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Expression and functional characterization of four aquaporin water channels from the European eel (*Anguilla anguilla*)

Bryce MacIver[1,2], Christopher P. Cutler[2,3], Jia Yin[1], Myles G. Hill[2], Mark L. Zeidel[2,4] and Warren G. Hill[2,5,*]

[1]Nephrology Division and [5]Division of Matrix Biology, [4]Department of Medicine, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA 02215, USA, [2]Mount Desert Island Biological Laboratory, Salisbury Cove, ME 06472, USA and [3]Department of Biology, Georgia Southern University, Statesboro, GA 30460, USA

*Author for correspondence (whill@bidmc.harvard.edu)

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SUMMARY

The European eel is a euryhaline teleost which has been shown to differentially up- and downregulate aquaporin (AQP) water channels in response to changes in environmental salinity. We have characterized the transport properties of four aquaporins localized to osmoregulatory organs – gill, esophagus, intestine and kidney. By sequence comparison these four AQP orthologs resemble human AQP1 (eel AQP1), AQP3 (eel AQP3) and AQP10 (AQPe). The fourth member is a duplicate form of AQP1 (AQP1dup) thought to arise from a duplication of the teleost genome. Using heterologous expression in *Xenopus* oocytes we demonstrate that all four eel orthologs transport water and are mercury inhibitable. Eel AQP3 and AQPe also transport urea and glycerol, making them aquaglyceroporins. Eel AQP3 is dramatically inhibited by extracellular acidity (91% and 69% inhibition of water and glycerol transport respectively at pH 6.5) consistent with channel gating by protons. Maximal water flux of eel AQP3 occurred around pH 8.2 – close to the physiological pH of plasma in the eel. Exposure of AQP-expressing oocytes to heavy metals revealed that eel AQP3 is highly sensitive to extracellular nickel and zinc (88.3% and 86.3% inhibition, respectively) but less sensitive to copper (56.4% inhibition). Surprisingly, copper had a stimulatory effect on eel AQP1 (153.7% activity of control). Copper, nickel and zinc did not affect AQP1dup or AQPe. We establish that all four eel AQP orthologs have similar transport profiles to their human counterparts, with eel AQP3 exhibiting some differences in its sensitivity to metals. This is the first investigation of the transport properties and inhibitor sensitivity of salinity-regulated aquaporins from a euryhaline species. Our results indicate a need to further investigate the deleterious effects of metal pollutants on AQP-containing epithelial cells of the gill and gastrointestinal tract at environmentally appropriate concentrations.

Key words: osmoregulation, transport, solutes.

INTRODUCTION

The European eel (*Anguilla anguilla*) is a catadromous species, breeding in the sea and migrating to near shore or freshwater habitats in order to grow, before returning to the sea to spawn. It therefore exhibits a remarkable ability to adapt its physiology as it transitions between seawater (hyperosmotic) and freshwater (hypoosmotic) environments. In order to survive it must completely shift its osmoregulatory system from one in which excess salt is eliminated and water conserved (in seawater; SW), to one in which the exact opposite is true (in freshwater; FW). The manner in which it does this is not well understood, although there are hormone-regulated pathways involving cortisol, which result in up- or downregulation of particular genes involved in salinity adaptation (Dean et al., 2003; Cutler et al., 2007b). At the gross anatomic level, the SW-adapted eel is known to drink copiously, to desalinate the ingested SW within the esophagus, thereby diluting it; passing the modified fluid through the stomach and then having water reabsorbed along with further salts in the intestine (Cutler and Cramb, 2000; Ando et al., 2003). There is little urine generated and the principal salt excretory pathway appears to be *via* highly metabolically active 'chloride cells' within the gill (Beyenbach, 2004; Evans et al., 2005; Marshall and Grosell, 2006; Hwang and Lee, 2007). In FW, however, the primary pathway for water uptake occurs across the gill epithelia by osmotically driven diffusion, coupled with the formation of large amounts of dilute urine which is excreted. In this environment salts are obtained from food eaten and ion absorptive mechanisms operating in the gill and gut (Marshall and Grosell, 2006).

Aquaporins (AQPs) are a family of water and solute transporting proteins that have been remarkably conserved throughout evolutionary history (Zardoya and Villalba, 2001). Although the functions and physiological importance of AQPs have received their fullest examination in humans, they are present throughout all kingdoms of life, from Archaea (Kozono et al., 2003; Lee et al., 2005) and eubacteria (Calamita, 2000; Froger et al., 2001), to fish (Cutler and Cramb, 2000) plants (Maurel et al., 2008) and to mammals (King et al., 2004). It has recently been shown that the eel possesses a number of aquaporins found within osmoregulatory tissues and which in some cases have their expression up- or downregulated depending upon the salinity of the environment. Four eel isoforms were identified, cloned and found to be located in the gill, esophagus, kidney and intestine (Cutler and Cramb, 2002; Lignot et al., 2002; Martinez et al., 2005b).

Two of the eel AQPs – eel AQP1 and AQP1dup (thought to be an AQP1 homolog derived from an ancient genome duplication event) share 58% and 51% identity with mammalian AQP1. Eel AQP1 has a wide tissue distribution and mRNA transcripts are found at high levels in brain, eye, heart, esophagus and to a lesser degree in the FW gill and kidney (Martinez et al., 2005b). The expression of eel AQP1 shows complex regulation by cortisol, where levels of mRNA and protein are upregulated in intestine but downregulated

in kidney in response to the hormone (Martinez et al., 2005b; Martinez et al., 2005a). AQP1dup expression is high in the esophagus and FW kidney and it appears to be downregulated in the kidney in response to SW acclimation or cortisol (Martinez et al., 2005b).

The eel AQPe protein shares highest amino acid identity with novel aquaporins recently isolated from the sea bream (*Sparus aurata*; 67%) and *Xenopus laevis* (58%), and slightly lower identity with other AQP isoforms, e.g. human AQP7, –9 and –10 (40–42%) – all of which are aquaglyceroporins (Martinez et al., 2005a). Eel AQPe is found at high levels in the intestine and the FW-adapted kidney. SW acclimation causes downregulation in the kidney but has no effect on levels within the intestine. Intestinal AQPe therefore, is unlikely to play a role in the osmoregulatory response to salinity adaptation.

Eel AQP3 has a broad tissue distribution and appears to exhibit complex expression patterns within subsets of cell types (Cutler and Cramb, 2002; Lignot et al., 2002; Cutler et al., 2007a). For example immunofluorescence was used to show it is highly expressed within chloride cells of gill epithelium; in the esophagus in surface epithelial cells within the anterior segment but mucus cells posteriorly; and within the kidney, where expression is restricted to a subset of renal tubules. Levels within the kidney and intestine are unaltered by FW to SW acclimation, however, mRNA levels are reduced by as much as 97% in the gill upon transition from FW to SW. Increases in circulating levels of cortisol appear to be at least partly responsible for the decrease in AQP3 branchial expression (Cutler et al., 2007b). The role of eel AQP3 in the gill is of particular interest since this organ is the site of much osmotically driven fluid exchange, the direction of which clearly depends on the environment. Additionally, the gill is responsible for acid–base regulation, and serves as the major excretory organ for salt and potentially for nitrogenous waste products too (Masoni and Payan, 1974; Engin and Carter, 2001). Thus it is clear that the eel (and other euryhaline teleosts) hormonally regulate AQP expression in novel ways through salt- or osmoregulated transcriptional pathways.

