Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater

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Grosell M, Gilmour KM, Perry SF. Intestinal carbonic anhydrase, bicarbonate, and proton carriers play a role in the acclimation of rainbow trout to seawater. Am J Physiol Regul Integr Comp Physiol 293: R2099–R2111, 2007. First published August 29, 2007; doi:10.1152/ajpregu.00156.2007.—Abrupt transfer of rainbow trout from freshwater to 65% seawater caused transient disturbances in extracellular fluid ionic composition, but homeostasis was reestablished 48 h posttransfer. Intestinal fluid chemistry revealed early onset of drinking and slightly delayed intestinal water absorption that coincided with initiation of NaCl absorption and HCO3− secretion. Suggestive of involvement in osmoregulation, relative mRNA levels for vacuolar H+-ATPase (V-ATPase), Na+/K+-ATPase, Na+/H+ exchanger 3 (NHE3), Na+/HCO3− cotransporter 1, and two carbonic anhydrase (CA) isoforms [a general cytosolic isoform trout cytoplasmic CA (tCAc) and an extracellular isoform trout membrane-bound CA type IV (tCAIV)], were increased transiently in the intestine following exposure to 65% seawater. Both tCAc and tCAIV proteins were localized to apical regions of the intestinal epithelium and exhibited elevated enzymatic activity after acclimation to 65% seawater. The V-ATPase was localized to both basolateral and apical regions and exhibited a 10-fold increase in enzymatic activity in fish acclimated to 65% seawater, suggesting a role in marine osmoregulation. The intestinal epithelium of rainbow trout acclimated to 65% seawater appears to be capable of both basolateral and apical H+ extrusion, likely depending on osmoregulatory status and intestinal fluid chemistry.

AN UNAVOIDABLE CONSEQUENCE of the osmotic gradient between seawater and the extracellular fluids of marine teleost fish is osmotic water loss. Dehydration is prevented, however, by ingestion of seawater and subsequent absorption of fluids across the intestinal epithelium (43, 56). The intestinal fluid absorption is intimately linked to absorption of NaCl (1, 59), and the ingestion of MgSO4-rich seawater results in the simultaneous uptake of MgSO4 by the intestine. The excess NaCl and MgSO4 arising from seawater ingestion are subsequently cleared by branchial and renal excretion, respectively (43).

The absorption of NaCl, which ultimately drives water absorption, relies on the activity of Na+/K+-ATPase (NKA), which establishes an electrochemical gradient favorable for apical Na+ uptake. Entry of Na+, Cl−, and K+ from the intestinal lumen across the apical membrane has long been attributed to two parallel carrier systems: Na+-Cl− and Na+-K+−2Cl− (NKCC) cotransporters (11, 27). However, recent reports have also demonstrated an important role for apical anion exchange in Cl− uptake (22, 25, 66). The contribution of this Cl− uptake pathway appears to explain the much higher net Cl− than Na+ uptake reported in all studies that have measured simultaneous fluxes of these two ions (20). In addition to Cl− uptake, the intestinal apical anion exchanger is responsible for the exceptionally high rate of transepithelial HCO3− secretion that is comparable to duodenal HCO3− secretion in mammals at much higher temperatures (37°C vs. 25°C) (22). The apical anion exchange system thus displays substantial base secretion, but does not appear to be directly involved in the dynamic regulation of acid-base balance (66). The cellular substrate for the apical anion exchanger is derived from either intracellular metabolic CO2 or serosal CO2 and HCO3− (20), with both sources contributing more or less equally to luminal HCO3− secretion (9, 22, 25). The hydration of endogenous CO2 is catalyzed by carbonic anhydrase (CA) (9, 22, 64).

Osmotic pressures in the intestinal fluids and blood plasma of marine teleosts are similar and appear to covary (24). The low osmotic pressure of intestinal fluids relative to the imbibed seawater is the result of selective NaCl absorption in the water-impermeable esophagus (32, 49) and of several processes occurring in the intestine (reviewed recently in Ref. 24). Perhaps of greatest importance for reduction of intestinal fluid osmotic pressure is the formation of CaCO3-rich precipitates in the lumen resulting from the combination of ingested Ca2+-rich seawater with highly alkaline intestinal fluids (66). This unique role of luminal biomineralization in water absorption in marine fish may rely on CA activity as seen in other biomineralization processes, such as bone growth (4), otolith formation (36, 50), shell deposition (16, 46), and coral calcification (17). A likely CA candidate for biomineralization in the marine fish intestinal lumen is extracellular CAIV, which is attached to the plasma membrane (57) by a glycosylphosphatidylinositol (GPI) anchor.

Considering the clear involvement of cytosolic CA activity in apical anion exchange (9, 22, 64), the possible role of extracellular, membrane-bound CA activity for luminal biomineralization, and the significance of both of these processes for marine fish osmoregulation, we hypothesized the following: 1) that trout cytoplasmic CA (tCAc) and CAIV would be closely associated with the apical membrane of the intestinal epithelium in trout acclimated to 65% seawater; 2) that relative mRNA expression of the genes coding for these enzymes would increase in trout intestine following transfer from freshwater to seawater; and finally, 3) that increased...
gene expression after salinity transfer would be reflected by increased cytosolic and membrane-bound CA activities.

Protons arising from intracellular CO$_2$ hydration are moved across the basolateral membrane at least in isolated epithelia under resting conditions (22, 24). Impairment of serosal H$^+$ extrusion leads to inhibition of luminal HCO$_3^-$ excretion, demonstrating that intestinal Cl$^-$ absorption (via Cl$^-$/HCO$_3^-$ exchange) relies on cellular H$^+$ extrusion (22). In the gulf toadfish, the basolateral H$^+$ extrusion is dependent on serosal Na$^+$ and relies on the activity of the basolateral NKA, suggesting the involvement of an Na$^+$/H$^+$ exchanger (NHE). However, the potential involvement of vacuolar H$^+$-ATPase (V-ATPase) in intestinal Cl$^-$ uptake has yet to be considered for marine fish. Moreover, luminal base secretion in seawater-acclimated rainbow trout has been reported to increase following the addition of luminal amiloride (64). An apparent stimulation of base secretion following the addition of amiloride, an inhibitor of NHE-type proteins, might indicate simultaneous luminal excretion of HCO$_3^-$ and H$^+$, explaining a net increase in apparent base secretion when H$^+$ secretion is impaired. Because H$^+$ extrusion is important for intestinal Cl$^-$ uptake in marine fish (22) and since an apical amiloride-sensitive NHE mechanism (64) might exist, we investigated the possible roles of V-ATPase and the apical NHE3 isoform in acclimation of trout to seawater by assessing relative mRNA expression.

