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ORIGINAL PAPER

# Roles of cortisol and carbonic anhydrase in acid–base compensation in rainbow trout, *Oncorhynchus mykiss*

**K. M. Gilmour · C. L. Collier · C. J. Dey · S. F. Perry**

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**Abstract** Fish compensate for acid–base disturbances primarily by modulating the branchial excretion of acid–base equivalents, with a supporting role played by adjustment of urinary acid excretion. The present study used metabolic acid–base disturbances in rainbow trout, *Oncorhynchus mykiss*, to evaluate the role played by cortisol in stimulating compensatory responses. Trout infused with acid (an iso-osmotic solution of 70 mmol $L^{-1}$ HCl), base (140 mmol $L^{-1}$ $NaHCO_3$) or saline (140 mmol $L^{-1}$ NaCl) for 24 h exhibited significant elevation of circulating cortisol concentrations. Acid infusion significantly increased both branchial (by 328 μmol $kg^{-1}$ $h^{-1}$) and urinary (by 5.9 μmol $kg^{-1}$ $h^{-1}$) net acid excretion, compensatory responses that were eliminated by pre-treatment of trout with the cortisol synthesis inhibitor metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone). The significant decrease in net acid excretion (equivalent to enhanced base excretion) of 203 μmol $kg^{-1}$ $h^{-1}$ detected in base-infused trout was unaffected by metyrapone treatment. Acid- and base-infusions also were associated with significant changes in the relative mRNA expression of branchial and renal cytosolic carbonic anhydrase (tCAc) and renal membrane-linked CA IV (tCA IV). Cortisol treatment caused changes in CA gene expression that tended to parallel those observed with acid but not base infusion. For example, significant increases in renal relative tCA IV mRNA expression were detected in both acid-infused ($\sim 2x$) and cortisol-treated ($\sim 10x$) trout, whereas tCA IV mRNA expression was significantly reduced ($\sim 5x$) in base-infused fish. Despite changes in CA gene expression in acid- or base-infused fish, neither acid nor base infusion affected CAc protein levels in the gill, but both caused significant increases in branchial CA activity. Cortisol treatment similarly increased branchial CA activity in the absence of an effect on branchial CAc protein expression. Taken together, these findings provide support for the hypothesis that in rainbow trout, cortisol is involved in mediating acid–base compensatory responses to a metabolic acidosis, and that cortisol exerts its effects at least in part through modulation of CA.

**Keywords** Cortisol · Carbonic anhydrase · Acid–base regulation · Rainbow trout · *Oncorhynchus mykiss* · Metyrapone

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## Introduction

Freshwater teleost fish compensate for acid–base disturbances primarily by modulating the branchial excretion of acid–base equivalents, with a supporting role played by adjustment of urinary acid excretion (for review, see Claiborne et al. 2002; Perry et al. 2003b; Evans et al. 2005; Perry and Gilmour 2006; Gilmour and Perry 2009). In general, a systemic acidosis is corrected by the increased output of acidic equivalents (thereby raising the plasma $HCO_3^-$ concentration), whereas the response to alkalosis consists of decreased net efflux of acid (equivalent to increased base output). At the gill, pH adjustments are achieved through the differential regulation of $H^+$ and $HCO_3^-$ effluxes that are coupled to the influx of, respectively, $Na^+$ and $Cl^-$ (Goss et al. 1992; Claiborne et al. 2002; Perry et al. 2003a, b; Evans et al. 2005). Thus, branchial net acid excretion can be adjusted by altering the rate of $H^+$ extrusion linked

to $Na^+$ uptake (e.g. Goss and Wood 1991), the rate of $Cl^-/HCO_3^-$ exchange (e.g. Hyde and Perry 1989; Goss and Perry 1994), or both, with the strategy employed depending both on fish species and on whether the acid–base disturbance is metabolic or respiratory in origin (see Gilmour and Perry 2009). The kidney responds to acid–base disturbances by modifying $HCO_3^-$ reabsorption from the filtrate as well as $NH_4^+$ production and excretion (King and Goldstein 1983; Wheatly et al. 1984; Perry et al. 1987b; Wood 1988; Wood et al. 1999; Georgalis et al. 2006a). As with the gill, metabolic versus respiratory acid–base challenges produce different renal responses (Wood et al. 1999).

At both the gill and the kidney, carbonic anhydrase (CA) plays an important role in acid–base regulation (reviewed by Gilmour and Perry 2009). Branchial cytosolic CA (CAc), a fish-specific isoform comparable to tetrapod CA II in structure and function (Esbaugh et al. 2005), catalyzes the hydration of $CO_2$ to provide $H^+$ and $HCO_3^-$ used by ion transporters involved in acid–base compensatory responses (Haswell et al. 1980; Perry and Laurent 1990). Treatment of rainbow trout with the CA inhibitor acetazolamide significantly reduced branchial net acid excretion (Kerstetter et al. 1970; Georgalis et al. 2006b), an effect that was enhanced by exposure of trout to hypercapnia (elevated water $CO_2$ tension) to induce a respiratory acidosis (Georgalis et al. 2006b). Similarly, proton extrusion was significantly reduced in zebrafish embryos and larvae in which cytosolic CA translation was knocked down using morpholino antisense oligonucleotides (Lin et al. 2008). The freshwater teleost kidney expresses at least two CA isoforms that are involved in $HCO_3^-$ reabsorption, cytosolic CAc and type IV CA (CA IV), a membrane-bound isoform with extracellular activity (Georgalis et al. 2006a). Filtered $HCO_3^-$ combines with $H^+$ derived from an apical membrane sodium/proton exchanger (NHE3; Hirata et al. 2003; Ivanis et al. 2008a) or V-type $H^+$-ATPase (Perry and Fryer 1997; Hirata et al. 2003) to form $CO_2$, a reaction that is catalyzed by CA IV. The newly formed $CO_2$ then enters the proximal tubule cell where it is hydrated, in a reaction catalyzed by CAc, to $H^+$ used to refuel apical membrane $H^+$ secretion, and $HCO_3^-$ that is transported across the basolateral membrane by $Na^+/HCO_3^-$ cotransporter isoform 1 (NBC1; Hirata et al. 2003; Perry et al. 2003a). Renal $HCO_3^-$ reabsorption was significantly reduced by the inhibition of either extracellular CA (Georgalis et al. 2006a) or both extracellular CA and CAc (Nishimura 1977; Georgalis et al. 2006a). Further evidence of the importance of branchial and renal CA to acid–base regulation comes from studies that have documented changes in CA gene expression, protein levels and/or activity in response to acid–base challenges (Hirata et al. 2003; Georgalis et al. 2006a, b).

Although the branchial and renal responses to a variety of acid–base disturbances have been examined, our understanding of the factors that regulate such compensatory effects remains poor. In both mammals (e.g. Welbourne 1976; Hulter et al. 1980, 1981; Wilcox et al. 1982; May et al. 1986) and fish (Wood et al. 1999), glucocorticoids have been implicated in the renal responses to metabolic acidosis. Elevated circulating concentrations of cortisol, the main glucocorticoid in fish, have been detected in acidotic fish (Brown et al. 1986; Wood et al. 1999; Warren et al. 2004; Ivanis et al. 2008a). In addition, the branchial and/or renal expression of several acid–base-relevant transporters has been found to be responsive to cortisol in freshwater teleost fish [e.g. NHE2 (Ivanis et al. 2008b); NHE3 (Ivanis et al. 2008a); V-type $H^+$-ATPase (Lin and Randall 1993 but also see Kiilerich et al. 2007)]. These observations suggest a role for cortisol in stimulating acid–base compensatory responses in fish. To test this hypothesis, the present study assessed cortisol and acid excretion responses to metabolic acid–base disturbances in rainbow trout, *Oncorhynchus mykiss*, under control conditions and following treatment with the cortisol synthesis inhibitor metyrapone. The hypothesis that cortisol triggers changes in CA expression that contribute to acid–base compensatory responses was examined by assessing CA mRNA expression, protein levels and activity in cortisol-treated trout and trout subjected to metabolic acid–base disturbances.

## Materials and methods

### Experimental animals

Rainbow trout (*O. mykiss* Walbaum) 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, and were fed to satiation on commercial trout pellets every second day. Trout were allowed to acclimate to these holding conditions for at least 2 weeks prior to experimentation. Food was withheld for 3 days prior to experimentation to minimize variation in acid excretion associated with feeding.

