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AQUAPORINS IN FISH – EXPRESSION, LOCALIZATION AND FUNCTIONAL DYNAMICS

Topic Editor
Steffen S. Madsen

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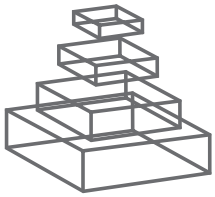

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AQUAPORINS IN FISH – EXPRESSION, LOCALIZATION AND FUNCTIONAL DYNAMICS

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Image by Courtney Trimble, University of Arkansas, Fayetteville, USA

By living in a 'world of water' fish are exposed to major challenges in maintaining water homeostasis. These are opposite in nature for fish living in marine and freshwater milieus; however, in both cases threatening, obligatory water fluxes due to global osmotic gradients must be compensated by opposite fluxes, driven by body fluid filtration and/or locally created osmotic gradients. In general, water may pass epithelia that are hydrophobic in nature by para- and/or transcellular pathways, the former mainly defined by the characteristics of tight junctions, the latter determined by the combined permeability of apical and baso-lateral cellular membranes. Transcellular water transport may occur by simple diffusion through lipid bilayers or become markedly improved by insertion of plasma membrane integral channel proteins of the Aquaporin (AQP) family. In mammals, 13 AQP subfamilies are present and several of these have been investigated structurally and functionally in >5000 publications since their discovery in 1992 by Agre and colleagues.

The first paper on AQPs in fish appeared in 2000 (Cutler and Cramb) but surprisingly few papers have addressed AQPs in fishes and other non-mammalian vertebrate classes since then. In zebrafish, 18 genes encode AQPs with homology to all but a few of the mammalian isoforms. Only few of these isoforms have been studied to some extent in this and other species.

AQPs most certainly play distinct osmoregulatory roles in fish as they do in mammals - both at the cellular and organismal level. However, there is a considerable lack of information from the fish world on this topic. At present, only ca. 50 papers have addressed AQPs in fish - most of these being concerned with basic investigations of isoform expression in various tissues of different teleosts.

This Research Topic will bring together original information as well as bring the field up-to-date on topics related to 'Aquaporins in fish - expression, localization and functional dynamics', hopefully thereby stimulating new research in this area. Contributions within the following areas are welcomed:

- Molecular biology of water transport
- AQP physiology and functionality (in vitro and in vivo studies)
- Cellular and subcellular localization of AQPs
- AQPs and cellular volume regulation/osmosensing
- AQPs and transepithelial water transport in kidney tubules and intestinal segments
- Endocrine regulation of AQPs
- Cellular trafficking of AQPs
- Pharmacological inhibition of AQPs
- The role of AQPs in handling "non-water" substances (toxic, waste etc.)
- Mini-reviews identifying areas of special interest.

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EDITORIAL
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Aquaporins in fishes—expression, localization, and functional dynamics

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By living in a "world of water" fishes are exposed to major osmotic challenges that are opposite in nature in the freshwater and marine environments. In both cases, obligatory water fluxes primarily due to osmotic gradients across respiratory surfaces are threatening to the internal milieu and must be compensated by bulk flows of water in the opposite direction. While the ionoregulatory mechanisms that generate the osmotic driving force for such water flows have been known for decades, the molecular pathways of compensatory water fluxes are still largely unravelled. Current models suggest that water passes hydrophobic epithelia by para- and/or transcellular pathways, the former being defined by the characteristics of tight junctions, the latter determined by the serial permeability of apical and basolateral cellular membranes. Transcellular water transport may occur by simple diffusion through lipid bilayers or become markedly improved by the insertion of integral channel proteins (aquaporins) in the plasma membrane. Thus, aquaporins can truly be conceived as the plumbing system of cells.

In mammals, 13 aquaporin subfamilies are present and several of these have been investigated structurally and functionally in >6600 publications since their discovery in 1992 by Agre and colleagues. The first paper on aquaporins in fishes appeared in Cutler and Cramb (2000) but surprisingly few papers have addressed aquaporins in fishes and other non-mammalian vertebrates during the ensuing decade. More recently, it has been established that the zebrafish and other teleosts retain up to 18 aquaporin genes with homologies to all but a few of the mammalian orthologues. However, the forthcoming publishing of the Atlantic salmon genome may add even more paralogues to this list (Finn, pers. communication). One of the major challenges ahead of us is therefore to describe and understand the differentiated functionality of such diversity.

Aquaporins most certainly play distinct roles in fishes as they do in mammals—both at the cellular, organ and organismal level. However, there is a considerable lack of information from the fish world on this topic, with only ca. 50 papers addressing aquaporins in fishes at the time of the call of this Research Topic in 2010. Most of these describe tissue expression patterns in various Teleosts, while Aqnathans and Chondrichthyes and the functionality of fish aquaporins have received very little focus. This ebook presents a collection of papers addressing the evolution and role of aquaporins during gametogenesis and embryonic development as well as for water transport across adult gill, kidney and intestinal epithelia using bony and cartilagenous fishes as models. Our aim is to stimulate new original research in this area as well as bringing together new collaborations across fields.

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Aquaporin evolution in fishes

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Aquaporins represent a primordial group of transmembrane solvent channels that have been documented throughout the living biota. This facet alone emphasizes the positive selection pressure for proteins associated with intracellular fluid homeostasis. Amongst extant Eukaryota the highest gene copy number can be found in plants and teleosts, a feature that reflects the genomic duplication history in both groups. In this minireview we discuss the discovery, structure, duplication, and diversification of the aquaporin superfamily. We focus on teleosts as the main models, but include data available for other organisms to provide a broader perspective.

Keywords: aquaporin, gene duplication, evolution, neofunctionalization, water transport

INTRODUCTION

Historically the transport of water across hydrophobic membranes was considered to occur via non-specific leakage. However, due to observations of increased water permeation in certain cells or tissues, such as human erythrocytes and the urinary bladder of frogs, specific water channels were also thought to exist (reviewed by Agre et al., 2001; Agre, 2005). The journey toward the discovery of aquaporins was aided by observations that junctional proteins were highly enriched in the plasma membrane of the bovine lens (Bloemendal et al., 1972; Broekhuyse and Kuhlmann, 1974). When purified and subjected to electrophoresis, such junctional complexes migrated as band III, MP26, or MP34 and were termed the main or major intrinsic protein (MIP) of the lens fiber (Broekhuyse et al., 1976; Vermorken et al., 1977; Gorin et al., 1984). However, it was by studying membranes purified from human erythrocytes that the first evidence of a bonafide water channel emerged (Benga et al., 1986a,b). The pure form of this ∼38–60 kDa glycosylated channel was isolated and shown to be a non-glycosylated 28 kDa MIP (CHIP28) of erythrocyte and renal membranes (Denker et al., 1988; Preston and Agre, 1991; Preston et al., 1992). These latter studies further provided direct evidence of water channel function through *ex vivo* injection of the CHIP28 transcript in *Xenopus laevis* oocytes followed by exposure of the oocytes to hyposmotic challenge. To concur with conventions established by the Human Genome Nomenclature Committee the term aquaporin-1 (AQP1) was coined for the CHIP28 protein (Agre et al., 1993) while MIP, which showed strong sequence homology to AQP1, became known as AQP0.

Since these early ground-breaking studies, aquaporins have been documented in all kingdoms of life, with several thousand sequences now available in public databases. The largest repertoire is currently found in plants with up to 71 paralogs reported in upland cotton (Park et al., 2010). In vertebrates the highest copy number is found in teleosts with up to 18 paralogs reported in zebrafish (Tingaud-Sequeira et al., 2010), while mammals run a close second due to tandem duplication of *AQP7* and *-12* resulting in up to 17 paralogs in humans (King et al., 2004; Cerdà and Finn, 2010). The precise copy number in Teleostei may increase as new genomes become available and novel annotations arise. For example the latest version of Ensembl (v63) predicts a second *aqp8b* paralog in zebrafish (ENSDARG00000089749) that is identical and closely linked to the *aqp8b* paralog (ENSDARG00000015512) characterized by Tingaud-Sequeira et al. (2010).

The vertebrate aquaporin complement has been classified according to molecular phylogeny, permeation preference for water, glycerol, or other small solutes and gases, tissue expression pattern and tertiary structure (Stahlberg et al., 2001; King et al., 2004; Takata et al., 2004; Zardoya, 2005; Tingaud-Sequeira et al., 2010). The superfamily thus consists of 13 subfamilies that include classical aquaporins (Aqp0, -1, -2, -4, -5, and -6), aquaglyceroporins (Aqp3, -7, -9, and -10), aquaporin-8 (Aqp8), and unorthodox aquaporins (Aqp11 and -12). A 14th subfamily suggested not to be present in mammals has also been identified in frogs (*AQPxlo*, Virkki et al., 2002). This gene is present in the Western-clawed frog genome (ENSXETG00000016307), but also exists in Prototheria (platypus: ENSOANG00000009732). Bayesian analysis of the amphibian and platypus sequences (data not shown) reveal that they are closely related to the aquaglyceroporins AQP3 and -9. To date, this gene has not been found in eutherian, metatherian, saurian, or piscine genomes.

STRUCTURAL DIVERSITY AND EXPRESSION OF PISCINE AQUAPORINS

By coupling the molecular phylogeny to linkage maps and comparing these data with structural and functional analyses it is

becoming possible to establish parsimonious models of the duplication history of a given superfamily of genes. This approach was recently adopted for the aquaporin superfamily in zebrafish (Tingaud-Sequeira et al., 2010). The study revealed that Teleostei mostly retain two or in some cases three orthologs of the human aquaporin repertoire. The exceptions include AQP2, -4, -5, -6, -7, and -12, which are present in Teleostei as single copy genes, a putative pseudogene or are absent. Alignment of the zebrafish primary structures shows that they retain the canonical features of the human counterparts, including the six transmembrane α-helices (1, 2, 4, 5, 6, and 8), five loops (A–E) and the two hemi-helices (3 and 7) that in most paralogs respectively retain the Asn-Pro-Ala (NPA) and Asn-Pro-Ala-Arg (NPAR) motifs that are the hallmark of the superfamily (**Figure 1A**). Comparison of the subdomains within each aquaporin group reveals that they have not experienced strong purifying selection. This is likely due to relaxed criteria associated with selection of amphipathic amino acids necessary for membrane integration. By contrast, hemi-helix 7 that bears the Arg-constriction residue shows the highest identity in all groups. This residue comprises one of a quartet that forms the aromatic-arginine (ar/R) selectivity filter in the outer third of the channel vestibule (de Groot and Grubmüller, 2005; Wu and Beitz, 2007). We have previously reported that amino acid residues involved in the ar/R constriction of teleost water channels are, like the mammalian counterparts, related to the different classes of aquaporins, but in Teleostei the underlying codons have experienced greater nucleotide substitution even in closely related paralogs (Cerdà and Finn, 2010). In some cases, this latter feature appears to have given rise to non-canonical residues associated with the selectivity filters. Examples include members of the teleost Aqp3a, -3b, -7, -8ab, -8b, and unorthodox aquaporins for the first NPA motif, and Aqp7, -8b, and the unorthodox aquaporins for the second NPA motif. Since there is a paucity of channel permeation preference studies in fishes (Fabra et al., 2006; Tingaud-Sequeira et al., 2008, 2010; MacIver et al., 2009; Hamdi et al., 2010; Chaube et al., 2011; Chauvigné et al., 2011), with a bias toward zebrafish, the functional and evolutionary significance of such core divergences remains to be determined. Interestingly, however, a recent study has revealed that the non-canonical Cys in the first NPA motif of murine AQP11 appears to augment oligomerization, suggesting that internal residues may play roles beyond permeation preference (Ikeda et al., 2011).

The regions of least conservation are the cytoplasmic N- and C-terminal domains. This is true both in terms of the number and identity of the encoded amino acids. The longest N-termini are present in teleost Aqp4, -8aa, -8ab, -8b, and the unorthodox aquaporins, the structural features of which are also conserved in the mammalian orthologs. With respect to fish, the molecular role of the N-terminus remains unknown. The role of this domain has, however, been associated with channel gating in plants (Törnroth-Horsefield et al., 2006), the intracellular localization of AQP6 (Beitz et al., 2006), and potentially the membrane trafficking and assembly of AQP4 variants in mammals (Neely et al., 1999). By contrast phosphorylation-mediated post-translational modifications of the C-terminal domain are well established for membrane trafficking and water permeability of mammalian AQP2 and amphibian AQP-h2/3 orthologs (Deen et al., 1994; Suzuki and Tanaka, 2009; Eto et al., 2011; Tamma et al., 2011). However, mutagenic studies addressing the role of this domain in teleost aquaporins has produced more divergent results (see below).

Expression data for piscine aquaporins have recently been reviewed by Cerdà and Finn (2010) and are schematically summarized here for zebrafish and other teleosts (**Figure 1B**). Three paralogs, Aqp1aa, -3a, and -12, are expressed ubiquitously or semi-ubiquitously in the range of tissues that have been examined to date. With the exception of Aqp12, which differs markedly from the near-exclusive sites in mammals (Itoh et al., 2005), such ubiquitous expression patterns might indicate constitutive roles associated transcellular fluid transport. Conversely, the more restricted and partially redundant expression profiles of the other paralogs suggest that novel functions have or are evolving. As an example, Aqp0a and -0b are both considered necessary for normal lens development and transparency, as found for mammals, but Aqp0b is thought to have subfunctionalized (Froger et al., 2010). This latter study further suggested that teleost Aqp0b could be involved in cell adhesion, which is inline with the earliest studies in bovine lens and emerging evidence for other animal orthologs such as mammalian AQP4 (Hiraoki et al., 2006) and dipteran big brain (Tatsumi et al., 2009).

DUPLICATION AND DUAL NEOFUNCTIONALIZATION OF PISCINE AQUAPORINS

Within the last decade, investigations of piscine aquaporins have advanced from identification of the first ortholog in European eel (Aqp3b, Cutler and Cramb, 2000) to characterization of the superfamily in zebrafish (Tingaud-Sequeira et al., 2010). The latter study utilized the multidisciplinary approach outlined above and proposed a new nomenclature in order to provide a common platform for future research. The phylogenetic data set primarily included Teleostei, but also incorporated more ancestral aquaporins available for Hyperotreti, Chondrichthyes, and members of the Sarcopterygii. There remains a clear absence of data available for ancient piscine lineages such as Polypteriformes, Acipenseriformes, Semionotiformes, and Amiiformes. Such data would provide a more complete picture of aquaporin evolution in the actinopterygian lineage.

The current theory for the origin of the majority of aquaporins in Teleostei is consistent with an ancient whole genome duplication (WGD) event at the root of the crown clade (Amores et al., 1998; Jaillon et al., 2004; Volff, 2005; Finn and Kristoffersen, 2007; Tingaud-Sequeira et al., 2010). However, two subfamilies (*aqp1* and *aqp8*) remained equivocal despite dichotomous and trichotomous clustering in relation to the human orthologs. The duplication history and putative neofunctionalization of the teleost *aqp8aa*, *-8ab*, and *-8b* paralogs has been explained on the basis of a combination of WGD and tandem replication (Cerdà and Finn, 2010), while the *aqp1* paralogs were suggested to have arisen via tandem duplication due to their juxtaposition in teleost genomes (Tingaud-Sequeira et al., 2008). The latter hypothesis, although seemingly parsimonious, did not incorporate the theory of diploidization and rearrangement or account for the loss of the WGD product. A new study has recently been conducted to address these issues (Zapater et al., 2011). Based upon extensive molecular phylogenetic, syntenic, and functional analyses, this

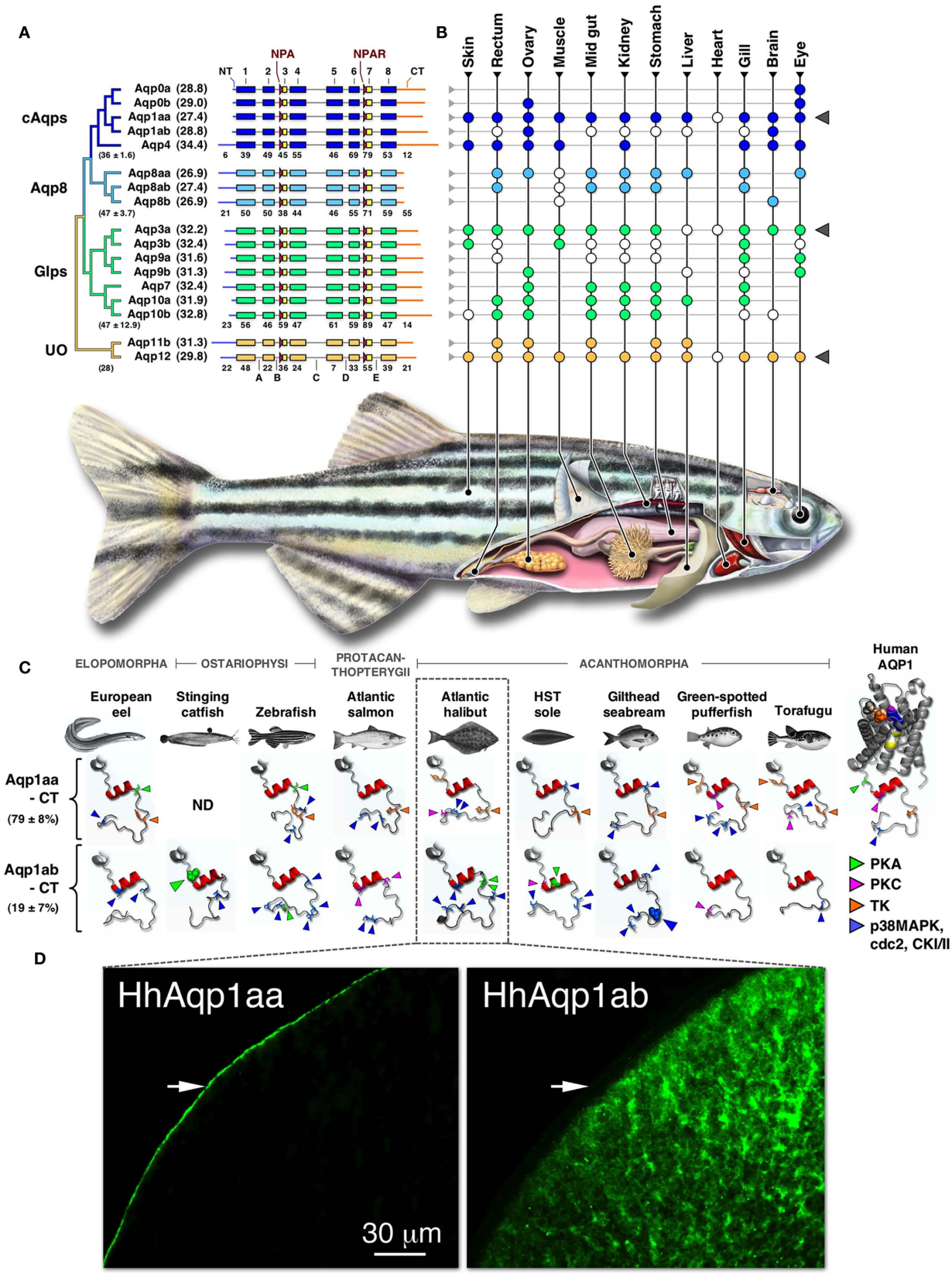

FIGURE 1 | Continued

FIGURE 1 | Phylogeny, structure, and expression of teleost aquaporins. **(A)** Bayesian midpoint rooted tree of the zebrafish aquaporin superfamily with predicted molecular masses of the deduced proteins given in parentheses. Linear-scale alignment of the superfamily shows the secondary structural conservation of α-helices 1–8 and the diversity of the N- (NT) and C-termini (CT) within each subfamily. The canonical Asn-Pro-Ala (NPA) and Asn-Pro-Ala-Arg (NPAR) motifs that are present in most paralogs are shown upstream of hemi-helices 3 and 7, respectively. Loops A–E are indicted below the unorthodox (UO) aquaporins. Identity values of the full-length (in parentheses ± SD), the α-helical, and the terminal subdomains of the classical aquaporins (cAqps), aquaporin 8 (Aqp8), aquaglyceroporins (Glps), and the UO aquaporins are shown below each group. **(B)** Summarized view of the tissue expression pattern of zebrafish aquaporins. Colored dots refer to the distribution in adults, while white dots represent data reported for other species of teleost. In some species the anterior intestine is indicated by the stomach, while the brain includes chemosensory and mechanosensory organs. Gray triangles to the right highlight ubiquitous or semi-ubiquitous expression patterns. **(C)** Three-dimensional cartoon renders of the cytoplasmic C-terminal regions of teleost Aqp1aa and -1ab compared to Human AQP1 (1H6I). Models were generated using ModWeb and rendered with MacPyMOL. For the human AQP1 channel, the NPA motifs are highlighted in yellow and the ar/R constriction residues (Phe, magenta; His, wheat; Cys, Orange; Arg, blue) are shown as spacefill. All proteins retain a tertiary helix (red H9) but otherwise fold as disordered loops extending intracellularly from α-helix 8. Putative phosphorylation sites are indicated as protein kinase A (PKA), protein kinase C (PKC), tyrosine kinase (TK), p38 mitogen-activated protein kinase (p38MAPK), cell division cycle 2 (cdc2), or casein type kinase (CKI/II). Phosphorylation sites known to influence membrane trafficking are rendered as spacefill for the stinging catfish (green) and gilthead seabream (blue). Identity (±SD) of the aligned teleost C-termini are given to the left. ND: no data. **(D)**. Localized cellular expression of Atlantic halibut (*Hippoglossus hippoglossus*) HhAqp1aa and -1ab (green) following *ex vivo* injection of transcripts in *Xenopus laevis* oocytes. HhAqp1aa is constitutively expressed in the plasma membrane (white arrows), while HhAqp1ab is retained in intracellular vesicles. Data for this figure are recompiled from Cerdà and Finn (2010), Sun et al. (2010), Tingaud-Sequeira et al. (2008, 2010), Tipsmark et al. (2010), Chaube et al. (2011), Zapater et al. (2011), and Zichichi et al. (2011).

latter study concluded that the teleost *aqp1* genes (formerly annotated as *aqp1a* and *-1b*) are indeed tandem duplicates and were respectively renamed *aqp1aa* and *aqp1ab* to match the *aqp8* terminology, while the *aqp5/1b* pseudogene in zebrafish is the most likely candidate for the lost WGD product.

The study by Zapater et al. (2011) further revealed that the cytoplasmic C-terminus of Aqp1ab is the most rapidly evolving subdomain in the vertebrate aquaporin superfamily (**Figure 1C**). Despite this degeneracy, Aqp1ab has been found to play a conserved role in the oocyte hydration of teleosts (reviewed by Cerdà, 2009; Finn and Fyhn, 2010). The novel role of Aqp1ab in oocyte hydration was first suggested for gilthead seabream (Fabra et al., 2005), and has recently been experimentally demonstrated in the acanthomorph teleost Atlantic halibut (Zapater et al., 2011). The data for Atlantic halibut show that the temporal insertion of the Aqp1ab paralog in the oocyte plasma membrane occurs during the phase of maximal osmolyte generation (Finn et al., 2002; Finn, 2007; Zapater et al., 2011) and differs markedly from the predominant thecal localization of AQP1 in pre-ovulatory stages of human follicles (Thoroddsen et al., 2011). An interesting aspect of the role of Aqp1ab in teleost oocytes is that it essentially functions as an inwardly rectifying channel due to the exclusive generation of intracellular osmolytes and the tight temporal regulation of the channel in the oolemma. Perhaps even more striking is that the rapid evolution of the Aqp1ab C-terminus has resulted in alternative control of the trafficking mechanism and in some species, such as the Atlantic halibut, loss of function when expressed in *X. laevis* oocytes (**Figure 1D**). It has been suggested that phosphorylation of Ser^{227} is associated with translocation of stinging catfish Aqp1ab (Chaube et al., 2011), while phosphorylation of Ser^{254} promotes recycling of gilthead seabream Aqp1ab (Tingaud-Sequeira et al., 2008), yet phosphorylation may not be involved in the trafficking mechanism of Atlantic halibut Aqp1ab (Zapater et al., 2011). These findings are revealing that for certain paralogs, gene duplication has resulted in constrained and relaxed traits controlling aquaporin function in teleosts. For the Aqp1ab paralogs, selection pressure has favored oocyte hydration, but has been relaxed with regard to the molecular control of membrane trafficking. The nature of these trafficking mechanisms and the intracellular pathways involved are not yet known, and remain intriguing avenues for future research.

FUTURE PERSPECTIVES

Investigations of the role of aquaporins in fishes are only just beginning. By comparing the results obtained from fishes to the more extensive data sets available for mammals, it seems likely that rapid advances can be achieved. From an evolutionary perspective, however, the piscine branch of vertebrates includes members that existed both prior to and after the second, third, and in some species of teleost the fourth round of WGD. Consequently by studying the superfamily in selected organisms, it should be possible to determine how gene duplication or gene loss has resulted in alternative evolution of aquaporins at the molecular, cellular, physiological, and ecological levels. Today, the number of extant vertebrates (∼55,000) is essentially equally split between aerial and aquatic environments. It can be expected that selection pressure has acted differently in these ecosystems.

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Aquaporin 4 is a ubiquitously expressed isoform in the dogfish (*Squalus acanthias*) shark

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The dogfish ortholog of aquaporin 4 (AQP4) was amplified from cDNA using degenerate PCR followed by cloning and sequencing. The complete coding region was then obtained using 5′ and 3′ RACE techniques. Alignment of the sequence with AQP4 amino acid sequences from other species showed that dogfish AQP4 has high levels (up to 65.3%) of homology with higher vertebrate sequences but lower levels of homology to Agnathan (38.2%) or teleost (57.5%) fish sequences. Northern blotting indicated that the dogfish mRNA was approximately 3.2 kb and was highly expressed in the rectal gland (a shark fluid secretory organ). Semi-quantitative PCR further indicates that AQP4 is ubiquitous, being expressed in all tissues measured but at low levels in certain tissues, where the level in liver > gill > intestine. Manipulation of the external environmental salinity of groups of dogfish showed that when fish were acclimated in stages to 120% seawater (SW) or 75% SW, there was no change in AQP4 mRNA expression in either rectal gland, kidney, or esophagus/cardiac stomach. Whereas quantitative PCR experiments using the RNA samples from the same experiment, showed a significant 63.1% lower abundance of gill AQP4 mRNA expression in 120% SW-acclimated dogfish. The function of dogfish AQP4 was also determined by measuring the effect of the AQP4 expression in *Xenopus laevis* oocytes. Dogfish AQP4 expressing-oocytes, exhibited significantly increased osmotic water permeability (P_f) compared to controls, and this was invariant with pH. Permeability was not significantly reduced by treatment of oocytes with mercury chloride, as is also the case with AQP4 in other species. Similarly AQP4 expressing-oocytes did not exhibit enhanced urea or glycerol permeability, which is also consistent with the water-selective property of AQP4 in other species.

Keywords: aquaporin 4, shark, dogfish, kidney, liver, rectal gland, gill, cardiac stomach

INTRODUCTION

Aquaporins (AQPs) are commonly known as cell membrane water channel proteins although various isoforms exist that exhibit other transport properties including having permeability's for small solutes such as glycerol and urea. In mammals, there are 13 isoforms known (numbered 0–12), where AQPs 0, 1, 2, 4, 5 fall into a sub-group of water-selective channels and where AQP1 is the most ubiquitously expressed in mammalian tissues (Ishibashi et al., 2009; Zelanina, 2010). Most AQPs are reversibly inhibitable by mercury, although one exception to this is AQP4, whose original name was MIWC (mercury insensitive water channel; Hasegawa et al., 1994; Jung et al., 1994). Mammalian AQP4 is expressed in a number of tissues but is not as ubiquitous as AQP1 (Ishibashi et al., 2009), AQP4 is particularly abundant in the brain (Amiry-Moghaddam et al., 2010; Brian et al., 2010; Zelanina, 2010), but is also expressed in the retina (Goodyear et al., 2009; Hirrlinger et al., 2011), salivary gland (Delporte and Steinfeld, 2006), respiratory tract (Matsuzaki et al., 2009), heart and muscle (Butler et al., 2006; Wakayama, 2010), gastrointestinal tract (Ma and Verkman, 1999; Xu et al., 2009), and kidney (Nejsum, 2005). The mammalian AQP4 gene is also known to produce alternative splice forms (Crane et al. (2009); Strand et al., 2009; Fenton et al., 2010). Several of these variants produce proteins with a variable sized N-terminal region.

In lower vertebrates very little is known about AQPs. The sequence of AQP4 has been determined in both Pacific and Atlantic hagfish (Cutler, 2007a; Nishimoto et al., 2007) and information is also available on hagfish AQP9 (Cutler, 2006a), but very little information is available in other lower vertebrate taxonomic groups such as in the elasmobranches (Cutler et al., 2005; Cutler, 2006b; Cutler, 2007b). The studies presented in this article (and its companion article) were performed to begin to rectify this situation. However at the outset when little is known, basic information needs to be determined. This includes (1) showing which organs or tissues express AQP4 and at what level (2) determining the functional properties of expressed AQP4 protein to show functional similarities or difference to AQP4 in other species and (3) undertaking an initial investigation to see if AQP4 might be involved in body fluid homeostatic mechanisms by perturbing the fish's osmotic

environment to determine whether this effects the level of AQP4 expression.

MATERIAL AND METHODS

CLONING AND SEQUENCING

Dogfish AQP4 was cloned and sequenced using well established methods (Cutler and Cramb, 2002a,b, 2008; Martinez et al., 2005) of degenerate PCR followed by 5′ and 3′ RACE. AQP4 degenerate primers were produced using an elasmobranch-targeted primer design with sequence information obtained from an aquaporin sequence cloned from the bull shark (Cutler et al., 2005). The sense primer was 5′-CAYRTIAGYGGI GCICAYRTIAAYCC-3′ and the antisense primer 5′-GGICCIACCCARTAIACCCART G-3′ the derived amino acid sequences of these primers are located at 117–125 and 257–264 of the alignment (as indicated) respectively (**Figure 1**.). Nucleotides using the base inosine (I) were incorporated into primers at positions of major sequence uncertainty. PCR conditions used with these primers varied but was essentially similar to: −96°C – 1 s, 55°C – 15 s, and 72°C – 30 s for 40 cycles. This amplifies an expected and actual cDNA fragment of 443 bp. cDNA for amplifications was made from dogfish total RNA from both rectal gland and kidney (extracted as in Cutler and Cramb, 2008), using Superscript III reverse transcriptase (Invitrogen) according to manufacturers instructions, with the exception that 65°C was used for RNA denaturation, as 1 μl of SUPERase•In thermostable RNase inhibitor was additionally added (Ambion). cDNA fragments were gel purified using a Gene clean kit (MP Biomedicals) and were cloned using a TOPO TA cloning kit for sequencing (Invitrogen). Sequencing reactions were performed by CUGI at Clemson University, S.C. 5′ and 3′ RACE primers were designed from the sequences obtained and RACE was performed using a Marathon RACE cDNA amplification kit (Clontech). Sequences were analyzed and aligned using Genejockey II software (Biosoft).

OSMOTIC MANIPULATION EXPERIMENTS

In order to determine whether AQP4 was directly involved in any kind of osmoregulatory or body fluid volume regulation processes in the shark, an attempt was made to disturb the shark regulatory system to try to cause changes in the expression of effector protein components. In other words, the external environmental salinity of the fish was manipulated to try to modulate the expression of aquaporin (and others) genes, such as AQP4. At the Mount Desert Island Biological Laboratory (MDIBL) at Salisbury Cove, in Maine, small (800–1500 g) adult mixed sex dogfish were (otherwise) randomly selected from a stock tank and were unfed during the experiment. All experimental animal protocols used were in compliance with IACUC regulations and had both GSU and MDIBL IACUC approval. The fish were placed in pairs in four-foot experimental tanks (approximately 1000 l). The acclimation of dogfish to dilute seawater (SW) in stages over 7 days was a protocol modified from Panabecker and Danzler (2005). A similar protocol to acclimate fish to 120% SW was then also devised. Two groups of sharks (six fish per group) were held in SW adjusted to either 75% salinity or 120% salinity in stages. The stages were 85% 3 days, 80% 2 days, and 75% 2 days or 110% 3 days, 115% 2 days, 120% 2 days. The acclimation of dogfish to dilute SW in stages over 7 days was a modified protocol from Panabecker and Danzler, 2005. A matching protocol to acclimate fish to 120% SW in stages was also then devised. Differing salinities were produced by the addition of de-chlorinated tap water, or sea salt (Instant Ocean) using a re-circulating system including a cooler to maintain temperature and biofilters. Control animals were kept in 100% salinity (normal) SW (around 31–32 ppt at MDIBL) for the same time period. Salinity was controlled using a model 85 dissolved oxygen, conductivity, salinity, and temperature meter (YSI). At the end of the 7 day experiment, fish were sacrificed and gill arches, kidney, rectal gland, esophagus (including the fundic stomach), and intestine were removed. Kidney and rectal gland were homogenized using a Polytron homogenizer (Kinematica). Gill epithelia were removed by scraping with a razor blade. Combined esophageal/cardiac stomach epithelia were removed by scraping with a microscope slide. The intestines were cleaned as much as possible and waste material removed. The intestinal epithelium was then also scraped using a microscope slide. Gill, esophageal/cardiac stomach, and intestinal epithelia were homogenized using a syringe and 16 gage needle. All tissues were homogenized in solution D for RNA extraction as previously outlined (Cutler and Cramb, 2008). Levels of AQP4 mRNA were then measured using Northern blots, performed using a ^{32}P labeled AQP4 DNA probe (purified degenerate PCR fragment). Blots were quantified as previously, using electronic autoradiography via an Instant Imager (Canberra Packard). As the level of AQP4 expression in gill was too low to be detectable by this technique, quantitative PCR (QPCR) was instead employed using total RNA samples normalized utilizing rRNA levels measured with a gel documentation and analysis system (Syngene). This technique was used instead of the use of housekeeping genes as (1) few housekeeping are available for *S. acanthias* (2) In any case supposed housekeeping genes such as GAPDH (and others) can vary in different circumstances (De Jonge et al., 2007; McCurley and Callard, 2008) and (3) testing of housekeeping genes in any case requires comparison to rRNA levels. QPCR was performed using a MX 4000 QPCR machine and Brilliant II QPCR master mix (Stratagene). Statistical analysis of quantitative results was performed using Statview statistical analysis software (Abacus Concepts) using ANOVA and a Fisher *post hoc* test.

OOCYTE EXPRESSION STUDIES

Production of aquaporin cRNA for micro-injection

A full-length cDNA encompassing the entire AQP4 coding region was amplified from rectal gland cDNA using PCR employing highly accurate Phusion DNA polymerase (Finnzymes), the resulting DNA band was gel purified and cloned into a pXT7 expression vector, which contains a T7 promoter upstream of the multiple cloning site. Plasmid DNA for dogfish AQP4 was cut with restriction enzyme *Xba*I to linearize it and prevent run on of transcription. cRNA transcripts were produced using a mMessage mMachine kit (Ambion) utilizing T7 DNA-dependent RNA Polymerase. The cRNA produced was purified using phenol/chloroform extraction followed by isopropanol precipitation, dissolved in RNAse free water and quantified using a Biophotometer spectrophotometer (Eppendorf).

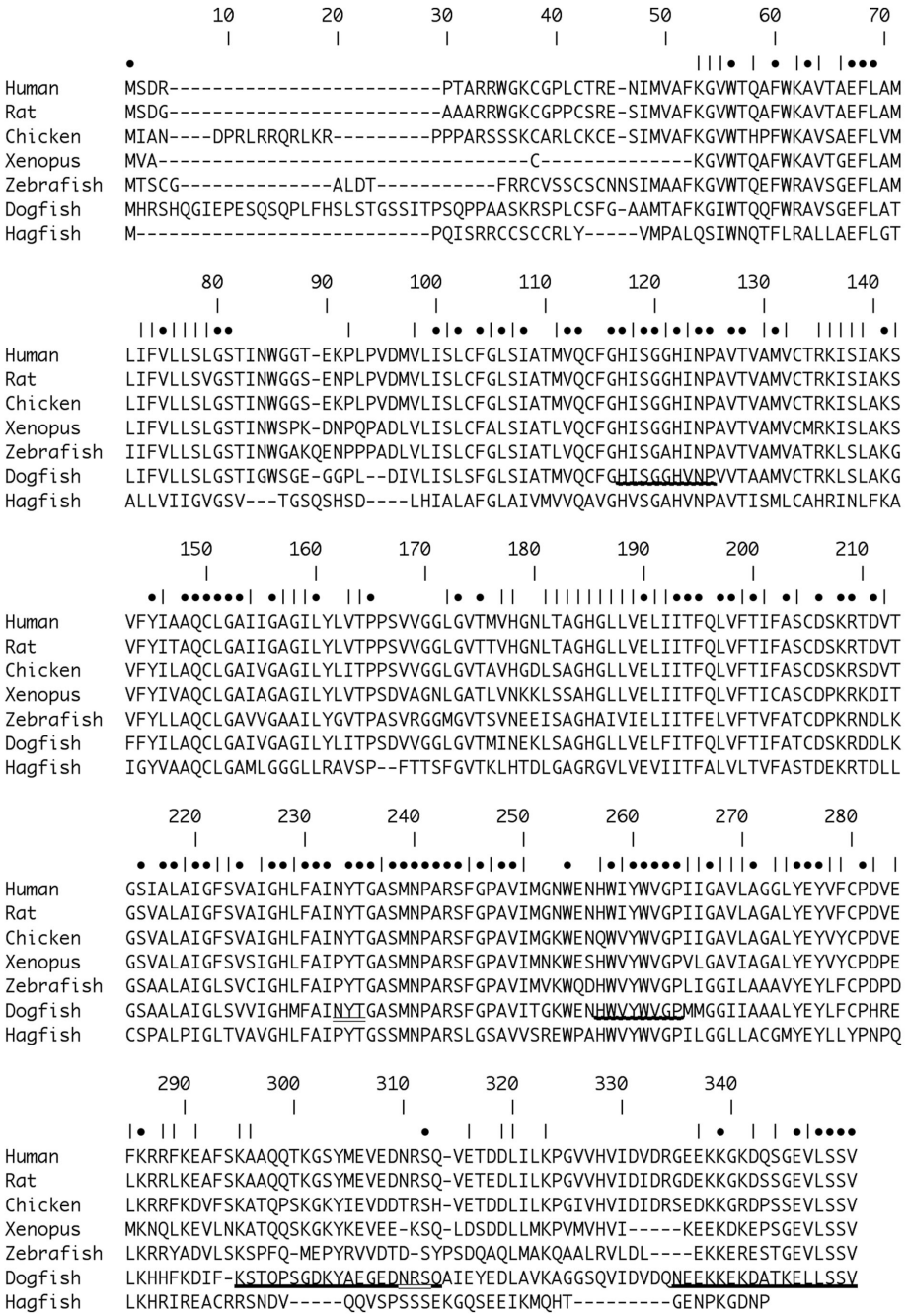

FIGURE 1 | Alignment of Dogfish AQP4 [Accession number (Ac. No.) JF944824] amino acid sequence with AQP4 sequences from Human (*Homo sapiens*; Ac. No. NM_001650.4; 63.0%), Rat (*Rattus norvegicus*; Ac. No. AF144082; 62.5%), Chicken (*Gallus gallus*; Ac. No. NM_001004765; 65.3%), African Clawed Toad (*Xenopus laevis*; Ac. No. NM_001130949.1; 56.7%), Zebrafish (*Danio rerio*; NM_001003749; 57.5%), and Hagfish (*Eptatretus burgeri*; Ac. No. AB258403.1; 38.2%). Percentages in parentheses represent amino acid homologies. Numbers indicate position within the alignment. • Symbols indicate positions with identical amino acids. | Symbols indicate positions with chemically similar amino acids. Bold underline _ indicates the position of the peptide sequences used to raise the polyclonal antibodies. Wavy underline indicates the position of amino acids sequences used to make degenerate primers for initial AQP4 OCR amplifications. Double underline indicates the positions of putative *N*-glycosylation sites with the dogfish AQP4 sequence.

Preparation of Xenopus laevis oocytes

All experiments were done in accordance with IACUC approved protocols at Beth Israel Deaconess Medical Center. *Xenopus laevis* frogs (Harvard Institute of Medicine, Boston, MA, USA) were anesthetized in 1 l 0.5% (w/v) 3-aminobenzoic acid ethyl ester methanesulfonate salt (Tricaine) containing ice for 20 min.

Oocytes were removed bilaterally from the abdominal cavity and the egg mass cut into small pieces and placed in calcium free ND96 buffer (in mM; 96 NaCl, 1 KCl, 1 $MgCl_2$ 5 Hepes, pH 7.5). Oocytes were then defolliculated in 2 mg/ml collagenase (Sigma-Aldrich), 0.2 mg/ml trypsin inhibitor (Sigma-Aldrich) in calcium free ND96 for 55 min with rotation on an Adams Nutator before washing three times with phosphate buffer [in mM; 100 K_2HPO_4, 0.1% (w/v) BSA, pH 6.5] and then allowing oocytes to incubate in phosphate buffer for 10 min at room temperature. Oocytes were transferred to calcium free ND96 and then to modified Barth's solution [MBS; in mM; 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, 10 Hepes, pH 7.4, supplemented with 1% v/v penicillin/streptomycin] where they were maintained at 18°C. cRNA (10 ng) of saAQP4s was injected into oocytes using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Co.). Control oocytes were either injected with water alone or were uninjected. After 3 days incubation at 18°C, oocytes were tested for their ability to transport water, urea, or glycerol. Water transport kinetics were assessed at room temperature by quantitation of oocyte swelling after placement in hypotonic buffer (65% of normal MBS). Time lapse video microscopy was used to capture oocyte images every 10 s for 3 min. using an Olympus SZX7 binocular microscope equipped with a Scion CFW 1308C digital camera (1360 × 1024 pixel resolution).

Aquaporin activity was tested over a pH range of 6.6–8.6. For all experiments other than pH 7.4 oocytes were placed in MBS at the tested pH for 5 min and then were swelled in hypotonic MBS (65%) at the same pH. Studies investigating the inhibition of water permeability by mercury were performed at pH 7.4 in MBS containing 1 mM $HgCl_2$.

Calculation of permeability coefficients

The images were converted to black and white in ImageJ (Rasband, 1997–2011) using the Binary function and the cross-sectional pixel area was calculated with the Analyze Particle function. Data from ImageJ was exported to Microsoft Excel and areas from each image were normalized to 1.0 relative to a starting value. To calculate the permeability coefficient (P_f) the data were adjusted from area (A) to volume (V) by $(A/Ao) = (V/Vo)^{3/2}$ (Zhang et al., 1990) then a second order polynomial equation was fit and the derivative of the equation was used to obtain the initial rate of swelling. P_f was calculated using the equation (Zhang et al., 1990):

$$P_f = d(V/Vo)/dt \times (1/S) \times (1/Vw) \times (1/\triangleq C)$$

Where $d(V/Vo)/dt$ is the rate from the curve fit, Vo is the initial volume of the oocyte, calculated as 5.2×10^{-4} cm^3 based on a 1 mm diameter, S is the surface area of the oocyte (0.4 cm^2 Zampighi et al., 1995), Vw is the molar volume of water (18 mol/cm^3), and ΔC is the concentration difference of the applied hypo-osmotic solution in mol/cm^3.

Urea and Glycerol uptake

Solute fluxes were measured by isotopic uptake of [^{3}H]glycerol and [^{14}C]urea (American Radiolabeled Chemicals, St. Louis, MO, USA). Oocytes were incubated for varying times in MBS to which either 10 μCi/ml [^{14}C]urea (55 mCi/mmol) and, or 10 μCi/ml [^{3}H]glycerol (20–40 Ci/mmol), and then made to a final concentration of 2 mM with unlabelled urea or glycerol respectively. At the end of the incubation period for uptake, oocytes were washed six times with ice-cold buffer containing 5 mM solute. Individual oocytes were then placed in scintillation vials, had 300 μl 20% SDS added and vortex mixed for 10–15 s before addition of 4 ml Scintisafe scintillation cocktail. Vials were counted for 2 min in a Packard 1500 liquid scintillation analyzer.

RESULTS

A translation of the putative dogfish AQP4 cDNA sequence (Accession number JF944824) resulting from degenerate PCR and 5′ and 3′ RACE DNA fragments is shown in **Figure 1**. The combined cDNA produced, aligns with AQP4 sequences from other species. The homology of the dogfish amino acid sequence shares homology between 38.2 and 66.3%, ranging from hagfish (lowest) to chicken (highest; see **Figure 1**.) Interestingly, using the same alignment the homology of hagfish to human AQP4 was only 43.3%. The dogfish AQP4 sequence also shows two putative *N*-glycosylation sites at positions 233–235 and 310–312 of the amino acid alignment (**Figure 1**.) that are common to both human and rat AQP4, but that are absent in some other species.

The first priority following cloning experiments was to establish an expression profile for AQP4 in dogfish. This was performed in two different ways, initially using Northern blotting where a mRNA band of around 3.2 kb was detected, particularly in the rectal gland, but also with lower levels in the eye, esophagus/fundic stomach, skeletal muscle, stomach, brain, and kidney with no apparent signal from liver, gill, or intestine (**Figure 2**). Secondly this was followed up by a semi-quantitative PCR survey, to increase the sensitivity of detection (**Figure 3**). Similar results were obtained but bands were also detected with the following intensity, liver > gill > intestine. Although a band could be detected in intestine the level of expression was clearly extremely low.

In conjunction with the Northern blotting and semi-quantitative PCR, the level of mRNA expression measured in (100%) SW fish (**Figure 4**) showed higher levels of AQP4 mRNA expression in rectal gland, with lower levels in kidney and

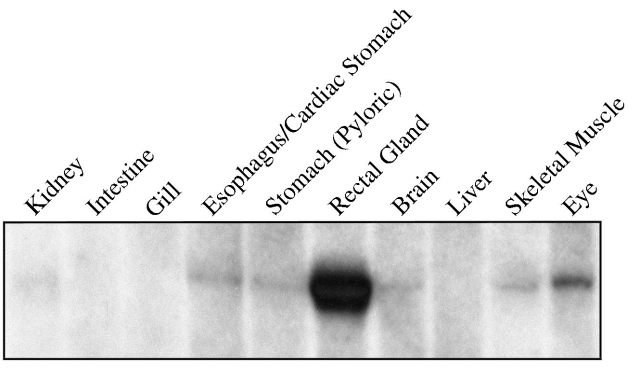

FIGURE 2 | Northern blot of 5 μg of total RNA extracted from various tissues of the dogfish (*S. acanthias*) and probed with a ^{32}P radioisotope-labeled cDNA fragment of the AQP4 gene. The probe hybridizes to a single band of 3.2 kbp.

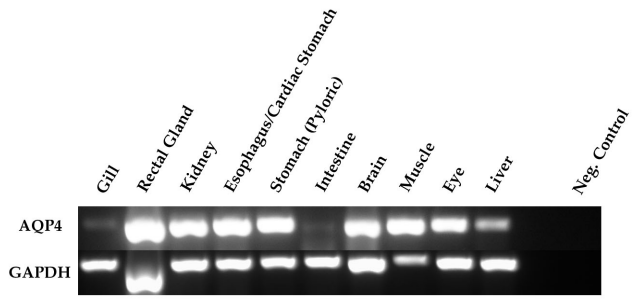

FIGURE 3 | Semi-quantitative PCR amplification of dogfish AQP4 (274 bp) and GAPDH (271 bp) cDNA fragments. The primers used were also used for quantitative PCR and were designed across conserved intron–exon splice junctions of the respective genes to avoid genomic DNA amplification. 0.5 μl of cDNA template (made with the following microgram's of total RNA; 1.15 gill; 0.67 rectal gland; 0.93 kidney; 0.88 esophagus/cardiac stomach; 0.99 stomach; 1.56 intestine; 0.41 brain; 0.21 muscle; 0.28 eye; 0.71 liver) was used in all reactions and GAPDH was used as a positive control. The negative (Neg.) control reactions were without cDNA.

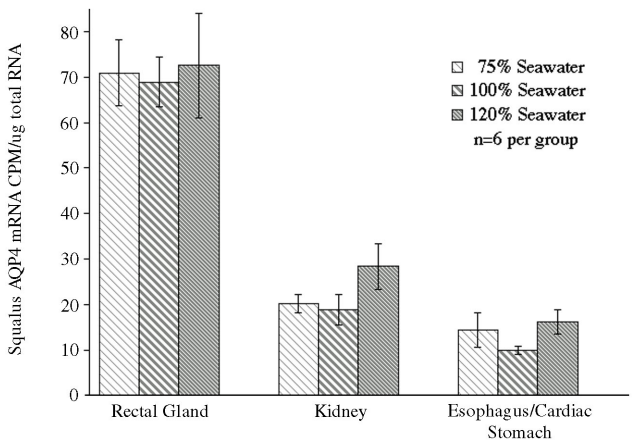

FIGURE 4 | Analysis of a Northern blot experiment measuring the mRNA expression of aquaporin 4 (AQP4) in the tissue of dogfish acclimated to different external environmental salinities. No significant differences were seen in any tissue between any of the salinities.