In addition to intracellular CO$_2$ hydration, it appears that HCO$_3^-$ may directly enter the epithelial cell across the serosal membrane thus providing additional substrate for extrusion. It was recently proposed that the entry of HCO$_3^-$ across the serosal membrane occurs via Na$^+$/HCO$_3^-$ cotransport (NBC) (22). The prediction of NBC involvement in seawater acclimation was tested in the present study by measuring the relative levels of trout Na$^+$/HCO$_3^-$ cotransporter-1 (NBC1) mRNA in intestinal tissue following seawater transfer.

Our results strongly suggest a role for tCAc, trout membrane-bound CA type IV (tCAIV), V-ATPase, NHE3, and NBC1 in intestinal osmoregulatory function of rainbow trout acclimated to partial seawater and lend further support to the recently identified role of intestinal HCO$_3^-$ secretion in water absorption.

**MATERIALS AND METHODS**

**Experimental Animals**

The animals used in this study were cared for in accordance with the principles of the Canadian Council for Animal Care, *Guide to the Care and Use of Experimental Animals*. Experimental protocols were approved by the University of Ottawa Animal Care Committee.

Rainbow trout (*Oncorhynchus mykiss*; approximate mass 150 g) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Prior to use, fish were maintained for a minimum of 2 wk in large fiberglass tanks supplied with flowing, aerated, freshwater (dechlorinated City of Ottawa tap water) at 13°C using a 12:12-h light-dark photoperiod. Fish were fed commercial trout pellets to satiation on alternate days.

**General Experimental Procedures**

Two different seawater exposures were performed in fiberglass tanks fitted with a recirculation system and a biological filter preventing accumulation of NH$_4^+$/NH$_3$ and NO$_3^-$, respectively. For the initial exposure, trout were acclimated to (aerated) 65% seawater by gradually increasing salinity to 65% over 2 wk, after which the fish were maintained at 65% seawater for an additional week. Fish from this exposure regimen are presented as 3-wk 65% seawater fish in Figs. 1–9. To investigate rapidly occurring changes in gene expression and intestinal and extracellular fluid compositions in response to a hyperosmotic environment, a second exposure regimen consisted of abrupt transfer from freshwater to 65% seawater. A maximal salinity of 65% was chosen based on pilot experiments revealing mortality at higher salinities. No mortality was observed in the gradual (2 wk) acclimation experiment while modest (<20%) mortality occurred during the first 48 h of the abrupt salinity transfer protocol.

For both exposures, food was withheld for 48 h prior to sampling, and trout were killed by a blow to the head and a blood sample was obtained by caudal puncture using a heparinized (50 IU/ml) 1-ml disposable syringe fitted with a 23-gauge needle. Plasma was obtained by immediate centrifugation (3 min at 10,000 g). Subsequently, the gastrointestinal tract was exposed by a longitudinal midline section and four intestinal segments were isolated by ligation (using silk ligatures) allowing for sampling of fluids from the pyloric, anterior (immediately distal to the pyloric caeca), mid-, and posterior regions of the intestine. Samples of tissue from these four intestinal regions were either immersed in fixation buffer (described below) for immunohistochemistry or immediately frozen in liquid nitrogen for subsequent RNA extraction (see *Gene Expression Analysis*).

A subset of fish was sampled for intestinal epithelium enzyme (CA, NKA, and V-ATPase) activity measurements. The intestines of freshwater- and 65% seawater-acclimated fish were obtained by dissection and opened by a longitudinal incision, exposing the mucosal surface (see *Gene Expression Analysis*). The intestine (not including the pyloric caeca) was divided into anterior and posterior portions and placed on an inverted petri dish (on ice). Epithelial cells were collected by scraping the mucosal surface with the edge of a glass microscope slide. The samples were frozen immediately in liquid N$_2$ and stored at −80°C for later analysis.

**Plasma and Intestinal Fluid Composition**

Intestinal fluid total CO$_2$ was analyzed using a Capnicon total CO$_2$ analyzer (model CC501; Cameron Instruments) and consists mainly of HCO$_3^-$ in marine teleost intestinal fluid (25), while anions (SO$_4^{2-}$ and Cl$^-$) were analyzed by anion chromatography (DIONEX 120, fitted with an AS14A anion column). The main cations were measured by fast sequential flame atomic absorption (Varian 220 FS) after LaCl$_3$ addition (0.1%) by using an air/acetylene flame. Samples were compared with certified standards and cross calibration of anion chromatography and atomic absorption instruments was performed using NaCl (not containing LaCl$_3$) and MgSO$_4$ reference material.

**Gene Expression Analysis**

Total RNA from the pyloric caeca, as well as the anterior, mid-, and posterior regions of the intestine was extracted from ~30 mg of tissue using Trizol reagent (Invitrogen) and resuspended in 30 μl of nuclease-free water. Reverse transcription was performed using Stratascript Reverse Transcriptase (Stratagene) as per kit instructions with the following changes: final reaction volume was adjusted to 12.5 μl, while 0.5 μg of total RNA was used with 0.15 μg of random hexamer primers added.

Real-time PCR was performed using an MX400 Multiplex Quantitative PCR system (Stratagene) and Brilliant SYBR Green QPCR Master Mix (Stratagene) as per the instructions of the manufacturer with the following modifications: 1) the total reaction volume was reduced to 25 μl, and 2) 0.5 μl of cDNA template was used in combination with primer concentrations of 0.15 nmol/l (see Table 1 for a description of primers). Initially, PCR products were sequenced to verify that primers were indeed amplifying the selected target genes. Dissociation curves were performed at the end of each PCR run to confirm the purity of amplicons. Randomly selected samples of
diluted RNA were examined and revealed no genomic DNA contamination. Relative expression of mRNA levels was determined by using 18s as a reference gene by the ΔΔCt method (52). Expression of 18s remained constant across treatments.

