Seawater acclimation and inositol monophosphatase isoform expression in the European eel (Anguilla anguilla) and Nile tilapia (Oreochromis niloticus)

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Submitted 24 January 2013; accepted in final form 1 June 2013

Kalunjaia S, Gellatly SA, Hazon N, Villasenor A, Yancey PH, Cramb G. Seawater acclimation and inositol monophosphatase isoform expression in the European eel (Anguilla anguilla) and Nile tilapia (Oreochromis niloticus). Am J Physiol Regul Integr Comp Physiol 305: R369–R384, 2013. First published June 5, 2013; doi:10.1152/ajpregu.00044.2013.—Inositol monophosphatase (IMPA) is responsible for the synthesis of inositol, a polyanol that can function as an intracellular osmolyte helping re-establish cell volume when exposed to hypertonic environments. Some epithelial tissues in euryhaline teleosts such as the eel and tilapia encounter considerable hypertonic challenge when fish move from freshwater (FW) to seawater (SW) environments; however, the roles played by organic osmolytes, such as inositol, have yet to be determined. Syntetic analysis has indicated that, as a result of whole genome- and tandem-duplication events, up to six IMPA isoforms can exist within teleost genomes. Four isoforms are homologs of the mammalian IMPA1 gene, and two isoforms are homologs of the mammalian IMPA2 gene. Although the tissue-dependent isoform expression profiles of the teleost isoforms appear to be species-specific, it was primarily mRNA for the IMPA1.1 isoform that was upregulated in epithelial tissues after fish were transferred to SW (up to 16-fold in eel and 90-fold in tilapia). Although up-regulation of IMPA1.1 expression was evident in many tissues in the eel, more substantial increases in IMPA1.1 expression were found in tilapia tissues, where SW acclimation resulted in up to 2,000-fold increases in protein expression, 16-fold increases in enzyme activity, and 15-fold increases in tissue inositol contents. Immunohistochemical studies indicated that the tissue distribution of IMPA1.1 protein differed slightly between eels and tilapia; however, in both species the basal epithelial cell layers within the skin and fin, and the branchial epithelium and interstitial cells within the kidney, exhibited high levels of IMPA1.1 protein expression.

Anguilla anguilla; Oreochromis niloticus; teleost; gene synteny; osmolyte; osmoregulation; inositol monophosphatase

THE IONIC AND OSMOTIC STRESSES faced by euryhaline fishes as they move between freshwater (FW) and seawater (SW) environments, and the osmoregulatory mechanisms employed to maintain body fluid homeostasis have been well documented in a number of recent review articles (12, 16, 17, 19, 32, 47). The major tissues involved in regulating body fluid composition in both FW and SW are the gills, the kidney, and the intestine. Individual cells distributed throughout these tissues are responsible for ion and water uptake or secretion to maintain the composition and osmolality of the body fluids to within certain finite limits to ensure the survival of fish at the extremes of habitat salinity. One question regarding teleost osmoregulation, which has received little attention so far, is how do epithelial cells on exposed body surfaces, such as the skin, fin, and gill, maintain their optimal cell volumes when directly exposed to either FW or SW? Vulnerable surface epithelial cells must be protected from the edematous or dehydrative effects of these respective environments. Protective elements include physical barriers to water and ion diffusion, such as the secretion of quite complex, and, as yet poorly characterized, mucins (11, 27, 34, 42) and the presence of a highly stratified skin layer covering most body surfaces (18, 38). The skin of ray-finned fishes is unlike that of tetrapods and sarcopterygian fishes, with the epidermis comprising only living cells with no surface keratinized dead cell layer to act as a water-impermeable barrier (7, 18). As a consequence, osmotic water flow will occur across the skin, causing the body surface epithelial cells to swell in FW and shrink in SW. The osmotic uptake or loss of water across the apical surfaces of the epithelia must be balanced by its respective removal or replacement by exchange with the body fluids. The consequences of cell swelling and cell shrinkage due to changes in the osmolality of the environment are well documented in animal cells and, in particular, in the mammalian kidney, where tubular epithelial cells can be exposed to osmolarities ranging from 200 to more than 1,500 mOsmol/kg and yet must still be able to retain their secretory and absorptive functions (5, 36). In animal cells, increases in environmental osmolality that cause water efflux, and subsequent cell shrinkage, are counteracted by a process known as regulatory volume increase (RVI) (1, 25). The acute response to cell shrinkage is the activation of a number of ion channels and transporters that mediate the retention of monovalent ions (mostly Na+, K+, and Cl−), increasing the intracellular ionic concentration, therefore, opposing and even correcting for osmotic water efflux. Although this process of RVI can help maintain cell volume, the retention of inorganic ions is not sustainable in the longer term, as high concentrations of intracellular cations disrupt the electrochemical gradients across the plasma membrane, thus affecting membrane potentials and the operation of voltage-sensitive channels, as well as altering chemical gradients and electrical potentials across the mitochondria, which can result in the production of reactive oxygen species, DNA damage, cell cycle arrest, and apoptosis (1, 5). For normal cell metabolism to be maintained, the elevated concentrations of these “incompatible” inorganic osmolytes must be quickly replaced by so-called “compatible” organic osmolytes, such as taurine, betaine, inositol, sorbitol, and free amino acids, such as glycine and glutamine (1, 4, 5, 49).

We have previously reported that the cyclic alcohol, inositol, may be an important compatible osmolyte in the SW-acclimated eel (22, 23). Inositol can be synthesized de novo in many animal cells from glucose 6-phosphate (G6-P) by the stepwise

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actions of two enzymes, myoinositol phosphate synthase, which converts G6-P to l-myoinositol 1-phosphate, which is then subsequently hydrolyzed to inositol by the enzyme inositol monophosphatase (IMPA). The cyclic polyol inositol can then be used for the purposes of cellular osmoregulation or regeneration of components of the membrane phosphatidylinositol pool (3). Two IMPA isoforms (IMPA1 and IMPA2) with different tissue distributions and slightly different enzyme characteristics are present in mammals (37, 43). Because of the long-term association of these enzymes with lithium treatments for manic-depressive psychosis there is a wealth of information now available on both isoforms, regarding their chemical structures, tissue distributions, substrate specificities, and physiological functions (2, 15, 29, 44). In direct contrast, very little is known about the structures and functions of IMPA isoforms in lower vertebrates, including teleost fish, other than what can be deduced from the mammalian studies.

