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Research article

Cloning and expression of three aquaporin homologues from the European eel (*Anguilla anguilla*): effects of seawater acclimation and cortisol treatment on renal expression

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Background information. The European eel (*Anguilla anguilla*) is able to osmoregulate over a wide range of environmental salinities from FW (freshwater) to hyperconcentrated SW (seawater). Successful acclimation is associated with strict regulation of ion and water transport pathways within key osmoregulatory epithelia to enable animals to survive the dehydrating or oedematous conditions. These observations suggested that homologues of the AQP (aquaporin) water channel family were expressed in the eel and that these proteins may contribute to the water transport and osmoregulation in all euryhaline teleosts.

Results. Complementary DNAs encoding a homologue of the mammalian aquaglyceroporins (termed AQPe) and two homologues of mammalian aquaporin-1 [termed AQP1 and AQP1dup (aquaporin-1 duplicate)] were isolated from the European eel. Northern-blot analysis revealed (i) two AQP1 transcripts exhibiting a wide tissue distribution, (ii) a single AQP1dup mRNA transcript found in the kidney and the oesophagus, and (iii) a single AQPe mRNA detectable mainly in the kidney and the intestine. The relative expression of isoforms within the kidney was AQP1dup > AQPe > AQP1. SW acclimation significantly reduced the abundance of AQP1, AQP1dup and AQPe transcripts in the kidney of yellow eels by approx. 72, 66 and 34% respectively, whereas the expression levels in silver eels were independent of salinity and equivalent to those observed in yellow SW-acclimated fish. AQP1 protein expression was primarily located within the vascular endothelium in yellow eels and the epithelial apical brush border in some renal tubules in silver eels. Infusion of cortisol into FW eels had no effect on AQPe mRNA expression, but induced significant decreases in AQP1 and AQP1dup mRNA levels in the kidney of yellow eels. Cortisol infusion had no effect on the expression of any isoform in the silver eels.

Conclusions. These results suggest that SW-acclimation or cortisol infusion induces a down-regulation of renal AQP expression in yellow eels. However, the lower levels of aquaporin expression found within the silver eel kidney were not further reduced by salinity transfer or steroid infusion. These differences in mRNA expression were accompanied by changes in the cellular distribution of the AQP1 protein between vascular endothelial and tubular epithelial cells.

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Key words: aquaporin water channel, corticosteroid, kidney, immunofluorescence, osmoregulation.
Abbreviations used: AP, adapter primer; AQP, aquaporin; AQP1dup, AQP1 duplicate; FW, freshwater; MDR, multidrug resistance; ORF, open reading frame; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SW, seawater; UTR, untranslated region.

Introduction

The kidney plays a major osmoregulatory role in euryhaline teleost fish such as the European eel as it helps in maintaining body fluid homeostasis during the

The nucleotide sequence data for the aquaporin isoforms of the European eel will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AJ564420, AJ564421 and AJ784153.

course of adaptation to different salinities (Cleveland and Trump, 1969; Karnaky, 1998). The kidney of the European eel (*Anguilla anguilla*) is fully glomerular and functions like any other euryhaline fish (Cleveland and Trump, 1969; Hentschel and Elger, 1989). In SW (seawater), the euryhaline fish kidney filters plasma at low rates to conserve water, and tubular secretion of electrolytes and fluid contribute significantly to urine formation. Although SW fish do not normally produce urine that is significantly hypertonic to plasma, the kidney's osmoregulatory role in SW is nonetheless essential, serving primarily as the main secretory route for absorbed Mg^{2+}, Ca^{2+} and SO_4^{2-} (Cleveland and Trump, 1969; Bone et al., 1995; Karnaky, 1998). In FW (freshwater) fish, the kidney filters at high rates and reabsorbs nearly all filtered solutes, thereby producing large volumes of dilute urine. In this way, the osmotic water loads of the FW habitat are excreted. The kidney therefore has an essential role in osmoregulation in both stenohaline FW and SW fishes, as well as euryhaline teleosts, such as European eel (Cleveland and Trump, 1969; Bone et al., 1995; Karnaky, 1998).

Although renal salt and water transport are central to the maintenance of body fluid homeostasis in euryhaline fish, especially during the course of adaptation to different salinities, there is currently a lack of information on the transporters involved and the mechanisms by which ions and water are transported across renal and extrarenal epithelia. AQPs (aquaporins) are a family of related membrane proteins, which have been identified in mammals to function as water channels that are responsible for water balance within cells, tissues and various osmoregulatory organs controlling body fluid homeostasis (Borgnia et al., 1999; Nielsen et al., 2002). AQPs have also been described in teleost fish implicating a role for these water transporters in the adaptation of euryhaline teleost species to both FW and marine environments (Borgnia et al., 1999; Lignot et al., 2002). To date, 13 AQPs have been identified in mammals (Ishibashi et al., 2000; Hatakeyama et al., 2001) and seven have been reported so far in teleost fish (Cutler and Cramb, 2001; Virkki et al., 2001; Aoki et al., 2003; Hirata et al., 2003; Santos et al., 2004). Recent work in our laboratory has resulted in the cloning of a number of isoforms from the European eel (*A. anguilla*) (Cutler and Cramb, 2000), including a homologue of AQP3 that exhibits high levels of expression within the branchial epithelium (Cutler and Cramb, 2001; Lignot et al., 2002).

Cortisol is the major corticosteroid found in euryhaline teleost fish, with release from the interrenal gland being stimulated as fish are transferred into SW (Hazon and Balment, 1998). Cortisol is often referred to as a 'SW-adapting' hormone, as it is heavily implicated in the ability of fish to maintain water and electrolyte balance when in the SW environment (Mommsen et al., 1999). The action of this hormone has been reported to include improved water absorptive capacity in both the intestine and the urinary bladder, and increased Na^+, K^+-ATPase activity in the gill, which together enable the fish to hypoosmoregulate in the hypersaline environment (Jobling, 1995). As part of our investigations into the mechanisms of water transport within euryhaline teleosts, we report the cloning of three AQP isoforms from the European eel [termed AQPe (a homologue of mammalian aquaglyceroporin), AQP1 and AQP1dup (aquaporin-1 duplicate)] that are homologues of known mammalian AQPs and expressed in the kidney. In addition, we have examined the effects of SW acclimation and cortisol treatment on the expression of these isoforms within the kidney at two different life stages of the eel. Experiments were conducted using both sexually immature 'yellow' eels that are indigenous to the FW waterways throughout Europe, and sexually maturing, 'silver' eels that have undergone a series of morphological and physiological changes before migration to SW in order to breed.

