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The ClC-3 chloride channel and osmoregulation in the European Sea Bass, *Dicentrarchus labrax*

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Abstract:

Dicentrarchus labrax migrates between sea (SW), brackish and fresh water (FW) where chloride concentrations and requirements for chloride handling change: in FW, fish absorb chloride and restrict renal losses; in SW, they excrete chloride. In this study, the expression and localization of ClC-3 and Na^+/K^+-ATPase (NKA) were studied in fish adapted to SW, or exposed to FW from 10 min to 30 days. In gills, NKA-α1 subunit expression transiently increased from 10 min and reached a stabilized intermediate expression level after 24 h in FW. ClC-3 co-localized with NKA in the basolateral membrane of mitochondria-rich cells (MRCs) at all conditions. The intensity of MRC ClC-3 immunostaining was significantly higher (by 50 %) 1 h after the transfer to FW, whereas the branchial ClC-3 protein expression was 30 % higher 7 days after the transfer as compared to SW. This is consistent with the increased number of immunopositive MRCs (immunostained for NKA and ClC-3). However, the ClC-3 mRNA expression was significantly lower in FW gills. In the kidney, after FW transfer, a transient decrease in NKA-α1 subunit expression was followed by significantly higher stable levels from 24 h. The low ClC-3 protein expression detected at both salinities was not observed by immunocytochemistry in the SW kidney; ClC-3 was localized in the basal membrane of the collecting ducts and tubules 7 and 30 days after transfer to FW. Renal ClC-3 mRNA expression, however, seemed higher in SW than in FW. The potential role of this chloride channel ClC-3 in osmoregulatory and osmosensing mechanisms is discussed.

Keywords: ClC-3 chloride channel ; Na+/K+-ATPase ; Dicentrarchus labrax ; Osmoregulation ; Osmosensing ;

1. Introduction

The European sea bass, *Dicentrarchus labrax* (Linnaeus, 1758), known for its euryhalinity, undergoes seasonal migrations from the sea (seawater, SW: 35–38‰) to lagoons and estuaries and tolerates a direct experimental transfer from SW to fresh water (FW) (0.3‰) (or *vice versa*) (Bodinier et al. 2009b; Lorin-Nebel et al. 2006). When salinity changes, sea bass, as other euryhaline teleosts, are able to regulate their blood osmolality at relatively constant values (Bossus et al. 2011; Evans and Claiborne 2006; Nebel et al. 2005a) due to efficient osmoregulatory mechanisms (Evans 2009). Osmoregulation in fish is mainly performed by sodium and chloride transport that occurs in the gills, the kidney and the intestine through ionocytes, also known in gills as mitochondrion-rich cells (MRC). These cells harbor different ion transporters and channels whose localization and expression have been extensively studied in the sea bass (Bodinier et al. 2009b; Boutet et al. 2006; Giffard-Mena et al. 2008; Lorin-Nebel et al. 2006; Nebel et al. 2005b; Varsamos et al. 2005). The main enzyme involved in osmoregulation in fish is the Na^+/K^+-ATPase (NKA) located in the basolateral membrane of ionocytes, and providing the driving force for ion transport in the osmoregulatory organs in SW and FW (Evans et al. 1999; Rankin and Davenport 1981).

Gills, considered as the major site to maintain the ionic balance, are responsible for salt excretion or uptake in respectively hyper- or hypotonic media. In the sea bass as in other teleosts (Bodinier et al. 2009b; Lorin-Nebel et al. 2006; Marshall et al. 2002), excretion of salt in hypertonic media is mediated by a co-transporter, $Na^+/K^+/2Cl^-$-1 (NKCC1), located in the basolateral membrane of MRCs, coupled to an apical Cl^- channel, the cystic fibrosis transmembrane conductance regulator (CFTR) channel that transports Cl^- outside the cell. The leaky junctions between MRCs and accessory cells allow a paracellular pathway for the Na^+ efflux, attracted by the negative charges (from Cl^- ions) temporarily trapped in the apical crypts. In contrast, the mechanisms of NaCl uptake in teleosts exposed to FW are more debated (see reviews: Hirose et al. 2003; Hwang and Lee 2007; Marshall 2002). The junctions between branchial cells are tight to avoid paracellular Na^+ efflux. In the sea bass, the electrochemical gradient created by the NKA pump results in the transport of NaCl via an apical NKCC isoform or Na^+/Cl^- co-transporter (NCC) (Lorin-Nebel et al. 2006) as suggested in MRCs of *Oreochromis mossambicus* (Hiroi et al. 2008; Inokuchi et al. 2008). The resulting increased chloride concentration within these cells coupled to the electrical gradient created

by NKA suggests the presence of a functional Cl^- channel at the basolateral membrane. The Cl^- channel allowing that basal Cl^- absorption still remains unknown.

In the sea bass kidney, active ion reabsorption in FW is mediated by a basolateral NKA and an apical NKCC or NCC co-transporter expressed in the collecting tubules and ducts (Lorin-Nebel et al. 2006; Nebel et al. 2005a). In SW, the same localization of these co-transporters is observed; however the highly expressed water channel aquaporin 1 (AQP1) (Giffard-Mena et al. 2008) would allow passive water uptake following the ionic reabsorption to compensate osmotic water loss. As mentioned in the branchial cells, the basal chloride channel is also unknown in the sea bass renal cells.

The intestine is also involved in osmoregulation, notably for water absorption in SW following active ion uptake in order to avoid dehydration. In FW, sea bass have a low drinking rate in order to restrict water invasion (Giffard-Mena et al. 2008; Varsamos et al. 2004); the intestine is rather involved in acid-base regulation. The chloride channel CFTR has been reported in the sea bass intestine at the apical membrane at both salinities. This channel could be involved in osmoregulatory mechanisms in SW and secrete Cl^- in FW to allow acid-base regulation (Bodinier et al. 2009a).

Three types of Cl^- channels could be expressed in the sea bass tissues: ligand-gated Cl^- channels, CFTR channels and ClC chloride channels (Estévez and Jentsch 2002). The sea bass CFTR is expressed in SW MRCs but not in FW MRCs (Bodinier et al. 2009a). Little information is available on the two other types of chloride channels. In mammals, nine ClCs are known, involved in diverse physiological functions, such as ion homeostasis, transepithelial chloride transport, electrical excitability and cell volume regulation (review in Jentsch et al. 2002). Some members of this ClC family have been recently suggested as potential channels for basolateral chloride transport in fish (Hirose et al. 2003). ClCs display a dimeric structure with two individual pores on each monomer contained within one subunit (Jentsch et al. 2005). They have 10 to 12 transmembrane domains among which the 9^{th} to 12^{th} form an extensive and almost uninterrupted hydrophobic region, and a long C-terminal tail containing two cystathionine-β synthase (CBS) domains (Estévez and Jentsch 2002; Mindell and Maduke 2001). The CBS domain has been found for the first time in the enzyme cystathionine-β synthase, which in human catalyzes the first step of the trans-sulfuration pathway, critical for cysteine synthesis (Meier et al. 2001). However, the roles of CBS domains are not well defined: they might be involved in protein targeting and

oligomerization, or bind to regulatory proteins (Ignoul and Eggermont 2005). It has also been reported that the CBS domain of ClCs interferes with ion gating and selectivity since mutations in this domain have been shown to change the selectivity of each pore, either through increase or loss (Dave et al. 2010; He et al. 2006).

ClC-3, ClC-K (K for kidney) and ClC-5 channels have been studied in fish, mainly for their potential role in osmoregulation (Miyazaki et al. 2002; Miyazaki et al. 1999; Shen et al. 2007; Tang et al. 2011; Tang et al. 2010; Tang and Lee 2007; Tang and Lee 2011). The first study on teleost ClC determined the presence of ClC-3 and 5 in the osmoregulatory organs of the tilapia *Oreochromis mossambicus* (Miyazaki et al. 1999). Thereafter, the OmClC-K was detected in the impermeable distal tubule of the kidney in FW, co-localized with NKA (Miyazaki et al. 2002). The authors suggested an involvement of OmClC-K, coupled with a NKCC or NCC co-transporter, in the reabsorption of NaCl, thus allowing the production of dilute urine in hypotonic environments. In fish MRCs, ClC-3 has been suggested as the basolateral chloride channel involved in chloride uptake (Hirose et al. 2003).

In mammals, ClC-3 is generally present in the membrane of organelles, vesicles (endoplasmic reticulum, Golgi, vesicles of exo- and endo-cytotic pathways, lysosomes and synaptic vesicles) and also in the plasma membrane (Huang et al. 2001; Jentsch 2008; Vessey et al. 2004). The addressing of the ClC-3 protein to the plasma membrane is most probably modulated by the medium osmolality (Vessey et al. 2004): an exposure of mammalian non-pigmented ciliary epithelial cells to hyposmoticity leads to an increased ClC-3 expression in the plasma membrane (Vessey et al. 2004). This channel has been highlighted as potentially involved in cell volume regulation, as it is activated by cell swelling in mammals (Duan et al. 1999; Duan et al. 1997; Duan et al. 2001). Its opening results in regulatory volume decrease (RVD) in various cells (Do et al. 2005; Duan et al. 1997; Duan et al. 2001; Hermoso et al. 2002; Kawasaki et al. 1994; Vessey et al. 2004; Wang et al. 2003; Wang et al. 2000). ClC-3 is also activated by electrochemical voltage and phosphorylation which could be due to cell swelling in a hypotonic environment (Duan et al. 1999; Mindell and Maduke 2001; Rutledge et al. 2002).

In *O. mossambicus* and *Tetraodon nigroviridis*, the ClC-3 mRNA was mainly expressed in the osmoregulatory organs and the brain without any difference in expression between SW and FW conditions (Miyazaki et al. 1999; Tang et al. 2010; Tang and Lee 2011). At the

protein level, ClC-3 expression was significantly higher in FW than in SW in *T. nigroviridis* (Tang et al. 2010; Tang and Lee 2007) and did not vary according to salinity in *O. mossambicus*. The ClC-3 protein was detected in the basolateral membrane of MRCs, co-localized with the NKA pump, in different teleost species in SW and FW (Shen et al. 2007; Tang et al. 2011; Tang et al. 2010; Tang and Lee 2011), making it a candidate in basal Cl^- uptake. However, there has been no patch clamp study performed in branchial cells of teleosts to confirm the operation of this channel in fish ion balance. A patch clamp study investigating a chloride channel located in the basolateral membrane of urinary bladder cells in the euryhaline goby, *Gillichthys mirabilis*, found a 75 pS outward rectifying channel (Chang and Loretz 1993). This channel has been blocked by an inhibitor, the diphenylamine-2-carboxylate (DPC), which has been shown to inhibit mammalian ClC-3 (Coca-Prados et al. 1996). The co-localization with NKA and expression of ClC-3 in fish osmoregulatory organs coupled with the ClC-3 role in cell osmosensing and regulation in various cells suggest that ClC-3 may have a role in hyper-osmoregulation in FW fish; other functions may include regulation of chloride intracellular concentration and acid-base homeostasis (Shen et al. 2007; Tang et al. 2011; Tang et al. 2010; Tang and Lee 2007; Tang and Lee 2011).

Furthermore, studing NKA, the driving force for osmoregulation, is essential to understand osmoregulatory mechanisms during salinity adaptation. Comparison between NKA and ClC-3 expression over time may lead to a better understanding of the kinetics of the response to an osmotic shock. The functional shift from hypo- to hyper-osmoregulation involves also structural modifications at the gill level, among them the migration of MRCs into the lamellar epithelium (Alvarado et al. 2006; Nebel et al. 2005b). In addition, the response within minutes of NKA mRNA expression levels following an exposure to hyposmotic medium is not well known in sea bass.

The purpose of this study conducted in *D. labrax*, was thus to characterize the ClC-3 and to investigate this channel in different osmoregulatory organs. The mRNA expression of ClC-3 and NKA was determined in the gills, the kidney and the brain of sea bass acclimated to SW and exposed to FW during various times. Their protein expression and localization was studied in the gills and the kidney. A morphometric analysis completes the protein and gene expression data for a better understanding of the plasticity of sea bass gills. The results are discussed regarding the potential role of ClC-3 in fish osmoregulation and cell volume regulation.

2. Materials and methods

2.1 Animals and experimental protocol

Juveniles of *Dicentrarchus labrax*, 8 months-old (length = 15.3 ± 0.9 cm; weight = 34.3 ± 9 g), were obtained from the Sète Marine Station (Hérault, France) where they were raised in seawater. After their transport to the laboratory in Montpellier, the fish were maintained in 3500 L tanks filled with aerated and mechanically/biologically filtered (Eheim system, Europrix Aquariophilie, Lens, Pas-de-Calais, France) natural SW from the Mediterranean Sea at 38 ppt and 19 ± 1 °C, under a 12 hrs L/12 hrs D photoperiod. They were fed daily to satiation with fish granules (Aphymar feed, Mèze, Hérault, France) until two days before sampling. Groups of 10 fish were directly transferred (with a landing net) to a tub filled with fresh water (FW: 0.3 ppt) and sampled at various times: 10 min, 1 hr, 24 hrs, 7 d and 30 d. 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). A control group was transferred from SW to SW. No mortalities have been reported during all experiments. At the end of the exposure times, fish were anesthetized by speed freezing and decapitated. These experiments were conducted according to the guidelines of the European Union (directive 86/609) and of the French law (decree 87/848) regulating animal experimentation.

