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The role of branchial carbonic anhydrase in acid–base regulation in rainbow trout (*Oncorhynchus mykiss*)

T. Georgalis, S. F. Perry and K. M. Gilmour*

Department of Biology and Centre for Advanced Research in Environmental Genomics, University of Ottawa, Ottawa, Ontario, K1N 6N5, Canada

*Author for correspondence (e-mail: Katie.Gilmour@science.uottawa.ca)

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Summary

The objective of the present study was to examine the branchial distribution of the recently identified rainbow trout cytoplasmic carbonic anhydrase isoform (tCAc) and to investigate its role in the regulation of acid–base disturbances in rainbow trout (*Oncorhynchus mykiss*). *In situ* hybridization using an oligonucleotide probe specific to tCAc revealed tCAc mRNA expression in both pavement cells and mitochondria-rich cells (chloride cells). Similarly, using a homologous polyclonal antibody, tCAc immunoreactivity was localized to pavement cells and mitochondria-rich cells in the interlamellar region and along the lamellae of the gills. Exposure of rainbow trout to hypercarbia (~0.8% CO_2) for 24 h resulted in significant increases in tCAc mRNA expression (~20-fold; quantified by real-time PCR) and protein levels (~1.3-fold; quantified by western analysis) but not enzyme activity (assessed on crude gill homogenates using the delta-pH CA assay). Inhibition of branchial CA activity *in vivo* using acetazolamide reduced branchial net acid excretion significantly by 20%. This effect was enhanced to a 36% reduction in branchial net acid excretion by subjecting the trout to hypercarbia (~0.8% CO_2) for 10 h prior to acetazolamide injection, an exposure that significantly increased branchial net acid excretion. The results of the present study support the widely held premise that branchial intracellular CA activity (tCAc) plays a key role in regulating acid–base balance in freshwater teleost fish.

Key words: carbonic anhydrase, acid–base balance, gill, rainbow trout, acetazolamide, hypercarbia, acid excretion.

Introduction

A plethora of evidence has clearly established the gills of teleost fish as the predominant site of acid–base relevant ion transfer, the transfer of H^+ and/or HCO_3^-, for the maintenance of systemic pH (reviewed by Perry and Laurent, 1990; Goss et al., 1992, 1995; Perry, 1997; Claiborne et al., 2002; Perry et al., 2003; Evans et al., 2005). Current models (e.g. Claiborne et al., 2002; Perry et al., 2003; Evans et al., 2005) postulate that CO_2 entering the gill epithelium is hydrated to HCO_3^- and H^+ in the presence of the enzyme carbonic anhydrase (CA). Regulation of systemic pH is then achieved by adjusting the rates of acid and/or base excretion, which in turn are linked to ion uptake through the involvement of a Na^+-coupled H^+ extrusion mechanism (Na^+/H^+ exchanger and/or Na^+ channel/V-type H^+-ATPase) and a Cl^-/HCO_3^- exchange mechanism. Morphological adjustments of the gill epithelium in freshwater teleosts may contribute to the regulation of systemic acid–base disturbances by modifying the availability of these exchange mechanisms (Goss et al., 1992, 1995). For example, a reduction in the exposed surface area of mitochondria-rich cells (MR cells; also termed chloride cells) under acidotic conditions serves to lower the availability of Cl^-/HCO_3^- exchangers, which are thought to be localized to MR cells, thereby contributing to the accumulation of HCO_3^- ions that is needed to compensate for the extracellular acidosis.

Although the involvement of ion uptake pathways in the regulation of acid–base balance has been well established experimentally (Payan and Maetz, 1973; Claiborne and Heisler, 1984, 1986; Perry et al., 1987a; Wood, 1988; Goss and Perry, 1993; Larsen and Jensen, 1997; Hirata et al., 2003), and evidence supporting the contribution of CA to ion uptake exists (e.g. Maetz, 1956; Maetz and Garcia-Romeu, 1964; Boisen et al., 2003; Chang and Hwang, 2004), experimental data for the role played by intracellular CA activity in acid–base regulation specifically are lacking. High levels of CA activity have been found in the gills of all fish species examined (reviewed by Perry and Laurent, 1990; Henry and Swenson, 2000). In teleost fish, the branchial CA activity is restricted to the cytoplasm (Henry et al., 1988; Gilmour et al., 1994, 2001; Perry et al., 1997; Gervais and Tufts, 1998; reviewed by Henry and Swenson, 2000), although in elasmobranch and chimaeran fish, branchial membrane-associated CA activity is also present (Gilmour et al., 1997, 2002; Wilson et al., 2000c). Immunohistochemical

evidence indicates that branchial cytoplasmic CA is present in both pavement and MR cells, with a generally apical location (Rahim et al., 1988; Sender et al., 1999; Wilson et al., 2000b). Rahim et al. (1988) also provided immunological evidence for the presence of distinct branchial and blood CA isoforms in rainbow trout and carp, a finding that was recently confirmed by the cloning of rainbow trout blood (tCAb) and cytosolic (tCAc) CA isoforms (Esbaugh et al., 2004, 2005). Northern and real-time PCR analyses identified the gills as a site of considerable tCAc mRNA expression (Esbaugh et al., 2005).

With this background in mind, the present study was conceived with three objectives. The first objective was to provide further support for the presence of a distinct CA isoform, tCAc, in rainbow trout gills by localizing tCAc mRNA expression and immunoreactivity within the gills using *in situ* hybridization and immunohistochemistry. The second and main objective was to test the hypothesis that branchial intracellular CA activity plays a significant role in the regulation of systemic acid–base balance. This goal was accomplished by assessing the branchial net excretion of acid–base equivalents during the inhibition of CA activity. Experiments were carried out under control conditions and during the imposition of an acid–base challenge (respiratory acidosis), a situation in which the importance of CA activity to branchial net acid excretion was predicted to be enhanced. The third and final objective of the present study was to test the hypothesis that upregulation of branchial CA activity (specifically tCAc) is one component of the response to an acid–base challenge. Relatively little information exists on the impact of environmental factors, particularly those imposing acid–base challenges, on branchial CA activity (e.g. see Henry and Swenson, 2000). Although significant increases in branchial CA activity were reported for rainbow trout exposed to hypercarbia for 20 days or more (Dimberg and Höglund, 1987), the time course of the changes was not consistent with the pattern typical of acid–base compensation in rainbow trout, in which extracellular pH is restored over 24–72 h (Perry et al., 1987a; Goss et al., 1992, 1993; Larsen and Jensen, 1997; reviewed by Goss et al., 1995). Branchial CA mRNA expression was enhanced by exposure to acidic water in the Osorezan dace, but whether this increase translated into changes in branchial CA activity was not investigated (Hirata et al., 2003). Thus, in the present study, the impact of acute (24 h) hypercarbic exposure on tCAc mRNA expression and immunoreactivity as well as branchial CA activity was assessed.

Materials and methods

Experimental animals

Rainbow trout (*Oncorhynchus mykiss* Walbaum; approximate mass 250 g, N=77) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Fish were maintained on a 12 h:12 h L:D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechloraminated city of Ottawa tap water at 13°C. Fish were allowed to acclimate to the holding conditions for at least 2 weeks prior to any experimentation and were fed to satiation on commercial trout pellets every second day.

Series I: molecular analysis of rainbow trout gill cytoplasmic CA (tCAc)

Experimental protocol and tissue collection

To expose rainbow trout to the acid–base challenge imposed by hypercarbia, fish were placed in individual opaque acrylic boxes for a 24 h acclimation period and were then exposed to external hypercarbia (final nominal water CO_2 tension, $P_{W_{CO_2}}$=6 Torr; 1 Torr=133.3 Pa) for up to 24 h. To achieve the final $P_{W_{CO_2}}$, a water equilibrium column supplying the experimental chambers was gassed with 2% CO_2 in air ($P_{W_{CO_2}}$=14 Torr) to achieve ~0.8% in the water in the holding boxes. Mixed gases were provided using gas mass flow controllers (Sierra C100L Smart-trak; SRB Controls, Markham, ON, Canada). The P_{CO_2} of water exiting the equilibration column to feed the experimental chambers was monitored using a CO_2 electrode (E201; Analytical Sensors, Sugarland, TX, USA) housed in a temperature-controlled cuvette and linked to a meter (BGM200 Blood gas meter; Cameron Instruments, Port Aransas, TX, USA) and data acquisition system (Biopac with AcqKnowledge v. 3.7.3 software; Harvard Apparatus Canada, Saint-Laurent, QC, Canada). Fish allocated to control groups were treated as described above, except that the equilibration column supplying the experimental chambers was gassed with air rather than CO_2-enriched air.

