The vertebrate Aqp14 water channel is a neuropeptide-regulated polytransporter

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The aquaporin superfamily of membrane proteins was first discovered almost 30 years ago through demonstration of the biophysical properties of human AQP1 in frog oocytes. The discovery validated the long search for molecular channels that evolved to specifically facilitate the rapid yet selective transport of water across biological membranes. With the subsequent discovery of other aquaporins, structural studies have revealed overall similarities of the monomeric proteins. This includes intracellular N- and C-termini, often involved in cytoplasmic protein trafficking, 6 transmembrane domains (TMD) linked by loops A–E, and two hemihelices that fold to oppose canonical Asp-Pro-Ala (NPA) motifs in the outer vestibules. In addition, four aromatic-arginine (ar/R) residues on TMD2, −5 and loop E typically form important selectivity filters surrounding the central channel. Each transmembrane protein functions in tetrameric assemblies to facilitate the passive single-file conductance of water or the other small uncharged solutes down their concentration gradients.

In the ~3 decades of research following the discovery of aquaporins, eutherian mammalian water channels have been classified according to the chronology of gene discovery, with 13 subfamilies (AQP0 – 12) initially identified. Broader phylogenetic studies in diverse ranges of eukaryotic organisms have supported this view for vertebrates. In a recent study of the evolution of this superfamily, however, we phylogenetically identified three entirely novel subfamilies (aqp14, −15 and −16) in vertebrates, and further showed that the AQP13 aquaporin subfamily, first documented as AQPdlo in Xenopus laevis oocytes, exists as a complete ortholog in Amphibia, but also as a pseudogene in the prototherian order of egg-laying mammals, the Monotremata, while aqp15 and aqp16 are restricted to specific lineages of fishes, turtles and crocodylians.

The vertebrate aquaporin superfamily is thus currently comprised of 17 subfamilies (AQP0 – 16) that are phylogenetically classified into four groups that can be traced to basal metazoan or parazoan lineages including Cnidaria (jelly fish and corals) or Porifera (sponges). This includes: classical aquaporins (AQP0, −1, −2, −4, −5, −6, −14 and 15) that primarily transport water, Aqp8-type aquaporins (AQP8 and −16) that primarily transport water, urea, ammonia and peroxide, aquaglyceroporins (AQP3, −7, −9, 10 and −13) that primarily function as water, urea and poliov transporters, and the intracellular unorthodox aquaporins (AQPN11 and −12), for which functional data are mostly lacking, except for AQP11 which seems to transport water and glycerol.

The physiological roles of the different channels have been best studied in eutherian mammals, with major roles demonstrated in vision (AQP0), erythrocyte volume regulation (AQP1), vasopressin-regulated antidiuresis (AQP2), transcellular fluid transport and skin hydration (AQP3), the blood–brain barrier (AQP4), sweat and tear production (AQP5) and adipocyte metabolism (AQP7). Studies of non-mammalian vertebrates have also revealed the physiological importance of AQP1 and AQP8 orthologs in the germ cell biology and osmoregulation of fishes and amphibians. To date, however, no functional data exist for the novel aqp14 gene subfamily, which has no specific annotation in available genome databases, yet is suggested to exist in a broad range of vertebrates.

To understand the genomic landscape and function of the novel aqp14 water channel subfamily, we focused on piscine genomes, which remain the least annotated, yet encode proteins that span >500 million years of evolution, and which represent species that have adapted to freshwater and marine environments. The homeosmotic biology of fishes that live in these opposing osmotic environments is fundamentally different, where the physiological task in freshwater species is to keep water out due to the hyperosmotic condition of their body fluids, while that of marine fishes is the reverse. These latter species, whose blood osmolality is about 1/4 that of seawater, need to obtain pure water from the dessicating saltwater environment. Within true bony fishes (Osteichthyes), the evolved solution amongst teleosts resulted in every species drinking seawater and managing the desalination and water transport of the imbibed fluid along the length of their intestines, while secondarily excreting excess salts from chloride cells in the gill. We therefore investigated the potential of Aqp14 proteins to function in fish osmoregulation, and here provide a comprehensive overview of the channel history by leveraging 190 and 87 piscine genomes and transcriptomes, respectively, to assemble >1000 exons into 179 full-length and 26 partial aqp14 coding sequences (CDS). This approach allowed us to identify lineage-specific pseudogenes and to reveal the origin, evolution and structure of the subfamily.

We further experimentally demonstrate the molecular function and neuropeptide regulation of the Aqp14 channel from ancient and modern lineages of fishes, and confirm the existence of the complete Aqp14 ortholog in all extant sarcopterygian lineages, except eutherian mammals.