In order to fully determine the physiological roles that these four transporters play and their part in the molecular cascades which are initiated by osmotic challenges, it is essential to understand their biophysical function. Once transport activities have been clearly defined rather than inferred from sequence homology data, we will be in a position to develop real insights into the role that these channel proteins play in osmoregulation and how they permit adaptation to different environmental conditions. The aim of this study was to define the substrates each aquaporin was capable of transporting and establish any similarities or differences with mammalian orthologs in terms of metal inhibition and pH regulation. Our findings establish the major functional transport properties of these aquaporin channels and in particular, show that eel AQP3 is sensitive to both external pH and several divalent metals.

MATERIALS AND METHODS

Harvesting oocytes

Xenopus laevis frogs were purchased from Xenopus Express (Plant City, FL, USA) and were maintained in aquarium tanks half filled with dechlorinated tap water at room temperature (~22°C) on a 12 h:12 h D:L cycle. Tank water was changed every 3–4 days. Animals were allowed to acclimatize for at least a week before any oocytes were harvested. Frogs 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 mmol l^{-1}; 96 NaCl, 1 KCl, 1 $MgCl_2$ 5 Hepes, pH 7.5). Oocytes were then defolliculated in 1.7% (w/v) collagenase (Sigma-Aldrich, St Louis, MO, USA), 0.17% (w/v) trypsin inhibitor (Sigma-Aldrich) in calcium-free ND96 for 55 min with rotation on an Adams Nutator before washing three times with hypotonic buffer [in mmol l^{-1}; 100 K_2HPO_4, 0.1% (w/v) BSA, pH 6.5]. The oocytes were then incubated in hypotonic buffer for 10 min at room temperature. Oocytes were transferred to calcium-free ND96 and then to modified Barth's solution [MBS; in mmol l^{-1}; 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] where they were maintained in an incubator at 18°C. All experiments were done in accordance with Institutional Animal Care and Use Committee approved protocols at Beth Israel Deaconess Medical Center and Mount Desert Island Biological Laboratories.

Cloning of eel aquaporin cDNAs for expression in *Xenopus laevis* oocytes

All four eel AQPs were RT-PCR amplified from several sources of eel mRNA and cloned into the *Xenopus* expression vector pXT7 using standard molecular cloning techniques. AQP1 and AQPe were amplified from SW-acclimated eel intestinal mRNA, whereas AQP1dup was isolated from FW-acclimated eel kidney mRNA, and AQP3 from SW-acclimated eel gill mRNA. Cloning sites used for AQP1, AQPe and AQP1dup were *Kpn*I and *Spe*I, whereas *Eco*RI was used for AQP3. A unique *Sma*I site within AQP3 was used to determine orientation. Sequence integrity was verified by DNA sequencing using standard dye-labeled dideoxy terminator techniques on an Applied Biosystems (Foster City, CA, USA) automated DNA sequencer at the Mount Desert Island Biological Laboratory core facility.

Production of eel aquaporin cRNAs for micro injection

Plasmid DNA for each aquaporin was cut with the restriction enzyme *Xba*I (for AQP1, AQP1dup and AQP3) and *Bam*HI (for AQPe) to linearize the plasmid and prevent run on of transcription. cRNA transcripts were produced using a mMESSAGE mMACHINE kit (Ambion, Austin, TX, USA) 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, Westbury, NY, USA).

Expression of eel aquaporins and transport assays

cRNA (10 ng) for each of the four AQPs was injected into oocytes using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific Co., Broomall, PA, USA). Control oocytes were injected with water alone. 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 quantification of oocyte swelling after placement in hypotonic buffer (67% of normal MBS). Time-lapse video microscopy was used to capture images of oocyte every 10 s for 3 min using an Olympus SZX7 binocular microscope equipped with a Scion CFW 1308C digital camera (1360×1024 pixel resolution) and ImageJ software (http://rsb.info.nih.gov/ij/). A threshold function was applied using ImageJ to convert the images to black and white, where the black portion of the image represented the oocyte cross-sectional area, which was then quantified. Data from ImageJ was exported to Microsoft Excel and areas from each image were normalized relative to a starting value of 1.0. Swelling curve data were fitted to a single exponential equation (three parameter, single exponential rising to maximum) [$f=y0+a(1-\exp^{-bx})$], where b is the derived rate constant used to

describe our results. Since the expression level of AQPs varied from oocyte batch to oocyte batch, cross-sectional area expansion (swelling) varied also. To normalize results from experiment to experiment, we used the curve that exhibited the maximal swelling amplitude i.e. whichever group of AQP-expressing oocytes swelled most on a given day was used as the reference. The value for a obtained from the exponential equation above, is the maximum relative excursion in cross-sectional area from the starting value of 1.0. We obtained a values for the oocytes that exhibited maximum swelling and then defined that value for all other exponential fitting of data obtained that day.

Some investigators have used P_f, the osmotic water permeability coefficient to describe data obtained from oocyte swelling experiments. However, in the absence of any information regarding the relative levels of aquaporin expression from isoform to isoform and the unknown surface density of aquaporins, this parameter has limited value. The rate constants (from which P_f would be derived) accurately reflect the relative water fluxes we actually measured in these experiments.

Solute fluxes were measured by isotopic uptake of [^{3}H]glycerol and [^{14}C]urea (American Radiolabelled Chemicals, St Louis, MO, USA). Oocytes were incubated for varying times in MBS to which either 10 μCi ml^{-1} [^{14}C]urea (55 mCi mmol l^{-1}) and 5 mmol l^{-1} urea; or 10 μCi ml^{-1} [^{3}H]glycerol (20–40 Ci mmol l^{-1}) and 5 mmol l^{-1} glycerol were added (values are final concentration). At the end of the incubation period for uptake (30s, 3 min and 6 min), oocytes were washed six times with ice-cold buffer containing 5 mmol l^{-1} solute. Individual oocytes were then placed in scintillation vials, with 200 μl 20% (w/v) sodium dodecyl sulfate and vortexed for 10–15 s before 4 ml Scintisafe scintillation cocktail was added (Fisher Scientific, Pittsburgh, PA, USA). Vials were counted for 2–4 min in a Packard liquid scintillation analyzer.