Gill tissue was sampled from trout exposed to hypercarbia or control conditions for analysis of tCAc mRNA expression by real-time PCR or *in situ* hybridization, for immunohistochemical localization of tCAc, for measurement of tCAc protein abundance by western analysis or for measurement of CA activity. In all cases, trout were killed by a blow to the head. Where tissue was used for western analysis, immunohistochemistry or CA assays, saline perfusion was carried out prior to tissue collection to clear the tissues of blood. The bulbus arteriosus was exposed and then cannulated with polyethylene tubing (Clay-Adams PE160 polyethylene tubing; VWR, Montréal, QC, Canada). Approximately 50 ml of ice-cold, heparinized (100 IU ml^{-1} heparin) modified (4.5 mmol l^{-1} $NaHCO_3$) Cortland's saline (Wolf, 1963) containing 10^{-5} mol l^{-1} isoproterenol was infused *via* the cannula; the ventricle was severed during this infusion to allow fluid in the circulatory system to drain from the body. In most cases, the collected tissues were immediately frozen in liquid N_2 and stored at –80°C until analysis. Tissues used for *in situ* hybridization or immunohistochemistry were immersion fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH=7.4) overnight, then transferred to 15% sucrose followed by 30% sucrose for 2 h in each case. Tissues were kept at 4°C throughout the fixation procedure and were then stored at –20°C until use.

Analysis of tCAc mRNA expression

Real-time PCR was used to quantify branchial tCAc mRNA expression in control *versus* hypercarbia-exposed trout, while *in situ* hybridization was used to localize tCAc mRNA within the branchial epithelium.

For the quantification of tCAc mRNA expression by real-time PCR, gill tissue was sampled from hypercarbia-exposed trout at 1, 2, 3, 6, 12 and 24 h (N=6 at each time) and from control trout at 3 h (representing a control point for 1–3 h), 6, 12 and 24 h (N=6 at each time). Total RNA was extracted from 30 mg aliquots of powdered tissue samples using the Absolutely RNA RT-PCR Miniprep Kit (Stratagene, Cedarlane Laboratories, Hornby, ON, Canada). To remove any remaining genomic DNA, the RNA was treated on-column using RNase-free DNase (5 μl) for 15 min at 37°C. The RNA was eluted in 70 μl of nuclease-free H_2O, and its quality was assessed by gel electrophoresis and spectrophotometry (Eppendorf Biophotometer; VWR). cDNA was synthesized from 2 μg of RNA using random hexamer primers and Stratascript reverse transcriptase (Stratagene).

tCAc mRNA levels were assessed by real-time PCR on samples of cDNA (0.5 μl) using a Brilliant SYBR Green QPCR Master Mix Kit (Stratagene) and a Stratagene MX-4000 multiplex quantitative PCR system. ROX (Stratagene) was used as reference dye. The PCR conditions (final reaction volume=25 μl) were as follows: cDNA template=0.5 μl; forward and reverse primer=300 nmol l^{-1}; 2× Master Mix=12 μl; ROX=1:30 000 final dilution. The annealing and extension temperatures over 40 cycles were 58°C (30 s) and 72°C (30 s), respectively. The primer pairs used were those designed and reported by Esbaugh et al. (2005): β-actin forward 5′-CCA ACA GAT GTG GAT CAG CAA-3′, β-actin reverse 5′-GGT GGC ACA GAG CTG AAG TGG TA-3′, tCAc forward 5′-CAG TCT CCC ATT GAC ATC GTA-3′, and tCAc reverse 5′-CGT TGT CGT CGG TGT AGG T-3′. The specificity of the primers was verified by the cloning (TOPO TA cloning kit; Invitrogen, Burlington, ON, Canada) and sequencing of amplified products. To ensure that SYBR Green was not being incorporated into primer dimers or non-specific amplicons during the real-time PCR runs, the PCR products were analyzed by gel electrophoresis in initial experiments. Single bands of the expected size were obtained at all times. The construction of SYBR Green dissociation curves after completion of 40 PCR cycles revealed the presence of single amplicons for each primer pair. To ensure that residual genomic DNA was not being amplified, control experiments were performed in which reverse transcriptase was omitted during cDNA synthesis. Relative expression of mRNA levels was determined (using actin as an endogenous standard) by a modification of the delta-delta Ct method (Pfaffl, 2001). Amplification efficiencies were determined from standard curves generated by serial dilution of plasmid DNA.

For the localization of tCAc mRNA by *in situ* hybridization, a digoxigenin-labelled 48-mer oligonucleotide probe (5′-CCAGGTACGATGTCAATGGGAGACTGGCGGGGTCCG TTGGCAACCC-3′) complementary to 48 nucleotides of the mRNA encoding tCAc was synthesized commercially (http://genedetect.com/). Cryoprotected tissue pieces (see above) were frozen in Shandon Cryomatrix embedding medium (Fisher Scientific, Ottawa, ON, Canada), and thin sections (8–10 μm) were prepared using a cryostat (CM 1850; Leica, Richmond Hill, ON, Canada) at –25°C, collected onto either poly-L-lysine-coated slides (Sigma, Oakville, ON, Canada) or electrostatically charged slides (SuperFrost Plus; Fisher Scientific), allowed to dry for 30 min and then stored at –20°C. *In situ* hybridization was then carried out according to the basic protocol provided by the manufacturer of the probes (http://www.genedetect.com/Merchant2/InsituFrozenDIGOXIN.pdf). Sections were hybridized with 400 ng ml^{-1} probe overnight at 37°C in a humid chamber. For detection of hybridization, sections were incubated first with a blocking solution containing 1% goat serum, 2 mg ml^{-1} bovine serum albumin (BSA) in 0.1 mol l^{-1} PBS with 0.3% Triton-X at room temperature for 1 h, followed by an overnight incubation at 4°C with anti-digoxigenin conjugated to alkaline phosphatase (Roche Molecular Biochemicals, Laval, QC, Canada) diluted 1:1000 in the above solution. Visualization was accomplished using nitroblue tetrazolium (NBT) and 5-bromocresyl-3-indolyl phosphate (BCIP) tablets (Sigma). Colour was allowed to develop in a humid chamber for at least 4 h at room temperature in the dark until satisfactory coloration was achieved. After washing, the slides were mounted with 60% glycerol and cover slipped. Sections were viewed using a Zeiss Axiophot light microscope fitted with a Hamamatsu C5985 chilled CCD camera (Hamamatsu Photonics, Hamamatsu, Japan). Images were captured using Metamorph v. 4.01 imaging system (Universal Imaging Corp., Downingtown, PA, USA).

Two types of negative control experiments were performed to assess the specificity of hybridization: probe was omitted from the hybridization protocol or sections were pre-treated with excess unlabelled probe. For the latter, sections were first incubated with 2000 ng ml^{-1} unlabelled probe in hybridization buffer for 3 h at 37°C. Sections were then subjected to the usual protocol, with the addition of 2000 ng ml^{-1} unlabelled probe to the hybridization buffer.

Quantification and localization of tCAc protein

Western blots were used to quantify tCAc protein expression across different tissues, and in gill tissue from control *versus* hypercarbia-exposed trout, while immunohistochemistry was used to localize tCAc protein within the branchial epithelium. A custom rabbit polyclonal antibody (Abgent, San Diego, CA, USA) was raised for this purpose against trout tCAc using a synthetic peptide (WNTKYPSFGDAASKSDGLA corresponding to amino acids 122–141 of the rainbow trout protein sequence; GenBank accession AAR99329) antigen conjugated to keyhole limpet protein. The antiserum was purified by protein G affinity chromatography (Abgent).