2.2 RNA extraction and reverse transcription (RT)

The gills, kidney and brain were collected from sea bass exposed to SW and FW. RNA extraction, purification, and RT were carried out according to the methods described in Bossus et al. (2011).

2.3 ClC-3 cloning and sequencing

A pool of branchial epithelium samples extracted from 10 fish acclimatized to SW and FW during 30 days was used for sequencing. The cDNA of the gene was first amplified by PCR using the degenerate primers ClC-3-d (Table 1) which were designed based on the nucleotide blast (basic local alignment search tool) alignments of ClC-3 on the NCBI website (the National Center for Biotechnology Information) from several species including *O. mossambicus* (Mozambique tilapia) (AF182215.1), *T. nigroviridis* (spotted green pufferfish) (FJ534534.1), *S. salar* (Atlantic salmon) (NM_001173586.1) and *H. sapiens* (AF172729.1).

After cloning into TOPO TA Cloning vector (Invitrogen, Cergy Pontoise, Val d'Oise, France) and sequencing, several primers were designed. The sequencing was performed with the BigDye® Terminator Sequencing kit (Applied Biosystems, Foster City, CA, USA).

ClC-3 5' and 3' RACE (Rapid Amplification of cDNA Ends) were performed according to the Roche 5'/3' RACE protocol (Roche, Mannheim, Germany) with some modifications. For the 5' end, 1 µL of total RNA was submitted to RT using the 5'RACE-ClC-3-SP1 specific primer (Table 1) and the M-MLV reverse transcriptase (Invitrogen). The cDNA was purified with a High Pure PCR Product purification kit (Roche) and a poly A tail was added using the terminal transferase (Promega, Southampton, UK). 5' RACE products were obtained after two nested PCR using the 5'RACE-ClC-3-SP2 specific primer and the oligo d(T)-anchor primer (Roche), and the second one with the 5'RACE-ClC-3-SP3 specific primer and the PCR anchor primer (Roche). For the 3' RACE, 1 µL of total RNA was submitted to RT using the RACE d(T)-anchor primer and M-MLV reverse transcriptase (Invitrogen). The resulting cDNA was submitted to amplification using the RACE anchor primer (Roche) and the specific 3'RACE-ClC-3-F primer. The PCR products of the 5'/3' RACE were then cloned into TOPO TA cloning vector (Invitrogen) and sequenced. All sequencing was performed on the Génotypage-Séquençage platform of IFR 119 (Montpellier, France) with an ABI Prism 3130 XL 16 capillary Genetic Analyzer instrument (Applied Biosystems).

2.4 Protein structure analysis

Sequences for comparative analysis were obtained from GenBank. The cDNA translation to protein, isoelectric point and theoretical calculation of relative molecular mass were achieved using ExPASy tools (Gasteiger et al. 2003) such as amino acid (aa) analysis. The multiple aa sequences alignments were performed with the BioEdit (version 7.0.9.0, Hall, 1999) computer program with Clustal X, such as the prediction of the membrane topology from the deduced aa sequence using the Kyte and Doolittle method (Kyte and Doolittle 1982).

2.5 Quantification of ClC-3 and Na^+/K^+-ATPase by quantitative real-time PCR (qRT-PCR)

The specific ClC-3-F-1 (forward) and ClC-3-R-1 (reverse) primers (Table 1) generated a PCR product of 146 bp and the specificity of these primers was controlled by PCR. Specific NKA-F and NKA-R primers, based on the sequence of NKA-α1 in *D. labrax* (AM419034), generated a PCR product of 152 bp (Table 1). qRT-PCR analyses were performed with a Light-Cycler™ system version 3.5 (Roche, Mannheim, Bade-Württemberg, Germany), using

2.5 µL of the LightCycler-FastStart DNA Master SYBR-Green I™ Mix (Roche), 0.75 µL of each primer (reverse and forward at 0.5 µM) and 1 µL of cDNA. The qPCR conditions were: denaturation at 95 °C for 10 min, repeat of amplification (95 °C, 15 s), hybridation (60 °C, 5 s) and elongation (72 °C, 10 s) 40 times, melting curve program at 60 °C for 1 min and final step at 40 °C for 30 s. The results were normalized with the elongation factor EF1α, a housekeeping gene used as a reference standard already used in the sea bass (Lorin-Nebel et al. 2006) and in other species (Frost and Nilsen 2003). The reverse and forward primers of EF1α generated a PCR product of 239 bp (Table 1). Ultra-pure water was used as a negative control in the qPCR. The relative expressions of ClC-3 and NKA to EF in gills, kidney and brain at each condition (in SW and at various times in FW) were calculated with the ΔΔCt method and statistical analyses were performed on 6 fish per condition with one reading per individual.

2.6 Antibodies

Immunocytochemistry and protein quantification were performed with a rabbit polyclonal primary antibody against the residues 592-661 of rat ClC-3 (provided by Alomone labs, Jerusalem, Israel). It was used at 4 µg mL^{-1} in immunocytochemistry procedures and at 2.7 µg mL^{-1} for Western blots. The immunoreaction specificity of the anti-ClC-3 antibody was tested by incubating it with a peptide control antigen of ClC-3 (molecular weight of 35 kDa) provided by Alomone Labs for Western blots and immunostaining procedures. Beta-Actin (43 kDa) was used as reference protein and its content in samples loaded to the gels proved to be similar throughout all analyses presented ($p<0.05$). The antibody against β-Actin, was developed in rabbit using a synthetic peptide corresponding to 11 amino acid residues of the C-terminal of the β-actin protein (provided by Sigma-Aldrich®, St. Louis, MO, USA), and it was used at 1.6 µg mL^{-1}. The primary monoclonal mouse NKA antibody (12 µg mL^{-1}) raised against the α-5 subunit of the chicken NKA (IgG α5) developed by Fambrough (Takeyasu et al. 1988) and purchased from the DSHB (Developmental Studies Hybridoma Bank; University of Iowa, USA), was used to visualize the mitochondrion-rich cells (MRCs). This antibody has already been successfully used in the sea bass (Giffard-Mena et al. 2006; Nebel et al. 2005b).

The secondary antibody for Western blots was an IRDye® 800 CW Infrared Dye antibody (Table 2) of donkey anti-rabbit IgG (H+L) (LI-COR® Biosciences™, Nebraska, USA) and it was used at 0.05 µg mL^{-1}. For immunocytochemistry, secondary antibodies were

Rhodamine donkey anti-rabbit (Tebubio, Le Perray en Yvelines, France), and AlexaFluor® 488 donkey anti-mouse (Invitrogen™); they were used at 10 µg mL^{-1} (Table 2).

2.7 Western blot

Protein extraction was adapted according to the protocol of Marshall et al. (2002). Following the dissection of gills (N=6) and kidney (N=3), the epithelia were scraped on ice and homogenized in 500 µL ice-cold SEI buffer (0.1 mol L^{-1} imidazole; 0.3 mol L^{-1}; 0.02 mol L^{-1} EDTA; pH 7.4) containing 75 µL of protease inhibitors (PI) (Complete™, Mini, EDTA-free, Boehringer Mannheim GmbH, Penzberg, Germany) with a 1 mL Wheaton glass potter and incubated 1 hr on ice. The homogenates were centrifuged at 2000 g for 6 min at 4°C. Pellets were resuspended in 100 µL of SEI-PI buffer and centrifuged a second time at 2000 g for 6 min at 4°C. The resulting supernatants were stored at -80°C. The protein concentration of the supernatants was determined using the Bradford method (Bradford 1976) with a BSA (bovine serum albumin) standard.

Each sample of protein was loaded with the 4X Protein Sample Loading Buffer (LI-COR®) according to the manufacturer instructions and separated under denaturing conditions on a 3% stacking gel and a 7% running polyacrylamide gel as described by Bollag et al., (1996). Protein size was estimated with an Odyssey® One-Color Protein Molecular Weight Marker (LI-COR®). Following the migration, proteins were transferred on a wet (incubated 1 min in 100% methanol, rinsed with ultra-pure water, transferred in PBS for 2 min) Millipore Immobilon® FL PVDF membrane (LI-COR®) for 2 hrs 30 min using a semi-dry transfer apparatus (Bio-Rad, Hercules, California, USA). Blots were blocked with the Odyssey® Blocking Buffer for 1 hr at room temperature with gentle agitation. The membrane was exposed to the primary antibodies diluted in Odyssey® Blocking Buffer with 0.2% Tween 20 overnight at 4°C. After a rapid wash and 4x5 min washes in PBS-T (0.1% Tween 20 in phosphate-buffered saline at pH 7.3) at room temperature, the membrane was incubated for 50 min at room temperature and obscurity with the IRDye 800CW secondary antibody diluted in Odyssey® Blocking Buffer with 0.2% Tween 20 and 0.1% SDS. Following washes with PBS-T and a final wash of 5 min in PBS, the blots were visualized on an Odyssey® Infrared Imaging System (LI-COR®) and analyzed with Image Studio Software for Odyssey v1.1.7 (LI-COR®). The ClC-3 expression was quantified using the ratio ClC-3/Actin of the sum of the individual pixel intensity (of both ClC-3 detected bands).

2.8 Histology

Three fish were used at each condition. The dissected organs (gills and kidney) from SW-and FW-exposed fish were immersed in Bouin's liquid for 24 to 48 hrs, washed and dehydrated in ascending grades of ethanol prior to impregnation and embedding in Paraplast® (Sigma-Aldrich, USA). Sections of 4 µm were obtained with a Leitz microtome (Leica, Rueil-Malmaison, Ile-de-France, France) and transferred on poly L-lysine coated slides.

For the observation of the gills, slides were stained with Masson's trichome. The images were taken using a Leica Diaplan microscope coupled to a Leica DC 300F digital camera and the FW4000 Software. Measures of the lamellae thickness were performed on 3 animals for each condition. The medium thickness of lamellae (N=10 by animals) (mean of each lamellae calculated with three measures taken to the base, to the center and to the extremity of the lamellae) was taken from randomly chosen filaments. All measurements were performed on lamellae located in the central area of filaments to avoid bias due to the differential shape between lamellae located at the tip of filaments relative to more central areas.

2.9 Immunocytochemistry

The immunocytochemistry procedures were carried out according to Bossus et al. (2011) with little modifications. Immunocolocalization of ClC-3 and Na^+/K^+-ATPase (NKA) was performed. Slides were hydrated, washed in PBS buffer solution and antigenic sites were revealed by a sodium citrate solution and heating in a microwave oven for 2 min (twice). Slides were then immersed for 10 min into 0.01% Tween 20, 150 mM NaCl in 10 mM PBS, pH 7.3. Tissue saturation was performed by incubation in a solution of PBS-5% skimmed milk (PBS-SM 5%) for 20 min. Following 3 washes, the slides were incubated overnight at 4°C in a moist chamber with the primary antibodies in PBS-SM 0.5%. Following washes, the slides were incubated with the secondary antibodies in PBS-SM 0.5% for 1 hr at room temperature. Control slides were placed in the same conditions but without primary antibody to rule out any nonspecific staining. After 3 washes, the slides were mounted in an anti-bleaching mounting medium (Gel/Mount, Permanent Aqueous Mounting, Biomeda, Plovdiv, Bulgaria) and observed with a Leica® SPE DM2500 confocal microscope and its software Leica Application Suite© Advanced fluorescence 2.0.1. The wavelengths of excitation and recorded emission for each antibody and pseudocoloring are given in Table 2. Confocal images were taken at 0.8 µm intervals to generate Z-stacks. MRCs were identified through their NKA immunostaining. The number of MRCs was determined for 40 randomly chosen

filaments on three animals per condition. Quantification on ClC-3 immunostaining were performed on three animals per condition, from the analysis of at least 35 randomly chosen MRCs. ImageJ software, v.1.45S was used to perform quantification.

2.10 Statistics

Results are expressed as the mean ± standard error of mean (s.e.m.). The normality was tested using the Shapiro-Wilk and Lilliefors tests. Multiple comparisons of mean values was performed with an ANOVA and Tukey test (HSD: honestly significant difference), with a significance level of $p < 0.05$. All statistical analyses were conducted using the XlStat Software (Addinsoft, Paris, France).

3. Results

3.1 Nucleotide and amino acid sequence of ClC-3 in the sea bass and its topology

Sequencing of ClC-3 in the sea bass *D. labrax* generated a nucleotide coding sequence of 2388 bp, available in GenBank under Accession Number JN998891. The deduced amino acid (aa) sequence of 761 residues had a predicted molecular mass of 84.89 kDa and a theoretical isoelectric point of 8.16. The deduced aa sequence of ClC-3 exhibited a high degree of identity of 95-97% with other fish ClC-3 sequences and of 90-91% with mammals (Table 3, Fig. 1). In the majority of mammals, the protein was longer in the N-terminus with 23 more aa than in the pufferfish *T. nigroviridis* and salmon *S. salar*, and 57 more aa than in the sea bass and in the tilapia *O. mossambicus*. The C-terminus appeared more conserved among the analyzed species, with some additional aa residues in *O. niloticus*, *T. nigroviridis* and in the common marmoset *Callithrix jacchus*. Three potential sites of N-linked glycosylation are highly conserved in fish and mammals (except for the third site, absent in the pufferfish): the first is located between TM1 and TM2 and the two following ones between TM8 and 9 (asterisks in Fig. 1). Conserved motifs within all ClCs were also found in the *D. labrax* sequence (in bold in Fig. 1).

