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Ionoregulatory changes during metamorphosis and salinity exposure of juvenile sea lamprey (*Petromyzon marinus* L.)

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SUMMARY

Ammocoetes of the anadromous sea lamprey *Petromyzon marinus* L. spend many years in freshwater before metamorphosing and migrating to sea. Metamorphosis involves the radical transformation from a substrate-dwelling, filter feeder into a free-swimming, parasitic feeder. In the present work we examined osmoregulatory differences between ammocoetes and transformers (metamorphic juveniles), and the effects of salinity acclimation. We measured the expression of key ion-transporting proteins [Na^+/K^+-ATPase, vacuolar (V)-type H^+-ATPase and carbonic anhydrase (CA)] as well as a number of relevant blood parameters (hematocrit, $[Na^+]$ and $[Cl^-]$). In addition, immunofluorescence microscopy was used to identify and characterize the distributions of Na^+/K^+-ATPase, V-type H^+-ATPase and CA immunoreactive cells in the gill. Ammocoetes did not survive in the experiments with salinities greater than 10‰, whereas survival in high salinity (≥25–35‰) increased with increased degree of metamorphosis in transformers. Plasma $[Na^+]$ and $[Cl^-]$ of ammocoetes in freshwater was lower than transformers and increased markedly at 10‰. In transformers, plasma ions increased only at high salinity (>25‰). Branchial Na^+/K^+-ATPase levels were ~ tenfold higher in transformers compared to ammocoetes and salinity did not affect expression in either group. However, branchial H^+-ATPase expression showed a negative correlation with salinity in both groups. Na^+/K^+-ATPase immunoreactivity was strongest in transformers and associated with clusters of cells in the interlamellar spaces. H^+-ATPase (B subunit) immunoreactivity was localized to epithelial cells not expressing high Na^+/K^+-ATPase immunoreactivity and having a similar tissue distribution as carbonic anhydrase. The results indicate that branchial Na^+/K^+-ATPase and salinity tolerance increase in metamorphosing lampreys, and that branchial H^+-ATPase is downregulated by salinity.

Key words: Ammocoete, transformer, Na^+/K^+-ATPase, vacuolar (V)-type H^+-ATPase, lamprey, ionoregulation.

INTRODUCTION

The anadromous sea lamprey *Petromyzon marinus* Linnaeus 1758 migrates twice during its life cycle between freshwater and seawater. Larval microphagous ammocoetes generally spend 3–7 years in the substrate of rivers and streams before undergoing a period of metamorphosis with marked morphological and physiological changes. In approximately 3 months they radically transform from a substrate-dwelling, filter feeder into a free-swimming, parasitic feeder and migrate downstream to the sea. In the marine environment sea lampreys experience a period of rapid growth and after 2 years adults re-enter freshwater and migrate upstream, where spawning and death occurs (Hardisty and Potter, 1971a; Beamish, 1980a).

As larvae of sea lampreys metamorphose into young adults a total of seven stages can be identified, based on several external morphological characteristics (Youson and Potter, 1979). The appearance and differentiation of the eye (up until stage 3), the detection and development of the tongue-like piston (beginning at stage 4), changes in body colouration, i.e. a progressive blue darkening of the dorsal and silvering of the ventral surfaces (occur with stage 5), and the shape and cornification of the teeth (stages 6 and 7) are some of the milestones and external criteria used to stage sea lampreys.

Early craniates such as hagfish are osmoconformers that are found only in marine habitats (Hardisty, 1979). Lampreys are the earliest extant vertebrate to have adopted an osmoregulatory strategy. As in teleost fish, the gills, gut and kidney are thought to be the main effector organs involved in the active regulation of salt and water balance, although firm data are scarce (Beamish, 1980b; Bartels et al., 1998). In teleost fishes, the Na^+/K^+-ATPase and V-type H^+-ATPase are the main drivers of active transepithelial ion movements in the gill. In freshwater, an apical H^+-ATPase drives Na^+ uptake *via* Na^+ channels and possibly Cl^- uptake *via* a Cl^-/HCO_3^- exchanger. The Na^+/K^+-ATPase drives secondary active Cl^- elimination in marine fishes and also has a role in Na^+ uptake in freshwater fishes (Evans et al., 2005; Marshall, 2002). In a number of fishes apical Na^+/H^+ exchangers (NHE 2 or 3) have been implicated in Na^+ uptake and acid–base regulation (Evans et al., 2005).

The cellular composition of the gill epithelium of lampreys differs from that of teleosts, particularly in freshwater (Bartels et al., 1998; Bartels and Potter, 2004; Choe et al., 2004). Detailed studies have described and located three types of mitochondria rich cell (MRC) in lamprey gill (Bartels et al., 1998; Bartels and Potter, 2004). The ammocoete MRC is present exclusively in the larval phase, the freshwater MRC (also referred to as the intercalated MRC, IMRC

or ICC) is present in ammocoetes and adults, and the third seawater type MRC (swMRC) is found only in seawater-adapted lampreys and downstream migrant transformers. The swMRC is also commonly referred to as a chloride cell (CC) due to morphological similarities with teleost CC. The latter cells are disc shaped and form a continuous row in the interlamellar region and possess a well-developed tubular system, which is an amplification of the basolateral membrane. In marine teleosts the tubular system is associated with Na^+/K^+-ATPase, and it is assumed that analogous activity occurs in lamprey CC and that they are responsible for secreting excess Na^+ and Cl^- in hypertonic environments.

Lamprey gill IMRCs are generally confined to the filament epithelium and occur singly at the base and between lamellae (interlamellar space) (Bartels et al., 1998). They are intercalated between ammocoete MRC pavement cells in larvae, CC and/or pavements in downstream juvenile migrants and marine residents and pavement cells in adult upstream migrants. Immunological and morphological studies have highlighted the presence of apical H^+-ATPase in IMRC cells as well as cytosolic carbonic anhydrase (CA) in adult freshwater Australian lamprey *Geotria australis*, which is consistent with a role of these cells in ion uptake (Choe et al., 2004). Freeze fracture microscopy has revealed rod shaped particles in the apical membrane of these cells that are similar to the V_1 domain of the H^+-ATPase complex (Bartels et al., 1998). In contrast, Na^+/K^+-ATPase has been immunolocalized to a separate cell type proposed to be a new IMRC type in freshwater *G. australis* (Choe et al., 2004).

The functional importance of the ammocoete MRC is still unknown, and may have a purpose other than osmoregulation. Considering that adult lampreys osmoregulate well in freshwater without feeding during this period and that these cells are only present in freshwater feeding ammocoetes, their involvement in ion and waste product excretion from food digestion in larval stages has been proposed (Bartels et al., 1998).

Before metamorphosis, ammocoetes are unable to osmoregulate in water with an osmolality exceeding that of their serum (Morris, 1980; Beamish et al., 1978). Once metamorphosis is complete, young downstream juvenile migrants of *P. marinus* are fully tolerant to 35‰ seawater (Beamish et al., 1978; Beamish, 1980b). However, the mechanisms of ion regulation in lampreys are still not well understood. Large gaps exist in the understanding of the ontogeny of ion regulation, namely on the regulation and development of salinity tolerance during metamorphosis. Osmotic regulation with metamorphosis has only been analyzed by Mathers and Beamish, who measured serum osmolality variations in landlocked *P. marinus* (Mathers and Beamish, 1974). Others have looked at osmoregulation in either ammocoetes or adult sea lampreys (Mathers and Beamish, 1974; Beamish et al., 1978; Beamish, 1980b; Morris, 1980), but studies on the expression of ion transport proteins are limited to freshwater spawning adults of *G. australis* (Choe et al., 2004).

The present study focused on the ionoregulatory changes that occur during metamorphosis in sea lampreys. Our aims were to examine the effects of salinity acclimation in ammocoetes and metamorphic larvae, both before and during transformation in order to characterize the osmoregulatory ability in different stages of metamorphosis and to detect developmental differences during transformation in the expression of key branchial ion transport proteins. This was done using *in vitro* activity measurements and western blot analysis. Immunohistochemical techniques were also applied to identify and characterize the distributions of Na^+/K^+-ATPase, V-type H^+-ATPase and CA immunoreactive cells in the gill. Achieving these goals will provide evidence for the mechanisms involved in gill ion transport in juvenile stages of the anadromous sea lamprey.