To allow repetitive blood sampling and/or infusion in trout, fish were anesthetized 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 anesthetic solution. An indwelling catheter (Clay-Adams PE50 polyethylene tubing; VWR) was then inserted into the dorsal aorta according to the basic method of Soivio

et al. (1975). In one experiment (see "Series I"), the caudal vein was also cannulated (Axelsson and Fritsche 1994). Following exposure of the hemal arch by means of a lateral incision at the level of the caudal peduncle, a catheter (PE50 polyethylene tubing) was inserted into the caudal vein in the anterior direction. Cannulae were flushed with heparinised (100 IU $mL^{-1}$ ammonium heparin; Sigma) modified (4.5 mmol $L^{-1}$ $NaHCO_3$) Cortland saline (Wolf 1963). To allow assessment of branchial and urinary net acid excretion, an external urinary catheter was sutured to the ventral surface around the urogenital papilla and anus of anesthetized trout 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) and were flushed with water. All fish were allowed to recover for 24 h prior to experimentation in individual chambers supplied with flowing, aerated water.

### Infusion regimes

Metabolic acidosis was achieved by infusing fish (syringe pump model 780220; KD Scientific) via the dorsal aortic cannula for 24 h with an iso-osmotic solution of 70 mmol $L^{-1}$ HCl + 70 mmol $L^{-1}$ NaCl at a rate of 2 mL $kg^{-1}$ $h^{-1}$ (total acid load = 3.4 mmol $H^+$ $kg^{-1}$). To elicit metabolic alkalosis, fish were infused with 140 mmol $L^{-1}$ $NaHCO_3$ at a rate of 2 mL $kg^{-1}$ $h^{-1}$ for a period of 24 h (total base load = 6.7 mmol $HCO_3^-$ $kg^{-1}$). Infusion of 140 mmol $L^{-1}$ NaCl for 24 h at a rate of 2 mL $kg^{-1}$ $h^{-1}$ served as a control for the effects of infusion. These treatments were based upon the work of Goss and Wood (1991) and were selected to achieve changes in blood pH that were of similar magnitude but opposite direction.

### Series I: Effects of metabolic acid–base disturbances on cortisol and CA expression

This series of experiments was designed to assess the impact of metabolic acid–base disturbances on circulating cortisol concentrations and on CA gene expression, protein levels and activity. Rainbow trout [mean weight 283 ± 9 g (mean ± SEM); $N = 73$] were assigned to one of five experimental groups. Trout fitted with a dorsal aortic cannula were infused with acid ($N = 20$), base ($N = 15$) or saline ($N = 15$) as described above. Blood samples were withdrawn before infusion commenced (time = 0 h; 400 μL) and at 2 h (400 μL) and 24 h (600 μL) of infusion. Blood samples were analyzed immediately for pH. The blood was then centrifuged at 10,000*g* for 30 s to obtain plasma for the analysis of total $CO_2$. The red blood cells were re-suspended in heparinised Cortland saline and re-injected via the cannula to minimize the effects of blood sampling on hematocrit. Plasma from the 24 h sample was frozen in liquid $N_2$ and stored at −80°C for later analysis of cortisol concentrations. At the end of the 24 h infusion protocol, fish were killed and gill and kidney tissue samples were collected. The sham treatment group ($N = 12$) consisted of cannulated fish that were held in the experimental chamber without infusion for 24 h. A single blood sample was then withdrawn, the fish were killed, and gill and kidney tissue samples were collected. A control group ($N = 11$) of non-cannulated fish was also included in the experimental design. These fish were removed from the holding tank, killed, and gill and kidney tissue samples were collected together with blood (withdrawn by caudal puncture). Blood samples were centrifuged at 10,000*g* for 30 s to obtain plasma which was frozen and stored at −80°C for later analysis of cortisol concentrations.

To obtain a more detailed time-course of the effects of infusion on circulating cortisol concentrations, an additional experiment was carried out using rainbow trout (384 ± 16 g, $N = 20$) fitted with cannulae in both the dorsal aorta and caudal vein, and infused with acid ($N = 8$), base ($N = 5$) or saline ($N = 7$) as described above. Fish were infused via the dorsal aortic cannula, and blood samples (200 μL) were withdrawn from the caudal vein cannula before infusion commenced (time = 0 h) and 0.5, 1, 2, 4 and 8 h after infusion was initiated. Blood samples were centrifuged at 10,000*g* for 30 s to obtain plasma which was frozen and stored at −80°C for later analysis of cortisol concentrations.

### Series II: Acid excretion during infusion

Series II was designed to assess the involvement of cortisol in regulating branchial and renal acid excretion during metabolic acid–base disturbances. Rainbow trout (273 ± 9 g; $N = 49$) were fitted with a dorsal aortic cannula and a urinary catheter. After the 24 h post-surgery recovery period, urine was collected continuously over the 27 h experimental period by allowing the urinary catheters to drain by gravity into vials located outside the holding boxes and ~5 cm below the water level. Urinary catheters were checked for leaks prior to initiation of the experiment by raising the catheter 5 cm above water level. Only fish for which there was no fall of the urine level in the catheter (i.e. no leak) were used. Fish were infused with acid ($N = 17$), base ($N = 15$) or saline ($N = 17$) as described above. The cortisol synthesis inhibitor metyrapone [2-methyl-1,2-di-3-pyridyl-1-propanone; 30 μg $g^{-1}$ body mass (Eros and Milligan 1996)] was administered via the arterial cannula to approximately half of the fish in each group ($N = 9$ acid-infused, $N = 7$ base-infused and $N = 9$ saline-infused fish) before the first measurement of net acid

excretion; the remaining fish in each group received an equal volume (1.5 μL $g^{-1}$ body mass) of saline. Branchial and renal net acid excretion was measured for a 3 h period prior to the initiation of infusion, and for the final 3 h of the infusion period. During each measurement period, water flow to the experimental chambers was halted and the volume of water in the boxes was set to a known value. Water samples (10 mL) were collected at the beginning and end of each flux period. Boxes were aerated during the flux period and maintained on flowing water between flux periods. Urine was collected over the same two 3 h periods. Water and urine samples were analyzed for titratable acidity ($J_{net}TA$) and ammonia levels within 24 h of sample collection. Net acid flux ($J_{net}H^+$) was calculated from $J_{net}TA$ and the change in water ammonia concentration or urinary ammonia excretion over the flux period (McDonald and Wood 1981). Blood samples (300 μL) were withdrawn prior to each flux period and analyzed for pH and total $CO_2$ concentration as described below.

The efficacy of metyrapone treatment in preventing elevation of circulating cortisol concentrations was tested in a separate experiment. Rainbow trout (244 ± 18 g; $N = 26$) were fitted with a dorsal aortic cannula and injected with metyrapone [30 μg $g^{-1}$ body mass (Eros and Milligan 1996); $N = 13$] or an equal volume (1.5 μL $g^{-1}$ body mass) of saline ($N = 13$). After 8 h ($N = 7$) or 24 h ($N = 6$), a blood sample (200 μL) was withdrawn and fish were then subjected to an emersion stress by lowering the water level in the experimental chamber to 1–2 cm depth. After 2 min, the boxes were refilled with water and a second blood sample (200 μL) was withdrawn 10 min later. Blood samples were centrifuged at 10,000*g* for 30 s to obtain plasma which was frozen and stored at −80°C for later analysis of cortisol concentrations.

### Series III: Effects of cortisol elevation on CA expression

This series was designed to assess the potential for an elevation of circulating cortisol concentrations to regulate CA gene expression, protein levels and activity. Rainbow trout (102 ± 8 g; $N = 36$) were lightly anesthetized (i.e. to the point of losing equilibrium) in an aerated solution of benzocaine (ethyl-*p*-aminobenzoate; 0.05 g $L^{-1}$) and weighed. Half of the fish ($N = 19$) then received an intraperitoneal injection of cocoa butter (0.005 mL cocoa butter $g^{-1}$ fish) containing dissolved cortisol (110 mg hydrocortisone 21-hemisuccinate $kg^{-1}$ fish) as per the study of DiBattista et al. (2005), which reported circulating cortisol concentrations of ~150 ng $mL^{-1}$ with this approach. The cocoa butter was injected as a liquid but solidified rapidly within the fish and acted as a solid implant for the remainder of the experiment. The remaining fish were otherwise untreated (control group; $N = 17$). A "sham" treatment group, i.e. injection of cocoa butter alone, was not included in the experimental design because trout often respond unpredictably to sham treatment, with cortisol concentrations being elevated in some fish but not others. This effect can obscure comparisons between cortisol-treated and control fish (DiBattista et al. 2005). After 48 h, fish were killed and gill and kidney tissue samples were collected.