For western analysis, proteins were prepared from frozen gill tissue samples (0.5 g ml^{-1} homogenization buffer). Tissues

were homogenized on ice in Tris-SO_4 buffer (25 mmol l^{-1} Tris-SO_4, 0.9% NaCl, pH 7.4) containing protease inhibitors (complete^{TM} Mini protease inhibitor cocktail tablets; Roche Molecular Biochemicals) and 2 μg ml^{-1} pepstatin A (Sigma). Samples were stored on ice for 15 min and then centrifuged at 7500 ***g*** for 10 min at 4°C. The supernatant containing soluble proteins was frozen and stored at –80°C until subsequent analysis. Total protein concentrations were assessed by the bicinchoninic acid method (Pierce Biotechnology Micro BCA protein assay; Fisher Scientific) using BSA as the standard. Samples (100–150 μg protein) were size fractionated by reducing SDS-PAGE using 10–14% separating and 4% stacking polyacrylamide gels. Fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) using a Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. After transfer, each membrane was blocked for 1 h in Tris-buffered Tween 20 (TBS-T) containing 5% milk powder. Membranes were then probed first with a 1:100 dilution of rabbit anti-trout tCAc for 1.5 h at 37°C and then with a 1:2000 horseradish peroxidase-conjugated goat anti-rabbit IgG (Pierce, Fisher Scientific). After each incubation, the membranes were washed for 3×5 min in TBS-T. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Pierce SuperSignal West Pico Chemiluminescent Substrate; Fisher Scientific) using a digital gel documentation system (Bio-Rad Chemi Doc) and digital image processing software (Quantity One, v. 4.1.1; BioRad). The protein size marker used was obtained from Fermentas Life Sciences (Burlington, ON, Canada). To demonstrate specificity of the tCAc antibody, membranes were incubated with the tCAc antibody in the presence of an excess (50 μg ml^{-1}) of the peptide against which the antibody was raised.

For the localization of tCAc by immunohistochemistry, tissue sections were prepared as described for *in situ* hybridization (see above). A hydrophobic barrier was created around each section with a PAP pen (Electron Microscopy Suppliers, Fort Washington, PA, USA). Mounted sections were incubated (3×5 min) in a blocking buffer containing 2% normal goat serum, 0.1 mol l^{-1} PB, 0.9% Triton-X, 1% gelatin and 2% BSA. Sections were then incubated for 2 h at room temperature in a humidified chamber with a 1:200 dilution (in blocking buffer) of the rabbit anti-trout tCAc or with a 1:100 dilution of α5, a mouse monoclonal antibody against the α_1 sub-unit of chicken Na^+/K^+-ATPase (University of Iowa Hybridoma Bank), or with both primary antibodies together. The α5 antibody has been used to describe the distribution of Na^+/K^+-ATPase in a wide range of organisms, including a variety of fish species (e.g. Wilson et al., 2000a,b, 2002; Piermarini et al., 2002; Choe et al., 2004). To demonstrate specificity of the tCAc antibody, sections were incubated with the tCAc antibody in the presence of an excess (20 μg) of the peptide against which the antibody was raised. Negative control sections were incubated with blocking buffer lacking primary antibodies. Following incubation, slides were washed (3×5 min) in 0.1 mol l^{-1} PB and incubated with a 1:400 dilution (in 0.1 mol l^{-1} PB) of Alexa 488-coupled goat anti-rabbit IgG (Fisher Scientific) for the detection of tCAc, or Alexa 546-coupled goat anti-mouse IgG (Fisher Scientific) for the detection of α5, or both, as appropriate, for 2 h at room temperature in a humidified chamber. The slides were then washed again (3×5 min in 0.1 mol l^{-1} PB) and mounted with a mounting medium (Vector Laboratories, Burlington, ON, Canada) containing 4′,6′-diamidino-2-phenylindole (DAPI) for the visualization of nuclei. The sections were viewed using a conventional epifluorescence microscope (Zeiss Axiophot) and CCD camera (Hamamatsu C5985). Images were captured using Metamorph v. 4.01 imaging software.

Assessment of branchial CA activity

CA activity was measured using the electrometric ΔpH method (Henry, 1991). Gill tissue (0.5–1 g; *N*=11 for control, *N*=11 for hypercarbia) was added to five volumes of assay reaction medium (in mmol l^{-1}: 225 mannitol, 75 sucrose, 10 Tris-base, adjusted to pH 7.4 using 10% phosphoric acid) and homogenized using a motor-driven Teflon–glass homogenizer. Crude homogenates were centrifuged briefly, and the supernatant (typically 50 μl of a 25-fold dilution) was then assayed for CA activity using 6 ml of reaction medium held at 4°C, and 200 μl of CO_2-saturated distilled water to initiate the reaction. The reaction velocity was measured over the initial 0.15 unit pH change. To obtain the true catalyzed rate, the uncatalyzed rate was subtracted from the observed rate. A pH electrode (GK2401C; Radiometer, London, ON, Canada) connected to a PHM84 pH meter (Radiometer) and data acquisition software (Biopac with AcqKnowledge v. 3.7.3 software; Harvard Apparatus Canada) was used to measure the pH of the reaction medium. Sample protein concentrations were measured using a protein assay (Bio-Rad), with BSA (Sigma) as a standard.

Series II: determination of the role of branchial CA activity in acid–base regulation

Experimental protocol

Rainbow trout were anaesthetized by immersion in an oxygenated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g l^{-1}), weighed and then placed on a surgical table that allowed continuous irrigation of the gills with the same anaesthetic solution. All trout were fitted with indwelling dorsal aortic catheters (PE50 tubing; VWR) according to the basic method of Soivio et al. (1975). At the same time, an external urinary catheter was sutured around the vent according to the procedure of Curtis and Wood (1991). Urinary catheters were modified Bard all-purpose urethral catheters (Bard size 12 French elastic rubber; Canada Care Medical, Ottawa, ON, Canada). Trout were then placed in experimental chambers served with flowing, aerated water to recover for 24 h. Cannulae were flushed with heparinised (100 IU ml^{-1} ammonium heparin; Sigma) modified (4.5 mmol l^{-1} $NaHCO_3$) Cortland saline (Wolf, 1963), and urinary catheters were flushed with water.

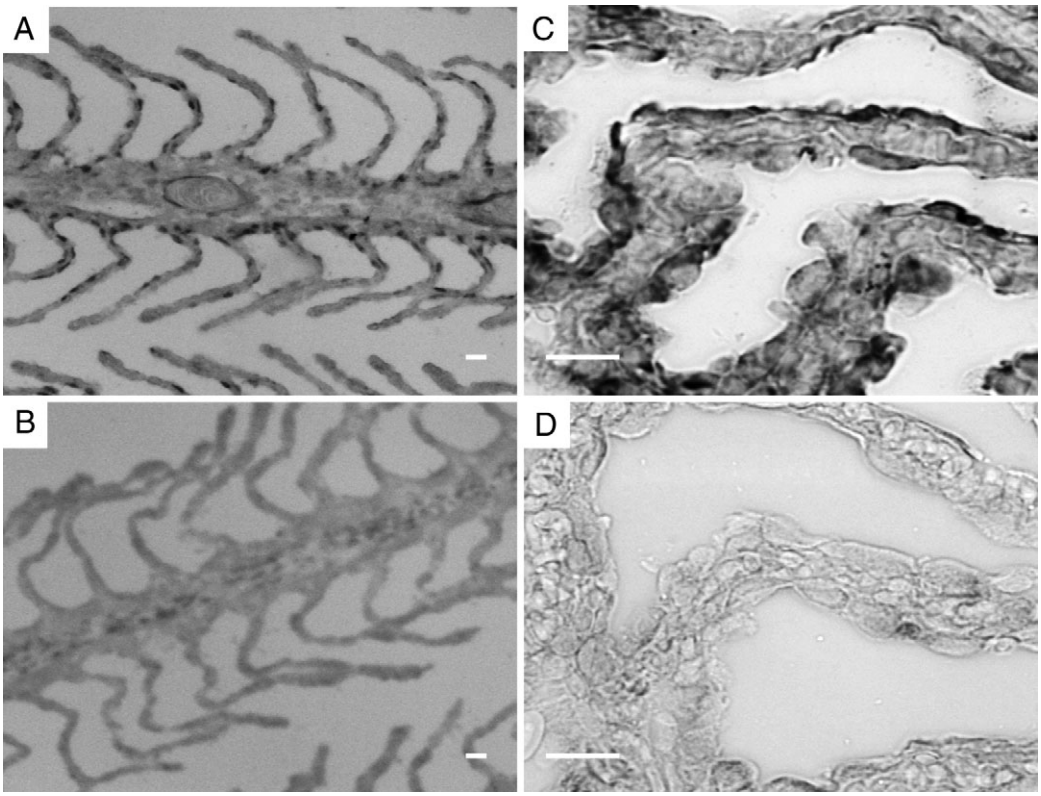

Fig. 1. Trout cytoplasmic carbonic anhydrase (tCAc) mRNA localization in the gills of rainbow trout (*Oncorhynchus mykiss*) by *in situ* hybridization. The images present two sets (A,B and C,D) of *in situ* hybridization results from serial sections of the gill incubated with probe (A,C), no probe (B) or probe in the presence of excess unlabelled probe (D) and are typical of the sections examined. Strong hybridization signals for tCAc are evident in epithelial cells of the lamellae at both low (A) and high (C) magnification. Scale bars, 40 μm (A,B) and 20 μm (C,D).