Protein Localization

Immunohistochemistry was used to localize cytosolic CA (cCA), CAIV, the α1-subunit of NKA and the V-ATPase within the pyloric ceca and the anterior, mid-, and posterior regions of the intestine from trout acclimated to 65% seawater (for details regarding the antibodies used for cCA, CAIV, and the V-ATPase, see Table 2). The NKAα1 antibody was raised against NKA purified from chicken kidney to detect a 100-kDa polypeptide in the α-subunit and has been used in a wide range of organisms, including several fish species (5, 53, 61–63). Intestinal segments obtained by dissection were immersion-fixed in 4% paraformaldehyde in PBS (pH 7.4) overnight and then transferred to 15% sucrose for 2 h in each case. Tissues were kept at 4°C throughout this procedure and were subsequently transferred to 30% sucrose for 2 h in each case. Fixed and cryoprotected tissue samples were embedded in Shandon Cryomatrix medium (Fisher Scientific, Ottawa, ON, Canada) and were sectioned (8- to 10-μm thickness) by using a cryostat (model CM1850; Leica, Richmond Hill, ON, Canada) and were allowed to air dry for 30 min prior to storage at −20°C.

A hydrophobic barrier was created around each section using a hydrophobic barrier pen (Electron Microscopy Suppliers, Fort Washington, PA), and sections were incubated (3 × 5 min) in blocking buffer containing 2% normal goat serum, 0.1 M phosphate buffer with 200 μM Triton-X, 1% gelatin, and 2% bovine serum albumin. Sections were then incubated for 2 h at room temperature in a humidified chamber with a 4%–diamidino-2-phenylindole for the visualization of nuclei. Sections were viewed by using a conventional epifluorescence microscope (Zeiss Axiohot) and charge-coupled device camera (Hamamatsu C5985). Images were captured using Metamorph version 4.01 imaging software.

Table 2. Sequences of antigens used for raising antibodies used for immunohistochemistry

<table>
<thead>
<tr>
<th>Gene</th>
<th>Peptide Sequence</th>
<th>Protein Sequence GenBank Accession No.</th>
</tr>
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<tbody>
<tr>
<td>tCAc</td>
<td>WNTKYPSFQDAASKSDGLA</td>
<td>AAR99329</td>
</tr>
<tr>
<td>tCAIV</td>
<td>TRKTLPDERLTPFTFTGY</td>
<td>AAR99330</td>
</tr>
<tr>
<td>V-ATPase</td>
<td>CAEMPADSGYPAYLGAR</td>
<td>BAB62103</td>
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The tCAc and tCAIV antibodies were generated by using synthetic antigens conjugated to keyhole limpet protein as described elsewhere (10). The antibody used for V-ATPase immunostaining was VATP317, a rabbit polyclonal antibody rabbit raised against a synthetic peptide (Cys-Ala-Glu-Met-Pro-Ala-Asp-Ser-Gly-Tyr-Pro-Ala-Tyr-Leu-Gly-Ala-Arg) that was based on a highly conserved and hydrophilic region in the A-subunit of V-ATPase. The antibody has been verified for its specificity (35) and was generously provided by Fumi Katoh.

Following incubation, slices were washed in 0.1 mol/l PB (3 × 5 min) and incubated for 2 h at room temperature in a humidified chamber with a 400-fold dilution (in 0.1 mol/l PB) of Alexa-Fluor 488-coupled goat anti-rabbit IgG (Fisher Scientific) for the detection of tCAc, tCAIV, and V-ATPase proteins and/or Alexa-Fluor 546-coupled goat anti-mouse IgG (Fisher Scientific) for detection of the NKA protein. The slides were washed (as above) and coveredslipped with mounting medium (Vector Laboratories, Burlington, ON, Canada) containing 4,6-diamidino-2-phenylindole for the visualization of nuclei. Sections were viewed by using a conventional epifluorescence microscope (Zeiss Axiohot) and charge-coupled device camera (Hamamatsu C5985). Images were captured using Metamorph version 4.01 imaging software.

Enzyme Activity Measurements

CA. Intestinal epithelium was homogenized and fractionated using standard procedures (19, 28, 30). Tissue (100–500 mg) was added to 3–5 volumes of cold assay reaction medium (in mmol/l: 225 mannitol, 75 sucrose, 10 Tris base, adjusted to pH 7.40 with 10% phosphoric acid) and homogenized in a microcentrifuge tube using a motor-driven pestle (Kontes Pellet Pestle with cordless motor). The homogenate was then subjected to differential centrifugation according to the procedure of Henry and coworkers (28, 29), and various fractions (initial supernatant or epithelial homogenate following removal of cell debris, final supernatant, or cytoplasm containing cytosolic CA activity, and final pellet or microsomes containing membrane-associated CA activity) were saved for analysis of CA activity (in duplicate or triplicate) by means of the electrometric ΔpH method (28). Typically, a 50-μl aliquot of sample (diluted up to 5-fold, as needed) was assayed for CA activity using 6 ml of reaction medium held at 4°C, with 200 μl of CO₂-saturated distilled water to initiate the reaction. The reaction velocity was measured over the initial 0.15 unit pH change. To obtain the catalyzed rate, the rate of the uncatalyzed CO₂ hydration reaction was subtracted from the observed rate. A pH electrode/meter combination (models GK2401C and PHM 84; Radiometer) and data acquisition software (Biopac with AcqKnowledge version 3.7.3 software) were used to measure the pH of the reaction medium.