### Tissue collection

Trout were killed by immersion in a solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g $L^{-1}$) and posterior kidney tissue and gill tissue (of the first gill arch) were dissected out, immediately frozen in liquid $N_2$ and stored at −80°C until analysis. Prior to collection, tissues used for western analysis or CA assays were perfused with saline to clear the tissues of blood. The bulbus arteriosus was exposed and cannulated with PE160 polyethylene tubing. Approximately 50 mL of ice-cold, heparinised (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 (a $\beta$-adrenoreceptor agonist used to facilitate vasodilation) was infused via the cannula; the ventricle was severed during this infusion to allow fluid in the circulatory system to drain from the body.

### Analytical techniques

#### *Blood analysis*

Arterial blood pH ($pH_a$) was determined on whole blood samples using a pH electrode and calomel reference (E301 glass pH electrode; Analytical Sensors) held within a low volume, temperature controlled (13°C), pH chamber (Cameron Instruments) and connected to a PHM 72 acid–base analyzer (Radiometer). Plasma total $CO_2$ concentrations were measured in duplicate on 50 μL samples using either a Capnicon (CC501; Cameron Instruments) or Corning (model 950) total $CO_2$ analyzer. The partial pressure of $CO_2$ ($PaCO_2$) and the $HCO_3^-$ concentration of arterial blood were calculated using the Henderson–Hasselbalch equation and appropriate constants (Boutilier et al. 1984). Plasma cortisol concentrations were measured using a commercial radioimmunoassay (MP Biomedicals).

#### *Determination of net acid fluxes*

Branchial $J_{net}TA$ was determined by titrating (using a Gilmont precision 2.0 mL microburet) 10 mL water samples collected at 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 $CO_2$. Urinary $J_{net}TA$ was measured by lowering the pH of a 200 μL aliquot of urine below 5.0 through the addition of a known volume of 0.02 mol $L^{-1}$ HCl. The sample was then aerated for 20 min to remove $CO_2$. While continuing to aerate, the pH of the urine sample was titrated back to the pH of blood representative of the particular sampling period through the addition of 0.02 mol $L^{-1}$ NaOH using a precision 2.0 mL microburet (Gilmont). The titratable component of renal net acid excretion is given by the difference in the quantities of acid and base added to the urine. A micro-modification of the salicylate–hypochlorite colorimetric assay of Verdouw et al. (1978) was used to measure total ammonia levels in water or urine samples. Branchial or renal net acid excretion was then calculated as the sum of $J_{net}TA$ and the ammonia net flux (water) or efflux (urine), signs considered, as described by McDonald and Wood (1981). Urine flow rates were determined gravimetrically.

*Branchial CA activity*

CA activity in gill tissue (that had been perfused clear of blood) was measured using the electrometric ΔpH method (Henry 1991); the presence of hematopoietic tissue in the kidney does not allow unambiguous measurement of renal tubule CA activity. The procedures and equipment used were identical to those described by Georgalis et al. (2006b).

*Quantification of CA protein levels*

Western blots were used to quantify tCAc protein expression in gill tissue (that had been perfused clear of blood). A rabbit anti-human erythrocyte CA II (Rockland) antibody was used for this purpose; this antibody has been used successfully in previous work on salmonid fish (e.g. Tohse et al. 2004). The presence of haematopoietic tissue in the kidney does not allow unambiguous measurement of renal tubule CA protein levels using the human CA II antibody because this antibody detects both trout cytosolic CA (CAc) and the blood-specific CA isoform (CAb; Esbaugh et al. 2005) in western blots. Although use of the custom rabbit polyclonal antibody raised against trout CAc (Georgalis et al. 2006a) would have overcome this difficulty, the western blots obtained with the trout CAc antibody were inadequate for quantification of protein levels. The procedures and equipment used were essentially the same as those described by Georgalis et al. (2006b) with the following exceptions. Proteins were prepared from frozen powdered gill tissue samples by homogenizing tissues (100 mg $mL^{-1}$ homogenization buffer) on ice in RIPA buffer (10 mmol $L^{-1}$ Tris–HCl, 30 mmol $L^{-1}$ NaCl, 0.2% NP-40, 0.02% SDS, 0.1% sodium deoxycholate) containing protease inhibitors (Complete™ Mini protease inhibitor cocktail tablets; Roche Molecular Biochemicals). Horseradish peroxidase-conjugated goat anti-rabbit IgG (Amersham Life Sciences) was used at a dilution of 1:12,000. To assess equality of protein loading, after recording a blot (see below), it was incubated in Re-Blot Plus mild stripping solution (Chemicon) for 20 min at room temperature, rinsed for 10 min in Tris-buffered Tween 20 (TBST), blocked 2 × 10 min in TBST containing 5% non-fat milk, and then probed with a 1:250 dilution of anti-β-actin antibody (Sigma) for 2 h at 37°C. The blot was then incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG at a 1:2,000 dilution for 1 h at room temperature. All western blots were recorded onto Kodak Scientific Imaging Film (X-Omat Blue XB-1) that was developed and fixed using Kodak GBX developer and fixer, respectively. Developed films were scanned and the digital images were processed using commercial software (ImageJ; http://rsbweb.nih.gov/ij/). CA protein levels were expressed relative to β-actin protein levels.

*Quantification of CA mRNA expression*

Real-time RT-PCR was used to quantify mRNA expression of tCAc in gill and kidney tissue, and tCA IV in kidney tissue. Total RNA was extracted using Trizol (Invitrogen) according to the manufacturer's instructions from 50 mg aliquots of frozen tissue samples that had been ground to a powder under liquid $N_2$ with a mortar and pestle, and then passed through a needle using a syringe. The extracted total RNA was re-suspended in 40 μL of nuclease-free water and treated with DNase (Sigma) as per the manufacturer's instructions to reduce genomic DNA contamination and with RNaseOut™ Ribonuclease Inhibitor (Invitrogen) for 15 min at room temperature to decrease the possibility of RNase contamination. RNA quality was assessed by gel electrophoresis and spectrophotometry (ND-1000; NanoDrop). To synthesize cDNA, 2 μg of RNA was used together with random hexamer primers (IDT DNA) and RevertAid M-MulV Reverse Transcriptase (Fermentas) according to the manufacturer's instructions. Real-time RT-PCR was carried using the procedures and equipment described by Georgalis et al. (2006a) with the exception that the total reaction volume was scaled to 12.5 μL and 18S was used as the control gene. The cDNA was diluted 1,000-fold for 18S and the primers used were forward 5′-GGCGGCGTTATTCCCATGACC-3′ and reverse 5′-GG TGGTGCCCTTCCGTCAATT-3′. For comparisons among treatments (control, sham, saline-infused, acid-infused, base-infused or control, cortisol-treated), mRNA expression of each gene relative to the appropriate control group

was calculated using the modified delta-delta Ct method (Pfaffl 2001) with 18S mRNA expression as a normalizing factor.

### Statistical analyses

Data are reported as mean values ± 1 standard error of the mean (SEM). The statistical significance of treatment effects was evaluated using paired or unpaired Student's *t* tests, or one-way analysis of variance (ANOVA) or one-way repeated measures (RM) ANOVA, as appropriate. Where ANOVA indicated that significant differences existed, post hoc multiple comparisons tests (Holm-Sidak method) was applied to identify the source of the differences. Equivalent non-parametric tests were employed in the event that the data did not meet the underlying assumptions of normality or equal variance for parametric tests. All statistical tests were carried out with SigmaStat v3.5 (Systat Software) with a fiducial limit of significance in all tests of 0.05.

## Results

### Series I: Effects of metabolic acid–base disturbances on cortisol and CA expression

Arterial blood acid–base measurements (Fig. 1) indicated that 24 h of acid infusion caused a metabolic acidosis in which $pH_a$ fell by 0.22 units (RM ANOVA, $P = 0.004$) in the absence of any significant change in $PaCO_2$ (RM ANOVA, $P = 0.077$) or plasma [$HCO_3^-$] (RM ANOVA, $P = 0.174$). Base infusion induced a pure metabolic alkalosis with an elevation in $pH_a$ of 0.22 units (RM ANOVA, $P < 0.001$) and a 1.6-fold increase of plasma [$HCO_3^-$] (RM ANOVA, $P = 0.004$). Saline infusion was without effect on acid–base status (RM ANOVA, $P = 0.355$, 0.198 and 0.278 for $pH_a$, $PaCO_2$ and [$HCO_3^-$], respectively).

