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Differential freshwater adaptation in juvenile sea-bass *Dicentrarchus labrax*: involvement of gills and urinary system

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Summary

The effects of long-term freshwater acclimatization were investigated in juvenile sea-bass *Dicentrarchus labrax* to determine whether all sea-bass juveniles are able to live in freshwater and to investigate the physiological basis of a successful adaptation to freshwater. This study particularly focused on the ability of sea-bass to maintain their hydromineral balance in freshwater and on their ion (re)absorbing abilities through the gills and kidneys. Two different responses were recorded after a long-term freshwater acclimatization. (1) Successfully adapted sea-bass displayed standard behavior; their blood osmolality was maintained almost constant after the freshwater challenge, attesting to their efficient hyperosmoregulation. Their branchial and renal Na^+/K^+-ATPase abundance and activity were high compared to seawater fish due to a high number of branchial ionocytes and to the involvement of the urinary system in active ion reabsorption, producing hypotonic urine. (2) Sea-bass that had not successfully adapted to freshwater were recognized by abnormal schooling behavior. Their blood osmolality was low (30% lower than in the successfully adapted sea-bass), which is a sign of acute osmoregulatory failure. High branchial Na^+/K^+-ATPase abundance and activity compared to successfully adapted fish were coupled to a proliferation of gill chloride cells, whose ultrastructure did not display pathological signs. The large surface used by the gill chloride cells might negatively interfere with respiratory gas exchanges. In their urinary system, enzyme abundance and activity were low, in accordance with the observed lower density of the kidney tubules. Urine was isotonic to blood in unsuccessfully adapted fish, ruling out any participation of the kidney in hyperosmoregulation. The kidney failure seems to generate a compensatory ion absorption through increased gill activity, but net ion loss through urine seems higher than ion absorption by the gills, leading to lower hyper-osmoregulatory performance and to death.

Key words: teleost, osmoregulation, gills, kidney, Na^+/K^+-ATPase, sea-bass, *Dicentrarchus labrax*.

Introduction

The sea-bass *Dicentrarchus labrax* (Linnaeus 1758) is a euryhaline marine teleost commonly found along the coasts of the north-east Atlantic Ocean and the Mediterranean Sea. It is also found in lagoons and estuaries where salinity is generally lower and more variable than in the open sea. Under natural conditions, reproduction always occurs in marine habitats. From the larva/juvenile transition and beyond (mean length ~15 mm), a fraction of the juvenile cohorts of *D. labrax* enters the brackish waters of the lagoons and estuaries, where they spend most of their early life (Kelley, 1988; Pickett and Pawson, 1994). Juveniles and adults seasonally concentrate at river mouths and in coastal lagoons, and some individuals are known to migrate several km up rivers to freshwater (FW; ~5–15 mOsmol kg^{-1}; Barnabé, 1989). However, whether or not all sea-bass are able to adapt long-term to FW is still unclear, as shown by different experimental studies (Cataudella et al., 1991; Allegrucci et al., 1994; Jensen et al., 1998; Lemaire et al., 2000; Varsamos et al., 2002b).

Adaptation to various levels of salinity, including FW, involves coordinated physiological responses based on the function of several osmoregulatory organs. It is well established that adult euryhaline teleosts are able to maintain their blood osmolality at about 300–350 mOsmol kg^{-1} in the range of tolerable salinities, due to an effective hydro-mineral regulation occurring mainly at the gill, urinary system, intestine and integument levels (see reviews by Evans et al., 1999; Greenwell et al., 2003; Varsamos et al., 2005). In FW, sea-bass are exposed to osmotic water entrance and diffusive ion loss. To compensate for the passive ion movements, the fish need to limit ion loss and to (re)absorb ions by specialized cells lining the osmoregulatory epithelia. These cells, called mitochondria-rich cells (MRC) or chloride cells in the gills, or

more generally ionocytes, are characterized by the abundance of several ion channels, transporters and enzymes. Among these, the Na^+/K^+-ATPase creates ionic and electrical gradients used for salt uptake from the external medium to the blood. At the cellular level, the Na^+/K^+-ATPase is located on a well-developed tubular system corresponding to an extension of the basolateral cell membrane; the cells are also called MRCs, due to the numerous mitochondria providing the energy required by the enzyme. In teleosts, the Na^+/K^+-ATPase activity and its abundance have often been used as indicators of the osmoregulatory ability in adults/juveniles acclimated to different salinities (Imsland et al., 2003) or in migratory species at specific ontogenetic stages (Uchida et al., 1996; Zydlewski et al., 2003). In FW, the main osmoregulatory organs are the gills (for active ion uptake) and the urinary system (for the production of large amounts of hypotonic urine). Previous studies have shown that these two organs progressively develop during the post-embryonic ontogeny of the sea-bass and are functional at the larva/juvenile transition (Varsamos et al., 2002a; Nebel et al., 2005), i.e. by the time young fish migrate towards low-salinity areas and FW.

The objectives of this study were thus: (1) to detect potential differences in the ability of juvenile sea-bass to successfully adapt to FW; (2) to determine different abilities of hyperosmoregulation between the fish assessed as able or unable to live in FW; and (3) to identify the physiological, histological and cellular basis of these differences at the gill and urinary system levels.

This study is a first step towards understanding the meaning of inter-individual variations in response to environmental fluctuations, and their bearings on population differential adaptation.

Materials and methods

Animals and experimental conditions

The study was carried out on the progeny of 6 (3 male + 3 female) unrelated sea-bass *Dicentrarchus labrax* L. breeders. Cultured sea-bass juveniles were obtained from the fish culture systems of the sea farm 'Les Poissons du Soleil' (Balaruc, Hérault, France) and of Ifremer (Palavas, Hérault, France) and transported to the 'Station Méditerranéenne de l'Environnement Littoral' (Sète, Hérault, France) located along the Thau lagoon, which communicates with the Mediterranean Sea. The salinity challenge was conducted using 2- (experiment 1) and 3- (experiment 2) month-old sea-bass juveniles, the first ones having hatched in November and the second ones in January. The osmolality measurements and the immunocytochemical and ultrastructural studies were conducted during experiment 1, and the other studies (quantitative histology, measurement of Na^+/K^+-ATPase activity and abundance, in gills and kidneys) during experiment 2. The rearing parameters did not vary between the two experiments. The fish were divided into eight tanks, each holding about 200 litres of aerated water at salinity 10‰ (294 mOsmol kg^{-1}), isosmotic to fish blood and corresponding to the initial transport medium. Each tank contained 200–250 fish. They were fed (Aphymar feed, Mèze, France) to apparent satiation twice daily throughout the study and subjected to a natural photoperiod and temperature regime. Following a 2-week acclimatization period, the fish were subjected to a progressive salinity change either to SW (≈950 mOsmol kg^{-1}) in four tanks or to FW (≈10–15 mOsmol kg^{-1}) in the four other tanks over a period of 3 weeks. The constantly renewed water was either filtered SW from the Thau lagoon or dechlorinated tapwater. Ionic composition (in mEq l^{-1}) of the FW was Na^+ (0.12), K^+ (0.04), Ca^{2+} (5.70), Mg^{2+} (0.29), Cl^- (0.98), NO_3^- (0.06) and SO_4^{2-} (0.61) (F. Persin, personal communication). Throughout the experiments, the behavior of the fish was recorded. Fish were sampled for further studies when different patterns of behavior appeared in FW (see Results). SW fish were sampled simultaneously. The sampled fish average total length was 4.7 cm (range: 3.8–5.7 cm). The experiments were conducted according to the French law on animal scientific experimentations. The fish were anaesthetized in a solution of phenoxy 2 ethanol (0.3 ml l^{-1}) prior to any manipulation.

Osmolality measurements

Blood and urine osmolalities were measured in the different categories of fish from FW and SW. Preliminary attempts at urine collection directly following capture and anaesthesia of fish failed, probably due to stress-induced urine emission. Therefore, the following procedure was used. The fish were captured with a hand-net and transferred to a black 10 l plastic container filled with FW or SW, aerated and covered by a black plastic sheet. The containers were kept still in a silent room for 2 h. The anaesthetic was then gently introduced into the water under the plastic cover. This method allowed about 80–90% successful urine collection. The urinary pore and the branchial chamber of each anaesthetized fish were quickly rinsed with distilled water and carefully dried with absorbent paper. Urine was collected following its emission induced by probing the urinary pore and gently inserting a 2 μl glass micropipette (Drummond microcaps, Bioblock, Broomall, PA, USA) into the pore. Blood was sampled from the ventral aorta using a 1 ml syringe. The urine and blood samples were immediately transferred into mineral oil to avoid evaporation. The osmolality of at least 30 nl of blood and urine was measured on a Clifton nano-osmometer (Clifton Technical Physics, Hartford, NY, USA).

Morphometric parameters of branchial chloride cells

The first gill arch of the right side of all fish was excised and immersed for at least 16 h into freshly mixed Champy-Maillet's fixative (0.4% osmium tetroxyde, 25 mg ml^{-1} iodine and saturated metallic zinc; Maillet, 1959). After rinsing with distilled water for at least 3 days, the samples were dehydrated in a series of ethanol and processed for embedding in Paraplast®. 7 μm-thick sagittal sections were cut and dewaxed with histochoice LMR. This simple and rapid method resulted in chloride cells being specifically stained in black due to their extensive basolateral membrane

system (Hartl et al., 2001). For all later analyses, at least 5 animals per group were used.