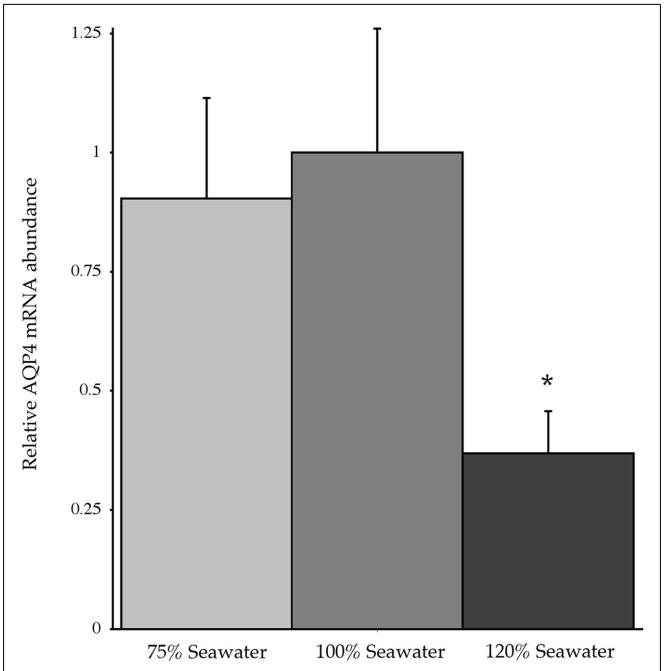

FIGURE 5 | Relative dogfish AQP4 mRNA abundance in the gills of fish acclimated to 75, 100, or 120% seawater (SW). mRNA expression was determined using quantitative PCR, with primers designed at conserved exon–intron splice junctions within the gene sequence to avoid amplification of genomic DNA. $n = 6$ Fish per group. * = Statistically significant difference between 75 and 100% SW fish, where $p < 0.05$.

esophagus/cardiac stomach. Expression in gill and intestine could not be detected using this technique. The effect of adjusting the external environmental salinity of the fish made no significant difference to the level of AQP4 mRNA expression in rectal gland, kidney, or esophagus/cardiac stomach. In order to carry out a similar type of analysis on the gill (more sensitive) QPCR was employed to determine mRNA expression levels in the 75, 100, and 120% SW fish. In this case, the fish held in 120% SW showed a significant 63.1% lower level of AQP4 mRNA in comparison to the 100% SW control dogfish (**Figure 5**).

Experiments measuring the function of AQP4 expressed in *Xenopus laevis* oocytes, showed that water permeability (P_f) was significantly higher in oocytes expressing AQP4 than in controls (**Figure 6**). The level of oocyte permeability also did not vary across a range of pH values. The effect of mercury on permeability was also measured (**Figure 7**), and this did cause a small reduction in permeability but this was not statistically significant. Additionally, as some AQP isoforms also show a urea and glycerol permeability, the uptake of isotopic versions of these molecules was tested and no significantly higher level of permeability was found in AQP4 expressing-oocytes compared to controls (**Figures 8** and **9**).

DISCUSSION

This article documents the characteristics of an elasmobranch aquaporin for the first time. Despite the relatively ancient origin of elasmobranchs, in common with Agnathans such as the hagfish (elasmobranchs, around 528 million years old and Agnathans, around 564 million years old; Kumar and Hedges, 1998), dogfish AQP4 shares a much higher level of amino acid homology with mammalian AQP4 sequences than does the hagfish AQP4 sequence. As has been seen with other elasmobranch gene sequences, dogfish AQP4 has a higher level of homology (63%) to human AQP4 than the somewhat more recently developed teleost fish (450 million years old; Kumar and Hedges, 1998) AQP4 sequence from zebrafish (60.4%). This therefore may represent another example of an apparently slower rate of evolutionary change in cartilaginous fish in comparison to teleost fish.

Due to the relatively high level of amino acid homology of AQP4 sequences, it might be expected that dogfish AQP4 might be functionally similar to homologs in other species such as mammals. The oocyte functional expression studies here show that

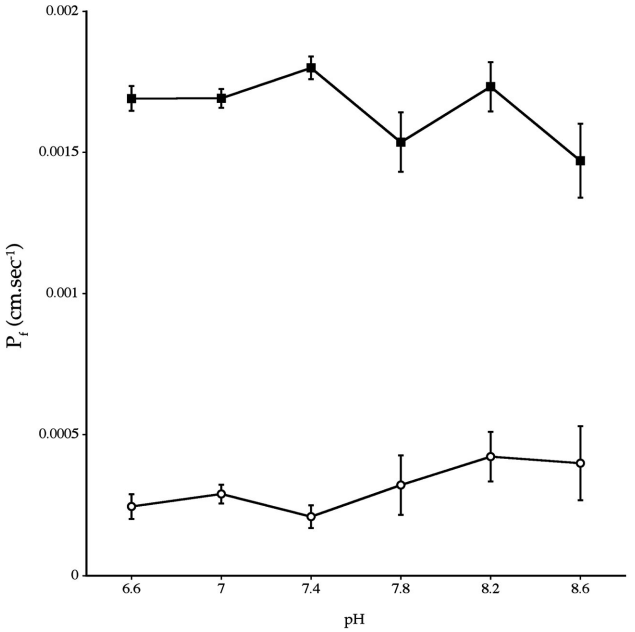

FIGURE 6 | Osmotic water permeability (P_f) of *Xenopus laevis* oocytes micro-injected with dogfish AQP4 cRNA (-■-) or with H2O (-o-). The data are averages of four experiments where measurements were made at six different pH values with an average of eight oocytes per group, at each pH value, in each experiment.

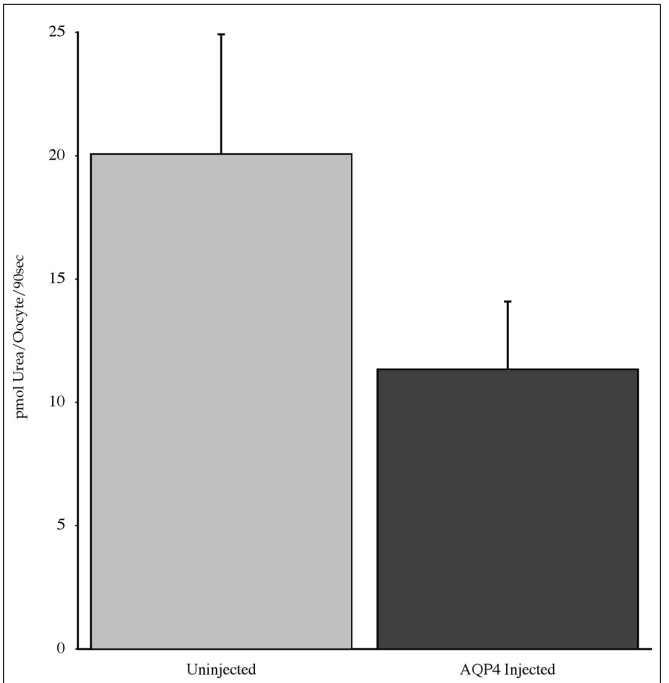

FIGURE 8 | The uptake of C-labeled urea in *Xenopus laevis* oocytes micro-injected with dogfish AQP4 cRNA or uninjected. Results are averages from three experiments with approximately eight oocytes used per group.

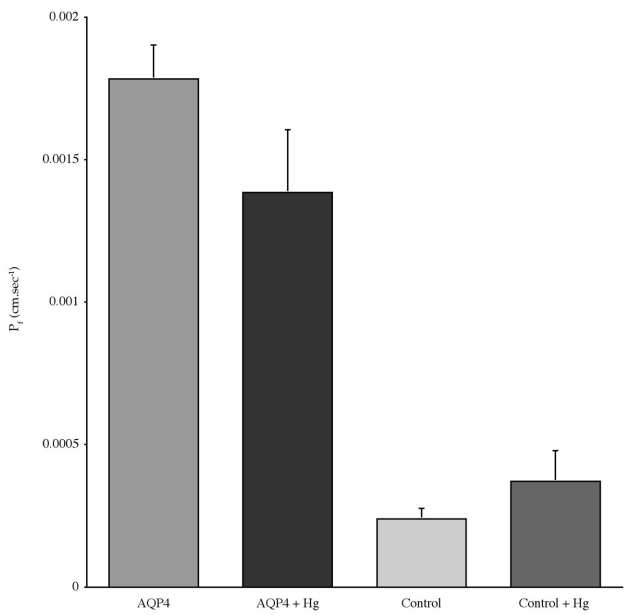

FIGURE 7 | The effect of mercury chloride (Hg) on the osmotic water permeability (P_f) of *Xenopus oocytes* micro-injected with dogfish AQP4 cRNA or in with H2O (Control). Data represent averages of three experiments, with approximately eight oocytes per group in each. There was no statistically significant difference in P_f with or without mercury chloride.

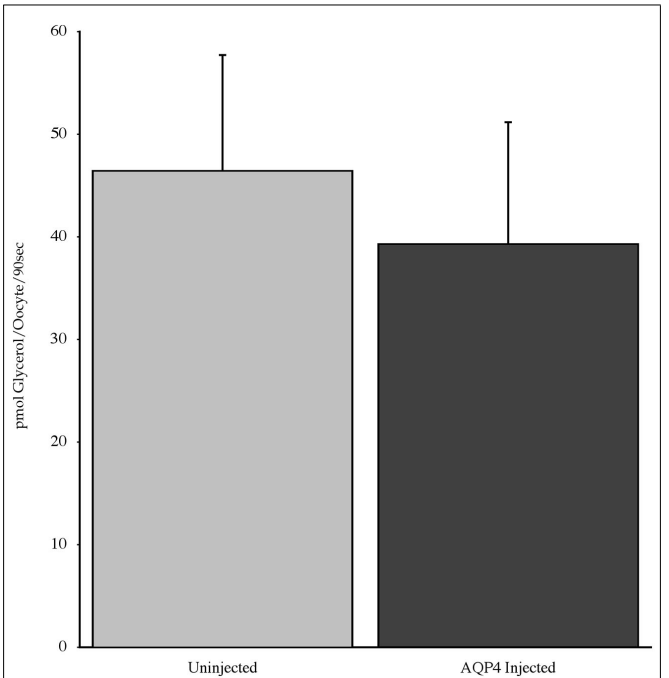

FIGURE 9 | The uptake of C-labeled glycerol in *Xenopus laevis* oocytes micro-injected with dogfish AQP4 cRNA or uninjected. Results are averages from three experiments with approximately eight oocytes used per group.

that was indeed the case in as much as, dogfish AQP4 expression produced significant oocyte water permeability irrespective of pH (other AQP isoforms can be affected by pH; Zelenina et al., 2003; MacIver et al., 2009), and it is permeability was insensitive to mercury (Hasegawa et al., 1994). The cysteine residue which is often present in most AQPs and is thought to be responsible for mercury-sensitive inhibition of AQPs is located at position 237 in the amino acid alignment (**Figure 1**; Yukutake et al., 2008). In AQP4 sequences from other species such as mammals, this residue is instead an alanine, hence explaining the mercury insensitivity of mammalian AQP4. Dogfish AQP4 also possesses an alanine residue at this position and this would therefore indicate that it is also likely to be insensitive to mercury. The Oocyte expression experiments also showed that dogfish AQP4 has no capacity to transport urea or glycerol in a similar way to mammalian AQP4 (Ishibashi et al., 2009).

In contrast to the tissue distribution of AQP4 mRNA expression in mammals, where there is significant expression in the GI tract but no expression in liver, in the dogfish there appears to be little (although still some) expression in intestine but definite expression seen in liver (Ishibashi et al., 2009). Dogfish AQP4 expression was seen at some level in every tissue tested and its expression is consequently more widespread than dogfish AQP1 (data not shown). Whereas, in mammals AQP1 is viewed as the ubiquitous aquaporin isoform (Zelanina et al., 2005; Ishibashi et al., 2009). There have been some suggestions that the intestine may play a role in osmoregulatory and/or body fluid homeostatic mechanisms in elasmobranch fish (Anderson et al. (2007)), but the low level of intestinal AQP4 mRNA expression in this study clearly suggests that this is unlikely to involve AQP4 at least in the dogfish.

Another aspect of interest is the presence of AQP4 expression in the rectal gland. Shark rectal gland has been a model for ion/fluid transport studies for decades, as the tissue is dedicated to regulated iso-osmotic fluid secretion. As many studies have concentrated on sodium and chloride ion transport over the years, it might be expected that the rectal gland function principally concerned the eradication of excess ions (Karnaky, 1997; Evans et al., 2004; Evans and Claiborne, 2008). However a series of nice studies by Solomon et al. (1984a,b, 1985) showed that ion secretion by the rectal gland was not stimulated when the animal was perfused with hypertonic shark ringer solution (they raised plasma salinity without changing body fluid volume), but was stimulated when body fluid volume was increased using isotonic shark ringer. This strongly suggests that the principle function of the rectal gland is actually to eradicate excess water but due to the passive nature of water transport, ions have to be transported to allow the water to follow by osmosis. Initial studies investigating osmotic water permeability of rectal gland cell membranes found little functional evidence to support the presence of AQPs (Zeidel et al., 2005).

However the fluid transport function of the rectal gland would suggest that this organ would likely contain AQPs. The Northern blot and PCR data in this study suggest that the rectal gland is likely to possess abundant aquaporin proteins but clearly further work needs to be done to determine what the role of AQP4 in the rectal gland is. A curious aspect of AQP4 rectal gland expression is that manipulation of the fish's environmental salinity did not significantly alter AQP4 mRNA expression. It would be anticipated that placing the fish in a hyper-osmotic environment such as 120% SW would result in loss of water across the gills by osmosis and compel the fish's regulatory system to retain both sodium chloride and urea to raise the internal osmotic concentration to match that of the external environment and to shut down rectal gland output. Placing the fish in a hypo-osmotic environment such as 75% SW, should have the opposite regulatory effect. Work in other Elasmobranch species shows that placing the fish in a dilute environment increases body fluid volume and consequently body mass (Anderson et al., 2007) as would be expected and this increases rectal gland secretion (Wong and Chan, 1977; Anderson et al. (2002)). The lack of change in AQP4 mRNA levels in either 75 or 120% SW suggests that either AQP4 is not involved directly in rectal gland fluid transport or that regulation occurs at another level (such as shutting down blood flow to the gland) eliminating any need to change AQP4 expression. The relatively high level of mRNA expression in the rectal gland might also indicate post-translational regulation of AQP4 protein production as seen with AQPs in other species (Ishibashi et al., 2009).

The change in AQP4 mRNA expression that was seen in the gill is also interesting. Its possible to hypothesize that in a hyper-osmotic environment such as 120% SW, there would likely tend to be a net outflow of water across the gills and if this produced osmotic problems (dehydration) for the fish, one possible response by the fish's regulatory system would be to decrease branchial AQP4 expression to reduce gill permeability to slow down water loss. Dogfish tend to hold their body fluid osmotic concentration slightly above that of the external environment (Karnaky, 1997; Evans et al., 2004; Evans and Claiborne, 2008), which normally allows a small continual water influx across the gill, which presumably matches outflows through urine production and rectal/rectal gland fluid output. Again its possible to hypothesize that if gill osmotic water inflows were reversed this may ultimately cause problems for urine production, some minimal level of which is necessary to remove toxic metabolites, and this may explain the need to down-regulate branchial AQP4 mRNA expression, to reduce gill permeability. However, one complicating factor for that idea is that some elasmobranch species are known to drink in response to dehydration and this would reduce the need to adjust gill permeability (Anderson et al. (2007)). The other result from gill is also curious because a hypo-osmotic external environment such as 75% SW might have been expected to have the opposite effect of 120% SW (i.e., increased AQP4 mRNA expression in gill). However in that case, again it is possible to hypothesize that if there are additional water inflows across the gill, the fish can merely increase urine output and decrease urine concentration to mitigate the increased branchial influx. While it is possible to speculate as to the possible reasons for the level of branchial AQP4 mRNA expression in different environments, its clear further studies needs to be performed to elucidate the actual regulatory mechanisms at work.

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Characterization of aquaporin 4 protein expression and localization in tissues of the dogfish (*Squalus acanthias*)

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The role of aquaporin water channels such as aquaporin 4 (Aqp4) in elasmobranchs such as the dogfish *Squalus acanthias* is completely unknown. This investigation set out to determine the expression and cellular and sub-cellular localization of Aqp4 protein in dogfish tissues. Two polyclonal antibodies were generated (AQP4/1 and AQP4/2) and these showed somewhat different characteristics in Western blotting and immunohistochemistry. Western blots using the AQP4/1 antibody showed two bands (35.5 and 49.5 kDa) in most tissues in a similar fashion to mammals. Liver had an additional band of 57 kDa and rectal gland two further faint bands of 37.5 and 38.5 kDa. However, unlike in mammals, Aqp4 protein was ubiquitously expressed in all tissues including gill and liver. The AQP4/2 antibody appeared much less specific in Western blots. Both antibodies were used in immunohistochemistry and showed similar cellular localizations, although the AQP4/2 antibody had a more restricted sub-cellular distribution compared to AQP4/1 and therefore appeared to be more specific for Aqp4. In kidney a sub-set of tubules were stained which may represent intermediate tubule segments (In-III–In-VI). AQP4/1 and AQP4/2 antibodies localized to the same tubules segments in serial sections although the intensity and sub-cellular distribution were different. AQP4/2 showed a basal or basolateral membrane distribution whereas AQP4/1 was often distributed throughout the whole cell including the nuclear region. In rectal gland and cardiac stomach Aqp4 was localized to secretory tubules but again AQP/1 and AQP/2 exhibited different sub-cellular distributions. In gill, both antibodies stained large cells in the primary filament and secondary lamellae. Again AQP4/1 antibody stained most or all the cell including the nucleus, whereas AQP4/2 had a plasma membrane or plasma membrane and cytoplasmic distribution. Two types of large mitochondrial rich transport cells are known to exist in elasmobranchs, that express either Na, K-ATPase, or V-type ATPase ion transporters. Using Na, K-ATPase, and V-type ATPase antibodies, Aqp4 was colocalized with these proteins using the AQP4/1 antibody. Results show Aqp4 is expressed in both (and all) branchial Na, K-ATPase, and V-type ATPase expressing cells.

Keywords: aquaporin 4, rectal gland, gill, stomach, kidney, dogfish

INTRODUCTION

Aquaporin 4 (*AQP4*) is a member of the water-selective sub-group of aquaporin water channel cell-membrane proteins found in all organisms so far investigated. This sub-group in mammals also includes other aquaporins such as *AQP0*, *AQP1*, *AQP2*, *AQP5*, and *AQP6* (Ishibashi et al., 2009; Zelanina, 2010). Other subgroups such as aquaglyceroporins (*AQP3*, *AQP7*, *AQP9*, and *AQP10*) have additional transport properties (e.g., transport of urea and glycerol, etc.). In mammals, AQP4 is widely expressed in a variety of tissues including the brain (Zelanina, 2010), retina (Hirrlinger et al., 2011), salivary gland (Delporte and Steinfeld, 2006), trachea (Borok and Verkman, 2002), heart and muscle (Butler et al., 2006; Wakayama, 2010), gastrointestinal tract (Ma and Verkman, 1999; Xu et al., 2009), and kidney (Nejsum, 2005), but is not expressed in the lung itself or liver tissues (Ishibashi et al., 2009). While aquaporins such as *AQP4* have been studied in a wide variety of (mostly) higher vertebrates, no complete studies have yet been published on the role of aquaporins in elasmobranch fish such as the dogfish shark (Cutler et al., 2005; Cutler, 2006, 2007). A companion paper (Cutler et al., 2012) has characterized *aqp4* mRNA expression in dogfish tissues, this article gives the first information on the Aqp4 protein and its localization in tissues. In mammals such as the rat, AQP4 appears as two bands on Western blots, a non-glycosylated form of 30–32 kDa (sometimes splicoforms with different N-terminal ends exist) and a putative 50 kDa glycosylated form (Terris et al., 1995; Nicchia et al., 2008). Immunohistochemical staining of mammalian epithelial cells generally yields a basolateral localization for AQ4 proteins, however, AQP4 staining both within the cell and in the apical membrane is thought to occur in some cells (Terris et al., 1995; Nejsum, 2005; Mobasheri et al., 2011). As indicated in the companion paper (Cutler et al., 2012), dogfish *aqp4* mRNA expression in tissues is largely ubiquitous. The questions this article sets out to address are, whether Aqp4 protein expression is similarly ubiquitous and if so, where

within the tissues that are important for the control of water balance in sharks (gills, kidney, rectal gland, etc.), is the Aqp4 protein located?

MATERIALS AND METHODS

FISH

All animal experiments were performed in accordance with IACUC regulations and had IACUC approval from both MDIBL and Georgia Southern University. Animals for experiments were held in a stock tank with running seawater at ambient temperature and were sacrificed by decapitation followed by immediate pithing of the spinal cord. Various tissues were then removed from the animal by dissection for further processing in Western blotting and immunohistochemistry experiments.

POLYCLONAL ANTIBODY PRODUCTION

Custom-made polyclonal antibodies were produced commercially against peptides whose amino acid sequence was derived from the dogfish *aqp4* nucleotide sequence. The first of these AQP4/1 (produced by ProSci, San Diego, CA, USA) was located at the C-terminal end of the protein (at positions 329–346 of the amino acid sequence) and had the sequence NH_2–CGGNEEKKEKDATKELLSSV–COOH. As part of that sequence, the two glycine amino acids were added as spacers at the N-terminal end. The second antibody AQP4/2 (produced by Genscript, Piscataway, NJ, USA), was produced much more recently and was located a little further in from the C-terminal end than the first peptide (at positions 290–308 of the amino acid sequence), and had the sequence NH_2–CKSTQPSGDKYAEGEDNRSQ–COOH. Peptides for both antibodies had an N-terminal cysteine amino acid added for coupling to the protein carrier. The Aqp4 peptides were coupled to keyhole limpet hemocyanin (KLH) prior to injections of the antigens into different pairs of rabbits. The resulting anti-sera were affinity purified using the same peptide (that was used for immunization), attached to a purification column.

TISSUE CELL-MEMBRANE PREPARATION

Dogfish tissues for Western blotting experiments were kept briefly on ice and then homogenized in Tris (25 mM), sucrose (0.25 M) buffer, also containing 78 mg/ml dithiotheritol (DTT), and either protease inhibitor cocktail I (Research Product International, Mount Prospect, IL, USA) or Halt protease inhibitor cocktail (Pierce), was used according to manufacturers instructions. Hard tissues such as muscle, kidney, liver, and rectal gland, etc., were homogenized using a polytron homogenizer (Kinematica, Luzern Switzerland). Soft tissues such as brain or scraped epithelia were homogenized using a syringe and 16 gage needle. Epithelia were scraped from gill arches using a single sided razor blade and from intestine and esophagus/cardiac stomach using a glass microscope slide.

Homogenized samples were then sieved through several layers of cotton gauze. The filtrate was then centrifuged in a SS-34 (Sorvall, Asheville, NC, USA) rotor at approximately 50,000 *g* max for 1 h at 4°C. The resulting crude membrane pellet was then resuspended in the same buffer as previously used and measured for protein content using a Bradford's protein assay (Boston Bioproducts, Ashland, MA, USA). Crude membrane homogenates were stored frozen at −20 or −80°C prior to use in Western blotting experiments.

WESTERN BLOTTING

Crude protein homogenates (300 μg protein/lane) were separated based on their size, on 10% Laemmli SDS-polyacrylamide gels (Laemmli, 1970) using a Protean II gel apparatus (Biorad). The gel was then transferred to a methanol-activated high protein capacity sequi-blot PVDF filter (Biorad) using a trans-blot cell electroblotter (Biorad, Taunton, MA, USA), at 30 V overnight. The resulting filters were then cut into strips for each experiment. Filter strips were incubated in TNT buffer [10 mM tris (pH 8.0), 150 mM NaCl, 0.05% Tween 20], containing 5% Blotto (fat-free dry milk powder) for 30 min room temperature. They were then washed four times in TNT buffer and primary antibody added (in TNT buffer) at 1 in 400 dilution (or 1 in 4000 dilution for peptide-blocking experiments; used because there can be a problem blocking high antibody concentrations sometimes due to the limits of peptide antigen solubility. Lower antibody concentrations allows the same result to be obtained with less peptide for 1 h at room temperature. The filters were then washed four times in TNT buffer and incubated in 1 in 4000 dilution of alkaline phosphatase enzyme cross linked – highly cross-absorbed – donkey anti-rabbit IgG secondary antibody for 1 h at room temperature. Filters were washed again twice in TNT buffer and twice in 10 mM tris (pH 8.0), 150 mM NaCl, and finally incubated in NBT/BCIP (nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indoly phosphate *p*-toluidine salt) alkaline phosphatase enzyme substrate containing 1 mM levamisole endogenous alkaline phosphatase inhibitor. The presence of the bound secondary antibody/alkaline phosphatase enzyme yielding a purple/blue colored product.

IMMUNOHISTOCHEMISTRY

Dissected tissues were fixed in filtered standard phosphate buffered saline (PBS; Oxoid, Lenexa, KS, USA) containing 4% paraformaldehyde, for 1 h at room temperature. Tissues were then cut into segments to fit in standard histological cassettes. The cassettes were rinsed twice in PBS and then dehydrated through a series of alcohols (50, 70, 85, 95, and 100% ethanol), 1 h in each. Subsequently, cassettes were placed twice in histochoice clearing medium (Amresco, Solon, OH, USA) and then into molten paraffin wax three times (Paraplast) held between 56 and 58°C in an oven, 1 h each wax solution. The tissue pieces were then placed in stainless steel molds, which were filled with molten wax and were finally mounted with the back of the cassette placed on top. Once the wax had cooled and set, the molds were removed, revealing the wax-embedded tissue blocks for section cutting. Five micron thick sections were cut using a microtome (Leica, Buffalo Grove, IL, USA), these were placed on the surface of a warm waterbath (37°C) and floated onto glass microscope slides (Superfrost plus). The slides were heated at 37°C for 1 h to adhere tissue sections to the positively charged surface of the slide. Slides for experiments were then taken back through two incubations (5 min each) in histochoice clearing agent to remove the wax and through a descending series of alcohol concentrations (5 min each; 100, 95, 85, 70, 50 ethanol) to re-hydrate the tissue. Finally slides were incubated in PBS.

Slides were then placed horizontally in a slide box that had moist tissue in the bottom for humidification. The tissue on the slides was ringed using a hydrophobic barrier pen (to retain subsequent solutions on the tissue) and a solution of PBS with sodium chloride (17.5 g/l) and 0.02% Tween 20 detergent was added for 10 min to permeabilize the tissue. The slides were washed twice with PBS and then incubated for 5 min in PBS containing (2.68 g/l) ammonium chloride, to block any free aldehyde groups of the fixative. The slides were washed again twice in PBS and then incubated in Image-iT FX blocking solution (Invitrogen, Grand Island, NE, USA) for 30 min. The slides were washed again twice in PBS and then incubated in a second blocking solution of PBS containing (10 g/l) bovine serum albumin (BSA; Promega, Madison, WI, USA) and (1 g/l) gelatin (Boston Bioproducts) for 10 min. The slides were washed again twice in PBS and then incubated in 1 in 100 dilution of primary antibody in PBS for 1 h, room temperature. The slides were then washed four times in PBS and then incubated in 1 in 1000 dilution of secondary antibody (Alexa 488-, Dylight 549-, or Alexa 555-labeled, highly cross-absorbed-anti-rabbit) in PBS for 1 h, room temperature. From this point onward slide boxes were kept closed in a draw between manipulations, to reduce light exposure. The slides were then washed four times in PBS and then were mounted in Prolong Gold mounting medium containing the nuclear counterstain, DAPI (Invitrogen). The slides were then covered with a coverslip ready for microscope viewing.

Four-color immunohistochemical co-localization studies, were carried out as above with the following modifications. A rabbit anti-sculpin V-type ATPase antibody (a gift from Dr. J. B. Claiborne) was used initially and detected using a highly cross-absorbed Alexa 488 (green) fluorescently labeled anti-rabbit secondary antibody. The secondary antibody was then blocked using normal rabbit serum (sections incubated for 1 h at room temperature). The rabbit anti-dogfish AQP4/1 antibody was directly labeled with a (red) Dylight 633 fluorescent dye (using a Pierce microscale antibody labeling kit) and then used on sections. Subsequently, a mouse anti-Na, K-ATPase a5 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was used and detected using a highly cross-absorbed (orange) Alexa 555 anti-mouse secondary antibody. A DAPI nuclear counterstain (blue) was also utilized as before.

RESULTS

Western blotting of crude membrane protein extracts from a variety of different dogfish tissues and using the AQP4/1 antibody showed that Aqp4 is expressed ubiquitously in all the tissues studied (**Figure 1A**). In most tissues, there were two protein bands on the blot with estimated molecular weights of 35.5 and 49.5 kDa respectively, and this tissue distribution was similar to that of *aqp4* mRNA (see companion paper; Cutler et al. (2012)). Additionally visible in the sample from rectal gland, there were two other faint bands of 37.5 and 38.5 kDa (see also **Figure 3**). Also in liver there was another strongly staining band at 57 kDa. The 35.5 kDa band common to most tissues is similar in size to the estimated molecular weight of the protein (37.2 kDa) based on the amino acid sequence derived from the gene sequence. The larger 49.5 kDa band was considerably more abundant in the rectal gland and appeared to be absent in the brain. The second AQP4/2 antibody was also used in Western blotting of tissue crude membrane extracts (**Figure 1B**). This blot showed a lot more bands than when using AQP4/1 antibody, although the 49.5 kDa was present

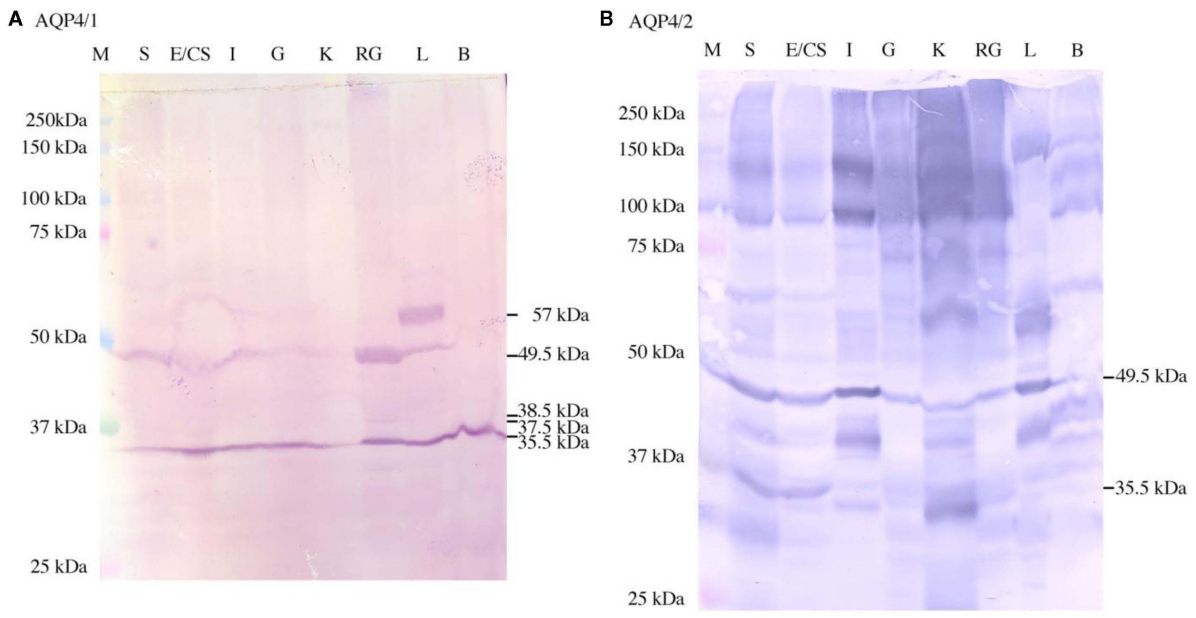

FIGURE 1 | Western blots showing the staining in dogfish tissues using the AQP4/1 (A) or AQP4/2 (B) antibodies. Three hundred micrograms of each tissue homogenate were used including stomach (S), esophagus/cardiac stomach (E/CS), intestine (I), gill (G), kidney (K), rectal gland (RG), liver (L), and brain (B). Bands were somewhat uneven, but this is normal when using relatively large amounts of protein per lane for detection purposes. Sizes were determined using kaleidoscope pre-stained molecular weight marker proteins (M).

in each tissue similar to the AQP4/1 antibody blot, except that the 49.5 kDa protein band was also present in the brain. There was also some indication of the 35.5 kDa band on the AQP4/2 blot, but the intensity of this was variable between tissues. There were additionally some higher molecular weight bands of 99 kDa and around 140 kDa suggesting the presence of dimers or Aqp4 aggregates. There was no indication of 37.5 and 38.5 kDa bands in the rectal gland sample as seen with the AQP4/1 antibody.

To test whether the antibody staining was specific, various control blots were performed (**Figure 2**). While the staining appeared as in the tissue blot (**Figure 1**) when using the AQP4/1 or AQP4/2 antibody alone, when the antibody was pre-blocked using its peptide antigen, staining was almost entirely abolished in the case of either antibody. Additionally, there was no similar staining when using either serum taken from the rabbit prior to immunization with the Aqp4 antigens (pre-immune serum) or from the secondary antibody used on its own (no primary Aqp4 antibody).

As the exact nature of the 49.5 and 57 kDa bands on the AQP4/1Western blot was worth further investigation, it was decided that, heat denaturation of the samples might cause some kind of aggregation (of the 35.5 kDa protein) to occur. Rectal gland crude membrane homogenate protein samples were therefore produced and blotted, that were either heated (as normal) and not heated (ambient temperature incubation, **Figure 3**). The lack of heat denaturation in comparison to normal had no effect on the 35.5 or 49.5 kDa bands, but the minor 37.5 and 38.5 kDa bands were absent in the un-heated protein sample lane and another diffuse band of around 32 kDa appeared instead. To further test whether any of the protein bands identified with the AQP4/1 antibody were glycosylated forms, crude membrane protein homogenates were treated with the enzyme PNGase F (New England Biolabs), which removes core N-glycosylated moieties from glycoproteins reducing their apparent molecular weight (**Figure 4**). However in either rectal gland or liver samples, PNGase F had no effect on the mobility of proteins identified by the AQP4/1 antibody, in comparison to similarly incubated control samples (no enzyme). This suggests that none of the protein bands represent glycosylated forms of Aqp4.

When the AQP4/1 and AQP4/2 antibodies were used for immunohistochemical staining of tissues, somewhat similar images were obtained with both antibodies but there were some differences, with the AQP4/2 antibody appearing to be more specific. In kidney (**Figure 5**), both antibodies labeled a sub-set of renal tubules both in the packed areas of the lateral bundle zone (**Figures 5A–C**) and in the sinus zone (**Figure 5D**). However, the AQP4/1 antibody (as is typical for other tissues also) stained the whole of the cell cytoplasm including in the region of the DAPI stained cell nucleus. Staining in renal tubule cells also was somewhat more intense toward the apical pole of the cell but was otherwise uniform throughout the cell. With the AQP4/2 antibody, fluorescence localized to the cytoplasm excluding the nuclear area stained by DAPI. Also there appeared to be no staining to the apical side of the nucleus in many cells. Serial sections stained with either AQP4/1 (**Figure 5E**) or AQP4/2 (**Figure 5F**) showed that the two antibodies stain the same segments of the similar renal tubule, although the type and intensity of staining was sometimes different between the two antibodies. With either antibody

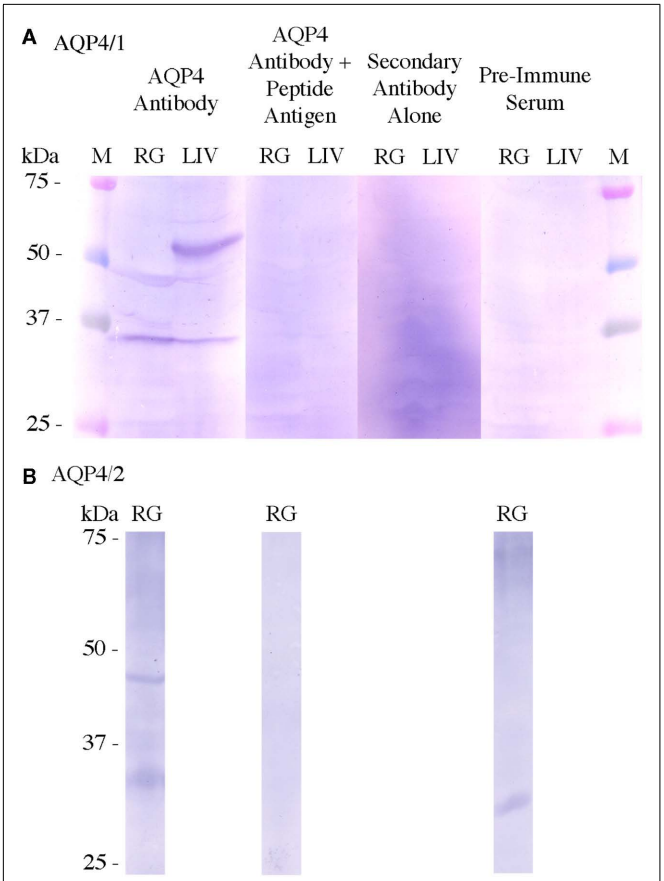

FIGURE 2 | Control Western blots using tissue homogenates (300 μg) from rectal gland (RG) and/or liver (LIV). Normal Western blots used either AQP4/1 **(A)** or AQP4/2 **(B)** primary antibodies (Aqp4 Antibody). Control Western blots used either the Aqp4 antibody pre-blocked with the peptide antigen used to raise it (Aqp4 Antibody + peptide antigen), no primary antibody, i.e., only TNT buffer (secondary antibody alone) or serum taken from the rabbit before immunization with dogfish Aqp4 antigen (pre-immune serum). Control blots used the same primary or secondary antibody concentrations as in the normal blots. All blots were incubated 1 min in NBT/BCIP substrate. Sizes were determined using kaleidoscope pre-stained molecular weight marker proteins (M).

there appeared to be a sub-set of renal tubules (approximately 1–10% of the total stained) that showed basolateral membrane staining but with little or no cytoplasmic staining (**Figure 6**). In **Figure 6B**, a tubule has been cut through in longitudinal section but due to the depth of the section (5 μm), the bottom of the tubule is also visible. Interestingly, the membranes of an apparent stellate-shaped tubule cell have also been stained by the AQP4/1 antibody.

Similar to the situation in renal tubule cells, in the rectal gland both antibodies fluorescently labeled all the secretory tubules of the gland. As in renal tubules AQP4/1 antibody stains the whole cell including the DAPI stained nuclear region with more intense labeling near the apical pole in many tubule cells (**Figures 7A,B**). The AQP4/2 antibody also stains the cytoplasm of tubule cells but with more intense staining toward the basal and apical poles of the cell and with lower intensity in the vicinity of the nucleus

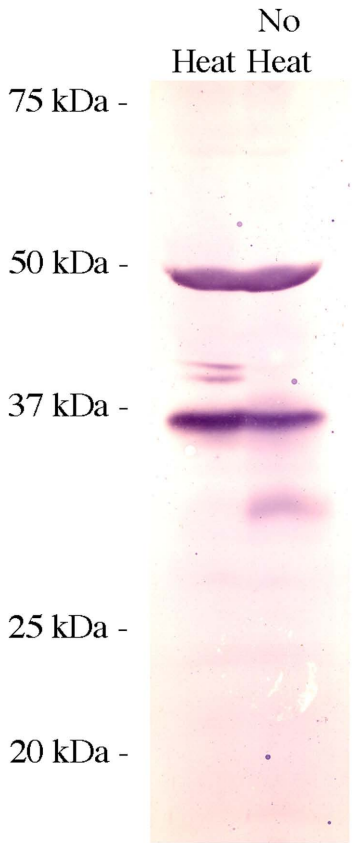

FIGURE 3 | Western blot using rectal gland tissue homogenates (300 μg/lane) with samples either heated for 5 min at 100°C prior to running on the gel (heat) or without being heat treated (no heat). The blot was incubated with the AQP4/1 primary antibody and otherwise processed as usual.

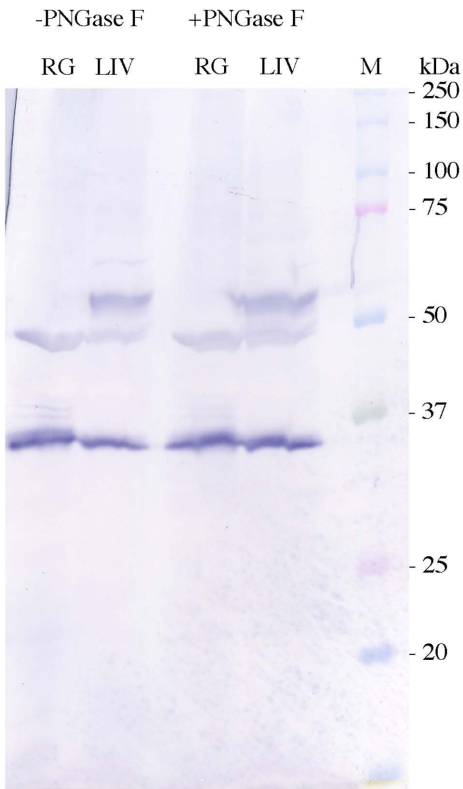

FIGURE 4 | Western blots performed using AQP4/1 antibody and tissue homogenates (300 μg) from rectal gland (RG) or liver (LIV), incubated either with (+) or without (−) the enzyme PNGase F (5500 units) for 1 h at 37°C prior to electrophoresis. Sizes were determined using kaleidoscope pre-stained molecular weight marker proteins (M).

(**Figures 7C,D**). Major staining was also seen in tubule-like structures in dogfish cardiac stomach (**Figure 8**). Again both antibodies stained these tubules strongly although there was also some less intense staining in cells underlying the epithelium (e.g., see **Figures 8A,C**). In these putative cardiac stomach secretory tubules, again the AQP1/4 antibody stained the whole of the cell with more intense staining toward the apical pole. Staining with the AQP4/2 antibody was somewhat more patchy and diffuse in the cytoplasm (than with the AQP1/4 antibody) but was of lower intensity in the nuclear region.

Lastly, strong staining was seen in large "chloride cell"-like cells of both the filament epithelium and the lamellae of the gill (**Figure 9**). The AQP4/1 antibody gave uniform cytoplasmic staining in many of these cells, and staining localized more to the plasma membrane and in the nuclear region in a minority of the other stained cells (**Figures 9C,D**). The AQP4/2 antibody also stained large cells in the primary filament epithelium and the secondary lamellae of the gill (**Figures 9E,F**) but here the staining in many of these cells was localized entirely in the region of the plasma membrane, while in others there was also some cytoplasmic staining.

Previously, Piermarini and Evans (2001), and Wilson et al. (2002), showed that there are different large mitochondria-rich (MR) or "chloride cell"-like cells present in elasmobranch gill, that stained either for the ion transport enzyme Na^+, K^+-ATPase, or V-type ATPase. Thus a four-color localization study was performed to determine whether the cells staining with the Aqp4 antibodies co-localize with either transport enzyme (**Figures 10** and **11**). Initial studies using AQP4/1, Na^+, K^+-ATPase, and V-type ATPase antibodies on serial dogfish gill sections suggested some co-localization of the three antibodies (data not shown). However, four-color staining with all the antibodies on the same section clarified the situation. Although it is not clear from the wide-field image of the gill (**Figure 10**), essentially all of the cells staining with the AQP1/4 antibody (red) co-localize with either Na^+, K^+-ATPase (orange), or V-type ATPase (green) fluorescence. Only one cell was seen that appeared to express Aqp4 alone (and this one may have occurred as a consequence of the sectioning technique used). An example higher magnification image (**Figure 11**), clearly shows the co-localization of AQP4/1 fluorescence with the Na^+, K^+-ATPase, or V-type ATPase staining. Additionally this study also showed that most of the V-type ATPase expressing cells were localized predominantly in the primary filament epithelium, with only a few cells on the secondary lamellae, whereas the majority of the

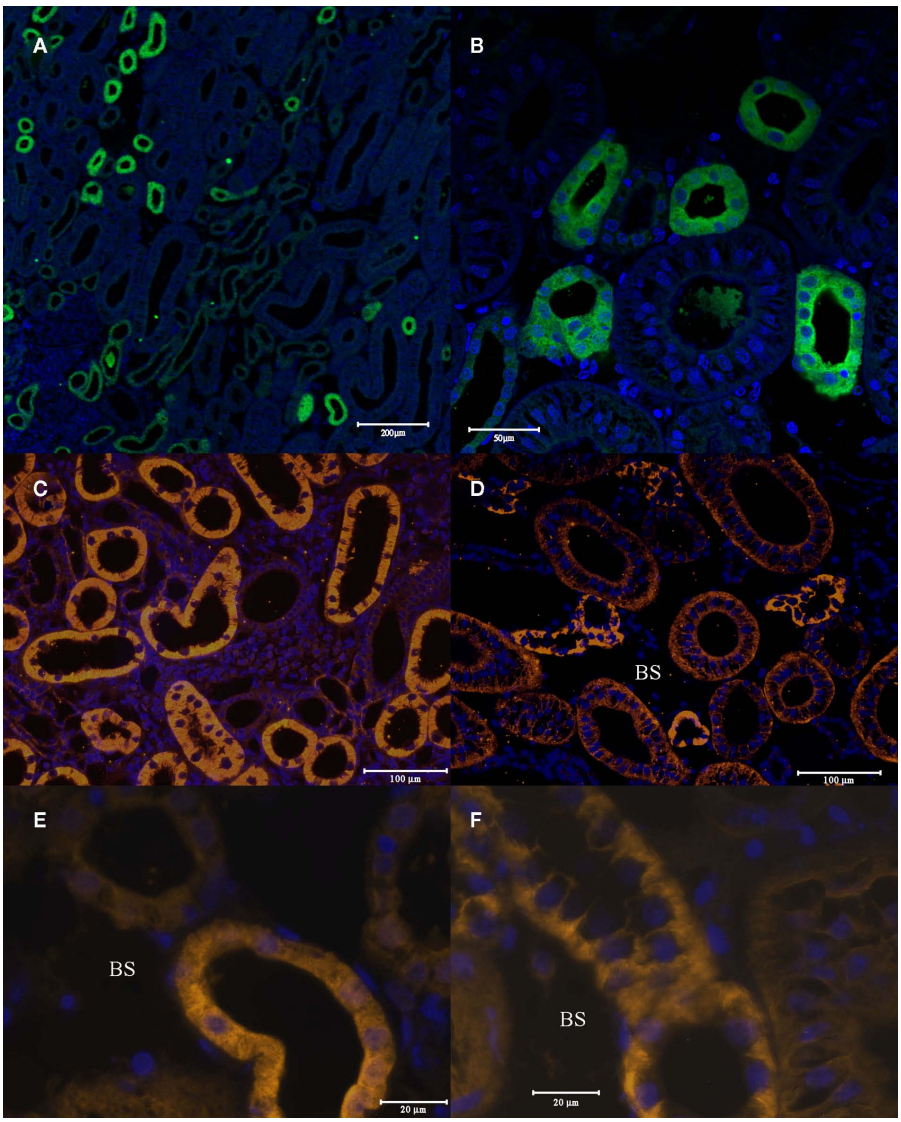

FIGURE 5 | Immunohistochemistry of 5 μm cross-sections through the dogfish kidney. These were incubated with the AQP4/1 antibody, an Alexa 488 anti-rabbit secondary antibody (green) and viewed with a Zeiss 510 Meta confocal microscope **(A,B)**. Or incubated with AQP4/2 antibody, an Alexa 555 anti-rabbit secondary antibody (orange) and viewed with a Zeiss Axiovert microscope **(C,D,F)**. Or incubated with AQP4/1 antibody, an Alexa 555 anti-rabbit secondary antibody and viewed with a Zeiss Axiovert microscope **(E)**. Nuclear counterstain, DAPI (blue). See scale bars for magnification. BS, blood sinus.

Na^+, K^+-ATPase expressing cells were on the secondary lamellae with only a few cells in the primary filament epithelium.

DISCUSSION

This study utilized two polyclonal antibodies that were raised against peptide antigens whose sequences were located in different non-overlapping regions of the dogfish Aqp4 derived amino acid sequence. Work with custom polyclonal antibodies is not always straightforward as they are often are more specific in immunohistochemistry than Western blotting or vice versa, or sometimes only work in one of those techniques. In this case, a second independent antibody was raised (AQP4/2) because unlike with the Western blot results with the AQP4/1 antibody, the immunohistochemical sub-cellular localizations, being largely cytoplasmic but also showing a nuclear location, were highly unusual for AQP4, which is normally a plasma membrane protein. To test the veracity of these results, AQP4/2 antibody was made and while it produces a larger array of bands in Western blotting (see **Figure 1**), as far as can be determined in this study, it produces staining in the same cells as the AQP4/1 antibody but shows a much more restricted sub-cellular localization, in particular showing either no or greatly reduced nuclear staining. It is possible to propose hypotheses that would explain the differences in results between the two antibodies (for example, it may be that only versions of Aqp4 with the C-terminal end removed (i.e., the region used to raise AQP4/1) can associate to form higher molecular weight dimers, trimers, and

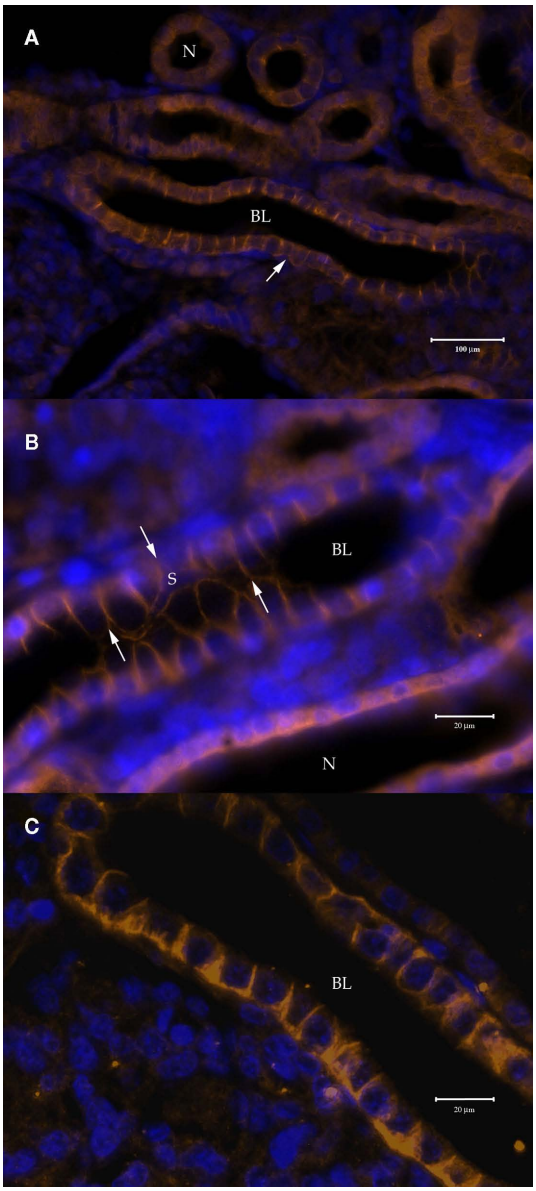

FIGURE 6 | Immunohistochemistry of 5 µm cross-sections through the dogfish kidney showing basolateral (BL) plasma membrane staining in around 1–10% of stained tubule segments, viewed with a Zeiss Axiovert microscope. (A,B) Stained with the AQP4/1 primary and a Dylight 549 anti-rabbit secondary antibody (orange). **(C)** Stained with the AQP4/2 primary and a Alexa 555 anti-rabbit secondary antibody (orange). Nuclear counterstain, DAPI (blue). *N* = Aqp4 staining throughout the cells of the tubule. Arrows indicate the extent of the cell processes of a stellate cell (S).

tetramers, hence explaining the high molecular weight banding seen with AQP4/2 (see **Figure 1**). However, the most parsimonious explanation is that the AQP4/1 binding is less specific in immunohistochemistry than AQP4/2, but that the opposite is true in Western blotting.

