The Journal of Experimental Biology 213, 368-379

doi:10.1242/jeb.034785

Aquaporin expression dynamics in osmoregulatory tissues of Atlantic salmon during smoltification and seawater acclimation

C. K. Tipsmark*, K. J. Sørensen and S. S. Madsen

Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark

*Author for correspondence (ckt@biology.sdu.dk)

Accepted 27 October 2009

SUMMARY

Osmotic balance in fish is maintained through the coordinated regulation of water and ion transport performed by epithelia in intestine, kidney and gill. In the current study, six aquaporin (AQP) isoforms found in Atlantic salmon (*Salmo salar*) were classified and their tissue specificity and mRNA expression in response to a hyperosmotic challenge and during smoltification were examined. While AQP-1a was generic, AQP-1b had highest expression in kidney and AQP-3 was predominantly found in oesophagus, gill and muscle. Two novel teleost isoforms, AQP-8a and -8b, were expressed specifically in liver and intestinal segments, respectively. AQP-10 was predominantly expressed in intestinal segments, albeit at very low levels. Transfer from freshwater (FW) to seawater (SW) induced elevated levels of intestinal AQP-1a, -1b and -8b mRNA, whereas only AQP-8b was stimulated during smoltification. In kidney, AQP-1a, -3 and -10 were elevated in SW whereas AQP-1b was reduced compared with FW levels. Correspondingly, renal AQP-1a and -10 peaked during smoltification in April and March, respectively, as AQP-1b and AQP-3 declined. In the gill, AQP-1a and AQP-3 declined in SW whereas AQP-1b increased. Gill AQP-1a and -b peaked in April, whereas AQP-3 declined through smoltification. These reciprocal isoform shifts in renal and gill tissues may be functionally linked with the changed role of these organs in FW compared with SW. The presence and observed dynamics of the AQP-8b isoform specifically in intestinal sections suggest that this is a key water channel responsible for water uptake in the intestinal tract of seawater salmonids.

Key words: AQP-1, AQP-3, AQP-8, AQP-10, gill, kidney, intestine.

INTRODUCTION

In teleost fish, osmotic balance is safeguarded by coordinated ion and water transport by the gill, intestinal tract and kidney (see Evans, 2008). Freshwater (FW) fish that are challenged by the continuous osmotic gain of water and loss of salt to the dilute surroundings counteract this by producing large volumes of dilute urine, retaining ions in the kidney along with compensatory uptake of ions from the food and by the gills. Seawater (SW) fish, by contrast, counter the osmotic loss of water to the concentrated environment by drinking and intestinal processing of SW in conjunction with greatly reduced glomerular filtration and urine production. To compensate for the overall salt gain, active secretion of NaCl by the gill is crucial. Euryhaline fish species can move between FW and SW habitats and accordingly are able to complete the major functional changes in their osmoregulatory tissues, as outlined above. Several studies have characterized how salinity changes may induce adjustment in ion-transporter expression in both gill (see Evans et al., 2005) and intestine (Aoki et al., 2003; Seidelin et al., 2000; Sundell et al., 2003; Veillette et al., 2005) whereas expressional changes in ion transporters in renal tissue are equivocal and may differ among species (e.g. McCormick et al., 1989; Tipsmark et al., 2008b).

Aquaporins (AQPs) play an integral role in cellular and transcellular water movement in mammals (Hill et al., 2004a). Up until now, 13 isoforms have been reported in mammals, whereas 17 isoforms have been identified in the pufferfish (*Fugu rubripes*) and zebrafish (*Danio rerio*) genomes. Yet in fish only five of these have received research attention (duplicate forms of AQP-1, AQP-4, AQP-3 and AQPe – the latter two belonging to the aquaglyceroporin subfamily). The current consensus is that duplicate isoforms of AQP-1 (a and b) are involved in intestinal uptake of water in marine fish since increased expression of both forms is found in SW compared with the levels in FW in several species [Japanese eel (*A. japonica*) (Aoki et al., 2003); European eel (Martinez et al., 2005a); European sea bass (*Dicentrarchus labrax*) (Giffard-Mena et al., 2007); gilthead sea bream (*Sparus aurata*) (Raldua et al., 2008)]. In the kidney, available data are less consistent, since studies in European sea bass (Giffard-Mena et al., 2007) and black porgy [*Acanthopagrus schlegel* (An et al., 2008)] report elevated renal AQP-1 expression in SW whereas the opposite results have been reported for both AQP-1 isoforms in juvenile European eel (Martinez et al., 2005b). In intestine and kidney, available evidence suggests that AQP-1 is localized exclusively in apical membranes. In the gill, data on AQP-1 expression is scarce. Expression levels were found to be low in European eel (Martinez et al., 2005b) and European sea bass (Giffard-Mena et al., 2007) and did not change with salinity. In black porgy, gill expression of AQP-1 was higher in FW than in SW but the function and localization remain unclear (An et al., 2008).

AQP-3 expression has been found at low levels in different intestinal regions, where a role in mucus secretion has been suggested (Cutler et al., 2007). In the kidney, low levels of AQP-3 have been reported in the apical membrane of tubule cells (Cutler and Cramb, 2002). In the gill, data consistently suggests that AQP-3 expression is elevated in response to hypo-osmotic challenge [European eel (Cutler and Cramb, 2002); silver sea bream (Deane and Woo, 2006); Japanese eel (Tse et al., 2006); European sea bass (Giffard-Mena et al., 2007)]. AQP-3 has been located in pavement

cells [using a heterologous antibody in Japanese eel (Tse et al., 2006)] and in basolateral membranes of mitochondrion-rich cells [MRCs; using homologous antibodies in European eel (Lignot et al., 2002); Mozambique tilapia (*Oreochromis mossambicus*) (Watanabe et al., 2005)] where it has been suggested to be involved in osmoreception used as a cue for MRC differentiation (Watanabe et al., 2005). Furthermore, Hirata et al. (Hirata et al., 2003) reported increased expression of gill AQP-3 mRNA during acid exposure in osorezan dace (*Tribolodon hakonensis*) and proposed a role in water supply to intracellular carbonic anhydrase.

AQP-4, also known as the mercurial-insensitive water channel has been examined in rainbow trout [*Oncorhynchus mykiss* (Bobe et al., 2006)], where it was assigned a role in oocyte swelling and maturation. Finally, an aquaglyceroporin related to the human AQP-10 gene has been reported in gilthead sea bream [gilthead sea bream, sbAQP (Santos et al., 2004)] and European eel [AQPe: Martinez et al. (Martinez et al., 2005b)] kidney and intestine. Its role and precise cellular location has yet to be defined; however, in sea bream its localization in the lamina propria and between the two smooth muscle layers within the gut seem to exclude a significant role in transepithelial water transport in the intestine.

Although there have been numerous studies on salmonid osmoregulation, no studies have, to our knowledge, examined the diversity and role of AQPs in this important teleost group. The available expressed sequence tag material for Atlantic salmon (currently ~500,000 sequences) with specific databases for gill, kidney and intestine is a very useful resource because it is possible to identify non-silent genes and in many cases determine if they are expressed in the organ of interest. Given this resource we decided to identify and classify expressed AQPs, determine their tissue distribution and dynamics during smoltification and osmotic challenge in the major osmoregulatory organs (intestine, kidney and gill). Our expectation was that new AQP isoforms of significance for osmoregulation might be discovered and that new modes of regulation would possibly be identified, in addition to a substantiation of what is known from other teleost species.

MATERIALS AND METHODS

Experimental animals and sampling

One-year old Atlantic salmon (*Salmo salar* L.; Vestjydske stock) were obtained in early February, 2008, from The Danish Centre for Wild Salmon (Randers, Denmark). Fish were kept in outdoor FW fibreglass tanks and exposed to natural variation in temperature and daylight conditions. Fish were sampled at five dates covering the smoltification period (29th February, 26th March, 23rd April, 20th May and 17th June). In July, post-smolts were moved to indoor FW tanks at 14°C with a 12 h:12 h light:dark photoperiod. The fish were fed *ad libitum* three times a week with pelleted trout feed. Food was withheld 1 day before sampling. When sampled, fish were stunned with a blow to the head and blood collected with a heparinized syringe from the caudal vessels, after which the fish was killed. Pyloric caecae, middle intestine, posterior intestine, gill and kidney were dissected and, when required, trimmed free of cartilage and visceral fat, thereafter frozen in liquid nitrogen and kept at –80°C until analysis. Sections of the intestine were distinguished with reference to the ileorectal valve. The sections immediately anterior and posterior to this reference were named middle and posterior intestine, respectively. Experimental protocols were approved by the Danish Animal Experiments Inspectorate being in accordance with The European convention for the protection of vertebrate animals used for experiments and other scientific purposes (#86/609/EØF).

Analysis of AQP expression in organs and initial comparison of FW and SW fish

Fish kept inside were sampled as indicated above in October, and oesophagus, pyloric caeca, middle intestine, posterior intestine, gill, kidney, brain, skeletal muscle and liver tissues were dissected and frozen for later analysis of mRNA levels. Two groups of fish kept at 14°C in FW or SW (24 p.p.t.; Red Sea Salt, Eliat, Israel) for 2 weeks were used for sampling of gill, kidney, pyloric caecae and middle intestine for comparison of FW and SW levels of AQP mRNA. Fish were fed once a day except for the day before sampling.