MATERIALS AND METHODS

Animals

Ammocoetes and metamorphic larvae of sea lamprey *Petromyzon marinus* Linnaeus 1758 were collected either by electrofishing or by hand from the exposed surface of the substrate at the Turners Falls or Holyoke power canals in the Connecticut River (MA, USA), while the water level was lowered for dam maintenance during the summer and autumn of 2004. Lampreys were transported to the laboratory where they were maintained in flow through tanks with Connecticut River water and a sandy silt substrate for burrowing. They were acclimated to laboratory conditions for at least 2 weeks prior to experimentation and larvae were fed a suspension of yeast once a week. Lampreys were also collected by electrofishing from the Fort River, a tributary of the Connecticut River (Hadley, MA, USA) in November 2004 for direct sampling in the field.

Experimental series

Three separate experimental designs were used. Groups of ammocoetes (pre-metamorphic larvae) and transformers (metamorphic larvae) in stages 3–5 [according to Youson and Potter (Youson and Potter, 1979)] were directly transferred to freshwater 0‰ (FW), brackish water 25‰ or full strength seawater 35‰ (SW) for 2 weeks (Experiment I). The second experiment consisted of field sampling ammocoetes and transformers collected from the Fort River (Experiment II). In the third experiment, ammocoetes and late stage transformers, stages 6 and later [according to Youson and Potter (Youson and Potter, 1979)] were acclimated for 2 weeks, after direct transfer, to either deionized water (DW), freshwater (FW), 10‰, 20‰, 30‰ or 35‰ (SW) (Experiment III).

In each experiment groups of eight ammocoetes or transformers were maintained in separate aquaria with aeration and filtration and partial water changes were made every 2 or 3 days. Dechlorinated tapwater (freshwater) was used during all experiments and seawater was produced by dissolving synthetic sea salt (Instant Ocean, Aquarium Systems Inc., Mentor, OH, USA) in dechlorinated tapwater. Deionized water was prepared using cation and anion exchange resins (Barnstead Model 04741, Boston, MA, USA). Conductivity in the deionized water aquaria was measured daily and kept below 12 μS during the course of the experiment. Temperature was kept constant at 15°C, water parameters and mortalities were monitored daily and animals were not fed during the experiments. All experiments were carried out in accordance with USGS-IACUC guidelines.

Sampling

At the end of the 2-week acclimation period animals were terminally sampled by overdosing with ethyl-m-amino benzoate (MS-222; Argent Chemical Laboratories, Redmond, WA, USA). Animals were measured, to the nearest mm (total length), weighed (±0.01 g) and the tail cut off in order to collect blood samples from the caudal vessels with heparinized capillary tubes. Following centrifugation at 5000 ***g*** for 5 min at 4°C, hematocrit was recorded and plasma stored at –80°C. Fulton's condition factor (K) was calculated as $K=1000$ [wet mass (in g)$\times$length^{-b} (in cm)], where b is the regression slope coefficient between mass and length (Bagenal and Tesch, 1978). Plasma Na^+ and Cl^- concentrations were measured by atomic absorption spectrophotometry (AAnalyst 100, Perkin

Elmer, Wellesley, MA, USA) and with a digital chloridometer (Labconco, Kansas City, MO, USA), respectively.

Gill pouches from each fish were excised and (1) placed in 100 μl SEI buffer (300 mmol l^{-1} sucrose, 20 mmol l^{-1} EDTA, 50 mmol l^{-1} imidazole, pH 7.5) and frozen at –80°C; (2) fixed in 3% PFA/phosphate buffered saline (PBS) at 4°C for 24 h and stored in 70% ethanol at 4°C and (3) fixed in 20% DMSO/methanol at –20°C for 48 h and transferred to 100% methanol at –20°C for storage. Fixed tissues were processed for paraffin embedding (type 6; Richard-Allan Scientific, Kalamazoo, MI, USA).

Gill Na^+/K^+-ATPase activity measurement

Gill Na^+/K^+-ATPase activity was measured *via* a kinetic microassay at 25°C (McCormick, 1993) using a THERMOmax microplate reader and SOFTmax software (Molecular Devices, Sunnyvale, CA, USA). Preliminary tests were made to optimize salt concentrations and sample dilutions for lamprey as previously outlined (McCormick and Bern, 1989). These tests resulted in peak activity occurring at the same ion concentrations as used for salmonids and transformer samples were diluted with an extra 150 μl of 1× SEID.

Samples stored in 100 μl SEI buffer were thawed on ice, sodium deoxycholate added to a final concentration of 0.1%, and homogenized using a motorized pestle. Homogenates were centrifuged at 3200 ***g*** for 30 s at 4°C and the supernatant decanted and used for the ATPase assay and immunoblotting experiments. Samples of 10 μl were run in two duplicate sets. In one set ouabain (0.5 mmol l^{-1}) was added to the assay mixture to specifically inhibit Na^+/K^+-ATPase activity. Total protein was measured using the bicinchoninic acid protein assay (BCA) with a bovine serum albumin (BSA) standard. Resulting ouabain-sensitive ATPase activity measurements were expressed in μmol ADP mg^{-1} protein h^{-1}.

Immunoblotting

The remaining homogenates (~50 μl) from the ATPase and protein assays were diluted with an equal volume of 2× Laemmli's buffer (Laemmli, 1970), vortexed and heated for 15 min at 70°C and then stored at – 20°C. Prior to loading on to gels, samples were thawed, the protein concentrations adjusted to 0.5 μg ml^{-1}, vortexed and centrifuged at 10 000 ***g*** for 5 min. Samples (20 μg per well) were loaded onto 1.5 mm thick mini vertical polyacrylamide gels (10% T resolving gels with 4% T stacking gels) and run at 150 V using a BioRad (Hercules, CA, USA) MiniProtean III system. The gels were equilibrated in transfer buffer (48 mmol l^{-1} Tris, 39 mmol l^{-1} glycine, 0.0375% SDS) and the protein bands were transferred to PVDF membranes (Hybond-P, GE Healthcare, Carnaxide, Portugal) using a semi-dry transfer apparatus for 1 h at 13 V (BioRad). The membranes were rinsed in TTBS (0.05% Tween-20 in Tris-buffered saline, pH 7.4) and blocked with 5% powdered skim milk in TTBS for 1 h. Following rinsing in TTBS, membranes were probed with either the α5 or αRbNKA (α subunit of the Na^+/K^+-ATPase), B2/BvA1 (H^+-ATPase B subunit), or CAIIb (carbonic anhydrase) antibodies diluted 1:1000 in Chemicon antibody dilution buffer overnight at 4°C. After rinsing with TTBS, membranes were incubated with either a goat anti-mouse or anti-rabbit HRP-conjugated secondary antibody (Sigma Chemical Co., St Louis, MO, USA) diluted 1: 20 000 v/v in TTBS for 1 h at room temperature. Membranes were then rinsed again with TTBS and the signal detected by ECL (GE Healthcare) using Kodak BioLight-1 film. The film was scanned (Agfa T1200) and band intensity was semi-quantified using an image analysis software program (SigmaScan Pro 5.0, SPSS Chicago, IL, USA). Since additional bands were detected with the B2/BcA1 antibody, preabsorption of this anti-peptide antibody with peptide was used as a negative control. However, preabsorption of the antibody eliminated all crossreactivity with the immunoblot.

Immunofluorescence microscopy

Paraffin sections (5 μm) were collected onto APS (3-aminopropyltriethoxysilane; Sigma)-coated slides, completely air dried, and dewaxed in Clear Rite (Richard-Allan Scientific). The sections were circled with a hydrophobic barrier (ImmunoPen, Sigma), and rehydrated with 5% normal goat serum in 0.1% BSA/TPBS (0.05%Tween-20/PBS, pH 7.4) for 20 min. Sections were then incubated with α5, αRbNKA, B2/BvA1 or CAIIb antibodies, or combinations of α5 with either B2/BvA1 or CAIIb, diluted 1:200 in BSA/TPBS overnight. Slides were rinsed in TPBS (5, 10, 15 min in Coplin jars), and incubated with goat anti-mouse Alexa Fluor 488 and/or goat anti-rabbit Alexa Fluor 594-conjugated secondary antibodies, both diluted 1:200 (Molecular Probes Inc, Eugene, OR, USA) in BSA/TPBS for 1 h at 37°C. Following a second round of rinses in TPBS, coverslips were mounted with 10% Mowiol, 40% glycerol, 0.1% DABCO, 0.1 mol l^{-1} Tris (pH 8.5). Sections were viewed on a Leica DM6000 B wide field epifluorescence microscope with a digital camera (DFC340FX, Leica Microsystems, Wetzlar, Germany). Optimal exposure settings were predetermined and all images captured under these settings.

