The pH sensitivity of Aqp0 channels in tetraploid and diploid teleosts

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ABSTRACT Water homeostasis and the structural integrity of the vertebrate lens is partially mediated by AQP0 channels. Emerging evidence indicates that external pH may be involved in channel gating. Here we show that a tetraploid teleost, the Atlantic salmon, retains 4 aqp0 genes (aqp0a1, -0a2, -0b1, and -0b2), which are highly, but not exclusively, expressed in the lens. Functional characterization reveals that, although each paralog permeates water efficiently, the permeability is respectively shifted to the neutral, alkaline, or acidic pH in Aqp0a1, -0a2, and -0b1, whereas that of Aqp0b2 is not regulated by external pH. Mutagenesis studies demonstrate that Ser38, His39, and His40 residues in the extracellular transmembrane domain of α-helix 2 facing the water pore are critical for the pH modulation of water transport. We find that both zebrafish Aqp0a and -0b are functional water channels with respective pH sensitivities toward alkaline or acidic pH ranges and that an N-terminal allelic variant (Ser19) of Aqp0b exists that abolishes water transport in Xenopus laevis oocytes. The data suggest that the alkaline pH sensitivity is a conserved trait in teleost Aqp0 a-type channels, whereas mammalian AQP0 and some teleost Aqp0 b-type channels display an acidic pH permeation preference.—Chauvigné, F., Zapater, C., Stavang, J. A., Taranger, G. L., Cerda, J., Finn, R. N. The pH sensitivity of Aqp0 channels in tetraploid and diploid teleosts. FASEBJ 29, 2172–2184 (2015). www.fasebj.org

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The vertebrate ocular lens is a transparent multifocal organ that refracts and transmits light to a retinal focal point to facilitate color vision (1). It is conserved from jawless lampreys (Hypanoartia) to modern aquatic and terrestrial animals, including sharks and rays (Chondrichthyes), ray-finned fishes (Actinopterygii), and lungfishes and tetrapods (Sarcopterygii) (2–6). The transparency of the lens arises during embryonic development because of the differentiation of the primary (cortical) and secondary (nuclear) lens fibers, which express high levels of soluble crystallins (7, 8). Subsequently, the differentiating nuclear lens fibers lose all membrane-bound cytoplasmic organelles including mitochondria and nuclei (9). In this state, the lens fibers are avascular and must survive undamaged for the lifetime of the organism (10). In the absence of vasculature, which would otherwise interfere with the transmission of light, an internal microcirculatory system is established between the equatorial epithelial cells and the inner “onion-like” layers of lens fibers (11, 12). This intrinsic circulation is suggested to be established via active Na+/K+ transport, which generates an osmotic gradient facilitating fluid movement through the interstitial space and into the lens fibers via membrane-spanning water channels (aquaporins (AQPs)) (12).

AQPs are a ubiquitous class of integral membrane protein that facilitate the transmembrane transport of water, glycerol, or other small solutes and gases (13, 14). Recent studies have shown that the deuterostome superfamily consists of 17 subfamilies (Aqp0–16) with 13 functional subfamilies (Aqp0–12) present in Eutheria (15). Although all 13 members of the eutherian aquaporin superfamily have been detected in different regions of mammalian eyes (10–18), only AQP0 and AQP5 are highly concentrated in the lens (19–21). Subcellular studies have shown that AQP0 is arranged in microdomains of the lens fibers and that the channels have multifunctional properties including cell-to-cell adhesion and water transport (22–27). Mammalian knockout models have further shown that AQP0 is essential for lens development and integrity and that its absence is sufficient to trigger the pathophysiologic condition of cataractogenesis (28). Other studies of mammalian AQP0 have revealed that a number of mutations in the coding regions of the transmembrane domains

Abbreviations: AQP, aquaporin; MS, modified Barth’s solution; TF, transcription factor; TMD, transmembrane domain; WGD, whole genome duplication

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(TMDs), the extracellular loop A, or in the intracellular C terminus can disrupt the trafficking of the protein to the plasma membrane resulting in loss of function, lens opacity, and impaired vision or blindness (28–30).

In contrast to mammals, teleosts lack an AQPS5 gene (15), but have been found to retain 2 copies of AQPO (aqp0a and -0b), both of which are highly concentrated in the lens (31–34). However, detectable levels of teleost aqp0 are found in other tissues, including aqp0b mRNA in the ovary (33) and Aqp0a protein in the Sertoli cells of the testis (35). As in mammals, morpholino-based knockdown experiments of aqp0b and -0b have revealed that both channels are essential for normal lens development and transparency (36, 37), indicating that the major physiologic role of AQPO is conserved in teleosts.

Functional studies of the duplicated Aqp0a and -0b paralogs have only been conducted for zebrafish (33, 36, 37), with additional measurements of water permeability tested for the Aqp0a channel of the common mummichog (Fundulus heteroclitus) (38) and gilthead seabream (Sparus aurata) (35). Each study found that, unlike mammalian AQPO, which has a low intrinsic water permeability (39), teleost Aqp0a transports water efficiently when heterologously expressed in Xenopus laevis oocytes. By contrast, the heterologous studies on zebrafish Aqp0b have produced conflicting results showing efficient water permeation (33) or dysfunctional channels (36, 37). These latter findings have led to the suggestion that Aqp0b has subfunctionalized and provides functions other than water permeability (12, 36, 37). A separate character of AQPO channels appears to be an inherent sensitivity to pH and Ca\(^{2+}\) (24, 38, 40–42). However, current evidence indicates that the mechanism of pH sensitivity may not be conserved between teleosts and mammals, because the water permeation of common mummichog Aqp0a is reduced by an acidic external pH, whereas the reverse is observed for bovine AQPO (38, 41). It thus remains to be established whether the alkaline permeation preference of teleost Aqp0a is altered in the Aqp0b paralog or represents an aquatic adaptation compared with the acidic shift of AQPO in terrestrial mammals.