FW-to-SW time-course experiment

To further investigate and confirm the FW and SW expression data and examine the temporal development in gene expression during SW acclimation, a second batch of fish was used in a SW transfer experiment in early February 2009. Fish were transferred directly to SW tanks by netting, or sham transferred to other FW tanks and eight fish from each group were sampled after 1 and 7 days after transfer. In addition eight fish were sampled from a control group at day 0. The water temperature was 14°C. Fish were not fed during the acclimation period in both FW and SW.

In order to make an approximate comparison of aquaporin expression in mucosal and serosal preparations of middle intestine, a longitudinal cut was made in the intestine and it was placed on a glass microscope slide and scraped carefully with another glass slide. The scrapings were collected and homogenized and RNA was extracted as described. This was compared with a homogenate of the residual layer of tissue which was composed mostly of smooth muscle.

Analyses

Plasma chloride and gill Na^+/K^+-ATPase activity

Plasma [Cl^-] was measured by a chloride titrator (Radiometer CMT10, Copenhagen, Denmark). Gill Na^+/K^+-ATPase activity was measured according to the method of McCormick (McCormick, 1993), using a microplate reader (SPECTRAmax PLUS, Molecular Devices, Sunnyvale, CA, USA), and was normalized to protein content of the homogenate.

Preparation of total RNA and cDNA synthesis

Total RNA was prepared by the TRIzol procedure (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendation. RNA concentrations were determined by measuring A_{260} in duplicate with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and purity (A_{260}/A_{280}) ranged from 1.8–2.0. RNA (1 µg) was treated with 1 i.u. RQ1 DNase (Promega, Madison, WI, USA) for 40 min at 37°C in a total volume of 10 µl followed by inactivation with 1 µl RQ DNase stop solution for 10 min at 65°C. Complementary DNA was synthesized by reverse transcription of 1 µg DNase-treated RNA using oligo$(dT)_{15}$ primers and DyNAmo™ cDNA synthesis kit (Finnzymes, Espoo, Finland) following the manufacturer's recommendations. At the end, sterile milli-Q H_2O was added to the cDNA to a total volume of 50 µl.

Aquaporin nucleotide sequences

Partial salmon AQP mRNA sequences were identified in the salmonid EST database in GenBank (Benson et al., 2000) by using BLAST similarity search (Altschul et al., 1997) with known teleost AQPs from the zebrafish and stickleback genome as search parameters. Sequences obtained from GenBank are here indicated by their UniGene and/or accession numbers in brackets: *AQP-1a*

(BT046625), *AQP-1b* (BT045044), *AQP3* (Ssa.27124), *AQP-8* (Ssa.15811). One *AQP* (DW573347) not automatically annotated in GenBank was most homologous to zebrafish *aquaporin 8a* [tblastx: Expect=7e-97; identities 151/220 (68%); acc. no. BC164834]. We therefore suggest that salmon *AQP-8* (Ssa.15811) is named *AQP-8b* since phylogenetic analysis (Fig. 1) groups it with the zebrafish homolog (acc. no. BC157381). Another *AQP* (Ssa.10089) was mostly homologous to eel *AQP-e* [tblastx: Expect=7e-126; identities 131/177 (74%); acc. no. AJ784153]. Since phylogenetic analysis (Fig. 1) groups this salmon isoform with stickleback and human AQP-10 we suggest that it is named accordingly.

Real-time quantitative PCR

Primers (Table 1) were designed using Primer3 software (Rozen et al., 2000). Testing for non-specific amplification and primer-dimer formation were done by analysis of melting curve and agarose gel verification of amplicon size. Quantitative PCR analysis was carried out using the Mx3000p instrument (Stratagene, La Jolla, CA, USA) with standard software settings, adaptive baseline for background detection, moving average and amplification-based threshold settings with built-in FAM/SYBR filter (excitation wavelength: 492 nm; emission wavelength: 516 nm). Complementary DNA obtained from reverse transcription of 20 ng total RNA, 150 nmol l^{-1} forward and reverse primer, 1× SYBR Green JumpStart (Sigma, St Louis, MO, USA) were used in each reaction in a total volume of 25 µl. Cycling conditions were 95°C for 30 s and 60°C for 60 s in 50 cycles and melting curve analysis was carried out with 30 s for each 1°C interval from 55°C to 95°C. Normalization of gene expression was done relative to elongation factor 1a (*EF-1a*) in accordance with Olsvik et al. (Olsvik et al., 2005). Amplification efficiency was determined for each gene and relative copy number for target and normalization genes was calculated according to Pfaffl (Pfaffl, 2001): Ea^{-Ct}, where E_a is the amplification efficiency and C_t the threshold cycle number. Normalized values were obtained by dividing the relative copy number of the target and normalization gene. Negative control reactions using DNase-treated total RNA from representative samples were used to analyse carry over of genomic DNA. In all samples and with all primers there was no genomic contamination detected.

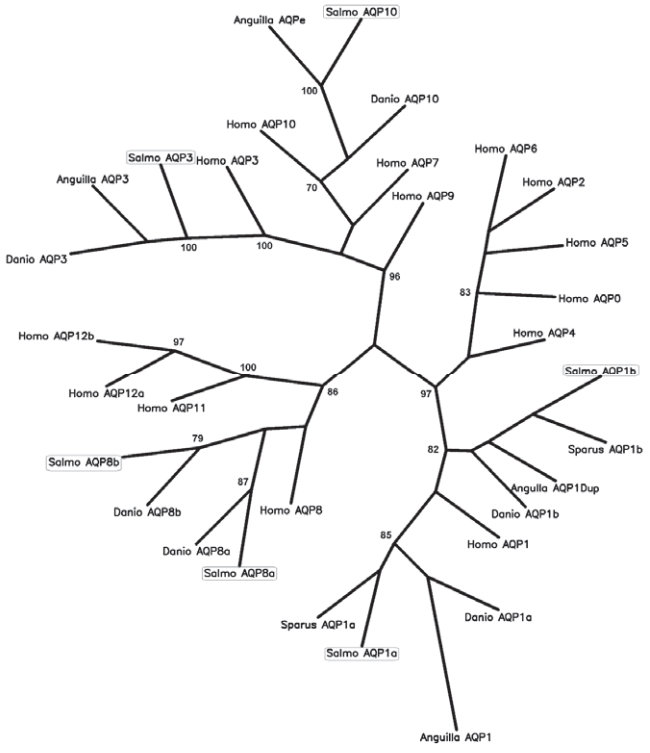

Fig. 1. A phylogenetic tree, comparing the salmon aquaporins (AQPs; salmon: *Salmo salar*) with relevant AQPs from European eel (Anguilla: *Anguilla anguilla*), zebrafish (Danio: *Danio rerio*) gilthead sea bream (Sparus: *Sparus aurata*) and all the isoforms found in man (Homo: *Homo sapiens*). Salmon isoforms are framed for emphasis. The tree is based on maximum likelihood analysis of amino acid sequences. Numbers represent bootstrap values in percent of 1000 replicates.

Phylogenetic analysis

Amino acid sequences were obtained from GenBank and Ensembl (EMBL-EBI) webpages. The predicted amino acid sequences were aligned using ClustalW. The phylogenetic tree was constructed using maximum likelihood analysis. Thousand bootstraps were used to test the consistency of grouping within the tree. The maximum likelihood majority rule consensus tree was created using SEQBOOT, PROML and CONSENSE, all programs of the PHYLIP package (Felsenstein, 1989).

Statistics

Organ expression and smoltification data of AQP isoforms were analysed by one-way ANOVA or nonparametric Kruskal–Wallis analysis as appropriate. When required, transformation of data was done to meet the ANOVA assumption of homogeneity of variances as tested by Bartlett's test. Testing for overall differences between FW and SW groups in the SW challenge experiment were done with two-way ANOVA. To minimize the total number of animals used, fish sampled at time zero were used as both the FW and SW group in the analysis. When the interaction between factors was significant this was followed by *post-hoc* analysis and otherwise the overall effects are indicated in the figure. When appropriate Tukey's or Dunn's test was used for *pos-hoc* analysis and a significance level of $P<0.05$ was chosen. All tests were performed using GraphPad Prism 4.0 software (San Diego, CA, USA).

RESULTS

We identified six aquaporin genes by blast search in the Atlantic salmon transcriptome available in GenBank. The salmon isoforms were classified according to an extensive phylogenetic analysis including all previously named isoforms from the stickleback, zebrafish and human genome along with sequences published for gilthead sea bream and European eel. For clarity the phylogenetic tree shown in Fig. 1 only includes teleost isoforms relevant for the current study along with all human AQPs for comparison. Two distinct homologous genes of both AQP-1 and -8 were found, here named a and b. Conversely, only one AQP-3 and one AQP-10 were found.