Circulating plasma [cortisol] was significantly (ANOVA, $P < 0.001$) elevated by 24 h of infusion (Fig. 2a), regardless of whether saline, acid or base was infused. The elevation of plasma [cortisol] reflected at least in part the effects of cannulation and confinement for 24 h in an experimental chamber, since plasma [cortisol] in sham-treated fish was between that of control and infused fish, although closer to the infused level (Fig. 2a). To contribute to the regulation of acid–base compensatory responses, plasma [cortisol] presumably would be expected to rise early in the infusion period, a pattern that was found to occur in acid-infused fish (Fig. 2b) but not base- or saline-infused fish (data not shown). The peak value over the initial 2 h of infusion (i.e. the maximum plasma [cortisol] from among the 0.5, 1 and 2 h sample times) was found to be significantly higher than the pre-infusion plasma [cortisol] in acid-infused fish (paired Student's *t* test, $P = 0.025$) but not base- (paired Student's *t* test, $P = 0.094$) or saline-infused fish (paired Student's *t* test, $P = 0.124$).

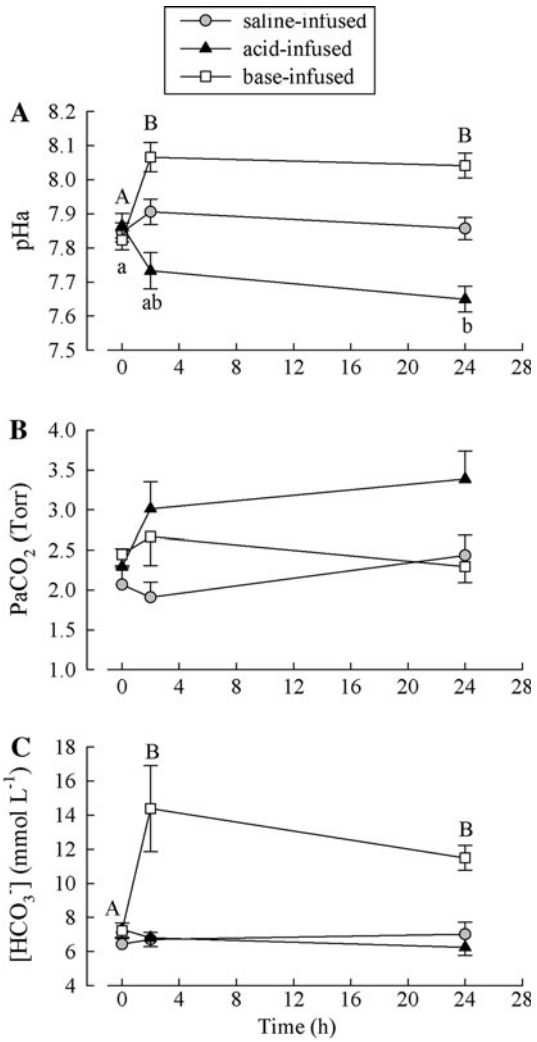


**Fig. 1** Blood acid–base status in rainbow trout (*Oncorhynchus mykiss*) infused with acid ($N = 16$), base ($N = 9$) or saline ($N = 9$) (see text for infusion conditions). Mean values (±SEM) of arterial blood **a** pH ($pH_a$), **b** partial pressure of $CO_2$ ($PaCO_2$), and **c** bicarbonate ion concentration ([$HCO_3^-$]) are depicted as a function of time over the 24-h infusion period; infusion was initiated immediately after collection of the time = 0 h sample. Within a treatment group, time points that share a *letter* are not significantly different from one another (one-way RM ANOVA; in **a** $P = 0.004$ and <0.001 for acid- and base-infused fish, respectively; in **c** $P = 0.004$ for base-infused fish; in all other cases $P > 0.05$)

Infusion also resulted in significant differences in CA mRNA expression (Fig. 3a–c). CA mRNA expression was normalized against 18S mRNA levels and then compared to the normalized CA mRNA expression of the control group, which was set to a value of one. Relative CA mRNA expression for sham-treated fish in no case differed from the

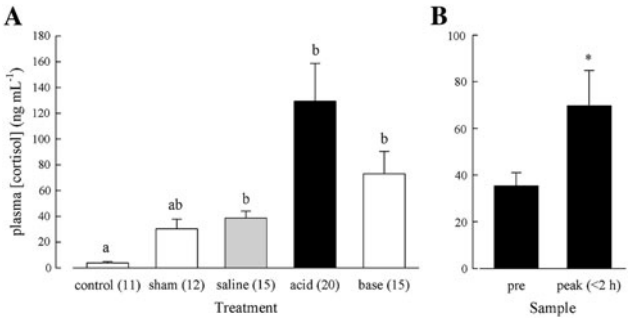


**Fig. 2** Circulating cortisol concentrations ([cortisol]) in rainbow trout (*Oncorhynchus mykiss*) subjected to metabolic acid–base disturbances. In **a**, mean (+SEM) plasma cortisol concentrations are presented for fish infused with acid, base or saline (see text for infusion conditions) as well as for fish that were cannulated but not infused (sham) and fish sampled by caudal puncture directly from the holding tank (control); *N* numbers for each treatment are listed in *parentheses*. *Bars* that share a *letter* are not significantly different from one another (one-way ANOVA; $P < 0.001$). **b** Data for a separate group of acid-infused trout ($N = 8$) in which plasma [cortisol] was followed as a function of time over the initial stages of acid infusion. Mean (+SEM) values of the peak plasma [cortisol] achieved over the initial 2 h of infusion are compared to the pre-infusion (time = 0 h) value. The *asterisk* indicates a significant difference (paired Student's *t* test, $P = 0.025$)

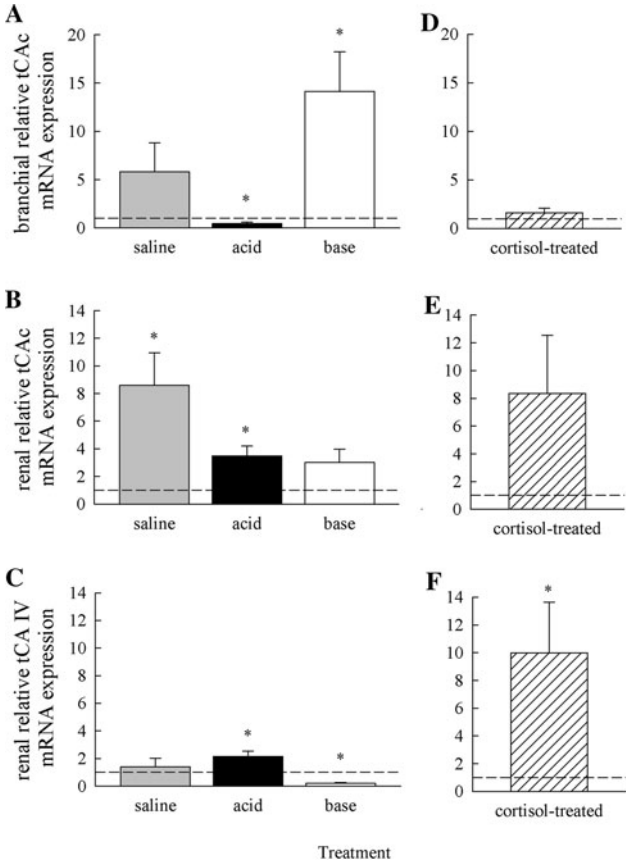


**Fig. 3** Relative mRNA expression of the cytosolic carbonic anhydrase (CA) isoform tCAc in gill (**a**, **d**) and kidney (**b**, **e**) and the membrane-bound CA isoform tCA IV in kidney (**c**, **f**) of rainbow trout (*Oncorhynchus mykiss*) subjected to metabolic acid–base disturbances (**a–c**; see text for infusion conditions) or treated with cortisol (**d–f;** see text for treatment details). All data are expressed relative to mRNA expression of the control 18S gene, and to the control group in each case which was assigned a relative value of one and is depicted as the *dashed line*. For the infusion experiment (**a–c**), the control group consisted of fish sampled directly from the holding tank while for the cortisol experiment (**d–f**), the control group consisted of untreated fish held for 48 h (see text for additional detail). Values are mean + SEM ($N = 8$ for all groups in **a**, **e** and **f**; $N = 6$ for all groups in **b–d**). An *asterisk* denotes a significant difference from the control value of one (one-sample Student's *t* test, $P < 0.05$)

control value of one (one-sample Student's *t* tests, $P > 0.05$ in all cases) and therefore only the control value is depicted in Fig. 3 (as the dashed line). At the gill, acid and base infusion had opposite effects (Fig. 3a). The relative mRNA expression of CAc was significantly lower than the control value in acid-infused fish, but significantly higher than the control value in base-infused fish (one-sample Student's *t* tests, $P < 0.05$ in both cases). At the kidney (Fig. 3b, c), the relative mRNA expression of both CAc and the membrane-bound CA IV was significantly higher than the control value in acid-infused fish (one-sample Student's *t* tests, $P < 0.05$ in both cases). In base-infused fish, CA IV mRNA expression was significantly lower than the control value (one-sample Student's *t* test, $P < 0.05$) although renal CAc mRNA expression did not differ significantly from the control value (one-sample Student's *t* test, $P > 0.05$). Saline infusion resulted in renal CAc mRNA expression that was significantly higher than the control value (one-sample Student's *t* test, $P < 0.05$), but did not affect branchial CAc or renal CA IV mRNA expression (one-sample Student's *t* tests, $P > 0.05$ in both cases).