It is well established that the majority of teleosts retain 2 gene copies arising from a fish-specific whole genome duplication event (R5 WGD) –320–350 million years ago (43–47). However, several lineages, including members of the Ostariophysi (48–50) and Protacanthopterygii (51), have experienced an independent R4 WGD. In the case of Salmonidae, this latter event is estimated to have occurred between 88 and 103 million years ago (52, 53). Considering that the average functional lifespan of duplicated non-functionalized genes is <8 million years (54), it is perhaps not surprising that gene fractionation in salmonids is very active (52). Nevertheless, an analysis of 9057 Atlantic salmon (Salmo salar) cDNA sequences has provided evidence that many gene duplicates have been retained (55). For the salmonid aquaporin superfamaly, this facet has recently been confirmed, with 35 and 42 paralogs, respectively, identified in the genomes of rainbow trout (Oncorhynchus mykiss) and Atlantic salmon (15). To date, only 7 of these paralogs have been investigated (56–58), but it is not known whether the Atlantic salmon retains 4 functional aquaporin paralogs in any given subfamily.

In the present study, we therefore researched the Atlantic salmon genome to facilitate isolation and cloning of 4 aqp0 genes. To address their interrelationships and the prevalence of duplicates in ray-finned fishes, we used Bayesian inference to reconstruct the phylogeny of 78 gnathostome aqp0 gene products. Subsequently we examined the molecular physiology of the tetraparalogous Aqp0 channels in the Atlantic salmon as a first step toward identifying their potential roles in cataractogenesis, which represents a welfare problem in farmed strains (59–61). We used site-directed mutagenesis to investigate the molecular basis of the pH sensitivity of the Atlantic salmon channels in relation to that of the zebrafish Aqp0a and -0b duplicates. The results suggest that the salmon Aqp0 paralogs may have been retained because of neofunctionalized pH sensitivities.

**MATERIALS AND METHODS**

**Animals**

Atlantic salmon between 1 and 5 kg of AquaGen origin were held in captivity at the Matredal Aquaculture Research Station (61°N) in Norway. The AquaGen strain is the most common salmon farming strain in Norway and has been under selective breeding for ~10 generations, originating from a range of wild Norwegian salmon populations collected in the early 1970s (http://aquagen.no/en/). Prior to sampling, fish were anesthetized with metomidate (Syndel, Victoria, BC, Canada) and immediately euthanized in accordance with the regulations approved by the governmental Norwegian Animal Research Authority (http://www.fda.no/fda/). Tissue samples were dissected from 5 fish, immediately frozen in liquid nitrogen, and stored at ~80°C for subsequent analyses.

**Cloning of Atlantic salmon aqp0 cDNAs and genomic sequences**

Initial searching of the Atlantic salmon genome database curated at the National Center for Biotechnology Information using the tblastn algorithm and zebrafish Aqp0a and -0b as input identified multiple aqp0bearing contigs from which gene-specific primers were designed. Four nonredundant cDNAs were isolated from the sampled lens tissues by RT-PCR using RNA and a high-fidelity DNA polymerase (EasyA high-fidelity PCR cloning enzyme; Agilent Technologies Santa Clara, CA, USA). Total RNA was purified using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich, St. Louis, MO, USA), according to the manufacturer’s instructions. cDNA synthesis was performed with 1 µg of total RNA using an oligo dT(12-18) primer (Life Technologies, Carlsbad, CA, USA) and SuperScript II RT enzyme (Life Technologies) as previously described (62). Oligonucleotide primers used to amplify the full-length mRNA sequences of aqp0a1 and -0a2 were designed from contigs AGKD01129317 and AGKD01164501 for aqp0a1 and AGKD01199297 and AGKD01157319 for aqp0a2. The PCR conditions were an initial denaturing step for 2 minutes at 94°C, followed by 35 cycles of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 2 minutes, ending with a final elongation at 72°C for 7 minutes. For aqp0b1 and -0b2, partial cDNA sequences bearing the 5’ and 3’ end, respectively, were found on 2 different contigs (AGKD01047775 for aqp0b1 and AGKD01117189 for aqp0b2), which were used for primer design and the cloning of the 5’ and 3’ cDNA ends using 3’ and 5’ RACE kits (Life Technologies). Subsequently, full-length aqp0b1 and -0b2 cDNAs were amplified using specific primers as described above. In all cases, the amplified cDNAs were cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) and sequenced by BigDye Terminator Version 3.1 cycle sequencing on ABI PRISM 377 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

Genomic sequences containing the full introns and exons of the aqp0a1, -0a2, -0b1, and -0b2 genes were amplified from
Sequence and phylogenetic analysis

The deduced amino acid sequences of the isolated Atlantic salmon cDNAs were aligned with other gnathostome Aqp0 orthologs retrieved from public sources, including Ensembl, GenBank, National Center for Biotechnology Information whole genome shotgun, transcriptome shotgun assemblies, and expressed sequence tag databases as described previously (15). Alignments were constructed using the multiple sequence alignment method of fast Fourier transform (v7.187) software package (63) and converted to codon alignments using Pal2Nal (64). Molecular phylogenies were inferred using Bayesian (MrBayes, v3.2.2; with 5 million Markov chain Monte Carlo generations) (65) and maximum likelihood (PAUP v4b10x86macoss) (66) protocols as described previously (15, 62, 67, 68).