Using specific primers (Table 1), we analyzed *AQP* expression pattern in a series of organs and tissues (oesophagus, pyloric caecae, middle intestine, posterior intestine, gill, kidney, brain, skeletal muscle and liver) from FW salmon. Expression of *AQP-1a* was found at roughly equal levels in most organs, but was higher in the brain (Fig. 2A). A relatively low level of *AQP-1b* was detected in all organs, except muscle, whereas a significantly higher expression was found in the kidney (Fig. 2B). Except for liver, *AQP-3* expression was detected in all organs with the highest levels in oesophagus, gill and muscle (Fig. 2C). Expression of *AQP-8a* was only detected in the liver (Fig. 2D), whereas *AQP-8b* appeared

Table 1. Primer sequences used for mRNA quantification of salmon aquaporins (AQP) by real-time PCR

Gene	Primer sequence (5′→3′)	Amplicon size (bp)	Gene sequence reference (UniGene acc. no.)
AQP-1a	Left: CTACCTTCCAGCTGGTCCTG Right: TGATACCGCAGCCTGTGTAG	141	BT046625
AQP-1b	Left: CTGTGGGTCTGGGACATCTT Right: TAAGGGCTGCTGCTACACCT	153	BT045044
AQP-3	Left: GTGACAGGAAGAGCCAGGAG Right: TGAGGCTGAGCTTAGGGGTA	138	Ssa.27124
AQP-8a	Left: TCATGACCCTCTTCCTGTCC Right: GGGTTCATACACCCTCCAGA	145	DW573347
AQP-8b	Left: GGAGCTGCCATGTCAAAGAT Right: CGCCCCTAGCAATACTACCA	159	Ssa.15811
AQP-10	Left: GGTGTTGGTGATCGGAGTCT Right: CGCCCTAAACACCTCATCC	121	Ssa.10089
EF-1a	Left: AGAACCATTGAGAAGTTCGAGAAG Right: GCACCCAGGCATACTTGAAAG	71	AF321836

specifically in intestinal tissues excluding the oesophagus (Fig. 2E). The gill was the only other organ with detectable *AQP-8b* transcript but this was three orders of magnitudes lower than intestinal levels. *AQP-10* expression was detected in pyloric caecae, middle and posterior intestine and kidney with the lowest level found in renal tissue (Fig. 2F).

In an initial experiment, AQP mRNA expression in gill, kidney, pyloric caecae and middle intestine were compared between FW- and fully SW-acclimated salmon (Fig. 3). In the gill, SW induced a decrease in *AQP-1a* and *-3* expression whereas *AQP-1b* transcript levels increased (Fig. 3A). In renal tissue, SW induced increased levels in *AQP-1a, -3* and *-10* mRNA while *AQP-1b* expression declined (Fig. 3B). In the intestine, a significant increase in *AQP-1a, -1b*, *-8b* and *-10* was observed in SW while *AQP-3* was unchanged (Fig. 3D). To substantiate and further investigate the observed changes in AQP mRNA expression, a time-course transfer experiment was conducted monitoring expression in the middle intestine, kidney and gill after 1 and 8 days. Plasma chloride, used to monitor the progress of acclimation, was elevated 1 day after SW transfer whereas the difference between FW and SW groups was

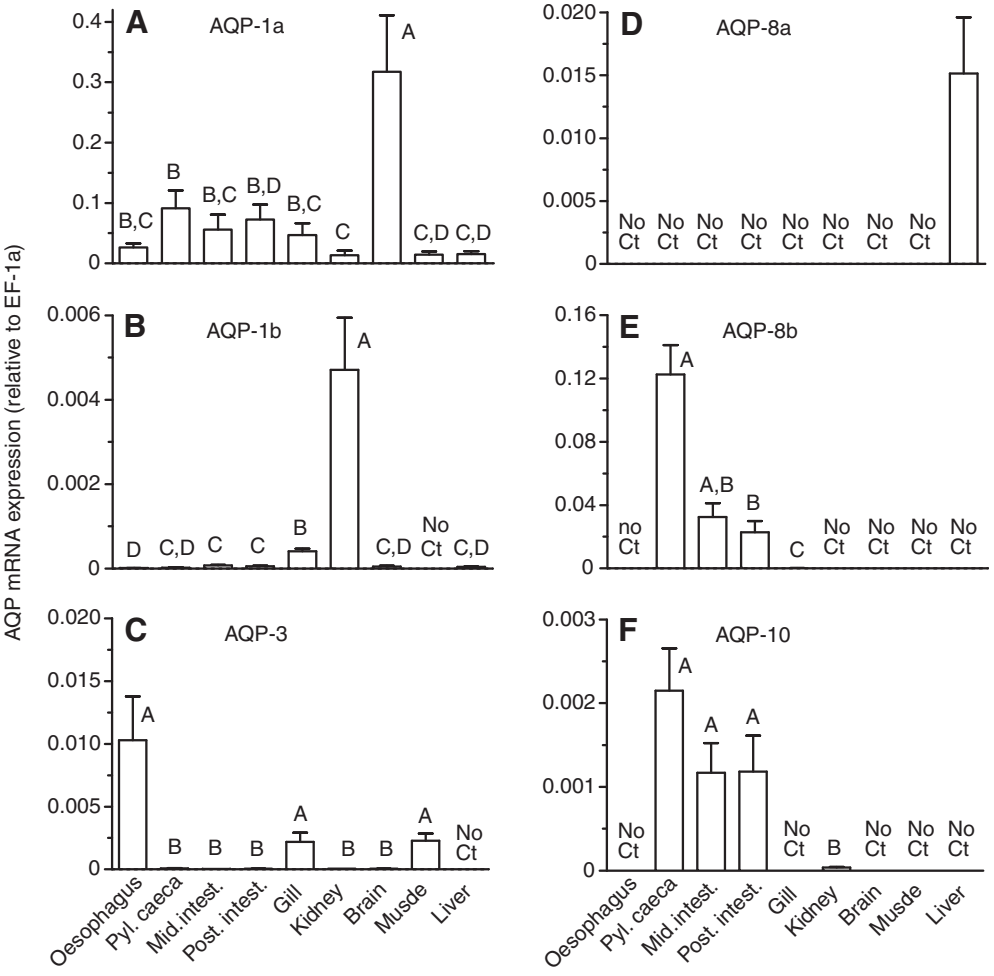

Fig. 2. Expression of aquaporin-1a (A), -1b (B), -3 (C), -8a (D), -8b (E) and -10 (F) mRNA in oesophagus, pyloric caecae, middle intestine, posterior intestine, gill, kidney, brain, skeletal muscle and liver tissue from freshwater salmon. Letters above data points indicate statistical relationships. Values with the same letters are not statistically different; means ± s.e.m. (*N*=3). No Ct means the level was too low to be detected by QPCR.

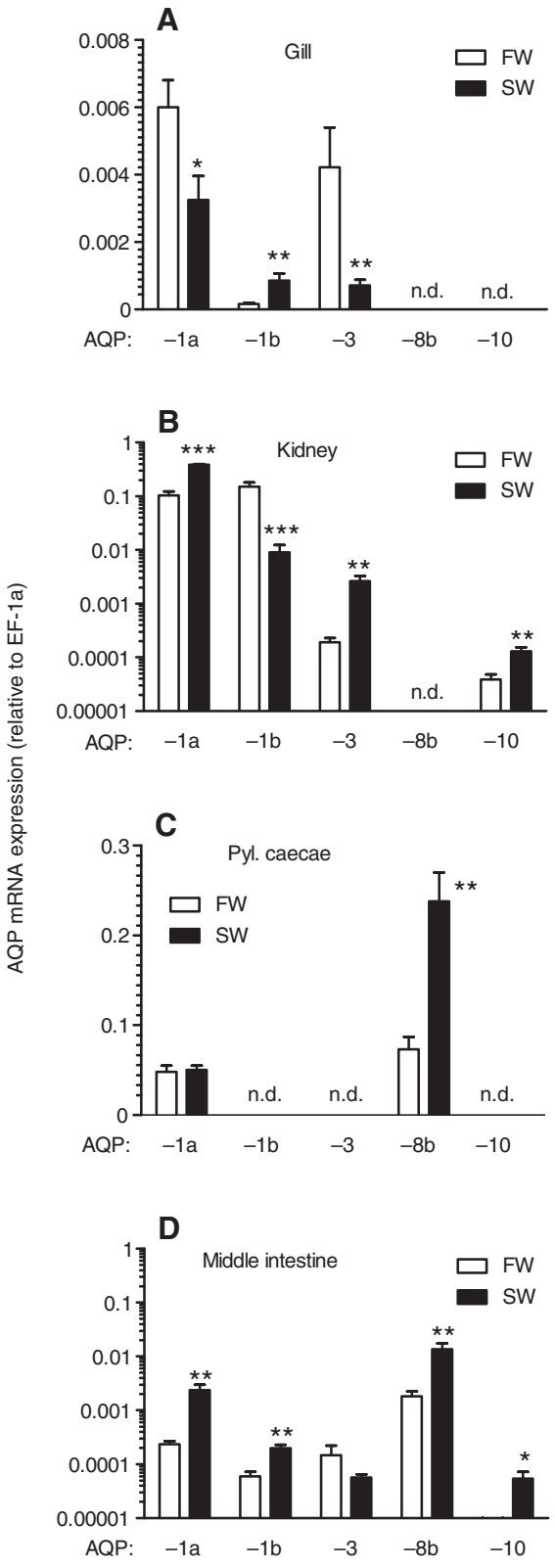

Fig. 3. Expression of aquaporin-1a, -1b, -3, -8b and -10 mRNA in gill (A), kidney (B), pyloric caecae (C) and middle intestine (D), from freshwater (FW)- and seawater (SW)-acclimated salmon. Asterisks indicate a significant difference between the FW and SW samples for a particular tissue. Values are means ± s.e.m. (*N*=6).

insignificant on day 8 (Fig. 4A). In the middle intestine *AQP-1a* and *-8b* were elevated after 8 days in SW both when compared with the group on day 0 and the relevant FW-sham group (Fig. 4B,E). Intestinal *AQP-1b* and *-10* were elevated on day 8 in SW compared to FW-sham control (Fig. 4C,F). Renal *AQP-1a* was elevated in response to SW on days 1 and 8 (Fig. 5A), whereas *AQP-1b* levels decreased after 8 days in SW (Fig. 5B). AQP-3 and AQP-10 transcript levels were elevated in SW-transferred fish after 1 and 8 days, respectively (Fig. 5C,D). In the gill both *AQP-1a* and *-3* decreased in SW groups after 1 and 8 days (Fig. 6A,C). *AQP-1b* was elevated after 8 days in SW when compared with the sham-FW control but not when compared with the time 0 group (Fig. 6B).