A preliminary experiment was carried out to determine the time period of maximum acid excretion elicited by exposure of rainbow trout to hypercarbic conditions (nominal water P_{CO2}=6 Torr). For this experiment, fish were neither cannulated nor catheterized, and overall net acid–base fluxes were estimated for control (N=9) and hypercarbia-exposed (N=9) trout prior to the initiation of hypercarbia (–3 to 0 h), and 1–4 h, 4–7 h, 9–12 h and 22–25 h after the onset of exposure to hypercarbia. Because net acid excretion in hypercarbia-exposed fish was significantly greater than that in control fish at 4–7 h (Student's t-test, P=0.048; hypercarbia 270.5±53.2 μmol H^+ kg^{-1} h^{-1}, control 13.8±107.5 μmol H^+ kg^{-1} h^{-1}) and 9–12 h (Student's t-test, P=0.049; hypercarbia 367.0±64.0 μmol H^+ kg^{-1} h^{-1}, control 148.4±80.1 μmol H^+ kg^{-1} h^{-1}), 8–12 h was selected as the period of maximum acid excretion induced by hypercarbia, and the subsequent experiment focused on this time period. As the focus of this experiment was branchial acid excretion, the purpose of the urinary catheter was simply to prevent urine elimination into the water, and therefore urine was allowed to drain by gravity into a vial placed outside the holding chamber and about 3–5 cm below the surface of the water. Branchial net acid–base fluxes were estimated for control (N=11) and hypercarbia-exposed (N=11) trout 8–10 h after the onset of hypercarbia. Trout were then treated with the CA inhibitor acetazolamide (Az), and branchial net acid–base fluxes were estimated for a further 2 h period, i.e. 10–12 h after the onset of hypercarbia. The acetazolamide dose (30 mg kg^{-1}; delivered as a bolus injection in 1 ml of saline *via* the dorsal aortic cannula) was calculated so as to elicit an initial circulating concentration of ~450 μmol l^{-1}, which would be expected to fully inhibit branchial tCAc activity, as the inhibition constant for Az against tCAc is ~1 nmol l^{-1} (Esbaugh et al., 2005). An arterial blood sample (600 μl) was withdrawn *via* the dorsal aortic cannula prior to the initiation of hypercarbia and at the beginning of each measurement period; any red cells not used for analysis were resuspended in saline and returned to the fish.

Hypercarbia was achieved by equilibrating the water feeding the experimental chambers with 2% CO_2 in air using a gas equilibration column as described above; water P_{CO_2} was monitored as described above. Experiments were carried out simultaneously on groups of hypercarbia-exposed and control fish by employing a second gas equilibration column supplied with air. Net acid–base fluxes were estimated for periods (2 or 3 h) in which water flow to the experimental chambers was halted; the volume of water in the boxes was set to a known value. During flux periods, the water in the boxes was aerated directly with either air (control group) or 0.8% CO_2 in air (hypercarbia-exposed group). The P_{CO_2} of water in the

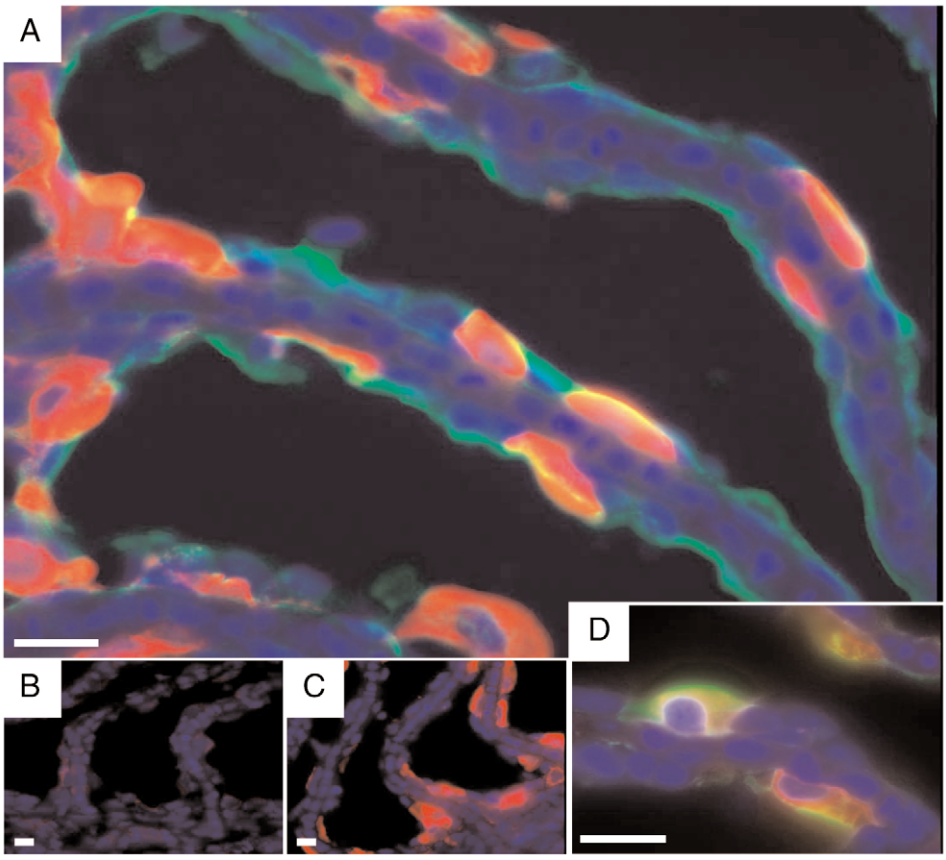

Fig. 2. Localization of trout cytoplasmic carbonic anhydrase (tCAc) protein in the gills of rainbow trout (*Oncorhynchus mykiss*) by immunohistochemistry. The images are overlays of three images collected individually for tCAc immunoreactivity (green), α5 immunoreactivity (red) and nuclei visualization (blue). Areas of overlap of tCAc and α5 immunoreactivity are indicated in yellow. Nuclei were visualized using 4′,6′-diamidino-2-phenylindole. Medium (A) and high (D) magnification images representative of the sections examined indicate that cells displaying only tCAc immunoreactivity, only α5 immunoreactivity or co-localization of tCAc and α5 immunoreactivities were present in the lamellar epithelia as well as in the interlamellar regions. Omission of primary antibodies eliminated all immunofluorescence (B). Pre-absorption of the tCAc antibody using the peptide against which the antibody was raised eliminated tCAc immunoreactivity without affecting α5 immunoreactivity (C). Scale bars, 20 μm.

