

**Transcriptional and post-translational regulation of cytosolic
carbonic anhydrase in rainbow trout (*Oncorhynchus mykiss*) and
zebrafish (*Danio rerio*)**

By

Daniel Carrie

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
In partial fulfillment of the requirements for the
M.Sc. degree in the
Ottawa-Carleton Institute of Biology
© Daniel Carrie, Ottawa, Canada, 2014

Abstract

The enzyme carbonic anhydrase (CA) contributes to multiple physiological processes by catalysing the reversible hydration of carbon dioxide. However, regulation of CA activity in response to homeostatic challenges remains poorly understood. The objectives of this thesis were to investigate whether CA is transcriptionally regulated by cortisol in fish and whether post-translational modification (PTM) of CA occurs in fish. The results of an *in vivo* reporter assay used to investigate potential transcriptional regulation of zebrafish, *Danio rerio*, cytoplasmic CA (CAc) were inconsistent, and it remains unclear whether zebrafish CAc is regulated transcriptionally by cortisol. Phosphorylation of rainbow trout, *Oncorhynchus mykiss*, CAc was predicted from *in silico* analysis of the putative amino acid sequence and confirmed by Western analysis of phosphoprotein levels following *in vitro* incubation of CA, purified from trout gill, under conditions designed to potentiate endogenous kinases. Again using *in vitro* incubations designed to potentiate endogenous kinases and phosphatases, changes to the phosphorylation state of CAc were found to modulate its enzymatic properties. These findings suggest that CA activity may be regulated by signalling pathways that activate cellular protein kinases, and future work should focus on identifying these pathways.

Resumé

L'anhydrase carbonique (AC) est une enzyme qui contribue à de multiples processus physiologiques en catalysant l'hydratation réversible du dioxyde de carbone. Cependant, la régulation de l'activité de l'AC en réponse aux défis homéostatiques demeure mal comprise. Les objectifs de cette thèse étaient d'investiger si le cortisol régule la transcription de l'AC chez les poissons. De plus, cette thèse cherchait à comprendre si la modification post-traductionnelle (MPT) de l'AC a lieu chez les poissons. Les résultats d'un essai rapporteur *in vivo* utilisé afin d'examiner la régulation de la transcription potentiel de l'AC cytoplasmique (ACc) chez les poissons zèbre *Danio rerio*, étaient variables. Ainsi il n'est encore pas clair si le cortisol régule la transcription de l'ACc des poissons zèbre. La phosphorylation de l'ACc chez la truite arc-en-ciel *Oncorhynchus mykiss* était prédite à partir d'analyse *in silico* de séquences d'acides aminés putatifs. Cette phosphorylation était confirmée par analyse Western de niveaux de phosphoprotéines à la suite d'incubations *in vitro* d'AC, purifiée à partir de branchies de truites, sous conditions conçues pour potentialiser les kinases endogènes. Suite à des incubations *in vitro* à nouveau conçues pour potentialiser les kinases et phosphatases endogènes, il a été établi que les changements d'états de phosphorylation de l'ACc pouvaient moduler les propriétés enzymatiques de l'ACc. Ces résultats suggèrent que l'activité de l'AC pourrait être régulée par des voies de signalisation activant les protéines kinases cellulaires et que les études futures devraient se concentrer sur l'identification de ces voies de signalisation.

Acknowledgments

I would first like to thank my supervisor Dr. Kathleen Gilmour, because without her this work would not have been possible. I have been fortunate enough to have spent the last four years as an undergraduate and graduate student in her lab, and the personal and professional growth I have derived from the experience is invaluable. Through all the challenges and adversity I've encountered, she has always been patient as a mentor, and provided me with the support and advice I've needed.

I would like to extend thanks to my committee members, Dr. Walsh and Dr. Storey for their helpful comments and suggestions. Thank you to Bill Fletcher and Vishal Saxena for their maintenance of the aquatic facility and experimental animals. Thank you to the Walsh lab for warmly welcoming me into their lab while I ran the thousands of gels and western blots that seemed to consume my entire graduate student experience at times! A great big thank you to all my labmates. Your advice, assistance and most importantly friendship made my time as a researcher an enjoyable experience. And of course I am extremely grateful to you for enduring the perpetual love/hate relationship I maintained with the CA assay.

Finally, thank you goes out to my friends and family. You were ever a source of encouragement and inspiration.

TABLE OF CONTENTS

Abstract	II
Resume	III
Acknowledgments	IV
TABLE OF CONTENTS	V
List of Figures	VII
List of abbreviations	X
CHAPTER 1: General Introduction	1
1.1. Introduction	2
1.2. Carbonic anhydrase	2
<i>1.2.1 Fish CAs</i>	4
1.3 Transcriptional regulation	7
<i>1.3.1. Cortisol and acid-base disturbances in fish</i>	9
1.4 Post-translational modification	11
<i>1.4.1 Protein phosphorylation</i>	12
<i>1.4.2 Glutathionylation</i>	13
<i>1.4.3 Post-translational modification of CA</i>	15
1.5 Hypotheses and predictions	17
CHAPTER 2: Post-translation modification of carbonic anhydrase in rainbow trout,	
<i>Oncorhynchus mykiss</i>	19
Abstract	20
2.1 Introduction	21
2.2 Materials and methods	25

2.3 Results	34
2.4 Discussion	56

CHAPTER 3: Transcriptional regulation of carbonic anhydrase in zebrafish, *Danio rerio*

	63
Abstract	64
3.1 Introduction	65
3.2 Materials and methods	68
3.3 Results	77
3.4 Discussion	82

CHAPTER 4: General Discussion

4.1 Introduction	89
4.2 zCAc regulation by cortisol	90
4.3 CA phosphorylation and modulation of enzymatic properties	91
4.3.1 <i>Physiological significance of CA phosphorylation</i>	95
4.4 Future Directions	98
4.5 Conclusions	99

REFERENCES

APPENDIX

124

List of Figures

CHAPTER 2

Figure 2-1.

Results of the *in silico* analysis of rainbow trout, *Oncorhynchus mykiss*, cytoplasmic CA for putative phosphorylation sites. 39

Figure 2-2.

Images of a representative (A) SDS PAGE gel and (B) western blot illustrating the effect of pAMBS purification of a crude homogenate of rainbow trout, *Oncorhynchus mykiss*, gill tissue to yield purified gill carbonic anhydrase (CA). 41

Figure 2-3.

The effect of incubation conditions that stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the (A) phosphorylation status and (B) activity of rainbow trout, *Oncorhynchus mykiss*, gill carbonic anhydrase. 43

Figure 2-4.

The effect of incubation conditions that stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the phosphorylation state of rainbow trout, *Oncorhynchus mykiss*, gill CA. 45

Figure 2-5.

The effect of incubation conditions that stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on kinetic parameters of rainbow

trout, *Oncorhynchus mykiss*, gill CA, specifically (A) turnover number (k_{cat}), (B) substrate affinity (K_m), (C) enzyme efficiency (V_{max}/K_m) and (D) the inhibition constant (K_i) for acetazolamide. 47

Figure 2-6. The effect of incubation conditions that stimulated specific protein kinases (protein kinase A, PKA; protein kinase C, PKC; protein kinase G, PKG; and AMP-activated protein kinase, AMPK) relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the (A) activity and (B) inhibition constant against acetazolamide (K_i) of rainbow trout, *Oncorhynchus mykiss*, gill CA. 49

Figure 2-7.

The effect of incubation conditions that stimulated specific protein kinases (protein kinase A, PKA; protein kinase C, PKC; protein kinase G, PKG; and AMP-activated protein kinase, AMPK) relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the (A) turnover number, k_{cat} , (B) substrate affinity constant, K_m , and (C) enzyme efficiency, V_{max}/K_m of rainbow trout, *Oncorhynchus mykiss*, gill CA. 51

Figure 2-8.

Glutathionylation of gill CA. (A) Detection of S-glutathionylation using an anti-GSH antibody before and after incubation with 100 mM DTT. (B) The effect of deglutathionylation (i.e. incubation with 100 mM DTT) on CA activity. 53

Figure 2-9.

The effect of exposure to hypercapnia (1% CO₂) on (A) relative CA phosphorylation status ($N = 6$), and (B) CA activity ($N = 4$). 55

CHAPTER 3**Figure 3-1.**

An 887 nucleotide DNA sequence of the promoter region for zebrafish (*Danio rerio*) cytosolic CA, zCAc. 75

Figure 3-2.

Features of a generic pGL4 vector. 76

Figure 3-3.

Effect of cortisol treatment (500 nmol L^{-1}) on mean relative luciferase activity (firefly/*Renilla*) for zebrafish (*Danio rerio*) larvae that were microinjected as one-cell embryos with the zebrafish cytosolic CA (zCAc) promoter construct. 79

Figure 3-4.

Effect of cortisol treatment (48 h exposure to 500 nmol L^{-1} cortisol) on (A) zebrafish cytosolic CA (zCAc) relative protein levels and (B) total CA activity in 96 hpf zebrafish (*Danio rerio*) larvae. 81

Figure 3-5.

NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>) prediction of phosphorylation sites for zCAc (top sequence) and tCAc (bottom sequence). Predicted phosphorylation sites are highlighted. 87

APPENDIX**Figure A-1.**

Representative kinetic curve for the effect of incubation conditions that modulated the phosphorylation status of rainbow trout, *Oncorhynchus mykiss*, gill CA. 126

List of Abbreviations

AMPK – AMP-activated protein kinase

ATP – Adenosine triphosphate

BCA - Bicinchoninic acid

BSA – Bovine serum albumin

CA – Carbonic anhydrase

CAc – Cytoplasmic carbonic anhydrase

cAMP – Cyclic adenosine monophosphate

cGMP – Cyclic guanosine monophosphate

CO₂ – Carbon dioxide

DMSO – Dimethyl sulphoxide

DNA – Deoxyribonucleic acid

DTT - Dithiothreitol

EDTA - Ethylenediaminetetraacetic acid

EGTA - Ethylene glycol tetraacetic acid

E_t - Total enzyme

eu – enzyme units

GPI - Glycosylphosphatidylinositol

GR – Glucocorticoid receptor

GRE – Glucocorticoid response element

GRE ½s - Glucocorticoid response element half site

GRU – Glucocorticoid response unit

GSH – Glutathione

GSSG – Glutathione disulphide

GTP – Guanosine triphosphate

H⁺ - Hydrogen ion

HCO₃⁻ – Bicarbonate ion

HEPES - 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hpf – hours post-fertilization

HR – Proton pump-rich cell (of the zebrafish gill or larval skin)

Ice-CA – Ice fish carbonic anhydrase

I_t – Total inhibitor concentration

k_{cat} – Turnover number

K_i – Inhibition constant

K_m – Substrate affinity constant

MRC – Mitochondrion-rich cells

mRNA – messenger ribonucleic acid

NADPH - Nicotinamide adenine dinucleotide phosphate

NHE – Sodium hydrogen exchanger

pAMBS – *p*-aminomethylbenzene-sulphonamide

PKA – Protein kinase A

PKB – Protein kinase B

PKC - Protein kinase C

PKG - Protein kinase G

PMA - Phorbol 12-myristate 13-acetate

PSH – Protein with free cysteine

PTM – Post-translational modification

PVC – Pavement cell

RBC - Red blood cell

RM ANOVA – Repeated measures analysis of variance

SEM – Standard error of the mean

SDS – Sodium dodecyl sulfate

TBS-T – Tris buffered Tween 20

tCAc – Trout cytoplasmic carbonic anhydrase

zCAc – Zebrafish cytoplasmic carbonic anhydrase

CHAPTER 1

General Introduction

1.1 Introduction

Carbonic anhydrase (CA) is a zinc metalloenzyme that catalyses the rapid, reversible inter-conversion of carbon dioxide (CO_2) and water to bicarbonate ions (HCO_3^-) and protons (H^+). Although CA has been implicated in numerous physiological processes (see Sobotka and Kann, 1941; Maren, 1967; Henry and Swenson, 2000), how the enzyme is regulated remains poorly understood. In fish, CA appears to be regulated transcriptionally (Georgalis et al, 2006a; Gilmour et al, 2011), but whether post-translational modifications have any regulatory role is unknown. The goals of this thesis were to expand our knowledge of the transcriptional regulation of CA in fish and to determine whether the enzyme undergoes post-translational modification; if post-translational modifications occur, an additional goal was to identify the impact of such modification(s) on enzymatic properties. The following pages encompass a concise review of CA, with a focus on gill CA in fish, as well as a discussion of transcriptional and post-translational modification and how these two forms of cellular control may participate in the regulation of CA.

1.2 Carbonic anhydrase

There are at least five different families (α , β , γ , δ and ϵ) of genetically unrelated CA isoforms (Xu et al, 2008). The vast majority of CA knowledge has been provided by the α -family. This family is the only one found in vertebrates and the most widely expressed in animals (see reviews by Chegwiddden and Carter, 2000; Hewett-Emmett, 2000; Tashian et al,

2000; Purkerson and Schwartz, 2007; Gilmour, 2010). In mammals, sixteen α -isoforms have been identified, and genomic evidence suggests that this encapsulates the total array of isoforms (Hilvo et al, 2008). Work in fish suggests that, at least in teleosts, this diversity of isoforms is matched and may even be exceeded, although this conclusion is based mainly on sequence information (Peterson et al, 1997; Lin et al, 2008; Gilmour and Perry, 2009).

The uncatalysed reaction proceeds as $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3 \rightleftharpoons \text{H}^+ + \text{HCO}_3^-$, which differs from the reaction when CA is present. In the catalysed reaction involving a zinc hydroxide nucleophile, H_2CO_3 is not generated, and H^+ and HCO_3^- are produced directly from H_2O and CO_2 (Lindskog, 1997). The reaction catalysed by CA reaches equilibrium quite slowly in the absence of the enzyme; for example, the half-time of the uncatalysed HCO_3^- dehydration reaction is 25-90 s at physiological pH and temperature (Edsall, 1969). For the uncatalysed reactions, hydration is approximately 365 times faster than dehydration (Gibbons and Edsall, 1963). However, in the presence of CA, the rates of the CO_2 hydration/dehydration reactions are increased between 13- and 25,000-fold (Forster and Itada, 1980; Forster et al, 1998). Thus, CA plays a fundamental role in many physiological processes that rely on inter-conversions between CO_2 and HCO_3^- , including metabolism, gas exchange, and acid-base balance (Maren, 1967). For example, studies in the mammalian kidney have demonstrated that CA isoforms associate with acid-base transporters to form $\text{H}^+/\text{HCO}_3^-$ transport metabolons (McMurtrie et al, 2004). This association markedly enhances $\text{H}^+/\text{HCO}_3^-$ transport, and thus CA provides a driving force for $\text{H}^+/\text{HCO}_3^-$ transport by favouring the accumulation or dissipation of substrate (Purkerson and Schwartz, 2007).

As an enzyme, the CA family broadly is characterized by an extremely high turnover number (k_{cat} : number of substrate molecules converted to product per catalytic site per second);

some isozymes exceed a turnover number of $1 \times 10^6 \text{ s}^{-1}$ (Chegwidden and Carter, 2000). As a comparison, citrate synthase has a turnover number of $2 \times 10^2 \text{ s}^{-1}$ (Moriyama and Srere, 1971). Different CA isoforms in mammals can be categorized on the basis of distinct features. For example, CA-related proteins (CAs VIII, X and XI) lack catalytic activity, as a result of substitutions in the histidine residues that coordinate the zinc ion necessary for the catalytic mechanism (Fujikawa-Adachi et al, 1999; Okamoto et al, 2001; reviewed by Tashian et al, 2000). Although the function of these proteins remains unknown, all three are expressed in the brain (and CA VIII in other tissues) (Tashian et al, 2000; Aspatwar et al, 2010). The CA isoforms possessing catalytic activity can be partitioned into intra- and extracellular isoforms. There are two mitochondrial (VA and VB) and five cytosolic isoforms (I, II, III, VII and XIII) which encapsulate the intracellular complement (Lehtonen et al, 2004; Chegwidden and Carter, 2000; Hewett-Emmett, 2000). These isoforms exhibit catalytic differences from very high activity in CAs II and VII, to low activity for CAs I and III, further subdividing the intracellular isoforms (Chegwidden and Carter, 2000; Supuran, 2004; 2008a). The remaining isoforms (IV, VI, IX, XII, XIV and XV) are extracellular (reviewed by Sly, 2000) and can be further characterized by their association with the cell membrane. A glycolipid (glycosylphosphatidylinositol or GPI) linkage anchors CAs IV and XV to the plasma membrane, whereas CAs IX, XII and XIV exist as transmembrane proteins (Opavský et al, 1996; Türeci et al, 1998; Mori et al, 1999; Fujikawa-Adachi et al, 1999; Schwartz et al, 2001). The last extracellular isoform, CA VI, is not associated with the membrane and is the only secreted CA (Fernley et al, 1988). This variation in the localization of different CA isoforms as well as differences in activity allow for their participation in a multitude of functions, and more than one CA isoform may be involved in a particular process (Chegwidden and Carter, 2000).

1.2.1 Fish CAs

Although mammalian CA isoforms have been widely investigated (Parkkila, 2000; Supuran, 2008), much less is known about CA in other vertebrate groups (Lin et al, 2008; Gilmour and Perry 2009; Gilmour, 2010). For example kinetics, inhibitor sensitivity and sequence data exist for many mammalian CAs, however only sequence information is available for most fish CA isoforms (Gilmour, 2010). Despite the lack of detailed information, the diversity of CA isoforms in fish is believed to at least equal, if not exceed that found in mammals (Gilmour and Perry, 2009). Although they exhibit orthologues to most extracellular CAs, fish lack the gene cluster for the intracellular CAs I, II, III and XIII found in mammals (Gilmour and Perry, 2009). Instead, fish express a cytoplasmic “b” isoform that is most prevalent in the red blood cell (RBC), and a distinct but closely related second cytoplasmic isoform (this “a” or “c” isoform has been given different names by different authors; here the “c” terminology is used) that is widely distributed throughout other tissues, but shows negligible RBC expression (Esbaugh et al, 2005; Lin et al, 2008). In rainbow trout (*Oncorhynchus mykiss*), kinetic differences were measured between the two isoforms, with the b isoform having a greater turnover number (approximately 3 times faster; Esbaugh et al, 2005).

Within the fish gill, there is an abundance of cytoplasmic CA (Sobotka and Kann, 1941) that is thought to contribute to both ionic regulation and the maintenance of acid-base balance (reviewed by Gilmour and Perry, 2009). The gill epithelium is the primary site of acid-base and ion exchange with the aquatic environment, and is composed of several cell types, including pavement cells (PVCs), mitochondrion-rich cells (MRCs), mucocytes, and neuroepithelial cells (Wilson and Laurent, 2002). Most of the above-mentioned cell types have had CA localized to

them (see Gilmour and Perry, 2009; Gilmour, 2012). In the several fish species that have been examined in detail, MRCs can be subdivided based on the types of transporters present (Gilmour and Perry, 2009; Hwang et al, 2011). For example, adherence of peanut lectin agglutinin (PNA) has been used to identify two types of MRCs in rainbow trout; MRCs positively stained with PNA are believed to contribute to base secretion, while PNA-negative MRCs are postulated to be involved in acid excretion (Goss et al, 2001; Galvez et al, 2002; reviewed by Evans et al, 2005; Perry and Gilmour, 2006; Tresguerres et al, 2006). Although CA is postulated to reside in both MRC subtypes in rainbow trout, this point remains to be confirmed (Gilmour and Perry, 2009). The vast majority of evidence indicates primarily intracellular localization of CA activity (Henry et al, 1988; Henry et al, 1993; Henry et al, 1997; Gervais and Tufts, 1998; Sender et al, 1999; Gilmour et al, 2001; Gilmour et al, 2002; Tufts et al, 2002; reviewed by Gilmour and Perry 2009; Gilmour 2012). However, whether this activity is associated with a single cytoplasmic isoform or a complement of different CAs remains unclear. For example, in zebrafish larvae (*Danio rerio*) proton pump-rich 'HR' cells in the gill, or skin/yolk sac epithelium (used as an experimental surrogate for the gill), two CA isoforms (zCAc and zCA15a) are present and appear to be involved in Na^+ uptake and acid base regulation (Lin et al, 2008). Sequence similarity between zCA15a and CA IV suggests that CA15a is anchored to the apical membrane of the HR cell by a GPI linkage (Lin et al, 2008; Gilmour, 2011, 2012). Thus, at least in the zebrafish gill, CA activity may not be strictly confined to the intracellular environment.

As an enzyme, CA functions in the gill epithelium by facilitating the provision of H^+ and HCO_3^- as counter-ions for Na^+ and Cl^- uptake through the catalysis of CO_2 hydration (Maetz, 1971). This hydration of CO_2 pairs CO_2 excretion and ionic regulation functionally, with both of these processes being linked to acid–base regulation. Fish rely primarily on modulation of Cl^-

HCO_3^- and Na^+/H^+ exchanges at the gill to adjust plasma HCO_3^- concentration and hence acid-base status (reviewed by Claiborne et al, 2002; Perry et al, 2003; Evans et al, 2005). In support of the role that CA plays in acid-base regulation, a significant reduction in branchial net acid excretion in rainbow trout was observed following administration of acetazolamide, a CA inhibitor (Kerstetter et al, 1970; Georgalis et al, 2006a), an effect that was enhanced by exposure of trout to hypercapnia (elevated water CO_2 tension) to induce a respiratory acidosis (Georgalis et al, 2006a). Similarly, proton extrusion was significantly reduced in zebrafish embryos and larvae in which translation of cytosolic CA mRNA was prevented using an antisense morpholino oligonucleotide (Lin et al, 2008). If CA contributes to the compensatory processes necessary to respond to acid-base disturbances, it seems likely that the modulation of CA activity would be an intrinsic component of acid-base regulation. Adjustment of CA activity could be achieved by increasing the total enzyme available through transcriptional regulation, or by modifying the activity of existing enzyme through post-translational modification.

1.3 Transcriptional regulation

Transcriptional regulation serves as a robust form of cellular control and occurs by altering transcription rates to increase or decrease the abundance of a protein (Carlson, 1997; Desvergne et al, 2008). Thus, transcriptional regulation is a vital component of an organism's ability to respond and adapt to its environment. For transcriptional control to occur, the cell must be able to detect external signals and transduce them into a cellular response at the level of the nucleus (Desvergne et al, 2008). The transduction of a signal to changes in gene expression

involves transcription factors, which are proteins that bind to specific DNA sequences, thereby controlling the flow (or transcription) of genetic information from DNA to mRNA (Latchman, 1997). Transcription factors may perform this function as a single protein or as a quaternary complex, by promoting (as an activator), or preventing (as a repressor) the recruitment of RNA polymerase to specific genes (Lee and Young, 2000). A superfamily of transcription factors is the nuclear receptor family (Aranda and Pascual, 2001). Members of this family include receptors for thyroid and steroid hormones, retinoids and vitamin D (Laudet, 1997), and are characterized by the presence of two zinc finger motif-type DNA binding domains, a ligand binding domain, as well as ligand-independent and ligand-dependent transactivation domains (Aranda and Pascual, 2001; Mangelsdorf et al, 1995). Three subgroups of receptors can be identified within the family; homodimeric, heterodimeric, and monomeric nuclear receptors (Oh et al, 2013). Homodimeric nuclear receptors normally reside in the cytosol through their interaction with molecular chaperone proteins such as heat shock proteins (Oh et al, 2013). Translocation to the nucleus occurs upon binding of a steroid-derived ligand to a homodimeric nuclear receptor, which dimerizes with an identical activated receptor and binds to the specific DNA sequences that constitute response elements within the promoter region of a target gene (Oh et al, 2013). The glucocorticoid hormones, cortisol and corticosterone, are examples of steroid-derived ligands that regulate numerous physiological processes (Mommesen et al, 1999). For example, the main glucocorticoid hormone in teleost fish, cortisol, plays key roles in the regulation of metabolism, stress responses, ionic and osmotic balance, and acid-base balance (Wendelaar Bonga, 1997; Mommsen et al, 1999; Takahashi and Sakamoto, 2013).

The model for glucocorticoid function centres on the binding of a hormone-bound glucocorticoid receptor (GR) dimer to a glucocorticoid response element (GRE) in the regulatory

region of a target gene, leading to altered expression of the gene (Schoneveld et al, 2004).

