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Acid-base Regulation in Rainbow Trout: The Roles of Carbonic Anhydrase and Cortisol

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Acid-base regulation in rainbow trout: The roles of carbonic anhydrase and cortisol.

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ABSTRACT

This study tests the hypotheses that compensation for metabolic acid-base disturbances involves regulation of carbonic anhydrase (CA) and that cortisol plays an important role in mediating the compensatory responses of the rainbow trout, *Oncorhynchus mykiss*.

Metabolic acidosis decreased mRNA expression of branchial trout cytosolic CA (tCAc) and increased mRNA expression of renal tCAc and membrane-bound CAIV (tCAIV). Metabolic alkalosis increased mRNA expression of branchial and renal tCAc, decreased renal tCAIV mRNA expression, and increased branchial CA activity.

Plasma cortisol was elevated during acidosis and alkalosis, indicating its possible involvement in regulating the response to an acid-base challenge. Cortisol may up-regulate CA, as treatment with exogenous cortisol increased tCAc and tCAIV mRNA expression, renal tCAc protein abundance, and branchial CA activity.

This study suggests that CA contributes to the compensation of metabolic acid-base disturbances in the rainbow trout, and that cortisol is involved in the regulation of acid-base status.

RÉSUMÉ

Cette étude test les hypothèses que la compensation pour les perturbations métaboliques acide-base implique la régularisation de l'anhydrase carbonique (CA) et que le cortisol joue un rôle important dans la médiation des réponses compensatoires.

L'alcalose métabolique a réduit l'expression dans l'mRNA de l'CA cytosolique branchial de la truite (tCAc), et a élevé l'expression dans l'mRNA de l'tCAc rénal et le CAIV membranaire de la truite (tCAIV). L'alcalose métabolique a élevé l'expression dans l'mRNA le tCAc branchial et rénal, a réduit l'expression dans l'mRNA du tCAIV rénal, and a élevé l'activité branchial de l'CA.

Le cortisol plasmique était élevé lors de l'acidose et de l'alcalose, ce qui indique la possibilité de sa contribution dans la régularisation de la réponse à une épreuve acide-base. Il se peut que le cortisol soit essentiel à l'haut-règlementation de l'CA, puisque le traitement avec le cortisol exogène a élevé l'expression dans l'mRNA de l'tCAc branchial et rénal et le tCAIV rénal, l'abondance de la protéine tCAc rénal, et l'activité de l'CA branchial.

Cette étude suggère que l'CA contribue à la compensation des désordres acide-base métabolique dans la truite arc-en-ciel et que le cortisol est impliqué dans la régularisation de l'état acide-base.

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ABBREVIATIONS

CO_2: Carbon dioxide
H_2O: Water
H^+: Protons (Hydrogen ions)
HCO_3^-: Bicarbonate
CA: Carbonic anhydrase
tCAc: Trout cytosolic carbonic anhydrase
tCAb: Trout blood carbonic anhydrase
tCAIV: Trout carbonic anhydrase IV
Pa_{CO2}: Arterial partial pressure of carbon dioxide
O_2: Oxygen
Cl^-: Chloride
Na^+: Sodium
NHE3: Sodium / hydrogen exchanger isoform 3
NBC1: Sodium / bicarbonate - cotransporter
Na^+-K^+-ATPase: Sodium / potassium ATPase
RT-PCR: Reverse transcriptase polymerase chain reaction
mRNA: Mitochondrial ribonucleic acid
cDNA: Complementary deoxyribonuclease
N_2: Nitrogen
CT: Threshold cycle
BSA: Bovine serum albumin
HCl: Hydrochloric acid
$NaHCO_3$: Sodium bicarbonate
SEM: Standard error of the mean
ANOVA: Analysis of variance
$J_{net}H^+$: Net rate of acid excretion
$J_{net}TA$: Net rate of total alkalinity
$J_{net}NH_3$: Net rate of ammonia excretion
SA: Surface area
$PNA^+/^-$: Peanut agglutinin staining (positive or negative)
MRC: Mitochondrial rich cell
PVC: Pavement cell

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INTRODUCTION

Carbonic anhydrase

In fish, like other vertebrates, the physiological processes of acid-base regulation and carbon dioxide (CO_2) excretion are coupled through the reversible hydration / dehydration reactions of CO_2 and protons (H^+) / bicarbonate ions (HCO_3^-) (Cameron, 1978; Claiborne et al., 2002; Swenson, 2003; Evans et al., 2005; Perry and Gilmour, 2006). This reaction, $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$, is catalyzed by carbonic anhydrase (CA), a ubiquitous zinc metalloenzyme (Maren, 1967). Because it catalyzes the reversible CO_2 reactions, CA is integral to a wide range of physiological processes including respiration, acid-base regulation, bone resorption and calcification, several biosynthetic pathways, and numerous processes that involve the transfer or transport of ions, gases, and fluids (Perry and Laurent, 1990; Randall and Val, 1995; Geers and Gros, 2000; Henry and Swenson, 2000). Recent evidence (Gilbert et al., 2006; Rini, 2006; Pastorekova et al., 2006; Dorai et al., 2006; Swietach et al., 2007) also supports the involvement of CA in cell growth, a finding that may, in the future, provide further insight into oncogenesis and cancer progression.

Carbonic anhydrase occurs in three distinct families of isoforms, designated the α-, β-, and γ-gene families (Chegwidden and Carter, 2000). Carbonic anhydrase, in one form or another, is prevalent in nearly all organisms: different CA isoforms from the different families have been discovered in vertebrates, lower eukaryotic species and invertebrates, plants, and even eubacteria and archaebacteria (Chegwidden and Carter, 2000). However, vertebrates express only CA isoforms from the α-gene family (Hewett-Emmett, 2005), and therefore only the α-gene CA family will be considered in this thesis.

The α-carbonic anhydrases are monomeric zinc metalloenzymes with an approximate molecular weight of 29 kDa (Sly and Hu, 1995; Chegwidden and Carter, 2000). In mammals, at least 15 different α-carbonic anhydrases have been discovered (Hewett-Emmett, 2000). Most of these isoforms exhibit catalytic activity, with only three appearing to lack activity owing to substitutions within one or more of the histidine residues that bind to the zinc ion within the active site (Chegwidden and Carter, 2000). The zinc ion is necessary for catalysis of CO_2 hydration / dehydration reactions (Lindskog and Silverman, 2000). The mutated and consequently inactive isoforms are designated CA-related proteins (Chegwidden and Carter, 2000). Of the enzymes that are active in the CO_2 hydration / dehydration reaction, the activity levels vary (Baird et al., 1997; Chegwidden and Carter, 2000). For example, the CAII isoform is one of the fastest enzymes studied, with a rate of catalysis (Kcat) reported to surpass one million moles CO_2 per mole CA per second (Chegwidden and Carter, 2000). In vertebrates, the different CA isoforms are an essential part of many biosynthetic pathways including the synthesis of glucose, fatty acids, amino acids, and urea (Henry, 1996). They also have multiple roles in cellular transport, facilitating the movement of CO_2 out of the cell, rapid ion exchange across cell membranes, and maintaining the acid-base equilibrium within the organism (Perry and Laurent, 1989). The active CA isoforms of the α-gene family have been identified and characterized through studies of their molecular structure and kinetic properties, cellular location and tissue distribution, inhibitor sensitivity, and physiological function, including catalytic activity (Sly and Hu, 1995; Hewett-Emmett and Tashian, 1996; Baird et al., 1997; Chegwidden and Carter, 2000; Tufts et al., 2003).

In mammals, eleven enzymatically-active CA isoforms have been identified and localized to a variety of tissues (Hewett-Emmett and Tashian, 1996; Baird et al., 1997; Whittington et al., 2001). Isoforms CAI and CAII are concentrated in the erythrocytes but have been reported in the cytosol of other tissues (Baird et al., 1997). Another cytosolic form of CA, CAIII, is localized to the cytoplasm of the muscle cell (Baird et al., 1997). The membrane-bound form of CA, identified as CAIV, is present in the lung, kidney, brain, and eye tissues and the ubiquitous CAV isoform, sub-classified as CAVA and VB, is present in the mitochondria of cells (Baird et al., 1997; Whittington et al., 2001). The two isoforms CAVI and CAVII are present in the saliva and salivary glands, respectively (Sly and Hu, 1995; Baird et al., 1997). Isoforms CAIX, XII, and XIV have all been characterized as membrane-associated (Sly and Hu, 1995), and CAIX and XII specifically have been associated with certain human cancers (Zavadá et al., 1993; McKiernan et al., 1997; Türeci et al., 1998).

Although CA enzymes within mammalian systems have been studied extensively, less is known about CA isoforms in the tissues of non-mammalian species, such as fish (Esbaugh et al., 2005; Esbaugh and Tufts, 2006). While the catalytic activity of CA has been studied in fish for over 60 years (Sobotka and Kann, 1941), few CA isoforms have been identified in non-mammalian species and fewer have been characterized with respect to their kinetics or molecular structure (Esbaugh et al., 2005). Although a number of different tissues exhibit CA activity (gill: Dimberg et al., 1981; Conley and Malalatt, 1988; liver: Sanyal, 1984; erythrocytes: Henry et al., 1988, 1993), research has focused on the CA activity of the erythrocytes (Esbaugh et al., 2005), which have been shown to be a vast source of CA (Henry and Swenson, 2000).

More diverse research into the gill tissue of a variety of fish species has revealed that branchial CA activity is mainly cytoplasmic, with the enzyme localized to the pavement cells, mucous cells, and mitochondrial rich cells (MRCs) of the gills (*Oncorhynchus mykiss, Cyprinus carpio*: Rahim et al., 1988; *Fundulus heteroclitus*: Flügel et al., 1991; *Squalus acanthias*: Wilson et al., 1997; *Platichthys flesus*: Sender et al., 1999; *Periophthalmodon schlosseri*: Wilson et al., 2000). Other studies have shown that the gills of some fish species contain membrane-bound CA isoforms (*Squalus acanthias*: Gilmour et al., 1997, 2001, 2007; *Raja rhina, Hydrolagus colliei*: Gilmour et al., 2002; *Chaenocephalus aceratus, Notothenia coriiceps*: Tufts et al., 2002).

In the rainbow trout, *Oncorhynchus mykiss*, a number of previous studies have revealed the presence of at least three distinct CA isoforms (Esbaugh et al. 2005; Georgalis et al., 2006a). These three isoforms are found to make a significant contribution to the maintenance of acid-base balance within the organism (Georgalis et al., 2006a, 2006b). Specifically, two cytosolic isoforms have been identified: trout cytosolic CA (tCAc) which is broadly distributed across a range of tissues, but found in high abundance in branchial and renal tissues, and trout blood CA (tCAb), which appears to be more specifically localized to erythrocytes (Esbaugh et al., 2005). A type IV membrane-bound isoform, tCAIV, has also been identified and is present in renal tissue as well as brain and heart (Georgalis et al., 2006a). This thesis explores the actions of the tCAc and the tCAIV isoforms with respect the maintenance of homeostasis during a systemic acid-base challenge.

Acid-base regulation

Any deviation from the homeostatic acid-base equilibrium of an organism is considered to be an acid-base disturbance. Animals can correct an acid-base disturbance through alterations of ventilation (termed respiratory compensation) or through the direct exchange of acid-base equivalents with the environment (termed metabolic compensation) (Perry and Gilmour, 2006). Respiratory compensation relies on the CA-catalyzed interconversions of CO_2 with H^+ and HCO_3^-, while the acid-base equivalents used in exchange with the environment (metabolic compensation) are often derived from CO_2 with the involvement of CA (Perry and Gilmour, 2006).

Environmental or physiological conditions that reduce ventilation (hypoventilation) such as exposure to a hyperoxic environment would result in a respiratory acidosis through CO_2 retention (Perry and Gilmour, 2006). Similarly, hyperventilation, an increase in the rate of ventilation, would cause a respiratory alkalosis, because the transfer of CO_2 would be increased (Gilmour, 2001; Perry and Gilmour, 2006). Thus, air-breathing vertebrates can regulate metabolic acid-base disturbances through finely-tuned adjustments in ventilation, because arterial blood PCO_2 (Pa_{CO2}) is high and air is an O_2-rich environment. Air-breathing vertebrates often employ alterations of ventilation in attempts to correct acid-base disturbances (Heisler, 1986; Swenson, 2000). It is important to note that this trend also extends to air-breathing fish species, such as the African lungfish, *Protopterus annectens* (Gilmour et al., 2007). In water-breathing vertebrates, however, the ability to use adjustments of ventilation to regulate metabolic acid-base disturbances is limited (Heisler, 1986; Claiborne et al., 2002; Perry and Gilmour, 2006).

In water-breathing fish, for example, Pa_{CO2} is low due to the relatively low O_2 content in water and the counter-current arrangement of blood flow through the gills and water flow across the gills. Thus, a change in ventilation to correct an acid-base disturbance would adversely impact O_2 delivery. That is, the low Pa_{CO2} values limit the effectiveness of hyperventilation as a strategy for correcting an acidosis, while the high convection requirement for O_2 uptake restricts the capacity to use hypoventilation as a strategy for correcting an alkalosis. Consequently, the acid-base status of water-breathing animals is predominantly regulated through metabolic compensation, where plasma HCO_3^- levels are adjusted at approximately constant values of Pa_{CO2} (Claiborne, 1998). Simply stated, metabolic compensation is achieved through the fine adjustments in the transfer rates of acid and base equivalents between internal and external compartments (Goss et al., 1992). Metabolic compensation occurs primarily at the gills (Evans et al., 2005) although the kidneys also play an integral role (Wood et al., 1999).

The gill and acid-base regulation

The gill is the principal organ for metabolic compensation owing to its ability to regulate net acid excretion (Evans et al., 2005) and because its ability to exchange acid-base equivalents with the environment is much greater than that of the renal system (Perry and Gilmour, 2006). Adjustments in HCO_3^- levels that form the basis for metabolic acid-base regulation reflect changes in net acid excretion that, in turn, rely on CA activity. CA is implicated in metabolic compensation by catalyzing the hydration of CO_2 within gill epithelial cells to provide the counter ions required for the ion exchange processes necessary to regulate systemic acid-base balance (Evans et al., 2005; Perry and Gilmour, 2006).

It is through such adjustments in net acid efflux, and, thus, plasma HCO_3^- levels, that the gills function to regulate blood pH. Although the precise mechanisms that alter branchial net acid movement have yet to be fully characterized, it is generally accepted that, in freshwater fish species, net branchial acid efflux is controlled by the relative rates of chloride (Cl^-) and sodium (Na^+) ion uptake, coupled to the HCO_3^- and H^+ effluxes, respectively (Fig. 1).

The epithelial cells of the gills that are involved in branchial acid-base regulatory mechanism are the mitochondrial rich cells and the pavement cells (PVCs) (Wilson and Laurent, 2002; Perry and Gilmour, 2006). Further experimentation has revealed distinct sub-types of the MRCs, which have since been sub-classified as those with peanut lectin agglutinin (PNA) binding sites on their apical membranes (PNA^+ MRCs) and those without these sites (PNA^- MRCs) (Goss et al., 2001; Galvez et al., 2002). This is an important distinction, as these two different cell types may function differently during an acid-base disturbance (Perry and Gilmour, 2006). The PNA^+ MRCs are believed to be base-excreting cells, and function to excrete basic equivalents into the environment (Perry and Gilmour, 2006). This is accomplished through the use of apical Cl^- / HCO_3^- exchangers to excrete HCO_3^- into the water and the use of basolateral V-type H^+-ATPases to reabsorb H^+ into the blood. The PNA^- MRCs are thought to be acid-excreting cells that function to excrete acidic equivalents into the environment. The PNA^- MRCs secrete acid through the use of apical Na^+ / H^+ exchangers and / or Na^+ channels linked to V-type H^+-ATPases, both of which function to excrete H^+ following the uptake of Na^+ from the water. These apically-located enzymes are coupled to basolaterally-located Cl^- / HCO_3^- exchangers or Na^+ / HCO_3^- co-transporters, which both function to reabsorb HCO_3^- into the blood (Fig. 1).

