.6	4.3	6.5	10.8	16.6
Mg^{2+} (mmol l^{-1})	7.7	24.3	38.9	61.6	93.4

Aquarium Systems, Sarrebourg, France). The osmolality and ionic composition of the water used for the different experimental groups are shown in Table 1. During the experiments the fish were maintained under natural photoperiod and constant temperature (18°C). Fish were fed once daily with 1% body weight commercial dry pellets (Dibaq-Diprotg SA, Segovia, Spain) and were fasted for 24 hr before sampling.

Trial 1—long-term exposure

Four different experimental salinities were tested and all experiments were conducted in duplicate tanks (100-l capacity) (7–8 fish per tank). After an initial 7-day acclimation period in SW, fish were exposed to gradually changing salinity over 2 hr until it reached 5‰ (130 mOsm kg^{-1} H_2O), 15‰ (366 mOsm kg^{-1} H_2O), 25‰ (613 mOsm kg^{-1} H_2O) or 60‰ (1494 mOsm kg^{-1} H_2O), while another group (SW control) was kept at 38‰ (1162 mOsm kg^{-1} H_2O). Fish were exposed to a specific environmental salinity for 2 weeks by recirculating tank water. At the end of the experiment, fish were anaesthetized in 2-phenoxyethanol (1 ml l^{-1} water, Sigma-Aldrich, Madrid, Spain) and samples were collected (see below).

Trial 2—short-term exposure

Fish were transferred directly from SW (38‰) to tanks containing water at 5‰, 15‰, 38‰ and 60‰ (n=7–8 per tank). Five tanks were used per salinity and were sampled consecutively at 2, 4, 24, 96 and 192 hr after transfer. At each time point all the animals from one tank per salinity were anaesthetized in 2-phenoxyethanol (1 ml l^{-1} water), and a blood sample and a biopsy of gill tissue were collected. Blood samples collected from fish previously withdrawn directly from the stocking tank were considered as representative for time zero.

During both trials the water salinity was checked daily and corrected when necessary by the addition of small volumes of either FW or SW. At the beginning and during the experiments, water samples were collected for ion composition determination. The experiments described comply with the Guidelines of the European Union Council (86/609/EU) and of the University of Cadiz (Spain) for the use of laboratory animals. No mortality was observed during the experiments.

Sampling

Fish were anaesthetized with 2-phenoxyethanol (Sigma-Aldrich) (1 ml l^{-1} water), weighed and sampled. The blood was collected from the caudal peduncle into 1-ml ammonia-heparinized syringes. Plasma was separated from cells by centrifugation of whole blood (5 min at 10,000g) and was immediately frozen in liquid nitrogen and stored at −80°C until analysis. A biopsy of gill tissue was placed in 100 µl of ice-cold sucrose-EDTA-imidazole (SEI) buffer (150 mM sucrose, 10 mM EDTA, 50 mM imidazole, pH 7.3) and frozen at −80°C. A piece of paraxial muscle was dissected for gravimetrical determination of total water content.

Immunolocalization and morphology of CC

Gill arches were removed and placed in freshly mixed Champy-Maillet's fixative (0.2% osmium tetraoxide, 25 mg ml^{-1} iodine, and saturated metallic zinc) for 16 hr (Avella et al., '87). The tissue was rinsed with deionized water, dehydrated and embedded in paraffin. Sections (6 µm thick) cut parallel to the long axis of the filament were obtained. In addition, gill filaments were placed for 24 h in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C. Tissues were dehydrated, embedded in paraffin and sections (6 µm thick) parallel to the long axis of the filament were obtained. The sections were stained with haematoxylin–eosin for histochemistry. For immunocytochemistry, tissue sections were immunostained using the unlabelled enzyme method of Sternberger ('86) with the monoclonal α5 antiserum raised in mouse against the chicken NKA α1-subunit (Developmental Studies Hybridoma Bank, Department of Biological Science, University of Iowa, Iowa, USA). This antiserum is specific for a cytosolic epitope, and reacts with all isoforms of the α-subunit of distant species (see Takeyasu et al., '88). All sections were incubated for 18 hr at 22°C in the primary antiserum at a dilution of 1:500. The second antiserum (anti-mouse IgG, raised in goat, Sigma M6898) was used at a dilution of 1:40 for 60 min at 22°C and the PAP complex (1:100) (peroxidase antiperoxidase soluble complex mouse, Sigma P2416) was used for 45 min at 22°C. The chromogen was 3.3′-diaminobenzidine tetrahydrochloride (DAB, Sigma). Antisera and the PAP complex were diluted in Tris buffer, pH 7.8, containing 0.7% non-gelling seaweed gelatin, lambda carrageenan (Sigma),

0.5% Triton X-100 (Sigma) and 0.02% sodium azide. Coplin jars were used for incubation of sections in the first and second antisera, whereas PAP incubation was carried out in a moist chamber. To test the specificity of the immunoreaction, adjacent sections were processed as described above, but incubation in the primera antisera was omitted.

Immunostained CC were analysed using a Leitz Fluovert inverted microscope with a CCD Kapa CF15/2 video camera. The size of cell bodies was measured using the image processor VIDS V program (AMD, Analitical Measuring Systems). The number of positively stained cells was expressed as cells per millimetre of filament (frequency; in both sides ventral and dorsal of a primary filament) measured in 5 transects per slide, 4 slides per fish and 6 fish per salinity. For CC size and shape, results are the average $\pm$SEM of at least 50 cells per slide, 4 slides per fish and 6 fish per salinity. Shape factor is defined as $4\pi A/P^2$ (where A is the area and P is the perimeter) such that values approaching 1 indicate a near-circular shape and lower values indicate a more elongated shape. The fish from which slides containing stained CC were prepared was not disclosed during the observations to avoid bias.