Inhibition studies

Aquaporin activity was tested at different pHs ranging from 6.5 to 8.6, and in the presence of divalent metals; mercury, copper, nickel and zinc. For pH inhibition and/or activity experiments, oocytes were placed in MBS titrated with HCl or NaOH to different pHs for 5 min and then were swollen in hypotonic MBS also at the same pH, or they were tested for [^{3}H]glycerol uptake at pH 6.5 as described above. Concerns about bicarbonate–CO_2 equilibria in acidified buffers led us to test the effect of removing $NaHCO_3$ from MBS. This had no effect on pH inhibition profiles leading us to conclude that the relatively low concentration of $NaHCO_3$ was not influencing our results. For mercury inhibition, oocytes were placed in MBS containing 1 mmol l^{-1} $HgCl_2$ for 30 min at room temperature (~22°C) and then swollen in hypotonic MBS. Mercury is not a potent inhibitor of aquaporins and different aquaporins exhibit differential sensitivity (Kuwahara et al., 1997). Therefore, concentrations in the range of 100 μmol l^{-1} to 1 mmol l^{-1} are typically employed (Lazowski et al., 1995). To obtain robust inhibition of the water channels we, like others (Mulders et al., 1997), chose 1 mmol l^{-1} for 30 min. For copper ($CuCl_2$), nickel ($NiCl_2$) and zinc ($ZnSO_4$) experiments, oocytes were preincubated in MBS containing 1 mmol l^{-1} of the divalent metal cation for 2 min and then placed in hypotonic MBS also containing divalent cation at the same respective concentration for swelling assessment. The addition of these metal ions did not alter the pH of the solution.

Statistical analysis

Two-sample Student's t-test with an assumption of equal population variance was used to compare means for statistical significance.

RESULTS

Oocytes injected with cRNAs for eel AQPs all showed elevated swelling kinetics when compared with water-injected controls, upon exposure to hypotonic MBS (Fig. 1). The data from a representative experiment show that oocyte cross-sectional area expanded from 6 to 8% compared with the starting circumference. The mean data fitted well to single exponential (rising) curves, which have been superimposed in Fig. 1. All four AQPs exhibited mercury-inhibitable water transport (Fig. 2). Incubation of oocytes in MBS containing 1 mmol l^{-1} $HgCl_2$ for 30 min at room temperature resulted in statistically significant reductions in the swelling rates of oocytes expressing all four aquaporins. The degree of inhibition for AQP1, AQP1dup, AQP3 and AQPe was 55%, 64%, 54% and 62%, respectively, after water-injected control rates were subtracted.

To examine the sequence-based predictions that the AQP3 and AQPe orthologs belonged to the aquaglyceroporin subclass of AQPs, we conducted urea and glycerol transport assays. Oocytes were exposed to isotopic glycerol or urea in the presence of 5 mmol l^{-1} external solute for varying times and were then rapidly washed in ice-cold MBS, after which internalized radioactivity was quantified. Eel AQP1 and AQP1dup showed low uptakes of each solute which were not different from water-injected controls, whereas eel AQP3 and AQPe were able to transport significantly greater quantities of glycerol and urea in a time-dependent manner (Fig. 3).

Human AQP3 has been reported to be gated by protons (Zeuthen and Klaerke, 1999). We tested whether any of the eel AQPs were sensitive to reduced extracellular pH (Fig. 4). The water transport activity of eel AQP3 was inhibited 91% (after subtraction of control rates) by 5 min exposure to pH 6.5 (Fig. 4A,B), indicating that in many respects the eel and human orthologs have similar functional characteristics. None of the other three AQP isoforms were sensitive to lowered extracellular pH (Fig. 4B). To determine whether low pH would impair the solute-transporting ability of either AQPe or eel AQP3, we conducted [^{3}H]glycerol uptake assays on oocytes incubated at pH 6.5 for 5 min. The combined data from three separate experiments is shown in Fig. 4C and demonstrates that glycerol transport by eel AQP3 is significantly impaired (P<0.001) but that the activity of AQPe is unaffected by low pH. Although these experiments pointed to a potential role for protons in regulating eel AQP3, plasma pH in eels is usually in the range of 7.7–7.9 (Marshall and Grosell, 2006). We therefore tested the pH dependence of water transport by eel AQPs at several pHs above and below 7.8. For eel AQP3, experiments performed on oocytes from two different frogs (Fig. 5), demonstrated that water flux rates were maximal around pH 8.2 with both lower and higher pHs leading to an inhibition of transport in the physiological range. Over the same pH range there was no evidence of any effect on the activities of eel AQP1, AQP1dup or AQPe (data not shown).

Human AQP3 has been shown to be inhibited by metals such as copper and nickel (Zelenina et al., 2003; Zelenina et al., 2004). We were therefore interested to test the biophysical properties of eel AQPs to metals after a brief exposure to a fairly high concentration (1 mmol l^{-1}). Data in the top two panels of Fig. 6 show that metals had stimulatory as well as inhibitory effects on certain AQPs. Most surprisingly, a brief exposure to copper appeared to stimulate the water transport activity of eel AQP1 (Fig. 6, top left). We confirmed that 1 mmol l^{-1} copper had no effect on the swelling kinetics of water-injected control oocytes, therefore eliminating the possibility that copper may have had non-specific effects on membrane permeability (not shown). Conversely, AQP3 exhibited a high degree of sensitivity to and was inhibited by, all of the metal cations tested