Negative controls consisted of pre-absorption of affinity purified anti-peptide antibodies with their respective peptides (αRbNKA, B2/BvA1) and substitution of the primary antibody with normal rabbit serum with an equivalent dilution for the CAIIb antibody, and either normal mouse serum, mouse IgG or isotyped culture supernatant for the α5 mouse monoclonal antibody. Omission of the antibody (dilution buffer only) in the immunolabeling protocol served as the null control. All these controls resulted in negligible background staining of the gill epithelium.

Antibodies

Na^+/K^+-ATPase was detected using the panspecific α5 mouse monoclonal antibody specific to the α subunit, developed by Douglas Fambrough (Johns Hopkins University) (Takeyasu, 1988) and αRbNKA affinity-purified anti-peptide rabbit polyclonal antibody (Wilson et al., 2007a) for immunoblotting and immunofluorescence microscopy, respectively. The αRbNKA antibody is based on the peptide described by Ura et al. (Ura et al., 1996), which also goes by the name NAK121 (Uchida et al., 2000). Both of these antibodies have been used in a number of studies on teleosts (see Wilson and Laurent, 2002). The α5 mouse monoclonal antibody was obtained as culture supernatant from Developmental Studies Hybridoma Bank, University of Iowa under contract N01-HD-7-3263 from National Institute for Child Health and Human Development (NICHD).

The vacuolar proton ATPase (H^+-ATPase) was detected using an affinity-purified rabbit anti-peptide polyclonal antibody (B2/BvA1) (Wilson et al., 2007a). The peptide is from a conserved region of the B1 and B2 subunit isoform of eel (*Anguilla anguilla*). Carbonic anhydrase was detected using a commercial rabbit anti-bovine erythrocyte CA polyclonal antibody (CA IIb) (Biogenesis, Poole, UK). This antibody has been used successfully in teleost fishes (Watrin and Mayer-Gostan, 1996).

Statistical analysis

Data are presented as mean ± standard error of the mean (s.e.m.). Statistical differences between sample groups were determined using one- or two-way ANOVA followed by the *post hoc* Student–Newman–Keuls (SNK) test (SigmaStat 3.0, SPSS).

Differences between ammocoetes and transformers from the same treatment group were tested using unpaired *t*-tests. The fiducial limit was set as 0.05.

RESULTS

Mortalities and biometric data

In Experiment I, 100% mortality occurred within 48 h in ammocoetes transferred to 25 and 35‰ and in transformers in the earlier stages of metamorphosis (stages 3–5) transferred to 35‰ SW. Early transformers survived in 25‰ but were inactive during the first 2 days. No mortality was registered in freshwater ammocoetes or early transformers in freshwater or 25‰, and at the end of the 2 weeks all individuals seemed fully acclimated to the experimental conditions. In Experiment III, ammocoetes only survived up to 10‰. Later staged transformers (stages ⩾6) survived in 35‰ and no mortalities were registered under any of the acclimation conditions.

Fulton's K was higher in ammocoetes in comparison to transformers in general, although significant differences between groups (P<0.01) were only detected in Experiments II and III. Overall condition factor was highest in ammocoetes from the Fort River (1.89) and FW ammocoetes of Experiment I (1.87) whilst the lowest values were found for transformers of Experiment III (minimum of 1.53 in 30‰) (Table 1). A decreasing trend in Fulton's K was observable between Experiment I and Experiment III (P<0.05). Significant differences in condition factor between ammocoetes and transformers within the same salinity treatment were detected in Experiments II and III, and *post hoc* analysis also revealed a significant difference in K between transformers at 20‰ and 30‰ salinities.

Table 1. Total length, mass, Fulton's condition factor K and hematocrit of ammocoetes and transformers of *Petromyzon marinus* in deionized water, freshwater, 10 ‰, 20‰, 25‰, 30‰, 35‰ salinity and field samples from the Fort River in experiments I, II and III

	Water	Length (cm)	Mass (g)	K	Hematocrit
Experiment I					
A	FW	9.0±1.1	1.1±0.4	1.87±0.06	27.2±1.7
T	FW	14.1±0.6	3.9±0.6	1.80±0.05	27.9±3.1
T	25	14.5±0.4	4.3±0.5	1.83±0.06	22.8±2.2
Experiment II					
A	FR	12.9±0.2	2.8±0.2	1.89±0.03	23.6±1.2
T	FR	13.1±0.3	3.0±0.3	1.70±0.06*	24.6±0.7
Experiment III					
A	DW	11.9±0.8	2.2±0.2	1.75±0.04	27.9±1.2
A	FW	11.7±0.6	2.1±0.3	1.78±0.04	23.2±1.2
A	10	12.7±0.5	2.5±0.3	1.77±0.03	22.1±1.4
T	DW	14.1±0.1	3.6±0.1	1.63±0.03*	23.8±0.5
T	FW	14.5±0.3	3.7±0.2	1.60±0.03*	24.4±1.5
T	10	14.6±0.3	3.9±0.3	1.61±0.03*	22.9±1.4
T	20	14.1±0.4	3.8±0.3	1.70±0.03	24.8±1.7
T	30	14.9±0.2	3.8±0.1	1.53±0.05†	22.3±1.9
T	35	14.3±0.3	3.6±0.3	1.56±0.04	18.8±0.9

Values are means ± s.e.m. (N=8–10).
A, ammocoetes; T, transformers; DW, deionized water; FW, freshwater; salinity, 10‰, 20‰, 25‰, 30‰, 35‰. Field samples were from the Fort River (FR).
*Significant difference from the corresponding ammocoetes at the same salinity; †significant difference between transformers at 20 and 30‰ salinity.

Blood variables

Ammocoetes had significantly lower plasma Na^+ and Cl^- concentrations (Fig. 1) than transformers in DW, FW and Fort River field collected animals. However, plasma ion levels of ammocoetes in 10‰ increased markedly and were significantly higher than those measured in FW or DW ammocoetes and transformers at the same salinity (although only for Cl^-). Transformer plasma ion levels generally increased with salinity although some variation occurred. The increase became particularly evident above 30‰ for Cl^-. There were no significant differences in hematocrit between ammocoetes and transformers or treatment effects (Table 1).

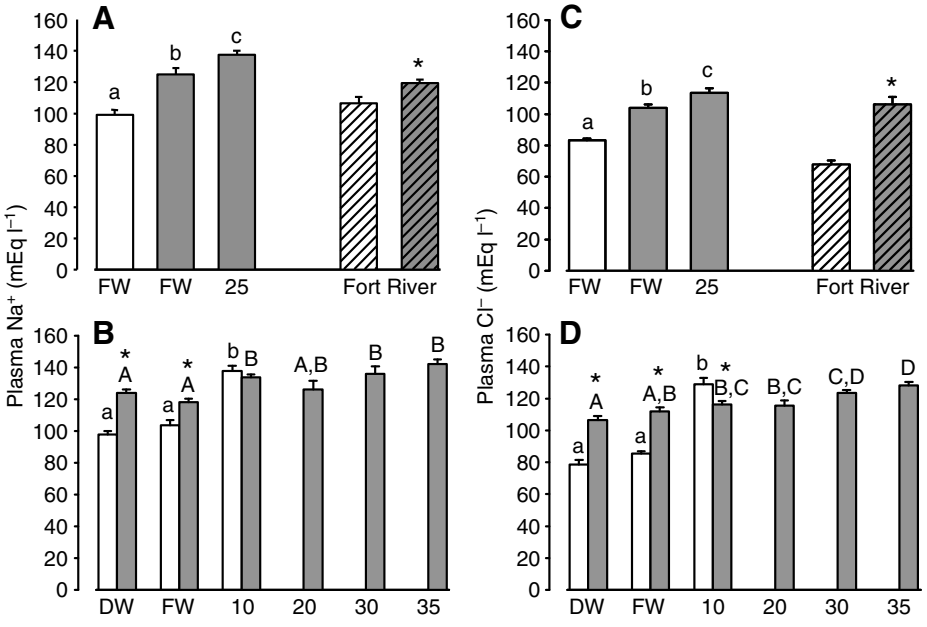

Fig. 1. Plasma sodium (Na^+) (mEq l^{-1}) (A,B) and plasma chloride (Cl^-) concentrations (μEq l^{-1}) (C,D) in ammocoetes (white bars) and transformers (grey bars) of *P. marinus*. (A,C) Ammocoetes and transformers (stages 3–5) acclimated to freshwater (FW) and 25‰ salinity and sampled from the Fort River, MA, USA (hatched bars). (B,D) Ammocoetes and transformers (stage ⩾6) acclimated to deionized water (DW), freshwater (FW) or saline water of 10, 20, 30 and 35‰. Bars with like characters are not significantly different (ANOVA; P<0.05). Upper and lower case characters are used for transformers and ammocoetes, respectively, in B and D. Asterisks indicate a significant difference from the ammocoetes at a given salinity.