Although most tissues express GRs, the set of target genes is tissue-specific (Schoneveld et al, 2004). An example of this tissue specificity is the selective expression of carbamoylphosphate synthetase in hepatocytes (Christoffels et al, 1998). The GREs are usually part of composite regulatory elements termed glucocorticoid response units (GRUs), composed of binding sites for several transcription factors (Zimmerman et al, 1997). The GRE contains the specific DNA sequence to which a hormone-bound GR dimer can bind; the consensus GRE consists of two partially palindromic hexanucleotide half-sites separated by 3 base pairs (bp), i.e.

GGTACAnnnTGTTCT (Scott et al, 1998). Additionally, several genes have been described where a GRE half-site (GRE ½s only one half of the classical palindrome) in the regulatory element is sufficient to relay glucocorticoid signalling (Segard-Maurel et al, 1996). An example of this situation was demonstrated in fish; specifically, the urea transporter in the gill of the Gulf toadfish, *Opsanus beta* appears to be regulated by cortisol through GRE ½s (Rodela et al, 2011).

1.3.1. Cortisol and acid-base disturbances in fish

An acid-base disturbance is a perturbation of pH homeostasis resulting from the accumulation or loss of acid-base equivalents, i.e. H^+ or HCO_3^- . Examples of acid-base disturbances include a decrease in systemic pH as a result of increased H^+ concentration from metabolic processes (e.g. lactic acid production) or the loss of bicarbonate ions leading to a metabolic acidosis. In fish, metabolic acidosis is associated with a rise in endogenous glucocorticoids (Brown et al, 1986; Wood et al, 1999; Warren et al, 2004; Gilmour et al, 2011). For example, plasma cortisol concentrations were observed to increase significantly during the initial 2 h of acid infusion into rainbow trout, but not during base infusion (Gilmour et al, 2011).

Similar to this acute response, chronic exposure of rainbow trout to acidic water also led to an increase in circulating cortisol concentrations (Brown et al, 1984, 1986; Wood et al, 1999), suggesting the elevated presence of this hormone over both short- and long-term acidosis. Increases in cortisol may be a component of compensatory responses to restore pH homeostasis. For example, cortisol administration modulated gene expression of two isoforms of Na^+/H^+ exchangers, NHE2 (Ivanis et al, 2008a) and NHE3 (Ivanis et al, 2008b); several studies have implicated NHE in acid-base regulation in teleost fish (Perry et al, 2003; Yan et al, 2007; Ivanis et al, 2008a, 2008b). Cortisol treatment also increased proton pump activity in rainbow trout gills (Lin and Randall, 1993). These modifications to key components of the acid-base regulatory machinery serve to enhance net acid excretion, thus leading to an increase in body pH and a return of pH homeostasis, and suggest a role for cortisol in stimulating acid–base compensatory responses in fish (Gilmour et al, 2011).

If indeed cortisol activates compensatory responses to acid–base disturbances, does the hormone act at least in part by regulating CA expression to maintain pH homeostasis? Previous work on rainbow trout reported that cortisol, CA mRNA and activity levels are increased during some acid-base challenges, suggesting that this regulation may exist (Gilmour et al, 2011). Administration of cortisol led to increased gene expression of the renal CAs (tCAc and tCA IV), but not gill tCAc (Gilmour et al, 2011). Together, these results suggest that cortisol may exert transcriptional regulation over CA at the level of the kidney but not the gill. It is interesting to note that while cortisol had no effect on either gill CA mRNA or protein expression, it did significantly increase gill CA activity (Gilmour et al, 2011). This increase in activity without a concomitant increase in protein levels suggests that the increase in activity might be attributed to post-translational modification (PTM) of existing CA rather than to transcriptional regulation to

increase CA protein levels. Relative to transcriptional regulation, PTMs tend to be more transient and rapidly invoked in response to external stimuli.

1.4 Post-translational modification

While transcriptional modulation is an important aspect of regulating protein function, it is not well suited to mediating rapid responses, because transcription and translation can be lengthy processes (Seet et al, 2006). Instead, rapid and flexible control is achieved by PTMs, which are post-synthesis, covalent modifications of the protein (Hoffman et al, 2008). A PTM must add or subtract a specific mass difference and not be particular to any one protein [e.g. methylation, the addition of a methyl group (-CH₃) to a protein] (Hoffman et al, 2008). Thus, PTMs change a protein's mass and they can also change the charge, structure and conformation of a protein, which can in turn influence the protein's enzymatic activity, binding affinity and hydrophobicity (Nishi et al, 2011). Therefore, PTMs offer an adaptable tactic to regulate protein activity, subcellular localization, and stability. Dynamic regulation is achieved through reversibility as well as the fast kinetics of post-translational modifications (Hoffman et al, 2008). The range of PTMs found within the cell is vast, with individual PTMs varying with respect to specificity and abundance (Uyss and Wold, 1977; Wold, 1981; Yang, 2005; Nishi et al, 2011). That is, some types of PTMs are universally employed, have a large number of target proteins, and are widespread, whereas others are very specific in their function, may only have a few target proteins (or target one protein at a time), and may be of very low occurrence. Moreover, some PTMs occur on only one specific amino acid residue, whereas others are associated with

numerous different residues (Yang, 2005). An individual polypeptide chain can be modified at several sites, thereby producing the possibility of many protein variants, each of which can have a distinct biological activity (Nishi et al, 2011). The most common PTMs include acetylation (i.e. addition of $-\text{CH}_2\text{CH}_3$), methylation (addition of $-\text{CH}_3$), and phosphorylation (addition of $-\text{PO}_3^{2-}$) (Yang, 2005; Seet et al, 2006; Hoffman et al, 2008).

1.4.1 Protein phosphorylation

Protein phosphorylation is one of the most widespread and versatile PTMs found in nature and an essential regulatory mechanism in both prokaryotes (Cozzone, 1988) and eukaryotes (Manning et al, 2002). Its ability to modify catalytic activity, macromolecular interactions, and protein stability (Johnson and Barford, 1993), grants phosphorylation the potential to regulate a plethora of cellular processes. Some of these processes include inter- and intracellular signaling, protein synthesis, gene expression, cell survival, and apoptosis (Hunter, 2000). Protein kinases form a family of enzymes that catalyze the transfer of a phosphoryl group (PO_3^{2-}) from a high energy organic compound, such as adenosine triphosphate (ATP) or guanosine triphosphate (GTP), to a hydroxyl group located on the side chain of serine, threonine, or tyrosine amino acid residues in proteins, thereby forming a phosphoester bond with the residue (Engholm-Keller and Larsen, 2013). By hydrolyzing this covalent phosphoester bond to release phosphate (PO_4^{3-}), protein phosphatases catalyze the enzymatic removal of these phosphate groups from a protein, returning the protein to a non-phosphorylated state (Barford, 1996). Protein phosphatases are estimated to have faster kinetics than protein kinases (Engholm-Keller and Larsen, 2013), which is of high importance in phosphoproteomics because labile

phosphorylations can be lost during sample preparation unless protein phosphatases are inactivated using denaturation or inhibitors (Thingholm et al, 2008).

Phosphorylation affects protein structure by changing the charge and hydrophobicity of the amino acid side chain (Nishi et al, 2011). Negatively charged amino acids in close proximity to the phosphorylated residue will be repelled, while positively charged amino acids will be attracted. Additionally, the phosphoryl moiety will incorporate into the affected protein a hydrophilic and polar region (Engholm-Keller and Larsen, 2013). Consequently, conformational changes can be induced, affecting the properties of the protein, and leading to activation, deactivation, or a change in protein interaction partners (Fuhrmann et al, 2009). For example, reversible protein phosphorylation often occurs in cascades intrinsic to signal transduction mechanisms used by both unicellular and multicellular organisms to respond to external stimuli (Pawson and Nash, 2000). These involve detection of the external stimulus, often as a ligand binding to a transmembrane receptor protein at the cell surface (Schlessinger, 2000). The ligand-receptor interaction induces a conformational change in the cytosolic domain of the receptor. This change induces signal transduction, involving activation of specific protein kinases or phosphatases that modulate other protein kinases further downstream in the cascade. Ultimately, the pathway will result in a specific cellular response such as a change in gene expression (Engholm-Keller and Larsen, 2013).

1.4.2 Glutathionylation

The addition of glutathione (GSH) to the side chain of a cysteine residue within a protein constitutes another PTM. Although not as common as phosphorylation, glutathionylation has gained increased attention in recent years owing to its role in the rapidly expanding field of

oxidative stress (Gao et al, 2009). Glutathione, the tripeptide gamma-glutamyl-cysteinyl-glycine, is the most abundant non-protein thiol present in the cell, and therefore plays a major role in maintaining a reduced state of protein thiols in the cytoplasm, i.e. it serves as a cellular antioxidant (Ghezzi, 2005). Glutathione serves as an antioxidant through its ability to act as a free radical scavenger, a term that specifies a molecule acting to trap reactive oxygen species that would otherwise react with protein sulphhydryls or other potential targets (Fahey et al, 1977). Glutathionylation constitutes the formation of a mixed disulphide linkage between a glutathione and a protein. The overall reaction of this thiol-disulphide exchange, where PSH indicates a protein with a free cysteine and GSSG is glutathione disulphide, is: $PSH + GSSG \rightarrow PSSG + GSH$. This reaction implies that the GSH/GSSG ratio is a key determinant of the glutathionylation status of proteins (Gilbert, 1984). Following oxidation, glutathione reductase can reduce GSSG back to GSH, using NADPH as an electron donor (Couto et al, 2013). Glutathionylated proteins can also be formed via direct oxidation or via sulphenic acid intermediates, s-nitrosylthiols, or thiyl radicals. Thus, several mechanisms may account for the formation of glutathionylated proteins, and it is not clear which mechanism is favoured *in vivo* (Ghezzi, 2005).

The susceptibility of a particular cysteine to glutathionylation is believed to be determined by two factors; steric accessibility, because GSH is not a small molecule, and the identity of amino acids in the immediate vicinity (Casagrand et al, 2002). The addition of GSH is favoured by basic amino acids, which can stabilize the protein-glutathione adduct by interacting electrostatically with the gamma-glutamyl group of GSH (Holmgren, 1985). Acidic amino acids adjacent to the site of glutathionylation have the opposite effect. These considerations suggest that some cysteines may form more stable mixed disulphides than others.

In general, however, mixed disulphides are unstable and can be reversed by the action of other thiols, indicating that glutathionylation is reversible, a feature that suggests its potential regulatory role (Holmgren, 1985).

Glutathionylation can modify protein function in many ways; the binding of the GSH tripeptide to a protein affects its activity through steric hindrance, or changes in its isoelectric point (Ghezzi, 2005). In most cases, glutathionylation leads to an inhibition of the protein's activity, which is particularly evident in case of enzymes (Klatt et al, 1999; Pineda-Molina et al, 2001). In this way, glutathionylation is different from other forms of S-thiolation, such as cysteinylolation (formation of a disulphide bond with a free cysteine), sulphenic (C-SOH), sulphinic (C-SO₂H) and sulphonic acids (C-SO₃H) (Ghezzi, 2005). For instance, glutathionylation inhibits several protein kinase C (PKC) isozymes, while cysteinylolation does not affect their activity (Chu et al, 2001). Glutathionylation may have functions other than those that are viewed as generally protective or antioxidant. For example, the susceptibility of a cysteine to other oxidative reactions is greatly reduced if it is glutathionylated. In this respect, glutathionylation is often considered to be a way to protect sensitive cysteines from other, possibly irreversible forms of oxidation, and can allow the cell to restore the normal function of the protein following oxidative stress (Holmgren 1985).

1.4.3 Post-translational modification of CA

The versatility and rapid onset of PTMs lend themselves to the regulation of enzyme function (Oliveira and Sauer, 2012), and an enzyme like CA that is involved in a large number of physiological processes is a prime candidate for regulation by PTM. However, relatively few studies have examined the occurrence and function of PTMs in CA isoforms. Phosphorylation of

CA can occur, and in some cases may regulate activity. For example, two CA isoenzymes, one highly active and one of lower activity, were isolated from rat gastric tissue (Bersimbaev et al, 1975). These isoforms could be phosphorylated *in vitro* by a protein kinase isolated from gastric mucosa, and this process was stimulated by AMP (Bersimbaev et al, 1975). Phosphorylation of the highly active CA isoenzyme increased its activity, whereas phosphorylation of the low activity isoenzyme did not affect its activity, or decreased it slightly (Bersimbaev et al, 1975). Similarly, two CA isoforms isolated from bovine erythrocytes were phosphorylated by protein kinase A (PKA), with the activity of one isoform increasing significantly following phosphorylation (Narumi and Miyamoto, 1974). Over the last decade, CA IX has been the focus of much research effort because of its nearly exclusive expression in the progressive front of tumours (Potter and Harris, 2003; Potter and Harris, 2004). This isoform is a single pass transmembrane protein and possesses extracellular enzymatic activity. In the hypoxic regions of a tumour, CA IX is an important contributor to bicarbonate transport, a necessary aspect in maintaining pH homeostasis (Ditte et al, 2011). Recently, PTM was investigated as a potential regulator of CA IX in cancer (Ditte et al, 2011). At the intracellular domain of CA IX, phosphorylation of T443 by PKA was found to be critical for CA IX activation in hypoxic cells, with the fullest activity also requiring dephosphorylation of S448 (Ditte et al, 2011). The mutation of PKA disrupted CA IX-mediated extracellular acidification, suggesting that this PTM has a functional outcome (Ditte et al, 2011). Collectively, these studies suggest that CA activity can be regulated by phosphorylation and that in the case of CA IX, PKA may be responsible for the transfer of the phosphoryl group.

In addition to phosphorylation, CA has been found to undergo other PTMs. For example, CA in human erythrocytes was methylated, although no regulatory role for methylation was

established (O'Connor and Clarke, 1984). Mammalian CA III has two surface-exposed sulphhydryl groups (C181 and C186), which can conjugate to glutathione through a disulphide link, that is, they can be S-glutathionylated (Chai et al, 1991). This isoform was rapidly glutathionylated *in vivo* and *in vitro* when cells were exposed to oxidative stress (Chai et al, 1991). Gill cytoplasmic CA of the haemoglobin-lacking Antarctic icefish *Chionodraco hamatus* (Ice-CA) was shown through western blotting also to be S-glutathionylated (Rizzello et al, 2007). Deglutathionylated Ice-CA maintained its enzyme activity but showed higher susceptibility to hydrogen peroxide, suggesting that the binding of glutathione to cysteine residues may have a role in defending enzyme function against oxidative damage.

1.5 Hypotheses and predictions

The present investigation sought to determine whether transcriptional and post-translational regulation of CA occur in fish. The study of transcriptional regulation focused on the relationship between cortisol and the general cytosolic isoform CAc in zebrafish. The zebrafish was chosen owing to the wealth of genomic and molecular tools available for this species (Patton and Zon, 2001). Previous work carried out in rainbow trout suggested that transcriptional regulation of several CA isoforms, including CAc, occurs in response to acid-base challenges such as exposure to hypercapnia, or acid- or base-infusion (Georgalis et al, 2006a; Georgalis et al, 2006b; Gilmour et al, 2011). Circulating levels of the stress hormone cortisol also can be elevated during acid-base disturbances in both rainbow trout (Gilmour et al, 2011) and zebrafish (Kumai et al, 2012), and changes in CA transcript abundance were reported in

cortisol-treated trout (Gilmour et al, 2011). Thus, it is hypothesized that CAc is transcriptionally regulated by cortisol in zebrafish. If CA mRNA expression is affected by cortisol, then GREs would be predicted to be present in the promoter region of zCAc; when the promoter is linked to a reporter gene (luciferase) and activated by cortisol bound to a GR, activation of the reporter should result. Moreover, cortisol treatment would be predicted to result in increases in CA transcript abundance, protein levels and activity.

The study of post-translational regulation focused on potential peptide modifications of rainbow trout gill CAc using *in silico*, *in vitro*, and *in vivo* approaches to first identify likely motifs, then to assess their effect on enzymatic properties, and finally to investigate their potential impacts on physiological function in rainbow trout. The rainbow trout was chosen for this work because its larger size provides an abundant source of the tissues necessary for enzyme purification. It is hypothesized that trout gill CAc is subject to PTM, and that the modification(s) regulates its enzymatic properties. If CA is modified by a PTM such as phosphorylation, then it would be predicted that changes in the phosphorylation state of CA should increase enzymatic activity. Based on previous work (Rizello et al, 2007), glutathionylation is predicted not to influence CA activity.

CHAPTER 2

Post-translation modification of carbonic anhydrase in rainbow trout, *Oncorhynchus mykiss*

Abstract

The ubiquitous zinc metalloenzyme carbonic anhydrase (CA) assumes a pivotal role in many physiological processes by catalysing the reversible reactions of carbon dioxide and water ($\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$). The present study investigated the potential for rainbow trout, *Oncorhynchus mykiss*, gill cytoplasmic CA (tCAc) to undergo post-translational modification (PTM). The activity of tCAc was significantly increased approximately two-fold by phosphorylation through stimulation of endogenous kinases. Additionally, kinase stimulation doubled phospho-threonine content from that observed in endogenous tCAc, whereas stimulation of phosphatase action halved phospho-threonine content from the endogenous amount. *In vitro* incubations to preferentially potentiate specific kinases implicated protein kinase G (PKG) as the kinase mediating the increase in tCAc activity. Whereas the kinetic parameters of turnover number (k_{cat}), and substrate affinity (K_m) were similarly affected by both phosphatase and kinase action, kinase action significantly increased the efficiency of tCAc (V_{max}/K_m) and this factor may have been of importance in increasing tCAc activity. Western analysis confirmed that tCAc is glutathionylated, but this modification did not impact enzyme activity. These results demonstrate that tCAc is subject to PTMs, and future work should focus on identifying the physiological situation in which PTM of trout branchial tCAc occurs.

2.1 Introduction

Post-translational modification is a form of cellular control that involves the addition or removal of a chemical moiety from a translated peptide (Hoffman et al, 2008). Because the addition or removal of a chemical group can influence electrostatics (e.g. addition of negative charge from phosphorylation) and overall structure, leading to changes in properties such as activity, cellular localization, and degradation, PTM serves as a method for rapid modulation of peptide function. Some PTMs are reversible, e.g. phosphorylation (Rogers and Overall, 2013), whereas other are not, e.g. proteolytic processing (Engholm-Keller and Larsen, 2013). An especially useful aspect of reversibility is that PTMs can function as molecular switches, allowing a control system to turn a given cellular process off or on (Cozzzone, 1988). One of the most common and widely used reversible PTMs is phosphorylation (Cohen, 2000). This modification involves the addition of an inorganic phosphate group to the hydroxyl group of serine, threonine, or tyrosine residues within a peptide (Johnson and Barford, 1993). Reversible phosphorylation is an intrinsic component of many signalling pathways and can have a profound effect on enzymatic function (Nishi et al, 2011).

The zinc metalloenzyme CA catalyses the reversible reactions of carbon dioxide and water, i.e. $\text{CO}_2 + \text{H}_2\text{O} \leftrightarrow \text{HCO}_3^- + \text{H}^+$. Although the enzyme is ubiquitously expressed and essential to many physiological processes (Hewett-Emmett, 2000; Chegwiddden and Carter, 2000), relatively little is known about the regulation of CA by PTM. Evidence from several studies suggests that phosphorylation can modify the activity and intracellular localization of CA. For example, cAMP stimulated the phosphorylation *in vitro* of two CA isoenzymes derived from rat gastric tissue (Bersimbaev et al, 1975). The activity of the more active CA isoenzyme

was increased by phosphorylation, whereas phosphorylation had negligible effect on the less active CA isoenzyme (Bersimbaev et al, 1975). Similarly, two CA isoforms isolated from bovine erythrocytes were phosphorylated by protein kinase A (PKA), with a significant rise in activity of one isoform (Narumi and Miyamoto, 1974). A cAMP-dependent increase in CA activity that was mediated by PKA also was found to occur in astroglial cell cultures from rat cerebellar tissues stimulated with norepinephrine (Church et al, 1980). Recent work in the green alga *Chlamydomonas reinhardtii* demonstrated the ability of phosphorylation to control the intracellular localization of CA. Phosphorylation not only increased CA activity, but also led to the concentration of CA in intrapyrenoid thylakoids of the chloroplast (centres of carbon dioxide fixation), a movement away from stroma thylakoids (Blanco-Rivero et al, 2012). Collectively, the results of these studies indicate that CA activity can be regulated by phosphorylation.

To extend our knowledge of the regulation of CA isoforms by phosphorylation, the present study focused on cytosolic CA (CAc) isolated from the gills of rainbow trout (*Oncorhynchus mykiss*). By catalyzing the hydration of CO₂ to produce H⁺ and HCO₃⁻ as counter-ions for ion transport processes (e.g. Na⁺/H⁺ exchange, Cl⁻/HCO₃⁻ exchange), this CA isoform participates in ionic, osmotic and acid-base regulation in freshwater teleost fish (Maren, 1967; Gilmour, and Perry 2009; see Gilmour 2012). Because these processes must be responsive to the animal's salt, water and acid-base status as well as environmental fluctuations in ion levels or pH (Evans et al, 2005; Perry, and Gilmour 2006), CAc would appear to be a likely candidate for regulation by PTM. The present study tested the hypothesis that rainbow trout gill CAc is regulated by post-translational mechanisms, specifically phosphorylation, and based on the results of previous studies in mammals, phosphorylation of tCAc was predicted to increase its activity.

One of the few studies that investigated post-translational regulation of CA in fish identified the occurrence of glutathionylation (Rizzello et al, 2007). Glutathionylation consists of the formation of a disulphide bond between the thiols of a peptidyl cysteine and the tripeptide glutathione (Ghezzi, 2005). This PTM is intimately associated with peptide redox chemistry (Hwang et al, 1992). Many enzymes are subject to glutathionylation, including CA. For example, mammalian CA III is a low activity isoform that is abundant in skeletal muscle and adipocytes (Chegwidden and Carter, 2000). This isoform possesses two surface-exposed cysteine residues that can form disulphide linkages to glutathione, and is therefore readily glutathionylated both *in vivo* and *in vitro* (Kim and Levine, 2005). Although the function of CA III remains unclear, the presence of free thiol groups and its ability to undergo glutathionylation suggest that it may function as a sensor of redox state and/or play a role in the cellular response to oxidative stress (Chegwidden and Carter, 2000; Kim and Levine, 2005). In icefish, glutathionylation had no effect on CA activity, but improved resistance of the protein to hydrogen peroxide (Rizzello et al, 2007). Similarly, non CA-expressing cells that were transfected with rat CA III exhibited significantly enhanced resistance to hydrogen peroxide (Räisänen et al, 1999). Thus, CA glutathionylation is believed to play a protective role during oxidative stress. The present study tested the hypothesis that rainbow trout gill CAc can undergo glutathionylation; glutathionylation would not be predicted to increase the activity of tCAc.

Sites of PTM are characterized by specific amino acid identities and motifs (Gnad et al, 2011). As a first step in investigating the possible post-translational regulation of tCAc, an *in silico* analysis of tCAc was conducted to identify likely PTMs. Established protocols (reviewed by Chegwidden, 1991) were then used to isolate and purify trout gill CAc. To investigate the capacity of trout gill CAc to undergo phosphorylation and glutathionylation, the enzyme was

incubated *in vitro* under conditions known to induce changes in phosphorylation or glutathionylation state. The impact of these PTMs on CA activity was tested. Finally, the question of whether PTMs identified *in vitro* occur or play a role *in vivo* was addressed. During exposure to hypercapnia, which causes a respiratory acidosis (Goss and Perry, 1993), gill CAC activity is believed to play a significant role in the regulation of systemic acid-base balance; inhibition of branchial CA activity during exposure to hypercapnia reduced branchial net acid excretion by 36% (Georgalis et al, 2006a). Exposure to hypercapnia increased gill CAC transcript abundance resulting in higher CAC protein levels by 24 h of exposure (Georgalis et al, 2006a). However, the possibility that CA activity is modulated more acutely by PTM to enhance the capacity for branchial net acid excretion has not been tested. Thus, the final experimental goal of the present study was to test the hypothesis that CA activity increases during short-term exposure (4 h) of rainbow trout to hypercapnia *in vivo*, and that such an increase in CA activity is linked to phosphorylation of CAC.