To correct for a systemic acidosis, the branchial system of teleost fish will either increase net acid efflux or decrease net base efflux across the gills (Kobayashi and Wood, 1980). This is carried out through either branchial remodeling, with an increase in the surface area of PNA^- MRCs or the exchange of PNA^+ MRCs for PVCs, or through the transcriptional activation of the H^+-excreting apically-located enzymes of the PNA^- MRCs (Sullivan et al., 1995, 1996). During a systemic alkalosis, the branchial system of the teleost fish will either decrease net acid efflux or increase net base efflux across the gills (Cameron and Kormanik, 1982). This is also accomplished through branchial remodeling, with the possible exchange of PVCs for PNA^+ MRCs (Perry and Gilmour, 2006). Previous studies have shown that gill remodeling can occur rapidly, within hours, in fish undergoing an acid-base disturbance (Goss et al., 1992; Goss and Perry, 1993). For example, Goss et al. (1992) reported that in the brown bullhead, *Ictalurus nebulosus*, the fractional area of the chloride cells was reduced by 50% after just 6 h exposure to hypercapnia.

Despite the considerable research on the role of branchial CA in acid-base regulation in teleost fish (Georgalis et al., 2006b), the complex cellular and molecular mechanisms that provide the basis for the necessary branchial ion transfers require further elucidation. Furthermore, because the gill is the primary organ system involved in the regulation of acid-base homeostasis in teleost fish, less emphasis has been placed on the potential roles of other organs, including the kidney, in acid-base regulation (Claiborne et al., 2002). Recent studies have revealed that the renal system may also be essential to the proper regulation of systemic acid-base balance in teleost fish (Wood et al., 1999; Georgalis et al., 2006a), although, once again, further study into the exact cellular and molecular mechanisms underlying this important function of the renal system is required.

The kidney and acid-base regulation

Studies have shown that, while adjustment of urine pH plays only a small role in net acid efflux during an acid-base disturbance (Cameron and Kormanik, 1982; Choe and Evans, 2003; Curtis and Wood, 1992), the changes in plasma HCO_3^- levels that are necessary for pH regulation, although initiated at the gills, require parallel changes in renal acid secretion. The ability to retain a high level of HCO_3^- in the plasma, in order to counter-act an acidosis, requires a corresponding increase in renal acid secretion to facilitate the reabsorption of filtered HCO_3^-. Thus, although the urine produced by the kidneys functions only slightly in whole body net acid efflux, a significant increase in renal acid secretion must occur to ensure that HCO_3^- ions accumulated via the gills are not lost via the urine.

In the mammalian kidney, filtered HCO_3^- is reabsorbed through a series of reactions that occurs within the proximal tubules of the kidneys (Romero and Boron, 1999; Boron, 2004). The filtered HCO_3^- ions that accumulate in the lumen of the kidney combine with H^+ provided by the apical membrane Na^+ / H^+ exchanger isoform 3 (NHE_3) (Wu et al., 1996) to form CO_2. This reaction is catalyzed by the membrane-bound CAIV enzyme (Schwartz et al., 2000). The CO_2 enters the proximal tubules and is hydrated to reform H^+ and HCO_3^- ions by the cytosolic CAII enzyme (Wistrand and Wahlstrand, 1977). To complete this reabsorption process, the HCO_3^- ions are returned to the bloodstream via the basolateral Na^+ / HCO_3^- cotransporter (NBC1) (Schmitt et al., 1999). Therefore, in mammalian systems, the HCO_3^- reabsorption process is dependent upon the presence of both the cytosolic and membrane-bound CA isoforms (Schwartz, 2002). A similar model has been proposed for freshwater fish species (Perry and Gilmour, 2006), although more experimental studies are required to support the model.

Thus, the kidneys play a supporting role to the gills during acid-base regulation, and function to retain HCO_3^- ions. The current model for this process is based upon mammalian models of HCO_3^- reabsorption (Steinmetz, 1974; Murer et al., 1976; McKinney and Burg, 1978; Friedman and Andreoli, 1982) and proposes that filtered HCO_3^- ions combine with H^+ to form CO_2, in a reaction catalyzed by membrane-bound tCAIV (Fig. 2) (Georgalis et al., 2006a). The newly produced CO_2 diffuses into the proximal tubule cells of the kidney, where it undergoes hydration, catalyzed by tCAc, to re-form H^+ and HCO_3^- ions (Georgalis et al., 2006a). Net HCO_3^- reabsorption is achieved by the basolateral export of HCO_3^- ions while protons are recycled into the lumen of the renal tubule (Georgalis et al., 2006a).

The kidneys of teleost fish respond to a systemic acidosis through an increase in the export of acidic equivalents through titratable acidity (TA) and ammonium (NH_4^+) (Wood et al., 1999). In the face of a systemic alkalosis, the kidney responds through an increase in the export of basic equivalents in the form of HCO_3^- (Wood et al., 1999). Renal responses are rapidly initiated, being detected within about 6 h of the onset of the acid-base disturbance, although frequently not peaking until 24 – 72 h of exposure (Perry et al., 1987b; Wood et al. 1999). It is interesting to note that these renal responses of the teleost fish parallel the responses made by the kidneys of mammals in similar situations (Wood et al., 1999).

Respiratory versus metabolic acid-base disturbances

The general conditions that challenge acid-base homeostasis are (1) a respiratory acidosis or alkalosis and (2) a metabolic acidosis or alkalosis (Rose, 2001). An acidosis occurs when the blood pH falls below the normal range and can occur naturally following exposure to an acidotic environment or after exhaustive exercise (Kakizawa et al., 1996).

A respiratory acidosis is caused by a rise of PCO_2, and a metabolic acidosis is caused by an increase in systemic H^+ production or a decrease in systemic HCO_3^- production (Rose, 2001). Alternatively, an alkalosis occurs when blood pH rises above the normal range of values and occurs naturally via the alkaline tide, following the consumption of a large meal (Tresguerres et al., 2007; Wood et al., 2007). A respiratory alkalosis reflects a lowering of the Pa_{CO2}, whereas a metabolic alkalosis is caused by a higher than normal level of plasma HCO_3^- ions (Rose, 2001). Each of these conditions has unique physiological characteristics. However, the conditions of interest in the present study are metabolic acidosis and alkalosis.

According to mammalian literature, the responses of an animal to a metabolic acid-base disturbance is often different from the responses to a respiratory acid-base disturbance (Carter et al., 1959; Rodriguez-Nichols et al., 1984; Krapf et al., 1991). For example, the excretion of net H^+ via the urine, in the form of NH_4^+, is much greater during a metabolic acidosis than during a respiratory acidosis (Rodriguez-Nichols et al., 1984). This is due to the fact that in mammals experiencing a systemic acidosis, the level of urinary NH_4^+ that results from plasma filtration is quite small in comparison to the amount of NH_4^+ excreted via the urine due to increased metabolic production by the kidney (Rodriguez-Nichols et al., 1984). Additionally, mammals have a differential TA response to a metabolic versus a respiratory acidosis. Mammals try to maintain urine pH by relating the rate of H^+ excretion to the availability of titratable buffer (Simpson, 1971; Valtin, 1973; Pitts, 1974). During a respiratory acidosis, the amount of TA excretion exceeds that of NH_4^+ excretion (Carter et al., 1959). The renal response of mammals to a metabolic alkalosis involves a modulation of the rate of H^+ excretion / HCO_3^- reabsorption (Emmett and Seldin, 1975; Sabatini and Kurtzman, 1989).

A number of previous studies (Wheatly et al., 1984; Perry et al., 1987b; Wood et al., 1999) have revealed that, similar to mammals, rainbow trout exhibit different responses to metabolic versus respiratory acid-base disturbances. Recent research (Georgalis et al., 2006a, 2006b) has explored the response of rainbow trout to respiratory acid-base disturbances. This study aims to ascertain the branchial and renal responses of the animal to metabolic acid-base disturbances.

Regulation of CA

A number of different studies have suggested that CA gene expression and protein abundance, as well as enzymatic activity, are regulated (Russell et al., 1976; Brion et al., 1991, 1994; Schwartz et al., 1993; Endroczi et al., 1994; Winkler et al., 1997; Quelo et al., 1998; Tsuruoka et al., 1998; Pelis et al., 2003). For example, a vitamin D3 responsive element was discovered in the promoter regions of the avian CAII gene (Quelo et al., 1998). To increase the net acid excretion from the kidney, rabbits undergoing an experimentally-induced metabolic acidosis experience a significant increase in renal CAII and CAIV catalytic activity (Brion et al., 1991, 1994; Winkler et al., 1997) and gene expression (Schwartz et al., 1993; Winkler et al., 1997; Tsuruoka et al., 1998). Recent studies on the Osorezan dace (*Tribolodon hakonensis*), a cyprinid teleost fish species that lives and grows in acidic waters, but spawns in a neutral environment, had similar findings (Hirata et al., 2003). The renal CAII mRNA expression of this teleost fish increased significantly after the exposure of the animal to an acidotic environment (Hirata et al., 2003).

The results of studies on mammals suggest that glucocorticoids (Welbourne, 1976; May et al., 1986) may play a critical role in the regulation of CA expression in the kidneys.

In vivo treatment with the cortisol agonist dexamethasone significantly induced CA activity in the brain of sexually-maturing rats (Endroczi et al., 1994). Further study revealed that *in vitro* and *in vivo* treatment with cortisol had a significant impact on erythrocytic CA activity (Russell et al., 1976). The findings of these studies in mammals suggest that further exploration into the action of cortisol on CA in teleost fish is worthwhile.

Cortisol

Cortisol secretion is the end product of the hypophyseal-pituitary-interrenal (HPI) stress axis and its overall function is to mobilize energy reserves for optimal performance during stressful conditions, i.e. situations where homeostasis is threatened (Wendelaar Bonga, 1997; Mommsen et al., 1999; Flik et al., 2006). A rise in plasma cortisol level is typically attributable to exposure to an acute stressor, although chronic stressors tend to increase cortisol levels and keep them elevated (Wendelaar Bonga, 1997). Research has shown that cortisol levels respond to such stressors as handling, toxin or pollutant exposure, drastic water temperature or acidity changes, and predation (Wendelaar Bonga, 1997).

In teleost fish, cortisol is the main corticosteroid hormone and has a variety of fundamental functions stemming from its dual role as a stress hormone and as a hormone involved in the regulation of salt and water balance (Wendelaar Bonga, 1997, Mommsen et al., 1999; McCormick, 2001). Cortisol has received particular attention during studies of the freshwater to ion-deficient (soft) water transitions in rainbow trout (Sloman et al., 2001). Other studies have focused on the possible role of cortisol in stimulating Na^+-K^+-ATPase activity within osmoregulatory organs such as the gills and kidney during fresh water to seawater transitions in coho salmon, *Oncorhynchus kisutch* (Bern and Madsen, 1992).

Additionally, cortisol has been shown to significantly impact both ionic and osmotic regulation, to stimulate the proliferation of mitochondrial rich cells (Perry and Laurent, 1990; Dang et al., 2000), and to increase the branchial ion influx in freshwater-acclimated rainbow trout (Perry and Laurent, 1989).

Previous research has revealed that cortisol levels in the plasma increase significantly in fish experiencing a metabolic acidosis, but not in fish experiencing a respiratory acidosis (Wood and LeMoigne, 1991; Wood et al., 1999), leading this current study to explore possible interactions between CA and cortisol during a metabolic acid-base disturbance. As previously stated, cortisol has been linked to control of CA expression and activity in a number of mammalian studies (Russell et al., 1976; Endroczi et al., 1994). Cortisol has also been shown to increase CA activity in the proximal tubule cells of winter flounder (Pelis et al., 2003). Additionally, because cortisol levels rise during metabolic acidosis (Wood et al., 1999), it is conceivable that renal CA is being targeted to aid acid-base regulation.

Hypotheses

The studies by Georgalis et al. (2006a; 2006b) explored the relationships between two CA isoforms (tCAc and tCAIV) and acid-base regulation in rainbow trout, focusing on urinary acidification (or renal HCO_3^- reabsorption) and branchial acid-base exchange in response to the imposition of a respiratory acidosis. With this in mind, and given the evidence from mammalian studies of different renal responses to metabolic versus respiratory acid-base disturbances, the present study was designed to focus on physiological acid-base compensation mechanisms that occur during metabolic acid-base disturbances.

Specifically, the present study tested the hypothesis that in rainbow trout, branchial and renal responses to the imposition of metabolic acid-base disturbances would involve the regulation of carbonic anhydrase expression and/or activity.

Based on current models for the involvement of gill CA in acid-base regulation, branchial tCAc mRNA, protein, and / or activity levels are predicted to fall during a metabolic acidosis as the activity of the base-secreting mitochondria-rich cells is reduced to promote the accumulation of HCO_3^- ions. By contrast, during a metabolic alkalosis branchial tCAc mRNA, protein, and / or activity are predicted to rise as the activity of the base-secreting mitochondria-rich cells is enhanced to promote the loss of HCO_3^- ions.

Following from current models for the involvement of renal CA isoforms in acid-base regulation, during a metabolic acidosis the mRNA expression, protein levels and/or activity of renal tCAc and tCAIV isoforms is predicted to increase so as to promote the reabsorption of HCO_3^- ions from the filtrate. Correspondingly, during a metabolic alkalosis the mRNA, protein, and / or activity levels of the renal CA isoforms (tCAc and tCAIV) are predicted to decrease so as to favour HCO_3^- loss.

A second hypothesis focused on cortisol as a possible regulator of CA activity during acid-base disturbances. That is, it was hypothesized that acid-base disturbances would result in an elevation of circulating cortisol levels that, in turn, would regulate branchial and / or renal CA activity through transcription or post-translational mechanisms. Following from this hypothesis, circulating cortisol levels were predicted to be elevated in fish subjected to metabolic acid-base disturbances, and mRNA expression, protein levels and / or the catalytic activity of both tCAc and tCAIV isoforms were predicted to be increased in trout treated with exogenous cortisol.

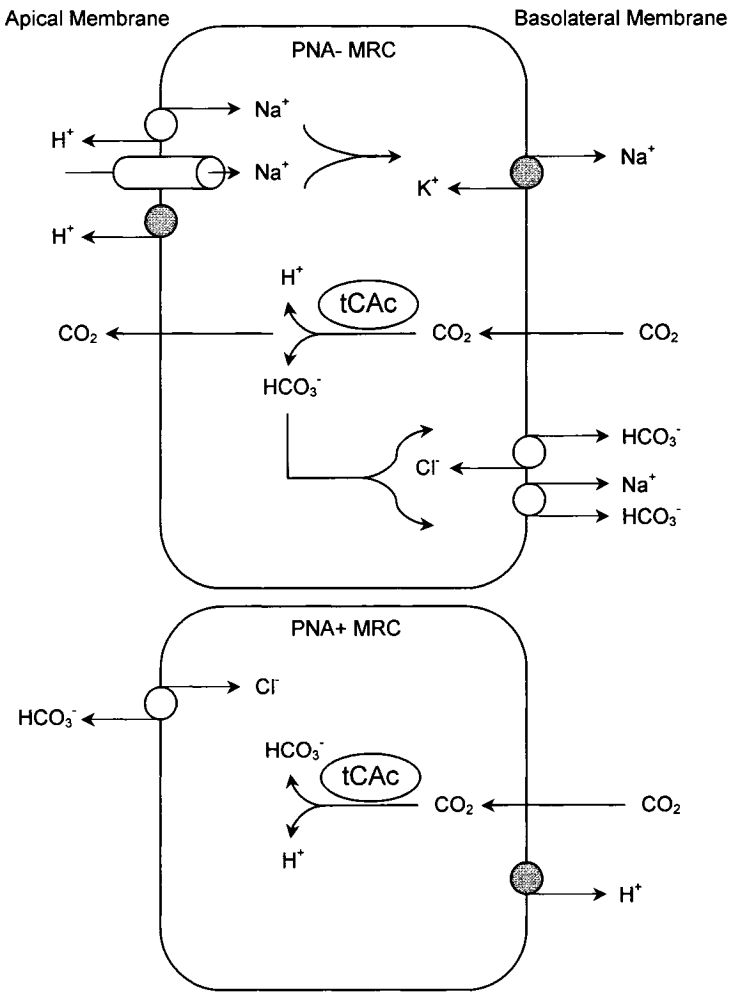


Figure 1: A model for acid-base regulation by the gill epithelium of the rainbow trout (adapted from Perry and Gilmour, 2006). The PNA^- mitochondrial rich cells (MRCs) excrete acid through the use of the apical Na^+ / H^+ exchangers, Na^+ channels, linked to V-type H^+ ATPase, coupled to the basolateral Cl^- / HCO_3^- exchangers or Na^+ / HCO_3^- co-transporters. The PNA^+ MRCs function to excrete base using the Cl^-/ HCO_3^- exchangers located at the apical membrane and the V-type H^+ ATPase located on the basolateral membrane. Research has revealed that acidosis can significantly increase the amount of exposed surface area of the PNA^- MRC cell type. Note that the filled circles indicate energy-consuming transporters. The enzyme tCAc (trout cytosolic carbonic anhydrase) is responsible for generating the counter-ions (shown in the figure) responsible for ionic and acid-base regulation.