For chloride cell numbering, 10 filaments per animal were observed to determine the number of positively stained chloride cells. On each filament, the chloride cell number was determined within 10 interlamellar spaces. An interlamellar space is defined as the distance on the filament between two adjacent lamellae. Chloride cell numbers of filaments and lamellae were counted separately.

For the evaluation of cell size and shape, 30 positively stained cells per animal were identified on photographs and their borders traced manually. Using a stage micrometer, their area (A), perimeter (P) and shape (S) was calculated using Optimas software (North Reading, MA, USA). The shape (defined as $S=4\pi AP^{-2}$) indicates the circularity of the chloride cells. The shape factor tends to 1 when the cell is spherical (Zydlewski et al., 2003).

Histomorphology of the urinary system

Whole animals were immersed into Bouin's liquid for 24–48 h. The fixative penetration was facilitated by longitudinal 2 mm-deep incisions and sections of the fins. They were washed and dehydrated in an ascending series of ethanol, and finally embedded in Paraplast®. The whole animals were cut in longitudinal horizontal 7 μm-thick sections, stained with Masson-trichrome and observed on a Leica Diaplan microscope (Leitz Wetzlar, Germany). A precise kidney zone was determined and 2–3 slides per animal used for further analysis. From photographs taken at a low magnification (×10 objective lenses), the borders of the kidney were manually traced using the Optimas software in order to measure the percentage of the area occupied by the urinary tubule sections compared to the 'total kidney area'. As the section areas of the dorsal aorta and of the collecting ducts were not comparable between animals, these areas were subtracted from the total kidney area in order to standardize the measurements. In this way, the density of urinary tubules was estimated compared to the 'total kidney area' (minus collecting ducts and dorsal aorta).

Transmission electron microscopy

Freshly dissected gill arches were fixed for 1 h in a solution of 5% glutaraldehyde buffered at pH 7.4 with 0.2 mol l^{-1} sodium cacodylate buffer at ambient temperature. Samples were then rinsed in sodium cacodylate buffer and post-fixed for 1 h in a mixture (v/v) of 2% osmium tetroxide and 0.45 mol l^{-1} sodium cacodylate buffer at 4°C. After extensive washing in distilled water, the samples were dehydrated in graded ethanol and embedded in Epon. Ultrathin sections were cut using a diamond knife on a Reichert–Jung ultramicrotome (Cambridge, UK), contrasted with uranyl acetate and bismuth tartrate and examined with a JEOL 1200 EX II transmission electron microscope (Tokyo, Japan), operated at 100 kV.

Immunolocalization of Na^+/K^+-ATPase in gills and kidney

Sections (4 μm) from Bouin's fixed samples of the gill and the kidney were immersed into 0.01% Tween 20, 150 mmol l^{-1} NaCl in 10 mmol l^{-1} phosphate-buffered saline (PBS), pH 7.3 for 10 min, treated for 5 min with 50 mmol l^{-1} NH_4Cl (to screen free aldehyde groups of the fixative), and finally incubated for 10 min in 1% BSA and 0.1% gelatin in PBS. The slides were incubated for 2 h at room temperature in a moist chamber with the specific monoclonal mouse antibody (diluted to 10 μg ml^{-1}) raised against the α-subunit of the chicken Na^+/K^+-ATPase (IgG α5) developed by Fambrough and purchased from the DSHB (Developmental Studies Hybridoma Bank; University of Iowa). This antibody has already been successfully used in the same species (Varsamos et al., 2002b; Nebel et al., 2005). Control sections were subjected to the same conditions, but without the monoclonal antibody. After rinsing, all sections were incubated for 1 h with the fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody. The slides were washed, mounted with anti-bleaching mounting medium and rapidly examined using a Leica Diaplan microscope equipped for fluorescence with the appropriate filter set (filters of 450 nm to 490 nm) and coupled to a digital camera and the FW4000 software.

Immunoblotting

Immunoblotting was carried out in gill and kidney homogenates according to the method of Pomport-Castillon et al. (1997), with modifications. The gills (without the gill arch) of the left side of the animals and the mid/posterior part of the kidney (urinary and collecting tubules/ducts) were dissected and stored in SEI buffer (150 mmol l^{-1} sucrose, 10 mmol l^{-1} Na_2EDTA, 50 mmol l^{-1} imidazole) at –80°C until use. The tissue was homogenized in 300 μl (kidney) and 500 μl (gills) ice-cold MIIM buffer (250 mmol l^{-1} sucrose, 5 mmol l^{-1} $MgCl_2$, 5 mmol l^{-1} Tris/Hepes, pH 7.3), centrifuged at 4000 ***g*** for 5 min (4°C), and the supernatant assayed for protein content by the Bradford method (Bradford, 1976). These homogenates were used for immunoassays and Na^+/K^+-ATPase activity measurements. Samples were diluted (1:8, 1:10) with PBS (phosphate buffered saline, pH 7.3). A 2 μl sample from each dilution was deposited in triplicate on a 0.45 μm-thick nitrocellulose membrane. Five known quantities of canine Na^+/K^+-ATPase (Sigma-Aldrich, St Louis, MO, USA), 0.3125 μg, 0.625 μg, 1.25 μg, 2.5 μg and 5 μg, were deposited in triplicate on the same membrane to standardize the measurements. After saturation in 5% skimmed milk (SM) in PBS at 37°C for 30 min, the SM powder was removed by washing the membrane twice with PBS. The strips were incubated for 2 h with the monoclonal mouse antibody (diluted at 3 μg ml^{-1} in 0.5% SM-PBS) raised against the α-subunit of the chicken Na^+/K^+-ATPase (IgG α5). After washing, the avidine peroxidase conjugate (Pierce Interchim; Rockford, IL, USA) at 2 μg ml^{-1} was added to the membrane for 1.5 h. The membrane was washed and the fractions were developed with α2-chloro-naphtol acetate (Sigma). Color development was stopped by rinsing the membrane with distilled water. The membrane was dried at room temperature and rapidly scanned. The immunoblots were analyzed using

the Software Scion Image and the colour intensity measured for each blot.

Na^+/K^+-ATPase activity measurements

Gill and kidney Na^+/K^+-ATPase activity was determined according to the method developed by Flik et al. (1983). Na^+/K^+-ATPase activity measurements, expressed in μmol Pi mg^{-1} protein h^{-1}, were based on the differences in ATP hydrolysis in the presence and absence of ouabain (1.4 mmol l^{-1}). The same homogenates as those for the immunoassays were used.

Statistical comparisons

Results are expressed as mean ± S.E.M. Student's *t*-tests were used for statistical comparisons of mean values.

Results

Differences in FW adaptability

In both experiments, sea-bass successfully (FWS) and unsuccessfully adapted to freshwater (FWU) were recorded in the FW tanks. The FWU were easily recognized by their erratic swimming behavior out of the shoal and their absent or decreased reactions to external movements. In particular, the presentation of a 30 cm×30 cm wooden plank at 50 cm above the water induced a swift change of swimming orientation by the shoal, except for fish labelled as FWU, which showed no reaction: they went on slowly swimming without altering their course and consequently became isolated from the shoal. These behavioural changes were simultaneously but independently recorded by two observers. About 48–72 h after the occurrence of this behavior, they ceased to swim and died. FWU were sampled as soon as the first signs of abnormal behavior were recorded. Each time FWU were sampled, fish displaying standard behavior in FW (FWS) and fish kept in SW (SWS) were also sampled. At the time of sampling, the fish were about 3.5 months (experiment 1) and 5 months (experiment 2) old. Among them, 26% of the 3.5 month-old sea-bass were FWU after the FW challenge and their occurrence was recorded starting 1 week after the FW acclimatization over a period of 1–2 weeks. In the 5 month-old sea-bass, FWU appeared about 3 weeks after the salinity challenge during 2–3 weeks. A low mortality (1–2%) was recorded in the SW fish (SWS) and the successfully adapted FW fish (FWS) survived over a period of at least 18 months with low mortality (5%).

Osmoregulation

Compared to SWS, blood osmolality was slightly but significantly lower (by 12%) in FWS. Most strikingly, blood osmolality was much lower in FWU (by 32%; 214±14 mOsmol kg^{-1}) compared to FWS (316±20 mOsmol kg^{-1}). By contrast, in SWS, urine (360±12 mOsmol kg^{-1}) was isotonic to blood (360±13 mOsmol kg^{-1}). In FWS, urine (227±43 mOsmol kg^{-1}) was markedly hypotonic to the blood (316±20 mOsmol kg^{-1}).

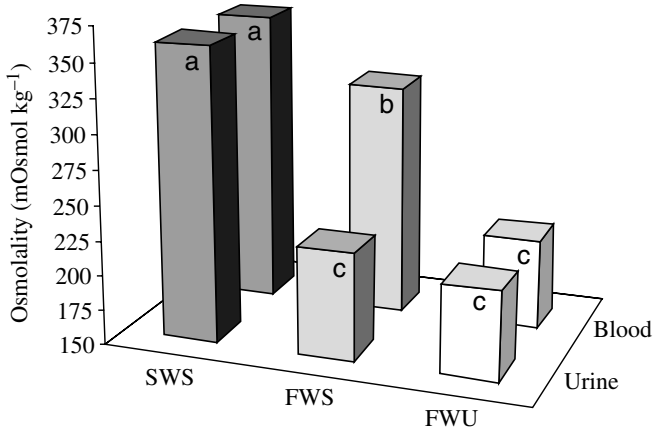

Fig. 1. Blood and urine osmolality in SWS, FWS and FWU *Dicentrarchus labrax* (*N*=12–19). Different letters indicate significant differences (P<0.001).