**Results**

**Phylogeny.** To illustrate the phylogenetic divisions of the major aquaporin subfamilies in vertebrates, we initially assembled the complete set of full-length transcripts from the prototherian platypus (Ornithorhyncus anatinus) and analysed them together with the established genomic repertoire of the zebrafish (Danio rerio). We selected the platypus, since we have previously shown that its genome also retains the AQP13 ortholog found in Amphibia, and each of the other subfamilies reported for tetrapods, except AQP10. Thus, with the exception of AQP16, which is an AQP8-type channel identified in amphibians, turtles and crocodylians, the platypus and zebrafish display each of the major divisions of aquaporin subfamilies (AQP0-15) that are representative for the two major lineages of osteichthyan vertebrates (Sarcopterygii and Actinopterygii) with the highest aquaporin gene copy numbers. Since Ensembl (v96) only lists four complete aquaporins for the platypus, we assembled the full CDS de novo from the genome based upon the manual identification of 67 exons (see materials and methods). This yielded 14 paralogs in the platypus compared to 19 in zebrafish. Phylogenetic analysis of the aligned codons shows that the platypus genome encodes seven classical aquaporins (AQP0, −1, −2, −4, −5, −6 and −14), one AQP8-type channel (AQP8), two unorthodox paralogs (AQP11 and −12) and four aquaglyceroporins (AQP3, −7, −9 and −13) (Fig. 1a). We did not detect AQP10 in the platypus genome, and therefore included the metatherian Tasmanian devil (Sarcophilus harrisii) AQP10 ortholog for comparison with the zebrafish repertoire. As previously shown, the zebrafish genome encodes single-copy or duplicated orthologs of all of the mammalian channels, except for AQP2, −5, −6 and −13, which are not found in the genomes of ray-finned (actinopterygian) or cartilaginous (chondrichthyan) fishes. In contrast to prototherians, the zebrafish encodes an Aqp15 channel, which is also present in the genomes of other actinopterygian or chondrichthyan fishes. This analysis shows the predominance of the classical-type aquaporins and aquaglyceroporins in mammalian and piscine genomes, with AQP14 clustering next to AQP4 within the classical grade of aquaporins.

We then identified and assembled 205 Aqp14 proteins and their respective CDS from 122 families within 59 orders of fishes encompassing ~68% of the known orders of actinopterygian fishes. Bayesian phylogenetic analyses (25 million MCMC generations) of the aligned codons without exon 1, which encodes the 3–7 variable N-terminal amino acids, provided a
high-resolution tree with ~70% of posterior probabilities >0.9 (Fig. 1b; see Supplementary Fig. 1 for the fully annotated tree together with accession numbers). The tree topology almost completely recovers the phylogenetic interrelations of actinopterygian fishes recently determined from transcriptomic and genomic data. This includes the position of anguilliforms (eels) relative to osteoglossiforms (bony tongues) as well as the clear hierarchical separation of the sister clade of Clupeocephala into Otomorpha and Euteleostei with respective subdivisions. The only major anomaly is the position of the Gobiiformes (mudskippers), which cluster with the Tetraodontiformes (box fishes) within the euceracean clade. This is despite the mudskipper aqp14 transcripts retaining lower identities (76.8 ± 0.2%; N = 4) than those of other Percomorphaceae (80.5 ± 1.9%; N = 87) in relation to the tetraodontiform CDS.

Examination of hagfish (Hyperotreti) databases (N = 2) revealed only 1 encoded classical aquaporin (aqp4), and consequently the tree shows that the oldest forms of aqp14 likely
arose in a common ancestor of jawed and jawed vertebrates as reflected by petromyzontiform lampreys (Hyperoartia) and cartilaginous fish (Chondrichthyes). The conserved aqp14 subfamily (<40% amino acid differences between sharks and modern acanthomorph teleosts encompassing >500 million years of evolution since their last common ancestor32) has remained encoded in the genomes of all three major lineages of extant gnathostome vertebrates, the Chondrichthyes, Actinopterygii and Sarcopterygii. The latter instance, this includes the Actinistia (coelacanths), Amphibia (caecilians, salamanders and frogs), Sauropsida (reptiles and birds), as well as prototherian and metatherian mammals (see below, Section on the Role of the C-terminus in the control of Aqp14 intracellular trafficking), but as noted previously, are fractionated into pseudogenes in eutherian mammals.16 Further, despite clear evidence that teleost fish retain many duplicated aquaporins resulting from a fish-specific whole-genome duplication (WGD)16,26,29,30, only single-copy aqp14 genes were discovered in the genomes of diploid species (N = 182), while intact duplicates and pseudogenes were detected in the genomes of paleotetraploid cyprinid (N = 7) and salmonid (N = 4) fishes, respectively.