2.2 Materials and methods

2.2.1 *In silico analysis*

On-line search engines were used to identify potential sites of PTM in the rainbow trout general cytosolic CA isoform, tCAc (Esbaugh et al, 2005). Analyses were carried out on the putative amino acid sequence (Q6R4A2_ONCMY; www.uniprot.org) translated from the mRNA coding sequence (GenBank accession number AY514870.1). Possible phosphorylation sites were assessed using NetPhos 2.0 Server (<http://www.cbs.dtu.dk/services/NetPhos/>). The NetPhosK 1.0 server (www.cbs.dtu.dk/services/NetPhosK) was used to predict kinase-specific phosphorylation sites. Lysine acetylation sites were predicted using <http://www.phosida.com>, and glycation sites were predicted by the NetGlycate 1.0 server (<http://www.cbs.dtu.dk/services/NetGlycate/>).

2.2.2 *Chemicals*

All chemicals were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA), unless otherwise noted.

2.2.3 *Experimental animals*

All experiments complied with University of Ottawa institutional guidelines, were approved by the Animal Care Committee (protocol BL-228), and were in accordance with guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching. Rainbow trout (*O. mykiss* Walbaum; approximate mass 250 g, N = 19) were obtained from Linwood Acres Trout Farm (Campbellcroft, ON, Canada). Fish were maintained on a 12

h:12 h L:D photoperiod in large fibreglass aquaria supplied with flowing, aerated and dechloraminated city of Ottawa tap water at 13°C, and were fed to satiation on commercial trout pellets every second day. Trout were allowed to acclimate to these holding conditions for at least 2 weeks prior to experimentation.

In most cases, trout were removed from the holding tank and euthanized, and tissues were collected. Trout were euthanized by immersion in a solution of benzocaine (ethyl-*p*-aminobenzoate; 0.1 g L⁻¹) and gill tissue was dissected out, immediately frozen in liquid N₂, and stored at -80°C until analysis. Prior to collection, the gills were perfused with saline to clear the tissues of blood. The bulbus arteriosus was exposed and cannulated with PE160 polyethylene tubing (Clay-Adams; VWR, Montréal, QC, Canada). Approximately 50 mL of ice-cold, heparinised (100 IU mL⁻¹ heparin) 0.9% NaCl containing 1x10⁻⁵ mol L⁻¹ isoproterenol (a synthetic beta-adrenoreceptor agonist used to promote vasodilation) was infused *via* the cannula. During saline infusion, the ventricle was severed to allow fluid in the circulatory system to drain.

In one experiment, rainbow trout (N = 6) were exposed to hypercapnia prior to being euthanized for tissue collection. Fish were placed in individual opaque acrylic boxes supplied with flowing, aerated, dechloraminated city of Ottawa tap water for a 24 h acclimation period. Water flow to the experimental chambers was then stopped, and the individual chambers were vigorously aerated with 1% CO₂ in air; mixed gases were supplied using a GF-3/MP gas mixing flowmeter (Cameron Instruments, Port Aransas, TX, USA). Fish allocated to the control group (N = 6) were treated identically, except that the experimental chamber was provided with air rather than CO₂-enriched air. Following 4 h of exposure to hypercapnia (or air in the control group), fish were euthanized and gill tissue was collected as described above.

2.2.4 Gill CA Purification

Carbonic anhydrase was isolated from trout gill tissue and purified by gravity flow affinity chromatography using *p*-aminomethylbenzene-sulphonamide (*p*-AMBS) immobilized on cyanogen-bromide-activated agarose gel as per the basic approach of Whitney (1974) and Rizello et al (2007; see also Chegwiddden 1991 for a review of the general approach). Gill samples (~1 g) were added 1:5 w/v to ice-cold buffer containing protein kinase and protein phosphatase inhibitors (30 mM HEPES, pH 7.0, 10% v/v glycerol, 5 mM 2-mercaptoethanol, 1.5 mM EDTA, 1.5 mM EGTA, 15 mM β -glycerophosphate). A few crystals of phenylmethylsulphonyl fluoride were added and the sample was immediately homogenized with a hand-held homogenizer (Pellet Pestle Cordless Motor, Kimble-Chase, Vineland, NJ, USA) in a 15 mL centrifuge tube. The gel column (10 mL Disposable Polypropylene, ThermoScientific, Waltham, MA) was equilibrated and rinsed with 15 mL of 0.25 M Tris, 0.1 M Na₂SO₄, adjusted to pH 8.7 with HCl, and then rinsed with 10 mL of 0.25 M Tris, 0.3 M NaClO₄, adjusted to pH 8.7 with HCl. Elution of CA was accomplished using 0.5 mL aliquots (15 mL total volume) of a solution of 0.1 M CH₃COOH, 0.5 M NaClO₄, pH 5.6 at 4°C (as per Rizello et al, 2007). Protein elution was monitored using Coomassie staining of SDS-PAGE gels loaded with aliquots of eluate; the single band viewed on such gels was later confirmed to be CA by Western analysis (for details see section 2.2.6 SDS-PAGE and Western analyses). Samples showing a single band at the molecular size of CA (~26 kDa) were pooled, desalted and concentrated by ultrafiltration with a 10 kDa centrifugation filter (Amicon Corp, Lexington, KY, USA). All purification steps were carried out at 0-4°C. This procedure yielded purified gill CA that could then be assayed for CA activity or probed by SDS-PAGE or Western analyses for PTMs of interest.

2.2.5 Assessment of CA activity and kinetic analyses

The electrometric ΔpH method of Henry (1991) was used to measure CA activity. Assays were carried out using 10-50 μL of desalted gill extract and 6 mL of reaction medium (in mmol L^{-1} : 225 mannitol, 75 sucrose, 10 Tris-base, adjusted to pH 7.4 using 10% orthophosphoric acid) held at 4°C . The reaction was initiated using 200 μL of ice-cold, CO_2 -saturated distilled water, and the rate of the reaction was measured over the initial 0.15 unit pH change. The true catalyzed rate was determined by subtracting the rate of the uncatalyzed hydration reaction from the rate of the reaction in the presence of the CA source. The buffer capacity of the reaction medium was then taken into account to express the reaction rate in units of $\mu\text{mol H}^+$ (or CO_2) per unit time. A pH electrode (GK2401C; Radiometer, London, ON) connected to a PHM84 pH meter (Radiometer) and data acquisition system (Biopac with AcqKnowledge v3.7.3 software; Harvard Apparatus Canada, Saint-Laurent, QC) was used to monitor the pH of the reaction medium. Enzyme activity was expressed as a function of sample protein concentration, which was measured using the bicinchoninic acid (BCA) protein assay (Sigma-Aldrich) with bovine serum albumin (BSA) as the standard.

The kinetic properties of CA under different conditions were investigated using the basic approach of Maren et al (1980). The maximum reaction rate (V_{max}) and substrate affinity value (K_m) were determined from Lineweaver-Burk plots (Lineweaver and Burk, 1934) of the reciprocals of the velocity of CO_2 hydration at increasing concentrations of CO_2 . The catalytic rate constant (k_{cat}) was then calculated using the formula $k_{cat} = V_{max}/E_t$, where E_t is the total enzyme concentration. The total enzyme concentration was determined by measuring CA activity in the presence of different concentrations of the potent CA inhibitor acetazolamide. These data were then plotted according to the equation (Morrison, 1969; Henderson, 1973;

Segel, 1975) $I_t/(1-v_o/v_i) = K_i(v_o/v_i) + E_t$, where I_t is the total inhibitor concentration and $(1 - v_o/v_i)$ is the fractional inhibition at a given concentration of inhibitor. For each sample, E_t and K_i values were calculated, the enzyme units (eu) value was determined from the equation $eu = (1/rate_{uncatalysed} - 1/rate_{catalysed}) / 1/rate_{catalysed}$, and a ratio of E_t/eu was then calculated; the E_t of further samples then could be determined based on the calculated eu (Maren et al, 1980, 1993).

2.2.6 SDS-PAGE and Western analyses

Phosphoprotein levels in purified CA samples were assayed using Pro-Q diamond phosphoprotein staining (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions. In brief, concentrated purified enzyme was mixed 1:1 v/v with SDS loading buffer (100 mM Tris buffer, pH 6.8, 4% w/v SDS, 20% v/v glycerol, 0.2% w/v bromophenol blue, 10% v/v 2-mercaptoethanol). A 40 μ L aliquot was added to a well of a 15% SDS-PAGE gel. Gels were exposed to 200 V for 60 min in running buffer containing 25 mM Tris-base, 250 mM glycine and 0.1% w/v SDS. Gels were then washed twice in fixing solution (50% v/v methanol, 10% v/v acetic acid), and left overnight in this solution at 4°C. The following day, gels were washed three times with distilled deionized H₂O (ddH₂O) for 10 min each time, and then stained with Pro-Q Diamond phosphoprotein stain (Invitrogen, Eugene, OR, USA) for 90 min at room temperature. During staining, the gel container was covered with tinfoil to minimize exposure of the light-sensitive stain to light; gels also remained covered for the next steps. Following staining, gels were de-stained by washing 3 x 30 min with Pro-Q Diamond de-staining solution (20% v/v acetonitrile, 50 mM sodium acetate, pH 4) followed by 2 x 5 min with ddH₂O. A Chemidoc XRS+ (Bio-Rad) was used to visualize UV bands, which were quantified by means of the accompanying Image Lab software. After visualization, the gel was stained for total protein

using Sypro Ruby protein gel stain (Invitrogen, Eugene, OR, USA) according to the manufacturer's instructions, and UV bands were visualized and quantified as described above. Phosphoprotein-positive CA band intensities (Pro-Q Diamond staining) were standardized against total protein intensity of the same band (Sypro Ruby staining) to correct for variations in protein concentration across purified enzyme preparations.

For Western analysis, purified CA samples (~10 µg) were first fractionated by SDS-PAGE using 15% separating and 4% stacking polyacrylamide gels; sample protein concentrations were assessed by the BCA protein assay with BSA as the standard. Fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) using a Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. After transfer, each membrane was blocked for 1 h in Tris-buffered Tween 20 (TBS-T; 20 mM Tris, 300 mM NaCl and 0.1% Tween 20) containing 5% BSA. Membranes were then incubated with primary antibody according to the goal of the specific experiment. To confirm CA isolation, membranes were probed with a 1:2,000 dilution of rabbit anti-trout tCAc (Georgalis et al, 2006a) for 2 h at room temperature and then with a 1:15,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad) for 1 h at room temperature. To test for amino acid residue-specific phosphorylation sites, membranes were incubated with phospho-serine, phospho-threonine or phospho-tyrosine antibodies (Abcam, Toronto, ON) at 1:1,000 dilution (1.5 h at room temperature) followed by 1:5,000 horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad) for 1 h at room temperature. Membranes were washed after each incubation for 4 x 5 min in TBS-T. Immunoreactive bands were in all cases visualized by enhanced chemiluminescence (Luminata Classico Millipore Corporation, Billerica, MA, USA) using a digital gel documentation system (Chemidoc XRS+, Bio-Rad) and digital image

processing software (Image Lab, Bio-Rad). The protein size marker used was obtained from Fermentas Life Sciences (Burlington, ON).

To standardize the density of phospho-positive bands, membranes probed with phospho-antibodies were stripped (ReBlot Plus Mild Antibody Stripping Solution, 10x; Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions and blocked for 1 h in Tris-buffered Tween 20 (TBS-T) containing 5% BSA. Membranes were then probed first with a 1:2,000 dilution of rabbit anti-trout tCAc (Georgalis et al, 2006a) for 2 h at room temperature, and then with a 1:15,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad) for 1 h at room temperature. After each incubation, membranes were washed for 3×10 min in TBS-T. Immunoreactive bands were visualized by enhanced chemiluminescence as described above. The intensity of the phospho-positive band was divided by the density of the same band probed for tCAc. All samples were assayed on a single gel.

2.2.7 *In vitro incubations*

To assess the potential effects of reversible phosphorylation on gill CA, gill homogenates were incubated under conditions that facilitated the activities of selected endogenous protein kinases or protein phosphatases (e.g. Dieni and Storey, 2010; Xiong and Storey, 2012). Gill homogenates were prepared as described in section 2.2.4 Gill CA Purification. Homogenates were centrifuged at 10,000 g at 4°C for 30 min, and the resultant supernatant was isolated and stored on ice. Low molecular weight metabolites and ions were removed from the supernatant by desalting with a 10 kDa ultracentrifugation filter (Amicon Corp, Lexington, U.S.A). Aliquots (500 µL) of sample were then mixed 1:1 v/v with an appropriate incubation buffer. Buffers

contained 60 mM HEPES, pH 7.0, 10 mM 2-mercaptoethanol, plus one of the three following mixtures:

(A) “*Stop*” conditions: 40 mM β -glycerophosphate, 4 mM EDTA and 4 mM EGTA to inhibit both protein kinase and protein phosphatase activities.

(B) *Promotion of endogenous protein kinases*: 40 mM β -glycerophosphate, 2 mM cAMP, 10 mM ATP, 2 mM cGMP, 2.6 mM CaCl_2 , $14 \mu\text{g mL}^{-1}$ phorbol myristate acetate (PMA), 1 mM AMP, 10 mM MgCl_2 and 10 mM Na_3VO_4 to stimulate PKA, PKG, protein kinase C (PKC), and AMP-activated protein kinase (AMPK). In some experiments, individual protein kinases were targeted. To promote PKA, 40 mM β -glycerophosphate, 2 mM cAMP, 10 mM ATP, 10 mM MgCl_2 , and 10 mM Na_3VO_4 was used. To selectively enhance PKG, the mix contained 40 mM β -glycerophosphate, 2 mM cGMP, 10 mM ATP, 10 mM MgCl_2 and 10 mM Na_3VO_4 . Protein kinase C was targeted using a mixture of 40 mM β -glycerophosphate, 1.3 mM CaCl_2 , $7 \mu\text{g mL}^{-1}$ PMA, 10 mM ATP, 10 mM MgCl_2 and 10 mM Na_3VO_4 . Finally, activation of AMPK was achieved using 40 mM β -glycerophosphate, 1 mM AMP, 10 mM ATP and 10 mM Na_3VO_4 .

(C) *Promotion of endogenous protein phosphatases*: 10 mM MgCl_2 and 10 mM CaCl_2 to stimulate the activities of protein phosphatases.

Samples were incubated overnight at 4°C . After incubation, an aliquot (500 μL) of the sample was desalted with a 10 kDa ultracentrifugation filter (Amicon Corp, Lexington, USA) and re-suspended in CA assay buffer for measurement of CA activity. The remaining volume (not desalted) was purified and concentrated as described above (see section 2.2.4 Gill CA Purification) to be used for Pro-Q Diamond staining or Western blotting.

To probe the role and possible effects of reversible glutathionylation on gill CA, concentrated, purified gill CA (50 μ L) was mixed with an equal volume of CA assay buffer containing dithiothreitol (DTT) to a final concentration of 0.1 M DTT (treated), or with CA assay buffer alone (control). Samples were incubated at room temperature for 2 h on an orbital shaker. Samples were then desalted (as above) and re-suspended in assay buffer for CA activity measurements. To test for glutathionylation of purified CA, membranes were probed first with a 1:1,000 dilution of mouse anti-GSH (Virogen, Watertown, MA) for 1.5 h at room temperature, and then with a 1:3,000 dilution of horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad) for 1 h at room temperature.

2.2.8 Statistical analyses

Data are reported as mean values \pm 1 standard error of the mean (SEM). The statistical significance of treatment effects was evaluated using paired Student's *t*-tests, or one-way repeated measures analysis of variance (RM ANOVA), as appropriate. Paired and repeated measures tests were used in statistical analyses where multiple treatments were applied to a sample derived from a single source. For example, a single enzyme extract was prepared for *in vitro* incubations and then divided into three treatment groups, so that the enzyme in each group was derived from a single source. Where ANOVA indicated that significant differences existed, *post hoc* multiple comparisons tests (Holm-Sidak method) were applied to identify the source of the differences. Equivalent non-parametric tests were employed in the event that the data did not meet the underlying assumptions of normality or equal variance for parametric tests. All statistical tests were carried out with SigmaStat v3.5 (in SigmaPlot 11.0; SPSS) with a fiducial limit of significance of 0.05.

2.3 Results

2.3.1 *In silico analysis*

Using online search engines, the putative amino acid sequence of trout CAc was probed for potential sites of PTMs. A total of 14 possible phosphorylation sites (7 serine, 3 threonine and 4 tyrosine) was predicted by NetPhos 2.0 software (Fig. 2-1). An acetylation predictor from <http://www.phosida.com/> found three possible lysine acetylation sites. Glycation of six sites was predicted by NetGlycate 1.0 (<http://www.cbs.dtu.dk/services/NetGlycate/>). There are 4 cysteine residues in the amino acid sequence, which are potential sites of glutathionylation. Subsequent experiments focused on phosphorylation of gill CA.

2.3.2 *Purification of gill CA*

To visually assess the success of the protocol used to purify CA from trout gill homogenates, samples were separated by SDS-PAGE and stained with Coomassie blue. A representative gel depicted in Fig. 2-2 illustrates that a single band at ~26kDa is visible in the concentrated eluate from the pAMBS column, in contrast to numerous bands that were present in gill supernatant prior to purification. Western blotting confirmed that the single band obtained from column eluates was tCAc (Fig. 2-1).

2.3.3 *Reversible phosphorylation of gill CA*

To determine whether trout gill CA was subject to reversible phosphorylation, crude gill homogenates were incubated *in vitro* under conditions designed to stimulate the action of endogenous protein kinases ('kinase' treatment), to inhibit endogenous kinases and phosphatases

(‘endogenous’), or to stimulate the action of endogenous protein phosphatases (‘phosphatase’). The standardized relative intensity of phospho-CA bands in homogenates incubated under kinase-stimulating conditions was significantly greater than one, the value that would be expected if kinase treatment was without effect (one-sample Student’s *t*-test, $P = 0.0368$), whereas that of homogenates incubated under phosphatase-stimulating conditions did not differ from one (one-sample Student’s *t*-test, $P > 0.05$; Fig. 2-3A). To distinguish among the relative importance of threonine, serine and tyrosine phosphorylation sites, the phosphorylation state of CA also was assessed using Western blots with phospho-specific antibodies (Fig. 2-4). Kinase-stimulating conditions resulted in significantly higher threonine phosphorylation content relative to the endogenous state, and phosphatase-stimulating conditions resulted in significantly lower threonine phosphorylation content relative to the endogenous state (one way RM ANOVA, $P = 0.001$). There were no significant differences among treatments in phospho-serine (one way RM ANOVA, $P = 0.764$) or phospho-tyrosine (one way RM ANOVA, $P = 0.094$) content.

Incubation under kinase-stimulating conditions resulted in significantly higher CA activity compared to the endogenous and phosphatase-stimulating conditions (one-way RM ANOVA, $P < 0.001$; Fig. 2-3B). To identify factors contributing to the higher activity of phosphorylated CA, kinetic analyses were carried out (Fig. 2-5). Incubation under kinase-stimulating conditions resulted in a significantly lower turnover number (k_{cat}) relative to the endogenous status (one-way RM ANOVA, $P = 0.023$). Incubation under phosphatase-stimulating conditions also tended to result in a lower k_{cat} value, although the difference from the endogenous value was not significant. Substrate affinity (K_m) was significantly lower under both kinase- and phosphatase-stimulating conditions than under endogenous conditions (one-way RM ANOVA, $P = 0.007$). The efficiency of the enzyme (V_{max}/K_m) as well as the inhibition constant

(K_i) against the potent CA inhibitor acetazolamide were significantly higher following kinase stimulation (one-way RM ANOVA, $P = 0.005$ for V_{max}/K_m and 0.007 for K_i) than the values for endogenous protein, but were unaffected by phosphatase stimulation.

Incubations designed to stimulate specific endogenous kinases then were used to investigate which kinase or kinases were most likely to have elicited changes in CA activity and kinetics (Figs. 2-6, 2-7). Conditions designed to stimulate PKG resulted in significantly higher CA activity than that under endogenous conditions (Fig. 2-6A); no other treatment significantly affected CA activity relative to the endogenous value (one-way RM ANOVA, $P < 0.001$). All conditions except stimulation of PKC elicited significantly lower k_{cat} values than that of CA incubated under endogenous conditions (one-way RM ANOVA, $P = 0.01$, Fig. 2-7A). Similarly, all incubation treatments resulted in significantly lower K_m values than that of CA incubated under endogenous conditions (one-way RM ANOVA, $P = 0.035$, Fig. 2-7B). There were no significant differences in V_{max}/K_m (one-way RM ANOVA, $P = 0.051$, Fig. 2-7C) or K_i for acetazolamide (one-way RM ANOVA, $P = 0.992$, Fig. 2-6B) with incubations designed to stimulate specific kinases.

2.3.4 Glutathionylation of gill CA

The specific binding of glutathione adducts to trout gill CA was demonstrated by Western blot analysis using a monoclonal anti-GSH antibody (Fig. 2-8A). Specific immunolabelling occurred as a result of glutathionylation under non-reducing conditions (i.e. without DTT); this labelling was eliminated by incubation with 0.1M DTT. Glutathionylation had no effect on the activity of CA because reduction by incubation with 0.1 M DTT did not significantly change activity (paired Student's t -test, $P = 0.189$; Fig. 2-8B).

2.3.5 Acid-base challenge *in vivo*

To investigate whether exposure to an acid-base challenge *in vivo* triggers phosphorylation of gill CAc, trout were exposed to hypercapnia for 4 h. Exposure to hypercapnia had no significant effect on either the relative phosphorylation status (Student's *t*-test, $P = 0.481$) or activity (Student's *t*-test, $P = 0.575$) of trout gill CA (Fig. 2-9).

Figure 2-1. Results of the *in silico* analysis of rainbow trout, *Oncorhynchus mykiss*, cytoplasmic CA for putative phosphorylation sites. The putative amino acid sequence of trout CAc (Q6R4A2_ONCMY) was obtained from UNIPROT, and analyzed using NETPHOS 2.0. Amino acid residues of the active site (derived from Gilmour and Perry, 2010) are underlined, potential sites of glutathionylation are highlighted in black and residues predicted to be phosphorylated are highlighted in grey.

MSHAWGYAPDNGPDKW**C**EGFPIANGPRQ**S**PIDIVPGEAADFDAALKATLK
YDPSTSIDILNNGHSFQ**V**TY**T**DDNDNSTLTGGPISGTYRLK**Q**F**H**F**H**WGAS
DDRG**S**E**H**T**V**A**G**T**K**Y**A**A**E**L**H**L**V**H**W**N**T**K**Y**P**S**F**G**D**A**A**S**K**S**D**G**L**A**V**V**G**V**F**L**Q**V**G
NENANLQKVLDAFDAIKAKG**Q**T**S**FENFDPTILLPKSLD**Y**W**T**Y**D**G**S**L**T**T**P**
PL**L**E**S**V**T**W**I**V**C**K**E**S**I**S**V**S**P**A**Q**M**G**K**F**R**S**L**L**F**S**G**E**G**E**A**A**C**C**M**V**D**N**Y**R**P**P**Q**P**L
K**G**R**A**I**T**A**R**S

Figure 2-2. Images of a representative (A) SDS PAGE gel and (B) western blot illustrating the effect of pAMBS purification of a crude homogenate of rainbow trout, *Oncorhynchus mykiss*, gill tissue to yield purified gill carbonic anhydrase (CA).