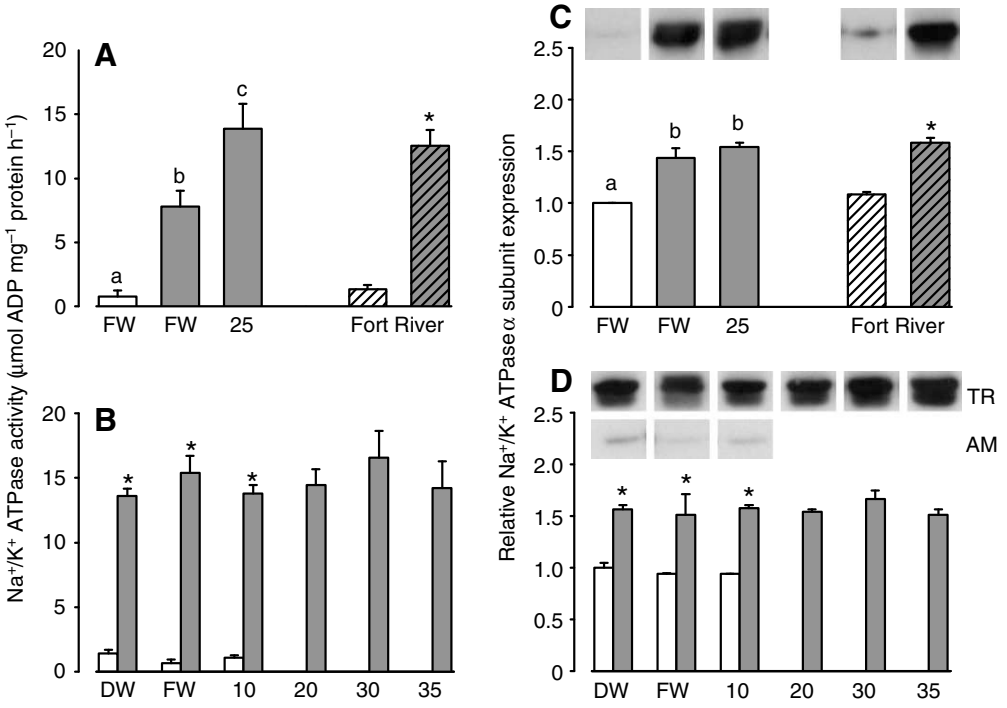

Fig. 2. Branchial Na^+/K^+-ATPase activity (μmol ADP mg^{-1} protein h^{-1}) (A,B) and α subunit expression determined by immunoblotting (C,D) in ammocoetes (white bars) and transformers (grey bars) of *P. marinus*. (A,C) Ammocoetes and transformers (stages 3–5) acclimated to freshwater (FW) and 25‰ salinity and sampled from the Fort River, MA, USA (hatched bars). (B,D) Ammocoetes and transformers (stage ⩾6) acclimated to deionized water (DW), FW or saline water of 10, 20, 30 and 35‰. Representative immunoblots of sea lamprey ammocoete and transformer gill probed with the α subunit antibody αRbNKA are shown above their respective graphs. Bars with like characters are not significantly different (ANOVA; $P<0.05$). Upper and lower case characters are used for transformers and ammocoetes, respectively, in B and D. Asterisks indicate a significant difference from the ammocoetes at a given salinity.

Gill ATPase activities

Gill Na^+/K^+-ATPase activities were highest in transformers when compared to ammocoetes at all test salinities in all experiments (Fig. 2). Activities were generally over tenfold higher in transformers, with a mean maximum of 16.5± 2.0 μmol ADP mg^{-1} protein h^{-1} in 30‰. Mean Na^+/K^+-ATPase activities measured in ammocoetes were between 0.75±0.2 in FW and 1.4±0.2 μmol ADP mg^{-1} protein h^{-1} in DW. A significant salinity-associated increase (78%) in Na^+/K^+-ATPase activities was detected with the earlier stage transformers (Experiment I). However, this tendency was not evident in the different acclimation groups with later stages of metamorphic lamprey (stage ⩾6). There was no increase in activity in response to DW acclimation in either ammocoetes or transformers.

High levels of residual (ouabain-insensitive) ATPase activity were detected in ammocoetes, which were significantly greater than in transformers from all experiments (Fig. 3). Residual activities were ~ ninefold and one-quarter relative to the ouabain-sensitive Na^+/K^+-ATPase activities in ammocoetes and transformers, respectively. Both ammocoete and transformer residual activities were unaffected by acclimation salinity.

Immunoblotting

In western blots, the Na^+/K^+-ATPase α5 and αRbNKA antibodies strongly immunoreacted with a pair of bands at approximately 100 kDa (Fig. 2C,D). This is within the observed molecular mass range of the Na^+/K^+-ATPase α subunit. Na^+/K^+-ATPase α subunit expression showed the same marked difference in expression between ammocoetes and transformers as Na^+/K^+-ATPase activities ($P<0.05$). Branchial Na^+/K^+-ATPase activity and α subunit expression patterns are similar, with the exception of Experiment I, in which α subunit expression in freshwater and 25‰ transformer were not significantly different.

The anti-peptide antibody (B2/BvA1) used to measure the expression of branchial H^+-ATPase B subunit reacted with a pair of bands of ~56 kDa in addition to a number of other high and low MW bands (Fig. 4). Analysis of the ~56 kDa bands, which corresponds to the predicted molecular mass of this subunit, indicates a decrease in expression with higher salinities in both late stage transformers and ammocoetes. This decrease was not apparent

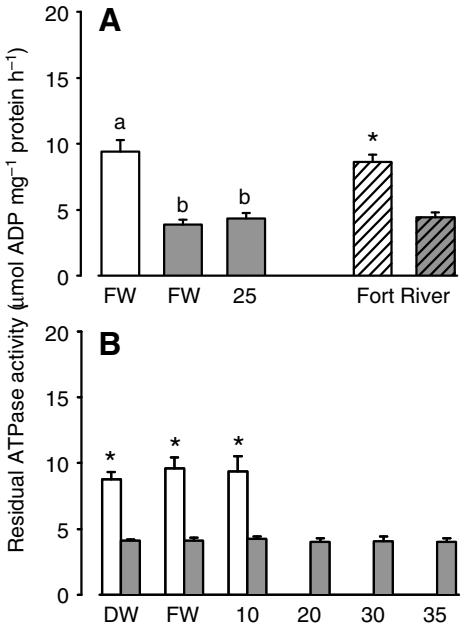

Fig. 3. Branchial residual (ouabain-insensitive) activity (μmol ADP mg^{-1} protein h^{-1}) in ammocoetes (white bars) and transformers (grey bars) of *P. marinus*. (A) Ammocoetes and transformers (stages 3–5) acclimated to freshwater (FW) and 25‰ salinity and sampled from the Fort River, MA, USA (hatched bars). (B) Ammocoetes and transformers (stage ⩾6) acclimated to deionized water (DW), FW or saline water of 10, 20, 30 and 35‰. Bars with like characters are not significantly different (ANOVA; $P<0.05$). Asterisks indicate a significant difference from the ammocoetes at a given salinity.