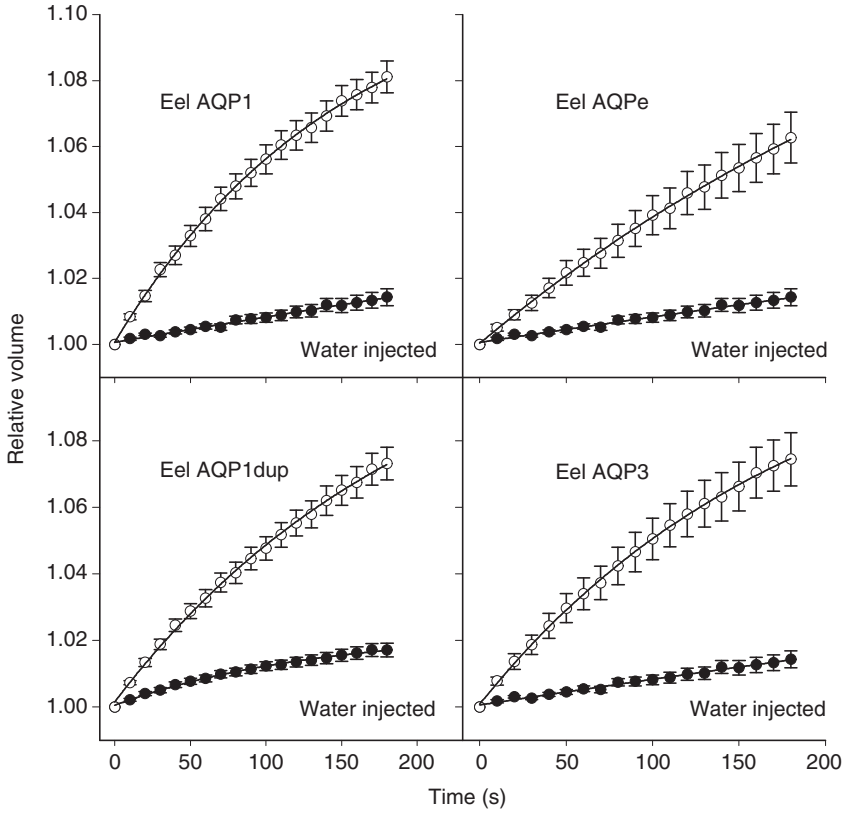

Fig. 1. All four eel aquaporins transport water. Oocytes injected with either 10 ng cRNA or water 3 days earlier, were placed in hypotonic MBS (2:1 MBS:water) on the stage of a low-power binocular microscope at room temperature and digital images were acquired every 10 s. Oocyte cross-sectional area was measured using the images and the data normalized to a relative starting volume of 1.0. Each data point represents the average response of 6–12 individual oocytes (usually 8). Error bars indicate the s.e.m. and the curves have been fitted with a single exponential function, the details of which are given in the Materials and methods. For all AQP-expressing oocyte non-linear regressions R^2=0.999.

(Fig. 6, top right). The combined results from three or four separate experiments with oocytes from different frogs are shown in Fig. 6, bottom panel. These data reveal that nickel and zinc did not significantly affect eel AQP1 function, but that copper appeared to enhance water channel activity by 53.7% (P<0.01) after subtracting control rates. AQP1dup and AQPe were unaffected by exposure to any of the metals, whereas eel AQP3 exhibited potent inhibition by nickel and zinc (88% and 86% inhibition, respectively; P<0.01) and a more modest inhibition by copper (56%; P<0.05).

DISCUSSION

AQPs have now been found in all orders of life from Archaea (Kozono et al., 2003) to eukaryotes (Zardoya and Villalba, 2001) where they play diverse roles and facilitate transport of a number of small molecule substrates. The euryhaline teleosts are a specialized class of vertebrates which are able to live in either freshwater – total osmolality less than 50 mOsm kg^{-1} – or the ocean, with an osmolality of 900–1000 mOsm kg^{-1}. Each environment poses its own unique osmoregulatory challenges, but the ability of animals like the eel to migrate from one to the other and completely alter salt and water handling across the epithelial surfaces of the gill, esophagus, intestine and kidney is remarkable.

The purpose of the experiments presented in this study was to integrate the molecular expression information available for a select group of AQPs from the eel, with functional measurements of their activity. These experiments establish definitively that four putative aquaporin water channels from osmoregulatory tissues of the eel do indeed function as predicted. Based on sequence alignments and homologies it was anticipated that eel AQP1 and AQP1dup would be classic water conducting channels whereas eel AQP3 and AQPe would probably conduct glycerol and urea as well. We have confirmed that all four are able to facilitate water flux in response to an osmotic gradient and that eel AQP3 and AQPe transport not only glycerol, but urea as well. The slow and partial effect of mercury inhibition is consistent with the mode of action of mercury which involves covalent modification of a cysteine within the selectivity filter region of the channel pore, resulting in a steric block (Savage and Stroud, 2007). AQP1dup showed consistently lower transport rates than the others. This suggests it may have an intrinsically lower

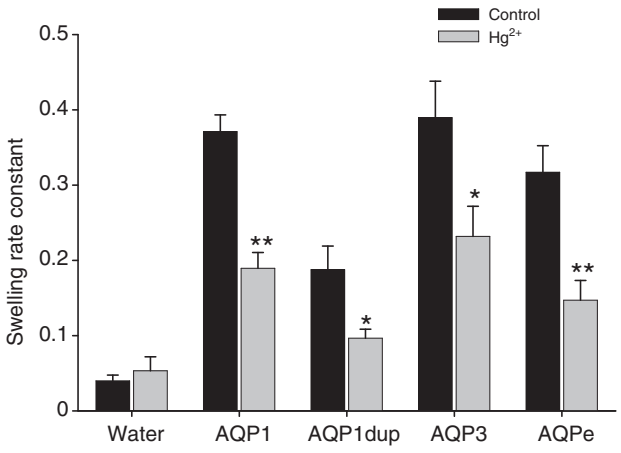

Fig. 2. All eel aquaporins are inhibitable by mercury. Oocytes expressing aquaporins or injected with water were exposed to 1 mmol l^{-1} $HgCl_2$ for 30 min and then tested for their swelling kinetics in hypotonic MBS. The swelling rate represents the rate constant derived from the single exponential fitted to the data as described in the Materials and methods. Values are means of three separate experiments, in which an average rate constant from the swelling of 8–12 oocytes was calculated. Error bars indicate the s.e.m. *P<0.05, **P<0.01

permeability to water and further suggests that the two aquaglyceroporins may have surprisingly good water transporting capabilities. This would be in contrast to human aquaglyceroporins such as AQP3 which transports glycerol efficiently, but has relatively low water permeability when compared with purely water-conducting channels such as AQP1 and AQP4 (Yang and Verkman, 1997). Interestingly, AQP1dup lacks a putative *N*-linked glycosylation site present in AQP1 (Martinez et al., 2005b), which might negatively impact its function.

AQP1dup is expressed at high levels in the kidney and within the esophagus (Martinez et al., 2005b). AQP1 is only seen sporadically in the kidney and usually at low levels, so the duplicated isoform is the predominant water channel of the kidney. Expression levels of both AQP1dup and AQP1 have been shown to diminish in the kidney upon SW acclimation (Martinez et al., 2005b), consistent with a role for both AQP1 and AQP1dup in fluid secretion, which is known to occur in many teleost fish species in the proximal tubule (Beyenbach, 2004).

AQP1 is found in many of the same tissues in eel as in humans. These include the eye, gill (equivalent in some ways to respiratory tract), gastrointestinal (GI) tract and brain. The one notable exception is its low level of expression in kidney. It is possible that upon genome duplication, expression of AQP1 was reduced or lost since AQP1dup was available to compensate.