In contrast, urine in FWU fish was isotonic to the blood (213±15 mOsmol kg^{-1} *vs* 214±14 mOsmol kg^{-1}). The results are illustrated in Fig. 1.

Gills

Branchial chloride cell distribution, morphometrical parameters and ultrastructural observations

Representative Champy-Maillet-prepared gill sections of SWS, FWS and FWU are shown in Fig. 2A–C. During the salinity challenge of the sea-bass from SW to FW, significant changes in branchial chloride cell distribution (Fig. 2A–C), abundance (Figs 2A–C, 3; Table 1) and morphology (Table 1) were noted. The chloride cells of SWS were exclusively located on the gill filaments (Fig. 2A). In the FWS and FWU groups, chloride cells were also located on the filaments, but

Table 1. *Morphometric parameters of the branchial chloride cells in SWS, FWS and FWU sea-bass juveniles*

	Area	Perimeter	Shape	Number
Filament				
SWS	86.8±11^{a}	37.1±2^{a}	0.78±0.02^{a}	17.9±1.8^{a}
FWS	64.3±7^{b}	32.7±2^{b}	0.75±0.03^{a,b}	18.3±3.8^{a}
FWU	77.8±19^{a,b}	36.3±4^{a,b}	0.73±0.02^{b}	24.1±2.2^{b}
Lamella				
FWS	56.5±7^{a}	36.4±3^{a}	0.55±0.09^{a}	9.7±2.7^{a}
FWU	74.4±14^{b}	40.4±2^{b}	0.58±0.07^{a}	23.8±8.6^{b}

Observations from Champy-Maillet-stained sections.

Values are means ± S.D. The cell area, perimeter and shape are expressed as the mean value of at least 30 cells per animal (*N*=5). The number of cells are expressed as the mean number of filamentary and lamellar cells within the length of 10 interlamellar spaces.

Different superscript letters indicate significant differences (P<0.05); statistical tests were effected separately between filamentary and lamellar data.

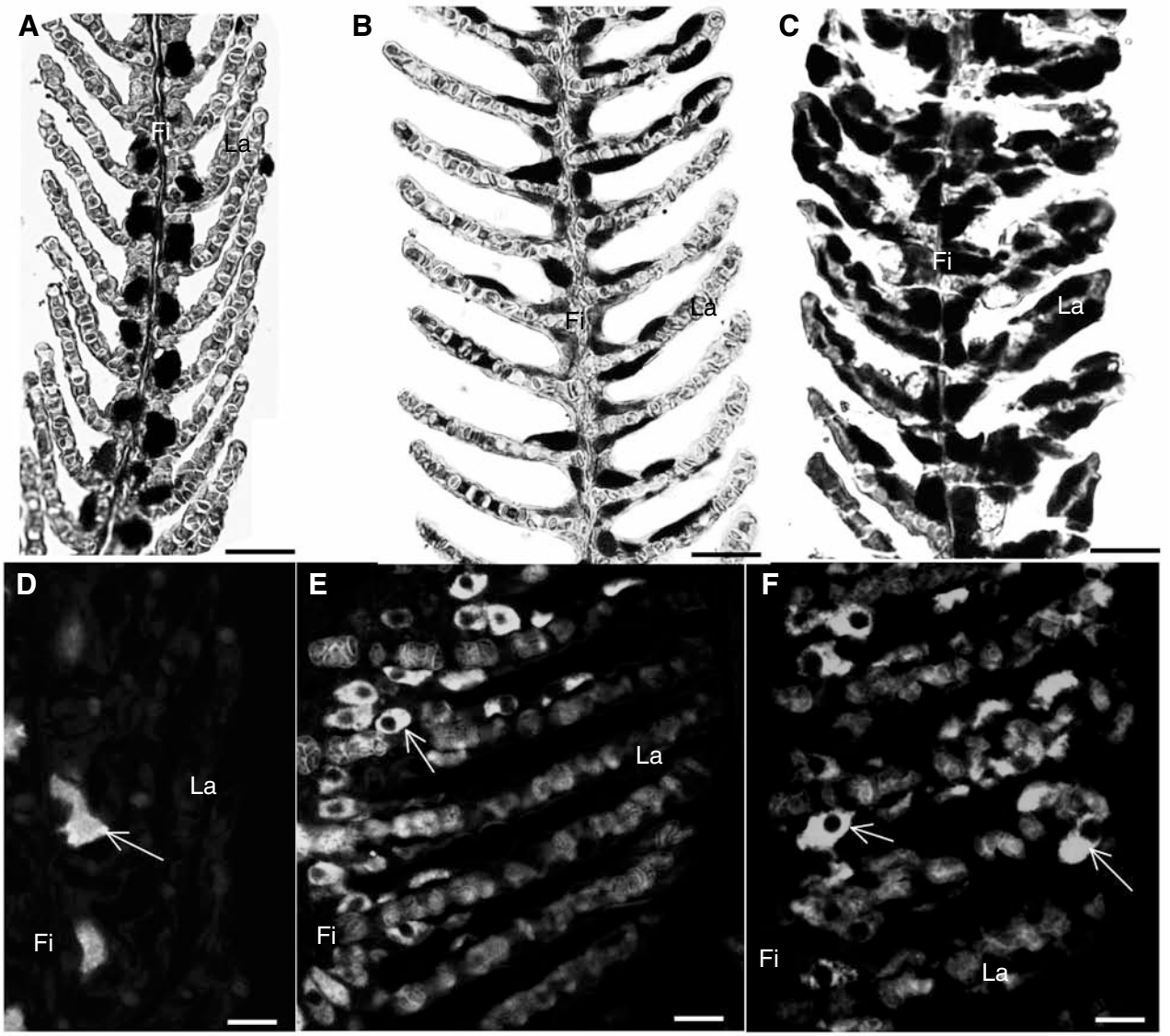

Fig. 2. Gill sections of SWS (A,D), FWS (B,E) and FWU (C,F) *Dicentrarchus labrax*. Chloride cells are specifically stained (black) with Champy-Maillet's fixative (A–C) or immunostained (arrows) by immunofluorescence to localize the Na^+/K^+-ATPase (D–F). Fi, filament; La, lamella. Scale bars, 20 μm (A–C), 10 μm (D–F).

other, more elongated chloride cells (shape factor decreased to 0.6; Table 1) were observed on the lamellae (Fig. 2B,C).

The mean total number of branchial chloride cells (including filamentary and lamellar chloride cells) within a distance of 10 interlamellar spaces increased significantly after the FW challenge, by 56% in FWS and by 167% in FWU, compared to SWS (Fig. 3). The mean number of filamentary chloride cells in FWU was significantly higher by 32% compared to FWS (F=2.965) and by 35% compared to SWS (F=1.517).

The number of lamellar ionocytes was much higher (by 245%) in the FWU compared to the FWS (Table 1). The ratio of the mean number of lamellar ionocytes to the mean number of filamentary ionocytes was 0.53 in the FWS and 0.97 in the FWU, which illustrates the much higher number of lamellar chloride cells in the FWU.

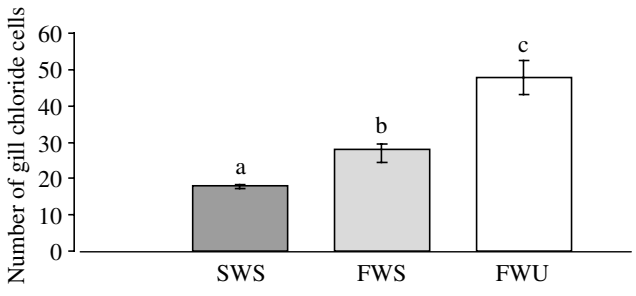

Fig. 3. Number of gill chloride cells (including all filamentary and lamellar ionocytes) in SWS, FWS and FWU *Dicentrarchus labrax* on a total length of 10 interlamellar spaces (N=5–11). Observations from Champy-Maillet-stained sections. Different letters indicate significant differences (SWS/FWS, SWS/FWU: P<0.001; FWS/FWU: P<0.01).