Previous studies from our laboratory indicated that an eel (Anguilla anguilla) homolog of IMPA1 (eIMPA1.1) was upregulated in all the major osmoregulatory tissues and also in the esophagus, skin, and fin when fish were transferred into SW (22, 23). Increases in eIMPA1.1 mRNA expression were accompanied by increases in the free inositol contents of gill, kidney, and fin (23). Cellular accumulation of inositol has also been reported in tilapia (Oreochromis mossambicus), in which levels in the kidney were elevated two- to four-fold and in the brain by 10- to 30-fold, after acclimation of fish to 200% SW (14). In a subsequent microarray study using the euryhaline goby (Gobius minutus), IMPA1 mRNA expression in the gill was reported to increase by more than 10-fold, 12 h after SW transfer (13). These results suggest that an osmoregulatory role for inositol might not be limited to the eel and that tilapia and possibly other SW-acclimated euryhaline, or stenohaline marine teleosts may also synthesize and/or accumulate the osmolyte. In addition to any functions to counteract increases in plasma osmolality in internal tissues, the induction of inositol synthesis may be even more important in the exposed epithelial cells of the gill, fin, and skin to counteract the dehydrative effects of the SW environment. To help characterize the osmoregulatory role of inositol during SW acclimation, we have identified all IMPA isoforms present in two euryhaline teleosts, the eel (Anguilla anguilla) and tilapia (Oreochromis niloticus), and characterized their tissue distribution and expression in both FW- and SW-acclimated fish.

MATERIALS AND METHODS

Reagents. All biochemicals, enzymes, horseradish peroxidase (HRP)-conjugated secondary antibodies and general chemicals were purchased from Sigma (Poole, Dorset, UK) unless otherwise stated. DNA primers were purchased from Eurofins MWG Operon (Ebersberg, Germany). General molecular cloning kits were from Invitrogen Life Technologies (Paisley, UK), rapid amplification of cDNA ends (RACE) kits were obtained from Clontech (Saint-Germain-en-Laye, France), DNAs-e and M-MLV-RT (RNase, H-) were purchased from Promega (Southampton, UK) and SYBR Green qPCR reagents were purchased from Applied Biosystems (Foster City, CA). Rabbit polyclonal anti-e (e)IMPA1.1 antibodies were raised commercially (21st Century Biochemicals, Marlboro, MA) against the KLH-conjugated peptide Ac-CIKEIEIFPAERDDVVKK-OH (P3: Supplemental Fig. S1), as described previously (23), and antibodies were affinity-purified before use using Western blot analysis and immunohistochemistry. Using maximum alignments, we found that the P3 antigen exhibited 29% and 47% amino acid identity with eel (e)IMPA1s 1.2 and 1.3, respectively, while it exhibited 77%, 35%, 53%, and 41% amino acid identity with the tilapia (t)IMPA1s 1.2, 1.3, and 1.4 proteins, respectively. The P3 antigen exhibited no homology to eIMPA2. Alexa Fluor 568-conjugated donkey anti-rabbit antibodies were obtained from Invitrogen Life Technologies (Paisley, UK).

Fish. Mixed sex, migratory “silver” European eels (Anguilla anguilla; 360–540 g) captured in river eel traps within the River Tay catchment area and tilapia (Oreochromis niloticus; red hybrid, 250–300 g) were purchased from local suppliers and kept in 300-liter tanks in fully aerated fresh water on a 12:12-h light-dark cycle either at ambient temperature (5–10°C; eels) or maintained at 24°C (tilapia) before experimentation. All protocols were conducted in accordance with the Animals (Scientific Procedures) Act, 1986 under a Home Office Project License (No. 603805) and all experiments were approved by the University of St. Andrews Animal Welfare and Ethics Committee. Because of the low water temperatures, eels were not fed. Tilapia were fed twice a day with standard tilapia chow (20 g/kg wet wt/day) throughout the experimental period. Eels were acutely transferred to FW or SW for the remainder of the acclimation periods, whereas tilapia were acutely transferred first to 30% SW for 1 wk before altering the inlet water flow rates and stepping the salinity to 50% then 75% and finally 100% SW every 7 days over the next 3 wk and then maintained at the final experimental salinity for either 10 (50% SW) or 8 wk (100% SW) before tissue sampling. Acute transfers were conducted by removal of 90% of the water from the experimental tanks and then free running, fully aerated, FW, SW, or appropriate FW/SW mixtures were gradually added over the next hour until the tanks were full. Eels were maintained as above without feeding for 3 wk after FW/FW or FW/SW transfer. A number of tissues were removed from FW-acclimated fish for the determination of tissue isoform distribution. A more limited group of osmoregulatory–relevant tissues were sampled from each of six FW control, six 50% SW (tilapia only), and six full SW-acclimated fish to examine the effects of environmental salinity. When sampling the eel gonadal tissue, it proved impossible to dissect this free from the surrounding adipose tissue; therefore, both gonadal tissue and adipose tissue were collected together. All tissue sampling was conducted between 3 h and 9 h of the light period of the light-dark cycle. Tissues were either frozen immediately in liquid nitrogen or subsequently stored at −80°C for RNA and protein extraction or fixed for 24 h in 4% paraformaldehyde in PBS before being processed for immunohistochemistry.

5′- and 3′-RACE cloning and sequencing. Initial cDNA fragments of all eIMPA isoforms (eIMPA1s 1.1, 1.2, 1.3, and 2) were obtained from a mixed kidney and gill SSH library (22, 24). Total RNA was prepared, treated with DNase, and reverse transcribed for use in standard PCR protocols (31). These initial fragments were then extended to 5′ or 3′ ends using a RACE technique (Clontech, Basingstoke, UK) and cloned for sequence analyses into the PCR4 vector using the TOPO-TA cloning system (Invitrogen, Paisley, UK) and at least three clones sequenced in both directions using a Big Dye Terminator sequencing kit (Perkin Elmer Biosystems, Cambridge, UK), as described previously (31). In some cases additional sequence information was also obtained from an eel genome library [http://www.zfgenomics.org/sub/eel; (35)] and confirmed by subsequent PCR amplification, cloning, and sequencing. Sequences were combined and initially analyzed using the GeneJockey II software package (Biosoft, Cambridge, UK). Sequences of the four eel IMPA isoforms have been deposited in the EMBL, GenBank under the accession numbers eIMPA1.1, FN599827.1; eIMPA1.2, HF679613; eIMPA1.3, HF657619; and eIMPA2, HF679195. The four tilapia IMPA1-like isoforms tIMPA1s 1.1, 1.2, 1.3, and 1.4 (accession nos. XM_003441555.1 and AY763793.1, respectively) were obtained from the NCBI GenBank.
**Biinformatics and comparative modeling.** Amino acid identity and similarity analyses for known IMPA protein sequences were calculated using MatGat2.01 software (6). For phylogenetic analysis, amino acid sequences were obtained from NCBI GenBank, Ensembl, TreeFam (Sanger Institute, Hinxton, Cambridge, UK) and the U.S. Department of Energy Joint Genome Institute (JGI) databases. The sequences were aligned using ClustalW and automatically edited in the software. Phylogenetic trees were constructed using Mega 5 software and visualized using the p-distance matrix of the neighbor-joining (N-J) method (41). The statistical reliability of individual nodes for sequence similarity of the newly constructed tree was assessed by bootstrap analyses with 1,000 replications.

**Syntenic analyses.** Synteny maps indicating the conserved genomic regions between human (*Homo sapiens*), mouse (*Mus musculus*), lizard (*Anolis carolinensis*), frog (*Xenopus tropicalis*), and selected teleost species were constructed using species information in the Ensembl database (http://www.ensembl.org/index.html), and the PhylowView program of the Genomicus v70.01 Web site (http://www.dyogen.ens.fr/genomicus-70.01/cgi-bin/search.pl). The synteny analyses of the eel genomic regions were determined manually using CLC DNA Workbench 6.8.2 software and information downloaded from the European eel genome database (http://www.zfgenomics.org/sub/eel/ (35)).