A defining characteristic of type IV CA is its GPI anchorage to the plasma membrane (57). The GPI anchor can be cleaved by phosphatidylinositol-specific phospholipase C (PI-PLC), and hence a diagnostic test for the presence of type IV CA activity is the release of CA activity from its membrane association by incubation with PI-PLC. To assess the sensitivity of intestinal epithelial microsomal CA activity to incubation with PI-PLC, microsomal fractions (final pellet) were resuspended in reaction medium, divided into two equal aliquots, and incubated (for 90 min at room temperature) with or without 1 unit of PI-PLC (Sigma). Following incubation, the suspensions were subjected to ultracentrifugation (100,000 g for 60 min at 4°C) to pellet the microsomes, and the CA activities of the resultant supernatants were assessed.
Enzymatic activity of ATPases. The NKA activity assay employed has been described previously (45) and was modified for H⁺-ATPase activity as outlined by Lin and Randall (39). In brief, tissue samples consisting of mucosal scrapings from the anterior and posterior regions of the intestine were homogenized in 25 µl of SEID (1 g sodium deoxycholate added to 20 ml sucrose-EDTA-imidazole buffer, see Ref. 38) by 10 strokes of a Dounce homogenizer, and the homogenate was centrifuged at 5,000 g for 30 s. The resultant supernatant was used for determination of protein concentration and ATPase activities. The ATPase activity was measured for 10–15 min by adding 10 µl of tissue homogenate to 200 µl of assay reagent (50 mmol/l imidazole buffer, 2 mmol/l phosphoenolpyruvate, 0.2 mmol/l NADH, 0.7 mmol/l ATP, 4.6 IU lactic dehydrogenase, 5.1 IU pyruvate kinase, pH 7.5) in a 96-well microplate. For determination of NKA activity, 13 mmol/l of ouabain was added to the assay reagent. For determination of V-ATPase activity, 17 mmol/l of N-ethylmaleimide was added to the assay reagent. Final NKA or V-ATPase activity was calculated by subtracting the rate of NADH hydrolysis in the presence of the inhibitor from that of the control. All samples were assayed in triplicate or quadruplicate, and absorbance was measured at 340 nm by using a Spectramax 340PC microplate reader (Molecular Devices). Protein concentrations were measured in triplicate using the Bio-Rad protein assay, and ATPase activity was expressed as micromole ADP per milligram of protein per hour.

Data Presentation and Statistical Evaluation

Numerical data are presented as means ± 1 SE throughout. Significant differences between post-65% seawater transfer and freshwater control values for plasma and intestinal fluid ion concentrations were assessed by Student’s t-test with the Bonferroni multisample comparison correction. Significant differences in mRNA expression as determined by real-time PCR as well as NKA and V-ATPase data were assessed using one-sample Student’s t-tests. The CA enzyme activity data were analyzed by two-way repeated-measures ANOVA, with treatment (control vs. seawater) and gut section (anterior vs. posterior) as the two factors. In all cases, values were considered significantly different at \( P < 0.05 \).

**Fig. 1.** Concentrations of selected electrolytes in rainbow trout plasma following abrupt transfer to 65% seawater; means ± 1 SE, \( n = 6 \) for time points up to 48 h and in plasma of fish gradually acclimated to 65% seawater for 3 wk. All ions except \( \text{SO}_4^{2-} \) were significantly (indicated by an asterisk) elevated from freshwater control values from 3 h posttransfer throughout the 500 h of exposure; \( \text{SO}_4^{2-} \) concentrations were significantly elevated after 24 h (Student’s t-test with Bonferroni multisample comparison correction, \( P < 0.05 \)).

**Fig. 2.** Concentrations of \( \text{Na}^+ \) and \( \text{Mg}^{2+} \) (A), \( \text{Cl}^- \) and \( \text{SO}_4^{2-} \) (B) and \( \text{K}^+ \), \( \text{Ca}^{2+} \) and total \( \text{CO}_2 \) (C) in fluids obtained from the anterior region of rainbow trout intestines following abrupt transfer to 65% seawater; means ± 1 SE, \( n = 6 \) for time points up to 48 h and in intestinal fluids of fish gradually acclimated to 65% seawater for 3 wk. Note that the intestine of freshwater-acclimated rainbow trout did not contain enough fluids for analysis. An asterisk denotes a significant difference from the corresponding 3-h posttransfer sample (Student’s t-test with Bonferroni multisample comparison correction, \( P < 0.05 \)).
RESULTS

Abrupt transfer to 65% seawater resulted in a transient loss of ionic homeostasis as indicated by increasing plasma electrolytes followed by a second steady state reached ~48 h posttransfer (Fig. 1). Values from samples obtained at 48 h and 3 wk posttransfer did not differ significantly (Fig. 1).

Insufficient volumes of fluid in the gastrointestinal tract of freshwater trout prevented analysis of its chemical composition. However, 3 h posttransfer, the pyloric ceca and the anterior intestine already contained significant amounts of fluid suggesting an early onset of drinking in response to the transfer to hyperosmotic conditions. The more distal segments of the intestine did not contain fluids until 6–24 h posttransfer (data not shown). At no point did the intestine contain food particles. The concentrations of Mg²⁺, SO₄²⁻, K⁺, and Ca²⁺ in the anterior intestine 3 h posttransfer were similar to corresponding concentrations in 65% seawater (~33, 20, 7, and 7 mM, respectively; Fig. 2). In contrast, Na⁺ and Cl⁻ concentrations in the anterior intestinal fluids 3 h after transfer were considerably lower than their values in 65% seawater (~280 and 306 mM, respectively; Fig. 2). The concentrations of K⁺ and Ca²⁺ did not change appreciably between 3 h and 3 wk posttransfer (Fig. 2).

Relative cytosolic tCAc mRNA expression was elevated in several intestinal segments following transfer to 65% seawater with the pyloric ceca showing highest expression 24 and 48 h posttransfer (Fig. 3A). The tCAc protein appeared to be preferentially located to the apical region of the intestinal epithelium (Fig. 3B). Similarly, relative tCAIV mRNA expression was markedly elevated in the pyloric ceca and the posterior gut 48 h posttransfer to 65% seawater. The tCAIV protein appeared to be localized to the apical region of the intestinal epithelium (Figs. 4, B and C).