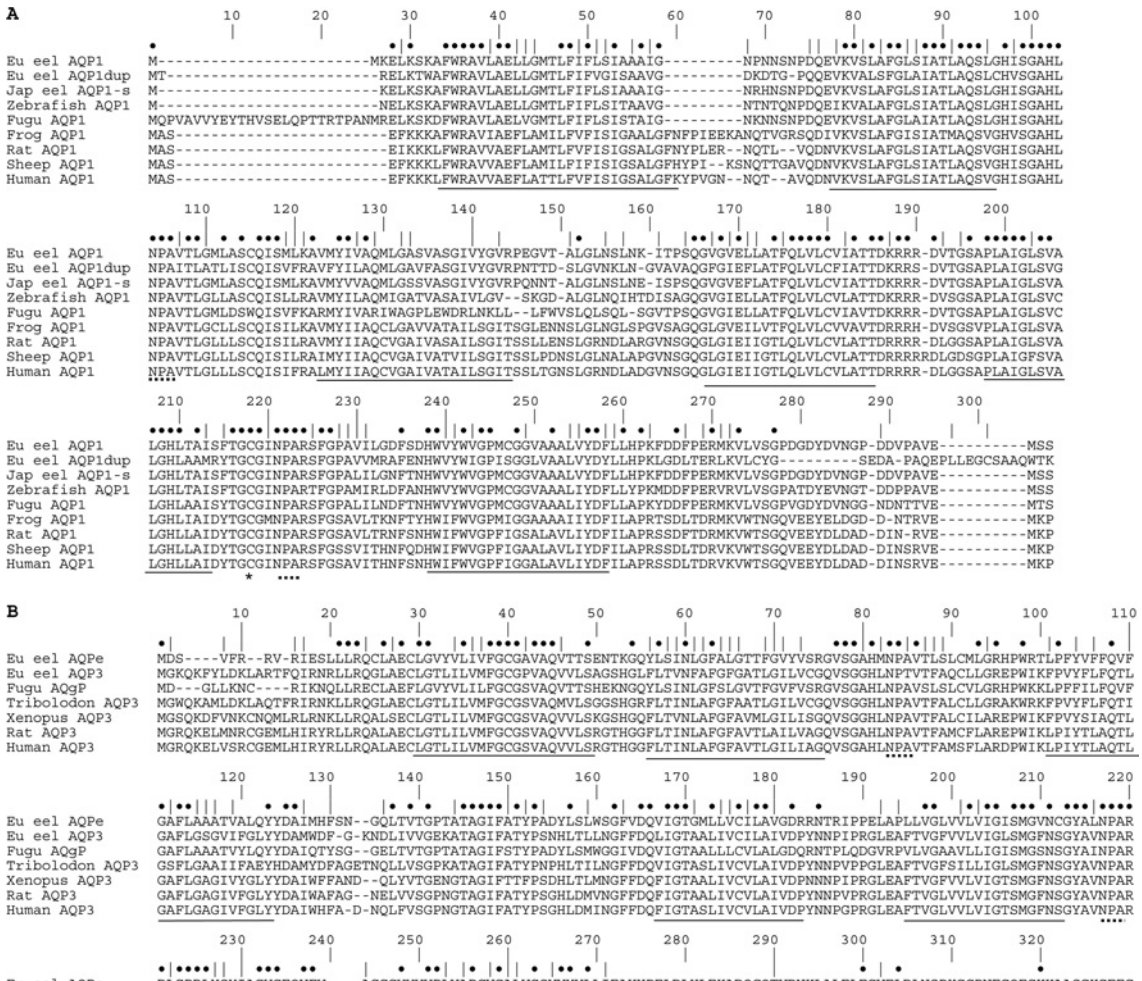
Results

Nucleotide and amino acid sequences of eel AQP1, AQP1dup and AQPe (Figure 1)

The eel AQP1 cDNA sequence comprises 1189 bp containing 24 bp of a 5′-UTR (untranslated region), 789 bp of an ORF (open reading frame) and 376 bp of 3′-UTR; the eel AQP1dup isoform cDNA sequence comprises 1066 bp containing 137 bp of a 5′-UTR, 789 bp of an ORF and 140 bp of a 3′-UTR; the eel AQPe cDNA comprises 1377 bp containing 79 bp of a 5′-UTR, 954 bp of an ORF and 344 bp of a 3′-UTR. The AQP1, AQP1dup and AQPe cDNAs encode proteins with 263, 263 and 318 amino acids respectively (Figures 1A and 1B) with calculated molecular

Figure 1 | Alignment of AQP1, AQP1dup (A) and AQPe (B) derived amino acid sequences from the European (Eu) eel in comparison with those from other species

Symbols in the alignment are as follows: •, conserved amino acid; |, amino acid with conserved similarity; ------, spaces introduced to give an optimal alignment; ••••, NPA motif; *, potential mercury-binding site; underlining indicates the approximate locations of membrane-spanning domains (TM 1–6; Kyte and Dolittle, 1982; Borgnia et al., 1999). Sequences were aligned using GeneJockey II software (Biosoft). The EMBL/Swiss-Prot accession numbers for sequences of various species are given in parentheses: Eu eel AQP (AJ564420), Japanese (Jap) eel AQP1-s (AB094502), Eu eel AQP1dup (AJ564421), zebrafish AQP1 (BQ783573), fugu AQP1 (http://genome.jgi-psf.org/fugu6), frog AQP1 (AB073315), rat AQP1 (NM012778), sheep AQP1 (AF009037), human AQP1 (BC022486), Eu eel AQP3 (AJ319533), Eu eel AQPe (AJ784153), fugu aquaglyceroporin (AQgP) (http://genome.jgi-psf.org/fugu6), tribolodon AQP3 (AB055465), xenopus AQP3 (AJ131847), rat AQP3 (NM031703) and human AQP3 (BC013566).



masses of 27.5, 27.8 and 34.1 kDa. The eel AQP1 protein shared highest levels of amino acid identity with the Japanese eel (94%), the zebrafish (76%) and the fugu (70%) AQP1 proteins, with lower identity to AQP1s from the higher mammals (between 56 and 58%). The eel AQP1 amino acid sequence exhibited 69% identity to a second AQP sequence cloned from the eel kidney, called AQP1dup

due to regions of relatively high homology (Figure 1A). The eel AQP1dup protein shared highest amino acid identity with the Japanese eel (*Anguilla japonica*) (61%), the zebrafish (61%) and the fugu (52%) AQP1s, with lower identity to AQP1s from the higher mammals (between 50 and 51%) (Figure 1A). The eel AQPe protein shared highest amino acid identity with a fugu aquaglyceroporin (61%) and to a lesser extent with mammalian (45–46%) and eel AQP3s (44%) (Figure 1B). A number of structural features typical of AQP water channels are present within the eel isoforms. The two channel-forming NPA motifs, six putative hydrophobic membrane-spanning domains with five hydrophilic intra- and extracellular loops and the retention of a cysteine residue at a putative mercury-inhibitory site present in a subgroup of mammalian AQPs (and in eel AQP1 and AQP1dup) are common features distributed throughout the eel AQP isoform protein sequences (Figure 1).

