

The $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter in the sea bass *Dicentrarchus labrax* during ontogeny: involvement in osmoregulation

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Summary

This study combines a cellular and molecular analysis of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) to determine the osmoregulatory role of this protein in different tissues during the ontogeny of the sea bass. We have characterized the complete sequence of the NKCC1 isoform isolated from the sea bass gills and have identified, by immunofluorescence, NKCC1, and other isoforms, within the epithelium of the major osmoregulatory organs. Different (absorptive and secretory) functions have been attributed to this protein according to the tissue and salinity. The effects of short- (1–4 days), medium- (7–21 days) and long (6 months)-term freshwater (FW) adaptations were investigated, in comparison with seawater (SW)-maintained sea bass. In adult sea bass after long-term adaptation to FW and SW, the gills had the highest expression of NKCC mRNA compared with the median/posterior kidney and to the posterior intestine. Expression of NKCC mRNA in the kidney was 95% (SW) and 63% (FW) lower, and in the intestine 98% (SW) and 77% (FW) lower. Compared to SW-maintained sea bass, long-term FW adaptation induced a significant 5.6-fold decrease in the branchial NKCC gene expression whereas the intestinal and renal expressions did not vary significantly. The cells of the intestine and collecting ducts as well as a part of the epithelium lining the urinary

bladder expressed NKCC apically. Within the gill chloride cells, NKCC was found basolaterally in SW-acclimated fish; some apically stained cells were detected after 7 days of FW exposure and their relative number increased progressively following FW acclimation. The appearance of FW-type chloride cells induces a functional shift of the gills from a secretory to an absorptive epithelium, which was only completed after long-term exposure to FW. Short- and medium-term exposure to FW induced a progressive decrease in total NKCC content and an increase in functionally different branchial chloride cells. During development, the cotransporter was already expressed in tegumentary ionocytes and along the digestive tract of late embryos. NKCC was recorded in the branchial chamber and along the renal collecting ducts in prelarvae and also in the dorsal part of the urinary bladder in larvae. The expression of NKCC along the osmoregulatory epithelial cells and the presence of Na^+/K^+ -ATPase within these cells contribute to the increase of the osmoregulatory capacity during sea bass ontogeny.

Key words: $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter, *Dicentrarchus labrax*, ontogeny, osmoregulation, immunofluorescence, quantitative expression.

Introduction

When exposed to salinity variations, teleosts adapt through osmoregulation, which results in the maintenance of their blood osmolality within a narrow range of ~280–350 mosmol kg^{-1} . Marine teleosts are subject to diffusive ion invasion and osmotic water loss. Their main osmoregulatory adaptations include the following. (1) In the digestive tract, high drinking rate of seawater (SW) followed by passive and active ion absorption, itself driving osmotic water intake that compensates dehydration; (2) in the excretory system, production of low volumes of isotonic urine; (3) in the gills,

active excretion of ions that compensate the ion load. In freshwater (FW), teleosts undergo ion loss and water invasion, to which they react through, (1) a low drinking rate; (2) production of a high volume of hypotonic urine following ion reabsorption along the excretory system; (3) active ion uptake through the gills (for reviews, see Evans, 1993; Evans et al., 1999; Varsamos et al., 2005). At the cellular level, ion transports are effected through ion transporting cells, called ionocytes. The ubiquitous Na^+/K^+ -ATPase, constantly located basolaterally in the cell, is one of the main enzymes involved in primary ion transport by creating an electrochemical

gradient. Different transmembrane proteins have been described that are involved in ion (ion channels, cotransporters) and water (aquaporins) exchanges following this gradient. The specificity, location (apical/basal) and the relative abundance and expression of these proteins within the ionocytes result either in ion absorption or excretion.

The $\text{Na}^+/\text{K}^+/2\text{Cl}^-$ cotransporter (NKCC) is one of these transmembrane proteins whose pattern of expression at different osmoregulatory sites is ultimately responsible for the adaptation of teleosts to salinity. The NKCC, a member of the chloride-cation cotransporter family, is widely distributed among different species of vertebrates (Haas, 1994; Gagnon et al., 2002). The coupled electrically neutral movement of sodium, potassium and chloride ions serves a number of different physiological functions according to the cell type. NKCC is generally recognized as playing a central role in cell volume homeostasis, maintenance of the electrolyte content and transepithelial ion and water movement in polarized cells (Russell, 2000; Cutler and Cramb, 2002). Two different isoforms of NKCC have been identified, the secretory isoform (NKCC1) and the absorptive isoform (NKCC2), located at the basolateral and apical sides of epithelial transporting cells, respectively. NKCC1 is the most widely distributed isoform whereas NKCC2 appears to be expressed in the kidney (Lytle et al., 1995) (review by Bachmann et al., 1999). In the present study, immunocytochemistry has been successfully used to locate NKCC, although a limitation of this technique lies in the relative nonspecificity of the antibody that recognizes both NKCC isoforms.

In fish, complete NKCC1 cDNAs have been cloned from the shark rectal gland (Xu et al., 1994), the gills of *Fundulus heteroclitus* (GenBank accession no. AY513737) and the intestine of *Anguilla anguilla* (Cutler and Cramb, 2002). Cutler and Cramb (Cutler and Cramb, 2002) have isolated two eel cDNAs, called NKCC1a and NKCC1b. NKCC1a is present in a wide range of tissues including the gills, intestine and kidney. Acclimation of yellow eels to SW induced an increase in NKCC1a expression in the gills, suggesting its involvement in branchial ion secretion. NKCC has been immunolocalized in the opercular (Marshall et al., 2002b) and branchial (Pelis et al., 2001; Cutler and Cramb, 2002; Tipsmark et al., 2002; McCormick et al., 2003) chloride cells (CC) of several teleosts. In the gills, NKCC mostly shows a basolateral cell location and its abundance increases after SW acclimation of salmonids (Pelis et al., 2001; Tipsmark et al., 2002), which shows its importance in hypo-osmoregulation through ion secretion.

As reported above, NKCC has been studied in the gills of various teleost species, but similar investigations are scarcer in the renal and intestinal epithelia (Suvitayavat et al., 1994; Cutler and Cramb, 2001; Masini et al., 2001; Marshall et al., 2002b). These studies have been mainly conducted in adults and very seldom in young fish or embryos (Pelis et al., 2001; Cutler and Cramb, 2002; Hiroi et al., 2005). As fish osmoregulate during their entire postembryonic life span (review in Varsamos et al., 2005), the localization of NKCC in the cells of different osmoregulatory organs must be studied

during the ontogeny of a teleost species. This is one of the objectives of the present study.

The European sea bass *Dicentrarchus labrax* is a euryhaline marine teleost, and the adults are able to tolerate salinities ranging from FW to hypersaline SW (Pickett and Pawson, 1994). The environmental salinity of its habitat varies during development. Sea bass hatch in the sea. The (pre)larvae first passively drift to coastal areas, then some juveniles move actively to nursery habitats in estuaries and lagoons where salinities vary rapidly and sometimes reach FW-like levels (Pickett and Pawson, 1994). Adult bass effect seasonal migrations in the sea or between the sea and lagoons/estuaries, the latter requiring a rapid shift in the osmoregulatory response (Pickett and Pawson, 1994).

At the cellular level, a high plasticity of the ion-transporting ability is thus necessary to cope with such environmental fluctuations. The salinity tolerance and osmoregulatory ability of this species vary according to the developmental stage. Previous studies have shown that sea bass are able to osmoregulate at very young stages with an increasing capacity in later stages (Varsamos et al., 2001). The resulting salinity tolerance provides an advantageous flexibility for the timing of migration towards lower salinity habitats. In sea bass, the salinity tolerance at least partially originates from the ion transporting activity of ionocytes (CC in the gills) rich in Na^+/K^+ -ATPase, first present at a high density in the tegument (Varsamos et al., 2002) and later in the gills, intestine and kidney (Nebel et al., 2005a). The basolateral Na^+/K^+ -ATPase generates a low intracellular Na^+ gradient, which may induce a transport of Na^+ , K^+ and Cl^- into the cell through the potential presence of a basolateral or apical NKCC cotransporter.

The objectives of this study, conducted in the sea bass *Dicentrarchus labrax* were thus to gather information on: (1) the molecular characterization of the branchial NKCC1 isoform in adult sea bass; (2) immunolocalization of the NKCC cotransporter (NKCC1 and other isoform/s) in the main osmoregulatory organs during ontogeny; (3) quantification of the expression of NKCC in adults after short-, medium- and long-term FW acclimation, in comparison to SW-maintained sea bass.

Materials and methods

Animals

Sea bass *Dicentrarchus labrax* L., at different developmental stages were obtained from the culture system at the Ifremer station at Palavas (Hérault, France). Following their transport to the Montpellier laboratory, they were maintained in 5 (prelarvae), 40 (larvae) and 3500 (adults) litre tanks. The constantly aerated water was either seawater (SW; $\approx 36\text{‰}$) from the Mediterranean or dechlorinated tap freshwater (FW; $\approx 0.3\text{‰}$). The ionic composition of FW in mEq l^{-1} 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). The waters were filtered through mechanical and biological filters (Eheim system; Europrix Aquariophilie, Lens, Pas-de-Calais, France). The different developmental stages and the

Table 1. *Developmental stages of D. labrax and salinities used in this study*

Developmental stage	Length (mass)	Age	Tested salinities
Embryos	–	0 days	SW
Prelarvae	3.5 mm	1 day	SW
	4 mm	3 days	SW
	5 mm	6 days	SW
Larvae	9 mm	30 days	SW
	18±2 cm (55±18 g)	14 months	SW + FW (long-term)
Adults	20±2 cm (118±47 g)	30 months	SW + FW (short- and medium-term)

SW, seawater; FW, freshwater.

tested salinities used in this study are reported in Table 1. For adults, the salinity transfers from SW to FW were either progressive (over 3 weeks) for observation of long-term (6 months) adaptation, or direct for observation of short-term (1–4 days) and medium-term (7–21 days) adaptation. Fish were anaesthetized in a solution of phenoxy 2 ethanol (0.3 ml l⁻¹) prior to any manipulation.