Total CA activity in homogenates of the intestinal epithelium was elevated after 3 wk of exposure to 65% seawater (Fig. 5A). Furthermore, total CA activity was significantly higher in the anterior compared with posterior region of the intestine (Fig. 5A). To test for the presence of membrane-bound CA activity, matched aliquots of microsomal suspensions were incubated with either PI-PLC or buffer, and the CA activity of the supernatant was analyzed. PI-PLC treatment resulted in significantly higher CA activities compared with buffer controls in supernatants from the anterior intestine of both fresh-}

![Figure 3](http://ajpregu.physiology.org/)

**Fig. 3.** A: relative expression of rainbow trout cytoplasmic carbonic anhydrase (tCAc) mRNA in the pyloric ceca, as well as anterior, mid-, and posterior regions of the intestine following abrupt transfer to 65% seawater for time points up to 48 h and in tissue from fish gradually acclimated to 65% seawater for 3 wk. Relative mRNA expression was determined by real-time PCR and expressed as fold change relative to the freshwater control value, which was set to 1 (gray horizontal bar). Values are means ± 1 SE (n = 6). An asterisk indicates a significant difference from the freshwater control value of 1 (Student’s one-sample t-test, P < 0.05). B: localization by immunohistochemistry of tCAc protein in the anterior intestinal epithelium of rainbow trout gradually acclimated to 65% seawater. The image consists of overlays of 3 images collected for tCAc immunoreactivity (green), Na⁺/K⁺-ATPase (NKA) immunoreactivity (red) and nuclei visualization (blue). C: omission of primary tCAc and NKA-5 antibodies eliminated all immunofluorescence. Scale bars = 100 μm.
water and 65% seawater-acclimated fish, as well as from the posterior region of seawater-acclimated fish, indicating the presence of significant membrane-bound CA activity in these samples. Additionally, acclimation to 65% seawater resulted in an increase in membrane-bound CA (Fig. 5B). The results of fractionation experiments revealed that the majority of the CA activity in the intestinal epithelium was cytoplasmic but that the membrane-associated fraction comprised 13–20% of the total activity, regardless of intestinal segment or ambient salinity (data not shown).

Relative NHE3 mRNA expression was greatly increased in the anterior intestine at 24 and 48 h posttransfer to 65% seawater (Fig. 6).

Relative V-ATPase mRNA expression was increased in all intestinal segments 48 h following transfer to 65% seawater (Fig. 7A) and correspondingly, an ~10-fold increase in intestinal epithelium V-ATPase enzymatic activity was detected after 3 wk in 65% seawater (Fig. 7B). The results of double immunolabeling of the V-ATPase and NKA (Figs. 7, C and D) suggest polarization of the proton pump protein to the apical regions and the NKA proteins to the basolateral regions of the intestinal epithelium. However, V-ATPase immunofluorescence, alone, revealed the presence of V-ATPase in the basolateral regions as well (Figs. 7, E and F).

Relative mRNA expression of NBC1 was significantly increased at 6 and 48 h posttransfer to 65% seawater, but only in the pyloric ceca and the anterior intestine (Fig. 8).

An increase in relative NKA mRNA expression was detected in the pyloric ceca and the anterior intestine after 6 and 48 h and after 3 wk posttransfer to 65% seawater, and in the posterior intestine 48 h after onset of exposure to 65% seawater (Fig. 9A). Despite this substantial transcriptional response, NKA activity was not significantly increased in epithelial homogenates (Fig. 9B). Immunolabeling of NKA in the anterior intestine of trout acclimated to 65% seawater revealed intense staining in the basolateral regions of the intestinal epithelium (Figs. 9, C and D).

**DISCUSSION**

Abrupt transfer of rainbow trout from freshwater to seawater clearly presented an osmoregulatory challenge as evidenced by the disturbance in extracellular fluid ionic composition. However, a second state of homeostasis was achieved within 48 h. The rapid response to the 65% seawater challenge included transient increases in relative mRNA levels of all genes investigated. Although direct association of gene expression, associated enzymatic activity levels, and fluid transport remains to be demonstrated, these transcriptional responses and observed changes in enzymatic activities suggest, for the first time, roles for tCA c and tCA IV as well as $\text{HCO}_3^-$ and $\text{H}^+$ transporters in intestinal transport processes relevant to osmoregulation in marine fish.

**Drinking and Ion Transport in the Intestine**

Although drinking and intestinal ion transport were not directly measured, the spot samples of intestinal fluids obtained after transfer to 65% seawater provided insight into the dynamic regulation of these processes. In these unfed fish, relatively high concentrations of $\text{Mg}^{2+}$ and $\text{SO}_4^{2-}$ in the fluids obtained from the anterior intestine after only 3 to 6 h suggest
onset of drinking inferred from luminal Mg²⁺ intestinal fluid absorption rates in marine fish (24). The early onset of drinking is in agreement with previous reports on other marine teleosts including rainbow trout (23, 56, 58, 64). The present study is in good agreement with earlier reports from Japanese and European eel have demonstrated immediate onset of drinking following seawater transfer (31, 37). Based on our observations of early appearance of high levels of Mg²⁺ and SO₄²⁻ in the intestinal lumen following transfer to 65% seawater and the abundant earlier reports of fast onset of drinking following transfer to seawater in euryhaline fish, there can be little doubt that the rainbow trout used in the present study exhibited a drinking response within few hours after transfer to 65% seawater.

Intestinal fluid absorption clearly was increased between 6 and 24 h following exposure to 65% seawater as indicated by the precipitous elevation in luminal concentrations of Mg²⁺ and SO₄²⁻. The approximately fivefold increase in the concentrations of these ions between 6 and 24 h after transfer demonstrates that at least 80% of the fluids entering the intestine were absorbed in the pyloric and anterior regions. The concomitant fall in luminal Na⁺ and Cl⁻ concentrations in this early phase of seawater acclimation suggests that fluid uptake is closely linked to NaCl absorption. During the period from 3 to 24 h post-65% seawater challenge, intestinal lumen total CO₂ concentrations almost doubled to reach levels of > 90 mM (note that total CO₂ exists mainly as HCO₃⁻ in the intestinal fluids see Ref. 25). The anion composition of intestinal fluids obtained in 65% seawater-acclimated animals in the present study is in good agreement with earlier reports from marine teleosts including rainbow trout (23, 56, 58, 64).