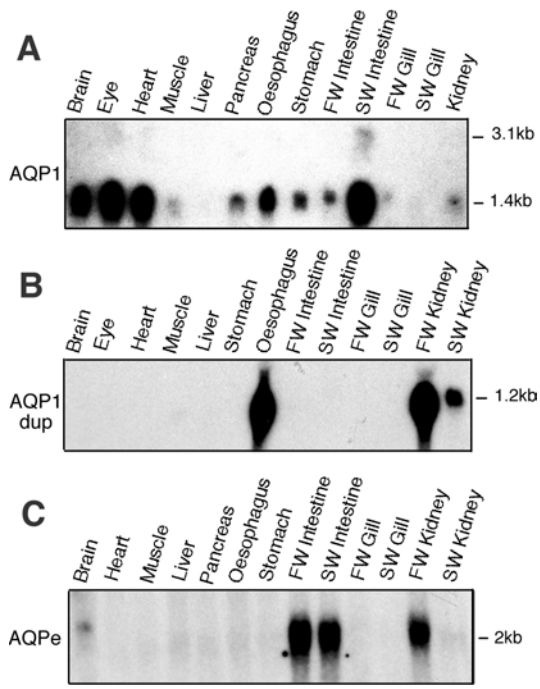
Tissue distribution of eel mRNAs (Figure 2)

Northern blots (Figure 2A) showed that the eel AQP1 homologue has a wide tissue distribution with expression of a 1.4 kb mRNA found in the brain, eye, heart, pancreas, oesophagus, stomach and intestine, with much lower levels in skeletal muscle, gill and kidney. A minor 3.1 kb transcript, which may reflect a precursor species or spliceform, was also present in some tissues. AQP1dup had a narrower tissue distribution, with a 1.2 kb mRNA expressed only in the oesophagus and in the kidney of FW-acclimated eels with much lower levels in the kidney of SW-acclimated animals (Figure 2B). Occasionally, weak signals were also detected in the intestinal samples of some fish. AQPe was expressed as a 2 kb mRNA mainly in the intestine and in the kidney with much lower levels also present in the brain (Figure 2C).

Quantitative expression of AQP1, AQP1dup and AQPe mRNAs in FW- and SW-acclimated yellow and silver eel kidney (Figures 3 and 4)

Quantitative Northern blots (Figure 3) were used to investigate AQP1, AQP1dup and AQPe mRNA expression in the kidneys of FW and 21-day-old SW-acclimated yellow and silver eels. All three AQPs exhibited highly variable expression levels among individual members of all fish groups (Figures 3 and 4). In the kidney of FW-acclimated yellow eels, AQP1dup mRNA levels were approx. 5–10-fold

Figure 2 | Northern blot using 10 μg of total RNA showing the tissue distribution of AQP1 (A), AQP1dup (B) and AQPe (C) mRNA expression in a SW-acclimated yellow eel, with the exception of RNA samples from a FW-acclimated yellow eel where labelled. RNA sizes (kb) were estimated from RNA standards (results not shown)



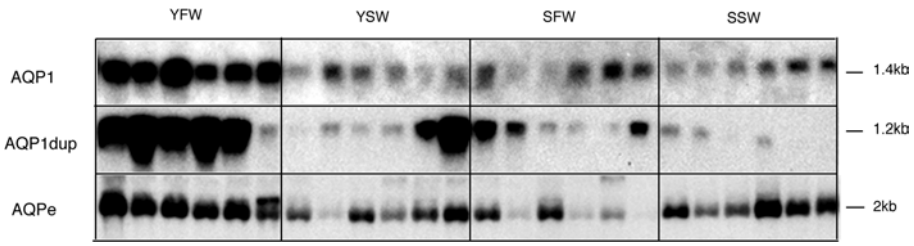
higher than AQPe which were approx. 2–5-fold that of AQP1 (Figure 4). Three weeks after SW-transfer, yellow eels exhibited significant decrease, 71.9, 66.1 and 33.5%, in the expression of mRNAs for AQP1 (Figures 3 and 4A), AQP1dup (Figures 3 and 4B) and AQPe (Figures 3 and 4C) respectively. Although SW transfer was accompanied by small decreases in AQP1 and AQP1dup mRNA abundance in silver eels, these levels were not significantly different from the existing low levels of expression found in the silver FW-acclimated group and similar to that found for the SW-acclimated yellow eels (Figures 3 and 4).

Cellular localization of AQP1 protein by immunofluorescence light microscopy (Figure 5)

The cellular distribution of an AQP1 protein isoform was characterized using a specific eel AQP1 antibody prepared as described in the Materials and methods section. AQP1 immunoreactivity was detected within the vascular endothelium in both FW- and

Figure 3 | Northern blot using 10 μg of total RNA showing the expression of AQP1, AQP1dup and AQPe mRNA in the kidney of FW- and 3-week-old SW-acclimated yellow (Y) and silver (S) eels

Each sample on the blot was taken from an individual fish such that $n = 6$ from each group. RNA sizes (kb) were estimated from RNA standards (results not shown). The AQP1 blot autoradiograph was exposed for 11 h, the AQP1dup blot for 1 h 20 min and the AQPe blot for 14 h 35 min at −80°C.