Gill Na^+,K^+-ATPase activity

Gill Na^+,K^+-ATPase activity was determined using the microassay method of McCormick ('93) adapted to gilthead sea bream (Mancera et al., 2002). Gill tissue was homogenized in 125 µl of SEI buffer with 0.1% deoxycholic acid and then centrifuged at 2,000*g* for 30 sec. Duplicate 10 µl homogenate samples were added to 200 µl assay mixture in the presence or absence of 0.5 mmol l^{-1} ouabain in 96-well microplates at 25°C and read at 340 nm for 10 min with intermittent stirring by vibration. Ouabain-sensitive ATPase activity was detected by enzymatic coupling of ATP dephosphorylation to NADH oxidation and expressed as µmol ADP mg protein^{-1} hr^{-1}. The Pierce BCA Protein kit (Pierce, Rockford, IL) was used with bovine albumin as standard. Both assays were run on a microplate reader (EL340i, Bio-Tek Instruments, Winooski, VT) using Delta Soft3 software for Macintosh (BioMetallics Inc., Princeton, NJ).

Blood chemistry

Plasma and water osmolality was measured with a vapour pressure osmometer (Fiske One-Ten Osmometer, Fiske, VT) and expressed as mOsm kg^{-1}. Plasma and water Na^+, K^+, Ca^{2+} and Mg^{2+} levels were measured using atomic absorption spectrophotometry (Philips PU7000) and Cl^- levels were measured with the Chloride Sigma-Aldrich kit (no. 461). Plasma glucose and lactate were measured using commercial kits from Sigma-Aldrich (Glucose HK #16-20UV; Lactate #735-10) adapted to microplates (Stein, '63; Iwama et al., '89). Plasma protein concentration was determined by diluting the plasma 1:40 and measuring protein concentration using the bicinchoninic acid method (Smith et al., '85) with a BCA protein kit (Pierce) for microplates, with bovine albumin as standard. The assays were read on a Bio Kinetics EL-340i Automated Microplate Reader (Bio-Tek Instruments) using DeltaSoft3 software for Macintosh (BioMetallics Inc.). Muscle water content (MWC) was determined gravimetically as percent weight loss after drying to a constant weight at 100°C over 2 days.

Cortisol assay

Plasma levels of cortisol were determined by radioimmunoassay (RIA) as described previously (Rotllant el al., 2005). Briefly, plasma samples were diluted in phosphate buffer containing 0.5 g l^{-1} gelatine, pH 7.6, and denatured at 70°C for 30 min. The antiserum used for the assay was raised in rabbits against cortisol-3-(O-carboxymethyl)oxime–bovine serum albumin conjugate (Sigma-Aldrich). This antiserum cross-reacts 100% with cortisol, 54% with 11-desoxycortisol, 10% with cortisone, 16% with 17,21-dihydroxy-5β-pregnan-3,11,20-trione, 5% with 11*b*,17,21-trihydroxy-5β-pregnan-3,20-dione, 0.05% with 11-hydroxytestosterone and <0.001% with testosterone. Although cross-reactivity exists with 11-desoxycortisol and cortisone (as observed when validating the assay), we have found that these substances are virtually non-existent in sea bream plasma: binding of the antisera to thin layer chromatography fractioned fish plasma confirmed the specificity of the assay by revealing a single peak in the cortisol-specific fraction and none in the region of the other compounds.

Statistics

Significant differences among groups were tested by one-way ANOVA, followed by the Student–Newman–Keuls multiple comparison test. For the study of gill density, size and shape, the effect of treatments was tested by a hierarchical (nested) ANOVA. The levels of variability

were the fish in each treatment ($n=6$) and the slides from each fish ($n=4$). Because no significant differences were found in the experimental units (fish and slides within treatments), one-way ANOVA was applied, using the pooled data of fish for each salinity (Zar, '84; Underwood, '97). Post hoc recommendation of Underwood ('97) before the use of pooled data was followed to reduce the risk of type I or II errors, increasing the power of the ANOVA test because of the large number of degrees of freedom associated with the pooled mean-square (Underwood '97). Results were considered significantly different at $P<0.05$.

RESULTS

Trial 1—long-term exposure

Substantial changes in plasma osmolality were observed after 2 weeks of exposure to altered external salinity (Fig. 1A). This parameter increased significantly from fish adapted at 5‰ to fish adapted at 60‰. Interestingly, the effect of environmental salinity on the activity of gill Na^+,K^+-ATPase after 2 weeks of exposure (Fig. 1B) was U-shaped and fish adapted to extremes of salinity (high and low) had significantly higher ($P<0.05$) gill Na^+,K^+-ATPase activity than fish exposed to other salinities. In fact, fish maintained in 60‰ had more than double the gill Na^+,K^+-ATPase than fish maintained in 15‰, 25‰ and 38‰.

Plasma concentrations of ions (sodium, chloride, calcium and potassium) increased in parallel with salinity, and were significantly higher in 60‰ acclimated fish than in fish adapted to all other salinities (Table 2). In contrast, MWC was significantly lower in 60‰ adapted fish than in fish adapted to lower salinities.