The changes in luminal concentrations of Na⁺, Cl⁻, and HCO₃⁻ during the period from 6 to 24 h posttransfer provide insight into the nature of the solute carriers involved in the absorption of Na⁺ and Cl⁻. During this period, luminal Na⁺ concentrations decreased by ~36 mM, Cl⁻ concentrations dropped by ~62 mM, and HCO₃⁻ concentrations increased by 23 mM. The simplest explanation for these changes is Na⁺ and Cl⁻ absorption in a stoichiometry of 1:1 (Na⁺−Cl⁻ cotransport) accounting for a change in concentration of both these ions of 36 mM, and anion exchange with a 1:1 stoichiometry.

Fig. 5. Total homogenate CA activity (A) and phosphatidylinositol-specific phospholipase C (PI-PLC; B) released CA activity (expressed as the difference in supernatant CA activity between microsomal suspensions incubated with PI-PLC vs. homogenization buffer) in intestinal epithelial cells (mucosal scrapings) from the anterior (not including pyloric ceca) and posterior regions of rainbow trout fully acclimated to freshwater (0 h) or gradually acclimated to 65% seawater (3 wk). Values are means ± 1 SE. *Significant difference between 65% seawater and freshwater samples (the "treatment" factor in 2-way repeated-measures ANOVA). A significant effect of region ("gut section" factor) was also detected for total homogenate CA activity, but the interaction term was not significant for either analysis (2-way repeated-measures ANOVA, P < 0.05 for significance). In B, an asterisk indicates a significant difference between buffer and PI-PLC incubated samples (paired Student’s t-test, P < 0.05).

Fig. 6. Relative expression of rainbow trout Na⁺/H⁺ exchanger 3 (NHE3) mRNA in the pyloric ceca, as well as the anterior, mid-, and posterior regions of the intestine following abrupt transfer to 65% seawater for time points up to 48 h and in tissue from fish gradually acclimated to 65% seawater for 3 wk. Relative mRNA expression was determined by real-time PCR and expressed as fold change relative to the freshwater control value, which was set to 1 (gray horizontal bar). Values are means ± 1 SE, *Significant difference from the freshwater control value of 1 (1-sample Student’s t-test, P < 0.05).
accounting for a change in HCO₃⁻ and Cl⁻ concentrations of 23 mM. Together, these transport processes would account for 95% of the overall net Cl⁻ absorption. A relative contribution of anion exchange to Cl⁻ absorption of 37% is in reasonable agreement with the value of 50% obtained through in situ measurements of net intestinal ion transport in the lemon sole (Parophrys vetulus) (21) and falls well within the range of 10 to 70% estimated in experiments using isolated intestinal epithelia (20). The 5% of Cl⁻ uptake unaccounted for by this scenario may be explained by errors associated with Na⁺, Cl⁻, and total CO₂ measurements but could also be attributed to activity of NKCC. The stoichiometry of NKCC would contribute to excess Cl⁻ absorption and because K⁺ concentrations were not changing in the intestinal lumen despite substantial water absorption, net K⁺ absorption must have occurred. In addition to NKCC, SO₄²⁻/Cl⁻ exchange may have contributed to Cl⁻ absorption (51). However, SO₄²⁻ secretion rates in the marine teleost intestine are in the order of a few nmol·cm⁻²·h⁻¹ compared with the net Cl⁻ absorption rates in the low range (20), and therefore SO₄²⁻/Cl⁻ exchange plays a quantitatively minor role in Cl⁻ absorption. Regardless of the possible involvement of NKCC and SO₄²⁻/Cl⁻ exchange,
Our data confirm a significant role for anion exchange in marine fish osmoregulation as recently suggested (20, 22, 25, 66).

**Gene Expression, Immunohistochemistry, and Enzyme Activities**

The findings of the present study implicate two CA isoforms, tCAc and tCAIV, in osmoregulatory transport processes in the intestine of rainbow trout during acclimation to seawater. Our hypothesis of tCAc and tCAIV being localized to the apical region of the intestinal epithelium was supported by immunohistochemistry results. Transient increases of relative apical region of the intestinal epithelium was supported by observations of in-situ hybridization of NBC1 mRNA following transfer to 65% seawater. While assay conditions were optimized for homogenized freshwater tissue (45), these conditions may not have been optimal for NBC1 activity in tissues obtained from 65% seawater-exposed fish.

NKA protein was highly abundant not only basolaterally, but also in the lateral regions of the intestinal epithelial cells, which is consistent with observations for water-absorbing tissues from other vertebrates, including the intestine, gall bladder, and renal tubules. Due to the stoichiometry of the NKA, this lateral localization results in the adjacent intercellular spaces becoming hyperosmotic and hyperbaric, conditions that are pivotal for transepithelial water absorption (38).

While some transcriptional responses (both CA isoforms, NHE3, and NBC1) and the overall CA enzymatic activity were clearly most pronounced in the pyloric ceca and the anterior intestine, the V-ATPase and NKA mRNA expression and enzyme activity responses appear to be robust in all intestinal segments. Although all intestinal segments apparently are capable of salt and water absorption in vitro, the anterior regions are likely exhibiting the highest HCO₃⁻ secretion.

The relatively intense V-ATPase immunostaining in basolateral regions of the intestinal epithelial cells, and the greatly elevated enzymatic activity attributable to this protein in epithelial homogenates from trout acclimated to 65% seawater are in agreement with reports that basolateral H⁺ secretion is necessary for apical Cl⁻/HCO₃⁻ exchange and thus osmoregulation in the gulf toadfish (22). However, V-ATPase staining in the apical intestinal epithelium, as well as elevated mRNA expression of NHE3, an apical NHE isoform, strongly suggest that the intestine of trout acclimated to 65% seawater is capable of luminal proton secretion. The functional contribution of an apical NHE-type protein is supported by observations of increased luminal base secretion in seawater-acclimated rainbow trout following treatment with amiloride (64); simultaneous apical anion- and Na⁺/H⁺-exchange would result in an apparent increase in base secretion following Na⁺/H⁺-exchange inhibition.