SW-acclimated yellow eels (Figures 5A–5C). Although no attempt was made to quantify the immunofluorescence, it was clear that the endothelial staining was less intense and consistently fewer blood vessels were found to be stained in sections taken from SW-acclimated fish. Unlike the variability seen with AQP1 mRNA expression, immunofluorescence studies showed a high level of consistency across tissue samples from all animal groups tested (four to six animals/condition). Although the endothelium was the main site of immunofluorescence in the yellow eel kidney, occasionally, very faint staining of the apical brush border of the epithelial cells of some tubules was also observed (results not shown). In contrast with that found in yellow eels, kidney sections taken from both FW- (Figure 5E) and SW-acclimated silver eels (Figure 5F) exhibited a strong AQP1 immunofluorescence primarily within the apical brush border of the epithelial cells, but only in a small subset of tubules (Figures 5E and 5F). In addition, much lower levels of immunofluorescence were also observed within the vascular endothelium (results not shown). Negative controls were also processed using either no primary antibody, a purified anti-flounder MDR (multidrug resistance) antibody or with incubations containing the peptide-negated AQP1 antiserum. No immunofluorescence was found with any of the control sections from the kidney of yellow or silver eels (Figure 5D). A non-specific autofluorescence was observed in the blood cells in all sections (Figure 5).

Plasma cortisol levels

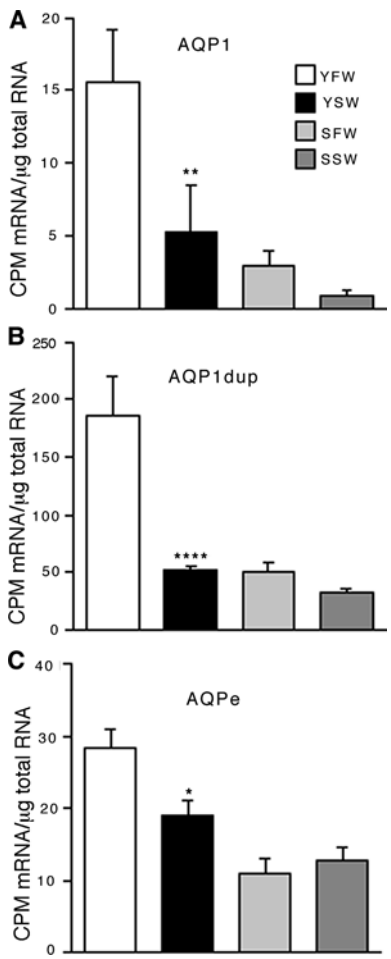
Plasma cortisol levels in FW-acclimated fish were 8.7 ± 2.7 ng/ml in silver and 7.4 ± 2.5 ng/ml in yellow eels. Long-term (3 week) SW-acclimated eels exhibited similar cortisol concentrations of 8.6 ± 2.4 and 10.5 ± 4.2 ng/ml for yellow and silver eels respectively; however, elevated levels averaging 20.4 ± 7.8 and 18.4 ± 7.2 ng/ml were measured for yellow and silver eels up to 2 days after acute FW–SW transfer. In the implanted animals (see the Materials and methods section), after 8 days, plasma cortisol levels for vehicle implanted fish had increased by approx. 4-fold from average values of 8.8 ± 1.7 and 9.6 ± 2.2 ng/ml to 33.4 ± 4.9 and 35.8 ± 5.3 ng/ml, for yellow and silver eels respectively.

Effect of cortisol on AQP1, AQP1dup and AQPe mRNA expression in yellow and silver eel kidneys (Figures 6 and 7)

Quantitative Northern blots (Figure 6) were used to investigate AQP1, AQP1dup and AQPe mRNA expression in kidneys of control (vehicle-treated) and cortisol-treated yellow and silver eels. As in previous experiments, all three AQPs, and in particular AQP1 and AQP1dup, exhibited highly variable expression profiles between individual members of all fish groups (Figures 6 and 7). Cortisol infusion in FW-acclimated yellow eels, resulted in (i) significant to highly significant decreases in mRNA expression of 82.8% for AQP1 (Figures 6 and 7A) and 80.3% for AQP1dup (Figures 6 and 7B) respectively and (ii) no significant change in AQPe mRNA expression (Figures 6 and 7C). However, cortisol infusion had no effect on the expression of any AQP isoform mRNA in the FW-acclimated silver eels, although levels were always lower when compared with the equivalent yellow eel group (Figures 6 and 7).

Figure 4 | Quantification of the radiolabelled AQP1 (A), AQP1dup (B) and AQPe (C) DNA probe bound to Northern blots of renal RNA samples isolated from FW- and 3-week-old SW-acclimated yellow (Y) and silver (S) eels (as shown if Figure 3)

Values are means ± S.E.M. ($n = 6$; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{****}P < 0.0001$; values for FW to SW comparisons).



Discussion

We report the cloning of three cDNAs called AQP1, AQP1dup and AQPe, encoding homologues of mammalian AQPs from the European eel. Their amino acid composition and certain structural features support their classification as putative AQP water channels: the presence of two NPA motifs, one within each half of the sequence, six putative membrane-spanning domains with five intertransmembrane loops, and the high percentage of sequence amino acid identity with other vertebrate AQPs (between 43 and 76%). Recently, Aoki et al. (2003) reported the cloning of two homologues of mammalian AQP1 from the Japanese eel (*A. japonica*) that exhibited 94% amino acid homology with the European eel AQP1 clone. The predicted protein molecular masses of the European eel AQPs range from 27.5 to 34.1 kDa, which is equivalent to the monomer size range for the mammalian AQPs (Borgnia et al., 1999; Verkman and Mitra, 2000). AQPs were originally defined as homologue proteins of the major intrinsic protein of the mammalian lens that rapidly and selectively allow the permeation of water across the lipid bilayer of the cell membrane (Agre et al., 1993). In mammals, the AQPs are a large gene family, presently with 13 members that are related by amino acid homology and genomic structure and which have been grouped into three broad subfamilies. These include the 'water-selective' AQPs, comprising AQP0–AQP2 and AQP4–AQP6 (although, AQP0 is also permeable to glycerol; Ishibashi et al., 2000), the aquaglyceroporin group containing AQP3, AQP7, AQP9 and AQP10 that are permeable to water and/or glycerol and urea (Echevarria et al., 1996; Ishibashi et al., 1997, 1998; Yang and Verkman, 1997; Hatakeyama et al., 2001), and finally AQP8, AQP11 and AQP12, which are an anomalous group of channels with lower amino acid homology, where AQP 8 is permeable to water and/or urea, AQP11 to water but not to glycerol, and the properties of AQP12 are unknown (Ishibashi et al., 2000). The eel AQPe sequence shows some specific characteristics of the aquaglyceroporin family with the AQP1 and AQP1dup sequences more similar to the orthodox 'water-selective' AQP family members. The overall characteristics of the eel isoforms include: (i) an aspartate residue after the second NPA motif (NPARD, amino acid 217; Figure 1B) in the AQPe sequence, which is characteristic of the aquaglyceroporins rather than the NPARS motif found in both eel AQP1 and AQP1dup sequences (amino acid 221; Figure 1A) and as also found in the orthodox AQPs (Figure 1A and Borgnia et al., 1999); (ii) a cysteine residue responsible for the mercury sensitivity of the AQPs, present in eel AQP1 (amino acid 218; Figure 1A), AQP1dup (amino acid 218; Figure 1A) and mammalian AQP1 sequences (Figure 1A and Borgnia et al., 1999), is replaced by a tyrosine residue in the eel AQPe sequence (amino acid 214; Figure 1B) as found in vertebrate AQP3 sequences (Figure 1B and Cutler and Cramb, 2002).