Table 3 shows the levels of plasma metabolites measured in fish adapted to different salinities. Plasma glucose levels were not significantly different among fish maintained at different salinities. However, plasma lactate levels were significantly higher ($P<0.05$) in fish adapted to

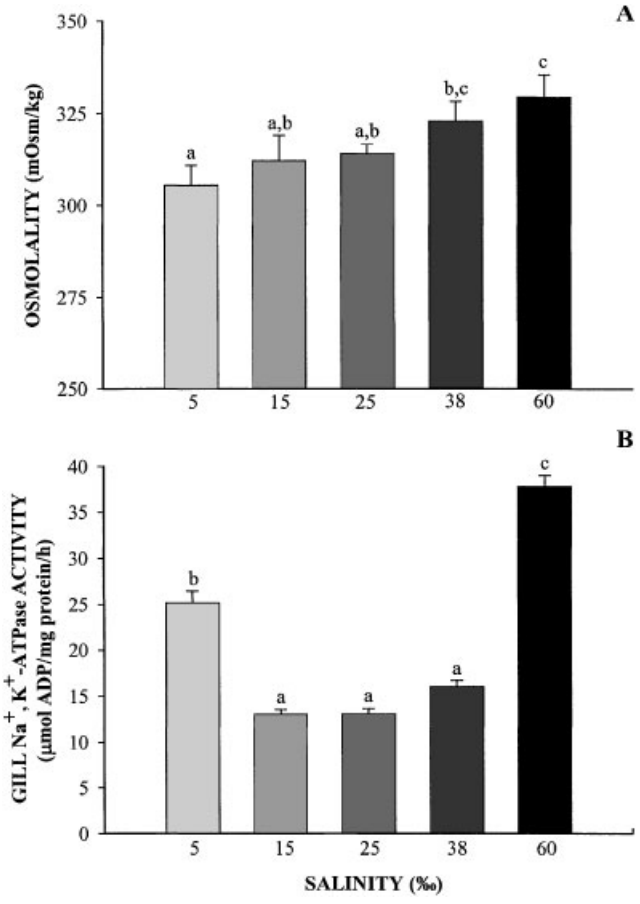

Fig. 1. Effect of different environmental salinities on osmolality (**A**) and gill Na^+,K^+-ATPase activity (**B**). Fish were acclimated to different salinities for 2 weeks. Data are expressed as mean±SEM ($n=7$–8). The letters indicate significant differences among groups ($P<0.05$).

TABLE 2 Plasma ion levels and muscle water content (MWC) in fish acclimated to different environmental salinities for 2 weeks

	Salinity				
	5‰	15‰	25‰	38‰	60‰
Na^+ (mmol l^{-1})	170 ± 3^a	$178\pm2^{a,b}$	$180\pm2^{b,c}$	$185\pm3^{b,c}$	196 ± 4^c
Cl^- (mmol l^{-1})	145 ± 3^a	148 ± 2^a	149 ± 1^a	152 ± 3^a	158 ± 2^b
Ca^{2+} (mmol l^{-1})	2.22 ± 0.21^a	2.35 ± 0.20^a	2.37 ± 0.19^a	2.51 ± 0.18^a	2.73 ± 0.23^b
K^+ (mmol l^{-1})	4.6 ± 0.4^a	4.8 ± 0.3^a	5.0 ± 0.2^a	5.1 ± 0.3^a	5.3 ± 0.3^b
MWC%	78.2 ± 0.6^a	77.4 ± 1.1^a	77.5 ± 0.5^a	76.9 ± 1.0^a	75.6 ± 1.1^b

[a]Data are shown as mean ±SEM ($n=7$–8).
[b]Groups that are significantly different ($P<0.05$) are indicated by a different letter.
[c]The same letter indicates that no significant differences exist.

TABLE 3. Plasma levels of glucose, lactate and proteins in fish acclimated to different environmental salinities for 2 weeks

	Salinity				
	5‰	15‰	25‰	38‰	60‰
Glucose (mmol l^{-1})	3.76 ± 0.29^{a}	3.18 ± 0.21^{a}	3.31 ± 0.19^{a}	3.66 ± 0.24^{a}	4.06 ± 0.30^{a}
Lactate (mmol l^{-1})	0.96 ± 0.10^{a}	0.58 ± 0.04^{b}	0.58 ± 0.03^{b}	$0.70 \pm 0.04^{b,c}$	$0.81 \pm 0.05^{a,c}$
Protein (mmol l^{-1})	32.2 ± 4.3^{a}	35.4 ± 4.1^{a}	37.5 ± 3.5^{a}	37.5 ± 4.3^{a}	40.3 ± 3.3^{b}

[a]Data are shown as mean ±SEM (n=7–8).
[b]Groups that are significantly different ($P<0.05$) are indicated by a different letter.
[c]The same letter indicates that no significant differences exist.

5‰ and 60‰ than in those maintained at 15‰, 25‰ and 38‰. The highest concentrations of plasma lactate were measured in fish maintained at the lowest salinity (5‰). Plasma protein levels increased in parallel with ambient salinity, and significant differences ($P<0.05$) were only observed between fish adapted to 60‰ and all other groups of fish adapted to lower salinities.