Cloning and sequencing of the branchial NKCC1

The gill arches from adult sea bass acclimatized to SW were excised and the branchial epithelium was isolated from scrapes of the gill arches using a sterile scalpel. Total RNA was extracted using the Trizol reagent (Invitrogen, Cergy Pontoise, Val d'Oise, France) according to the manufacturer's instructions and quantified based on the absorbance at 260 nm. Total RNA (2 µg) was treated with RNase-free DNase (Invitrogen) and the reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen) and an oligo(dT). The partial sequence was amplified by PCR using degenerate primers NKCCd (Table 2) the design of which was based on nucleotide blast alignments of the NKCC cotransporter from several species including *Oreochromis mossambicus* NKCC1α (AY513737), *Anguilla anguilla* NKCC1b (AJ486859), *Mus musculus* NKCC (U94518.1) and *Squalus acanthias* NKCC2 (AF521912.1). These original primers amplified a 1198 bp fragment. After cloning into TOPO TA Cloning vector (Invitrogen) and sequencing, several primers were designed. The 5' and 3' flanking regions were amplified using the RACE

method (Roche, Basel, Switzerland) using specific (Table 2) and non-specific primers (Roche). For the 3' end, 1 µg of branchial RNA was submitted to reverse transcription using the RACE dT anchor primer and M-MLV reverse transcriptase. 1 µl of the synthesized cDNA was submitted to amplification using the RACE anchor primer and the specific NKCC-3, -4, -5 or -6 forward primers under the following conditions: 1 cycle at 94°C, 5 min; 35 cycles at 94°C, 30 s; 55°C, 30 s; 72°C, 2 min, and a final cycle at 72°C, 5 min. For the 5' end, 1 µg of branchial RNA was submitted to reverse transcription using the specific NKCC1 reverse primer. The cDNA was purified (Invitrogen) and an A-tail was added to the 3' end of the purified cDNA using the terminal transferase (Promega, Charbonnières, Eure-et-Loire, France). 1 µl of cDNA was submitted to amplification using the specific NKCC-2 reverse primer and the SKdT anchor primer (Roche). The same amplification conditions were used as described above but the annealing temperature was fixed at 56°C. The PCR products of the 3' and 5' RACE were then cloned into TOPO TA cloning vector (Invitrogen) and sequenced.

Western immunoblots

According to the study of Lytle et al. (Lytle et al., 1995), the T4 antibody recognizes a well conserved epitope between different isoforms (NKCC1 and NKCC2) of distantly related animal species (e.g. shark NKCC1 vs human NKCC1). The use of the antibody is warranted by the fact that other researchers have used it to identify NKCC in teleosts (Wilson et al., 2000b; Marshall et al., 2002b; Tipsmark et al., 2002). Gill tissues were dissected from adult sea bass acclimated for 6 months to either FW or SW. The gill epithelium was scraped from arches I to IV from the left branchial chamber using a scalpel. The tissues were then homogenized on ice with a 1-ml Wheaton glass potter in 500 µl of ice-cold SEI buffer (0.3 mol l⁻¹ sucrose; 0.02 mol l⁻¹ EDTA; 0.1 mol l⁻¹ imidazole, pH 7.3) containing 75 µl of a mix of protease inhibitors (PI) (Complete™, Mini, EDTA-free, Boehringer Mannheim GmbH, Penzberg, Germany). The homogenate was then centrifuged at 2000 g for 5 min at 4°C. The pellet was resuspended in 200 µl of SEI-PI and centrifuged a second time. The pellets were resuspended and centrifuged a third time and the supernatants were retained. The protein content of the supernatants was determined using the Bradford method (Bradford, 1976) with a BSA (bovine

Table 2. *Primer sequences used in this study*

Primer name	Nucleotide sequences (from 5' to 3')
NKCCd forward	ATTCTGGCTGGWGCYAACAT
NKCCd reverse	TCATYTCTCCDTCCCTCCAG
NKCC1 reverse	AGAGAAACCCACATGTTGTA
NKCC2 reverse	GAATGTCAGGATGTAGCCTC
NKCC3 forward	TCAAGAACGACTGGAGGGAC
NKCC4 forward	GCCACCAGCCTCCAGAACAGC
NKCC5 forward	TCATCACTGCTGGAATCTT
NKCC6 forward	TGGAGGCGTTGTCTAAGGAC

The sequences used standard IUPAC code: W: A/T; D: A/G/T; Y: C/T.

serum albumin) standard. Similar amounts of proteins were separated under denaturing conditions on a 3% stacking and a 6% running polyacrylamide gel as described by Bollag et al. (Bollag et al., 1996). Proteins were then transferred for 2 h 15 min on a PVDF membrane (WESTRAM Clear Signal, Schleicher and Schuell, VWR, Val-de-Marne, France) using a semi-dry transfer apparatus (Bio-Rad, Marnes la Coquette, Hauts-de-Seine, France). Blots were blocked in 5% skimmed milk (SM)/PBS-Tween 20 (0.05%) for 3 h and then incubated with the primary antibody (T4; Iowa Hybridoma Bank, University of Iowa, USA) at $1 \mu\text{g ml}^{-1}$ in phosphate-buffered saline (PBS)-Tween 20 (0.05%) in SM (0.5%) overnight at 4°C . Following washing in PBS-Tween 20 (0.05%), membranes were incubated for 1 h at room temperature with the secondary antibody at $0.27 \mu\text{g ml}^{-1}$ (peroxidase-conjugated goat anti-mouse IgG (H+L); Jackson ImmunoResearch, Westgrove, PA, USA). The blots were washed and visualized in a peroxidase substrate (4-chloro-1-naphthol; Sigma-Aldrich, St Louis, MO, USA) for 20 min and finally stored in distilled water.

Immunocytochemistry

Whole eggs, prelarvae, larvae and organs dissected from adults (following anaesthesia) were immersed into Bouin's liquid for 24 to 48 h, washed and dehydrated in an ascending ethanol series for embedding in Paraplast. Prior to embedding, the eggs were incised and the vitellus was removed. Longitudinal and transverse $4 \mu\text{m}$ -thick sections were cut on a Leitz microtome and transferred onto glass slides. The slides were immersed in 0.01% Tween 20, 150 mmol l^{-1} NaCl in 10 mmol l^{-1} phosphate-buffered saline (PBS), pH 7.3 for 10 min. After saturation in 5% SM in PBS at 37°C for 20 min, the SM powder was removed by rinsing the slides twice with PBS. The slides were incubated for 2 h at room temperature in a moist chamber with the monoclonal mouse antibody (T4; Iowa Hybridoma Bank, University of Iowa, IA, USA) at a concentration of $12 \mu\text{g ml}^{-1}$ in 0.5% SM/PBS. This antibody was directed against the carboxyl terminus of the human colonic $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) and has been shown to bind to several isoforms of NKCC across several species (Lytle et al., 1995). Control sections were subjected to the same conditions, but without the monoclonal antibody. After rinsing in PBS, all sections were incubated for 1 h with the fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse antibody (Jackson ImmunoResearch Labs) at $6.7 \mu\text{g ml}^{-1}$. The slides were washed, mounted with anti-bleaching mounting medium and rapidly examined with a Leica Diaplan microscope equipped for fluorescence with the appropriate filter set (filters of 450 nm to 490 nm) and coupled to a Leica DC 300F digital camera and FW4000 Software.

Dot blots

Dot blots were realized on gills of sea bass maintained in SW and following short-term (1 to 4 days) or medium-term (7 to 22 days) acclimation to FW, in order to compare the relative NKCC protein expressions ($N=3-5$). Immunoblotting

was carried out according to the method used by Nebel et al. (Nebel et al., 2005b) with modifications. The gill samples were prepared as described above. The supernatants were assayed for protein content by the Bradford method (Bradford, 1976). The samples were diluted in PBS-PI in SM (0.5%) and $2 \mu\text{l}$ of proteins were applied in triplicate to two $0.45 \mu\text{m}$ -thick nitrocellulose membranes (BioRad). After saturation in 5% SM in PBS at 37°C for 30 min, the SM powder was removed by twice washing the membranes with PBS. The strips of one membrane were incubated for 2 h with the monoclonal T4 mouse antibody (diluted at $3 \mu\text{g ml}^{-1}$ in 0.5% SM-PBS). The second membrane was incubated in the same conditions without the first antibody. After washing, the avidine peroxidase conjugate (Pierce Interchim; Rockford, IL, USA) at $2 \mu\text{g ml}^{-1}$ was added to the membranes for 1 h 30 min. The membranes were washed and the fractions were developed with α 2-chloro-naphthol acetate (Sigma-Aldrich, Lyon, Switzerland). Colour development was stopped by rinsing the membranes with distilled water. The membranes were dried at room temperature and rapidly scanned. The immunoblots were analyzed using the Scion Image Software and the colour intensity was measured for each blot. For semiquantification, the negative standards (without the primary antibody) were deducted from each sample. For statistical comparisons, SW samples ($N=3$) were compared to samples from short-term ($N=4$) and medium-term ($N=5$) acclimated sea bass.

Quantification of NKCC expression by real-time PCR

The posterior intestine, the median/posterior kidney and the gills were dissected from 10 adult sea bass following acclimation for 6 months in SW and FW. Total RNA was extracted from the three organs using the TRIzol reagent (Invitrogen) according to the manufacturer's instruction and quantification of RNA was based on the absorbance at 260 nm. After verification of the integrity of the RNA samples on the gel, $2 \mu\text{g}$ of total RNA were treated with RNase-free DNase (Invitrogen) to remove any genomic DNA contamination. The reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using M-MLV reverse transcriptase (Invitrogen) and an oligo(dT) primer. The NKCC5 (forward) and NKCC1 (reverse) primer were then used to generate a PCR product of 346 bp. The results were normalized with the elongation factor EF1 α . This housekeeping gene has been validated in other species (Frost and Nilsen, 2003). The forward (EF1 α -F) and reverse (EF1 α -R) primers of the elongation factor (provided by S. Varsamos) generated a PCR product of 239 bp. Water was used as negative control in the real-time PCR. A mix of the following reaction components was prepared as follows (final concentrations): $5.5 \mu\text{l}$ of water, $1 \mu\text{l}$ of forward primer ($0.5 \mu\text{mol l}^{-1}$), $1 \mu\text{l}$ of reverse primer ($0.5 \mu\text{mol l}^{-1}$), $2 \mu\text{l}$ of the Mastermix FastStart DNA Master^{PLUS} SYBR Green I (Roche Applied Science, Basel, Switzerland). The LightCycler glass capillaries were filled with $9.5 \mu\text{l}$ of the mix, and $0.5 \mu\text{l}$ of cDNA was added as PCR template. The cycling conditions were: denaturation program

(95°C for 10 min), amplification, hybridization and elongation programs repeated 40 times (95°C for 15 s; 60°C for 5 s; 72°C for 10 s), melting curve program (60°C for 1 min) and a final cooling step of 30 s to 40°C. For each reaction, the crossing point (CP) was determined according to the 'Fit Point Method' of the LightCycler Software, version 3.5 (Roche Molecular Biochemicals). All samples were analyzed in triplicates and the mean CP was calculated. Standard curves were generated for each primer set to calculate the amplification efficiencies (E) from the given slope according to the equation $E=10^{(-1/\text{slope})}$. According to the method described in Scott et al. (Scott et al., 2004), the absolute mRNA expression was semiquantitatively estimated using the formula E^{-CP} . The results were normalized to the estimated absolute expression of EF1 α in order to compare the expression levels between different organs and salinities.

Statistical comparisons

Results are expressed as the mean \pm standard deviation (s.d.). Student's *t*-tests were used for statistical comparisons of mean values.