The present evidence shows that the structures of the aqp14 genes are split into five exons in basal lineages of actinopterygian, chondrichthyan and hyperoartian fishes as well as in all sarcopterygian animals that retain the genes, but into 6 exons in teleost fishes (Fig. 1c). Gene lengths are polymorphic, with the longest currently identified in the great white shark (Carcharodon carcharias, 20.74 kb), and the most compact in the Fugu (Takifugu bimaculatus, 1.35 kb), with the majority of aqp14 gene structures in Euteleostei spanning <4.6 kb. All aqp14 genes are located within a small, but conserved gene cassette upstream of each animal’s aqp0 paralog with rearrangement of the flanking piges3 and baz2 paralogs to upstream loci in teleost fishes (Fig. 1d). While diploid teleost fishes are also known to retain two aqp0 paralogs (aqp0a and –0b) and paratetraploid teleosts such as Atlantic salmon four paralogs (aqp0a1, –0a2, –0b1, –0b2)33, the aqp14 genes are only found upstream of the aqp0b paralogs. This includes the aqp14_1 pseudogenes upstream of the paratetraploid salmonid aqp0b1 paralogs and the complete aqp14 gene upstream of a fractionated aqp0b pseudogene in the electric eel (Electrophorus electricus). These observations suggest loss of a WGD aqp14(a) gene shortly after the fish-specific WGD.

Permeability properties of teleost Aqp14. To investigate the functional properties of the aqp14 genes, we isolated and cloned aqp14 CDNAs from the freshwater ostariophyian zebrafish as a representative of the ancient lineage of Otophorma, and two euteleostean species, the freshwater protacanthopterygian Atlantic salmon (Salmo salar) and the marine acanthopterygian gill- head seabream (Sparus aurata). Tertiary renders of the deduced proteins against a crystallographically resolved AQP4 structure mask (PDB: 3GD8), revealed that the Aqp14 channels retain the canonical six transmembrane domains, together with two helices bearing NPA motifs, the 5 linking loops (3 external: A, C; 2 internal: B, D) and intracellular N- and C-termini (Fig. 2a).

Some variation of the first NPA motif was noted amongst gadiiform, ophidiform and kurtiform teleosts, which encode NPS in that position. In contrast to other classical aquaporins, which retain four ar/R residues, the Aqp14 renders indicate that a fifth residue (Val29 in seabream, or Val30 in zebrafish and salmon) on TMD1 participates in the outer vestibule constriction of this subfamily (Fig. 2a). In addition, although the Arg constriction residue following the second NPA motif is fully conserved, none of the other Aqp14 constriction residues are aromatic.

The biophysical properties of the zebrafish, salmon and seabream channels were investigated using the Xenopus laevis oocyte-swelling and radioactive or fluorescent substrate uptake assays. Oocytes expressing either the zebrafish, seabream or salmon channels (DrAqp14, SaAqp14 or SsAqp14, respectively) show an approximately 5-fold increase in osmotic water permeability (Pf) with respect to the water-injected oocytes, as well as a conserved inhibition sensitivity to mercury, which is reversed by the reducing agent β-mercaptoethanol (Fig. 2b). Oocytes injected with SaAqp14 and SsAqp14 are also slightly permeable to urea, uric acid and hydrogen peroxide and the ammonia analogue methylamine (Fig. 2c–f). By contrast, oocytes expressing DrAqp14 are highly permeable to urea and hydrogen peroxide, but as for SaAqp14 and SsAqp14 show a lesser permeability to glycerol and methylamine (Fig. 2c–f). These data show that in contrast to other classical aquaporins, Aqp14 channels share some of the permeation properties typically elicited by aquaglyceroporins.

Tissue distribution and cellular localization. As a first insight to the physiological function of the Aqp14 channels, we investigated the tissue distribution and cellular localization in the three selected species. Expression of aqp14 transcripts was investigated by reverse-transcriptase PCR (RT-PCR) using paralog-specific primers on extracts from the brain, eye, lens, ovary, testis, gills, kidney, liver, anterior intestine, mid intestine, rectum and skin (Fig. 3a–c; and Supplementary Fig. 2 for biological replicates). In each species, prominent aqp14 expression is detected in the brain, lens and testis, but in other tissues it shows greater variability between the species, and in some cases also between animals. For example, although aqp14 mRNA is found in the gills, kidney, liver, gut and skin of zebrafish and salmon, the expression of aqp14 in these tissues from seabream is less prominent or undetectable in some animals (Fig. 3a–c).