The protein topology of ClC-3 was predicted according to the hydrophobic profile analyzed with the Kyte and Doolittle method (Fig. 2a). At least 10 to 12 hydrophobic regions may be transmembrane domains (see also Fig. 1, underlined aa). The predicted topology illustrated in Figure 2b shows that the first loop and the loop located between TM 8 and 9 are

longer than the others. The last two couples of TM domains (9-10 and 11-12) are long, difficult to distinguish, and they might cross the membrane several times. The two N- and C-termini are intracellular and the long C-terminus is composed of two cystathionine-β synthase (CBS) domains (indicated in italics in Fig. 1).

The CBS1 and 2, following each other, are separated by only thirty aa residues. The CBS1 domain of *D. labrax* is composed of 67 aa and when compared to mammals, this domain shows variations in some amino acids that are common to most fish in the alignment, particularly to the pufferfish sequence. The aa sequence homology of sea bass CBS1 ranges from 95% to 99% compared to other fish species, and this domain shares about 80% aa homology compared to mammals (Table 4). Furthermore, the CBS2 domain of 59 aa is identical to the majority of species present in this alignment of sequences in the first 52 aa (with an average of 97% aa homology for CBS1 sequences), except for the common marmoset, the tilapia (*O. niloticus*) and the pufferfish (an average of only 83% of aa homology). These species show a higher homology between them than with other species, with the same seven aa variations from the 793th to the 808th aa. The extremity of the C-terminus is the most variable section of the protein.

3.2 Expression of ClC-3 and α1 subunit NKA mRNA according to salinity

The expression level of the housekeeping gene, the elongation factor EF1α, did not change in any analyzed organ and salinity condition (results not shown), as previously shown in this species (Lorin-Nebel et al. 2006) and in other fish species (Frost and Nilsen 2003).

The mRNA expression levels of ClC-3 and of the α1-subunit of NKA in the gills, kidney and brain from SW-acclimated sea bass and from fish exposed to FW for 30 days are illustrated in Figure 3a. The relative expression of ClC-3 was higher in the brain than in the kidney (by about 91% in SW and 90% in FW) and gills (by about 90% in SW and 94% in FW). The mRNA expression of ClC-3 was significantly lower in FW than in SW in these three organs, by 44, 37 and 69% in the brain, kidney and gills, respectively.

Compared to SW conditions, the branchial ClC-3 mRNA expression (Fig. 3b) was significantly lower (by 83%) 10 min after the FW transfer, and it remained constant for longer exposures times with a low inter-individual variability. Compared to SW, the branchial NKA expression was significantly higher in FW by 70-74% 10 min and 1 hr after the FW transfer. From 24 hrs to 30 d in FW, the NKA expression reached an intermediate expression level,

significantly higher than in SW by about 42-54%. The inter-individual variability was also higher from FW-10 min to FW-7 d than in SW and FW-30 d.

The renal ClC-3 expression significantly decreased 10 min after FW exposure (by 42%) and it remained stable up to 30 days (Fig. 3c). The expression of NKA was significantly lower at 1 hr in FW compared to SW conditions, and it was significantly higher by about 30% at longer exposure times (from 24 hrs).

3.3 ClC-3 antibody specificity

Two main immunoreactive bands of about 84 and 95 kDa were detected either alone or together in the gill samples of sea bass exposed to SW or FW (Fig. 4a). The gill samples from SW fish and from fish maintained for 10 min to 1 hr in FW showed mainly the 84 kDa band. Fish maintained for 24 hrs in FW showed both bands whereas fish maintained from 7 d to 30 d in FW showed mainly the 95 kDa band. In the kidney, one band of about 95 kDa was found in all conditions (Fig. 4b). A slight band was also visible at about 63-67 kDa in gills and kidney samples. The samples incubated with antibody/antigen of ClC-3 did not show any band in Western blots (results not shown) such as did the samples incubated with only the secondary antibody.

3.4 Immunostaining of NKA and ClC-3 in gills of sea bass exposed to SW and FW

In SW fish and in fish maintained from 10 min to 1 hr in FW, the branchial MRCs are located in the filament at the basis of lamellae (Fig. 5a.a'") which are thin and have a regular thickness of about 13 µm (Fig. 5a'"). The thickness was less regular from 24 hrs to 7 d after a direct transfer to FW and lamellae were 30% thicker (about 17 µm) after 30 d FW-exposure (Fig. 5b'"). The thickness of lamellae was significantly higher in sea bass maintained for 30 d in FW compared to SW and to short-term FW conditions (Fig. 6a). A temporary slight but significant decrease was noted after one day in FW (Fig. 6a).

In gills, ClC-3 (Fig. 5a'.b') is co-localized with NKA (Fig. 5a.b.a".b") in the basolateral membrane of all MRCs in SW and at all times of FW exposure. Following transfer to FW, the total number of immunostained MRCs was stable over 24 hrs then significantly increased by 69% at 7 d in FW compared to the SW condition and by 83% at 30 d in FW (Fig. 6b). This increase was notably due to the migration of MRCs into the lamellae from 24 hrs (Fig. 5b.b'.b"). The intensity of ClC-3 immunostaining was quantified in MRCs of fish acclimated to SW and FW for various times. It increased significantly by 33 % 1 hr after the FW transfer

(Fig. 6c), then slightly decreased from 24 hrs to 30 d in FW but remained significantly higher than in SW by about 20%.

The slides incubated with antibody/antigen of ClC-3 did not show any staining (results not shown). None of the negative control slides showed immunostaining when using only secondary antibodies (results not shown).

3.5 Western blot quantification of ClC-3 in the gills of sea bass exposed to SW and FW

The ClC-3 protein quantification by Western blot was performed on branchial epithelium homogenates in SW-adapted sea-bass and in fish exposed to FW at various times (Fig. 6d).

A significant increase in ClC-3 protein expression was detected in the samples from 7 to 30 d in FW (Fig. 6d). At these exposure times, ClC-3 protein expression was about 31% higher than in SW or shortly after FW exposure (10 min to 24 hrs).

3.6 Immunostaining of NKA and ClC-3 in the kidney of sea bass exposed to SW and FW

NKA immunostaining was observed at all salinity conditions in all kidney ducts and tubules: the collecting duct (CD), the urinary tubules (UT) and the collecting tubules (CT) (Fig. 7a.b.c). NKA staining was different according to the tubule/duct and with salinity. In SW, NKA was slightly expressed in the basolateral membrane of the UT, CD cells (Fig. 7a). NKA staining was similar in fish exposed to SW and for short-term in FW (from 10 min to 24 hrs) (data not shown). In fish exposed for longer exposure times in FW, NKA staining was higher in all of the above-mentioned kidney sections compared to the SW samples (Fig. 7b.c) and to short-term FW samples, with a strong and homogeneously distributed fluorescence in the CD and CT cells.

ClC-3 immunostaining, which was not observed in the SW kidney (Fig. 7a'), was detected in FW from 7 to 30 d (Fig. 7b',c'). ClC-3 was basolaterally located in the CD and the CT, co-localized with NKA with a homogeneously distributed immunofluorescence throughout the cell (Fig. 7b".c").

3.7 Western blot quantification of ClC-3 in the kidney of sea bass exposed to SW and FW

The ClC-3 protein quantification by Western blot was performed on kidney homogenates in SW-adapted sea bass and in fish exposed to FW at various times (Fig. 8).

Quantification of the immunoreactive band in the kidney showed no remarkable variation between salinity conditions, except at FW-7 d were the protein expression was slightly higher.

4. Discussion

In FW, fish need to compensate the passive ionic loss by active uptake of ions in gills and by ion reabsorption in the kidney. In these organs, the apical transport of chloride in FW-exposed sea bass is mediated by a NKCC/NCC co-transporter. The mechanisms of basal chloride absorption still remains only partly understood (Hirose et al. 2003; Hwang and Lee 2007; Marshall 2002). The chloride channel 3 (or ClC-3), activated by hypotonicity and involved in cell volume regulation in mammals (Duan et al. 1997; Hoffmann et al. 2007; Wang et al. 2000), might be involved in hyper-osmoregulation and osmosensing in fish osmoregulatory organs. The sea bass is an unique model where the net movements of chloride ions clearly differ according to salinity. This is probably related to a differentially expressed ClC-3 channel, which might be a key channel involved in osmoregulation.

4.1 NKA expression in *D. labrax*

NKA is responsible for the electrochemical gradient activating osmoregulatory mechanisms in cells, and its activity reflects the level of osmoregulation in fish. NKA activity has often been reported to be lowest at the isosmotic salinity and to increase when fish including sea bass are exposed to higher or lower salinities (Evans and Claiborne 2006; Nebel et al. 2005b). NKA is constituted by a catalytic α-subunit and a glycoprotein β-subunit. Several α-subunits and their isoforms (α1a, α1b, α1c, α3) are known in fish species, some (for example α1a and α1b in salmonids, α1 and α3 in tilapia and milkfish) being differentially expressed according to salinity (Bystriansky et al. 2006; Hwang et al. 2011; Marshall 2002; McCormick et al. 2009; Richards et al. 2003). In the gills of some euryhaline salmonids, the NKA α1a was more expressed in FW than in SW whereas NKA α1b was more expressed in SW than in FW (Bystriansky et al. 2006; Hwang et al. 2011; McCormick et al. 2009; Richards et al. 2003), suggesting a role for the α1b isoform in ion secretion and for α1a in ion uptake. No evidence on differentially expressed α1a and α1b isoforms has been available in *D. labrax* so far. In the

present study, the primers used seems not specific to either α1a or α1b. Whether a specific α1a isoform is involved remains to be investigated in future studies.

The α1-NKA mRNA expression in the gills and kidney changed almost immediately (10 min) after FW exposure (increased in the gills and decreased in the kidney). Its expression was stable and higher in FW compared to SW in both organs after 24 hrs to 30 d, a fact which is consistent with previous studies in sea bass (Giffard-Mena et al. 2008; Jensen et al. 1998; Nebel et al. 2005b). This increase in mRNA expression of the NKA α1-subunit in FW was followed by an increase in the protein abundance and/or activity in sea bass gills and kidney (Nebel et al. 2005b; Varsamos et al. 2002); it has also been reported in *T. nigroviridis* (Lin et al. 2004). In previous studies, significant changes in the NKA mRNA expression have been shown as early as 6 hrs after a salinity shock in a tilapia (Lin et al. 2006) and as early as 1 day in the sea bass (Jensen et al. 1998). In this study, NKA expression was measured in the sea bass 10 min after a hyposmotic exposure and its significant change after such a short time is worth considering. Since gills are in direct contact with the external medium, NKA expression might be up-regulated through a local branchial osmosensor potentially activating transcription factors. The change in external osmolality is thus sensed without delay and it triggers rapid gene expression changes. This regulation could also be mediated via hormonal regulation, for instance by cortisol (McCormick 2011). Plasma cortisol levels are higher in FW-adapted sea bass (after 1 month of acclimation) than in SW-adapted fish (Varsamos et al. 2006); to our knowledge no data are available after a short-term FW transfer. After a long-term acclimation to FW, the increase in branchial NKA expression, triggered by hormonal changes, is probably also linked to the increase in the number of FW-type MRCs shown in the sea bass (Nebel et al. 2005b; Varsamos et al. 2002).

In this study, the impact of salinity changes on NKA expression was slower in the kidney, which might be related to the fact that this organ is not in direct contact with the environment. NKA expression changes might be triggered by the hormonal pathway or by osmosensors of blood osmolality changes. In SW, active ionic uptake by the kidney tubules and ducts would allow water reabsorption by osmosis to compensate the passive water loss, resulting in the production of a limited volume of urine isotonic to the blood as shown in this species (Nebel et al. 2005a; Nebel et al. 2005b) and in other teleosts (Evans 2009; Marshall and Grosell 2005; Richards et al. 2003). In FW, the high expression of α1-NKA mRNA and of the protein respectively from 24 hrs and 7 d, which coincides with a significant blood

osmolality decrease (Bossus et al. 2011), may result in an increased ionic uptake which is necessary to partly compensate the ion losses that occur at low salinity. As previously reported, the collecting tubules and ducts express more NKA than anterior segments, a fact that might be related to differential regulation pathways among kidney segments (Nebel et al. 2005b). A further study on α1-NKA isoforms in *D. labrax* should allow a clarification of the precise role and involvement of each isoform in osmoregulation.

4.2 ClC-3 sequence analysis and structural features

The *D. labrax* ClC-3 gene encodes a protein of 761 aa, as in *O. mossambicus* (Miyazaki et al. 1999). This protein is highly conserved in fish and in mammals, with amino acid homologies ranging from 90 to 97% and with similar structural features (Jentsch et al. 2002). The ClC-3 predicted topology in the sea bass contains about 13 hydrophobic regions and at least 12 putative transmembrane domains (TM) as shown in other ClC channels (Jentsch et al. 1999), including ClC-3 in *O. mossambicus* (Miyazaki et al. 1999). This observation sustains the intracellular localization of N- and C-termini (Dutzler et al. 2002; Schmidt-Rose and Jentsch 1997a; Schmidt-Rose and Jentsch 1997b). The ClC-3 of *T. nigroviridis* however has been predicted to contain only 11 TM and a N-terminal located in the extracellular compartment (Tang et al. 2010).