The 3-dimensional structure of bovine AQP0 (2b6p) was obtained from the protein data bank (http://www.pdb.org) (66) protocols as described previously (15, 62, 67, 68).

In silico analysis of cis-acting regulatory sequences in aqp0 5′-flanking regions

The 5′ flanking regions of the 4 Atlantic salmon aqp0 genes were analyzed using TRANSFAC 7.0 Public 2005 software (http://www.gene-regulation.com).

TABLE 1. Primer pair sequences, amplicon size, annealing temperature, and efficiency for Atlantic salmon aqp0 and reference gene used in quantitative RT-PCR

| Gene     | GenBank accession no. | Forward/reverse (5′ to 3′ end) | Amplicon (bp) | Annealing temperature (°C) | Primer efficiency<sup>*</sup> |
|----------|-----------------------|---------------------------------|---------------|-----------------------------|-----------------------------|
| aqp0a1   | KM829661              | TGAACCTTACCCCAACACACA/          | 109           | 60                          | 2.05                        |
|          |                       | TGGAGGGGTTGAAAGGTTG            |               |                             |                             |
| aqp0a2   | KM677198              | CGACTGGTCCTTACCGCTA/          | 128           | 60                          | 2.04                        |
|          |                       | GAGGAGTGCACCTTCCCTTA           |               |                             |                             |
| aqp0b1   | KM677199              | TTAGTTTTTGTAGCGCTTTCC/         | 112           | 60                          | 2.03                        |
|          |                       | AGCCAACTGACTGAGAAGAGCA         |               |                             |                             |
| aqp0b2   | KM677200              | AGCTAGCTGACCTGCAAGGG/          | 142           | 60                          | 1.93                        |
|          |                       | GCGGAAATGTCTTGGGAAA            |               |                             |                             |
| rps18    | AJ427629              | TACCTGGAATCGCAATG/            | 153           | 60                          | 1.99                        |
|          |                       | GATGCCCTTGTGGCTCTG             |               |                             |                             |

<sup>*</sup>Efficiency was determined from standard curves generated from 10-fold serial dilutions of first-stranded cDNA template from lens. In all cases, the correlation coefficients were 0.99.

Real-time quantitative RT-PCR

Total RNA from adult tissues (lens, eye, brain, gills, kidney, middle intestine, rectum, ovary, and testis) was isolated as described above, and first-strand cDNA was synthesized from 0.5 μg total RNA. After 15 minutes of heating at 70°C in the presence of 0.5 μg oligo(dT) (12-18) and 1 mM dNTPs, 40 μl RNase out and 10 μl SuperScript II enzyme were added, and the reaction was completed for 1.5 hours at 42°C. Real-time quantitative RT-PCR amplifications were performed in a final volume of 10 μl with 5 μl Platinum SYBR Green qPCR Supermix-UDG with ROX (Life Technologies), 1 μl of 1:20 diluted cDNA, and 0.5 μM of each specific primer (Table 1 and Supplemental Fig. S1). The sequences were amplified in duplicate for each sample on 384-well plates using the ABI PRISM 7900HT sequence detection system (Applied Biosystems). The amplification protocol was an initial denaturation and activation step at 50°C for 2 minutes and 5°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 63°C for 1 minute. After the amplification phase, a temperature-determining dissociation step was carried out at 95°C for 15 seconds, 60°C for 15 seconds, and 95°C for 15 seconds. For normalization of cDNA loading, all samples were run in parallel using the 18s ribosomal protein (rps18) as a reference gene, because its expression between experimental samples did not show significant differences (data not shown). To estimate the primer efficiencies, a standard curve was generated for each primer pair from 10-fold serial dilutions (from 1 to 0.0001) of a pool of mixed lens cDNA templates. Standard curves represented the cycle threshold value as a function of the logarithm of the number of copies generated, defined arbitrarily as 1 copy for the nondiluted standard. All calibration curves exhibited correlation coefficients >0.99, and the corresponding quantitative RT-PCR efficiencies ranged from 1.9 to 2.0.