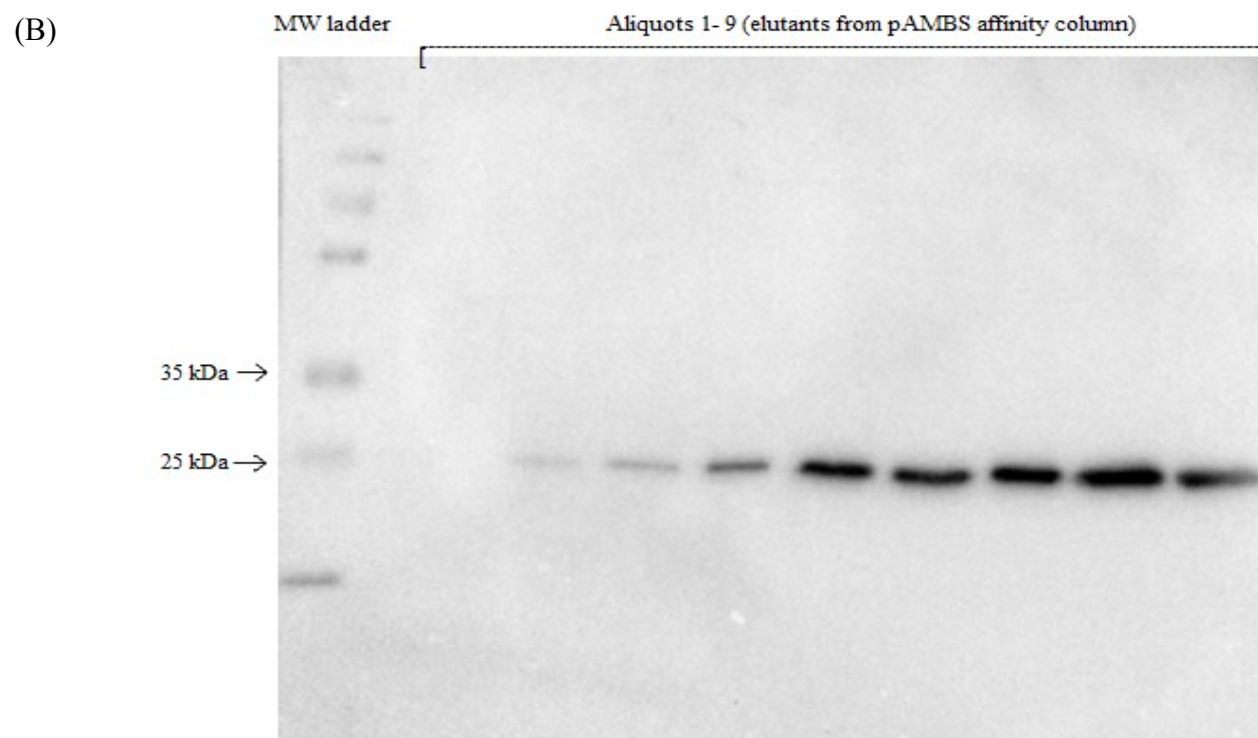
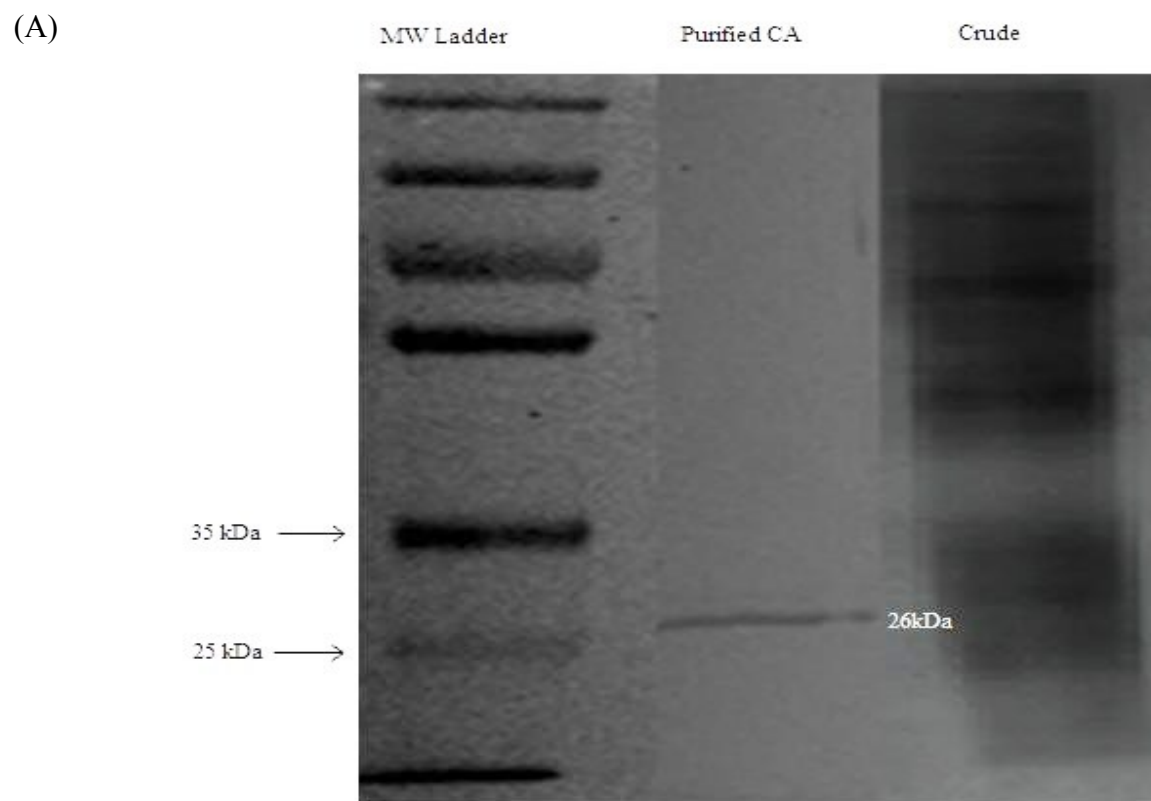


Figure 2-3. The effect of incubation conditions that stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the (A) phosphorylation status and (B) activity of rainbow trout, *Oncorhynchus mykiss*, gill carbonic anhydrase. In panel A, band intensities for phosphoprotein staining (Pro-Q Diamond) were standardized against the corresponding intensity for Sypro Ruby stain, and values for kinase or phosphatase stimulation were then standardized to the endogenous value; the figure presents mean values \pm SEM, $N = 6$. The inset image presents representative bands for one sample. An asterisk (*) denotes a treatment in which the relative intensity was significantly different from one, which is marked on the figure by the dashed line (one-sample Student's t -test, $P = 0.0368$ for kinase and > 0.05 for phosphatase). Values in panel B are means \pm SEM, $N = 6$. Treatment groups that do not share a letter were significantly different from one another (one way RM ANOVA, $P < 0.001$).

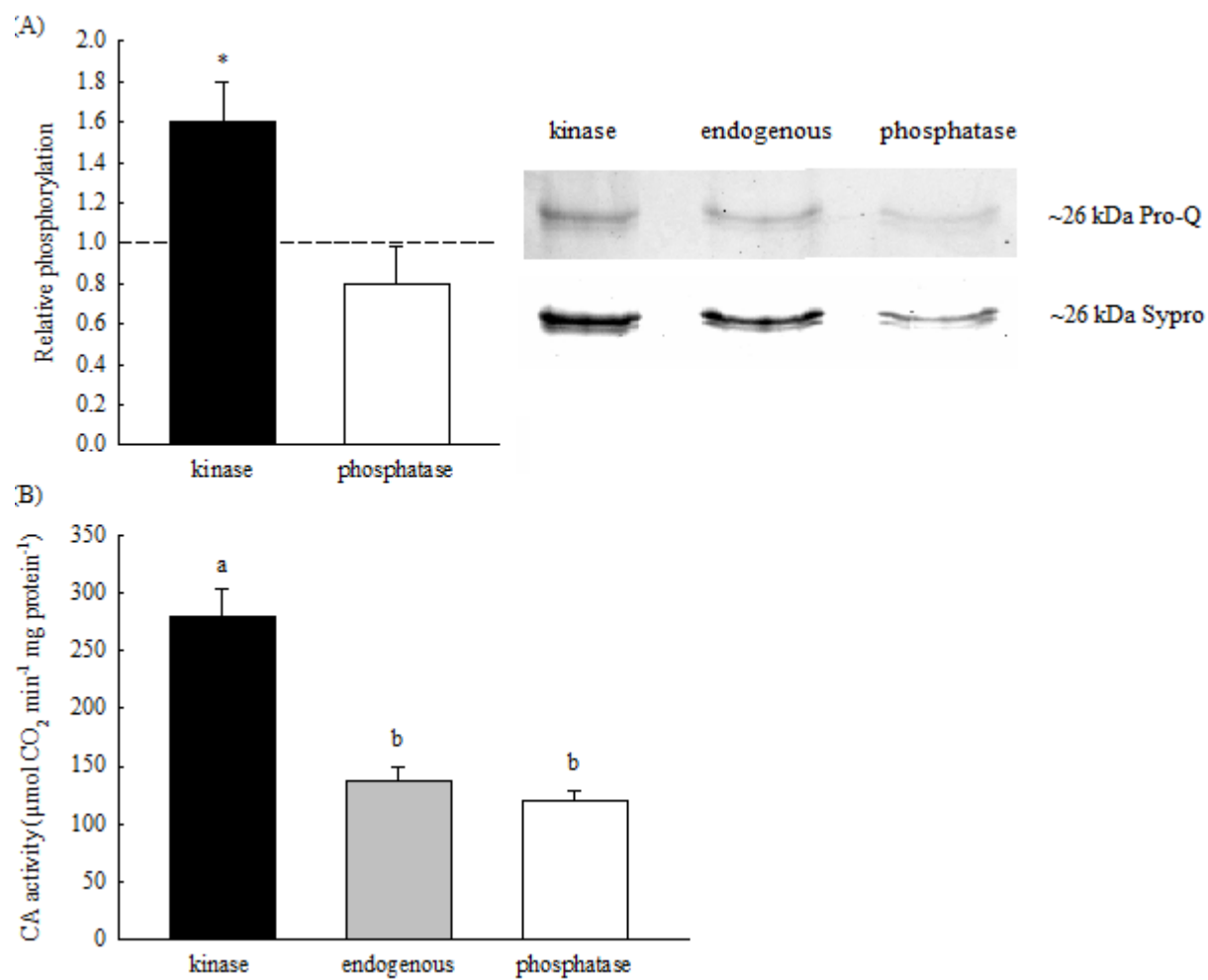


Figure 2-4. The effect of incubation conditions that stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the phosphorylation state of rainbow trout, *Oncorhynchus mykiss*, gill CA. Western blotting was carried out using phospho-specific antibodies against (A) phospho-threonine, (B) phospho-serine, and (C) phospho-tyrosine. Phospho-specific bands were normalized to the corresponding band detected by a trout CAC antibody. Data are mean values \pm SEM, $N = 4$. Treatment groups that do not share a letter were significantly different from one another (one way RM ANOVA, $P = 0.001$ for panel A, 0.764 for panel B, and 0.094 for panel C).

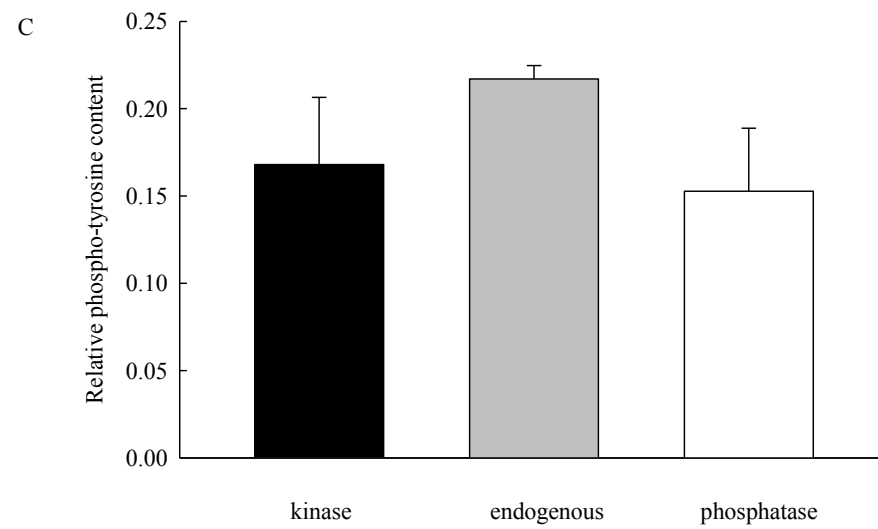
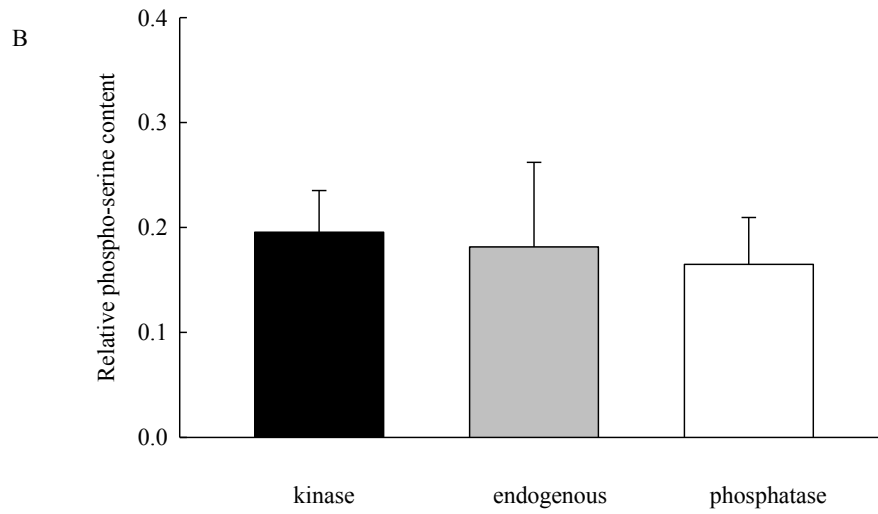
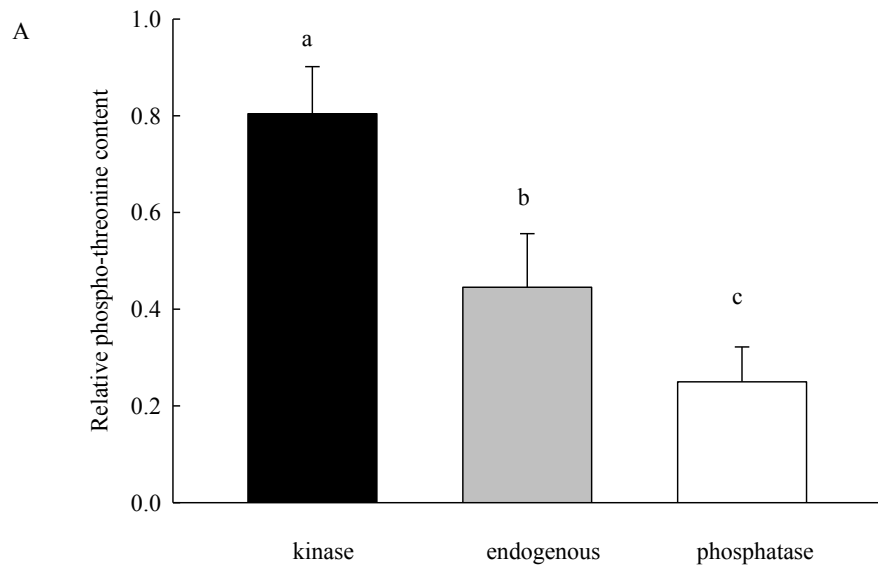


Figure 2-5. The effect of incubation conditions that stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on kinetic parameters of rainbow trout, *Oncorhynchus mykiss*, gill CA, specifically (A) turnover number (k_{cat}), (B) substrate affinity (K_m), (C) enzyme efficiency (V_{max}/K_m) and (D) the inhibition constant (K_i) for acetazolamide. Data are mean values \pm SEM, with $N = 3$ (panels A, B and C) or 4 (panel D). Treatment groups that do not share a letter are significantly different from one another (one-way RM ANOVA, $P = 0.023, 0.007, 0.005$ and 0.007 for panels A-D, respectively).

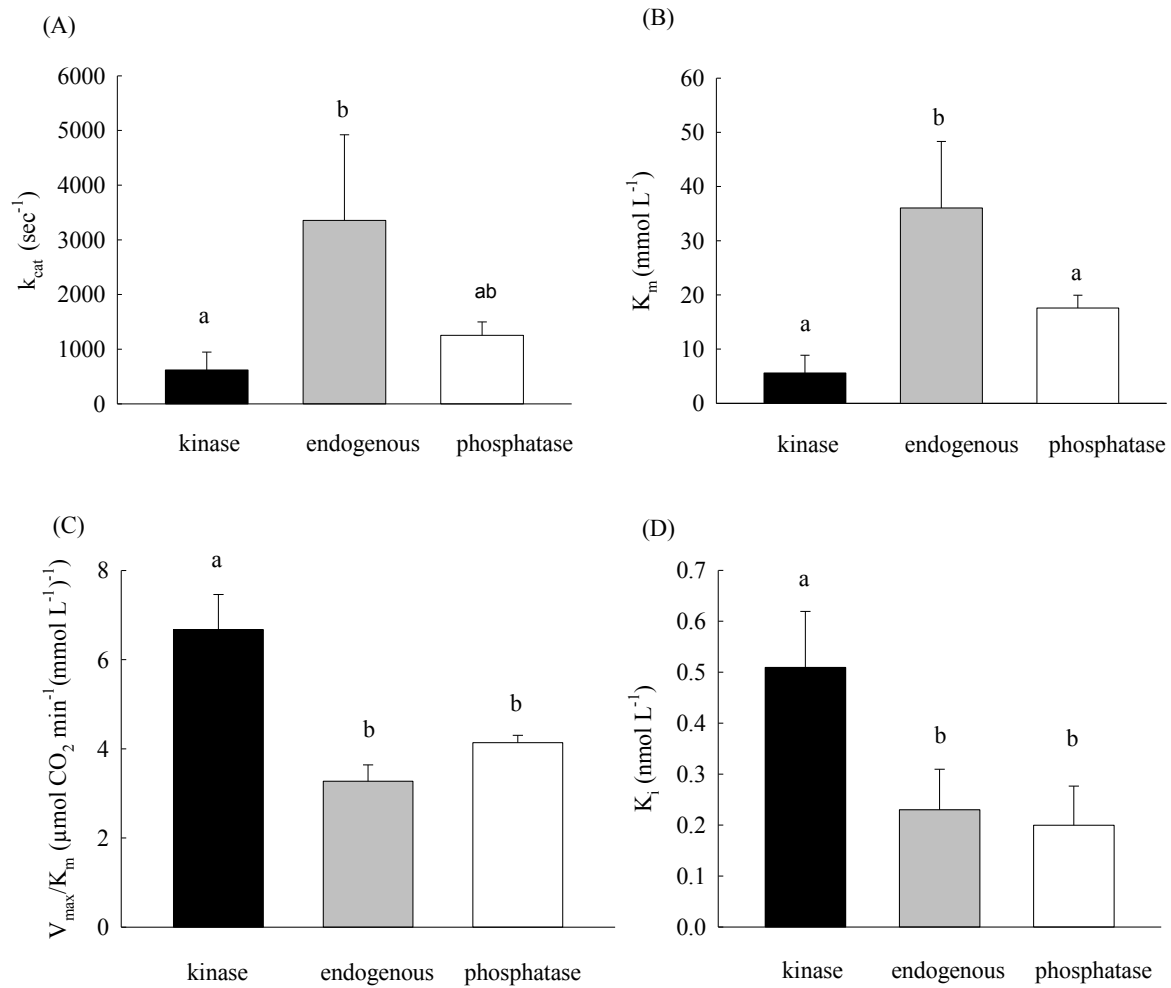


Figure 2-6. The effect of incubation conditions that stimulated specific protein kinases (protein kinase A, PKA; protein kinase C, PKC; protein kinase G, PKG; and AMP-activated protein kinase, AMPK) relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the (A) activity and (B) inhibition constant against acetazolamide (K_i) of rainbow trout, *Oncorhynchus mykiss*, gill CA. Data are mean values \pm SEM; in panel A, $N = 5$ except PKC where $N = 4$ and in panel B, $N = 4$. An asterisk indicates a value that is significantly different from that for the endogenous incubation conditions (one-way RM ANOVA, $P < 0.001$ for panel A and 0.992 for panel B).

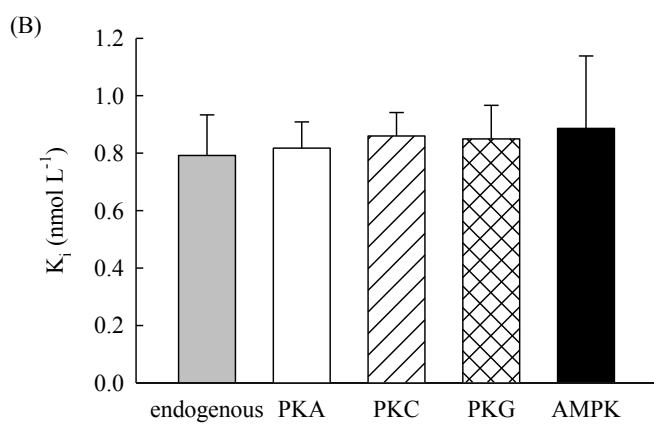
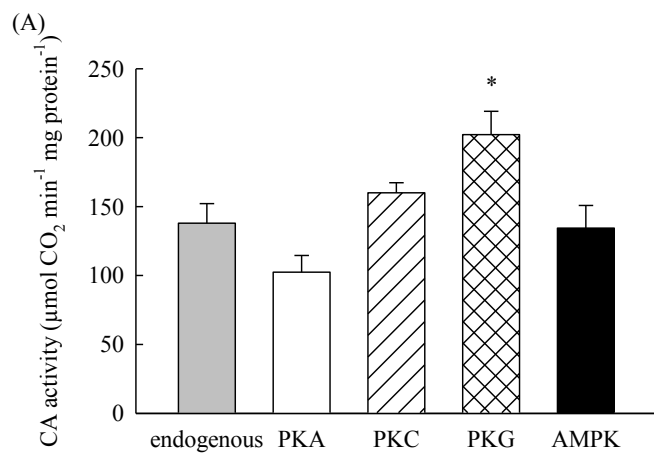


Figure 2-7. The effect of incubation conditions that stimulated specific protein kinases (protein kinase A, PKA; protein kinase C, PKC; protein kinase G, PKG; and AMP-activated protein kinase, AMPK) relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous status ('endogenous') on the (A) turnover number, k_{cat} , (B) substrate affinity constant, K_m , and (C) enzyme efficiency, V_{max}/K_m of rainbow trout, *Oncorhynchus mykiss*, gill CA. Data are mean values \pm SEM; $N = 4$ throughout. An asterisk indicates a value that is significantly different from that for the endogenous incubation conditions (one-way RM ANOVA, $P = 0.01$ for panel A, 0.035 for panel B and 0.051 for panel C).