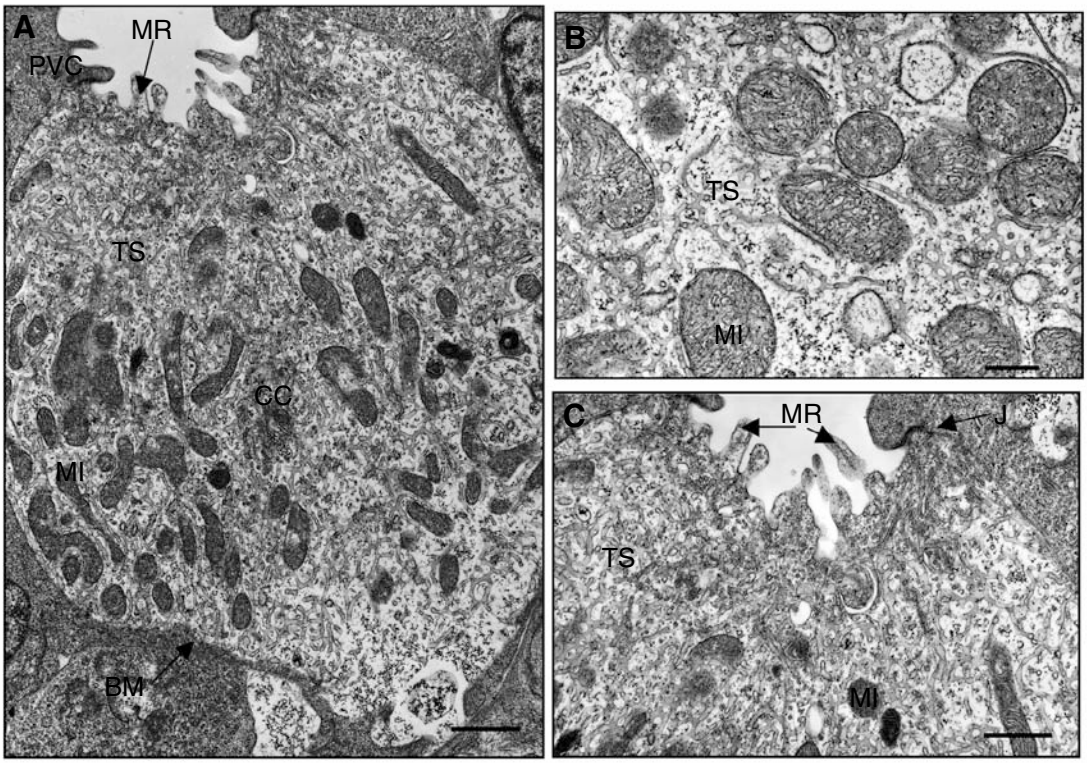

Fig. 4. Ultrastructure of a gill filamentary chloride cell of FWU *Dicentrarchus labrax*. Note the presence of numerous mitochondria in the median part of the cell and the well-developed tubular system that is an extension of the basolateral cell membrane (A,B). The apical cell part presents a few microridges (A,C). BM, basement membrane; CC, chloride cell; J, junction; MI, mitochondria; MR, microridges; PVC, pavement cell; TS, tubular system. Scale bars, 2 μm (A), 0.7 μm (B), 1 μm (C).

The mean area and perimeter of the filamentary chloride cells were significantly higher in the SWS than in the FWS. There was no significant difference in the filamentary cells morphometry (area, perimeter and shape factor) between the FW groups (FWS and FWU; Table 1), but the lamellar cells of the FWU had a larger area and perimeter than the FWS cells. Ultrastructural observations showed that the chloride cells of the FWU displayed the same features as observed in FWS (results not shown) and described in previous studies (Varsamos et al., 2002b). No sign of cell degeneration was detected in filamentary (Fig. 4A) and lamellar FWU chloride cells. The chloride cells of FWU contained numerous mitochondria characterized by an electron-dense matrix (Fig. 4A,B). The apical part of the cells, which was devoid of mitochondria, displayed a few microridges and presented a dense vesiculotubular system (Fig. 4A,C). An extensive tubular system, distributed throughout the whole cytoplasm, was continuous with the basolateral cell membrane (Fig. 4A). Tight junctions were observed between chloride cells and pavement cells (Fig. 4C).

Branchial Na^+/K^+-ATPase immunofluorescence, immunoassays and specific activity

Control sections without primary antibody showed no immunolabeling (results not shown). In SWS, the filamentary chloride cells were densely immunostained (Fig. 2D). The

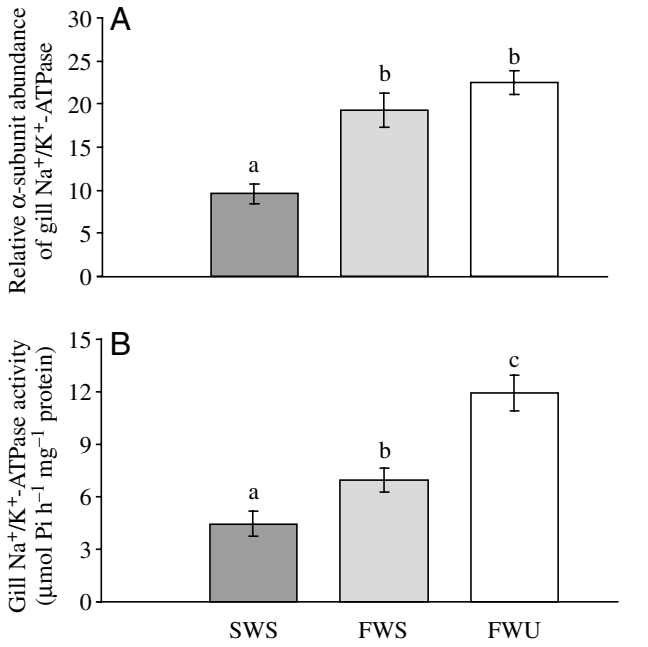

Fig. 5. Gill Na^+/K^+-ATPase abundance (A) and activity (B) in SWS, FWS and FWU *Dicentrarchus labrax*. Different letters indicate significant differences. (A) N=7; SWS/FWS: P<0.01, SWS/FWU: P<0.001; (B) N=9; SWS/FWS: P<0.05, FWS/FWU: P<0.01, SWS/FWU: P<0.001.

filaments and the lamellae of all FW-exposed fish observed presented positively immunostained chloride cells (Fig. 2E,F).

For the dot-blot assays, the 5 known Na^+/K^+-ATPase control samples displayed a linear response (gills: r^2=0.9928; kidney: r^2=0.9792). No immunoblotting was detected in the absence of the monoclonal antibody directed against the Na^+/K^+-ATPase and no crossreaction was detected between the avidine peroxidase conjugate and the nitrocellulose membrane. In SWS gills, Na^+/K^+-ATPase levels were significantly lower than in the FWS and the FWU (Fig. 5A). The Na^+/K^+-ATPase level was slightly but not significantly higher (by 17%) in the FWU than in the FWS (Fig. 5A).

Following the salinity challenge of *D. labrax* from SW to FW, the branchial specific Na^+/K^+-ATPase activity was increased by 56% in FWS and by 168% in FWU (Fig. 5B). The Na^+/K^+-ATPase activity was 72% higher in the FWU than in the FWS (Fig. 5B).

Urinary system

Morphometric analyzis of the urinary system

Examinations of sea-bass kidney sections revealed differences between the three groups of fish. In the SWS kidney, the lumen of the collecting ducts seems larger than in FWS and FWU (Fig. 6B,D,F). More strikingly, the renal tissue appears less dense in FWU (Fig. 6E,F) compared to FWS (Fig. 6C,D) and SWS (Fig. 6A,B). In order to quantify the latter observations, the area of the kidney tubules was measured and compared to the total kidney area (minus the ducts and the dorsal aorta) in the three groups of fish. The resulting data show similar values in SWS and FWS, but they reveal a lower tubular density (18–23% lower), in the FWU kidney (Fig. 7).

Na^+/K^+-ATPase immunofluorescence, immunoassays and specific activity in the urinary system

Na^+/K^+-ATPase was detected through immunofluorescence in all urinary tubules and ducts of the three fish categories (Fig. 8). The urinary tubules were immunostained on their basolateral cell membrane and no apparent difference in the cell staining intensities was detected between the SWS, FWS and FWU (Fig. 8A,C,E). The collecting ducts present a fluorescence distributed over the whole cytoplasm and not only on the