Trial 2—short-term exposure

At 2 hr after transfer, the plasma osmolality was slightly higher in fish transferred to 60‰ than in those maintained at 38‰ (although not statistically different from the values from undisturbed fish, 312 ± 3.2 mOsm kg^{-1}), whereas those transferred to lower salinities (5‰ and 15‰) had significantly lower plasma osmolality levels (Fig. 2A). Values of plasma osmolality between the 5‰ and the 60‰ groups were significantly different 2 hr after transfer and thereafter. There was a clear relationship between plasma osmolality and external salinity after 8 days of exposure, and in fish maintained at 5‰, 15‰ and 60‰, plasma osmolality averaged 83‰, 89‰ and 109% of SW fish, respectively. In all groups, 4 hr after transfer plasma osmolality increased slightly over that measured 2 hr after transfer and had returned to pre-transfer levels within 24 hr. Interestingly, the range of plasma osmolality values of the fish assayed throughout trial 2 was greater than that measured at the end of trial 1.

Gill Na^+,K^+-ATPase activity of gilthead sea bream increased significantly between 24 and 96 hr after transfer from 38‰ to 5‰ or 60‰ and remained elevated at 192 hr (Fig. 2B). At this time point, the activity in the extreme salinity two groups was almost 2.5-fold higher than in the control group. Transfer to 15‰ did not cause significant modifications in gill Na^+,K^+-ATPase activity at any time point.

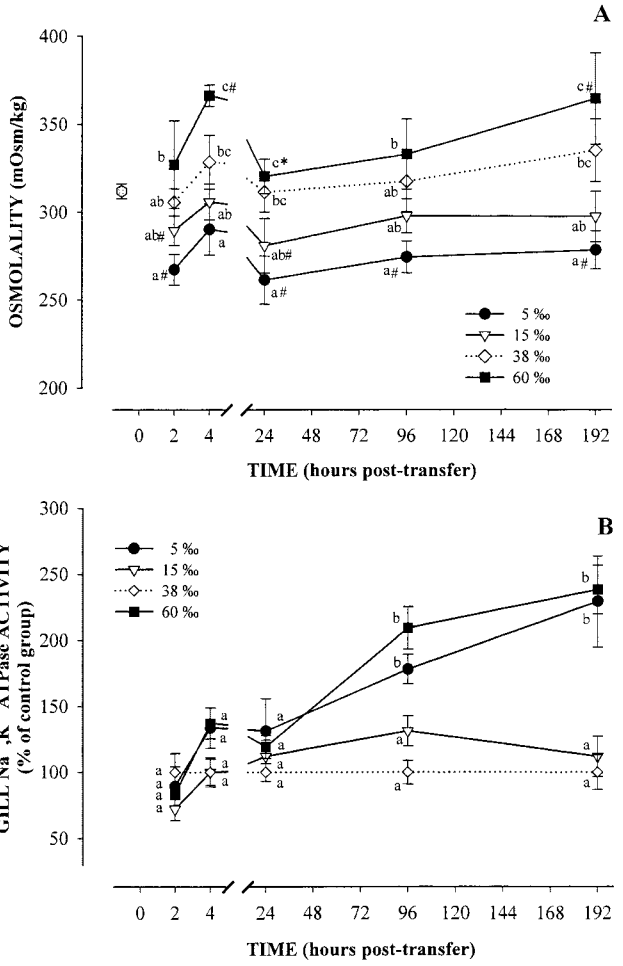

Fig. 2. Time-course effect of different environmental salinities in osmolality (**A**) and gill Na^+,K^+-ATPase activity (**B**) at 2, 4, 24, 96 and 192 hr after transfer. The grey dot before time zero indicates the osmolality value for undisturbed fish in the stocking tank. Na^+,K^+-ATPase activity data are shown as % of control (control=100%). Each value is the mean±SEM of n=7–8 fish per group in each sampling time. Different letters indicate significant differences ($P<0.05$) between groups within the same time point. ∗ indicates significant differences between the current and preceding time point. # indicates significant differences from undisturbed fish.

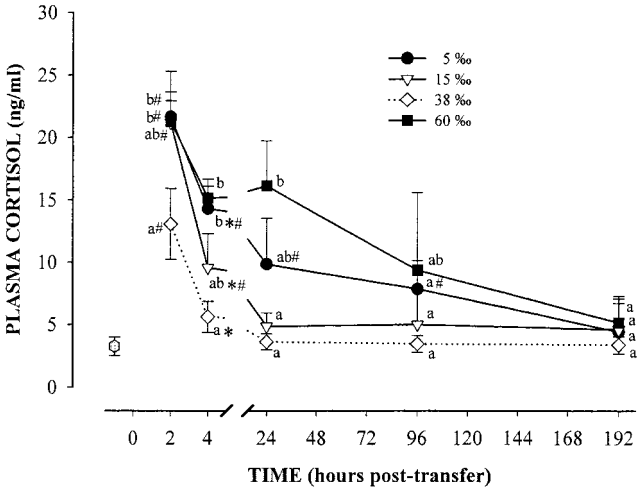

Fig. 3. Effects of different environmental salinities on plasma cortisol levels at 2, 4, 24, 96 and 192 hr after transfer to different salinities. The grey dot before time zero indicates the cortisol value for undisturbed fish in the stocking tank. Each value is the mean $\pm$ SEM of n=7–8 fish per group in each sampling time. Different letters indicate statistical differences ($P<0.05$) among groups within the same time point. * indicates significant differences between the current and preceding time point. # indicates significant differences from undisturbed fish.