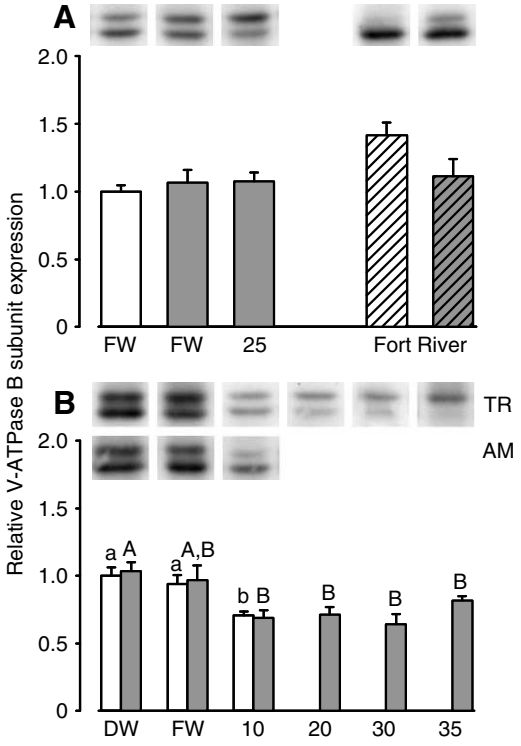

Fig. 4. H^+-ATPase B subunit expression determined by immunoblotting in ammocoetes (white bars) and transformers (grey bars) of *P. marinus*. (A) Ammocoetes and transformers (stages 3–5) acclimated to freshwater (FW) and 25‰ salinity and sampled from the Fort River, MA, USA (hatched bars). (B) Ammocoetes and transformers (stage ⩾6) acclimated to deionized water (DW), FW or a salinity of 10, 20, 30 and 35‰. Bars with like characters are not significantly different (ANOVA; $P<0.05$). Upper and lower case characters are used for transformers (TR) and ammocoetes (AM), respectively, in B. Representative immunoblots of sea lamprey ammocoete and transformer gill probed with the H^+-ATPase B subunit antibody B2/BvA1 are shown above their respective bars.

in earlier stage transformers (Experiment I). There were no significant differences between ammocoetes and transformers (Fig. 4).

The carbonic anhydrase antibody CAIIb reacted strongly with a single band of ~30 kDa; however, expression did not change with treatment or differ between groups in any of the experiments (data not shown).

Immunofluorescence

Na^+/K^+-ATPase

In ammocoete gills, Na^+/K^+-ATPase was immunolocalized to the basolateral membrane of epithelial cells in both the filament and lamellae (Fig. 5). In some cells, additional weaker cytoplasmic staining was also observed. In general, the Na^+/K^+-ATPase immunoreactive (IR) cells were distinct from those immunoreactive for H^+-ATPase. There was no apparent change in the labelling pattern with salinity and the Na^+/K^+-ATPase labelling intensity was much weaker in comparison with the immunolabeling in transformer gills (Fig. 6). In transformers, intense immunoreactivity was found within clusters (2–5 cells) of large cells in the filament epithelium with the fluorescence being present throughout the body of these cells. These cells have been previously identified as seawater-type chloride cells (CC) in adult lampreys (Bartels and Potter, 2004) and were absent in ammocoetes. The apparent cytoplasmic pattern of cell fluorescence is likely associated with the tubular system characteristic of this cell type. In transformers the weakly Na^+/K^+-ATPase immunoreactive cells were largely absent from the lamellar epithelium, being mainly restricted to the extreme efferent side of the filament epithelium where there were few CCs. However, due to the strong fluorescent Na^+/K^+-ATPase signal of the CC it was not possible to determine if there were closely neighbouring weakly immunocreactive cells in areas of CC abundance due to their substantially weaker signal (Fig. 7).

H^+-ATPase

H^+-ATPase B subunit was immunolocalized to a small population of cells scattered throughout the lower lamellar and filament epithelia (Fig. 5, Fig. 6 and Fig. 8D). For the most part these cells had a diffuse whole cell signal, although there are clear examples of labelling concentrated in the apical region of these cells (Fig. 6). In general qualitative observations indicate that there was a decrease in the frequency of these cells with an increase in salinity in both ammocoetes and transformer, although these cells were still present in the latter group, even at the highest salinities tested (data not shown).

Carbonic anhydrase

Carbonic anhydrase immunoreactivity was greatest in erythrocytes and also in a small population of epithelial cells with a similar staining pattern as the H^+-ATPase (Fig. 8). In both these cells, the labelling pattern was diffuse. In addition there was also a weaker general staining of epithelial tissue. Qualitative observations indicate that the frequency and distribution of the CA-IR cells did not change with salinity (data not shown).

DISCUSSION

We demonstrate for the first time that osmoregulatory competence in anadromous lampreys increases dramatically during metamorphosis and correlates well with the expression of branchial Na^+/K^+-ATPase and chloride cell abundance. In contrast, the pre-metamorphic ammocoete larvae are poor hypo-osmoregulators, which is reflected in their inability to survive in the experiments with salinities above 10‰, and to tightly regulate plasma ions or to upregulate ion excretory mechanisms through increased Na^+/K^+-ATPase expression. Branchial H^+-ATPase (B subunit) expression decreases in both ammocoetes and transformers with increasing salinity, indicating its potential role in driving ion uptake under hypo-ionic conditions.

In the present study, ammocoetes were only able to survive acute transfer and successfully acclimate to 10‰. The higher salinities tested (20–35‰) were acutely lethal. This is largely in agreement with previous work with the anadromous form of this species in which a LC_{50} value of 14‰ was calculated (Beamish et al., 1978). In freshwater, plasma levels of Na^+ and Cl^- of ammocoetes were low compared to metamorphic and post metamorphic lampreys as well as freshwater teleosts in general (see Morris, 1980; Beamish, 1980b), but increased dramatically so as to be nearly iso-ionic with the media following acclimation to 10‰.

The onset and progression of metamorphosis was marked by clear improvements in hypo-osmoregulatory ability. In early transformers (stages 3–5), freshwater plasma ion levels and branchial Na^+/K^+-ATPase expression were markedly higher than in ammocoetes, and these animals were capable of tolerating transfer to 25‰, accompanied by increases in plasma ions and Na^+/K^+-ATPase. However, the initial levels of preparedness and compensatory response were insufficient for coping with direct seawater (35‰)

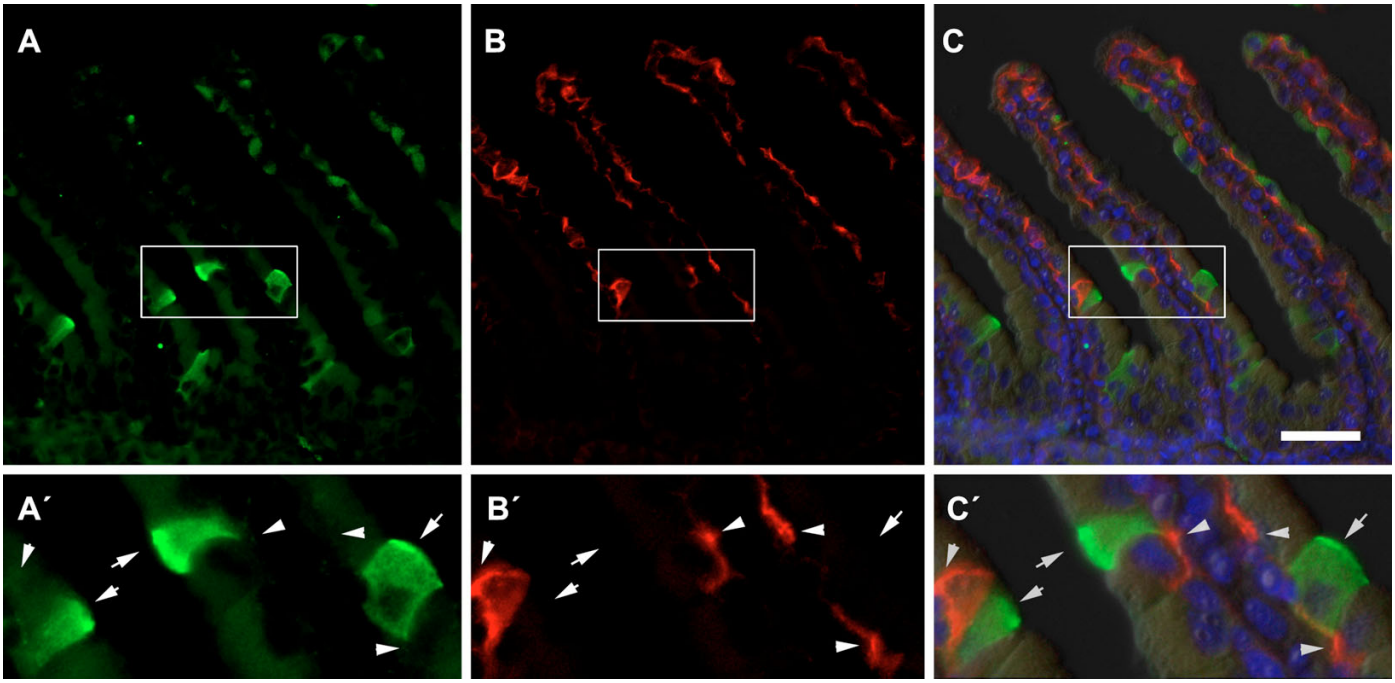

Fig. 5. Double immunofluorescence localization of H^+-ATPase (green; A,A′) and Na^+/K^+-ATPase (red; B,B′) in the gills of a freshwater ammocoete of *P. marinus* with the corresponding merged image overlaid with DAPI nuclear staining (blue) and differential interference contrast (DIC) for orientation (C,C′). The area within the box has been enlarged 3×. Arrows indicate apical H^+-ATPase labelling and arrowheads indicate basolateral Na^+/K^+-ATPase labelling. Scale bar, 50 μm.

transfer as 100% mortality resulted. Later stage transformers (⩾ stage 6) were capable of successfully acclimating to higher salinities without mortality (maximum tested 35‰). At this stage branchial Na^+/K^+-ATPase in freshwater was at its maximal level and no further increase was seen with higher acclimation salinities. Results indicate a strong positive correlation between developmental stage salinity tolerance and branchial Na^+/K^+-ATPase levels in sea lamprey, as found with smolting salmonids (Hoar, 1988).