To determine the expression and cellular localization of Aqp14 proteins in the different tissues we produced an affinity-purified rabbit antibody against a C-terminal 16 amino acid region of the seabream channel (Supplementary Fig. 3a–c). The specificity of the antiserum was confirmed through western blot analysis of total membrane protein extracts from oocytes injected with equal...
amounts of seabream aqp0a, −1aa, −1ab, −4a, −8bb, −3a, −9b, −7, −10b and −14 cRNA (Supplementary Fig. 3d). The cross-reactivity of the antiserum against DrAqp14 and SsAqp14, which, respectively, show 63 and 81% identity in the region of the SaAqp14 C-terminus selected for immunization, was also tested by Western blot analysis on total membrane protein extracts from X. laevis oocytes injected with equal amounts of aqp14 cRNA. The results show that the antiserum generated was able to specifically recognize Aqp14 from all three species, although with less intensity to that of SaAqp14 according with their lower level of amino acid identity within the immunization region (Supplementary Fig. 3e).

Immunoblotting analyses of zebrafish tissues using the seabream Aqp14 antibody revealed a single immunoreactive polypeptide band of approximately the same molecular mass as the predicted Aqp14 monomer (29.5 kDa) in the brain, eye, lens, testis, rectum and gills, somewhat more intense when normalized to prohibitin expression in the brain, eye and gills (Fig. 3d, Supplementary Figs. 5–8). In the brain, Aqp14 is distributed exclusively in the optical tectum in the stratum marginale and stratum periventriculare (Fig. 4a,b), whereas in the eye the channel is detected in the lens epithelium but not in the lens fiber cells (Fig. 4c). The Aqp14 channel is also expressed in ganglion cells located in the inner surface (the ganglion cell layer) and in neurons of the inner nuclear layer of the retina, which extend...
their axons across the inner plexiform layer (Fig. 4d). In the gills, Aqp14 immunostaining is found in the apical plasma membrane of epithelial cells along the length of gill lamellae (Fig. 4e). In the testis, Aqp14 expression is found exclusively in the myofibroblasts and smooth muscle cells of the tunica albuginea (Fig. 4f and inset), whereas the channel is also expressed in the myocytes of white muscle fibers (Fig. 4g). Interestingly, in both the zebrafish and salmon that were maintained in freshwater, very low levels of Aqp14 are detected in rectal enterocytes, while prominent Aqp14 expression is observed both in the cytoplasm and apical plasma membrane of these cells in seawater-held seabream (Fig. 5a–c). In the seabream rectum, the channel is also detected in some cells of the submucosa, but not in the mucus secreting goblet cells spread in the luminal epithelium (Fig. 5c). The sections of all these tissues incubated with the preabsorbed affinity-purified Aqp14 antiserum did not show any positive signals (Figs. 4h–n and 5d–f), confirming the specificity of the antibody. In contrast, in the three species investigated no consistent Aqp14 immunostaining is observed in the ovary, kidney, liver, anterior and mid intestine, and skin.

C-terminal control of Aqp14 intracellular trafficking. The observation that Aqp14 in a marine teleost, such as the seabream, is strongly expressed in both the cytoplasm and apical plasma membrane of rectal enterocytes, which is a major site of intestinal water resorption in marine teleosts, prompted us to investigate the potential role of the Aqp14 cytoplasmic C-terminus in the control of the channel’s intracellular trafficking. Amino acid sequence alignment of zebrafish, salmon and seabream Aqp14 revealed the presence of two conserved Thr residues (Thr240/Thr241 and Thr259/Thr260) and one Ser (Ser256/Ser257) in the C-terminus of the channels as potential sites for protein kinase C (PKC) and protein kinase A (PKA) phosphorylation, respectively (Fig. 6a). To experimentally assess if PKC and PKA regulate the trafficking of Aqp14, X. laevis oocytes expressing DrAqp14, SaAqp14 or SsAqp14 were exposed to the PKC activator phorbol 12-myristate 13-acetate (PMA) or the cAMP-PKA activator forskolin (FSK), the latter after preincubation of oocytes with the phosphodiesterase inhibitor 3-isobutyl-1-methylxanthine (IBMX). Interestingly, the results of these experiments showed that the PPM of Aqp14 oocytes is inhibited by ~45% by PMA regardless of the ortholog expressed, whereas treatment with IBMX and FSK stimulates water permeability by ~40–60% (Fig. 6b). Immunoblots of total and plasma membrane extracts of these oocytes revealed that the opposite effects of PMA and IBMX/FSK on oocyte permeability is caused by the PKC-mediated negative and PKA-mediated positive regulation of Aqp14 trafficking to the oocyte surface (Fig. 6c, Supplementary Fig. 9).