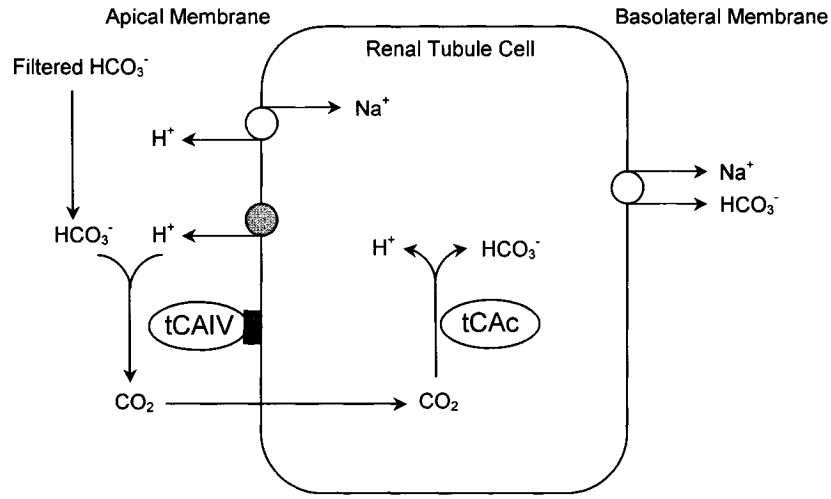


Figure 2: A model for HCO_3^- reabsorption by the rainbow trout nephron (adapted from Perry and Gilmour, 2006). Protons that are added to the filtrate by the V-type H^+-ATPases and Na^+ / H^+ exchanger isoform 3 (NHE3) titrate filtered HCO_3^- ions in the lumen of the kidney tubule. This reaction is catalyzed by the membrane-bound tCAIV. The CO_2 that is produced from this reaction diffuses into the epithelial cell and undergoes hydration by tCAc to reform H^+ and HCO_3^- ions. The HCO_3^- ions are then transported across the basolateral membrane by Na^+ / HCO_3^- cotransporter (NBC1) and the protons get recycled into the tubule lumen. Note that the filled circles indicate energy-consuming transporters.

MATERIALS AND METHODS

Experimental animals

Rainbow trout (*Oncorhynchus mykiss*, Walbaum) of approximately 75 g ($N = 38$) or 250 g ($N = 50$) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). The fish were maintained on a 12 h : 12 h light : dark photoperiod regime in large fibreglass aquaria that were supplied with City of Ottawa tapwater (13°C) that had been aerated and dechloraminated. The rainbow trout were fed to satiation daily with a commercial trout diet (Purina Trout Chow, St. Louis, MO, USA). Fish were acclimated to these holding conditions for at least 2 weeks prior to any experimentation.

Cortisol treatment

Fish (102 ± 8 g, $N = 38$) were lightly anaesthetized (i.e. to the point of losing equilibrium) in a benzocaine solution (ethyl-*p*-aminobenzoate; 0.05 g L^{-1}) and their weights were recorded. Fish were then "randomly" assigned to either control or cortisol-treated experimental groups. Cortisol-treated fish received an injection into the intraperitoneal cavity of cocoa butter (0.005 mL cocoa butter g^{-1} fish) containing dissolved cortisol (110 mg hydrocortisone 21-hemisuccinate kg^{-1} fish). The cocoa butter was warmed slightly so that at the time of injection it was liquid, but at the 13°C body temperature of the fish it quickly solidified and remained a solid implant throughout the remainder of the experiment. The cortisol dose used in the present study was chosen on the basis of previous research that showed this dose to increase cortisol concentrations by a physiologically-relevant amount over the desired experimental period (DiBattista et al., 2005).

Control fish were left untreated and both control and cortisol-treated fish were sampled 48 h post-injection. Because the objective of the present experiment was to explore possible interactions between circulating cortisol concentrations and the expression and activity of carbonic anhydrase (CA), sham treatment groups (fish injected with cocoa butter alone) were not included in the experimental design. Previous studies indicated that sham-treated fish respond to treatment in an unpredictable manner, in that cortisol levels in sham-treated fish are variable, an effect that can diminish comparisons between cortisol-treated and control fish (DiBattista et al. 2005).

Acid-base infusions

Rainbow trout (308 ± 9 g, $N = 50$) were anaesthetized by immersion in an oxygenated solution of benzocaine (ethyl-p-aminobenzoate; 0.1 g L^{-1}) that was maintained at a temperature of 13°C. Anaesthetized fish were weighed and placed on a surgical table equipped to allow the continuous irrigation of the gills with the oxygenated anaesthetic solution. Following the basic protocol of Soivio et al. (1975), each fish received an in-dwelling dorsal aortic cannula (Clay-Adams PE50 polyethylene tubing; VWR) which was used for both blood sampling and infusion of an acid, base, or saline. After revival in fresh water, the fish were placed in individual opaque, experimental chambers supplied with fresh, aerated, 13°C water and left to recover for 24 h. Cannulae were flushed with heparinized (100 IU mL^{-1} ammonium heparin; Sigma) modified (4.5 mmol L^{-1} $NaHCO_3$) Cortland saline (Wolf, 1963).

Cannulated fish were assigned to one of four experimental groups. Acid-infused fish ($N = 21$) were infused (780220 syringe pump; KD Scientific) with 70 mmol L^{-1} HCl (made up in 70 mmol L^{-1} NaCl solution to yield a solution that was approximately iso-osmotic) at a rate of 2 mL kg^{-1} h^{-1} for 24 h so as to achieve a total acid load of 3.4 mmol H^+ kg^{-1}. Similarly, base-infused fish ($N = 14$) were infused with 140 mmol L^{-1} $NaHCO_3$ at a rate of 2 mL kg^{-1} h^{-1} for 24 h to achieve a total base load of 6.7 mmol HCO_3^- kg^{-1}. These treatments, which were based upon the work of Cameron and Kormanik (1982) and Goss and Wood (1991), were selected to achieve changes in blood pH that were of similar magnitude but opposite direction. A third group of fish ($N = 14$) was infused with 140 mmol L^{-1} NaCl at a rate of 2 mL kg^{-1} h^{-1} for 24 h (saline-infused fish), while the sham group ($N = 12$) was not infused. In addition to these four experimental groups, a final control group ($N = 12$) of non-cannulated fish was included in the experimental design.

Blood samples (500 μL) were withdrawn before infusion commenced (time = 0 h) and at 12 h and 22 h of infusion. The dorsal aortic cannula was thoroughly flushed with heparinized saline prior to the collection of blood samples to prevent contamination of the blood sample with the infusion solution. Net acid-base fluxes were estimated for 2 h flux periods (-2 to 0 h, 12 to 14 h, and 22 to 24 h) in which water flow to the experimental chambers was halted (boxes were aerated). Water volume in the boxes was adjusted to a known value and water samples (10 mL) were collected at the beginning and end of the flux period. Between flux periods, the experimental chambers were maintained on flowing water. Net acid flux ($J_{net}H^+$) was determined from measurements of net titratable acid flux ($J_{net}TA$) and the change in water ammonia concentration over the flux period (McDonald and Wood, 1981).

Following the infusion protocol (for acid-, base- or saline-infused experimental groups), or 24 h in the experimental chamber without infusion (control group), or removal from the holding tank (non-cannulated group), fish were euthanized and gill and kidney tissue samples were collected.

Haematocrit was measured in duplicate using microcapillary tubes centrifuged at ~6,000 *g* for 5 min. Arterial blood pH (pHa) was determined on whole blood samples using a pH electrode and calomel reference (E301 glass pH electrode; Analytical Sensors) that were contained within a low-volume, temperature-controlled (13°C) pH chamber (Cameron Instruments) and connected to a PHM 72 acid-base analyzer (Radiometer). Blood was centrifuged (~10,000 *g* for 30 s) to obtain plasma, and red blood cells were re-suspended in heparinized Cortland saline and re-injected into the fish to reduce the effects of sampling on haematocrit. Plasma total CO_2 concentration was determined on 50 μL samples using a Capnicon total CO_2 analyzer (CC501; Cameron Instruments). The partial pressure of CO_2 (PCO_2) and the HCO_3^- concentration of the arterial blood were calculated using the Henderson-Hasselbalch equation, using appropriate values for $\forall CO_2$ and pK for rainbow trout plasma (Boutilier et al., 1984). $J_{net}TA$ was calculated by titrating 10 mL water samples from the beginning and end of each 2 h flux period to pH 4.00 with 0.02 mol L^{-1} HCl and considering the difference in titrant added. HCl titrations were carried out using a precision microburet (Gilmont), and water samples were continuously bubbled with air during the titration to ensure complete mixing of the titrant and removal of CO_2. Total ammonia concentration in the water was determined using a micro-modification of the salicylate-hypochlorite colorimetric assay, following the procedure 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).

Tissue collection

Posterior kidney tissue and gill tissue (of the first gill arch) were sampled from rainbow trout from each experimental group for 1) analysis of tCAc and tCAIV mRNA expression through real-time PCR, 2) determination of the abundance of tCAc protein through western blotting, and 3) measurement of the total catalytic activity of CA enzymes using the electrometric delta-pH method (Henry, 1981). Fish were euthanized by immersion in a solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g L^{-1}). A blood sample (~1 mL) was withdrawn by caudal puncture and the blood was centrifuged (~10,000 *g* for 1 min) to obtain plasma which was immediately frozen in liquid nitrogen (N_2) and stored at !80°C for later analysis of cortisol concentrations (by commercial RIA; ICN).

Prior to collection, tissues used for western blot analysis and CA assays were perfused with saline to remove blood. The bulbus arteriosus was exposed and cannulated with PE160 polyethylene tubing (VWR). Up to 50 mL of ice-cold, heparinized (100 IU mL^{-1} heparin), modified (4.5 mmol L^{-1} HCO_3^-) Cortland's saline was infused via the canula to clear the blood from the tissues. The ventricle was severed to allow drainage of the perfusate.

After collection, all tissue samples were immediately frozen in liquid N_2 and stored at !80°C until analysis. Immediately prior to use, tissue samples were ground to a fine powder using a mortar and pestle chilled with liquid N_2 and dry ice.

Cortisol Analysis

The concentration of cortisol in the plasma was determined using a commercially available radioimmunoassay cortisol assay kit (MP Biomedicals, LLC, Diagnostic Division, Solon, OH, USA), as per the manufacturer's instructions.

Analysis of tCAc and tCAIV mRNA expression

To quantify mRNA expression of tCAc and tCAIV in gill and kidney tissue, real-time PCR was performed. Total RNA was extracted from 50 mg aliquots of frozen ground tissue using Trizol (Invitrogen) and re-suspended in 40 μL of nuclease-free water. To reduce genomic DNA contamination, the RNA was treated with DNase (Sigma) as per the manufacturer's instructions. To increase the success of cDNA production, the RNA was treated 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 (NanoDrop, ND-1000, Wilmington). To obtain cDNA, reverse transcription was performed on ~100 mg RNA using RevertAid™ Minus M-MuLV Reverse Transcriptase (Fermentas) with 0.2 μg of random hexamer primers, as per the manufacturer's instructions.

The mRNA levels of tCAc and tCAIV were assessed by real-time PCR on samples of cDNA (1.0 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 the reference dye. The manufacturer's instructions were followed, with the modification that the total reaction volume was reduced to 12.25 μL. The cDNA used for quantification of 18S (control gene) mRNA levels was diluted 1:1000.

The annealing and extension temperatures over 45 cycles were 57°C (30 s) and 72°C (25 s), respectively. The following primer pairs (Georgalis et al., 2006a) were used:

18S	FWD	5' – GGCGGCGTTATTCCCATGACC – 3'
18S	REV	5' – GGTGGTGCCCTTCCGTCAATT – 3'
tCAc	FWD	5' – CAGTCTCCCATTGACATCGTA – 3'
tCAc	REV	5' – CGTTGTCGTCGGTGTAGGT – 3'
tCAIV	FWD	5' – ATACGAATCCATCATAAATGCT – 3'
tCAIV	REV	5' – CGGAAGTAGCTGGTCATATTACTCTG – 3'

Primer specificity was verified through cloning techniques (TOPO TA cloning kit; Invitrogen) and gene sequencing of the amplified PCR products (refer to Georgalis et al., 2006a). To assess the amplification efficiency of the primers, standard curves were produced, using kidney cDNA from control fish as a template. Serial dilutions spanning 1 to 10^{-4} µl were used to construct the standard curves. After completion of 45 PCR cycles, SYBR Green dissociation curves were constructed to ensure that SYBR Green was not being incorporated into any non-specific amplicons or primer-dimers. For each primer pair, the dissociation curves revealed the presence of single amplicons. To guard against amplification of any residual genomic DNA, negative control experiments were performed in which cDNA synthesis was performed without reverse transcriptase.

The relative mRNA expression of the two genes of interest (i.e. tCAc and tCAIV) was determined using 18S as an endogenous standard by a modification of the delta-delta Ct (threshold cycle) method (Pfaffl, 2001) in which expression in an experimental treatment group was compared to that in a designated control group.

Quantification of tCAc protein

Western blot analysis was conducted to quantify tCAc protein levels in renal and branchial tissue. To extract protein from tissue samples, approximately 100 mg of frozen, pre-ground tissue was homogenized with a battery-operated hand-held homogenizer, on ice in 1 mL of 1X extraction buffer for 10 min. The extraction buffer contained 2 mL 5X RIPA buffer, 8 mL double-distilled water, and protease inhibitors (Complete^{TM} Mini protease inhibitor cocktail tablets; Roche Molecular Biochemicals). The 5X RIPA buffer contained 50 mmol L^{-1} Tris-HCl, pH = 8.0, 150 mmol L^{-1} NaCl, 1% NP-40, 0.1% SDS, and 0.5% sodium deoxycholate. Following homogenization, samples were centrifuged at 14,000 *g* for 10 min at 4°C. The supernatant containing soluble proteins was collected and stored at !20°C for future use. Total protein concentration was determined colorimetrically using the standard BC solution technique (1 mL bicinchoninic acid and 20 µl of copper (II) sulfate) with bovine serum albumin (BSA) as a standard.

Acrylamide gels (12% resolving and 4% stacking) were used to size fractionate samples by reducing SDS-PAGE. The 12% resolving gel contained 12% acrylamide / bis solution (Bio Basic Inc), 2.5% buffer B (1.5 mol L^{-1} Tris, pH = 8.8, and 0.4% SDS), 0.2% TEMED, and 0.005% ammonium persulphate. The 4% stacking gel contained 4% acrylamide / bis solution, 2.5% buffer C (0.5 mol L^{-1} Tris, pH = 8.8, and 0.4% SDS), 0.2% TEMED and 0.005% ammonium persulphate. Protein samples were diluted to 7 µg μL^{-1} and ~50 µg of protein were loaded onto the gel [7 µL of protein was mixed with 7 µL of 2X sample buffer (25% buffer C, 20% glycerol, 4% SDS, 0.005% bromophenol blue, and 10% beta-mercaptoethanol) and incubated at 95°C for 5 min]. A ColorBurst^{TM} Electrophoresis Marker (Sigma) was run on each gel for band size comparisons.

Gels were run at room temperature, in a BioRad system, following the manufacturer's instructions, using a Tris-glycine running buffer (0.302% Tris base, 1.88% glycine, and 0.1% SDS). Fractionated proteins were then transferred to nitrocellulose membranes (Bio-Rad) using a Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. The transfer buffer consisted of 0.58% Tris base, 0.29% glycine, 20% methanol, and 0.038% SDS.