Our results contrast markedly with those of Beamish and co-workers (Beamish et al., 1978), which is the only other study in which lamprey branchial Na^+/K^+-ATPase activities have been reported. They were only able to find consistently detectable Na^+ and K^+ stimulated ATPase activities in juvenile lampreys acclimated to seawater. They were unable to consistently detect Na^+/K^+-ATPase activity in animals of any stage (post metamorphic juvenile, or adults on their spawning migration) in freshwater. Although the method used in the present study is more sensitive than that available to Beamish et al. (Beamish et al., 1978), the levels of branchial Na^+/K^+-ATPase activity reported here for lamprey are similar to teleosts, so the source of this discrepancy is unclear. In the present study, ouabain-sensitive Na^+/K^+-ATPase activity was detected in both ammocoetes and transformers in freshwater. This was confirmed by immunodetection of the Na^+/K^+-ATPase α subunit by immunoblotting and immunofluorescence microscopy. The improvement in salinity tolerance in transformers is clearly correlated with the development of branchial chloride cells in freshwater, which were previously reported only to be fully differentiated at stages 6/7 (Peek and Youson, 1979a; Peek and Youson, 1979b). We have demonstrated for the first time that these cells have high Na^+/K^+-ATPase expression, which had only previously been inferred from morphological similarities with teleost chloride cells that are known to express high levels of Na^+/K^+-ATPase (Peek and Youson, 1979a; Peek and Youson, 1979b; Bartels and Potter, 2004). The intense Na^+/K^+-ATPase immunoreactivity in clusters of disk shaped cells in continuous rows along the interlamellar space found in transformers is consistent with the distribution and morphology of lamprey CCs (Bartels et al., 1993; Bartels and Potter, 2004). The high levels of Na^+/K^+-ATPase associated with the tubular system of the basolateral membrane result in the labelling pattern of the entire cell, which has also been reported in teleost fish branchial CCs (Wilson and Laurent, 2002).

The lower branchial Na^+/K^+-ATPase activity in ammocoetes relative to transformers was consistent with the Na^+/K^+-ATPase labelling intensities found by immunofluorescence microscopy. In ammocoetes, electron microscopy has been used to determine that the lamellar epithelium is composed predominantly of ammocoete MRCs, with squamous pavement cells limited to the tips, and that the filament epithelium is composed mainly of IMRC and pavement cells (Bartels and Potter, 2004). The weak Na^+/K^+-ATPase immunofluorescence in ammocoetes occurred only in the basolateral region of filament and lamellar epithelial cells that lack an extensive tubular system of CCs, as has been described in adult *G. australis* in freshwater (Choe et al., 2004). Conley and Mallatt (Conley and Mallatt, 1988) were unable to detect Na^+/K^+-ATPase activity in ammocoetes using enzyme histochemistry, which is likely due to a lack of sensitivity of the technique. The Na^+/K^+-ATPase staining in ammocoetes may be associated with one subtype of IMRC, as proposed by Choe et al. (Choe et al., 2004), or alternatively pavement cells or ammocoete MRCs. However, it is unclear if the Na^+/K^+-ATPase IR IMRCs (A-type MRC[1]) identified by Choe et

[1]To avoid any confusion it should be noted that this nomenclature is based on their elasmobranch gill work (Evans et al., 2005) and is different from that used by Bartels and Potter (Bartels and Potter, 2004), which is based on IMRCs from acid–base regulating epithelia.

al. (Choe et al., 2004) are indeed not a pavement cell subtype. Bartels and Potter (Bartels and Potter, 2004) note the presence of columnar pavement cells in the filament epithelium interspersed with IMRC and that apical studding, indicative of the presence of H^+-ATPase, is present in the majority of IMRCs. They suggested that by analogy to other acid-secreting epithelia, lamprey pavement cells would possess Na^+/K^+-ATPase, though direct evidence for this was lacking. Thus in the double labelling experiment (Choe et al., 2004) for Na^+/K^+-ATPase and H^+-ATPase the labelled cells could be interpreted as PVC and IMRC, respectively, and not the existence of a new IMRC subtype. Alternatively, the lamellar epithelial Na^+/K^+-ATPase staining may be associated with ammocoete MRCs, which predominate in this region. The function of ammocoete MRCs still remains poorly characterized (Bartels and Potter, 2004). Ultimately, immunoelectron microscopy would be needed to resolve these discrepancies.

Na^+/K^+-ATPase is likely involved in branchial Na^+ absorption in series with an apical Na^+/H^+ exchanger in either IMRCs, as has

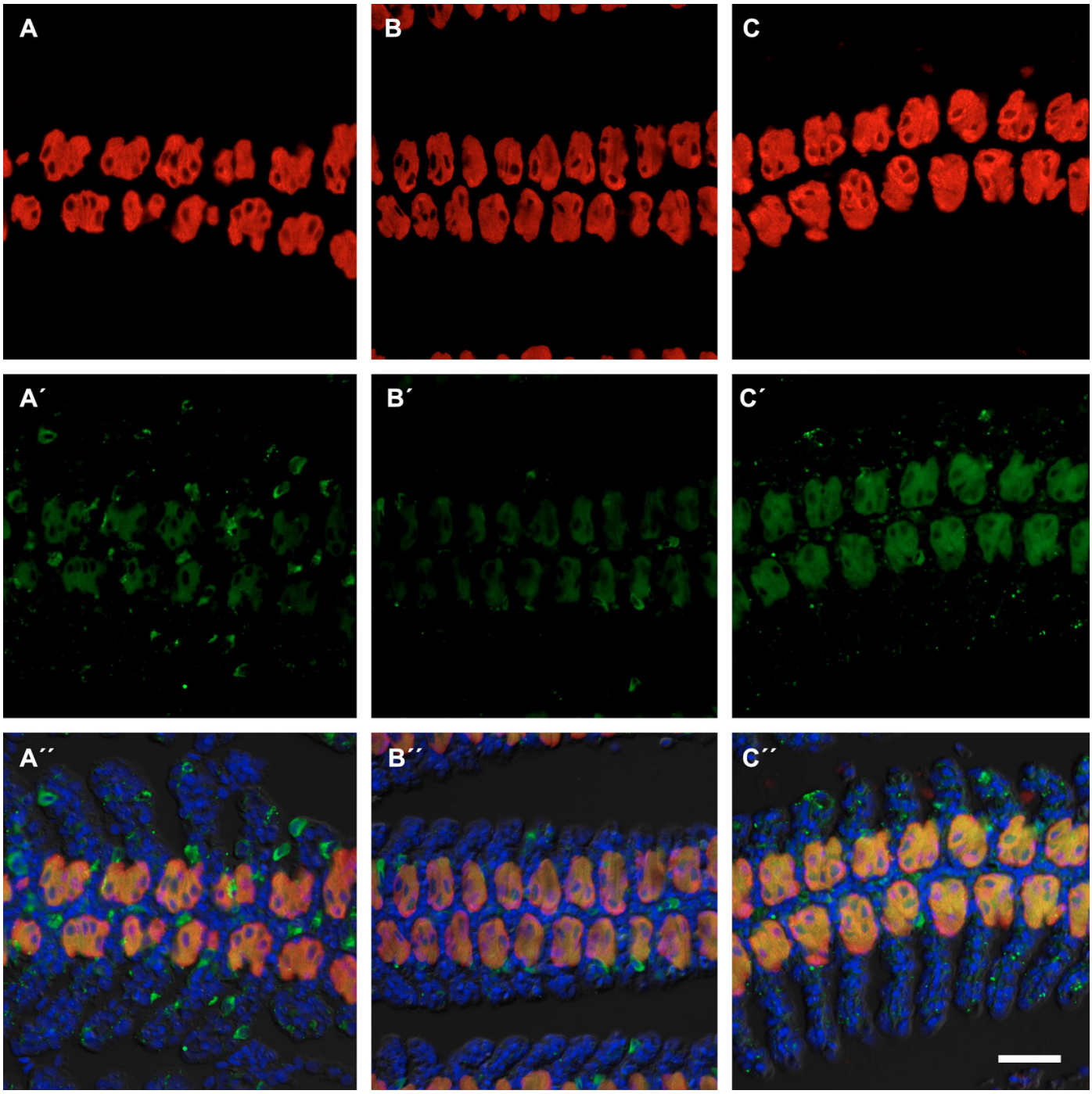

Fig. 6. Immunolocalization of Na^+/K^+-ATPase (red; A,B,C) and H^+-ATPase (green; A′,B′,C′) in gill filament sagital sections of transformers of *P. marinus* acclimated to freshwater (FW; A,A′,A″), 10‰ (B,B′,B″) and 25 ‰ (C,C′,C″) salinity. Images were merged with DAPI nuclear staining (blue) and differential interference contrast (DIC) images for orientation (A″,B″,C″). Scale bar, 50 μm.