In the smoltification sampling experiment, gill Na^+/K^+-ATPase activity increased in late March and April with peak levels observed in May (Fig. 8H), thus signifying smolt development. The most marked and consistent change in intestinal AQP expression during smoltification was the four- to sixfold elevation of *AQP-8b* expression in all intestinal sections during April to June (Fig. 7D,I,N). In addition, *AQP-1a* and *-10* in pyloric caecae and AQP-10 in the posterior intestine increased in June. All other AQPs showed no changes. In the kidney, a peak in *AQP-1a* and *-10* expression was observed in late April and March, respectively (Fig. 8A,D). Conversely, *AQP-1b* and *-3* levels declined during the later stages of smoltification in May and June (Fig. 8B,C). In the gill, a peak in *AQP-1a* and *-1b* was observed in April (Fig. 8E,F), while a continuous reduction in gill *AQP-3* occurred throughout the sampling period (Fig. 8F).

A comparison between mucosal and serosal expression of aquaporin isoforms in the middle intestine revealed that *AQP-1a* was 50-fold more abundant in the serosal layer (Table 2). *AQP-1b* and *-3* were equally abundant in the two layers, and *AQP-8b* and *-10* were approximately 20-fold more abundant in the mucosal layer.

A summary of the main effects of SW acclimation and trends during smolt development is given in Table 3.

DISCUSSION

The AQP family of integral membrane proteins all form physical pore structures that allow the permeation of water and in the case of some isoforms, solutes such as urea, glycerol, ammonia and carbon dioxide (Hill et al., 2004a; Krane and Goldstein, 2007). Thus, tissue- and cell-type-dependent and developmentally controlled expression of specific isoforms helps to define the properties of a given tissue. This is nicely illustrated in the mammalian kidney, where a series of isoforms is successively expressed in distinct sections of the nephron (Nielsen et al., 2002). In the current study, we report both generic and organ-specific distribution of AQPs isoforms in salmon together with their coordinated regulation during salinity acclimation in the key osmoregulatory organs. To our knowledge, this is the first study to examine AQPs in a salmonid fish and to suggest a key role of the AQP-8b in intestinal function of marine teleosts, in general.

Aquaporin isoforms and their organ distribution

Six AQP isoforms were found in the available salmon transcriptome. Salmon AQP-1a and -1b are homologues to the reported AQP-1a and -1b from gilthead sea bream (Tingaud-Sequeira et al., 2008) and AQP-1 and AQP-dup in European eel (Martinez et al., 2005b), respectively. AQP-1a was expressed in all examined organs with a slightly higher level in the brain and this distribution is similar to what has been reported for eel AQP-1a (Martinez et al., 2005b). Salmon AQP-1b was predominantly expressed in the kidney similar to what has been observed in eel (Martinez et al., 2005b). By

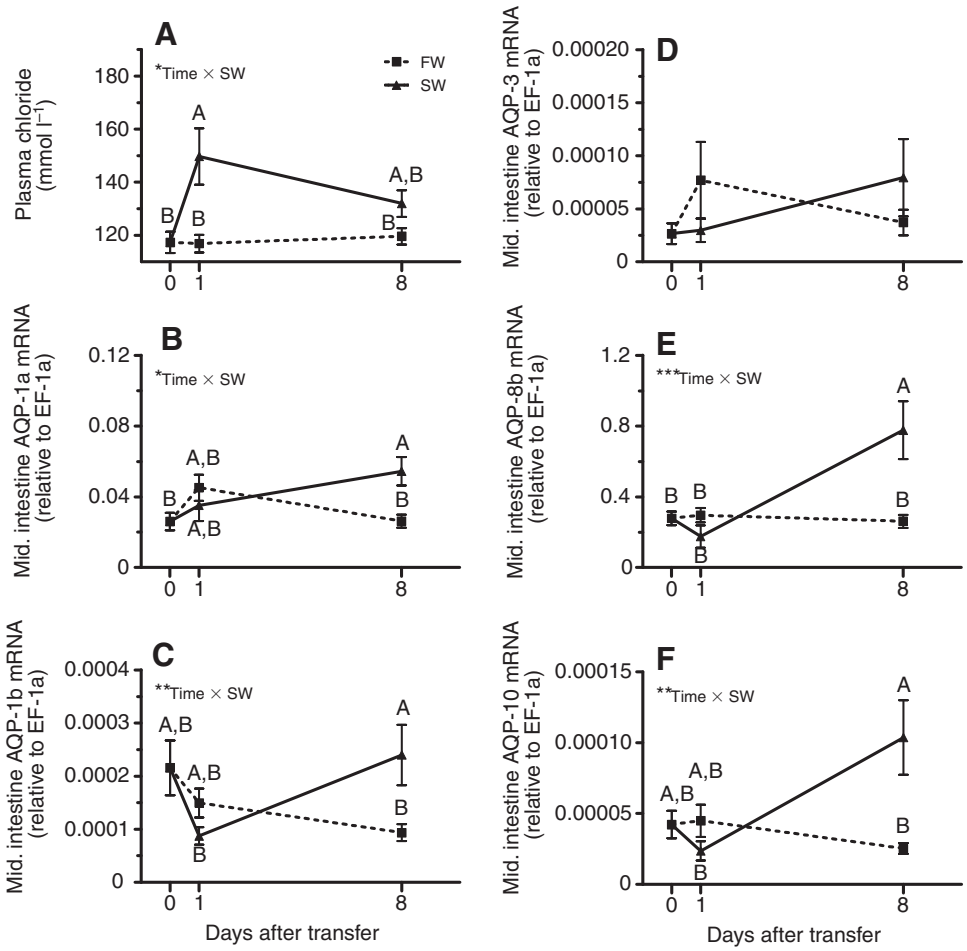

Fig. 4. Time course of plasma chloride (A) and expression of aquaporin-1a (B), -1b (C), -3 (D), -8b (E) and -10 (F) mRNA in middle intestine following transfer of freshwater (FW) salmon to seawater (SW; triangles), or sham transfer to FW (squares). Significant interaction between time and salinity is indicated by asterisks next to 'Time × SW' (*P<0.05, **P<0.01, ***P<0.001). Letters above data points indicate statistical relationships. Values with the same letters are not statistically different; means ± s.e.m. (N=8).

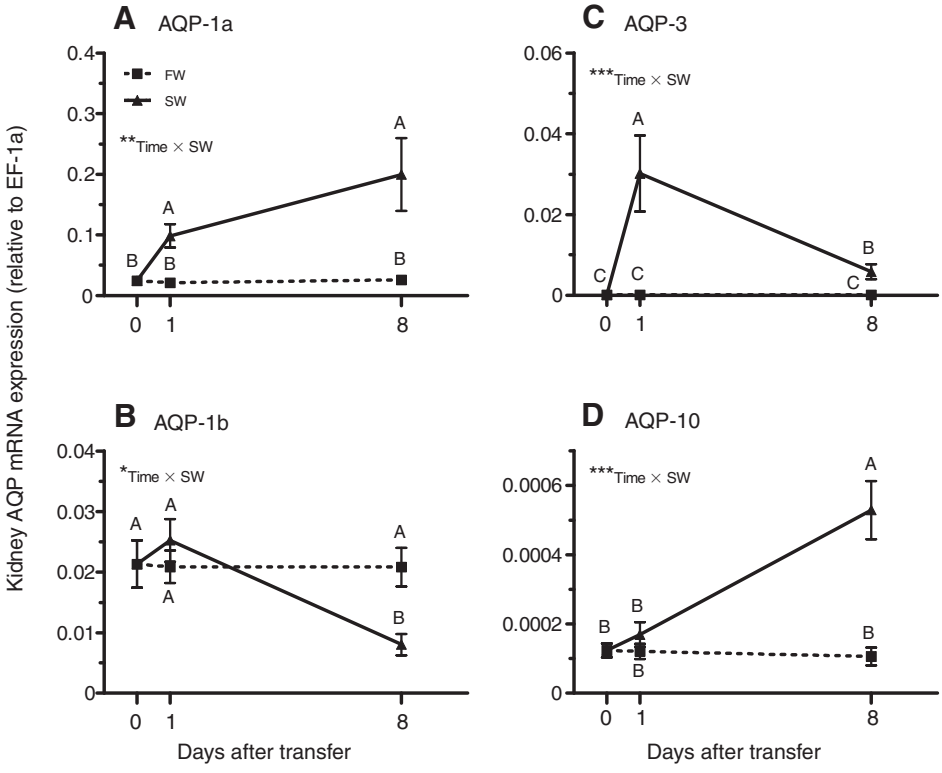

Fig. 5. Time course of renal aquaporin-1a (A), -1b (B), -3 (C) and -10 (D) mRNA expression after transfer of freshwater (FW) salmon to seawater (SW; triangles), or sham transfer to FW (squares). Significant interaction between time and salinity is indicated by asterisks next to 'Time × SW' (*P<0.05, **P<0.01, ***P<0.001). Letters above data points indicate statistical relationships. Values with the same letters are not statistically different; means ± s.e.m. (N=8).