Immediately after transfer, membranes were incubated in 5% non-fat milk blocking solution [25 g skim milk powder in 50 mL TBST buffer (485 g Tris base, pH = 7.4, 3% NaCl, and 0.002% Tween 20)] for 1 h at room temperature. Membranes were then washed twice with TBST buffer and incubated for 2 h at room temperature with primary antibody; a 1:100 dilution (in 5% non-fat milk blocking solution) of rabbit anti-human erythrocyte CAII (Rockland). The human CAII antibody has been used successfully in previous work on salmonid fish (Tohse et al. 2004). Following incubation with the primary antibody, membranes were washed 3 x 5 min with TBST buffer and then incubated for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:12,000, Amersham Life Sciences). After additional washing (3 x 5 min with TBST buffer), immunoreactive protein bands were visualized with enhanced chemiluminescence (ECL; Pierce; SuperSignal West Pico Chemiluminescent Substrate, Rockford, IL, USA), following the manufacturer's instructions. As a negative control, blots were incubated with buffer lacking primary antibodies. Kodak Scientific Imaging Film (X-Omat Blue XB-1) was used to record the Western blot; films were developed and fixed using GBX developer and fixer, respectively (Kodak). Developed films were scanned (CanoScan LiDE 500F) and the digital images were processed using commercial software (ImageJ).

To assess equality of protein loading, blots were then incubated in 1X Re-Blot Plus mild stripping solution (CHEMICON) for 20 min at room temperature, rinsed for 10 min in TBST buffer, blocked 2 x 10 min in 5% non-fat milk blocking solution, and then probed with a 1:250 dilution of anti-ß-actin antibody (Sigma) for 2 h at 37°C. The blot was then incubated with horseradish peroxidase-conjugated sheep anti-mouse IgG (1:2,000; Amersham Life Sciences) for 1 h at room temperature. Subsequent washing, protein visualization and quantification of band density were as described above.

Assessment of branchial and renal CA activity

CA activity in branchial or renal tissue was measured using the electrometric delta-pH method (Henry, 1981). Gill (0.25 – 1 g) or kidney (0.25 – 1 g) tissue was combined with two volumes of assay reaction medium (in mmol L^{-1}, 225 mannitol, 75 sucrose, and 10 Tris-base, adjusted to pH 7.4 using 10% phosphoric acid) and homogenized with a motor-driven, Teflon-glass homogenizer. The crude homogenate was centrifuged briefly and the supernatant (50 µL of an approximately 16-fold dilution) was assayed for CA activity using 6 ml of assay reaction medium held at 4°C and 125 – 250 µL of CO_2-saturated distilled water to initiate the reaction. Note that all samples of a given set were assayed using a consistent volume of CO_2-saturated water. The reaction velocity was measured over the initial 0.15 unit pH change and corrected by subtracting from it the rate of the uncatalyzed reaction. A pH electrode (GK2401C; Radiometer) and pH meter (PHM 84; Radiometer) together with a data acquisition system (Biopac with AcqKnowledge v.3.7.3 software; Harvard Apparatus Canada) were used to monitor the pH of the reaction medium.

Statistical analyses

Data are reported as mean values ± 1 standard error of the mean (SEM). Data from the cortisol experiment were analyzed by (unpaired) Student's *t*-tests. The blood and acid-flux data from the infusion experiment were analyzed using a two-way repeated-measures analysis of variance (RM ANOVA) with the treatment group (acid-, base- or saline-infused) and the sample time (0, 12 or 22 h) as factors. If a statistical difference was identified, then a post-hoc multiple comparisons test was performed (the Holm-Sidak's test). Plasma cortisol concentrations and measures of CA (relative mRNA expression, protein levels or activity) from the infusion experiments were assessed by one-way ANOVA followed by post-hoc multiple comparisons, as appropriate. All statistical tests were carried out using commercial software (SigmaStat, v.3.0; Systat Software Inc.).

RESULTS

Acid-base infusion

Effects on blood acid-base status and excretion

Acid-, base-, or saline-infusion produced the expected changes in arterial blood acid-base status (Fig. 3.1). Base-infusion elicited a metabolic alkalosis in which arterial pH and $[HCO_3^-]$ increased significantly in the absence of a change in PCO_2. The fall in pH in acid-infused fish was accompanied by a significant increase in arterial PCO_2, indicative of an acidosis of mixed (metabolic and respiratory) origin. Acid-base status was unaffected by saline-infusion. All groups exhibited significant reductions in haematocrit as a result of repeated blood sampling (Fig. 3.2). Despite the clear effects of acid- or base-infusion on blood acid-base status, the impact of infusion on whole body net acid-base excretion was not obvious. No obvious trend was identified in any of the data relating to $J_{net}TA$ (Fig. 3.3A), $J_{net}[NH_3]$ (Fig. 3.3B), or $J_{net}H^+$ (Fig. 3.3C), and there were no significant differences between samples or time points.

Finally, plasma cortisol concentrations ([cortisol]) after 24 h of infusion were compared with values for fish that underwent the surgical implantation of a cannula but did not receive an infusion treatment (to control for surgery effects, sham group) and with values for fish that were sampled directly from the large holding tanks (control group). The plasma cortisol levels for the acid- and base-infused fish were significantly higher than those of the control, sham and saline-infused fish (Fig. 3.4).

Figure 3.1: Mean arterial plasma pH (A), plasma [HCO_3^-] (B), and plasma PCO_2 (C) in acid-infused ($N = 16$), base-infused ($N = 10$), and saline-infused ($N = 9$) rainbow trout, *Oncorhynchus mykiss*. Values represent means ±SEM. For pH, there is a difference in the mean values among the different time periods of treatment (two-way RM ANOVA followed by a Holm-Sidak post hoc multiple comparisons test, $P \leq 0.001$) but there is no significance due to time ($P = 0.127$). Additionally, there is a significant interaction between treatment and time ($P \leq 0.001$). For PCO_2, there is a significant difference in the mean values among the different time periods of treatment ($P \leq 0.001$) but there is no significance due to time ($P = 0.210$) and there is no significant interaction between treatment and time ($P = 0.203$). For HCO_3^-, there is a statistically significant difference with respect to treatment ($P \leq 0.001$) and time ($P = 0.004$). There is also a significant interaction between treatment and time. Asterisks indicate a significant difference within a treatment from the 0 h value. Crosses indicate a significant difference within a sampling time from the saline-infused group.

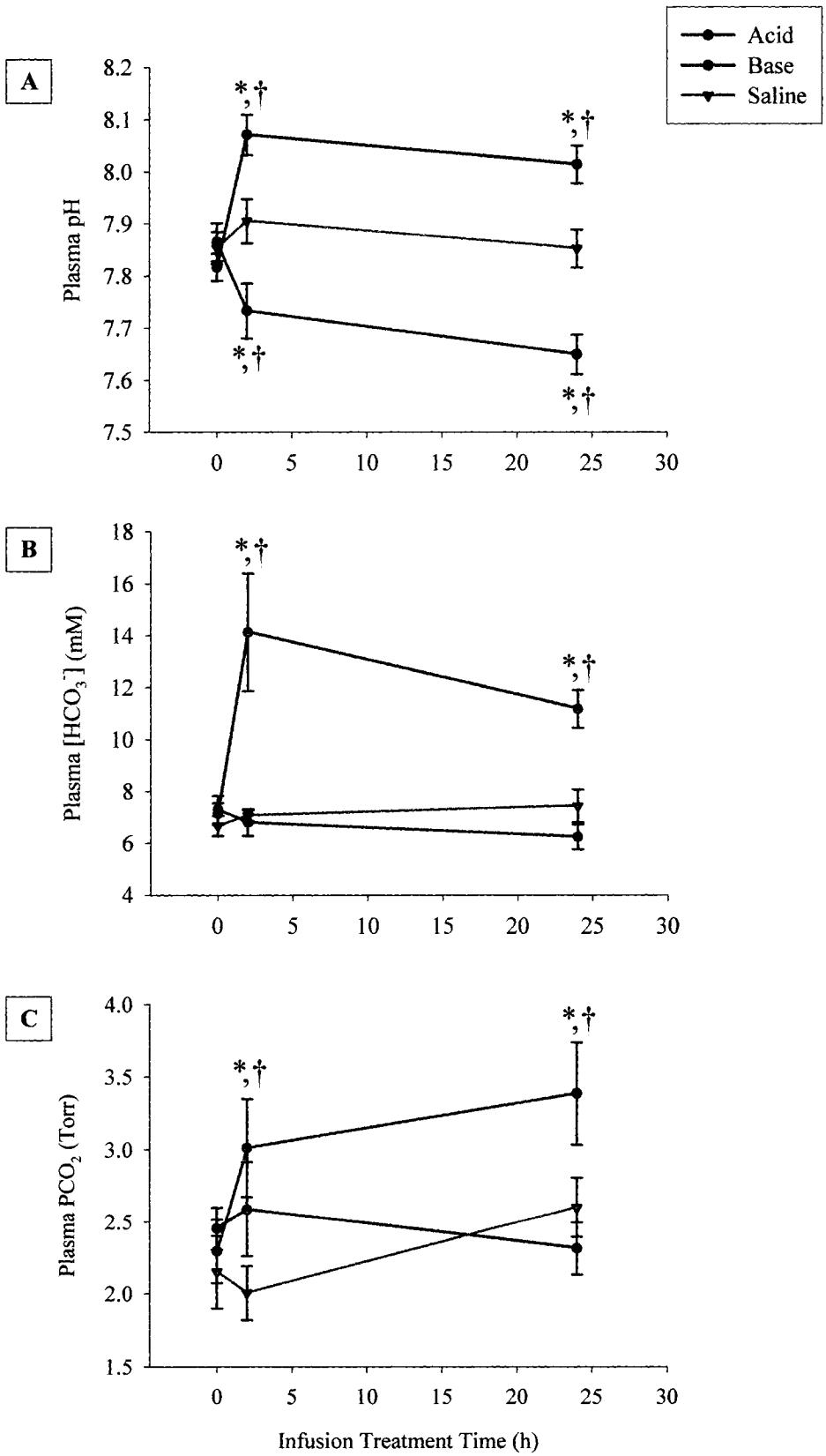

A
B
C
Acid
Base
Saline
Plasma pH
Plasma [HCO3-] (mM)
Plasma PCO2 (Torr)
Infusion Treatment Time (h)
*,†

Figure 3.2: Mean haematocrit (%) as a function of time in acid-infused (N = 16), base-infused (N = 10), and saline-infused (N = 9) rainbow trout, *Oncorhynchus mykiss*. Values represent means ± SEM. The difference in the mean values among the different types of treatment is not significantly different (two-way RM ANOVA, P = 0.206). The difference in the mean values among the different time points is statistically significant (two-way RM ANOVA followed by a Holm-Sidak *post hoc* multiple comparisons test, P < 0.001). Please note that the effect of different levels of treatment is not dependent upon the period of time (two-way RM ANOVA, P = 0.584). Asterisks indicate a significant difference within a treatment from the 0 h value.

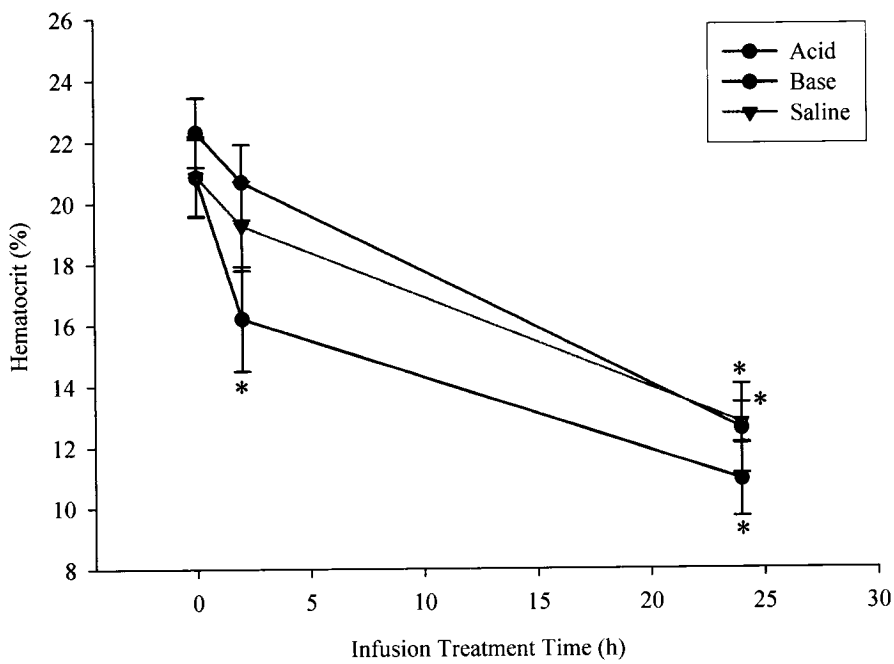

Acid
Base
Saline
Hematocrit (%)
26
24
22
20
18
16
14
12
10
8
0
5
10
15
20
25
30
Infusion Treatment Time (h)
*
*
*
*

Figure 3.3: Mean $J_{net}H^+$ (A), $J_{net}TA$ (B), and $J_{net}[NH_3]$ (C) in acid-infused ($N = 5$), base-infused ($N = 4$), and saline-infused ($N = 5$) rainbow trout, *Oncorhynchus mykiss*. Values represent means ±SEM. For A, B, and C, there was no significant difference between treatments (two-way RM ANOVA, P = 0.615, 0.164, and 0.330, respectively) or time (P = 0.306, 0.105, and 0.171, respectively). Additionally, there was no significant interactions between treatments and time (P = 0.056, 0.105, and 0.299, respectively).