As with the tissue distribution of dogfish *aqp4* mRNA expression (see Cutler et al., 2012), dogfish Aqp4 protein expression was ubiquitous and consequently dogfish Aqp4 is more widely expressed than is the case in mammals (Ishibashi et al., 2009). In particular, unlike in mammals, Aqp4 is expressed in dogfish gill and liver.

The AQP4/1 antibody produced two bands (49.5 and 35.5 kDa) in Western blots, which are essentially similar to the two bands obtained with mammalian AQP4 (Terris et al., 1995). The abundance of the 35.5 kDa protein band was similar between tissues but there was somewhat more Aqp4 protein in rectal gland, liver and brain, and with lower levels in (pyloric) stomach and kidney. In the case of mammalian AQP4, the larger band was suggested to be a glycosylated form of AQP4. While the Aqp4 amino acid sequence from dogfish as well as other species, possess putative consensus N-glycosylation sites, often glycoproteins on gels/blots yield broad diffuse bands and the 49.5 kDa band here and the 50 kDa AQP4 band in mammals are both discrete bands. It would also be expected that if the 49.5 kDa proteins were glycosylated, these bands would have their molecular weights reduced by the enzyme PNGase F, and this was not the case in this study (see **Figure 4**). There is some evidence from unpublished work from this laboratory concerning eel aquaporins expressed in *Xenopus* oocytes (i.e., the presence of dimers, trimers and tetramers), that standard Laemmli SDS-reducing gels do not abolish all interactions between proteins and also that some proteins appear to run apparently smaller than their molecular weight, suggesting incomplete unfolding (Lignot et al., 2002). Consequently it would seem likely that the 49.5 kDa band either represents Aqp4 with an accessory protein still attached to it, or an Aqp4 dimer that has run much smaller than its expected size (74.4 kDa). Lastly the 49.5 kDa band could represent a dimer that has undergone partial protease digestion. Similarly the 57 kDa protein seen in liver homogenates is likewise likely to occur due to one of the three aforementioned options. Finally it is not clear why the 37.5 and 38.5 kDa bands were absent from the un-heated rectal gland crude membrane homogenates (see **Figure 3**), but its likely the diffuse 32 kDa band present instead, represents some form of folded Aqp4 protein that when heat denatured runs at a larger more accurate molecular weight. It is also not clear why the 37.5 and 38.5 kDa bands were absent when using the AQP4/2 antibody on blots of rectal gland crude homogenates, although the region of Aqp4 used as an antigen to raise the AQP4/2 antibody, contains predicted serine and tyrosine kinase phosphorylation sites (NetPhos 2.0; Blom et al., 1999) and if 37.5 and 38.5 kDa represent phosphorylated Aqp4 proteins, then the AQP4/2 antibody might not be able to bind them.

KIDNEY

Clearly from the immunohistochemistry performed with either antibody, dogfish Aqp4 is expressed in a sub-set or particular parts of renal tubules. Marine elasmobranch renal tubules are complex with two loops [with various neck (I–II), proximal (I–IV), intermediate (I–VI), distal (I–II), and collecting duct (I–II) segments] compared to the single loop of Henle found in mammals (Lacy and Reale, 1995). There are also lateral bundle zones with tightly packed tubules and sinus zones with blood sinuses seen as open areas. Based on the work of Lacy and Reale (1995), very tentative localizations for dogfish renal Aqp4 staining can be made. The

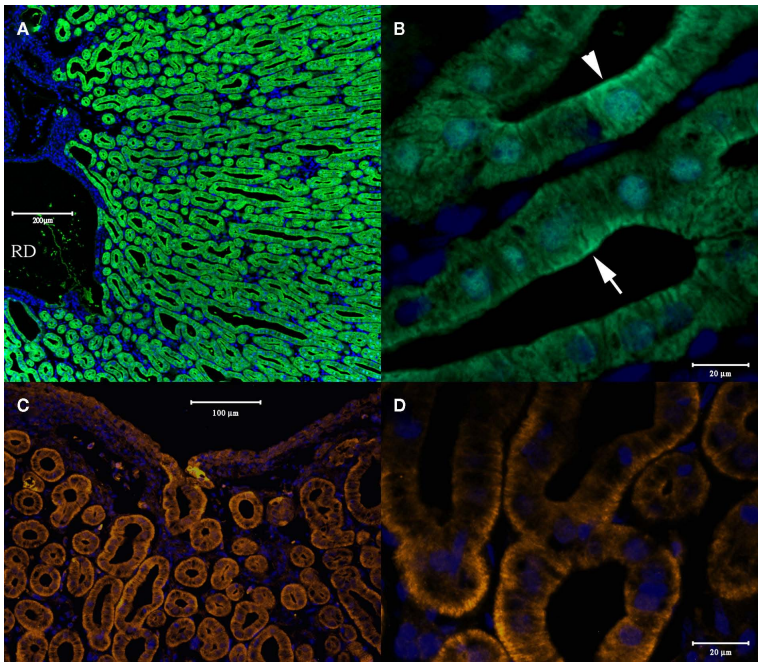

FIGURE 7 | Cross-sections through dogfish rectal gland stained with AQP4/1 (A,B) or AQP4/2 (C,D). A, lower magnification image acquired with a Zeiss 510 Meta confocal microscope showing tubule staining and the gland's central duct (RD). **(B)** Higher magnification image using a Zeiss Axiovert microscope, showing AQP/1 – Alexa 488 secondary antibody (green) staining throughout the cytoplasm of tubule cells with higher intensity in the apical pole (arrows). **(C,D)** Images showing AQP4/2 – Alexa 555 secondary antibody (orange) staining using a Zeiss Axiovert microscope, and showing stronger staining toward the basal pole of cells. Nuclear counterstain, DAPI (blue).

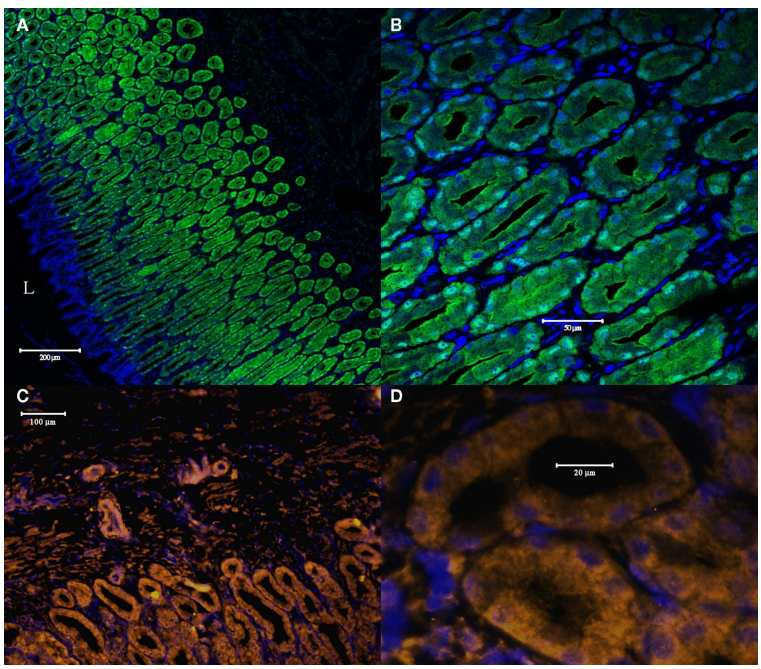

FIGURE 8 | Cross-sections through dogfish cardiac stomach stained with AQP4/1 (A,B) and acquired with a Zeiss 510 Meta confocal microscope or with AQP4/2 (C,D) and acquired with a Zeiss Axiovert microscope. (A) Lower magnification image showing tubule staining and the cardiac stomach lumen (L). **(B)** Higher magnification image, showing AQP4/1 – Alexa 488 secondary antibody (green) staining throughout the cytoplasm of tubule cells with higher intensity in the apical pole. **(C,D)** Images showing AQP4/2 – Alexa 555 secondary antibody staining (orange), and showing patchy cytoplasmic staining but with less staining in the vicinity of the nucleus than in the case of the AQP4/1 antibody. Nuclear counterstain, DAPI (blue).

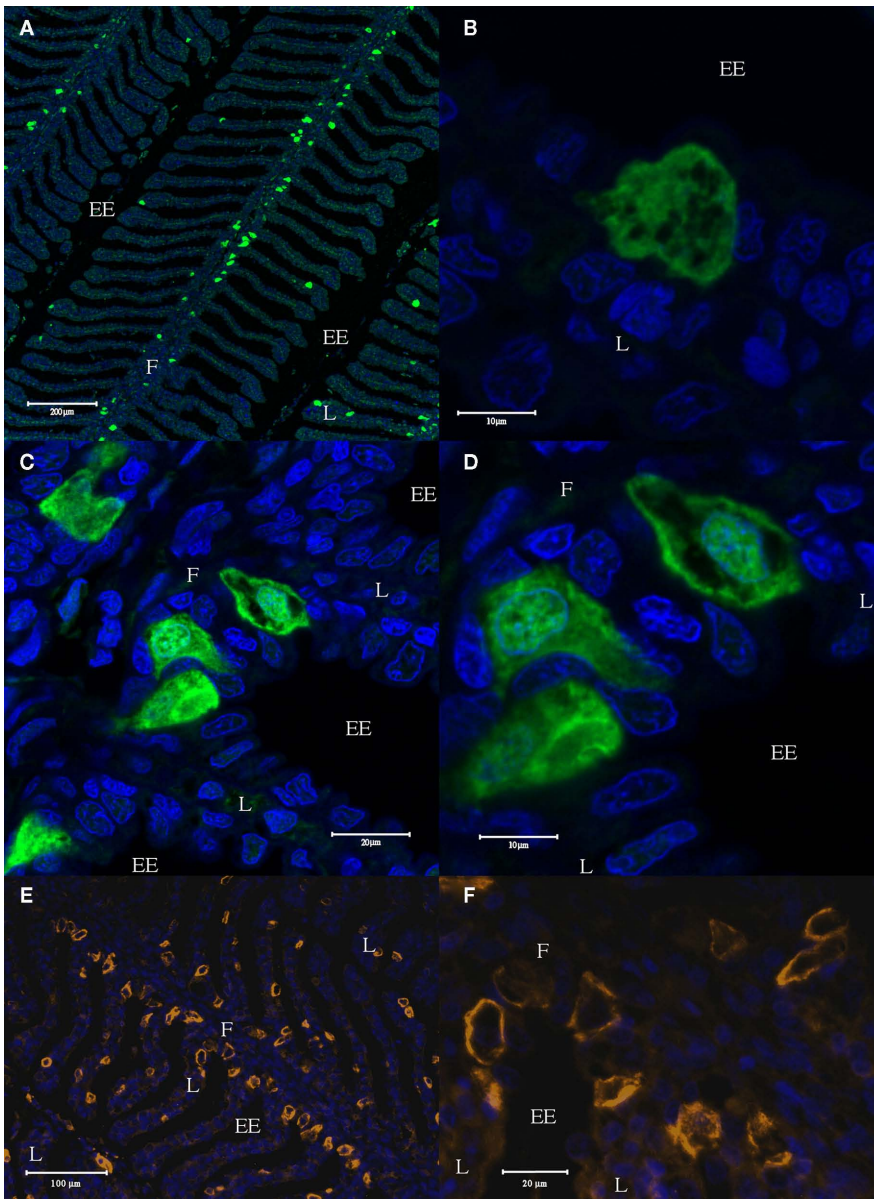

FIGURE 9 | Longitudinal sections cut through the dogfish gill, stained with the AQP4/1 antibody and an Alexa 488 secondary antibody (green), and viewed with a Zeiss 510 Meta confocal microscope (A–D). Further sections were stained with the AQP4/2 antibody and an Alexa 555 secondary antibody (orange), and viewed with a Zeiss Axiovert microscope **(E,F)**. Nuclear counterstain, DAPI (blue). Where F, filament, L, lamellae, and EE, external environment.

majority of tubules segments staining are reminiscent of intermediate tubule segments, such as In-IV and In-V (or possibly In-II though In-IV) of the second renal tubule loop. This is because the Aqp4 antibodies stain open tubules without any apparent brush border (found in proximal segments) and these stained tubules are largely found in the lateral bundle zone. The In-IV segment is known as the "diluting segment" due to the occurrence of sodium chloride re-absorption in this region of the nephron (Friedman and Hebert, 1990). The presence of Aqp4 in this region would be curious as its thought to have low water permeability (Friedman and Hebert, 1990). If Aqp4 was trafficked into the basolateral membrane as occurs with some AQPs in mammals, provided their was no apical water conduit this would be consistent with low tubule water permeability. Additionally this would suggest Aqp4 may be involved in cell volume regulation in this segment. Because some tubules showing Aqp4 staining are also found in the sinus zone this suggests that these regions are likely to be other intermediate segments (i.e., the first part of In-VI or less likely In-I). The presence of Aqp4 in the In-VI would make sense, as this segment is thought to have high water permeability and may be involved in osmotic equilibration due to water egress from the renal tubule (Friedman and Hebert, 1990). However Aqp4 would have to reside

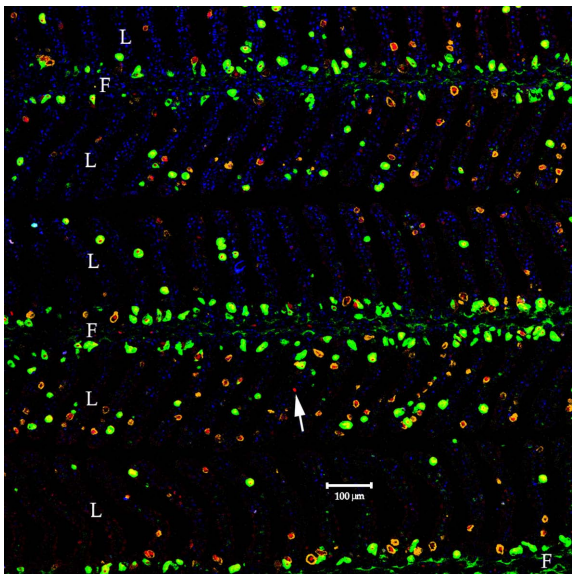

FIGURE 10 | Wide-field view of a longitudinal section cut through the dogfish gill, stained with the AQP4/1 antibody directly labeled with Dylight 633 (red), a rabbit anti-sculpin V-ATPase antibody detected with highly cross-absorbed Alexa 488 secondary antibody (green), a mouse Na, K-ATPase monoclonal antibody detected with highly cross-absorbed anti-mouse Alexa 555 secondary antibody (orange) and nuclear counterstain, DAPI (blue). Image viewed with a Zeiss 510 Meta confocal microscope, where PF = primary filament and SL = secondary lamellae. The arrow indicates the only cell staining with AQP4 but not V-ATPase or Na, K-ATPase antibodies.

in the plasma membrane for this to be the case and its currently not clear that that is the case. Again regulated trafficking of Aqp4 to the plasma membrane might also be an explanation for that as mention above. Additionally, the very occasional tubule showing Aqp4 staining, did appear to have some brush border material present, this suggests Aqp4 may also stain a small part of the proximal tubule. Lastly, the tubule segments showing basolateral staining probably represent a different part of the intermediate segment (In-III?). However the localization of Aqp4 to particular tubule segments currently remains difficult. Different renal tubule segments have been identified using various lectins (Althoff et al., 2006) but the results of this study suggest it would not be trivial to reliably localize Aqp4 (or other proteins) to particular tubule segments using their methods, and that is therefore beyond the scope of the current article.

RECTAL GLAND

The fact that Aqp4 was expressed in the dogfish rectal gland is also of interest as the gland has been a major model tissue historically for studies involving fluid secretion (Burger and Hess, 1960; Bonting, 1966; Hayslett et al., 1974; Epstein et al., 1983; Greger et al., 1988; Riordan et al., 1994; Forest, 1996; Silva et al., 1996; Evans, 2010). Despite the many studies concerning ion transport, the only study to look at water permeability of the gland suggested that it probably did not express aquaporins due to low apparent membrane water permeability (Zeidel et al., 2005). However, a series of illuminating studies by Solomon et al. (1984a,b, 1985) showed that ion secretion by the rectal gland was not stimulated when animals were perfused with hypertonic shark ringer solution (plasma salinity was raised without changing body fluid volume), but was stimulated when body fluid volume was increased using isotonic shark ringer. This strongly suggests that the principle function of the rectal gland is actually to remove excess water but due to the fact that water transport is passive, ions have to be transported to allow the water to follow by osmosis. Additionally, almost every example of secretory tissues/cells investigated has shown that the cells involved (in fluid secretion) invariably express some kind of aquaporin isoform. So it might be expected that elasmobranch rectal gland secretory tubule cells would express aquaporins. However, in the case of the staining with either of the AQP4/1 or AQP4/2 antibodies, with the respective apical or basal staining, in neither case does staining appear be present in the plasma membrane itself to any great extent, and this may explain why the study of Zeidel et al. (2005), found no significant water permeability associated with rectal gland plasma membranes. However, the question would be, why have Aqp4 then? The dogfish used in this study were normal unfed animals whose rectal glands are unlikely to have been particular active. The answer to the question therefore may be that a significant amount of Aqp4 may not reside in the plasma membranes of tubule cells until the gland is stimulated to secrete, whereupon Aqp4 may be inserted into the plasma membrane. Regulated insertion of aquaporins has been shown to occur in mammals and is a particularly important mechanism for renal AQP2 (Nejsum, 2005). The possibility of regulated trafficking of dogfish rectal gland Aqp4 may be tested by further experiments in the future.

CARDIAC STOMACH

During a screen of different dogfish tissues to see where Aqp4 was expressed, particularly strong staining was found in the cardiac stomach, which is an extension of the esophagus (anterior to the pyloric stomach) that has a totally distinct morphology in comparison to the esophagus itself (smooth brown epithelium rather than a surface covered with white cartilaginous conical structures). The Aqp4 staining appears to be localized particularly to secretory tubule structures that are likely to represent acid secreting gastric glands. The cardiac stomach of dogfish has been shown to have an acidic lumen with a pH in the range of 2–4 (Wood et al., 2007). Elasmobranchs have also been shown to express the H^+, K^+-ATPase enzyme in proximal stomach, which is associated with stomach acid secretions in mammals (Smolka et al., 1994; Choe et al., 2004; Shin et al. (2009)). As often occurs with ion secretions, they usually represent fluid secretions and consequently water is also secreted. This may move via the paracellular pathway, but a transcellular route via aquaporin water channels is more easily controlled. In dogfish cardiac stomach gastric glands, the immunohistochemical results in this study suggest fluid secretion may well involve Aqp4, but again there is no particular staining clearly associated with the gland cell plasma membranes although the AQP4/1 antibody shows staining toward the apical pole of cells. As mentioned previously, the dogfish in this study were unfed and it may be that Aqp4 is only inserted into the plasma membranes

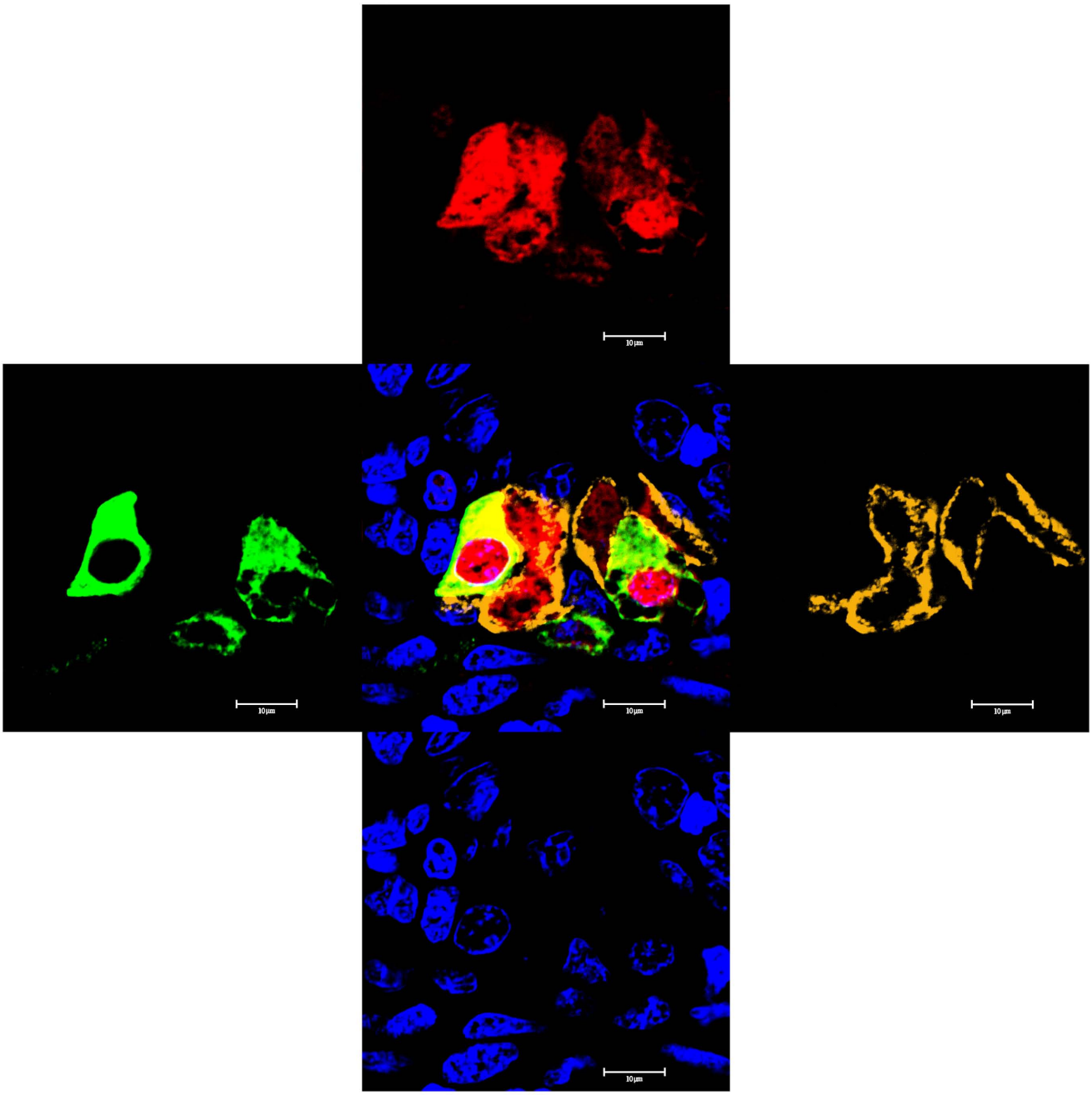

FIGURE 11 | High magnification image of a longitudinal section cut through the dogfish gill primary filament epithelium, stained with the AQP4/1 antibody directly labeled with Dylight 633 (red), a rabbit anti-sculpin V-ATPase antibody detected with highly cross-absorbed Alexa 488 secondary antibody (green), a mouse Na, K-ATPase monoclonal antibody detected with highly cross-absorbed anti-mouse Alexa 555 secondary antibody (orange) and DAPI nuclear stain (blue). Image viewed with a Zeiss 510 Meta confocal microscope.

after secretion is stimulated by feeding. This is therefore another avenue for further study.

GILL

Another location showing strong Aqp4 staining was the epithelial cells of the gill. Studies in the even more ancient Agnathan hagfish show staining for Aqp4 only in the gill but not other tissues, suggesting this may be the original cell localization for Aqp4 in vertebrates (Nishimoto et al. (2007)). In these cyclostomes, however, Aqp4 was found only in the pavement cells of lamellae. In this study, Aqp4 staining was found in both the filament epithelium and the in lamellae, in large "chloride cell"-like or "MR"-like cells. In particular with the AQP4/2 antibody (but also to some extent the AQP4/1 antibody) there were two different staining patterns

in these large cells, staining either located exclusively in the plasma membrane, or in the plasma membrane and interior cytoplasmic regions of the cell. Previous work has shown that there are two different types of "chloride cell"-like or "MR"-like cells which express either of the ion transporting enzymes, Na^+, K^+-ATPase, or V-type H^+-ATPase (Piermarini and Evans, 2001; Wilson et al., 2002). To determine whether either of these cell types corresponded to the cells expressing Aqp4, a four-color localization study was undertaken and this showed that Aqp4 staining localizes to both the Na^+, K^+-ATPase, and the V-type H^+-ATPase expressing cells. While the function of these cells has still not been determined, a similar situation exists in the gill of freshwater teleosts, where the V-type H^+-ATPase expressing (HR) cells are thought to be concerned with acid–base balance, whereas the Na^+, K^+-ATPase (NaR) expressing cells are involved in calcium transport (Hwang, 2009). Whether the Aqp4-expressing dogfish gill cells have the same functions and transport properties as these teleost gill cells remains to be determined.

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Expression and localization of Aquaporin 1a in the sea-bass (*Dicentrarchus labrax*) during ontogeny

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The successful establishment of a species in a given habitat depends on the ability of each of its developing stages to adapt to the environment. In order to understand this process we have studied the adaptation of a euryhaline fish, the sea-bass *Dicentrarchus labrax*, to various salinities during its ontogeny. The expression and localization of Aquaporin 1a (AQP1a) mRNA and protein were determined in different osmoregulatory tissues. In larvae, the sites of AQP1a expression are variable and they shift according to age, implying functional changes. In juveniles after metamorphosis (D32–D48 post-hatch, 15–25 mm) and in pre-adults, an increase in AQP1a transcript abundance was noted in the digestive tract, and the AQP1a location was observed in the intestine. In juveniles (D87–D100 post-hatch, 38–48 mm), the transcript levels of AQP1a in the digestive tract and in the kidney were higher in sea water (SW) than at lower salinity. These observations, in agreement with existing models, suggest that in SW-acclimated fish, the imbibed water is absorbed via AQP1a through the digestive tract, particularly the intestine and the rectum. In addition, AQP1a may play a role in water reabsorption in the kidney. These mechanisms compensate dehydration in SW, and they contribute to the adaptation of juveniles to salinity changes during sea-lagoon migrations. These results contribute to the interpretation of the adaptation of populations to habitats where salinity varies.

Keywords: AQP1a, fish larvae, osmoregulation, intestine, water channel

INTRODUCTION

Aquaporin 1 (AQP1) was the first water channel to be identified following its cloning and isolation from a human bone marrow library (Preston and Agre, 1991; Agre, 1997). Since then, a number of AQP homologs with wide tissue distributions (Takata et al., 2004) have been found in all living organisms (Hohmann et al., 2001; Cerdà and Finn, 2010). These proteins allow the fast flux of water across the cell membrane (Parisi et al., 1997). In mammals, 13 homologs of this protein are known, with some also involved in the exchange of glycerol and other low molecular weight solutes such as urea, CO_2, or NH_4 (Verkman and Mitra, 2000; Takata et al., 2004). The AQPs have been divided into two groups according to their transport selectivity, the aquaporins (e.g., AQPs Z, 0, 1, 2, 4, 5, 6, and 8) and aquaglyceroporins (AQPs 3, 7, 9, 10, and GlpF; Kozono et al., 2002). A third group, named the subcellular aquaporins, has been proposed for other related AQP-like proteins called AQP 11 and AQP 12 (Ishibashi, 2006). The distribution, function, structure, and molecular characteristics of AQP1 have been extensively reviewed (Jung et al., 1994; Walz et al., 1997; Verkman and Mitra, 2000; Stahlberg et al., 2001; Kozono et al., 2002; Agre, 2006; Cerdà and Finn, 2010).

Homologs of AQP1 have been identified in several species of teleost fish including the European eel (*Anguilla anguilla*; AQP1 and AQP1dup; Cutler and Cramb, 2000; Martínez et al., 2005c), the Japanese eel (*Anguilla japonica*; S-AQP1, L-AQP1, AQP3, AQP8, and AQP10; Aoki et al., 2003; Kim et al., 2010), the gilthead sea-bream (*Sparus aurata*; AQP1, AQP1-like SaAQP1o, SaAQP1a and SaAQP1b, AQP1a; Fabra et al., 2006; Raldúa et al., 2008; Cerdà and Finn, 2010), the sole (*Solea senegalensis*; AQP1), the zebrafish (*Danio rerio*; AQP1), the black sea-bass (*Centropristis striata*; AQP1; Fabra et al., 2005), the silver sea-bream (*Sparus sarba*; AQP3; Deane and Woo, 2006), the European sea-bass (*Dicentrarchus labrax*; AQP1; Giffard-Mena et al., 2007), the black porgy (*Acanthopagrus schlegeli*; AQP1; An et al., 2008), the killifish (*Fundulus heteroclitus*; FhAQP0, FhAQP1a, and FhAQP3; Tingaud-Sequeira et al., 2009), the rainbow wrasse (*Coris julis*; AQP1 and AQP3; Brunelli et al., 2010), and recently in the catfish (*Heteropneustes fossilis*; AQP1b; Chaube et al., 2011). As in other euryhaline teleosts, the European sea-bass larvae are confronted with salinity variations as they drift to coastal waters (Sabriye et al., 1988; Beyst et al., 2001) and subsequently when they enter lagoons and estuaries (Jennings and Pawson, 1992; Brehmer et al., 2006). This migration is accomplished after the metamorphic transition from larva to juvenile that occurs between D40 and D72 (18–25 mm; Varsamos et al., 2001, 2002, 2004; Saillant et al., 2003b). Metamorphic changes include morphological and physiological changes, one of them being an increase in the capacity to osmoregulate (Balon, 1999; Varsamos et al., 2001; Falk-Petersen, 2005). In this species, the osmoregulatory abilities tend to increase during ontogenesis with a particular increase of low-salinity tolerance

following the metamorphic larva/juvenile transition (Lasserre, 1971; Jensen et al., 1998; Varsamos et al., 2001). The organs involved in osmoregulation in the sea-bass have been studied (Nebel et al., 2005a,b; Varsamos et al., 2005; Giffard-Mena et al., 2006). As in other teleosts, the sea-bass hyper-osmoregulate at salinities lower than 10–11 ppt, and they hypo-osmoregulate at salinities higher than these iso-osmotic values. In fresh water the fish gains water and loses ions; in order to compensate for these movements, ions are absorbed mainly by the gills, and the kidneys produce and excrete relatively large volumes of hypotonic urine. In sea water (SW), where the opposite situation occurs, the fish gains ions and loses water. Under these conditions, the fish drinks SW which is then desalinated in the esophagus and stomach, before the water and remaining salts are absorbed through the intestine following an osmotic ion gradient established in epithelial cells by the activity of Na^+/K^+ ATPase (Marshall and Bryson, 1998; Marshall and Grosell, 2005). Excess plasma ions are excreted by the gills, and the kidney produces low volumes of isotonic urine (Marshall and Grosell, 2005; Nebel et al., 2005a; Giffard-Mena et al., 2006).

Several studies in the eel have shown that AQP1 is expressed more strongly in the intestine of SW-acclimated fish than in those fish acclimated to fresh water (FW; Martínez et al., 2005a,c). These results are correlated with an increase in drinking rate and with the absorption of water by the intestine (Aoki et al., 2003). Similar studies have shown that AQP1 transcript abundance is higher in the intestine of SW- compared to FW-acclimated sea-bass (Giffard-Mena et al., 2007), gilthead sea-bream (Raldúa et al., 2008), and Atlantic salmon (Tipsmark et al., 2010). However, a study conducted with the black porgy reported higher levels of AQP1 mRNA levels during FW acclimation (An et al., 2008). The functional water transport capacity of AQP1 in fish has been shown in the gilthead sea-bream. In this species the SaAQP1a and SaAQP1b are both water selective channels being true human AQP1 paralogs (Raldúa et al., 2008). It also has been shown that four eel AQPs have similar transport specificities as their human orthologs: as in humans, the eel AQP1, as well as killifish AQP1, transports water but not urea or glycerol (Hill et al., 2007; MacIver et al., 2009; Tingaud-Sequeira et al., 2009). Nevertheless, zebrafish AQP1 transports water, glycerol and urea (Tingaud-Sequeira et al., 2010) indicating paralogy among species.

Studies concerning AQPs during the post-embryonic development of fish are scarce. To our knowledge, the only work on this subject relates to the sea-bream *Sparus sarba*, where AQP3 has been detected from day 14 (D14) until D46 post-hatch. This protein becomes important from D28 and is expressed in several tissues (including the kidney, liver, brain, heart, and spleen). Its expression is not significantly different in SW and FW, except for the gills where it is more abundant in FW, perhaps as means of protection against the osmotic swelling of the cells (Deane and Woo, 2006). Its regulation during larval development has been related not only to osmoregulatory processes, but also in cell shape changes, migration, proliferation during metamorphosis, and sperm motility (Papadopoulos et al., 2008; Zilli et al., 2009; Cerdà and Finn, 2010).

In this study, we present results on the expression and localization of AQP1a during the ontogeny of the sea-bass *Dicentrarchus labrax* (Linné, 1758), from hatching larvae (day 0; D0, 3.5 mm) to pre-adult fish, during acclimation to SW, diluted sea water (DSW) or FW. An important role for the gut in water absorption is identified. The AQP1 gene has been found during screening of the complete zebrafish (*Danio rerio*) genome for functional aquaporins (Tingaud-Sequeira et al., 2010). According to this data, a recent classification proposes that genes have evolved from a teleost-specific local duplication of an ancestral AQP1 gene during evolution. Therefore, the so-called sea-bass AQP1 (Giffard-Mena et al., 2007) should be named AQP1a from now on, and we will use this terminology thereafter.

MATERIALS AND METHODS

ANIMALS AND EXPERIMENTAL CONDITIONS

European sea-bass *Dicentrarchus labrax* were provided by a local hatchery ("Les Poissons du Soleil," Balaruc) located in the south-west of France. In the present study five different developmental stages were investigated: three larval stages (3.5–25 mm in total length), one stage following the metamorphic transition to the juvenile phase (38–48 mm), and 2-year-old pre-adults (133 mm long; **Table 1**). The choice of these developmental stages was based on specific associated physiological events well described previously (Barnabé et al., 1976; Barnabé, 1989; Chatain, 1994; Pickett and Pawson, 1994; Varsamos et al., 2001; Saillant et al., 2003a) and on the fact that they cover all the main post-embryonic phases including the larval, juvenile and pre-adult phases.

Hatching was carried out in the fish farm from naturally spawned eggs maintained in SW at 34 ppt and 15°C. Larvae at different ages were transported to culture facilities of the University of Montpellier 2. Each group of larvae was divided in two 20 l aquaria, and was progressively conditioned over a period of 5 h to two different salinity strengths: SW (1029 $mOsm \cdot kg^{-1} \sim 35$ ppt) and DSW (147 $mOsm \cdot kg^{-1} \sim 5$ ppt), obtained by addition of dechlorinated fresh tap water. All larval stages were kept at each salinity for 48 h. Juveniles were kept for 48 h (D80), or for 10 (D87 and D96) and 15 (D100) days at each salinity (D, days post-hatch). Pre-adults (133 ± 14 mm, 28 ± 7 g) were maintained for 2 years in SW or in FW at 0.3 ppt (9 $mOsm \cdot kg^{-1}$). Temperature and photoperiod were set at 18 ± 0.5°C and at 12 h light/12 h dark. The osmotic pressure of the media was measured with a

Table 1 | *Dicentrarchus labrax* stages used for immunofluorescence, quantitative PCR (Q-PCR) and Western blot.

Stage	Length (mm)	Immuno fluorescence	Q-PCR	Western blot
Larvae	3.5–5	D1, D3	D0, D2	–
Preflexion larvae	5–7.5	D10	D6	–
Postflexion larvae	15–25	D33, D42	D32, D48	–
Juveniles	38–48	D96	D80, D87, D100	–
Pre-adults	133 ± 14	Two-year-old	–	Two-year-old

Size (total length range, n = 10).

D, days after hatch; –, not evaluated in this work.

micro-osmometer Model 3300 (Advanced Instruments, Needham Heights, MA, USA) and their salinity was evaluated from the relation 100 mOsm·kg^{-1} ∼ 3.4 ppt.

Following mouth opening (D5), the individuals received *Artemia* nauplii and fine particle artificial fish food (Gemma/Nutreco Aquaculture, Vervins, Picardie, France; particle diameters according to fish length: 50–250 μm from 10 mm, 180–400 μm from 20 mm, and 315–500 μm from 25 mm). Juveniles and pre-adults were fed with Aphymar granulates (Aphytec, Mèze, France) to apparent satiation once a day. The fish were unfed 24 h before sampling and they were anesthetized using phenoxy-2-ethanol (150 μg·l^{-1}). Samples from different stages were processed for immunohistological studies, quantitative (real-time) PCR or Western blot analyses (**Table 1**). All procedures were carried out according to the French law concerning animal scientific experimentation.

RNA EXTRACTION AND REVERSE TRANSCRIPTION

Total RNA was extracted using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA) from whole animal sub-pools of 30 larvae (D0), 10 larvae (D2), 10 preflexion larvae (D6), 5 postflexion larvae (D32, D48), with three pools for each stage. In juveniles (D80, D87), extracts were made from six whole animals. In juveniles at D100, the gut (from esophagus to rectum), kidney, and gill (filament and lamella removed from four gill arches on the right side of the body) were dissected separately from three individual fish. The animal and tissue quantities were calculated in order to have approximately 0.1 g to maintain a constant value for extraction according to manufacturer.

A treatment with DNase I (Invitrogen) was applied to the total RNA to prevent genomic DNA contamination. Total RNA concentration was determined by OD_{260} measurements in a NanoDrop ND-1000 Spectrophotometer V3.3 (NanoDrop Technologies Inc., Wilmington, USA), and its purity was verified using the 260/280 absorbance ratio. The integrity and relative quantity of total RNA were checked by electrophoresis. Total RNA (350 ng) from each developmental stage were reverse transcribed into cDNA in a reaction mixture containing 500 μg·ml^{-1} of oligo (dT) primer and 200 U of M-MLV RT (Invitrogen) following the manufacturer's instructions.

QUANTITATIVE REAL-TIME PCR

In order to quantify AQP1a transcript abundance throughout development and across salinities, the relative abundance of AQP1a transcripts (DQ924529), in each sample, was normalized to the amount of an endogenous reference, the gene encoding the sea-bass elongation factor gene (EF1α, AJ866727). This kind of normalization also takes into account the efficacy of the reverse transcription reaction. The EF1α expression levels did not change between salinities (data not shown) and it has been previously validated in other species and in sea-bass as a housekeeping gene (Nebel et al., 2005b). The AQP1a primers were designed using the Primer 3 software v 0.4.0 (National Human Genome Research Institute, USA; AQP1F, 5′-CAA-GGC-AGT-CAT-GTA-TAT-TG-3′ and AQP1R, 5′-AGA-GAG-TTG-AGC-CCC-AGT-3′). Quantitative PCR (Q-PCR) analyses were performed with a LightCycler™system version 3.5 (Roche Molecular Biochemicals) using 1× of the Lightcycler-FastStart DNA Master SYBR-Green I™Mix (Roche Applied Science), 0.5 μM of each forward (F) and reverse (R) primers and 0.5 μl of transcribed cDNA. Q-PCR reactions were achieved for 40 cycles in 10 μl volume. Melting curve analysis was performed with continuous fluorescence acquisition (65–95°C) at a temperature transition rate of 0.05°C/s to determine the amplification specificity. The relative transcript level of each gene at each stage and salinity was calculated for 100 copies of the housekeeping gene (EF1α) using the following formula: $N = 100 \times 2^{(\mathrm{Ct\ EF1\alpha} - \mathrm{Ct\ AQP1})}$ (Rodet et al., 2005) where *N*, means the copy numbers and Ct is the cycle threshold (defined as the number of cycles required for the fluorescent signal to cross the threshold, i.e., exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample.

PHYLOGENETIC ANALYSIS AND ANTIBODY PRODUCTION

BLAST alignment was made from public GenBank for sea-bass AQP1 DQ924529 (Giffard-Mena et al., 2007; AQP1a according to phylogeny nomenclature analysis as stated before). Neighbor joining (NJ) methods were used for comparisons of paralogs trees rendered with Geneious v5.3 (Drummond et al., 2010). Final trees were annotated with species names using Adobe Photoshop. Phosphorylation sites were searched with NetPhos 2.0 Server (Technical University of Denmark). A polyclonal antibody was raised in rabbit against a synthetic peptide corresponding to part of the carboxyl terminus region of the sea-bass *Dicentrarchus labrax* AQP1 molecule (amino acid residues 248–261 in sea-bass. The antigen C*NGGNDATTVEMTSK was conjugated with keyhole limpet hemocyanin (KLH*). The antiserum was obtained after three booster injections, and the IgG fraction was obtained after affinity purification by a commercial company (Genosphere Biotechnologies, Paris, France). Since extensive work has been done recently on piscine (Cerdà and Finn, 2010) and teleost (Raldúa et al., 2008; Tingaud-Sequeira et al., 2008, 2010) aquaporins, we presented only the alignment of sequences that have been used to develop antibodies for fish AQP1a for cellular location.

WESTERN BLOTTING

The gills, the kidney, and the digestive tract (specifically rectum) were used to verify the AQP1a antibody specificity. These tissues were dissected from SW sea-bass pre-adults. Their epithelia were scraped off with a sterile blade and homogenized with a 5-ml syringe and sterile needle (gage 22) in a lysis buffer consisting of SEI buffer (0.5 M sucrose, 0.01 M Na_2 EDTA, 0.05 M imidazole) pH 7.4, containing 20% of enzymatic protease inhibitors (Complete™ MINI EDTA-free, Roche, Mannheim, Germany) for inhibition of serine and cysteine proteases and therefore for stabilizing and protecting isolated proteins. The lysate was centrifuged at 3000 *g* for 5 min at 4°C. The supernatant was frozen at −20°C for later Western blotting. The protein concentration was quantified by the Bradford method (Bradford, 1976). Samples (30 μg protein) were solubilized and denatured by addition of 0.33 volumes of 4× loading buffer (0.25 M Tris, 8.33% SDS, 40% glycerol, 2.8 M β-mercaptoethanol, and 0.02% bromophenol blue) and incubated at 100°C for 15 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis (15% polyacrylamide).

After electrophoresis, the proteins were transferred to a polyvinylidene difluoride (PVDF) membrane (0.45 μm; Schleicher and Schuell, Saint Marcel, France). After blocking with phosphate buffered saline (PBS, pH 7.3) containing 5% skimmed milk for 3 h at room temperature, the membrane was incubated overnight at 4°C with the anti-sea-bass AQP1a polyclonal antibody at 5 $\mu g \cdot ml^{-1}$ (1:1780) in a PBS-containing 0.5% skimmed milk and 0.05% Tween 20. After washing two times for 5 min in PBS containing 0.5% Tween 20 and one time for 10 min in PBS, the membrane was incubated for 1 h at room temperature with the peroxidase-conjugate anti-rabbit IgG (Sigma) at 3.7 $\mu g \cdot ml^{-1}$ (1:2000) in PBS, 0.5% skimmed milk, and 0.05% Tween 20 solution. For negative controls, membranes were incubated with a solution lacking the primary antibody but supplemented with the same dilution of rabbit pre-immune serum. Membranes were washed and developed by chemiluminescence with luminol (0.25 M luminol diluted in DMSO, 0.09 M coumaric acid diluted in DMSO, 0.1 M Tris pH 8.5, and 0.0035% H_2O_2) for 1 min to visualize the immunoreactive bands with a UV-transilluminator (Roche) and LumiImage software (Boehringer, Mannheim).

INDIRECT IMMUNOFLUORESCENCE

Tissues extracted from fish at different developmental stages (**Table 1**) were fixed in Bouin solution over 48 h, then rinsed with 30% ethanol until elimination of the strong yellow color, and then replaced with 50 and 70% ethanol. The fixed material was dehydrated, embedded in Paraplast medium (Sigma), and sectioned as described previously (Giffard-Mena et al., 2006). Sections were incubated for 2 h at room temperature with the sea-bass specific anti-AQP1a primary antibody diluted 1:30. For negative controls, sections were incubated with a solution lacking the primary antibody but with equivalent dilutions of pre-immune serum instead. Immunofluorescence was detected after incubating the sections with a 1:150 dilution of fluorescein 5′ isothiocyanate (FITC) anti-rabbit antibody (Sigma, MO, USA). The sections were observed with a Leitz Diaplan fluorescence-fitted microscope (450–490 nm filters; Leica Microsystems, Rueil-Malmaison, France) in order to localize the cells immunoreactive to AQP1-a (called thereafter AQP1a-cells).

MORPHOMETRIC ANALYSIS

Intestinal sections from the anterior intestine, posterior intestine, and rectum stained as described above were examined using the Leitz Diaplan fluorescence-fitted microscope (six sections from three fish in SW, DSW, or FW were used). Photos from randomly selected areas (74×10^3 μm^2) were taken at 25× magnification with a Leica DC300F digital camera adapted to the microscope, and a Leica FW4000 software (Leica Microsystems, Rueil-Malmaison, France). Digital images were analyzed using web-based imaging software (Image J v1.34s, Wayne Rasband, National Institute of Health, USA) to determine the number and surface areas of AQP1a-cells.

STATISTICS

Analysis of variance (ANOVA) and non-parametric Student's *t*-test were used for statistical comparisons of the mean values ($p < 0.05$) for three replicates of larvae and post-larvae pools (D0, D2, D6, D32, and D48) or six individual fish at juvenile stage (D80 and D87) in each salinity condition (SW and DSW).

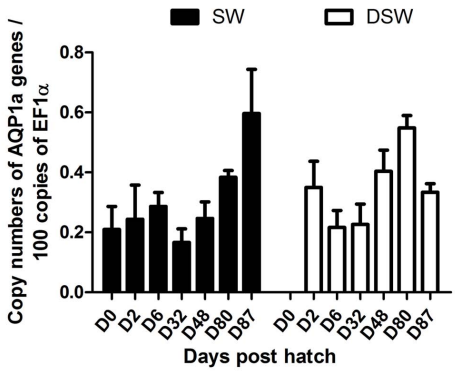
RESULTS

DIFFERENTIAL AQP1a ABUNDANCE DURING ONTOGENY ACCORDING TO SALINITY

The abundance of AQP1a was followed by Q-PCR at different developmental stages in sea-bass acclimated to SW and DSW for 48 h (D2, D6, D32, D48, and D80), 10 days (D87), and 15 days (D100). Transcript levels of the housekeeping gene EF1α did not change when two salinities where compared (not shown).

The abundance of AQP1a transcripts was similar between stages and salinities from D0 SW larvae until D48 (**Figure 1**). At D80, the AQP1a transcript levels showed significant increases (0.4-fold) in DSW- compared to SW-acclimated fish. Although there was a steady and progressive increase in AQP1a transcript abundance in SW-acclimated fish through developmental stages D32–D87, a similar progressive increase was only maintained until D80 in DSW-acclimated fish, but then it was followed by a significant fall in mRNA abundance at juvenile D87 stage. As a consequence, at D87, the AQP1a transcript level was significantly higher (0.8-fold) in SW-acclimated fish compared to DSW-acclimated fish (**Figure 1**).

In juveniles (D100; **Figure 2**), the AQP1a transcript level in the gut was significantly higher (by 2.6-fold) in SW-acclimated fish than in DSW-acclimated fish. In the kidney, which exhibited the highest levels of expression, the AQP1a level was also significantly higher (by 2.2-fold) in SW- than in DSW-acclimated fish. In the gill, the AQP1a transcript level was lower than in intestine or kidney and no significant difference was observed following salinity transfer.



Ttest	D2	D6	D32	D48	D80*	D87*
SW/DSW	0.50	0.39	0.49	0.12	0.01	0.04

FIGURE 1 | ***Dicentrarchus labrax.*** AQP1a transcript abundance evaluated by quantitative PCR, during sea-bass ontogeny, following a 48-h acclimation period in SW and DSW, only D87 was acclimated for 10 days. D0 larvae (hatching time) were only exposed to SW. Each value is the mean ± standard error of larvae pools from three samples (D0, D2, and D6, with 30, 10, and 10 fish respectively) or post-larvae pools (D32 and D48, with five fish in each pool) or juvenile individual animals (D80 and D87, six individual fish for each stage). *Asterisk* (*) indicates differences between SW and DSW samples ($p < 0.05$). D, days post-hatch. SW, sea water (1029 $mOsm \cdot kg^{-1}$ ~ 35 ppt); DSW, diluted sea water (147 $mOsm \cdot kg^{-1}$ ~ 5 ppt).

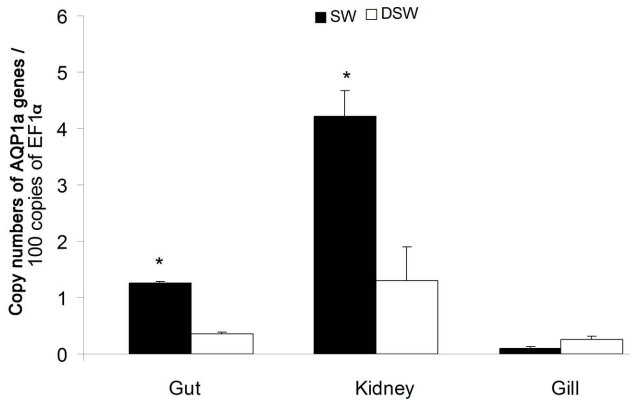


FIGURE 2 | *Dicentrarchus labrax.* AQP1a transcript abundance in tissues of juvenile sea-bass (D100) in SW and DSW evaluated by quantitative PCR. Each value is the mean ± standard error of three measurements (each measurement with three fish). *Asterisk* (*) indicates significant differences between SW and DSW samples ($p < 0.05$). D, days post-hatch. SW, sea water (1029 mOsm·kg^{-1} ∼ 35 ppt); DSW, diluted sea water (147 mOsm·kg^{-1} ∼ 5 ppt).