Plasma cortisol levels were elevated at 2 hr after transfer in all groups (Fig. 3), well above the values measured for undisturbed sea bream before this experiment (3.3 ± 0.33 ng ml^{-1}) and in similar stocking conditions (Rotllant et al., 2001; Tort et al., 2001). At this time point, in the extreme salinity two groups, fish had higher cortisol levels (21.7 ± 1.99 and 21.3 ± 4.02 ng ml^{-1}, respectively) than fish at 38‰ salinity (13.1 ± 2.84 ng ml^{-1}), whereas fish at 15‰ had intermediate values close to the extreme groups (21.2 ± 1.78 ng ml^{-1}) but not statistically different from the control. At 4 hr after transfer cortisol levels had decreased significantly, and from 24 hr onwards these were constant in the intermediate salinities and similar to those of undisturbed animals. The restoration of normal cortisol levels was slower in fish at 5‰ and 60‰, in which values were only identical to those fish in the control after 192 hours post-transfer.

Branchial CC density and morphology

There was complete overlap in the results obtained with the two CC identification methods utilized, namely, immunolocalization with an antiserum against Na^+,K^+-ATPase and the osmium tetraoxide technique. CC were present only on the surface of the gill primary filament but never in the secondary lamellae, and this distribution was not affected by salinity challenge (Fig. 4).

Fish acclimated to SW (38‰) showed an average of 106.4 ± 1.73 CC mm^{-1} and adaptation to hypersaline SW (60‰) significantly increased the number of CC present in fish gills (Figs 4 and 5A). In fish exposed to intermediate salinities (15‰ and 25‰), a significant reduction in CC number relative to the SW group was observed. However, the CC frequency in fish acclimated to 5‰ was similar to that observed in the fish at 38‰. Nevertheless, at 5‰, CC cell size increased markedly and they were 1.3 times larger than those in control fish. CC in fish maintained at 60‰ were also larger than in control fish, whereas in fish exposed to 15‰ and 25‰ the area of the CC decreased significantly (Fig. 5B). In fish maintained in full-strength and hypersaline SW, CC had an oblate ellipsoid and contrasted with that of fish in diluted SW, which were more closely spheroidal (ratio of area to perimeter closer to 1; Fig. 5C).

DISCUSSION

This study shows that gilthead sea bream can adapt to a wide range of salinities with adjustment in body fluids osmolytes, plasma lactate and cortisol, which are more evident at the extreme range of salinities experienced in nature (5–60‰). The gill, a major osmoregulatory organ in fish, undergoes large morphological changes, even at intermediate salinities, but Na^+,K^+-ATPase only showed increased activity at the low and high extremes of salinity.

Time course of salinity adaptation

After an abrupt salinity transfer, an adaptive period involving changes in osmotic variables is expected (Holmes and Donaldson, '69; Maetz, '74; Goswami et al., '83). In our experiments (trial 2), the transfer from 38‰ to the different experimental salinities induced immediate (2 hr after transfer) changes in plasma osmolality that were significantly different from control in the extreme salinities throughout the duration of the experiment. Almost instant changes in plasma osmolality and/or electrolyte concentration after salinity transfer have also been described by Wood and Marshall ('94) and Marshall et al. ('99) in *F. heteroclitus*, by Kelly and Woo ('99) in *Sparus*

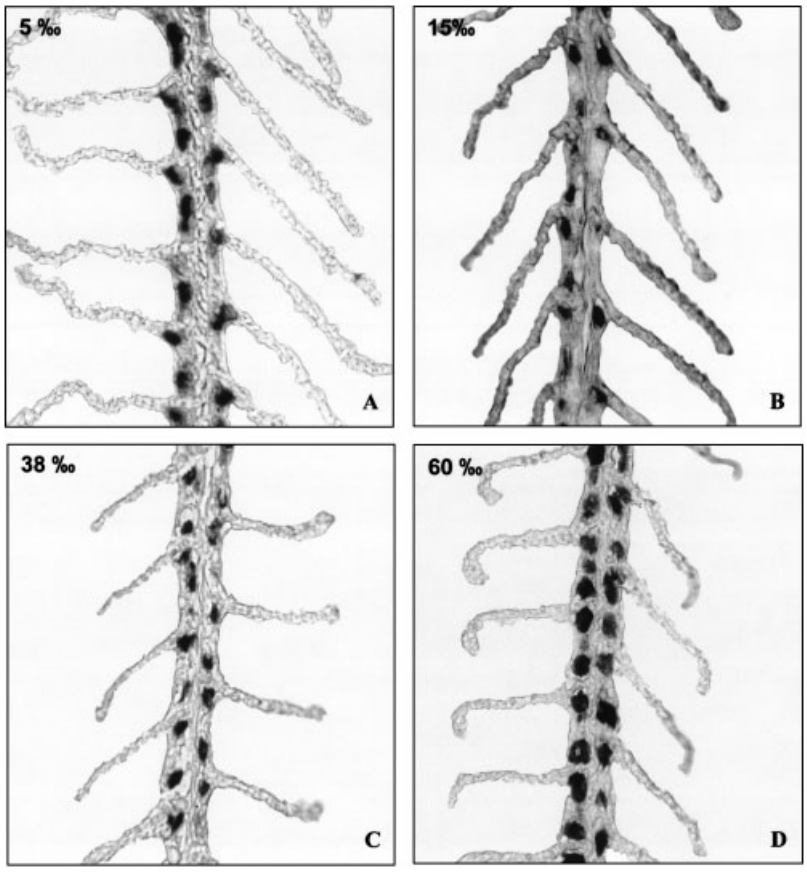

Fig. 4. Section through the gill showing the immunolocalization of chloride cells (CC) in sea bream juveniles adapted to (**A**) 5‰, (**B**) 15‰, (**C**) 38‰ and (**D**) 60‰ environmental salinities. 350 ×.

sarba and by Lin et al. (2004) in *Oreochromis mossambicus*.