**Quantitative real-time-PCR (qPCR).** Quantitative real-time-PCR (qPCR) was performed using RNA samples extracted from all tissues. Samples containing 2 μg of total RNA from each fish were DNase-treated before being reverse-transcribed and 100–280-bp fragments from the coding regions of all IMPA isoforms and Rpl-p0 transcripts were amplified. Primer sequences are indicated in Table 1.

Rpl-p0 was used for data normalization, as it has previously been shown to exhibit no difference in tissue-specific expression between SW- and FW-acclimated eels (22), and this was also confirmed in tilapia tissues (data not shown). The relative abundance values for each target gene were expressed as standard quantity of gene of interest [determined from the derived cycle threshold (Ct) and the efficiency curve for the target gene] divided by the standard quantity of the reference gene (determined from the derived Ct and the efficiency curve for the reference gene) to obtain normalized relative expression values. The cDNA samples for each fish were prepared in triplicate on the same qPCR reaction plate, and both template-negative and reverse transcriptase-negative controls were routinely run for each sample. Results were collected and data were analyzed using the 7300 System SDS detection software (Applied Biosystems, Paisley, UK).

**Protein preparation.** Supernatant and membrane fractions from tissue homogenates were processed for Western blot analysis and enzymatic analysis. Tissues were sampled and stored for 2–3 wk at −80°C prior to processing. Frozen tissue samples (100–150 mg) were homogenized using a Precellys 24 homogenizer (6 × 2.8 mm ceramic beads per tube; 4 × 20 s at 6,500 rpm; PeqLab, Fareham, UK) in an ice-cold buffer (1/10 wt/vol) comprising 50 mM HEPES, 1 mM MgCl2, 0.25 M sucrose pH 7.4 containing a 1/100 dilution of EDTA-free cOmplete ULTRA protease inhibitor cocktail (Roche Diagnostics, West Sussex, UK). After tissue disruption, homogenates were centrifuged at 20,000 g for 1 min at 4°C to pellet insoluble particulate matter, and supernatants were recentrifuged at 30,000 g for 45 min at 4°C before decanting the supernatants and sampling for storage at −20°C. The remaining membrane pellets were resuspended in 0.2 × the original homogenizing volume of buffer, aliquoted and stored frozen at −20°C. Protein concentrations in supernatant and pellet fractions were determined by the Bradford method (Bio-Rad Laboratories, Hercules, CA) following the manufacturer’s protocol.

**Western blot analysis.** Protein samples extracted from the tissues (2 mg/ml) were diluted with an equal volume of sample preparation buffer (125 mM Tris-HCl, 20% glycerol, 4% SDS and 90 mM β-mercaptoethanol, pH 6.8), and denatured by heating to 100°C for 5 min. Denatured proteins (10 μg) were separated by NuPAGE (Invitrogen) gradient (4–12%) PAGE and electroblotted for 1 h at 0.3 Amp onto PVDF membranes (VWR, Leicestershire, UK). Membranes were blocked for 1.5 h at room temperature in PBS containing 10% nonfat milk and 0.2% Tween-20. Membranes were then incubated overnight at 4°C in PBS, 1% nonfat milk, 0.5% Tween-20 using the appropriately diluted anti-IMPA1.1 antibody and then washed for 4 × 10 min in PBS, 0.2% Tween-20 before the addition of the secondary antibody. The original anti-eel IMPA1 antiseraum (2914, at dilutions of 1/20K to 1/80K; see ref. (23)) and affinity-purified antibodies to the P3 peptide, at final concentrations between 0.5 and 2 ng/ml both detected the same immunoreactive proteins in all eel and tilapia tissues tested. The binding of all primary antibodies was detected by incubation of blots for 1.5 h at room temperature with HRP-conjugated anti-rabbit IgG secondary antibodies (1/20K dilution) in PBS, 5% nonfat milk, 0.5% Tween-20. Blots were then washed for 4 × 10

### Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sense (S)/Antisense (AS)</th>
<th>Sequence</th>
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<tr>
<td>Tilapia</td>
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| tIMPA1.1 S  |                        | GCGGCCTGGCTCAATACGTCAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA

Primer sequences used and size of DNA fragments amplified in quantitative RT-PCR analyses.
min at room temperature in PBS, 0.2% Tween-20 and conjugated secondary antibodies visualized using an ECL detection method, as described by the manufacturer (Pierce Dura ECL, Thermo Fisher Scientific, Loughborough, UK) (31, 33).

**Immunohistochemistry.** Immunohistochemical localization of IMPA1.1 protein was performed on kidney, gill, skin, and fin samples removed from FW- and SW-acclimated tilapia and stomach and gonad/adipose tissue from FW- and SW-acclimated eels, as previously described (28). Fixed tissue sections were incubated overnight at 4°C with the rabbit anti-eel IMPA1.1 P3 antibody (50 ng/ml) in PBS containing 1% BSA, 0.1% gelatin, and 0.1% Triton X-100 and detected following incubation for 1 h at room temperature with a 1:800 dilution of secondary, Alexa Fluor 568-conjugated donkey anti-rabbit antibodies (Invitrogen, Life Technologies, Paisley, UK) in the same buffer. Sections were counterstained with DAPI to allow detection of cell nuclei. Sections were viewed with a fluorescence microscope (Zeiss Axioscan, Welwyn Garden City, Hertfordshire, UK) equipped with appropriate filters, and images were collected and analyzed using Zeiss Axiovision Software. Control sections were run routinely using either peptide-negated primary antibody (preincubated overnight at 4°C with 50 μg/ml P3 antigen) or in the absence of primary antibody.

**Measurement of IMPA enzyme activity.** Enzymatic activities were determined at room temperature (21°C) in supernatant samples from tilapia gill and fin homogenates in a 100-g/ml PBS assay, as described previously (28). Briefly, tissue sections were incubated overnight at 4°C with the rabbit anti-eel IMPA1.1 P3 antibody (50 ng/ml) in PBS containing 1% BSA, 0.1% gelatin, and 0.1% Triton X-100 and detected following incubation for 1 h at room temperature with a 1:800 dilution of secondary, Alexa Fluor 568-conjugated donkey anti-rabbit antibodies (Invitrogen, Life Technologies, Paisley, UK) in the same buffer. Sections were counterstained with DAPI to allow detection of cell nuclei. Sections were viewed with a fluorescence microscope (Zeiss Axioscan, Welwyn Garden City, Hertfordshire, UK) equipped with appropriate filters, and images were collected and analyzed using Zeiss Axiovision Software. Control sections were run routinely using either peptide-negated primary antibody (preincubated overnight at 4°C with 50 μg/ml P3 antigen) or in the absence of primary antibody.