Functional characterization of Aqp0 paralogs in X. laevis oocytes

Constructs for heterologous expression in X. laevis oocytes were generated by subcloning full-length aqp0 cDNAs from...
Atlantic salmon and zebrafish (GenBank accession numbers NM_001003534 and NM_001020520) into the pT7Ts expression vector (69) by introducing EcoRV and *Spel* sites at the 5' and 3' end, respectively. Point mutations in the sequences were introduced using the Quickchange site-directed mutagenesis kit (Stratagene; Agilent Technologies). All constructs in pT7Ts were resequenced as above to assure that the correct mutations were present. Isolation of stage V–VI oocytes and cRNA synthesis were carried out as previously described (69, 70). Oocytes were transferred to modified Barth’s solution (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM NaHCO₃, 0.82 mM MgSO₄, 0.33 mM Ca(NO₃)₂, 0.41 mM CaCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and 25 μg/ml gentamycin, pH 7.5) and injected with 50 nl of distilled water (negative control) or 50 nl of water solution containing 10 ng of cRNA. Two days after injection, the oocytes were transferred to isotonic MBS (200 mOsm) at pH 6, 7.5, or 8.5 for 15 minutes and then transferred to 10-fold diluted MBS (20 mOsm) at the same pH. Oocyte swelling and determination of the osmotic water permeability (*P*) were calculated as previously described (70) using an estimated geometric area. Each experiment was carried out ≥3 times on separate oocyte batches.

**Statistics**

Data are presented as the mean ± SEM. Data analyses were carried out by 1- or 2-way ANOVA, after log-transformation of the data when needed, followed by the Duncan multiple range test. A value of *P* < 0.05 was considered significant.

**RESULTS**

**Structure and genomic organization of Atlantic salmon *aqp0* genes**

Initial searching of the Atlantic salmon genome identified 4 paralogous *aqp0* genes, which, following molecular phylogenetic characterization (see below), were termed *aqp0a1*, -0a2, -0b1, and -0b2 (Fig. 1). The genomic sequences were used to design paralog-specific primers to amplify the full-length cDNAs from total RNA isolated from lens tissues, as well as the complete genes from genomic DNA. These experiments revealed that the 4 Atlantic salmon *aqp0* genes were each split into 4 exons of conserved lengths (360, 165, 84, and 183 bp for exons 1, 2, 3, and 4, respectively), whereas the size of the intronic regions were more divergent (581–1618, 180–661, and 205–406 bp for introns 1, 2, and 3, respectively) (Fig. 1). Compared with the *aqp0a1* mRNA, nucleotide identity varied between 83–85% for the *aqp0b* paralogs and 94% for the *aqp0a2* paralog.

A region of ~2.2 kb corresponding to the 5' flanking region of each *aqp0* gene was further amplified by PCR. For *aqp0a1* and -0b1, however, 2 different variants of the 5' flanking region, with a deletion of ~200 bp, were found. The 4 salmon *aqp0* paralogs each encode protein products of 263 amino acids, showing 6 TMDs and 2 Asn-Pro-Ala (NPA) motifs, typical for members of the aquaporin superfamily. The amino acid identities reflected the mRNAs with 84–87% between the Aqp0a and -0b types and 96% between the Aqp0a1 and -0a2, and Aqp0b1 and -0b2 R1 duplicates.

**Phylogenetic relationships**

Bayesian inference of 78 gnathostome *aqp0* codon and deduced amino acid sequences revealed that Chondrichthyes, Sarcopterygii, and actinopterygian Holostei harbor single genes, whereas the majority of Teleostei retain 2 *aqp0* paralogs (*aqp0a* and -0b). Although only single paralogs are shown for someteleost species, such as common mummichog *aqp0a*, it is not yet possible to determine whether the absence of *aqp0b* represents gene loss, because of the lack of an available genome. The high posterior probability (97%) separating the teleost *aqp0a* and -0b clusters is thus consistent with R3 WGD at the root of the crown clade (Fig. 2). The 4 Atlantic salmon paralogs (*aqp0a1*, -0a2, -0b1, and -0b2), clustered as duplicated members of the Protoactinopterygii within the respective teleost *aqp0a* and -0b subfamilies. Comparison of the 4 *aqp0* paralogs assembled from the rainbow trout genome revealed that the interspecific amino acid identities between each of the Aqp0a1, -0a2, -0b1, and -0b2 orthologs were 98.1%, 99.6%, 98.9%, and 99.2%, respectively, and thus consistent with an extra tetraploidization in Salmonidae.

**The 5' flanking regions of Atlantic salmon *aqp0* genes contain high numbers of putative sites for lens development-related transcription factors**

The 2.2-kb 5' flanking genomic sequence of the 4 Atlantic salmon *aqp0* genes were analyzed for putative cis-acting regulatory sequences using the TRANSFAC 7.0 software. These analyses revealed the presence of consensus

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**Figure 1.** Genomic organization of Atlantic salmon *aqp0* genes. Schematic representation of *aqp0a1*, -0a2, -0b1, and -0b2 gene loci. Gray boxes indicate exons with coding regions only.
sequences for core promoter elements important for the interaction with the basal transcription machinery, such as several TATA boxes and Sp1 and AP1. The 4 \(aqp0\) flanking sequences also contained a high number (>100) of putative binding sites for 13 transcription factors, including musculoaponeurotic oncogene homolog (MAF), myeloid ecotropic viral integration site (MEIS), paired-like homeodomain 3 (PITX3), or sex determining region Y-box (SOX), that are known to be involved in ectodermal signaling for lens differentiation, proliferation,
and survival (Table 2 and Supplemental Table S1). A lower number (<15) of sites for factors related to retina development, such as Optix or VSX2, were also found in the 4 genes, with aqp0b1 harboring the majority of these factors (Supplemental Table S1). Interestingly, the sequences potentially involved in lens development were more conserved among the 4 aqp0 paralogs than those related to eye development (Table 2; Supplemental Table S1). Another, 42 different putative regulatory elements, including DMBX1, NEUROD1, and NEUROG2, that are specifically involved in brain and central nervous system development were identified among the aqp0 genes, with the aqp0b1 5’ flanking region showing the highest number of different potential sites (38) compared with the other aqp0 genes (11, 24, and 22 in aqp0a1, -0a2, and -0b2, respectively; Supplemental Table S1). The flanking region of the aqp0a1, -0a2, -0b1, and -0b2 also contained 10, 14, 13, and 18 different consensus sites, respectively, for TFs associated with gonad development, including doublesex and mab-5 related transcription factor 1 (DMRT1), Wilms tumor 1 (WT1), or AR for testicular differentiation and FOXL2, FIGLA, or ER for ovarian development (Table 2; Supplemental Table S1). In addition, a variable number of putative binding sites for other tissue-specific transcription factors, such as in the muscle or kidney, were differentially found in the promoter regions of the 4 Atlantic salmon aqp0s (Supplemental Table S1).