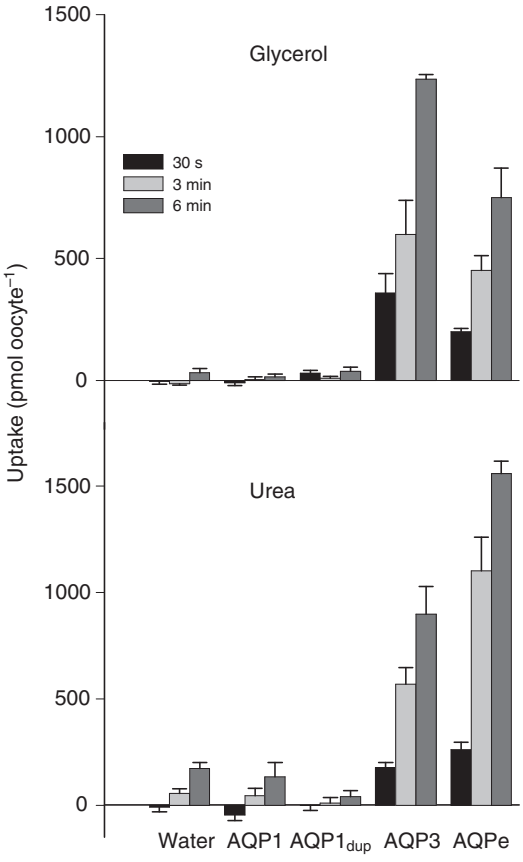

Fig. 3. AQP3 and AQPe are aquaglyceroporins. Time-dependent uptake of [^{14}C]urea and [^{3}H]glycerol was measured in oocytes expressing eel aquaporins. Oocytes were placed in solutions containing 5 mmol l^{-1} solute supplemented with 10 μCi ml^{-1} radioisotope for varying times and were then washed in ice-cold MBS, lysed and the radioactivity counted. Data shown are mean of eight oocytes ± s.e.m. The experiment was performed three times with similar results each time.

AQPe and eel AQP3 are now defined functionally as being members of the aquaglyceroporin family. The role of this class of AQPs has remained unclear, although in several instances studies on knockout mice have revealed interesting and unexpected roles for these channels. In skin for example, AQP3 is expressed within the basal keratinocytes and has now been shown to be important for epidermal hydration by virtue of its glycerol transporting properties (Verkman, 2005). Another mammalian aquaglyceroporin, AQP7 is now known to be important for triglyceride storage in adipocytes (Verkman, 2005). In the eel it would not be surprising – given the usual location of these channels on the basolateral membranes of epithelia – if they were important for osmoprotection of epithelia by virtue of their solute transporting ability. AQPe is expressed in the intestine and the kidney; however, in the kidney it was found at one-tenth of the levels of AQP1-dup. Upon shifting eels from FW to SW there is little change in AQPe levels in the

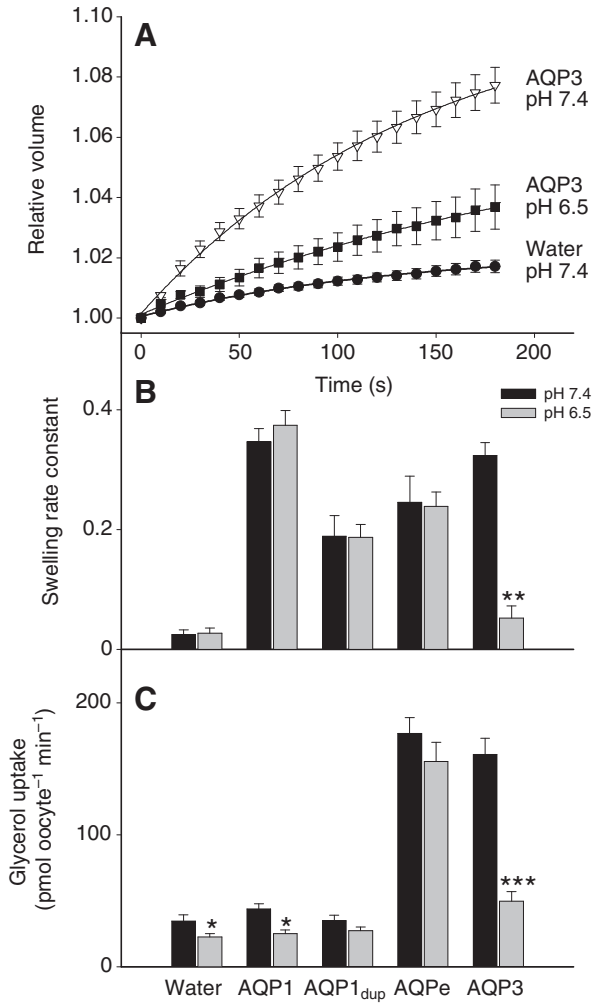

Fig. 4. AQP3 is pH sensitive. Oocytes expressing eel aquaporins were incubated in MBS at either pH 7.4 or pH 6.5 for 5 min and then placed in hypotonic MBS (of the same pH) or tested for uptake of isotopic glycerol. (A) Swelling curves for AQP3 at pH 7.4 and pH 6.5 compared with water-injected controls (6–12 oocytes; values are means ± s.e.m.). (B) Combined results from three experiments showing that AQP3 is 91% inhibited at low pH whereas the other AQPs are unaffected. (C) [^{3}H]Glycerol uptake at two different pHs. AQPe and eAQP3 both transport glycerol at pH 7.4 but eAQP3 is inhibited by 69% at pH 6.5 (20–25 oocytes from three experiments). **P*<0.05, ****P*<0.001.

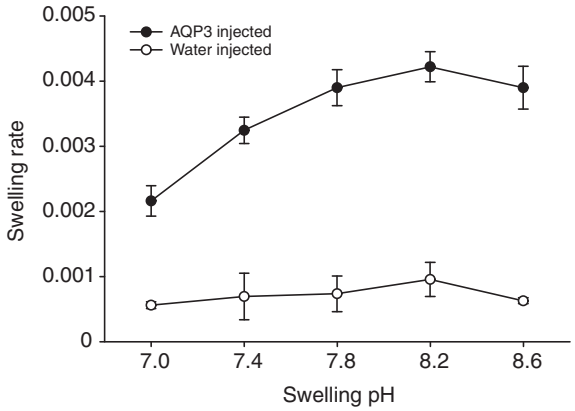

Fig. 5. Dependence of AQP3 activity on pH. Oocytes expressing AQP3 or injected with water were exposed for 5 min to MBS titrated with NaOH to different pHs, and the oocytes were then subjected to diluted buffer of the same pH. Swelling rates were calculated as described in the Materials and methods from the mean swelling curve of 4–8 oocytes (in each experiment). Three experiments were performed using oocytes from two different frogs. Values are means ± s.e.m.

gut, unlike AQP1. It appears, therefore, not to play a role in salinity adaptation. Of interest was the finding that despite similar solute transport profiles, AQPe was not regulated by extracellular pH whereas AQP3 was. Maximal activity of AQP3 occurs close to the normal blood pH of the eel and with inhibition at pHs lower than 8.0 (Fig. 5), it is tempting to speculate that plasma pH may be an important regulator of AQP3 and could possibly fine tune its activity. Since plasma pH in the eel is between 7.7 and 7.9, subtle shifts in plasma pH equilibrium would be expected to impact on water and solute transport. Although SW is well buffered and varies little in pH, temperature has been shown to influence blood pH in teleosts and elasmobranchs. For example a typical plasma pH at 10°C is 7.95, whereas at 20°C pH decreases to 7.8 (Marshall and Grosell, 2006). Perhaps more relevant for eels living in relatively isothermal conditions, plasma pH varies with both activity [aerobic and anaerobic metabolism causing acidosis (Kieffer et al., 1994; Richards et al., 2003)] and digestion, which leads to postprandial alkalosis (Bucking and Wood, 2008; Cooper and Wilson, 2008).