Sustained elevation of plasma cortisol is known to induce CC proliferation and the increase of Na^+,K^+-ATPase activity (*O. mossambicus* opercular membrane, McCormick, '90, '95; Dang et al., 2000). In addition, cortisol injections significantly elevated gill Na^+,K^+-ATPase activity in gilthead sea bream in full-strength SW and BW (Mancera et al., 2002; Laiz-Carrión et al., 2003). However, the mechanisms underlying this effect are not universal, and in the marine *S. sarba* hypercortisolaemia did not affect the branchial osmoregulatory response (Deane et al., 2000).

The dynamics of plasma cortisol in our experiments were similar to those caused by handling stress in this species (Arends et al., '99; Rotllant et al., 2001). Cortisol levels peaked just after transfer and rapidly recovered to basal levels, suggesting that the transfer itself and not salinity was the main cause for the profile observed. It would be tempting to associate the short-term alteration in plasma osmolality (within 4 hr after transfer) with the increase in plasma cortisol, which may have increased the leakage of the gill epithelia as part of its SW-adapting effects (McCormick, '95). However, in fish adapted to 38‰, plasma osmolality remains unchanged despite the increase in plasma cortisol, as a good indication that in addition to handling stress other mechanisms are involved in short-term modifications of epithelial permeability. The reestablishment of normal circulating levels of cortisol was slower in fish exposed to 5‰ and 60‰ than in those at 15‰ and 38‰, indicating an effect of salinity, the relevance of which cannot be effectively determined.

In the present study a direct relationship between cortisol and either Na^+,K^+-ATPase activity or CC distribution could not be established because, after following an initial generalized increase in plasma cortisol subsequent to salinity transfer, all groups recovered rapidly to basal levels. This result was similar to that observed in

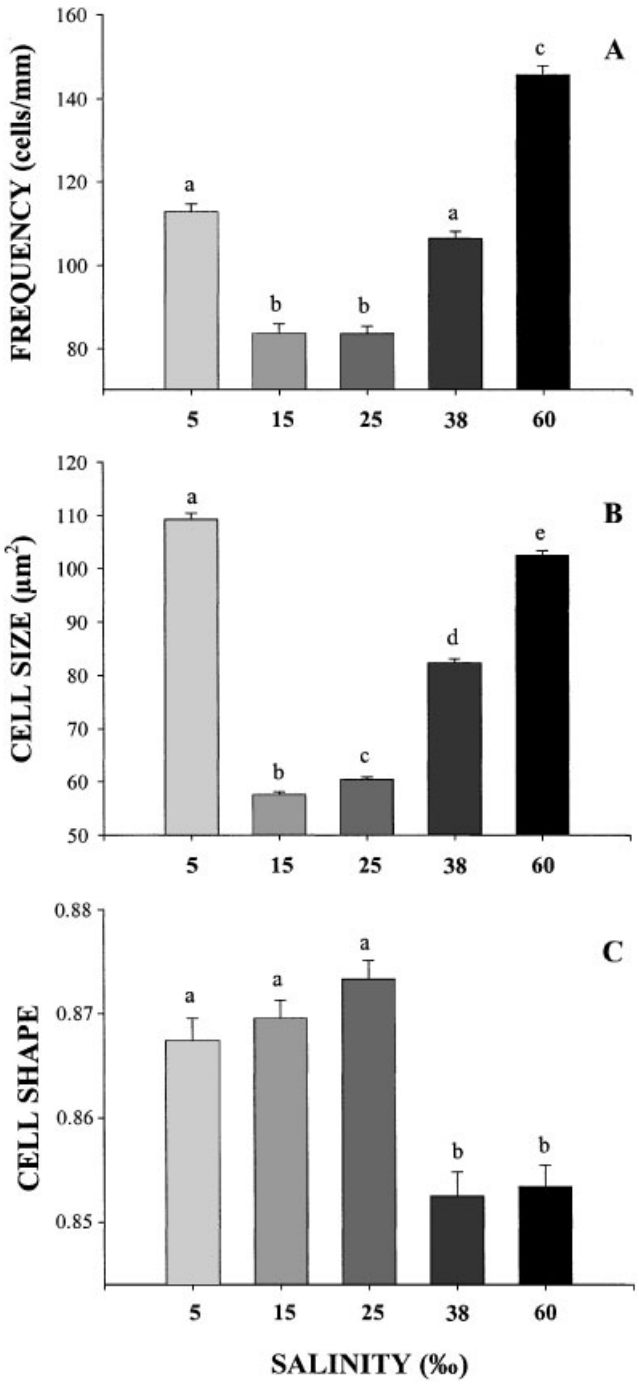

Fig. 5. Chloride cell (CC) distribution (**A**), size (**B**) and shape ($4\pi A/P2$) (**C**) in the gills of sea bream adapted to the different salinities for 2 weeks. Distribution was measured in 120 gill transects per treatment. For cell size and shape, results are the average $\pm$SEM of at least 50 cells per slide, 4 slides per fish and 6 fish per salinity. Cell shape index=1 corresponds to a perfect circumference. Different letters indicate statistical differences ($P<0.05$) among salinity groups.

S. sarba transferred to different environmental salinities (Kelly and Woo, '99), in which changes in CC distribution occurred only after the reestablishment of normal plasma cortisol. However, the possibility that the initial increase in cortisol can trigger indirect stimulation of Na^+,K^+-ATPase activity and proliferation of CC cannot be ruled out.