N-glycosylation sites are exclusively located out of the cytoplasm in transmembrane proteins (Gupta and Brunak 2002; Jentsch et al. 2002) and studies on other ClC channels (Schmidt-Rose and Jentsch 1997b) corroborate the predicted topology of ClC-3 in *D. labrax*. Two potential N-linked glycosylation sites are common with mammals and fish and correspond to the 120^{th} (after TM1) and 394^{th} aa (between TM8 and 9) in *D. labrax*. This last site has been demonstrated in other ClC channels (Kieferle et al. 1994) as glycosylated, confirming its outer localization (Jentsch et al. 1995). These findings and the cytoplasmic location of N- and C-termini imply that one of the TM domains of ClC-3 in the sea bass does not span the membrane. The insertion of TM4 in the plasma membrane has been broadly discussed (Fahlke et al. 1997; Jentsch et al. 1999; Jentsch et al. 2002; Waldegger and Jentsch 2000) and whether this domain spans the membrane or not still remains unclear. In our study, TM4 seems less hydrophobic than other regions, which could point to an outside location. In the same way, the broad hydrophobic region from TM9 to TM12 is unclear (Jentsch et al. 1999; Jentsch et al. 2002; Waldegger and Jentsch 2000) and should cross the membrane 3 or 5 times to allow an intracellular location of both protein extremities. Modifications in

glycosylation sites in fish might change the functional properties or the protein stability of ClC-3 in fish compared to mammals. Further studies on ClC-3 topology, using glycosylation scanning, should clarify the sites of glycosylation in the sea bass ClC-3.

The sequencing of ClC-3 in *D. labrax* has shown two CBS domains contained in the long C-terminal tail, as in other eukaryotic cells (Jentsch et al. 1999). The precise role of CBS domains is still not fully known, but some mutations in these domains lead to the loss of function or to gating alteration (Estévez et al. 2004; He et al. 2006; Jentsch et al. 2002; Nehrke et al. 2000; Schmidt-Rose and Jentsch 1997b). CBS domains have a crucial role in the conformation and function of ClC proteins, such as ion selectivity (Dave et al. 2010; He et al. 2006; Jentsch et al. 1999), and their role varies according to the considered ClC channel (Carr et al. 2003; Hebeisen et al. 2004). CBS1 and 2 domains together in ClC could bind nucleosides such as ATP (Bennetts et al. 2005; Ignoul and Eggermont 2005; Scott et al. 2004b) and they have been suggested as regulating ion transport by gating control (Kemp 2004; Vanoye and George 2002).

In this study, the amino acid alignment of the CBS2 domain showed a high aa homology with the majority of ClC-3 sequences in our alignment (from 83 to 97% homology). The percentage of homology of the CBS1 domain among the fish species in the alignment was high (with 97% of aa homology) compared to mammals (with 81% of aa homology with the sea bass). Phylogenetic analyses of CBS domains seem to reflect the evolutionary history of these domains in organisms and they suggest differential evolution resulting in the adaptation to specific functions, including responses to osmotic stress (Kushwaha et al. 2009). The distinction between mammals and fish, based on the aa homology of the CBS1, might indicate a variation in the involvement of this domain in the ClC-3 function between these two groups. Further studies on the CBS domains of ClC-3 in teleosts should determine whether mutations in nucleotides of the CBS domains that are specific to teleosts involve modifications in the protein function and possibly in osmoregulation.

4.3 ClC-3 expression in *D. labrax*

4.3.1 *Western blot*

The ClC-3 antibody used in this study was directed against the highly conserved CBS-1 domain of rat ClC-3, which is 83% homologous to the residues 593-662 of the sea bass. It has

been successfully used in other teleosts species (Shen et al. 2007; Tang et al. 2011; Tang et al. 2010; Tang and Lee 2007; Tang and Lee 2011). The slight band of about 63-67 kDa found in both organs could be an immature ClC-3 protein or an artifact due to a degradation of the protein samples used in this study.

In the gills of SW sea bass, the predominant band was detected at 85 kDa. In FW, however, the predominant band was detected at 95 kDa. This change in molecular weight according to salinity suggests the presence of different ClC-3 isoforms that might both be recognized by the antibody and whose expression is differentially activated by salinity. In the kidney, the 95 kDa band was detected at all salinity conditions. In other fish and in mammals, the ClC-3 molecular mass ranges from 80 to 120 kDa (Robinson et al. 2004; Tang et al. 2010; Tang and Lee 2007; Tang and Lee 2011) according to the species, or in a same species according to the organ (Schmieder et al. 2001), which is consistent with the present results. The potential sites of N-linked glycosylation might also induce a variation in the molecular weight of ClC-3 in *D. labrax*. Molecular mass in *T. nigroviridis* was about 102 kDa and fell to about 90 kDa after deglycosylation by removal of the N-linked sugar of the protein (Tang et al. 2010). N-glycosylation would confer a better stability to the ClC-3 protein, or it could act in signal transduction via a change in the structure or function of the protein (Taylor and Drickamer 2003). In this case, ClC-3 might be consistently more glycosylated in the kidney or in gills after a hypotonic shock, explaining higher molecular weight bands under these conditions.

4.3.2 *Protein and mRNA expressions*

Surprisingly, the branchial and renal ClC-3 mRNA expression, measured in SW and after FW transfer, do not correlate with the protein expression. In gills, following a hypotonic shock, ClC-3 mRNA expression decreased rapidly from 10 min, while the protein expressions significantly increased from 7 d. This expression pattern according to salinity does not seem to be conserved in teleost species. In *O. mossambicus* and *T. nigroviridis*, the branchial ClC-3 mRNA expression seemed not to change or to slightly decrease between SW and FW (Miyazaki et al. 1999; Tang et al. 2010; Tang and Lee 2011). In the kidney, even if mRNA expression decreased in FW, the amount of protein remained relatively stable after exposure to FW with a slight increase at 7 d, when ClC-3 immunostaining increased. These results are in agreement with those reported in *T. nigroviridis*, in which no variation in branchial ClC-3 mRNA expression were recorded according to salinity while the ClC-3 protein amount was

significantly higher in hyposmotic conditions (Tang et al. 2010). In fact, the mRNA expression does not necessarily reflect the amount and/or the activity of proteins (Bossus et al. 2011; Scott et al. 2004a; Tang et al. 2010). In *D. labrax*, the low expression of ClC-3 mRNA in FW compared to SW may not reflect a lower protein quantity and a lower chloride transport in branchial cells. This lack of correlation between mRNA and protein expressions has already been reported for other proteins, including transmembrane channels such as CFTR in *Fundulus heteroclitus* (Chen et al. 2002; Greenbaum et al. 2003; Scott et al. 2004a). In this study, the localization and quantification of the ClC-3 protein at both salinities rather suggests a post-transcriptional regulation or it may originate from the existence of different ClC-3 isoforms and separate isoform-specific activation mechanisms (Chen et al. 2002), as suggested for CFTR in *F. heteroclitus* (Scott et al. 2004a). If this is the case, the mRNA expression previously measured might have recognized the 85 kDa SW-type isoform only, which was not upregulated in FW. The potential FW-type isoform, detected by Western blot, is thus possibly involved in hyper-osmoregulation and might also be up-regulated at the mRNA level, but this hypothesis must be tested in future studies. The existence of two isoforms of ClC-3 has been demonstrated in the plasma membrane of rat hepatocytes (Shimada et al. 2000) with different gating properties and potential involvement in native volume-sensitive outwardly rectifying anion currents (VSOAC), as is the case for other ClC-family channels (Chu and Zeitlin 1997; Eggermont et al. 1997; Loewen et al. 2000).

4.4 ClC-3 in the brain

In the brain, the expression of the ClC-3 protein in the sea bass was higher in SW than in FW, which is in agreement with its expression determined in *O. mossambicus* (Miyazaki et al. 1999). ClC-3 is highly expressed in the brain of mammals (Kawasaki et al. 1994) where possible functions include growth, vision and behavior (Stobrawa et al. 2001). In *Clcn3*$^{-/-}$ knockout mice, a rapid retina and hippocampus degeneration has been reported after birth (Stobrawa et al. 2001) followed by a larger neuro-degeneration. The main consequences are blindness and severe neurological damage, such as metabolic and behavior abnormalities (Jentsch 2008). In fish, the lateral pallium, which receives the main olfactory input, is homologous to the hippocampus in mammals and is crucial for spatial learning (Braithwaite 2011; Vargas et al. 2009). The high expression of ClC-3 in this tissue suggests that this channel could be essential to the operation of the brain, particularly the pallium, the ancestral structure of the hippocampus. Further studies on the location and functional characterization are thus necessary for a better understanding of the role of ClC-3 in the fish brain.

4.5 ClC-3 and osmoregulation in gills

In SW MRCs, basolateral NKCC-1 enables the transport of Na^+, Cl^- and K^+ from blood to the cell (Marshall et al. 2002), mediated by the electrochemical gradient generated by the NKA pump. Cl^- exits the cell apically through an apical CFTR, whereas Na^+ is transported to the outer medium via a paracellular pathway. The basolateral localization of ClC-3 reported here in the sea bass MRCs has also been recently detected in *O. mossambicus, T. nigroviridis* and *C. chanos* (Tang et al. 2011; Tang et al. 2010; Tang and Lee 2007; Tang and Lee 2011). The mechanisms of ion excretion in SW are well defined and raise the question of the role of ClC-3 in the basolateral membrane of SW MRCs. Chloride transport to the blood by branchial ClC-3 may contribute to acid-base regulation or intracellular chloride homeostasis, as suggested by the above-cited authors. This hypothesis is supported by an increase of mRNA and protein expression in gills of fish exposed to a low-Cl^- medium (Tang et al. 2010; Tang and Lee 2011). ClC-3 may also be present but not activated in SW; the gating properties might be modified following a hyposmotic shock triggering N-glycosylation of the channel. In mammals, ClC-3 was strongly activated by a hypo-osmotic environment and involved in cell volume regulation (Duan et al. 1997; Duan et al. 2001; Wang et al. 2000). The mean blood osmolality of sea bass at about 375 ±3 mOsm kg^{-1} in SW slowly decreased after 10 min of FW-exposure and the difference became significant from 24 hrs (at about 321 ±10 mOsm kg^{-1}) (Bossus et al. 2011), suggesting that the ClC-3 in fish may well be as responsive as in mammals to the hypo-osmoticity of the external medium. A hypotonic shock results in an immediate increase in cell volume due to osmotic water invasion. Considering the hypothesis of the presence of two isoforms, a SW and FW-type ClC-3, the decrease in the expression of the SW-type ClC-3 in SW MRCs seems physiogically relevant. Also, the higher mRNA expression in SW than in FW may originate from a role of ClC-3 in cell volume regulation; ClC-3 would act as a booster to promptly respond to an osmolality decrease by a Cl^- transport to the blood, triggering a rapid regulatory volume decrease. This hypothesis has been already suggested for another protein, the transient receptor potential vanilloid 4 (TRPV4), which is activated, as ClC-3, by hypotonicity and involved in the regulatory volume decrease process of the prolactin cells (Bossus et al. 2011) and in hypo-osmoreception in the euryhaline Mozambique tilapia, *O. mossambicus* (Seale et al. 2012).

The rapid decrease in the ClC-3 mRNA expression within a few minutes of FW exposure could be due to a rapid degradation of mRNA, the half-life of mRNA generally being around minutes or hours (Decker and Parker 1994). This mRNA change could also be

controlled by an inhibition of specific transcription factors by local branchial osmosensors (Evans 2009). An endocrine regulation might differentially activate the potential ClC-3 SW- and FW-type. This last hypothesis could be strengthened if there is a glucocorticoid regulatory element in the promoter region of ClC-3, which should be investigated in future studies.

In FW, Cl^- uptake from the environment in euryhaline teleosts is debated and often considered to be effected via the apical Cl^-/HCO_3^- exchanger protein AE1 (Evans et al. 2005; Shen et al. 2007; Tang et al. 2011; Wilson and Laurent 2002; Wilson et al. 2000a; Wilson et al. 2000b). In sea bass maintained in FW, a NKCC/NCC co-transporter was detected in the apical membrane of the MRCs (Lorin-Nebel et al. 2006). This co-transporter may be the NCC co-transporter, since the latter is expressed in sea bass FW gills (Boul, pers. comm.). NCC is apically located in MRCs of *O. mossambicus* (Hiroi et al. 1999; Inokuchi et al. 2009; Inokuchi et al. 2008). In this species, as in *D. labrax*, the basolateral location of ClC-3 should complete ionic uptake by allowing basal chloride efflux from the cell to the blood (Tang et al. 2011; Tang et al. 2010; Tang and Lee 2007; Tang and Lee 2011), contributing to hyper-osmoregulation.