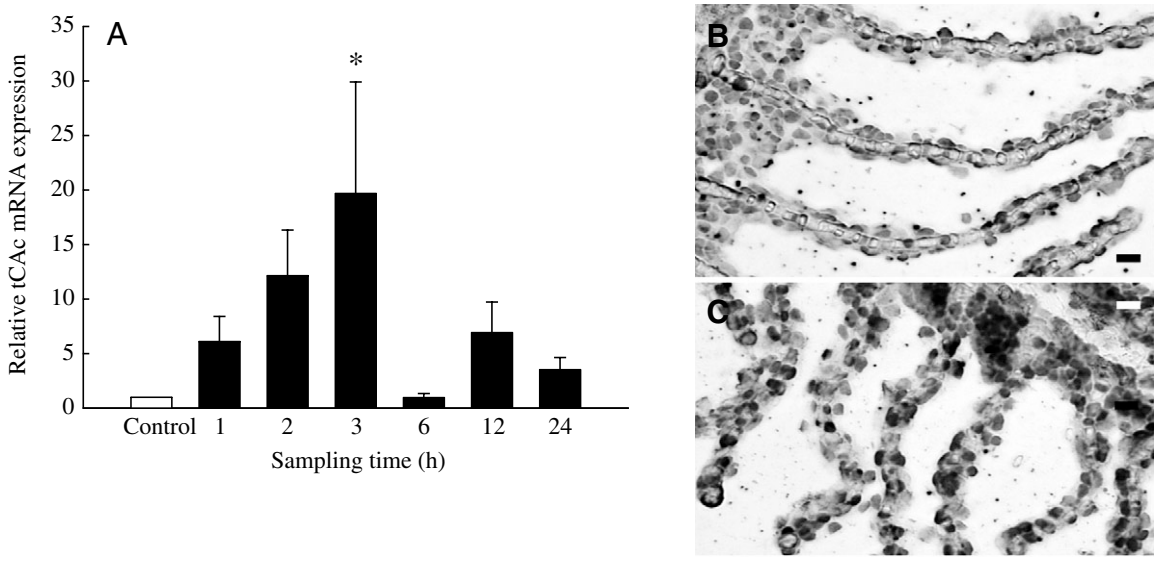

Fig. 3. (A) The effect of exposure to hypercarbia (filled bars), for periods ranging from 1 h to 24 h, on trout cytoplasmic carbonic anhydrase (tCAc) mRNA expression in the gills of rainbow trout (*Oncorhynchus mykiss*) relative to the expression of β-actin mRNA (relative tCAc mRNA expression), as determined by real-time PCR. Values are means ± 1 s.e.m. and N=6 for each period of exposure to hypercarbia. For statistical analysis, values were compared to corresponding relative tCAc mRNA expression values for fish (N=6 in each case) held under control (normocarbic) conditions for the corresponding period of time; a single group of control fish sampled at 3 h served as the control for 1, 2 and 3 h of exposure to hypercarbia. In each case, mRNA expression in the control group was given a relative value of 1 (open bar), and an asterisk therefore indicates relative tCAc mRNA expression in the hypercarbic fish that was significantly different from 1 (one-sample Student's t-test, P<0.05). (B,C) Representative images of tCAc mRNA localization by *in situ* hybridization in the gills of (B) control and (C) hypercarbic (24 h at a nominal $P_{W_{CO_2}}$ of 6 Torr; 1 Torr=133.3 Pa) trout. Scale bars, 20 μm.

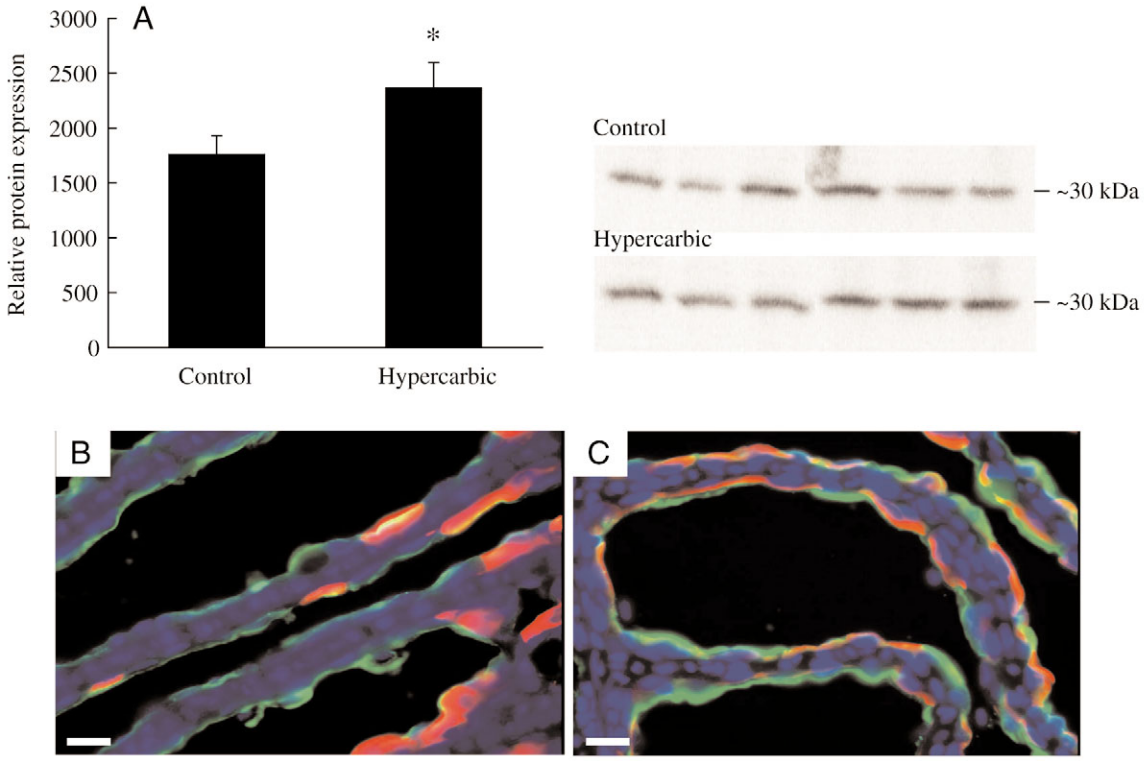

Fig. 4. The effects of exposure to hypercarbia on the expression of trout cytoplasmic carbonic anhydrase (tCAc) protein in the gills of rainbow trout (*Oncorhynchus mykiss*). An antibody raised against a peptide sequence specific to tCAc was used to detect tCAc protein expression by (A) western analysis and (B,C) immunohistochemistry in perfused gills from fish held under control conditions or exposed to hypercarbia (nominal $P_{W\mathrm{CO_2}}$=6 Torr; 1 Torr=133.3 Pa) for 24 h. In A, expression of a ~30 kDa protein was significantly greater (one-tailed Student's *t*-test, *P*=0.031) in gills extracted from hypercarbic trout (*N*=6) than in those extracted from normocarbic fish (*N*=6). Protein expression from the immunoblots presented on the right was quantified by digital image processing software and depicted in the figure on the left. In B and C, representative overlay images visualizing tCAc immunoreactivity (green), α5 immunoreactivity (red) and nuclei (4′,6′-diamidino-2-phenylindole; blue) are presented for gill sections from trout exposed to (B) control conditions and (C) hypercarbia for 24 h. Co-localization of tCAc and α5 is indicated in yellow. Scale bars, 20 μm.

experimental chambers was monitored during flux periods using a CO_2 electrode as described above, and any deviations from the desired $P_{W\mathrm{CO_2}}$ of 6 Torr were corrected by adjusting the CO_2 composition of the gas mixture used to aerate the boxes. After each flux period, the experimental chambers were flushed with flowing water from the gas equilibration column for 10–15 min; experimental chambers were maintained on flowing water between flux periods. At the beginning and end of each flux period, 10 ml of water were collected from each experimental chamber. Net acid base flux ($J_{net}H^+$) was determined from measurements of titratable net acid flux (J_{net}TA) and the change in ammonia concentration in these water samples. Water samples were analyzed within 6 h of sampling.

Analytical techniques

Following withdrawal, blood samples were centrifuged (~10 000 ***g*** for 1 min) to obtain plasma. Plasma total CO_2 concentrations were determined in duplicate on 50 μl samples using a Capnicon total CO_2 analyzer (CC501; Cameron Instruments), while pH was assessed using a pH electrode and calomel reference (E301 glass pH electrode; Analytical Sensors) housed in a temperature controlled low-volume pH chamber (Cameron Instruments) and connected to a PHM 72 acid–base analyzer (Radiometer). The arterial blood $P_{\mathrm{CO_2}}$ ($Pa_{\mathrm{CO_2}}$) and bicarbonate concentration ($[HCO_3^-]$) were then calculated from the Henderson–Hasselbalch equation using appropriate values for αCO_2 and pK′ (Boutilier et al., 1984).

J_{net}TA was determined by titrating 5 ml water samples from the beginning and end of each flux period to pH 4.00 with 0.02 mol l^{-1} HCl and considering the difference in titrant added. Samples were continuously aerated during titration to ensure mixing and removal of CO_2 (see McDonald and Wood, 1981). Total ammonia in the water samples was analyzed using a micro-modification of the salicylate-hypochlorite colorimetric assay of Verdouw et al. (1978). $J_{net}H^+$ was then calculated as the sum of J_{net}TA and the ammonia flux ($J_{net}NH_3$), signs considered, as described by McDonald and Wood (1981).

Statistical analyses

Data are reported as mean values ± 1 standard error of the mean (s.e.m.). The statistical significance of effects of exposure to hypercarbia on gill tCAc mRNA expression as determined

by real-time PCR was assessed using one-sample Student's *t*-tests. The impact of hypercarbia on gill tCAc protein levels as determined by western analysis was assessed by Student's *t*-tests. A two-way repeated measures (RM) analysis of variance (ANOVA) with sampling period (pre-exposure, pre-Az and post-Az, or simply pre- and post-Az) and treatment group (control or hypercarbia-exposed) as factors was used to analyze the effects of acetazolamide injection and treatment (hypercarbia) on blood acid–base variables and branchial net acid–base fluxes. The two-way RM ANOVA was followed by Bonferroni tests for *post hoc* multiple comparisons, as appropriate. The fiducial limit of significance in all tests was 0.05, and all statistical analyses were carried out using SPSS SigmaStat v3.0 (Systat Software, Point Richmond, CA, USA) software.