been demonstrated in elasmobranchs [NHE2 (Edwards et al., 2002), NHE3 (Choe et al., 2004; Choe et al., 2007)] or pavement cells [as proposed by Bartels and Potter (Bartels and Potter, 2004)], fulfilling the lampreys' needs for ion uptake in freshwater. The contributions to Na^+/K^+-ATPase total tissue activity levels from this IMRC subtype are likely to be very low, as both the number of cells and immunofluorescent signal were low, consistent with the low branchial Na^+/K^+-ATPase activity in ammocoetes.

We show for the first time that H^+-ATPase expression decreases with increasing salinity in both ammocoetes and transformers, suggesting that this enzyme is involved in driving Na^+ and/or Cl^- uptake. The presence of H^+-ATPase immunoreactive cells in the gills was also demonstrated using a heterologous antibody to a conserved region of eel H^+-ATPase B subunit developed by Wilson et al. (Wilson et al., 2007a). These cells tended to decrease in abundance with salinity, although they did not disappear entirely. In some of these cells there was clear apical immunoreactivity, indicative of an apical plasma membrane localization. Previously, the presence of H^+-ATPase has been inferred from freeze-fracture studies demonstrating rod-shaped particles similar to the H^+-ATPase V_1 domain in acid–base regulating epithelia (Bartels and Potter, 2004). These particles were found apically in most IMRC, with a smaller subset having a basolateral location. Apical H^+-ATPase expression has also been confirmed in adult lampreys on their spawning migration in freshwater (Choe et al., 2004), similar to the H^+-ATPase IR cells that we detected in ammocoetes and transformers. Since no data exist on the presence or expression of coupled Na^+ and Cl^- uptake mechanisms, the exact role of the H^+-ATPase is unknown, although various hypotheses have been proposed and remain untested (Choe et al., 2004; Bartels and Potter, 2004).

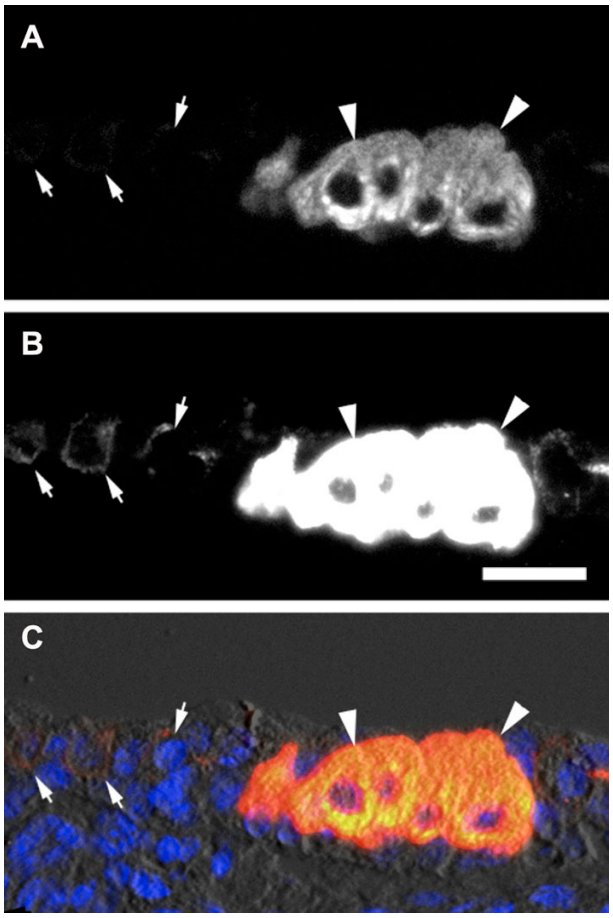

Fig. 7. Localization of Na^+/K^+-ATPase immunoreactive (IR) cells on the afferent edge of the gill filament of a freshwater transformer. Image capture conditions were optimized for (A) the cluster of strongly IR chloride cells (CCs) and (B) weakly IR cells indicated by arrowheads and arrows, respectively. (C) The corresponding merged image overlaid with DAPI nuclear staining (blue) and differential interference contrast (DIC) is given for orientation. Scale bar, 25 μm.

The importance of the H^+-ATPase in both Na^+ and Cl^- uptake in freshwater teleosts and elasmobranchs has, however, been established for a number of species (Evans et al., 2005). *In vivo* bafilomycin A1, a specific V-type H^+-ATPase inhibitor (Bowman et al., 1988), significantly inhibits Na^+ uptake [carp (Fenwick et al., 1999), zebrafish (Esaki et al., 2007)] as well as Cl^- uptake (Fenwick et al., 1999). Knockdown of H^+-ATPase subunit A in zebrafish significantly decreases Na^+ accumulation (Horng et al., 2007). Sodium uptake may be through a phenamil-sensitive Na^+ channel (Parks et al., 2007) or possibly by NHE (Evans et al., 2005). In elasmobranchs the H^+-ATPase basolateral expression pattern is found in cells with apical Cl^-/HCO_3^- exchanger (SLC26A4-like) expression and thus considered to be the driving force for Cl^- absorption (Piermarini et al., 2002). A similar mechanism has been proposed for teleost fishes but the identity of the apical anion exchanger is unresolved (Evans et al., 2005). It remains to be determined whether lampreys employ similar transport mechanisms for ion and acid–base regulation.

In the gill epithelium, strong carbonic anhydrase immunoreactivity is evident in a pattern similar to H^+-ATPase, suggesting a co-localization to IMRCs. Intracellular CA is important for providing an intracellular supply of H^+ for the pump through the catalysis of the CO_2 hydration reaction (Evans et al., 2005; Bartels and Potter, 2004). A very similar labelling pattern has been reported in adult lamprey (Choe et al., 2004). In the present study, erythrocyte CA immunoreactivity was also detected and a weaker, general immunoreactivity associated with the gill epithelium. Choe et al. (Choe et al., 2004) found only weak immunoreactivity with erythrocytes and Conley and Mallatt (Conley and Mallatt, 1988) were unable to detect CA activity by enzyme histochemistry in ammocoete erythrocytes. These findings are consistent with the low levels of CA activity that are associated with lamprey erythrocytes (Esbaugh and Tufts, 2006). In ammocoetes, Conley and Mallatt (Conley and Mallatt, 1988) reported weak lamellar staining associated with ammocoete MRCs using the same technique, and in adult lamprey in freshwater CA immunoreactivity was also found in the squamous pavement cells towards the tips of the lamellae (Choe et al., 2004). In contrast, in the present study no preferential staining in ammocoete MRCs or the lamellar tips in transformers was found. This may represent differences in sensitivities of the techniques and antibodies used. No developmental stage differences or salinity related changes in tissue levels of CA expression were found by immunoblotting, which is generally consistent with the immunofluorescence results.