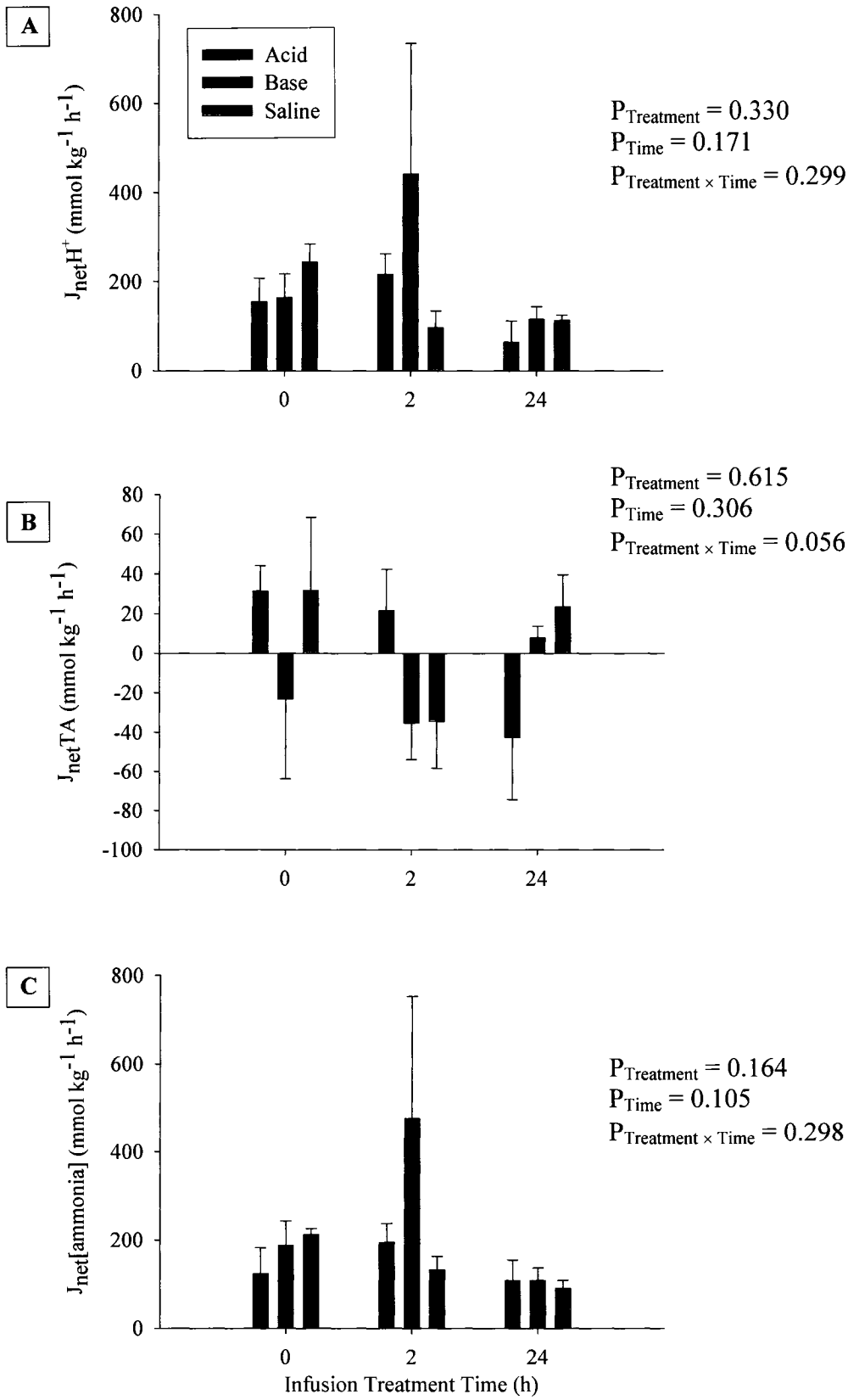
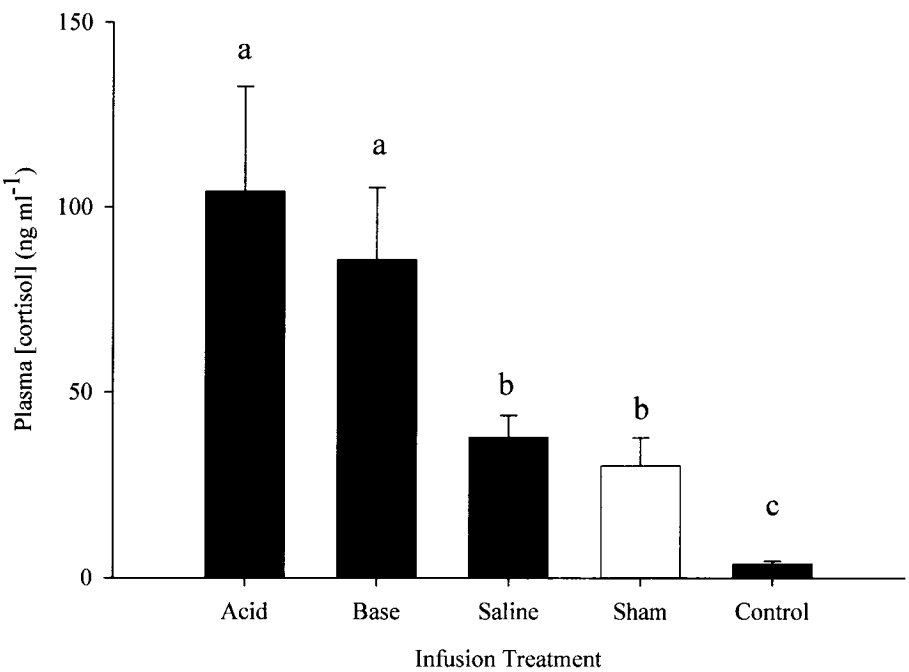

A
800
600
400
200
0
Acid
Base
Saline
$J_{net}H^+$ (mmol kg^{-1} h^{-1})
$P_{Treatment}$ = 0.330
P_{Time} = 0.171
$P_{Treatment \times Time}$ = 0.299
0
2
24
B
80
60
40
20
0
-20
-40
-60
-80
-100
$J_{net}TA$ (mmol kg^{-1} h^{-1})
$P_{Treatment}$ = 0.615
P_{Time} = 0.306
$P_{Treatment \times Time}$ = 0.056
0
2
24
C
800
600
400
200
0
J_{net}[ammonia] (mmol kg^{-1} h^{-1})
$P_{Treatment}$ = 0.164
P_{Time} = 0.105
$P_{Treatment \times Time}$ = 0.298
0
2
24
Infusion Treatment Time (h)

Figure 3.4: Mean plasma cortisol concentrations in acid-infused ($N = 14$), base-infused ($N = 14$), and saline-infused fish ($N = 14$), as well as sham ($N = 12$) and control ($N = 12$) rainbow trout, *Oncorhynchus mykiss*. Blood was sampled 24 h after the start of infusion. Values represent means +SEM. Groups that do not share a letter are significantly different from one another (one-way ANOVA followed by a Holm-Sidak *post hoc* multiple comparisons test, $P < 0.001$).


150
100
50
0
Plasma [cortisol] (ng ml-1)
a
a
b
b
c
Acid
Base
Saline
Sham
Control
Infusion Treatment

Effects on branchial and renal carbonic anhydrase

Real-time PCR was performed to assess the mRNA expression of the tCAc gene in branchial and renal tissue, and the tCAIV gene in renal tissue. In both cases the mRNA expression data are relative to the corresponding gene expression in the control group, which was set to a value of 1.

Acid- or base-infusion resulted in, respectively, significantly lower and higher relative mRNA expression of tCAc in gill tissue (Fig. 3.5A), whereas in kidney tissue, the mRNA expression of tCAc was elevated in all treatment groups relative to the expression in the sham group (Fig. 3.5B). The membrane-bound CA isoform tCAIV is not present in trout gills. In the kidney, mRNA expression of tCAIV was significantly higher and lower, respectively, in acid-infused and base-infused fish in comparison to the corresponding expression of the sham group (Fig. 3.6).

Western blot analysis was used to quantify the expression of tCAc protein in gill and kidney tissue. Unfortunately, difficulties with the trout-specific tCAIV antibody (see Georgalis et al., 2006a) precluded a corresponding quantification of renal tCAIV protein expression. The expression of the tCAc protein was calculated relative to the corresponding expression of the control gene, β-actin (Fig 3.7).

For branchial tCAc expression, acid- or base-infusion did not result in statistically different protein expression than that of the saline-infused, sham, or control treatment groups (Fig 3.7A). Additionally, for renal tCAc expression, acid- or base-infusion did not significantly affect the protein expression of tCAc, relative to the protein expression of the saline-infused, sham, or control treatment groups (Fig 3.7B).

Finally, total CA activity in crude homogenates of gill and kidney tissue was assessed using the electrometric delta-pH CA assay. Acid- or base-infused fish exhibited significantly higher branchial CA activities than the control group, sham or saline-infused groups (Fig. 3.8A). Saline infusion itself also impacted on branchial CA activity, with saline-infused fish exhibiting significantly higher branchial total CA activity than the sham or control groups (Fig. 3.8A). No differences in renal total CA activity levels were detected among any of the treatment groups (Fig. 3.8B).

Figure 3.5: The mRNA expression of the tCAc gene in the branchial (A) and renal (B) tissues of acid-, base-, saline-infused and sham-treated rainbow trout (*Oncorhynchus mykiss*) relative to that in the untreated control fish as assessed by real-time PCR. Tissue was sampled 24 h after the start of infusion. Values represent means +SEM ($N = 8$ and $N = 6$ for A and B, respectively). The control gene 18S was used as an endogenous standard. For statistical analysis, the mRNA expression for the control fish was set to 1 and is indicated on the graph by the dashed line. An asterisk indicates a value that is significantly different from the sham value (one-sample Student's *t*-test; $P < 0.05$).

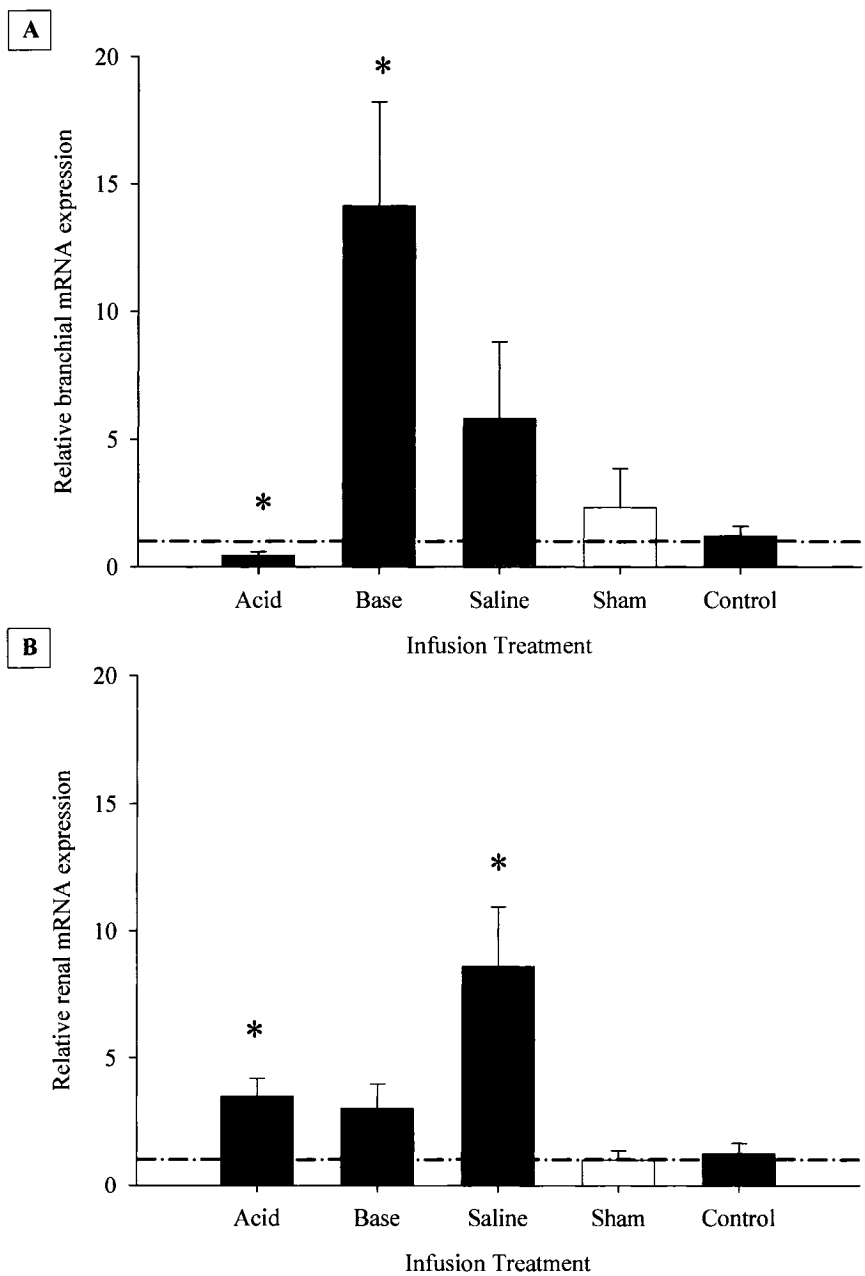

A
20
15
10
5
0
Relative branchial mRNA expression
*
*
Acid
Base
Saline
Sham
Control
Infusion Treatment
B
20
15
10
5
0
Relative renal mRNA expression
*
*
Acid
Base
Saline
Sham
Control
Infusion Treatment

Figure 3.6: The mRNA expression of the tCAIV gene in the renal tissue of acid-, base-, saline-infused and sham-treated rainbow trout (*Oncorhynchus mykiss*) relative to that in the untreated control fish as assessed by real-time PCR. Tissue was sampled 24 h after the start of infusion. Values represent means +SEM ($N = 6$). The control gene 18S was used as an endogenous standard. For statistical analysis, the mRNA expression for the control fish was set to 1 and is indicated on the graph by the dashed line. An asterisk indicates a value that is significantly different from the control value of 1 (one-sample Student's *t*-test; $P < 0.05$).

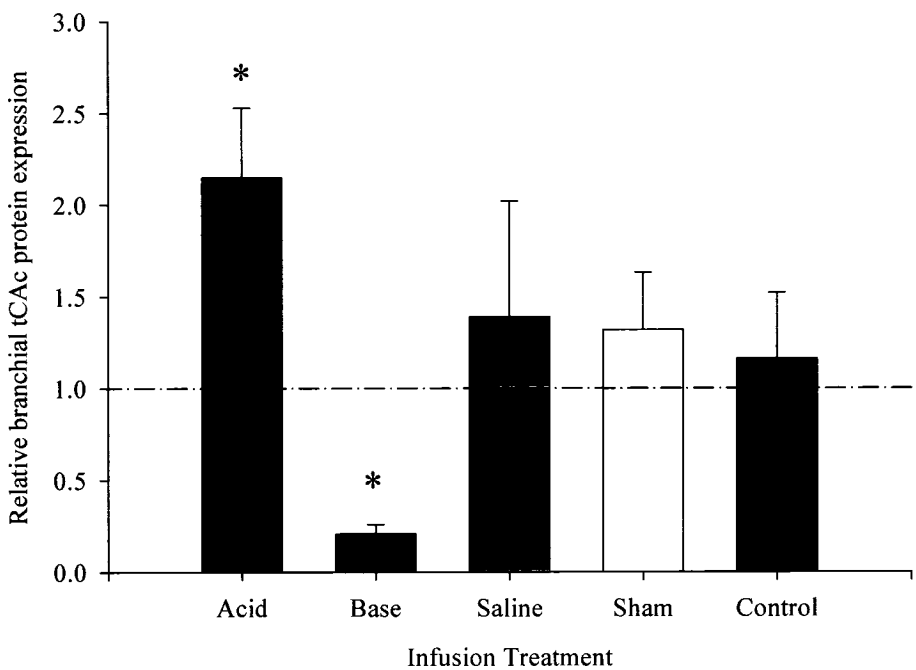
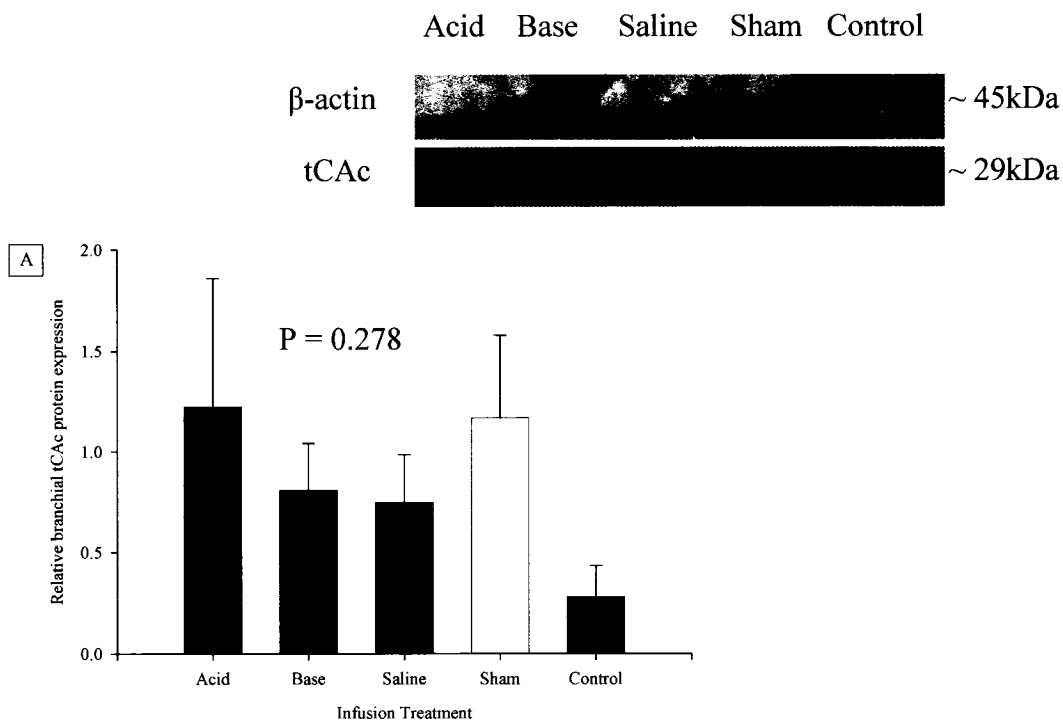

3.0
2.5
2.0
1.5
1.0
0.5
0.0
Relative branchial tCAc protein expression
*
*
Acid
Base
Saline
Sham
Control
Infusion Treatment

Figure 3.7: The effects of acid-, base- or saline-infusion on the expression of trout cytoplasmic carbonic anhydrase (tCAc) protein in the gills (A) and kidney (B) of rainbow trout (*Oncorhynchus mykiss*). Tissue was sampled 24 h after the start of infusion. Values represent means +SEM ($N = 6$). Protein expression from the representative immunoblots presented on the right was quantified by digital image processing software and depicted in the figure on the left. For A and B, there is no significant difference between treatments (one-way ANOVA, on Ranks, P = 0.278 and P = 0.734, for A and B, respectively).