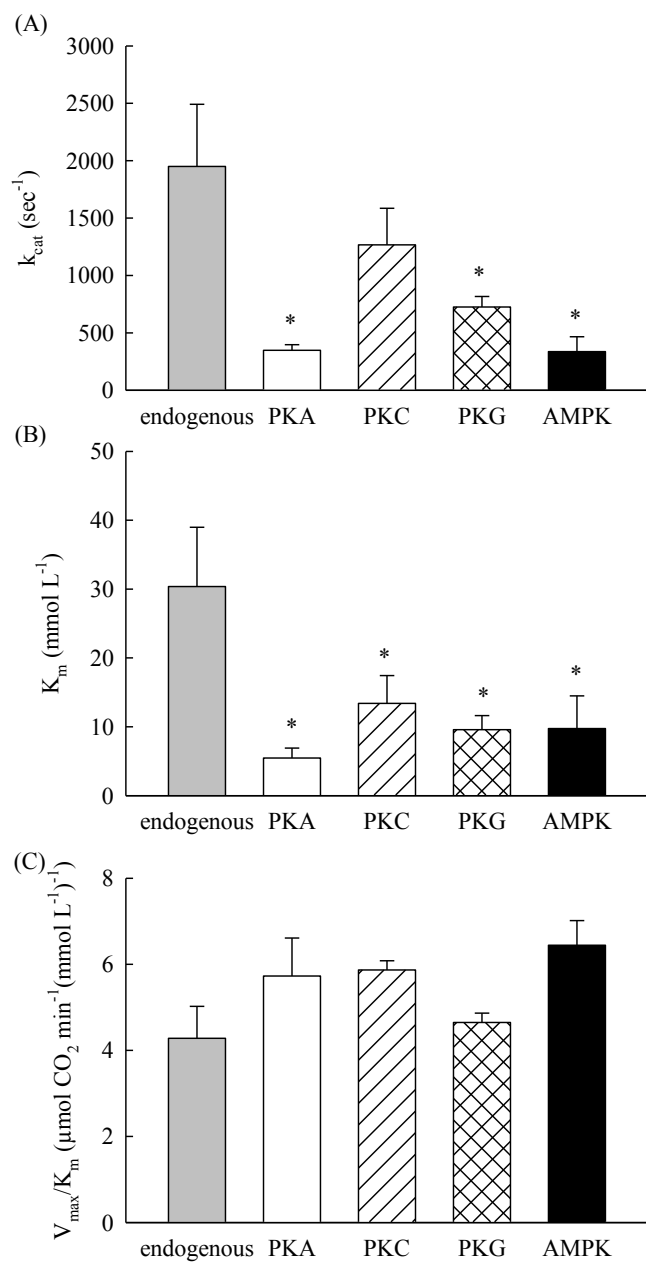
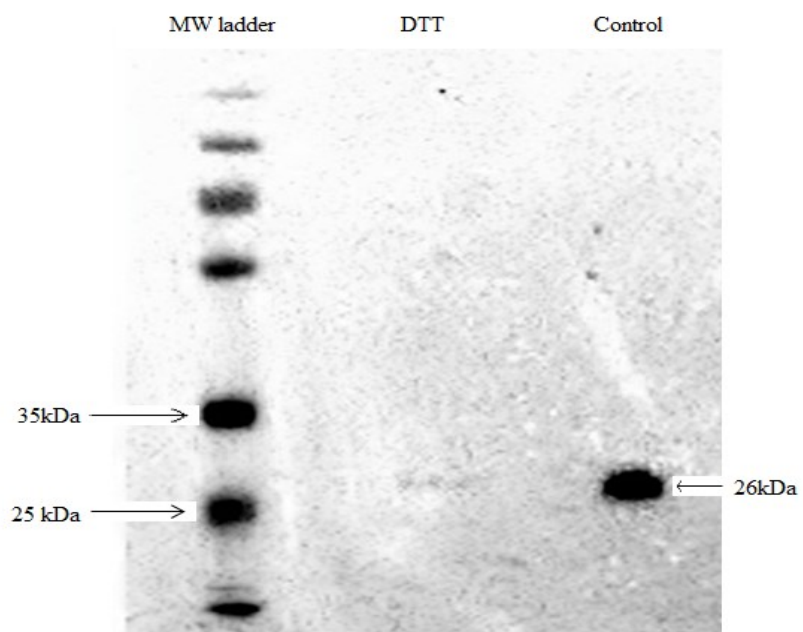


Figure 2-8. Glutathionylation of gill CA. (A) Detection of S-glutathionylation using an anti-GSH antibody before and after incubation with 100 mM DTT. (B) The effect of deglutathionylation (i.e. incubation with 100 mM DTT) on CA activity. Data are mean values \pm SEM, $N = 5$. No significant difference between control and DTT incubation conditions was detected (paired Student's t -test, $P = 0.189$).

(A)



(B)

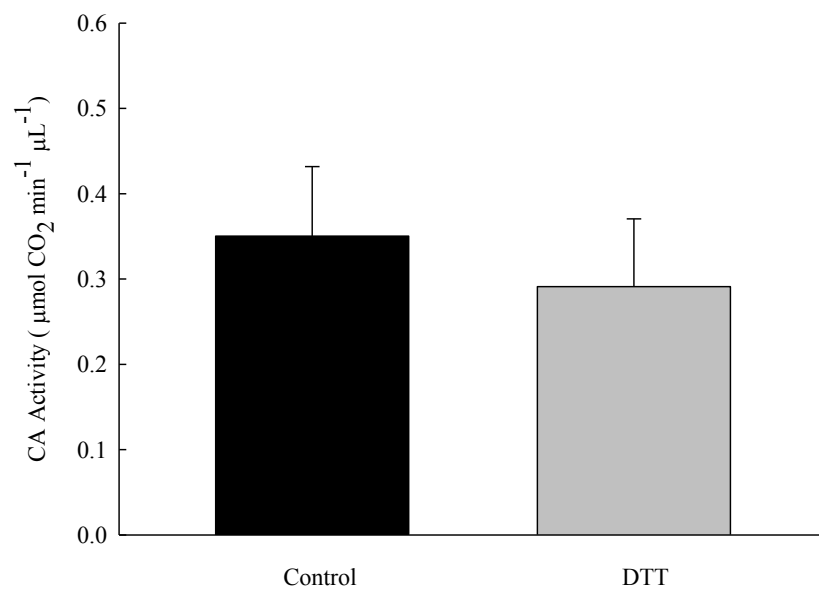
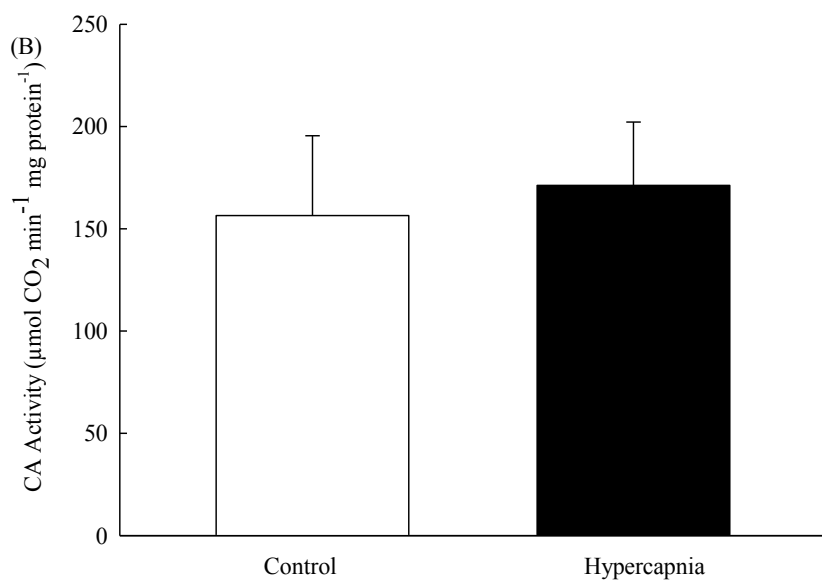
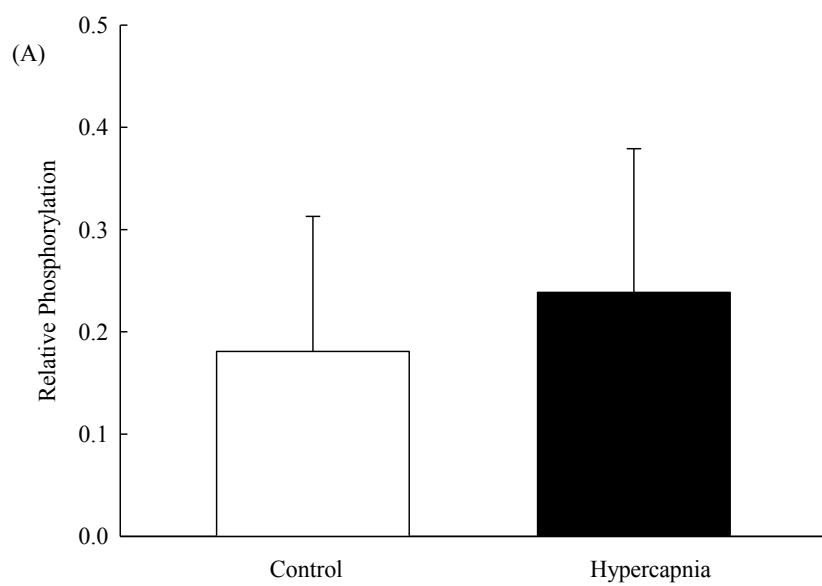


Figure 2-9. The effect of exposure to hypercapnia (1% CO₂) on (A) relative CA phosphorylation status ($N = 6$), and (B) CA activity ($N = 4$). Band intensities for phosphoprotein staining were standardized against the corresponding intensity of Sypro Ruby staining for the same band. Data are mean values \pm SEM. No statistically significant effects of hypercapnic exposure were detected (Student's t -test, $P > 0.05$ in both cases).



2.4 Discussion

The findings of the present investigation strongly suggest that cytoplasmic CA in rainbow trout can be regulated by PTM. Analysis of the trout gill CAc amino acid sequence identified threonine, serine and tyrosine residues with a high possibility for phosphorylation. Incubation of gill homogenates under kinase-stimulating conditions promoted phosphorylation of trout CAc, specifically, phosphorylation at threonine residue(s). Kinase-stimulating conditions also increased CA enzyme activity, by increasing the efficiency of the enzyme. Incubation conditions designed to enhance activity of the serine/threonine kinase PKG similarly increased CA activity. The finding that phosphorylation increased CA activity suggests that there is a transduction pathway present to phosphorylate CA in response to some as yet unknown stimulus.

The *in silico* analysis identified 14 predicted phosphorylation sites in the amino acid sequence of tCAc. Collectively, this protein contains 22 serine, 17 threonine and 9 tyrosine residues; of these, 7 serine, 3 threonine and 9 tyrosine residues were identified as having a high probability of phosphorylation. Of the residues predicted to undergo phosphorylation, only a single residue, serine 29, is expected to be located within the active site of tCAc. However, several of the predicted phospho-sites are in proximity to an active site amino acid residue. The catalytic mechanism of CA relies on an electron rich hydroxide ion coordinated to the central zinc ion, which acts as a nucleophile, attacking CO₂ (see Lindskog and Silverman, 2000). This addition reaction results in HCO₃⁻ bound to the zinc ion, which is subsequently displaced by a water molecule, and leaves the enzyme inactive (Lindskog and Silverman, 2000). The active form of the enzyme is re-established by a proton transfer, regenerating the nucleophilic hydroxide (Lindskog and Silverman, 2000). The removal of the proton appears to be the rate

limiting step, and higher activity enzymes accelerate this step with a histidine residue promoting the proton transfer (Gilmour, 2012). Phosphorylation changes the electrostatic environment near the modified amino acid residue owing to the addition of negative charge (Tarrant and Cole, 2009). Sites of phosphorylation within or neighbouring to the active site pocket might be expected to affect enzyme kinetics by altering the physical shape or electrostatic environment of the active site.

Similarly to previous studies that used *p*-aminomethylbenzene-sulphonamide to purify CA (e.g. Whitney, 1974; Maffia et al, 2002; Rizello et al, 2007), the approach permitted isolation of a homogenous preparation of tCAc as confirmed using SDS PAGE to visualize a single band, which was subsequently confirmed to be tCAc by western analysis. At ~26 kDa, this band was slightly smaller than the tCAc band at 29 kDa in a previous study (Georgalis et al, 2006b). This discrepancy may have arisen due to differences in preparing protein samples for western analysis. As in other studies investigating reversible phosphorylation (e.g. Bell and Storey, 2010; Dieni and Storey, 2010; Dawson et al, 2013), the present study relied on endogenous kinases and phosphatases present in the prepared enzyme extract to modify CA phosphorylation state. The incubation mixtures allow kinases or phosphatases to function, but do not vigorously stimulate their action. However, the changes observed in phosphorylation state and activity in the present study indicated that the kinases and phosphatases present in the incubation mixtures were sufficiently active to elicit changes. Incubations that stimulated kinases significantly increased tCAc phosphorylation content from the endogenous state, and use of phospho-specific antibodies implicated threonine sites as the most likely to be involved in regulation of CA activity by phosphorylation. However, incubations designed to allow phosphatases to be active did not reduce overall phosphorylation status, although a reduction in threonine phosphorylation

specifically was detected. Although not significantly different among treatments, purified CA was immuno-positive for both phospho-tyrosine and phospho-serine. Therefore, tCAc may contain multiple types of phospho-residues, and this factor could account for the difference observed between total phosphoprotein content and phospho-threonine content under phosphatase-stimulating incubation conditions. Because phospho-tyrosine and phospho-serine did not change during the kinase and phosphatase treatments, the change in phospho-threonine content might not have been sufficient to be detected as a significant difference in total content of phosphorylated CA.

Phosphorylation of trout gill CAc was associated with significantly higher enzyme activity, and to probe the basis of this effect, the kinetic properties and inhibitor sensitivity of trout gill CAc were examined following incubation under kinase-stimulating and phosphatase-stimulating conditions. The measured k_{cat} value for endogenous tCAc in the present study was lower (~10-fold), while the K_m was ~4-fold higher than published values for trout CAc (Esbaugh et al, 2005). These differences may be related to procedural variations in preparing the enzyme for CA assays. Substantial differences in kinetic constants for a single species are present in the literature (e.g. Maren et al, 1980; Esbaugh et al, 2004; Esbaugh et al, 2005). Surprisingly, the turnover value k_{cat} , a measure of catalytic activity (Maren, 1967) of phosphorylated CAc was significantly lower than that of the endogenous enzyme, even though enzyme activity was higher following incubation under kinase-stimulating conditions. However, phosphorylation also led to a significantly lower substrate affinity constant (K_m), indicative of higher affinity of the enzyme for its substrate (CO_2), and examination of enzyme efficiency (V_{max}/K_m ; Bell and Storey, 2010) revealed that it was significantly higher for phosphorylated CAc, probably accounting for the higher enzyme activity. Phosphorylation of trout CAc also increased its resistance to inhibition

by acetazolamide, as indicated by the significantly greater inhibition constant following kinase-stimulation. It is highly unlikely that the phosphorylation state of tCAc changed during addition to the CA reaction medium. The medium is a simple buffer at pH 7.4, and the chemical modulation of phosphorylated amino acids requires strongly alkaline conditions for β -elimination of the phosphate group (Meyer et al, 1986; Chen et al, 2010). Thus, the findings of the present study indicate that the increase in CA activity caused by phosphorylation results from an increase in enzyme efficiency.

Stimulation of endogenous phosphatases also tended to lower k_{cat} , although not significantly. This finding is difficult to interpret because kinase- and phosphatase-stimulation were expected to produce opposite effects on k_{cat} . Similarly, the substrate affinity constant of phosphatase-stimulated CA was decreased significantly from the endogenous value. However, neither enzyme efficiency nor the inhibition constant for acetazolamide were affected by phosphatase-stimulating conditions. Although phosphatase-stimulating conditions did not appear to affect overall phosphorylation status of trout gill CAc, this incubation condition was associated with significantly lower threonine phosphorylation. It remains unclear, however, how similar changes in turnover number and substrate affinity constant could be achieved by opposite changes in the phosphorylation status of threonine residues.

Modulation of protein phosphorylation state *in vivo* is elicited by the action of specific signal transduction pathways (Olsen et al, 2006). Phospho-residue specific antibodies identified a significant difference in relative phospho-threonine content between the *in vitro* incubation groups. Consequently, pathways that activate serine/threonine kinases were investigated. The results of several other preceding studies supported PKA-mediated phosphorylation of CA (Narumi and Miyamoto, 1974; Bersimbaev et al, 1975; Ditte et al, 2011). However, the results

of the present study suggest that PKG is a likely candidate for the phosphorylation of trout gill CAC and the associated increase in CA activity. Following PKG stimulation, CA activity was significantly increased relative to the endogenous conditions. Additionally PKG lowered k_{cat} and K_m , as did kinase-stimulating conditions, but this did not lead to an increase in efficiency. Many of the specific conditions lowered k_{cat} and K_m from the endogenous state, but none affected efficiency or K_i . This may indicate that the action of more than one kinase is necessary to produce the changes observed. Additional caution must be used in interpreting data from kinase-specific incubation conditions. Although the incubation conditions were designed to preferentially stimulate a particular kinase, other kinases were not inhibited.

To test the possible significance of phosphorylation as a regulatory mechanism in allowing fish to respond to an acid-base disturbance, rainbow trout were acutely exposed to hypercapnia. However, no significant differences in CA activity or relative phosphorylation were detected as a result of hypercapnic exposure. It is possible that hypercapnia does not stimulate changes in CA phosphorylation status and/or that the exposure time chosen (4 h) was inappropriate. Alternatively, the focus on whole gill homogenates may have been problematic. Current models of ionic and acid–base regulation for the rainbow trout gill postulate the existence of base-secreting ionocytes that take up Cl^- (the peanut lectin agglutinin-positive mitochondrion-rich cell or PNA^+ -MR cell), and acid-secreting ionocytes that take up Na^+ (PNA^- -MR cell) (Goss et al, 2001; Galvez et al, 2002; see Evans et al, 2005; Perry and Gilmour, 2006; Tresguerres et al, 2006). It is possible that only the CAC of a specific ionocyte is phosphorylated to increase enzyme activity during a particular acid-base disturbance. For example, activation of a signalling pathway during acidosis may lead to the phosphorylation of CAC in acid-secreting ionocytes, and dephosphorylation in base-secreting cells. Such cell-specific responses would be

difficult to detect using whole-gill homogenates. Similar arguments apply to studies that have attempted to detect changes in CA transcription and/or protein levels in response to acid-base challenges (reviewed by Gilmour and Perry 2009). Clearly, identifying *in vivo* conditions under which phosphorylation occurs must be a high priority for future work.

In addition to providing evidence for phosphorylation of trout gill CAC, the results of the present study support the possibility that trout gill CAC may be glutathionylated.

Glutathionylation of cysteine residues has been described in several other CAs, including rat CA III, where C181 and C186 can be glutathionylated (Chai et al, 1991; Mallis et al, 2000, 2002), the red blood cell CA of tiger shark, where at least one of the eight cysteine residues can undergo glutathionylation (Maynard and Coleman, 1971; Bergenhem et al, 1986), and gill CAC of icefish (Rizello et al, 2007). In trout gill CAC, as in icefish CA (Rizello et al, 2007), no effect on enzyme activity of removing the glutathione adduct was detected. Deglutathionylated icefish CA was found to be more susceptible to oxidation with a resultant loss of enzyme activity than was the glutathionylated enzyme (Rizello et al, 2007). This finding suggests that glutathionylation of icefish CA may serve as a protective mechanism during periods of oxidative stress. Whether a similar protective effect of glutathionylation occurs in trout gill CAC remains to be determined. Future work might monitor the glutathione content of trout gill CAC following exposure to oxidative stress to investigate this possibility.

The findings of the present study indicate that trout gill CAC can undergo at least two PTMs; reversible phosphorylation and glutathionylation. The results further implicate reversible phosphorylation as a potential regulatory mechanism controlling trout gill CAC activity. Clearly a high priority for future work must be to establish the physiological situations in which phosphorylation regulates CA activity; these situations are expected to link phosphorylation of

trout gill CAc to a requirement for elevated CA activity and may occur in a cell type-specific fashion.

CHAPTER 3

Transcriptional regulation of carbonic anhydrase in zebrafish, *Danio rerio*

Contributions:

Jocelyn Black carried out the analysis of the zCAc promoter region for GREs, and amplification by PCR of the zCAc promoter region. The zCAc promoter-luciferase reporter gene construct was created by Tamara Rodela. All other experimental work was carried out by Daniel Carrie.

Abstract

Cortisol has been implicated as a regulator of compensatory responses designed to maintain pH. Carbonic anhydrase (CA) contributes to acid-base regulation by catalyzing the hydration of CO₂ to provide acid-base equivalents, H⁺ and HCO₃⁻. The present study tested the hypothesis that cortisol regulates the transcription of zebrafish cytoplasmic CA (zCAc) by acting through glucocorticoid response element (GRE) half sites in the promoter sequence of zCAc. A DNA construct consisting of the zCAc promoter linked to a luciferase reporter gene was microinjected into zebrafish embryos at the one cell stage. Relative luciferase activity was significantly increased by 3.6-fold in microinjected larvae sampled at 72 hours post-fertilization (hpf) after 24 h of cortisol treatment, and by 6.3-fold in larvae sampled at 96 hpf after 48 h of cortisol exposure. However, no effect of 24 h of cortisol treatment was detected at 48 hpf, whereas relative luciferase activity was decreased significantly by 3.5-fold in microinjected larvae treated with cortisol for 24 h and sampled at 96 hpf. Despite positive results in the reporter gene assay, zCAc protein levels and CA enzyme activity in wild-type larvae sampled at 96 hpf were not affected by 48 h of cortisol treatment. Collectively, these results suggest that the potential for transcriptional regulation of zCAc by cortisol exists.

3.1 Introduction

Acid-base balance in fish is maintained by fine-tuning the branchial excretion of H^+ and/or HCO_3^- ions, with a supporting role for renal net acid excretion (Claiborne et al, 2002; Evans et al, 2005; Perry and Gilmour, 2006; Gilmour and Perry, 2009). Carbonic anhydrase has an important role in acid-base regulation because it catalyzes the reversible reactions, $H_2O + CO_2 \leftrightarrow HCO_3^- + H^+$. The hydration reaction provides the acid-base equivalents required by ion transporters for compensatory excretion processes at the gill and kidney (Gilmour and Perry, 2009). At the gill, cytosolic CA contributes H^+ and HCO_3^- for export to the water in exchange for the uptake of Na^+ and Cl^- , respectively (Claiborne et al, 2002; Marshall, 2002; Perry et al, 2003; Evans et al, 2005). At the kidney, cytosolic and membrane-bound CA isoforms have been implicated in HCO_3^- reabsorption and urine acidification (Georgalis et al, 2006b; see Gilmour and Perry, 2009). Because of its involvement in providing the acid-base equivalents necessary for compensation, CA is expected to be regulated during acid-base disturbances. However, the mechanisms responsible for regulation of CA during acid-base challenges remain unclear.

Several studies on rainbow trout (*Oncorhynchus mykiss*) reported changes in CA mRNA abundance in response to acid-base challenges, suggesting that transcriptional regulation of CA occurs. For example, the mRNA abundances of gill and renal cytosolic CA (tCAc) increased significantly at 3 h of exposure to hypercapnia (Georgalis et al, 2006a,b). After 24 h of acid infusion, branchial tCAc mRNA abundance was significantly lower, and renal tCAc mRNA abundance significantly higher, than the control value; 24 h of base infusion was associated with a significant increase in the mRNA abundance of gill tCAc (Gilmour et al, 2011). Cortisol levels also were increased during some acid-base challenges, and cortisol treatment increased branchial

CA activity, suggesting cortisol as a potential regulator of CA (Gilmour et al, 2011). Cortisol is well-known as a transcriptional regulator of enzymatic function in both mammals and fish (Schoneveld et al, 2004; Mommsen et al, 1999). Previous work has implicated cortisol in the regulation of numerous physiological processes including metabolism (Vijayan et al, 1994, 1996a, 1996b, 1997), maintenance of ionic homeostasis (McCormick, 1995), and immune function (Wendelaar Bonga, 1997). For example, cortisol can act as a salt water-adapting hormone by stimulating gill Na^+/K^+ -ATPase activity through increases in the mRNA expression of the α -subunit of Na^+/K^+ -ATPase (Madsen et al, 1995; see McCormick, 1995).

The genomic effects of cortisol in fish are mediated through cytoplasmic receptors that act as ligand-inducible transcription factors (reviewed by Bury and Sturm, 2007). Binding of cortisol to its receptor allows nuclear translocation and the creation of a homodimer with a second cortisol-receptor complex. The homodimer then interacts with a specific DNA motif, a glucocorticoid response element (GRE) that is an imperfect palindrome, GGTACA nnn TGTTCT. Binding of the homodimer to the GRE facilitates the recruitment of additional nuclear factors, resulting in changes in the transcription of target genes (Bury and Sturm, 2007). Although the available evidence for rainbow trout suggests that cortisol may play a role in regulating the transcription of CA during acid-base challenges in fish (Gilmour et al, 2011), this hypothesis has not been tested directly. Several previous investigations have used the presence of GREs within the promoter region of a gene of interest together with a reporter gene assay to implicate cortisol as a transcriptional regulator of a gene (Schulte et al, 2000; Esbaugh and Walsh, 2009; Rodela et al, 2011). A construct consisting of the promoter of the gene of interest coupled to a reporter gene (luciferase) was injected into the liver of the experimental species,

which was then treated with cortisol or exposed to a stressor to elevate endogenous cortisol levels.