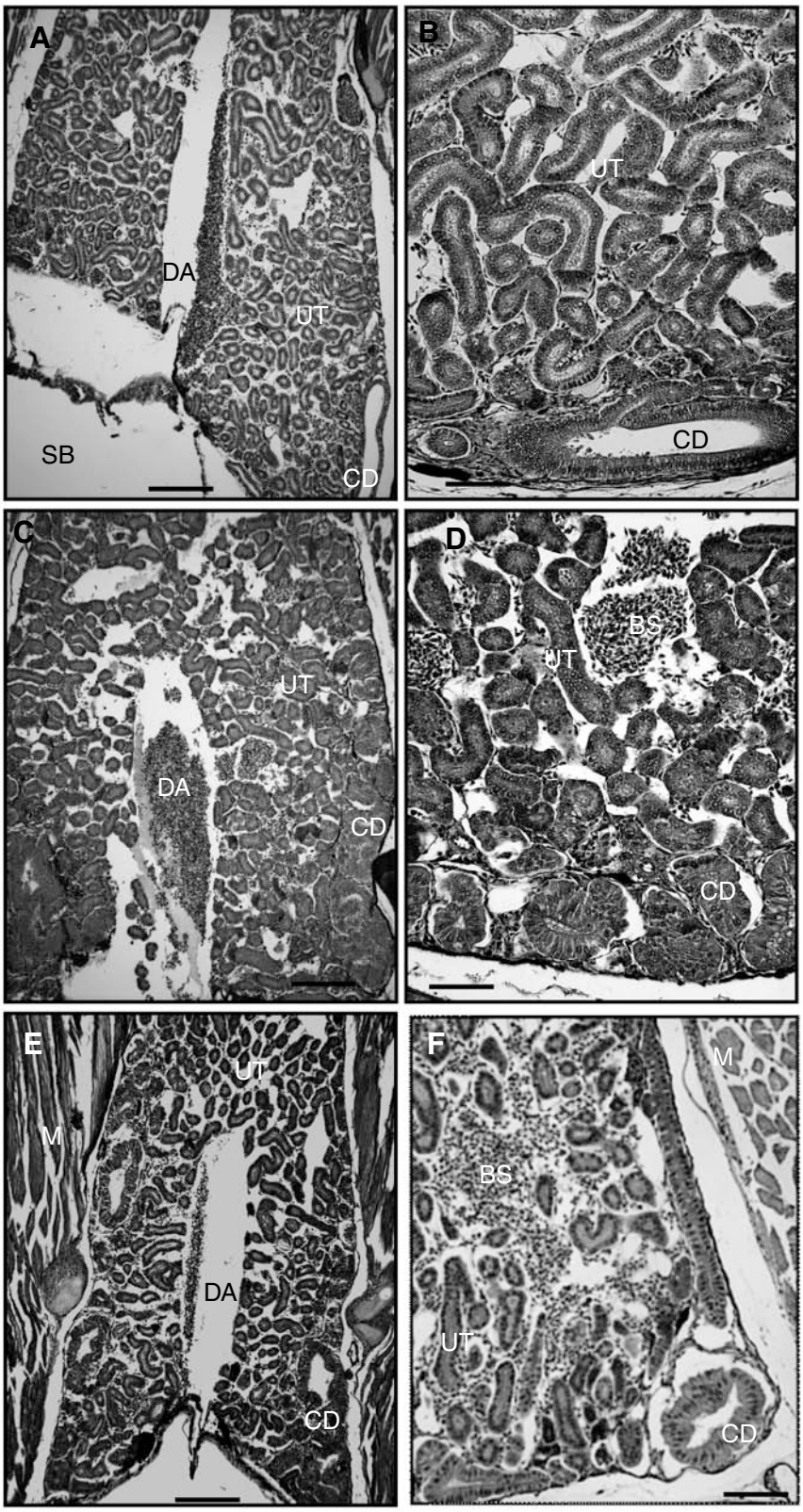

Fig. 6. Longitudinal horizontal sections of *Dicentrarchus labrax* kidney in SWS (A,B), FWS (C,D) and FWU (E,F). BS, blood sinus; CD, collecting duct; DA, dorsal aorta; M, muscle; SB, swim bladder; UT, urinary tubule. Scale bars, 120 μm (A,C,E), 60 μm (B,D,F).

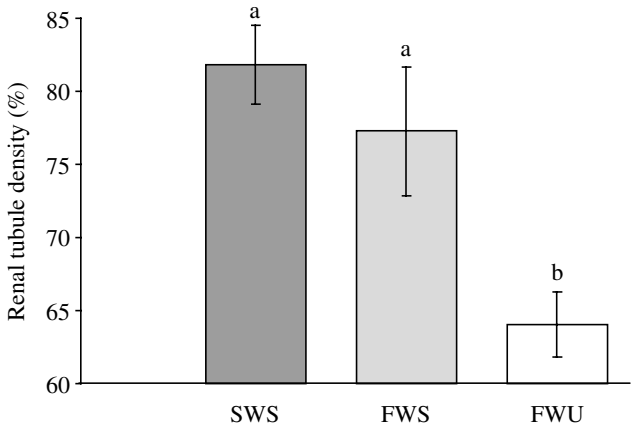

Fig. 7. Percentage area of the kidney tubules compared to the total kidney area (minus the ducts and the dorsal aorta) in SWS, FWS and FWU *Dicentrarchus labrax* (N=4–5). Different letters indicate significant differences (P<0.05).

basolateral cell side. They present the same immunostaining in FWS (Fig. 8C,D) and FWU (Fig. 8E,F) and a lesser staining in SWS (Fig. 8A). The dorsal part of the urinary bladder was strongly stained in the FW fish (FWS, FWU) (Fig. 8D,F); the fluorescence was localized in the whole cytoplasm, as reported in the collecting ducts. In the SWS, the urinary bladder showed no immunostaining (Fig. 8B). The Na^+/K^+-ATPase content of the urinary system, measured by dot-blot assays, was higher in FWS than in SWS; it was 44% lower in FWU compared to FWS; the enzyme content was similar in FWU and SWS (Fig. 9A). Similarly, the Na^+/K^+-ATPase activity of the urinary system was significantly higher in FWS than in SWS (by 144%); the enzyme activity was much lower (by 65%), in FWU compared to FWS; the activities were similar in FWU and SWS (Fig. 9B).

Discussion

The acclimatization of sea-bass from SW to low salinities and FW has been investigated in several studies (Dendrinos et al., 1985; Venturini et al., 1992; Allegrucci et al., 1994; Pickett et al., 1994; Jensen et al., 1998; Eroldogan et al., 2002). It is generally agreed that sea-bass tolerate low salinities after a progressive acclimatization but, following a challenge to FW, different consequences have been reported. In some cases, the sea-bass are able to successfully adapt to FW (Cataudella et al., 1991; Venturini et al., 1992; Jensen et al., 1998; Varsamos et al., 2002b). But other experiments have resulted in low (Jensen et al., 1998) or high mortality rates (Allegrucci et al., 1994), or even complete mortality (Dendrinos and Thorpe, 1985; Pickett et al., 1994; Eroldogan et al., 2002). In the present study, the developmental stages tested follow the period of acquisition of adult levels of osmoregulatory ability (Varsamos et al., 2001). The FW challenge generated different behavior and mortality within the sea-bass juveniles. Most of them (FWS) displayed no apparent alteration of their behavior and they lived in FW for at least 18 months. Unsuccessfully adapted sea-bass (FWU) also occurred in both experiments; they died 48–72 h after the first appearance of abnormal behavior. The validity of these observations was confirmed by their repeatability over 2 consecutive years.

Blood osmolality measurements were carried out in order to estimate the osmoregulatory ability of the different sea-bass groups. In the well-acclimated sea-bass juveniles (SWS and FWS), the blood osmolality was maintained within a range of 320–360 mOsmol kg^{-1} in FW and SW, respectively, demonstrating an efficient hyper- or hypo-osmoregulation. These values are in agreement with other studies carried out in the sea-bass (Lasserre, 1971; Varsamos et al., 2001) and in other euryhaline teleosts such as striped bass *Morone saxatilis* (Jackson et al., 2005), *Chanos chanos* (Lin et al., 2003) and *Crenimugil labrosus* (Lasserre, 1971). In the FWU, the blood osmolality decreased by 30–40% to 213 mOsmol kg^{-1}, an osmotic imbalance which may be considered a symptom of non-adaptation (Franklin et al., 1992) leading to death. In similar experiments, the transfer of sea-bass to FW by Jensen et al. (1998) resulted in a loss of Na^+ and Cl^- and in a decrease of the plasma osmolality to 240 mOsmol kg^{-1}; the fish died within 10 days. The sea-bass used by these authors are physiologically close to the FWU fish in this study. A similar blood osmolality value was measured in *Tetraodon nigroviridis* in FW (Lin et al., 2004), where no mortality was detected, and in hypophysectomized *Morone saxatilis* maintained in FW (Jackson et al., 2005). In the latter, an injection of prolactin was followed by a recovery of the blood osmolality to normal values. A possible hormonal deficiency might cause the osmoregulatory failure in the FWU and should be considered in further studies. The blood osmolality recorded in all these fish including the FWU *D. labrax* (200–240 mOsmol kg^{-1}) may be considered as the lower limit of tolerable blood osmolality in the sea-bass.

These observations raise the question of the physiological cause(s) of the osmoregulatory failure in the FWU fish. The decrease in plasma osmolality might originate from a failed regulation of the passive ion and water flow between their blood and the external medium. In other fish species, changes in body water content have been reported, which caused severe osmotic water loss at high salinities (Sclafani et al., 1997; Moustakas et al., 2004). Thus, in the FWU, an osmotic water entrance might result in a lower blood osmolality, but their ability to limit water entrance was not examined. In our study, the possible causes of osmoregulatory failure were addressed at the gill and urinary system levels.

Owing to their numerous chloride cells and their enzymatic equipment, the gills are considered as the major osmoregulatory site in euryhaline teleosts (Evans et al., 1999; Wilson and Laurent, 2002; Lin et al., 2003, 2004). In the successfully FW-adapted sea-bass (FWS) the FW challenge induced changes in the chloride cells, which were smaller and more numerous than in SW, particularly through their occurrence on the lamellae. At the ultrastructural level, the surfaces of their apical openings increased and the leaky