Figure 5 | Immunolocalization of AQP1 in the kidney of yellow (A–D) and silver (E, F) eels

Typical AQP1 immunohistochemistry of both the renal tissue of FW- (**A**, **B**, **E**) and SW-acclimated (**C**, **F**) eels. Control sections were incubated without the primary antibody, with a purified antibody anti-flounder MDR or the immune serum preincubated with the peptide antigen (**D**). ABB, apical brush border; BV, blood vessel; RBC, red blood cells; T, tubule; TL, tubular lumen. Scale bar, 10 μm.

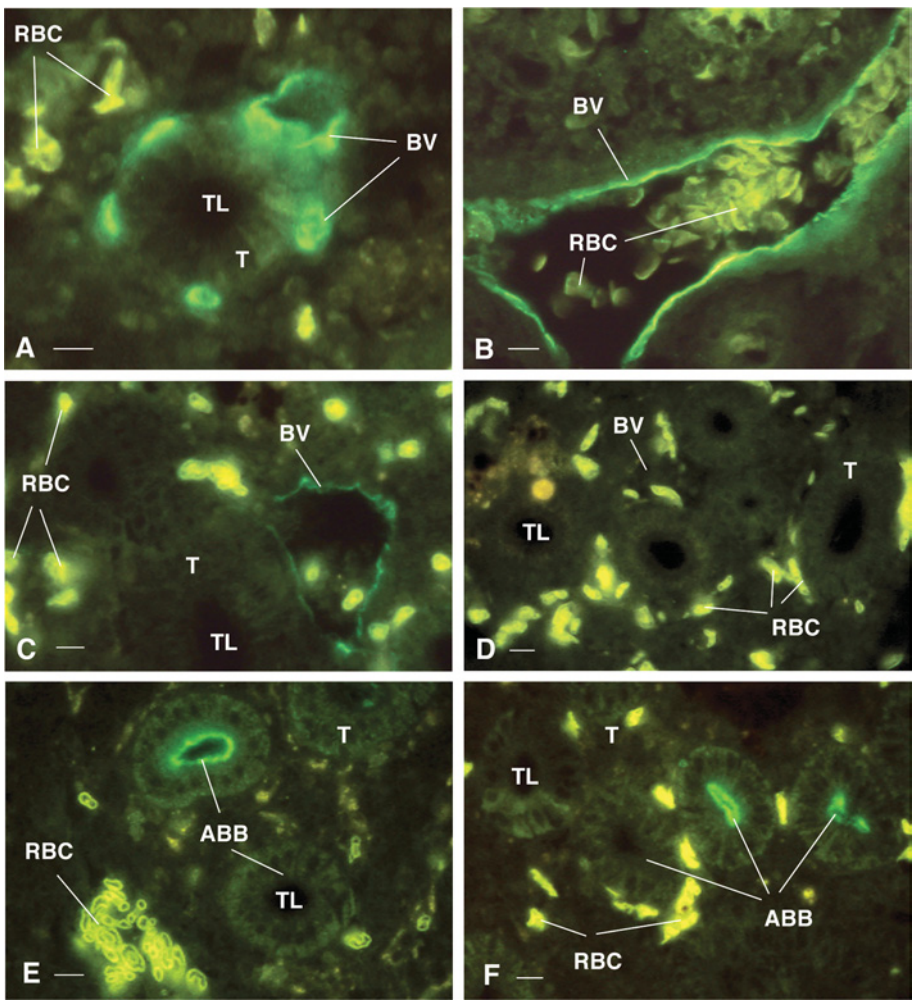


Figure 6 | Northern blot using 10 μg of total RNA showing the expression of AQP1, AQP1dup and AQPe mRNA in the kidney of vehicle- (Cont) and cortisol-treated (Cort) yellow (Y) and silver (S) eels ($n = 6$ for each group)

RNA sizes (kb) were estimated from RNA standards (results not shown). The AQP1 blot for autoradiograph was exposed for 21 h, the AQP1dup blot for 20 h and the AQPe blot for 45 h at −80°C.

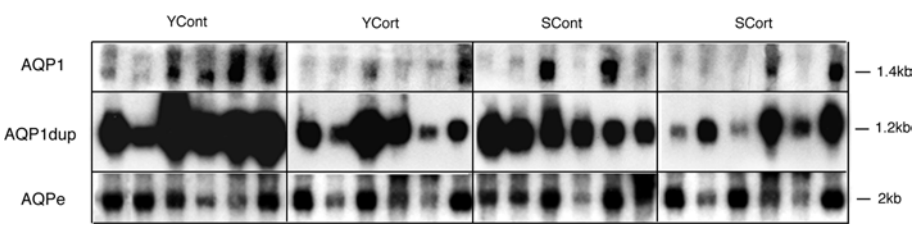
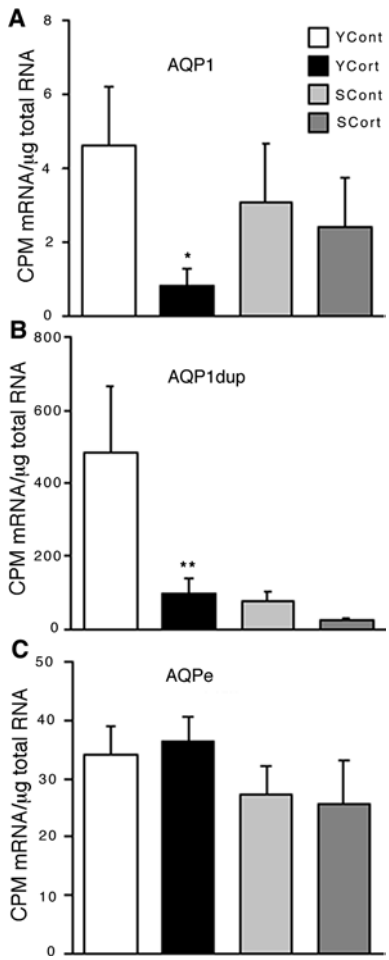


Figure 7 | Quantification of the radiolabelled AQP1 (A), AQP1dup (B) and AQPe (C) DNA probe bound to Northern blots of kidney RNA samples isolated from vehicle- (Cont) and cortisol-treated (Cort) yellow (Y) and silver (S) eels (as shown in Figure 6)

Values are means ± S.E.M. ($n = 6$). *$P < 0.05$ and **$P < 0.01$, values for control versus cortisol-treated comparisons.