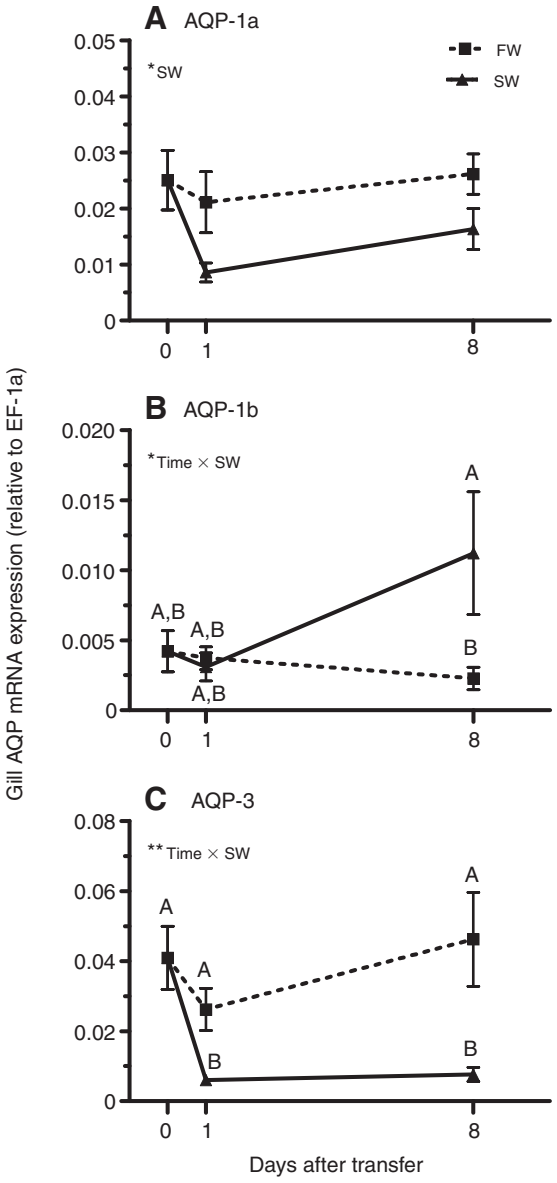

Fig. 6. Time course of aquaporin-1a (A), -1b (B) and -3 (C) mRNA expression in the gill after transfer of freshwater (FW) salmon to seawater (SW; triangles), or sham transfer to FW (squares). Significant interaction between time and salinity is indicated by asterisks next to 'Time × SW' (*P<0.05, **P<0.01, ***P<0.001). Letters above data points indicate statistical relationships. Values with the same letters are not statistically different. A significant overall effect of SW is indicated by an asterisk (*P<0.05). Values are means ± s.e.m. (N=8).

contrast, strong AQP-1b expression was detected in the eel oesophagus, whereas we detected only negligible expression in salmon oesophagus. AQP-3 was detected in all but hepatic tissue, with a prominently higher level in gill, skeletal muscle and oesophagus. The significant gill expression is similar to observations in eel (Cutler and Cramb, 2002); however, the eel study reported only minimal detection in oesophagus and muscle using northern blots. The present work is the first complete study to report expression patterns of AQP-8 in any lower vertebrate, and the two salmon isoforms are homologous to the annotated zebrafish AQP-8a and -8b gene sequences available in GenBank. Zebrafish AQP-8a and -8b are encoded by distinct genes that probably originated specifically in the teleost lineage by duplication from a shared ancestor gene similar to what appears to be the case for the AQP-1 paralogues (Tingaud-Sequeira et al., 2008). Although AQP-8a is exclusively detected in the liver, AQP-8b appears to be limited to the gastrointestinal tract excluding the oesophagus and this discrete distribution strongly suggests participation in organ-specific functions. In mammals, AQP-8 is highly expressed in the liver and intestinal tract (Elkjaer et al., 2001), where it may be involved in water transport across canalicular membranes (Larocca et al., 2009) and the apical domain of intestine epithelial cells (Tritto et al., 2007), respectively. Intriguingly, our data suggests that in salmon these functions in fluid transport of hepatocytes and intestinal epithelia may be conducted by two separate and specialized teleost AQP-8 paralogues.

Previous studies in eel (Martinez et al., 2005b) and gilthead sea bream (Santos et al., 2004) described AQPe and sbAQP, respectively, and these appear to be homologous to zebrafish, salmon and mammalian AQP-10 as indicated by the phylogenetic analysis. As in eel we found the highest expression of this aquaglyceroporin in the intestine and kidney. Mammalian variants of this isoform can conduct both water and small solutes, and studies in human tissues suggest that it may be involved in the apical absorption of nutrients and water in the small intestine (Mobasheri et al., 2004). The present distribution analysis gives a first clue of their relative importance in the various organs examined. However, a narrowing down to tissue and cellular expression patterns will have to await further progress.

Intestinal aquaporins

Previous studies in eel and gilthead sea bream reported that AQP-1a and -1b in the intestine are localized in the apical membrane of enterocytes, suggesting that they may act as the water entry site in epithelial cells (Aoki et al., 2003; Martinez et al., 2005a; Raldua et al., 2008). These studies have also shown that the abundance of these AQPs are increased in SW fish, when intestinal uptake of imbibed water becomes critical for retaining osmotic balance. Our data confirm this response in salmon at the mRNA level. In all intestinal sections mRNA of both isoforms were expressed. However, expression level was higher in the serosal cell layers (muscle) compared with the mucosa (enterocytes), which seems to suggest a minor role in enterocyte water transport (see Table 2). Salmon AQP-1a expressed in the deeper cell layers may be localized in and facilitate water transport across endothelial cells, which was found to be a major site of expression in eel intestine (Martinez et al., 2005a). We identified two likely candidate pores for enterocyte water transport. AQP-8b, which was almost exclusively expressed in the mucosal layer of intestinal segments and showed a substantial increase after SW exposure. We also found an increase in AQP-10 after SW exposure. This isoform was preferentially expressed in scrapings from the mucosal layer, which possibly includes part of the lamina propria, where this isoform is localized in sea bream (Santos et al., 2004). The transcript is generally present at very low levels, suggesting a minor role in enterocyte water balance. At present, it is possible that AQP-1a, -1b, -8b and -10 may all be involved in the handling of imbibed water in marine fish. However, AQP-8b is the only isoform stimulated at the peak of smoltification – a process that is often seen as a preparation for entry into SW for the juvenile FW salmon. In addition, AQP-8b is the only isoform predominantly expressed in the intestine, which emphasizes a particular significance of this pore in water uptake in SW. The result was consistent in pyloric caecae, middle and posterior intestine, which are all engaged in the absorption of water. Our data therefore