Results

Molecular characterization of NKCC1 in the gills

The RACE PCR generated a nucleotide coding sequence of 3578-bp which has been registered with GenBank (accession no. AY954108). The deduced 1161-residue protein had a predicted molecular mass of about 126.3 kDa (Expasy-TrEMBL). The alignment of the NKCC amino acid sequence with NKCC1 sequences of other vertebrates is shown in Fig. 1. The amino acid homologies of the sea bass NKCC1 with other species is shown in Table 3. Hydropathy analysis (Kyte and Doolittle, 1982) showed a central hydrophobic region flanked by the large hydrophilic N and C termini. The 12 putative transmembrane TM domains of the NKCC are indicated in Fig. 1. The potential N-linked glycosylation consensus sites between the TM domains 7 and 8 are conserved in the sea bass (Fig. 1, bold N at positions 494 and 504) suggesting that the sea bass NKCC1 could be a glycosylated protein. The interacting sequence of the NKCC1 amino-terminal with the stress-related kinases SPAK and OSR1 is indicated in Fig. 1 (Piechotta et al., 2002).

Table 3. Percentage amino acid homology of the branchial sea-bass NKCC1 cotransporter sequence with other vertebrates

Species/isoform	Amino acid homology
<i>Oreochromis mossambicus</i> NKCC1 α	88%
<i>Anguilla anguilla</i> NKCC1a	84%
<i>Rattus norvegicus</i> NKCC1	71%
<i>Mus musculus</i> NKCC1	70%

Homologies were calculated according to the NCBI blast.

Western blot detection of branchial NKCC in adult *D. labrax* exposed to seawater and freshwater

Western blots were performed on branchial homogenates in adult sea bass following long-term exposure to SW and FW (Fig. 2). A major immunoreactive band of about 225 kDa was detected in the gills of adult sea bass acclimated to SW. Two additional lower molecular mass bands, at about 110 kDa and 120 kDa, also were detected (Fig. 2, lane A). In FW-adapted sea bass gills, the same bands were detected, but the 225 kDa and to a lesser extent the 120/110 kDa bands were weaker than those from the SW-adapted fish (Fig. 2, lane B).

Distribution of NKCC in *D. labrax* organs during the ontogeny

NKCC was immunolocalized in embryonic (Fig. 3), prelarval (Fig. 3) and larval (Figs 4, 5) sea bass. In late embryos, the cotransporter was localized in the apical region of the digestive tract cells and basolaterally in the tegumentary ionocytes (Fig. 3A). After hatching, the operculum developed and a few immunostained opercular cells were present in 1-day-old prelarvae (Fig. 3B). A few immunoreactive cells were also visible along the branchial slits (Fig. 3B). In the opercular and branchial cells, immunostaining appeared evenly distributed.

In 3-day-old prelarvae, the intestine showed a fluorescence restricted to the apical brush-border membrane, revealed in longitudinal (Fig. 3C) and transverse (Fig. 3C') sections. No immunostaining was observed in the anterior and median parts of the kidney, but the collecting ducts presented a weak apical staining in posterior sections of the kidney (Fig. 3D).

In 6-day-old larvae (Fig. 4), the epithelium of the branchial chamber presented some immunopositive cells located at the junction with the operculum (Fig. 4A). A few immunopositive chloride cells (CC) were already present on the developing branchial filaments (Fig. 4A). The oesophagus was not immunofluorescent (not illustrated) and the intestinal cells showed an apical immunostaining, more diffuse than in previous stages (Fig. 4B). Stained tegumentary cells were still present at this developmental stage. In the posterior kidney, the

Fig. 1. Amino acid alignment of the NKCC1 cotransporter from sea bass, other teleosts, *Oreochromis mossambicus* (NCBI accession no. AAR97731), *Anguilla anguilla* (NKCC1 α , NCBI accession no. CAD31111) and mammals, *Rattus norvegicus* (NCBI accession no. AAC27557), *Mus musculus* (NCBI accession no. AAC778332). Amino acid positions are numbered on the right. The 12 putative transmembrane TM domains are bold-underlined (Delpire et al., 1994); dashes indicate where spaces have been added to improve alignment; asterisks indicate positions where alignments show complete conservation between species; colons and semicolons indicate conserved and semi-conserved nucleotide substitution, respectively. Potential N-linked glycosylation sites (bold N, positions 494, 504, 792, 951) are indicated. The potential phosphorylation sites (underlined) were determined by NetPhos 2.0 (Blom et al., 1999). The potential site of SPAK and OSR1 interaction (Piechotta et al., 2002) is indicated (bold, italic letters).