Acid Base Saline Sham Control
β-actin
~ 45kDa
tCAc
~ 29kDa
A
Relative branchial tCAc protein expression
2.0
1.5
1.0
0.5
0.0
P = 0.278
Acid
Base
Saline
Sham
Control
Infusion Treatment

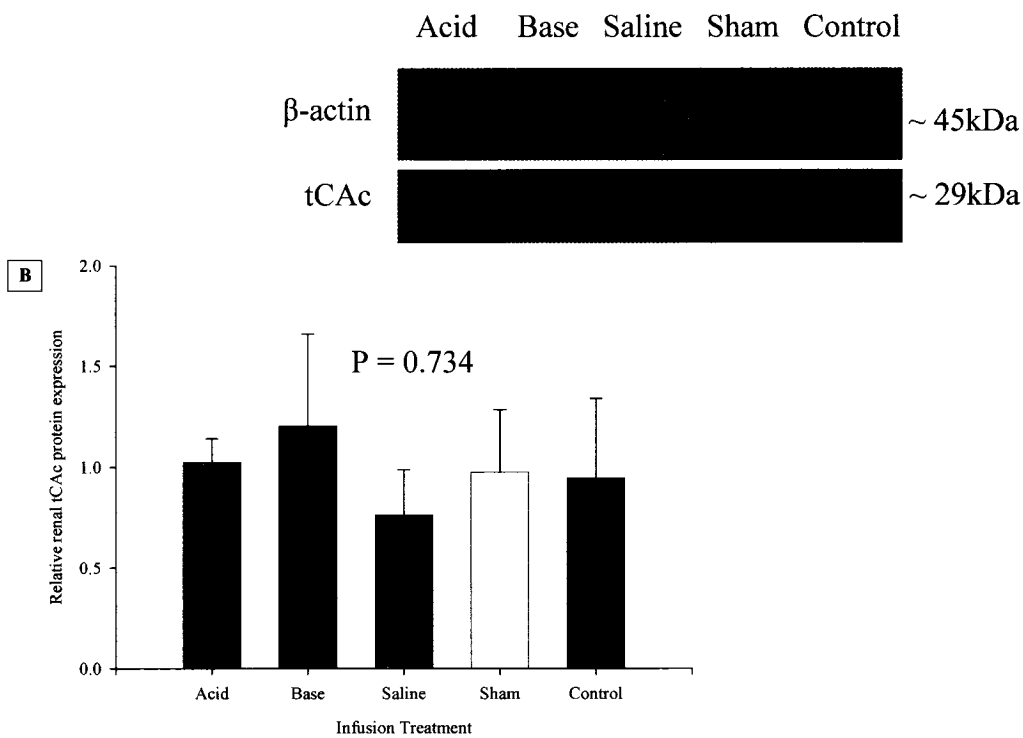
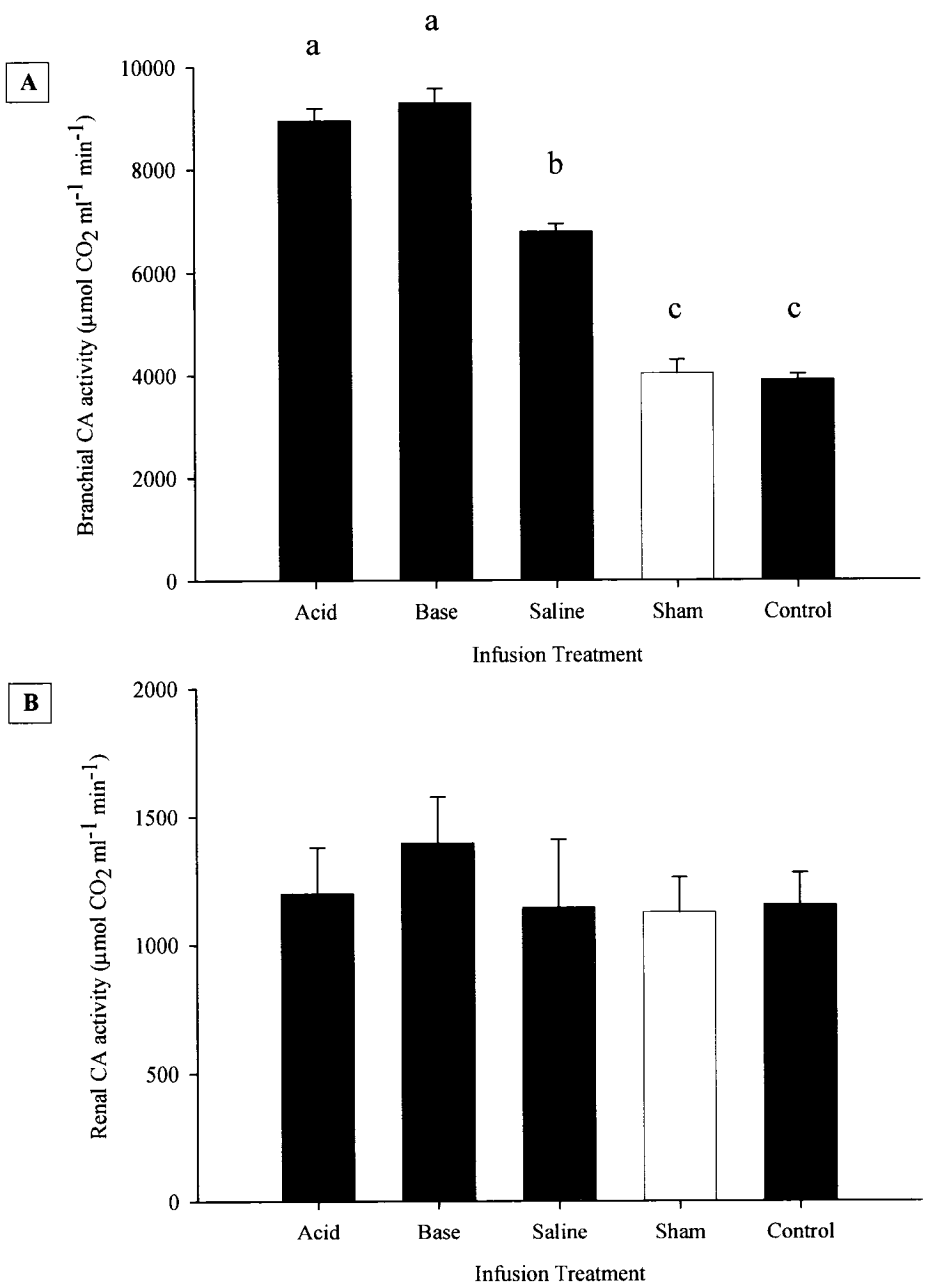

Acid Base Saline Sham Control
β-actin
~ 45kDa
tCAc
~ 29kDa
B
Relative renal tCAc protein expression
2.0
1.5
1.0
0.5
0.0
P = 0.734
Acid
Base
Saline
Sham
Control
Infusion Treatment

Figure 3.8: Effects of acid-, base-, or saline-infusion on the total CA activity of branchial tissue (A) and renal tissue (B) crude homogenates of rainbow trout (*Oncorhynchus mykiss*). Tissue was sampled 24 h after the start of infusion. Values represent means +SEM ($N = 6$). Groups that do not share a letter are significantly different from one another (one-way ANOVA, $P < 0.05$).


A
a
a
b
c
c
10000
8000
6000
4000
2000
0
Branchial CA activity (µmol CO_2 ml^{-1} min^{-1})
Acid
Base
Saline
Sham
Control
Infusion Treatment
B
2000
1500
1000
500
0
Renal CA activity (µmol CO_2 ml^{-1} min^{-1})
Acid
Base
Saline
Sham
Control
Infusion Treatment

Cortisol treatment

Effects on branchial and renal carbonic anhydrase

Because the acid- and base-infused fish exhibited significantly elevated circulating cortisol concentrations (Fig. 3.4), a second experiment was carried out to distinguish between the effects of elevated cortisol levels and infusion-specific effects. The rainbow trout were sampled after 48 h of exposure to elevated circulating cortisol concentrations achieved by administration of a cortisol-containing intraperitoneal implant, and the effects of this exogenous cortisol treatment on CA mRNA expression, protein abundance and activity were assessed.

Administration of exogenous cortisol was successful in significantly elevating circulating cortisol concentrations at 48 h (Fig. 3.9). In the cortisol-treated fish, the mRNA expression of tCAc in both the gill and kidney tissue was significantly higher than the control value of 1, with the extent of the elevated relative mRNA expression being greater in kidney than in gill tissue (Fig. 3.10A). Correspondingly, renal tCAc protein abundance in the cortisol-treated fish was significantly higher than that in the control group (Fig. 3.11B), but branchial tCAc protein levels did not differ significantly between control and cortisol-treated fish (Fig. 3.11A). In kidney tissue, tCAIV relative mRNA expression was also significantly elevated by the cortisol treatment (Fig. 3.10B); again, difficulties with the tCAIV antibody prevented the quantification of renal tCAIV protein levels. The assessment of the CA activity of crude homogenates of gill and kidney tissue revealed elevated branchial CA activity in cortisol-treated fish but no significant difference in renal CA activity (Fig. 3.12).

Figure 3.9: Mean plasma cortisol concentrations in control (untreated) rainbow trout, *Oncorhynchus mykiss* ($N = 18$) compared to those fish that received an intraperitoneal cortisol-containing implant ($N = 18$). Blood was sampled 48 h post-injection. Values are means +SEM. The asterisk indicates a statistically significant difference from the control value (unpaired Student's *t*-test, $P < 0.001$).

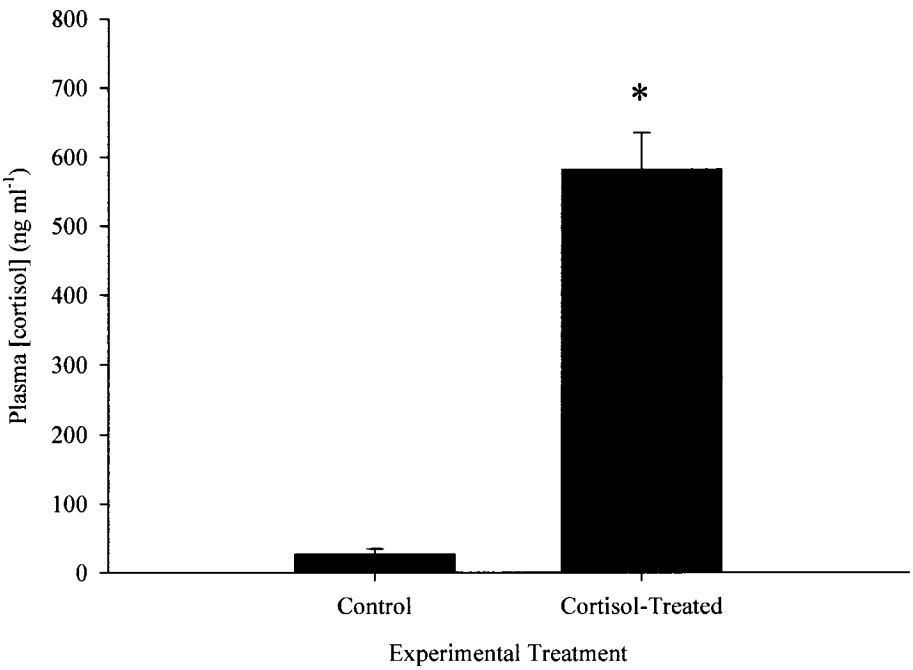

800
700
600
500
400
300
200
100
0
*
Plasma [cortisol] (ng ml-1)
Control
Cortisol-Treated
Experimental Treatment

Figure 3.10: The mRNA expression of tCAc and tCAIV in branchial and/or renal tissues of cortisol-treated rainbow trout (*Oncorhynchus mykiss*) relative to those of control fish. Tissue was sampled 48 h post-injection. The control gene 18S was used as an endogenous standard. Values represent means +SEM ($N = 6$). The mRNA expression for control fish was set to 1 and is indicated on the graph by the dashed line. An asterisk indicates a value that is significantly different from the control value of 1 (one-sample Student's *t*-test; $P < 0.05$).

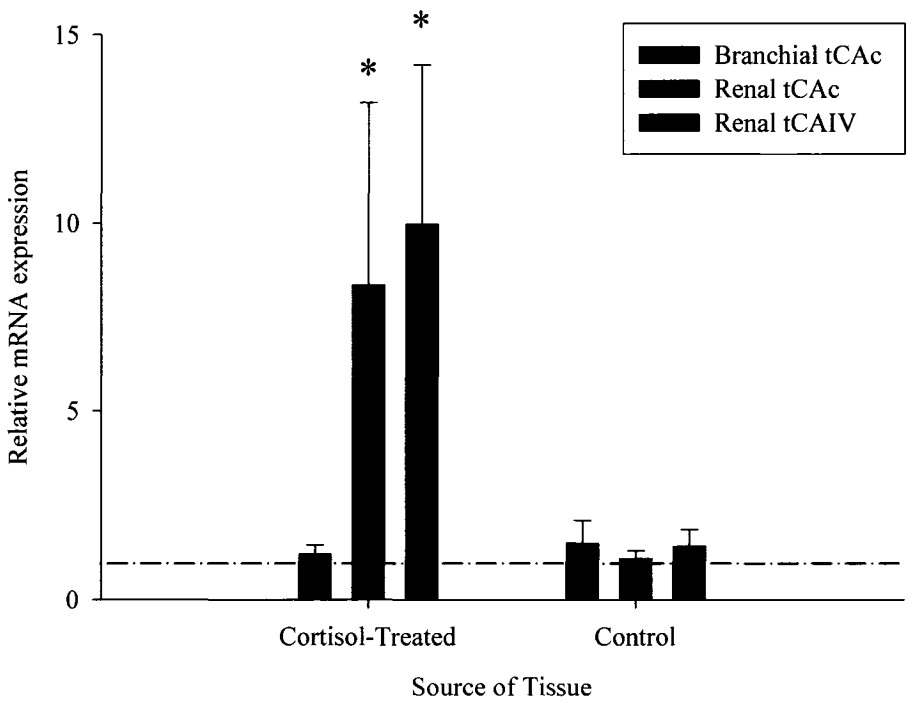
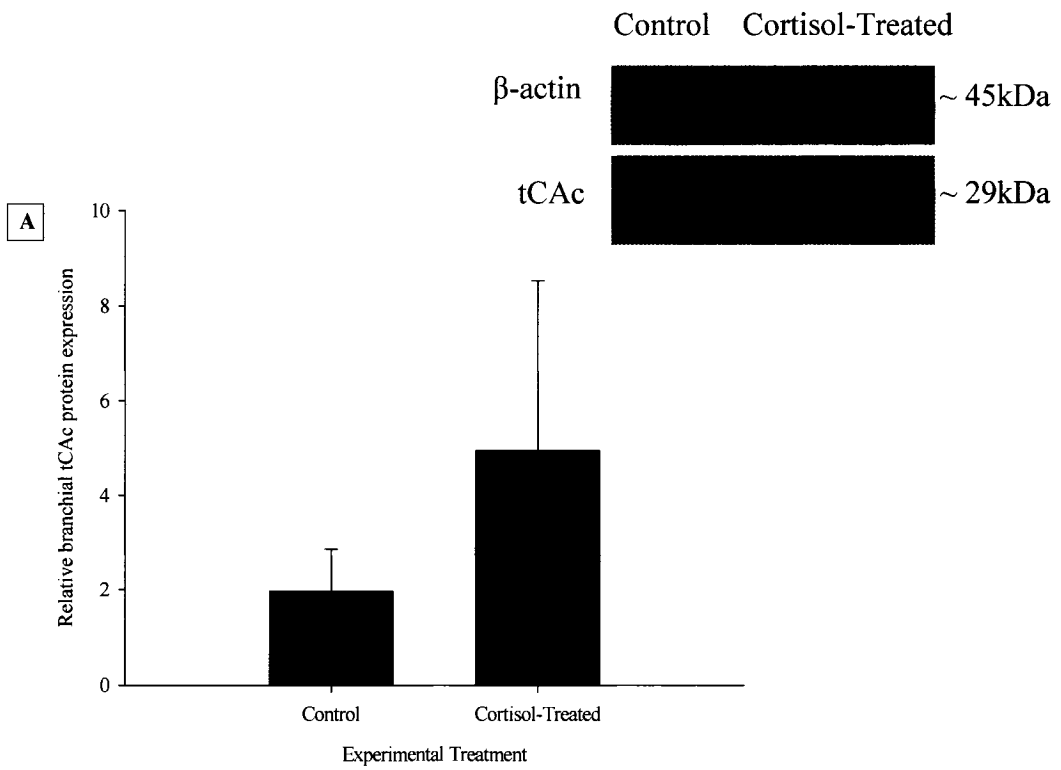

15
10
5
0
Relative mRNA expression
*
*
Branchial tCAc
Renal tCAc
Renal tCAIV
Cortisol-Treated
Control
Source of Tissue

Figure 3.11: The effects of cortisol-treatment on the expression of trout cytoplasmic carbonic anhydrase (tCAc) protein in the gills (A) and kidney (B) of rainbow trout (*Oncorhynchus mykiss*). Tissue was sampled 48 h post-injection. Protein expression from the representative immunoblots presented on the right was quantified by digital image processing software and depicted in the figure on the left. For A, there was no significant difference between treatments (Student's *t*-test, $P > 0.001$). For B, an asterisk indicates a value that is significantly different from the control value (Student's *t*-test, $P < 0.001$).