Despite opposing changes in branchial CAc mRNA expression in acid- versus base-infused fish, CA protein levels in the gill were unaffected (ANOVA, $P = 0.058$) by infusion (Fig. 4a), yet branchial CA activity was significantly increased (ANOVA, $P < 0.001$) by both acid and base infusion (Fig. 4b). Saline infusion also elevated gill CA activity significantly, although not to the extent (~1.7-fold) found in acid- or base-infused fish (~2.3-fold).

## Series II: Acid excretion during infusion

Administration of metyrapone was used to investigate the role of cortisol in regulating acid–base compensatory responses. To establish the period over which metyrapone treatment was effective, plasma [cortisol] responses to a standardized stressor (2 min of air emersion) were assessed 8 and 24 h after injection of saline (control) or metyrapone (Fig. 5). Although saline-injected fish always mounted a robust plasma [cortisol] response to the stressor (paired Student's *t* test, $P \leq 0.001$ and 0.039 for 8 and 24 h, respectively), no elevation of plasma [cortisol] was detected in fish to which metyrapone had been administered

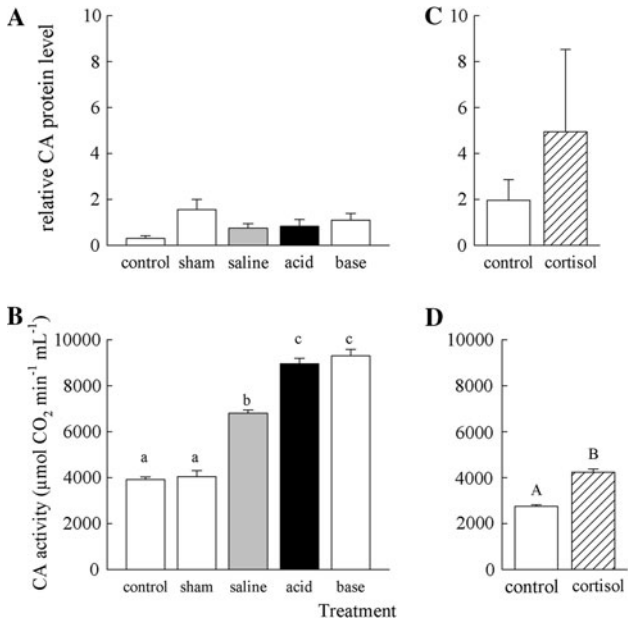


**Fig. 4** Carbonic anhydrase (CA) relative protein levels (**a**, **c**) and activity (**b**, **d**) in the gills of rainbow trout (*Oncorhynchus mykiss*) subjected to metabolic acid–base disturbances (**a**, **b**; see text for infusion conditions) or treated with cortisol (**c**, **d**; see text for treatment details). CA protein levels were expressed relative to $\beta$-actin protein levels. CA activity was assayed using crude homogenates of gill tissue. Values are mean + SEM ($N = 6$ in all cases except **d** where $N = 10$ and 11 for control and cortisol-treated fish, respectively). Groups that do not share a *letter* are significantly different from one another (**a**, **b**, one-way ANOVA, $P = 0.058$ and $<0.001$; **c**, **d**, Student's $t$ test, $P = 1$ and $<0.001$)

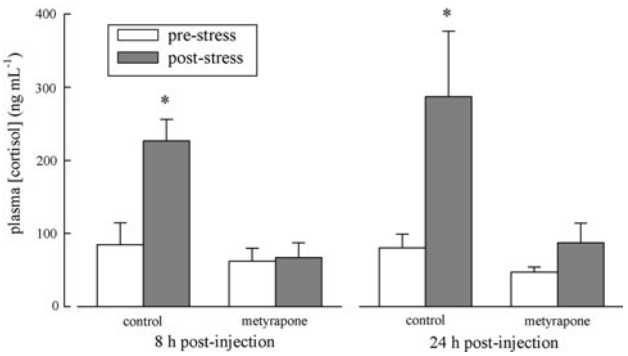


**Fig. 5** Circulating cortisol concentrations ([cortisol]) in rainbow trout (*Oncorhynchus mykiss*) before (pre-stress) and 10 min after (post-stress) 2 min of air emersion. Fish were subjected to the stressor 8 or 24 h after receiving an injection of saline (control) or the cortisol synthesis inhibitor metyrapone (see text for metyrapone dose). Values are mean + SEM ($N = 6$ and 7 for the 8 and 24 h trials, respectively). An *asterisk* indicates a significant difference between the post-stress value and its corresponding pre-stress value (one-tailed paired Student's $t$ tests, $P < 0.001$ for 8 h control, 0.267 for 8 h metyrapone, 0.039 for 24 h control, 0.065 for 24 h metyrapone)

(paired Student's $t$ test, $P = 0.533$ and 0.131 for 8 and 24 h, respectively).

Acid-, base- and saline-infusion elicited the expected adjustments of branchial and urinary net acid excretion, and these were altered by metyrapone administration in acid- but not saline- or base-infused fish (Fig. 6; Table 1). Figure 6 depicts branchial and urinary net acid excretion for control and metyrapone-treated fish, while the components of net acid excretion are summarized in Table 1. To test for effects of metyrapone treatment on blood acid–base status, changes in blood acid–base status were calculated as the difference between values at 24 h of infusion and pre-infusion values. Comparison of such delta values between fish from *Series I* and metyrapone-treated fish indicated that whether fish were treated with metyrapone did not influence the effects of infusion on acid–base status (Student's $t$ tests, $P > 0.05$ in all cases; data not shown).

Trout responded to acid infusion by increasing net acid excretion via the gill and kidney (Fig. 6a), primarily through $NH_4^+$ excretion (Table 1), which increased 1.6-fold at the gill. In metyrapone-treated fish, by contrast, neither branchial nor urinary net acid excretion was altered by acid infusion. Trout responded to base infusion with a significant reduction in branchial net acid excretion, which became highly negative (i.e. net excretion of basic equivalents; Fig. 6c). This response was driven by changes in titratable acidity; $NH_4^+$ excretion did not change (Table 1). Parallel changes were observed at the kidney, including a significant reduction in urine titratable acidity although this did not translate into a significant effect on urinary net acid excretion (Fig. 6c). The branchial and urinary responses of metyrapone-treated trout to base infusion were comparable to those of control fish. Saline infusion had no significant impact on branchial or urinary net acid excretion in control or metyrapone-treated trout (Fig. 6b). In metyrapone-treated fish, however, a significant reduction in branchial $NH_4^+$ excretion was detected over the infusion period (Table 1).

## Series III: Effects of cortisol elevation on CA expression

Cortisol treatment had significant effects on renal CA mRNA expression (Fig. 3d–f) and branchial activity (Fig. 4d) but not protein levels (Fig. 4c). Relative CA mRNA expression was compared to the control value of one, depicted in Fig. 3 as the dashed line. At the gill, relative CAc mRNA expression in cortisol-treated trout was not significantly different from the control value of one (one-sample Student's $t$ test, $P > 0.05$), nor did CA protein levels differ between control and cortisol-treated trout (Student's $t$ test, $P = 1$). However, branchial CA activity in cortisol-treated trout was 1.5-fold higher than the control value (Student's $t$ test, $P < 0.001$). At the kidney, relative mRNA expression of both CAc and CA IV in cortisol-treated trout appeared to be elevated, but only the mRNA