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**Determination of plasma osmolalities, plasma chloride concentrations, and tissue osmolyte contents.** Plasma osmolalities were determined using a freezing-point depression osmometer (Roebeling Osmometer, Camlab, Cambridge, UK), and plasma chloride concentrations were determined using a chloride meter (Corning Chloride Analyser 925, Ciba Corning Diagnostics, Halstead, UK). Frozen gill, kidney (eel only), and caudal fin samples (120–180 mg wet wt) from FW-acclimated and 100% SW-acclimated fish were homogenized in 9 vol of ice-cold 7% perchloric acid using a Polytron homogenizer for 2 × 15 s at full speed, and left on ice for >3 h. Samples were centrifuged at 30,000 g for 40 min at 4°C, and supernatants were removed and neutralized with 2 M KOH and left on ice overnight before centrifugation once more at 30,000 g for 40 min at 4°C. Osmolyte concentrations (inositol, taurine, betaine, glycine, glycerophosphocholineGPC, creatine, and glucose) in the neutralized extracts were determined by HPLC as described previously (48).

**Statistical analyses.** Values are presented as means ± SD or means ± SE. Statistical significance within the qPCR and Western blot analyses (eel samples) was determined using a nonpaired Student’s t-test or by two-way ANOVA (tilapia Western blot samples) followed by Fisher’s paired least significant difference post hoc analysis of significance (StatView 4.01 software; Abacus Concepts, CA). Statistical significance within the osmolyte and enzymatic analyses was determined using a nonpaired Student’s t-test. Significant differences are considered as P < 0.05, P < 0.01, and P < 0.001.

**RESULTS**

**Sequences, gene synteny, and phylogenetic analyses of teleost IMPA isoforms.** Partial cDNA fragments for four eel IMPA isoforms were initially identified in an eel multtissue library used for an earlier microarray study to determine differential mRNA expression following salinity transfer (24). Together, with additional information in the eel genome database [http://www.zfgenomics.org/sub/eel; (35)], full-length cDNA fragments for all eel IMPA isoforms were subsequently amplified and sequenced. Details on the sequence and structure of the eel IMPA1.1 protein isoform have been presented previously (23). In this study, two other eel IMPA1-like isoforms (eIMPA1.2 and eIMPA1.3) have been identified that exhibit 62.8% and 73.4% amino acid identity, respectively, with eIMPA1.1 and 62.1% identity with each other. Up to four IMPA1-like isoforms are present in other teleost databases, including tilapia. Syntenic analysis has indicated that the genes for teleost IMPAs 1.1 and 1.2 and for IMPAs 1.3 and 1.4 are contiguous within the genomes of all fish species in which they have been identified. To date, IMPA1.4 isoforms have only been found in tetraodon and tilapia genomes, whereas a single IMPA2 ortholog has only been identified in the eel, salmon, and zebrafish. In vertebrates, IMPA1-like and IMPA2-like isoforms can be distinguished by a number of isoform-specific motifs, including X193GT/SAAXMC201 (IMPA1; human sequence) and D204GSSTLA/S121 (IMPA2; human sequence) (Supplemental Fig. S1). Phylogenic analysis (Fig. 1) indicates that the IMPA1-like proteins in teleosts group into four distinctive clusters, which are separate from the cluster of single IMPA1 protein isoforms reported in the higher vertebrates. Teleost IMPA1.1 and IMPA1.2 isoforms exhibit closest homology to the IMPA1.3 isoforms (60–69%) and 60–63%, respectively, slightly lower homology to each other (55–60%), and all three isoforms exhibit much lower homology to IMPA1.4 subtypes (48–54%). The teleost IMPA1.2 isoforms exhibit closest homology with mammalian IMPA1s (64–70%) followed by fish IMPA1.3 (60–69%), IMPA1.1 (59–66%), and finally IMPA1.4 (54–56%) isoforms. The teleost IMPA1.3 isoforms segregate into two subgroups with the evolutionary ancient fishes (the eel, zebrafish, salmon, and rainbow trout) forming a distinct cluster (that exhibits higher homology with fish IMPA1.1s) from the other, evolutionary more modern species. It is interesting that it is only members of the former group, the eel, salmon, and zebrafish, that appear to have retained an IMPA2 isoform. The teleost IMPA2 isoforms also form a completely separate clade aligning more closely with the higher vertebrate IMPA2 group. The eel IMPA2 isoform shares 76% and 63% amino acid identity, respectively, with zebrafish and human IMPA2 and only 50–51% identity with the other eIMPA1s. Across all vertebrates, IMPA2 isoforms generally exhibit <55% amino acid homology with IMPA1 isoforms. Syntenic analyses (Fig. 2) indicated that teleost IMPA1.1 and IMPA1.2 genes and IMPA1.3 and (where present) 1.4 genes are juxtaposed within the teleost genomes. Macrosynteny around the single zebrafish IMPA gene exhibited some similarities with genomic segments harboring both IMPA1.1/1.2 and IMPA1.3/1.4 genes, although phylogenetic analysis grouped the zebrafish gene with the eel and salmon IMPA1.3 isoforms. Macrosynteny of teleost IMPA1 genes with tetrapod IMPA1s was very low with only the RALYL (Fig. 2) and carboxic anhydrase (CA; not shown) genes found upstream of both teleost IMPA1.1/1.2 and tetrapod IMPA1 sequences. In some higher mammals, including humans, pseudogenes for IMPA1 were also present immediately upstream of the functional IMPA1 gene, although no additional pseudogenes were detected in any lower vertebrate, including all teleosts (results not shown).
**IMP**A isoforms in tissues from FW-acclimated eels (Fig. 3) and tilapia (Fig. 4). The expression of the **IMP**A isoforms appears species-specific with the **IMP**A1.1 homolog in eel and the **IMP**A1.3 homolog in tilapia generally exhibiting highest expression across most tissues.

In the eel, the exception to this was the gonad/adipose tissue sample where both e**IMP**A1.2 and more-so e**IMP**A1.3 expression predominated. In the other eel tissues investigated, e**IMP**A1.1 mRNA expression predominated with high levels being found in the major osmoregulatory tissues (gill, kidney, esophagus), as well as the fin and skin, although much lower concentrations were found in the intestine and stomach (Fig. 3A). The spleen, skeletal muscle, and gonad/adipose tissue also exhibited relatively high levels of expression. In most tissues, IMPA isoforms were found to be expressed at comparable levels in the other species investigated.
eIMPA1.2 and eIMPA1.3 mRNA expression levels were much lower or in some cases just above consistently detectable levels (Ct values) with only the gonad and adipose tissue, skeletal muscle, and brain exhibiting levels equivalent to eIMPA1.1 (Fig. 3, B and C). Although detectable in most tissues tested, eIMPA2 mRNA expression was generally much lower than that of the eIMPA1 isoforms and predominated in brain/gonad/adipose tissue/heart/eye/intestine/skeletal muscle, with much lower levels of expression in the other osmoregulatory tissues, skin, and fin, and below detectable levels in head kidney, liver, and spleen (Fig. 3D).