Pattern of gill Na^+,K^+-ATPase activity in salinity adapted fish

In gilthead sea bream the relationship between gill Na^+,K^+-ATPase activity and environmental salinity was ''U-shaped'', with the highest activities observed in hyper- and hyposaline water. In most anadromous species and other migratory fish, this relationship has been observed to be linear (McCormick, '95), but in other euryhaline species such as *F. heteroclitus* (Towle et al., '77), *Chelon labrosus* (Gallis et al., '79) and *D. labrax* (Jensen et al., '98) this relationship is also ''U-shaped''. This has been suggested to be a general characteristic of marine euryhaline species or euryhaline FW species living in intertidal waters with rapid and frequent changes in environmental salinity (Jensen et al., '98). However, it should be noted that gilthead sea bream never enter full-strength FW and the transfer to environments with very low salinity ($<$2–3 ppt) results in mortality within a few hours (Chervinski, '84; author's personal observation).

The typical ''U-shape'' salinity dependence of the gill Na^+,K^+-ATPase activity described after 14 days of acclimation was first observed at 96 hr after transfer, but the initial changes in gill Na^+,K^+-ATPase activity occurred between 24 and 96 hr after exposure to the new environmental conditions. This is in agreement with the time-course studies for most euryhaline fish, in which responses are delayed 2–3 days after the onset of the alterations in environmental salinity (Forrest et al., '73; Evans and Mallery, '75; Jacob and Taylor, '83; Jensen et al., '98). Quantitative expression of Na^+,K^+-ATPase mRNA or protein was not studied, but the interval between transfer and enzyme activity response is in good agreement with the processes of reshuffling of existing protein and/or the synthesis of novel protein described for other species (*D. labrax*: Jensen et al., '98; *F. heteroclitus*: Mancera and McCormick, 2000; *Salmo truta*: Seidelin et al., 2000; *Chanos chanos*: Lin et al., 2003). Indeed, expression of alpha and beta subunits of the

Na^+,K^+-ATPase mRNA in the gill (Deane and Woo, 2004) follow the same pattern in *S. sarba* that our measurements of enzyme activity in response to similar salinity challenge in *S. auratus*.

In some euryhaline teleosts a rapid activation of gill Na^+,K^+-ATPase activity has been reported during the adaptive period after transfer to environments of different salinity (Mancera and McCormick, 2000; Tipsmark and Madsen, 2001; Lin et al., 2004). This rapid increase in activity is not likely due to an increase in the number and/or size of CC, but could involve modifications of pump catalytic subunits, changes in the subcellular distribution of pump units, or increase in translational or post-translational kinetics (see Mancera and McCormick, 2000; Tipsmark and Madsen, 2001). Our results with gilthead sea bream (trial 2) showed a slight increase in Na^+,K^+-ATPase activity after transfer to hyperosmotic or hypoosmotic environments visible as early as 4 hr after transfer, although no significant changes were observed before 96 hr. In the euryhaline *F. heteroclitus*, the increase in gill Na^+,K^+-ATPase activity observed during the first adaptive period after transfer from FW to SW is also transitory (Mancera and McCormick, 2000). It has not been determined whether a similar short-term increase in gill Na^+,K^+-ATPase activity takes place in the gilthead sea bream. Further detailed in vivo and in vitro studies during the initial adaptation period are being carried out to clarify this hypothesis.

FW species have a tight gill epithelium that prevents water entry and ion loss (McCormick, '95; Marshall, 2002), and it is generally accepted that an apical V-type H^+-ATPase generates the driving force for a channel mediated apical Na^+ uptake (Lin and Randall, '95; Marshall, 2002; Kirschner, 2004). Katoh et al. (2002) indicated that the V-type H^+-ATPase is present in the basolateral membrane of killifish adapted to a low ion environment, supposedly joining forces with the Na^+,K^+-ATPase to promote a gradient for Na^+ uptake. Marine fish living in FW or dilute SW maintain higher Na^+ fluxes via paracellular routes than demanded by the environmental conditions, and this has led to the suggestion that they are unable to adapt totally in these conditions (McCormick, '95), probably by lacking elements in the osmoregulatory machinery. The increase in gill Na^+,K^+-ATPase and reduction in plasma Na^+ and osmolality observed in the present study with gilthead sea bream maintained at low salinity (5‰) suggests that this fish is unable to maintain homeostasis at such low salinities. Similar findings have been observed in other euryhaline fish such as the sheepshead minnow *Cyprinodon variegates* and flounder *Paralichthys orbignyanus* (Nordlie, '85; Sampaio and Bianchini, 2002), and it is not clear whether this could reflect an ill-adaptation of the H^+-ATPase in these fish. In this context, the increase in gill Na^+,K^+-ATPase activity observed in gilthead sea bream adapted to 5‰ salinity may be a compensatory mechanism to balance the increased branchial ion loss setting the conditions for an inward Na^+ flux, at an increased energy cost.

The energetic cost of NaCl transport across the gill in FW and SW represents a relatively small proportion (4%) of the animal's total energy budget in cut-throat trout, *Oncorhynchus clarki clarki* (Morgan and Iwama, '99). However, gilthead sea bream juveniles cultured in 6‰ salinity grow substantially less than fish maintained at salinities of 12‰ or 38‰ (Laiz-Carrión et al., 2005). This observation and those reported in other studies (reviewed by Boeuf and Payan, 2001) indicate that a rather large proportion of the total energy budget is allocated to osmoregulation in the sea bream maintained at low salinity.