The goal of the present study was to directly determine whether cortisol regulates CAC expression in the zebrafish *Danio rerio*. Zebrafish were chosen owing to the availability for this species of the genomic and molecular tools necessary to produce and utilise a reporter gene construct for the zCAC promoter. Zebrafish express a number of CA isoforms, including a cytosolic isoform [variously termed zCAC (Gilmour et al, 2009) or zCA2-like a (Lin et al, 2008)] that is involved in proton extrusion in embryonic and larval zebrafish (Lin et al, 2008). Moreover, zebrafish larvae exposed to water of pH 4 for 24 h exhibited elevated whole-body cortisol levels (Kumai et al, 2012), and adult zebrafish fed cortisol-supplemented food for 5 d exhibited an increase in branchial zCAC mRNA abundance (J Black & KM Gilmour, unpublished data). These observations lend support to the hypothesis that zCAC is transcriptionally regulated by cortisol, which is elevated in response to an acid-base challenge. If cortisol regulates CA expression, then GREs would be predicted to be present in the promoter region of zCAC; when the promoter is linked to a reporter gene (luciferase) and activated by cortisol bound to a glucocorticoid receptor (GR), activation of the reporter should result. Finally, cortisol treatment of wild-type zebrafish larvae should result in increases in zCAC transcript abundance, zCAC protein levels, and CA activity.

3.2 Materials and methods

3.2.1 *Experimental animals*

Adult zebrafish (*Danio rerio*) were obtained from a commercial supplier (Big Al's, Montreal, QC, Canada). Fish were housed in the University of Ottawa Aquatic Care Facility in plastic 4 L aquaria (~15 fish per aquarium) supplied with a constant flow of aerated, dechloraminated city of Ottawa tap water at 28°C. Fish were fed daily to satiation with commercial feed (No. 1 crumble-Zeigler; Aquatic Habitats, Apopka, FL, USA) and kept on a 14 h:10 h light:dark cycle. Zebrafish were housed under these conditions for at least 2 weeks before experimentation. Newly spawned eggs were collected from breeding tanks containing equal numbers of male and female fish, according to standard methods (Westerfield, 2000). Collected embryos were reared in 50 mL petri dishes of dechloraminated city of Ottawa tap water supplemented with 0.05% ethylene blue and held in incubators set at 28.5°C. Daily water changes were carried out together with the removal of dead embryos. All experiments were terminated by 96 hours post-fertilization (hpf), and therefore fish were not fed during experiments. All experiments complied with University of Ottawa institutional guidelines, were approved by the Animal Care Committee (protocol BL-228), and were in accordance with the guidelines of the Canadian Council on Animal Care for the use of animals in research and teaching.

3.2.2 *Development of a zCac reporter assay*

To determine whether cortisol influences putative GREs in the promoter region of zCac to modulate zCac expression, an *in vivo* reporter assay was used. The general approach was that

developed by Schulte et al (2000) for killifish and subsequently used in toadfish (Esbaugh and Walsh, 2009; Rodela et al, 2011), but the present study focused on zebrafish embryos and larvae; previous work using this approach utilized adult fish.

Identification of GREs within the zCac promoter. The entire genomic sequence of zCac (ENSDART00000038364) was obtained from the ENSEMBL database, and a 1000 bp fragment upstream of the zCac translation start site on chromosome 24 was analyzed using the online Transcriptional Element Search System (TESS; <http://www.cbil.upenn.edu/cgi-bin/tess/tess>) to identify putative transcription factor binding sites. Specifically, the program was used to identify putative GRE half sites (i.e. AGAACA or TGTTCT). Analysis was focused on half-palindrome sequences because of variation in the “nnn” nucleotides making up the classical palindrome, 5'-AGAACA nnn TGTTCT- 3'. Although no complete GRE palindrome was identified within the zCac promoter region (Fig. 3-1), the effects of cortisol may be mediated through GRE half-sites or even glucocorticoid response units (GRUs) that are composed of several GRE half-sites (Schoneveld et al, 2004).

Creation of a zCac reporter construct. Genomic DNA was extracted from zebrafish gill using a standard phenol/chloroform protocol. Using primers 5'-GTT VTG TAA TAG CAG ATT TTT GCA TCA TTG-3' (forward) and 5'-TCC GCC ACG TGT GCT GCA GTC CTC AAG-3' (reverse), an 887 bp fragment of the promoter region upstream of the zCac translation start site was amplified by PCR. Gel-purified PCR products (Minelute gel extraction kit, Qiagen) were ligated into a pDrive vector (Qiagen, Mississauga, ON, Canada) and transformed in chemically

competent DH5 α *Escherichia coli* (Invitrogen, Eugene, OR, USA). The insert in the resulting plasmids was verified by sequencing.

The luciferase reporter assay construct was generated by adding BglII (Fermentas, Burlington, ON, Canada) and HindIII (Fermentas) restriction sites via PCR to the zCAc promoter [using primers 5'-AGA TCT GTA ATA GGA TAT TTT TGC ATC ATT G-3' (forward) and 5'-AAG CTT TCC GCC AGC TGT GCT GCA GTC CTC AAG-3' (reverse)]. The construct with the added restriction sites was gel purified (Minelute gel extraction kit, Qiagen), ligated into a pDrive vector (Qiagen), and transformed into DH5 α *E. coli* (Invitrogen). The plasmids were sequenced to confirm the addition of the restriction sites to the zCAc promoter fragment and to check that no errors were incorporated.

The new zCAc plasmid was linearized with restriction enzymes and sub-cloned into the luciferase PGL4.10 vector (Promega, Madison, WI, USA; Fig. 3-2) at the BglII and the HindIII restriction sites. The ligated plasmid was transformed into DH5 α *E. coli* (Invitrogen). The plasmids were sequenced prior to experimental use for the *in vivo* reporter assay. To create a sufficient yield of reporter construct (2-3 mg mL⁻¹) for *in vivo* injection, the zCAc reporter plasmid and a *Renilla* luciferase PGL4.75 plasmid (Promega) were isolated using a maxi-prep kit (Qiagen) with all procedures following the manufacturer's specifications. The PGL4.75 plasmid contained a cytomegalovirus promoter linked to *Renilla* luciferase and was used as a control for transfection efficiency.

3.2.3 *The regulatory role of the zCAc promoter*

Zebrafish embryos at the one cell stage were microinjected (model IM 300, Narishige, Long Island, NY) with 4 nL of a cocktail solution of the zCAc promoter construct (PGL4.10)

and a control construct; the final solution also contained 0.05% phenol red. The control PGL4.75 construct contained a cytomegalovirus promoter linked to *Renilla* luciferase, and was co-injected with the PGL4.10 construct to normalize for transfection efficiency. The zCAc PGL 4.10 construct was injected at a concentration of $1.5 \text{ ng } \mu\text{L}^{-1}$ (maximum concentration following mixing with control plasmid) while the control PGL 4.75 construct was injected at a concentration of $500 \text{ ng } \mu\text{L}^{-1}$ (the concentration necessary to produce measurable *Renilla* luciferase activity). The microinjected eggs were then incubated as described above until the desired developmental stage. No significant mortality was associated with microinjection, nor were obvious deformities observed in microinjected larvae.

Each batch of microinjected embryos was divided; half of the embryos were treated with cortisol [hydrocortisone 21-hemisuccinate sodium salt, Sigma, St. Louis, MO, USA; 500 nmol L^{-1} made up in embryo medium containing 0.05% dimethyl sulfoxide (DMSO) final concentration] while the other half was maintained in embryo medium without cortisol (control, contained 0.05% DMSO). This cortisol concentration (500 nmol L^{-1} or 240 ng mL^{-1}) was reported previously to significantly increase Na^+ uptake in zebrafish larvae (Kumai et al, 2012). Four different combinations of cortisol exposure/sampling time were examined; embryos were sampled at 48, 72 and 96 hpf following 24 h of cortisol exposure, or at 96 hpf following 48 h of cortisol exposure. At the end of each treatment, embryos (3 embryos per microcentrifuge tube = one N for the purposes of measuring luciferase activity; each N was derived from a separate batch of microinjected embryos) were washed twice in phosphate-buffered saline (PBS), flash frozen in liquid N_2 , and stored at -80°C until assayed for luciferase activity (stored no longer than 4 months).

To assay luciferase activity, samples were thawed on ice and then immediately homogenized in 50 μ L passive lysis buffer (Promega) using a handheld tissue homogenizer (Pellet Pestle Cordless Motor, Kimble-Chase, Vineland, NJ, USA). The homogenate was centrifuged twice at 13,000 g for 15 min, and the resulting supernatant was assayed according to the manufacturer's specifications for the Dual Luciferase Assay System (Promega, Madison, WI, USA) using an LMax-II luminometer (Molecular Devices, Sunnyvale, CA, USA). Data were expressed as normalized ratios of firefly luciferase (PGL4.10 construct) activity to *Renilla* luciferase (PGL4.75) activity, and reported in relative light units.

3.2.4 CA activity and zCAc protein levels following cortisol treatment

To assess the ability of cortisol to modulate zCAc protein levels and CA activity, wild-type embryos were raised to 48 hpf. Embryos from a single batch were then divided; half of the embryos were treated with cortisol (500 nmol L^{-1} , dissolved in 0.05% DMSO), while the other half was maintained in embryo medium without cortisol (control, containing 0.05% DMSO, as described above). Embryos (10 embryos per microcentrifuge tube = one N for the purposes of measuring CA activity or zCAc protein levels) were sampled at 96 hpf, i.e. following 48 h cortisol exposure, and were immediately homogenized in 100 μ L of CA assay reaction medium (in mM: 225 mannitol, 75 sucrose, 10 Tris-base, adjusted to pH 7.4 using 10% phosphoric acid), and centrifuged for 15 min at 10,000 g to remove cellular debris. The resulting supernatant was used for western blotting to measure zCAc protein levels, and in the electrometric Δ pH assay for measurement of CA activity.

Measurement of zCAc protein levels. Protein samples in CA assay buffer (50-75 µg protein) were fractionated by SDS-PAGE using 15% separating and 4% stacking polyacrylamide gels. Sample protein concentrations were measured using the bicinchoninic acid (BCA) protein assay with bovine serum albumin (BSA; Sigma) as the standard. All samples were assayed in a single gel. Fractionated proteins were transferred to nitrocellulose membranes (Bio-Rad, Mississauga, ON, Canada) using a Trans-Blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's instructions. After transfer, each membrane was blocked for 1 h at room temperature in Tris-buffered Tween 20 (TBS-T: 20 mM Tris, 300 mM NaCl and 0.1% Tween 20) containing 5% BSA. Membranes were then probed first with a 1:4,000 dilution of a custom anti-zCAc primary antibody (kindly provided by Dr. S.F. Perry) for 2 h at room temperature, and then with a 1:15,000 dilution of secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit IgG (Bio-Rad) for 1 h at room temperature. Following each incubation, membranes were washed for 3×10 min in TBS-T.

Immunoreactive bands were visualized by enhanced chemiluminescence (Luminata Classico Millipore Corporation, Billerica, MA, USA) using a digital gel documentation system (Chemi Doc XRS+; Bio-Rad) and digital image processing software (Image Lab; Bio-Rad). The protein size marker was obtained from Fermentas Life Sciences (Burlington, ON, Canada). To standardize the density of bands immunoreactive for CA, membranes probed for CA were stripped (ReBlot Plus Mild Antibody Stripping Solution, 10x; Millipore Corporation, Billerica, MA, USA) according to the manufacturer's instructions, and then blocked for 1 h in Tris-buffered Tween 20 (TBS-T) containing 5% BSA. Membranes were then probed first with a 1:4,000 dilution of anti-beta actin (Abcam, Toronto, ON, CA) for 2 h at room temperature and then with a 1:15,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (BioRad)

for 1 h at room temperature, after which immunoreactive bands were visualized by enhanced chemiluminescence as above. The density of the band probed for CA was determined and normalized to the density of the band probed for beta actin in the same lane.

Assessment of CA activity. CA activity was measured using the electrometric Δ pH method (Henry, 1991). Supernatants (25-50 μ L) were assayed for CA activity using 6 mL of CA assay reaction medium held at 4°C, and 200 μ L of ice-cold CO₂-saturated distilled water to initiate the reaction. The reaction velocity was measured over the initial 0.15 unit pH change. The true catalyzed rate was then obtained by subtracting the uncatalyzed rate from the observed rate. A pH electrode (GK2401C; Radiometer, London, ON, Canada) connected to a PHM84 pH meter (Radiometer) and data acquisition software (Biopac with AcqKnowledge v3.7.3 software; Harvard Apparatus Canada, Saint-Laurent, QC) was used to measure the pH of the reaction medium.

3.2.5 Statistical analyses

Data are reported as mean values \pm 1 standard error of the mean (SEM). To control for batch-to-batch differences in microinjection efficiency, paired Student's *t*-tests were used to compare relative luciferase activities between cortisol-treated and control larvae microinjected with the zCAc reporter construct. Similarly, paired Student's *t*-tests were used to compare zCAc protein levels and CA activity between cortisol-treated and control larvae. The fiducial limit of significance in all tests was 0.05. Statistical analyses were carried out using SigmaStat v3.5 (in SigmaPlot v11; SPSS).

```

GTAATAGGATATTTTTGCATCATTGCCTTTAAGGCGAATGACTGAATGATTATTTCAACA 60
ATCTATATTTTAAATCAATTAACAAATAATTAGCTTCTGACAAATAGTGCATCTGTAGAA 120
GTTTGAGTTCCATTGGTGAATGATTATTAACCTATATTAATTCTTATTATTTTATGTAAAT 180
ATGAGTGATCTTACAATTTTACCACTAATGAAAATACAAAATATATATATATATATATA 240
TATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATATTTTTTTT 300
TTTTTTTTTTTTTTTTTATAGTAAGATACAAAGCTGGCTATTAATAGTGCTTCTGTAAACG 360
CGTACGAAAATAGCCTCAATAGAACAAGTTAATAATACGCTACCCCTGTCTCCAACCACA 420
ACGTGTTTCGCTGTAAATCGTCAAATATTAGCCAAAGCAGCGCAGTGTTGCGTAAACTGC 480
CACTGAGTCCGCGCGCGGCTCGTTCACAGTGATGATGAATCATTACCGCACTCATTGTCA 540
TTACTATGTACACCCGTCATCGACGTCACCAGGACCAGGCGCGCCACAAGTCTCCGATTG 600
CGCAATCGCAGAATGTTCAGAACGTGTTTCGAATGTCGGCGTATCTGTAGATCAAAGCAG 660
CAGCGCGCAGATTCATAATGATGGAACCGCGCATTCCATGAGATGGATCGAGCGCGCGCG 720
TGAGGGGACAGGAGAGCGCGCATTATTGATCGCACGCGCGGCTCCTCCTGCTGATCACACTT 780
AATGTACGGTCATGTGGGCAAACAGCGGCTATATAAACCACAGTCTCATCTGTGTGAGGAC 840
ACTGACAGTGTTCAGGACACTTGAGGACTGCAGCACAGCTGGCGGA 887

```

Figure 3-1. An 887 nucleotide DNA sequence of the promoter region for zebrafish (*Danio rerio*) cytosolic CA, zCAc. Predicted **GRE half-sites** are highlighted in black with white text.

Predicted sites for several other transcription factors are also indicated; these include **C/EBP** in grey, **SP-1** in a solid outline, and **NF-1** in a dashed outline. These transcription factors are accessory factors required to mediate a glucocorticoid response because GRE half sites cannot act as a GRE acts to regulate transcription (Schoneveld et al, 2004).

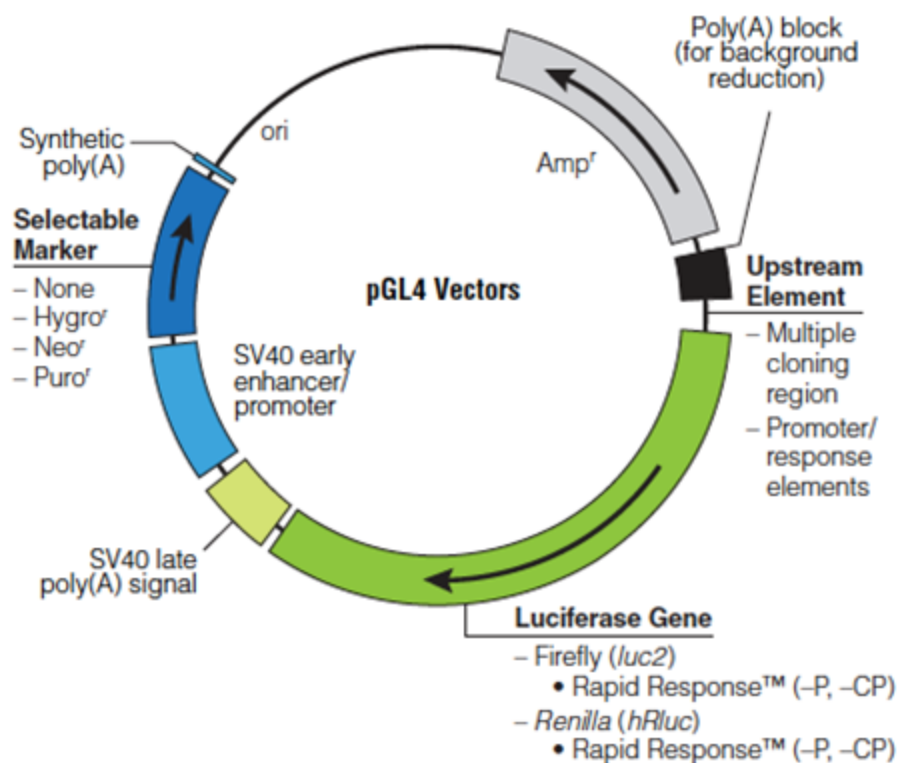


Figure 3-2. Features of a generic pGL4 Vector. The zCAc promoter was inserted upstream of the luciferase gene to couple luciferase activity with zCAc promoter activity (pGL4 Luciferase Reporter Vectors – Technical Manual – Promega;

<http://www.promega.ca/~media/Files/Resources/Protocols/Technical%20Manuals/0/pGL4%20Luciferase%20Reporter%20Vectors%20Protocol.pdf>).

3.3 Results

On the basis of previous work suggesting the presence of GRE half-sites in the 5' flanking region of zCAc (see above), functional analysis of the putative GRE sequences was carried out using an *in vivo* reporter assay. Luciferase activity was detected in all samples, and was significantly affected by cortisol treatment in larvae sampled at 72 and 96 hpf but not 48 hpf (paired *t*-test, $P = 0.156$) (Fig. 3-3). Relative luciferase activity was significantly greater than the control value in larvae treated with cortisol for 24 h and sampled at 72 hpf (paired *t*-test, $P = 0.013$), and in larvae treated with cortisol for 48 h and sampled at 96 hpf (signed rank sum test, $P = 0.002$). By contrast, relative luciferase activity was significantly less than the control value for larvae treated with cortisol for 24 h and sampled at 96 hpf (paired *t*-test, $P = 0.034$). To further pursue the apparent transcriptional regulation of CA in 96 hpf larvae after 48 h of cortisol treatment, wild-type larvae were treated with cortisol for 48 h and sampled at 96 hpf, and zCAc protein levels (by Western blot) and total CA activity were measured. Somewhat surprisingly given the ~6-fold difference in relative luciferase activity detected between control and cortisol-treated larvae with this treatment, no significant differences in zCAc protein levels (*t*-test, $P = 0.169$) or total CA activity (*t*-test, $P = 0.479$) were detected between control and cortisol-treated larvae (Fig. 3-4).

Figure 3-3. Effect of cortisol treatment (500 nmol L^{-1}) on mean relative luciferase activity (firefly/*Renilla*) for zebrafish (*Danio rerio*) larvae that were microinjected as one-cell embryos with the zebrafish cytosolic CA (zCAc) promoter construct. Larvae were sampled at 48 ($N = 5$), 72 ($N = 9$) or 96 ($N = 4$) hpf following 24 h of cortisol exposure, or at 96 hpf following 48 h of cortisol exposure ($N = 10$). Data are means + SEM, and an asterisk denotes a statistically significant difference from the control group in the same exposure (paired Student's *t*-test, $P < 0.05$; see text for details).

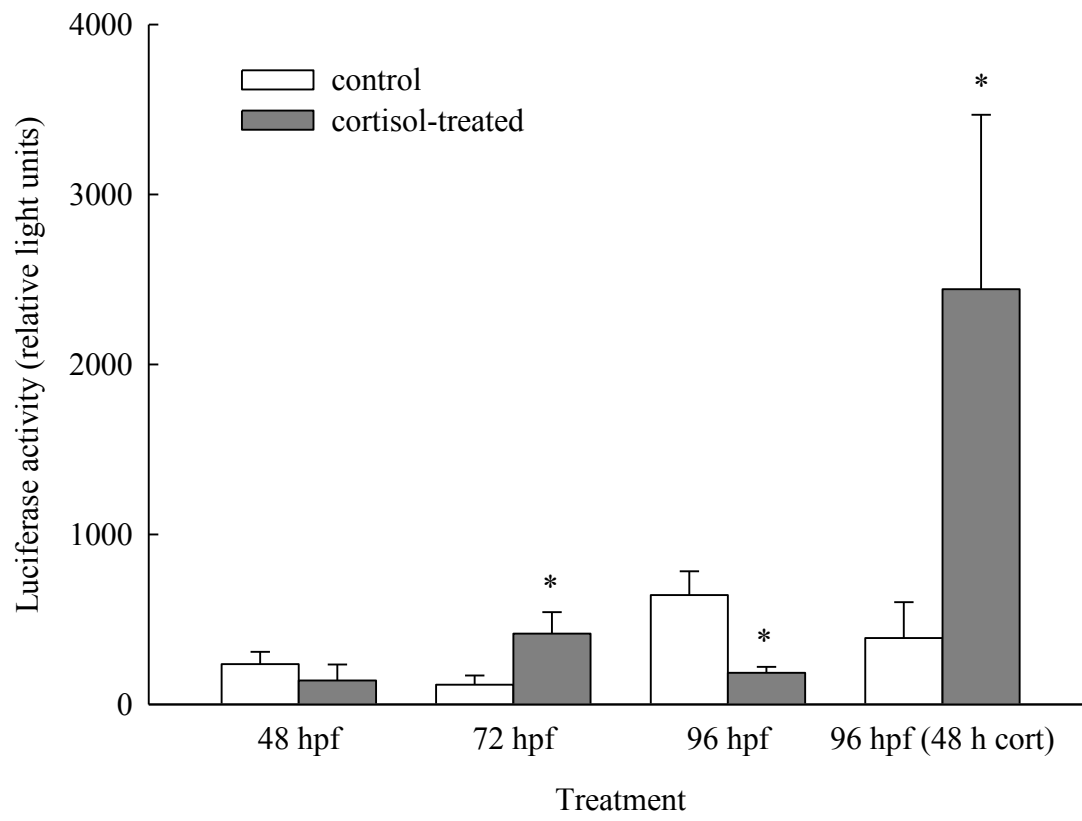
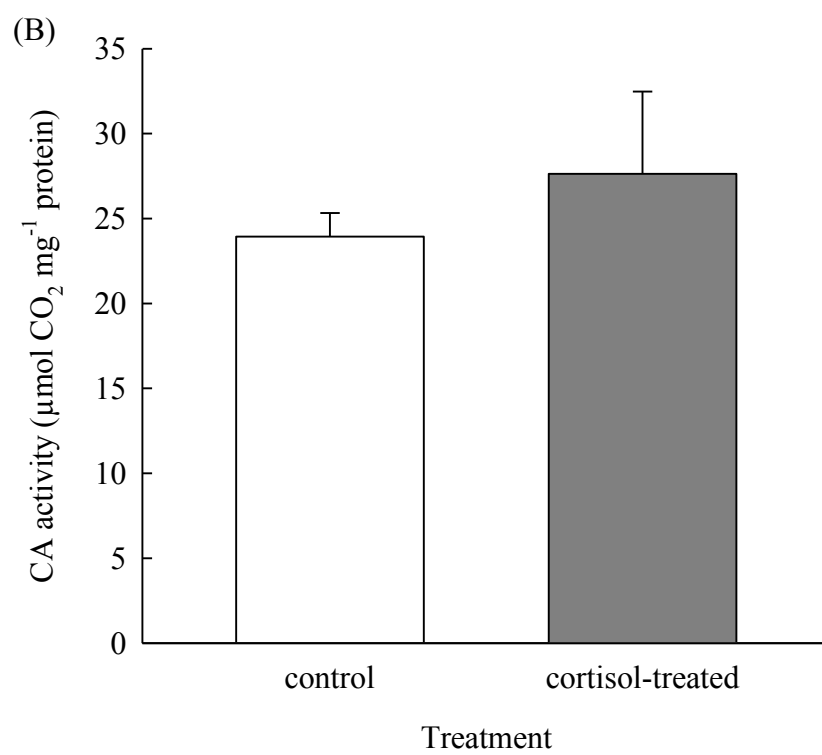
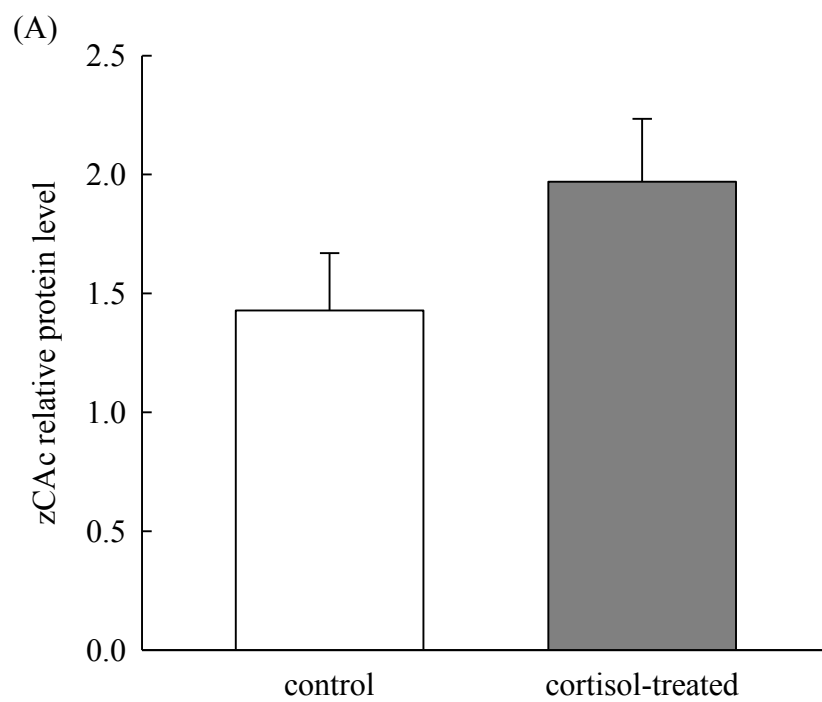


Figure 3-4. Effect of cortisol treatment (48 h exposure to 500 nmol L⁻¹ cortisol) on (A) zebrafish cytosolic CA (zCAc) relative protein levels and (B) total CA activity in 96 hpf zebrafish (*Danio rerio*) larvae. Values are means + SEM; *N* = 5 in panel A and 6 in panel B. No significant differences were detected between control and cortisol-treated larvae (paired *t*-tests, *P* > 0.05; see text for details).