The expression levels and tissue distribution of eel IMPA1 isoforms were quite different from found in tilapia. In tilapia, tIMPA1.3 mRNA predominated in all tissues tested and was especially highly expressed in brain/spleen/heart/intestine (Fig. 4C). Expression of this isoform also predominated in all osmoregulatory tissues and fin and was equivalent to that found for tIMPA1.1 in the skin (Fig. 4A). The abundance of tIMPA1.1 and 1.2 mRNAs was generally much lower in all tissues tested with the skin and brain exhibiting highest levels of expression of 1.1 and 1.2 isoforms, respectively (Fig. 4, A and B). In a number of tissues tIMPAs 1.1 and 1.2 were at, or below, detection levels. tIMPA1.4 expression could be detected, again at low levels, in most tissues with highest levels in the brain, stomach, and heart (Fig. 4D).
In Western blots using supernatant samples from selected tissue homogenates, antibodies raised against the C-terminal 17 amino acid peptide antigen from eIMPA1.1 (P3) detected immunoreactive protein bands of the expected molecular weight (31 kDa) in both eel and tilapia samples (Fig. 5, A and B, respectively). The P3 antigen shares 29% amino acid identity with eIMPA1.2, 47% identity with eIMPA1.3 and no obvious homology with eIMPA2. As qPCR results indicated that eIMPA1.1 expression was more than 10-fold higher than mRNAs for eIMPA1.2, 1.3, and 2 in most tissues examined,
the potential for cross-reactivity with these isoforms is unlikely. Tissues where cross-reactivity may potentially be a problem are the brain, stomach, intestine, skeletal muscle, and gonads and adipose tissue where mRNAs for eIMPA1.2 and 1.3 are expressed at higher or near-equivalent levels to eIMPA1.1. The low levels of immunoreactivity found in FW gonadal/adipose tissue samples using the P3 antibody (Fig. 5A), where eIMPA1.3 mRNA expression is 14-times that of eIMPA1.1, suggest that cross-reactivity is minimal. The eel anti-P3 IMPA1.1 antibody is also more likely to detect the tilapia tIMPA1.1 isoform (77% amino acid identity) than the other tilapia isoforms, in which antigen identity is much lower (35–53%). Although qPCR data suggest that the tIMPA1.3 isoform is expressed in all tissues at much higher levels than tIMPA1.1, substantial cross-reactivity with tIMPA1.3 is unlikely, as virtually no immunoreactivity was detected in Western blots from both gill and fin samples from FW-acclimated fish [where mRNA for tIMPA1.3 is at least 10-fold higher than mRNA for tIMPA1.1 (Fig. 5B)]. In addition to the expected 31-kDa tIMPA1.1 protein, Western blots processing tilapia tissue supernatants also identified two less prominent lower molecular weight bands of 28 and 24 kDa in gill and fin samples taken from some fish (Fig. 5B). The nature of these immunoreactive proteins is unknown, but they possibly represent proteolytic fragments of the tilapia IMPA1.1 protein. Similar Western blots run for most eel tissues identified a single immunoreactive protein band of the expected size of eIMPA1.1 (31 kDa). The exceptions to this were the esophagus and stomach, where an additional but less intense immunoreactive species with slightly higher molecular weight (~34 kDa) was present in most, but not all samples (Fig. 5A). Doubling dilutions of the eIMPA1.1 antibody resulted in a progressive and similar reduction in intensity of all immunoreactive proteins. As all other eel IMPA1 isoforms are also ~31 kDa in size, the nature of this immunoreactive protein is currently unknown.

Quantitative evaluation of IMPA expression in eels and tilapia following salinity transfer. In the eel, IMPA1.1 mRNA expression was increased in SW-acclimated fish in all tissues tested (gill, three-fold; kidney, 16-fold; esophagus 16-fold; stomach, 9-fold; intestine, 3-fold; fin, 1.7-fold; skin, 5.8-fold)
The expression of all tTIMPA mRNA and IMPA isoforms in tilapia was determined in gill, kidney, intestine, and fin samples taken from FW-, 50% SW-, and 100% SW-acclimated tilapia. tTIMPA1.1 mRNA levels were significantly upregulated in gill and fin samples from fish acclimated to 50% SW, as well as in all tissue samples taken from 100% SW-acclimated fish, with expression levels increased by ~90%-100% SW-acclimated group (Fig. 7A). SW acclimation resulted in significant decreases in tTIMPA1.2 mRNA expression in the gill, kidney, intestine, skin, and gonad and adipose tissue following SW transfer, although no significant changes in protein expression were found in either the esophagus or fin (Fig. 5C).

The expression of all tTIMPA isoform mRNAs was determined in gill, kidney, intestine, and fin samples taken from fish acclimated to 50% SW, with expression levels increased by ~90%, 10-, 20-, and 100-fold, respectively, for gill, kidney, fin, and intestine in the 100% SW-acclimated group (Fig. 7A). tTIMPA1.1 mRNA levels were significantly upregulated in gill and fin samples from fish acclimated to 50% SW, as well as in all tissue samples taken from 100% SW-acclimated fish, with expression levels increased by ~90%-100% SW-acclimated group (Fig. 7A). tTIMPA1.1 mRNA levels were significantly upregulated in gill and fin samples from fish acclimated to 50% SW, as well as in all tissue samples taken from 100% SW-acclimated fish, with expression levels increased by ~90%-100% SW-acclimated group (Fig. 7A). tTIMPA1.1 mRNA levels were significantly upregulated in gill and fin samples from fish acclimated to 50% SW, as well as in all tissue samples taken from 100% SW-acclimated fish, with 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Fig. 7. Quantitative PCR analyses of IMPA isoform mRNAs in tissues from FW-, 50% SW-, and 100% SW-acclimated tilapia. The relative expression of tIMPA isoform mRNAs: tIMPA1.1 (A), tIMPA1.2 (B), tIMPA1.3 (C), and tIMPA1.4 (D), was determined from Ct values obtained from selected tissues removed from FW- (stippled bars) 50% SW- (gray bars), and 100% SW- (black bars) acclimated fish and normalized to Rpl-p0 mRNA expression, as detailed in MATERIALS AND METHODS. A: expression values near zero appear on the ordinate scale and are indicated at the top of the bars. Results are expressed as means ± SE of duplicate measures taken from tissue samples extracted from six different fish. *P < 0.05; **P < 0.01.

Fig. 8. IMPA enzyme activity in FW- and SW-acclimated tilapia gill and fin. IMPA enzyme activities represent the magnesium-dependent, lithium-sensitive phosphate release in supernatant samples from the gill and pectoral fin of FW- and SW-acclimated tilapia. Results indicated are the means ± SE of triplicate measures taken from tissue samples extracted from six different fish. *P < 0.05.

DISCUSSION

The enzymes and transporters involved in the metabolism of inositol have critical roles in the regulation of both the intra- and extracellular concentrations of this key cyclic alcohol that has major functions in many biological processes, including cell-cell signaling (3) and cell volume regulation (5). Although osmoregulatory roles for inositol have been documented across many animal and plant species, it is only recently that this organic osmolyte has been implicated in osmoregulation in a restricted number of euryhaline teleosts (13, 14, 22, 23). Euryhaline teleost fish are capable of movement between habitats with wide-ranging salinity, and SW acclimation is accompanied by a myriad of molecular, physiological, cellular, and behavioral changes, which are essential for successful adaptation not only to the increased salinity (19) but also to the possibility of temperature change (21), as well as to exposure to a different cocktail of environmental toxins and pathogens (10). The role that organic osmolytes play in the adaptive responses to higher salinity and other environmental stresses has only recently come under investigation. It is
possible that successful adaptation following SW transfer is dependent on rapid changes in expression or activity of the enzymes and transporters required for the synthesis and accumulation of these essential metabolites.