ALIGNMENT AND WESTERN BLOT DETECTION OF AQP1A

The molecular phylogeny of five teleost AQP1a to sea-bass brings out differences on specific amino acids (positions 252, 255–257 of alignment; **Figure 3A**). AQP1a sea-bass protein sequence has several high score phosphorylation sites (Ser^{6,38,188,224,239}, Thr^{255,259}, and Tyr^{245}), the residues S^{38,224} with 0.99 score. **Figure 3A** shows only Y^{245}, T^{255}, and T^{259} (0.82, 0.63, and 0.71). The phylogenetic tree indicates a closer genetic relation with gilthead sea-bream than eels and greater distance with zebrafish (**Figure 3B**). The immunoreactive bands found in the three tissues (gill, kidney, and gut) with molecular masses of 28 kDa correspond to the calculated molecular mass of the AQP1a channel (**Figure 3C**). A smaller band at 25 kDa was also detected in all tissues. These specific bands were absent in control blots (the rectum negative lane is shown).

AQP1a IMMUNOLOCALIZATION AND MORPHOMETRIC ANALYSIS DURING ONTOGENY ACCORDING TO SALINITY

The cellular localization of AQP1a was detected by immunofluorescence during the ontogeny of the sea-bass acclimated during 48 h, from SW to DSW (D1, D3, D10, D33, and D42),

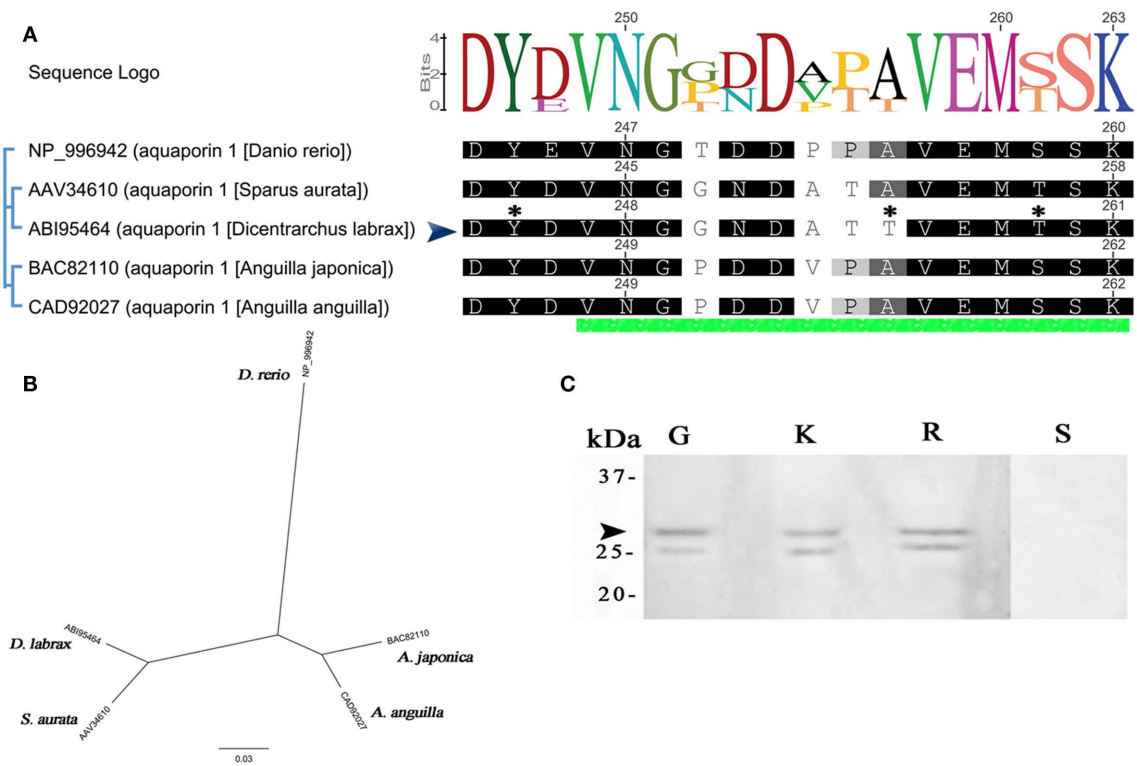


FIGURE 3 | *Dicentrarchus labrax.* Alignment, phylogenetic tree, and antibody specificity for AQP1a. **(A)** Complete AQP1a amino acid sequence alignment from some fishes. Only C termini is shown, the green bar at the bottom of alignment indicates the section chosen for developed antibodies (sea-bass peptide developed in this work is indicated by an arrow). Non-conserved residues are unboxed. The consensus sequence logo is scaled according to amino acid conservation, numbers on top correspond to this alignment. Numbers next to sequences corresponds to original sequence numbering. Potential phosphorylation sites for sea-bass are indicated with an asterisk. **(B)** Phylogenetic relationships among aligned AQP1a aquaporins. The unrooted phylogenetic tree was constructed using the NJ method. The bar indicates the mean distance of 0.03 changes per amino acid residue. **(C)** Representative Western blot of protein extracts (30 μg) from gills (G), kidney (K), and rectum (R). Negative control without the primary antibody (S). The apparent molecular masses (kDa) are indicated on the left. The arrow indicates the ∼28 kDa sea-bass AQP1a protein.

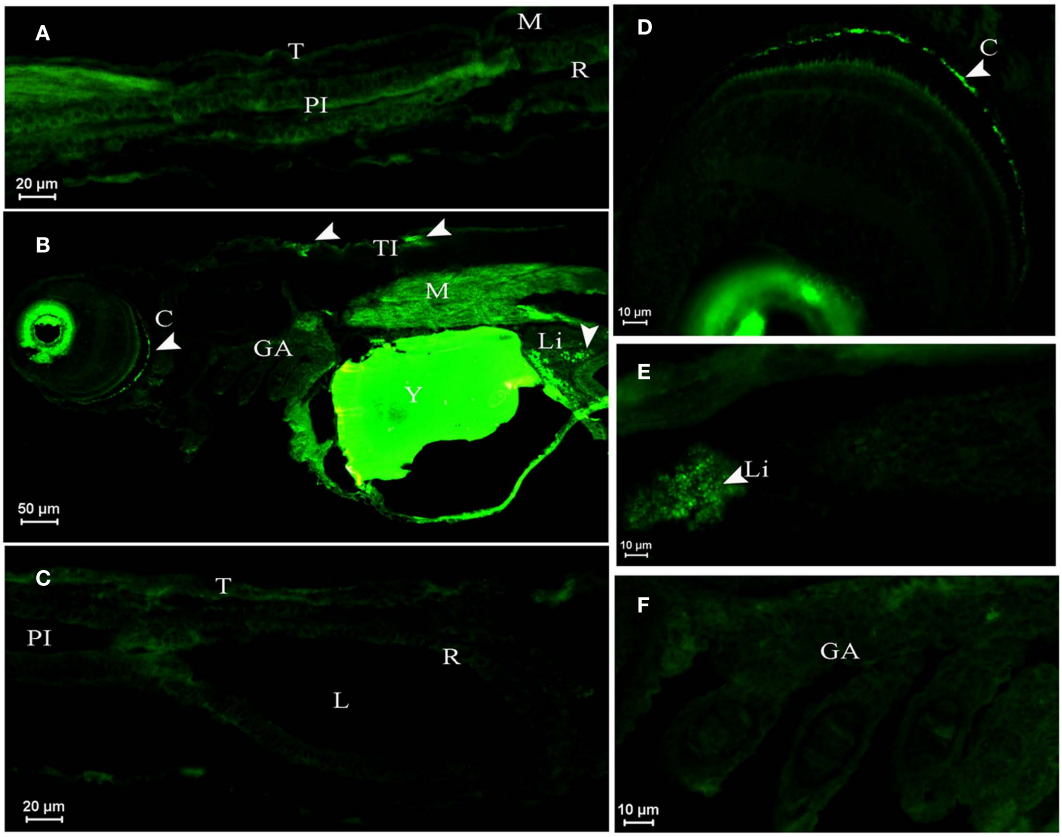


FIGURE 4 | ***Dicentrarchus labrax.*** AQP1a immunolocalization in SW- **(A,B,C,F)** and DSW-acclimated **(D,E)** animals. **(A)** Posterior intestine section of a larva at D1 displaying no fluorescence. **(B)** Larva at D3 with autofluorescence in eyes and yolk, showing immunopositive reaction to AQP1a in crystalline, muscle, liver, and in tegumentary cells (the presence of AQP1a is indicated by arrows). **(D)** Crystalline detail in a D3 larva. **(E)** Liver detail at D10. No fluorescence at D3 **(F)** in gills and **(C)** in digestive tract. C, crystalline; GA, gill arch; L, lumen; Li, liver; M, muscle; PI, posterior intestine; R, rectum; T, tegument; TI, tegumentary cell; Y, yolk; SW, sea water; DSW, diluted sea water.

10 days (D96), and 2-year-old. Positive immunoreactive cells are subsequently designated AQP1a-cells. Negative control sections at each developmental stage in the absence of AQP1 primary antibody exhibited no fluorescence. Control negatives display a lemon pale coloration very different from the strong green fluorescence of labeled protein (gills and gut are showed in **Figure 3C** respectively). However, autofluorescence was noted in the eyes, the yolk (in larvae; **Figures 4B,D**), muscle (**Figure 6D**), and in blood cells, particularly visible in the gills and in blood vessels of the intestine (**Figures 6A,B** and **7A,F**).

In larvae at D1 sampled about 8 h after hatch in SW, AQP1a-cells were not detected in any organ (as an example, see the posterior intestine, **Figure 4A**). In SW-acclimated larvae at D3 (**Figure 4B**) and in both SW- and DSW-acclimated larvae at D10 (**Figure 4E**), AQP1a immunofluorescence was noted in the crystalline lens epithelium of the eye and in the liver. However, specific immunofluorescence was not detected in the digestive tract (**Figure 4C**) nor the gills (**Figure 4F**) at any salinity (only shown for SW). At D3, a few AQP1a-positive tegumentary cells were also present in SW-fish (**Figure 4C**). At D33, AQP1a-cells were observed at the same location as at D10 but only in SW-acclimated fish (not illustrated).

In postflexion larvae at D42, AQP1a-cells were detected in the epithelium of the anterior intestine (**Figures 5A,B**), posterior intestine (**Figures 5C,D**), and in the rectum (not illustrated) at both salinities. Moreover, AQP1a immunoreactivity in the brush border was detected in the anterior intestine and rectum in SW (**Figures 5A** and **7F,G**). This signal was verified several times in different sections and individuals in order to rule out an artifact. Some AQP1a-cells were detected in the liver of SW- and DSW-acclimated fish (not illustrated).

In juveniles at D96 (**Figure 6**), AQP1a-cells were observed in the gills at both salinities (**Figures 6A,B**) and also in the submucosa and epithelial cells of the esophagus (**Figures 6C,D**). AQP1a-cells were present in the anterior intestine submucosa of SW-acclimated fish, but no signal was observed in the posterior intestine and rectum (not illustrated). At this developmental stage, no AQP1a immunoreactivity was observed in the crystalline epithelium of the eye, nor in the liver at any salinity (not illustrated).

In pre-adult fish (2-year-old fish exposed from 2 months of age to either SW or FW), AQP1a-cells were observed in the gills. They were mainly located in the filaments, but some AQP1a-cells were also present along the lamellae in both SW- and FW-acclimated fish (**Figures 6E,F**). AQP1a-cells were observed in the anterior

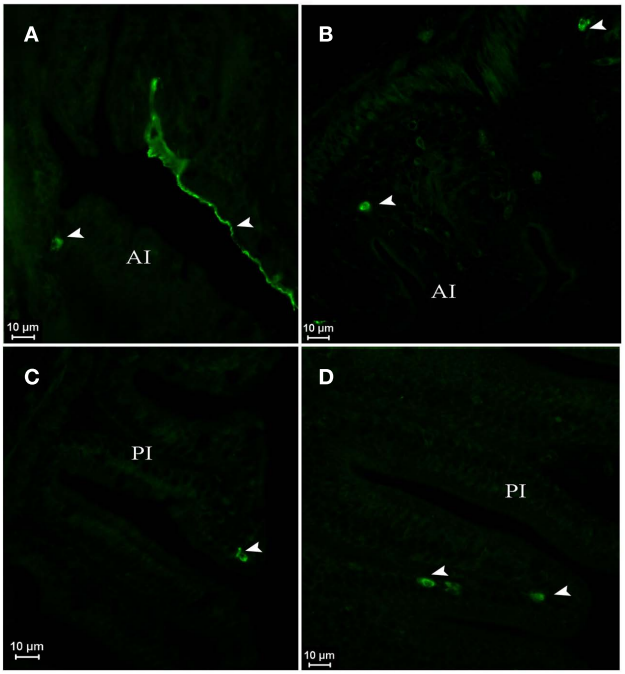


FIGURE 5 | *Dicentrarchus labrax.* AQP1a immunolocalization in anterior **(A,B)** and posterior intestine **(C,D)** of SW- **(A,C)** and DSW-acclimated **(B,D)** postflexion larvae at D42. The presence of AQP1a-cells and epithelial cell fluorescence is indicated by arrows. AI, anterior intestine; PI, posterior intestine; SW, sea water; DSW, diluted sea water.

intestine (**Figures 7A,B**), posterior intestine (**Figures 7C–E**), and rectum (**Figures 7F–H**). Most AQP1a-cells were located in the submucosa for all tissues in both SW- and FW-acclimated fish, but some immunoreactive cells were also present at the base of the columnar epithelial cells in the three segments (**Figures 7B,H**). In the rectum, immunostained cells were predominantly located at the base of the fold between intestinal villi, while in the anterior intestine AQP1a-cells were also abundant at the apical side of intestinal villi (**Figures 7F–H**). Immunoreactivity was also detected in the brush border apical section of the anterior intestine and rectum in both salinities, although it appeared stronger in the rectum of SW-acclimated fish (**Figure 7G**).

Morphometric analyses revealed several differences in the number and size of AQP1a-cells (**Figure 8**). These cells were more abundant in pre-adults than in juveniles. In juveniles (D96), AQP1a-cells were found in the anterior intestine only, and only in SW-acclimated fish. No immunoreactive cells were found in the anterior intestine of DSW-acclimated juvenile fish (**Figure 8A**). In pre-adult fish, the relative number of AQP1a-cells was highest in the anterior intestine, and it was higher in the rectum than in the posterior intestine. At each segment of the digestive tract, the number of AQP1a-cells was significantly higher in SW than in FW, by 49% in the anterior intestine, 50% in the posterior intestine, and 46% in the rectum (**Figure 8A**). The size of the cells was not different in the anterior intestine of SW-acclimated juveniles and pre-adults (**Figure 8B**). In pre-adults, slight but significant changes in the AQP1a-cell size were observed, with the largest cells found in the rectum and the smallest in the posterior intestine at both salinities. In the three sections of the digestive tract, the cell size was significantly higher (+16%) in SW than in FW (**Figure 8B**).

DISCUSSION

AQP1a is an important water channel involved in whole body osmoregulation in teleosts. This study shows the expression and localization of AQP1a at different osmoregulatory sites during the ontogeny of the sea-bass acclimated to different environmental salinity changes. One of the main results of this study indicates that a significant increase in AQP1a mRNA levels is detected around the time of metamorphosis of larvae into juveniles (D48–D80 in this work). Also, the number and size of AQP1a-cells is significantly higher in the gut sections of SW-acclimated fish compared to DSW-/FW-acclimated fish, strongly suggesting an important role of AQP1a for water absorption in SW-acclimated fish gut. In early larvae, the protein is also expressed in tegument, eyes, and liver and from D96 in gills and esophagus.

Western blot analyses suggest that *Dicentrarchus labrax* gill, kidney, and rectal tissues contain an AQP1a of around 28 kDa. This value is similar to those reported in mammals (Preston and Agre, 1991; Verkman and Mitra, 2000) and fish (Aoki et al., 2003; Martínez et al., 2005a). The 25 kDa band may correspond to a non-phosphorylated form of the protein (Verkman and Mitra, 2000; King and Agre, 2001; Fabra et al., 2006). Similar two-band profiles have been observed in gilthead sea-bream SaAQP1o (Fabra et al., 2006). Although further functional analysis of SaAQP1o (AQP1b; Tingaud-Sequeira et al., 2008) and discovery of AQP1a in eggs of this species, revealed that only AQP1b is able to be phosphorylated, several potential sites are present in AQP1a from the sea-bass, particularly S^{38} and S^{224} (0.99) indicating that sea-bass AQP1a has a strong probability of being a regulated form by phosphorylation. Another explanation is that aquaporins contains a number of glycosylation sites which may account for different molecular sizes as reported for AQP3 (Lignot et al., 2002a; Deane and Woo, 2006).

The profiles of AQP1a expression during the ontogeny of sea-bass show that transcripts are already expressed at hatch and during the larval and juvenile stages. The level of transcripts does not vary significantly from D0 and after mouth opening between D6, and is lower during the larval period than in juveniles (D80–D87). The protein has been detected at most observed stages in the crystalline epithelia of the eye and in the liver. The potential roles of mammalian AQP1 in the crystalline epithelium include the regulation of tear volume, the ionic composition, intraocular pressure and size of the sub-retinal compartment, and the hydration and transparency of the cornea (Levin and Verkman, 2006). In the rat, AQP1 expression was observed in the liver in the endothelial capillary cells, where the protein has been proposed to be involved in the transcellular movement of water, and in the formation of bile (Matsuzaki et al., 2004). The corresponding functions of AQP1a in the eyes and liver of fish are unknown. Although AQP1a is not expressed in these organs in the sea-bass after D33, the water transporting functions in these tissues might be taken over by another aquaporin.

At D3, immunofluorescence has revealed the presence of AQP1a in some tegumentary cells, but only in SW-acclimated larvae. At this larval stage, tegumentary ionocytes, that are active in

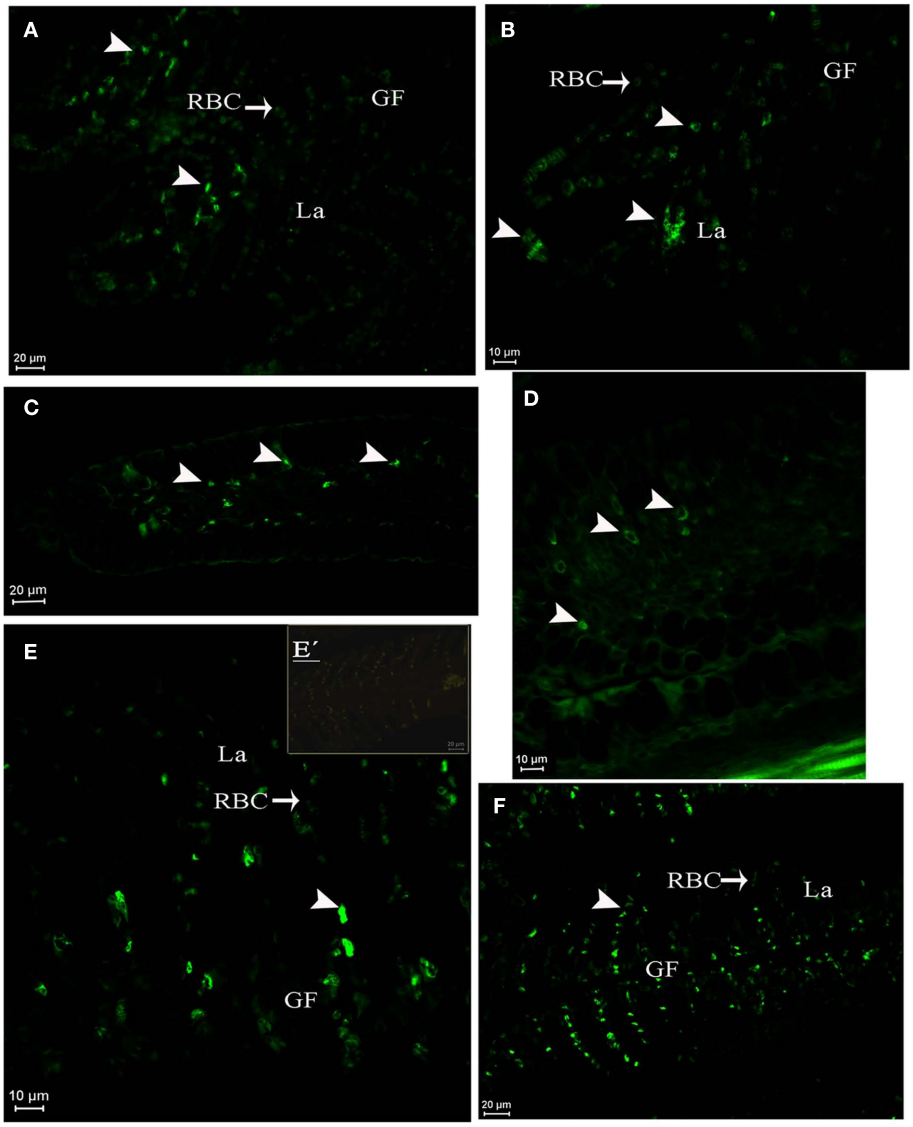


FIGURE 6 | ***Dicentrarchus labrax.*** AQP1a immunolocalization in juveniles at D96 in gill lamellae and filament **(A,B)**, esophagus **(C,D)**, and in pre-adult gill **(E,E′,F)**. Acclimation in SW **(A,C,E,E′)**, DSW **(B,D)**, or FW **(F)**. The presence of AQP1a-cells is indicated by arrows. GF, gill filament; La, lamellae; RBC, red blood cell; SW, sea water; DSW, diluted sea water. **E′** is a negative control without primary antibody.

ion transport, as indicated by the abundance of Na^+/K^+ ATPase, represent the main site of osmoregulation (reviewed by Varsamos et al., 2005). The precise osmoregulatory role for AQP1a in these cells at this stage is difficult to determine, especially since water would tend to flow out of the cell when larvae are in the marine environment. The reverse process, necessary to maintain the water content of these hypo-osmoregulating larvae submitted to dehydration (Varsamos et al., 2001), would necessitate the establishment of a strong ionic gradient against full SW at the basal side of tegumentary cells, which is unlikely. If sea-bass AQP1a sea-bass is also able to transport urea and or glycerol (as zebrafish does; Tingaud-Sequeira et al., 2010), this protein could also function to transport osmolytes to maintain osmotic pressure and therefore regulate cell volume. A functional analysis is required in order to understand this point.

Since AQP1a was not detected in the digestive tract or the gills in early larval stages, and as at stages D3–D48, there was no difference between fish kept at differing salinities, AQP1a does not appear to have a major functional role before metamorphosis. However, the maintenance of water balance remains necessary as the larvae are then completing their passive marine drift toward the coasts and are exposed to salinity fluctuations. The mechanism of water absorption, if carried out by the developing digestive tract as in later stages (Aoki et al., 2003; Giffard-Mena et al., 2007; Raldúa et al., 2008; Brunelli et al., 2010; Cerdà and Finn, 2010; Kim et al., 2010), could be based on the presence of an aquaporin isoform different from AQP1a, as described in the sea-bream *Sparus aurata* whose SaAQP1o (AQP1b) may facilitate hydration in oocytes (Fabra et al., 2005, 2006; Tingaud-Sequeira et al., 2010). Another candidate would be AQP3, detected in several tissues of

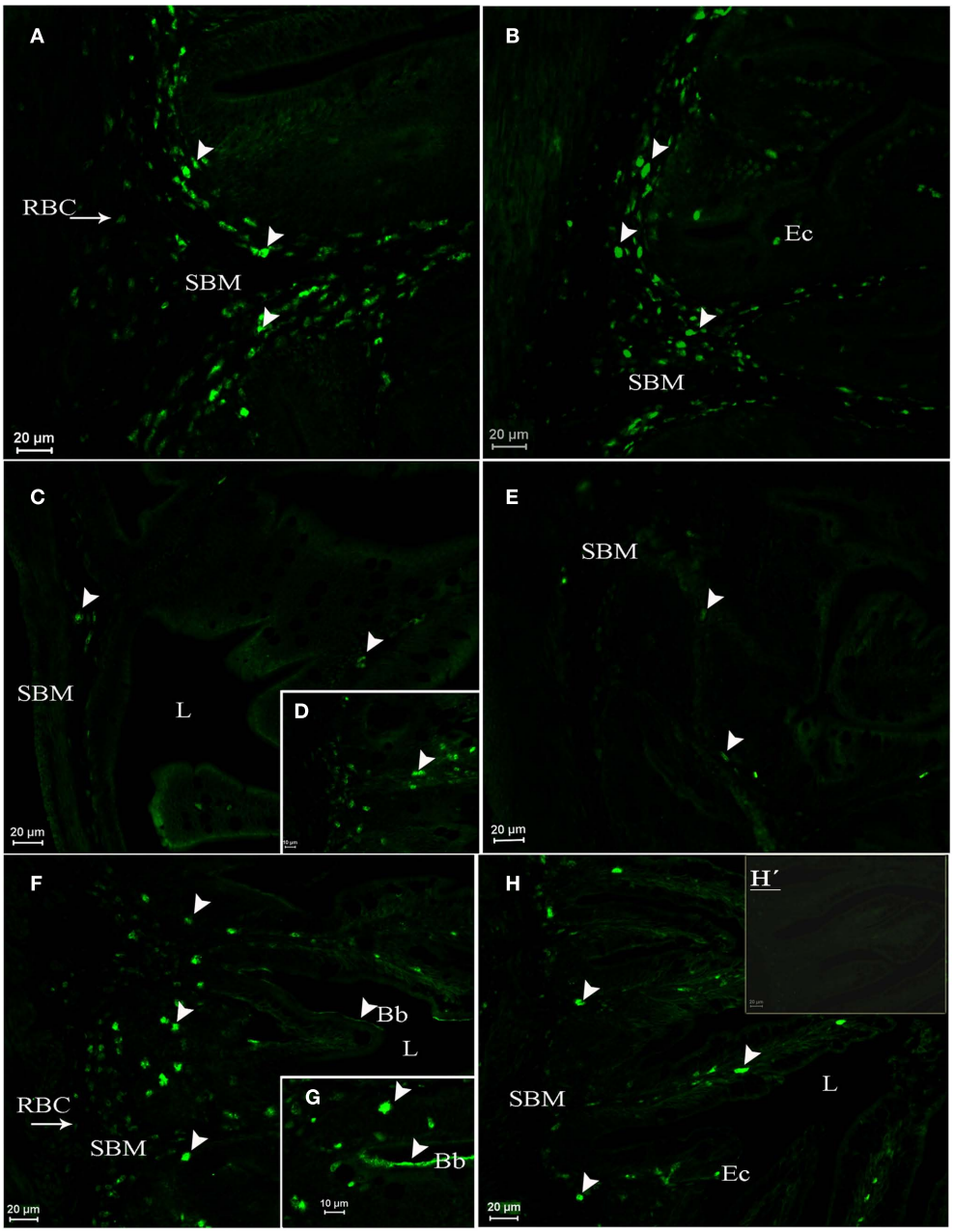


FIGURE 7 | *Dicentrarchus labrax.* AQP1a immunolocalization in the anterior intestine **(A,B)**, posterior intestine **(C,D,E)**, and rectum **(F,G,H,H′)** from SW-**(A,C,D,F,G)** and FW-acclimated **(B,E,H,H′)** 2-year-old pre-adults. The presence of AQP1a-cells is indicated by arrows. Immunoreaction was mainly observed in the submucosa (SBM), but also in cells present among columnar epithelial cells (Ec). Immunoreaction for AQP1a in the apical brush border cells (Bb) was stronger in the rectum of SW-acclimated fish than in FW-acclimated fish. Bb, brush border; Ec, epithelial cells; L, lumen; RBC, red blood cell; SBM, submucosa; SW, sea water; FW, fresh water. **H′** is a negative control without primary antibody.

Sparus sarba larvae and particularly in FW gills, which could act in the regulation of the cell volume (Deane and Woo, 2006). A similar function had been postulated for AQP3 in *Dicentrarchus labrax* (Giffard-Mena et al., 2007).

When *Dicentrarchus labrax* reach the juvenile stage (environ 25 mm, D80), a low but significant increase in AQP1a transcript abundance corresponded to the first occurrence of AQP1a protein in the gut (anterior intestine, posterior intestine, and rectum). The increase of AQP1a mRNA, detected in larvae at D48 and D80 after 48 h of acclimation to DSW is probably not associated with water uptake, since additional water absorption would impose an additional osmoregulatory burden to the fish. This fact strengthens the hypothesis of a specific function for this paralog at low salinities and at a developmental stage that corresponds to the metamorphic transition (Giffard-Mena et al., 2006). As whole fish have been used for this quantification, further measures on separate organs should

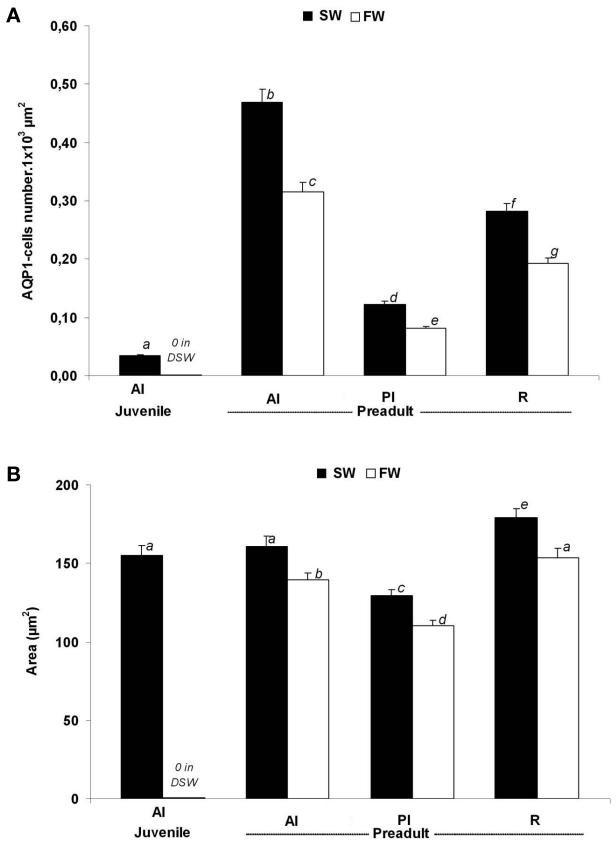


FIGURE 8 | ***Dicentrarchus labrax.*** Morphometric analysis of AQP1a-cells in selected organs of juvenile (D96) and 2-year-old pre-adult sea-bass acclimated to SW and DSW or FW. The number of AQP1a-cells **(A)** and their surface area **(B)** are shown. Values are mean ± standard error. Different letters indicate significant differences between values ($p < 0.05$, $n = 6$). AI, anterior intestine; PI, posterior intestine; R, rectum; SW, sea water; DSW, diluted sea water; FW, fresh water.

be conducted. Longer acclimation periods for larval stages are not possible since this would change the developmental stage; also, and it has been shown for other proteins such as Na^+/K^+-ATPase that 48 h acclimation trials are long enough to detect changes and stable responses in expression (Varsamos et al., 2004; Giffard-Mena et al., 2007).

In juveniles at D96–D100, when salinity acclimation was extended to 10–15 days, the expression of AQP1a in the digestive tract was consistently significantly higher in SW than in DSW. This fact reflects the capacity of juvenile fish at this age to cope with sustained salinity changes over long periods. Similar changes occurred in the kidney, but not in the gills. The protein is detectable through immunofluorescence in the gills, but the lack of change of its expression or localization according to salinity suggests that AQP1a has a limited osmoregulatory role in this organs, unlike AQP3 which seems to have a key role in adult sea-bass, particularly in FW (Giffard-Mena et al., 2007).

Within the digestive tract of juvenile sea-bass (D87), the highest AQP1a mRNAs abundance is found in SW-acclimated fish, which is in accordance with a high level of AQP1a protein expression in the anterior intestine. This is in consistent with the model of water absorption within the gut of adult fish, and which compensate for the dehydration effects of the environment (Aoki et al., 2003; Marshall and Grosell, 2005). It confirms the specific localization of AQP1a protein for this species. In sea-bass, the anterior intestine appears as the first section involved in osmoregulation. A previous study of its ontogeny has shown that it develops faster than other gut sections (Giffard-Mena et al., 2006). The AQP1a C-terminus is the most divergent region of sequence and it is known to play a role in AQPs intracellular trafficking (Raldúa et al., 2008). The differences in the teleost peptides (see consensus sequence, **Figure 3**) seems to be determining on tissue AQP1a distribution, mainly in gut sections.

In the digestive tract of 2-year-old pre-adult sea-bass, the number and size of AQP1a-cells were markedly higher in SW than in DSW, with highest values in the anterior intestine and rectum, respectively. Higher levels of this protein in SW were also found in other fishes (Aoki et al., 2003; Martínez et al., 2005a; Raldúa et al., 2008; Tipsmark et al., 2010), but its localization in some of them was restricted to the apical epithelia. In the sea-bass, the immunoreactive cells constitute a continuous cellular layer at the base of folds, in the conjunctive tissue (submucosa), the blood vessels and also on the apical brush border of the epithelium, especially in the anterior intestine and rectum. Apical localizations of AQP1a have previously been reported in the European eel (Martínez et al., 2005a), Japanese eel (Aoki et al., 2003), and in the gilthead sea-bream (Raldúa et al., 2008; Cerdà and Finn, 2010). The apical localization of AQP1a in the epithelial cells of the intestine points to a water flux through these cells. Another type of AQP is most probably present at the basolateral side of these cells, allowing a flux of water through the conjunctive tissue (Aoki et al., 2003; Tipsmark et al., 2010). The strong cellular density of AQP1a-cells in the submucosa of the sea-bass gut in SW is notable. Although these conjunctive cells do not form a continuous layer, several of them are very close at certain locations: they may increase the permeability of the submucosa, allowing a water flux from the intestinal lumen to the blood as suggested for salmon AQP1a (Tipsmark et al., 2010) and eel (Martínez et al., 2005a). Water would follow an osmotic gradient established by ionocytes located in the intestinal epithelium, with a basolateral localization of Na^+/K^+ ATPase (Giffard-Mena et al., 2006, 2007). Conversely, the presence of AQP1a in these cells may indicate that they regulate their own cellular volume or that they are involved in accelerating cell migration, particularly during the build-up of new blood vessels (Papadopoulos et al., 2008).

In the sea-bass, AQP1a has been observed in esophageal cells from juveniles (D96) in SW and DSW. This is the first report of AQP1a in this organ of that species. The esophagus water permeability is low in marine fish: this organ would mainly participate in the desalinization of imbibed SW (Venturini et al., 1992; Ando et al., 2003). However the earlier discovery of aquaporins in this organ (Lignot et al., 2002b) has led to suggestions of other physiological roles for this water transporter in this gut segment, including cell volume homeostasis (Martínez et al., 2005b), water trafficking associated with mucus secretion or maintenance of wetness of the epithelium (Lignot et al., 2002b), passive transcellular water fluxes from or to the luminal fluid imbibed from either FW

or SW, or protection of epithelial cells from the effects of swelling or shrinkage (Martínez et al., 2005b). Similar roles could also exist in the sea-bass.

In the present study, AQP1a expression was detected in pre-adult kidney of sea-bass by Western blot analysis and by Q-PCR. However, due to the high levels of autofluorescence within this tissue, the specific signals were not strong enough to determine a cellular localization of AQP1a. Although, this protein was been apical located within the renal tubules of eel and sea-bream (Martínez et al., 2005a; Raldúa et al., 2008). The high levels of AQP1a transcripts in the sea-bass kidney in SW may be related to required increases in water reabsorption from the renal tubules into the blood, contributing to a decrease in water loss via the urine (Marshall and Grosell, 2005). The presence of AQP1a in the kidney of both the adult eel (Martínez et al., 2005c) and pre-adult sea-bass (Giffard-Mena et al., 2007) is probably related to the reabsorption of water from the tubular fluids, a process of particular importance to prevent dehydration in SW-acclimated fish. This hypothesis is supported by lower urinary excretion rates found in sea-bass acclimated to SW rather than DSW (Nebel et al., 2005a). In FW fish, urinary excretion is important and allows to eliminate the excess of water, while reabsorption of ions by the kidney and bladder ionocytes limits ion loss (Nebel et al., 2005a). Results concerning the detection of AQP1 and AQP1 dup in the kidney of eels acclimated in SW are contradictory; the increased abundance of AQP1 transcripts found in FW-acclimated yellow eels is difficult to link with the need for eliminating water excess (Martínez et al., 2005c). Interestingly, increases in expression of AQP1 transcripts were not observed in migratory silver eels when still in FW, indicating that expression of AQP1 within the kidney is regulated more by developmental changes than environmental salinity variations (Martínez et al., 2005c). Therefore AQP1 might have a more important role in the control of cellular volume within the kidney rather than the bulk transepithelial movement of water (Martínez et al., 2005c). Conversely, AQP1 may also be involved in the absorption or secretion of water in the renal proximal tubules (Cutler et al., 2006). A recent study (Cerdà and Finn, 2010) has shown that these were actually paralogs including AQP1a, AQP1b, and AQP10b.

CONCLUSION

The regulation of AQP1a in the euryhaline sea-bass begins at the metamorphic transition. Elevated AQP1a mRNA levels in juvenile gut and kidney in SW-acclimated sea-bass suggest a key role in water absorption. The expression of AQP1a protein in the gut starts at metamorphosis (D42, 21 mm) and increases in juveniles (from D96, 43 mm) indicating an approximate time for water regulation through AQP1a-cells in developing fish. The importance of other aquaporin family members still remains to be elucidated. These results contribute to the interpretation of the adaptation of the sea bas to their habitats and their variability, as metamorphosis occurs just prior to the migration from the sea to estuaries and lagoons, i.e., to areas where salinity fluctuates.

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Aquaporin 1a expression in gill, intestine, and kidney of the euryhaline silver sea bream

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This study aimed to investigate the effects of chronic salinity acclimation, abrupt salinity transfer, and cortisol administration on aquaporin 1 (AQP1) expression in gill, intestine, and kidney of silver sea bream (*Sparus sarba*). An AQP1a cDNA was cloned and found to share 83–96% amino acid sequence identity with AQP1 genes from several fish species. Tissue distribution studies of AQP1a mRNA demonstrated that it was expressed in gill, liver, intestine, rectum, kidney, heart, urinary bladder, and whole blood. Semi-quantitative RT-PCR analysis was used to measure AQP1a transcript abundance in sea bream that were acclimated to salinity conditions of 0, 6, 12, 33, 50, and 70 ppt for 1 month. The abundance of gill AQP1a transcript was highest in sea bream acclimated to 0 ppt whereas no differences were found among 0–50 ppt groups. For intestine, the highest AQP1a transcript amounts were found in sea bream acclimated to 12 and 70 ppt whereas the transcript abundance of kidney AQP1a was found to be unchanged amongst the different salinity groups. To investigate the effects of acute salinity alterations on AQP1a expression, sea bream were abruptly transferred from 33 to 6 ppt. For intestine AQP1a levels were altered at different times, post transfer, but remained unchanged in gill and kidney. To study the effects of cortisol on AQP1a expression, sea bream were administered a single dose of cortisol followed by a 3-day acclimation to either 33 or 6 ppt. The findings from this experiment demonstrated that cortisol administration resulted in alterations of AQP1a transcript in gill and intestine but not in kidney.

Keywords: fish, aquaporin, gene, gill, intestine, kidney, salinity, cortisol

INTRODUCTION

The aquaporin family consists of relatively small intrinsic membrane proteins that are hydrophobic and are important in a range of physiological processes in living organisms, including water and solute transport (Borgnia et al., 1999). The diversity of aquaporins has been well reviewed and phylogenetic analysis suggests that these proteins can be grouped into three subfamilies (Heymann and Engel, 1999; Verkman and Mitra, 2000; Zardoya, 2005; Campbell et al., 2008; Ishibashi et al., 2009). The subfamily "aquaporins" are highly selective for the passage of water and are comprised of AQPs 0, 1, 2, 4, 5, 6, and 8 whereas the subfamily "aquaglyceroporins" allows for the passage of water, glycerol, and solutes such as urea and are comprised of AQPs 3, 7, 9, and 10. A third, less studied subfamily, designated as "superaquaporins" are comprised of AQPs 11 and 12 that are subcellular and have low amino acid homology to other aquaporins. Whilst most studies on aquaporins have been performed, using mammalian models, teleosts also represent excellent animal models in which to investigate their regulation and expression due to the continuous requirement toward maintaining both ion and water homeostasis. Several aquaporin families have been identified and studied, in fish, including AQP0 (killifish, Virkki et al., 2001), AQP8 (European eel, Cutler et al., 2009; Atlantic salmon, Tipsmark et al., 2010), AQP10 (Atlantic salmon, Tipsmark et al., 2010), and AQP3 from European eel (Cutler and Cramb, 2002; Lignot et al., 2002), Mozambique tilapia (Watanabe et al., 2005), Japanese dace (Hirata et al., 2003), and silver sea bream (Deane and Woo, 2006a).

Whilst several aquaporin families are known to exist, in fish, the most ubiquitous and extensively studied family is AQP1 and topological studies have revealed that this is a tetrameric protein consisting of six transmembrane spanning domains that are joined by two hydrophobic loops to form the classic hour-glass shaped structure (Jung et al., 1994). For teleosts the AQP1 family has been studied in Japanese eel (Aoki et al., 2003), European eel (Martinez et al., 2005a,b,c), black porgy (An et al., 2008), gilthead sea bream (Fabra et al., 2005, 2006; Raldúa et al., 2008) zebrafish and Senegalese sole (Tingaud-Sequeira et al., 2008), and Atlantic salmon (Tipsmark et al., 2010). Aquaporin regulation and expression studies in gill, intestine, and kidney have also provided for important insights on water transport in fish (Cutler and Cramb, 2000). In terms of understanding the effects of altered salinity conditions on water regulation, AQP1 expression profiles from several fish have been reported. For Atlantic salmon the expression profiles of AQP1a and AQP1b, were assessed during freshwater and seawater acclimation (Tipsmark et al., 2010). The findings from this study demonstrated that AQP1a transcript amounts were highest in gill following freshwater acclimation whereas amounts of this transcript were highest in kidney and middle intestine following seawater acclimation. The transcript profiles of Atlantic salmon AQP1a and AQP1b were also assessed after rapid transfer from

freshwater to seawater and it was found that AQP1a amounts were increased in the middle intestine and kidney whereas AQP1b transcript amounts increased in middle intestine and gill but decreased in kidney within the first 8 days following transfer (Tipsmark et al., 2010). The effects of chronic seawater and freshwater salinity acclimation on gill and intestinal AQP1 expression was reported for black porgy (An et al., 2008) and for both tissues the highest amounts of AQP1 transcript was found in fish acclimated to freshwater conditions. For European eel, intestinal AQP1 mRNA expression, of seawater acclimated yellow and silver eels, was found to be higher than eels that were acclimated to freshwater (Martinez et al., 2005b). Similar observations were also found for intestinal AQP1 in Japanese eels (Aoki et al., 2003), sea bass (Giffard-Mena et al., 2007), and gilthead sea bream (Raldúa et al., 2008). By using Northern blot analysis, transcript abundance of AQP1, AQP1dup, and AQPe were shown to be significantly reduced in renal tissue of yellow eels after transfer from seawater to freshwater. Conversely, renal expression levels of these aquaporins in silver eels were determined to be independent of salinity alterations (Martinez et al., 2005a).

The endocrine system is critical for regulating teleostean osmoregulatory function and amongst the plethora of hormones that have been investigated cortisol has received much attention due to its key role as a "seawater adapting hormone" (McCormick, 1995; Mommsen et al., 1999). Cortisol is a corticosteroid hormone which is synthesized by the interrenal tissue of teleost fish and is known to regulate hypoosmoregulatory processes via various ion-exchange mechanisms (McCormick, 1995). To date most of our knowledge concerning the role and importance of corticosteroids, on aquaporin expression, has been derived from studies on mammals (Tanaka et al., 1997; Wintour et al., 1998; Liu et al., 2003; Stoenoiu et al., 2003). Presently there is a paucity of information regarding the role of cortisol on fish aquaporin regulation and thus far our knowledge has been confined to a study on the freshwater yellow European eel where it was found that the administration of cortisol increased intestinal AQP1 expression levels (Martinez et al., 2005a). Interestingly AQPe, was found to be unresponsive to cortisol treatment in kidney and intestine of European eels (Martinez et al., 2005a,b) whereas another aquaglyceroporin (AQP3) was found to be significantly down-regulated in branchial tissues, following cortisol infusion (Cutler et al., 2007). It is clear that studies concerning the role and importance of cortisol, on teleost aquaporin expression, are certainly warranted.

As part of our investigations into understanding osmoregulatory strategies for euryhaline fish we report on the cloning of a silver sea bream (*Sparus sarba*) AQP1a and its expression profile during chronic salinity acclimation and after an abrupt salinity transfer. As our knowledge regarding the influence of hormones on aquaporin expression in fish is still in its infancy we have also undertaken experiments aimed at defining the importance of cortisol on silver sea bream AQP1a expression.

MATERIALS AND METHODS

FISH AND SALINITY ACCLIMATION

Silver sea bream (*S. sarba*) weighing 100–150 g, were purchased from a local fish farm, and were kept in a recirculating seawater (33 ppt) system in the Simon F. S. Li Marine Science Laboratory, Chinese University of Hong Kong for not less than 3 weeks to allow recovery from transportation stress. The fish were then divided into six groups randomly ($n = 7$) and put into six 1 ton seawater (33 ppt) tanks with individual recirculating filter systems. The salinity of each tank was adjusted to freshwater (0 ppt), hypoosmotic (6 ppt), isoosmotic (12 ppt), seawater (33 ppt), hypersaline (50 ppt), or extreme hypersaline (70 ppt) by flushing gradually with either tap water or hypersaline water. These salinity adjustments were completed within 7 days and the fish were acclimated for a further 28 days at their respective final salinities. The fish were fed *ad libitum* with fish meal pellets (Woo and Kelly, 1995) throughout the experiment and feeding was terminated 24 h before fish were sacrificed. Silver sea bream were removed from the tanks and were firstly anesthetized by MS-222 (Sigma, USA). Perfusion was used to remove the blood from gills and kidney by inserting a PE-50 polyethylene cannula (Clay-Adams, USA) filled with heparinized physiological saline [composition in mmol/L: NaCl, 1.43; KCl, 3.35; Na_2HPO_4, 1.4; $MgSO_4{\cdot}7H_2O$, 4; $CaCl_2$, 1.3; $(NH_4)_2SO_4$, 0.3; KH_2PO_4, 0.3; glucose, 5.6] into the first afferent branchial artery and then attaching the tube to a peristaltic pump. The aorta, slightly anterior to bulbus arteriosus, was tied off by a silk suture and an incision was made between the bulbus arteriosus and the heart to allow the blood/perfusate mixture to run out. The saline was kept in an ice-bath throughout the perfusion operation. Perfusion was also performed via the dorsal aorta on several occasions when blood inside the kidney had not been removed properly by the branchial afferent artery perfusion. The perfusion operation was deemed complete when the red color of the organs disappeared. For subsequent mRNA studies small pieces of gill, intestine, and kidney from all salinity acclimated groups were placed into 1 ml of Tri-reagent (Molecular Research Center, USA). For tissue mRNA studies, samples of gill, intestine, kidney, brain, heart, liver, rectum, urinary bladder, and whole blood were also kept in 1 ml Tri-reagent and all samples were then stored at −70°C.

ABRUPT SALINITY TRANSFER

Silver sea bream weighing 100−150 g, were purchased and maintained in seawater as described above. To perform an abrupt salinity transfer, twelve 1 ton tanks, with independent recirculating systems, were prepared such that six of these contained seawater (33 ppt), and six contained hypoosmotic (6 ppt) water. Groups of randomly selected fish ($n = 7$) were abruptly transferred from seawater conditions to either seawater or hypoosmotic conditions and sacrificed at 2, 6, 12, 72, and 168 h following transfer. Pre-transferred fish were used as time = 0 h group and feeding was terminated 24 h prior to performing the experiment. To assess mRNA amounts, small pieces of gill, intestine, and kidney tissues were prepared and collected as described above.

CORTISOL TREATMENT

For these sets of experiments silver sea bream weighing between 50 and 100 g, were purchased from a local fish farm and maintained in seawater as described above. In order to minimize stress, the administration, and dosage of cortisol followed previously described procedures (Pelis and McCormick, 2001). Silver sea

bream were initially divided into two groups ($n = 14$) and each group of fish were lightly anesthetized with MS-222 (Sigma), weighed, and either intraperitoneally injected with 10 μl/g body weight of a slow releasing oil implant (mixture 1:1 coconut oil and olive oil) or an oil implant containing cortisol (hydrocortisone, Sigma) to a final amount that was equal to 50 μg/g body weight. The subsequent abrupt transfer followed the procedure as described for gilthead sea bream (Sangio-Alvarellos et al., 2006) whereby after 3 days, following implantation, each treatment group was further divided into two sub-groups ($n = 7$), transferred to 6 ppt or to 33ppt (transfer control), and sampled at 3 days post transfer. Serum cortisol levels were measured using an ELISA kit purchased from Cayman Chemical Company (Michigan USA). The cortisol implantation procedure used was sufficient to maintain a state of hypercortisolemia as the average levels of cortisol were measured (as ng/ml) and found to be 11.4 ± 5.4 (33 ppt transfer/oil), 232 ± 49.9 (33 ppt transfer/cortisol), 24.11 ± 8.7 (6 ppt transfer/oil), and 186 ± 31.4 (6 ppt transfer/cortisol).