Both water and glycerol transport were shown to be inhibited at low pH (Fig. 4B,C) and therefore we can also conclude that there are important differences in the pore structures of AQPe and eel AQP3 which allow differential regulation to occur. In this experiment it was noted that there was a small, but significant reduction in isotopic counts at low pH for water-injected controls and AQP1-expressing oocytes (Fig. 4C). This is likely to be due to non-specific effects of low pH on the membrane surface (e.g. charge).

Finally, we show that eel AQP3, like its human counterpart, can be inhibited by several metal ions (Zelenina et al., 2003; Zelenina et al., 2004) and that AQP1 in the eel is uniquely stimulated by copper ions. That the swelling rates of water-injected controls were not affected by any of the treatments indicates that the stimulatory and inhibitory activities were mediated by direct effects of metals on the AQP channels themselves.

Human AQP3 has been shown to be inhibited by very low levels of copper (Cu^{2+}, 25 μM in bicarbonate buffer) and at higher concentrations by nickel (Ni^{2+}, 1 mmol l^{-1}), but not at all by zinc (Zn^{2+}) (Zelenina et al., 2003; Zelenina et al., 2004). We were therefore interested to know if eel AQP3 would exhibit a similar inhibitory profile and whether any of the other eel AQPs might be

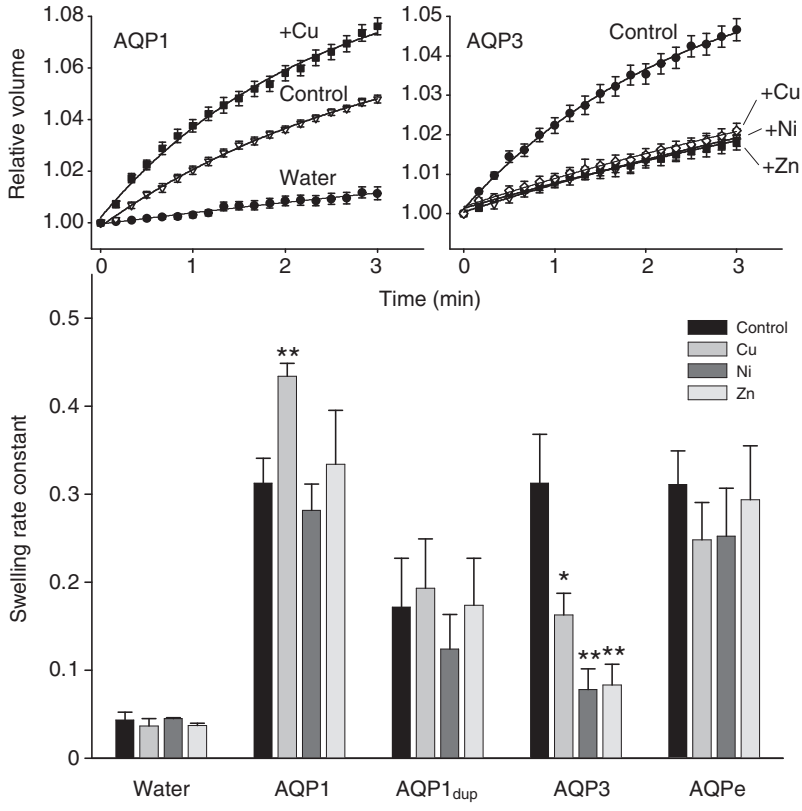

Fig. 6. AQP1 is stimulated by copper whereas AQP3 is inhibited by zinc, nickel and copper. Oocytes expressing eel aquaporins were incubated in MBS containing 1 mmol l^{-1} metal ion for 2 min and then placed in hypotonic MBS also containing 1 mmol l^{-1} of the metal ion, for swelling assays. (Top left) Effect of copper on AQP1-expressing oocytes. The curve labeled 'Control' shows the swelling rate of AQP1-expressing oocytes in the absence of copper; that labeled 'Water' shows the swelling rate of water-injected oocytes. (Top right) Swelling curves for AQP3-expressing oocytes exposed to metals. Each curve represents the average of eight oocytes. (Bottom panel) Combined data from three or four experiments demonstrating that copper appears to have a stimulatory effect on AQP1 and that AQP3 is inhibited by all three metals tested. *P<0.05, **P<0.01.

sensitive to heavy metals. The interest in these modulators of AQP channel activity is twofold. First, identification of additional AQP inhibitors would improve our ability to determine the physiological function of AQP3 *in vivo*. Aquaporin research has been hindered by the lack of high potency, high specificity blockers. Mercury is still the inhibitor of choice despite its lack of both specificity and potency. Second, there is considerable interest because of the toxicological effect of metal ions on fish and the presence of various metals as major pollutants in aquatic environments. Although it is clear the present studies have employed pharmacological doses rather than the markedly lower environmental levels likely to be encountered by the eel, several points are worth noting. Firstly, our protocol used high metal concentrations but only an acute exposure (2 min). The data are intended to shed light on the biophysical properties of these four proteins. However, the possibility exists that long-term exposure of the eel to low levels of heavy metals could result in cumulative effects on susceptible channels. Furthermore, drinking of seawater for osmoregulation in marine fish creates a chronic exposure scenario for esophageal and intestinal epithelia (Zhang and Wang, 2007). If there are transport pathways within the digestive tract for uptake, accumulation and concentration of these cations, then blood and tissue levels could become higher than the nominal environmental concentrations encountered, although the pharmacological availability for modulating channel activity is unclear because of the likelihood of cations being largely complexed by organic molecules.

Studies show that exposure to copper, zinc or nickel ions can all have effects on osmoregulation in fish (Lewis and Lewis, 1971; Denton and Burdon-Jones, 1986; Evans, 1987; Terreros et al., 1988; Grosell et al., 2007; Zhang and Wang, 2007; Pandey et al., 2008). The effect of copper on osmoregulation and nitrogen metabolism in fish are consistent with either inhibition of AQP3 or stimulation of AQP1. Copper has been shown to affect plasma sodium levels in both freshwater and seawater (Lauren and McDonald, 1985; Grosell et al., 2002; Blanchard and Grosell, 2006; Grosell et al., 2007). The effect on plasma sodium levels may result from inhibition of branchial Na^+/K^+-ATPase although it has also been suggested it may be due to an increase in gill permeability by an unknown mechanism (Beyenbach, 2004). If AQP3 inhibition was involved in a change in gill permeability (AQP3 expression is much higher in FW), the effect of copper on plasma sodium would be, and is, greater in FW than in SW (Beyenbach, 2004; Blanchard and Grosell, 2006). As well as changes in branchial osmoregulatory parameters, copper also affects gill nitrogen excretion in both FW and SW (Lauren and McDonald, 1985; Grosell et al., 2004; Blanchard and Grosell, 2006). Plasma ammonia increases with copper exposure and branchial ammonia effluxes are decreased (Lauren and McDonald, 1985; Grosell et al., 2004; Blanchard and Grosell, 2006). Similarly plasma urea is also elevated following copper exposure in the toadfish (*Opsanus beta*) (Grosell et al., 2004). If AQP3 was involved in any way in urea or ammonia excretion across gill, inhibition of the channel by copper might be expected to elevate plasma levels of these nitrogenous waste products.