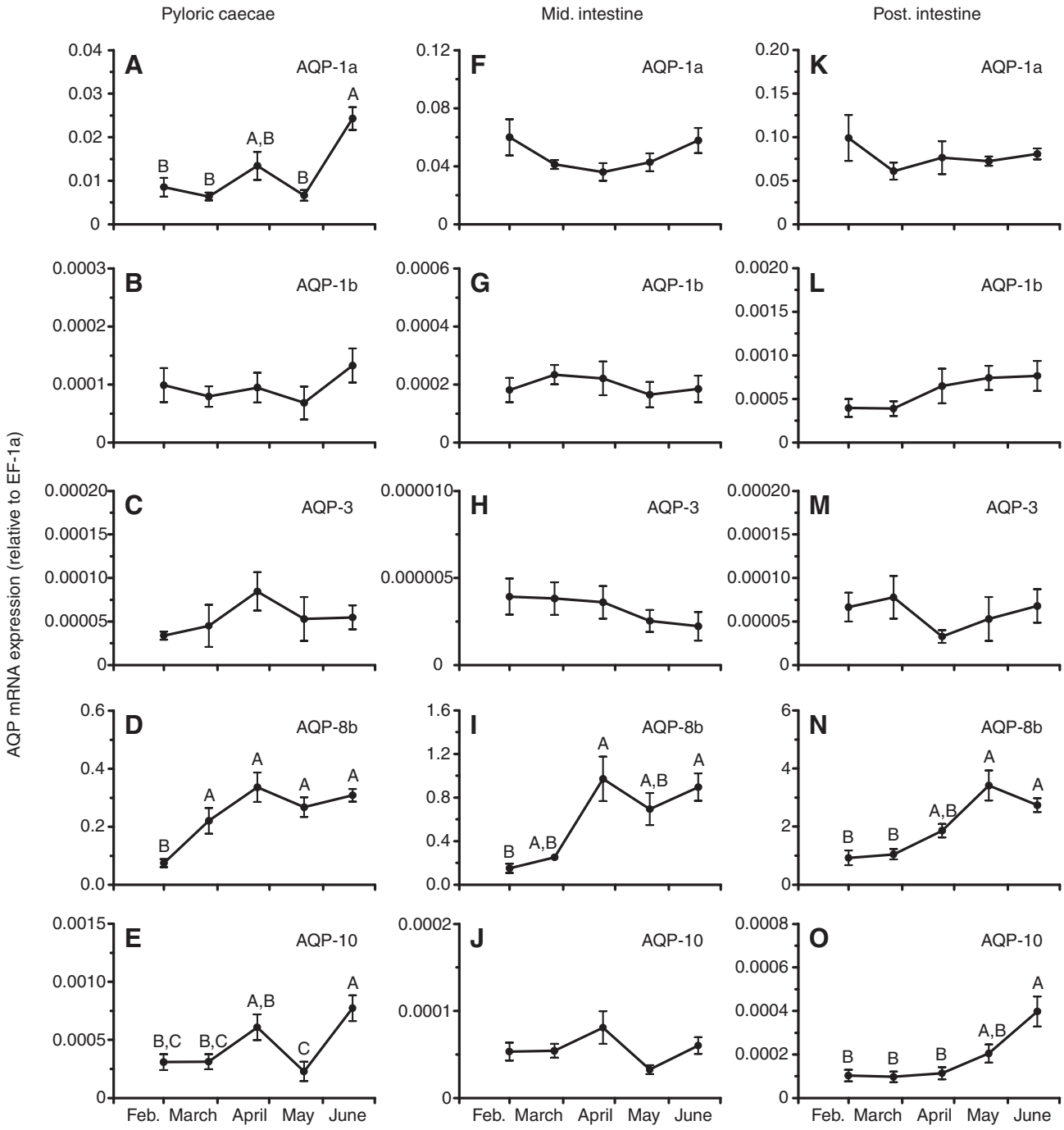

Fig. 7. Time course of aquaporin (AQP) expression in pyloric caecae (A–E), middle- (F–J) and posterior (K,O) intestine during the period of smoltification from February to June. AQP-1a (A,F,K); AQP-1b (B,G,L); AQP-3 (C,H,M); AQP-8b (D,I,N) and AQP-10 (E,J,O) were analysed. Letters above data points indicate statistical relationships. Values with the same letters are not statistically different; means ± s.e.m. (*N*=8–10).

suggests a mechanism to account for previous findings of increased iso-osmotic water uptake capacity, J_v in isolated intestinal preparations during smoltification [brown trout (Nielsen et al., 1999), Atlantic salmon (Sundell et al., 2003)]. It is possible that in order for the intestine to develop full functionality in water absorption as well as cell volume regulation, all four AQP isoforms may have to be induced simultaneously. During smoltification in the FW environment this seems only partly achieved by increasing AQP-8b expression and not AQP-1a, -1b and -10. Eventually, initiation of drinking and direct exposure of the intestine to imbibed SW may be a critical stimulus needed to finalize development of the hypo-osmoregulatory capacity of the intestine.

In mammals, another potential substrate for AQP-8 is ammonia. Even though AQP-8 knockout experiments in mice have failed to demonstrate significant phenotypic effects (Yang et al., 2006), reconstituted AQP-8 into lipid bilayers facilitates neutral ammonia transport, even with a twofold preference over water molecules (Saparov et al., 2007). Intriguingly, this aquaporin may, in combination with members of the non-erythroid Rhesus glycoprotein family (Marini et al., 2000), account for most ammonium transport

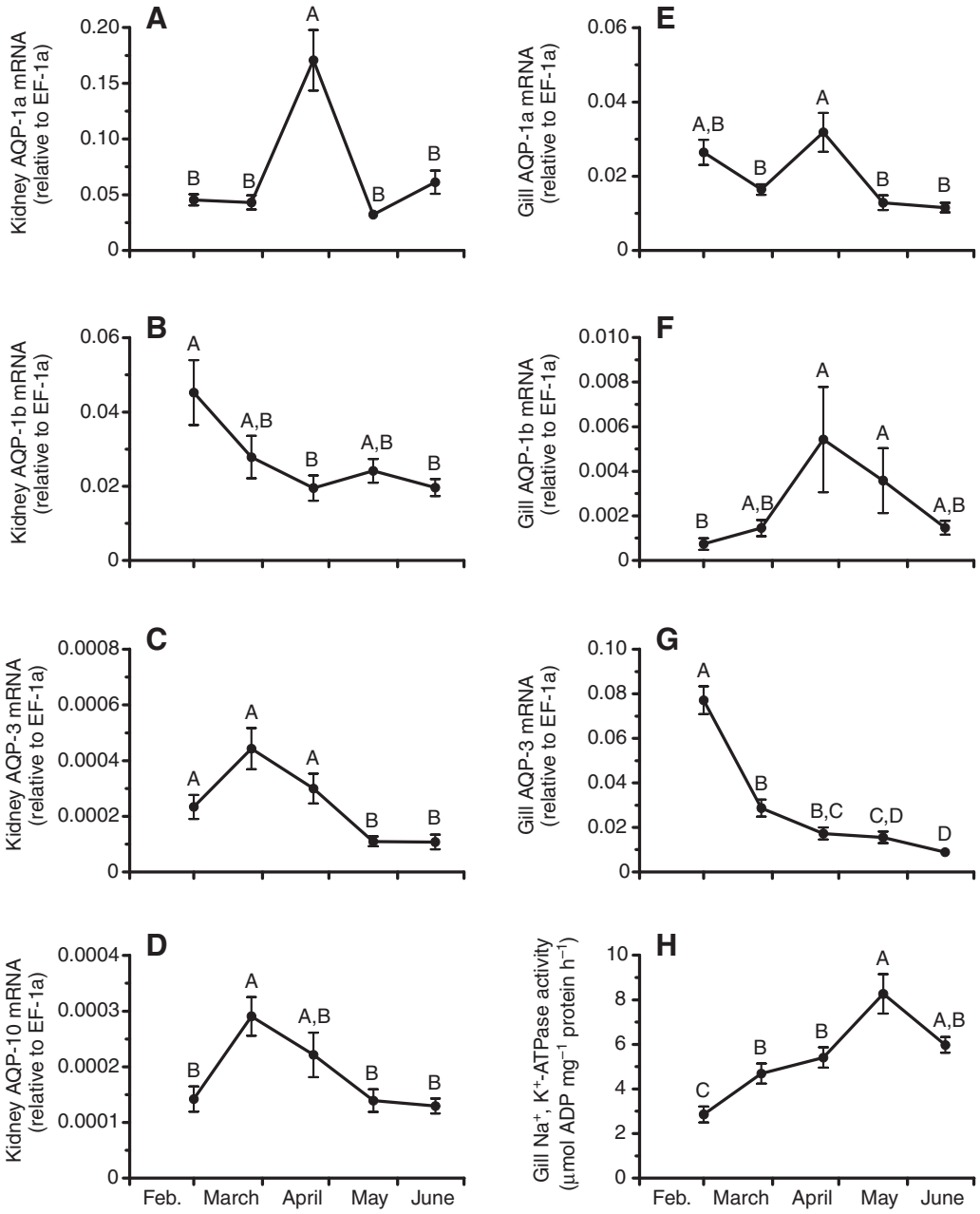

Fig. 8. Time course of aquaporin (AQP) expression in kidney (A–D) and gill (E–G) and gill Na^+/K^+-ATPase activity (H) during the period of smoltification from February to June. AQP-1a (A,E); AQP-1b (B,F); AQP-3 (C,G); AQP-10 (D) were analysed. Letters above data points indicate statistical relationships. Values with the same letters are not statistically different; means ± s.e.m. (*N*=8).

across cell membranes; in the case of the digestive tract this means ammonia absorption from intestinal chyme (Handlogten et al., 2005). In fish, it is generally accepted that the majority of ammonia excretion takes place across the gill (Wood, 1993). However, ammonia handling in the digestive tract and how it is affected by salinity or starvation has largely been ignored. On the basis of the present high expression of AQP-8 in the intestinal tract it may be speculated that this AQP may play a role in nitrogen metabolism.

Kidney aquaporins

In mammals, it is well known that AQP-1 is essential for urinary concentration in the descending thin limb of Henle (see Nielsen et al., 2002). Teleost kidneys are unable to make hypertonic urine; however, isotonic conditions may be reached in the urine of euryhaline fish during acclimation to hyper-saline conditions. This is largely explained by increased reabsorption of water from the glomerular filtrate in the distal segments of nephrons (Marshall and Grosell, 2006). Therefore it is somewhat paradoxical that in European eel, SW transfer induces decreased mRNA expression in both renal AQP-1 paralogues (Martinez et al., 2005b). The eel AQP-1s were localized in the apical brush border of proximal, rather than distal, tubule cells, and accordingly the authors suggested that they are involved in fluid secretion in early sections of the tubule. There is also evidence of AQP-1 in the vascular endothelium of the kidney, suggesting a further role in fluid transport to/from the blood. Assuming the present mRNA data can be extended to functional

Table 2. Transcript levels in mucosal and serosal cell layers from the middle intestine of freshwater salmon

Gene	Relative transcript levels	
	Mucosal scraping	Residual layer
AQP-1a	0.018±0.0045	1.0±0.34*
AQP-1b	0.46±0.33	1.0±0.30
AQP-3	1.4±0.41	1.0±0.26
AQP-8b	35.0±11.6	1.0±0.41*
AQP-10	24.0±1.5	1.0±0.41*

Asterisks denote significant differences as determined by Student's *t*-test ($P<0.01$). Values are target mRNA relative to EF-1a normalized to 1 in the residual layer for each AQP isoform (means±s.e.m.) $N=3$.
Mucosal cell layers were scraped off the interior of the intestine using a glass slide, and the residual layer consisted mostly of smooth muscle.

protein, our data both confirm and oppose the observations in eel. As in eel, we found decreased levels of AQP-1b in SW kidney whereas salmon AQP-1a was clearly elevated in the SW environment. Increased renal AQP-1a expression in SW has also been reported in sea bass (Giffard-Mena et al., 2007). The observed convergence of salinity acclimation and smoltification seen in the current study makes a strong case that AQP-1a, to a higher extent than AQP-1b, is defining the ability of the SW kidney to produce iso-osmotic urine.