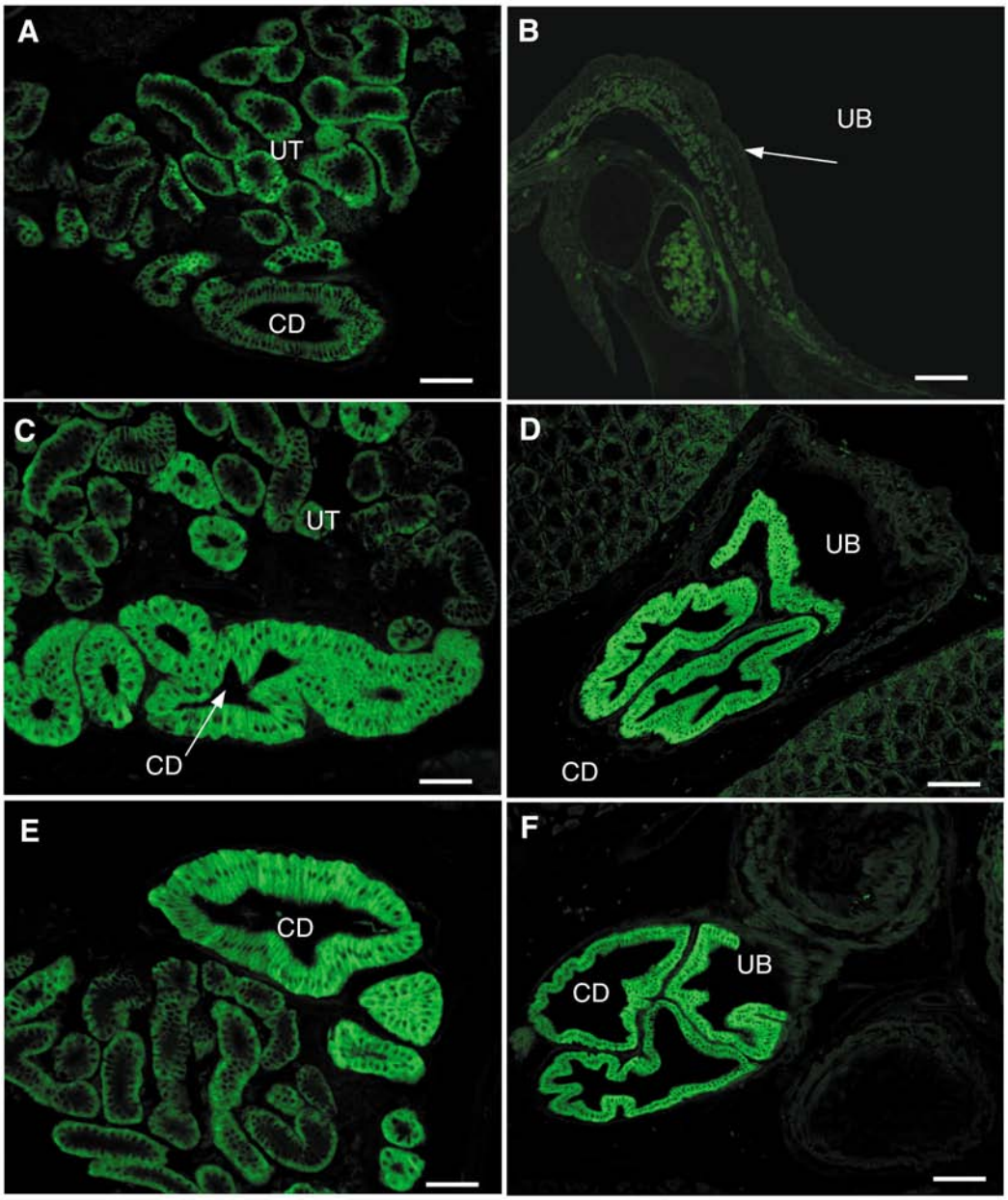

Fig. 8. Localization of the Na^+/K^+-ATPase through immunofluorescence in the urinary system in SWS (A,B), FWS (C,D) and FWU (E,F) *Dicentrarchus labrax* juveniles. Note the absence of immunostaining at the dorsal part of the SWS urinary bladder (arrow). CD, collecting duct; UB, urinary bladder; UT, urinary tubule. Scale bars, 100 μm (A,C,E), 20 μm (B,D,F).

junctions between adjacent cells were replaced by tight junctions (Varsamos et al., 2002b). In all analyzed fish, the branchial chloride cells displayed high amounts of Na^+/K^+-ATPase, apparently located within the deep basolateral membrane infoldings, as shown through immunofluorescence. A significantly higher Na^+/K^+-ATPase abundance was measured through immunoblotting in FWS compared to SWS fish, and the Na^+/K^+-ATPase activity followed the same trend. In the same species, a similar increase in enzyme activity has been reported in FW (Jensen et al., 1998), probably related to the higher number of chloride cells. According to Jensen et al. (1998), the branchial enzyme activity of *D. labrax* is minimum in an iso-osmotic environment and increases after transfer to hypo- and hyperosmotic media. This pattern of regulation of gill Na^+/K^+-ATPase activity, also reported in *Tetraodon nigroviridis* (Lin et al., 2004), seems to be mostly found in euryhaline species living in an environment where salinity fluctuates (Jensen et al., 1998). This reaction differs from the majority of teleosts in which the Na^+/K^+-ATPase activity increases with salinity (McCormick et al., 1989; Uchida et al., 1996). These examples illustrate the generally accepted view that changes in Na^+/K^+-ATPase activity correlate with transepithelial transport, although this enzyme is found in all animal cells for volume regulation.

As the osmoregulatory failure observed in FWU might hypothetically have originated from branchial chloride cell dysfunction or degeneration, the gill structure was compared between FWS and FWU fish. But, contrary to this hypothesis, the FW challenge induced a significantly higher increase in filamentary, and mainly in lamellar, chloride cell numbers in

the FWU compared to the FWS. The resulting increase in total chloride cell number might generate higher ion transporting activities in the branchial epithelium of FWU. It is worth noting that the ultrastructure of chloride cells in FWU did not show any sign of abnormality or degeneration compared to similar cells in FWS. FWU thus clearly possess more apparently normal functioning gill chloride cells than the FWS. Whether this increase in number results from a signal-induced proliferation (possibly hormonal, involving cortisol; Dang et al., 2000; Sloman et al., 2001) remains to be investigated. Although tight junctions were observed here between FWU chloride and pavement cells, a temporary leakiness of the branchial epithelium during the chloride cell proliferation cannot be excluded and would contribute to the decrease in blood osmolality.

Chloride cells, whose function has been extensively studied in fish (see reviews by Evans et al., 1999; Marshall, 2002; Varsamos et al., 2005), may be differently involved in osmoregulation according to their lamellar or filamentary location in some species (Shikano and Fujio, 1998). In *D. labrax*, only filamentary cells have been observed in SW (this study), suggesting their function as ion excretory sites. We hypothesize that lamellar chloride cells are involved in hyper-osmoregulation in FW, in FWS and in FWU (this study). They would also be involved in hypo-osmoregulation at doubly concentrated seawater (Varsamos et al., 2002b). Since the lamellae are also the site responsible for O_2 and CO_2 exchanges, a high proliferation of chloride cells, as observed in FWU, may result in a reduced lamellar area for respiration, as shown in *Oncorhynchus mykiss* (Bindon et al., 1994). In fact, gas and ion transfer is closely linked in FW teleosts, as reported by Randall and Brauner (1998). According to Perry (1998), lamellar chloride cell proliferation is a common response of freshwater fish to enhance the ion transporting capacity, but also has negative effects on respiratory gas transfer because of a thickening of the blood-to-water diffusion barrier. Moreover, the lamellar chloride cells of FWU are larger than those of FWS, which supports this hypothesis. In the sea-bass, these circumstances may contribute to the FWU mortality. A much higher Na^+/K^+-ATPase abundance and activity were recorded in FWU compared to FWS, thus generating an increased energy expenditure associated to hyperosmoregulation in FWU. This highly energy-demanding process, competing with other physiological requirements, may also compromise survival.

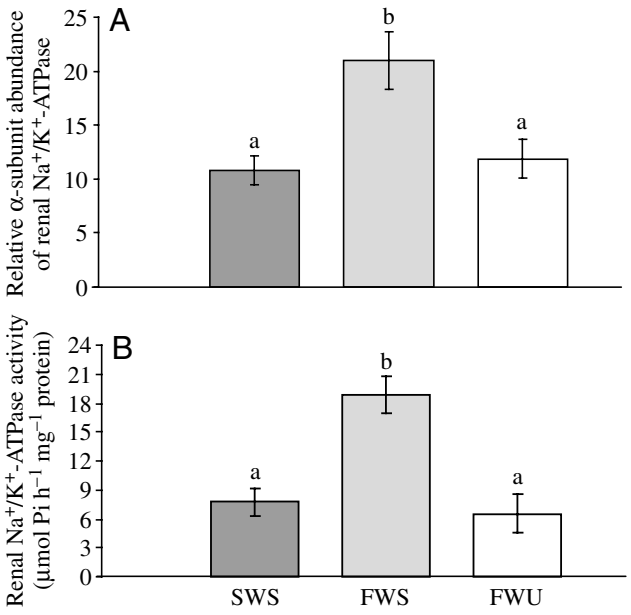

Fig. 9. Renal Na^+/K^+-ATPase abundance (A) and activity (B) in SWS, FWS and FWU *Dicentrarchus labrax*. Different letters indicate significant differences. (A) N=48; SWS/FWS: P<0.01, FWS/FWU: P<0.05; (B) N=9–14; P<0.001.

In summary, the gill chloride cells of FWU seem to possess all required features to efficiently hyperosmoregulate. Their higher number in FWU compared to FWS suggests a compensatory process that will be discussed later. But this abundance of chloride cells may in turn reduce the surface available for gas exchanges, and thus negatively interfere with respiration. Concurrently, the increased branchial Na^+/K^+-ATPase abundance and activity may worsen the respiratory problem through increased O_2 requirement. The high amounts of energy expenditure for ion absorption from FW may contribute to the high mortality recorded in the FWU. But since the results discussed above tend to rule out any direct degeneration process of gill chloride cells as a contributive factor to the osmoregulatory failure in FWU, further investigations were performed on the urinary system.