The effects of copper on fish described above, are largely consistent with what would be expected if AQP3 was involved in branchial permeability and/or fluid transport and nitrogenous waste transport, however, because AQP3 is also inhibited by zinc and nickel ions, it would expected that these metals would also have similar effects. At present there is only limited evidence that supports this idea. Zinc ions have been shown to inhibit branchial Na^+/K^+-ATPase activity and may cause changes in plasma NaCl and gill permeability (Lewis and Lewis, 1971; Evans, 1987), however, the main effect of zinc exposure appears to be on calcium homeostasis (Hogstrand et al., 1998; Niyogi and Wood, 2006).

Limited data are available on the effect of nickel ion exposure on fish, but the studies done so far suggest that nickel impacts plasma calcium levels, causes changes in the balance of sodium and magnesium ions in intestinal fluids and affects the renal handling of magnesium (Pane et al., 2005; Pane et al., 2006). Other plasma ion levels and nitrogen excretion were unaffected following nickel exposure (Pane et al., 2006).

The present studies establish the transport selectivities of four eel aquaporins and the biophysical effects of pH and certain metals on their activity. Further *in vivo* studies, will be required to determine whether exposure of AQPs to these metals at the low concentrations found in the environment results in adverse physiological consequences for the eel.

LIST OF ABBREVIATIONS

AQP	aquaporin
BSA	bovine serum albumin
FW	fresh water
MBS	modified Barth's solution
SW	sea water

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REFERENCES

Ando, M., Mukuda, T. and Kozaka, T. (2003). Water metabolism in the eel acclimated to sea water: from mouth to intestine. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **136**, 621-633.
Beyenbach, K. W. (2004). Kidneys sans glomeruli. *Am. J. Physiol. Renal. Physiol.* **286**, F811-F827.
Blanchard, J. and Grosell, M. (2006). Copper toxicity across salinities from freshwater to seawater in the euryhaline fish *Fundulus heteroclitus*: is copper an ionoregulatory toxicant in high salinities? *Aquat. Toxicol.* **80**, 131-139.
Bucking, C. and Wood, C. M. (2008). The alkaline tide and ammonia excretion after voluntary feeding in freshwater rainbow trout. *J. Exp. Biol.* **211**, 2533-2541.
Calamita, G. (2000). The *Escherichia coli* aquaporin-Z water channel. *Mol. Microbiol.* **37**, 254-262.
Cooper, C. A. and Wilson, R. W. (2008). Post-prandial alkaline tide in freshwater rainbow trout: effects of meal anticipation on recovery from acid-base and ion regulatory disturbances. *J. Exp. Biol.* **211**, 2542-2550.
Cutler, C. P. and Cramb, G. (2000). Water transport and aquaporin expression in fish. In *Molecular Biology and Physiology of Water and Solute Transport* (ed. S. Hohmann and S. Nielsen), pp. 431-444. London: Kluwer Academic Press.
Cutler, C. P. and Cramb, G. (2002). Branchial expression of an aquaporin 3 (AQP-3) homologue is downregulated in the European eel *Anguilla anguilla* following seawater acclimation. *J. Exp. Biol.* **205**, 2643-2651.
Cutler, C. P., Martinez, A. S. and Cramb, G. (2007a). The role of aquaporin 3 in teleost fish. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 82-91.
Cutler, C. P., Phillips, C., Hazon, N. and Cramb, G. (2007b). Cortisol regulates eel (*Anguilla anguilla*) aquaporin 3 (AQP3) mRNA expression levels in gill. *Gen. Comp. Endocrinol.* **152**, 310-313.
Dean, D. B., Whitlow, Z. W. and Borski, R. J. (2003). Glucocorticoid receptor upregulation during seawater adaptation in a euryhaline teleost, the tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* **132**, 112-118.
Denton, G. R. W. and Burdon-Jones, C. (1986). Environmental effects on toxicity of heavy metals to two species of tropical marine fish from Northern Australia. *Chem. Ecol.* **2**, 233-249.
Engin, K. and Carter, C. G. (2001). Ammonia and urea excretion rates of juvenile Australian short-finned eel (*Anguilla australis australis*) as influenced by dietary protein level. *Aquaculture* **194**, 123-136.
Evans, D. H. (1987). The fish gill: site of action and model for toxic effects of environmental pollutants. *Environ. Health Perspect.* **71**, 47-58.
Evans, D. H., Piermarini, P. M. and Choe, K. P. (2005). The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. *Physiol. Rev.* **85**, 97-177.
Froger, A., Rolland, J. P., Bron, P., Lagree, V., Le Caherec, F., Deschamps, S., Hubert, J. F., Pellerin, I., Thomas, D. and Delamarche, C. (2001). Functional characterization of a microbial aquaglyceroporin. *Microbiology* **147**, 1129-1135.
Grosell, M., Nielsen, C. and Bianchini, A. (2002). Sodium turnover rate determines sensitivity to acute copper and silver exposure in freshwater animals. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **133**, 287-303.
Grosell, M., McDonald, M. D., Walsh, P. J. and Wood, C. M. (2004). Effects of prolonged copper exposure in the marine gulf toadfish (*Opsanus beta*) II: copper