To corroborate that the effects of PKC and PKA on Aqp14 trafficking occur via the Thr and Ser residues identified in the...
C-terminus of the piscine channels, oocytes were injected with wild-type DrAqp14, or with DrAqp14 independently mutated into Ala or Asp at Thr\textsuperscript{241}, Ser\textsuperscript{257} and Thr\textsuperscript{260} residues to, respectively, mimic nonphosphorylated and phosphorylated states, and treated with PMA or IBMX/FSK as above. Swelling assays showed that DrAqp14-T241A and -S257D constitutively increase water permeability with respect to the wild-type by ~50% and ~90%, respectively (Fig. 6d, Supplementary Fig. 10e). In addition, these mutants respectively prevent the effect of PMA or FSK, whereas the DrAqp14-T260A and -T260D mutants have no effect. According to these observations, the DrAqp14-T241D mutant reduces the permeability with respect to the wild-type by
~53%, and blocks the inhibition by PMA, while it does not interfere with the positive effect of FSK. By contrast, the DrAqp14-S257A mutant does not change the negative effect of PMA on channel trafficking, but abolishes the positive effect of FSK. Immunoblot analysis confirmed that the wild-type and mutant channels were expressed in oocytes at similar levels (Fig. 6e). The same results were obtained when oocytes expressed wild-type SaAqp14 or the SaAqp14-T240A, -T240D, -S256A and S256D mutants (Fig. 6f, g, Supplementary Fig. 10g). These data therefore suggest that the intracellular trafficking of teleost Aqp14 is dually regulated by PKC and PKA pathways through phosphorylation of Thr240/Thr241 and Ser256/Ser257, respectively. Strikingly, these residues are not only fully conserved in the C-termini of the Aqp14 orthologs of all agnathan and gnathostome fishes, but also in nearly all of the sarcopterygian orthologs (see Supplementary Fig. 11). Available genomic data thus reveal that all extant lineages of tetrapods, except eutherian mammals, also encode the AQP14 channels upstream of the AQP0 gene, but indicate that some birds and the platypus have substituted the Thr240/Thr241 for an Ala. These observations suggest strong purifying selection of the PKC and PKA targeted phosphorylation sites in Aqp14 for >500 million years of evolution.

Vasotocinergic and isotocinergic regulation of Aqp14. Since seawater acclimation in euryhaline teleosts is associated with the increased expression of some aquaporin orthologs in the intestinal and rectal epithelial cells34, the Aqp14 immunostaining pattern in seabream rectum raised the question of a potential regulation of Aqp14 trafficking by environmental salinity. In teleosts, osmoregulatory adaptations are regulated by the pituitary neurohormones vasotocin (AVT) and isotocin (IT), which evolutionarily, are amongst the most primitive molecules involved in vertebrate osmoregulation35. AVT and IT are orthologs of mammalian arginine vasopressin and oxytocin, respectively, which act on target tissues through specific AVT1a and AVT2 receptors (AVT1aR and AVT2R), preferentially coupled to PKC- or PKA-mediated pathways, respectively, or the IT receptor (ITR) that also acts via PKC36–38. We therefore investigated the possible control of seabream Aqp14 intracellular transport by AVT and IT, and whether this regulation involves the same C-terminal Thr and Ser residues identified above.

To examine this hypothesis, X. laevis oocytes were co-injected with seabream wild-type Aqp14, Aqp14-T240A or -S256A and seabream AVT1a2R, AVT2R or ITR. The cDNAs of the latter neuropetide receptors were previously isolated from publically
available data\textsuperscript{39}. Oocytes were then exposed to AVT or IT in the presence or absence of the PKC inhibitor Bisindolylmaleimide (Bim-II) or the PKA inhibitor H89. The results show that the P\textsubscript{f} of oocytes expressing the wild-type Aqp14 plus AVT1a2R or ITR are inhibited by ~84% and ~65% by AVT and IT, respectively, regardless of the presence of H89, whereas both effects are abolished when oocytes are exposed to Bim-II, or they co-express the AVT1a2R or ITR with the Aqp14-T240A mutant (Fig. 7a, c). In contrast, water permeability of oocytes expressing wild-type Aqp14 and AVT2R is increased by ~70% after AVT treatment, while this effect is prevented with H89, but not with Bim-II, or by coexpressing the Aqp14-T240A mutant instead of the wild-type (Fig. 7b). Oocytes expressing the Aqp14-T240A or -S256A mutants alone show more and less P\textsubscript{f}, respectively, than those injected with the wild-type, suggesting some level of endogenous activation of PKC or PKA in the oocytes (Fig. 7a–c). These data are therefore consistent with previous mutagenesis experiments, and suggest that activation of the AVT2R can induce the insertion of Aqp14 into the plasma membrane through PKA-mediated Ser\textsuperscript{256} phosphorylation, whereas the AVT1a2R and ITR inhibit channel trafficking to the oocyte surface through PKC-mediated Thr\textsuperscript{240} phosphorylation.