A number of IMPA isoforms have been reported in many different organisms. In mammals, the expression and functions of two isoforms (IMPA1 and IMPA2) have been reported that exhibit slightly different tissue distributions and enzyme characteristics (37). In mammals, the IMPA1 isoform generally exhibits a specific and more widespread tissue distribution with higher expression levels than IMPA2 (37). Previous studies from our laboratory have shown that the expression of an eel IMPA1 homolog (eIMPA1.1) is upregulated in a number of tissues following SW transfer, and this has highlighted a potential osmoregulatory role for inositol in salinity adaptation (22, 23). Current investigations have resulted in the identification of three further IMPA isoforms in the eel, two additional eIMPA1-like isoforms (designated eIMPAs 1.2 and 1.3), and one eIMPA2-like isoform. In the eel, it is the eIMPA1.1 homolog that appears to be ubiquitously distributed and exhibit-

Table 2. Plasma osmolalities and chloride concentrations in FW- and SW-acclimated eels and tilapia

<table>
<thead>
<tr>
<th></th>
<th>Plasma Osmolality mOsmol/kg</th>
<th>Plasma [Cl\textsuperscript{-}] mM</th>
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<tbody>
<tr>
<td>Eel</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW</td>
<td>299.1 ± 3.8</td>
<td>94.2 ± 2.7</td>
</tr>
<tr>
<td>SW</td>
<td>353.6 ± 3.5***</td>
<td>138.9 ± 2.3***</td>
</tr>
<tr>
<td>Tilapia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FW</td>
<td>359.0 ± 4.9</td>
<td>140.3 ± 1.6</td>
</tr>
<tr>
<td>SW</td>
<td>442.1 ± 10.7***</td>
<td>180.0 ± 5.5***</td>
</tr>
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</table>

Values are expressed as means ± SEs for \( n = 8 \) fish. FW, freshwater; SW, seawater. ***\( P < 0.001 \).
its the highest levels of mRNA expression in most tissues, including the gill, kidney, GI tract, skin, and fin. Genetic analysis has indicated that up to four IMPA1-like isoforms can exist in other teleosts, including tilapia, where it is the tIMPA1.3 isoform that exhibits the highest and widest tissue expression profile. Much lower levels of expression were found with the other tilapia isoforms, which exhibited more tissue selectivity (e.g., tIMPA1.1 found mainly in the skin, tIMPA1.2 found mainly in the brain and tIMPA1.4 mainly in brain, heart, and stomach). Phylogenetic analysis has mapped the teleost IMPA1 homologs into four distinct groups with similar evolutionary distance from the IMPA1 clade of the higher vertebrates. Syntenic analysis has determined that teleost IMPAs 1.1 and 1.2 and also IMPAs 1.3 and (where present) 1.4 are juxtaposed within fish genomes. This suggests that subsequent to the two rounds of whole genome duplication (WGD) that gave rise to IMPA1 and IMPA2 in all vertebrate species (43), the IMPA1 gene in the teleost lineage underwent a tandem duplication event prior to the third WGD event that was restricted to the ray-finned fishes (20). Therefore, there is the potential for up to six different IMPA isoforms to be present within teleost genomes.

From the limited information available, it would appear that most teleost species have lost one or more IMPA isoforms during evolution resulting in the isoform distribution presented in Fig. 1. IMPA1.4 isoforms were only found in tetraodon and tilapia genomes, whereas IMPA2 isoforms were only identified in the eel, salmon, and zebrafish. The zebrafish genome has retained only one IMPA1 isoform, with gene synteny suggesting evolutionary association with both IMPA1.1/1.2 and IMPA1.3/1.4 genes, although phylogenetic analysis groups this isoform with teleost IMPA1.3s. Phylogenetic analyses together with assessment of amino acid sequence homologies suggest that the IMPA1 homologs of the evolutionary more ancient fishes (the eel, salmon, rainbow trout, and zebrafish) have collectively retained more homology than the IMPA1 isoforms from the other, more evolutionary modern species. Indeed, the IMPA1.3 isoforms from the more ancient fishes appear to group closer to the cluster of IMPA1.1 isoforms than the IMPA1.3 isoforms of the more modern fishes that form a distinct subgroup with slightly lower homology. Although it has been reported that teleost genomes have evolved at a much greater rate than those of most other vertebrates (26), these results suggest that the rates of genome remodeling may be highest in the more modern fishes. This may also explain why IMPA2 isoforms have been retained only in the more modern fish lineages. As also suggested in other vertebrate species, a number of IMPA spliceforms are also predicted in teleost databases and in zebrafish, one potential transcript comprises exons from both IMPAs within teleost genomes. Therefore, there is the potential for up to six different IMPA isoforms to be present within teleost genomes.

Sequence analysis has indicated that IMPA isoforms from all teleost species have retained most of the amino acids known to contribute to the binding and three-metal ion catalysis of inositol phosphate, including Asp47, Glu70, amino acids 90 to 100, Glu213, and Asp220 (human IMPA1 sequence; highlighted in red in Supplemental Fig. S1) (2, 23, 29, 44). Although all amino acids involved in the catalytic mechanism are retained, amino acids associated with substrate binding have mutated in some teleost isoforms. One notable difference

Table 3. Osmolyte contents of gill, fin, and kidney (eel only) from FW- and SW-acclimated eels and tilapia

<table>
<thead>
<tr>
<th>Tissue</th>
<th>FW SW</th>
<th>FW SW</th>
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<th>FW SW</th>
<th>FW SW</th>
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</thead>
<tbody>
<tr>
<td>Gomphostoma morio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Gill</td>
<td>2.91</td>
<td>0.62</td>
<td>5.25</td>
<td>1.10</td>
<td>0.86</td>
<td>23.50</td>
<td>5.19</td>
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<tr>
<td>Fin</td>
<td>2.96</td>
<td>2.33</td>
<td>2.81</td>
<td>2.77</td>
<td>2.91</td>
<td>2.48</td>
<td>0.23</td>
</tr>
<tr>
<td>Kidney</td>
<td>1.40</td>
<td>0.51</td>
<td>3.61</td>
<td>1.00</td>
<td>0.25</td>
<td>1.50</td>
<td>0.87</td>
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Tilapia