This suggests that if the eel AQPe is sensitive to mercurial reagents, this is probably a result of interactions at some other site. Another interesting eel AQP sequence feature concerns the single putative N-linked glycosylation site, (i) present in the AQP3 sequences of the higher vertebrates, but absent from teleost fish AQP3 and eel AQPe sequences (amino acid 143; Figure 1B) and (ii) present in vertebrate AQP1 sequences including teleost fish and the European eel (amino acid 68; Figure 1A) but absent from the eel AQP1dup sequence (Figure 1A and Borgnia et al., 1999). This suggests that the eel AQPe and AQP1dup isoforms may not be glycosylated.

The tissue distribution of eel AQP1 is also similar, although not identical, to that found for AQP1 in mammals where it is primarily expressed in tissues such as the eye, respiratory tract (equivalent to the teleost gill), kidney, brain, gastrointestinal tract and liver (Borgnia et al., 1999; Ma and Verkman, 1999) and to that reported for AQP1 in the Japanese eel with an exception that no mRNA transcripts were detected in the stomach or the kidney (Aoki et al., 2003). There are also one or two notable exceptions between the tissue distribution of eel and mammal AQP1. Firstly, eel AQP1 was only expressed in an inconsistent fashion and at a relatively low level in the kidney, whereas in mammals the kidney is a major site of AQP1 expression, where it is found in abundance in the proximal tubules and descending thin limb (Borgnia et al., 1999). As the mammalian kidney is known to express a number of different AQP isoforms, and given the inconsistent and low levels of renal AQP1 in the eel, there was a high probability that other AQP isoforms were present in the teleost kidney. Indeed, a further search in the kidney led to the discovery of another AQP with high amino acid homology to eel AQPs and AQP1 in particular, and which therefore was called AQP1dup. Northern-blot analysis revealed that AQP1dup mRNA expression was mainly confined to the kidney and the oesophagus of the eel. As the latter tissue has been reported to have low water permeability in marine fish (Nagashima and Ando, 1993), the finding of AQP1dup and two other AQP isoforms (Cutler and Cramb, 2001) in this tissue in eels has caused some ambiguity about the physiological role of the oesophagus in osmoregulation. Eel AQP1 was also found to be expressed at high levels throughout the gastrointestinal tract of the eel (oesophagus, stomach and intestine), whereas in mammals the gastrointestinal tract is not a major site of AQP1 expression. In this tissue, at least four other AQPs are expressed with only limited AQP1 abundance within the capillary and lymphatic endothelia being reported (Koyama et al., 1999; Ma and Verkman, 1999). The tissue distribution of eel AQPe was very similar to that found for the mammalian aquaglyceroporin AQP3 with expression primarily within the kidney and the gastrointestinal tract (Borgnia et al., 1999; Koyama et al., 1999; Ma and Verkman, 1999).

In quantitative expression studies, AQP1, AQP1dup and AQPe mRNAs were all expressed at different levels within the kidney: AQP1dup was consistently the most abundant isoform, with expression being approx. 10-fold higher than AQPe which in turn was approx. 2–5-fold more abundant than AQP1. Although highly variable expression levels were observed between individual fish, in yellow eels, FW to SW transfer induced statistically significant decreases in transcript abundance for all isoforms. This finding was not repeated for the silver eels with SW-acclimation failing to further reduce the already low levels of expression found for all isoforms in the FW group. These results suggest that some as yet unknown developmental changes associated with the 'silvering' process are responsible for the down-regulation of AQP gene expression in the eel kidney. This, however, brings into question the role of these kidney AQP isoforms in osmoregulation especially in FW. One possible explanation is that as a consequence of the 'silvering' process, the permeable body surfaces of the eel, and the gills in particular, become 'tighter' with respect to water movement and as a consequence, osmotic water loading from the FW environment is reduced. If this was the case, then there would be a reduced need to maintain the high glomerular filtration rate and the associated tubular ion and water transport necessary for diuresis in the kidney.

To date, at least seven AQPs (AQP1–AQP4, AQP6–AQP8) have been found to be expressed in the kidney of humans, mainly in the proximal tubules, descending thin limb and collecting ducts (Nielsen et al., 2002). The physiological role of the decrease in AQP1, AQP1dup and AQPe expression in yellow eels after SW transfer is difficult to comprehend, when considering that the role of the kidney in FW fish is to produce copious quantities of dilute urine, presumably with a minimal level of water reabsorption (Cleveland and Trump, 1969; Bone et al., 1995; Karnaky, 1998). However, the production of large amounts of dilute urine in FW-acclimated fish involves either relatively high levels of glomerular filtration and/or tubular fluid secretion to account for the high urine flow rates (Cleveland and Trump, 1969; Schmidt-Nielsen and Renfro, 1975; Beyenbach, 1995; Bone et al., 1995; Karnaky, 1998). Indeed, it is possible that individual renal tubules may function primarily as absorptive or secretory epithelia with differential expression of ion transporters and AQP isoforms within the epithelial cells. Irrespective of the major driving forces for urine production, the tubules of FW-acclimated fish will be involved in the transcellular movement of large quantities of salts, which consequently requires concomitant changes in water transport. As a consequence, the level of water transport is presumably much greater in FW-acclimated fish compared with SW-acclimated fish. If, as we hypothesized above, a certain amount of water transport must parallel the absorption/secretion of salts (and also amino acids, sugars etc.) in FW conditions, it might not be surprising to find AQP1, AQP1dup and AQPe in the epithelium of the renal proximal tubules, where substantial univalent ion absorption and secretion and 'nutrient' absorption is known to occur (Cleveland and Trump, 1969; Schmidt-Nielsen and Renfro, 1975; Beyenbach, 1995). Immunohistochemical studies have shown that AQP1 is expressed within the apical brush border of a subset of tubules within the renal epithelium in both FW- and SW-acclimated eels. Immunoreactivity associated with the epithelial cells in silver eels was much greater than in yellow eels, where the predominant staining was within the endothelial cells of the blood vessels. The location of AQP1 within the epithelium was not unexpected, as in mammals AQP1 has been reported to be present in both apical and basolateral plasma membranes of the proximal tubules and descending thin limb (Nielsen et al., 2002). At present, there is no functional evidence to determine whether this apically located AQP1 has an absorptive or secretive role in the eel kidney; however, differential immunofluorescence intensities would suggest that water transport through this mechanism is developmentally regulated with staining more abundant in silver eels rather than in yellow eels, irrespective of the environmental salinity. Roles for the two other renal isoforms, AQPe and AQP1-dup, may be associated with (i) the apical transport of water within other tubules or tubular segments and/or (ii) the protection of tubular epithelial cells from shrinkage or swelling by providing a basolateral pathway for water movement. Such asymmetrical distributions of AQP isoforms have previously been reported within epithelial cells of the mammalian kidney (Nielsen et al., 2002). Such an arrangement of the different isoforms in the apical and basolateral membranes of the epithelial cells would generally provide a transcellular pathway for water movement.