Discussion

In the present contribution, we uncover the broad genomic diversity and function of a novel subfamily of vertebrate water channel genes that currently lack formal annotation in genome databases. In accordance with the chronology of aquaporin gene discovery, the subfamily is named \textit{AQP14}, after \textit{AQP13}, which was originally isolated from frog oocytes\textsuperscript{15}, and shown to exist in amphibians and prototherian mammals\textsuperscript{16}. Our previous phylogenetic analyses revealed that \textit{aqp14} genes are members of the classical Aqp4-related grade of aquaporins, with multiple \textit{aqp4}-like genes traced to cnidarian lineages of jellyfishes and corals\textsuperscript{16}. The classical AQP4-related grade of aquaporins, thus evolved prior to the separation of Deuterostomia and Protostomia, and is distinct from the AQP8-related grade of channels, which also evolved prior to the separation of these lineages\textsuperscript{16,19,20,40}. Our new data show that the \textit{aqp14} genes are located upstream of the \textit{aqp0} paralog in nearly all vertebrate lineages, except hagfishes. The current evidence that hagfishes lack the \textit{aqp14} channel is consistent with the absence of the genomic region based upon synteny to other vertebrate genomes, and the absence of an \textit{aqp0} paralog in these jawless craniates. The observed paucity of classical aquaporins in hagfish may reflect their reduced
The Aqp14 channel is highly expressed in rectal enterocytes of shes, which is the major site of water absorption in shes, revealing that the neuropeptide regulation of Aqp14 channels co-evolved with vasotocinergic and isotocinergic neuropeptide systems to regulate water homeostasis throughout vertebrate evolution.

In contrast to other classical aquaporins, our data show that Aqp14 is a polytransporter, with permeabilities to urea, ammonia, H₂O₂ and glycerol in addition to water. This is unusual for classical aquaporins, which primarily transport water while preventing proton flux due to the specific arrangement of the ar/R and NPA constriction residues. Molecular renders of the piscine Aqp14 channels revealed that they each present a quintet of small non-aromatic constriction residues with hydrophobic side chains. This provides a more open and less polar arrangement compared to AQP1 and AQP4 channels, which in vertebrates only present four ar/R constriction residues that are highly selective for water due to the conserved His on TMD5. The arrangement of the five constriction residues in the Aqp14 channels also differs from other aquaporins that present ar/R quintets, including bacterial AqpZ, plant TIP2;1 and vertebrate AQP8. In these latter channels, an interacting fifth residue on loop C contributes to the selectivity filter, while in Aqp14, the fifth residue is located on TMD1. This novel topology generates the hydrophobic pockets in the Aqp14 channels to functionally resemble the selectivity filters of aquaglyceroporins, and although transport efficiencies differ between species, it likely explains their broader permeability properties for urea and glycerol. Since the Aqp14 channels are expressed in the cell membranes of neurons, muscles and gill epithelia, this feature indicates that the channels may also play important roles in metabolite and nitrogen metabolism.

In conclusion, we uncover the genomic diversity, structure and function of a novel subfamily of water channels in vertebrates. The aqp14 gene is located upstream of the aqp0 paralog in all extant vertebrates, except agnathan hagfishes and eutherian mammals, which in some of the latter lineages retain AQP14 pseudogenes. Our phylogenetic data show that the aqp14 subfamily clusters with the aqp4-related grade of classical aquaporins, but in contrast to other classical aquaporins, the proteins form hydrophobic selectivity filters through the presentation of five constriction residues on TMD1, –2, –5 and loop E. This unique arrangement supports our experimental data demonstrating that the channel is a polytransporter facilitating the molecular permeation of water, urea, ammonia, H₂O₂ and glycerol. We further show that channel trafficking is tightly controlled by the ancient vasotocinergic and isotocinergic neuropeptide systems to activate PKC and PKA transduction pathways for the phosphorylation of highly conserved Thr and Ser residues in the C-terminus. These data coupled with observations that the Aqp14 channel is highly expressed in rectal enterocytes of a marine fish, which is the major site of water absorption in seawater adapted fishes, reveal that the neuropeptide regulation of the Aqp14 channels predates the vasotocinergic/vasopressin regulation of AQP2, AQP5 or AQP6 observed in tetrapods and evolved under strong selective pressure to mediate osmoregulatory mechanisms. We identify two residues in the C-terminus of Aqp14 that provide a clue to this trait. We show that a Thr induces the retention of Aqp14 through AVT-AVT1a2R and IT-ITR mediated activation of the PKC pathway, while a Ser alternatively induces membrane insertion of Aqp14 through AVT-AVT2R-mediated activation of the PKA pathway. In addition, our data reveal that the Aqp14 channel is highly expressed in the cytoplasm and apical plasma membranes of rectal enterocytes of the marine teleost, gilthead seabream, a region of the intestine that plays a major role in the resorption of water in seawater adapted teleosts. The high conservation of the Thr and Ser residues in both piscine and tetrapod orthologs, and their respective regulation by the PKC and PKA transduction pathways in distantly related fishes, indicates that the Aqp14 channels co-evolved with vasotocinergic and isotocinergic neuropeptide systems to regulate water homeostasis throughout vertebrate evolution.