Sea-bass Tilapia Bel Rat Mouse	AFILIAQNVIAPIISNFIASVALINFSVPHASLANSPGWRPFSFKYNNMVSLAGAILC 666 AFILIAQNVIAPIISNFIASVALINFSVPHASLANSPGWRPFSFKYNNMVSLAGAILC 666 AFILIAQNVIAPIISNFIASVALINFSVPHASLANSPGWRPFSFKYNNMVSLAGAILC 666 AFILIAQNVIAPIISNFIASVALINFSVPHASLANSPGWRPFSFKYNNMVSLAGAILC 666 AFILIAQNVIAPIISNFIASVALINFSVPHASLANSPGWRPFSFKYNNMVSLAGAILC 666 AFILIAQNVIAPIISNFIASVALINFSVPHASLANSPGWRPFSFKYNNMVSLAGAILC 666	666 666 666 666 666 666
Sea-bass Tilapia Bel Rat Mouse	CVVMPFNNSAALLTNNVI VLGILFYIYSYKPKDVMNNGSSTQALTYHQALHTLTLHLSGVEDH 726 CVVMPFNNSAALLTNNVI VMAIYIYSYHKKPDMNNGSSTQALTYHQALHTLTLHLSGVEDH 716 CVVMPFNNSAALLTNNVI VMAIYIYSYHKKPDMNNGSSTQALTYHQALHTLTLHLSGVEDH 716 CVVMPFNNSAALLTNNVI VMAIYIYSYHKKPDMNNGSSTQALTYHQALHTLTLHLSGVEDH 716 CVVMPFNNSAALLTNNVI VMAIYIYSYHKKPDMNNGSSTQALTYHQALHTLTLHLSGVEDH 716 CVVMPFNNSAALLTNNVI VMAIYIYSYHKKPDMNNGSSTQALTYHQALHTLTLHLSGVEDH 716	726 716 716 716 716 716
Sea-bass Tilapia Bel Rat Mouse	VKNRFPQCLWMTGPNRSPRALLLDLIVHSFTKNVGLMCGHVFTGYRRPNFKDLATDQARYQ 786 VKNRFPQCLWMTGPNRSPRALLLDLIVHSFTKNVGLMCGHVFTGYRRPNFKDLATDQARYQ 786 VKNRFPQCLWMTGPNRSPRALLLDLIVHSFTKNVGLMCGHVFTGYRRPNFKDLATDQARYQ 786 VKNRFPQCLWMTGPNRSPRALLLDLIVHSFTKNVGLMCGHVFTGYRRPNFKDLATDQARYQ 786 VKNRFPQCLWMTGPNRSPRALLLDLIVHSFTKNVGLMCGHVFTGYRRPNFKDLATDQARYQ 786 VKNRFPQCLWMTGPNRSPRALLLDLIVHSFTKNVGLMCGHVFTGYRRPNFKDLATDQARYQ 786	786 786 786 786 786 786
Sea-bass Tilapia Bel Rat Mouse	RWLLKNEKAFYTFVFAEDMROGTQYLLQAAAGLGRKPKNTLVLGFKNDWRGDMWNVETY 846 RWLLKNEKAFYTFVFAEDMROGTQYLLQAAAGLGRKPKNTLVLGFKNDWRGDMWNVETY 846 RWLLKNEKAFYTFVFAEDMROGTQYLLQAAAGLGRKPKNTLVLGFKNDWRGDMWNVETY 846 RWLLKNEKAFYTFVFAEDMROGTQYLLQAAAGLGRKPKNTLVLGFKNDWRGDMWNVETY 846 RWLLKNEKAFYTFVFAEDMROGTQYLLQAAAGLGRKPKNTLVLGFKNDWRGDMWNVETY 846 RWLLKNEKAFYTFVFAEDMROGTQYLLQAAAGLGRKPKNTLVLGFKNDWRGDMWNVETY 846	846 846 846 846 846 846
Sea-bass Tilapia Bel Rat Mouse	ISWIYDAFDFQFGAVILRLKLEGLDVSHIQODGDSLSQKSSGKMDVIVSITDKSDSDG 906 ISWIYDAFDFQFGAVILRLKLEGLDVSHIQODGDSLSQKSSGKMDVIVSITDKSDSDG 906 ISWIYDAFDFQFGAVILRLKLEGLDVSHIQODGDSLSQKSSGKMDVIVSITDKSDSDG 906 ISWIYDAFDFQFGAVILRLKLEGLDVSHIQODGDSLSQKSSGKMDVIVSITDKSDSDG 906 ISWIYDAFDFQFGAVILRLKLEGLDVSHIQODGDSLSQKSSGKMDVIVSITDKSDSDG 906 ISWIYDAFDFQFGAVILRLKLEGLDVSHIQODGDSLSQKSSGKMDVIVSITDKSDSDG 906	906 906 906 906 906 906
Sea-bass Tilapia Bel Rat Mouse	SSKFSKATLQNSPFAIQKDDDDKRAITQPLKLDKDKSPTVPLNVSQRLLLEASQFQK 966 SSKFSKATLQNSPFAIQKDDDDKRAITQPLKLDKDKSPTVPLNVSQRLLLEASQFQK 966 SSKFSKATLQNSPFAIQKDDDDKRAITQPLKLDKDKSPTVPLNVSQRLLLEASQFQK 966 SSKFSKATLQNSPFAIQKDDDDKRAITQPLKLDKDKSPTVPLNVSQRLLLEASQFQK 966 SSKFSKATLQNSPFAIQKDDDDKRAITQPLKLDKDKSPTVPLNVSQRLLLEASQFQK 966 SSKFSKATLQNSPFAIQKDDDDKRAITQPLKLDKDKSPTVPLNVSQRLLLEASQFQK 966	966 966 966 966 966 966
Sea-bass Tilapia Bel Rat Mouse	KGGKGTVDVWMLFDDGGGLTLLPVLPLNKKRWDCKIRVFIGGKINRIDHRRAMATLLS 1026 KGGKGTVDVWMLFDDGGGLTLLPVLPLNKKRWDCKIRVFIGGKINRIDHRRAMATLLS 1026 KGGKGTVDVWMLFDDGGGLTLLPVLPLNKKRWDCKIRVFIGGKINRIDHRRAMATLLS 1026 KGGKGTVDVWMLFDDGGGLTLLPVLPLNKKRWDCKIRVFIGGKINRIDHRRAMATLLS 1026 KGGKGTVDVWMLFDDGGGLTLLPVLPLNKKRWDCKIRVFIGGKINRIDHRRAMATLLS 1026 KGGKGTVDVWMLFDDGGGLTLLPVLPLNKKRWDCKIRVFIGGKINRIDHRRAMATLLS 1026	1026 1026 1026 1026 1026 1026
Sea-bass Tilapia Bel Rat Mouse	KPRIDFSDIIVLGDINTKPKKHHVAFBELEPYRLEKDDMEQFAAERLNKNSFPWRITDN 1086 KPRIDFSDIIVLGDINTKPKKHHVAFBELEPYRLEKDDMEQFAAERLNKNSFPWRITDN 1086 KPRIDFSDIIVLGDINTKPKKHHVAFBELEPYRLEKDDMEQFAAERLNKNSFPWRITDN 1086 KPRIDFSDIIVLGDINTKPKKHHVAFBELEPYRLEKDDMEQFAAERLNKNSFPWRITDN 1086 KPRIDFSDIIVLGDINTKPKKHHVAFBELEPYRLEKDDMEQFAAERLNKNSFPWRITDN 1086 KPRIDFSDIIVLGDINTKPKKHHVAFBELEPYRLEKDDMEQFAAERLNKNSFPWRITDN 1086	1086 1086 1086 1086 1086 1086
Sea-bass Tilapia Bel Rat Mouse	EELEYRAKNTNRQIRLANELLKHSSTANLIIWMSLPLARKGAVSSALYMWLEALS KDLPP1 1146 EELEYRAKNTNRQIRLANELLKHSSTANLIIWMSLPLARKGAVSSALYMWLEALS KDLPP1 1146 EELEYRAKNTNRQIRLANELLKHSSTANLIIWMSLPLARKGAVSSALYMWLEALS KDLPP1 1146 EELEYRAKNTNRQIRLANELLKHSSTANLIIWMSLPLARKGAVSSALYMWLEALS KDLPP1 1146 EELEYRAKNTNRQIRLANELLKHSSTANLIIWMSLPLARKGAVSSALYMWLEALS KDLPP1 1146 EELEYRAKNTNRQIRLANELLKHSSTANLIIWMSLPLARKGAVSSALYMWLEALS KDLPP1 1146	1146 1146 1146 1146 1146 1146
Sea-bass Tilapia Bel Rat Mouse	LLVRGNHQSVLTFYS 1161 LLVRGNHQSVLTFYS 1151 LLVRGNHQSVLTFYS 1158 LLVRGNHQSVLTFYS 1203 LLVRGNHQSVLTFYS 1205	1161 1151 1158 1203 1205
Sea-bass Tilapia Bel Rat Mouse	-----MSAPSPTSS-APAESAAA-----ENDF 21 -----MSAPSPTSS-APAESAAA-----ENDF 21 -----MSAPSPTSS-APAESAAA-----ENDF 21 -----MSAPSPTSS-APAESAAA-----ENDF 21 -----MSAPSPTSS-APAESAAA-----ENDF 21 -----MSAPSPTSS-APAESAAA-----ENDF 21	21 21 21 21 21 21
Sea-bass Tilapia Bel Rat Mouse	LAPDAGLKPPEGTTPSQRFRQVLDLVAEAAQTEKEDSKSPSDASITVPSDKKATPTPADSDQGP 81 LAPDAGLKPPEGTTPSQRFRQVLDLVAEAAQTEKEDSKSPSDASITVPSDKKATPTPADSDQGP 81 LAPDAGLKPPEGTTPSQRFRQVLDLVAEAAQTEKEDSKSPSDASITVPSDKKATPTPADSDQGP 81 LAPDAGLKPPEGTTPSQRFRQVLDLVAEAAQTEKEDSKSPSDASITVPSDKKATPTPADSDQGP 81 LAPDAGLKPPEGTTPSQRFRQVLDLVAEAAQTEKEDSKSPSDASITVPSDKKATPTPADSDQGP 81 LAPDAGLKPPEGTTPSQRFRQVLDLVAEAAQTEKEDSKSPSDASITVPSDKKATPTPADSDQGP 81	81 81 81 81 81 81
Sea-bass Tilapia Bel Rat Mouse	S-DPAGGEBAKGRFVNFADPSGAGSA--ASPEG-----PADLQNG-DTVMSB-T 129 S-DPAGGEBAKGRFVNFADPSGAGSA--ASPEA-----PTMGQNG-DTVMSB-T 119 GPEPFAANGEAKGRFVNFADPSGAGSA--SPDVA-----PAEGQNG-DTVMSB-T 129 ESGVAKGSEBAKGRFVNFADPSGAGSA--SSDLSDAAGVGDGPNVFSQNGGDTVLSEGS 176 ESGVAKGSEBAKGRFVNFADPSGAGSA--SSDLSDAAGVGDGPNVFSQNGGDTVLSEGS 178	129 119 129 176 178
Sea-bass Tilapia Bel Rat Mouse	SLHSGTGGQHH--YHYDTHNTYLRFTGHNTIDAVPNIDFRQTAAPLGRKLRPTLSE 187 SLHSGTGGQHH--YHYDTHNTYLRFTGHNTIDAVPNIDFRQTAAPLGRKLRPTLSE 177 SLHSGTGGQHH--YHYDTHNTYLRFTGHNTIDAVPNIDFRQTAAPLGRKLRPTLSE 187 SLHSGTGGQHH--YHYDTHNTYLRFTGHNTIDAVPNIDFRQTAAPLGRKLRPTLSE 187 SLHSGTGGQHH--YHYDTHNTYLRFTGHNTIDAVPNIDFRQTAAPLGRKLRPTLSE 187 SLHSGTGGQHH--YHYDTHNTYLRFTGHNTIDAVPNIDFRQTAAPLGRKLRPTLSE 187	187 177 187 187 187 187
Sea-bass Tilapia Bel Rat Mouse	LHDELEKPEDFGANGDELTPAEAAKESKGVYKGVKGVLRVRCMLNIGWMLF 247 LHDELEKPEDFGANGDELTPAEAAKESKGVYKGVKGVLRVRCMLNIGWMLF 237 LHDELEKPEDFGANGDELTPAEAAKESKGVYKGVKGVLRVRCMLNIGWMLF 247 LHDELEKPEDFGANGDELTPAEAAKESKGVYKGVKGVLRVRCMLNIGWMLF 247 LHDELEKPEDFGANGDELTPAEAAKESKGVYKGVKGVLRVRCMLNIGWMLF 247 LHDELEKPEDFGANGDELTPAEAAKESKGVYKGVKGVLRVRCMLNIGWMLF 247	247 237 247 247 247 247
Sea-bass Tilapia Bel Rat Mouse	IRMSVIVGQAGIHALCVIVAMATVVTTITGLSTSAITANGFRGGGAYLISRSGLPFGF 307 IRMSVIVGQAGIHALCVIVAMATVVTTITGLSTSAITANGFRGGGAYLISRSGLPFGF 297 IRMSVIVGQAGIHALCVIVAMATVVTTITGLSTSAITANGFRGGGAYLISRSGLPFGF 307 IRMSVIVGQAGIHALCVIVAMATVVTTITGLSTSAITANGFRGGGAYLISRSGLPFGF 307 IRMSVIVGQAGIHALCVIVAMATVVTTITGLSTSAITANGFRGGGAYLISRSGLPFGF 307 IRMSVIVGQAGIHALCVIVAMATVVTTITGLSTSAITANGFRGGGAYLISRSGLPFGF 307	307 297 307 307 307 307
Sea-bass Tilapia Bel Rat Mouse	GSIGLIFAPANAVAMVYVGFABTVELLQAGVDVMTDEINDIRIGITITVILLGHSV 367 GSIGLIFAPANAVAMVYVGFABTVELLQAGVDVMTDEINDIRIGITITVILLGHSV 357 GSIGLIFAPANAVAMVYVGFABTVELLQAGVDVMTDEINDIRIGITITVILLGHSV 367 GSIGLIFAPANAVAMVYVGFABTVELLQAGVDVMTDEINDIRIGITITVILLGHSV 367 GSIGLIFAPANAVAMVYVGFABTVELLQAGVDVMTDEINDIRIGITITVILLGHSV 367 GSIGLIFAPANAVAMVYVGFABTVELLQAGVDVMTDEINDIRIGITITVILLGHSV 367	367 357 367 367 367 367
Sea-bass Tilapia Bel Rat Mouse	AGMWEAKAQIFLVLVITAIINVFICHTPI SVSKBEPFGYVDSIMWENMGDFR-GE 426 AGMWEAKAQIFLVLVITAIINVFICHTPI SVSKBEPFGYVDSIMWENMGDFR-GE 416 AGMWEAKAQIFLVLVITAIINVFICHTPI SVSKBEPFGYVDSIMWENMGDFR-GE 426 AGMWEAKAQIFLVLVITAIINVFICHTPI SVSKBEPFGYVDSIMWENMGDFR-GE 426 AGMWEAKAQIFLVLVITAIINVFICHTPI SVSKBEPFGYVDSIMWENMGDFR-GE 426 AGMWEAKAQIFLVLVITAIINVFICHTPI SVSKBEPFGYVDSIMWENMGDFR-GE 426	426 416 426 426 426 426
Sea-bass Tilapia Bel Rat Mouse	TFFSVFAIFFAAQTGILAGANISGDLADPOQAIPRGTLLAALLTGVIVGAVSTGSCIV 486 TFFSVFAIFFAAQTGILAGANISGDLADPOQAIPRGTLLAALLTGVIVGAVSTGSCIV 476 TFFSVFAIFFAAQTGILAGANISGDLADPOQAIPRGTLLAALLTGVIVGAVSTGSCIV 487 TFFSVFAIFFAAQTGILAGANISGDLADPOQAIPRGTLLAALLTGVIVGAVSTGSCIV 486 TFFSVFAIFFAAQTGILAGANISGDLADPOQAIPRGTLLAALLTGVIVGAVSTGSCIV 487 TFFSVFAIFFAAQTGILAGANISGDLADPOQAIPRGTLLAALLTGVIVGAVSTGSCIV 486	486 476 487 486 487 486
Sea-bass Tilapia Bel Rat Mouse	RDAQSHVNDTVSQQFANCTDAA CNFQYDFITCKSENSCRVGLHNDFOVMSLVSGRGP11 546 RDAQSHVNDTVSQQFANCTDAA CNFQYDFITCKSENSCRVGLHNDFOVMSLVSGRGP11 536 RDAQSHVNDTVSQQFANCTDAA CNFQYDFITCKSENSCRVGLHNDFOVMSLVSGRGP11 543 RDAQSHVNDTVSQQFANCTDAA CNFQYDFITCKSENSCRVGLHNDFOVMSLVSGRGP11 543 RDAQSHVNDTVSQQFANCTDAA CNFQYDFITCKSENSCRVGLHNDFOVMSLVSGRGP11 543 RDAQSHVNDTVSQQFANCTDAA CNFQYDFITCKSENSCRVGLHNDFOVMSLVSGRGP11 543	546 536 543 543 543 543
Sea-bass Tilapia Bel Rat Mouse	TAGIFSATLSSALASLVSAPKVFQALCKNDLIPGLGMFAKGYGNNEPLRGYLLITFLIAL 606 TAGIFSATLSSALASLVSAPKVFQALCKNDLIPGLGMFAKGYGNNEPLRGYLLITFLIAL 596 TAGIFSATLSSALASLVSAPKVFQALCKNDLIPGLGMFAKGYGNNEPLRGYLLITFLIAL 603 TAGIFSATLSSALASLVSAPKVFQALCKNDLIPGLGMFAKGYGNNEPLRGYLLITFLIAL 654 TAGIFSATLSSALASLVSAPKVFQALCKNDLIPGLGMFAKGYGNNEPLRGYLLITFLIAL 656 TAGIFSATLSSALASLVSAPKVFQALCKNDLIPGLGMFAKGYGNNEPLRGYLLITFLIAL 656	606 596 603 654 656 656

Fig. 1. See previous page for legend.