AQPe, as a member of the aquaglyceroporin family, could also be associated with the transport of small polar solutes such as urea and glycerol. Despite being ammoniotelic, most teleosts also produce and excrete a small amount of urea (Wood, 1993). In the European eel, urea excretion (i) occurs at the same rate as in the gill and the kidney and (ii) is 3-fold lower in SW compared to FW environments (Masoni and Payan, 1974). The decrease in renal AQPe expression correlates with the reported changes in urea excretion that accompany SW transfer, suggesting that this AQP isoform may be involved in the clearance of this nitrogenous waste product within the kidney. However, despite these possible explanations, further investigations including the use of specific antibodies to AQP1dup and AQPe are required before any further conclusions can be reached.

In yellow eels, AQP1 appears to have a different role in water transport compared with the silver eels. Indeed, immunohistochemical studies in yellow eels indicate that AQP1 is predominantly expressed within the endothelium of the blood vessels in both FW- and SW-acclimated fish, with a slightly stronger and more frequent staining in FW conditions. A similar localization has recently been reported for AQP1 in the intestine of the SW-acclimated Japanese eel (Aoki et al., 2003). In mammals, AQP1 has been observed in the microvascular endothelia of various tissues (Verkman, 2002), including the intestine (Koyama et al., 1999; Ma and Verkman, 1999), respiratory system (Nielsen et al., 1997) and kidney (Nielsen et al., 1995).

The study of possible influences of the interrenal tissue on the hydromineral regulation of fish has received extensive attention, but most studies to date are centred around the effects of cortisol on the movement of ions in the gills and the gastrointestinal tract and very little is known about possible actions on water transport in the kidney. Results of the present study suggest that cortisol, at plasma concentrations similar or slightly higher than those reported during FW–SW acclimation, induces a similar down-regulation of AQP1 and AQP1dup gene expression in the yellow eel kidney as found after SW acclimation. However, this was not also true for renal AQPe expression that remained unchanged after the cortisol treatment. The FW silver eels, which already exhibited a lower expression of the renal AQP isoform mRNAs, appeared to be less sensitive to cortisol and although small reductions in expression were found with AQP1 and more so in AQP1dup, data analyses indicated that these reductions were not statistically significant. Cortisol is the major corticosteroid released by the interrenal gland in both FW and SW fish and is responsible for the regulation of both mineral balance (as associated with aldosterone in mammals) as well as glucocorticoid effects. The steroid has largely been identified as a 'SW-adapting' hormone, primarily promoting a number of SW-adapting functions (Hazon and Balment, 1998). When administered to FW-acclimated fish, cortisol has been shown to induce epithelial features similar to those of SW fish such as (i) chloride cell proliferation and differentiation associated with an increase in the Na^+, K^+-ATPase activity (Hazon and Balment, 1998; Nishimura and Fan, 2003), (ii) an increase in intestinal fluid absorption and in the permeability of ions in the oesophagus and the intestine (Hazon and Balment, 1998). Studies on the effects of adrenal steroids on water and mineral balance in the kidney have mainly concentrated on the actions of aldosterone in mammals. The most well-documented actions of the mineralocorticoids is the salt reabsorption function within the distal and collecting tubules, which potentiates the secondary osmotic retention of water (Maetz, 1968). In non-mammalian vertebrates, studies on this subject are scarce and mainly concern the effects of mineralocorticoids in birds, amphibian and reptiles (Braun and Dantzler, 1987). Moreover, there is no clear pattern of renal responses to the adrenocorticosteroids in this diverse group of vertebrates as the effects are often contradictory in different species and no uniform picture emerges (Braun and Dantzler, 1987). For example, aldosterone apparently stimulates renal sodium reabsorption in one species of lizard, inhibits sodium reabsorption in another and has no effect in a third (Dantzler, 2003). In teleost fish, indirect studies suggest a possible stimulatory effect of aldosterone on sodium reabsorption and water secretion in the kidney of FW-acclimated fish, but no direct studies have been made at the molecular level (Jones et al., 1969; Braun and Dantzler, 1987; Dantzler, 2003). The few reported studies regarding the effects of corticosteroids on AQP expression have only concerned mammalian AQPs with the steroids up-regulating expression in the kidney (Wintour et al., 1998; Jonassen et al., 2000; Kang et al., 2003), lung (King et al., 1996; Tanaka et al., 1997; Liu et al.,

2003), peritoneal epithelium (Stoenoiu et al., 2003) and red blood cells (Moon et al., 1997). Taking into account these reports, our results seem difficult to reconcile with any other study. However, they provide new information that might help to elucidate the role and mechanisms of action of corticosteroids on water transport in the kidney of vertebrates in general and of eels in particular, especially in association with migration to the SW environment.