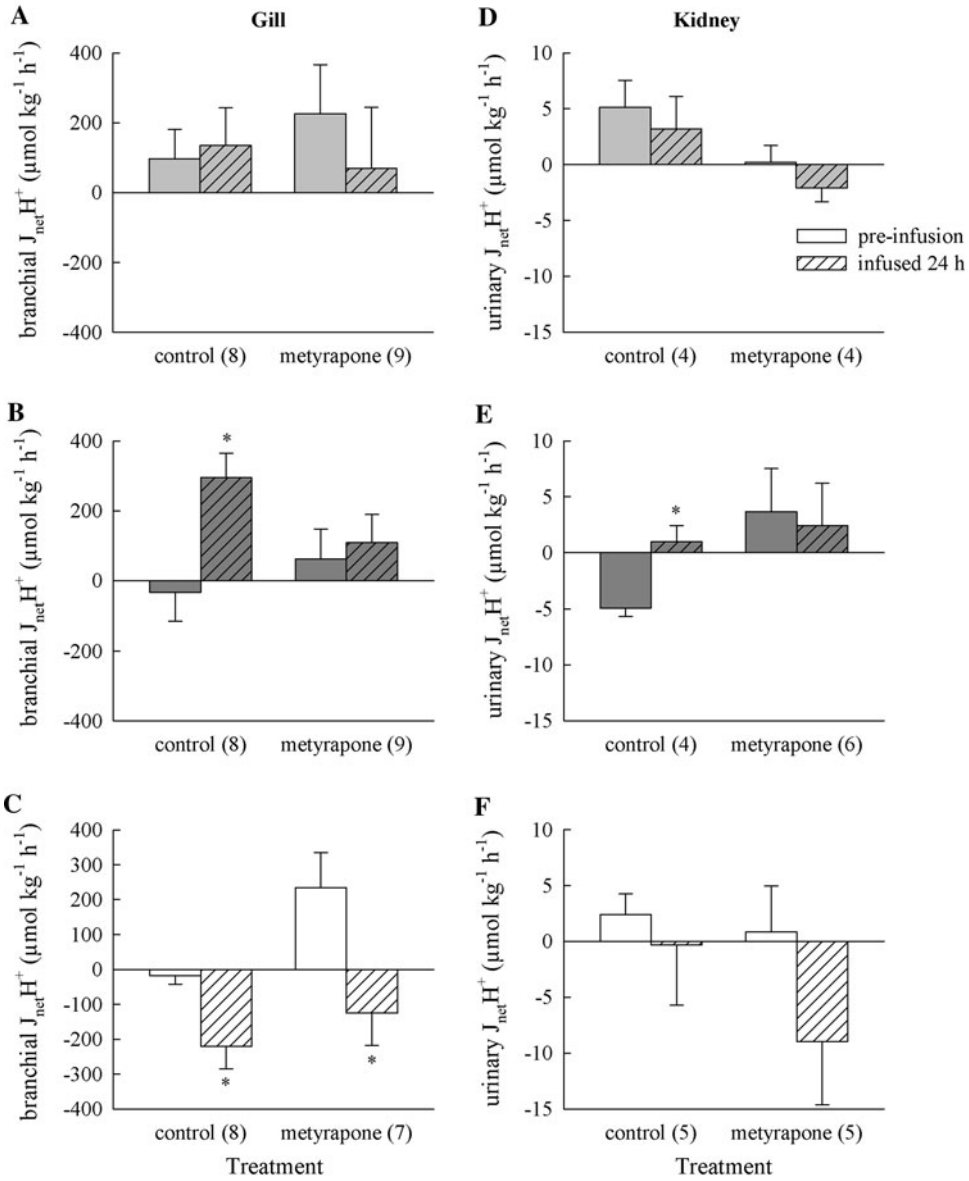


**Fig. 6** Net acid excretion ($J_{net}H^+$) via the gill (**a**–**c**) and urine (**d**–**f**) for rainbow trout (*Oncorhynchus mykiss*) subjected to saline infusion (**a**, **d** *light grey fill*), metabolic acidosis via acid infusion (**b**, **e** *dark grey fill*), or metabolic alkalosis via base infusion (**c**, **f** *white fill*) (see text for infusion conditions). Net acid excretion was measured immediately prior to infusion (pre-infusion; *non-hatched bars*) or over the final 3 h of the 24 h infusion period (infused 24 h; *hatched bars*). Measurements were carried out in fish that received an injection of saline (control) or the cortisol synthesis inhibitor metyrapone (see text for metyrapone dose). Values are mean + SEM (*N* numbers are indicated in *parentheses*). An *asterisk* indicates a significant difference between the 24 h infusion value and its corresponding pre-infusion value (paired Student's *t* tests, *P* values were **b** 0.031; **c** control 0.003, metyrapone 0.035; **e** 0.004; in all other cases $P > 0.05$)

expression of CA IV was significantly higher than the control value of one (one-sample Student's *t* test, $P < 0.05$); the relative mRNA expression of CAc in cortisol-treated fish was quite variable.

## Discussion

In the present study, the blood metabolic acid–base disturbances elicited by acid or base infusion, as well as the compensatory responses initiated by rainbow trout, were comparable to those reported previously (e.g. Cameron and Kormanik 1982; Goss and Wood 1990b, 1991; Curtis and Wood 1992; Goss and Perry 1994; Wood et al. 1999). What the present study adds to this body of literature is information on the cellular mechanisms underlying the compensatory responses, focusing specifically on CA, an enzyme that is a key player in acid–base regulation in both fish (Gilmour and Perry 2009) and mammals (Swenson 2000). The current findings also implicate cortisol in the regulation of compensatory responses to metabolic acidosis.

### Acid–base compensatory responses and CA

The gill is widely acknowledged to play a dominant role in restoring systemic pH during acid–base disturbances in teleost fish (reviewed by Claiborne et al. 2002; Perry et al. 2003b; Evans et al. 2005; Perry and Gilmour 2006). Current models for rainbow trout postulate the involvement of two cell types in acid–base regulation, a base-secreting cell termed the peanut lectin agglutinin-positive ($PNA^+$) mitochondrion-rich cell (MR) cell for its abundance of

**Table 1** Net excretion of acid as titratable acidity and ammonia via the gills and kidneys, together with urine flow rates, for acid-, saline- and base-infused rainbow trout *Oncorhynchus mykiss* in the absence or presence of the cortisol synthesis inhibitor metyrapone

| | Branchial excretion | | | | Urinary excretion | | | |
|---|---|---|---|---|---|---|---|---|
| | Control | | Metyrapone | | Control | | Metyrapone | |
| | Pre-infusion | Infused 24 h | Pre-infusion | Infused 24 h | Pre-infusion | Infused 24 h | Pre-infusion | Infused 24 h |
| Saline-infused fish | | | | | | | | |
| $J_{net}TA$ | −368.7 ± 87.6 (8) | −343.2 ± 52.9 (8) | −451.5 ± 156.5 (9) | −360.1 ± 144.2 (9) | −6.4 ± 2.3 (4) | −5.6 ± 3.2 (4) | −4.0 ± 1.4 (4) | −6.5 ± 0.6 (4) |
| $J_{net}NH_4^+$ | 465.7 ± 132.0 (8) | 477.9 ± 120.0 (8) | 677.8 ± 162.9 (9) | **430.0 ± 73.7**[a] **(9)** | 11.6 ± 4.2 (4) | 8.8 ± 0.7 (4) | 4.2 ± 0.9 (4) | 4.4 ± 1.3 (4) |
| UFR | | | | | 4.1 ± 0.5 (4) | 3.3 ± 0.8 (4) | 5.4 ± 1.0 (4) | 4.3 ± 0.3 (4) |
| Acid-infused fish | | | | | | | | |
| $J_{net}TA$ | −305.4 ± 90.6 (8) | −130.7 ± 71.0 (8) | −446.9 ± 85.0 (9) | −326.7 ± 62.5 (9) | −7.5 ± 0.3 (4) | −5.4 ± 3.8 (4) | −5.0 ± 2.6 (6) | −4.3 ± 2.2 (6) |
| $J_{net}NH_4^+$ | 272.1 ± 33.7 (8) | **425.7 ± 83.3**[b] **(8)** | 508.9 ± 108.4 (9) | 435.5 ± 87.1 (9) | 2.6 ± 0.9 (4) | 6.4 ± 5.2 (4) | 8.7 ± 1.8 (6) | 6.7 ± 3.5 (6) |
| UFR | | | | | 4.9 ± 0.8 (4) | 2.1 ± 0.9 (4) | 3.6 ± 0.6 (6) | 3.6 ± 1.5 (6) |
| Base-infused fish | | | | | | | | |
| $J_{net}TA$ | −350.8 ± 55.3 (8) | **−594.0 ± 70.5**[c] **(8)** | −338.3 ± 113.2 (7) | −659.2 ± 137.4 (7) | −2.9 ± 1.6 (5) | **−8.3 ± 2.6**[d] **(5)** | −6.6 ± 4.5 (5) | −20.7 ± 6.1 (5) |
| $J_{net}NH_4^+$ | 333.5 ± 58.5 (8) | 373.3 ± 40.7 (8) | 573.0 ± 84.2 (7) | 533.9 ± 86.9 (7) | 5.3 ± 0.7 (5) | 8.0 ± 3.3 (5) | 7.5 ± 1.9 (5) | 11.7 ± 2.3 (5) |
| UFR | | | | | 3.1 ± 0.6 (5) | 3.7 ± 0.5 (5) | 4.6 ± 0.5 (5) | 4.7 ± 0.4 (5) |