Atlantic salmon aqp0 paralogs are highly, but not exclusively, expressed in the lens

To design paralog-specific oligonucleotide primers for quantitative RT-PCR experiments, the 3’ UTR experiments of the aqp0a1, -0a2, and -0b1 mRNAs were amplified by 3’ rapid amplification of cDNA ends. Because of the high sequence similarity of the fourth exon of aqp0b1 and -0b2 (97.3% identity), the 3’UTR of aqp0b2 could not be specifically amplified, and the 5’ UTR was used instead. The 5’ and 3’ terminal sequences were then aligned and specific primers with similar efficiencies designed for each paralog (Table 1). Quantitative RT-PCR analyses of the pattern of mRNA expression in different adult tissues subsequently revealed that the 4 aqp0s transcripts were highly concentrated in the lens, with no significant difference between the paralog titers (Fig. 3). However, aqp0a1, -0a2, -0b1, and -0b2 mRNAs could also be detected in the deelened eye, brain, gills, kidney, mid intestine, rectum, ovary, and testis, although at much lower expression levels (~1000-fold less) than in the lens (Fig. 3). Compared with the other transcripts, aqp0a1 was more concentrated in the brain, gills, and ovary, whereas in testis, the expression levels of all 4 mRNAs were elevated, with aqp0b1 showing the highest levels (Fig. 3). These data confirmed that, although the mRNA levels in the lens are greatly concentrated with respect to the other tissues, aqp0 expression in Atlantic salmon and other species of teleost (33, 35) is not less specific.

Atlantic salmon Aqp0 paralogs retain unique pH sensitivities

The amino acid alignment of the 78 gnathostome Aqp0 orthologs revealed that His40 at the beginning of TMD2 close to loop A, which is known to affect the pH sensitivity of the water channel activity of bovine AQP0 (40, 41), is conserved in Eutheria, Metatheria, and the teleost b-type Aqp0s. However, it is not found in Chondrichthyes, Actinistia (coelacanths), Dipnoi (lungfishes), Amphibia, Sauropsida (reptiles and birds), Prototheria (platypus), Holostei (spotted gar), or the teleost a-type aquaporins. In all cases, an Asn40 is encoded instead of the His40, whereas in the teleost a-type Aqp0s, a His is encoded at position 39. This latter His39 is also prevalent in the b-type water channels of Acanthomorpha (spiny ray-finned teleosts). Interestingly, the Atlantic salmon displays the a-type His39 in both of the Aqp0a1 and -0a2 paralogs, but with a
nonconserved Ser encoded 1 amino acid upstream in the Aqp0a1 paralog. Conversely the salmon Aqp0b1 paralog displays the conserved His\(^{39}\), whereas the Aqp0b2 encodes both His\(^{39}\) and His\(^{40}\) as found in the majority of Acantho- morpha (Fig. 4A). Based on the crystal structure of bovine AQP0 (Fig. 4B) (71), in silico modeling of Aqp0a2 and -0b1 (Fig. 4C, D) revealed that the His\(^{39}\) residues of bovine AQP0 and Atlantic salmon Aqp0b1 face the inner vestibular opening of the water pore, whereas His\(^{39}\) in Aqp0a2 lies outside of the pore.

The functional properties of the 4 salmon Aqp0 paralogs were investigated using X. laevis oocytes as a heterologous expression system. Oocytes expressing Aqp0a1, -0a2, -0b1, or -0b2 showed a 20- to 25-fold increase in \(P_{f}\) with respect to water-injected (control) oocytes (Fig. 5). However, each paralog displayed a different pH sensitivity (Fig. 5). Aqp0a1 oocytes were more permeable at external neutral pH (7.5) and showed lower permeability at acidic and alkaline pH (6.0 and 8.5), whereas Aqp0a2 oocytes showed a progressive increase of water transport associated with an increase in pH, with the highest \(P_{f}\) at alkaline pH. In contrast, the \(P_{f}\) of oocytes expressing Aqp0b1 increased under acidic conditions (pH 6.0), whereas that of Aqp0b2 oocytes was not affected by changes in the external pH. These data therefore indicated a unique pH regulation for each of the 4 Atlantic salmon Aqp0 paralogs.