The kidney is known to play an important role in osmoregulation in euryhaline teleosts, by changing the rate of urine flow and controlling the balance between ion secretion and reabsorption according to the environmental salinity (see reviews by Hickman and Trump, 1969; Dantzler, 1992). As reported in this study, urine is isotonic to blood in SWS; the FWS are able to produce hypotonic urine, starting at least in 2- month-old juveniles (Nebel et al., 2005), probably through active ion reabsorption by the cells lining the urinary epithelia. Other euryhaline teleosts like *Platichthys flesus* (Lahlou, 1967) and *Paralichthys lethostigma* (Hickman and Trump, 1969) also possess this capacity to vary the urine osmolality according to salinity. In contrast, the FWU not only show a low blood osmolality, but have also lost the ability to save ions at the kidney level since their urine is isotonic to their blood. We suggest that the FWU urinary system is unable to actively reabsorb ions, particularly Na^+ and Cl^-, in order to produce hypotonic urine.

Experiments were carried out to test whether this decreased capacity of ion reabsorption from the filtrate originated from a lower amount and/or activity of the Na^+/K^+-ATPase within the urinary epithelia. This enzyme is located within the deep basolateral membrane infoldings of the cells lining the urinary tubules and ducts at both salinities, and of the dorsal part of the bladder in FW (Nebel et al., 2005). The localization of Na^+/K^+-ATPase revealed by immunofluorescence varies

according to the kidney section; in this study, a mostly basal cell location was observed in all urinary tubules, whereas the collecting ducts presented homogeneously distributed cell fluorescence. These characteristics have also been observed during the ontogeny of the sea-bass (see discussion in Nebel et al., 2005). The high amount of Na^+/K^+-ATPase in the collecting ducts and dorsal bladder in FW suggests their increased involvement in active ion transport. The tubular fluid has been shown to become progressively diluted along the distal tubule, the collecting tubule/collecting duct system (CT/CD) and the succeeding urinary bladder through Na^+ and Cl^- reabsorption (review in Hentschel and Elger, 1989). In SWS sea-bass, the ducts seemed to be less stained and the urinary bladder showed no staining, which helps to explain the lower Na^+/K^+-ATPase content and activity reported in kidney homogenates. At high salinities, the physiological need for ion retention decreases and instead the fish secrete/excrete ions (mainly divalent ions) in order to avoid ion invasion.

When comparing the FWS and FWU urinary systems, no apparent difference in Na^+/K^+-ATPase immunostaining was noted in the urinary tubule, duct and bladder between the two fish categories. But the enzyme abundance and activity were noticeably lower in FWU compared to FWS. The relative tubular density in the different sea-bass groups was thus investigated and found to be lower in the FWU than FWS. This observation means either a lower number or a lower length of nephrons in FWU than in FWS. Cell apoptosis might occur in the FWU kidney, as reported in the proximal tubules of *Cyprinus carpio* (Fischer and Dietrich, 2000). In the present study, no distinction was made between the different tubule sections, so which of these is likely to be less represented in FWU is therefore not known. The urinary tubules enclose at least proximal tubules I and II, as well as collecting tubules (Nebel et al., 2005), which have different functions (Hickman and Trump, 1969). The low blood osmolality in the FWU suggests a possible degeneration of the urinary tubules mainly involved in ion reabsorption. These may be the first proximal tubules in the sea-bass, since their cells possess a dense system of apical tubules, endocytotic vesicles and vacuoles, all suggesting a high activity of reabsorption (Nebel et al., 2005). These tubules are known to be the site of ion reabsorption in *Pseudopleuronectes americanus* (Elger et al., 1998). Ultrastructural observations of the FWU tubules are thus necessary.

We also note that the Na^+/K^+-ATPase content and activity were similar in SWS and FWU fish. The apparent similarity between these data may lead to the hypothesis that the proper (possibly hormonal) signal for FW adaptation was not given, or not received, at the kidney level, in the FWU sea-bass. However, Na^+/K^+-ATPase immunolocalization is quite different between SWS and FWU, particularly in the collecting ducts and the dorsal bladder. In addition, the renal tubule density is much lower in FWU than in SWS fish. Thus, according to these available data, the signal hypothesis may be ruled out.

The kidney, and particularly the distal segments, play an essential role in the regulation of water balance (Nishimura et al., 1983). Active ion reabsorption is often coupled to an osmotic water transport to the blood, depending on the water permeability of the urinary epithelium. The collecting ducts are lined by a membrane impermeable to water, and the rate of water transport depends on hormonal stimulation, on the presence of aquaporins and on the rate of water delivery by the nephrons (reviewed by Nishimura and Fan, 2003). In FW teleosts, the water permeability of the collecting ducts is very low (Nishimura et al., 1983). In FWU, an increased permeability of the collecting ducts to water cannot be ruled out, and might be another factor contributing to the decrease in blood osmolality.

In summary, the ion transporting pump is less abundant and less active in the FWU urinary system, because of a lower density of urinary tubules, possibly due to their partial degeneration. If the reabsorbing tubules are less numerous or damaged, this may cause, over several weeks, a decrease in the net active ion reabsorption from the filtrate, resulting in a net loss of ions by the organism. This altered function may in turn result in a decreased blood osmolality, which must be readjusted by ion absorption from the external medium by other osmoregulatory organs like the gills. This physiological attempt at osmoregulatory compensation would explain the high number and activity of the branchial chloride cells in active ion transports in FWU. During chronic exposure to FW, the branchial ion uptake is not sufficient to compensate for the urinary ion loss, resulting in an osmoregulatory imbalance leading to a decrease of the blood osmolality to critical levels and, finally, to the death of the FWU.

In the wild, a fraction of the juvenile sea-bass population is believed to undergo seasonal low salinity and/or to migrate to FW and there is indirect evidence of differential mortality (Lemaire et al., 2000). These differential mortalities observed in FW probably result from the existence, in the sea-bass stock from which our experimental animals were drawn, of a polymorphism for osmoregulatory capacity, as suggested by Allegrucci et al. (1994). The existence of this polymorphism and the ensuing selective pressure when juveniles enter brackish waters would explain the genetic divergence observed between marine and lagoon sea-bass samples in the western Mediterranean (Lemaire et al., 2000). Correlates of these differential physiological abilities at the genetic level will be investigated in future studies. It still remains to be determined whether individuals select their habitat as a function of their osmoregulatory ability (in this case, fish found upstream rivers would be assimilated to the FWS in our study), or passively undergo the selective mortalities following their migration to highly unpredictable environments such as lagoons, in which case local adaptation would be achieved at a high genetic cost.

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References

Allegrucci, G. C., Fortunato, C. S. and Sbordoni, V. (1994). Acclimatation to fresh water of the seabass: evidence of selective mortality of allozyme genotypes. In *Genetics and Evolution of Aquatic Organism* (ed. A. R. Beaumont), pp. 487-502. London: Chapman & Hall.

Barnabé, G. (1989). L'élevage du loup et de la daurade. In *Aquaculture,* vol. 2 (ed. G. Barnabé), pp. 675-720. Paris: Lavoisier Technique et Documentation.

Bindon, S. D., Gilmour, K. M., Fenwick, J. C. and Perry, S. F. (1994). The effects of branchial chloride cell proliferation on respiratory function in the rainbow trout *Oncorhynchus mykiss*. *J. Exp. Biol.* **197**, 47-63.

Bradford, M. M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Cataudella, S., Allegrucci, G., Bronzi, P., Cataldi, E., Cioni, C., Corti, M., Crosetti, D., De Merich, D., Fortunato, C., Garibaldi, L. et al. (1991). Multidisciplinary approach to the optimisation of sea bass (*Dicentrarchus labrax)* rearing in freshwater – Basic morpho-physiology and osmoregulation. *Aquacult. Env.* **14**, 56-57.

Dang, Z., Balm, P. H. M., Flik, G., Wendelaar Bonga, S. E. and Lock, R. A. C. (2000). Cortisol increases Na^+/K^+-ATPase density in plasma membranes of gill chloride cells in the freshwater tilapia *Oreochromis mossambicus. J. Exp. Biol.* **203**, 2349-2355.

Dantzler, W. H. (1992). Comparative aspects of renal function. In *The Kidney: Physiology and Pathophysiology* (ed. D. W. Seldin and G. Giebisch), pp. 885-942. New York: Raven Press.

Dendrinos, P. and Thorpe, J. P. (1985). Effects of reduced salinity on growth and body composition in the European bass *Dicentrarchus labrax* (L.). *Aquaculture* **49**, 333-358.

Elger, M., Werner, A., Herter, B., Kohl, B., Kinne, H. and Hentschel, H. (1998). Na-P_i cotransport sites in proximal tubule and collecting tubule of winter flounder (*Pleuronectes americanus*). *Am. J. Physiol.* **274**, F374-F383.

Eroldogan, O. T. and Kumlu, M. (2002). Growth performance, body traits and fillet composition of the european sea bass (*Dicentrarchus labrax*) reared in various salinities and freshwater. *Turk. J. Vet. Anim. Sci.* **26**, 993-1001.

Evans, D. H., Piermarini, P. M. and Potts, W. T. W. (1999). Ionic transport in the fish gill epithelium. *J. Exp. Biol.* **283**, 641-652.