Acclimation of FW animals to DW has been used to stimulate ion uptake under this extreme hypo-osmotic condition in order to investigate the underlying mechanisms (Krogh, 1937; Wilson et al., 2007a; Esaki et al., 2007). In lampreys, however, no osmoregulatory stress was evident in either ammocoetes or transformers in DW since no decreases in plasma ion concentrations were detected, nor were there increases in either H^+-ATPase or Na^+/K^+-ATPase levels. The ability of both non-feeding ammocoetes and transformers to effectively ionoregulate in DW would argue against solely a NHE mediated Na^+ uptake mechanism driven by Na^+/K^+-ATPase (Choe

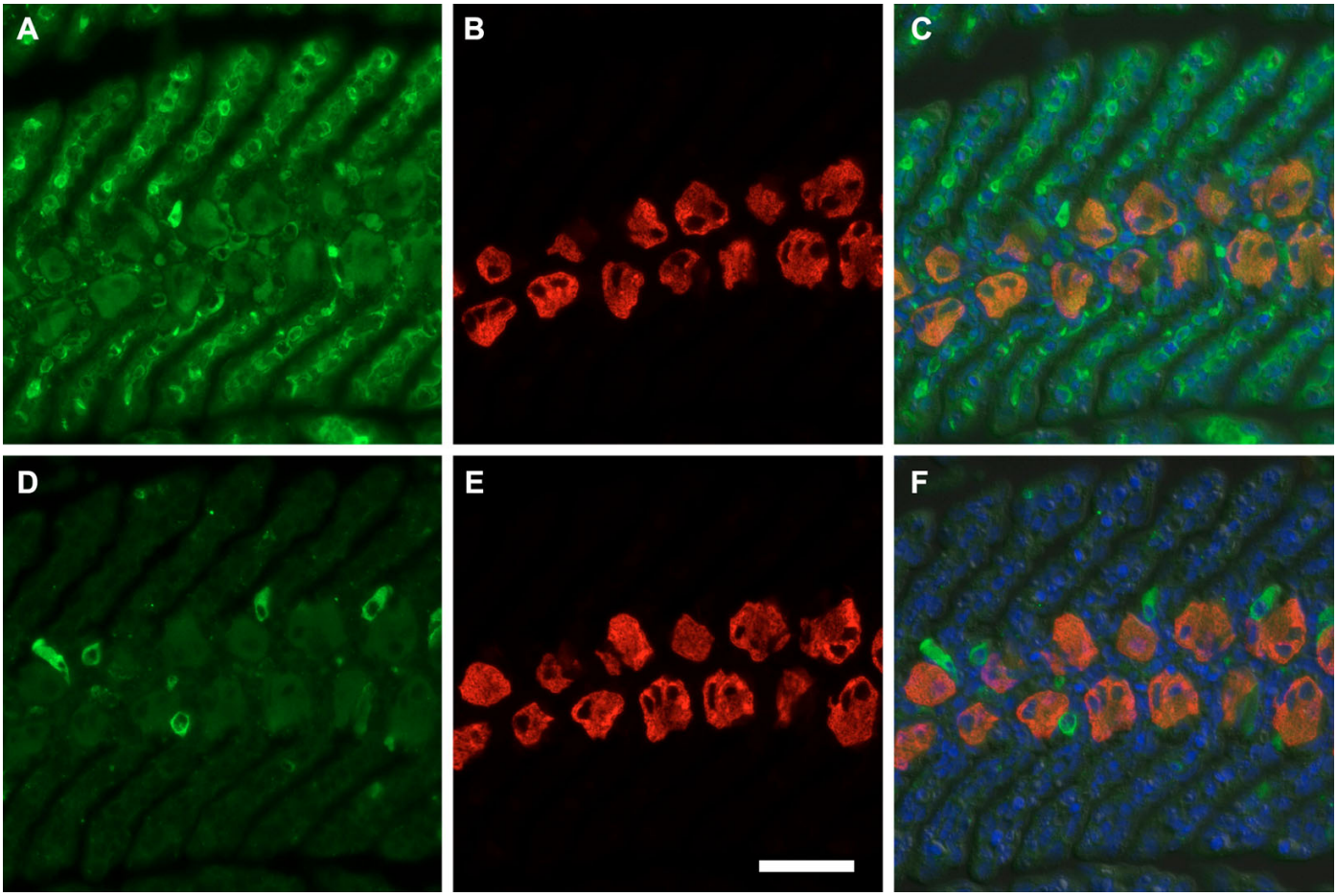

Fig. 8. Indirect immunofluorescence double labelling of a gill filament sagittal sections of a Fort River *P. marinus* transformer for (A) carbonic anhydrase (green) or (D) H^+-ATPase B subunit (green) double labelled for Na^+/K^+-ATPase α subunit (red; B and E, respectively) with the corresponding merged images with the overlaid with DAPI nuclear staining (blue) and differential interference contrast (DIC) images for orientation (C and F, respectively). Scale bar, 50 μm.

et al., 2004) since the H^+ gradient necessary to drive Na^+ uptake under these conditions is not thermodynamically realistic. This is also indirectly supported by the observation that Na^+/K^+-ATPase, which is necessary for maintaining low intracellular Na^+ levels, does not increase during DW exposure. However, neither does branchial H^+-ATPase expression, so it remains to be determined if existing levels of these ATPases are sufficient to drive uptake processes, or if lamprey possess a novel mechanism. In freshwater teleost fishes acclimated to ion-poor water conditions, there are reports of MRC proliferation (e.g. Greco et al., 1996), but no corresponding increase in branchial Na^+/K^+-ATPase activity [*Oncorhynchus mykiss* (Sloman et al., 2001); *A. anguilla* (Wilson et al., 2007a); *Danio rerio* (Craig et al., 2007)]. In trout, H^+-ATPase expression appears to be higher in animals from ion-poor environments (Wilson et al., 2000) although in glass eels acclimated to ion-poor conditions no difference in expression is evident (Wilson et al., 2007a).

In ammocoetes, high residual (ouabain-insensitive) ATPase activities were detected. These activity levels were not affected by salinity change and immunoblotting data strongly suggest that it is not H^+-ATPase or Na^+/K^+-ATPase activity. If the high residual ATPase activity was H^+-ATPase, then lower levels of residual activity with increasing salinity would be expected (based on the western blots for H^+-ATPase), but this was not the case. It also seems unlikely this residual activity is an ouabain-insensitive Na^+/K^+-ATPase because Na^+/K^+-ATPase protein levels measured by western blots were also very low in ammocoetes. This low branchial Na^+/K^+-ATPase expression correlates with the weak basolateral Na^+/K^+-ATPase IR in the ammocete gill epithelium. However, it remains possible that lamprey ammocoetes may express another α isoform that is not recognized by either of the panspecific α subunit antibodies used in this study. It is also possible that another yet to be identified ATPase may account for the high residual activity in crude gill homogenates. This residual activity may be related to ammocoete MRCs, which are present only during the larval phase and compromise 60% of the branchial epithelial surface (Bartels and Potter, 2004). Although ammocoete MRCs were initially considered to be involved with ion uptake, their absence in returning adult lampreys, which overcome the same ionoregulatory challenge as ammocoetes in freshwater, has led to the proposition that they may have functions other than osmoregulation (Bartels et al., 1998; Bartels and Potter, 2004). Ammocoetes are the only life stage in lamprey that filter feed in freshwater and ammocoete MRC are thought to be related to feeding and waste metabolism (Bartels et al., 1998).

During metamorphosis lamprey do not feed, and are mostly sedentary and have low metabolic rates until downstream migration (Hardisty and Potter, 1971b). As a result, there is a decrease in condition factor (K), which explains the differences found between ammocoetes and transformers in these experiments, as well as the

decrease in transformers' condition factor throughout the experiments as metamorphosis progresses. When analysing the movements of ammocoetes and transformers in river beds, a similar decline in K has been found (Quintella et al., 2005) as well as between non-feeding glass eels (*Anguilla anguilla*) and feeding resident estuarine elvers (Wilson et al., 2007b). Condition factor also decreases during the parr–smolt transformation, and has been attributed both to the metabolic demands of transformation and alterations in growth patterns favouring an increase in length over mass (McCormick and Saunders, 1987).

In the present study we have demonstrated an increase in survival and hypo-osmoregulatory ability during the transformation of juvenile lampreys. We have also shown an increase in branchial Na^+/K^+-ATPase activity and abundance, and the appearance of Na^+/K^+-ATPase-rich chloride cells as clusters in the primary filament. Developmental increases in salinity tolerance, gill Na^+/K^+-ATPase and chloride cell abundance also occur in downstream migrating juvenile salmon (Hoar, 1988; McCormick and Saunders, 1987), American shad (Zydlewski and McCormick, 1997) and in adult eel (Epstein and Katz, 1967) and may be a common feature of diadromous species that make a limited number of migrations from freshwater to seawater. It will be of interest to determine if the environmental and endocrine factors that control these developmental changes in teleosts are also shared with lampreys.

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