The observed increase in NBC1 mRNA expression following transfer to 65% seawater is consistent with recent suggestions of a role for basolateral NBC in intestinal HCO₃⁻ secretion in marine fish (22) and fits with early observations of intestinal HCO₃⁻ secretion by the Japanese eel being dependent on Na⁺ in the serosal medium (2). These recent suggestions were based on observations of elevated luminal HCO₃⁻ secretion in the presence of physiologically relevant levels of molecular CO₂ and HCO₃⁻ in the serosal salines (22). However, because molecular CO₂ and HCO₃⁻ were covaried, contributions to cellular CO₂ hydration from serosal CO₂ could not be distinguished from those due to HCO₃⁻ uptake across the basolateral membrane. The greatly increased relative expression of NBC1 mRNA following transfer to 65% seawater supports a contribution of HCO₃⁻ uptake from the extracellular fluids to luminal HCO₃⁻ secretion without excluding intracellular hydration of molecular CO₂ as an important source of exchangeable HCO₃⁻.

This finding of increased relative expression of NKA mRNA following transfer to 65% seawater is consistent with previous reports of elevated NKA mRNA expression in euryhaline species after seawater exposure (8, 34, 55). However, in contrast to earlier findings (7, 15, 33, 42) elevated relative mRNA curiously did not translate into increased enzymatic NKA activity in the present study, although activity in the anterior segment of the intestine appeared to be somewhat elevated following 65% seawater exposure. It is unclear why increased NKA activity was not observed in the present study, although it should be noted that NKA activity measurements were performed using the same assay protocol for samples obtained from control fish in freshwater and from fish transferred to 65% seawater. While assay conditions were optimized for homogenized freshwater tissue (45), these conditions may not have been optimal for NKA activity in tissues obtained from 65% seawater-exposed fish.
suggested by transcriptional differences seen in the present study and documented at least for Cl\textsuperscript{−} transport in earlier studies on freshwater-acclimated rainbow trout (6). The conceptual transport model presented in Fig. 10 and discussed below is a general model for the seawater-acclimated trout intestine, but it is likely that differences exist among different intestinal regions.

**Conceptual Epithelial Transport Model**

A conceptual model for intestinal ion and water transport is presented in Fig. 10. The identity of the apical anion exchange protein(s) remains to be determined, and it should also be noted that apical anion exchange occurs in concert with different apical Na\textsuperscript{+}-Cl\textsuperscript{−} cotransport proteins, included in the Fig. 10. Once absorbed by the intestinal epithelia cells Cl\textsuperscript{−} exits across the basolateral membrane via Cl\textsuperscript{−} channels (41) or K\textsuperscript{+}-Cl\textsuperscript{−} cotransporters (26) or, in some cases, is secreted into the lumen via apical CFTR-type Cl\textsuperscript{−} channels (44). A novel aspect of intestinal transport physiology in marine teleosts suggested by the present findings is the involvement of a V-ATPase in intestinal transport physiology in marine teleosts suggested by transcriptional differences seen in the present study and documented at least for Cl\textsuperscript{−} transport in earlier studies on freshwater-acclimated rainbow trout (6). The conceptual transport model presented in Fig. 10 and discussed below is a general model for the seawater-acclimated trout intestine, but it is likely that differences exist among different intestinal regions.

![Conceptual Epithelial Transport Model](image)

**A** relative expression of rainbow trout NKA mRNA in the pyloric ceca, as well as the anterior, mid-, and posterior regions of the intestine following abrupt transfer to 65% seawater for time points up to 48 h and in tissue from fish gradually acclimated to 65% seawater for 3 wk. Relative mRNA expression was determined by real-time PCR and expressed as fold change relative to the freshwater control value, which was set to 1 (gray horizontal bar). Values are means ± SE, (n = 6), and an asterisk indicates a significant difference from the freshwater control value of 1 (1-sample Student’s t-test, P < 0.05). B: activity of NKA (nmol ADP/mg protein h\textsuperscript{−1}) in intestinal tissue from freshwater (0 h) and in rainbow trout gradually acclimated to 65% seawater (3 wk). Values are means ± SE, (n = 8 and 5 for 0 h and 3 wk, respectively). No significant differences between treatments or intestinal region were detected (Student’s t-test with Bonferroni multisample comparison correction). C and D: low- and high-magnification images of the localization by immunohistochemistry of NKA protein in the anterior intestinal epithelium of 65% seawater-acclimated rainbow trout. The images consist of overlays of 2 images collected for NKA immunoreactivity (red) and nuclei visualization (blue). Insert: omission of the primary NKA\textsubscript{αA}-5 antibody eliminated all immunofluorescence. Scale bars = 25 μm.

This suggests that full polarization of HCO\textsubscript{3}{−} and H\textsuperscript{+} secretion, as detected in the isolated toadfish intestine under resting conditions, may not apply uniformly across environmental conditions and/or species. While anion exchange provides for Cl\textsuperscript{−} uptake, regardless of basolateral or apical H\textsuperscript{+} secretion, luminal alkalization serves to promote CaCO\textsubscript{3} formation, which contributes significantly to water absorption by the marine teleost intestine (20, 65, 66). Luminal CaCO\textsubscript{3} precipitation and the consequent reduction in osmotic pressure (as much as 70 mOsm, see Ref. 66) would be most effective if H\textsuperscript{+} arising from endogenous CO\textsubscript{2} hydration were secreted across the basolateral membrane. However, apical H\textsuperscript{+} extrusion via NHE3, which clearly shows increased mRNA expression following seawater acclimation, would contribute to Na\textsuperscript{+} absorption, which ultimately favors water transport and thus would be beneficial to marine osmoregulation. In the gulf toadfish, NKA provides energy for the secondary active HCO\textsubscript{3}{−} secretion by facilitating basolateral proton extrusion via an NHE-like mechanism. Considering the increase in NBC1 expression following seawater transfer, transepithelial HCO\textsubscript{3}{−} transport in rainbow trout intestine also appears to depend on Na\textsuperscript{+} gradients and, thus, ultimately NKA.