Plasma lactate levels were significantly higher in fish exposed to the extreme salinities (5‰ and 60‰) and correlate well with the higher gill Na^+,K^+-ATPase activity. The increase in lactate could indicate, as suggested for other teleosts (Mommsen, '84), that this metabolite is important in fuelling the osmoregulatory mechanisms. The data of Sangiao-Alvarellos et al. (2003) describing high plasma lactate, high gill Na^+,K^+-ATPase activity and high plasma glucose in gilthead sea bream acclimated to 55‰ are in good agreement with the present data. Hyperglycaemia at 55‰ and hypoglycaemia at 12‰ suggest that glucose is mobilized to satisfy the increased energetic of the higher gill Na^+,K^+-ATPase activity demand observed in the gills at extreme salinities. A comparable situation could explain the higher values of plasma glucose observed in fish acclimated to hypoosmotic conditions (this study), which also showed elevated values of gill Na^+, K^+-ATPase activity.

CC distribution and morphology

Long-term changes in gill Na^+,K^+-ATPase activity usually reflect differences in the synthesis

of new Na^+,K^+-ATPase units as well as an increase in the biogenesis or apoptosis of CC (see McCormick, '95; Jensen et al., '98, Marshall, 2002). CC in the sea bream are localized solely in the primary filament, which is in keeping with the assumption that this area of the branchial epithelia is involved in ion exchange while the secondary lamellae are mainly responsible for gas transfer (Perry, '97; Marshall, 2002). In contrast with other species such as *Oncorhynchus keta* (Uchida and Kaneko, '96), *Lateolabrax japonicus* (Hirai et al., '99), *D. labrax* (Varsamos et al., 2002) and *C. chanos* (Lin et al., 2003), adaptation to altered salinities does not change CC location but promotes changes in the number, size and shape of these cells (Figs. 4 and 5). Adaptation to a hyperosmotic environment induces the proliferation of CC, an effect described in most teleost species studied during the transition from FW to SW (see Sakamoto et al. (2001) for a review). This response has been linked with the need for an enlarged ion transport capacity as presumably reflects an increase in ion pumps and transporters. Concomitantly, acclimation of gilthead sea bream to a more diluted environment (15‰ and 25‰) was followed by a reduction in CC number. However, the transfer of gilthead sea bream to 5‰ did not significantly change the number of CC in the branchial epithelia, unlike what has been observed in *S. sarba* (Kelly and Woo, '99) and *D. labrax* (Varsamos et al., 2002).

Cell size was lower at the intermediate salinities and significantly higher at the extreme salinities with some degree of parallelism to gill Na^+,K^+-ATPase activity, although the largest cells were detected not in the 60‰ but in the 5‰ acclimated fish. The size of the CC in fish adapted to 5‰ and 60‰ was almost twice that of fish in a near-isosmotic situation (15‰). A similar situation was described for sea bass adapted to FW, SW and doubly concentrated SW (Varsamos et al., 2002). It is tempting to speculate that larger cells can accommodate a higher number of pumps and therefore to relate cell size to increased enzyme activity. In fact, a rough estimate of the total gill area occupied by CC at each salinity, obtained by multiplying the number of CC by their surface area, results in a pattern almost identical to that of the gill Na^+,K^+-ATPase activity.

After salinity acclimation, the CC of fish adapted to full-strength SW (38‰) and those of fish in hypersaline water (60‰) showed an angular and elongated shape whereas those of fish exposed to reduced salinities had a more circular profile. It is well established that CC in gills and opercular epithelium of FW- and SW-adapted fish have different morphofunctional characteristics (Pisam and Rambourg, '91; Sakamoto et al., 2001), and that polymorphism is believed to play crucial roles in the uptake of diverse ions as well as in acid–base regulation (Perry, '97). Recently Chang et al., (2003) have demonstrated that the number and apical size of a specific type of mitochondria-rich cell in tilapia gills are positively associated with the level of Cl^- influx, but not to Na^+ or Ca^{2+}. Further studies should aid in clarifying whether that is the case in the sea bream.

The time course of CC dynamics was not evaluated in this study, but previous studies (Marshall et al., '99; Sakamoto et al., 2001; Kaneko et al., 2002; Katoh and Kaneko, 2003; Lin et al., 2004) indicate that transformations in cell structures can occur within hours after the osmotic challenge and that replacement of FW- by SW-type cells (or vice versa) as well as the onset of cell proliferation usually take a few days.

In conclusion, the sea bream *S. auratus* is able to withstand the range of their normal environmental salinities with minor changes in plasma osmoregulatory variables and the effective regulation of the gill Na^+,K^+-ATPase. At extreme low salinities, only a moderate adaptive capacity is observed, possibly related to insufficient or absent expression of specific components of the CC transport machinery (e.g., H^+-ATPase). Therefore, the U-shape response observed in gill Na^+,K^+-ATPase in relation to salinity in *S. auratus* and the parallel activation of alpha and beta subunit mRNA expression of Na^+,K^+-ATPase described in *S. sarba* (Deane and Woo, 2004) reflect an alternative for the inadequate capacity of the H^+-ATPase to generate enough Na^+ uptake in the gill as occurs in stenohaline FW species.

ACKNOWLEDGMENTS

The authors are grateful to CUPIMAR SA (San Fernando, Cádiz, Spain) for providing the fish. The authors are indebted to Dr. C. García Jimenez for his help in using the apparatus for image analysis, Dr. C. Valero and Dr. F. Brun (Universidad de Cádiz) for the statistical assistance, and Dr. D. M. Power for a critical reading of the manuscript.

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