Values are mean ± SEM (*N*). Bold font indicates a significant difference between the value after infusion for 24 h and the pre-infusion value (paired Student's *t* test; *P* values were [a]0.004, [b]0.034, [c]0.005, and [d]0.032; $P > 0.05$ in all other cases)

$J_{net}TA$ net excretion of acid as titratable acidity (in μmol $kg^{-1}$ $h^{-1}$), $J_{net}NH_4^+$ net excretion of acid as ammonia (in μmol $kg^{-1}$ $h^{-1}$), *UFR* urine flow rate (in mL $kg^{-1}$ $h^{-1}$)

mitochondria and ability to bind PNA, and an acid-secreting $PNA^-$ MR cell (reviewed by Evans et al. 2005; Perry and Gilmour 2006; Tresguerres et al. 2006; Gilmour and Perry 2009). The $PNA^+$ MR cell is hypothesized to excrete $HCO_3^-$ in exchange for $Cl^-$ uptake via an anion exchanger that remains to be characterized (see Tresguerres et al. 2006) whereas the $PNA^-$ MR cell is hypothesized to excrete $H^+$ in exchange for $Na^+$ uptake via a $Na^+/H^+$ exchanger (likely NHE2 or NHE3) and/or coupled proton pump/$Na^+$ channel mechanism (see Galvez et al. 2002; Reid et al. 2003). In both ionocytes, catalysis of $CO_2$ by cytosolic CA has been proposed to provide the necessary acid–base equivalents for the transport mechanisms (see Perry and Gilmour 2006; Gilmour and Perry 2009), and in support of this hypothesis, CA inhibition during respiratory acidosis significantly reduced branchial net acid excretion (Georgalis et al. 2006b). Interestingly, whereas 24 h exposure to hypercapnia had no impact on branchial CAc mRNA expression (although a transient increase in mRNA expression was detected at 3 h) or activity but resulted in elevated CAc protein levels (Georgalis et al. 2006b), 24 h of acid infusion in the present study increased branchial CA activity in the absence of a change in protein levels, while decreasing CAc mRNA expression.

It is possible that these differences in CA expression and activity reflect differences in the branchial response to respiratory versus metabolic acidosis. The trout kidney responds differently to respiratory versus metabolic acidosis (Wood et al. 1999), and scrutiny of the literature reporting acid–base compensatory responses of rainbow trout to hypercapnia or hyperoxia (to induce respiratory acidosis) versus acid infusion (to induce metabolic acidosis) suggests that the same may be true of the gill. Respiratory acidosis in rainbow trout is compensated by increases in branchial net acid excretion in which excretion of acid as titratable acidity is usually more important than ammonia excretion (Goss and Wood 1990a; Wood 1991; Goss and Perry 1993; Georgalis et al. 2006b; but see Wood et al. 1984; Perry et al. 1987a). Based on measurement of ion fluxes, this net acid excretion has been attributed primarily to a reduction in $Cl^-/HCO_3^-$ exchange with a smaller contribution of enhanced $Na^+/H^+$ exchange (Wood et al. 1984; Perry et al. 1987a; Goss and Wood 1990a; Goss et al. 1995); reduced $Cl^-/HCO_3^-$ exchange is thought to reflect at least in part morphological changes at the gill in which MR cells, presumed to be base-secreting MR cells, are covered by pavement cells (Goss and Perry 1993; Goss et al. 1994). With acid–base compensation achieved mainly by physical covering of base excreting cells, there is little obvious need for a change in branchial CA activity, in accordance with the results of Georgalis et al. (2006b). By contrast, metabolic acidosis in rainbow trout elicits a substantial increase in ammonia excretion that, together with increased acid excretion as

titratable acidity, raises branchial net acid excretion (Goss and Wood 1991; present study). This net acid excretion appears to stem from enhanced $Na^+/H^+$ exchange with little or no contribution of reduced $Cl^-/HCO_3^-$ exchange (Goss and Wood 1991), and is accompanied by an increase in MR cell exposed surface area at the gill (Goss et al. 1994). Ionocyte subtypes were only identified in later work (see Goss et al. 2001), but assuming the increase represented acid-secreting MR cells (e.g. see Goss et al. 1995), the increased branchial CA activity observed in the present study would be consistent with the morphological mechanism to enhance acid excretion. CA protein levels presumably would have increased in concordance with the number of acid-secreting MR cells but this increase would not be detected by the methods used in the present study, in which CA protein was expressed relative to $\beta$-actin (which also would have been expected to increase with the number of acid-secreting MR cells). Finally, the significant reduction of CAc mRNA expression might represent a homeostatic mechanism to re-establish the normal situation. Interpretation of the mRNA and protein data is hampered by the lack of time-course information. Clearly, a head-to-head comparison of respiratory versus metabolic acidosis is needed to verify this scenario and more fully characterize the branchial compensatory responses and their underlying mechanisms.

The branchial response of rainbow trout to a metabolic alkalosis comprises a reduction in net acid excretion effected by modulation of the titratable acidity flux rather than ammonia excretion (Goss and Wood 1990b, 1991; Goss and Perry 1994), a pattern also observed in the present study. Ion flux studies suggest that $Cl^-/HCO_3^-$ exchange is enhanced (Goss and Wood 1990b, 1991; Goss and Perry 1994), at least in part through increases in the gill epithelium in the exposed surface area of MR cells that are presumed to be base-secreting MR cells (Goss et al. 1994; Goss and Perry 1994). With this model, base secretion would benefit from increased CA activity, and indeed, base-infused trout in the present study exhibited elevated branchial CA mRNA expression and enzymatic activity.

Branchial compensatory responses in the acid- or base-infused trout of the present study were quantitatively dominant over renal responses, a pattern that is consistent with previous reports that have compared branchial and renal compensation in freshwater teleosts (Kobayashi and Wood 1980; Cameron and Kormanik 1982; Wheatly et al. 1984; Wood et al. 1984; Perry et al. 1987b; Goss and Wood 1990b). Nevertheless, the kidney clearly contributes to acid–base compensatory responses. In mammals, the kidney responds to systemic acidosis by increasing ammonia synthesis and excretion, as well as $HCO_3^-$ reabsorption/$H^+$ secretion (e.g. Hamm and Nakhoul 2008; Hamm et al. 2008; Koeppen 2009), through increases in the amount and/or activity of key enzymes and transporters such as renal glutaminase (Wright and Knepper 1990), NHE3 (Preisig and Alpern 1988; Laghmani et al. 1997), and CA II and CA IV (Brion et al. 1994; Tsuruoka et al. 1998). Similarly, rainbow trout and other teleost fish compensate for systemic acidosis by increasing renal ammonia synthesis and $HCO_3^-$ reabsorption/$H^+$ secretion (Cameron and Kormanik 1982; King and Goldstein 1983; Perry et al. 1987b; Wood et al. 1999). Metabolic acidosis, in particular, strongly stimulates renal ammoniagenesis, a response that reflects increased renal activity of glutaminase and other enzymes involved in ammonia synthesis from glutamine (King and Goldstein 1983; Wood et al. 1999; Hirata et al. 2003). Enhanced tubular $HCO_3^-$ reabsorption/$H^+$ secretion during acidosis is probably supported by increases in NHE3, $H^+$-ATPase and NBC1; increases in the mRNA expression of all three transporters and protein levels of NHE3 have been detected in fish exposed to hypercapnia or water of low pH (Hirata et al. 2003; Perry et al. 2003a; Ivanis et al. 2008a; reviewed by Gilmour and Perry 2009). Georgalis et al. (2006a) used selective CA inhibitors to demonstrate roles for both cytosolic (CAc) and luminal membrane-bound (CA IV) CA isoforms in renal $HCO_3^-$ reabsorption in rainbow trout, and reported that the mRNA expression of both CAc and CA IV as well as CAc protein levels increased during hypercapnia. Similarly, in the present study, the mRNA expression of both CAc and CA IV was elevated in the kidney of rainbow trout after 24 h of acid infusion; enhanced mRNA expression of cytosolic CA was also detected in the kidney of Osorezan dace exposed to pH 3.5 water (Hirata et al. 2003). Thus, both respiratory and metabolic acidoses in teleost fish stimulate renal CA expression, a response that should enhance tubular $HCO_3^-$ reabsorption.