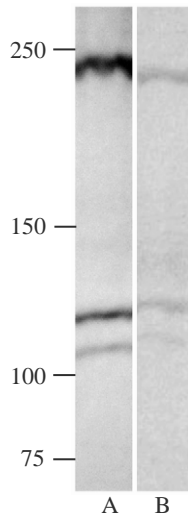


Fig. 2. Western blot analysis of the branchial $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) in *D. labrax* after long-term acclimation to (A) seawater and (B) freshwater. Molecular masses in kDa are indicated on the left.

renal collecting tubules were not stained (Fig. 4B), however, the collecting ducts (Fig. 4C,D) and the dorsal part of the urinary bladder (Fig. 4D,E) were stained apically.

In 30-day-old larvae (Fig. 5), the gill filaments and lamellae were differentiated (Fig. 5A), but only the filaments showed CC, characterized by an intense basolateral NKCC staining in SW-acclimatized fish (Fig. 5A,A'). The branchial chamber and the opercular epithelium were lined by some similarly stained immunoreactive cells (Fig. 5A). In the anterior intestine,

immunostaining was mostly apical (Fig. 5B,C). In some parts of the intestine, immunostaining was slightly diffuse in the apical and subapical parts of the cell (Fig. 5B), and in other parts, immunostaining was localized on the apical brush-border only, particularly in the dorsal region of the intestine (Fig. 5C,D). The renal collecting tubules were not immunostained whereas the ducts and the dorsal part of the urinary bladder were intensely stained apically (Fig. 5D,D',E,F).

*NKCC protein immunolocalization and expression in adult *D. labrax* according to salinity*

The effects of long- (Fig. 6), medium- and short-term (Figs 7, 8) exposure to FW on the localization and expression of the NKCC protein in adult sea bass were investigated.

Long-term adaptation of sea bass to seawater and freshwater

In sea bass exposed long-term to either SW (Fig. 6A) or FW (Fig. 6B) immunostaining for NKCC was localized along the brush-border of the anterior intestine. Immunoreactivity appeared higher in enterocytes of SW-acclimated fish. In a few single cells, probably mucous cells, a basolateral staining was recorded in SW as well as in FW fish, but the identification of these cells has to be confirmed (Fig. 6A,B). In the kidney, NKCC was localized in the subapical and apical part of the cells lining the collecting ducts of both groups of fish (Fig. 6C,D,D'). Immunostaining seemed more intense in FW than in SW fish. In the gills of fish exposed to SW, the CC showed intense basolateral immunostaining (Fig. 6E,E'). The long-term FW acclimatized fish showed a thickening of the filamentary epithelium from about 15 to 30 μm due to an increase in the number of CC (Fig. 6E,F). A shift of the NKCC localization

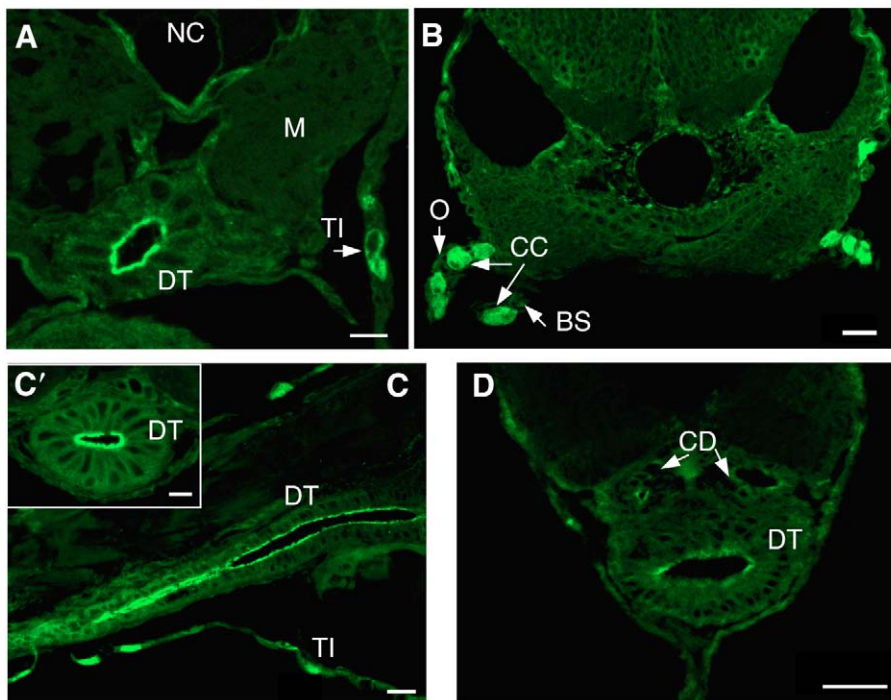


Fig. 3. Distribution of NKCC during the ontogeny of *Dicentrarchus labrax*. of (A) Embryo; (B) 1-day-old prelarva; (C,C',D) 3-day-old prelarvae. (A) Transverse section showing apical staining in the cells lining the digestive tract (DT) and basolateral staining in the tegumentary ionocytes (TI). (B) Transverse section of the developing branchial chamber, showing immunostained chloride cells (CC) are already present on the opercular membrane (O) and the branchial slits (BS). (C) Longitudinal and (C') transverse sections of prelarvae with intense staining of the intestinal brush-border. Note the immunopositive tegumentary ionocytes. (D) Transverse section showing weak apical staining of the renal collecting ducts (CD) and apical staining of the digestive tract. M, muscle; NC, notochord. Scale bars, 10 μm (B,C,D), 20 μm (A,C').

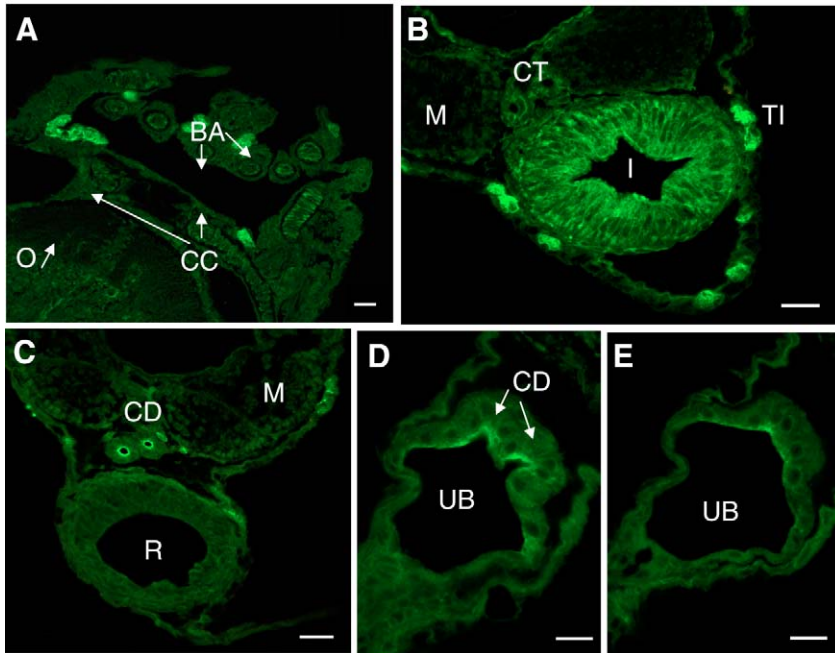
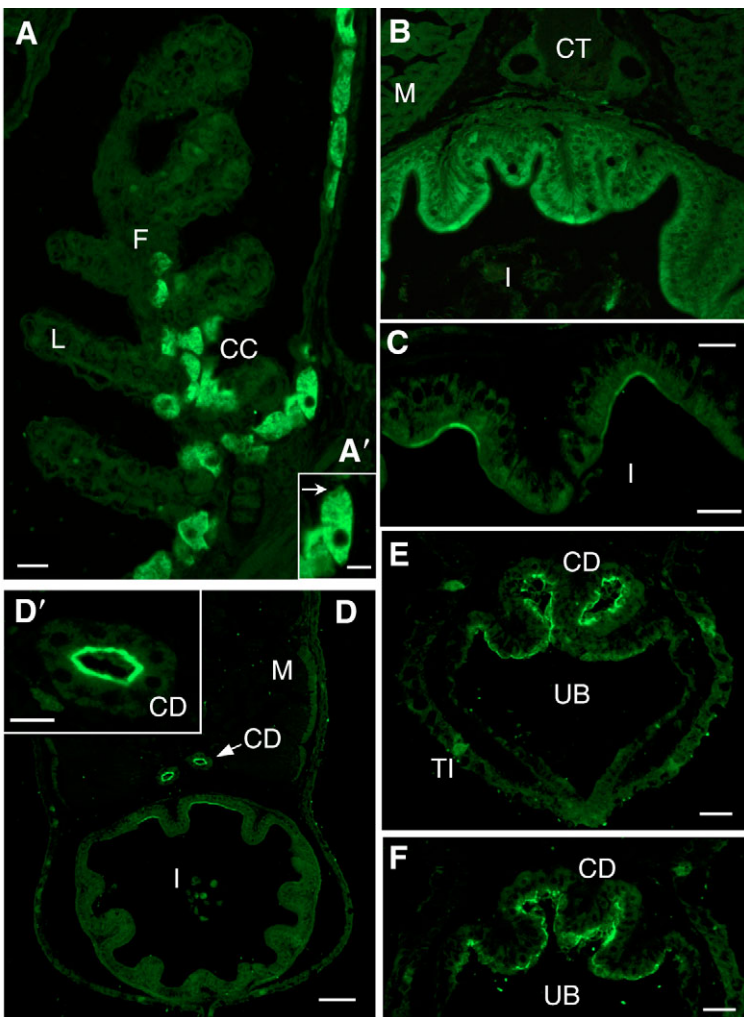


Fig. 4. Distribution of NKCC during the ontogeny of *Dicentrarchus labrax*. (A–E) Transverse sections of 6-day-old larvae. (A) Basolateral immunostaining of the branchial and opercular chloride cells (CC). (B) Apical diffuse staining of the intestinal cells (I) and basolateral staining of the tegumentary ionocytes (TI). (C–E) Renal collecting ducts (CD) before (C), at (D) and after (E) their junction with the urinary bladder (UB); apical immunostaining is visible in the collecting duct cells (C,D) and in the cells lining the dorsal part of the urinary bladder (D,E). BA, branchial arch; CT, collecting tubule; M, muscle; O, operculum; R, rectum. Scale bars, 20 μm (A–C), 10 μm (D,E).