CLONING AND SEQUENCE ANALYSIS OF SILVER SEA BREAM AQP1

Total RNA from silver sea bream tissues were prepared using Tri-Reagent (Molecular Probes), treated with DNase I and then quantified spectrophotometrically. Total RNA (1 μg) was mixed with 0.5 μg oligo-dT primer (Proligo) and MMLV reverse transcriptase (InVitrogen) and first strand cDNA synthesis was allowed to proceed at 42°C for 2 h. Following synthesis the reaction mix was incubated at 70°C for 10 min and the cDNA preparations were stored at −20°C. In order to perform PCR amplification of AQP1 gene fragments we compared available fish AQP1 gene sequences on GenBank and identified two highly conserved regions. From the sequence information of these regions two sense primers designated as A1S1 (5′-CTTCTGGAGGGCCGTTCT-3′) and A1S2 (5′-TGTTCAAGGCGGTCATGTA-3′) and three antisense primers designated as A1AS1 (5′-ATGTACATGACCGCCTTGA-3′), A1AS2 (5′-CACCCAGTACACCCAGTGGT-3′), and A1AS3 (5′-GGGAAGTCGTCGAATTTGG-3′) were synthesized (Genset, Singapore). For amplification of silver sea bream AQP1, PCR reactions were carried out in a volume of 25 μl containing 2 μl first strand cDNA, 5 U of *Taq* polymerase (Promega), 0.25 μl dNTP mix (10 mM), 2.5 μl $MgCl_2$ (25 mM), 2.5 μl of PCR reaction buffer, and 0.8 mM of each primer. PCR amplifications were performed using an Eppendorf 9600 Thermal Cycler (Eppendorf, Germany) with a gradient of annealing temperatures from 45 to 60°C. Subsequent PCR reactions, cloning of gene fragments, and sequencing of putative clones were performed as described previously (Deane and Woo, 2004). A single DNA fragment of about 690 bp was obtained with the primer combination of A1S1 and A1AS3 under a high stringency annealing temperature of 60°C. As initial sequence analysis demonstrated that this fragment had a high degree of homology to known fish AQP1 sequences we proceeded to isolate the entire open reading frame (ORF) using 5′ and 3′ RACE systems (InVitrogen) and the cloned fragments were fully sequenced. The sequence data was analyzed using Clustal W[1] and Expasy[2].

[1]www.ebi.ac.uk
[2]www.expasy.org

SEMI-QUANTITATIVE AQP1A RT-PCR

Extraction of total RNA from each sea bream tissue and reverse transcription were performed as described above. Reverse transcriptase products of negative control for each tissue were also prepared with the same amount of RNA without MMLV reverse transcriptase. The primers used for RT-PCR were A1S1 and A1AS3 as described above and each RT-PCR reaction mix (25 μl) contained 1 × PCR buffer, 0.5 mM each dNTP, 2.5 mM $MgCl_2$, 0.8 mM each primer, 0.5 U of *Taq* polymerase. Polymerase chain reaction was performed using an Eppendorf 9600 Thermal Cycler with parameters of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The PCR products were separated on 2% (w/v) agarose gel (with ethidium bromide) and the band intensities were quantified with a Gel-Doc 1000 system (Bio-Rad) and analyzed using Quantity One Molecular Analyst Software (Bio-Rad). The optimum cycle number was obtained by plotting an amplification profile, with the optimum cycle number being the mid cycle number of the exponential phase in the amplification profile for all organs. The optimal cycle number used for tissue distribution analysis was 30 cycles as it fell at the exponential phases for most tissues tested. After validation, PCR was performed with the same profile for 30 cycles and as a normalization control, primers for β actin were used according to previously described procedures for sea bream (Deane et al., 2002).

STATISTICAL ANALYSIS

All data were expressed as mean values ± SEM. Data from each chronic salinity acclimation and cortisol treatment groups were subjected to a one-way analysis of variance (ANOVA), followed by a Tukey's test (SPSS) with $p < 0.05$ to delineate significance. Data from abrupt transfer experiments were subjected to two-way ANOVA and to delineate the significance of the interaction between salinity and time.

RESULTS

CHARACTERIZATION OF SILVER SEA BREAM AQP1a cDNA

The silver sea bream AQP1 cDNA clone was 904 base pairs (bp) containing 51 bp of a 5′-untranslated region (UTR), 774 bp of an ORF, and 79 bp of 3′-UTR. The nucleotide sequence encodes a protein with 258 amino acids with a calculated mass of 27.1 kDa and has been submitted on GenBank under accession number JF803845. Importantly the two channel-forming NPA (asparagine–proline–alanine) signature motifs are located at amino acid positions 67–69 and 181–183. Sequence comparison of the encoded protein, with known AQP1 proteins, also indicated the presence of amino acid residues of the pore-forming region at positions 56 (phenylalanine), 180 (histidine), and 195 (arginine). A cysteine residue is located before the second NPA motif at position 178, which is the potential site responsible for the inhibition of water permeability by mercurial compounds. The silver sea bream AQP1 protein shared high levels of amino acid identity with AQP1a from gilthead sea bream (97.7%) black porgy (95.8%), and European eel (83.6%; **Figure 1**). Also the cloned silver sea bream AQP1 amino acid sequence shares relatively low identity to AQP1b of the gilthead sea bream (56%) and human AQP1 (57.8%).

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SsAQP1       1  -MREFKSKDFWRAVLAELVGMTLFIFLSISTAIGS--------ANPNQEVKVSLAFGLAI  51
SaAQP1a      1  -MREFKSKDFWRAVLAELVGMTLFIFLSISTAIGS--------TNPDQEVKVSLAFGLAI  51
AsAQP1       1  -MREFKSKDFWRAVLAELVGMTLFIFLSISTAIGN-----ANNTNPDQEVKVSLAFGLAI  54
AaAQP1       1  MMKELKSKAFWRAVLAELLGMTLFIFLSIAAAIGN-----PNNSNPDQEVKVSLAFGLSI  55
SaAQP1b      1  -MTEVKSWAFWRAVAAEFVGMLLFIFAGLTAIIGS-----VEK-GVAQELKVALAFALAI  53
hsAQP1       1  MASEFKKKLFWRAVVAEFLATTLFVFISIGSALGFKYPVGNNQTTVQDNVKVSLAFGLSI  60
                   *.*.  ***** **::.  **:* .: : :*            :::**:***.*:*

SsAQP1      52  ATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVYGTRPST-  110
SaAQP1a     52  ATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVYGTRPST-  110
AsAQP1      55  ATLAQSLGHISGAHLNPAVTLGMLASCQISVFKAVMYIVAQMLGSALASGIVYGTRPDT-  113
AaAQP1      56  ATLAQSLGHISGAHLNPAVTLGMLASCQISMLKAVMYIVAQMLGASVASGIVYGVRPEG-  114
SaAQP1b     54  ATLVQSLGHVSGAHFNPAVTLGLLVSGQISALRCVCYILAQMLGAVAASAIVNGYA--Q-  110
hsAQP1      61  ATLAQSVGHISGAHLNPAVTLGLLLSCQISIFRALMYIIAQCVGAIVATAILSGITSSLT  120
                ***.**:**:****:*******:* * *** ::.: **:** :*:  *:.*: *

SsAQP1     111  TAELGLNTLT-GVTPSQGVGIELLATFQLVLCVIAVTDKRRRDVTGSAPLAIGLSVCLGH  169
SaAQP1a    111  TDKLGLNALT-GVTPSQGVGIELLATFQLVLCVIAVTDKRRRDVTGSAPLAIGLSVCLGH  169
AsAQP1     114  TGGLGLNALT-GVTPSQGVGIELLATFQLVLCVIAVTDKRRRDVTGSAPLAIGLSVCLGH  172
AaAQP1     115  VTALGLNSLN-KITPSQGVGVELLATFQLVLCVIATTDKRRRDVTGSAPLAIGLSVALGH  173
SaAQP1b    111  IGSLGVNELN-RVTKAQGFIIEFLATLQLVLCVIAVTDKRRSDVKGSAPLAIGLSVGLGH  169
hsAQP1     121  GNSLGRNDLADGVNSGQGLGIEIIGTLQLVLCVLATTDRRRRDLGGSAPLAIGLSVALGH  180
                   ** * *   :. .**. :*::.*:******:*.**:** *:  ********** ***

SsAQP1     170  LAAISYTGCGINPARSFGPALILNDFTNHWVYWVGPMCGGVAAALTYDFLLSPKFDDFPE  229
SaAQP1a    170  LAAISYTGCGINPARSFGPALILNNFTNHWVYWVGPMCGGVAAALTYDFLLSPKFDDFPE  229
AsAQP1     173  LAAISYTGCGINPARSFGPALILNNFTNHWVYWVGPMCGGVAAALTYDFLLSPKFDDFPE  232
AaAQP1     174  LTAISFTGCGINPARSFGPAVILGDFSDHWVYWVGPMCGGVAAALVYDFLLHPKFDDFPE  233
SaAQP1b    170  FAAISFTGCGINPARSFGPALIRSKMENHWVYWLGPMCGGIAAALIYDFLLCPRAQNFRT  229
hsAQP1     181  LLAIDYTGCGINPARSFGSAVITHNFSNHWIFWVGPFIGGALAVLIYDFILAPRSSDLTD  240
                : **.:************.*:*  .: :**::*:**: **  *.* ***:* *: .::

SsAQP1     230  RMKVLVSGPVGD-YD---------VNGGNDATAVEMTSK                     258
SaAQP1a    230  RMKVLVSGPVGD-YD---------VNGGNDATAVEMTSK                     258
AsAQP1     233  RMKVLVSGPVGD-YD---------VNGGNDATAVEMPSK                     261
AaAQP1     234  RMKVLVSGPDGD-YD---------VNGPDDVPAVEMSSK                     262
SaAQP1b    230  RRNVLLNGSEDEDAGFDAPREGNSSPGPSQGP-SQWPKH                     267
hsAQP1     241  RVKVWTSGQVEE-YD---------LDADDINSRVEMKPK                     269
                * :*  .*   :  .          . .  .  :  :
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FIGURE 1 | Comparison of amino acid sequences of the AQP1 of silver sea bream (SsAQP1), gilthead sea bream (SaAQP1a), black porgy (AsAQP1), European eel (AaAQP1), human (hsAQP1), and AQP1b of gilthead sea bream (SaAQP1b). Approximate locations of the six transmembrane domains are underlined and two signature NPA motifs are double underlined. Asterisks denote identical amino acid residues among different AQP1 sequences.

TISSUE DISTRIBUTION OF SILVER SEA BREAM AQP1a TRANSCRIPT

Using RT-PCR it was found that the AQP1a transcript was expressed in gill, liver, intestine, rectum, kidney, heart, urinary bladder, and whole blood but not in brain tissue of silver sea bream (**Figure 2**). For each tissue studied no amplified signals were detected in MMLV-negative controls therefore eliminating the possibility of false positive results due to genomic DNA contamination.

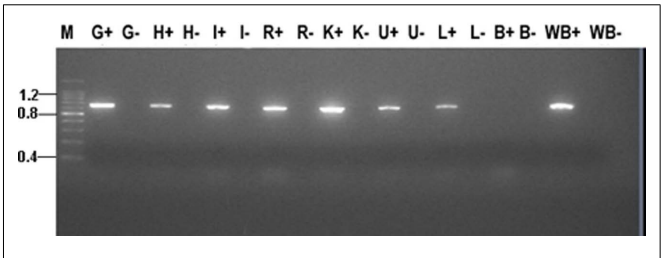

FIGURE 2 | Tissue distribution of AQP1a transcript in seawater acclimated silver sea bream. Abbreviations: M, molecular marker (sizes indicated kb); G, gill; H, heart; I, intestine; R, rectum; K, kidney; U, urinary bladder; L, liver; B, brain; and WB, whole blood. A (+) or (−) after an abbreviation indicates reverse transcriptase reactions that were performed with or without MMLV respectively.

EFFECTS OF CHRONIC SALINITY ACCLIMATION ON AQP1a TRANSCRIPT ABUNDANCE

Silver sea bream were acclimated, for 1 month, to salinities ranging from freshwater (0 ppt) to extreme hypersaline (70 ppt) and transcript amounts of AQP1a were semi-quantified using RT-PCR. In gill, the abundance of AQP1a transcript was found to be the highest in freshwater acclimated sea bream, whereas no significant difference in the expression levels was observed between 6 and 70 ppt salinity groups (**Figure 3A**). For intestine, the expression of AQP1a was found to be the highest in fish that were acclimated to 70 ppt being approximately 2.1–7.9 fold higher than amounts from sea bream acclimated between 6 and 50 ppt (**Figure 3B**). It was also found that intestinal AQP1a transcript abundance was increased to a smaller but significant extent in sea bream that were acclimated to an isosmotic salinity (12 ppt) when compared to levels from 0, 6, and 33 ppt acclimated sea bream (**Figure 3B**). In kidney, the expression level for AQP1a was not significantly different over the salinity range tested (**Figure 3C**).

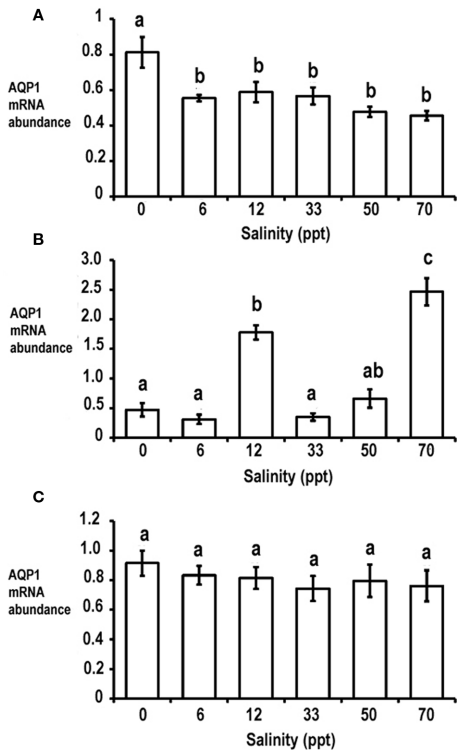

FIGURE 3 | Expression of AQP1a transcript in gill (A), intestine (B), and kidney (C) of silver sea bream that were acclimated to freshwater (0 ppt), hypoosmotic (6 ppt), isoosmotic (12 ppt), seawater (33 ppt), hypersaline (50 ppt), and extreme hypersaline (70 ppt) for 1 month. Data is presented as normalized AQP1a transcript abundance (arbitrary units) and values are expressed as mean ± SEM ($n = 7$). Different letters above a bar denote significant differences among salinity acclimated groups ($p < 0.05$).

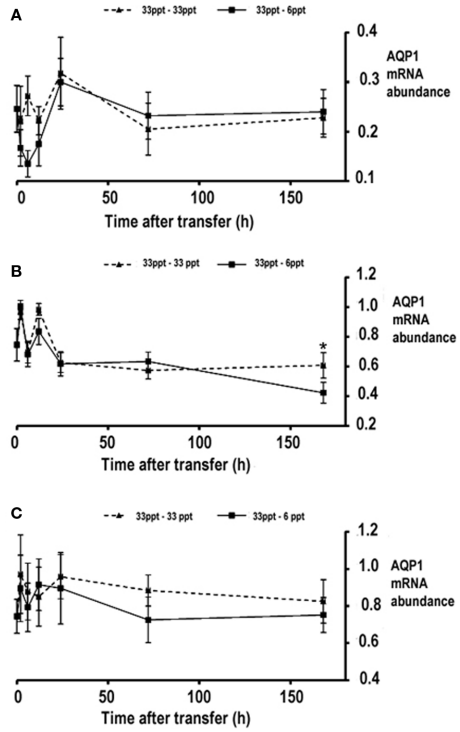

FIGURE 4 | Expression of AQP1a transcript in gill (A), intestine (B), and kidney (C) following an abrupt transfer of silver sea bream. Two transfers were performed, a 33- to 6-ppt abrupt hypoosmotic transfer and a control 33–33 ppt transfer. Data is presented as normalized AQP1a transcript abundance (arbitrary units) and values are expressed as mean ± SEM ($n = 7 - 4$). There was no significant interaction between salinity and time ($p < 0.05$).

EFFECTS OF ABRUPT SALINITY TRANSFER ON AQP1a TRANSCRIPT ABUNDANCE

To study the effects of acute salinity change on AQP1a transcript expression, sea bream were abruptly transferred from seawater (33 ppt) to hypoosmotic (6 ppt) conditions. Gill AQP1a transcript amounts started to decline at 2 h post transfer and continued to fall until 12 h post transfer. The abundance of gill AQP1a transcript was lowest at 12 h post transfer as the amount of AQP1a mRNA was less than half of that found in the control transfer group at the same time point (**Figure 4A**). However following statistical analysis it was found that there was not a significant interaction between salinity and time for gill AQP1a transcript amounts. Intestinal AQP1a transcript abundance exhibited transient perturbations in both the hypoosmotic transferred group and the seawater transfer control group but there was not a significant interaction between time and salinity. However it was found that time following transfer had a significant effect on AQP1a transcript abundance (**Figure 4B**). There was no significant interaction between salinity and time for kidney AQP1a transcript amounts when compared to transfer control groups (**Figure 4C**).

EFFECT OF CORTISOL ADMINISTRATION ON AQP1a TRANSCRIPT ABUNDANCE

In order for us to define the importance of hormones on regulating silver sea bream AQP1a expression we performed experiments whereby silver sea bream were treated with cortisol and then transferred to either 33 or 6 ppt for 3 days. Gill AQP1a transcript abundance remained unchanged between the oil control and cortisol treated fish, regardless of salinity (**Figure 5A**). Administration of cortisol caused approximately a 1.8-fold increase in AQP1a transcript abundance in the intestine of sea bream acclimated to 33 and 6 ppt when compared to the oil control group (**Figure 5B**). Comparable to our findings with gill, cortisol treatment did not modulate the abundance of AQP1a transcript in silver sea bream kidney when compared to oil control groups (**Figure 5C**).

DISCUSSION

AQP1a GENE CHARACTERIZATION AND TISSUE EXPRESSION

Toward our molecular studies a cDNA of the silver sea bream AQP1 homolog was isolated and found to share high identity with several previously isolated fish AQP1 genes. Hydropathy analysis predicted that it consists of six transmembrane domains which are key characteristics found in the major intrinsic protein (MIP)

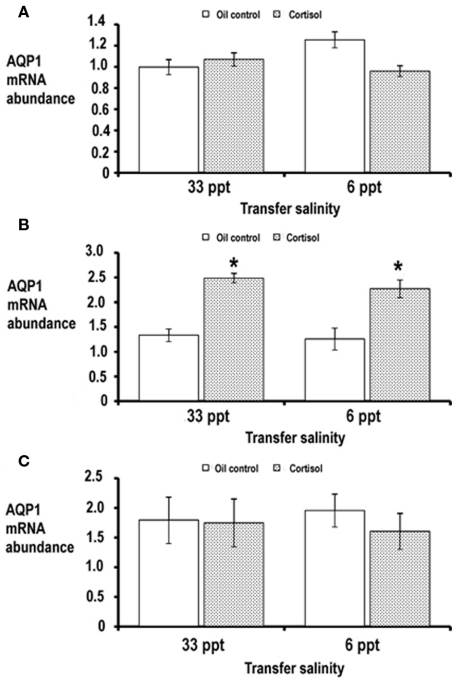

FIGURE 5 | Expression of AQP1a in gill (A), intestine (B), and kidney (C) of silver sea bream following administration of oil alone (control) or cortisol and then transferred to either 33 or 6 ppt salinity. Data is presented as normalized AQP1a transcript abundance (arbitrary units) and values are expressed as means ± SEM ($n = 7$). An asterisk above a bar indicates values that were found to be different between oil and cortisol treated groups ($p < 0.05$).

family as well as amongst aquaporins (Borgnia et al., 1999). The repeat amino acid sequences of loops B and E in silver sea bream AQP1 were highly conserved with respect to other mammalian and fish AQP1 homologs and are considered to be functionally important for water permeability (Jung et al., 1994; Shi et al., 1994; Borgnia et al., 1999). Also present in loops B and E were the signature NPA motifs that appear to act mainly as size exclusion filters (De Groot and Grubmuller, 2001). The cysteine residue (amino acid 178) was also located in loop E at the putative mercurial inhibiting site found in human AQP1 homologs (Borgnia et al., 1999). The silver sea bream AQP1 protein shared highest levels of amino acid identity with AQP1a from gilthead sea bream (97.7%) but relatively low identity to AQP1b of the gilthead sea bream (56%) therefore it would seem most likely that we had isolated an AQP1a from silver sea bream. Transcript analysis revealed the presence of AQP1a in gill, heart, intestine, rectum, kidney, liver, urinary bladder, and whole blood which is in general agreement with studies in mammals (Ishibashi et al., 2009) and teleosts (Aoki et al., 2003; Martinez et al., 2005a; An et al., 2008; Raldúa et al., 2008). In contrast, AQP1 transcript was not detected in sea bream brain but has been detected in the brain of the European eel (Martinez et al., 2005a) and black porgy (An et al., 2008). Taken together these findings suggest that the tissue expression profile of AQP1 is not constant amongst different fish species.

EFFECT OF CHRONIC SALINITY ACCLIMATION ON AQP1a TRANSCRIPT ABUNDANCE

We investigated AQP1a transcript abundance in gill, intestine, and kidney tissues taken from fish that were acclimated for 1 month to salinities ranging from freshwater (0 ppt) to extreme hypersaline (70 ppt). For gill, it was found that freshwater acclimation resulted in an elevated abundance of AQP1a transcript only whereas acclimation to salinities ranging from 6 to 70 ppt did not have any significant effect on gill AQP1a expression. This finding is in general agreement with those reported for black porgy AQP1 (An et al., 2008) and Atlantic salmon AQP1b (Tipsmark et al., 2010) where transcript amounts were higher in freshwater acclimated fish compared to those maintained in seawater. The elevated AQP1a transcript abundance in freshwater acclimated silver sea bream may contribute to the higher osmotic water permeability known to occur in gills of freshwater fish (Motais and Isaia, 1972). Preliminary immunohistochemical analysis has indicated that AQP1 could be localized within the chloride cells of sea bream gill (unpublished data). Although these findings await further and more stringent confirmation it could be plausible that AQP1a may act as an exit point to release water through the basolateral membrane of these cells to the serosal fluid in order to prevent cell swelling during hypoosmotic exposure. However, we cannot overlook the possibility that AQP1a may act in concert with other AQP homologs to prevent excessive cell swelling, in gill cells, as the amounts of the AQP3 homolog have been shown to increase upon freshwater acclimation of yellow and silver European eel (Cutler and Cramb, 2002) and sea bass (Giffard-Mena et al., 2007). Also a previous study on silver sea bream found that low (6 ppt) salinity acclimation caused a four-fold increase in gill AQP3 protein amounts in comparison to fish acclimated to seawater conditions (Deane and Woo, 2006a). We also studied intestinal AQP1a expression in silver sea bream and it was found that the largest and most significant increase in intestinal AQP1a transcript occurred following acclimation to extreme hypersaline (70 ppt) conditions. With regards to the increased AQP1a transcript in 70 ppt conditions our results generally support previous findings on European yellow and silver eels (Martinez et al., 2005b) and Japanese eels (Aoki et al., 2003) where seawater acclimation resulted in elevated AQP1 in comparison to fish acclimated to freshwater conditions. The elevated intestinal AQP1a expression would likely contribute to increased water permeability and hence a higher intestinal water absorption rate that would occur as silver sea bream were acclimated to hypersaline environments. It was also of interest that we found a significant increase in AQP1a transcript during isosmotic salinity (12 ppt) acclimation. Whilst there are no other studies that have reported similar findings we speculate that this may be a consequence of altered hormonal status in particular increased amounts of circulating growth hormone (GH). GH is well established to play a key role during seawater acclimation of fish (McCormick, 1995; Sakamoto and McCormick, 2006; Deane and Woo, 2009) and in several species, mainly belonging to the Sparidae, the highest amounts of GH have been shown to occur following isosmotic salinity acclimation (Mancera et al., 1995; Deane and Woo, 2004, 2006b, 2009). As we have shown previously that isosmotic salinity acclimation

of silver sea bream causes an increase in pituitary GH transcript then it is possible that the higher availability of GH could have altered intestine functions through increased water absorption and hence elevated AQP1a expression. To date no studies have examined the role and importance of GH on teleostean AQP expression and research within this context would be very informative in aiding our understanding of hormonal control of water regulation in fish. Together with the gill and intestine the kidney may also play a key role during salinity acclimation of fish particularly in relation to maintaining water balance via adjustments of glomerular filtration rate (McDonald and Grosell, 2006). Although aquaporins could be important in such processes, in the present study AQP1a transcript abundance remained unchanged in kidney from silver sea bream acclimated between freshwater to extreme hypersaline conditions. In this regard our data contrast to those reported for AQP1 from black porgy (An et al., 2008) and AQP1a from Atlantic salmon (Tipsmark et al., 2010) where transcript amounts were highest in freshwater acclimated fish in comparison to those maintained in seawater. Also renal AQP1b transcript amounts were found to be significantly lower in freshwater acclimated Atlantic salmon when compared to those maintained in seawater and indicates differences in AQP isoform expression (Tipsmark et al., 2010). The alterations in renal AQP1 expression levels, during salinity acclimation, may also exhibit species specificity as yellow and silver eels were found to have lowered or unchanged renal AQP1 transcript abundance, respectively, when fish were transferred from freshwater and acclimated to seawater for 3 weeks (Martinez et al., 2005a). Whilst we could not detect alterations in renal AQP1 for silver sea bream it is possible that other renal AQP homologs may be modulated and therefore play key roles in maintaining water balance. Indeed studies on European eel have demonstrated the existence of a renal AQP1 isoform (AQP1a) and an aquaglyceroporin (AQPe) both of which exhibit markedly higher transcription levels than AQP1 (Martinez et al., 2005a).

EFFECT OF ABRUPT SALINITY TRANSFER ON AQP1a TRANSCRIPT ABUNDANCE

To complement our chronic salinity acclimation studies we also investigated whether an abrupt transfer of silver sea bream from seawater (33 ppt) to hypoosmotic (6 ppt) conditions could have an effect on the transient expression of AQP1a. In gill, it was found that during the first 6 h, following hypoosmotic transfer, the abundance of branchial AQP1a transcript was lower than the seawater transfer control group although no statistical significant differences were found between salinity and/or time. However it is apparent when comparing our AQP1a expression data from chronic salinity acclimation and abrupt transfer experiments that during the hours following abrupt transfer different data are derived. It is possible that the rapid post exposure decrease in AQP1a transcript, following abrupt hypoosmotic transfer, indicates that there was a "shut-down" of water permeation across the gill epithelium to prevent further osmotic water load entry. Indeed a previous study on isolated Japanese eel gill preparations demonstrated that water permeability decreased within the first few hours after transfer from seawater to freshwater (Ogasawara and Hirano, 1984). It was also found from studies on Japanese eel that chloride cell number and apical pit diameter were also decreased within the first 6 h following transfer from seawater to freshwater (Ogasawara and Hirano, 1984). Similarly, in a previous study on abrupt transferred silver sea bream a transient reduction in gill chloride cell numbers was observed after a 6-h hypoosmotic exposure (Kelly and Woo, 1999). Also the morphology of the apical membranes of gill chloride cells changed from invaginated to co-existing on the same plane or protruding above pavement cells (Kelly and Woo, 1999). Given that our preliminary immunohistochemical studies (unpublished data) indicate that the majority of AQP1 is associated within the gill chloride cell then it is plausible that the decreased AQP1a transcript, found during early hypoosmotic transfer, is most likely associated with decreased chloride cell number as well as modifications of chloride cell morphology. For intestine, AQP1a transcript amounts were observed to fluctuate quite widely during the first 12 h in both seawater and hypoosmotic transferred groups but stabilized at 168 h post transfer. After statistical analysis it was found that the abundance of intestinal AQP1a was not significantly changed due to the interaction of salinity. However it was found that time did have a significant effect on transcript amounts which suggests that AQP1a expression is prone to wide temporal fluctuations during abrupt salinity transfer. In accordance with our findings for chronic salinity acclimated silver sea bream, renal AQP1a transcript amounts were unchanged following an abrupt hypoosmotic salinity transfer. Whilst it is possible that functional protein amounts could have been altered it would appear from transcript studies that there is probably little importance for renal AQP1a during conditions that can cause rapid hydration of silver sea bream. In freshwater teleosts the glomerular filtration rate is known to be very high in order to remove excess water (Nishimura and Imai, 1982) and therefore we cannot exclude the possibility that other aquaporin homologs are involved in this process but further and more comprehensive studies will be needed to investigate this conjecture.

MODULATION OF AQP1a TRANSCRIPT ABUNDANCE FOLLOWING CORTISOL ADMINISTRATION

The final part of this study aimed to investigate whether cortisol treatment could modulate AQP1a transcript amounts in silver sea bream. The circulating levels following cortisol administration demonstrated that the fish were hypercortisolemic with serum cortisol levels approximately 8–20 fold higher than controls. Similar conditions of hypercortisolemia have been previously shown to alter expression profiles of Na^+–K^+–ATPase and stress proteins in silver sea bream (Deane et al., 1999a,b, 2006; Deane and Woo, 2011). Even though fish were in a hypercortisolemic state we did not detect any significant alterations for both branchial and renal AQP1a transcript. To date no studies have examined the importance of cortisol on branchial AQP1a expression but our data on renal AQP1a are different from those reported for European eels where cortisol treatment caused an increase in AQP1 transcript abundance (Martinez et al., 2005a). Presently we cannot explain this disparity but the functional

role of cortisol may be dependent upon its receptors which in turn may exhibit species specificity. We did however observe that cortisol treatment caused an increase in AQP1a transcript in the intestine of silver sea bream that were transferred to either seawater or hypoosmotic conditions. A similar finding has been reported for freshwater acclimated yellow eels where cortisol treatment caused a three-fold increase in AQP1 transcript in intestinal epithelial scrapes (Martinez et al., 2005b). Cortisol has been shown to influence water transport in fish as the fluid uptake rate across the posterior intestine of Atlantic salmon was approximately doubled following cortisol treatment (Cornell et al., 1994). Also earlier studies have reported that the injection of cortisol mimicked changes in intestinal water movement during seawater transfer of European eel (Gaitskell and Chester Jones, 1970) and Japanese eel (Hirano and Utida, 1968). Therefore the findings from our study suggest that cortisol can modulate intestinal AQP1a expression in silver sea bream and this may be related to the cortisol enhanced intestinal water transport previously found in Atlantic salmon, European eel, and Japanese eel.

CONCLUSION

In this study, we have shown that the expression profile of sea bream AQP1a is significantly altered during chronic salinity acclimation although abrupt salinity transfer has little effect on transcript abundance. We cannot overlook the possibility that AQP1a may only play a partial role in regulating water exchange and the importance of other AQPs need to be fully understood and investigated. Studies on the effects of hormones on AQP expression, in fish, are few and in this study we were able to demonstrate that cortisol administration can modulate AQP1a in silver sea bream intestine. However studies aimed at addressing the role of other key osmoregulatory important hormones such as prolactin, GH, and insulin-like growth factor 1 would certainly prove to be worthwhile in aiding our understanding of how fish AQPs are regulated.

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Functional characterization of water transport and cellular localization of three aquaporin paralogs in the salmonid intestine

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Intestinal water absorption is greatly enhanced in salmonids upon acclimation from freshwater (FW) to seawater (SW); however, the molecular mechanism for water transport is unknown. We conducted a pharmacological characterization of water absorption in the rainbow trout intestine along with an investigation of the distribution and cellular localization of three aquaporins (Aqp1aa, -1ab, and -8ab) in pyloric caeca, middle (M), and posterior (P) intestine of the Atlantic salmon. *In vitro* iso-osmotic water absorption (J_v) was higher in SW than FW-trout and was inhibited by (mmol L^{-1}): 0.1 KCN (41%), 0.1 ouabain (72%), and 0.1 bumetanide (82%) suggesting that active transport, Na^+, K^+-ATPase and Na^+, K^+, $2Cl^-$-co-transport are involved in establishing the driving gradient for water transport. J_v was also inhibited by 1 mmol L^{-1} $HgCl_2$, serosally (23% in M and 44% in P), mucosally (27% in M), or both (61% in M and 58% in P), suggesting involvement of both apical and basolateral aquaporins in water transport. The inhibition was antagonized by 5 mmol L^{-1} mercaptoethanol. By comparison, 10 mmol L^{-1} mucosal tetraethylammonium, an inhibitor of certain aquaporins, inhibited J_v by 20%. In the presence of glucose, mucosal addition of phloridzin inhibited water transport by 20%, suggesting that water transport is partially linked to the Na^+-glucose co-transporter. Using polyclonal antibodies against salmon Aqp1aa, -1ab, and -8ab, we detected Aqp1aa, and -1ab immunoreactivity in the brush border and sub-apical region of enterocytes in all intestinal segments. The Aqp8ab antibody showed a particularly strong immunoreaction in the brush border and sub-apical region of enterocytes throughout the intestine and also stained lateral membranes and peri-nuclear regions though at lower intensity. The present localization of three aquaporins in both apical and lateral membranes of salmonid enterocytes facilitates a model for transcellular water transport in the intestine of SW-acclimated salmonids.

Keywords: aquaporin, osmoregulation, salmon, intestine, enterocyte, water absorption

INTRODUCTION

Animals living in a dehydrating environment need compensatory mechanisms in order to maintain osmotic homeostasis. Terrestrial animals largely overcome this challenge by drinking water followed by intestinal absorption. Accordingly, the intestine in these animals is developed in order to absorb quite large amounts of fluid. Per day the intestine of a human individual absorbs ca. Two liter of ingested water in addition to approximately 7 L of digestive juice (Ma and Verkman, 1999). Among aquatic animals, marine fishes use one of two strategies to avoid dehydration. Cartilaginous species actively build up the internal osmotic pressure by means of organic osmolytes to slightly exceed the external osmotic pressure. Thereby they may gain water passively across the gill epithelium. Teleost fishes, whether marine or euryhaline, maintain hypo-osmotic body fluids by drinking seawater (SW) and actively excreting surplus ions. It has been known since the classical paper by Smith (1932) that they swallow an appreciable amount of water (drinking rates typically in the range of 1–5 mL^{-1} kg^{-1} h^{-1}, Perrott et al., 1992), which is processed and partially absorbed in the gastrointestinal tract (GIT). Water absorption is basically solute linked as proposed in the original standing gradient model by Diamond and Bossert (1967). Several details are known with regard to the processing of imbibed SW along the length of the intestine, in particular concerning the accompanying transport of dissolved ions (see Grosell, 2011). In short, the fluid is desalinated in the esophagus and anterior parts of the GIT by absorption of the major monovalent ions Na^+ and Cl^-. While being moved peristaltically through the more posterior parts, monovalent ions are actively absorbed by enterocytes via a mechanism involving basolateral Na^+, K^+-ATPase and cystic fibrosis transmembrane conductance regulator (CFTR)-like Cl^--channels and apical Na^+, Cl^- and Na^+, K^+, $2Cl^-$-co-transporters as well as HCO_3^-/Cl^- exchangers. Across the basolateral membrane, Na^+ ions may be recycled as shown in the toad small intestine by Nedergaard et al. (1999). Divalent ions such as Mg^{2+} and Ca^{2+} are largely untouched and precipitate with bicarbonate and sulfate ions due

to the secretion of bicarbonate and successive osmotic removal of water (Grosell, 2011). Precipitation leads to a reduced osmotic pressure and thus eases the osmotic absorption further.

Whereas the molecular mechanism of salt transport in the GIT is known in some detail, the exact pathway by which water follows is not well established. Theoretically, it may occur via paracellular or transcellular pathways or a combination thereof (Laforenza et al., 2005). Fish and mammalian intestinal epithelia are generally considered as being leaky, thus allowing some paracellular passage of water (Loretz, 1995; Ma and Verkman, 1999). In mammals, the rectum on the other hand has higher transepithelial resistance (TER) and thus a tighter epithelium with low paracellular water permeability (Ma and Verkman, 1999). Sundell et al. (2003) found an increase in the intestinal iso-osmotic fluid uptake rate *in vitro* (J_v) during SW-acclimation of Atlantic salmon, *Salmo salar*. This was associated with an increase in TER probably caused by a tightening of the junctions between the enterocytes, and suggests that the increased water absorption occurs via a transcellular rather than paracellular pathway in SW-acclimated salmon.

Transcellular water flux may involve three different pathways: passive diffusion across the lipid bilayers, co-transport with ions and nutrients, and diffusion through aquaporins in the membranes. Water transport across biological membranes was long considered to occur by simple diffusion through the lipid bilayer. But the discovery of the first aquaporin by Preston et al. (1992) showed that water transport across membranes may indeed be greatly enhanced by insertion of molecular water channels. Furthermore, trafficking of aquaporins to and from membranes makes a powerful mechanism to regulate epithelial and cellular water flux both in the short and long term (Gradilone et al., 2003; Valenti et al., 2005; Tingaud-Sequeira et al., 2008). In mammals, it has been shown that the majority of intestinal water absorption occurs through aquaporins present in both apical and basolateral membranes of enterocytes. Aquaporins 1, 3, 4, 7, 8, and 10 have all been demonstrated in enterocytes or surrounding associated endothelial tissue in mammals (Ma and Verkman, 1999; Ishibashi et al., 2009). In addition to aquaporins, it has been proposed that substantial amounts (50%) of water may be transported by the apical Na^+-glucose cotransporter (SGLT1; Loo et al., 2002). This requires, however, that glucose is present in the intestinal lumen. The SGLT1 has been demonstrated by immunocytochemistry (ICC) in the rainbow trout enterocytes (Polakof et al., 2010) but its role in water absorption has to our knowledge never been studied in fish. However, since fish can undergo extended periods of starvation without losing homeostatic control of water balance, it is reasonable to speculate that the role may be minor or at least transient.

Our current knowledge about aquaporins in fish is still rather fragmentary and gathered from many different species. In the whole genome of zebrafish, *Danio rerio*, 10 aquaporin subfamilies are found, some of which have duplicate or triplicate paralogs (reviewed by Cerdà and Finn, 2010). As in mammals, the expression of specific paralogs appears to be related to tissue type. Some paralogs are expressed more generally whereas others are restricted to certain tissues. In teleosts, the following aquaporins have been demonstrated at mRNA level, protein level, or both in various intestinal segments (esophagus, anterior, posterior region, rectum): Aqp1aa/ab (European eel, *Anguilla anguilla*: Martinez et al., 2005a; Japanese eel, *A. japonica*: Aoki et al., 2003; Kim et al., 2010; European seabass, *Dicentrarchus labrax*: Giffard-Mena et al., 2007; Atlantic salmon, *S. salar*: Tipsmark et al., 2010; seabream, *Sparus aurata*: Raldúa et al., 2008), Aqp3 (European eel: Cutler et al., 2007; European seabass: Giffard-Mena et al., 2007; Atlantic salmon: Tipsmark et al., 2010); Aqp4, -7, -8aa (zebrafish: Tingaud-Sequeira et al., 2010), Aqp8ab (zebrafish: Tingaud-Sequeira et al., 2010; Atlantic salmon: Tipsmark et al., 2010), Aqp10 (European eel: Martinez et al., 2005a; Japanese eel: Kim et al., 2010; seabream: Santos et al., 2004; Atlantic salmon: Tipsmark et al., 2010), and Aqp11b and -12 (zebrafish: Tingaud-Sequeira et al., 2010). Only Aqp1aa/ab have been localized in enterocytes and thus directly associated with water transport (European eel: Martinez et al., 2005b; Japanese eel: Aoki et al., 2003; seabream: Raldúa et al., 2008). All other aquaporins have either been found in other cell types (mucus cells, lamina propria) or not been localized yet.

On this basis, the present study investigated the mechanism for water transport in the intestine of two salmonids acclimated to SW: rainbow trout, *Oncorhynchus mykiss*, and Atlantic salmon. We first characterized water transport in isolated intestinal segments in rainbow trout and showed how it was affected by pharmacological agents known to interfere with ion and metabolite transport and aquaporins. Next we developed homologous antibodies against Atlantic salmon aquaporins-1aa, -1ab, and -8ab (formerly Aqp8b in Tipsmark et al., 2010) and investigated their cellular localization by ICC and confocal microscopy. Whereas the intestinal cellular localization of Aqp1aa/ab has been investigated in other species, Aqp8ab is a novel paralog in any teleost in this respect.

MATERIALS AND METHODS

FISH AND MAINTENANCE

For characterization of intestinal water transport we used 1^+-year-old rainbow trout (ca. 200 g) from an all-female stock obtained from a trout hatchery (Lime Hatchery, Randbol, Denmark). Upon arrival to the Institute of Biology at the University of Southern Denmark (SDU) they were acclimated for at least 4 weeks to 28 ppt re-circulated and biofiltered artificial SW (Red Sea Salt, Eliat, Israel) in 400 L tanks (14°C, 12:12 light:dark period). For immunolocalization studies we used 1-year-old Atlantic salmon (ca. 25 g) obtained as pre-smolts from The Danish Centre for Wild Salmon (Randers, Denmark). After spending the spring in outdoor tanks on the SDU Campus, they were transferred to indoor 400 L tanks in late June with constant temperature and light conditions (14°C, 12:12 light:dark). The tanks were supplied with re-circulated and biofiltered fresh water. In September, a batch of fish was transferred directly to 25 ppt SW (14°C) and acclimated for 3 weeks. All fish were fed 1% body weight^{-1} per day and food was generally withheld 4 days before an experiment. All experimental protocols were approved by the Danish Animal Experiments Inspectorate and in accordance with the European convention for the protection of vertebrate animals used for experiments and other scientific purposes (#86/609/EØF).

MEASUREMENT OF ISO-OSMOTIC *IN VITRO* WATER ABSORPTION RATE (J_V)

For historical reasons, two slightly different techniques were used to measure water transport capacity under iso-osmotic conditions

in vitro. The methods were previously cross-validated and gave values in the same range (not shown). In both, the intestine was isolated from a freshly killed rainbow trout and perivisceral fat was carefully removed. The lumen was rinsed with Ringer's solution (in mmol L^{-1}: 140 NaCl, 2.5 KCl, 15 $NaHCO_3$, 1 KH_2PO_4, 1.5 $CaCl_2$, 0.8 $MgSO_4$, 10 D-glucose equilibrated with 99% O_2/1% CO_2, pH 7.8) and the intestine was transferred to a Petri dish with Ringer's containing 5 mg L^{-1} of the smooth muscle relaxant papaverine for 10–20 min at 15°C. In some experiments (ouabain, bumetanide, KCN, mercury) J_v was analyzed separately in the middle (light colored) and posterior (darker colored) segments with reference to the ileo-rectal valve (**Figure 1A**). This was done using the non-everted sac method as described by Collie and Bern (1982). In short, a polyethylene tube (inner diameter 1.1 mm, outer diameter, 1.6 mm) with a bulged end was inserted into the anterior end of the segment and fixed by cotton thread. After further rinsing of the lumen with Ringer's, the other end was either tied with cotton thread and the segment was filled with Ringer's through a syringe in the tube. Each sac was carefully checked for leaks before closing the tube with a plastic plug. The samples were pre-incubated for 30 min in 15°C Ringer's solution with 5 mg L^{-1} papaverine and bubbled with 99% O_2/1% CO_2. After that, the contents were replaced with the test solution and the rate of water transport, J_v (in μL cm^{-2} h^{-1}) was calculated from the decrease of the blotted wet weight of the sacs (linear regression) normalized to surface area of the cut open segment. The weight loss of each sac was monitored every 15 min for 1.5 h. At the end of an experiment, the surface area was measured by spreading out the opened segment on a glass slide and pressing between two slides with clamps. The outline was marked and transferred to paper with a given weight per square centimeter, cut out, weighed and the area calculated.

In other experiments (phloridzin and tetraethylammonium) the whole (i.e., middle + posterior) intestine was used intact according to the method of Usher et al. (1991). In this method the intestine was held in place in a 500-mL chamber and a 10-cm polyethylene tube (same dimensions as above) was inserted into the anterior end connected to a syringe. The posterior end was connected to a 35-cm vertically placed tube. The intestine and tubing were filled with Ringer's and the decrease in the meniscus height was measured every 15 min for 1 h (control) and converted into μL h^{-1} (8.67 μL cm^{-1} tube). The intestine was rinsed every 30 min to prevent mucus accumulation. After 1 h the Ringer's solution was replaced with the test solution and the measurement was continued for 2.5 h. Control incubation with standard Ringer's was made in a separate series and used for comparison with the test agents. Thus for each preparation J_v was measured before and after pharmacological treatment and the effect of the agent was calculated in percent.

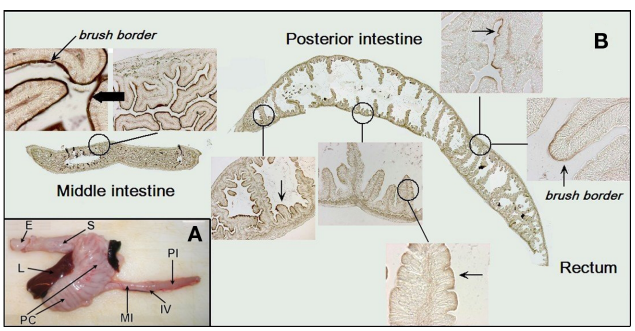

FIGURE 1 | (A) An overview of the morphology of the gastrointestinal tract in Atlantic salmon. **(B)** A longitudinal section of the entire middle and posterior intestine from SW-acclimated salmon with higher magnifications shown as inserts. The brush border is visualized by staining for alkaline phosphatase activity with NBT/BCIP. Staining of the brush border (dark brown) is seen throughout the intestine, however, more intensely in the middle segment. Abbreviations: E, esophagus; L, liver; S, stomach; PC, pyloric caeca; MI, middle intestine; PI, posterior intestine; IV, ileo-rectal valve.

EXPERIMENTS

In different series of experiments the effects of the following pharmacological agents dissolved in Ringer's solution were tested: ouabain (0.5 mmol L^{-1} serosally; blocks Na^+, K^+-ATPase), bumetanide (0.5 mmol L^{-1} mucosally; blocks Na^+, K^+, $2Cl^-$ co-transport), KCN (0.5 mmol L^{-1} serosally; metabolic poison), phloridzin (1 mmol L^{-1} mucosally; blocks Na^+-glucose co-transport), mercury (1 mmol L^{-1} $HgCl_2$ mucosally, serosally, or both; blocks certain aquaporins), tetraethylammonium (TEA, 10 mmol L^{-1} mucosally, blocks certain aquaporins). An experiment typically included the following steps after the initial pre-incubation in Ringer's (30 min): (1) replacement of mucosal solution with fresh Ringer's solution and gravimetric measurement of J_v for 1.5 h; (2) replacement of mucosal/serosal solution with test solution (above) and pre-incubation for 30 min; (3) replacement with fresh test solution and gravimetric measurement of J_v for 1.5 h. Following the experiments with mercury, the mercury Ringer's was replaced with Ringer's containing 5 mmol L^{-1} 2-mercaptoethanol in an attempt to antagonize the mercury effect. After a 30-min pre-incubation, the mercaptoethanol Ringer's was replaced with a fresh solution and the J_v measured for the next 1.5 h.

ANTIBODIES

Messenger RNA sequences coding for salmon aquaporins were found in the Atlantic salmon EST (expressed sequence tags) database in GenBank using the following UniGene accession numbers: BT046625 (*aqp1aa*), BT045044 (*aqp1ab*), Ssa.15811 (*aqp8ab*). The *aqp8ab* paralog corresponds to the former *aqp8b* paralog reported by Tipsmark et al. (2010) and was renamed in order to match the nomenclature used in zebrafish (Cerdà and Finn, 2010). The mRNA sequences were translated into protein sequences with the following predicted molecular weights: Aqp1aa: 27.3 kDa, Aqp1ab: 27.8 kDa, Aqp8ab: 27.5 kDa. Based on antigenicity plots, the following sequences were used for immunization: Aqp1aa: C-terminal residues 243–256: GDYDVNGEETAAVE; Aqp1ab: C-terminal residues 240–253: GPDKENDAPEEGSS; Aqp8ab: N-terminal residues 11–24: GHSTLMSGTKKPTP. Polyclonal antibodies were produced in rabbits by BioGenes (Berlin, Germany). For each antibody the serum preparation was affinity purified and the titer was measured by BioGenes. The affinity purified antibodies were tested in Western blotting and used for immunofluorescence.

WESTERN BLOTTING ANALYSIS

For validation of the antibodies homogenates of pyloric caeca were prepared as described below. For Aqp1aa a whole-kidney homogenate was used for comparison, since Aqp1aa is expressed at higher levels in kidney tubules (Madsen, unpublished data). SW-acclimated salmon were anesthetized in MS222 and killed by decapitation. Pyloric caeca (and kidney tissue) were homogenized in sucrose–EDTA–imidazole buffer (SEI; in mmol L^{-1}: 300 sucrose, 20 Na_2EDTA, 50 imidazol, pH 7.3) and a cocktail of protease inhibitors (P8340; Sigma-Aldrich, St. Louis, MO, USA) with a Polytron PT 1200 CL for 20 s. The homogenate was centrifuged 10 min at 2000 *g* and the pellet containing whole cells, mitochondria and nuclei was discarded. The supernatant was then centrifuged 40 min at 60,000 *g* at 4°C and the membrane fraction and supernatant was separated. The membrane fraction was resuspended in SEI buffer containing 2% (w/v) sodium deoxycholate and the supernatant was added deoxycholate to a concentration of 2% (w/v). Supernatant (pyloric caeca) or the membrane fraction (kidney) was used for Western blotting of the individual aquaporins depending on the abundance of the proteins in the fraction. An aliquot of the homogenate was saved for measuring protein concentration using the method described by Lowry et al. (1951). The NuPAGE system (Invitrogen, Carlsbad, CA, USA) was used for electrophoresis and blotting according to manufacturer's protocols. The samples were mixed with reducing sample buffer (NuPAGE® LDS Sample Buffer, Invitrogen) containing 3% dithiothreitol (Merck chemicals, Darmstadt, Germany) and heated at 70°C for 10 min ensuring reduction of the proteins. The samples were loaded on a 4–12% gradient Bis–Tris polyacrylamide gel (NuPAGE® 4–12% Bis–Tris Gel, Invitrogen) in a volume corresponding to 150 μg protein. The electrophoresis was run at 200 V for 35 min with MES-running buffer (Invitrogen). The proteins were blotted on a nitrocellulose membrane in a tris–glycine transfer buffer (in mmol L^{-1}: 7.5 tris-base, 60 glycine, and 20% methanol) for 120 min at 25 V. The membrane was blocked 1 h in blocking buffer containing 2% bovine serum albumin (BSA) in TBS-T (in mmol L^{-1}: 20 tris-base, 140 NaCl, 1% Tween 20) followed by incubation overnight at 4°C with primary antibody (1–5 μg mL^{-1}) in blocking buffer. After four washes in TBS-T, the membrane was incubated with goat anti-rabbit IgG antibody (1:5000) coupled to the fluorescent dye Cy5 (Invitrogen). After four washes and drying overnight, the fluorescence was measured using in a Typhoon Trio Variable mode Imager (GE Healthcare, Little Chalfont, UK) set to the Cy5 excitation and emission wavelengths (649 and 670 nm respectively). As control for antibody specificity, strips of membranes were incubated with up to 400-fold molar excess of the corresponding immunization peptide. These membranes were compared to membranes incubated with the corresponding antibody alone.