Alkaline and acidic pH sensitivities of salmon Aqp0a2 and -0b1 are conserved in zebrafish Aqp0 channels

To determine which of the pH sensitivities observed in the salmon Aqp0 paralogs could be potentially conserved in other teleosts, we reexamined the water permeation properties of the duplicated zebrafish Aqp0a and -0b paralogs. Two different forms of zebrafish Aqp0b have been reported, which differ in a Gly or Ser at position 19 in the N terminus (accession numbers BC098535 and NM_001020520, respectively; Fig. 4A). The form bearing Gly\(^{39}\), isolated in our laboratory, is conserved in other teleosts, and functional when expressed in X. leavis oocytes (33), whereas the form with a Ser\(^{19}\) residue has been reported to lack water transport (36, 37). To confirm that this single nucleotide polymorphism can alter the functional properties of zebrafish Aqp0b, a zebrafish Aqp0b-G19S mutant was constructed by site-directed mutagenesis and tested in oocytes exposed to different pH. The results of these experiments showed that zebrafish Aqp0a and -0b display opposite pH sensitivities, with the highest \(P_{f}\) elicited by Aqp0a expressing oocytes at alkaline pH, whereas that of the Aqp0b expressing oocytes was at acidic pH, thus closely reflecting the pH permeation properties observed for the Atlantic salmon Aqp0a2 and -0b1 orthologs (Fig. 6). As expected, the water permeability of oocytes expressing zebrafish Aqp0b-G19S was completely abolished (Fig. 6), confirming that zebrafish may harbor a nonfunctional Aqp0b allele (36, 37).

Single His at the entrance of the water pore regulates pH sensitivity

The preceding experiments, together with previously published data (38, 40, 41), indicated that teleost Aqp0 a-type with His at position 39, such as Atlantic salmon Aqp0a2, zebrafish Aqp0a, and killifish Aqp0a, show maximum water channel activity at alkaline pH, whereas teleost Aqp0 b-type, such as Atlantic salmon Aqp0b1, zebrafish Aqp0b, and bovine AQP0, all bearing the His\(^{40}\) residue, are more permeable at acidic pH. However, salmon Aqp0a1 showed the highest permeability at neutral pH, despite a His residue at position 39. Reinspection of the amino acid alignment revealed that the nonconserved Ser\(^{38}\) of Aqp0a1 is usually represented by a conserved Pro at position 38 (Fig. 4A). Mutation of the Ser\(^{38}\) into a Pro in Aqp0a1 (Aqp0a1-S38P) recovered maximum permeability

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Figure 3. Tissue expression pattern of Atlantic salmon aqp0 genes. Tissue distribution of aqp0a1, -0a2, -0b1, and -0b2 transcripts determined by quantitative RT-PCR and using rps18 as reference gene. Data are means ± SEM (n = 5 fish). Significant differences (*P < 0.05; **P < 0.01; ***P < 0.001) between paralogs in each tissue are indicated. The bracket indicates significant differences of the expression levels in the lens with respect to the other tissues. NS, not significant.
Figure 4. Structural analysis of tetrapod and teleost AQP0. A) Amino acid alignment of Atlantic salmon and representative teleost Aqp0 sequences in relation to bovine AQP0. Fully conserved residues are boxed in dark gray, whereas the His residue involved in pH sensitivity is shaded in magenta. The zebrafish allelic variant at position Gly19 is circled in red. Conserved Asn-Pro-Ala (NPA) motifs (pale red arrows) are highlighted with α-helical regions shown for TMDs 1–6 (light blue arrows), hemihelices 3 (yellow arrow), 7 (green arrow), and 9 (orange arrow), intra-and extracellular loops (A–E; pink lines) with the N (NT) and C (CT) termini (pale orange lines). Taxa as follows: Bt, Bos taurus; Fh, Fundulus heteroclitus; Ss, Salmo salar; Dr, Danio rerio. B–D) Extracellular (upper) and lateral (lower) views of bovine AQP0 (B) and Atlantic salmon Aqp0a2 (C) and Aqp0b1 (D) are rendered with MacPymol.
The high expression of Atlantic salmon *aqp0a1*, *-0a2*, *-0b1*, and *-0b2* in the lens agrees with the finding that the 5′ flanking regions of each of the 4 genes bear elevated numbers of putative binding sites for TFs important for lens development and differentiation in mammals, including PITX3 (72, 73), SOX and MEIS (74), or avian-MAF (75). Such equally abundant expression of *aqp0* paralogs in the salmon lens is intriguing because it implies that each has been positively selected for nonredundant functions. In zebrafish it has been established that both *aqp0a* and *-0b* are necessary for lens development and transparency with the water permeability mediated by *aqp0a* being essential; however, the *aqp0b* paralog may play additional roles not related to water transport (12, 37). Therefore, in Atlantic salmon, it is possible that each *aqp0b* paralog plays distinct but complementary functions in the lens, although this hypothesis needs further investigation.

Despite the high expression titers of *aqp0* genes in the Atlantic salmon lens, *aqp0*1, *-0a2*, *-0b1*, and *-0b2* transcripts were also detected in other tissues, particularly in the gonads, as previously documented for other teleosts (33, 35). These observations confirm that *aqp0* in Teleostei is not lens specific. The extraocular *aqp0* expression in salmon is supported by the presence of putative sites for TFs related to gonad development, and in particular Sertoli cell function in the tests, such as ETV5 (76), androgen receptor (77, 78), DMRT1 (79), or WT1 (80), in the 5′ flanking region of the *aqp0* paralogs. Although the role of *aqp0* in the gonads is unknown, this channel has been localized in Sertoli cells of gilthead seabream (35), and the rat (81), where it might regulate the secretion of intratubular fluid in addition to reinforcing Sertoli cell junctions (82).