3.4 Discussion

The results of the present study provide limited and conflicting evidence for the regulation of zCAc mRNA expression by cortisol through GRE $\frac{1}{2}$ s. Although the results of an *in vivo* reporter gene assay suggested that 48 h of cortisol treatment should result in significantly higher CA expression in 96 hpf zebrafish larvae, neither zCAc protein levels nor total CA activity were found to be elevated in wild-type larvae under these conditions. This difference might reflect intrinsic mechanisms present to regulate CA protein levels that are not active against the luciferase protein. For example, the mRNA transcript of zCAc might be subject to more stringent degradation compared to the transcript for the luciferase protein. Reporter mRNA and protein can accumulate due to low clearance rates (Voon et al, 2005), and such effects might explain the disparity between wild-type larval protein levels and construct-injected larval luciferase activity. Additionally, zebrafish have been shown to possess a β -GR isoform that is a splice variant of the α -GR isoform (Schaaf et al, 2008). In humans, β -GR has been shown to act as a dominant-negative inhibitor of the transactivation nature of α -GR (Bamberger et al, 1995; Oakley et al, 1996; Oakley et al, 1999). It is speculated that inhibitory action of β -GR is due to competition between α -GR and β -GR for transcriptional coactivator proteins (Charmandari et al, 2005) and/or the formation of inactive α -GR/ β -GR heterodimers (Hauk et al, 2002). Potential variation in the effects of β -GR between wild-type and construct larvae might have produced the differences between the two experiments. In addition, 96 hpf larvae treated with cortisol for only 24 h exhibited significantly lower relative luciferase activity than the control group, a response opposite to that detected with 48 h of cortisol treatment. Explanations for these discrepancies remain elusive.

Previous work using an *in vivo* luciferase reporter assay to assess whether gene expression in fish is cortisol inducible injected the plasmid containing the promoter and luciferase reporter into the liver of adult fish (Schulte et al, 2000; Esbaugh and Walsh, 2009; Rodela et al, 2011). Owing to the small size of adult zebrafish and the availability of microinjection technology for zebrafish embryos, in the present study the construct was microinjected into zebrafish embryos at the one-cell stage. An advantage of this approach was higher transfection efficiency, as indicated by the higher luciferase relative activity in the present study (>100 relative light units) versus previous studies (<0.1; Schulte et al, 2000; Esbaugh and Walsh, 2009; Rodela et al, 2011). A potential complicating factor with this approach, however, is changes in the expression of genes or proteins of interest with development. For example, Gilmour et al (2009) reported that the relative mRNA expression of zCac was detectable as early as 0 hpf and increased markedly during early development, becoming significantly elevated over the 0 hpf value at 72 and 96 hpf. The extent to which the microinjected plasmid was affected by this developmental program is unclear. Moreover, cortisol may regulate zCac mRNA expression differentially, depending on the stage of development and the length of exposure. In zebrafish, endogenous cortisol levels increase from 49 to 72 hpf and are significantly higher at 97 hpf than 48 hpf (Alsop and Vijayan, 2007). This developmental increase in cortisol levels may account for the somewhat higher luciferase activity detected in 96 hpf control larvae relative to earlier time points. It is also possible that the elevated cortisol levels used in this study may have triggered compensatory processes (e.g. down-regulation of the stress axis via negative feedback), accounting for the variability present in the results.

For cortisol to regulate gene expression, it must complex with a GR, which activates the receptor, leading to nuclear translocation. The activated receptor homodimerizes before binding

to a GRE; once bound to a GRE it can, in association with other transcription factors, regulate the expression of the target gene (Mommsen et al, 1999; Kumar and Thompson, 2005; Kumar et al, 2006; Bury and Sturm, 2007). In developing zebrafish, GR mRNA of maternal origin is present at 1.5 hpf; GR mRNA decreases in abundance from 1.5 to 25 hpf, but then rebounds and is stable from 49 to 146 hpf (Alsop and Vijayan, 2007). Zygotic expression of GR mRNA begins around 15 hpf (Nesan et al, 2012) and probably accounts for the stable expression of GR mRNA after hatch. Protein expression of GR is widespread at 24, 36 and 48 hpf in developing zebrafish, and translational knockdown of GR using antisense oligonucleotide morpholinos resulted in developmental defects that could in general be rescued by simultaneous injection of GR mRNA (Nesan et al, 2012). These data suggest that a functional GR signalling system is present during early development in zebrafish. At the time points chosen in the present study (24 – 96 hpf), GR protein levels should have been sufficient to allow responses to cortisol. Nevertheless, it remains possible that at the earliest time point examined in the present study (48 hpf), the expression of GRs during the period of cortisol exposure (24 – 48 hpf) may not have been sufficient to allow activation of the construct, accounting for the lack of response to cortisol at 48 hpf.

In the present study, cortisol treatment of wild-type zebrafish larvae had no significant impact on zCAc protein levels or total CA activity. Similarly, previous work on adult trout reported that cortisol treatment had no significant effect on branchial CA protein levels (Gilmour et al, 2011). However, cortisol treatment significantly increased branchial CA activity in trout (Gilmour et al, 2011). Clearly, there are substantial differences between these studies in the organism used (adult trout versus larval zebrafish), the method of cortisol delivery (intraperitoneal implants versus waterborne), and the CA measured (branchial CA in trout is

probably dominated by CAc but this is not certain, versus whole-body zCAc and whole body total CA activity in zebrafish). However, it is also tempting to speculate that the differences may reflect post-translation modification activated by a signal transduction pathway in one species, but not the other. Cortisol can elicit rapid non-genomic effects, including changes in the phosphorylation state of both protein kinase A (PKA), and protein kinase C (PKC) substrates (Dindia et al, 2012, 2013). The amino acid sequences of zCAc and trout CAc differ in the placement and number of potential phosphorylation sites. There are 16 potential sites in zCAc (10 serine, 5 threonine and 1 tyrosine), versus 14 in trout CAc (7 serine, 3 threonine and 4 tyrosine), and these sites also differ in the placement of potential phospho-residues (Fig. 3-5). For example, the presence of a C-terminal serine (S258) in zCAc, but a C-terminal threonine (T257) in trout CAc might indicate species-specific regulation of CAc. However, until our understanding of how such sequence differences affect the regulation of CA increases, it is only possible to speculate about the significance of sequence differences between species.

In summary, the present work demonstrated that cortisol may regulate CA mRNA expression through GRE $\frac{1}{2}$ s. However, the results were not straightforward; more sampling time points and different lengths of cortisol exposure may help in clarifying the events taking place. Whether the developing zebrafish was a good model in which to address this hypothesis remains an open question. Though appealing from a technical standpoint, ultimately the complexity of events occurring during early development may conflict with an already intricate regulatory pathway. It is possible that results obtained from adult zebrafish, in which the stress axis is operational, would be more clear-cut than results from larvae in which the stress pathways are still in development.

Figure 3-5. NetPhos 2.0 (<http://www.cbs.dtu.dk/services/NetPhos/>) prediction of phosphorylation sites for zCAc (top sequence) and tCAc (bottom sequence). Predicted phosphorylation sites are highlighted.

zCac MAHAWGYGPADGPESWAESFPIANGPRQSPIDIVPTQAQHDPSELKHLKLLK
tCac MSHAWGYAPDNGPDKWCEGFPIANGPRQSPIDIVPGEAAFDAALKALTLK

YDPATTKSILNNGHSFQVDFVDDNSSTLAGGPITGIYRLRQFHFHWGSS
YDPSTSIDILNNGHSFQVTYTDNDNSTLTGGPISGTYRLKQFHFHWGAS

DDKGSEHTIAGTKFPCELHLVHWNTKYPNFGEAASKPDGLAVVGVFLKIG
DDRGEHTVAGTKYAAELHLVHWNTKYPNFGDAASKSDGLAVVGVFLQVG

AANPRLQKVLDAALDDIKSKGRQTTFANFDPKTLTPASLDYWTYEGSLTTP
NENANLQKVLDAFDAIKAKGKQTSFENFDPTILLPKSLDYWTYDGSLLTTP

PLLESVTWIVLKEPISVSPAQMAKFRSLLFSSEGETPCCMVDNYRPPQPL
PLLESVTWIVCKESISVSPAQMGKFRSLLFSGEGEAACCMVDNYRPPQPL

KGRKVRASFK
KGRAITARS

Chapter 4

General Discussion

4.1 Introduction

Numerous studies have investigated the sequence variants, structure, inhibitor sensitivity and kinetics of the enzyme CA, particularly in mammals (reviewed by Maren, 1967; Parkilla, 2000; Supuran, 2008a,b). Fewer studies have investigated the regulation of CA and indeed, the mechanisms through which the enzyme is regulated remain unknown and/or unclear. The overall goal of this thesis was to determine whether the general cytosolic CA isoform in fish (CAc) is regulated transcriptionally by cortisol and/or through post-translational modification. While the data obtained in the present study provide conflicting evidence as to whether CA is transcriptionally regulated by cortisol, the data clearly demonstrate that rainbow trout CAc can be regulated by PTMs, and the study is the first to show phosphorylation of a fish CA.

Both transcriptional and post-translational regulation are important tools for responding to changing environmental conditions (Yang, 2005; Choudhuri, 2006), but their relative importance may vary according to the specific situation. For example, transcriptional regulation may be more appropriate in response to longer-term exposures, where environmental parameters such as salinity, pH or oxygen content have changed (Evans, 2002; Liu and Simon, 2004). By contrast, PTMs are more likely to be prevalent during transient changes in conditions, where there is an acute challenge to homeostasis and rapid modulation of protein function is required (Seet et al, 2006). Additionally, the two forms of regulation can act in tandem (Jensen et al, 2006); PTM might serve to enhance protein function until changes to transcriptional rates can sufficiently adjust protein levels. With this background in mind, the following discussion will focus on the methodology employed in the present study, and on the significance of the experimental findings. First, potential complications that may have arisen in the zCAc construct

experiments will be addressed, and this discussion will be followed by consideration of the consequences and significance of tCac phosphorylation. The discussion will conclude by considering future research that might stem from the present work.

4.2 zCac regulation by cortisol

Cortisol was hypothesized to regulate zCac transcription by binding to GRE $\frac{1}{2}$ s in the zCac promoter. The results of experiments designed to test this hypothesis were inconsistent; although data from a pair of sampling times supported the hypothesis, changes at a third sampling time were in the direction opposite to that predicted. Additionally, zCac protein levels and activity were unaffected at a sampling time where cortisol treatment should have increased zCac mRNA levels based on the zCac promoter-luciferase construct data. Potential sources of these discrepancies are discussed below.

The use of a reporter assay for the zCac promoter produced conflicting results that were difficult to interpret. Although the zebrafish genome contains only a single gene that encodes the GR (Alsop and Vijayan, 2007), alternative slicing can produce two isoforms; the canonical α -GR and the C-terminal splice variant β -GR, which can inhibit the activity of α -GR (Schaaf et al, 2008). It is possible that differences observed over developmental time in the effect of cortisol on reporter gene expression may have arisen owing to variation in the α -GR/ β -GR ratio during zebrafish development. A decrease in the α -GR/ β -GR ratio led to suppression of the activity of α -GR, and from 24 hpf to 72 hpf, the α -GR/ β -GR ratio decreased (Shaaf et al, 2008). Such an effect may explain why after 24 h of cortisol treatment, zebrafish larvae sampled at 72 hpf had significantly increased luciferase activity, whereas the group sampled at 96 hpf did not; the

decreased α -GR/ β -GR ratio at 72 hpf compared to 48 hpf may have allowed β -GR to exert a stronger inhibitory effect on α -GR during cortisol treatment. Larvae exposed to cortisol for 48 h and sampled at 96 hpf may have attained increased luciferase activity during the initial 24 h of exposure, when there would be a greater α -GR/ β -GR ratio. Unfortunately, the reason why luciferase activity was reduced in larvae treated with cortisol for 24 h and sampled at 96 hpf is still unclear.

A prior study on rainbow trout suggested that cortisol could regulate CA; both cortisol and CA mRNA levels were increased during challenges to pH homeostasis (Gilmour et al, 2011). Specifically, treatment with exogenous cortisol increased the mRNA abundance of tCAc and tCA IV in renal tissue, but branchial tCAc was unaffected (Gilmour et al, 2011). Similarly, in adult zebrafish fed cortisol-treated food, the mRNA abundance of branchial zCAc but not renal zCAc was significantly elevated (J. Black and KM Gilmour, unpublished data). These results suggest that regulation of CA by cortisol may be tissue specific. In the present investigation, zCAc activity and protein levels in zebrafish larvae were unaffected by cortisol treatment. If tissue-specific regulation of CA occurs in response to cortisol, then assessing whole body zCAc activity and protein levels, as in the present study, may have diminished the differences produced by cortisol administration. More measurements of mRNA levels of zCAc in renal and branchial tissues in adult zebrafish treated with cortisol would help to determine whether there is tissue-specific regulation of CA in this species.

4.3 CA phosphorylation and modulation of enzymatic properties

Phosphorylation is one of the most prevalent components of signal transduction pathways and its reversibility allows for the rapid modulation of protein function within a cell (Cohen, 2000). In the present study, it was hypothesized that tCAc is regulated by reversible phosphorylation. *In vitro* incubations containing chemical agents known to potentiate cellular kinases and phosphatases were predicted to change endogenous tCAc enzymatic properties and phosphorylation content. The results of the present investigation clearly demonstrated that tCAc is phosphorylated, with a concomitant increase in activity. Additional work is needed, however, to identify the signalling pathway and kinase(s) responsible for phosphorylation as well as the physiological situations in which phosphorylation occurs.

Incubations designed to favour a specific kinase indicated that the increase in tCAc activity was most likely the result of phosphorylation by PKG; conditions designed to enhance PKG activity were the only treatment conditions that significantly elevated tCAc activity. However, the kinetic assays following incubations designed to favour a specific kinase produced results that were less straightforward. Decreases in the kinetic parameters of substrate affinity (K_m) and turnover number (k_{cat}) occurred with enhancement of several kinases. Indeed, treatments designed to enhance PKA and AMPK produced comparable effects on kinetic parameters. This result was somewhat unexpected because PKA and AMPK often have opposing effects in signalling pathways (Hallows et al, 2000, 2003; Hurley et al, 2006). These results suggest that the kinase-specific incubation conditions may have been unsuccessful in stimulating only a single kinase of interest.

It is interesting to note that *in silico* analysis of tCAc suggested the presence of multiple potential phosphorylation sites. This observation raises questions about whether the changes in CA activity and kinetics were due to a single phosphorylation, or to phosphorylation at multiple

sites, and further, whether the endogenously-present tCAc is phosphorylated. The use of phospho-specific antibodies indicated that changes in phosphorylation state occurred at threonine residues. Trout tCAc contains three potential phospho-threonine sites. However, the experimental techniques used in the present study could not discern which site or sites were phosphorylated. Kinase stimulation increased phospho-threonine content from the endogenous state, which in turn exhibited a higher phospho-threonine content than tCAc subjected to phosphatases. These results suggest that the endogenous pool of tCAc contains a population of phosphorylated tCAc and this population can be increased by the action of kinases, or decreased by phosphatases. Although not significantly different among treatments, purified tCAc stained for both phospho-tyrosine and phospho-serine. This finding suggests that tCAc may contain multiple phospho-residues, but the residue(s) responsible for the modulation of activity and enzyme kinetics was (were) threonine. It has been estimated that approximately 65% of sites detected by phosphoproteomic experiments might be non-functional (Landry et al, 2009). Thus, the phospho-tyrosine and phospho-serine residues may be of no functional significance.

While there is no simple explanation for the comparable adjustments in tCAc kinetics observed with both kinase and phosphatase stimulation, insight may be provided by adopting the premise that multiple threonine phosphorylation sites exist for tCAc. A protein can potentially be phosphorylated at several sites, thereby producing many phospho-isoforms, each of which can potentially have a distinct biological activity (Yang, 2005). It seems counter-intuitive that antagonistic changes in tCAc phospho-threonine content should produce equivalent changes in enzymatic kinetics. However, any modification in the electrostatic profile of a protein may result in peptide conformational changes. Three phospho-threonine sites were identified in tCAc, and the phospho-residue-specific antibody results suggested that three distinct tCAc phospho-

threonine populations can be generated. With three possible phosphorylation sites, up to eight phospho-isoforms may exist. For example, if tCAc is endogenously phosphorylated at a single residue, the removal of that phosphate or the phosphorylation of an additional residue could produce two structurally distinct isoforms from the endogenous isoform. Potentially, any deviation from endogenous tCAc electrostatics may alter the conformation of the enzyme and cause the same change to kinetics, i.e. any change in tCAc conformation may decrease k_{cat} .

Phosphorylation of tCAc increased the inhibition constant K_i against acetazolamide, where K_i is the dissociation constant of the enzyme-inhibitor complex (Cheng and Prussoff, 1973). The higher the K_i value, the lower the affinity of the inhibitor for an enzyme. Acetazolamide displaces the $\text{H}_2\text{O}/\text{OH}^-$ necessary for enzyme catalysis by direct binding to the zinc atom of the active site (Sippel et al, 2009). Phosphorylation of an amino acid changes the electrostatics in its vicinity because of the addition of negative charge (Pawson and Nash, 2000). The addition of negative charge may induce steric and/or electrostatic barriers, reducing the affinity of acetazolamide for phospho-CA and leading to a higher concentration of inhibitor being necessary to produce the same decrease in the rate of the reaction. Alternatively, the increased K_i for acetazolamide may have resulted from a change in the conformation of CA. Negatively-charged amino acids in close proximity to the phosphorylated residue can be repelled, while positively charged amino acids are attracted, leading to protein structure variation (Tarrant and Cole, 2009). One or more of these factors may have reduced the affinity of acetazolamide for phospho-CA, accounting for the decreased potency of acetazolamide to inhibit phospho-CA observed in the inhibitor experiments. Although not investigated in the present study, CA also can be inhibited by anions that bind to the zinc centre of the enzyme's active site (Tibell et al, 1983). Phosphorylation may act as a protective mechanism for conserving CA

activity in the presence of anions, because the negative charge added to the enzyme's structure may serve as a deterrent to anion binding. However, this hypothesis must be tested experimentally.

4.3.1 Physiological significance of CA phosphorylation

Incubations designed to stimulate endogenous protein kinases phosphorylated CA and significantly increased enzymatic activity, indicating that phosphorylation is a mechanism that could be used *in vivo* to regulate CA activity. What, then, is the purpose of increasing CA activity, and when physiologically does this phenomenon occur? Ultimately, there must be some context *in vivo* that requires enhanced CA activity. For example, increased CA activity has been hypothesized to occur during acid-base challenges (Gilmour and Perry, 2009). At the proximate level, it is likely that a signal transduction pathway is activated to trigger the phosphorylation of CA to increase its activity. In rat astroglial cells, norepinephrine and histamine administration led to a doubling of CA activity together with increased incorporation of ^{32}P , a result that suggests phosphorylation of CA occurred (Church et al, 1980). The accumulation of ^{32}P by CA was associated with increased cAMP levels, pointing to PKA as the likely kinase responsible for phosphorylation (Church et al, 1980). The results of several other studies similarly support the possibility of PKA-mediated phosphorylation of CA (Narumi and Miyamoto, 1974; Bersimbaev et al, 1975; Ditte et al, 2011). While the results of kinase-specific incubations on tCAc activity strongly implicated tCAc phosphorylation by PKG, tCAc kinetic parameters were altered in a similar fashion by incubations designed to preferentially stimulate PKA or PKG. Based on this result and the fact that previous studies have identified PKA-mediated phosphorylation of CA, it is intriguing to postulate a hypothetical situation based on existing research on zebrafish. Similar

situations may exist in trout, but the extent of available data for zebrafish makes a more compelling model. The adult fish gill is extensively innervated (for review, see Nilsson, 1984), and the proton pump-rich (HR) cells of zebrafish larvae are adrenergically innervated and appear to express β -adrenergic receptors (Kumai et al, 2012; Kumai and Perry, 2012). The HR cells contain zCAc (Lin et al, 2008), and therefore it is plausible to hypothesize adrenergic regulation of CA activity. In this scenario, the activation of β -adrenergic receptors would lead to the synthesis of cAMP, which in turn activates PKA, resulting in the phosphorylation of target proteins. If zCAc phosphorylation is governed by PKA as in other instances of CA phosphorylation (Narumi and Miyamoto, 1974; Bersimbaev et al, 1975; Church et al, 1980; Ditte et al, 2011), then environmental stimuli that elevate cAMP levels may identify conditions under which CA phosphorylation occurs. For example, Na^+ uptake by larval zebrafish appears to be under adrenergic control because a β -adrenergic receptor agonist stimulated Na^+ uptake (Kumai et al, 2012). Carbonic anhydrase is believed to contribute to Na^+ uptake (see Gilmour, 2012), and inhibition of the enzyme leads to a reduction in Na^+ accumulation (Esaki et al, 2007). Thus, one mechanism through which Na^+ uptake may be increased during adrenergic stimulation is via PKA-mediated phosphorylation of CA to enhance activity, increasing the production of H^+ for excretion (which is linked to Na^+ uptake). Clearly such hypotheses require experimental investigation.

In the present study, exposure of trout to acute hypercapnia (1% CO_2) did not affect tCAc activity or phosphorylation state. Fish were sampled after 4 hours of exposure to hypercapnia, a time when acid excretion should be increased (Wood and LeMoigne, 1991), and CA activity might be enhanced by rapidly-acting PTM rather than more slowly acting transcriptional regulation. Based on the rapid time course on which phosphorylation can operate, this trial-and-

error approach may be a rather onerous way of pinpointing conditions in which tCAc phosphorylation occurs. A more rewarding plan of attack may be to focus on identifying the signal transduction pathways that are activated by acid-base or other challenges, and that could phosphorylate tCAc. It seems plausible that the phosphorylation of tCAc and the concomitant increase in its activity are linked to some compensatory response in which enhanced CA activity is necessary for a return to, or the maintenance of, homeostasis.