GENERAL HISTOLOGICAL PROCEDURES

Pyloric caeca, anterior and posterior intestines were sampled from SW-acclimated salmon, immediately fixed in paraformaldehyde (4% PFA, 0.9% NaCl in 5 mmol L^{-1} NaH_2PO_4, pH 7.4) overnight at 4°C. After being washed four times in 70% ethanol, the tissues were processed through graded series of ethanol and xylene, and finally embedded into paraffin. Serial sections were cut on a Zeiss Microtome (Microm HM355; Carl Zeiss, Jena, Germany) and placed onto Superfrost Plus glass slides (Thermo Fisher Scientific Inc., Waltham, MA, USA) for subsequent analysis.

OVERVIEW OF THE GROSS MORPHOLOGY OF THE ATLANTIC SALMON INTESTINE

A deparaffinized 10 μm longitudinal section of the entire middle and posterior intestine of a SW salmon was used to visualize the apical brush border. The section was incubated with a combination of 0.4 mmol L^{-1} 5-bromo-4-chloro-3′-indolyphosphate *p*-toluidine salt (BCIP) and 0.4 mmol L^{-1} nitro-blue tetrazolium chloride (NBT) in Tris–HCl buffer (in mmol L^{-1}: 0.1 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride, 0.1 NaCl, 10 $MgCl_2$, pH 9.8) for 10 min which yields an intense, insoluble black–purple precipitate when reacted with alkaline phosphatase (AP). Since AP is a marker enzyme of the intestinal brush border, this method can be used to visualize the presence of microvilli.

IMMUNOFLUORESCENCE LIGHT MICROSCOPY AND CONFOCAL MICROSCOPY

Microslides with 5 μm paraffin sections were placed vertically in an oven at 55°C overnight after which they were deparaffinized and demasked by boiling 10 min in TEG buffer (in mmol L^{-1}: 10 tris-base, 1 EGTA, pH 9). Subsequently they were transferred to 50 mmol L^{-1} NH_4Cl in phosphate buffered saline (PBS, in mmol L^{-1}: 137 NaCl, 2.7 KCl, 4.3 Na_2HPO_4, 1.4 KH_2PO_4, pH 7.3) for 30 min and blocked with 3% (BSA) in PBS for 30 min at room temperature. The slides were dual labeled by incubating overnight at 4°C with a cocktail (in PBS with 1.5% BSA) of primary polyclonal rabbit antibodies for one of the aquaporins (1–5 μg mL^{-1}) and a monoclonal mouse antibody recognizing all isoforms of the Na^+, K^+-ATPase alpha subunit (0.5–1 μg mL^{-1}; alpha-5, The Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA). After two washes in PBS at room temperature, the slides were incubated for 2 h at 37°C with a cocktail of two fluorescent secondary antibodies (Alexa Fluor® 568 goat anti-rabbit and Oregon Green® 488 goat anti mouse IgG, 1:600 dilution; Invitrogen). After two washes in PBS, nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI, 0.1 μg mL^{-1} in PBS) for 10 min. Before being sealed with Vectashield (Vector Labs, Burlingame, CA, USA), the slides were washed four times in PBS and two times in milli-Q water. Negative control incubations with 3% BSA in PBS instead of primary antibodies were made routinely. The fluorescence was inspected on a Leica HC microscope and pictures of representative areas were captured using a Leica DC 200 camera. Confocal images were taken on a Zeiss LSM510 META confocal microscope (Carl Zeiss) using objectives at 63× and 100× magnification.

STATISTICAL ANALYSES

The effect of mercury and mercaptoethanol on J_v were evaluated by repeated measures one-way ANOVA followed by a Bonferroni-adjusted Fishers least significant differences test ($p < 0.05$). For all other pharmacological agents, the (negative) effects on *in vitro* water transport capacity (J_v) were evaluated by a paired one-tailed Student's *t*-test with a significance level of $p < 0.05$. Statistics were

carried out using the GraphPad Prism 5.0 software (San Diego, CA, USA).

RESULTS

OVERALL MORPHOLOGY

The gastrointestinal system of Atlantic salmon is shown in **Figure 1A** with the segments involved in water absorption indicated on the figure: the anterior region with numerous pyloric caeca, the middle and posterior segments. The longitudinal section in **Figure 1B** shows the intensive folding of the luminal epithelium with a clear indication of the apical brush border judged from the positive reaction for AP. The AP reaction is more intense in the middle than in the posterior brush border. The intense brush border is also clearly seen in the transmission electron micrograph in **Figure 2**, together with other characteristics of enterocytes: apical tight junctions, lateral membrane, several mitochondria, and vesicles in the sub-apical compartment.

VALIDATION OF ANTIBODIES

The specificity of the affinity purified polyclonal antibodies was validated by Western blotting of tissue homogenates as shown in **Figure 3**. All three antibodies (Aqp1aa, Aqp1ab, and Aqp8ab) detected a protein around 30 kDa in addition to both lower and higher molecular weight bands. For Aqp1aa the strongest band was observed around 40 kDa. The 30-kDa band matches the expected Mr of the native aquaporin whereas the higher molecular weight bands may represent various glycosylated forms of the aquaporin. The 30-kDa band and those of higher Mr disappeared in the neutralization experiment with 400× molar excess of the immunization peptide.

EFFECT OF PHARMACEUTICALS ON J_V

The iso-osmotic water transport rate (J_v) tested in the middle intestine segment was higher in SW than in freshwater (FW) fish (**Figure 4A**). All pharmacological agents significantly inhibited J_v when tested in FW and/or SW intestines: ouabain (FW): 66%; bumetanide (FW): 67%; ouabain (SW): 72%; bumetanide (SW): 82%; KCN (SW): 68%; phloridzin (SW): 20%; TEA (SW): 20% (**Figure 4**).

In the middle intestine, mercury (**Figure 5**) inhibited J_v, when added mucosally (42%) and when added to both sides simultaneously (70%). In the posterior segment the effect of mercury was somehow different: there was a tendency that mucosal addition of mercury increased J_v ($p = 0.062$), had no effect when added serosally, whereas a strong inhibitory effect (78%) was observed when added to both sides simultaneously. Mercaptoethanol completely abolished all observed effects of mercury except when added mucosally in the posterior segment, where it reinforced the mercury effect.

IMMUNOHISTOCHEMISTRY AND CONFOCAL MICROSCOPY

In pyloric caeca there were distinct immunoreactions to all three aquaporins (Aqp1aa, Aqp1ab, and Aqp8ab) in the brush border membrane (**Figure 6**). Aqp1aa and -1ab also stained positive in the sub-apical domain of enterocytes, whereas Aqp8ab was restricted to the outermost zone with microvilli.

Aqp1aa immunoreaction was confined to the apical brush border zone in the middle and posterior intestine (**Figure 7**). The intensity of the reaction was generally not very strong. Occasionally, goblet cells also stained positive for this aquaporin in the middle but not in the posterior segment or pyloric caeca.

Staining for Aqp1ab was also found in the brush border throughout the intestine (**Figure 8**). In the middle segment it was confined to the outermost zone with microvilli, with almost no sub-apical staining. The sub-apical cytoplasmic zone was

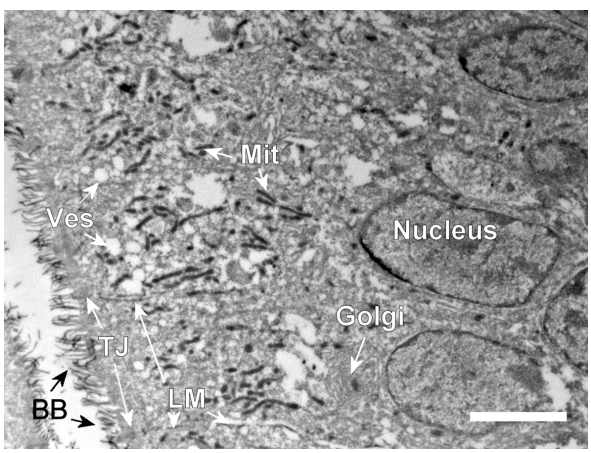

FIGURE 2 | Transmission electron micrograph from the middle intestine of SW-acclimated Atlantic salmon. The apical brush border is seen to the left (BB). Tight junctions (TJ), lateral membrane (LM), cytoplasmic vescicles (Ves), mitochondria (Mit), nucleus, and Golgi apparatus are indicated. Size bar = 5 μm.

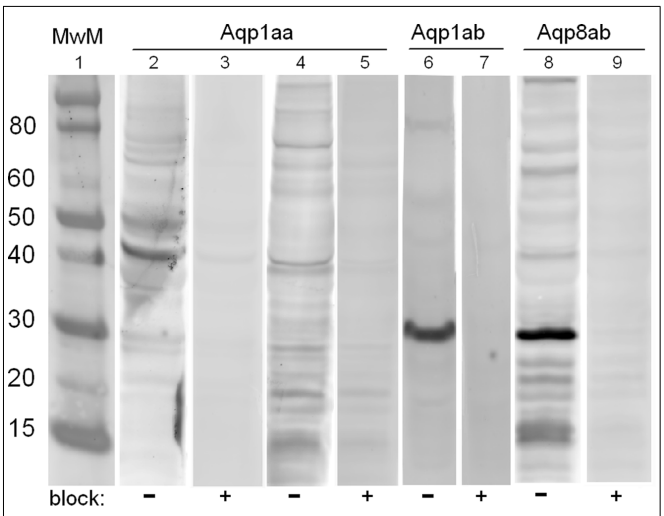

FIGURE 3 | Representative Western blots showing immunoreactive bands using homologous, affinity purified antibodies against Aqp1aa (lanes 2–5), Aqp1ab (lanes 6–7) and Aqp8ab (lanes 8–9) in homogenates from pyloric caeca (lanes 4–9), or kidney (lanes 2–3) from SW Atlantic salmon. In lanes 3, 5, 7, and 9, where the antibodies were preadsorbed with 400× molar excess of the respective immunizing peptide, the band intensities were much reduced. Molecular weight markers of the indicated sizes (MwM, in kDa) are shown in lane 1.

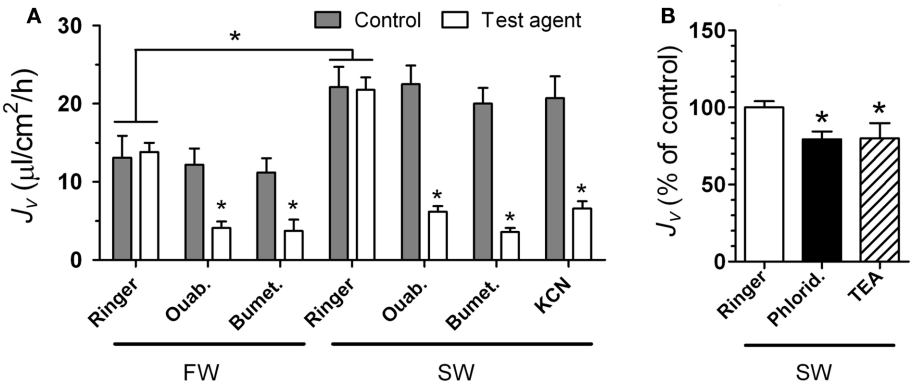

FIGURE 4 | The effect of various inhibitors on iso-osmotic water transport (J_v) in the intestine of rainbow trout. In **(A)** trout were acclimated to either FW or SW and non-everted sacs of the posterior intestine were used for the measurements of J_v (shown in $\mu L\,cm^{-2}\,h^{-1}$): ouabain (Ouab) was added to the serosal side; bumetanide (Bumet), and KCN were added to the mucosal side. Following the control measurement, intestinal sacs were incubated for 30 min with the test agent before the effect was evaluated. All inhibitors were applied at 10^{-4} M concentrations. In **(B)** whole (i.e., middle + posterior) intestines from SW trout were used to measure J_v (shown in % of control): phloridzin (1 mmol L^{-1}) or TEA (10 mmol L^{-1}) were added to the mucosal side. Asterisk indicates difference from control value (ringer; $p < 0.05$). All values represent mean ± SEM, $n = 6$–8.

significantly stained, however, in the posterior intestine – especially in the most distal part near the rectum.

Aqp8ab gave very strong staining intensity of the intestinal brush border (**Figure 9**), and in the posterior segment staining of peri-nuclear and lateral membrane structures was also revealed especially when using higher magnification (**Figure 10**). In the lateral membrane Aqp8ab co-localized with the Na^+, K^+-ATPase. No such lateral staining was seen using the Aqp1aa and −1ab antibodies.

The aquaporin sequences were subject to analysis for potential phosphorylation sites by using the NetPhos 2.0 server (www.cbs.dtu.dk; Blom et al., 1999; **Figure 11**). Aqp1aa has one tyrosine residue with high phosphorylation probability, whereas Aqp1ab has four serine residues with high probability near the cytoplasmic C-terminal. Aqp8ab has no phosphorylation sites in the cytoplasmic C-tail; however, a single threonine residue near the N-terminus and a single serine residue in the second cytoplasmic loop have high phosphorylation potential.

DISCUSSION

In the intestine of marine fish, osmotic water absorption across the luminal epithelium may occur via one or more of different routes: paracellularly between enterocytes or transcellularly across the lipid bilayers, through aquaporins or as co-transport with ions and nutrients (Laforenza et al., 2005; see **Figure 12**). The present study focused on a pharmacological characterization of this transport mechanism and suggests the contribution of three Aqp paralogs in the salmon intestine: Aqp1aa, -1ab, and -8ab. Among these the role of Aqp8ab is particularly interesting, since it has not been demonstrated before. It is strongly increased in response to SW-transfer (Tipsmark et al., 2010) and it is expressed both apically and laterally in enterocytes.

PHARMACOLOGICAL CHARACTERIZATION OF WATER TRANSPORT

In a series of *in vitro* experiments, we characterized the nature of iso-osmotic water transport across the intestinal epithelium in rainbow trout. The reason for using rainbow trout was that they could be obtained in a size that was reasonable for studies of this kind. Furthermore, there is no reason to speculate different mechanisms for water absorption in different salmonids. Iso-osmotic solutions were used on both sides of the intestine, thus any observed net water transport must occur as a result of osmotic gradients established on a micro scale. SW-fish generally had higher J_v than FW fish, indicating that the intestinal epithelium is modified in response to hyper-osmotic exposure. This is in accordance with earlier studies in salmonids (Collie and Bern, 1982; Veillette et al., 1993) and is also seen during smoltification in preparation to seaward migration (Sundell et al., 2003). As expected, J_v was significantly inhibited by serosal addition of ouabain and mucosal addition of bumetanide indicating the involvement of both basolateral Na^+, K^+-ATPase and apical absorptive Na^+, K^+, $2Cl^-$-co-transport in the process. Furthermore, the metabolic poison KCN also inhibited J_v. None of the agents inhibited J_v by 100% within the timescale used here but the findings are in accordance with the standing gradient model for transepithelial water transport (see Larsen and Møbjerg, 2006). This mechanism has also been suggested, although not directly proven for coho salmon, *O. kisutch*, and Atlantic salmon, where ouabain was shown to decrease the J_v across intestinal sac preparations by 67–100% (Collie and Bern, 1982; Veillette et al., 1993).

Our experiments with mercury chloride revealed that water transport was significantly inhibited by this compound, both when added serosally and mucosally. A puzzling exception to this was seen in the posterior segment, where mercury did not have a significant effect when added only mucosally. The highest degree of inhibition was, however, obtained when added to both sides simultaneously in both middle and posterior segments. This effect was reversibly blocked by mercaptoethanol. Based on osmotic swelling assays with heterologously expressed aquaporins in *Xenopus* oocytes, mercury has been shown to inhibit water transport by certain Aquaporin orthologs (AQP1 in mammals: e.g., Savage and Stroud, 2007; Aqp1, Aqp1dup, Aqp3, Aqpe in European eel:

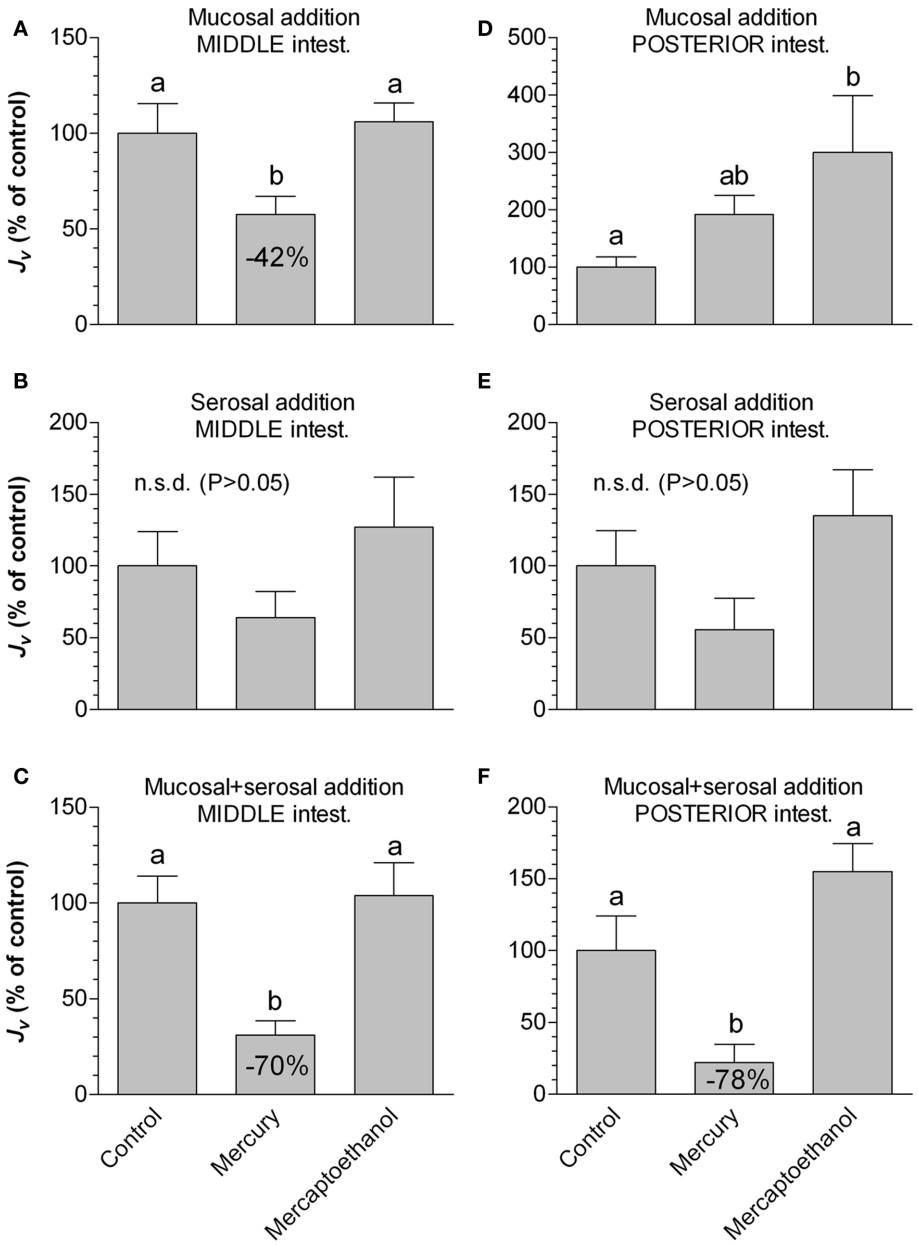

FIGURE 5 | The effect of mercury and subsequent mercaptoethanol on water transport rate (J_v in % of control values) in non-everted sacs of middle (A–C) and posterior (D–F) segments of intestine from SW-acclimated rainbow trout. In different series the test agents were added to either the mucosal **(A–D)**, serosal **(B–E)**, or both sides simultaneously **(C–F)**. Bars sharing identical letters are not significantly different ($p > 0.05$). Values represent mean ± SEM, $n = 8$.

MacIver et al., 2009) by interacting with the central pore (Savage and Stroud, 2007). In the eel study, the degree of inhibition by mercury was 54–64% for each individual aquaporin. It has also been reported that mercury inhibits water transport through AQP8 100% when reconstituted into proteoliposomes (Liu et al., 2006). Therefore, our findings support the involvement of aquaporins in water transport in the salmonid intestine. However, data based on studies in intact epithelia should be interpreted with great care, as mercury is likely to interact with other important elements of water transport such as Na^+, K^+-ATPase (Wang and Horrisberger, 1996), Na^+, K^+, $2Cl^-$-co-transporter (Kinne-Saffran and Kinne, 2001) and also the status of tight junction proteins (Kawedia et al., 2008). Therefore alternative aquaporin inhibitors should be tested as well. In some studies it has been reported that quaternary ammonium compounds such as TEA inhibit water transport through AQP1, -2 and -4 but not AQP3 and -5 when expressed in *Xenopus* oocytes (AQP1: Brooks et al., 2000; Detmers et al., 2006) and mammalian cells (AQP1: Yool et al., 2002). We found

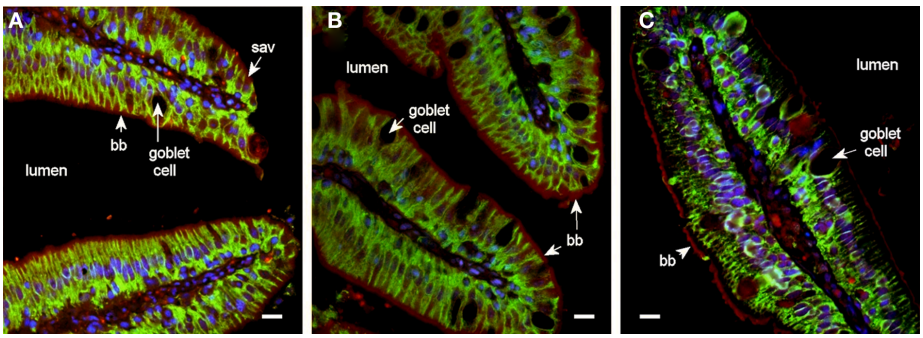

FIGURE 6 | Fluorescent microscope images (40× objective) showing localization of Aqp1aa (A), Aqp1ab (B), and Aqp8ab (C) in pyloric caeca from SW-acclimated Atlantic salmon. Sections were incubated with antibodies against the aquaporin (red), Na^+, K^+-ATPase (green), and nuclei were counterstained with DAPI (blue). Lumen, goblet cells, nuclei, brush border (bb), and sub-apical vesicles (sav) are indicated. Note the distinct immunoreaction to all three aquaporins in the brush border and for Aqp1aa and −1ab also in the sub-apical cytoplasmic domain. Size bar = 20 μm.

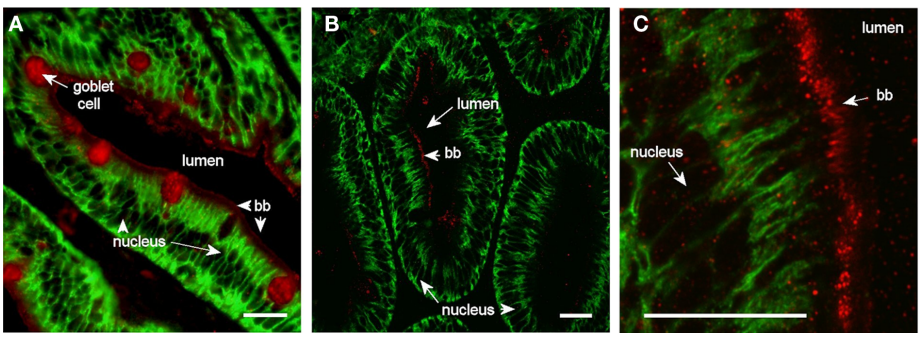

FIGURE 7 | Confocal images (63× objective) showing localization of Aqp1aa in middle (A) and posterior (B,C) segments of intestine from SW-acclimated Atlantic salmon. Sections were incubated with a cocktail of antibodies against Aqp1aa (red) and Na^+, K^+-ATPase (green). Lumen, goblet cells, nuclei, and brush border (bb) are indicated. Size bar = 20 μm.

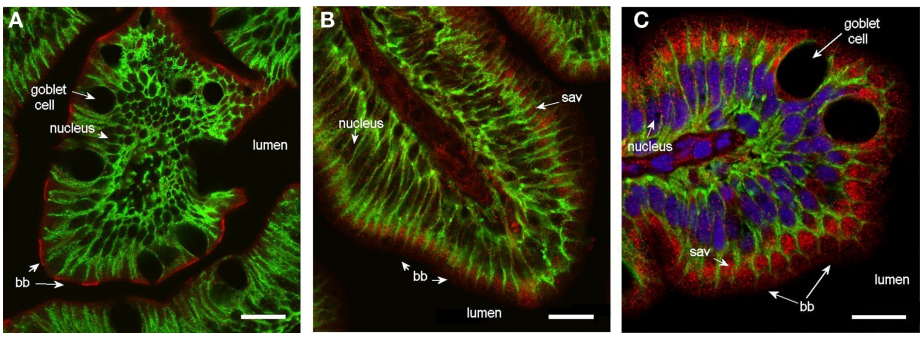

FIGURE 8 | Confocal images (63× objective) showing localization of Aqp1ab in middle (A) and posterior segments (B,C) of intestine from SW-acclimated Atlantic salmon. (B,C) are from the proximal and distal region of the posterior segment, respectively, with reference to the ileo-rectal valve. Sections were incubated with a cocktail of antibodies against Aqp1ab (red), Na^+, K^+-ATPase (green), and nuclei were counterstained with DAPI [blue, only in **(C)**]. Lumen, goblet cells, nuclei, brush border (bb), and sub-apical vesicles (sav) are indicated. Size bar = 20 μm.

a 14% inhibition of J_v by 10 mmol L^{-1} TEA, thus adding to the evidence that aquaporins are involved in water transport. A possible explanation for the relatively small inhibition by TEA is the fact that several aquaporins are expressed in the intestine, some of which may not be sensitive to TEA. TEA also inhibits potassium channels even though the potency is 1000-fold lower (Detmers et al., 2006).

We also tested the effect of phloridzin which is a recognized inhibitor of the apical sodium–glucose symporter (SGLT1; Xia et al., 2003). The effect was marginal when compared to the effects

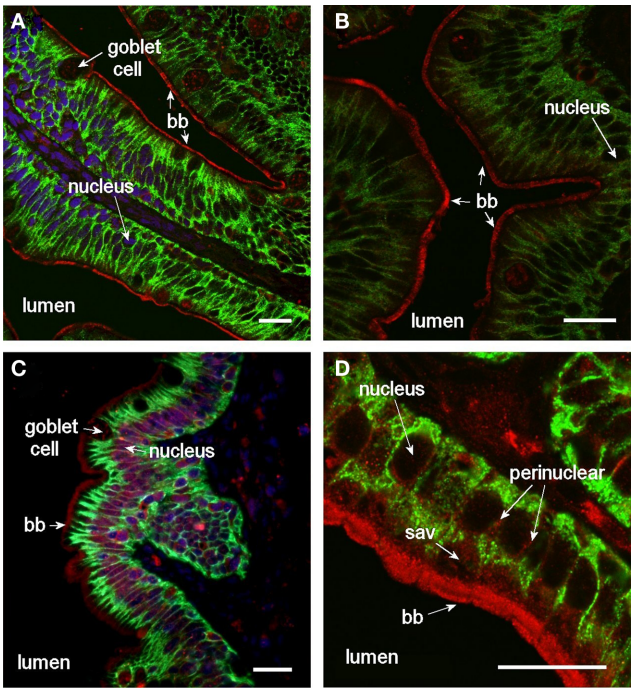

FIGURE 9 | Confocal images (63× objective) showing localization of Aqp8ab in middle (A,B) and posterior (C,D) segments of intestine from SW-acclimated Atlantic salmon. Sections were incubated with a cocktail of antibodies against Aqp8ab (red), Na^+, K^+-ATPase (green), and nuclei were counterstained with DAPI [blue, in **(A–C)**]. Lumen, goblet cells, nuclei, brush border, (bb) and sub-apical vesicles (sav) are indicated. Size bar = 20 μm.

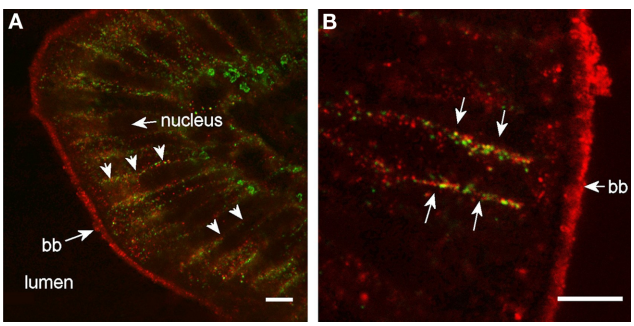

FIGURE 10 | High magnification confocal images (100× objective) showing localization of Aqp8ab in the posterior segment of intestine from SW-acclimated Atlantic salmon. Sections were incubated with a cocktail of antibodies against Aqp8ab (red) and Na^+, K^+-ATPase (green). Lumen, nuclei, brush border (bb), and lateral membranes (arrows) are indicated. Size bar = 20 μm.

of all other agents, indicating that water transport accompanying the function of this protein is not a major route. We tested the effect on both fed and starved fish but found no difference (Madsen, unpublished data). We suspect that this transporter may have a more significant role in water transport in the anterior part of the intestine (including pyloric caeca), where nutrient absorption is assumed to be higher. Unpublished observations in our laboratory have shown that the SGLT1 transcript is expressed in all intestinal segments – the order of expression being pyloric caeca > middle > posterior intestine. This supports a previous observation of the presence of the SGLT1 protein in the brush border of absorptive enterocytes in the rainbow trout (Polakof et al., 2010).

VALIDATION OF AFFINITY PURIFIED ANTIBODIES

Western blotting experiments on homogenates from intestinal tissues revealed immunoreactive bands around 30 kDa for all three aquaporins: Aqp1aa, Aqp1ab, and Aqp8ab in addition to both lower (15 kDa: Aqp8ab) and higher (40 kDa: Aqp1aa) molecular weight bands. The bands around 30 were efficiently blocked when the antibodies were preadsorbed with 400× molar excess of the specific antigenic peptide, suggesting that the affinity purified antibodies recognize specific aquaporin isoforms with the expected Mr around 30 kDa. However, some degree of blocking was also observed for both higher and lower Mr bands. We cannot presently explain the bands at 15–20 kDa, whereas it is well known that aquaporins appear in various glycosylated forms that may give rise to multiple bands >30 kDa on Western blots (e.g., Christensen et al., 2000; Pandey et al., 2010). Aquaporins are also known to be functionally arranged in tetramers in biological membranes; however, there was no indication of oligomeric forms when using the present reducing conditions. The Aqp1ab and -8ab antibodies seemed to have a better quality for Western blotting than the Aqp1aa antibody, which also gave the weakest signals. The data for Aqp1aa thus have to be interpreted with some care.

LOCALIZATION OF INTESTINAL AQUAPORINS: Aqp1

Several studies have shown that expression of Aqp1 is higher in SW-acclimated fish compared to FW fish (Aoki et al., 2003; Martinez et al., 2005b; Raldúa et al., 2008; Kim et al., 2010), suggesting that it is involved in the absorption of imbibed water in these species. This is also the case in Atlantic salmon judged from higher mRNA levels in SW-fish than FW fish (Tipsmark et al., 2010). The present study used SW salmon for the localization of aquaporins. Aqp1aa was localized in the apical brush border membranes of enterocytes along the whole length of the intestine. Even though fluorescence staining intensities must be compared with care, it generally appeared that the staining intensity for Aqp1aa was weaker than for the other aquaporins investigated. This matches the weaker bands observed on the Western blots when probing for this aquaporin and we cannot exclude that it is due to a poorer antibody quality (see above). In the pyloric caeca, staining was also observed in the sub-apical zone of enterocytes, suggesting the presence of cytoplasmic vesicles with an internalized pool of Aqp1aa. This was less prominent in the other regions. In the middle intestine a significant immunoreaction was also observed in goblet cells, which has not been shown in previous studies. Interestingly, this was not seen in other segments of the intestine even though goblet cells were numerous there. Nor did the antibodies for Aqp1ab and Aqp8ab produce a similar reaction. Localization of Aqp3 in goblet cells has been reported in the posterior esophagus and the rectum of European eel (Cutler et al., 2007), suggesting that aquaporins may to some degree be involved in mucus production or maintenance of the mucus layer fluidity.

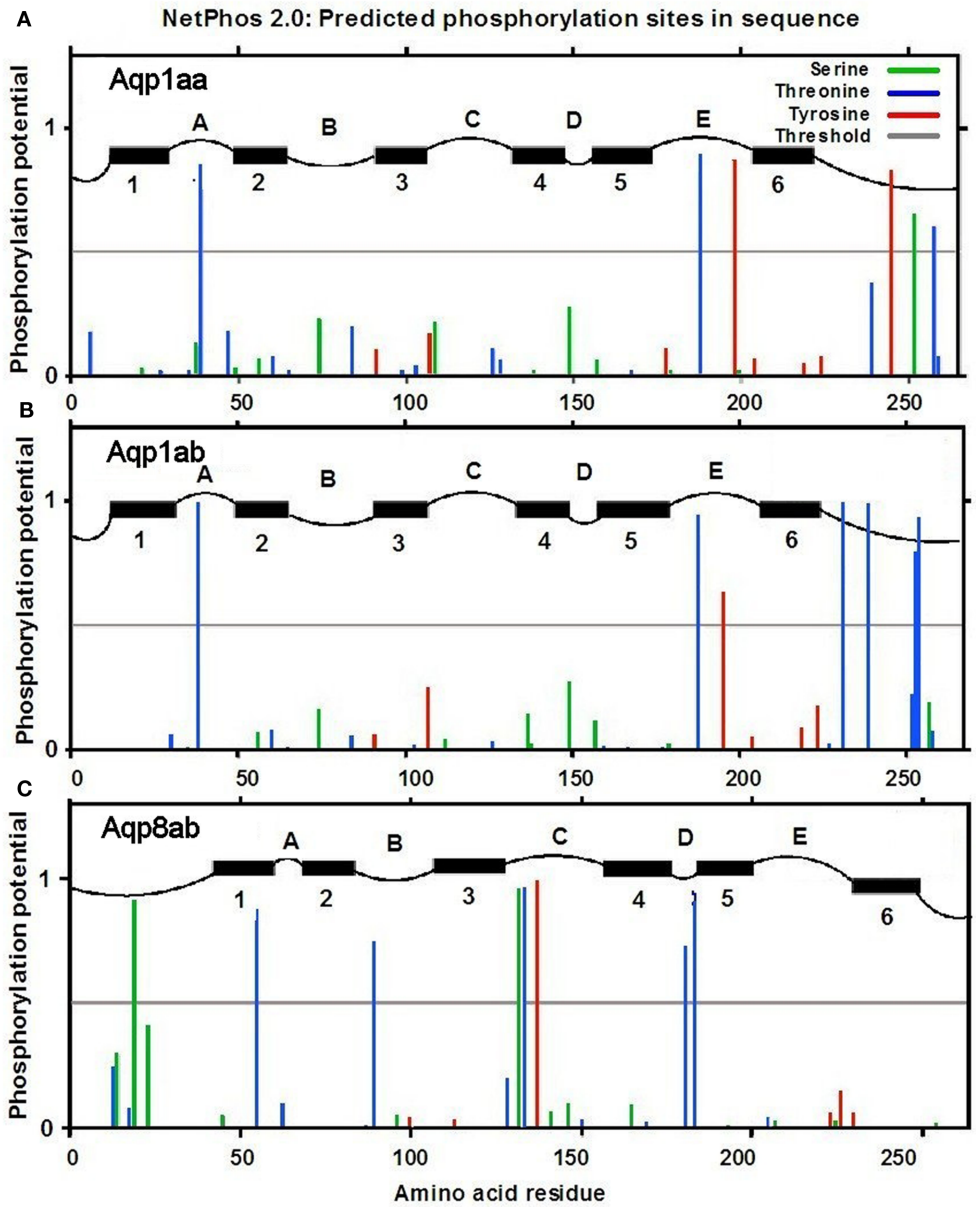

FIGURE 11 | Analysis of predicted serine, threonine and tyrosine phosphorylation sites in Aqp1aa (A), Aqp1ab (B), and Aqp8ab (C) (from NetPhos 2.0 server at www.cbs.dtu.dk; Blom et al., 1999). Black horizontal bars indicate predicted transmembrane domains with numbering below. Letters (A–E) indicate extracellular domains; letters (B–D) indicate cytosolic domains (from TMPred at www.ch.embnet.org; Hofmann and Stoffel, 1993).

Aqp1ab was also present in the apical brush border in all segments of the intestine, with a very distinct staining of the outermost zone of microvilli in the pyloric caeca and middle intestine. In the posterior intestine the localization moved into a more sub-apical position, and was most pronounced in the distal region of the posterior intestine, where Aqp1ab was located almost exclusively in sub-apical compartments. Based on the transmission electron microscopic picture (**Figure 2**) this region was judged to contain intracellular vesicles which may form a pool of recruitable membrane proteins including aquaporins.

Other fish studies have also localized Aqp1 in the enterocytes of the intestinal epithelium: Aqp1aa was demonstrated in the apical membrane of the enterocytes in Japanese and European eel (Aoki et al., 2003; Martinez et al., 2005a). In gilthead seabream, Raldúa et al. (2008) investigated the localization of both Aqp1aa and -1ab, and found immunoreactivity of both paralogs in the apical brush borders in the duodenum and hindgut, with Aqp1aa being most abundant. This aquaporin may thus facilitate water entry into enterocytes. Aqp1aa also showed weak staining of the lateral membranes and the peri-nuclear region in their study, suggesting that this paralog may also offer an exit pathway for absorbed water in

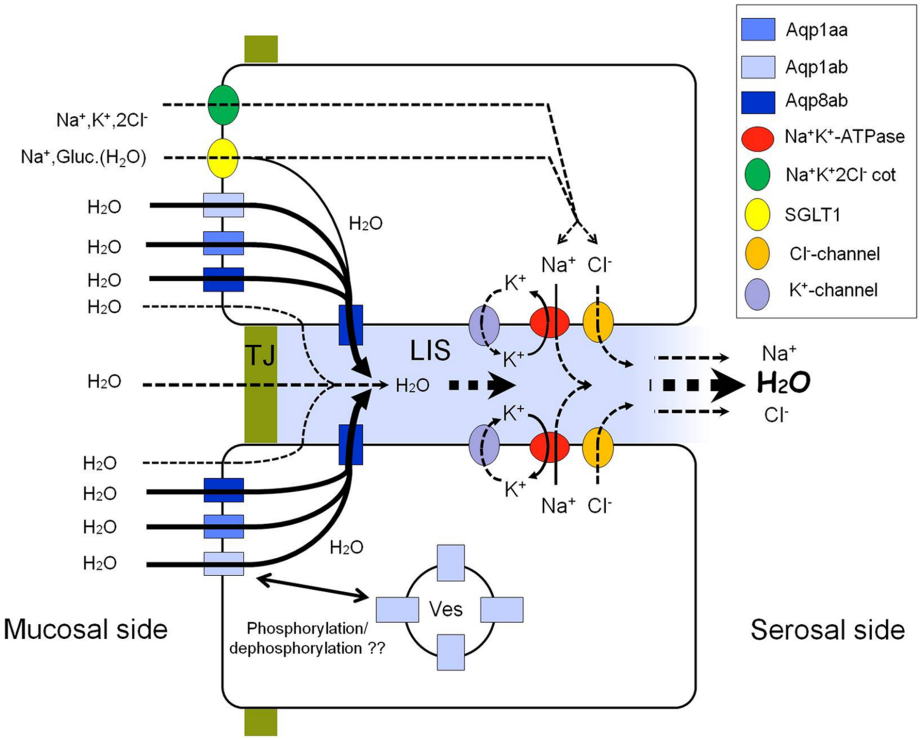

FIGURE 12 | Tentative model of the pathways of water absorption across the intestinal epithelium in salmonids. An osmotic gradient is set up across the lateral membrane by means of the Na^+, K^+-ATPase. Water may be drawn osmotically through four pathways: (1) diffusion through the lipid bilayer of the enterocyte; (2) paracellular diffusion through the TJ; (3) symport with Na^+ and glucose by the SGLT1; (4) diffusion through aquaporins apically as well as laterally. Aquaporins may be translocated between sub-apical vesicles and the cell membrane possibly regulated by phosphorylation events. The model is based on the elements investigated in the present paper. For simplicity apical ion-transporters are only shown in the upper enterocyte. LIS, lateral intercellular space; TJ, tight junction; Ves, intracellular vesicles.

the seabream. In the seabream rectum (equals the posterior intestine investigated in the present study) Aqp1aa was preferentially localized in sub-apical intracellular compartments of enterocytes. Aqp1ab on the other hand stained the apical brush borders in the rectal epithelium intensively (Raldúa et al., 2008).

The results of the present study are thus overall in agreement with previous fish studies with respect to localization of Aqp1 paralogs in enterocytes. However, the relative staining intensities between segments seem to vary between species (seabream: Raldúa et al., 2008; European eel: Martinez et al., 2005a). The presently observed change of Aqp1ab localization when moving from the middle into the posterior segment, and especially *within* the posterior segment shows, that the definition of intestinal "segments" is purely based on gross morphology and does not necessarily imply similar function or cellular histology within a given segment. Taken together, all available data show that Aqp1 paralogs may be important for trans-enterocytic water transport, and in most species offers an entry pathway through the apical membranes.

In the GIT of mammals, AQP1 is typically localized in the serosa, in endothelia of the lymphatic vessels and in the lamina propria but not in the epithelium lining the intestinal lumen (rats: Koyama et al., 1999; Ma and Verkman, 1999). On the other hand studies on dolphins (Suzuki, 2010) and the desert rodent *Octodon degus* (Gallardo et al., 2002) have localized AQP1 in the apical membranes of the epithelial cells lining the gut, similar to what is seen in marine fish. Thus this aquaporin may be of greater importance for water uptake in the GIT of animals living in more water-deprived environments.

Aqp8

The transport characteristics of mammalian AQP8 is different from the orthodox aquaporins as it is able to transport ammonia (Liu et al., 2006; Carbrey and Agre, 2009) and urea (Ma et al., 1997; Cerdà and Finn, 2010) in addition to water. Our study demonstrated that the Aqp8ab protein is abundant in the brush border membrane of enterocytes in all three intestinal segments of Atlantic salmon. The Aqp8ab antibody gave a very strong band around 30 kDa and seemed the most abundant of the three aquaporins investigated. This observation strongly supports a physiological role of Aqp8ab in water absorption across the apical membrane of the intestinal epithelia in SW salmon. Three other studies have reported *aqp8* mRNA expression in fish. Kim et al. (2010) recently reported expression of an *aqp8* paralog in the intestine of Japanese eel, which increased after SW-acclimation. Tingaud-Sequeira et al. (2010) found expression of two paralogs (*aqp8aa* and *aqp8ab*) in the intestine and one paralog (*aqp8b*) in the brain of zebrafish. And finally Tipsmark et al. (2010) reported two paralogs of *aqp8* in Atlantic salmon: *aqp8aa*,

which is exclusively expressed in the liver and *aqp8ab* (formerly named AQP8b), which is expressed exclusively in the intestine (pyloric caeca, middle and posterior intestine). At present, two observations suggest that Aqp8ab is important for intestinal water transport in Atlantic salmon: (1) *aqp8ab* mRNA is up-regulated during SW-acclimation in pyloric caeca, middle, and posterior intestine, and (2) *aqp8ab* mRNA expression is increased during smoltification in preparation for SW-entry (Tipsmark et al., 2010). From the present study, it is further evident that Aqp8ab co-localizes with Na^+, K^+-ATPase in the lateral membranes in the middle and posterior intestine, thus Aqp8ab may be important for both water entry and exit through the enterocyte. We also found occasional peri-nuclear staining using the Aqp8ab antibody, which may be related to recognition of Aqp8ab protein in association with the Golgi apparatus and endoplasmic reticulum. Investigations using colloidal gold staining and transmission electron microscopy are currently performed in order to verify this lateral staining.

Mammalian studies have detected AQP8 protein expression in liver, testis, epididymis, kidney, bronchi/trachea, duodenum, jejunum, and colon (Elkjær et al., 2001). AQP8 is found to be important in the secretion of bile from liver hepatocytes, where it is recruited from intracellular compartments to the plasma membrane following stimulation with glucagon (Gradilone et al., 2003) or cAMP (Carcia et al., 2001). The molecular mechanism behind this trafficking is not yet fully understood, even though there is evidence that phosphorylation of AQP8 initiates its translocation from the intracellular compartment to the plasma membrane (Carbrey and Agre, 2009). A study by Elkjær et al. (2001) and Calamita et al. (2001) localized AQP8 in the intestinal epithelium; however, it was located almost exclusively in intracellular compartments. Later studies on rats by Tritto et al. (2007) and Laforenza et al. (2005) also revealed the expression of AQP8 in the small intestine and colon, respectively. In these studies AQP8 was localized in the brush borders of the epithelial cells. All these studies suggest that AQP8 may play a role in the movement of water across the apical membranes of the intestinal cells, and that trafficking of AQP8 between intracellular compartments and the plasma membrane might be involved in the regulation of this aquaporin.

ANALYSIS OF POTENTIAL PHOSPHORYLATION SITES

In the evolutionary history of teleosts, it is suggested that the group arose in FW and later inhabited the marine environment (Fyhn et al., 1999). This event required new molecular adaptations to maintain water homeostasis. Among such adaptations, gene duplication of *aqp1* at a local gene level rather than at the chromosome level has been suggested (Tingaud-Sequeira et al., 2008, 2010; Finn and Cerdà, 2011). One mechanism that is known to regulate trafficking of aquaporins is phosphorylation events, exemplified in the scenario of fish oocyte hydration. In both the marine gilthead seabream (Fabra et al., 2005, 2006; Tingaud-Sequeira et al., 2008) and the FW catfish (*Heteropneustes fossilis*, Chaube et al., 2011) the Aqp1ab paralog is expressed in oocytes, where it has a particular role to facilitate oocyte swelling during meiotic maturation. In seabream, this is initiated when Aqp1ab is recruited to the oocyte cell surface induced by *de*-phosphorylation of its C-terminal serine-254, whereas in catfish phosphorylation of serine-227 induces membrane insertion of this paralog. Interestingly, in seabream, only the Aqp1ab paralog has a phosphorylation site, Aqp1aa does not. In salmon, both Aqp1aa and −1ab have predicted phosphorylation sites in the cytosolic domain between residues 230 and 260 (**Figure 11**), however, the phosphorylation potential is stronger for Aqp1ab than Aqp1aa at especially serine residues. We suspect that some of these sites may be involved in regulatory phosphorylation, which may affect the cellular distribution of these aquaporins. Judged from the transmission electron microscopy picture (**Figure 2**) vesicles are abundant in the sub-apical zone of the enterocyte. This is also where especially Aqp1ab is located in the enterocytes of the distal part of the posterior intestine, suggesting that trafficking may be an important aspect of the function of this aquaporin in this region. Sup-apical localization of Aqp1aa was not seen to the same degree. Aqp8ab was also localized in sub-apical domains in the present study (**Figure 9D**) suggesting a vesicular location. However, no phosphorylation residues were present near the C-terminus of this aquaporin; only a serine, a threonine and a tyrosine residue with a high phosphorylation potential were present in intracellular domains of Aqp8ab, and it thus differs markedly from the aquaporin 1aa and -1ab in this respect. It shall be interesting to investigate the role of these residues in intracellular trafficking of Aqp8ab.

CONCLUSION AND PERSPECTIVES: PHYSIOLOGICAL ROLE OF AQUAPORINS IN INTESTINAL WATER ABSORPTION

There is increasing circumstantial evidence of a role of aquaporins in intestinal water uptake in SW-fish. The definite proof is, however, still missing – not least due to the pharmacology of aquaporins and the difficulty of specifically inhibiting these in live tissue. In salmon, the significant up regulation of *aqp1aa* and especially -*8ab* mRNA in pyloric caeca, middle and posterior intestine, suggests their importance in intestinal water absorption in hypo-osmoregulating fish (Tipsmark et al., 2010). In addition, there may be a small contribution to apical water transport through supplementary pathways such as the SGLT1, though depending on the nutritional condition. This study has shown that Aqp1aa, -1ab, and -8ab are localized in the apical brush borders of enterocytes lining the entire intestine from the pyloric caeca through the anus, thus allowing entry of water from the lumen into the enterocytes. In salmon, the pyloric caeca may have a particular significance in quantitative uptake of water (and nutrients; Veillette et al., 2005). We could not confirm a lateral location of Aqp1 as reported in seabream (Raldúa et al., 2008) but give evidence that in salmon (and rainbow trout: Madsen, unpublished data) Aqp8ab occupies this location – in addition to being present in both apical and sub-apical locations. Thus at least for salmonids it is possible to propose a model for transcellular water transport involving three aquaporin paralogs (**Figure 12**). The reason for having (at least) three different paralogs accounting for apical water entry can only be speculated but such diversity is also common in mammalian tissues. One obvious possibility is that paralogs may have different permeability characteristics and regulatory potential and thus contribute differently to long- and short-term regulation. We are currently investigating the permeability characteristics of the expressed isoforms by heterologous expression in *Xenopus* oocytes.