accumulation, drinking rate and Na^+/K^+-ATPase activity in osmoregulatory tissues. *Aquat. Toxicol.* **68**, 263-275.
Grosell, M., Blanchard, J., Brix, K. V. and Gerdes, R. (2007). Physiology is pivotal for interactions between salinity and acute copper toxicity to fish and invertebrates. *Aquat. Toxicol.* **84**, 162-172.
Hogstrand, C., Webb, N. and Wood, C. M. (1998). Covariation in regulation of affinity for branchial zinc and calcium uptake in freshwater rainbow trout. *J. Exp. Biol.* **201**, 1809-1815.
Hwang, P. P. and Lee, T. H. (2007). New insights into fish ion regulation and mitochondrion-rich cells. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **148**, 479-497.
Kieffer, J., Currie, S. and Tufts, B. (1994). Effects of environmental temperature on the metabolic and acid-base responses of rainbow trout to exhaustive exercise. *J. Exp. Biol.* **194**, 299-317.
King, L. S., Kozono, D. and Agre, P. (2004). From structure to disease: the evolving tale of aquaporin biology. *Nat. Rev. Mol. Cell Biol.* **5**, 687-698.
Kozono, D., Ding, X., Iwasaki, I., Meng, X., Kamagata, Y., Agre, P. and Kitagawa, Y. (2003). Functional expression and characterization of an archaeal aquaporin. AqpM from methanothermobacter marburgensis. *J. Biol. Chem.* **278**, 10649-10656.
Kuwahara, M., Gu, Y., Ishibashi, K., Marumo, F. and Sasaki, S. (1997). Mercury-sensitive residues and pore site in AQP3 water channel. *Biochemistry* **36**, 13973-13978.
Lauren, D. J. and McDonald, M. D. (1985). Effects of copper on branchial ionoregulation in the rainbow trout, *Salmo gairdneri* Richardson. *J. Comp. Physiol.* **155**, 635-644.
Lazowski, K. W., Li, J., Delporte, C. and Baum, B. J. (1995). Evidence for the presence of a Hg-inhibitable water-permeability pathway and aquaporin 1 in A5 salivary epithelial cells. *J. Cell Physiol.* **164**, 613-619.
Lee, J. K., Kozono, D., Remis, J., Kitagawa, Y., Agre, P. and Stroud, R. M. (2005). Structural basis for conductance by the archaeal aquaporin AqpM at 1.68 A. *Proc. Natl. Acad. Sci. USA* **102**, 18932-18937.
Lewis, S. D. and Lewis, W. M. (1971). The effect of zinc and copper on the osmolality of blood serum of the channel catfish, *Ictalurus punctatus* Rafinesque, and Golden Shiner, *Notemigonus crysoleucas* Mitchill. *Trans. Am. Fish. Soc.* **100**, 639-643.
Lignot, J. H., Cutler, C. P., Hazon, N. and Cramb, G. (2002). Immunolocalisation of aquaporin 3 in the gill and the gastrointestinal tract of the European eel *Anguilla anguilla* (L.). *J. Exp. Biol.* **205**, 2653-2663.
Marshall, W. S. and Grosell, M. (2006). Ion transport, osmoregulation and acid-base balance. In *The Physiology of Fishes* (ed. D. H. Evans and J. B. Claiborne), pp. 177-230. Boca Raton, FL: CRC Press.
Martinez, A. S., Cutler, C. P., Wilson, G. D., Phillips, C., Hazon, N. and Cramb, G. (2005a). Regulation of expression of two aquaporin homologs in the intestine of the European eel: effects of seawater acclimation and cortisol treatment. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **288**, R1733-R1743.
Martinez, A. S., Cutler, C. P., Wilson, G. D., Phillips, C., Hazon, N. and Cramb, G. (2005b). Cloning and expression of three aquaporin homologues from the European eel (*Anguilla anguilla*): effects of seawater acclimation and cortisol treatment on renal expression. *Biol. Cell* **97**, 615-627.
Masoni, A. and Payan, P. (1974). Urea, inulin and para-amino-hippuric acid (PAH) excretion by the gills of the eel, *Anguilla anguilla* L. *Comp. Biochem. Physiol.* **47A**, 1241-1244.
Maurel, C., Verdoucq, L., Luu, D. T. and Santoni, V. (2008). Plant aquaporins: membrane channels with multiple integrated functions. *Annu. Rev. Plant Biol.* **59**, 595-624.
Mulders, S. M., Rijss, J. P., Hartog, A., Bindels, R. J., van Os, C. H. and Deen, P. M. (1997). Importance of the mercury-sensitive cysteine on function and routing of AQP1 and AQP2 in oocytes. *Am. J. Physiol.* **273**, F451-F456.
Niyogi, S. and Wood, C. M. (2006). Interaction between dietary calcium supplementation and chronic waterborne zinc exposure in juvenile rainbow trout, *Oncorhynchus mykiss*. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* **143**, 94-102.
Pandey, S., Parvez, S., Ansari, R. A., Ali, M., Kaur, M., Hayat, F., Ahmad, F. and Raisuddin, S. (2008). Effects of exposure to multiple trace metals on biochemical, histological and ultrastructural features of gills of a freshwater fish, *Channa punctata* Bloch. *Chem. Biol. Interact.* **174**, 183-192.
Pane, E. F., Bucking, C., Patel, M. and Wood, C. M. (2005). Renal function in the freshwater rainbow trout (*Oncorhynchus mykiss*) following acute and prolonged exposure to waterborne nickel. *Aquat. Toxicol.* **72**, 119-133.
Pane, E. F., McDonald, M. D., Curry, H. N., Blanchard, J., Wood, C. M. and Grosell, M. (2006). Hydromineral balance in the marine gulf toadfish (*Opsanus beta*) exposed to waterborne or infused nickel. *Aquat. Toxicol.* **80**, 70-81.
Richards, J. G., Heigenhauser, G. J. and Wood, C. M. (2003). Exercise and recovery metabolism in the Pacific spiny dogfish (*Squalus acanthias*). *J. Comp. Physiol. B* **173**, 463-474.
Savage, D. F. and Stroud, R. M. (2007). Structural basis of aquaporin inhibition by mercury. *J. Mol. Biol.* **368**, 607-617.
Terreros, D. A., Knight, J. A. and Ashwood, E. R. (1988). Nickel inhibition of the osmotic-sensitive ionic cellular channels. *Ann. Clin. Lab. Sci.* **18**, 444-450.
Verkman, A. S. (2005). More than just water channels: unexpected cellular roles of aquaporins. *J. Cell Sci.* **118**, 3225-3232.
Yang, B. and Verkman, A. S. (1997). Water and glycerol permeabilities of aquaporins 1-5 and MIP determined quantitatively by expression of epitope-tagged constructs in Xenopus oocytes. *J. Biol. Chem.* **272**, 16140-16146.
Zardoya, R. and Villalba, S. (2001). A phylogenetic framework for the aquaporin family in eukaryotes. *J. Mol. Evol.* **52**, 391-404.
Zelenina, M., Bondar, A. A., Zelenin, S. and Aperia, A. (2003). Nickel and extracellular acidification inhibit the water permeability of human aquaporin-3 in lung epithelial cells. *J. Biol. Chem.* **278**, 30037-30043.
Zelenina, M., Tritto, S., Bondar, A. A., Zelenin, S. and Aperia, A. (2004). Copper inhibits the water and glycerol permeability of aquaporin-3. *J. Biol. Chem.* **279**, 51939-51943.
Zeuthen, T. and Klaerke, D. A. (1999). Transport of water and glycerol in aquaporin 3 is gated by H(+). *J. Biol. Chem.* **274**, 21631-21636.
Zhang, L. and Wang, W. X. (2007). Gastrointestinal uptake of cadmium and zinc by a marine teleost *Acanthopagrus schlegeli*. *Aquat. Toxicol.* **85**, 143-153.