<table>
<thead>
<tr>
<th>Tissue</th>
<th>FW SW</th>
<th>FW SW</th>
<th>FW SW</th>
<th>FW SW</th>
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<tbody>
<tr>
<td>Gill</td>
<td>0.87</td>
<td>0.25</td>
<td>0.98</td>
<td>0.98</td>
<td>0.25</td>
<td>1.54</td>
<td>0.25</td>
</tr>
<tr>
<td>Fin</td>
<td>0.98</td>
<td>1.94</td>
<td>2.77</td>
<td>3.35</td>
<td>1.94</td>
<td>3.35</td>
<td>0.98</td>
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<tr>
<td>Kidney</td>
<td>0.98</td>
<td>0.51</td>
<td>3.61</td>
<td>1.00</td>
<td>0.25</td>
<td>1.50</td>
<td>0.87</td>
</tr>
</tbody>
</table>

Caudal fin was sampled from the eel, and the pectoral fin was sampled from tilapia. Betaine levels in all eel samples were very low and just at the detection limits of the analyzer and are, therefore, reported as “trace.” Values are presented as mmol/kg wet wt tissue, and are means ± SDs for n = 6 fish. P < 0.05, † P < 0.01, ‡ P < 0.001.
in teleost IMPA1.2 and 1.4 isoforms is the presence of a histidine residue at position 213 rather than glutamic acid (highlighted in blue in Supplemental Fig. S1). Structural modeling of the IMPA enzyme active site suggests that the glutamic acid at this position (and the equivalent glutamine residue at the equivalent position 224 in IMPA2; highlighted in green in Supplemental Fig. S1) contributes to the binding and stabilization of the inositol monophosphate (IP) moiety at the active site by forming H-bonds with OH groups on carbon atoms 4 and 5 of the polyol ring (15, 29). Although histidine is still capable of forming H-bonds with inositol, the exchange of an acidic side chain for a basic and more rigid imidazole group is likely to alter the binding kinetics with IP and other potential substrates. A serine residue at position 165 (highlighted in lilac in Supplemental Fig. S1), conserved in all vertebrate IMPA1 isoforms so far reported, is also implicated in stabilizing IP binding by H-bonding with the OH group at position 3 in the inositol ring. In teleost IMPA1.1 and IMPA1.3 isoforms, this amino acid is either retained or mutated to the compatible threonine residue (also highlighted in lilac in Supplemental Fig. S1), whereas hydrophobic residues occupy this position in teleost IMPA1.2 and IMPA1.4 isoforms (highlighted in cyan in Supplemental Fig. S1). This serine residue is also lost in all vertebrate IMPA2 isoforms, where it is replaced by alanine in the teleosts and by a proline in all other species (again highlighted in cyan in Supplemental Fig. S1). The change of serine 165 to proline may help explain the differences in substrate specificity reported between mammalian IMPA1 and IMPA2 enzymes (37) and likewise, the change to alanine, leucine, or phenylalanine in teleost IMPA1.2, IMPA1.4, and IMPA2 isoforms may also result in substrate and/or functional adaptations. Indeed, given these two amino acid changes near the active site, it is not clear whether inositol phosphate will still be the preferred physiological substrate for teleost IMPA1.2 and IMPA1.4 isoforms. The significance of this and other sequence differences on structure and function of the IMPA isoforms remains to be determined.

In previous work on the eel, qPCR, Western blot analysis and immunohistochemical methods indicated that eIMPA1.1 expression was increased in the epithelial tissues of the gill, esophagus, and anterior intestine, as well as throughout the nontubular interstitial cells of the renal kidney, the basal epithelial cells of the squamous epithelium of fin and skin, and in the branchial and fin ray chondrocytes, following SW acclimation (22, 23). However, the extent of any SW-induced increases in eIMPA1.1 expression is still somewhat uncertain in the esophagus and fin. Although earlier investigations detected 20-fold increases in eIMPA1.1 mRNA expression, and immunohistochemical studies indicated marked increases in eIMPA1.1 immunoreactive protein in the basal epithelial layers in the esophagus of SW-acclimated fish (23), in the present study, no significant increases were detected in eIMPA1.1 protein expression in Western blot analyses. Similarly in the fin, no significant increases in eIMPA1.1 protein were detected in Western blots; yet previous immunohistochemical analyses (23) suggested substantial increases in eIMPA1.1 protein expression were present in the basal epithelial layers and in the fin ray chondrocytes of SW-acclimated fish. The reasons for the apparent lack of consistency in protein expression in the Western/immunohistochemical analyses in the esophagus and fin are unknown. Additional immunohistochemical studies with eel tissues indicated that eIMPA1.1 expression was present in single basal epithelial cells underlying the columnar epithelium of the stomach and also within blood vessel endothelial cells, which were particularly highlighted in vessels deep within the stomach epithelium and throughout the gonad/adipose tissue sections. Therefore, unlike that previously seen in the eel intestine (23), the large columnar epithelial cells of the stomach do not appear to express high levels of eIMPA1.1 despite the salinity of the imbibed SW in the stomach being at least equivalent to that found in the anterior parts of the intestine. The detection of eIMPA1.1 protein expression in vascular endothelial cells, and the increased abundance following SW transfer, suggests that blood vessels may be a source of plasma inositol in SW-acclimated eels.

Increases in IMPA expression have previously been linked to salinity adaptation in the euryhaline goby (Gillichthys mirabilis) (13), and the enzyme has also been associated with the increased tissue inositol concentrations found in various tissues from SW-acclimated tilapia (Oreochromis mossambicus) (14). The studies described in this paper further support these findings and suggest that salinity-induced increases in tissue IMPA1.1 expression and inositol contents are not confined to the eel, as similar and even larger increases in these parameters were found tilapia acclimated to either 50% or 100% SW. Although q-PCR analyses indicates that the tiMPA1.3 isoform is expressed at 15–50 × higher levels than the tiMPA1.1 isoform in most tissues from FW-acclimated fish, it is the latter isoform that is up-regulated following transfer of fish to SW. Despite the fact that salinity-induced increases in tiMPA1.1 mRNA expression are of the order 25 to 90-fold across the tissues tested; the major osmoregulatory tissues still express relatively high levels of tiMPA1.3 mRNA, and therefore it is likely that the tiMPA1.3 isoform will make a substantial contribution to intracellular inositol production. Consequently, even although SW-acclimation appears to have no effect on tiMPA1.3 mRNA expression, the functional role of tiMPA1.3 in cell volume regulation and osmoregulation in SW-acclimated fish remains to be established.

As found for tiMPA1.1, the levels of tiMPAs 1.2 and 1.4 are generally much lower than tiMPA1.3 in FW-acclimated fish. Following SW transfer the already low tiMPA1.2 mRNA levels in the gill are increased 2-fold suggesting that this isoform may also have a potential, although probably minor, role in inositol production and osmoregulation in SW-acclimated fish. Perhaps unexpectedly, in both eel and tilapia the expression of some IMPA isoforms decreased following SW transfer. The levels of tiMPA1.4 mRNA in gill, fin and intestine decreased by up to 60% and levels of tiMPAs 1.3 and 1.4 fell by up to 94% in a number of tissues following SW transfer. The reasons for this are unclear, however, the results may indicate that the increased expression of eel or tilapia IMPA1.1 may partially fulfill the roles of these other isoforms when fish are in SW. Indeed the tiMPA1.4 isoform is not present in most of the teleost species for which we have genomic information, indicating that any role that it may have can be accommodated by other isoforms. No significant changes were found in the expression of any other tilapia isoforms in any tissue tested.