During the progress of this work we have identified yet another Aqp8 paralog – termed Aqp8b in the extensive EST library of Atlantic salmon. This leads to three paralogs of Aqp8, which is also the case for zebrafish (Tingaud-Sequeira et al., 2010). The Aqp8b isoform is highly expressed in the GI tract and kidney (Madsen, unpublished data) and future studies will reveal the localization and contribution of this novel paralog to intestinal water transport.

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The role of aquaporins in the kidney of euryhaline teleosts

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Water balance in teleost fish is maintained with contributions from the major osmoregulatory organs: intestine, gills, and kidney. Overall water fluxes have been studied in all of these organs but not until recently has it become possible to approach the mechanisms of water transport at the molecular level. This mini-review addresses the role of the kidney in osmoregulation with special emphasis on euryhaline teleosts. After a short review of current knowledge of renal functional morphology and regulation, we turn the focus to recent molecular investigations of the role of aquaporins in water and solute transport in the teleost kidney. We conclude that there is much to be achieved in understanding water transport and its regulation in the teleost kidney and that effort should be put into systematic mapping of aquaporins to their tubular as well as cellular localization.

Keywords: kidney, aquaporin

INTRODUCTION

Multiple aquaporins have been functionally characterized and mapped along the nephron in the mammalian kidney, where they play a pivotal role in the maintenance of water homeostasis (Nielsen et al., 2002). Teleost kidneys are unable to produce hyperosmotic urine due to the lack of a loop of Henle, yet the role of aquaporins is suspected to be related to conservation of water in seawater (SW) and possibly excretion of water in freshwater (FW). In 2010 Cerdà and Finn summarized that 11 orthologs have been reported in whole kidney tissue (incl. vasculature and supporting tissues) of various teleosts: *aqp-1aa*, *-1ab*, *-3a*, *-3b*, *-7*, *-8aa*, *-8ab*, *-9a*, *-10a*, *10b*, and *-12*. We have recently localized yet a paralog of *aqp8* – termed *aqp8b* in the kidney of Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*; **Figures 1E,F**). Only a few aquaporins have been localized in specific tubule cells, and their dynamics and functional role are far from known (**Table 1**).

FUNCTIONAL MORPHOLOGY OF THE KIDNEY IN EURYHALINE TELEOSTS

Freshwater-fish encounter osmotic water load from the environment and the primary function of the filtrating kidney is to produce large amounts of hypotonic urine. Salts are inevitably lost in this process as well as across gills and skin, which is compensated for by branchial and dietary NaCl uptake. SW fish are threatened by dehydration and compensate by ingesting and absorbing SW followed by excretion of excess salts in the gill. The kidney switches to water saving with excretion of divalent ions as the main function. Thus euryhaline fish are able to make major adjustments in renal function as the salinity changes (Beyenbach, 2004).

The mesonephric teleost kidney has a poor 3-dimensional organization: It lacks a well defined cortex, medulla, and a loop of Henle which is characteristic of the metanephric kidney of mammals. Vasculature, glomeruli, and tubular segments are interwoven and interspersed with hematopoietic tissue especially in the anterior part (Anderson and Loewen, 1975; Resende et al., 2010). The number and size of glomeruli as well as the differentiation of functional segments of teleost nephrons are reduced compared to the mammalian type, and vary according to evolutionary origin and habitat. In euryhaline teleosts, glomeruli with closed Bowman's capsules precede proximal tubule segments I and II followed by distal and collecting tubules and collecting ducts which drain into the mesonephric duct. Paired mesonephric ducts then merge distally forming the urinary bladder, which further drains through the urogenital papilla (see Hickman and Trump, 1969).

Glomerular filtration (GFR) varies between species and is absent in a few aglomerular marine species (Beyenbach, 2004). GFR is typically 7–10× higher in FW than in SW, reflecting the contrasting demands in the two habitats (Brown et al., 1978; Elger et al., 1987; McDonald, 2007). This active adjustment of GFR according to hydrational status is markedly different from mammals and is achieved through vascular adjustments serving to change perfusion pressure and recruitment of individual nephrons (McDonald, 2007). In rainbow trout (*Oncorhynchus mykiss*) <50% of the nephrons are filtering in FW fish (Brown et al., 1980). This number decreases to about 5% in SW-acclimated fish (McDonald, 2007) but the average single nephron GFR (SNGFR) is almost three times higher in SW- than in FW-acclimated trout (Brown et al., 1978). This illustrates that fish kidneys may undergo a major switch from being filtratory in FW to being predominantly secretory in SW.

The function of the proximal segment(s) of the kidney tubule is somewhat controversial. There is, however, consensus that one primary function is secretion of Mg^{2+} especially in marine and SW-acclimated species. Mg^{2+} secretion involves apical exocytosis of vesicles enriched with Mg^{2+} (Renfro, 1999). Accompanying fluid secretion may occur in both filtering and non-filtering nephrons but would be expected to occur particularly in SW fish

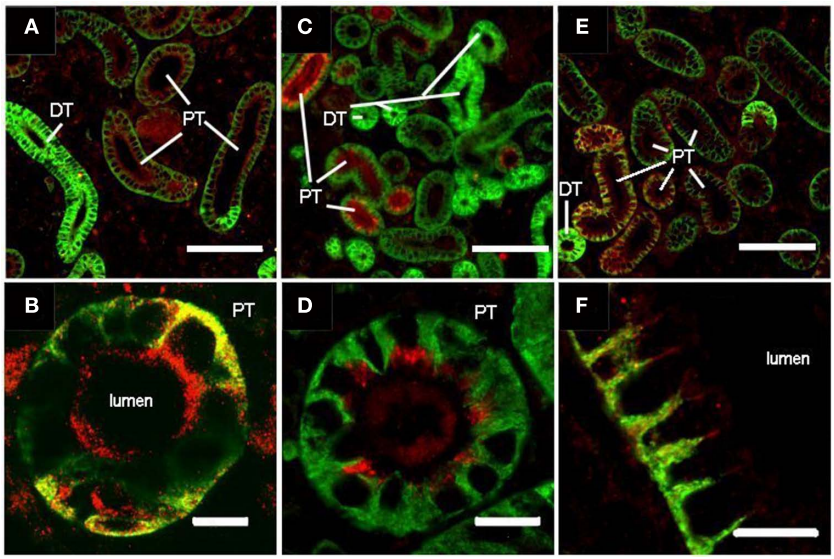

FIGURE 1 | Localization of three aquaporins (Aqp) isoforms in the FW rainbow trout kidney: Aqp1aa (A,B), Aqp1ab (C,D), and Aqp8b (E,F). Sections were prepared as described in Madsen et al. (in review). Sections were double stained with polyclonal rabbit antibodies against *S. salar* Aqps (red) and monoclonal mouse α-5 against the α-subunit of Na^+, K^+-ATPase (green; The Developmental Studies Hybridoma Bank developed under auspices of the NICHD and maintained by The University of Iowa, Department of Biological Sciences, Iowa City, IA, USA) and visualized using a cocktail of secondary goat antibodies (Alexa Fluor® 568 anti-rabbit and Oregon Green® 488 anti-mouse. Yellow color appears where the two antibodies co-localize. Letters show different tubule segments distinguished by the cellular localization and abundance of Na^+, K^+-ATPase (basolateral, less abundant: proximal tubules, PT; entire cell, more abundant: distal tubules, DT). Aqp1aa stains the brush border and some basolateral membrane of PT, Aqp1ab is present subapically and in brush border of PT and Aqp8b is present in both apical and basolateral positions in some proximal tubules. Size bars are 100 and 10 μm in upper and lower panels, respectively.

in order to facilitate Mg^{2+}-secretion in tubules with low GFR. In FW-kidneys it could play an additional role in excretion of excess water (Beyenbach, 2004). With regard to NaCl and water transport, available data are more diverging. In some species, there is evidence that the proximal segments of both FW and SW fish are responsible for absorption of Na^+ and Cl^- as wells as glucose and other important osmolytes (Nishimura and Imai, 1982; Dantzler, 2003; Beyenbach, 2004). The mechanism for this builds on basolateral Na^+, K^+-ATPase (Katoh et al., 2008; Teranishi and Kaneko, 2010). The apical Na^+-entry pathway into proximal cells is yet unclear but may involve Na^+/H^+-exchange (Braun and Dantzler, 1997; Ivanis et al., 2008) and Na^+-glucose cotransporters (SGLT1). The role of SGLT1 has generally not been investigated much in teleost kidneys but its mRNA expression is indeed very high in mixed renal tissue of rainbow trout (Madsen, unpublished). In little skate (*Leucoraja erinacea*) SGLT1 is present in proximal as well as distal tubules (Althoff et al., 2007). Like in mammals, the absorptive-type Na^+, K^+, $2Cl^-$ cotransporter (NKCC2) is absent in fish in this segment (Katoh et al., 2008). In accordance with the mammalian model it was earlier believed that some trans- and para-cellular water absorption occurred in the proximal segments driven by a suspected locally established osmotic gradient (Hickman and Trump, 1969). This would make good sense in a SW fish but not in FW fish, where body water is excessive. This model is, however, contradicted by more recent demonstrations of basolateral secretory-type NKCC1 at least in killifish (*Fundulus heteroclitus*) proximal segments (Katoh et al., 2008), suggesting a pathway for NaCl secretion which may occur in conjunction with Mg^{2+} secretion and thus set up a reverse osmotic gradient facilitating water secretion in this segment (Beyenbach, 2004). Secreted NaCl would be expected to be reabsorbed in the distal and collecting segments. In SW fish, branchial NaCl secretion would easily compensate for this recycling of salt in the kidney. It is further possible that processes operate differently in PI and PII segments and in different teleosts.

Distal tubules and collecting ducts together make up a variable percentage of whole nephrons in different fish (Katoh et al., 2008) and the primary activity here is reabsorption of NaCl in both FW and SW. This is favored by extensive expression of basolateral Na^+, K^+-ATPase, apical NKCC2 (Katoh et al., 2008) and NaCl cotransporter (NCC; Kato et al., 2011), and basolateral kidney-specific Cl-channels (Miyazaki et al., 2002). Accordingly the distal segment is absent in the majority of truly marine species (Hickman and Trump, 1969). In FW fish the distal segments are proposed to have low water permeability in order to minimize water reabsorption and promote the formation of hypotonic urine (McDonald, 2007). Upon acclimation to SW, the fractional reabsorption of water increases along the nephron by increasing tubular water permeability as seen in mammalian collecting tubules. Reabsorption of NaCl may promote the osmotic removal of water – thereby creating isotonic urine primarily consisting of $MgSO_4$ and other unwanted osmolytes (Beyenbach, 2004).

Surprisingly little information is available about the molecular mechanisms of tubular water movement and how it is controlled in fish. Among the most obvious candidates for renal control are arginine vasotocin and angiotensin which have vascular effects that

Table 1 | Overview of aquaporin expression and localization in fish kidneys.

	*D. rerio**	*S. salar*	*O. mykiss*	*O. mossambicus*	*A. anguilla*	*A. japonica*	*D. labrax*	*S. aurata*	*S. sarba*	*A. schlegeli*	*P. annectens**
aqp1aa	m	m/p, SW (↑)	p		m/p, SW (↓)	–	m, SW (↑)	m/p		m, SW (↓)	
TS			PT		Unknown			Unknown			
SL			A + BL		A			A			
aqp1ab	–	m/p, SW (↓)	p		m, SW (↓)			m			
TS			PT (FW)								
			PT + DT (SW)								
SL			SA + A								
aqp2											m/p(estivation) LDT
TS											A
SL											
aqp3a	m	m, SW (↑)		m, SW (↑)			m, SW (↑)		m/p		
aqp3b	–				m/p SW (↑)	–					
TS					Unknown						
SL					A						
aqp7	m										
aqp8aa	m	–				–					
aqp8ab	m	–									
aqp8b	–	m/p	p								
TS			PT (FW + SW)								
SL			BL								
aqp9a	m										
aqp10a	m							–			
aqp10b	m	m, SW (↑)			m, SW (↓)	–		m			
aqp12	m										
Reference	Tingaud-Sequeira et al. (2010)	Tipsmark et al. (2010), Engelund and Madsen (unpublished)	Engelund and Madsen (unpublished)	Watanabe et al. (2005)	Martinez et al. (2005), Cutler et al. (2007)	Aoki et al. (2003), Kim et al. (2010)	Giffard-Mena et al. (2007, 2008)	Santos et al. (2004), Fabra et al. (2005), Cerdà and Finn (2010)	Deane and Woo (2006)	An et al. (2008)	Konno et al. (2010)

**All species listed in the table are euryhaline with the exception of D. rerio and P. annectens which are freshwater fishes. TS, tubule segment; SL, subcellular localization; m, mRNA expression; p, protein expression by immunocytochemistry or immunoblotting; –: not detected; blank: not investigated; ↑ or ↓: up- or down-regulation of mRNA upon seawater exposure; A, apical membrane; SA, subapical membrane; BL, basolateral membrane; PT, proximal tubules; DT, distal tubules LDT, late distal tubules; FW, freshwater acclimated fish; SW, seawater acclimated fish.*

decrease SNGFR and number of filtering nephrons and thereby exert a profound antidiuretic effect (Amer and Brown, 1995; Brown et al., 2000). Prolactin, cortisol, and growth hormone are also suspected to play important roles in modulation of renal water and ion homeostasis (Manzon, 2002; Martinez et al., 2005; Breves et al., 2011), however, there is very little concrete evidence.

AQUAPORINS IN THE TELEOST KIDNEY

AQUAPORIN 1

Aquaporin 1 exists as two paralogs in teleosts: *aqp1aa* and *aqp1ab* (Cerdà and Finn, 2010). The first report of kidney aquaporins was made by Martinez et al. (2005), who found two classic aquaporins (*aqp1* and *aqp1dup* – now *aqp1ab*) and one aquaglyceroporin (*aqpe* – now *aqp10b*, Tingaud-Sequeira et al., 2010) in FW- and SW-acclimated European eels (*Anguilla anguilla*). The mRNA expression of these declined during SW-acclimation – and also partly during the pre-migratory metamorphosis from the yellow to silver form. A somewhat similar expression pattern was reported in black porgy (*Acanthopagrus schlegeli*; An et al., 2008), where *aqp1* expression varied according to salinity in the order: 10% SW > FW > SW. This response is opposite of what would be expected, if these aquaporins are involved in tubular water absorption, and accordingly the authors concluded that *aqp1* may be involved in water secretion. In contrast, the expression of *aqp1* mRNA in the kidney of sea bass (*Dicentrarchus labrax)* was four to five times higher in SW- than in FW-acclimated fish (Giffard-Mena et al., 2007). In Atlantic salmon, Tipsmark et al. (2010) reported increasing mRNA levels of *aqp1aa* during SW-acclimation (and smoltification), but this was accompanied by a concomitant decrease in the mRNA of *aqp1ab*, suggesting that these paralogs play differential roles in water homeostasis in the Atlantic salmon kidney. The bass and salmon studies agree well, since the sea-bass *aqp1* ortholog is more similar to the aa- than the ab-ortholog of Atlantic salmon *aqp1*. In stenohaline zebrafish kidney only one *aqp1* paralog is found (*aqp1aa*), which adds further complexity to the physiological roles of *aqp1* (Tingaud-Sequeira et al., 2010). At the moment it is unclear to what degree the diverging results obtained for *aqp1* can be explained by species differences or the varying experimental designs.

There is very little knowledge of the localization of Aqp1 protein isoforms along the teleost nephron. Martinez et al. (2005) found positive immunoreaction in the eel kidney using a homologous antibody. Staining was generally present in the endothelium in yellow eels; but in both FW and SW silver eels a "subset" of renal tubules (presumably proximal) exhibited strong staining in the brush border zone of epithelial cells. No attempts were made to further determine the segmental origin of these tubules. Aqp1aa also appeared in apical localization of renal tubules of SW-acclimated gilthead sea bream (*Sparus aurata*, Cerdà and Finn, 2010). Initial studies of rainbow trout show a clear segmentation of Aqp1 protein isoforms in renal tubules. Irrespective of salinity, Aqp1aa is present in the apical and basolateral membrane of proximal tubules, judged from the intensity and pattern of Na^+, K^+-ATPase localization (**Figures 1A,B**). The Na^+, K^+-ATPase is present basolaterally in all tubule segments but is more abundant in the highly folded membranes of distal and collecting tubules than in proximal segments of the nephron (Katoh et al., 2008) allowing differentiation between tubule segments. Aqp1ab is found in the apical brush border of proximal and distal tubules in SW-acclimated rainbow trout (not shown), while being predominantly withdrawn to a subapical position in proximal tubules in FW-acclimated rainbow trout (**Figures 1C,D**). This suggests that trafficking may contribute to the regulation of this isoform as has been reported for fish oocytes (Fabra et al., 2005, 2006; Chaube et al., 2011) and also discussed for intestinal Aqp1ab elsewhere in this Special Topic (Madsen et al., in review).

Only one study has reported hormonal effects on kidney *aqp1* mRNA. Martinez et al. (2005) found that expression of *aqp1aa* and *aqp1ab* in yellow eels was suppressed by cortisol, whereas the hormone had no effect in silver eels, where the mRNAs were already at lower levels. This confirms the conception of cortisol being a SW-adapting hormone in eel.

AQUAPORIN 2

In strong contrast to mammals, this isoform has not been detected in teleosts (Cerdà and Finn, 2010). However, one study has documented aquaporin 2 in the kidney of African lungfish (*Protopterus annectens*; Konno et al., 2010), where mRNA and protein levels increased during aestivation. Moreover, Aqp2 was localized to the apical membrane of late distal tubule cells, where the vasopressin/vasotocin-V2 type receptor was present basolaterally. This suggests that water deficiency in the fish world may have promoted convergent evolution of a mechanism for renal water conservation similar to that responsible for urine concentration in mammals (Nielsen et al., 2002).

AQUAPORIN 3

The aquaglyceroporin *aqp3* exists as duplicate paralogs in zebrafish: *aqp3a* and *aqp3b* (Cerdà and Finn, 2010) but only *aqp3a* mRNA is present in the kidney of zebrafish. *Aqp3b* mRNA was first reported absent from kidneys of SW-acclimated eel using Northern blotting (Cutler and Cramb, 2002). Later, the same group used RT-PCR to detect low levels of *aqp3b* mRNA in renal tissues which increased upon SW-acclimation (Cutler et al., 2007). This agrees with tilapia (*Oreochromis mossambicus*), where *aqp3a* mRNA is present in higher levels in SW- than FW-kidney ($n = 1$; Watanabe et al., 2005), and with Atlantic salmon where renal *aqp3a* mRNA increased >10-fold within 24 h after FW- to SW-transfer and stayed elevated for 2 weeks (Tipsmark et al., 2010). The same trend was seen in sea bass (Giffard-Mena et al., 2007), though not confirmed in a subsequent study (Giffard-Mena et al., 2008). In silver sea bream (*Sparus sarba*) the Aqp3a protein was detected by immunoblotting using homologous antibodies but no change was seen upon acclimation to a range of salinities (Deane and Woo, 2006). Finally, Kim et al. (2010) found no expression of *aqp3b* in kidney of Japanese eel (*A. japonica*). From this comparison, it seems that the *aqp3b* isoform is either absent or present in rather low levels in fish kidneys. In those species where *aqp3a* is expressed, the abundance is higher and mostly increases in response to hyperosmotic conditions. Thus Aqp3a may have a role in water conservation. A supplemental role in glycerol and urea transport is another putative function of Aqp3 (MacIver et al., 2009).

There is only one preliminary report of renal Aqp3 localization. Using a homologous antibody Cutler et al. (2007) detected Aqp3b protein in the apical domain of eel renal tubule cells (Cutler et al., 2007). This contrasts the basolateral location in distal segments of

the mammalian kidney, where it serves a role in cellular water exit (Nielsen et al., 2002).

AQUAPORIN 8

In mammals, the role of renal AQP8 is unclear, since it is predominantly located in intracellular vesicles in proximal tubule and collecting duct cells (Elkjær et al., 2001). In addition to water, this isoform may also transport ammonium and thus be involved in acid–base control (Liu et al., 2006). In fish, there is very little information regarding the expression and function of this isoform – mostly related to intestinal function (Cutler et al., 2009; Kim et al., 2010; Madsen et al., in review). In zebrafish kidney, two paralogs of *aqp8* are expressed: *aqp8aa* and *aqp8ab* (Tingaud-Sequeira et al., 2010), whereas no expression of these paralogs was detected in Atlantic salmon (*aqp-8aa* and *-8ab*: Tipsmark et al., 2010) or Japanese eel (*aqp8aa*: Kim et al., 2010). However, we have recently discovered a third paralog of *aqp8* (*aqp8b*) in salmon, which is abundantly expressed in both intestinal and renal tissue. This paralog is also found in other teleost species but surprisingly not in the intestine or kidney of zebrafish (Cerdà and Finn, 2010; Tingaud-Sequeira et al., 2010). Preliminary results using homologous antibodies show a predominant staining of the basolateral membrane of some renal tubule cells in FW-acclimated rainbow trout (**Figures 1E,F**). We suspect that these tubules are proximal based on the moderate basolateral staining for Na^+, K^+-ATPase. This is the first demonstration of a basolateral Aqp8 in fish kidney tubules, which may serve together with the Aqp1aa isoform as important exit pathways for reabsorbed water.

AQUAPORIN 10

Aquaporin 10, another aquaglyceroporin, is not expressed in the kidneys of mammals but is located in the apical membrane of the small intestine, where it is suspected to participate in transport of water and small solutes (Hatakeyama et al., 2001; Mobasheri et al., 2004). In zebrafish kidney, *aqp10* is expressed as two paralogs: *aqp10a* and *aqp10b* (Cerdà and Finn, 2010). Orthologs of *aqp10b* were also identified in kidneys of SW-acclimated gilthead sea bream (*sbaqp*: Santos et al., 2004), European eel (*aqpe*: Martinez et al., 2005), and Atlantic salmon (Tipsmark et al., 2010). In sea bream, *aqp10b* mRNA was demonstrated by *in situ* hybridization in tubule cells of undefined origin. Functional assays using *Xenopus* oocytes have further shown that this aquaglyceroporin is capable of water, glycerol and urea uptake (Santos et al., 2004; MacIver et al., 2009). In the eel study, SW-acclimation was associated with lower levels of *aqp10b* mRNA in yellow eels, whereas silver eels showed no response. In salmon, the effect of salinity was opposite; *aqp10b* expression was increased fivefold at 8 days after transfer to SW. Even though species differences seem to exist, all available evidence points to a salinity dependent expression and thus a role of Aqp10b in fluid handling in the teleost kidney. The endocrine regulation of this isoform remains unknown.

ADDITIONAL AQUAPORINS

Aquaglyceroporins 7 and 9 and one unorthodox aquaporin 12 have been reported in the zebrafish kidney (Tingaud-Sequeira et al., 2010) but no information is available on their functional characteristics, tubular localization, nor their expression dynamics. In mammals, AQP7 is present in brush border membranes of proximal tubules, where it is suspected a role in glycerol metabolism (Noda et al., 2010). AQP9 and -12 have not been located in the kidney of mammals (Nielsen et al., 2002).

CONCLUSION AND PERSPECTIVES FOR FUTURE RESEARCH

Euryhaline teleosts have a capacity to acclimate to salinities ranging from strongly hypotonic to strongly hypertonic. In doing so their kidney function is dramatically altered. Despite this fact, surprisingly few studies have addressed the role and dynamics of aquaporins in fish kidneys. These have so far only probed for transcriptional regulation of few aquaporins and found interesting though conflicting results. Most data have demonstrated apical aquaporin expression in renal tubules, and future studies are encouraged to systematically investigate the specific protein isoforms, their dynamics and cellular expression as well as their hormonal and environmental regulation. This review has tried to show that there is still much to learn about water homeostasis in fish.

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Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins

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The anadromous salmonid life cycle includes both fresh water (FW) and seawater (SW) stages. The parr-smolt transformation (smoltification) pre-adapt the fish to SW while still in FW. The osmoregulatory organs change their mode of action from a role of preventing water inflow in FW, to absorb ions to replace water lost by osmosis in SW. During smoltification, the drinking rate increases, in the intestine the ion and fluid transport increases and is further elevated after SW entry. In SW, the intestine absorbs ions to create an inwardly directed water flow which is accomplished by increased Na^+, K^+-ATPase (NKA) activity in the basolateral membrane, driving ion absorption via ion channels and/or co-transporters. This review will aim at discussing the expression patterns of the ion transporting proteins involved in intestinal fluid absorption in the FW stage, during smoltification and after SW entry. Of equal importance for intestinal fluid absorption as the active absorption of ions is the permeability of the epithelium to ions and water. During the smoltification the increase in NKA activity and water uptake in SW is accompanied by decreased paracellular permeability suggesting a redirection of the fluid movement from a paracellular route in FW, to a transcellular route in SW. Increased transcellular fluid absorption could be achieved by incorporation of aquaporins (AQPs) into the enterocyte membranes and/or by a change in fatty acid profile of the enterocyte lipid bilayer. An increased incorporation of unsaturated fatty acids into the membrane phospholipids will increase water permeability by enhancing the fluidity of the membrane. A second aim of the present review is therefore to discuss the presence and regulation of expression of AQPs in the enterocyte membrane as well as to discuss the profile of fatty acids present in the membrane phospholipids during different stages of the salmonid lifecycle.

Keywords: intestinal fluid absorption, aquaporin, claudin, paracellular permeability, salmonids, Ussing chambers, osmoregulation

INTRODUCTION

Anadromous salmonids are born in fresh water (FW), migrate to the sea to forage and grow before becoming sexually mature and return to their natal rivers to spawn (McCormick et al., 1998). Thus, the typical life cycle of anadromous salmonids includes two important transitions between FW and seawater (SW). The current overview will focus on the first osmotic transitional stage, i.e., the pre-adaptation of the hyperosmoregulatory parr in FW to become a hypoosmoregulatory smolt in SW.

The anadromous salmonids show a wide spectrum of complex changes in physiology, morphology, biochemistry, and behavior that take place in FW, during the parr-smolt transformation, pre-adapting the fish for a life in SW. The developmental changes are governed by a number of endocrine systems, of which cortisol is a major component together with growth hormone (GH), insulin-like growth factor-I (IGF-I), and thyroid hormones (McCormick et al., 1998).

In teleost fish, continuously exposed to osmotic forces across all epithelia, transitions between environmental salinities requires marked changes in osmoregulatory mechanisms in order to maintain osmotic homeostasis. In FW, active absorption of ions and excretion of the excess amounts of water diffusing into the fish is crucial. In SW, on the other hand, uptake of water in combination with secretion of ions is needed (see Marshall and Grosell, 2005; Evans, 2008). For both passive and active movements of ions and water, all epithelia: intestine, gills, kidney, and skin, are involved, but the main epithelia responsible for a regulated fluid intake is the intestine. A prerequisite to perform this task is to have access to ingested water. Already in 1930, Smith demonstrated that eel (*Anguilla anguilla*) in SW had higher drinking rates compared to FW conspecifics. High drinking rates have since been described as a general feature for several stenohaline marine and euryhaline SW acclimated species when compared to fish living in FW (Perrott et al., 1992). However, water cannot be actively absorbed and therefore the water uptake was suggested to be coupled to an uptake of monovalent ions (Smith, 1930). For stenohaline SW fish, solute linked water absorption has been extensively investigated and molecular mechanisms including active monovalent ion transporters, ion-channels and co-transporters have been presented (**Figure 1**; see Grosell, 2011 for details). Several of the active transport mechanisms suggested for stenohaline SW fish are present also in anadromous salmonids but the detailed

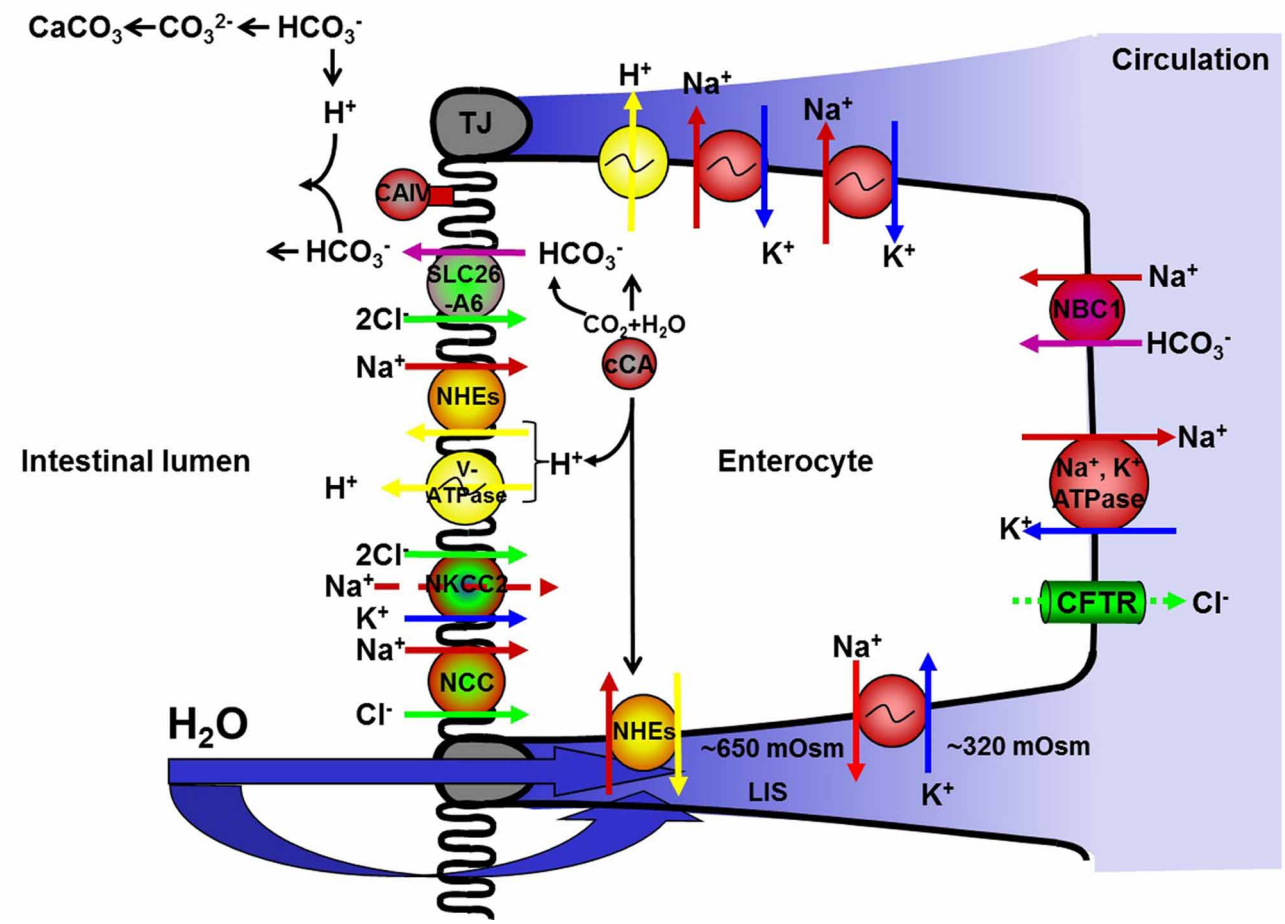

FIGURE 1 | A simplified version of the current hypothesis regarding ion-driven intestinal fluid absorption in stenohaline marine and seawater acclimated euryhaline teleosts (see text for details). NKA; Na^+, K^+-ATPase, NKCC; Na^+:K^+:$2Cl^-$ co-transporter, NCC; Na^+:Cl^- co-transporter, NHEs; Na^+/H^+ exchangers, CAc; cytosolic carbonic anhydrase, tCAIV; membrane bound carbonic anhydrase type IV, V-ATPase; V-type H^+-ATPase, Na^+:HCO_3^- co-transporters; NBCs, Cl^-/HCO_3^- exchanger; SLC26A6. CFTR; cystic fibrosis transmembrane conductance regulator.

characterization and localization of all various transporters are not yet performed. Furthermore, not only the transporting function of the solutes determines the intestinal fluid uptake, but equally important for an efficient absorption of fluid across the intestinal epithelium are the characteristics and regulation of the intestinal epithelial permeability. Present models for intestinal fluid uptake in stenohaline teleosts do not reveal the relative importance of paracellular versus transcellular pathways or the components responsible for allowing the movement of water across the epithelia. In this respect the intestine of anadromous salmonids, which changes physiological mechanisms to meet FW and SW environments, respectively, can provide a valuable model system. The salmonid intestine offers a powerful and general model to study regulatory mechanisms of and pathways for water movement during intestinal fluid absorption also beyond the boundaries of teleost osmoregulation.

METHODOLOGICAL CONSIDERATIONS

In order to study intestinal fluid uptake in salmonids, several methods have been used. The classical everted and non-everted gut sac preparations have been used to measure intestinal fluid uptake in fish from different external salinities as well as in different regions of the gastrointestinal tract (Collie and Bern, 1982; Usher et al., 1991; Veillette et al., 1993; Cornell et al., 1994; Kerstetter and White, 1994; Veillette et al., 1995; Madsen et al., 2011). To assess the contribution of active ion transporters to the intestinal fluid uptake, the main focus has been directed toward the intestinal Na^+, K^+-ATPase (NKA) activity, using NKA enzyme specific *in vitro* assays (Colin et al., 1985; Madsen, 1990; Bisbal and Specker, 1991; Seidelin et al., 2000; Stefansson et al., 2003; Sundell et al., 2003; Veillette and Young, 2004; Veillette et al., 2005). The recent expansion in knowledge regarding the genomes of multiple salmonid species has led to a rapid increase in development of both molecular and immunological tools. These tools have opened up for the assessment of specific target genes for several other transporters also involved in the ion coupled fluid transport, i.e., NKA, Na^+/H^+ exchangers (NHEs), cytosolic carbonic anhydrase (CAc), membrane bound carbonic anhydrase type IV (tCAIV), V-type H^+-ATPase (V-ATPase), Na^+:HCO_3^- co-transporters

(NBCs), FXYDs, claudins, aquaporins (AQPs), Na^+:K^+:$2Cl^-$ co-transporters (NKCCs), Na^+:Cl^- co-transporter (NCC) and Cl^-/HCO_3^- exchanger (SLC26A6) (Grosell et al., 2007, 2009; Tipsmark and Madsen, 2007; Tipsmark, 2008; Tipsmark et al., 2008, 2010a,b; Madsen et al., 2011) as well as immunostaining of specific target proteins (AQPs, CAc, tCAIV, V-ATPase) which has been important to determine the abundance and localization of these ion transporters (Seidelin et al., 2000; Grosell et al., 2007; Madsen et al., 2011).

In order to get an overall picture of the entire intestinal epithelia and the physiological mechanisms involved in fluid transport including the barrier creating proteins, live epithelia can be studied, *in vitro*, using the Ussing chamber methodology (Ussing and Zerahn, 1951). This is an established technique for measurements of active and passive transports and transfer of solutes and water across a live epithelial tissue. The Ussing chambers were first described by the Danish physiologist Hans H. Ussing, who studied the capacity of an epithelium to actively move ions and nutrients against an electrochemical and/or concentration gradient using the frog skin as a model (Ussing and Zerahn, 1951). Today, modified Ussing chambers, or diffusion chambers (Grass and Sweetana, 1988), are widely used to study epithelial physiology in a multitude of species and tissues for applications such as ion transport, nutrient uptake, protein absorption, drug absorption, host pathogen interactions, and pathophysiological mechanisms (Santos et al., 2000, 2001; Saunders et al., 2002; Sundell et al., 2003; Velin et al., 2004; Jutfelt et al., 2006, 2007, 2008; Moeser et al., 2007; Clarke, 2009). Within the pharmacuetical sciences and industry, diffusion chambers are routinely used for high troughput screening of substance absorption across epithelia. This has led to a simplification of the classical Ussing chamber technique by exclusion of one or both electrode set ups, a clear disadvantage when investigating detailed physiological processes within epithelia. One of the great advantages with the Ussing-type of diffusion chambers is the two electrode pairs equipping the chambers and measuring the electrical characteristics of the epithelium. These electrical characteristics will provide information on both preparation viability and valuable information on transporting activities and permeability. Each chamber is equipped with one pair of KCl-electrodes to measure the potential difference across the epithelium and one pair of inert (e.g., platinum) electrodes that can be used to apply currents or voltages. The measuring electrodes are continuously monitoring the transepithelial potential (TEP) and in the original Ussing chamber set up, increasing currents were applied across the epithelium until a TEP of zero was reached. That current was named the short circuit current (SCC) and is equivalent to the sum of the ion movements induced by active transport. The transepithelial resistance (TER) could then be calculated from the TEP and the SCC, using Ohms' law (Ussing and Zerahn, 1951). However, the classical Ussing method has been further developed with time, and one main improvement of the methodology is the use of alternating small currents after which the resulting voltages are recorded, at time intervals, instead of applying a larger current clamping the epithelium. This way, electrical charging of the epithelium is prevented and more undisturbed electrical measures possible (see Wikman-Larhed and Artursson, 1995 and Sundell et al., 2003). Our laboratory has further refined this methodology by the development of a new Ussing chamber measurement system: UCC-401 (UCC-Labs Ltd.) that applies alternating adaptive DC voltages (U) to the epithelium generating corresponding currents (I). The voltages are randomly applied to generate currents that alternate between positive and negative values, within the range of a fixed min and max value, resulting in zero net charge. The range of currents is manually defined, i.e., between -30 and $30\,\mu A$ and the currents are generated during four consecutive cycles to generate mean values. The DC voltages are applied through the use of platinum electrodes, every 5th minute, to minimize electrical loading of the epithelium. The U/I pairs obtained are fitted to a straight line using the least square method. The slope of the line represents the TER and the voltage where it intercepts $U = 0$, show the SCC. Undisturbed TEP is continuously measured using the pair of KCl electrodes immersed in 3 M KCl and connected to a beaker with 0.9% NaCl using KCl-agar bridges (4% agar in 3 M KCl) from the NaCl beaker a second set of agar bridges made in 0.9% NaCl connect to the Ussing chambers as close as possible to the epithelium (Sundell et al., 2003).

Using the Ussing chamber methodology, the TEP obtained is a result of the whole epithelium and will thus be a sum of ion transfer both through the paracellular pathway and via the electromotive forces of the basolateral and apical membrane in series (Halm et al., 1985a). A serosa-negative potential would reflect a net uptake of negative charges and most likely a diffusion of positive ions (Na^+) back to the lumen through possible cation-selective tight junctions (TJ), whereas a serosa-positive potential would reflect a net uptake of positive charges and may indicate a lower permeability for cations across TJ. The SCC across the epithelium reflects an overall net ion transport activity and includes apart from the major ions, Na^+ and Cl^- , also K^+ and HCO_3^- .

The TER equals the sum of the paracellular shunt resistance and the transcellular resistance, in which the transcellular resistance is determined by the apical and the basolateral membrane resistances in series. The fish intestine is mostly defined as a leaky epithelium (Powell, 1981; Loretz, 1995), and thus the TER is regarded to reflect the paracellular permeability, i.e., the conductance across the TJ but can under certain conditions also be influenced by the lateral intercellular space (LIS) (Blikslager et al., 2007). However, this assumption is dependent on the relative contribution of the para- and transcellular resistances to the TER, which differs between regions of the gastrointestinal tract as well as between the species and environmental conditions (Sundell et al., 2003; Jutfelt et al., 2006, 2008; Sundh et al., 2010). The permeability of the paracellular pathway can additionally be studied by the use of hydrophilic permeability markers such as ^{14}C-mannitol, which is a hydrophilic molecule, suggested to be passing only through the paracellular pathway (Bjarnason et al., 1995). Urea, a smaller but equally uncharged and hydrophilic molecule, has also been used as a paracellular marker (Artursson et al., 1993). However, urea may also have a transcellular uptake route through specific urea transporters. In fish, the specific urea transporter (UT-b) is present in kidneys and gills (Walsh et al., 2000; Mistry et al., 2005), but has to our knowledge not yet been demonstrated in the intestine. Therefore urea may still be a

relevant paracellular marker in this epithelium (Artursson et al., 1993). On the other hand, the UT-b transporter belongs to the same family as the Na^+-coupled glucose transporter (SGLT1) (Leung et al., 2000) which is an important transporter in the intestinal epithelium and interactions may be possible making the route of urea transfer a bit more unpredictable also in the intestine.

SALMONIDS AND INTESTINAL ION TRANSPORT

As shown for stenohaline SW fish, salmonids in SW display elevated drinking rates (Usher et al., 1988; Fuentes et al., 1996) in association with elevated intestinal fluid absorption (Collie and Bern, 1982; Veillette et al., 1993, 2005; Kerstetter and White, 1994; Nielsen et al., 1999; Genz et al., 2011; Madsen et al., 2011). The major driving force and hence the first step in intestinal fluid transport (**Figure 1**) in salmonids is considered to be basolateral located NKA (Loretz, 1995; Veillette et al., 2005; Madsen et al., 2011). The selective NKA inhibitor, ouabain, decreases the Jv across intestinal sac preparations by 67–100% in coho salmon (*Oncorhynchus kisutch*; Collie and Bern, 1982), Atlantic salmon (*Salmon salar* L.; Veillette et al., 1993) and rainbow trout (*Oncorhynchus mykiss*; Madsen et al., 2011) and the elevated fluid absorption in SW is associated with elevated NKA activities throughout the whole intestinal canal (see **Figure 2** for description of the intestinal regions), from the pyloric caeca (Rey et al., 1991; Seidelin et al., 2000; Veillette et al., 2005) to the proximal (Colin et al., 1985; Veillette et al., 1995; Sundell et al., 2003) and distal intestine (Colin et al., 1985; Sundell et al., 2003).

During smoltification, the intestine, as well as the other osmoregulatory tissues, will pre-adapt for a life in SW, while the fish are still in FW. A developmental increase in drinking rates (Nielsen et al., 1999) and increased fluid absorption have been observed in the distal intestine at the peak of smoltification in Atlantic salmon (Veillette et al., 1993) and brown trout (*Salmo trutta* L.) (Nielsen et al., 1999), whereas the proximal intestine does not seem to show the same increase (Veillette et al., 1993; Nielsen et al., 1999). Increased fluid absorption at the peak of smoltification is accompanied by increased NKA activity, which in Atlantic salmon is apparent in both proximal and distal intestine (Sundell et al., 2003).

The second step in the solute coupled fluid absorption (**Figure 1**) is the intake of Cl^- ions into the enterocytes. In marine teleosts, this is mainly governed by an apically situated NKCC2 or for some species by a NCC, both driven by the inwardly directed Na^+-gradient (Field et al., 1978; Frizzell et al., 1979, 1984; Musch et al., 1982; Halm et al., 1985b; Tresguerres et al., 2010; Watanabe et al., 2011). The presence and expression profile of NKCC2 or NCC in Atlantic salmon intestines during smoltification and SW acclimation is poorly investigated but is under assessment in our lab. In salmonids, Cl^- absorption in exchange for HCO_3^- (SLC26A6) has received increased attention the last decade following its first demonstration in rainbow trout (Shehadeh and Gordon, 1969). This exchange is suggested to contribute to the solute coupled fluid absorption both by intake of Cl^- by the enterocytes and by reducing the luminal osmolality through an increases supply of HCO_3^- for precipitation of luminal Ca^{2+} into $CaCO_3$ (see Grosell, 2010, 2011). Several intracellular sources for HCO_3^- have been suggested, including a basolateral NBC1 as well as intracellular CO_2 hydration facilitated by CAc (Grosell et al., 2007). Hydration of CO_2 generates a surplus of H^+ which have to be excreted to avoid cellular acidification and a basolateral as well an apical V-ATPase together with NHEs has been suggested in rainbow trout acclimated to SW (Grosell et al., 2007). As far as we know, the expression profile and significance of NBC, SLC26A6, and CAc during smoltification and SW acclimation of Atlantic salmon intestine, is mainly unexplored and certainly needs attention.

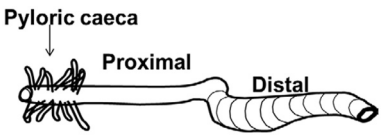

FIGURE 2 | A schematic drawing of the gastrointestinal tract of salmonid fish. In this review the intestinal region just after the pyloric caeca is termed as the proximal intestine. The region just behind the ileo-rectal valve is termed the distal intestine.

THE PERMEABILITY OF THE SALMONID INTESTINAL EPITHELIUM—IMPLICATIONS FOR FLUID ABSORPTION

While the major mechanisms for the ion transport in SW fish, i.e., the driving force behind the fluid transport across the intestine is thoroughly investigated, the main route for water flow, is under debate and has not yet been established (Alves et al., 1999; Hill et al., 2004; Fischbarg, 2010; Laforenza, 2012). The permeability for both transcellular and paracellular routes can be physiologically regulated. The paracellular permeability is mainly regulated by affecting the TJs (Madara and Pappenheimer, 1987; Daugherty and Mrsny, 1999; Anderson et al., 2004), whereas the transcellular permeability to water can be regulated by the composition of the polar membrane lipids (Haines, 1994; Hill et al., 1999) and/or by incorporation of AQPs into the membranes (Ma and Verkman, 1999; Nedvetsky et al., 2009; Laforenza, 2012). Thus, depending on the relative permeability of these two pathways the fluid absorption could be shunted between the paracellular and the transcellular route. In this aspect the anadromous salmonids are very interesting to study as they migrate between a hypoosmotic and hyperosmotic environment also including dramatic changes in degree of exposure of the environment to the intestinal epithelium.

PARACELLULAR PERMEABILITY AT DIFFERENT ENVIRONMENTAL SALINITIES AND DURING SMOLTIFICATION

In studies on rainbow trout, the electrical characteristics of the intestinal epithelium have been investigated at different salinities. Rainbow trout, provided by Anten fish farm (Alingsås, Sweden), were of both sexes (body weight 100–150 g; $n = 88$) and maintained in re-circulated, filtered, and aerated FW for at least 10 days after transfer to the laboratory. Subsamples of fish were transferred to SW (25‰) and allowed to acclimate for at least 3 weeks to the new environment. Intestines of fish maintained in FW as well as acclimated to SW were sampled

and mounted in Ussing chambers as described by Sundell et al. (2003). The TER, TEP, and SCC were monitored together with radiotracer studies assessing the apparent permeability (P_{app}) of three different sized hydrophilic markers: ^{14}C-urea; MW:62, ^{14}C-erytritol; MW:124 and ^{14}C-mannitol; MW:184. SW acclimated trout showed higher TER than the FW acclimated, in all intestinal regions examined (**Figure 3A**). This was confirmed by measurements of P_{app} for the three hydrophilic marker molecules. P_{app} was lower for all three markers in SW than in FW acclimated trout and followed a trend where the P_{app} decreased with increasing molecular size in all cases (**Figure 3B**). This suggests that decreased intestinal paracellular permeability is a hallmark for salmonids after acclimation to SW. In agreement, increased TER in both proximal and distal intestine and reduced P_{app} for mannitol after acclimation to SW have been observed in several studies on Atlantic salmon (Sundell et al., 2003; Sundh, Nielsen, Stefansson, and Sundell, in preparation; Sundh, Nielsen, Stefansson, Andersson, Taranger, and Sundell, in preparation; Sundh, Nielsen, Andersson, Taranger, Schultz, Prunet, Stefansson, and Sundell, in preparation). Considering the importance of an osmotic gradient in the LIS which creates the driving force for fluid transport (see Grosell, 2010, 2011), the decrease in the permeability of the paracellular pathway after migration to SW may be advantageous to the fish. By reducing conductance for ions through TJs when the fish are in SW, this would increase the ability of the fish to build up the osmotic gradient in the LIS. Moreover, a higher TER and considerably lower P_{app} for all paracellular marker molecules were observed in the rainbow trout distal compared to the proximal intestine (**Figures 3A,B**). This is normally observed also in Atlantic salmon, making this regional differentiation a common feature for these two salmonids (Sundell et al., 2003; Jutfelt et al., 2006, 2008; Sundh et al., 2010). Since the distal intestine appears to be the dominating water absorbing region in salmonids in SW, this suggests that the higher TER in this region is of functional importance for fluid absorption, probably by decreasing the leakage of especially positive ions back into the intestinal lumen. This is however, somewhat contradictory to one earlier study on Coho salmon (Collie, 1985) where no change in TER was seen in the proximal intestine whereas a decrease was seen in posterior intestine after SW acclimation.

The increase in TER observed in SW acclimated trout is also reflected in the other electrical parameters. Proximal intestines from SW acclimated fish show a more serosa positive TEP than intestines from FW acclimated trout (**Figure 4A**). This is an expected consequence of the increase in TER and can be explained by reduced conductance and thus a reduction in the cation leakage from the hyperosmotic LIS to the intestinal lumen. Further, there were higher absolute values in SCC of SW acclimated trout compared to FW acclimated trout (**Figure 4B**). No difference was observed between FW and SW acclimated trout in the distal intestinal region, which can be a result of the distal region being less prone to have gradient building active transport mechanisms as this region do not actively absorb nutrients to a significant extent (Loretz, 1995; Bakke-McKellep et al., 2000; Jutfelt et al., 2007). The serosa positive TEP in SW seems to be quite specific for salmonids (**Figure 4A**; (Oxley et al., 2007; Sundh et al., 2010, 2011), as when comparing with electrical data from more stenohaline SW fish, most previous literature show serosa negative TEP (see Loretz, 1995). However, this is not a totally universal pattern, since small serosa-positive potentials have been reported also for one marine teleost *Cottus scorpius* (House and Green, 1965). In FW acclimated fish, on the other hand, the TEP is often more close to zero and can fluctuate between serosa positive and serosa negative values even within the same species (**Figure 4A**; Sundh, Nielsen, Andersson, Taranger,

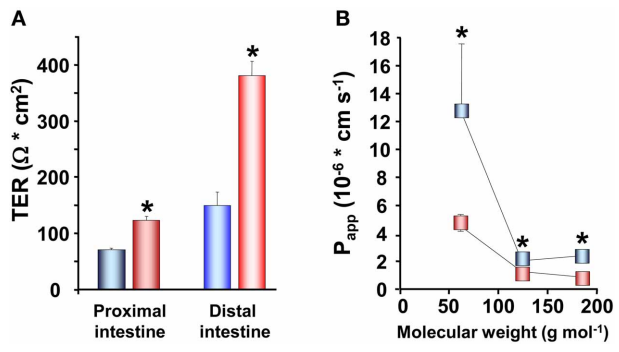

FIGURE 3 | Transepithelial resistance (TER; $n = 8$) in the proximal and distal intestine (A) and the apparent permeability coefficient (P_{app}; $n = 16$) (B) for three different sized hydrophilic permeability markers; ^{14}C-urea, MW:62, ^{14}C-erytritol, MW:124 and ^{14}C-mannitol, MW:184, in the proximal and distal intestine of fresh water (■, ■) and seawater (■, ■) adapted rainbow trout. (Values presented are means ± SEM). Asterisks indicate significant differences between FW- and SW-acclimated trout. All data are expressed as mean values ± SEM. Differences in the parameters measured are analyzed using Two-Way ANOVA followed by Student Neuman Keuls *post-hoc* procedure. Significance was accepted at $p < 0.05$ (see Sundell et al., 2003; Jutfelt et al., 2007; Sundh et al., 2009).