AQP-3 and AQP-10 are expressed in the kidney at much lower levels than the AQP-1s. Classified as aquaglyceroporins in mammals, these two isoforms could be involved in transport of small molecules (glycerol, urea, NH_3) as well as water. However, their elevation during SW acclimation suggests that they, along with AQP-1a, are involved in renal fluid balance and reabsorption in SW salmon. This is in accord with reports of higher renal expression of AQP-3 in SW specimens of European sea bass (Giffard-Mena et al., 2007) and in tilapia [$N=1$ (Watanabe et al., 2005)]. In order for the elevated kidney AQP-1a, -3 and -10 expression observed in our study to evoke increased re-absorptive capacity of the SW nephron, the expressed AQPs must be localized in some combination in both apical and basal membranes within the same tubular cells. So far, there are no reports of cellular AQP-10 localization in fish tissues; interestingly, AQP-1a has been identified in apical membranes in teleost kidney and intestine (Aoki et al., 2003; Martinez et al., 2005b), whereas AQP-3, at least in the case of gill cells, is localized basolaterally (Cutler et al., 2007). Future investigations are needed to reveal the pattern of combination which allows for transcellular water transport.

Gill aquaporins

In contrast to the epithelia of the intestine and kidney tubules, the gill is in direct contact with the surrounding medium and has a potential risk of large transepithelial water fluxes due to the osmotic gradient. From an osmoregulatory perspective, transepithelial water permeability should therefore be kept to a minimum; however, this is compromised by the high demand for diffusive gas transfer at this site. The cells of the branchial epithelium, however, probably need a well-developed capacity for regulatory volume adjustments, as they are defending osmotic gradients between blood and surrounding water. This could be accomplished by having low apical membrane permeability to reduce apical fluid flow and high basolateral permeability, which in combination with the expanded surface area of this region would allow for regulatory volume changes (Hill et al., 2004b). Water permeability of the gill is further influenced by a mucus layer lining the apical surface, which is reported to be less abundant in gills adapted to fresh water than in those adapted to seawater (Gilmour, 1997). Realistic measures of water permeability of the branchial epithelium are hard to obtain, and only a few studies have reported such data and the impact of salinity change. In general, the osmotic water permeability of fish gills is in the range of a barrier forming epithelium (Motais et al., 1969). No change was found in the osmotic water permeability in trout gill during salinity changes (Isaia et al., 1979), whereas in other euryhaline teleosts such as eel and flounder higher permeability was detected in FW than in SW (Motais et al., 1969). We measured mRNAs of three AQPs in the gill. AQP-1a and -3 decreased whereas AQP-1b increased upon SW acclimation. This reciprocal change in isoform composition in the gill related to salinity, may correspond to the cellular reorganization occurring during salinity shifts. By comparison with previous studies, there seems to be consensus that AQP-3 is less abundant in gills of SW-acclimated euryhaline fish [eel (Cutler and Cramb, 2002); tilapia (Tse et al., 2007); European sea bass (Giffard-Mena et al., 2007)]. AQP-3 has been localized in both pavement cells and MRCs (Cutler et al., 2007), and in the case of MRCs the protein has been nicely demonstrated in the basolateral tubular network of these cells by electron microscopic immunocytochemistry (Watanabe et al., 2005). Thus this isoform may be responsible for the regulatory cell volume control, and may also partly explain why osmotic water permeability in eel gill decreases during SW acclimation (Isaia and Hirano, 1976; Evans, 1984). The cellular and subcellular location of the two AQP-1 isoforms in the gill is unknown. However, in most vertebrate tissues AQP-1 is located apically and serves as a gate for water exchange on the mucosal side. The changes observed in AQP-1b and -3 after transfer to SW are also

Table 3. A summary of the regulation of aquaporin (AQP) transcript levels in osmoregulatory tissues (middle intestine, kidney and gill) by seawater acclimation and during smoltification

Gene	Middle intestine		Kidney		Gill	
	SW	Smoltification	SW	Smoltification	SW	Smoltification
AQP-1a	↑	0	↑	↑	↓	0
AQP-1b	↑	0	↓	↓	↑	↑
AQP-3	0	0	↑	↓	↓	↓
AQP-8b	↑	↑	n.d.	n.d.	n.d.	n.d.
AQP-10	↑	0	↑	↓	n.d.	n.d.

SW, seawater.
An elevation (↑) or decline (↓) in expression levels are indicated by arrows while no changes compared with initial values are indicated by a zero. n.d., not detected.

seen in FW salmon during smoltification. This adds to our perception of smoltification as being preparatory to facing the hyperosmotic environment in the ocean.

Conclusion and perspectives

Euryhaline fish are useful models to study ion and water transport, since they have very 'plastic' osmoregulatory epithelia in the gill and intestine. Although active ion transport in teleost epithelia has been studied carefully, it remains important to characterize passive elements such as the AQPs and critical tight junction elements (Tipsmark et al., 2008a). Interestingly, we found reciprocal shifts in AQP isoform expression in both gill and renal tissue which may have functional significance and provide important clues to further understanding of changes in organ function in FW *versus.* SW. In future studies on intestine function of marine teleosts it may be useful to direct attention to the novel AQP-8b isoform, given its organ-specific distribution and stimulation by SW and during smoltification. It will also be important to include analyses of protein expression and in particular to clarify subcellular localization patterns of each isoform, since this may have a strong impact on the functional properties of AQPs involved in either cell volume homeostasis, vectorial water movement or both.

ACKNOWLEDGEMENTS

The research was supported by grants from The Danish Natural Research Council (272-06-0526), The Novo Nordisk Foundation (10584) and a Fellowship grant from The Carlsberg Foundation (2007-01-0213).

REFERENCES

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389-3402.

An, K. W., Kim, N. N. and Choi, C. Y. (2008). Cloning and expression of aquaporin 1 and arginine vasotocin receptor mRNA from the black porgy, *Acanthopagrus schlegeli*: effect of freshwater acclimation. *Fish Physiol. Biochem.* **34**, 185-194.

Aoki, M., Kaneko, T., Katoh, F., Hasegawa, S., Tsutsui, N. and Aida, K. (2003). Intestinal water absorption through aquaporin 1 expressed in the apical membrane of mucosal epithelial cells in seawater-adapted Japanese eel. *J. Exp. Biol.* **206**, 3495-3505.

Benson, D. A., Karsch-Mizrachi, I., Lipman, D. J., Ostell, J., Rapp, B. A. and Wheeler, D. L. (2000). GenBank. *Nucleic Acids Res.* **28**, 15-18.

Bobe, J., Montfort, J., Nguyen, T. and Fostier, A. (2006). Identification of new participants in the rainbow trout (*Oncorhynchus mykiss*) oocyte maturation and ovulation processes using cDNA microarrays. *Reprod. Biol. Endocr.* **4**, 39.

Cutler, C. P. and Cramb, G. (2002). Branchial expression of an aquaporin 3 (AQP-3) homologue is downregulated in the European eel *Anguilla anguilla* following seawater acclimation. *J. Exp. Biol.* **205**, 2643-2651.

Cutler, C. P., Martinez, A. S. and Cramb, G. (2007). The role of aquaporin 3 in teleost fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 82-91.

Deane, E. E. and Woo, N. Y. S. (2006). Tissue distribution, effects of salinity acclimation and ontogeny of aquaporin 3 in the marine teleost, silver sea bream (*Sparus sarba*). *Mar. Biotechnol.* **8**, 663-671.

Elkjaer, M. L., Nejsum, L. N., Gresz, V., Kwon, T. H., Jensen, U. B., Frokiaer, J. and Nielsen, S. (2001). Immunolocalization of aquaporin-8 in rat kidney, gastrointestinal tract, testis, and airways. *Am. J. Physiol. Renal. Physiol.* **281**, F1047-F1057.

Evans, D. H. (1984). The roles of gill permeability and transport mechanisms in euryhalinity. In *Fish Physiology* (ed. W. S. Hoar and D. J. Randall), pp. 239-283. Orlando, FL: Academic Press.

Evans, D. H. (2008). Teleost fish osmoregulation: what have we learned since August Krogh, Homer Smith, and Ancel Keys? *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R704-R713.

Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid-base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.

Felsenstein, J. (1989). PHYLIP-Phylogeny inference package (version 3.2). *Cladistics* **5**, 164-166.

Giffard-Mena, I., Boulo, V., Aujoulat, F., Fowden, H., Castille, R., Charmantier, G. and Cramb, G. (2007). Aquaporin molecular characterization in the sea-bass (*Dicentrarchus labrax*): the effect of salinity on AQP1 and AQP3 expression. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 430-444.

Gilmour, K. M. (1997). Gas exchange. In *The Physiology of Fishes* (ed. D. H. Evans). New York: CRC Press.