Control
Cortisol-Treated
β-actin
~ 45kDa
tCAc
~ 29kDa
A
Relative branchial tCAc protein expression
10
8
6
4
2
0
Control
Cortisol-Treated
Experimental Treatment

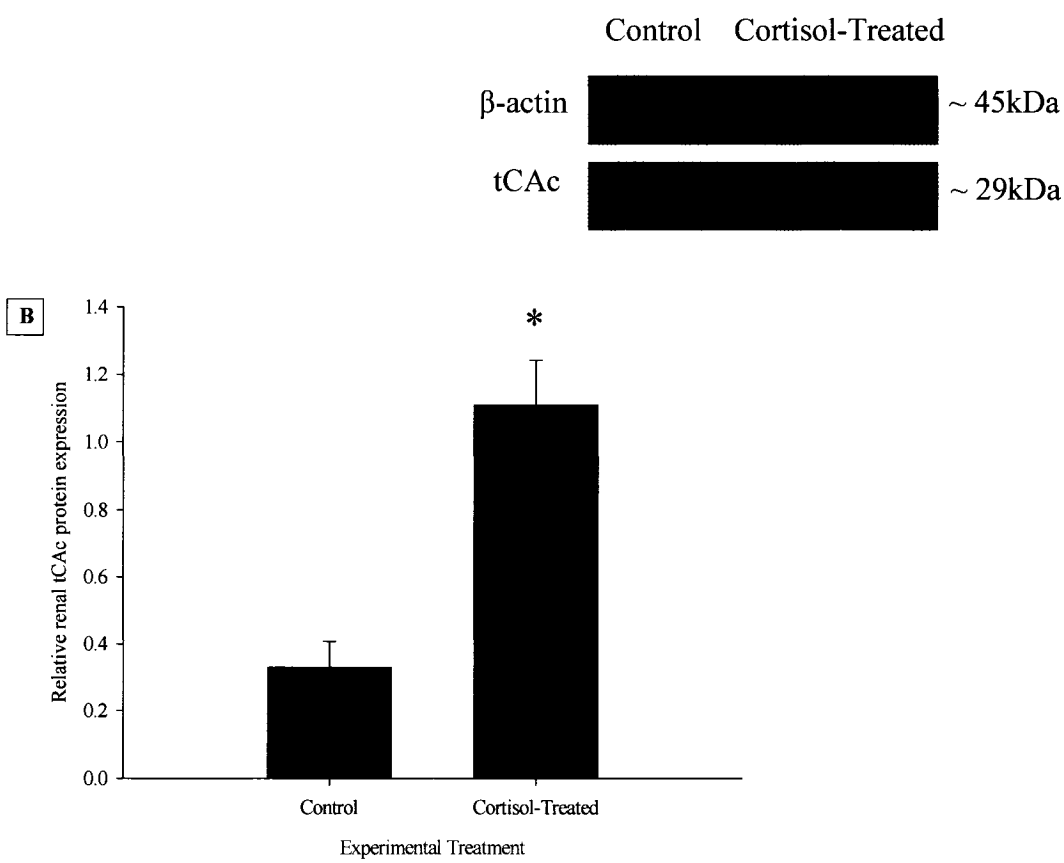
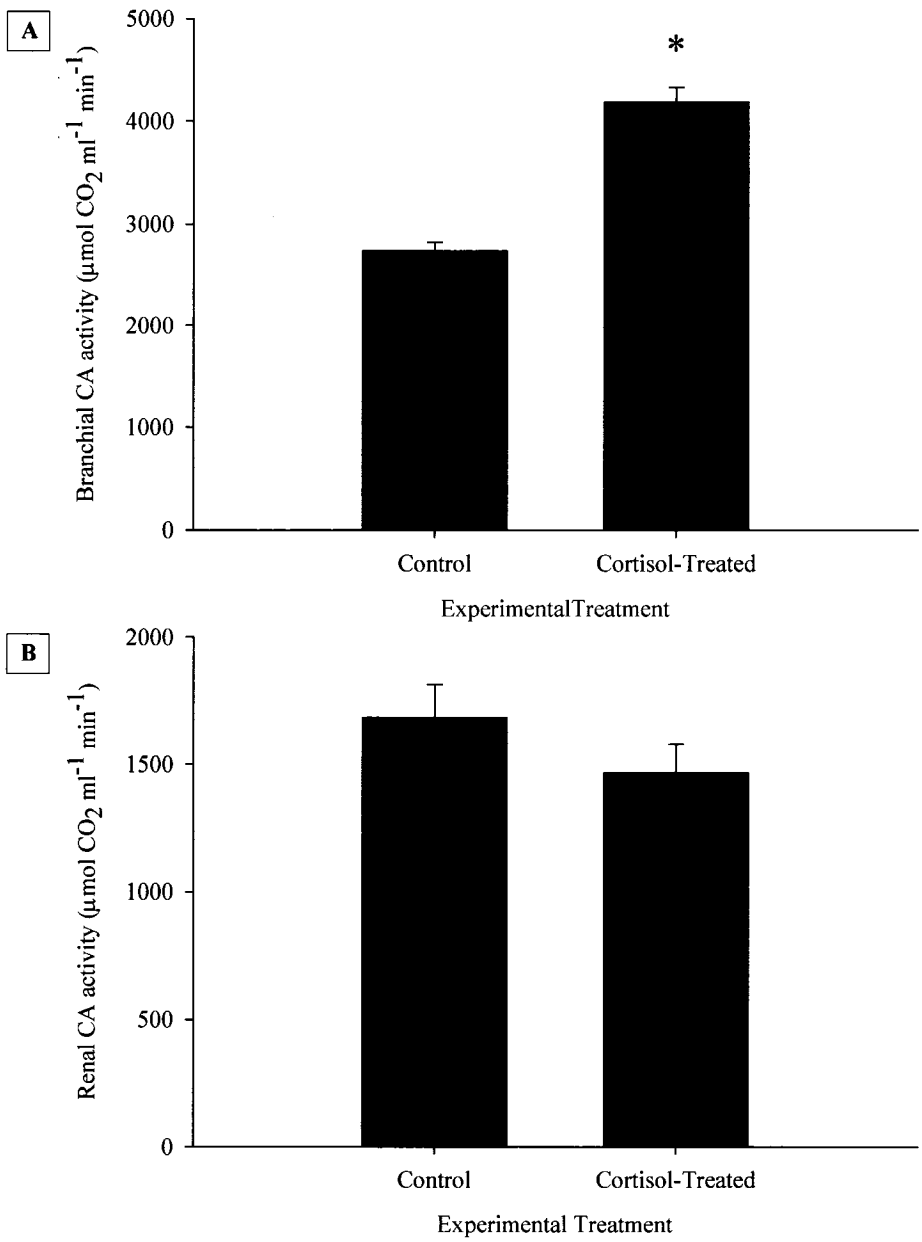

Control
Cortisol-Treated
β-actin
~ 45kDa
tCAc
~ 29kDa
B
*
Relative renal tCAc protein expression
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Control
Cortisol-Treated
Experimental Treatment

Figure 3.12: The effect of cortisol treatment on total CA activity in crude homogenates of branchial (A) and renal (B) tissue of rainbow trout (*Oncorhynchus mykiss*). Tissue was sampled 48 h post-injection. Values represent means + SEM (N = 10). The asterisk indicates a significant difference (unpaired Student's t-test, $P < 0.001$) in CA activity between control and cortisol-treated groups.


A
5000
4000
3000
2000
1000
0
*
Branchial CA activity (µmol CO_2 ml^{-1} min^{-1})
Control
Cortisol-Treated
ExperimentalTreatment
B
2000
1500
1000
500
0
Renal CA activity (µmol CO_2 ml^{-1} min^{-1})
Control
Cortisol-Treated
Experimental Treatment

DISCUSSION

The present study demonstrated that cytosolic carbonic anhydrase (tCAc) and membrane bound carbonic anhydrase (tCAIV) are dynamically regulated in gill (tCAc only) and kidney in response to metabolic acid-base disturbances in the rainbow trout. In addition, the stress hormone cortisol appears to be involved in this process, as cortisol levels are elevated during metabolic acid-base challenges and CA expression and activity are altered by high circulating cortisol levels. The results of this study confirm and extend our understanding of the role played by CA in acid-base regulation in the rainbow trout, specifically with respect to the roles of the gill and kidney in regulating acid-base status.

A number of studies have focused on the potential roles played by CA in the regulation of acid base disturbances (Dimberg and Höglund, 1987; Lin and Randall 1991; Hirata et al., 2003; Tresguerres et al., 2005, 2006, 2007; Georgalis et al., 2006a, 2006b). For example, Georgalis et al. (2006b) demonstrated that branchial tCAc functions to supply the necessary acid-base equivalents, derived from the hydration of CO_2, for excretion into the environment. Additionally, Georgalis et al. (2006a) showed that the inhibition of one or both of the sources of renal CA (tCAc and / or tCAIV) affects the excretion of HCO_3^- via the urine, thereby impairing the ability of fish to compensate respiratory acidosis. This supports the idea that both renal tCAc and tCAIV contribute to the reabsorption of filtered HCO_3^- ions. While previous studies on rainbow trout have focused on the role of CA in mediating responses to **respiratory** acid-base disturbances, the present study is the first to explore the roles of branchial and renal CA in rainbow trout experiencing **metabolic** acid-base disturbances.

Methods – rationale and critique

The infusion of an acid or base directly into the arterial blood of the fish elicits a metabolic acidosis or alkalosis, respectively (Cameron and Kormanik, 1982). A metabolic acid-base disturbance is characterized by changes in pH at a constant Pa_{CO2}. In the present study, the infusion of base produced a pure metabolic alkalosis but the acid infusion was accompanied by an elevation of Pa_{CO2}, suggesting that the acidosis was actually of a mixed respiratory / metabolic origin. It is likely that the elevation of Pa_{CO2} in the acid-infused fish reflected the titration of plasma HCO_3^- by the infused H^+ so as to produce CO_2, a phenomenon that has been observed in other studies using similar techniques (Thomas and Perry, 1992; Perry and McKendry, 2001; Gilmour et al., 2007).

Despite the evidence of acid-base disturbances provided by the assessment of blood acid-base status, no significant changes in net acid flux ($J_{net}H^+$) were observed in either the acid- or base-infused fish. This result was unexpected, as previous studies have documented increased net acid excretion in response to acidosis and correspondingly, negative net acid excretion (i.e. base excretion) in response to alkalosis (Perry et al., 1987a; Goss and Wood, 1991; Goss and Perry 1993; Wood et al., 1999; Georgalis et al., 2006b). However, measurements of net acid excretion are often variable (McDonald and Wood, 1981), and the present study was no exception. Despite the inconsistencies in net acid excretion, the use of a well-established protocol (Cameron and Kormanik, 1982) coupled with the detection of the expected changes in the blood acid-base status, suggests that the infused fish were subjected to the desired acid-base challenges. Additionally, the fish experienced a steady state with respect to their pH between 2 and 24h of infusion, strongly suggesting that the infused acid or base was being excreted.

In all treatment groups, the initial haematocrit was ~22% and fell gradually to about 12% after 24 h, presumably largely because of repeated blood sampling. It is important to note that haematocrits greater than 12% are above the threshold of 5 – 10% (Wood et al., 1982) reported to cause adverse health effects (Wood et al., 1982, Iwama et al., 1987) in rainbow trout. Therefore, the reduction in haematocrit probably had little effect on the results of this study.

The goal of the present study was to investigate the impact of metabolic acid-base disturbances on CA gene expression, protein abundance, and catalytic activity in the gills and kidneys of rainbow trout. To assess the underlying causes of changes in CA expression or activity, a variety of controls were incorporated into the experimental design. A saline-infused group controlled for the effect of infusion alone, while a "sham" group in which fish were cannulated but not infused controlled for any effects of surgery and confinement. Plasma cortisol concentrations in these groups were similar and somewhat elevated over the accepted "unstressed" value of ~10 ng mL^{-1} (Gamperl et al., 1994), which was the level detected in the "control" group in which fish were sampled directly from the holding tank.

The experimental acid and base infusions were accompanied by significantly greater elevations of plasma cortisol concentrations. To distinguish between the effects of acid- or base-infusion per se, and the effect of accompanying elevation of plasma cortisol levels, a second experiment was carried out in which plasma cortisol levels were raised by exogenous cortisol administration. Although the desired cortisol concentration of ~100 ng mL^{-1} was exceeded, a wide range of circulating cortisol concentrations was achieved, allowing for the investigation of possible relationships between plasma cortisol concentration and CA expression or activity. However, attempts to establish such relationships were unsuccessful.

Significant relationships were detected when variables such as CA expression were correlated with plasma cortisol concentrations, but these relationships reflected the presence of two groups, one with low and the other with high cortisol concentrations. Attempts to establish correlations within a group (either control fish or cortisol-treated fish) were unsuccessful, probably in large part owing to the low N values for CA expression or activity (typically, $N = 6$).

Quantification of CA gene expression by real-time PCR was accompanied by the measurement of tCAc protein abundance by western analysis, and the assessment of total CA activity using the electrometric delta-pH assay. The use of all three approaches provided a complete picture of CA mRNA expression, protein abundance, and catalytic activity in the gill. However, technical difficulties prevented a similarly complete assessment of CA expression and activity in kidney tissue. Difficulties were experienced in using the tCAIV antibody developed in a previous study (Georgalis et al., 2006a), preventing the quantification of renal tCAIV protein abundance. In addition, measurement of total CA activity in kidney homogenates failed to reveal any significant differences among treatments. There are several reasons why the measurements of total CA activity in kidney homogenates should be treated with caution. Generally low levels of CA activity in the kidneys (Esbaugh and Tufts, 2006) coupled with the inherent difficulty of adequately clearing the renal tissue of blood and the presence of haematopoetic tissue in the kidney (Swenson and Maren, 1986; Gehnrich et al., 1994; 1995; Swenson et al., 1996; Renfro et al., 1999) make it difficult to attribute any crude homogenate CA activity to renal tCAc and tCAIV specifically. In addition, the use of the crude homogenates did not allow relative changes in the cytoplasmic *versus* membrane-bound CA activities to be distinguished.

Further characterization of renal CA activity is required, using differential centrifugation to separate cytoplasmic and membrane-bound activities (Henry et al., 1988). Thus, discussion of renal CA is limited to changes in gene expression and tCAc protein abundance.