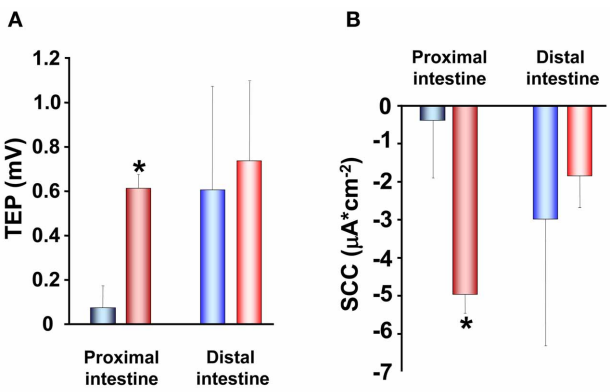

FIGURE 4 | Transepithelial potential (TEP; A) and short-circuit current (SCC; B) in proximal and distal regions of the intestinal tract of fresh water (■, ■) and seawater (■, ■) adapted rainbow trout ($n = 8$). Asterisks indicate significant differences between FW- and SW-acclimated trout. All data are expressed as mean values ± SEM. Differences in the parameters measured are analyzed using Two-Way ANOVA of variance, followed by Student Neuman Keuls *post hoc* procedure. Significance was accepted at $p < 0.05$ (see Sundell et al., 2003; Jutfelt et al., 2007; Sundh et al., 2009).

Schultz, Prunet, Stefansson, and Sundell, in preparation; Huang and Holt, 1974; Ando, 1975; Ando et al., 1975). The combined pattern of responses in electrical parameters after acclimation of FW trout to SW, supports each other and suggests a model in which the ion transporting activities increases (as seen by a higher absolute value for the SCC in SW) in order to desalt the ingested SW and thereby reducing the luminal osmolality to allow for fluid uptake. Concomitantly the paracellular permeability decreases to allow for building up a sufficient osmotic gradient in the LIS, and thus creates a strong driving force for fluid absorption.

Another important aspect of a tighter intestinal epithelium, i.e., increased TER in SW acclimated fish, is an increased physical barrier function. This would favor the disease resistance, as SW is a thriving habitat for both bacteria and viruses (Wilhelm and Suttle, 1999) and with increasing drinking rates in SW the load of potentially harmful substances will increase. Hence, a second advantage of decreased paracellular permeability in SW would be to reduce the ability for antigens and other harmful substances to get access to the host via the paracellular pathway thus increasing the disease resistance by creating a stronger intestinal barrier (Sundh et al., 2009; Ahrne and Johansson Hagslätt, 2011; Segner et al., 2012).

CLAUDIN EXPRESSION AT DIFFERENT SALINITIES AND DURING SMOLTIFICATION—THE LINK TO PARACELLULAR PERMEABILITY

The TJ consists of several physiologically regulated proteins forming the circumferential seals around adjacent epithelial cells. Three of the main protein families found in the TJs are occludins, claudins, and junction-associated membrane proteins (JAM). The claudins and occludins form the backbone of the TJ, and the number of TJ strands is suggested to be proportional to the permeability (Schneeberger and Lynch, 2004; Van Itallie et al., 2008). However, the selective permeability to different both charged and uncharged molecules are much more complex and discrepancies between the measures of the paracellular pathway when using the electrical parameter TER or the apparent molecular permeability of a hydrophilic molecule, P_{app}, are not infrequent (Sundell et al., 2003; Van Itallie et al., 2008; Sundh et al., 2010, 2011). One probable explanation for this is that TER is measured within milliseconds, whereas P_{app} is measured as fluxes over hours and therefore also represents the dynamics of the TJs. TJs have been shown to frequently break, migrate and reconnect which would allow for alternating movement of molecules over time (Anderson et al., 2004; Van Itallie and Anderson, 2006). Claudins, as well as occludin and other adjacent proteins affect the selective permeability for different molecules, and this selectivity is not only dependent on molecular size but also on electrical charge (Van Itallie et al., 2008; Vikström et al., 2009; Cummins, 2012).

The claudins constitute a large protein family, with several different isoforms in fish. In the pufferfish (*Fugu rubripes*), 56 different claudin isoforms are described (Loh et al., 2004) and the number of isoforms presently known in Atlantic salmon are 26 (Tipsmark et al., 2008). The different claudin isoforms display different number and types of charged amino acid residues lining the pore that is formed between the adjacent cells which constitute the passage way for molecules using the paracellular pathway. The differential expression of claudin isoforms has therefore been suggested to be the main determiners of TJ ion and size selectivity (Van Itallie and Anderson, 2006; Amasheh et al., 2011). This suggestion is based on detailed functional studies in mammals, whereas in fish, the knowledge on the physiological characteristics of the different claudin isoforms is limited. As far as we know, one study in zebrafish (*Danio rerio*) has suggested claudin-15 to be a cation pore forming isoform (Bagnat et al., 2007) and another study has demonstrated that claudin-30 reduces sodium permeability in the gills of Atlantic salmon (Engelund et al., 2012). In mammals, claudin-1, -3, -4, -5, -8, -11, -14, and -19 are all described as barrier builders, claudin-2 and -10 are suggested to create cation selective pores, whereas the role for claudin-7, -12, -15, and -16 is still obscure (Amasheh et al., 2011). Interestingly, claudin-2 appears, in addition to increase the cation permeability, also to create a paracellular water channel through the TJ (Rosenthal et al., 2010). In the Atlantic salmon intestine, recent work has revealed the mRNA expression of several claudin isoforms. These includes claudin-3a, -3b, -3c, claudin-15, and claudin-25b of which the two latter are suggested as isoforms specific for the intestine (Tipsmark et al., 2008, 2010a; Tipsmark and Madsen, 2012). Based on comparison of the charged amino acid residues in the part of claudin sequences located within the paracellular space between adjacent enterocytes, claudin-15 in Atlantic salmon was suggested to be similar to zebrafish and mammalian claudin-15, a pore forming isoform, while Atlantic salmon claudin-25b displayed similarities with mammalian claudin-4, a barrier building claudin (Tipsmark et al., 2010a). Moreover, two other proteins that are known to be important in the formation of TJs are occludin and tricellulin and the presence of these in the salmonid intestine have been shown at the mRNA level (Tipsmark and Madsen, 2012). Thus, these proteins are also believed to be important players in determining the structure and function of the TJ complex.

Baring the nature of the claudins in mind, an increased TER after SW transfer could thus be the result of an increased expression of barrier builders and/or a reduced expression of pore forming claudins. Throughout the whole intestinal length, the mRNA levels of claudin-25b was >10 times more abundant compared to claudin-15 and claudin-3 (Tipsmark et al., 2010a; Tipsmark and Madsen, 2012) suggesting that claudin-25b is the dominating claudin in the intestine of Atlantic salmon. Moreover, the expression of claudin-25b was 10–20 times higher in the distal region compared to the proximal region which would support the suggestion that claudin-25b is, similarly to mammalian claudin-4, a barrier building claudin, as the permeability is normally lower in distal compared to the proximal intestine (Sundell et al., 2003; Jutfelt et al., 2006, 2008; Sundh et al., 2010; Tipsmark et al., 2010a). Altogether, this suggests that claudin-25b is one important determiner of the character of TJ properties and that changes in this isoform determine/dominate the physiological effects observed in epithelial permeability measured as TER. Thus, the higher TER normally observed in the distal intestine compared to the proximal intestine (**Figures 3A** and **6A**; Sundell et al., 2003; Jutfelt et al., 2006, 2008; Sundh et al., 2010) could be explained by the higher expression of claudin-25b in this

intestinal region. Further, the increased expression of claudin-25b in the proximal intestine of SW acclimated Atlantic salmon (Tipsmark et al., 2010a) clearly supports and also provides an explanation to the increase in TER seen in SW exposed salmonids (**Figure 2A**; Sundell et al., 2003; Sundh, Nielsen, Stefansson, and Sundell, in preparation).

CORTISOL AS A REGULATOR OF INTESTINAL EPITHELIAL TRANSPORT AND PARACELLULAR PERMEABILITY

Cortisol has a major developmental role in the smoltification of salmonids and the increase in plasma levels during this life stage is well established (Specker, 1982; Specker and Schreck, 1982; Young, 1986; Shrimpton and McCormick, 1998; Sundell et al., 2003). *In vivo* injections of cortisol stimulate NKA activity in the intestine of rainbow trout (Madsen, 1990) as well as increases intestinal fluid absorption of several salmonid species (Cornell et al., 1994; Specker et al., 1994; Veillette et al., 1995, 2005). Both *in vivo* and *in vitro* treatment with cortisol stimulates NKA activity and fluid absorption in the pyloric caeca of Chinook salmon (*Oncorhynchus tshawytscha*; Veillette and Young, 2004; Veillette et al., 2005). Moreover, administration of the corticosteroid antagonist RU486 abolished the increased fluid absorption observed during natural smoltification as well as after *in vivo* treatment with slow release cortisol implants (Veillette et al., 1995). Thus, the intestinal fluid transport seems to be fully regulated by cortisol and in order to provide a more detailed view on the mechanisms by which cortisol act to increase the intestinal fluid uptake, Ussing chambers have been used to study electrophysiology of rainbow trout intestines after treatment with slow release implant of cortisol as described by Specker et al. (1994). The cortisol implant procedure has been shown to chronically elevate plasma cortisol concentrations in a physiological range over 7 days and significantly stimulate intestinal fluid uptake in post-smolt stage Atlantic salmon 5–7 days after administration of the implant (Cornell et al., 1994; Specker et al., 1994; Veillette et al., 1995). After 7 days of cortisol treatment the SCC showed a higher absolute value with similar effect for both intestinal regions (**Figure 5A**). This suggest that cortisol stimulates the epithelial ion transporting activity, which is in line with previously shown cortisol stimulation of fluid transport, increases in NKA activity in salmonids during smoltification as well as the concomitant increase in NKA activity and plasma cortisol levels after SW transfer (Colin et al., 1985; Rey et al., 1991; Veillette et al., 1995, 2005; Seidelin et al., 2000; Sundell et al., 2003). A stimulation of SCC was also observed when FW acclimated trout was acclimated to SW, which presents further support for the role of cortisol as a main regulator of the increased ion-transporting capacity occurring during parr-smolt transformation.

The electrical parameter reflecting intestinal permeability, TER, as well as the P_{app} both demonstrated increased paracellular permeability in response to cortisol treatment. The proximal and distal intestine showed decreased TER (**Figure 6A**) and increased P_{app} for mannitol (**Figure 6B**) in the cortisol treated trout compared to controls, suggesting that cortisol modulates the composition of proteins in the TJ complex. Indeed, Atlantic salmon receiving injections with cortisol downregulate claudin-25b, the probable barrier building claudin, in the proximal intestine of

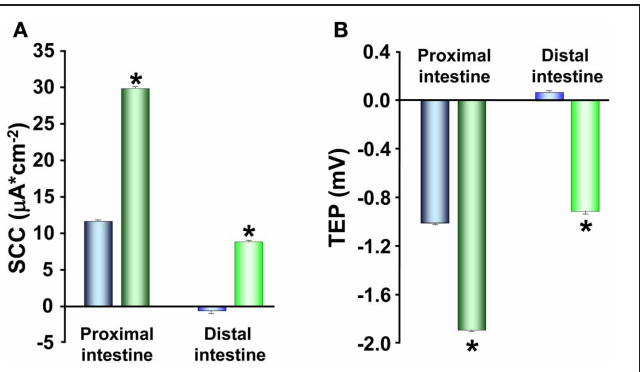

FIGURE 5 | Short-circuit current (SCC; A) and transepithelial potential (TEP; B) in proximal and distal intestine of control (■, ■) and cortisol (■, ■) implanted rainbow trout acclimated to fresh water. Values presented are means ± SEM ($n = 8$). Asterisks indicate significant differences between cortisol treated and control trout, in each region of the intestinal tract. All data are expressed as mean values ± SEM. Differences in the parameters measured are analyzed using Two-Way ANOVA of variance, followed by Student Neuman Keuls *post hoc* procedure. Significance was accepted at $p < 0.05$ (see Sundell et al., 2003; Jutfelt et al., 2007; Sundh et al., 2009).

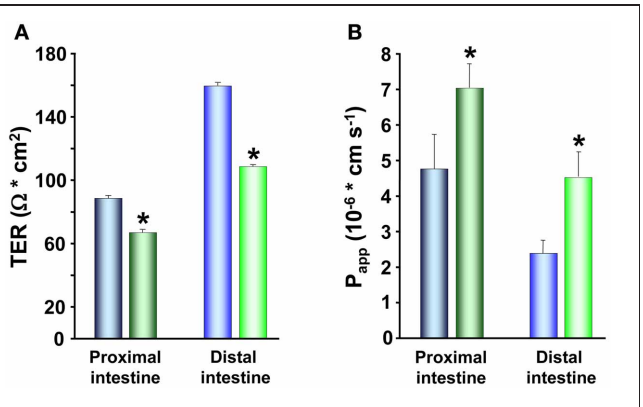

FIGURE 6 | Transepithelial resistance (TER; A) and the apparent permeability coefficients (P_{app}; B) of ^{14}C-mannitol, MW:184, in proximal and distal intestine of control (■, ■) and cortisol (■, ■) implanted rainbow trout acclimated to fresh water. Values presented are means ± SEM ($n = 8$). Asterisks indicate significant differences between cortisol treated and control trout, in each region of the intestinal tract. All data are expressed as mean values ± SEM. Differences in the parameters measured are analyzed using Two-Way ANOVA of variance, followed by Student Neuman Keuls *post hoc* procedure. Significance was accepted at $p < 0.05$ (see Sundell et al., 2003; Jutfelt et al., 2007; Sundh et al., 2009).

FW acclimated fish and in both proximal and distal intestine of SW acclimated fish (Tipsmark et al., 2010a). Thus, a reduction in claudin-25b may be an explanation behind the increased paracellular permeability seen in the cortisol treated rainbow trout. Moreover, a reduction in TER can be observed close to the peak of smoltification (Sundell et al., 2003; Sundh, Nielsen, Stefansson, and Sundell, in preparation) when the plasma cortisol levels are peaking. In agreement, mRNA levels of claudin-15 and -25b decrease during smoltification with lowest levels just prior to SW

transfer (Tipsmark et al., 2010a), suggesting that cortisol might be responsible for these developmental changes seen in paracellular permeability.

When implanting rainbow trout with cortisol, TEP show a more serosa negative value (**Figure 5B**) which in parallel to the findings in paracellular permeability is contradictory to the response observed after transfer to SW (**Figures 3** and **4**; Sundell et al., 2003). Taken together, cortisol seems to be the main regulatory hormone of the increased intestinal fluid uptake necessary for salmonids when transferred to SW. This is achieved through a stimulation of enterocyte NKA activity during smoltification as well as after SW transfer. However, cortisol treatment of rainbow trout and increasing circulating levels of cortisol in Atlantic salmon during smoltification instead result in increased paracellular permeability, which is counter to the pattern seen after SW transfer. A possible explanation to these discrepancies is that cortisol stimulates the active transporting activities during smoltification, while the fish are still in FW, thus equipping the intestine with the right set of transporting proteins for a SW environment. The effects of cortisol on the paracellular permeability on the other hand, is to remain a high or even increased permeability, and the increase in serosa negative TEP suggests that this increased paracellular permeability is cation selective. A leakage of positive ions back to the intestinal lumen would thereby prevent the buildup of an osmotic gradient in the LIS and thus allow for a preparatory increase of ion transporting activities without creating a too high fluid absorption while the fish is still in FW.

TRANSCELLULAR PERMEABILITY AT DIFFERENT SALINITIES AND DURING SMOLTIFICATION

In salmonids, during smoltification as well as during cortisol treatment, the paracellular permeability is maintained high and any fluid absorption would probably mainly occur through a paracellular route. However, after SW transfer there is a decrease in the paracellular permeability of the epithelia together with an increased NKA activity of the basolateral enterocyte membrane. This clearly suggests a re-direction of the water flow from a paracellular route in FW to a more transcellular route in SW, anticipating increased transcellular permeability for water in SW. An increase in the water permeability of the enterocyte membrane could be due to altered permeability of the lipid bilayer or to incorporation and/or activation of AQPs.

THE ROLE OF THE LIPID BILAYER

The enterocyte membrane, being an epithelial cell with major transporting functions, contains a large fraction of transport proteins, but also, a large area of lipid bilayer. In the enterocyte plasmamembrane phospholipids play a major role for fluidity and permeability (Stubbs and Smith, 1984; Seo et al., 2006) and changes in the composition of fatty acids in this cell membrane may thus have a major impact on the transcellular water permeability. Since the gastrointestinal tract is the first organ to encounter ingested feed, the lipid composition of the fish diet has shown to influence the lipid composition of the enterocyte membrane (Houpe et al., 1997; Cahu et al., 2000; Ruyter et al., 2006). Moreover, it is clear that also the external environment have an impact on enterocyte membrane composition as the fatty acid profiles can change after SW acclimation even though the same diet is maintained. Transfer of masu salmon (*Oncorhynchus masou*) and rainbow trout from FW to SW resulted in an increased level of $n-3$ poly unsaturated fatty acids ($n-3$ PUFA) of the intestinal brush border membrane (Leray et al., 1984) and total intestinal tissue (Li and Yamada, 1992). This increased proportion of $n-3$ PUFA in the brush border membrane was concomitant with an increased fluidity of the membrane (Leray et al., 1984). Alteration of PUFA incorporation into cell membranes is a physiological control mechanism to alter fluidity of the membranes in response to changes in temperature and hydrostatic pressure. Regarding the intestinal epithelial membranes this increase is suggested to result in increased water permeability (Brasitus et al., 1986; Lande et al., 1995). However, our most recent and preliminary results using NMR diffusometry of lipid vesicles prepared from intestinal mucosa of Atlantic salmon reared in FW or SW, show no major differences in water permeability (Bernin, Claesson, Sundh, Olsen, Andersson, Nydén, and Sundell, in preparation). This suggests that the protein part of the cell membranes have a larger influence than the lipid bilayer, on the transcellular water permeability. The most plausible explanation for an increased protein mediated transcelluar fluid absorption would be through incorporation of AQPs. This has elegantly been shown to be the physiological regulation of water transport in other transporting epithelia, like the classical trafficking model of AQP2 in mammalian kidney cells (Nedvetsky et al., 2009).

THE ROLE OF AQPs

The existence of cellular water channels was heavily disputed until evidence was presented in 1992 (Preston et al., 1992). AQPs are divided in two subfamilies; orthodox AQPs transporting only water and aquaglyceroporins that in addition, transport solutes like glycerol. In the human genome, 13 AQPs has been identified (AQP0-12) and an intense period of research have followed the AQP discovery showing the importance of AQPs in, e.g., absorption of water in the kidney, balance of the osmotic pressure in the brain tissue, tumor growth, and oocyte maturation (Verkman, 2009, 2012). Also, the importance of AQPs in the gastrointestinal tract of mammals has been highlighted (Ma and Verkman, 1999; Matsuzaki et al., 2004; Laforenza, 2012). Much less is knowm about AQPs in fish but data on the importance of AQPs in the gastrointestinal tract is growing (see Cutler et al., 2007; Cerda and Finn, 2010). However, in order to elucidate the importance of AQPs in salmonids and their role in intestinal fluid absorption during smoltification as well as in different environmental salinities, more studies are essential. The protein abundance and localization of different AQP isoforms at the cellular and subcellular levels must be known and supplemented by functional and structural characterization and regulation of the proteins (Walz et al., 2009; Cerda and Finn, 2010).

Recently, initial steps have been undertaken in order to elucidate the role of AQPs in Atlantic salmon during smoltification and after SW transfer. AQP-1aa, -1ab, -8ab and 10 have all been suggested as possible players in transepithelial water transport due to their existence at the mRNA transcript level

(Tipsmark et al., 2010b). During Atlantic salmon smoltification, mRNA expression increased for AQP-1aa (pyloric caeca), AQP-8ab (pyloric caeca, proximal, and distal intestine) and AQP-10 (pyloric caeca and distal intestine). After transfer to SW, the expression of these isoforms was up-regulated in the proximal intestine whereas no expression data were reported for the distal intestine (Tipsmark et al., 2010b). This indicates increased importance of the above mentioned AQPs in intestinal fluid absorption in a hyperosmotic environment. Indeed, the expression of AQP-1aa, -1ab, and -8ab at the protein level have been verified in SW acclimated Atlantic salmon by immunostaining by Madsen and co-workers (2011), whereas no protein expression patterns are available for salmon in FW or during smoltification. Although the protein abundance and cellular distribution has yet to be described during smoltification and compared between FW and SW, AQP-1aa and AQP-1ab in SW can be located to the brush border and sub-apical region of pyloric caeca, proximal and distal intestine membrane (Madsen et al., 2011). AQP-8ab was localized to the same area as 1aa and -1ab but in addition it was found also in the lateral regions of the enterocytes (Madsen et al., 2011). Moreover, a functional importance of AQPs in intestinal fluid absorption was suggested after a >50% reduction of intestinal fluid transport in non-everted gut-sac preparations treated with $HgCl_2$, a potent AQP inhibitor (Madsen et al., 2011). Convincing data for the predominance of a transcellular route for intestinal fluid absorption has recently been shown in another euryhaline teleost, the killifish (*Fundulus heteroclitus*). In this species, osmotic clamping conditions increased the net mucosal to serosal water flux 10-fold, whereas the flux of different sized polyethylene glycols (PEG; 400, 900, and 4000) was unaffected (Wood and Grosell, 2012). Under these conditions, addition of $HgCl_2$ reduced the fluid absorption by 60%, whereas the PEG permeability was increased 6–8 times. Thus, this study concludes that water and PEGs uses separate pathways to transfer across the intestinal epithelia and that the fluid absorption mainly uses a transcellular route, presumably through AQPs (Wood and Grosell, 2012). If the same relation can be observed in the intestine of salmonids remains to be determined.

An extensive amount of work is needed to fully elucidate the role of AQPs in intestinal fluid absorption of salmonids. Nevertheless, the expression of several AQPs at mRNA and protein level as well as decreased fluid absorption by $HgCl_2$, clearly point toward a major importance of AQPs and the transcellular route for intestinal fluid absorption also in salmonids.

THE ROLE OF SGLT1

Significant volumes of water have been suggested to be transported via SGLT1 in the mammalian intestine (Loo et al., 2002). In rainbow trout, glucose homeostasis appears to be dependent on intestinal absorption and the presence of SGLT1 in the enterocytes has been verified at both mRNA and protein level (Polakof et al., 2010). Transport kinetics of glucose in the intestine of Atlantic salmon show highest transport rate in the pyloric caeca, intermediate in the proximal intestine and low in the distal intestine (Bakke-McKellep et al., 2000) which correlates well to the mRNA expression of SGLT1 in rainbow trout (Madsen et al., 2011). If the salmonid SGLT1 is involved in water flux in a similar manner as described for mammals, a regional difference in the contribution of SGLT1 to water transport would be expected. Interestingly, SGLT1 also appears to have a role in intestinal water transport of rainbow trout as Madsen et al. (2011) showed that the water transport could be reduced by 20% by blocking the SGLT1 transport with phlorizin. However, gut sac preparations from the whole intestinal tract, proximal and distal intestine together, were used in this study. Thus, no differentiation between intestinal regions was possible. High fluid uptake has been observed in pyloric caeca of chinook salmon (Veillette et al., 2005). It can be speculated that SGLT1 may be a major contributor to water absorption in this region as well as in the proximal intestine.

CONCLUSIONS AND FUTURE PERSPECTIVES

The life cycle of anadromous salmonids makes these fish interesting to study as they are able to acclimate to both hypo- and hyperosmotic environments. In FW, smoltification prepares the hyperosmoregulatory parr for a life as a hypoosmoregulatory smolt in SW by increasing the drinking rate, intestinal NKA activity and ion co-transports and subsequently fluid transport (**Figure 7**). These changes are mediated, to a large extent, by the developmental increase in circulating plasma cortisol levels. Even though some important transporters behind the intestinal fluid absorption has been characterized and localized, expression at both mRNA and protein level of others, such as NKCC2, NHEs, SLC26A6, NCC, and NBC1 remains to be determined during the smoltification. The developmental elevation in plasma cortisol levels further results in increased paracellular permeability, probably through a down regulation of the barrier building claudin-25b in the intestine (Tipsmark et al., 2010a). In order to understand how the different isoforms of fish claudins modulate the intestinal permeability, functional studies needs to be assessed for each isoform to assign different claudins barrier building and/or pore forming characteristics. Recently, an intestinal epithelial cell line from the distal region, exhibiting epithelial-like structure, has been developed from rainbow trout (Kawano et al., 2011). If this cell line is suitable for Ussing chamber studies, over expression of the different claudins followed by monitoring of TER and P_{app} will provide information on how these proteins regulate the charge and size selectivity within the TJ. Nevertheless, the increased paracellular permeability observed during smoltification in FW, is suggested to result in an increased leakage of positive ions from the LIS back to the intestinal lumen and thus prevent the buildup of the osmotic ion gradient in the LIS essential for the SW adaptive fluid absorption. Thus, the increased paracellular permeability in FW allows for a preparatory increase of ion transporting activities (i.e., increased NKA activity) in the enterocytes without creating an efficient fluid absorption while the fish is still in FW. After transition to SW, the drinking rates, intestinal NKA activity and fluid absorption are maintained high. In parallel, the paracellular permeability decreases, probably due to up-regulation of claudin-25b (Tipsmark et al., 2010a). This decreases the leakage of ions into the lumen which in turn allows for the buildup of the fluid driving osmotic gradient in the LIS. The tightening of the paracellular permeability further

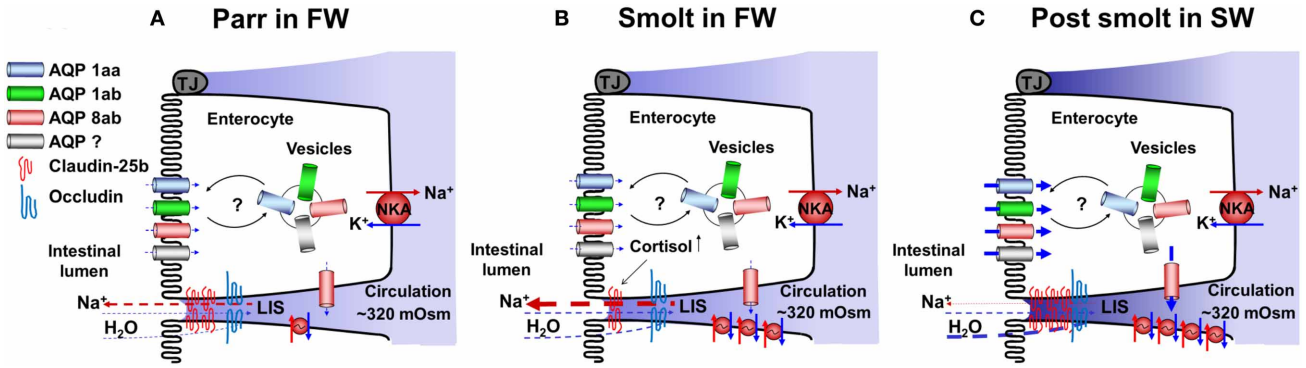

FIGURE 7 | A tentative model showing possible roles and positions of aquaporins (AQPs) and claudins in FW parr (A), during smoltification (B), and after acclimation to SW (C). In SW post-smolts, protein expression of AQPs 1aa, 1ab, and 8ab are present in the apical membrane, AQPs 1aa and 1ab in vesicles and AQP 8ab in the basolateral membrane **(C)**, with basis in these experiments and results of transcriptome analyses possible AQP positions in FW parr and smolts are suggested (**A** and **B**). Regarding tight junction proteins no protein expression has, as far as we know, been demonstrated. Thus the present model is based on transcriptome analyses of different claudin and occluding genes. NKA; Na^+, K^+-ATPase, LIS; lateral intercellular space.

suggests that the water flow is redirected from a paracellular route, to a more transcellular route, which can be accomplished either through the lipid bilayer and/or by incorporation and/or trafficking of AQPs into the intestinal epithelium. The relative importance of transcellular water permeability through the enterocyte lipid bilayer should be further investigated using polar lipid vesicles derived from intestinal enterocyte membranes of FW and SW acclimated salmonids, respectively. The protein expression of AQPs at the enterocyte cellular and subcellular levels should be investigated during smoltification and after SW acclimation. Moreover, crystallization studies can be used to dissolve the high resolution structures of AQPs. This information can in turn be used to study the function and regulation of AQPs in artificial liposomes where different extracellular conditions can be mimicked. These detailed mechanistic, molecular approaches should be complemented by functional studies using pharmacological tools for trans- and paracellular transporters and pathways respectively, while simultaneously monitoring fluid fluxes. This approach would provide important information on the relative importance of the paracellular and the transcellular pathway in salmonid fluid absorption in FW, during smoltification and after SW acclimation.

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Role of aquaporins during teleost gametogenesis and early embryogenesis

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Aquaporins are believed to be involved in homeosmotic mechanisms of marine teleosts. Increasing data suggest that these molecular water channels play critical roles associated with the adaptation of gametes and early embryos to the external spawning environment. In this mini-review, we discuss recent studies suggesting the function of aquaporin-mediated fluid homeostasis during spermatozoa activation and egg formation in teleosts. In addition, we address the potential role of water channels in osmosensing and cell migration during early embryonic development.

Keywords: teleost, aquaporin, oocyte, spermatogenesis, spermatozoa, embryo

INTRODUCTION

Marine and freshwater teleosts are constantly exposed to opposite osmotic gradients leading respectively to passive influx or efflux of water and ions. Such gains and losses are compensated through the coordinated control of osmoregulatory organs that mediate opposite fluxes within body fluids in order to maintain homeosmotic balance (Marshall and Grosell, 2006). Amongst other mechanisms, water channels or aquaporins have been implicated in this process. Recent studies have advanced this notion by revealing that teleosts harbor a large repertoire of water-selective aquaporins and water and solute (glycerol, urea) permeable aquaporins, also known as aquaglyceroporins (Cerdà and Finn, 2010; Tingaud-Sequeira et al., 2010). An overview of the evolution and nomenclature of piscine aquaporins, including the recently renamed aquaporin-1 paralogs, as well as their permeability properties and expression profiles, is provided by Cerdà and Finn (2010) and Finn and Cerdà (2011).

In this mini-review, we focus on the early stages of teleost development. Oviparous marine and catadromous teleosts release their gametes into the marine environment where external fertilization takes place. Considering that the gametes lack the adult organs that deal with ion and water balance, it seems likely that molecular adaptations should be present in the reproductive organs, gametes, and early embryos to cope with the external osmotic challenges. We therefore discuss recent studies that highlight the mechanistic roles of aquaporins, and how such channels may be associated with the adaptation of teleosts to diverse life histories and habitats.

AQUAPORIN EXPRESSION AND FUNCTION DURING SPERMATOGENESIS

During spermatogenesis diploid spermatogonia associated with somatic Sertoli cells enter meiosis to become primary and secondary spermatocytes, and eventually haploid spermatids, which elongate and differentiate into flagellated spermatozoa. In teleosts, this process is regulated by pituitary gonadotropins, via specific receptors in Leydig and Sertoli cells, through the synthesis and release of sex steroids (androgens and progestins) and growth factors, respectively (Schulz et al., 2010). The final stage of spermatogenesis or "spermiation" principally involves the production of hydrated seminal fluid that facilitates the acquisition of motility and the passage of spermatozoa through the sperm ducts (Scott et al., 2010).

In mammals, different aquaporins are found in spermatozoa, as well as in testicular germ and somatic cells (Yeung, 2010). Aquaporin-7 (AQP7) is localized in late spermatids, both in the cytoplasm and later in the plasma membrane, whereas AQP8 shows a more variable distribution from restriction to certain spermatogenic cell types to all germ cells. Elongating spermatids also express the intracellular AQP11 in the caudal cytoplasm. These three aquaporin isoforms are subsequently found in spermatozoa, as well as AQP3, although they are differentially localized along the sperm tail (Yeung, 2010; Chen et al., 2011a). Although a role of AQP8 in volume regulation of murine spermatozoa has recently been suggested (Yeung et al., 2009), knockout mice models for AQP7 and -8, have failed to show clear phenotypes during spermatogenesis or sperm viability (Yang et al., 2005; Sohara et al., 2007). The function of AQP11 is also yet uncovered because *AQP*11 knockout mice die of renal failure before puberty (Morishita et al., 2005). However, a recent study shows that *AQP*3-deficient sperm displays defects in volume regulation and excessive cell swelling upon physiological hypotonic stress in the female reproductive tract (Chen et al., 2011a).

In teleosts, as suggested for mammals, aquaporins may be involved in the hydration of the seminal fluid as well as in sperm

physiology. However, although mRNAs of different aquaporin paralogs have been found in the teleost testis (Cerdà and Finn, 2010), their specific cellular localization and the function of the protein products during spermatogenesis remain to be investigated. Recently, Zilli et al. (2009) have investigated the role of aquaporins during the activation of spermatozoa motility in marine teleosts. In these species, the hyperosmotic challenge faced by the spermatozoa when discharged into seawater leads to a rapid water efflux. This rapid efflux, which causes membrane hyperpolarization resulting in the activation of cell motility (Alavi and Cosson, 2006; Zilli et al., 2008), was suggested to be mediated by aquaporins (Cosson et al., 2008). By using specific antibodies, Zilli et al. (2009) showed that both the water-selective Aqp1aa (Raldúa et al., 2008) and the aquaglyceroporin channel Aqp10b (formerly named GLP; Santos et al., 2004) are indeed expressed in the head and flagellum of gilthead sea bream (*Sparus aurata*) spermatozoa. Further functional expression and sperm activation assays in the presence of mercury chloride, a non-selective inhibitor of aquaporin permeability, suggested that Aqp1aa might mediate sperm activation (Zilli et al., 2009). The model proposed by the authors suggests that the hyperosmotic stimulus upon release of spermatozoa into seawater induces the accumulation of Aqp1aa in the plasma membrane to facilitate the rapid water efflux. This results in the reduction of cell volume and concomitant rise of the intracellular ion concentration, which in turn activates the cAMP signaling-pathway leading to the downstream phosphorylation of the flagellar proteins and the initiation of sperm motility. This model may be premature, however, since it is based on sperm activation assays in the presence of the aquaporin inhibitor $HgCl_2$ and the reducing compound β-mercaptoethanol (which can not reverse the mercurial inhibition of Aqp10b expressed in *Xenopus laevis* oocytes; Santos et al., 2004; Zilli et al., 2009), and therefore further studies using more specific inhibitors are needed. In addition, a number of key questions remain to be addressed, such as the role of Aqp10b-mediated water and/or solute transport in spermatozoa and of other aquaporins during germ cell differentiation, and the distribution and function of aquaporins in Sertoli and Leydig cells.

Whether the expression of aquaporins during teleost spermatogenesis is hormonally controlled still needs to be clarified. In mammals, AQP8 is expressed in the Sertoli cells of all testicular tubules, whereas AQP0 is only expressed within Sertoli cells in tubules containing elongating spermatids just before being released into the lumen (Hermo et al., 2004). These observations suggest that AQP0 might be hormonally regulated. However, whether AQP0 or -8 in Sertoli cells are modulated by steroids, as it occurs for AQP1 and -9 in the epididymis (Oliveira et al., 2005) or for AQP2 in the uterus (Jablonski et al., 2003), remains to be investigated. In teleosts, the hydration of the seminal fluid during spermiation and acquisition of sperm motility seems to be regulated by progestins such as 17,20β-dihydroxypregn-4-en-3-one (17,20βP) or 17,20β,21-trihydroxypregn-4-en-3-one (20β-S; Scott et al., 2010). These steroids can potentially activate nuclear progestin receptors expressed in Sertoli and Leydig cells, spermatogonia and/or spermatocytes (Miura et al., 2006; Chen et al., 2010b, 2011b; Hanna et al., 2010). In addition, progestins can directly stimulate spermatozoa hypermotility through membrane receptors coupled to adenylyl cyclase located in the plasma membrane (Tubbs et al., 2011). Therefore, it will be of interest to investigate if testicular and sperm aquaporins may be under 17,20βP- or 20β-S-mediated transcriptional and/or posttranslational regulation.

ROLE AND MOLECULAR REGULATION OF AQUAPORINS IN THE OOCYTE

In mammalian oocytes, expression of mRNAs encoding different aquaporins has been described (e.g., Edashige et al., 2000), although so far detection of the corresponding polypeptides remained elusive, except for AQP3 in bovine oocytes (Jin et al., 2011). In the granulosa and theca cells associated to the oocyte, however, several functional aquaporins such as AQP1-4, -5, and -9 are differentially localized (Skowronski et al., 2009; Thoroddsen et al., 2011).

As an adaptation to the hyperosmotic condition of seawater, the oocytes of oviparous marine teleosts hydrate during meiosis resumption (oocyte maturation). This mechanism provides a water reservoir in the embryo to compensate for the passive water efflux until osmoregulatory organs develop, and improves oxygen exchange and egg dispersal in the ocean (Fyhn et al., 1999; Finn and Kristoffersen, 2007; Cerdà, 2009; Finn and Fyhn, 2010). Early studies in the gilthead sea bream (Fabra et al., 2005, 2006), and later in the Japanese eel (*Anguilla japonica*; Kagawa et al., 2009, 2011), identified the novel role of Aqp1ab in this process. This duplicated paralog facilitates the temporal water permeation and the preovulatory swelling of the oocyte. The mechanism is coregulated with yolk proteolysis and ion fluxes that generate the intracellular osmotic driving force for fluid transport, a feature well established in both old and modern teleost species (Cerdà et al., 2007; Kristoffersen et al., 2009). Further functional, genomic and phylogenetic analyses revealed that Aqp1ab belongs to a teleost-specific subfamily of water-selective aquaporins, which evolved by tandem duplication of a common ancestor (Martinez et al., 2005; Raldúa et al., 2008; Tingaud-Sequeira et al., 2008, 2010; Zapater et al., 2011). Interestingly, *aqp1ab* transcripts have also been found in the ovary of the freshwater teleost stinging catfish (*Heteropneustes fossilis*), in which oocytes moderately hydrate during meiotic maturation (Singh and Joy, 2010), although the role of Aqp1ab in the oocyte of this species is yet unknown (Chaube et al., 2011).

The role of Aqp1ab during oocyte hydration in marine teleosts is supported experimentally by the observation that the swelling of oocytes is blocked by aquaporin inhibitors such as mercury and tetraethylammonium (Fabra et al., 2005, 2006; Kagawa et al., 2009). However, these compounds can also affect K^+ channels and other ion transport proteins (Armstrong, 1990; Jacoby et al., 1999), which may play a role for inorganic osmolyte accumulation in the oocyte (Cerdà et al., 2007; Kristoffersen and Finn, 2008). More conclusive data have been recently obtained in the Atlantic halibut (*Hippoglossus hippoglossus*), a marine teleost that reproduces at low temperature and spawns one of the largest pelagic eggs known (Zapater et al., 2011). In this study, Atlantic halibut oocytes undergoing hydration were microinjected with an antiserum specific for halibut Aqp1ab, resulting in a dose-dependant inhibition of oocyte hydration in the presence of yolk hydrolysis. The immunological inhibition could be fully reversed by the artificial expression of halibut Aqp1aa which is not recognized by

the antibody. Therefore, these findings indicate that the decrease of oocyte hydration of Atlantic halibut oocytes can be directly related to the loss of function of Aqp1ab, providing for the first time functional evidence of the essential physiological role of this water channel.

Recent studies in the gilthead sea bream have begun to dissect the molecular mechanisms involved in the physiological regulation of Aqp1b in the oocyte. Preliminary data suggest that transcriptional activation of the *aqp1ab* promoter in primary growth oocytes may be dependent on Sry-related high mobility group [HMG]-box (sox) genes, as well as on the nuclear progestin receptor, which are highly expressed in oogonia (Zapater et al., unpublished data). This mechanism likely results in the accumulation of high levels of *aqp1ab* transcripts and Aqp1ab peptides in primary growth oocytes. Subsequently, Aqp1ab-containing vesicles are transported toward the oocyte cortex throughout the period of oocyte growth, and during meiotic maturation and hydration they are temporarily inserted into the oocyte plasma membrane (Fabra et al., 2006). Structural analyses have revealed that the cytoplasmic tail of Aqp1ab, although highly divergent among teleosts, retains specific motifs that regulate vesicular trafficking, and therefore they may be involved in the control of Aqp1ab translocation into the oocyte plasma membrane during hydration (Tingaud-Sequeira et al., 2008; Chaube et al., 2011). These processes appear to involve alternative mechanisms of phosphorylation and/or dephosphorylation of specific C-terminal residues, but the specific intracellular signaling pathways involved are yet unknown. These observations thus indicate that Aqp1ab in the oocyte is tightly regulated at the transcriptional and post-translational level during oogenesis, oocyte growth, and meiotic maturation.

The studies carried out so far are revealing that the Aqp1ab-mediated mechanism of oocyte hydration in marine teleosts is a conserved and highly regulated process, based on the interplay between osmolyte generation and the controlled synthesis and insertion of Aqp1ab at the oocyte surface. However, there are still many unresolved issues, such as the transductional pathways activated in the oocyte during hormone-induced meiotic maturation and hydration that coordinate osmolyte generation, Aqp1ab intracellular trafficking and meiosis resumption. In this regard, it will be of interest to investigate the role of G protein-coupled progestin receptors on the oocyte surface, as well as the classical nuclear progestin receptor, during the control of Aqp1ab trafficking, as these receptors may be the physiological transducers of progestins to activate meiosis resumption (Thomas et al., 2004). However, it is known that in the teleost ovary, in addition to *aqp1ab*, mRNAs encoding many other aquaporin paralogs are found (Cerdà and Finn, 2010), although the cellular sites of expression have not yet been defined. Whether the accumulation of these transcripts corresponds to maternal messengers stored in oocytes required for early development, or reflect the coordinated role of different aquaporins in ovarian fluid homeostasis, remains to be investigated.

AQUAPORINS IN EMBRYONIC DEVELOPMENT

Very limited information is available on the localization and function of aquaporins during mammalian and teleost early embryonic development. In the mammalian morula, AQP3, -8, and -9 are detected in the cell–cell contact domains of blastomeres, despite observations that these embryos show very low water and solute permeability (Barcroft et al., 2003; Edashige et al., 2007), although this may differ among species (Jin et al., 2011). At the blastula stage, blastoderm cells express only AQP3, whereas AQP3 and -8 are accumulated at the basolateral membranes of trophectodermal epithelial cells of blastocysts, which also express AQP9 at the apical membrane (Barcroft et al., 2003). The change in the subcellular localization of these aquaporins coincides with the enhanced water permeability of blastula stage embryos, and consequently it was suggested that these channels may mediate transepithelial water and solute movements. (Barcroft et al., 2003). The function of these aquaporins could compensate each other because *AQP3* knockout mice can develop to term (Ma et al., 2000). Moreover, solute permeability is not completely abolished in embryos in which AQP3 expression has been suppressed by injection of AQP3 double-stranded RNA (Edashige et al., 2007).

During teleost embryonic development, changes in the expression and cellular localization of aquaporins have only been investigated in zebrafish (*Danio rerio*) and common mummichog (*Fundulus heteroclitus*; Tingaud-Sequeira et al., 2009; Chen et al., 2010a). In zebrafish, *aqp1aa*, -3*a*, -7, and -10*b* transcripts are detected at the 2–4 cell and morula stages (**Figure 1A**), suggesting that these aquaporins are maternally inherited, as reported for mummichog *aqp1aa* (Tingaud-Sequeira et al., 2009). Interestingly, transcripts encoding zebrafish *aqp3b* and mummichog *aqp3a* are first noted at the onset of gastrulation, during which they are apparently accumulated in embryos (Tingaud-Sequeira et al., 2009; **Figure 1A**). Later in development, aquaporin expression is generally enhanced where the expression of other paralogs such as *aqp0a, -0b,* -8*aa*, -10*a*, and -11*b* may be detected. This latter expression appears to be associated with organogenesis and tissue differentiation. For example, Aqp1aa and -8aa have been related to somitogenesis and vascular development (Sumanas et al., 2005; Tingaud-Sequeira et al., 2009; Chen et al., 2010a), Aqp0a and -0b to normal lens development and transparency (Froger et al., 2010), and Aqp11b to tail differentiation (Ikeda et al., 2011).

In the early embryos of common mummichog (Tingaud-Sequeira et al., 2009) and zebrafish (**Figures 1B–E**), immunolocalization experiments using specific antibodies have respectively revealed the presence of Aqp3a and -3b in the blastoderm cells as well as in the enveloping layer (EVL). In the mummichog, further accumulation of the Aqp3a protein is evident in the basolateral membrane of the EVL epithelium as well as in the membrane of migrating blastomeres at the marginal region of the blastoderm (Tingaud-Sequeira et al., 2009). The localization of Aqp3a in the EVL of teleost embryos may indicate a role of this channel in water and solute transport during epiboly. Interestingly, the embryos of the common mummichog respond to environmental desiccation by accelerating development (Tingaud-Sequeira et al., 2009). Under these conditions Aqp3a expression in the EVL is reduced, which could be a mechanism to reduce evaporative water loss through the EVL, while membrane localization of Aqp3a in migrating blastomeres remains unchanged (Tingaud-Sequeira et al., 2009). In recent years, water and solute transport

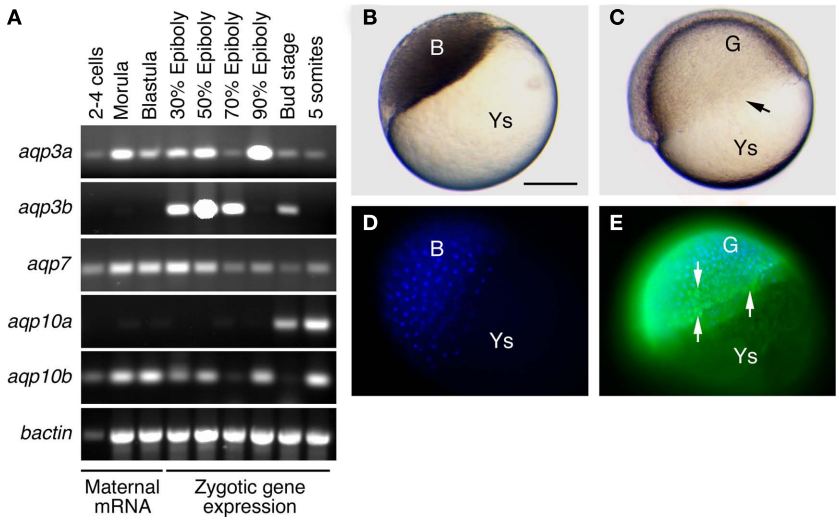

FIGURE 1 | Expression of aquaporins during teleost embryonic development. (A) Standart RT-PCR analyses of aquaporin transcriptional expression in zebrafish embryos reveals that *aqp3a*, *-7*, and -10*b* transcripts are detectable throughout development, whereas *aqp3b* and *aqp*10*a* expression is only detected during gastrulation and onward, and at the onset of organogenesis, respectively. **(B–E)** Whole-mount immulocalization of Aqp3b in zebrafish blastula embryos (left) and 50% epiboly embryos (right) using specific antibodies (Chauvigné et al., 2011). **(C)** The involuting edge of the gastrula is indicated with an arrow. **(D–E)** Aqp3b is not detected in the blastula **(D)**, whereas it appears in migrating blastomeres [**(E)** arrows] during gastrulation. Nuclei of blastomeres are counterstained with DAPI. Bar, 500 μm. Ys, yolk sac; B, blastomeres; G, gastrula. Bar, 500 μm.

mediated by mammalian AQP1, -3, or -4 has been proposed to be involved in cell migration and proliferation by driving water influx, thus facilitating lamellipodia extension and cell migration (Papadopoulos et al., 2008; Monzani et al., 2009). A similar role for AQP3 has been proposed during neural tube closure in *X. laevis* embryos (Cornish et al., 2009). In teleost embryos, the specific pattern of Aqp3a expression in the plasma membrane of migrating blastomeres, and its persistence in mummichog embryos showing an accelerated rate of epiboly under desiccation conditions, also suggests the involvement of this aquaporin in cell migration during gastrulation. Preliminary experiments in common mummichog have shown delayed epiboly of embryos injected with an specific antibody against Aqp3a, and thus these observations may support the role of this aquaporin during cell migration (Chauvigné and Cerdà, unpublished data). This potentially conserved role requires however further investigation. Moreover, teleost embryos also express other aquaporins such as *aqp*1*aa*, -7, and -10*b* during gastrulation (Tingaud-Sequeira et al., 2009; **Figure 1A**), and therefore their functional relationships need to be elucidated.

CONCLUSION AND FUTURE PERSPECTIVES

Although available information remains scarce, increasing data indicate that aquaporins are involved in water homeostasis in reproductive organs and gametes of teleosts in addition to osmoregulatory mechanisms. At least one paralog (e.g., Aqp1ab), appears to be tightly regulated both at the gene and protein level during gametogenesis, underlining its importance for the production of gametes well adapted to the reproductive habitats. However, more studies are necessary in teleosts, particularly those using genetic and molecular approaches, as well as specific aquaporin blockers, to elucidate aquaporin function during reproduction. An understanding of the involvement of aquaporins in fluid movement in the teleost gonads and gametes may also lead to improved cryopreservation protocols to assist breeding and species conservation programs.

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