It has long been established that a neuropeptide-regulated water channel response governs antiurea or transepithelial water uptake in tetrapods, whereby vasopressin/vasotocin and the V2-type receptor induces cAMP-mediated trafficking of AQP2 in the kidney collecting tubules of mammals, birds and frogs, and of AQP5 and –6 channels in the skin of frogs. However, these aquaporin gene clusters are unique to sarcopterygian animals, and consequently, the broad prevalence and conservation of the aqp14 gene in vertebrate genomes suggests that this subfamily of water channels represents a much older system that osmoregulatory demands, since unlike other vertebrates, hagfishes are marine osmoconformers with blood osmolalities isosmotic to seawater. By contrast, extant lampreys are osmoregulators in both freshwater and marine environments, and encode the aqp14 gene upstream of their fused aqp0 paralog. Indeed as in marine teleosts, lampreys that live in marine environments are hyposmotic to seawater and drink to compensate for water loss revealing that such an osmoregulatory adaptation is likely ancient.

![Figure 7. Vasotocin (AVT) and isotocin (IT) regulation of seabream Aqp14 in X. laevis oocytes.](https://example.com/figure7)

**Fig. 7 Vasotocin (AVT) and isotocin (IT) regulation of seabream Aqp14 in X. laevis oocytes.** Osmotic water permeability (Pₒ) of oocytes expressing or not the seabream AVT1a2R (a), AVT2R (b) or ITR (c) receptors, and injected with water, wild-type Aqp14 (WT), or the Aqp14-T240A or -S256A mutants. Oocytes were treated with DMSO (controls) or 10 µM AVT or IT, in the presence or absence of 10 µM of PKC and PKA inhibitors (Bim-II and H89, respectively). Data are box and whisker plots with the number of biologically independent oocytes indicated above each plot. *P < 0.05; **P < 0.01; ***P < 0.001 (unpaired Student’s t-test), with respect to control oocytes, with respect to the WT (in parenthesis), or as indicated in brackets.
thus represents an ancestral mechanism of neuropeptide-regulated water homeostasis in vertebrates.

**Methods**

**Fish and chemicals.** Adult ~1 kg gilthead seabream (Sparus aurata) raised in captivity were maintained in seawater (~3.0% salinity) at the facilities of the Instituto de Marismas, Spanish Council for Scientific Research (CSIC, Spain) following previously described procedures. Adult zebrafish were obtained from the PRBB Animal Facility (Barcelona, Spain), whereas ~1.5 kg Atlantic salmon were collected from the Matredal Aquaculture Research Station (61°N in Norway). Both species were maintained in freshwater (~0.05% salinity). For tissue collection, fish were euthanized with 500 ppm of pentoxyphylline or 10 mg/l metomidate (Syndal, Victoria, BC, Canada), weighted and immediately euthanized by decapitation.

Biases of different tissues were either frozen in liquid nitrogen and stored at −80 °C for RNA and protein extraction, or processed for histology and immunofluorescence microscopy (see below). Procedures relating to the care and use of animals and sample collection were carried out in accordance with the protocols approved by the Ethics Committee (EC) of the Instituto de Recerca i Tecnologia Agroalimentaries (IRTA, Spain) following the European Union Council Guidelines (86/609/EU), or in accordance with the regulations approved by the governmental Norwegian Animal Research Authority (http://www.fdu.no/fdu/).

All chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) unless indicated otherwise.