Branchial and renal responses to acidosis

The main responses of the rainbow trout to a systemic acidosis are to reduce the rate of HCO_3^- excretion at the gills and to increase the rate of HCO_3^- reabsorption at the kidney (Perry et al., 1987a; 1987b; Wood et al., 1999). However, the effect of a metabolic acidosis on branchial CA expression and activity is difficult to predict owing to the heterogenous cell population of the gills. The cell population of the gills involves two main types of mitochondria-rich cells (MRCs) (Fig. 1), distinguished by their binding affinities for peanut lectin agglutinin, PNA (Goss et al., 2001). It is believed that MRCs that do not bind PNA (PNA^- MRCs) function to excrete acid and that MRCs that show a binding affinity for PNA (PNA^+ MRCs) function to excrete base (Perry and Gilmour, 2006). Because of the presence of presumptive acid- and base-secreting cells in the fish, different and potentially opposing responses from the different cell types must be expected to occur.

Current models postulate that tCAc plays a pivotal role in branchial acid-base regulation by providing the H^+ and HCO_3^- ions necessary for acid and base excretion. Acidotic rainbow trout reduce the rate of branchial HCO_3^- excretion, a process aided by physical covering of the base-excreting PNA^+ MRCs by pavement cells (Goss and Perry, 1993). Thus, the expression and activity of branchial tCAc would be predicted to decrease in the face of a metabolic acidosis, in an attempt to reduce the activity of the base-secreting MRCs, so as to promote the accumulation of HCO_3^- ions (Georgalis et al., 2006b).

Additionally, renal tCAc and tCAIV expression and activity would be predicted to increase in response to a metabolic acidosis, to promote the reabsorption of HCO_3^- from the filtrate. These predictions are summarized in Tables 4.1 (gills) and 4.2 (kidney).

The recent work of Georgalis et al (2006a) provided strong evidence for the involvement of tCAc and tCAIV in the maintenance of acid-base status. The proposed model for the kidney (Georgalis et al., 2006a) shows that both tCAc and tCAIV function to reabsorb filtered HCO_3^- ions from the filtrate. As previously noted, the changes in plasma HCO_3^- levels required for pH regulation are achieved via increases in net branchial acid excretion, but necessitate parallel changes in renal acid secretion.

Following from the above predictions, the relative mRNA expression of branchial tCAc was reduced in the acid-infused fish and the relative mRNA expression of both renal tCAc and tCAIV was significantly increased. In the gill, the tCAc protein levels did not parallel the changes in mRNA level, while the branchial CA activity in the acid-infused fish was significantly increased. Correspondingly, the abundance of the renal tCAc protein was unaffected by any experimental treatment (Tables 4.1, 4.2). These finding differ markedly from the responses of rainbow trout to respiratory acidosis (Georgalis et al., 2006b), where the tCAc mRNA expression and protein levels were significantly elevated but there was no observable impact on tCAc enzyme activity. It is possible that the different cell types of the gill respond differently, depending on whether the acidosis is of a respiratory or metabolic origin. The most striking renal response to metabolic acidosis was a pronounced increase in the expression of tCAIV mRNA that was accompanied by a more modest increase in tCAc mRNA (Table 4.2); similar results were reported for rainbow trout experiencing respiratory acidosis (Georgalis et al., 2006b).

Table 4.1: A summary table of the results predicted and obtained with respect to the response of the branchial system of the rainbow trout, *Oncorhynchus mykiss*, to the imposition of an acid-base disturbance. The arrows indicate the direction of change and the dashed lines indicate the control values, to which all other values are compared. The red blocks on the table show where the observed result agrees with the prediction.

APPENDIX

Treatment	*mRNA Level*		*Protein Level*		*Activity Level*	
	Predicted	*Observed*	*Predicted*	*Observed*	*Predicted*	*Observed*
Acid			↓	×	↓	↑
Base			↑	×		
Saline					×	↑
Sham						
Control	-	-	-	-	-	-

Table 4.2: A summary table of the results predicted and obtained with respect to the response of the renal system of the rainbow trout, *Oncorhynchus mykiss*, to the imposition of an acid-base disturbance. The arrows indicate the direction of change and the dashed lines indicate the control values, to which all other values are compared. The red blocks on the table show where the observed result agrees with the prediction. As CA activity in the renal tissue could not be accurately measured, the observed results for these experiments are not included.

Treatment	*mRNA Level*		*Protein Level*		*Activity Level*	
	Predicted	*Observed*	*Predicted*	*Observed*	*Predicted*	*Observed*
Acid			↑	×	↓	
Base	↓	×	↓	×	↓	
Saline	×	↑			×	
Sham					×	
Control	-	-	-	-	-	

The discrepancies among gene expression, protein abundance, and CA activity observed in the present study (Tables 4.1, 4.2) are difficult to reconcile, and may reflect a diversity of factors. In particular, tissue samples for all analyses were collected at 24 h of infusion, yet the impact of the acid-infusion on gene expression *versus* protein abundance *versus* enzymatic activity might occur with different time courses. For example, 24 h of acid infusion may be a sufficient stimulus to trigger appropriate changes in gene transcription, as detected, but may not be sufficient to allow an impact on protein abundance. Results of other studies support this possibility (Henry et al., 2002; 2003; 2006).

Henry et al. (2006) found that CA mRNA expression in the euryhaline green crab, *Carcinus maenas*, increased in the posterior gills 24 h after transfer of animals to lowered salinity, but that CA activity did not increase until 48 h post-transfer, and the greatest increase in CA protein concentration did not occur until 10 days post-transfer. These discrepancies suggest that a time-course for mRNA expression, protein abundance, and catalytic activity for CA should be performed in future studies. Additionally, potential post-translational effects that enhance CA activity in the short term, resulting in elevated branchial CA activity at 24 h of infusion, could over a longer time course be superseded by transcriptional regulation of tCAc gene expression (Henry et al., 2003; 2006). For example, the results of Henry et al. (2006) demonstrate that the elevation in CA activity levels is caused by an increase in the CA mRNA abundance, as exposure to an environment of low salinity caused a rapid 8-fold increase in CA mRNA that preceded the increase in CA activity by 24 h. Additionally, this increase in activity persisted through 7 days, as the newly produced protein persisted for an extended period of time after the decline in CA mRNA levels.

In addition to possible temporal differences in the responses to acidosis, there may also be a confounding influence of the infusion, itself, on CA expression and / or activity. For example, the saline infusion caused a significant increase in renal tCAc relative mRNA expression, suggesting that the similar increase in acid-infused fish may have reflected (at least in part) the infusion procedure rather than the actual acidosis. Enhanced HCO_3^- reabsorption from the filtrate may be favoured during the infusion if the volume load is cleared via the kidneys (Curtis and Wood, 1992). This response may be mediated, at least partially, by the elevation of circulating plasma cortisol concentrations, as the saline-infusion was accompanied by a moderate increase in plasma cortisol levels, and cortisol injection was accompanied by a significant elevation of renal tCAc relative mRNA expression (Table 4.3). However, whereas cortisol administration also resulted in a corresponding increase in renal tCAc protein abundance, neither acid nor saline infusion affected renal tCAc protein levels (Tables 4.2, 4.3).

Previous research has revealed possible links between cortisol and CA activity and expression in rabbit (Russell et al., 1976) and winter flounder (Pelis et al., 2003; Pelis and Renfro, 2004). Pelis et al. (2003) demonstrated that the protein level of CAII (their terminology – see Georgalis et al., 2006a, for a discussion of CA nomenclature in fish *versus* mammals) in cultured renal proximal tubule cells of winter flounder was reduced by ~65% and the catalytic activity of CA was reduced by ~30% in cells that were maintained in cultures with reduced cortisol levels for 5 days. The level of cortisol that was normally used in the cultures was 7.3 $H10^{-6}$ M, and the cortisol was reduced to the lower end of the physiological range (6.3 H 10^{-9} M) 5 days before the experimental analysis was performed (Pelis et al., 2003).

Table 4.3: A summary table of the results predicted and obtained with respect to the response of the gills and the kidney of the rainbow trout, Oncorhynchus mykiss, to an increase in circulating cortisol level. The arrows indicate the direction of change and the dashed lines indicate the control values, to which all other values are compared. The red blocks on the table show where the observed result agrees with the prediction. As CA activity in the renal tissue could not be accurately measured, the observed results for these experiments are not included.

Treatment	*mRNA Level*		*Protein Level*		*Activity Level*	
	Predicted	*Observed*	*Predicted*	*Observed*	*Predicted*	*Observed*
Gills						
Cortisol	↑	×	↑	×		
Control	-	-	-	-	-	-
Kidneys						
Cortisol					↑	
Control	-	-	-	-	-	

In agreement with these findings, renal tCAc and tCAIV gene expression and renal tCAc protein abundance were significantly increased in the present study following administration of exogenous cortisol *in vivo* (Table 4.3), providing further evidence of an involvement of cortisol in renal CA regulation.

In the present study, branchial tCAc gene expression was unaffected while catalytic activity increased with cortisol treatment (Table 4.3). While the protein abundance of branchial tCAc was not significantly affected by injection of cortisol, a trend toward an increase in protein expression with elevated plasma cortisol was apparent (Fig. 3.11A). However, a linear regression analysis revealed no significant relationship or correlation between cortisol level and branchial tCAc protein abundance. The high variability in the protein levels of cortisol-treated fish likely masked any significant difference between control and cortisol-treated animals. Further experimentation is required to reduce this variability and hence to determine whether branchial tCAc protein abundance is affected by increased plasma cortisol concentrations.

Goss et al. (2001), reported that PNA binding to the epithelium of a gill filament was increased by pretreatment with cortisol, suggesting that cortisol stimulates the proliferation of the PNA^+, presumptive base-excreting MRCs. Furthermore, Laurent et al. (1994) demonstrated that cortisol promoted the differentiation within 12 h, but not division, of branchial epithelial cells. Thus, the increase in CA activity caused by cortisol treatment that was observed in the current study could be caused by an increase in MRC abundance (Laurent and Perry, 1990), rather than a specific up-regulation of CA, which could account for the lack of significant effect on either tCAc gene expression or protein abundance.

Branchial and renal responses to alkalosis

The main response of rainbow trout to a systemic alkalosis is an increase in the rate of branchial HCO_3^- excretion and a decrease in the rate of renal HCO_3^- reabsorption (Goss and Wood, 1991; Wood and LeMoigne, 1991; Curtis and Wood, 1992; Wood et al., 1999). Thus, it was predicted that branchial CA expression and activity would be increased, to facilitate the excretion of HCO_3^-, and renal CA expression and activity would be decreased, to promote the loss of HCO_3^- via the urine (see Tables 4.1, 4.2). Again, however, the presence of various cell types in the gills makes it difficult to accurately predict how the CA expression and activity might respond to a metabolic alkalosis.

As predicted, the branchial tCAc relative mRNA expression was significantly higher in the rainbow trout subjected to a metabolic alkalosis, as was the branchial CA activity. However, the branchial tCAc protein abundance failed to increase in accordance with the increases in gene expression and activity (Table 4.1). Also in keeping with the predictions outlined above, a significant decrease in renal tCAIV relative mRNA expression was observed. Renal tCAc relative mRNA expression in the base-infused fish did not differ from the control value of one, but this finding could be considered a relative decrease in gene expression given that saline-infusion alone resulted in significantly elevated renal tCAc relative mRNA expression. Renal tCAc protein abundance was not altered by base infusion (Table 4.2).

Interestingly, base infusion was accompanied by significant elevation of plasma cortisol concentration. The effects of cortisol, namely the observed increase in renal CA expression and tCAc protein abundance, are opposite to those expected to benefit acid-base regulation following a base load (compare predicted responses in Tables 4.1, 4.2 and 4.3)

and indeed, such responses were not observed during base-infusion. It would appear that cortisol is but one of several factors regulating CA expression and activity in the kidney during metabolic alkalosis. To determine whether the elevation of circulating cortisol concentrations in either acid-infused or base-infused fish contributes to acid-base regulation, it will be necessary to block the cortisol response either by inhibiting cortisol synthesis or by blocking cortisol receptors.

Future directions

Previous studies have shown that the effects of cortisol in fish are mediated by both glucocorticoid and mineralocorticoid receptors (Bern and Madsen, 1992; Sturm et al., 2005). It may be worth investigating how cortisol impacts acid-base regulation in the rainbow trout by studying which receptor type is involved. However, the roles of the different receptors have yet to be fully elucidated in fish and, indeed, there is evidence for glucocorticoid receptors playing roles in salt and water balance – roles that do not fit with the traditional view of this receptor as being involved in stress responses and the regulation of metabolism (Yudt and Cidlowski, 2002; Gilmour, 2005).

In the absence of information about the receptor types involved in mediating the effects of cortisol on CA activity and expression, a more useful experiment to investigate the possibility of a causal relationship might be to reduce circulating cortisol levels using the inhibitor metyrapone (Milligan, 2003). Metyrapone is an inhibitor of 11ß hydroxylase, an enzyme that converts 11-deoxycortisol to cortisol in the final step of cortisol synthesis (Bennett and Rhodes, 1986).

Although there exists uncertainty about the what receptor type is involved, use of a glucocorticoid receptor inhibitor such as RU486, that has been shown to be very effective at blocking the actions of cortisol in the rainbow trout (McDonald and Wood, 2004), could clarify the role of cortisol in acid-base maintenance by determining the relative involvement of cortisol *versus* acid-base status in inducing CA changes.

More experimentation with CA inhibitors such as acetazolamide (Maren, 1967), or F3500 (Conroy et al., 1996), may further elucidate the functions of the various CA isoforms with respect to cortisol during an acid-base disturbance. Acetazolamide is a membrane-permeant CA inhibitor that acts on both the cytosolic and membrane-bound CA isoforms (Georgalis et al., 2006a). F3500 is a membrane-impermeant inhibitor and permits tCAc activity while inhibiting the activity of tCAIV (Conroy et al., 1996). This selective CA inhibition could reveal the relative importance of the different CA isoforms in regulating acid-base homeostasis in the rainbow trout. This approach was taken by Georgalis et al. (2006a) to provide the existing evidence for the involvement of the two different CA isoforms (tCAc and CAIV) in regulating acid-base homeostasis in the rainbow trout. By reducing the activity of CA through inhibitors, further studies could support the importance of CA in regulating acid-base homeostasis and possibly reveal alternative response mechanisms of the animal to a systemic acid-base challenge. Additionally, studies involving the exploration for a cortisol responsive element in the gene sequence of the CA isoforms may be worthwhile.

Conclusions

The findings of the present study support the hypothesis that the expression and action of two CA isoforms (tCAc and tCAIV), in the gill (tCAc only) and kidney, play an important role in regulating systemic metabolic acid-base disturbances. In addition, cortisol also appears to be involved in the regulation of acid-base status. The present study has documented a correlation between cortisol and effects on CA expression and activity, but more studies are required to determine the mechanisms underlying this relationship. Additionally, by documenting significant changes in the response of rainbow trout to the imposition of metabolic acid-base disturbances, as opposed to respiratory acid-base disturbances, the findings of the present study extend the understanding that different regulatory mechanisms are involved, depending on the form of acid-base challenge.

The changes in the branchial tCAc mRNA expression during both metabolic acid-base challenges followed from the predictions based on the current model of the system and the possible roles of the enzyme. The changes in the renal tCAc mRNA expression during a metabolic acidosis and the renal tCAIV mRNA expression during both the metabolic acidosis and alkalosis are exactly in line with the predicted role of these enzymes in facilitating HCO_3^- reabsorption from the filtrate. Moreover, cortisol caused a marked increase in renal tCAIV mRNA and protein levels. It is possible that elevated cortisol, when coupled with a metabolic acidosis, acts to promote the increased expression of renal tCAIV that then facilitates HCO_3^- reabsorption.

As previously stated, the observed discrepancies among changes in gene expression, protein abundance, and catalytic activity observed in the present study are problematic, and likely reflect the wide range of complex factors that act upon CA expression and activity.

While confirming the important roles of branchial and renal CA isoforms in acid-base regulation established in previous studies (Georgalis et al., 2006a, b), the results of the present study advance our knowledge and understanding in two important directions. First, by comparison with the results of Georgalis et al. (2006a, b), the present study establishes both similarities and differences in the branchial and renal CA responses to respiratory *versus* metabolic acid-base disturbances. Documenting such similarities and differences is an important step in understanding the regulation of acid-base disturbances by the gill and kidney of fish. Second, the role of cortisol as a possible mediator of changes in CA expression and activity during acid-base disturbances is examined. To date, CA research has focused largely on establishing the presence and function of different CA isoforms – the present work takes an important step towards exploring mechanisms underlying the dynamic regulation of CA expression and activity.

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