FW adaptation involves morphological modifications, such as the occurrence of "FW-like" MRCs in lamellae, which are characterized by an apically located NKCC/NCC co-transporter involved in Na^+ and Cl^- absorption (Lorin-Nebel et al. 2006). In our study, the ClC-3 was co-localized with the NKA in the basolateral membrane of all MRCs in the same way and in all conditions. In some teleosts such as in *O. mossambicus* (Choi et al. 2011; Hwang et al. 2011; Tang and Lee 2011), several types of MRCs with a differential immunostaining of the osmoregulatory proteins (NKCC, NCC, CFTR, NHE3 and ClC-3) have been found. In contrast, in the sea bass, all MRCs at each salinity condition seem to present the same protein immunostaining properties (ClC-3, CFTR, NKA and NKCC1 in SW; ClC-3, NKCC/NCC and NKA in FW) except for the so-called immature MRCs found in 7 d FW-transferred sea bass expressing NKA but only low levels of NKCC/NCC (Bodinier et al. 2009a; Bodinier et al. 2009c; Lorin-Nebel et al. 2006; Nebel et al. 2005b; Sucré et al. 2012). The first lamellar MRCs were detected from 24 hrs after FW exposure, coupled with a significant blood osmolality decrease (Bossus et al. 2011) and the increase of NKA mRNA expression. The slight decrease in the lamellae thickness at 24 hrs in FW could be related to the temporary gill remodeling and increased apoptosis during the salinity challenge (Nebel et al. 2005b; Nilsson 2011). The density of lamellar MRCs progressively and significantly

increased until 30 d. The occurrence of these cells was probably responsible for the increased thickness of the lamellae, significantly higher in fish exposed to FW-30 d than in SW, and for the significant increase in the branchial ClC-3 protein amount. These results are similar to those reported in *C. chanos* where the number of lamellar MRCs was more important in FW than in SW (Tang et al. 2011), with a probable link to enhanced hyperosmoregulatory abilities. The increase in the lamellae thickness would raise the water-to-blood distance of O_2 diffusion and it suggests an impairment in oxygen uptake as previously suggested in the sea bass (Nebel et al. 2005b). However, in fish living in well aerated water, this proliferation of MRCs could be compensated by the increase in cardiac frequency, blood pressure and flow (Gonzalez 2011). The decrease in blood diffusion could be also followed by a slow-down in general fish activity as previously observed in the tilapia (Sardella et al. 2004).

Our and previous results points out that at least one week (time to allow significant increase in MRC density) is required to allow the highly plastic gill to shift to an efficient hyper-osmoregulatory epithelium. The increase in the ClC-3 protein amount linked to the occurrence of "FW-type" MRCs (Lorin-Nebel et al. 2006) is consistent with a role of ClC-3 in hyper-osmoregulation in the sea bass.

Furthermore, measurements of the immunostaining intensity of ClC-3 has shown a significant increase of ClC-3 in each cell from 1 hr in FW, followed by a slight decrease but still at a higher level than in SW. This rapid increase in the amount of ClC-3 may allow a rapid response following the FW exposure. The increased ClC-3 amount within each cell would then reflect an increased need for Cl^- flux through the basolateral membrane immediately after a hypotonic shock, either for transepithelial transport or for cell volume regulation. This is in agreement with the hypothesis of an inactive ClC-3 in SW.

4.6 ClC-3 and osmoregulation in the kidney

The kidney is important in fish osmoregulation through its control of urine flow and of its ionic composition (Evans and Claiborne 2006). In SW, euryhaline fish such as the sea bass produce a low volume of urine isotonic to the blood (Hickman and Trump 1969; Nebel et al. 2005a; Nishimura and Imai 1982) to retain water and excrete ions. As shown in other teleosts, the sea bass renal NKA amount and activity are lower in SW than in FW which is the result of the lower need of ion reabsorption in SW (Lasserre 1971; Nebel et al. 2005b). The low necessity to reabsorb ions in the kidney of euryhaline fish exposed to SW is consistent with the low expression of ClC-3. The absence of basal ClC-3 immunodetection in SW could be

due to a lower sensitivity of the immunocytochemical detection system compared to Western blots.

In sea bass maintained in FW, urine is hypotonic to blood as a result of active ion reabsorption notably by the collecting ducts (CD) and the collecting tubules (CT) (Nebel et al. 2005a). The renal NKA protein amount, activity and mRNA expression are significantly increased in FW (Lasserre 1971; Nebel et al. 2005a). The electrochemical gradient created by NKA enables apical ion entry through an apical NKCC/NCC co-transporter (Lorin-Nebel et al. 2006). The predominant ionic reabsorption takes place in segments of the kidney which are less permeable to water, and which allow the excretion of excess water in urine while retaining filtered solutes. In sea bass, NKA is highly expressed in collecting tubules and ducts in FW (Nebel et al. 2005b). This study shows for the first time the presence of the basolateral chloride channel ClC-3 in renal cells of fish maintained in FW, probably involved in transepithelial chloride transport to the blood.

The potential role of ClC-3 in the kidney of fish and mammals is unknown. ClC-K channels have been reported as involved in basolateral Cl^- uptake in the mammalian thick ascending limb of Henle's loop (Jentsch et al. 2005), to which the distal tubule of fish is homologous (Miyazaki et al. 2002). In *O. mossambicus*, ClC-K was almost exclusively expressed in the kidney on the basolateral membrane of the distal tubule, with a higher expression in FW than in SW (Miyazaki et al. 2002). Its role in Cl^- reabsorption by the distal tubule of the kidney, allowing the production of diluted urine, has thus been suggested. In the sea bass, ClC-3 occurred in the kidney from 7 d in FW, which may support the hypothesis of its involvement in chloride transport. However, the low mRNA and protein expression of ClC-3 in FW conditions in this species also point to other roles such as acid-base regulation, intracellular chloride regulation or blood chloride homeostasis.

The decrease in blood osmolality is significant only from 24 hrs of FW-exposure (Bossus et al. 2011); cells expressing ClC-3 in the kidney are therefore in contact with a lower blood osmolality only after this time lapse, which can be related to the fact that an increased amount of ClC-3 is detected only after 7d of FW exposure. The low amount of ClC-3 after the short-term transfer suggests that the cell volume regulation by the kidney probably does not involve ClC-3. The Western blot shows the same higher band of about 95 kDa as the one found mainly in FW gills. The "FW-type" ClC-3 (another isoform or the same protein glycosylated) is the only one type found in the kidney in all conditions, and this supports the role of ClC-3 in hyper-osmoregulation in the kidney.

5. Conclusion

Our results indicate two levels of sequential adaptation of the gills to hyposmotic environment, - a short-term reaction, with a slight increase of ClC-3 immunostaining allowing the cells to quickly transport chloride to the blood, - and a long-term response, with deep morphological modifications of the branchial epithelium allowing the fish to efficiently hyper-osmoregulate in FW. These adaptations appear of utmost importance for the migrations of *D. labrax* between environments at different salinities, e.g. between the sea and estuaries or lagoons, i.e. on either sides of the physiological barrier represented by the isosmotic salinity of about 11-12‰ (320-350 mOsm kg^{-1}). The short-term reaction would permit short incursions in such low salinity media, while longer residence in these environments would trigger the long-term response. The co-localization of ClC-3 with NKA in osmoregulatory tissues and its high abundance in FW gills, and to a lesser extent in the kidney, points to an involvement in hyper-osmoregulation. In SW gills, this protein seems rather inactivated and involved in rapid cell volume regulation in case of a FW transfer. The detection of two sizes of ClC-3 by Western blot, depending on the salinity, suggests two different isoforms in gills, which could be linked to the shift in the direction of net ion transports following a salinity challenge. However, only one band was detected in the kidney in both salinities, and thus only one isoform seems involved, suggesting that the direction of ion transport remains unchanged.

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Wilson JM, Randall DJ, Donowitz M, Vogl AW, Ip AK (2000b) Immunolocalization of ion-transport proteins to branchial epithelium mitochondria-rich cells in the mudskipper (*Periophthalmodon schlosseri*). J Exp Biol 203 (15):2297-231

Table 1.

Primer sequences used in this study.

Primer name	Nucleotide sequences (from 5' to 3')	Use
ClC-3-d1-F	TAACTACRHCATGTCWAAYGG	CS
ClC-3-d2-F	GGAYGACMTGACDGTGGAGG	CS
ClC-3-d3-R	CYGTYCARGAGGCMCACC	CS
ClC-3-d4-R	GGAARGAGSKGAYYAGTTGAAC	CS
ClC-3-d5-R	CAGAGCRAAGCCMACCAG	CS
ClC-3-R-3	AACACGCCCAGGAGGATGAA	CS
5'RACE-ClC-3-SP1	CGTTCATCCAGTCAGCAGCA	RACE
5'RACE-ClC-3-SP2	GGCGTCGTACAGGCTCTTTG	RACE
5'RACE-ClC-3-SP3	GGAAGTCCAACAGGTGGGTG	RACE
3'RACE-ClC-3-F	CTTCCCTCTCAAGACGCTGTG	CS, RACE
ClC-3-F-1	CAAGTACAGCAAGAACGAGGC	CS, qPCR
ClC-3-R-2	ACAGCGTCTTGAGAGGGAAG	CS, qPCR
NKA-F	AGAGGGATGTTGGCGATGAT	qPCR
NKA-R	CTGCTGGACGACAACTTTGC	qPCR
EF1α-F	GGCTGGTATCTCTAAGAACG	qPCR
EF1α-R	CCTCCAGCATGTTGTCTCC	qPCR

The sequences used standard IUPAC code: D: A/G/T; H: A/C/T; K: G/T; M: A/C; R: A/G; S: C/G; W: A/T; Y: C/T. CS: primer used for cloning and sequencing; d: degenerate; F: forward; qPCR: primer used for real-time PCR; R: reverse; RACE: primer used in RACE amplification.

Table 2.

Secondary antibodies used to stain anti-NKA and anti-ClC-3 antibodies (immunochemistry and Western blot).

1st antibody	2nd antibody	Ex. (nm)	Em. (nm)	Pseudocolor
NKA	Alexa Fluor 488	488	500-520	Green
ClC-3	Rhodamine	532	560-610	Red
	IRDye® 800CW	778	795	Green
Actin	IRDye® 800CW	778	795	Green

ClC-3: chloride channel 3; Em.: wavelength of emission; Ex.: wavelength of excitation; NKA: Na^+/K^+-ATPase.

Table 3.

Percentage of amino acid homology of the branchial *D. labrax* ClC-3 sequence with ClC-3 from other vertebrates.

Species	Common name	GenBank Accession number	Amino acid homology
Oreochromis mossambicus	Mozambique tilapia	AAD56388.1	97%
Oreochromis niloticus	Nile tilapia	XP_003447641.1	96%
Tetraodon nigroviridis	Pufferfish	ACL79521.1	95%
Salmo salar	Atlantic salmon	NP_001167057.1	95%
Rattus norvegicus	Common rat	NP_445815.2	91%
Callithrix jacchus	Common marmoset	XP_002745267.1	90%
Monodelphis domestica	Opossum	XP_003341450.1	91%
Ornithorhynchus anatinus	Platypus	XP_001506627.1	90%
Homo sapiens	Human	AAI36511.1	91%

Homologies were calculated according to the NCBI blast.

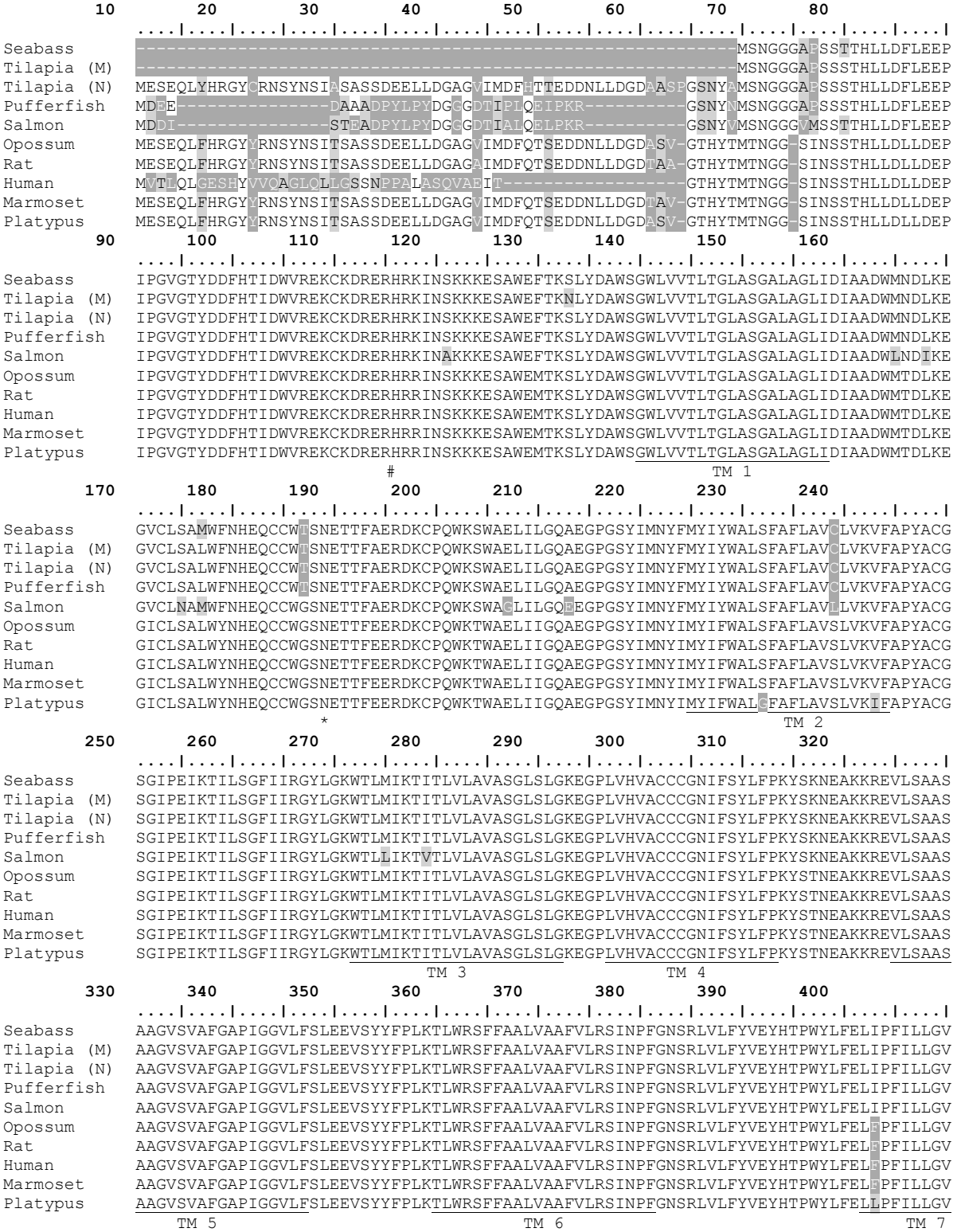
Table 4.