cDNA cloning. Gilthead seabream and Atlantic salmon aqp14 genes were identified by screening the corresponding genomes and gene specific oligonucleotide primers flanking the first and last exon were designed to isolate the full-length mRNA coding sequences by reverse transcriptase-(RT)-PCR. For zebrafish aqp14, 5’ and 3’ end specific primers were designed using a publicly available sequence (GenBank accession no. XM_003174125). The sequences of the primers EcoRV and SpeI restriction sites were as follows: for seabream, forward and reverse primers were 5′-gatatcGGCTGCTCTCATCAAAGGAC-3′ and 5′-actagtGATC AACGGCTGATCTACAGA-3′, respectively; for Atlantic salmon, forward and reverse primers were 5′-gatatcCATGGCTCTGAAGGAC-3′ and 5′-actagtGATTCGGTATGAGTA-3′, respectively; and for zebrafish, forward and reverse primers were 5′-gatatcGATAGACCAACTGACGGATT-3′ and 5′-actagtGATTCGGTATGAGTA-3′, respectively. Total RNA from brain (seabream and zebrafish) or kidney (Atlantic salmon) was purified using the GenElute mammalian total RNA miniprep kit (Sigma-Aldrich), 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 Corp., Carlsbad, CA, USA) and SuperScript II RT enzyme (Life Technologies Corp.) as previously described. The PCR was carried out with the EasyA™ high-fidelity PCR cloning enzyme (Agilent Technologies, Santa Clara, CA, USA), with an initial denaturing step for 2 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 2 min, ending with a final elongation at 72 °C for 7 min. 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). The full-length cDNAs encoding the gilthead seabream vasotocin and isotocin receptors (V1a2R, V2R and ITR) were isolated from renal total RNA by RT-PCR as above using specific primers designed based on deposited sequences (GenBank accession nos. KC159974, KC604888 and KC159973, respectively). For the V1a2R forward and reverse primers were 5′-ACTATGCGCTTGTCCTGAGC-3′ and 5′-TGGTACCCCTTGTGATGCCAGACA-3′, respectively; for the V2R forward and reverse primers were 5′-AAGGACACCCGATGGTGAAG-3′ and 5′-CCGGTCACTTCTTTCTAACA-3′, respectively; and for the ITR forward and reverse primers were 5′-GACCAGCCACTTGTGGTGTG-3′ and 5′-GGGTCCACCGAGCTACT-3′, respectively.

**Phylogenetic, syntonic and sequence analyses.** Using the full-length deduced proteins of the clones or exon-deduced peptides from our initial analyses as tblast queries, >1000 piscine Aqp14 peptide fragments were identified by an unpaired Student’s t-test using the STATGRAPHICS PLUS 4.1 software (Statistical Graphics, USA). A P-value < 0.05 was considered statistically significant.

**Gene expression analysis.** Expression of total RNA from different tissues of gilthead seabream, zebrafish and Atlantic salmon (N = 3 for each species) and cDNA synthesis was carried out as described above. RT-PCR was performed using 1 μl of cDNA, 1 μl of Taq polymerase enzyme, and 0.5 μl of forward and reverse primers specific for each aqp14 ortholog. For seabream, forward and reverse primers were 5′-ATGCAAGAACGAGGAAACTC-3′ and 5′-CAGTCAACCGGCTTCTACAGA-3′, respectively; for zebrafish forward and reverse primers were 5′-ATGACCGGCGTAAGCCTCT-3′ and 5′-GTCTCATGGCTCTGTGAGA-3′, respectively; and for Atlantic salmon forward and reverse primers were 5′-GCCGGGCTCCTACTCTC-3′ and 5′-ACTGGGGGCAAGGAAGAATC-3′, respectively. The amplification protocol was composed of an initial denaturing step for 2 min at 94 °C, followed by 35 cycles of 94 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min, ending with a final elongation step for 7 min at 72 °C. PCR products were run on 1% agarose gels. For seabream and zebrafish, the reference gene was 18s ribosomal protein (ips18; forward and reverse primers were 5′-ACTAAGAA CGCCGATCGACCC-3′ and 5′-GAAGTGCAGGGGGCCACCAC-3′, respectively), whereas β-actin (bactin; forward and reverse primers were 5′- CCAAAGGCAACAGGGAAGA-3′ and 5′-GATGTTGTACTGTTGGTTC-3′, respectively) was the reference gene for Atlantic salmon.

**Antibody production.** An Aqp14 antisera was raised in rabbits against a synthetic peptide corresponding to a region of the seabream Aqp14 C-terminus (amino acid residues 261–276; TNQAMRAQKANQENN; Agrisera AB, Vännäs, Sweden).
Isolated as described previously62. Fish tissues were dissociated with a glass dounce Immunoblotting

Rad Laboratories, Hercules, CA, USA), as previously described52. The membranes (SDS-PAGE), and blotted onto Immun-Blot nitrocellulose 0.2 µm Membrane (Bio-electrophoresed in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis

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MK883753, MK883754 and MK883755, respectively. All data generated or analysed

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available at https://paup.phylosolutions.com. MAFFT used for multiple sequence

alignments is available athttps://mafft.cbrc.jp/alignment/software.

Reporting summary

Code availability

Mr Bayes software used for phylogenetic inference is available at https://github.com/ NRSweden/MrBayes/releases. PAUP software used for phylogenetic inference is available at https://paup.phylosolutions.com. MAFFT used for multiple sequence alignments is available at https://mafft.cbrc.jp/alignment/software.

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Author contributions

R.N.F. and J.C. designed the research; F.C., O.Y., A.F. and R.N.F. performed the research; P.G.F. contributed with animal samples; F.C., O.Y., R.N.F. and J.C. analyzed the data; and F.C., R.N.F. and J.C. wrote the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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