Our finding that the tetraploid Atlantic salmon retains 4 functional *aqp0* water channels derived from the R4 WGD event in Salmonidae indicates that *aqp0* paralogs have been positively selected in this lineage for ~100 million years.

**Figure 5.** Functional characterization of Atlantic salmon *Aqp0* paralogs. Osmotic water permeability (*Pₜ*) of *X. laevis* oocytes injected with water (W) or cRNA encoding *Aqp0a1*, *-0a2*, *-0b1*, and *-0b2* and exposed to different pHs before and during the swelling assays. The *Pₜ* was calculated using an estimated surface area of 9X the geometric area. Data are the mean ± SEM (*n = 12 oocytes per treatment) of 3 independent experiments. Significant differences (**P < 0.05; ***P < 0.01; ****P < 0.001) for each construct at the 3 pHs are indicated. The bracket indicates significant differences with respect water-injected oocytes. NS, not significant.

at alkaline pH, thus converting the channel into a bona fide a-type *Aqp0* (Fig. 7). Similarly, when one of the His⁹⁰ and His⁹⁰ residues of Atlantic salmon *Aqp0b2*, which is not affected by pH, was replaced by an Asn (Aqp0b2-H39N or Aqp0b2-H40N), the channel became more permeable at acidic or basic pH, respectively (Fig. 7). These data therefore demonstrate that the pH sensitivity of the Atlantic salmon *Aqp0* paralogs is primarily determined by the relative position of a single His in the second transmembrane domain close to loop A facing the entrance of the pore.

**DISCUSSION**

It has recently been reported that almost half of the duplicated protein-coding genes arising from the R4 WGD event in Salmonidae are lost in extant species (52). In the trout genome, the cluster of genes with retained duplicates, which neofunctionalized, subfunctionalized, or acquired different expression patterns, is mostly related to visual perception (52). The present study in Atlantic salmon supports these findings because we found that tetraparalogous *aqp0* genes encode functional water channels that are equally expressed in the ocular lens at high titers, but with unique pH sensitivities.

The high expression of Atlantic salmon *aqp0*1, *-0a2*, *-0b1*, and *-0b2* in the lens agrees with the finding that the 5′ flanking regions of each of the 4 genes bear elevated numbers of putative binding sites for TFs important for lens development and differentiation in mammals, including PITX3 (72, 73), SOX and MEIS (74), or avian-MAF (75). Such equally abundant expression of *aqp0* paralogs in the salmon lens is intriguing because it implies that each has been positively selected for nonredundant functions. In zebrafish it has been established that both *aqp0a* and *-0b* are necessary for lens development and transparency with the water permeability mediated by *aqp0a* being essential; however, the *aqp0b* paralog may play additional roles not related to water transport (12, 37). Therefore, in Atlantic salmon, it is possible that each *aqp0b* paralog plays distinct but complementary functions in the lens, although this hypothesis needs further investigation.

Despite the high expression titers of *aqp0* genes in the Atlantic salmon lens, *aqp0*1, *-0a2*, *-0b1*, and *-0b2* transcripts were also detected in other tissues, particularly in the gonads, as previously documented for other teleosts (33, 35). These observations confirm that *aqp0* in Teleostei is not lens specific. The extraocular *aqp0* expression in salmon is supported by the presence of putative sites for TFs related to gonad development, and in particular Sertoli cell function in the tests, such as ETV5 (76), androgen receptor (77, 78), DMRT1 (79), or WT1 (80), in the 5′ flanking region of the *aqp0* paralogs. Although the role of *aqp0* in the gonads is unknown, this channel has been localized in Sertoli cells of gilthead seabream (35), and the rat (81), where it might regulate the secretion of intratubular fluid in addition to reinforcing Sertoli cell junctions (82).

Our finding that the tetraploid Atlantic salmon retains 4 functional *Aqp0* water channels derived from the R4 WGD event in Salmonidae indicates that *Aqp0* paralogs have been positively selected in this lineage for ~100 million years.
The additional confirmation that both zebrafish Aqp0a1 and -0b2 paralogs are functional water transporters when heterologously expressed in X. laevis oocytes (33) further suggests that such positive selection has existed since the R3 WGD event, and thus for \(-350\) million years. These findings contrast another study, however, which reported that cell-to-cell junctional strength is dependent on the positioning of pH sensitivities to encompass a broad range of aquatic conditions. For example, the paralog-specific pH sensitivities may provide compensatory water transport between the lens fibers when juveniles migrate from acidic freshwater to alkaline seawater during smoltification or when adults reenter freshwater from their oceanic sojourns during the spawning migration. It is also known that the pH of the lens varies from the superficial layers to the nuclear layers (83, 84). However, the potential differential localization of the salmon Aqp0 paralogs in the lens remains to be investigated. In addition, it has been reported that cell-to-cell junctional strength is dependent on acid-base homeostasis (85). The reconstitution of AQP0 into large unilamellar liposomes promotes a fast liposome permeation of pH 8.6, which is the pH at which the permeability of the channel is the highest. Therefore, the potential role of pH in regulating the water transport and permeability might not be required for Aqp0b function in the zebrafish lens.