The increases in protein expression of tiMPA1.1 in gill and fin were accompanied by respective 6- and 16-fold increases in measured enzyme activities in tissue supernatant samples. Although these fold-changes are not as large as seen with the
increases in tIMPA1.1 protein as determined by the Western blots, they are equivalent to that previously reported for the eel (23) and probably reflect the presence of other tIMPA isofoms proteins (and particularly tIMPA1.3) in FW-acclimated fish. These results are consistent with a recent report that two transcripts of a tilapia IMPA1 gene were upregulated in the branchial epithelia of fish exposed to salinities of up to 90 ppt (40). The same group reported the presence of another tilapia tIMPA transcript (designated tIMPA2), the expression of which was not affected by environmental salinity; however, the sequence of this and the other tIMPA1 isoforms were not presented. Taken together, these results indicate that, like that found in the eel, it is predominantly the tIMPA1.1 isoform that exhibits salinity-regulated expression and, therefore, is likely to be responsible for the required synthesis of inositol as an osmolyte in the major osmoregulatory tissues, as well as the skin and fin following SW transfer. It is interesting to note that the only stenohaline FW fish for which we have genomic information available, the zebrafish, does not appear to express an IMPA1.1-like isoform, suggesting that this particular gene may be a prerequisite for SW acclimation.

Again, like that found previously in the eel (23), tIMPA1.1 immunoreactivity was located to the nontubular interstitial cells within the kidney, as well as in the squamous epithelial cells of skin and fin in which the highest levels were found within the basal epithelial cell layers of fish acclimated to SW. The high levels of tIMPA1.1 immunoreactivity found within the basal epithelial cell layers of the skin, fin, and esophagus of SW-acclimated fish is gradually reduced in the supraepithelial layers, and immunoreactivity is totally absent in the mesenchymal cells below the basal lamina and within the deeper dermal layers. This suggests that this basal stem cell layer is responsible for the generation of the majority of IMPA expressed and, therefore, potentially the inositol accumulated within the dermal and epidermal tissues of skin, fin, and esophagus. If, and how, the inositol is subsequently distributed to the other cells within the dermis is unknown and awaits future investigations. Slight differences in IMPA1.1 expression between the two fish species were apparent, however. Unlike the eel, immunoreactivity was not universally distributed throughout all tilapia renal interstitial cells, but it was confined to clusters of cells, or in some cases a few single cells, usually juxtaposed to the glomeruli and larger tubules. No immunoreactivity was apparent in the chondrocytes in tilapia gill or fins in which high levels of immunoreactivity were detected previously in the eel. Therefore, tilapia chondrocytes appear not to act as a specialized tissue source of inositol for the branchial and systemic circulations, as suggested in the eel (23).

In tilapia, the increase in tissue inositol content following SW acclimation were much greater than those seen with other well-known osmolytes, such as taurine and glycero-phosphocholine, and are consistent with previous reports (14), indicating that inositol may be the principal organic osmolyte used for cell volume regulation in this euryhaline teleost. Although increases in eIMPA1.1 expression and cellular inositol levels were not as prominent in eels following SW transfer, the results still suggest that the upregulation of IMPA expression and activity may be important in all euryhaline teleosts, and a prerequisite for successful adaptation to hypersaline environments. It should be noted that the changes in inositol (and other osmolyte) contents reflect the average concentrations across all the cells extracted from the gill, kidney, or fin tissue. On the basis of the cellular distribution of IMPA1.1 from the immunohistochemical analyses, it is highly likely that intracellular inositol levels will show a similar asymmetrical distribution, with concentrations being several-fold (and potentially several orders of magnitude) higher within the peripheral epithelial cell layers directly exposed to the SW environment. In both eel and tilapia, the ubiquitous expression of IMPA1.1 suggests that all branchial epithelial cells are capable of synthesizing inositol for osmoregulation. Previous studies in the eel have suggested that another organic osmolyte, taurine, may have a cell-specific osmoregulatory role in the branchial epithelium, as the sodium-dependent taurine transporter Tau-T is selectively expressed primarily in the mitochondria-rich cells following SW transfer (8, 9). This may explain the modest increase in the levels of this metabolite found in the SW-acclimated eel gill. A number of amino acids, and especially glutamine, have been shown to increase in the plasma, muscle, and liver of the swamp eel (Monopterus albus) following transfer to brackish (25 ppt) water (45). Indeed, the tissue osmolyte analyses in this study indicate that a number of other organic metabolites, including the amino acid glycine, are likely to be important for successful osmoregulation in SW-acclimated fish, and particularly so in the tilapia where nearly all metabolites determined exhibited substantial increases in concentration.

Perspectives and Significance

These studies have shown that as a result of tandem duplication and whole genome duplication events, up to four IMPA1 and two IMPA2 genes can be found in teleost genomes. The tissue expression of IMPA1 isoforms appears species-specific with IMPA1.1 predominating in tissues from the eel, whereas IMPA1.3 is the main isoform expressed in tilapia. Salinity challenge experiments have determined that, in both species, it is primarily the IMPA1.1 isoform that is upregulated in a variety of epithelial, epidermal, and interstitial cells within the major osmoregulatory tissues, skin, and fin of SW-acclimated fish. Immunohistochemical analyses indicate that in increases in IMPA1.1 isoform expression are restricted to specialized cell types within the tissues, and particularly to epithelial cell layers in gill, fin, and skin, which are directly exposed to the SW environment. The increases in IMPA1.1 expression are paralleled by increases in inositol content of the tissues, which presumably have the same cellular distribution as IMPA1.1 expression. The differences found between the eel and tilapia with respect to expression of IMPA isoforms (and especially in FW-acclimated fish) may reflect the difference in osmoregulatory plasticity of some euryhaline fish, which can survive both acute and large changes in environmental salinity (such as Anguilla anguilla) and those that can only adapt more slowly to more limited and gradual increases in environmental osmolality (such as Oreochromis niloticus). Alternatively, the differences in gene expression and tissue inositol contents may reflect the variable experimental acclimation periods used for the two species, and the findings may be a consequence of temporal changes in gene expression that are inherent in a universal adaptive response. Further experiments will be required to determine the full nature of any species-specific characteristics of salinity-induced regulation in IMPA isoform expression and inositol metabolism in teleost fish.
It is likely that similar osmoregulatory mechanisms exist in other euryhaline species, as well as stenohaline marine fish exposed to SW environments. Increases in cytosolic inositol concentrations resulting from increases in the intracellular production of the osmolyte could be responsible for the osmotic retention of water within the kidney, the protection of peripheral epithelial, and epidermal cells from the dehydrating effects of the aquatic SW environment and the maintenance of cell growth and cell division under hypertonic conditions.

ACKNOWLEDGMENTS
We would like to thank Jill McVee for technical support associated with the immunohistochemical analyses.

GRANTS
The work was funded by a research grant awarded to GC and NH by the Natural Environment Research Council (NE/J010081/1). SAG is a recipient of a School of Medicine PhD Studentship.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