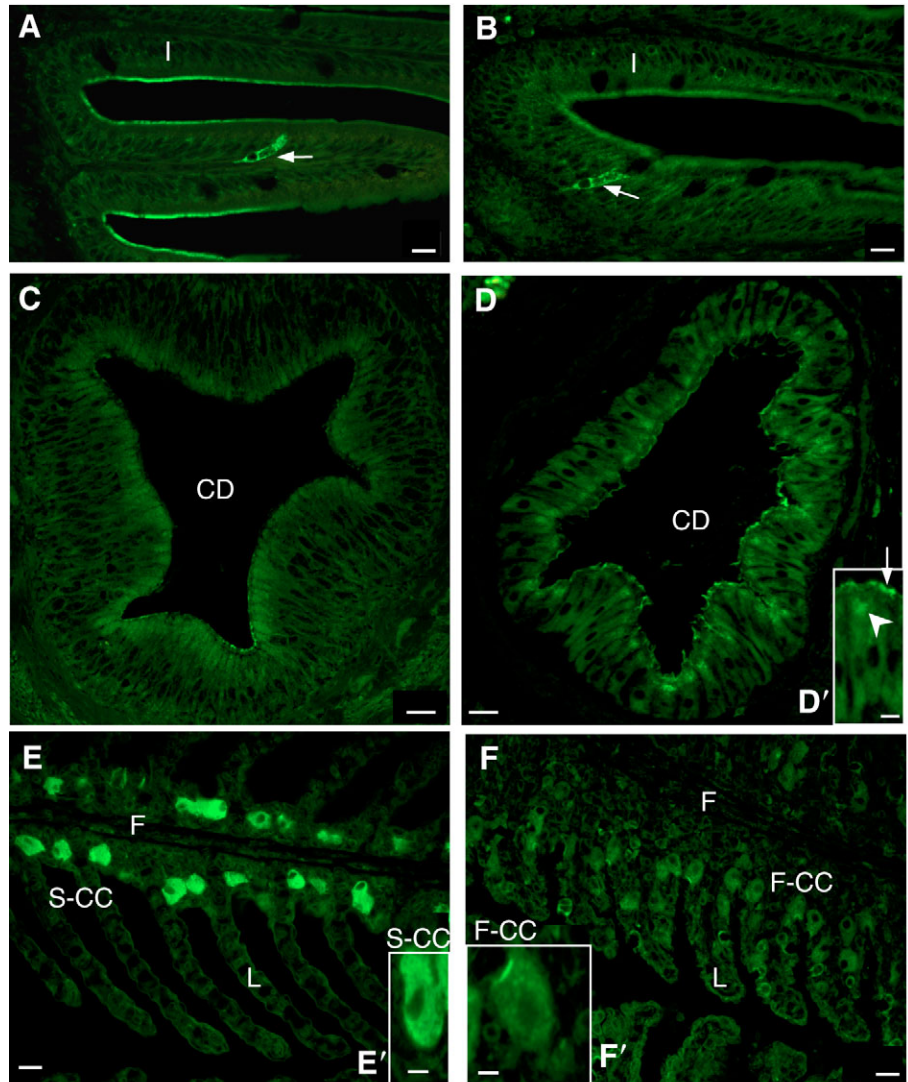
from the basolateral to the apical cell part was detected in FW (Fig. 6F,F'). A subapical punctate immunostaining was observed in some cells suggesting the presence of NKCC in vesicular membranes.

Short- and medium-term adaptation of sea bass to freshwater

To compare the effects of the SW challenge with those of FW, fish were analyzed after 1, 4 (short-term), 7, 14 and 22 (medium-term) days of exposure to FW through immunoblotting (Fig. 7) and immunocytochemistry (Fig. 8). SW-acclimated *D. labrax* presented high amounts of NKCC as shown by semiquantification from the dot blots (Fig. 7) and the intensely stained filamentary CC (Fig. 6E). After a short-term FW acclimation (1–4 days) there was a low but not significant decrease in the total NKCC (Fig. 7, S-FW). There was an additional significant

Fig. 5. Distribution of NKCC during the ontogeny of *Dicentrarchus labrax*. (A–F) Longitudinal section (A) and transverse sections (B–D) of 30-day-old larvae; (A) Predominant basolateral staining in branchial filamentary and opercular chloride cells (CC) (longitudinal section). (A') Larger magnification of a CC showing that the apical pit (arrow) presents no or weak immunostaining. (B,C,D,D',E,F) Apical immunostaining of the anterior (B) and posterior (C,D) intestine (I), of the collecting ducts (CD) and of the dorsal part of the urinary bladder (UB) (D,D',E,F). CT, collecting tubule; F, filament; L, lamellae; M, muscle; TI, tegumentary ionocyte. Scale bars, 5 μm (A'), 10 μm (A), 15 μm (D'), 20 μm (B,C,E,F), 50 μm (D).

Fig. 6. Distribution of NKCC in adult *D. labrax* following long-term adaptation in seawater (A,C,E,E') and freshwater (B,D,D',F,F'). (A,B) The anterior intestine (I) is stained along the brush-border membrane and the staining intensity appears stronger in the seawater-acclimated fish. (A). Note the presence of single basolaterally stained cells in both types of fish (A,B, arrows). (C,D) The renal collecting ducts (CD) have a (sub)apical staining (C,D,D') which is stronger in freshwater-acclimated fish (D,D'). Note the clearly distinguishable apical (D' arrow) and subapical (D' arrowhead) immunostaining. (E) Seawater-type chloride cells (S-CC) located on the filaments (F) are basolaterally stained (E'). (F) Freshwater-type chloride cells (F-CC) located on both the filaments and the lamellae (L), are (sub)apically stained (F'). Scale bars, 3 μm (D'), 5 μm (E',F'), 10 μm (A-F).



24% decrease in NKCC after a medium-term acclimation, i.e. 7–22 days following FW exposure (Fig. 7, M-FW). Immunocytochemical analysis of the gills showed a rapid colonization of the lamellae by immunopositive chloride cells (CC) after only 1 day in FW (Fig. 8A). In most CC, immunostaining was exclusively located basolaterally. Some CC (filamentary and lamellar), called SW-type CC, had an intense basolateral immunostaining; others, called intermediate CC (Fig. 8; arrowheads), showed a low basolateral immunostaining. Some filamentary cells were not immunostained (Fig. 8, asterisks). Following 7 days in FW (Fig. 8B), the proportion of SW-type CC cells decreased, particularly in the filaments, and a few apically stained CC appeared, called FW-type CC. After 14 days in FW (Fig. 8C), a few lamellar immunopositive SW-type CC and intermediate CC were still detected. The FW-type CC were still rare whereas numerous immunonegative cells were observed (Fig. 8C, asterisk).

NKCC gene expression in adult *D. labrax* exposed to seawater and freshwater

Absolute NKCC mRNA expression level in long-term SW- and FW-acclimated sea bass was determined in

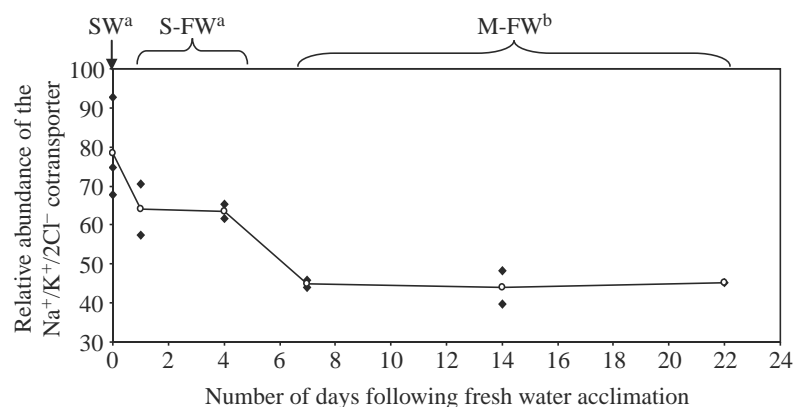


Fig. 7. Relative branchial NKCC abundance in *D. labrax* in seawater (SW; day 0) and following short-term (1 and 4 days; S) and medium-term (7, 14 and 22 days; M) freshwater (FW) acclimation. The line joins the means (open circles) of the individual measurements (black diamonds). Statistical tests were used to compare SW-acclimated sea bass ($N=3$) to short-term ($N=4$)- and medium-term ($N=5$)-acclimated sea bass to FW. Different letters indicate significant differences ($P<0.005$).

the gills (Fig. 9A), kidney (Fig. 9B) and intestine (Fig. 9C). mRNA levels of the housekeeping gene EF1 α did not change after salinity transfer in any analyzed organ (results not shown). A much higher NKCC expression (by 15–20 times in SW) was measured in the gills compared to the other organs at both salinities (Fig. 9A–C). In the gills, the NKCC expression was significantly lower in FW than in SW

($P < 0.001$) (Fig. 9A). In the kidney and the intestine, NKCC expressions were not significantly different between SW and FW (Fig. 9B,C).

Discussion

The NKCC cotransporter is one of the most important ion transport proteins involved in whole body osmoregulation in teleosts. This study shows for the first time in a teleost species the localization and expression of NKCC in different osmoregulatory sites of adults and during ontogeny. As underlined in the Introduction, the antibody used in immunolocalization does not differentiate between the secretory NKCC1 isoform (supposedly basolateral) and the absorptive NKCC2 isoform (apical).