Our data further demonstrate that teleost a-type Aqp0 water channels display maximum water permeability at alkaline pH, whereas some of the b-type channels are more permeated at acidic pH as found for bovine AQP0. This latter acidic permeation preference is therefore likely conserved in metatherian and eutherian mammals because of their retention of His\(^{39}\). In Atlantic salmon, however, Aqp0a1 is more permeable at neutral pH, whereas Aqp0b2 does not display pH sensitivity. Previous studies on bovine and common mummichog AQP0 suggested that external His residues in loops A and C that span the outer vestibule of the channel contribute to pH sensitivity (41). In the present work, site-directed mutagenesis experiments show that the differential pH regulation of Atlantic salmon Aqp0 paralogs is determined by the position of a single His located at the entrance of the water pore. However, the sensitivity of Aqp0a1 at neutral pH can be shifted to alkaline pH by the S38P mutation, suggesting that the effect of His\(^{39}\) protonation on the permeability of the pore is also modulated by the microenvironment surrounding this residue. Considering that the \(K_e\) of the His imidazole group is 6.0, we speculate that charge may play a gating role in Aqp0b1 at pH values > 6, whereas protonation of His\(^{40}\) at acidic pH would not only alleviate the negative electrostatic gating, but may move His\(^{40}\) away from the central pore because of additional Van der Waals forces, thus facilitating water transport. Conversely, protonation of His\(^{39}\), which lies on the outside of Aqp0a2 TMD2, could have the opposite effect at acidic pH, which together with an allosteric realignment of loop A may partially occlude the pore entrance. The significance of the S38P substitution in Aqp0a1 is likely associated with the conformational unwinding of the \(\alpha\)-helical terminus of TMD2 and thus the positioning of His\(^{40}\) over the pore entrance in a-type channels, thus altering pH sensitivity, whereas the opposing arrangement of His\(^{39}\) and His\(^{40}\) present in Aqp0b2 cancel each other’s effect, leaving this type of channel insensitive to pH modulation.

The physiologic implications of the different pH sensitivities of Atlantic salmon Aqp0 paralogs remain intriguing. It seems reasonable to suggest that the retention of the tetraparalogous salmon Aqp0 channels may be caused by positive selection associated with the neofunctionalization of pH sensitivities to encompass a broad range of aquatic conditions. For example, the paralog-specific pH sensitivities may provide compensatory water transport between the lens fibers when juveniles migrate from acidic freshwater to alkaline seawater during smoltification or when adults reenter freshwater from their oceanic sojourns during the spawning migration. It is also known that the pH of the lens varies from the superficial layers to the nuclear layers (83, 84). However, the potential differential localization of the salmon Aqp0 paralogs in the lens fiber cells remains to be investigated. In addition, it has been reported that cell-to-cell junctional strength is dependent on acid-base homeostasis (85). The reconstitution of AQP0 into large unilamellar liposomes promotes a fast liposome aggregation at acid pH (86), which is the pH at which the permeability of the channel is the highest. Therefore, the potential role of pH in regulating the water transport and permeability might not be required for Aqp0b function in the zebrafish lens.

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Figure 7. Role of Ser\(^{38}\) and His\(^{39}\)-His\(^{40}\) on Atlantic salmon Aqp0a1 and -0b2 pH sensitivity. Osmotic water permeability (\(P_t\)) of X. laevis oocytes injected with water or cRNA encoding wild-type Aqp0a1 or Aqp0b2 or different mutants in which Ser\(^{39}\) was replaced by Pro in Aqp0a1 (Aqp0a1-S39P) or in which a single His in position 39 or 40 replaced the double His in Aqp0b2 (Aqp0b2-H40N and Aqp0b2-H39N, respectively). The \(P_t\) was calculated using an estimated surface area of \(9\times\) the geometric area. Oocytes were exposed to different pH before and during the swelling assays. Data are the mean ± SEM (\(n = 12\) oocytes per construct) of a representative experiment. Significant differences (*\(P < 0.05\); **\(P < 0.01\); ***\(P < 0.001\)) for each construct at the 3 pHs are indicated. The bracket indicates significant differences with respect water-injected oocytes. NS, not significant.
adhesive function of each salmon Aqp0 paralog should be investigated in the future.

In summary, the present study shows that, in contrast to the single aqp0 genes in Chondrichthyes, Sarcopterygii, Holostei, and the duplicated Aqp0a and -0b paralogs found in the majority of Teleostei, Atlantic salmon retains 4 aqp0 genes that encode functional water channels with paralog-specific pH sensitivities. Site-directed mutagenic experiments demonstrate that the position of a histidine at the extracellular end of TMD2 is critical for pH-regulated water permeability. Comparison of the salmon Aqp0 permeation properties with the zebrafish orthologs confirm that both zebrafish Aqp0a and -0b are functional water transporters and that the zebrafish channels, respectively, display the alkaline and acidic sensitivities determined for the salmon Aqp0a2 and -0b2 paralogs. Based on the position of the histidine in the amino acid alignment of 78 gastronomie Aqp0 proteins, the present findings suggest that the alkaline pH control of water permeation is conserved in teleost a-type channels, whereas the acidic pH sensitivity regulated by His40 in some teleost b-type channels is conserved in mammals but not in other Tetrapoda, basal Sarcopterygii, Holostei, or Chondrichthyes.

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