Percentage of amino acid homology of each CBS domain of *D. labrax* ClC-3 with CBS domains sequences of ClC-3 from other vertebrates.

Species	Amino acid homology for CBS1 domain	Amino acid homology for CBS2 domain
Oreochromis mossambicus	96%	98%
Oreochromis niloticus	95%	81%
Tetraodon nigroviridis	99%	85%
Salmo salar	97%	97%
Rattus norvegicus	**82%**	97%
Callithrix jacchus	**81%**	82%
Monodelphis domestica	**82%**	98%
Ornithorhynchus anatinus	**79%**	95%
Homo sapiens	**81%**	97%

Homologies were calculated according to the NCBI blast. Percentage in bold indicates a lower aa homology with *D. labrax* in the CBS1 domain.

Figure 1.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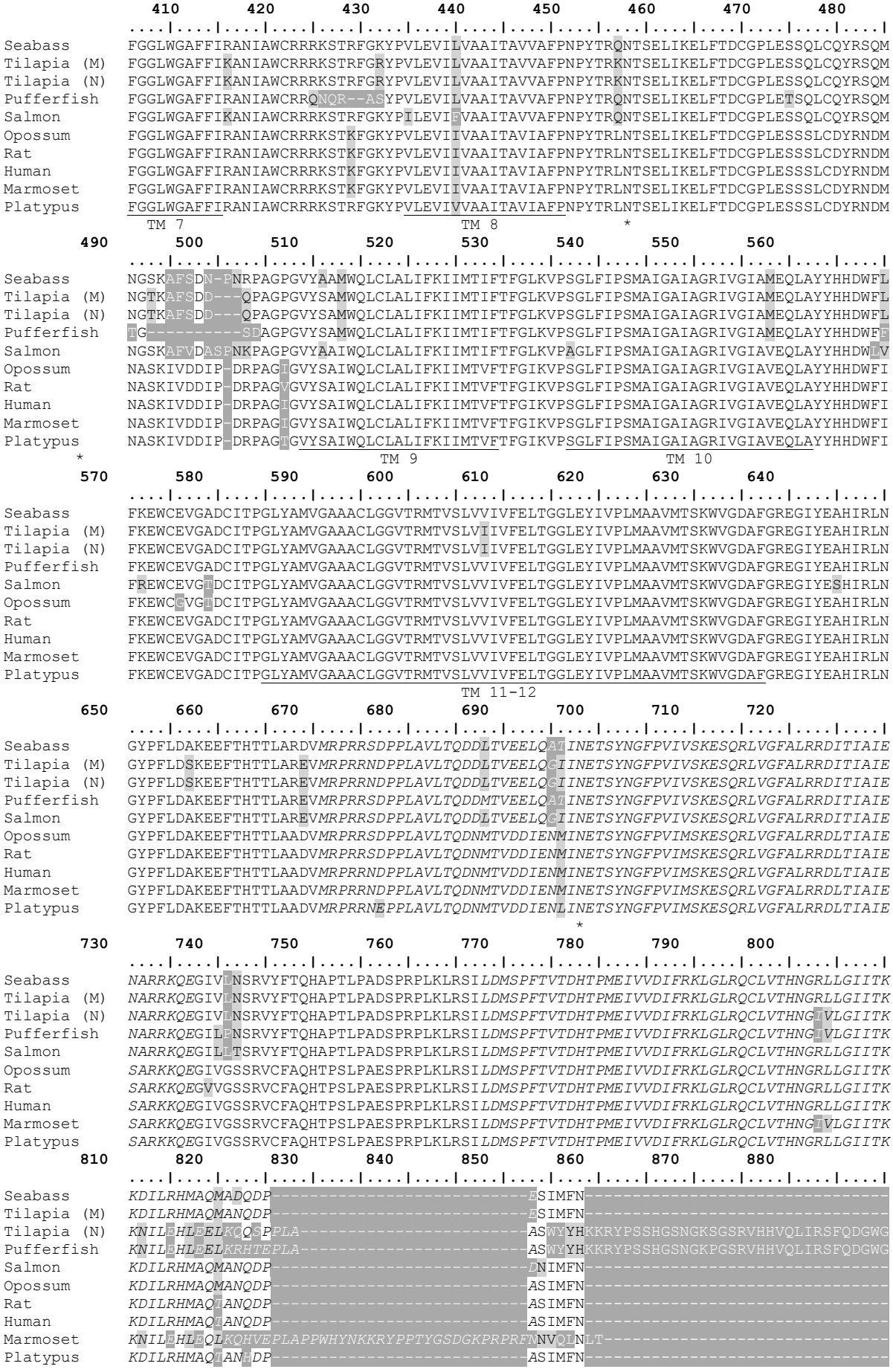
Tilapia (M)
Tilapia (N)
Pufferfish
Salmon
Opossum
Rat
Human
Marmoset
Platypus
90 100 110 120 130 140 150 160
170 180 190 200 210 220 230 240
250 260 270 280 290 300 310 320
330 340 350 360 370 380 390 400
#
TM 1
*
TM 2
TM 3
TM 4
TM 5
TM 6
TM 7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Tilapia (M) FGGLWGAFFIKANIAWCRRRKSTRFGRYPVLEVILVAAITAVVAFPNPYTRKNTSELIKELFTDCGPLESSQLCQYRSQM
Tilapia (N) FGGLWGAFFIKANIAWCRRRKSTRFGRYPVLEVILVAAITAVVAFPNPYTRKNTSELIKELFTDCGPLESSQLCQYRSQM
Pufferfish FGGLWGAFFIRANIAWCRRQNQR--ASYPVLEVILVAAITAVVAFPNPYTRQNTSELIKELFTDCGPLETSQLCQYRSQM
Salmon FGGLWGAFFIKANIAWCRRRKSTRFGKYPILEVIFVAAITAVVAFPNPYTRQNTSELIKELFTDCGPLESSQLCQYRSQM
Opossum FGGLWGAFFIRANIAWCRRRKSTKFGKYPVLEVIIVAAITAVIAFPNPYTRLNTSELIKELFTDCGPLESSSLCDYRNDM
Rat FGGLWGAFFIRANIAWCRRRKSTKFGKYPVLEVIIVAAITAVIAFPNPYTRLNTSELIKELFTDCGPLESSSLCDYRNDM
Human FGGLWGAFFIRANIAWCRRRKSTKFGKYPVLEVIIVAAITAVIAFPNPYTRLNTSELIKELFTDCGPLESSSLCDYRNDM
Marmoset FGGLWGAFFIRANIAWCRRRKSTKFGKYPVLEVIIVAAITAVIAFPNPYTRLNTSELIKELFTDCGPLESSSLCDYRNDM
Platypus FGGLWGAFFIRANIAWCRRRKSTRFGKYPVLEVIVVAAITAVIAFPNPYTRLNTSELIKELFTDCGPLESSSLCDYRNDM
TM 7
TM 8
*
490 500 510 520 530 540 550 560
Seabass NGSKAFSDN-PNRPAGPGVYAAMWQLCLALIFKIIMTIFTFGLKVPSGLFIPSMAIGAIAGRIVGIAMEQLAYYHHDWFL
Tilapia (M) NGTKAFSDD---QPAGPGVYSAMWQLCLALIFKIIMTIFTFGLKVPSGLFIPSMAIGAIAGRIVGIAMEQLAYYHHDWFL
Tilapia (N) NGTKAFSDD---QPAGPGVYSAMWQLCLALIFKIIMTIFTFGLKVPSGLFIPSMAIGAIAGRIVGIAMEQLAYYHHDWFL
Pufferfish TG----------SDAGPGVYSAMWQLCLALIFKIIMTIFTFGLKVPSGLFIPSMAIGAIAGRIVGIAMEQLAYYHHDWFF
Salmon NGSKAFVDASPNKPAGPGVYAAIWQLCLALIFKIIMTIFTFGLKVPAGLFIPSMAIGAIAGRIVGIAVEQLAYYHHDWLV
Opossum NASKIVDDIP-DRPAGIGVYSAIWQLCLALIFKIIMTVFTFGIKVPSGLFIPSMAIGAIAGRIVGIAVEQLAYYHHDWFI
Rat NASKIVDDIP-DRPAGVGVYSAIWQLCLALIFKIIMTVFTFGIKVPSGLFIPSMAIGAIAGRIVGIAVEQLAYYHHDWFI
Human NASKIVDDIP-DRPAGIGVYSAIWQLCLALIFKIIMTVFTFGIKVPSGLFIPSMAIGAIAGRIVGIAVEQLAYYHHDWFI
Marmoset NASKIVDDIP-DRPAGIGVYSAIWQLCLALIFKIIMTVFTFGIKVPSGLFIPSMAIGAIAGRIVGIAVEQLAYYHHDWFI
Platypus NASKIVDDIP-DRPAGIGVYSAIWQLCLALIFKIIMTVFTFGIKVPSGLFIPSMAIGAIAGRIVGIAVEQLAYYHHDWFI
*
TM 9
TM 10
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Seabass FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Tilapia (M) FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVIIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Tilapia (N) FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVIIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Pufferfish FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Salmon FREWCEVGTDCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYESHIRLN
Opossum FKEWCGVGTDCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Rat FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Human FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Marmoset FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
Platypus FKEWCEVGADCITPGLYAMVGAAACLGGVTRMTVSLVVIVFELTGGLEYIVPLMAAVMTSKWVGDAFGREGIYEAHIRLN
TM 11-12
650 660 670 680 690 700 710 720
Seabass GYPFLDAKEEFTHTTLARDVMRPRRSDPPLAVLTQDDLTVEELQATINETSYNGFPVIVSKESQRLVGFALRRDITIAIE
Tilapia (M) GYPFLDSKEEFTHTTLAREVMRPRRNDPPLAVLTQDDLTVEELQGIINETSYNGFPVIVSKESQRLVGFALRRDITIAIE
Tilapia (N) GYPFLDSKEEFTHTTLAREVMRPRRNDPPLAVLTQDDLTVEELQGIINETSYNGFPVIVSKESQRLVGFALRRDITIAIE
Pufferfish GYPFLDAKEEFTHTTLAREVMRPRRSDPPLAVLTQDDMTVEELQATINETSYNGFPVIVSKESQRLVGFALRRDITIAIE
Salmon GYPFLDAKEEFTHTTLAREVMRPRRSDPPLAVLTQDDLTVEELQGIINETSYNGFPVIVSKESQRLVGFALRRDITIAIE
Opossum GYPFLDAKEEFTHTTLAADVMRPRRSDPPLAVLTQDNMTVDDIENMINETSYNGFPVIMSKESQRLVGFALRRDLTIAIE
Rat GYPFLDAKEEFTHTTLAADVMRPRRSDPPLAVLTQDNMTVDDIENMINETSYNGFPVIMSKESQRLVGFALRRDLTIAIE
Human GYPFLDAKEEFTHTTLAADVMRPRRNDPPLAVLTQDNMTVDDIENMINETSYNGFPVIMSKESQRLVGFALRRDLTIAIE
Marmoset GYPFLDAKEEFTHTTLAADVMRPRRNDPPLAVLTQDNMTVDDIENMINETSYNGFPVIMSKESQRLVGFALRRDLTIAIE
Platypus GYPFLDAKEEFTHTTLAADVMRPRRNEPPLAVLTQDNMTVDDIENLINETSYNGFPVIMSKESQRLVGFALRRDLTIAIE
*
730 740 750 760 770 780 790 800
Seabass NARRKQEGIVLNSRVYFTQHAPTLPADSPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
Tilapia (M) NARRKQEGIVLNSRVYFTQHAPTLPADSPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
Tilapia (N) NARRKQEGIVLNSRVYFTQHAPTLPADSPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGIVLGIITK
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Salmon NARRKQEGILLTSRVYFTQHAPTLPADSPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
Opossum SARKKQEGIVGSSRVCFAQHTPSLPAESPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
Rat SARKKQEGVVGSSRVCFAQHTPSLPAESPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
Human SARKKQEGIVGSSRVCFAQHTPSLPAESPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
Marmoset SARKKQEGIVGSSRVCFAQHTPSLPAESPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGIVLGIITK
Platypus SARKKQEGIVGSSRVCFAQHTPSLPAESPRPLKLRSILDMSPFTVTDHTPMEIVVDIFRKLGLRQCLVTHNGRLLGIITK
810 820 830 840 850 860 870 880
Seabass KDILRHMAQMADQDP----------ESIMFN----------
Tilapia (M) KDILRHMAQMANQDP----------ESIMFN----------
Tilapia (N) KNILEHLEELKQQSPPLA----------ASWYYHKKRYPSSHGSNGKSGSRVHHVQLIRSFQDGWG
Pufferfish KNILEHLEELKRHTEPLA----------ASWYYHKKRYPSSHGSNGKPGSRVHHVQLIRSFQDGWG
Salmon KDILRHMAQMANQDP----------DNIMFN----------
Opossum KDILRHMAQMANQDP----------ASIMFN----------
Rat KDILRHMAQTANQDP----------ASIMFN----------
Human KDILRHMAQTANQDP----------ASIMFN----------
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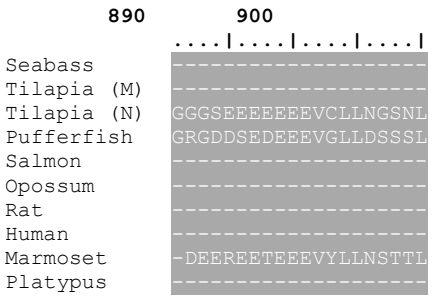

Fig1. Alignment of the deduced amino acid sequence for ClC-3 from *Dicentrarchus labrax* with fish and mammalian ClC-3. Species names and GenBank accession numbers are given in Table 1. In tilapias, m stands for *Oreochromis mossambicus* and n for *O. niloticus*. Percentage of conserved residues are filled from white to dark gray blocks from respectively the most to the least conserved residues. The 12 putative membrane-spanning segments (TM) are underlined according to the original topology (Jentsch et al. 1990) and the potential glycosylation sites are indicated by an asterisk. The location of the two CBS domains is indicated in italics. Dashes indicate spaces introduced to give an optimal alignment and regions in bold indicate the most conserved sequences of the ClC chloride channel family.