Results

Localization of tCAc mRNA and protein

In situ hybridization was used to examine the distribution of tCAc mRNA expression within the gills of rainbow trout. Positive hybridization signals were observed in epithelial cells of the lamellae, as well as within the interlamellar region (Fig. 1). No hybridization was evident in the negative control sections from which probe was omitted (Fig. 1B). In addition, sections treated with excess unlabeled probe failed to exhibit positive hybridization signals (Fig. 1D), indicating that the signal was specific.

The distribution of tCAc protein in gill tissue was examined in conjunction with that of Na^+/K^+-ATPase using immunohistochemical techniques. Immunoreactivity against both proteins was detected within the lamellar epithelia as well as the interlamellar region of the gill (Fig. 2). Double-labelling allowed the relative distributions of the two proteins to be compared. Cells exhibiting immunoreactivity for only one of the two proteins were present in both the lamellar epithelia and interlamellar regions, as were cells that displayed co-localization of tCAc and α5. Whereas tCAc immunoreactivity tended to be localized most intensely at the apical membrane of the lamellar epithelial cells, Na^+/K^+-ATPase (α5) immunoreactivity was localized principally in the basolateral regions (Fig. 2D). Immunoreactivity for tCAc was eliminated by preincubation with excess peptide against which the antibody was raised (Fig. 2C), and all immunofluorescence was eliminated when primary antibodies were omitted (Fig. 2B).

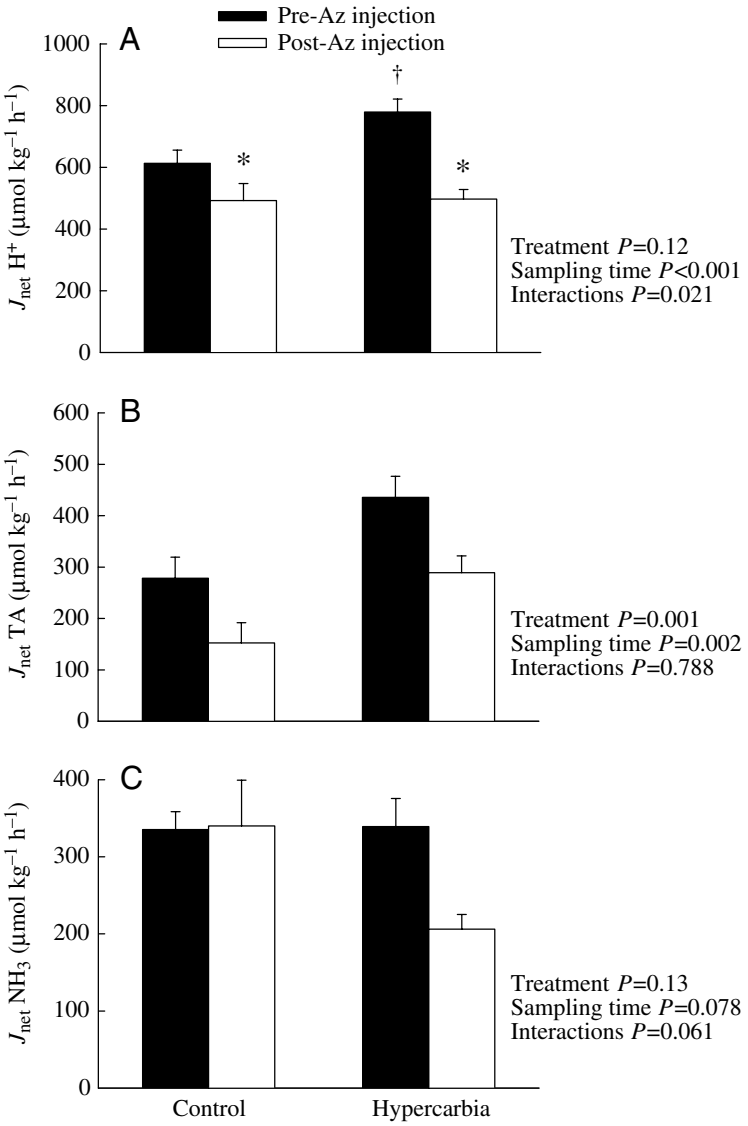

Fig. 5. The effects of exposure to hypercarbia (nominal $P_{W_{CO_2}}$=6 Torr; 1 Torr=133.3 Pa) and acetazolamide treatment (30 mg kg^{-1}) on branchial (A) net excretion of acidic equivalents ($J_{net}H^+$), (B) titratable net acid flux (J_{net}TA) and (C) net ammonia excretion ($J_{net}NH_3$) in rainbow trout (*Oncorhynchus mykiss*). $J_{net}H^+$ was calculated as the sum of J_{net}TA and $J_{net}NH_3$, signs considered. Data are mean values ± 1 s.e.m.; N=11 for both control and hypercarbia treatment groups. Data were analyzed by two-way RM ANOVA with treatment group (control *versus* hypercarbic) and sampling time [pre- or post-acetazolamide (Az) injection] as factors; P values are indicated on the figure. An asterisk (*) indicates a significant difference as a result of Az injection within a treatment group, while a dagger (†) indicates a significant difference between control and hypercarbia treatment groups within a given sampling time. Where significant interactions did not occur, only the P values are indicated on the figure.

Effects of hypercarbia on tCAc expression

Exposure of rainbow trout to hypercarbia (nominal $P_{W_{CO_2}}$=6 Torr) for periods ranging from 1 h to 24 h generally resulted in increases in relative tCAc mRNA expression in the gills as assessed by real-time PCR (Fig. 3). Branchial tCAc mRNA expression relative to that of β-actin appeared to increase during the initial stages of hypercarbic exposure, peaking at 3 h, the only time point at which the change in relative mRNA expression was significantly different from the

Table 1. *The effects of exposure to hypercarbia (nominal Pw_{CO_2}=6 Torr) and acetazolamide treatment (30 mg kg^{-1}) on arterial blood pH, CO_2 tension and HCO_3^- concentration*

	Control			Hypercarbia-exposed		
	Pre-exposure	Pre-Az	Post-Az	Pre-exposure	Pre-Az	Post-Az
pHa	7.78±0.02[a]	7.75±0.02[a]	7.57±0.01[b]	7.76±0.02[a]	7.58±0.01[b,*]	7.40±0.01[c,*]
Pa_{CO_2} (Torr)	2.97±0.24[a]	3.36±0.30[a]	4.96±0.22[b]	3.35±0.29[a]	7.73±0.15[b,*]	12.15±0.17[c,*]
[HCO_3^-] (mmol l^{-1})	7.68±0.20[a]	7.94±0.28[a]	7.89±0.26[a]	8.07±0.27[a]	12.3±0.07[b,*]	12.6±0.43[b,*]

Mean values ± 1 s.e.m., N=8 for both control and hypercarbia-exposed fish.

1 Torr=133.3 Pa. pHa, arterial pH; Pa_{CO_2}, arterial blood partial pressure of CO_2; [HCO_3^-], arterial blood HCO_3^- concentration; Az, acetazolamide.

Data were analyzed by two-way RM ANOVA with treatment group (control *vs* hypercarbia-exposed) and sampling time (pre-exposure, pre-Az or post-Az) as factors. For all three variables (pHa, Pa_{CO_2} and [HCO_3^-]), P<0.001 for both factors and interactions between these factors. Within a treatment group, values that do not share a letter are significantly different from one another, while an asterisk (*) indicates a significant difference from the corresponding value for the control group at a given sampling time.

control value of 1 (one-sample Student's t-test, P<0.05), before declining again over the subsequent 21 h. Even at 24 h, however, relative tCAc expression in the gills of hypercarbia-exposed trout appeared to be higher (although the difference was not statistically significantly) than that in control fish, a trend that was supported qualitatively by examination of tissue sections from the gills of fish exposed to normocarbia and hypercarbia for 24 h by *in situ* hybridization (Fig. 3B,C).