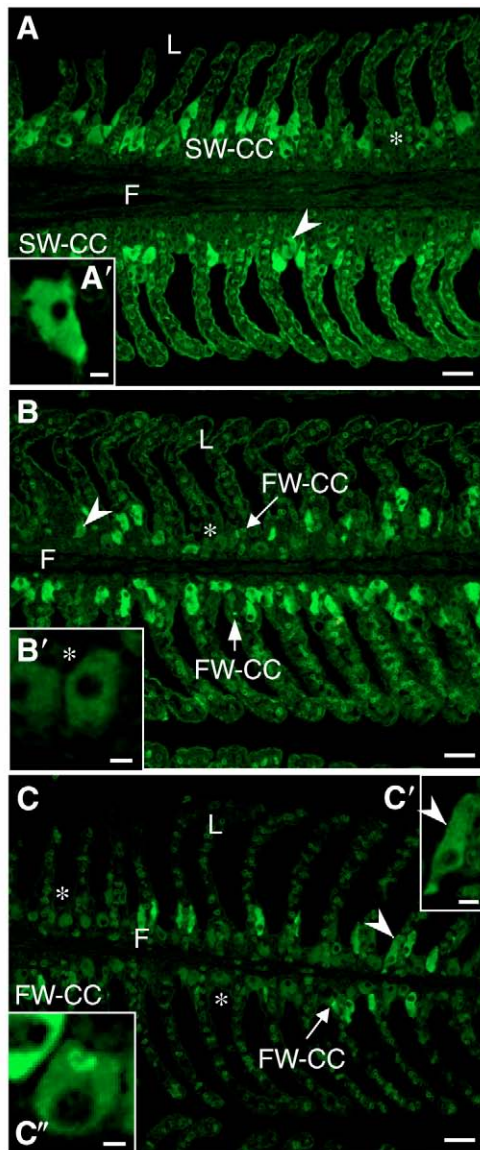


Fig. 8. Distribution of NKCC in branchial chloride cells of adult *D. labrax* following direct transfer from seawater to freshwater and exposure to freshwater for 1 day (A,A'), 7 days (B,B') and 14 days (C,C',C''). Note the presence of four different cell types. The SW-type chloride cells (SW-CC; A') are rich in basolateral NKCC. The intermediate cells (arrowheads, C') have moderate amounts of basolateral NKCC, the immunonegative cells (asterisks, B') lack NKCC staining and the FW-type cells (FW-CC, C'') have an apically stained cell pit. F, filament; L, lamellae. Scale bars, 20 μm (A–C), 4 μm (A',B'), 5 μm (C'), 2 μm (C'').

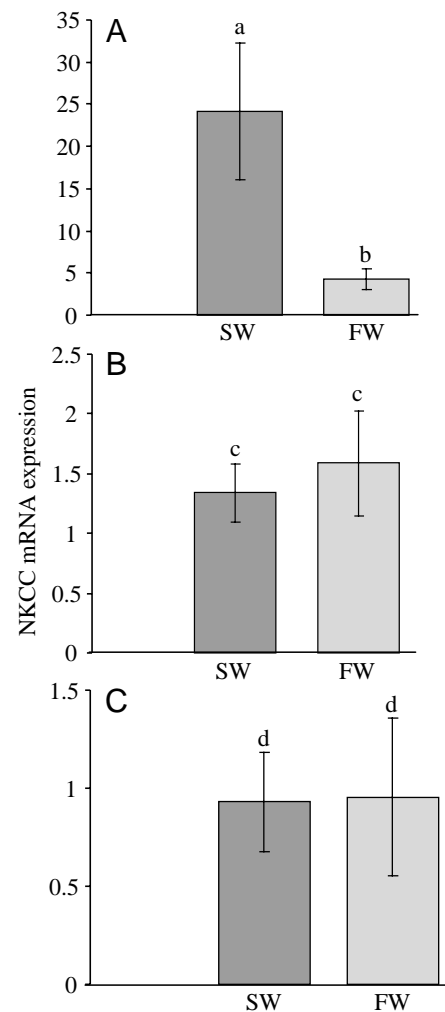


Fig. 9. $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter mRNA expression ($N=10$) in different organs of adult SW- and FW-acclimated *Dicentrarchus labrax*. (A) Gills, (B) kidney, (C) posterior intestine. Absolute expression ($E^{-\text{ct}}$) has been normalized to expression of the elongation factor (EF1 α). Data are expressed as the mean \pm s.d. Different letters indicate significant differences ($P < 0.001$). FW, freshwater; SW, seawater.

NKCC expression in adult D. labrax

The high homology of the sea bass protein sequence with NKCC1 sequences of other vertebrates (Table 3) as well as the similarity of the hydropathy profile with other NKCC proteins (Xu et al., 1994) suggest that the cDNA encodes the NKCC1 cotransporter. The protein homology of the sea bass branchial NKCC1 was 88% with *Oreochromis mossambicus* NKCC1 (Table 3) and still about 70% with that of mammals (Table 3). NKCC1 thus appears as a conserved protein in vertebrates. Previous studies have shown that NKCC1 and NKCC2 are regulated by direct phosphorylation in the NH₂ terminus during osmotic and oxidative stress (Lytle and Forbush, 1992; Giménez and Forbush, 2005). In the sea bass, the presence of numerous potential phosphorylation sites (Fig. 1) and particularly the conservation of a SPAK and OSR1 interaction site in the NH₂ terminus, identified by Piechotta et al. (Piechotta et al., 2002) indicate a possible activation of the sea bass NKCC1 by stress kinases as an initial sensor of the osmotic stress response.

Gills

In western blots, three bands were detected in the gills extracts of *D. labrax*. The most intense band from SW-acclimated fish, corresponding to a molecular mass of 225 kDa, might be NKCC1 in a glycosylated form. The sea bass protein sequence deduced from the NKCC1 nucleotide sequence was estimated to be about 126.3 kDa. The reported molecular masses of NKCC1 in chloride-secreting epithelia range from 153 to 205 kDa, this variability being probably due to different degrees of glycosylation (Lytle et al., 1995). The presence of two potential N-linked glycosylation sites between transmembrane domains 7 and 8 (Fig. 1) reinforce this hypothesis. The 225 kDa band may also correspond to the association of NKCC1 with a smaller peptide as in *Salmo trutta* and *S. salar* (Tipsmark et al., 2002). In sea bass, the 120 kDa and particularly the 110 kDa bands are less intense than the 225 kDa band and correspond either to the same isoform (NKCC1) lacking glycosylation or to different isoforms. Lytle et al. (Lytle et al., 1995) reported that the NKCC2 isoforms found in absorptive epithelia had lower molecular masses than NKCC1, ranging from 150 to 175 kDa, which suggests that the 110 and 120 kDa bands recorded in this study may correspond to the (sub) apical NKCC isoform without glycosylation.

In FW-acclimated sea bass, the three bands, but particularly the 225 kDa band, were much less intense, suggesting a higher proportion of the 110/120 kDa isoform in FW fish than in SW fish. Since no upregulation of these lower molecular mass forms has been recorded in FW fish, they do not correspond exclusively to the apical NKCC isoform.

At the branchial level, NKCC has been found on the basolateral side of the chloride cells (CC) in several fish, including *Fundulus heteroclitus* (Marshall et al., 2002b), *Periophthalmodon schlosseri* (Wilson et al., 2000b), *S. trutta* and *S. salar* (Tipsmark et al., 2002).

In most models of ion transport cells, NKCC is present only in the SW-acclimated fish, i.e. in conditions of hypo-

osmoregulation, requiring ion secretion by the CC. The basolateral NKCC enables the uptake of Na⁺, Cl⁻ and K⁺ from blood into the cell (Marshall, 2002). This cotransport is mediated by the electrochemical gradient generated by the basolateral Na⁺/K⁺-ATPase. Cl⁻ exits the cell apically through a CFTR-type anion channel, whereas Na⁺ is transported *via* the Na⁺/K⁺-ATPase to the blood and is secreted *via* a paracellular pathway.

In FW models, the absorption of Cl⁻ and Na⁺ from the medium is chemically coupled to NH₄⁺, H⁺ or HCO₃⁻ transepithelial transport (Evans et al., 1999; Wilson et al., 2000a; Hirose et al., 2003). In *Oncorhynchus mykiss*, NKCC is present in the gills of both FW and SW-adapted fish on the basolateral side of the CC (Flik et al., 1997). In *F. heteroclitus*, there is a slow redistribution of NKCC from eccentric portions of the cytosol in FW to a more diffuse basolateral location in SW, but no strictly apical staining has been reported (Marshall et al., 2002b). Flik et al. suggested that in FW basolateral NKCC might be involved in cell volume regulation (Flik et al., 1997) as in nonepithelial cells (Geck and Pfeiffer, 1985; O'Neill, 1999). Wu et al. (Wu et al., 2003) showed for the first time an apical NKCC location in branchial CC of FW-acclimated *Oreochromis mossambicus* and the authors suggest the presence of at least two functionally different CC: FW-type cells and SW-type cells. In embryos of the same species, a FW-type cell was observed with an apical NKCC and a basolateral Na⁺/K⁺-ATPase (Hiroi et al., 2005).

In sea bass maintained in SW, the gill CC had an intense diffuse basolateral immunostaining for NKCC. The NKCC colocalization with the Na⁺/K⁺-ATPase (Nebel et al., 2005b) suggested the involvement of NKCC (supposedly NKCC1) in ion secretion. In agreement with this hypothesis, a short- and medium-term challenge to FW induced a decrease in the NKCC protein abundance. This rapid adjustment of branchial NKCC content following a transfer from FW to SW has also been reported in the gills of *F. heteroclitus*, with an initial increased NKCC1 mRNA expression then a rise in NKCC protein abundance (Scott et al., 2004).

In the sea bass, the NKCC adjustment was related to the rapid downregulation in basolateral NKCC in some CC, that we called intermediate cells in this study. New immunonegative cells developed on the filament resulting in an increase in the thickness of the epithelium where some NKCC-rich CC (SW-CC) were still present. One week after the salinity challenge, a few apically stained CC appeared (FW-CC), but their number remained low, even after medium-term FW exposure. Our current hypothesis of how the observations from the current study relate to ion regulation in the sea bass are as follows. Short- and medium-term FW acclimation in sea bass induced a neoformation of CC devoid of NKCC, a differential CC distribution through the branchial epithelium correlated to a decrease of basolateral NKCC in some CC, which probably resulted in a decrease of net ion secretion. The majority of CC did not seem involved in ion absorption, at least not through NKCC. There are other apical proteins involved in ion transport (Na⁺ channels,

HCO₃⁻/Cl⁻exchanger ...). The transition of a SW-type epithelium with high amounts of basolaterally stained CC into a FW-type epithelium with CC with essentially apical NKCC (supposedly NKCC2) occurred progressively and was completed only after long-term acclimation. The gills of sea bass in FW have been shown to overexpress Na⁺/K⁺-ATPase with a twofold higher activity compared to gills from fish in SW (Nebel et al., 2005b), suggesting that the FW-type CC observed in this study are involved in ion uptake through NKCC driven by the electrochemical gradient of the Na⁺/K⁺-ATPase. The existence of this mechanism has been shown in two freshwater species (Preest et al., 2005). Future studies will be necessary to test if this hypothesis in the sea bass.