At least in rainbow trout, the V-ATPase offers an additional driving force for the secondary active HCO\textsubscript{3}{−} secretion mech-
anism. The benefit of basolateral V-ATPase activity is easily perceived, as it contributes to luminal alkalization and hence CaCO₃ precipitation. Although possible benefits of apical V-ATPase activity are less clear, one possibility could be related to the titration of luminal HCO₃⁻ by apical H⁺ extrusion (via both NHE3 and V-ATPase). Apical anion exchange removes Cl⁻ from the intestinal lumen by replacement with HCO₃⁻, and hence the net effect of this exchange process on luminal osmotic pressure may be benign. Anion exchangers of the SLC26 family display different transport stoichiometries (47), but in all cases, anion exchange contributes osmolytes (HCO₃⁻) to the intestinal lumen. However, anion exchange combined with apical H⁺ extrusion may contribute to Cl⁻ absorption without the addition of osmolytes to the intestinal lumen because the titration of HCO₃⁻ by extruded H⁺ would yield molecular CO₂. This scenario would elevate the CO₂ partial pressure in the intestinal lumen, promoting CO₂ diffusion into the epithelial cell, much like the case in the proximal tubule during acidosis (18). Such recycling of HCO₃⁻ ions may provide additional substrate for the apical anion exchange, thus favoring Cl⁻ uptake.

Therefore, it appears that two possible means of reducing luminal osmotic pressure exist, but that they might be mutually exclusive as one occurs under alkaline conditions (CaCO₃ precipitation), while the other relies on apical H⁺ extrusion (HCO₃⁻ titration). The relative importance of these two mechanisms may be influenced by the availability of luminal Ca²⁺ for CaCO₃ precipitation. Concentrations of Ca²⁺ in seawater are ~10 mM and should be much higher in the intestinal lumen given limited intestinal Ca²⁺ uptake and substantial water absorption. However, measured luminal Ca²⁺ concentrations range between 2 and 5 mM in most cases (23, 66) owing to CaCO₃ precipitation (65). Presumably, a certain level of luminal Ca²⁺ is required for epithelial function and integrity, and it appears likely that Ca²⁺ may become limiting for CaCO₃ formation and epithelial function in the presence of a large excess of HCO₃⁻ (approaching 100 mM in the present study). In situations of luminal Ca²⁺ limitations, continued apical anion exchange would be best accompanied by apical H⁺ extrusion to reduce luminal HCO₃⁻ concentrations, osmotic pressure, and Ca²⁺ precipitation.

Our observations support the role of CA (tCAc) in providing substrate for apical anion exchange (22, 64) and provide the first evidence that it plays a role in the dynamic acclimation response to elevated ambient salinity. In addition, we provide the first evidence that extracellular, membrane-bound CAIV is involved in acclimation to hypersaline conditions. We propose that membrane-bound CAIV may contribute to both mechanisms reducing luminal osmotic pressure by facilitating either biomineralization (CaCO₃ precipitation), as seen in many other organ systems across the animal kingdom (see INTRODUCTION), or HCO₃⁻ dehydration in the face of apical H⁺ extrusion as suggested for the trout proximal tubule (18).

The novel, but admittedly speculative, role suggested for apical H⁺ extrusion in controlling luminal HCO₃⁻ concentrations, Ca²⁺ precipitation, and osmotic pressure has some empirical support from our measurements of intestinal fluid chemistry. Between 24 and 48 h posttransfer to 65% seawater, Cl⁻ concentrations continued to drop, while Na⁺ concentrations remained constant, suggesting Cl⁻ uptake via a Na⁺-independent pathway, such as anion exchange. However, luminal HCO₃⁻ concentrations fell rather than increased, which may reflect activation of apical H⁺ extrusion during the period of 24 to 48 h post-65% seawater transfer. Interestingly, the fall from maximal HCO₃⁻ concentrations in the intestinal lumen 24 h after transfer to steady-state levels after 48 h coincided with the fall from luminal Ca²⁺ concentrations continued to drop, while Na⁺ concentrations fell rather than increased, which may reflect activation of apical H⁺ extrusion during the period of 24 to 48 h post-65% seawater transfer. Interestingly, the fall from maximal HCO₃⁻ concentrations in the intestinal lumen 24 h after transfer to steady-state levels after 48 h coincided with the fall from luminal Ca²⁺ concentrations.
eurynale flounder revealed that elevated luminal Ca\(^{2+}\) stimulates HCO\(_3^-\) secretion, presumably to enhance CaCO\(_3\) precipitation. Accepting the proposed role of apical H\(^+\) secretion, it seems possible that reduced luminal Ca\(^{2+}\) concentrations might also influence apical H\(^+\) secretion to further reduce luminal alkalization. In addition to the possibility of Ca\(^{2+}\)-mediated regulation, luminal osmotic pressure and/or HCO\(_3^-\) concentrations may trigger shifts in the direction (apical vs. basolateral) of H\(^+\) extrusion. The prediction would be that low Ca\(^{2+}\) concentrations, high osmotic pressure, and high HCO\(_3^-\) concentrations in the intestinal lumen would facilitate apical H\(^+\) extrusion, while high luminal Ca\(^{2+}\) would trigger basolateral H\(^+\) secretion.

**Perspectives and Significance**

While the direction of H\(^+\) transport from epithelial cells is likely subject to complex regulation, the fish intestine exhibits net base secretion, ultimately leading to rectal base excretion in hyperosmotic environments. The magnitude of this rectal base secretion, ultimately leading to rectal base excretion in hyperosmotic environments. The magnitude of this rectal base secretion, ultimately leading to rectal base excretion in hyperosmotic environments. The magnitude of this rectal base secretion, ultimately leading to rectal base excretion in hyperosmotic environments. The magnitude of this rectal base secretion, ultimately leading to rectal base excretion in hyperosmotic environments. The magnitude of this rectal base secretion, ultimately leading to rectal base excretion in hyperosmotic environments.

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