By contrast, the role of the kidney during systemic alkalosis is to eliminate $HCO_3^-$. In the present study, as in previous reports (Goss and Wood 1990b; Curtis and Wood 1992; Wood et al. 1999; Gilmour et al. 2007), base infusion was accompanied by a significant increase in renal base loss as titratable acidity. Although renal mRNA expression of CAc was unaltered by metabolic alkosis, CA IV mRNA expression was reduced in base-infused rainbow trout of the present study. The differential responses of CAc and CA IV to base infusion can be considered in light of the roles these isoforms play in the mammalian kidney. In mammals, CA IV is expressed by acidifying nephron segments, including the proximal tubule and $\alpha$- ($H^+$ secreting) but not $\beta$-intercalated cells ($HCO_3^-$ secreting) of the distal tubule, and functions exclusively in $HCO_3^-$ reabsorption (reviewed by Schwartz 2002; Purkerson and Schwartz 2007). Assuming a comparable situation exists in fish, which is reasonable based on the available evidence (Georgalis et al. 2006a), decreased CA IV expression would be an appropriate response to limit $HCO_3^-$ reabsorption. CA II, on the other

hand, is expressed throughout the mammalian nephron and is found in both α- and β-intercalated cells (see Schwartz 2002), where it catalyzes the hydration of $CO_2$ to provide acid–base equivalents for, respectively, $H^+$ secretion and $HCO_3^-$ secretion (Hamm et al. 2008). $HCO_3^-$ secretion is stimulated in metabolic alkalosis (McKinney and Burg 1977; Atkins and Burg 1985; Wesson and Dolson 1993; reviewed by Galla et al. 1991) and, like $HCO_3^-$ reabsorption (see Hamm et al. 2008 for review), is impaired by CA inhibition (McKinney and Burg 1978). Maintenance of CAc expression in alkalotic rainbow trout may therefore benefit $HCO_3^-$ elimination by supporting a $HCO_3^-$ secretion mechanism. Additional work is required to fully characterize the mechanisms underlying tubular acid secretion and $HCO_3^-$ transport in fish, particularly in the distal nephron, and to understand the responses of these mechanisms to acid–base challenges, particularly metabolic alkalosis. Indeed, even in mammals, the renal responses to alkalosis have been much less well studied than those to systemic acidosis (Hamm and Nakhoul 2008; Koeppen 2009).

Isotonic saline infusion was used in the present study, as in previous work (e.g. Goss and Wood 1990b; Curtis and Wood 1992; Goss et al. 1994; Goss and Perry 1994), as a control for the effects of infusion, while "sham" fish that were cannulated but not infused were incorporated into the experimental design as a control for the effects of surgery. Unlike in the study of Nawata and Wood (2009), cannulation itself had no effect on any measured parameter (except, arguably, plasma cortisol concentration, see below). Saline infusion, however, resulted in elevated branchial CA activity and elevated renal CAc mRNA expression. These effects may have occurred in response to the expansion of the extracellular fluid volume that accompanies saline infusion, because this treatment generally has little effect on acid–base status (Goss and Wood 1990b; Curtis and Wood 1992; Goss and Perry 1994), as was also the case here. The need to clear excess extracellular fluid via the urine while minimizing urinary ion losses could account for the elevation of renal CAc mRNA expression; elevated branchial CA activity may aid in replacing ions lost in elimination of the excess fluid.

## Does cortisol contribute to acid–base regulation?

The results of the present study support the hypothesis that cortisol plays a role in regulating acid–base compensatory responses to metabolic acidosis in rainbow trout. Not only was the pattern of circulating cortisol concentrations during acid infusion consistent with a role in triggering compensatory responses, but elimination of the cortisol response to acid infusion by administration of metyrapone correspondingly prevented the increases in both branchial and urinary net acid excretion that accompanied acid infusion under control conditions. Cannulation and confinement in an experimental chamber cause increases in circulating cortisol levels (Brown et al. 1986; Gamperl et al. 1994), and may account for the elevated cortisol levels in all fish infused for 24 h as well as the sham group (cannulated but not infused). In acid-infused fish, however, plasma [cortisol] increased significantly during the initial 2 h of acid infusion, a time frame appropriate for initiating compensatory responses to an acid–base disturbance. Saline- and base-infused fish did not exhibit this cortisol response, suggesting that it was specific to the onset of metabolic acidosis. Similarly, green sturgeon exhibited a rapid, transient cortisol response to the bolus infusion of acid but not saline (Warren et al. 2004). Elevated plasma cortisol concentrations have also been reported over a longer time frame (≥3 days) in trout in which metabolic acidosis resulted from exposure to water of low pH (Brown et al. 1984, 1986; Wood et al. 1999). Whether a cortisol response to respiratory acidosis occurs is less clear. Plasma cortisol concentrations were not elevated in hyperoxic rainbow trout (Wood and LeMoigne 1991; Wood et al. 1999), which experience respiratory acidosis owing to hypoventilation and consequent $CO_2$ retention, but were elevated by exposure of trout to hypercapnia (Ivanis et al. 2008a). Few studies have examined cortisol responses to alkalosis in fish; however, trout exposed to relatively hard water of high pH exhibited little (1–3 ng $mL^{-1}$) or no increase in circulating cortisol concentrations (Yesaki and Iwama 1992; Wilkie et al. 1996). Collectively, these data suggest that fish mount a cortisol response to acidosis but not alkalosis.

In mammals, glucocorticoids (cortisol or corticosterone) and aldosterone have been implicated in the renal ammoniagenic and $H^+$ secretory responses to metabolic acidosis (Wilcox et al. 1982; see also Hamm and Nakhoul 2008; Hamm et al. 2008). Glucocorticoids promote both ammonia synthesis and $HCO_3^-$ reabsorption/$H^+$ secretion (Welbourne 1976; Hulter et al. 1980, 1981; Wilcox et al. 1982; Kinsella et al. 1984; Baum and Quigley 1993) through actions on several enzymes and transporters, including NHE3 (Baum et al. 1993; Ambühl et al. 1999) and NBC (Ruiz et al. 1995; Ali et al. 2000). Aldosterone stimulates $H^+$ secretion (Koeppen and Helman 1982; Stone et al. 1983; Winter et al. 2004), lowering urine pH (Wilcox et al. 1982). Teleost fish lack aldosterone (see Prunet et al. 2006 for review), and while the possibility of an alternative mineralocorticoid such as 11-deoxycorticosterone has garnered significant research attention (e.g. Sturm et al. 2004; McCormick et al. 2008), experimental evidence suggests that cortisol can serve both glucocorticoid and mineralocorticoid functions (e.g. McCormick et al. 2008; see McCormick 2001; Evans et al. 2005 for review).

Evidence from the present study and the literature suggests several pathways through which cortisol may

promote net acid excretion during acidosis in fish. In the present study, cortisol treatment elicited changes in CA mRNA expression and activity that, particularly for the kidney, paralleled those observed in acid-infused fish. Ivanis et al. (2008a) reported increased renal NHE3 mRNA and protein expression in trout treated with cortisol, and cortisol treatment increased NHE2 mRNA expression (Ivanis et al. 2008b) and proton pump activity (Lin and Randall 1993) in trout gills. All of these responses are appropriate to enhance net acid excretion. In addition, cortisol has been implicated in the regulation of ammonia excretion. Cortisol increases muscle proteolysis, leading to adjustments in amino acid metabolism (e.g. Andersen et al. 1991) that can include increased ammonia output (Mommsen et al. 1999). Cortisol also appears to play a role in regulating key elements of the branchial ammonia excretion pathway, notably the gill Rhesus glycoproteins (Wright and Wood 2009). In this regard it is interesting that branchial ammonia excretion was significantly reduced by metyrapone treatment in saline-infused trout of the present study.

## Conclusions

In summary, the findings of the present study provide support for the hypothesis that cortisol plays a role in stimulating acid–base compensatory responses to metabolic acidosis in rainbow trout. Plasma [cortisol] and net acid excretion increased during metabolic acidosis, and the increase in net acid excretion was prevented by blocking the cortisol response. Moreover, parallel changes in renal CA IV mRNA expression and branchial CA activity in cortisol-treated fish and fish subjected to a metabolic acidosis suggest that CA expression during metabolic acidosis may be regulated at least in part by cortisol. Finally, the data provide insight into the role of CA in acid–base compensatory responses, and, in particular, highlight differences between compensatory responses to metabolic versus respiratory acid–base disturbances.

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