Handlogten, M. E., Hong, S. P., Zhang, L., Vander, A. W., Steinbaum, M. L., Campbell-Thompson, M. and Weiner, I. D. (2005). Expression of the ammonia transporter proteins Rh B glycoprotein and Rh C glycoprotein in the intestinal tract. *Am. J. Physiol. Gastrointest. Liver Physiol.* **288**, G1036-G1047.

Hill, A. E., Shachar-Hill, B. and Shachar-Hill, Y. (2004a). What are aquaporins for? *J. Membr. Biol.* **197**, 1-32.

Hill, W. G., Mathai, J. C., Gensure, R. H., Zeidel, J. D., Apodaca, G., Saenz, J. P., Kinne-Saffran, E., Kinne, R. and Zeidel, M. L. (2004b). Permeabilities of teleost and elasmobranch gill apical membranes: evidence that lipid bilayers alone do not account for barrier function. *Am. J. Physiol. Cell Physiol.* **287**, C235-C242.

Hirata, T., Kaneko, T., Ono, T., Nakazato, T., Furukawa, N., Hasegawa, S., Wakabayashi, S., Shigekawa, M., Chang, M. H., Romero, M. F. et al. (2003). Mechanism of acid adaptation of a fish living in a pH 3.5 lake. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **284**, R1199-R1212.

Isaia, J. and Hirano, T. (1976). Effect of environmental salinity change on osmotic permeability of isolated gill of eel, *Anguilla anguilla* L. *Journal de Physiologie* **70**, 737-747.

Isaia, J., Payan, P. and Girard, J. P. (1979). A study of the water permeability of the gills of freshwater- and seawater-adapted trout (*Salmo gairdneri*): mode of action of epinephrine. *Physiol. Zool.* **52**, 269-279.

Krane, C. M. and Goldstein, D. L. (2007). Comparative functional analysis of aquaporins/glyceroporins in mammals and anurans. *Mamm. Genome* **18**, 452-462.

Larocca, M. C., Soria, L. R., Espelt, M. V., Lehmann, G. L. and Marinelli, R. A. (2009). Knockdown of hepatocyte aquaporin-8 by RNA interference induces defective bile canalicular water transport. *Am. J. Physiol. Gastrointest. Liver Physiol.* **296**, G93-G100.

Lignot, J. H., Cutler, C. P., Hazon, N. and Cramb, G. (2002). Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel (*Anguilla anguilla* L.). *J. Exp. Biol.* **205**, 2653-2663.

Marini, A. M., Matassi, G., Raynal, V., Andre, B., Cartron, J. P. and Cherif-Zahar, B. (2000). The human Rhesus-associated RhAG protein and a kidney homologue promote ammonium transport in yeast. *Nat. Genet.* **26**, 341-344.

Marshall, W. S. and Grosell, M. (2006). Ion transport, osmoregulation and acid-base balance. In *The Physiology of Fishes*, 3rd edn (ed. D. H. Evans and J. B. Claiborne), pp. 177-230. Boca Raton, FL: CRC Press.

Martínez, A. S., Cutler, C. P., Wilson, G. D., Phillips, C., Hazon, N. and Cramb, G. (2005a). Regulation of expression of two aquaporin homologs in the intestine of the European eel: effects of seawater acclimation and cortisol treatment. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R1733-R1743.

Martínez, A. S., Cutler, C. P., Wilson, G. D., Phillips, C., Hazon, N. and Cramb, G. (2005b). Cloning and expression of three aquaporin homologues from the European eel (Anguilla anguilla): effects of seawater acclimation and cortisol treatment on renal expression. *Biol. Cell* **97**, 615-627.

McCormick, S. D. (1993). Methods for nonlethal gill biopsy and measurements of Na^+,K^+-ATPase activity. *Can. J. Fish. Aquat. Sci.* **50**, 656-658.

McCormick, S. D., Moyes, D. and Ballantyne, J. S. (1989). Influence of salinity on the energetics of gill and kidney of Atlantic salmon (*Salmo salar*). *Fish Physiol. Biochem.* **6**, 243-254.

Mobasheri, A., Shakibaei, M. and Marples, D. (2004). Immunohistochemical localization of aquaporin 10 in the apical membranes of the human ileum: a potential pathway for luminal water and small solute absorption. *Histochem. Cell. Biol.* **121**, 463-471.

Motais, R., Isaia, J., Rankin, J. C. and Maetz, J. (1969). Adaptative changes of the water permeability of the teleoatean gill epithelium in relation to external salinity. *J. Exp. Biol.* **51**, 529-546.

Nielsen, C., Madsen, S. S. and Björnsson, B. T. (1999). Changes in branchial and intestinal osmoregulatory mechanisms and growth hormone levels during smolting in hatchery-reared and wild brown trout. *J. Fish Biol.* **54**, 799-818.

Nielsen, S., Frokiaer, J., Marples, D., Kwon, T. H., Agre, P. and Knepper, M. A. (2002). Aquaporins in the kidney: from molecules to medicine. *Physiol. Rev.* **82**, 205-244.

Olsvik, P. A., Lie, K. K., Jordal, A. E., Nilsen, T. O. and Hordvik, I. (2005). Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon, *BMC Mol. Bio.* **6**, Art. No. 21.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.

Raldúa, D., Otero, D., Fabra, M. and Cerdà, J. (2008). Differential localization and regulation of two aquaporin-1 homologs in the intestinal epithelia of the marine teleost Sparus aurata. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R993-R1003.

Rozen, S. and Skaletsky, H. J. (2000). Primer3 on the WWW for general users and for biologist programmers. In *Bioinformatics Methods and Protocols: Methods in Molecular Biology* (ed. S. Krawetz and S. Misener), pp. 365-386. Humana Press: Totowa, NJ.

Santos, C. R., Estêvão, M. D., Fuentes, J., Cardoso, J. C., Fabra, M., Passos, A. L., Detmers, F. J., Deen, P. M., Cerdà, J. and Power, D. M. (2004). Isolation of a novel aquaglyceroporin from a marine teleost (*Sparus auratus*): function and tissue distribution. *J. Exp. Biol.* **207**, 1217-1227.

Saparov, S. M., Liu, K., Agre, P. and Pohl, P. (2007). Fast and selective ammonia transport by aquaporin-8. *J. Biol. Chem.* **282**, 5296-5301.

Seidelin, M., Madsen, S. S., Blenstrup, H. and Tipsmark, C. K. (2000). Time-course changes in the expression of Na^+,K^+-ATPase in gills and pyloric caeca of brown trout (*Salmo trutta*) during acclimation to seawater. *Physiol. Biochem. Zool.* **73**, 446-453.

Sundell, K., Jutfelt, F., Agustsson, T., Olsen, R. E., Sandblom, E., Hansen, T. and Bjornsson, B. T. (2003). Intestinal transport mechanisms and plasma cortisol levels during normal and out-of-season parr-smolt transformation of Atlantic salmon, *Salmo salar. Aquaculture* **222**, 265-285.

Tingaud-Sequeira, A., Chauvigné, F., Fabra, M., Lozano, J., Raldúa, D. and Cerdà, J. (2008). Structural and functional divergence of two fish aquaporin-1 water channels following teleost-specific gene duplication. *BMC Evol. Biol.* **8**, 259.

Tipsmark, C. K., Kiilerich, P., Nilsen, T. O., Ebbesson, L. O. E., Stefansson, S. O. and Madsen, S. S. (2008a). Branchial expression patterns of claudin isoforms in Atlantic salmon during seawater acclimation and smoltification. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1563-R1574.

Tipsmark, C. K., Luckenbach, J. A., Madsen, S. S., Kiilerich, P. and Borski, R. J. (2008b). Osmoregulation and expression of ion transport proteins and putative

claudins in the gill of southern flounder (*Paralichthys lethostigma*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **150**, 265-273.
Tritto, S., Gastaldi, G., Zelenin, S., Grazioli, M., Orsenigo, M. N., Ventura, U., Laforenza, U. and Zelenina, M. (2007). Osmotic water permeability of rat intestinal brush border membrane vesicles: involvement of aquaporin-7 and aquaporin-8 and effect of metal ions. *Biochem. Cell Biol.* **85**, 675-684.
Tse, W. K. F., Au, D. W. T. and Wong, C. K. C. (2006). Characterization of ion channel and transporter mRNA expressions in isolated gill chloride and pavement cells of seawater acclimating eels, *Biochem. Biophys. Res. Commun.* **346**, 1181-1190.
Veillette, P. A., White, R. J., Specker, J. L. and Young, G. (2005). Osmoregulatory physiology of pyloric ceca: Regulated and adaptive changes in Chinook salmon. *J. Exp. Zool.* **303A**, 608-613.
Watanabe, S., Kaneko, T. and Aida, K. (2005). Aquaporin-3 expressed in the basolateral membrane of gill chloride cells in Mozambique tilapia *Oreochromis mossambicus* adapted to freshwater and seawater. *J. Exp. Biol.* **208**, 2673-2682.
Wood, C. M. (1993). Ammonia and urea metabolism and excretion. In *The Physiology of Fishes* (ed. D. Evans), pp. 379-425. Boca Raton, FL; CRC Press.
Yang, B., Song, Y., Zhao, D. and Verkman, A. S. (2005). Phenotype analysis of aquaporin-8 null mice. *Am. J. Physiol. Cell Physiol.* **288**, C1161-C1170.