The differences between short- medium- and long-term responses in sea bass gills reflected two different strategies to cope with salinity changes. Given the ecology of the sea bass, adult fish migrating between coastal and estuarine waters often encounter salinity fluctuations: a rapid modulation of ion fluxes is thus necessary. In the sea bass, a decrease in salinity results in a rapid decrease of branchial NKCC due to a decrease in basolateral NKCC within numerous CC. This short- (medium-) term response probably enables sea bass to rapidly decrease net ion secretion and to move between waters of different salinities. A long-term adaptation to low salinity not only requires a decrease in basolateral NKCC expression, but also the formation of morphologically and functionally different CC differentiating into FW-type cells, able to efficiently reabsorb ions from low salinity media or FW, and to maintain constant blood osmolality after a long-term exposure (Nebel et al., 2005b). These osmoregulatory mechanisms enable adult sea bass to swim upstream in rivers and to stay in those habitats for longer periods. These deeper intracellular changes might not be rapidly reversible. In the other studied species, an increased level of NKCC expression follows SW transfer (Flik et al., 1997; Cutler and Cramb, 2002; Tipsmark et al., 2002; Scott et al., 2004). Long-term adapted sea bass showed the same trend, since NKCC gene expression and protein abundance were significantly higher in SW-type than in FW-type gills. Whether CC alter their functions in response to salinity change or SW-type CC degenerate and are replaced by newly differentiated FW-type CC has not yet been determined. Both types of response might occur, the first and second respectively corresponding to short- (medium-) and long-term adaptation. In the yolk-sac membrane CC of *O. mossambicus* the CC were able to alter their ion-transporting function (Hiroi et al., 1999), whereas both types of responses have been recorded in *F. heteroclitus* CC by CFTR expressions studies (Kato and Kaneko, 2003).

Urinary system

In adult sea bass, the collecting ducts expressed NKCC apically and subapically. Immunostaining was particularly intense in FW-acclimated fish, both apically (probably in cilia and microvilli) and subapically (probably in intracellular vesicles). The localization of NKCC in intracellular vesicles may represent a reservoir of transporters to be recruited during

regulation of apical NaCl transport as suggested in the rat kidney (Nielsen et al., 1998). In FW, several teleosts (FW fish and euryhaline teleosts including sea bass) produce urine hypotonic to blood as a result of ion reabsorption by the collecting ducts, the distal tubules and the urinary bladder (Renfro, 1975; Hentschel and Elger, 1987; Marshall, 1995; Nebel et al., 2005a). In the sea bass, these renal cells are rich in mitochondria, basolateral Na⁺/K⁺-ATPase (Nebel et al., 2005a) and (sub)apical NKCC. They are thus equipped to reabsorb ions. In SW fish, NKCC seems less abundant, perhaps in relation to a lower need to reabsorb ions from the urine, which is isotonic to blood (Nebel et al., 2005a). Only slight differences were detected in t mRNA expression after the long-term salinity challenge. Analyzing the short- and medium-term response by measuring mRNA expression at different times of acclimation is thus interesting. In *Salmo gairdneri*, furosemide (the NKCC inhibitor) added to the lumen reduced the transepithelial ion transport, which suggests the presence of NKCC2 (Nishimura et al., 1983). But in other species, ion transport in the kidney is insensitive to NKCC inhibitors (Marshall, 1986), which reflects a diversity of the osmoregulatory mechanisms between teleost species. Other channels [Cl⁻ channel in *O. mossambicus* (Miyazaki et al., 2002)] or transporters (Na⁺/Cl⁻ cotransporter (Marshall, 1995)] are probably involved in ion reabsorption. The proximal tubules of fish are thought to express NKCC in the basolateral part of the cell in order to secrete ions (Masini et al., 2001; Beyenbach, 1995; Beyenbach, 2004). This was not observed in the sea bass at any salinity, and other ion transporters or channels might be expressed in these tubules. It is also worth noting that the strong immunostaining of the apical part of the urinary bladder corresponds to an equally strong presence of Na⁺/K⁺-ATPase at the same location (Nebel et al., 2005a), thus confirming the involvement of the bladder in ion reabsorption.

Intestine

In the intestine of sea bass, the cotransporter is present in the brush-border membrane, which suggests the involvement NKCC (possibly NKCC2) in ion uptake. Immunostaining seemed stronger in SW-acclimated sea bass, which drink much higher volumes of water (Varsamos et al., 2004), a mechanism followed by an active reabsorption of ions and an osmotic water uptake to avoid dehydration. In *F. heteroclitus*, two types of intestinal cells were identified according to their NKCC immunolocalization (apical or basal) (Marshall et al., 2002a), whereas in *Pseudopleuronectes americanus* only the apical NKCC has been detected (Suvitayavat et al., 1994). In the intestinal cells of *Anguilla anguilla*, two secretory and one absorptive NKCC isoform have been identified (Cutler and Cramb, 2002). The expression of NKCC2 increased with salinity whereas the expression of NKCC1 did not vary significantly. NKCC1 was suggested to be involved in luminal fluid secretion for digestive purposes or cell volume regulation (Cutler and Cramb, 2001). In the sea bass, the NKCC localization and expression did not vary between SW-

acclimated fish and FW-acclimated fish, suggesting that intestinal NKCC may have other functions than osmoregulation.

Ontogeny of NKCC in D. labrax

Few studies have dealt with the ontogeny of ion transport proteins in fish. In the mammal kidney, the onset of expression of various transport proteins including NKCC2 has been analyzed recently and a distinct pattern of protein expression at different nephrogenic stages has been reported (Igarashi et al., 1995; Bachmann et al., 1999; Schmitt et al., 1999). In fish, the differential ontogenetical expression of NKCC was studied in migratory groups such as salmonids (Pelis et al., 2001; Tipsmark et al., 2002) and eels (Cutler and Cramb, 2002). In *Anguilla anguilla*, SW acclimation induced a sixfold increase in the branchial secretory isoform NKCC1a in yellow eels, but in later silver eels, only a slight decrease in mRNA expression was recorded following SW acclimation (Cutler and Cramb, 2002). NKCC1a expression decreased during development from yellow eels to mature silver eels in the kidney and the authors suggested a preacclimation of the mRNA levels to lower tubular/fluid secretion (Cutler and Cramb, 2002). In *Salmo salar*, the branchial NKCC level increased by 3.3-fold in smolts and decreased in postsmolts below presmolt values (Pelis et al., 2001). These values coincide with the occurrence of SW tolerance.

In this study, NKCC (without differentiation between NKCC1 and 2) has been identified in the three major osmoregulatory organs during the ontogeny of the sea bass. In late embryos, NKCC was already present in the intestinal and tegumentary ionocytes, the latter probably being involved in osmoregulation in prelarval stages prior to the full development of the gills, the intestine and the kidney (reviewed by Varsamos et al., 2005). According to Alderdice, the integument appears as the primary site of osmoregulation and gas exchange (Alderdice, 1988). Later, the skin thickens and becomes less permeable to gases and ions. According to Burggren, this functional shift of an organ during development is called 'prosynchronotropy', which is a general vertebrate trait (Burggren, 2005).

At 1 day post-hatch, CC appear in the branchial chamber of sea bass prelarvae. At this stage, the lamellae are not yet formed, which suggests that the sea bass gills are primarily involved in ion exchanges rather than in gas exchanges as suggested in other species (Li et al., 1995; González et al., 1996; Rombough, 1999; van der Heijden et al., 1999; Rombough, 2002). In *Danio rerio* prelarvae, ion exchange by the gills become limiting before gas exchanges does (Rombough, 2002). The basolateral location of NKCC suggests the involvement of the branchial CC in ion secretion. As sea bass hatch in marine waters (Pickett and Pawson, 1994), ion secretion by the tegumentary and branchial ionocytes probably enables prelarvae to restrain the variability of blood osmolality. In *O. mossambicus* embryos, different types of CC exist, and the relative numbers vary according to salinity (Hiroi et al., 2005). The differentiation of various CC

types according to salinity has not yet been determined in sea bass (pre)larvae.

In 3-day-old sea bass, the brush-border of the intestine was intensely stained apically. As the mouth is not yet open (Barnabé et al., 1976), this early expression of NKCC appears as a preadaptation to reabsorb ions at mouth opening (day 5). Ion absorption by the intestine is energized by the Na⁺/K⁺-ATPase (personal observation) and enables a secondary water absorption by osmosis required in SW environments.

The collecting ducts of the prelarval kidney present low amounts of apical NKCC, whose abundance increases a few days later. The high amounts of Na⁺/K⁺-ATPase in the cells lining the collecting ducts and the dorsal part of the urinary bladder, the numerous mitochondria (Nebel et al., 2005a) and the presence of apical NKCC in these cells are typical features that point to the involvement of the kidney in ion reabsorption in 6-day-old sea bass. Moreover, the primary urinary system appears to possess all required features (glomus, urinary tubules and urinary bladder) (Nebel et al., 2005a) to excrete urine from this stage on.

Beside the mouth opening and the apparent onset of kidney functionality, the increase in the number of branchial CC rich in basolateral Na⁺/K⁺-ATPase (Varsamos et al., 2002) and NKCC is another key factor for the sudden increase in osmoregulatory capacity reported at that stage (Varsamos et al., 2001). At the larva/juvenile transition, a further increase in osmoregulatory capacity was detected (Varsamos et al., 2001), which could be related to the increase in branchial CC expressing basolateral Na⁺/K⁺-ATPase (Varsamos et al., 2002) and NKCC. The differentiation of the digestive tract (Hernández et al., 2001), characterized by apical NKCC, probably enables ion reabsorption and a modulation of the drinking rate according to salinity (Varsamos et al., 2004). The development of the mesonephros from day 20 (Nebel et al., 2005a), in which the collecting ducts express the absorptive NKCC isoform (probably NKCC2), as does a part of the bladder, enables sea bass larvae to produce dilute urine at low salinities, at least from the juvenile stage on (Nebel et al., 2005a).

In summary, we have identified NKCC in the main osmoregulatory organs of the sea bass during ontogeny. This cotransporter is detected in late embryos and its cell localization has shown that secretory (tegumentary ionocytes) and absorptive (intestine) NKCC isoforms are already expressed. In prelarvae, the kidney expresses NKCC apically along the collecting ducts. From the stage when the mouth opens, the cells lining the dorsal part of the bladder express NKCC apically, and the branchial CC, rich in basolateral NKCC, increase in number. These mechanisms are correlated with a high increase in osmoregulatory ability and they enable a shift of the osmoregulatory function from the tegument (and gills) to the adult-type organs. We have also shown that NKCC is involved in osmoregulation in adult sea bass, at least in branchial chloride cells, as its expression and cell localization change according to salinity. Furthermore, the capacity of the gills to switch from a hypo- to a hyperosmoregulatory function is at least partially based on the plasticity of the chloride cells

to adjust their NKCC content and probably on their capacity to synthesize predominantly FW-type CC as a long-term response. This study advances our understanding of the mechanisms of osmoregulation in sea bass, which undergo high salinity fluctuations in natural habitats during their life cycle.

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