The abundance of tCAc protein in the gills was also significantly increased by exposure to hypercarbia. Quantification of immunoblots of gill tissue from trout exposed to control conditions or hypercarbia for 24 h indicated that branchial tCAc protein levels were ~1.3-fold higher, a significant difference (one-tailed Student's t-test, P=0.031), in hypercarbic over control fish (Fig. 4A). Qualitative support for this finding was provided by scrutiny of tCAc immunofluorescence in gill sections from control (Fig. 4B) and hypercarbic (Fig. 4C) trout. Interestingly, α5 immunofluorescence also appeared to be enhanced in sections from hypercarbic trout, although this effect was not quantified.

The differences in tCAc mRNA and protein expression did not, however, translate into differences in branchial CA activity. The CA activity of crude homogenates derived from hypercarbia-exposed fish (11 641±1100 μmol CO_2 ml^{-1} min^{-1}) was not significantly different (Student's t-test, P=0.844) from that for control fish (13 231±1830 μmol CO_2 ml^{-1} min^{-1}).

The role of branchial CA in acid–base regulation

As expected on the basis of the preliminary experiment (see above), branchial $J_{net}H^+$ was significantly higher in trout exposed to hypercarbia than in those held under normocarbic conditions (Fig. 5A; two-way RM ANOVA with treatment group and sampling time as factors, P=0.12 for treatment group, P<0.001 for sampling time and P=0.021 for the interaction of these two terms). Branchial $J_{net}H^+$ was decreased significantly by acetazolamide treatment in both the control (P=0.015) and hypercarbic (P<0.001) groups (Fig. 5A), and the extent of the decrease in branchial $J_{net}H^+$ was significantly greater (Student's t-test, P=0.021) in hypercarbic (281.7±52.03 μmol H^+ kg^{-1} h^{-1}; N=11) over control fish (121.0±37.2 μmol H^+ kg^{-1} h^{-1}, N=11). These differences were the result primarily of changes in branchial $J_{net}TA$ (Fig. 5B; two-way RM ANOVA, P=0.001 for treatment group, P=0.002 for sampling time and P=0.788 for the interaction of these two terms), as branchial $J_{net}NH_3$ was not significantly affected by either exposure to hypercarbia or injection of acetazolamide (Fig. 5C; two-way RM ANOVA, P=0.13 for treatment group, P=0.078 for sampling time and P=0.061 for the interaction of these two terms). Branchial $J_{net}TA$ was significantly higher in hypercarbic than control fish (P=0.001) and was reduced significantly by acetazolamide injection (P=0.002).

Analysis of the acid–base status of arterial blood (Table 1) revealed patterns typical of exposure to hypercarbia (e.g. Perry et al., 1987a) and acetazolamide treatment (e.g. Hoffert and Fromm, 1973), both of which exerted a significant impact on all three variables measured: arterial pH (pHa), Pa_{CO_2} and [HCO_3^-] (two-way RM ANOVA with treatment group and sampling time as factors, P<0.001 for both factors and the interaction of these two factors for all three variables). No significant differences existed between the control and hypercarbia-exposed groups prior to the onset of hypercarbia ('pre-exposure' values, P>0.05). Within the control group, pHa fell significantly as a result of acetazolamide injection (P<0.001), while Pa_{CO_2} rose (P<0.001). Within the hypercarbia treatment group, exposure to hypercarbia and acetazolamide injection both elicited significant falls in pHa together with increases in Pa_{CO_2} (P<0.001 in all cases). Arterial [HCO_3^-] was significantly elevated by exposure to hypercarbia (P<0.001), but no further increase occurred following acetazolamide injection. Consequently, pHa values were significantly lower, and Pa_{CO_2} and [HCO_3^-] values significantly higher, in hypercarbic fish in comparison with those in control fish at both the pre-Az and post-Az sampling times (P<0.001 in all cases).

Discussion

The results of the present study provide compelling evidence that branchial CA plays an important role in acid–base

regulation in rainbow trout, a role that is enhanced under conditions of acid–base challenge. It has long been postulated that branchial CA contributes to ionic and acid–base regulation in freshwater teleost fish by catalyzing the hydration of CO_2 within the branchial epithelium to the acid–base equivalents, H^+ and HCO_3^-, that are used as counter-ions for Na^+ and Cl^- uptake, respectively (reviewed by Perry and Laurent, 1990; Goss et al., 1992, 1995; Henry and Swenson, 2000; Claiborne et al., 2002; Perry et al., 2003; Hirose et al., 2003; Evans et al., 2005). The contribution of branchial CA to ion uptake is supported by the significant reductions in Na^+ or Cl^- influx that have been documented in most, although not all (Kerstetter and Kirschner, 1972), studies following inhibition of branchial CA by acetazolamide *in vivo* (Maetz, 1956; Maetz and Garcia-Romeu, 1964; Boisen et al., 2003; Chang and Hwang, 2004), *in situ* (Kerstetter et al., 1970) or in perfused gill preparations (Payan et al., 1975). Fewer studies have focused on the role of branchial CA in acid–base regulation. Henry et al. (1988) attributed the lack of pH compensation in response to a respiratory acidosis induced by red blood cell CA inhibition to the concomitant inhibition of branchial CA activity, while Lin and Randall (1991) demonstrated that net proton excretion across the gills was reduced by acetazolamide addition to the external water. Additional evidence was obtained from studies on marine dogfish, which utilize branchial Na^+/H^+ and Cl^-/HCO_3^- exchanges for acid–base regulation, although not ionic regulation (e.g. Tresguerres et al., 2005; see reviews by Perry and Laurent, 1990; Claiborne et al., 2002; Evans et al., 2005). The branchial clearance of an infused HCO_3^- load was impaired in dogfish treated with benzolamide to selectively inhibit gill CA activity (Swenson and Maren, 1987). In addition, a preliminary report suggested that compensation for a respiratory acidosis induced by exposure to hypercarbia was similarly impaired in benzolamide-treated dogfish (Swenson and Claiborne, 1985).

In the present study, branchial acid excretion and blood acid–base status were monitored directly and simultaneously to provide a comprehensive picture of the role of branchial CA activity in acid–base regulation. The significant reduction in branchial net acid excretion following acetazolamide treatment (Fig. 5) provides the clearest evidence to date that branchial CA functions in acid–base regulation. Branchial net acid excretion was reduced despite the significant respiratory acidosis induced by inhibition of red blood cell CA activity (Table 1) and was accounted for by decreases in the titratable net acid flux rather than changes in branchial ammonia excretion. Moreover, branchial net acid excretion was inhibited by acetazolamide treatment to a significantly greater extent in trout exposed to environmental hypercarbia (nominal $P_{W\,CO_2}$=6 Torr; Fig. 5). Exposure to hypercarbic water elicits an initial respiratory acidosis that is regulated at constant $P_{a\,CO_2}$ through the accumulation of HCO_3^- *via* the gills and its retention at the kidney (Claiborne and Heisler, 1984, 1986; Perry et al., 1987a,b; Goss and Perry, 1993; Larsen and Jensen, 1997; Choe and Evans, 2003; see reviews by Goss et al., 1995; Perry et al., 2003). The changes in net acid excretion and blood acid–base status detected in the present study were consistent with this pattern. Significantly higher branchial net acid excretion in hypercarbia-exposed trout (Fig. 5) resulted in HCO_3^- accumulation (Table 1), such that at 8–10 h of hypercarbic exposure, a metabolic alkalosis was superposed on the respiratory acidosis induced by hypercarbia, yielding partial pH compensation (Fig. 6). Injection of acetazolamide at this point not only exacerbated the respiratory acidosis but added to it a component of metabolic acidosis (base deficit of ~1.4 mmol l^{-1}; Fig. 6) that likely reflected the inhibition of branchial net acid excretion as well as the inhibition of the renal CA-dependent HCO_3^- retention mechanism (T. Georgalis, K. M. Gilmour, J. Yorston and S. F. Perry, submitted). While adjustments of acid or base excretion at the gill constitute the main mechanism for acid–base regulation in fish (Cameron and Kormanik, 1982; Wheatly et al., 1984; Perry et al., 1987a,b; Wood, 1988; Goss and Perry, 1993; Choe and Evans, 2003), accounting for ~90% of the net movement of acid–base equivalents (Claiborne et al., 2002), the kidney plays an important complementary role in acid–base regulation by reabsorbing accumulated HCO_3^- ions from the filtrate (Perry et al., 2003). Inhibition of this CA-dependent process results in HCO_3^- loss in the urine, contributing to the base deficit of acetazolamide-treated hypercarbic trout.