Figure 2.

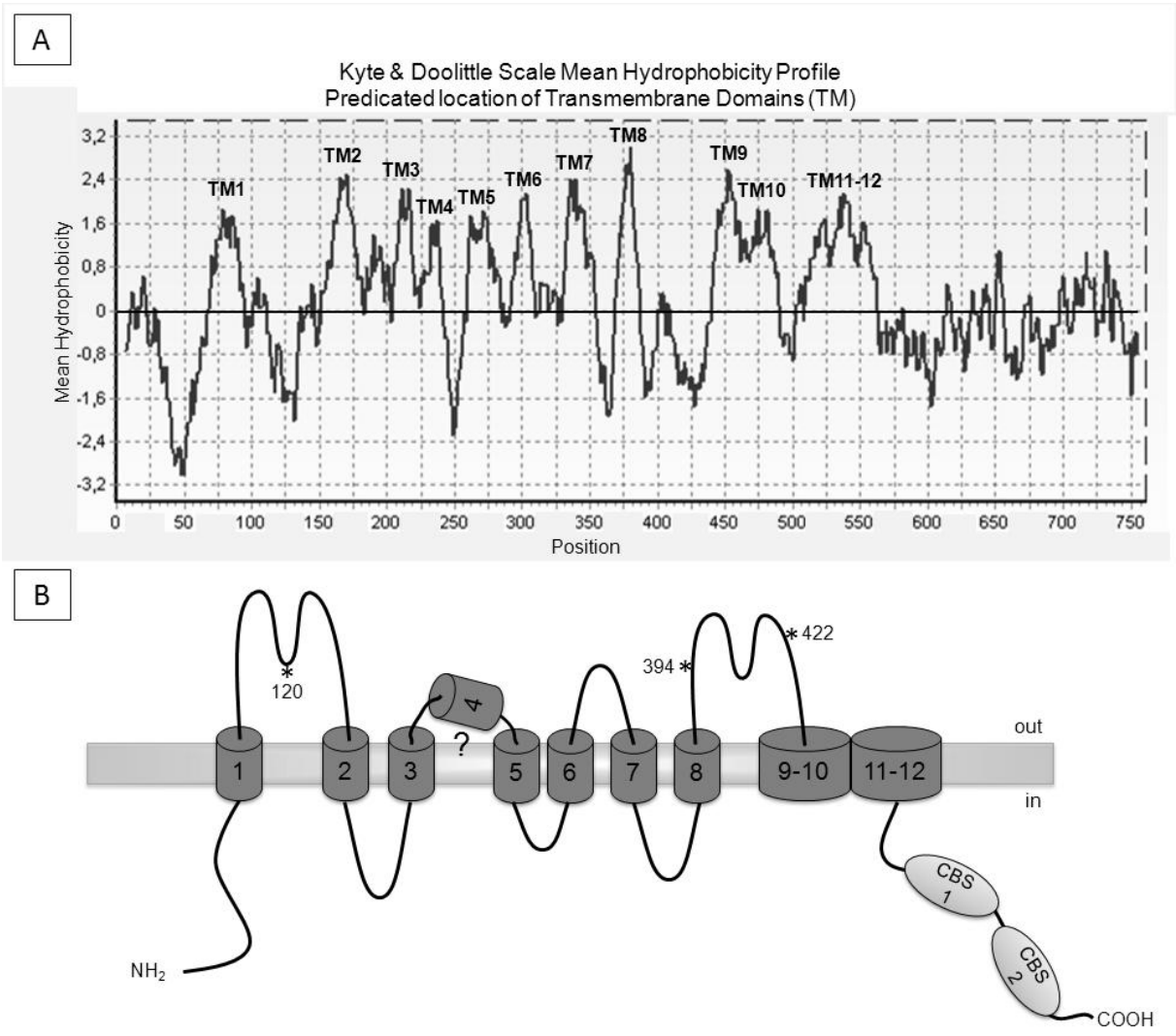

Fig2. Hydropathy plot (a) and predicted topology (b) of the ClC-3 protein in *Dicentrarchus labrax*. a: the mean hydropathy index after computation according to the algorithm of Kyte and Doolittle (1982). The transmembrane domains are determined using the most hydrophobic domains. They are numbered. b: transmembrane domains are numbered and the two CBS domains in the long-C-tail are indicated. Potential sites of N-linked glycosylation are indicated by asterisks. This topology was designed based on the present work and on previous studies on ClC proteins.

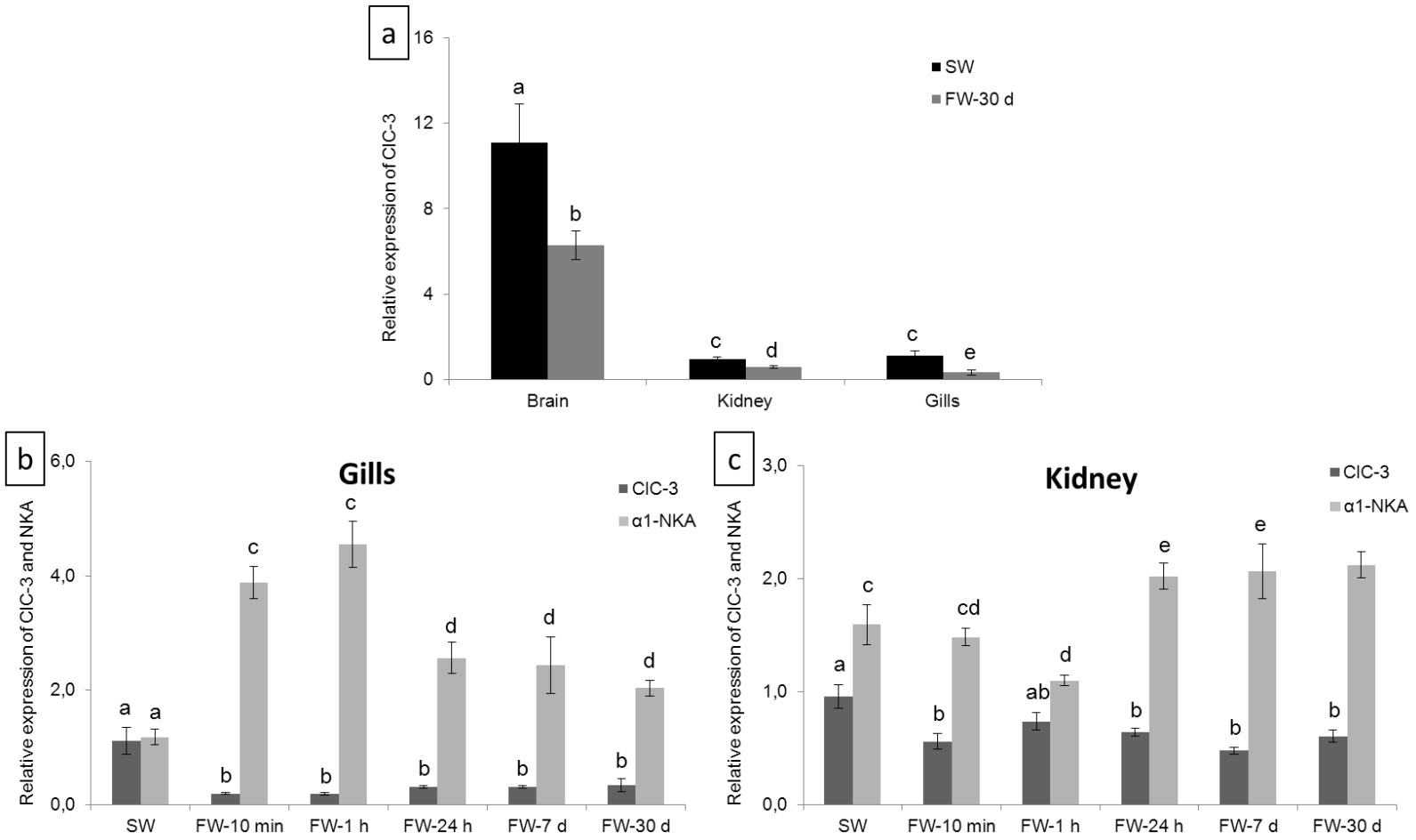

Fig3. *Dicentrarchus labrax* juveniles. Relative expression of ClC-3 and α1-NKA mRNA in different organs of fish exposed to SW and to FW for different exposure times. a: comparison of the ClC-3 mRNA expression in the brain, kidney and gills of fish exposed to SW (in black) and to FW during 30 days (in gray). b and c: relative expression of ClC-3 and NKA mRNA in gills (b) and kidney (c). The expression has been normalized according to the expression of the elongation factor (EF1α). N = 6. Data are expressed as the mean ± s.e.m. For each organ, different letters indicate significant differences ($p < 0.05$).

Figure 4.

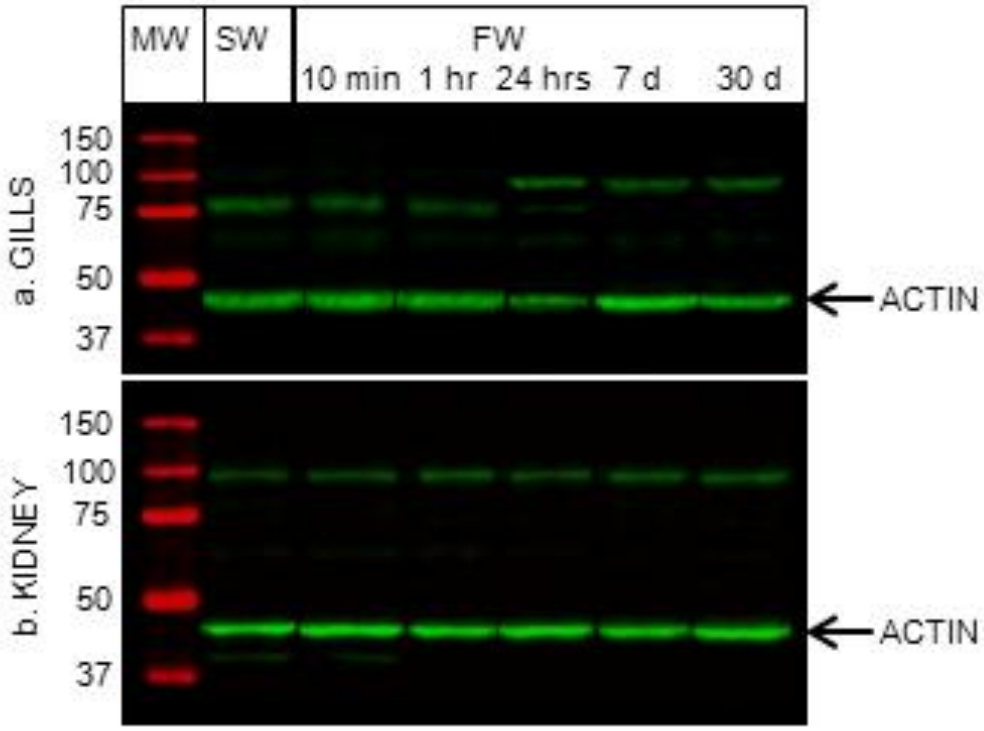

Fig4. *Dicentrarchus labrax* juveniles. Western blot analysis of the branchial and renal ClC-3 in fish exposed to SW and to FW- 30 d. Actin was used as loading control. Molecular weights (MW) in kDa are indicated on the left. FW: fresh water; SW: seawater.