Fischer, W. J. and Dietrich, D. R. (2000). Pathological and biochemical characterization of microcystin-induced hepatopancreas and kidney damage in carp (*Cyprinus carpio*). *Toxicol. Appl. Pharmacol.* **164**, 73-81.

Flik, G., Wendelaar Bonga, S. E. and Fenwick, J. C. (1983). Ca^{2+}-dependant phosphatase and ATPase activities in eel gill plasma membranes. I. Identification of Ca^{2+}-activated ATPase activities with non-specific phosphatase activities. *Comp. Biochem. Physiol.* **76B**, 745-754.

Franklin, C. E., Forster, M. E. and Davison, W. (1992). Plasma cortisol and osmoregulatory changes in sockeye salmon transferred to sea water: Comparison between successful and unsuccessful adaptation. *J. Fish Biol.* **41**, 113-122.

Greenwell, M. G., Sherrill, J. and Clayton, L. A. (2003). Osmoregulation in fish. Mechanisms and clinical implications. *Vet. Clin. Exot. Anim.* **6**, 169-189.

Hartl, M., Hutchinson, S., Hawkins, L. E. and Grand, D. J. (2001). Environmental levels of sediment-associated tri-n-butyltin chloride (TBTCI) and ionic regulation in flounders during seawater adaptation. *Mar. Biol.* **138**, 1121-1130.

Hentschel, H. and Elger, M. (1989). Morphology of glomerular and aglomerular kidneys. In *Comparative Physiology: Structure and Function of the Kidney,* vol. 1 (ed. R. K. H. Kinne), pp. 1-63. Basel: Karger.

Hickman, C. P. and Trump, B. F. (1969). The kidney. In *Fish Physiology*, vol. I (ed. W. S. Hoar and D. J. Randall), pp. 91-239. New York: Academic Press.

Imsland, A. K., Gunnarsson, S., Foss, A. and Stefansson, S. O. (2003). Gill Na+/K+-ATPase activity, plasma chloride and osmolality in juvenile turbot (*Scophthalmus maximus*) reared at different temperatures and salinities. *Aquaculture* **218**, 671-683.

Jackson, L. F., McCormick, S. D., Madsen, S. S., Swanson, P. and Sullivan, C. V. (2005). Osmoregulatory effects of hypophysectomy and homologous prolactin replacement in hybrid striped bass. *Comp. Biochem. Physiol.* **140**, 211-218.

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

Kelley, D. F. (1988). The importance of estuaries for sea-bass, *Dicentrarchus labrax* (L.). *J. Fish Biol.* **33**, 25-33.

Lahlou, B. (1967). Excrétion rénale chez un poisson euryhalin, le flêt (*Platichthys flesus* L.): caractéristiques de l'urine normale en eau douce et en eau de mer et effets des changements de milieu. *Comp. Biochem. Physiol.* **20**, 925-938.

Lasserre, P. (1971). Increase of (Na^++K^+)-dependent ATPase activity in gills and kidneys of two euryhaline marine teleosts, *Crenimugil labrosus* (Risso, 1826) and *Dicentrarchus labrax* (Linnaeus, 1758), during adaptation to fresh water. *Life Sci.* **10**, 113-119.

Lemaire, C., Allegrucci, G., Naciri, M., Bahri-Sfar, L., Kara, H. and Bonhomme, F. (2000). Do discrepancies between microsatellite and allozyme variation reveal differential selection between sea and lagoon in the sea bass (*Dicentrarchus labrax*)? *Mol. Ecol.* **9**, 457-467.

Lin, C. H., Tsai, R. S. and Lee, T. H. (2004). Expression and distribution of Na, K-ATPase in gill and kidney of the spotted green pufferfish, *Tetraodon nigroviridis*, in response to salinity challenge. *Comp. Biochem. Physiol.* **138A**, 287-295.

Lin, Y. M., Chen, C. N. and Lee, T. H. (2003). The expression of gill Na^+/K^+-ATPase in milkfish, *Chanos chanos*, acclimated to seawater, brackish water and freshwater. *Comp. Biochem. Physiol.* **135A**, 489-497.

Maillet, M. (1959). Modification de la technique de Champy au tetroxyde d'osmium-iodure de potassium. Résultats de son application à l'étude des fibres nerveuses. *CR Soc. Biol.* **153**, 939-940.

Marshall, W. S. (2002). Na^+, Cl^-, Ca^{2+} and Zn^{2+} transport by fish gills: retrospective review and prospective synthesis. *J. Exp. Zool.* **293**, 264-283.

McCormick, S. D., Saunders, R. L. and MacIntyre, A. D. (1989). Mitochondrial enzyme activity and ion regulation during parr-smolt transformation of Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **6**, 231-241.

Moustakas, C. T., Watanabe, W. O. and Copeland, K. A. (2004). Combined effetcs of photoperiod and salinity on growth, survival, and osmoregulatory ability of larval southern flounder *Paralichthys lethostigma*. *Aquaculture* **229**, 159-179.

Nebel, C., Nègre-Sadargues, G., Blasco, C. and Charmantier, G. (2005). Morpho-functional ontogeny of the urinary system of the European sea bass *Dicentrarchus labrax*. *Anat. Embryol.* **209**, 193-206.

Nishimura, H. and Fan, Z. (2003). Regulation of water movement across vertebrate renal tubules. *Comp. Biochem. Physiol.* **136A**, 479-498.

Nishimura, H., Imai, M. and Ogawa, M. (1983). Sodium chloride and water transport in the renal distal tubule of the rainbow trout. *Am. J. Physiol.* **244**, F247-F254.

Perry, S. F. (1998). Relationships between branchial chloride cells and gas transfer in freshwater fish. *Comp. Biochem. Physiol.* **119A**, 9-16.

Pickett, G. D. and Pawson, M. G. (1994). Biology and ecology. In *Sea Bass: Biology, Exploitation and Conservation* (ed. T. J. Pitcher), pp. 9-146. London: Chapman & Hall.

Pomport-Castillon, C., Gasc, C. and Romestand, B. (1997). Development, characterization and future prospects of monoclonal antibodies against spores of *Glugea atherinae* (protozoa-microsporidia-fish parasites). *J. Eukaryat. Microbiol.* **44**, 643-648.

Randall, D. J. and Brauner, C. (1998). Interactions between ion and gas transfer in freshwater teleost fish. *Comp. Biochem. Physiol.* **119A**, 3-8.

Sclafani, M., Stirling, G. and Leggett, W. C. (1997). Osmoregulation, nutritional effects and buoyancy of marine larval fish: a bioassay for assessing density changes during the earliest life-history stages. *Mar. Biol.* **129**, 1-9.

Shikano, T. and Fujio, Y. (1998). Immunolocalization of Na^+/K^+-ATPase and morphological changes in two types of chloride cells in the gill epithelium during seawater and freshwater adaptation in a euryhaline teleost, *Poecilia reticulata*. *J. Exp. Zool.* **281**, 80-89.

Sloman, K. A., Desforges, P. R. and Gilmour, K. M. (2001). Evidence for a mineralocorticoid-like receptor linked to branchial chloride cell proliferation in freshwater rainbow trout. *J. Exp. Biol.* **204**, 3953-3961.

Uchida, K., Kaneko, T., Yamauchi, K. and Hirano, T. (1996). Morphometrical analysis of chloride cell activity in the gill filaments and lamellae and changes in Na^+, K^+-ATPase activity during seawater adaptation in chum salmon fry. *J. Exp. Biol.* **276**, 193-200.

Varsamos, S., Connes, R., Diaz, J.-P., Barnabé, G. and Charmantier, G.

(2001). Ontogeny of osmoregulation in the European sea bass *Dicentrarchus labrax* L. *Mar. Biol.* **138**, 909-915.

Varsamos, S., Diaz, J.-P., Charmantier, G., Blasco, C., Connes, R. and Flik, G. (2002a). Location and morphology of chloride cells during the postembryonic development of the European sea bass, *Dicentrarchus labrax*. *Anat. Embryol.* **205**, 203-213.

Varsamos, S., Diaz, J.-P., Charmantier, G., Flik, G., Blasco, C. and Connes, R. (2002b). Branchial chloride cells in sea bass (*Dicentrarchus labrax)* adapted to fresh water, seawater, and doubly concentrated seawater. *J. Exp. Zool.* **293**, 12-26.

Varsamos, S., Nebel, C. and Charmantier, G. (2005). Ontogeny of osmoregulation in post-embryonic fish: a review. *Comp. Biochem. Physiol.* **141A**, 401-429.

Venturini, G., Cataldi, E., Marino, G., Pucci, P., Garibaldi, L., Bronzi, P. and Cataudella, S. (1992). Serum ions concentration and ATPase activity in gills, kidney and oesophagus of European sea bass (*Dicentrarchus labrax*, Pisces, Perciformes) during acclimation trials to fresh water. *Comp. Biochem. Physiol.* **103A**, 451-454.

Wilson, J. M. and Laurent, P. (2002). Fish gill morphology: Inside out. *J. Exp. Zool.* **293**, 192-213.

Zydlewski, J. and McCormick, S. D. (2001). Developmental and environmental regulation of chloride cells in young American shad *Alosa sapidissima*. *J. Exp. Biol.* **290**, 73-87.

Zydlewski, J., McCormick, S. D. and Kunkel, J. G. (2003). Late migration and seawater entry is physiologically disadvantageous for American shad juveniles. *J. Fish Biol.* **63**, 1521-1537.