The greater impact of acetazolamide treatment on branchial net acid excretion in hypercarbia-exposed trout reflects the increased requirement for net acid excretion to compensate for the hypercarbia-induced respiratory acidosis. Assessment of Na^+ and Cl^- fluxes during hypercarbia in rainbow trout

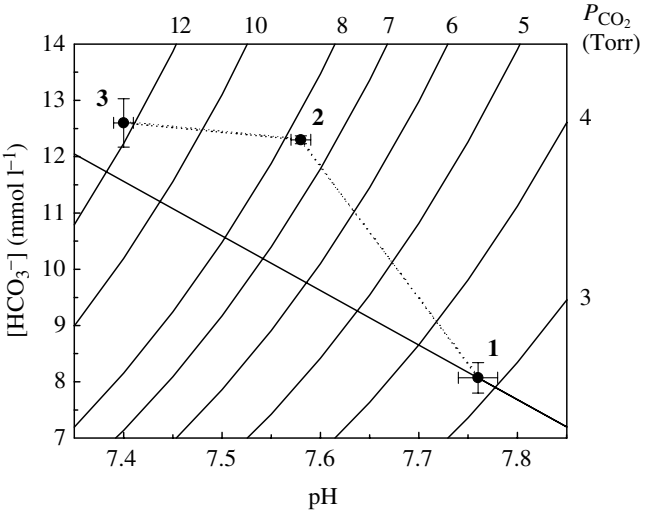

Fig. 6. A pH–[HCO_3^-] diagram for rainbow trout at 13°C. The P_{CO_2} for a given combination of pH and [HCO_3^-] was calculated using the Henderson–Hasselbalch equation and the appropriate values for pK′ and the solubility coefficient αCO_2 (Boutilier et al., 1984). The buffer line for rainbow trout true plasma was constructed using buffer values derived by Wood et al. (1982). The labelled points represent mean data (±1 s.e.m., N=8) for (1) pre-exposure, (2) pre-acetazolamide injection and (3) post-acetazolamide injection sampling points for trout exposed to hypercarbia (nominal $P_{W\,CO_2}$ of 6 Torr; 1 Torr=133.3 Pa).

suggests that the necessary net acid excretion (or HCO_3^- accumulation) is achieved primarily through the reduction of Cl^-/HCO_3^- exchange, which is coupled with a quantitatively less important enhancement of Na^+-linked proton extrusion (Perry et al., 1987a; Goss and Perry, 1993; reviewed by Goss et al., 1992, 1995). Morphological adjustment of the branchial epithelium, specifically a reduction in the exposed surface area of MR cells, the presumed site of branchial Cl^-/HCO_3^- exchangers (see below), constitutes one mechanism through which this reduction in Cl^-/HCO_3^- exchange is achieved (Goss and Perry, 1993; reviewed by Goss et al., 1992, 1995; Perry, 1997; Perry et al., 2003). The minor enhancement of proton extrusion during hypercarbia is supported by increased branchial H^+-ATPase activity (Lin and Randall, 1993; Sullivan et al., 1995; Galvez et al., 2002) that reflects, at least to some degree, greater gene expression of the vacuolar H^+-ATPase (Sullivan et al., 1996; Perry et al., 2000a,b). The results of the present study indicate that the abundance of the trout gill cytoplasmic CA isoform (tCAc) is also increased, albeit modestly, during hypercarbia (Fig. 4), at least in part owing to enhanced gene transcription (Fig. 3). The increased expression was not, however, translated into augmented branchial CA activity, suggesting that existing CA activity is sufficient to prevent limitations on the provision of protons for H^+-ATPase activity even under hypercarbic conditions. Increases in CA expression during hypercarbic acidosis may reflect increased enzyme turnover in response to the acid–base challenge. Dimberg and Höglund (1987) detected increased gill CA activity in rainbow trout exposed to hypercarbia, but as the higher activity was measured at 20 and 80 days of hypercarbia, it was probably not involved in acute acid–base regulation. The significant induction of gill CA activity in response to hypercarbic exposure indicates, however, that branchial CA expression is sensitive to acid–base challenges. A similar conclusion was reached by Hirata et al. (2003) for the Osorezan dace, which exhibited increased branchial CA mRNA expression following exposure to acidic (pH 3.5) water.

Although the cellular location and molecular nature of acid and base translocating proteins in the gills of freshwater teleosts remain topics of considerable debate, current models (see reviews by Claiborne et al., 2002; Perry et al., 2003; Hirose et al., 2003; Evans et al., 2005) suggest that acid excretion is accomplished by a vacuolar H^+-ATPase that is located in the apical membrane of MR cells that do not bind peanut lectin agglutinin (PNA^-; Galvez et al., 2002); these cells are also termed MR pavement cells. Proton secretion is linked to Na^+ uptake that may occur through apically located Na^+-selective channels. Base excretion, on the other hand, is linked to Cl^- uptake, probably through a Cl^-/HCO_3^- exchanger that is apically located in PNA^+ MR cells (MR cells or chloride cells). Given the role played by CA in providing H^+ and HCO_3^- for acid and base excretion, a broad distribution of tCAc expression would be expected to occur in the gills of rainbow trout. Examination of tCAc mRNA and protein distribution in the present study revealed patterns of expression that were consistent with current models. Positive hybridization signals for tCAc mRNA were visible both along the lamellae and in the interlamellar regions (Fig. 1). Because MR cells in freshwater rainbow trout are typically concentrated in the interlamellar regions and only sparsely distributed along the lamellae (Perry, 1997), the distribution of positive hybridization signal suggests that both pavement cells and MR cells expressed tCAc mRNA. Similarly, immunoreactivity for tCAc was present equally in Na^+/K^+-ATPase immunopositive and immunonegative cells (Fig. 2). The abundant tubular system that characterizes MR cells houses ion-transporting enzymes, such as Na^+/K^+-ATPase (Jürss and Bastrop, 1995; Perry, 1997), and therefore high levels of this enzyme, assessed biochemically or through fluorescence microscopy, are frequently used as a tool to identify MR cells (e.g. Li et al., 1995; Wilson et al., 2000b). Co-localization of tCAc and Na^+/K^+-ATPase immunoreactivity was therefore indicative of CA-containing MR cells, whereas cells that were immunopositive for tCAc alone were probably pavement cells (Fig. 2). Interestingly, not all putative MR cells exhibited tCAc immunofluorescence. Equally interesting was the apparent, although not quantified, increase in Na^+/K^+-ATPase immunofluorescence in the gills of hypercarbia-exposed trout (Fig. 4B,C). In view of the reduction in exposed MR cell area that typically occurs during hypercarbic exposure (see above), further investigation of the apparent increase in Na^+/K^+-ATPase abundance is clearly warranted.

The branchial distribution of tCAc observed in the present investigation is in agreement with that observed previously and complements the results of earlier studies by focusing on the specific CA isoform that is present in the gills (Esbaugh et al., 2005) and by examining the impact of hypercarbic exposure on this isoform. CA was localized to both pavement and MR cells in rainbow trout using Hansson's technique, a histochemical approach based on a cobalt phosphate/cobalt sulphate vital stain (Conley and Mallatt, 1988). Immunocytochemical localization of branchial CA using an antibody raised against purified trout gill CA confirmed the broad distribution reported by Conley and Mallatt (1988) and additionally indicated that CA was concentrated in the apical region of both pavement cells and MR cells (Rahim et al., 1988). However, these patterns are not true of all species examined to date; in some species CA appears to be confined to either pavement cells or MR cells, while in others it is found in both (Dimberg et al., 1981; Conley and Mallatt, 1988; Flügel et al., 1991; Sender et al., 1999; Wilson et al., 2000b,c; Hirata et al., 2003; Choe et al., 2004).

In summary, the findings of the present study provide three significant contributions to our knowledge of the distribution and function of CA in the gills of freshwater fish. First, evidence for expression of the trout general cytoplasmic CA isoform (tCAc) in both pavement cells and MR cells was obtained. The results confirm and extend the findings of Esbaugh et al. (2005), who identified the tCAc isoform but focused exclusively on its mRNA expression, by examining the cellular localization of the protein as well as mRNA. Second, trout branchial CA (tCAc) was demonstrated to play

an important role in the branchial regulation of acid–base balance, as CA inhibition significantly decreased branchial net acid excretion. Finally, the larger impact of CA inhibition on branchial net acid excretion in trout exposed to hypercarbia, coupled with the significantly higher tCAc mRNA and protein expression in hypercarbic trout, provides evidence that branchial CA expression is regulated in response to acid–base challenges, further supporting the conclusion that this enzyme is a key contributor to acid–base regulation in freshwater rainbow trout.

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