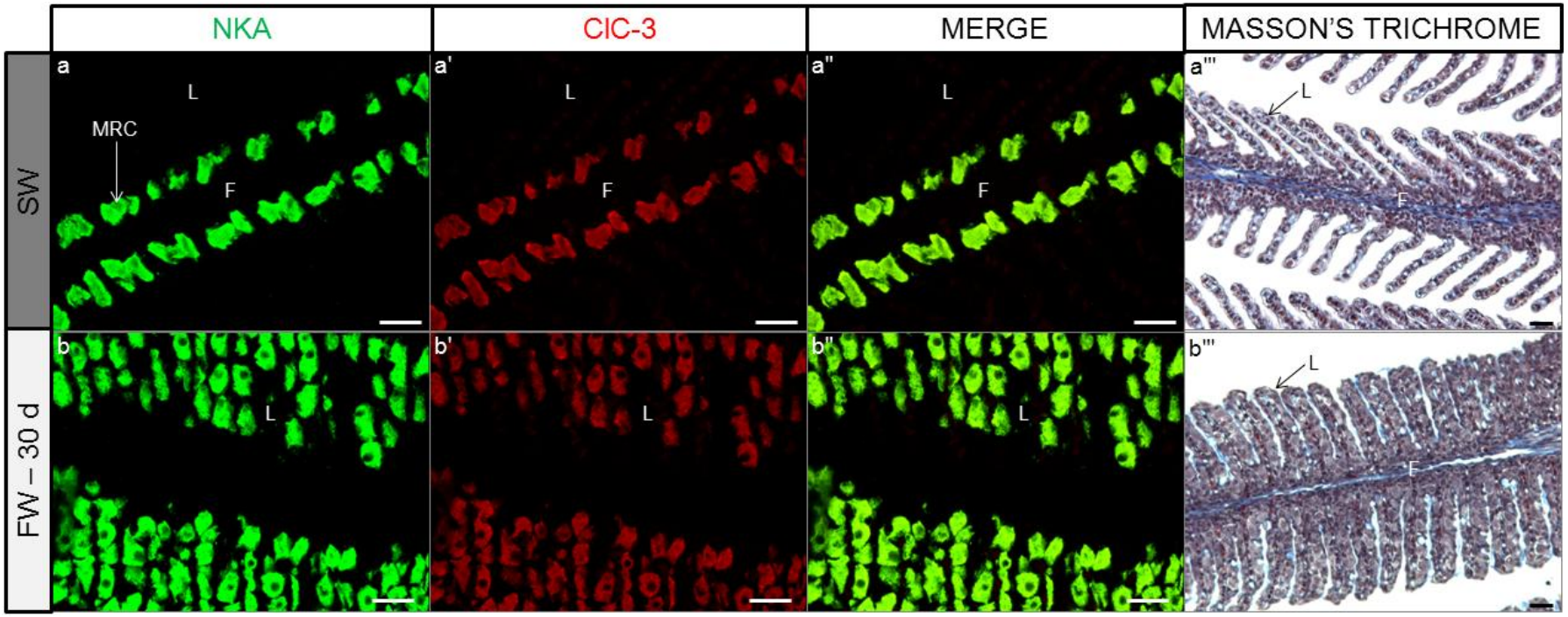

Fig5. *Dicentrarchus labrax* juveniles. Immunolocalization of NKA and ClC-3, respectively green and red fluorescence, in gills of fish exposed to SW (a, a', a") or in FW- 30 d (b, b', b"). ": merge of NKA and ClC-3. Histology of gills with a Masson's Trichrome staining in fish exposed to SW (a'") or to FW- 30 d (b'"). N = 3. Scale bars: 20 µm. F: filament; FW: freshwater; MRC: mitochondria-rich cells; L: lamellae; SW: seawater.

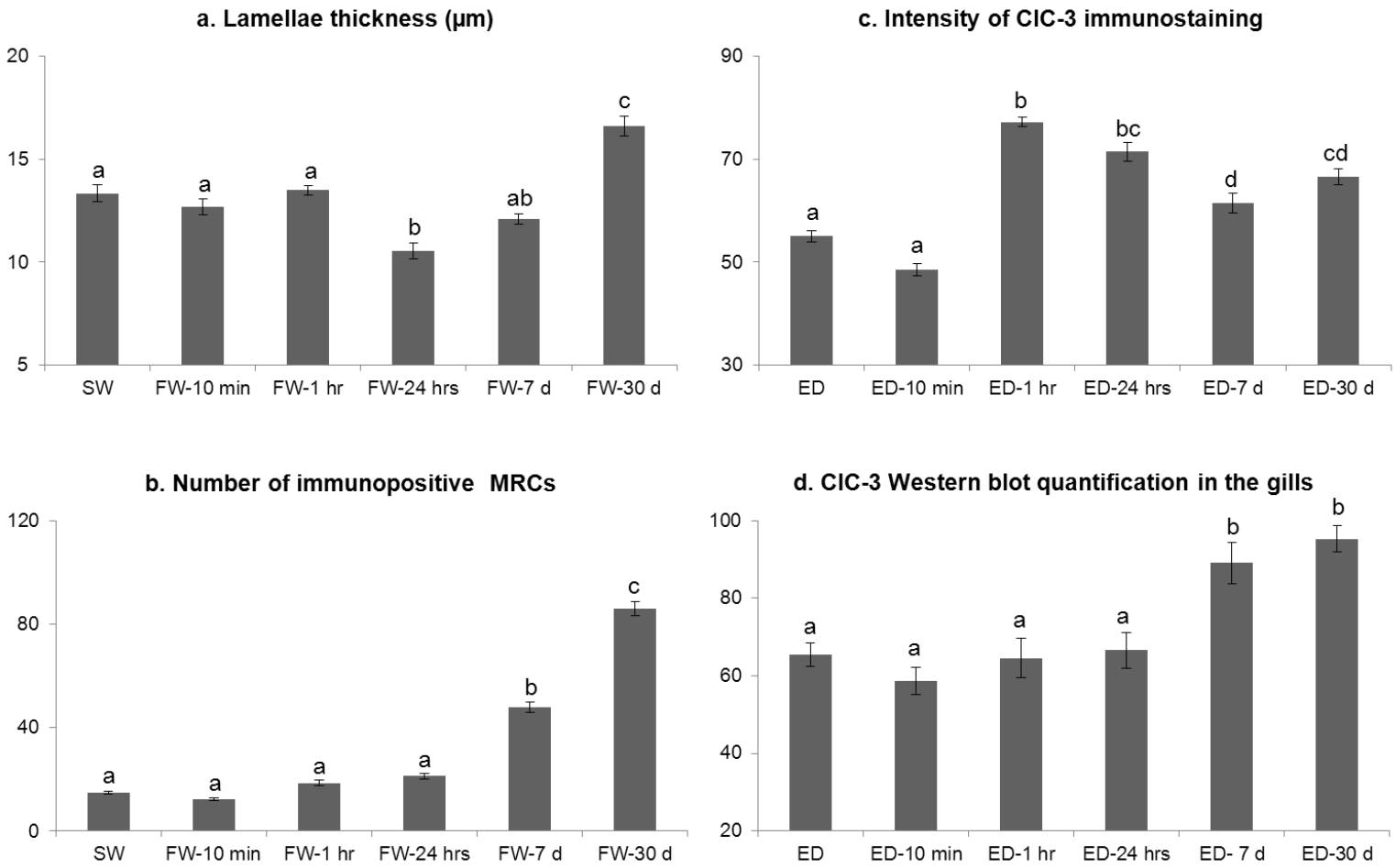

Fig6. *Dicentrarchus labrax* juveniles. a: Thickness of lamellae in fish exposed to SW and to FW for different times (N = 3). b: Number of branchial MRCs immunopositive for NKA and ClC-3 in fish exposed to SW and to FW for different times (N = 3). c: Quantification of the ClC-3 immunofluorescence in gill MRCs in fish exposed to SW and to FW for different times (N = 3). d: Western blot quantification of ClC-3 in gills of fish exposed to SW and FW for different times (N = 6). Data obtained from the Image Studio v1.1 program (LI-COR®). Data are expressed as the mean ± standard error of mean (s.e.m.). Different letters indicate significant differences ($p < 0.05$). FW: fresh water; SW: seawater.

Figure 7.

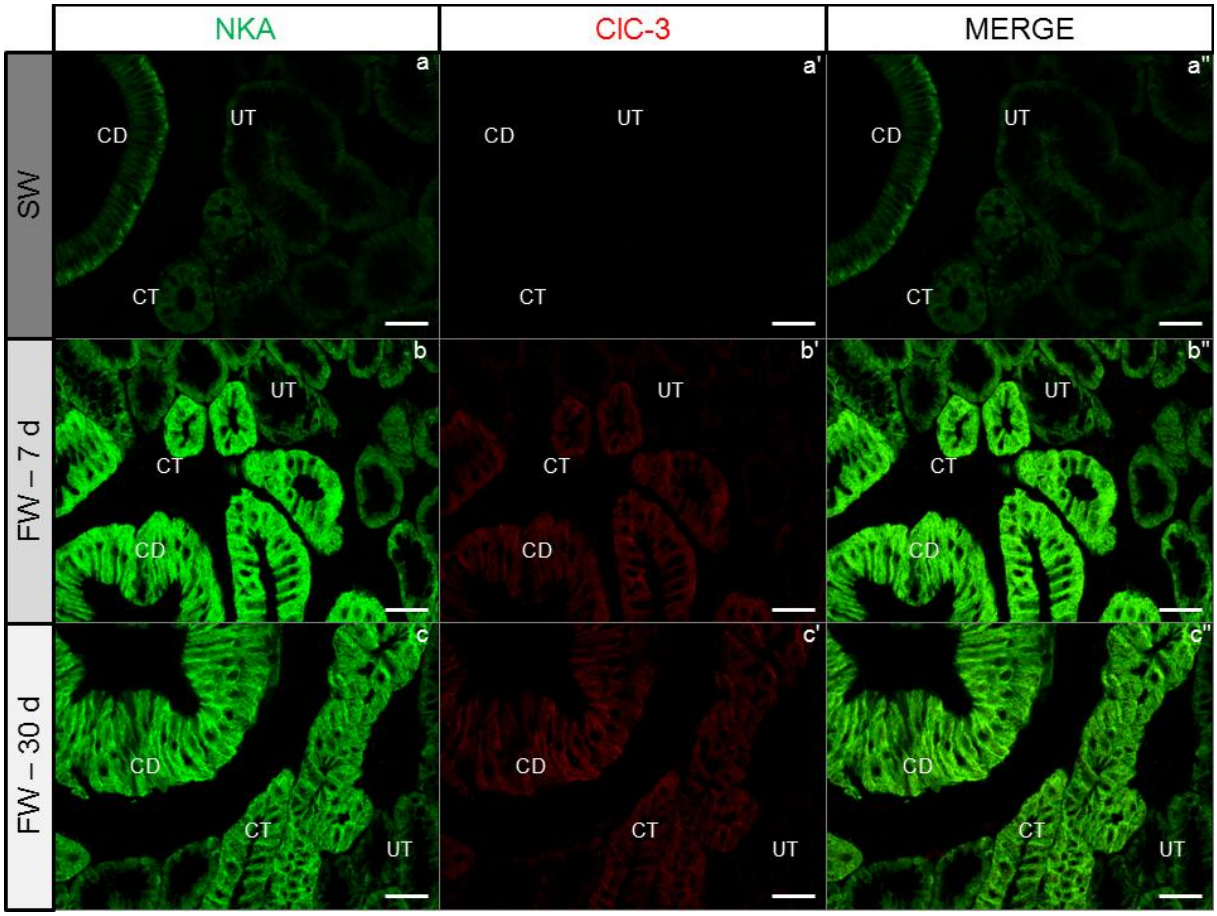

Fig7. *Dicentrarchus labrax* juveniles. Immunolocalization of NKA and ClC-3, respectively green and red fluorescence, in the kidney of fish exposed to SW (a, a', a") and to FW during 7 d (b, b', b") or during 30 d (c, c', c"). ": merge of NKA and ClC-3. N = 3. Scale bars: 20 µm. CD: collecting duct; CT: collecting tubule; FW: fresh water; SW: seawater; UT: urinary tubule.

Figure 8.

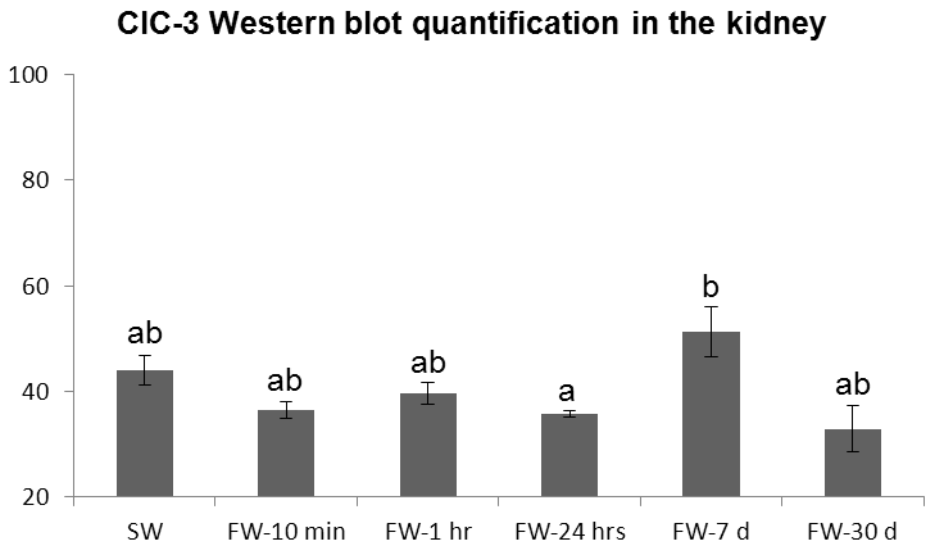

Fig8. *Dicentrarchus labrax* juveniles. Western blot quantification of ClC-3 in the kidney of fish exposed to SW and FW for different times. Data obtained from the Image Studio v1.1 program (LI-COR®). N = 3. Data are expressed as the mean ± standard error of mean (s.e.m.). Different letters indicate significant differences ($p < 0.05$). FW: fresh water; SW: seawater.