Materials and methods

Fish

Adult FW sexually immature yellow and sexually maturing migratory silver eels were obtained from local suppliers in Inverness, Blairgowrie and Kelso and transferred to laboratory aquariums at the Gatty Marine Laboratory (St. Andrews, Scotland, U.K.). Unfed eels were maintained at ambient temperature (5–14°C) in aerated natural FW (FW: 0–10 mosmol/kg) before experiments and then acclimated to SW (SW: 960–1020 mosmol/kg) (or FW for controls) for a period of 21 days before use in experiments. A 12 h light/12 h dark photoperiod was maintained. Fish were decapitated and pithed before removal of tissues.

Cortisol infusion and RIA

Silver and yellow FW-acclimated eels were anaesthetized (48 p.p.m. of 3-aminobenzoic acid ethyl ester), weighed and implanted with Alzet mini osmotic pumps (model 1003D) releasing vehicle alone [a 30% (w/v) solution of 2-hydroxypropyl-β-cyclodextrin; Fluka, Gillingham, U.K.] (control animals) or cortisol dissolved in the vehicle [10 mg of cortisol · (kg wet wt. fish)$^{-1}$ · pump^{-1} = rate of delivery of cortisol 15 μg · (kg wet wt)$^{-1}$ · h^{-1}]. After 8 days, blood was collected, centrifuged and the resulting plasma was frozen and stored at −80°C until analysis. Plasma concentrations were measured by RIA essentially as described by Van Anholt et al. (2003). Sheep cortisol antibody was obtained from National Diagnostics Scotland (Lanarkshire, U.K.).

Total RNA extraction

Gut and kidney RNAs used for cloning experiments were extracted by a modified LiCl procedure as described in Cutler et al. (1995). RNAs (brain, eye, heart, skeletal muscle, liver, pancreas, oesophagus, intestine, kidney, gills and stomach) for Northern-blot experiments were extracted by a modification of the Chomczynski and Sacchi method (Chomczynski and Sacchi, 1987) as described in Cutler et al. (2000). Gut and kidney mRNAs were purified for RT (reverse transcriptase)–PCR experiments as described previously (Cutler et al., 1995).

Cloning and sequencing

Initial fragments for all AQPs were amplified by RT–PCR using synthetic sense and antisense degenerate primers as described previously in Cutler and Cramb (2002). 5′- and 3′-RACE (rapid amplification of cDNA ends) DNA fragments were produced from 3-week old SW-acclimated silver eel kidney and intestine mRNA using a Marathon cDNA amplification kit (ClonTech Laboratories, Basingstoke, U.K.) as described previously (Cutler et al., 2000). 5′-RACE products were produced in nested PCR reactions using eel AQP1-, AQP1dup- or AQPe-specific antisense primer 1 and then primer 2 in conjunction with the Marathon kit nested AP1 (adapter primer 1) and AP2 primers (Clontech). 3′-RACE products were obtained using the same procedure using eel AQP1-, AQP1dup- or AQPe-specific sense primer 1 and then primer 2 in conjunction with the Marathon kit AP1 and AP2 primers. PCR fragments generated using the degenerate primers or by 5′- and 3′-RACE amplification were cloned and sequenced as described previously in Cutler et al. (2000).

Northern blotting and analysis

Northern blotting and quantitative determination of transcript abundance were performed by electronic autoradiography as described previously (Cutler et al., 2000; Mahmmoud et al., 2003). The probes used for Northern-blot analysis were colony PCR-amplified plasmid inserts of the original fragments of AQP1 (387 bp), AQP1dup (395 bp) and AQPe (445 bp) produced using the original degenerate sense and antisense primers. Probes (25 ng cDNA) were radiolabelled to specific activities between 2 and 2.6 × 10^9 d.p.m./μg DNA using [α-^{32}P]dCTP (6000 Ci/mmol) and a Megaprime DNA labelling kit (Amersham Biosciences, Little Chalfont, Bucks., U.K.) according to the manufacturer's instructions and purified by size exclusion chromatography on a Sephadex G50 column before its use in the hybridizations. Following stringent washing instructions, the specific radioactivity hybridizing to the blots was quantified using electronic autoradiography using an Instant Imager (Canberra Packard, Meriden, CA, U.S.A.) and individual mRNA abundance expressed as c.p.m./μg total RNA after first correcting for the relative amounts of total RNA loaded for each sample by quantification of the combined 18 and 28 S ribosomal RNAs in each lane, as described previously (Mahmmoud et al., 2003). The blots were finally exposed for various times to X-ray film (Kodak BioMax MS from Sigma–Aldrich Chemicals, Poole, U.K.) at −80°C and developed for autoradiography.

AQP1 antibody production and purification

Commercially prepared (Pepceuticals, Leicester, U.K.) AQP1 antibodies were raised against a 15 amino acid C-terminal peptide (VNGPDDVPAVEMSSK) as described previously (Lignot et al., 2002). Anti-AQP1 antibodies were affinity-purified from the polyclonal antiserum by affinity-adsorption of the peptide antigen cross-linked to activated thiol Sepharose 4B as instructed by the manufacturer (Amersham Biosciences). The antiserum (20 ml) was continually mixed with 4 ml of the peptide-conjugated beads for 40 min at room temperature (19–21°C), cooled to 4°C and poured into a column. The eluted serum was collected and the beads washed with 5 vol. of ice-cold wash buffer (50 mM Tris, 0.5 M NaCl and 1 mM EDTA, pH 7.5). The bound AQP1 antibodies were eluted from the column with 15 ml of ice-cold 0.1 M glycine/HCl (pH 2.5) and 1.2 ml fractions were collected into 0.3 ml of 1 M Tris (pH 8.0). Fractions containing antibody (as determined by SDS/PAGE; Laemmli, 1970) were pooled together, aliquoted and stored at −20°C until use for immunohistochemistry.

Immunofluorescence light microscopy

Immunohistochemical localization of AQP1 was performed on kidney samples removed from yellow and silver eels as

described previously by Lignot et al. (2002). The dilutions of the purified primary antibody and the secondary antibody FITC-conjugated donkey anti-rabbit IgG (H&L); Jackson Immunoresearch Laboratories, West Baltimore, MD, U.S.A.) were respectively 1:2000 and 1:200. The control sections were incubated with (i) phosphate buffered saline (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, pH 7.3) without the primary antibody or equivalent dilutions of (ii) a purified anti-flounder MDR antibody or (iii) the immune serum preincubated overnight at 4°C with 50 μg/ml of the peptide antigen (peptide-negated antiserum).

Statistical analysis

The data were analysed using Stat View 4.01 software (Abacus Concept, Berkeley, CA, U.S.A.), using ANOVA with levels of significance determined using Fisher's test.

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