One potential regulator of CA phosphorylation state may be cortisol. Aside from its effects on the transcriptional machinery in fish, cortisol can also elicit rapid non-genomic effects (Wehling, 1997; Mommsen et al, 1999). Furthermore, the phosphorylation state of the targets of PKA, PKA, and protein kinase B(PKB) are modified by cortisol (Dindia et al, 2012; 2013). In these studies an acute stressor (leading to endogenously raised plasma cortisol levels) or treatment with stress-induced levels of exogenous cortisol increased the activity of intracellular stress signalling pathways. The amino acid sequence of tCAc contains potential phospho-sites for all three of these kinases at threonine residues, the amino acid identified as being modified by phosphorylation in the present investigation. A previous study in rainbow trout linked an increase in CA activity to cortisol administration, without an increase in enzyme levels (Gilmour et al, 2011). This finding supports the idea that a PTM may be responsible for the increase in enzyme activity; the result of the present study suggest that this PTM should be phosphorylation. Collectively, these findings suggest a role for cortisol as a regulator of CA phosphorylation. Future work could seek to determine whether cortisol modulates CA phosphorylation state. Indeed, cortisol would appear to be an ideal candidate to regulate CA function in a dual fashion, through both transcriptional control and PTM. By synergizing post-translational and transcriptional regulation in response to a homeostatic disturbance, cortisol might act non-

genomically through a kinase signalling pathway to raise CA activity until its action on transcription can come in effect to adjust CA protein levels sufficiently.

4.4 Future directions

The results of the present study have opened up an intriguing research area and several possible research directions could be adopted. One option would be to focus on *in vitro* investigations using isolated cell cultures. The advantage of this approach is the possibility of identifying the specific signalling pathways that can lead to CA phosphorylation. Identifying these pathways would provide valuable insight into the physiological context in which CA is phosphorylated. A second possibility would be to continue searching for physiological situations in which CA may be phosphorylated (but see above). In the present study, tCAc activity was hypothesized to contribute to acid-base compensatory responses necessary to correct pH disturbances. The PNA⁺ and PNA⁻ MR cells of the trout gill are believed to be responsible for base- and acid-secretion, respectively (Goss et al, 2001; Galvez et al, 2002; Evans et al, 2005; Perry and Gilmour, 2006; Gilmour, 2012). These cell types can be separated and isolated using a Percoll density gradient (Galvez et al, 2002). Using an array of pharmacological agents on isolated cells would allow an *in vitro* approach to be developed to delve into the types of agonists that might modify CA activity in the two different cell types. When attempting to determine the physiological context in which CA may be phosphorylated, it may be necessary to isolate tCAc from either PNA⁺ or PNA⁻ MR cells because the two cell types may have distinct or even opposite responses to different circumstances. For example, an acidosis induced by hypercapnia increased the relative H⁺-ATPase expression of PNA⁻ MR cells, but not PNA⁺ MR cells (Galvez

et al, 2002). Therefore, CA might be regulated by a specific receptor or pathway in PNA⁺ MR cells, but a different one in PNA⁻ MR cells.

The present study demonstrated that the phosphorylation of CA increased its ability to catalyze CO₂ hydration. Aside from its ability to catalyse the reversible hydration of CO₂, CA can also act as an esterase (see Maren, 1967). In both reactions there is nucleophilic attack by a Zn-OH⁻ ion and the stabilization of an oxyanionic intermediate (Gould and Tawfik, 2005). This similarity between the mechanisms of carbonyl hydration of an ester versus CO₂ suggests that because phosphorylation increases hydase activity, it may also increase esterase activity. From a technical standpoint, it is both simpler and faster to measure the esterase activity of CA than it is to measure the ability of CA to catalyze CO₂ hydration. Confirmation that phosphorylation of fish CAc increases its esterase activity would allow subsequent studies to use *p*-NPA hydrolysis as an alternate method for measuring CA activity when manipulating CA phosphorylation state. This method would increase experimental efficiency.

4.5 Conclusions

To summarize the major findings of the present study; tCAc is subject to at least 2 types of PTM, phosphorylation and glutathionylation. Phosphorylation of tCAc was accompanied by an increase in enzymatic activity and an increase in phospho-threonine levels. The present study determined that there appear to be mechanisms in place to regulate CA, but the physiological context in which they are employed remains unidentified, and requires further investigation. It seems likely that phosphorylation of CA is an intrinsic compensatory response in situations

where increased CA activity is necessary. This phenomenon may serve a transient role, enhancing CA activity until other forms of regulation can be implemented.

REFERENCES

References

- Alcaraz-Pérez, F., Mulero, V., Cayuela, ML. 2008. Application of the dual-luciferase reporter assay to the analysis of promoter activity in zebrafish embryos. *BMC Biotechnol.* 8, 81.
- Alsop, D., Vijayan, MM. 2008. Development of the corticosteroid stress axis and receptor expression in zebrafish. *Am J Physiol Reg Integr Comp Physiol.* 294, R711–R719.
- Aranda, A., and Pascual, A. 2001. Nuclear hormone receptors and gene expression. *Physiological Reviews.* 81, 1269–1304.
- Aspatwar, A., Tolvanen, MEE., Parkkila, S. 2010. Phylogeny and expression of carbonic anhydrase-related proteins. *BMC Mol Biol.* 11, 25.
- Bamberger, CM., Bamberger, AM., de Castro, M., Chrousos, GP. 1995. Glucocorticoid receptor β , a potential endogenous inhibitor of glucocorticoid action in humans. *J Clin Invest.* 95, 2435–2441.
- Barford, D. 1996. Molecular mechanisms of the protein serine/ threonine phosphatases. *Trends Biochem Sci.* 21, 407–412.
- Bergenheim, N., Carlsson, U., and Strid, L. 1986. *Biochim Biophys Acta.* 87, 155–160.
- Bersimbaev, RI., Konstantinov, YM., Arguyinskaya, SV., Salganik, RI. 1975. Activation of carbonic anhydrase from rat gastric tissue as a result of phosphorylation by 3'5'-AMP-dependent protein kinase. *Biokhimiia.* 40, 570–577.
- Boron, WF. 2010. Evaluating the role of carbonic anhydrases in the transport of HCO_3^- related species. *Biochim Biophys Acta.* 1804, 410–421.
- Bottcher, K., and Siebers, D. 1993. Biochemistry, localization, and physiology of carbonic anhydrase in the gills of euryhaline crabs. *J Exp Zoo.* 265, 397-409.

- Brown, SB., Eales, JG., Evans, RE., Hara, TJ. 1984. Interrenal, thyroidal, and carbohydrate responses of rainbow trout (*Salmo gairdneri*) to environmental acidification. *Can J Fish Aquat Sci.* 41, 36–45.
- Brown, SB., Eales, JG., Hara, TJ. 1986. A protocol for estimation of cortisol plasma clearance in acid-exposed rainbow trout (*Salmo gairdneri*). *Gen Comp Endocrinol.* 62, 493–502.
- Bury, N., Sturm, A. 2007. Evolution of the corticosteroid receptor signalling pathway in fish, *Gen Comp Endocrinol.* 153, 47–56.
- Carlson, M. 1997. Genetics of transcriptional regulation in yeast: connections to the RNA polymerase II CTD. *Annu Rev Cell Dev Biol.* 13, 1.
- Casagrande, S., Bonetto, V., Fratelli, M., Gianazza, E., Eberini, I., Massignan, T., Salmona, M., Chang, G., Holmgren, A., Ghezzi, P. 2002. Glutathionylation of human thioredoxin: A possible crosstalk between the glutathione and thioredoxin systems. *Proc Natl Acad Sci USA.* 99, 9745–9749.
- Chai, YC., Jung, CH., Lii, CK., Ashraf, SS., Hendrich, S., Wolf, B., Sies, H., and Thomas, JA. 1991. Identification of an abundant S-thiolated rat liver protein as carbonic anhydrase III. Characterization of S-thiolation and dethiolation reactions. *Arch Biochem Biophys.* 284, 270–278.
- Charmandari, E., Chrousos, GP., Ichijo, T., Bhattacharyya, N., Vottero, A., Souvatzoglou, E., Kino, T. 2005. The human glucocorticoid receptor (hGR) β isoform suppresses the transcriptional activity of hGR α by interfering with formation of active coactivator complexes. *Mol Endocrinol.* 19, 52–64.

- Chegwidden, WR. 1991. Purification of the carbonic anhydrases. In *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*, Dodgson, SJ.; Tashian, RE.; Gros, G.; Carter, N. (Eds), Plenum Press, NY, pp 101-118.
- Chegwidden, WR., and Carter, ND. 2000. Introduction to the carbonic anhydrases. *EXS* 90, 13–28.
- Cheng, Y., Prusoff, W. 1973. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50 per cent inhibition (I_{50}) of an enzymatic reaction *Biochemical Pharmacology*. 22, 3099-3108.
- Choudhuri, S. 2006. Gene regulation and molecular toxicology. *Toxicology Mechanisms and Methods*. 15, 1-23.
- Christoffels, V., Grange, T., Kaestner, K., Cole, T., Darlington, G., Croniger, C., Lamers, W. 1998. Glucocorticoid receptor, C/EBP, HNF3, and protein kinase A coordinately activate the glucocorticoid response unit of the carbamoylphosphate synthetase I gene. *Mol Cell Biol*. 18, 6305.
- Chu, F., Ward, NE., O'Brian, CA. 2001. Potent inactivation of representative members of each PKC isozyme subfamily and PKD via S-thiolation by the tumor-promotion/progression antagonist glutathione but not by its precursor cysteine. *Carcinogenesis*. 22, 1221–1229.
- Church, G., Kimelberg, H., and Sapirstein, V. 1980. Stimulation of carbonic anhydrase activity and phosphorylation in primary astroglial cultures by norepinephrine. *Journal of Neurochemistry*. 34(4), 873-879.
- Claiborne, JB., Edwards, SL., Morrison-Shetlar, AI. 2002. Acid–base regulation in fishes: cellular and molecular mechanisms. *J Exp Zoo*. 293, 302–319.

- Clark, RS., Bayir, H., Jenkins, LW. 2005. Posttranslational protein modifications. Crit Care Med. 33, 407-409.
- Cohen, P. 2000. The regulation of protein function by multisite phosphorylation--a 25 year update. Trends Biochem Sci. 25, 596-601.
- Cohen, P. 2002. The origins of protein phosphorylation. Nat. Cell Biol. 4, E127–E130.
- Couto, N., Malys, N., Gaskell, S., Barber, J. 2013. Partition and turnover of glutathione reductase from *Saccharomyces cerevisiae*: a Proteomic Approach. Journal of Proteome Research. 12, 2885–94.
- Cozzone, AJ. 1988. Protein phosphorylation in prokaryotes. Annu Rev Microbiol. 42, 97–125
- Desvergne, B., Michalik, L., Wahli, W. 2006. Transcriptional regulation of metabolism. Physiol Rev. 86(2), 465-514.
- Dieni, C., Storey, KB. 2010. Regulation of glucose-6-phosphate dehydrogenase by reversible phosphorylation in liver of a freeze tolerant frog. Journal of Comparative Physiology. 180, B1133-B1142.
- Dindia, L., Murray, J., Faught, E., Davis, TL., Leonenko, Z., Vijayan, MM. 2012. Novel nongenomic signaling by glucocorticoid may involve changes to liver membrane order in Rainbow Trout. PLoS ONE 7(10): e46859. doi:10.1371/journal.pone.0046859.
- Dindia, L., Faught, E., Leonenko, Z., Thomas, R., Vijayan, MM. 2013. Rapid cortisol signaling in response to acute stress involves changes in plasma membrane order in rainbow trout liver. Am J Physiol. 304, E1157-E1166.
- Ditte, P., Dequiedt, F., Svastova, E., Hulikova, A., Ohradanova-Repic, A., Zatovicova, M., Csaderova, L., Kopacek, J., Supuran, CT., Pastorekova, S., Pastorek, J. 2011.

- Phosphorylation of carbonic anhydrase IX controls its ability to mediate extracellular acidification in hypoxic tumors. *Cancer Research*. 71, 7558-7567.
- Edsall, JT. 1969. Carbon dioxide, carbonic acid, and bicarbonate ion: physical properties and kinetics of interconversion. In: *CO₂: Chemical, Biological and Physiological Aspects*, Forster, RE, Edsall, JT.; Otis, AB.; and Roughton, FJW, (Eds), NASA Spec. Publ., Washington, pp 15-27.
- Engholm-Keller, K. and Larsen, MR. 2013, Technologies and challenges in large-scale phosphoproteomics. *Proteomics*. 13, 910–931.
- Esbaugh, A., Perry, SF., Bayaa, M., Georgalis, T., Nickerson, JG., Tufts, BL., and Gilmour, KM. 2005. Cytoplasmic carbonic anhydrase isozymes in rainbow trout *Oncorhynchus mykiss*: comparative physiology and molecular evolution. *J Exp Biol*. 208, 1951–1961.
- Esbaugh, AJ., Walsh, PJ. 2009. Identification of two glucocorticoid response elements in the promoter region of the ubiquitous isoform of glutamine synthetase in gulf toadfish, *Opsanus beta*. *Am J Physiol*. 297, R1075–R1081.
- Esaki, M., Hoshijima, K., Kobayashi, S., Fukuda, H., Kawakami, K., Hirose, S., 2007. Visualization in zebrafish larvae of Na⁺ uptake in mitochondria-rich cells whose differentiation is dependent on foxi3a. *Am J Physiol*. 292, R470–R480.
- Evans, DH. 2002. Cell signaling and ion transport across the fish gill epithelium. *J Exp Zool*. 293, 336 -347.
- Evans, DH., Piermarini, PM., Choe, KP. 2005. The multifunctional fish gill: dominant site of gas exchange, osmoregulation, acid–base regulation, and excretion of nitrogenous waste. *Physiol Rev*. 85, 97–177.

- Fahey, RC., Hunt, JS., Windham, GC. 1977. On the cysteine and cystine content of proteins. Differences between intracellular and extracellular proteins. *J Mol Evol.* 10, 155–160.
- Forster, RE., Itada, N. 1980. Carbonic anhydrase activity in intact red cells as measured by means of ^{18}O exchange between CO_2 and water. In: *Biophysics and Physiology of Carbon Dioxide*, Bauer C.; Gros G.; Bartels H, (Eds), Springer-Verlag, Heidelberg, Germany.
- Forster, RE., Gros, G., Lin, L., Onon, Y., Wunder, M. 1998. The effect of 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate on CO_2 permeability of the red blood cell membrane. *Proc Natl Acad Sci USA.* 95, 15815–15820.
- Fuhrmann, J., Schmidt, A., Spiess, S., Lehner, A. 2009. McsB is a protein arginine kinase that phosphorylates and inhibits the heat-shock regulator CtsR. *Science.* 324, 1323–1327.
- Fujikawa-Adachi, K., Nishimori, I., Taguchi, T., Yuri, K., Onishi, S. 1999. cDNA sequence, mRNA expression, and chromosomal localization of human carbonic anhydrase related protein CA-RP XI. *Biochim Biophys Acta.* 1431, 518–524.
- Galvez, F., Reid, SD., Hawkings, GS., Goss, GG., 2002. Isolation and characterization of mitochondria-rich cell types from the gill of freshwater rainbow trout. *Am J Physiol.* 282, R658–R668.
- Gao, X., Bedhomme, M., Veyel, D., Zaffagnini D., and Lemaire, SD. 2009. Methods for analysis of protein glutathionylation and their application to photosynthetic organisms. *Molecular Plant.* 2, 218–235.
- Georgalis, T., Perry, SF., Gilmour, KM., 2006a. The role of branchial carbonic anhydrase in acid–base regulation in rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol.* 209, 518–530.

- Georgalis, T., Gilmour, KM., Yorston, J. and Perry, SF. 2006b. The roles of cytosolic and membrane bound carbonic anhydrase in the renal control of acid-base balance in rainbow trout *Oncorhynchus mykiss*. *Am J Physiol.* 291, F407-F421.
- Gervais, MR., Tufts, BL. 1999. Characterization of carbonic anhydrase and anion exchange in the erythrocytes of bowfin (*Amia calva*), a primitive air-breathing fish. *Comp Biochem Physiol.* 123, A343–A350.
- Ghezzi, P. 2005. Regulation of protein function by glutathionylation. *Free Radical Research.* 39, 573–580.
- Gibbons, BH., and Esdall, JT. 1963. Rate of hydration of carbon dioxide and dehydration at 25C. *J Biol Chem.* 238, 3501-3507.
- Gilbert, HF. 1984. Redox control of enzyme activities by thiol/disulfide exchange. *Methods Enzymol.* 107, 330–351.
- Gilmour, K., and Perry, SF. 2009. Carbonic Anhydrase and acid-base regulation in fish. *J Exp Biol.* 212, 1647-1661.
- Gilmour, KM., and Perry, SF. 2010. Gas transfer in dogfish: a unique model of CO₂ excretion. *Comp Biochem Physiol.* 155, A476–A485.
- Gilmour, K. 2010. Perspectives on carbonic anhydrase. *Comp Biochem Physiol.* 157, A193-A197.
- Gilmour, KM., Collier, CL., Dey, CJ., Perry, SF. 2011. Roles of cortisol and carbonic anhydrase in acid–base compensation in rainbow trout, *Oncorhynchus mykiss*. *J Comp Physiol.* 181, B501–B515.
- Gilmour, KM. 2012. New insights into the many functions of carbonic anhydrase in fish gills. *Respir Physiol Neurobiol.* 184, 223-230.

- Gnad, F., Gunawardena, J., Mann, M. 2011. PHOSIDA 2011: the posttranslational modification database. *Nucleic Acids Res.* 39, 253-260.
- Goss, GG., and Perry, SF. 1993. Physiological and morphological regulation of acid-base status during hypercapnia in rainbow trout (*Oncorhynchus mykiss*). *Can J Zool.* 71, 1673-1680.
- Goss, GG., Adamia, S., Galvez, F. 2001. Peanut lectin binds to a subpopulation of mitochondria-rich cells in the rainbow trout gill epithelium. *Am J Physiol.* 281, R1718–R1725.
- Gould, S., and Tawfik, D. 2005. Directed evolution of the promiscuous esterase activity of carbonic anhydrase II. *Biochemistry.* 44, 5444-5452.
- Hallows, KR., Raghuram, V., Kemp, BE., Witters, LA., Foskett, JK. Inhibition of cystic fibrosis transmembrane conductance regulator by novel interaction with the metabolic sensor AMP-activated protein kinase. *J Clin Invest.* 105, 1711–1721.
- Hallows, KR., McCane, JE., Kemp, BE., Witters, LA., Foskett, JK. 2003. Regulation of channel gating by AMP-activated protein kinase modulates cystic fibrosis transmembrane conductance regulator activity in lung submucosal cells. *J Biol Chem.* 278, 998–1004.
- Hauk, PJ., Goleva, E., Strickland, I., Vottero, A., Chrousos, GP., Kisich, KO., Leung, DY. 2002. Increased glucocorticoid receptor β expression converts mouse hybridoma cells to a corticosteroid-insensitive phenotype. *Am J Respir Cell Mol Biol.* 27, 361–367.
- Henderson, PJF. 1973. Steady-state enzyme kinetics with high affinity substrates or inhibitors. A statistical treatment of dose-response curves. *Biochem J.* 135,101–107.
- Henry, RP. 1991. Techniques for measuring carbonic anhydrase activity in vitro. In: *The Carbonic Anhydrases: Cellular Physiology and Molecular Genetics*, Dodgson, SJ., Tashian, RE.; Gros, G.; Carter, ND. (Eds), Plenum, New York, pp. 119–131.

- Henry, RP., Gilmour, KM., Wood, CM., Perry, SF. 1997. Extracellular carbonic anhydrase activity and carbonic anhydrase inhibitors in the circulatory system of fish. *Physiological Zoology*. 70, 650–659.
- Henry, RP., Swenson, ER. 2000. The distribution and physiological significance of carbonic anhydrase in vertebrate gas exchange organs. *Respiration Physiology* 121, 1–12.
- Hewett-Emmett, D. 2000. Evolution and distribution of the carbonic anhydrase gene families. *EXS* 90, 29–76.
- Hilvo, M., Innocenti, A., Monti, SM., De Simone, G, Supuran, CT., Parkkila, S. 2008. Recent advances in research on the most novel carbonic anhydrases, CA XIII and XV. *Curr Pharm Des*. 14, 672–678.
- Hoffman, M.D., Sniatynskia M.J., Kasta, J. 2008. Current approaches for global post-translational modification discovery and mass spectrometric analysis. *Analytica Chimica Acta*. 627, 50–61.
- Holmgren A. 1985. Thioredoxin. *Annu Rev Biochem*. 54, 237–271.
- Hunter, T. 2000. Signaling—2000 and beyond. *Cell*. 100, 113–127.
- Hurley, RL., Barre, LK., Wood, SD., Anderson, KA., Kemp, BE., Means, AR., Witters LA. 2006. Regulation of AMP-activated protein kinase by multisite phosphorylation in response to agents that elevate cellular cAMP. *J Biol Chem*. 281, 36662–36672.
- Hwang C, Sinskey AJ, Lodish HF. 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. *Science*. 257, 1496–1502.
- Hwang, P., Lee, T. Lin, L. 2011. Ion regulation in fish gills: recent progress in the cellular and molecular mechanisms. *Am J Physiol*. 301, R28–R47.

- Ivanis, G., Braun, M., Perry, SF. 2008a. Renal expression and localization of SLC9A3 sodium/hydrogen exchanger and its possible role in acid–base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *Am J Physiol.* 295, R971–R978.
- Ivanis, G., Esbaugh, A., Perry, SF. 2008b. Branchial expression and localization of SLC9A2 and SLC9A3 sodium/hydrogen exchangers and their possible role in acid–base regulation in freshwater rainbow trout (*Oncorhynchus mykiss*). *J Exp Biol.* 211, 2467–2477.
- Jensen, L., Jensen, T., Lichtenberg, U., Brunak, S., Bork, P. 2006. Co-evolution of transcriptional and posttranslational cell-cycle regulation. *Nature.* 443, 594-598.
- Johnson, LN., Barford, D. 1993. The effects of phosphorylation on the structure and function of proteins. *Annu Rev Biophys Biomol Struct.* 22, 199–232.
- Kerstetter, TH., Kirschner, LB. 1972. Active chloride transport by the gills of rainbow trout (*Salmo gairdneri*). *J Exp Biol.* 56, 263–272.
- Kim, G., Levine, R. 2005. Molecular determinants of S-glutathionylation of carbonic anhydrase 3. *Antioxidants and Redox Signalling.* 7, 849-854.
- Klatt, P., Molina, EP., Lamas, S. 1999. Nitric oxide inhibits c-Jun DNA binding by specifically targeted S-glutathionylation. *J Biol Chem.* 274, 15857–15864.
- Kumai, Y., Nesan, D., Vijayan, MM., and Perry SF. 2012. Cortisol regulates Na⁺ uptake in zebrafish, *Danio rerio*, larvae via the glucocorticoid receptor. *Mol Cell Endocrinol.* 364, 113-125.
- Kumai, Y., and Perry, SF. 2012. Mechanisms and regulation of Na⁺ uptake by freshwater fish. *Respir Physiol Neurobiol.* 184, .249-256.
- Kumar, R., Thompson, EB. 2005. Gene regulation by the glucocorticoid receptor: Structure: function relationship. *J Steroid Biochem Mol Biol.* 94, 383–394.

- Kumar, S., Saradhi, M., Chaturvedi, N.K., Tyagi, R.K., 2006. Intracellular localization and nucleocytoplasmic trafficking of steroid receptors: an overview. *Mol Cell Endocrinol.* 246, 147–156.
- Landry, C., Levy, E., Michnick, S. 2012. Weak functional constraints on phosphoproteomes. *Trends in Genetics.* 25, 193-197.
- Lant, B., Storey, KB. 2011. Glucose-6-phosphate dehydrogenase regulation in anoxia tolerance of the freshwater crayfish *Orconectes virilise*. *Enzyme Research.* 11, 1-8.
- Latchman, DS. 1997. Transcription factors: an overview. *Int J Biochem Cell Biol.* 29(12), 1305–12.
- Lee, TI., Young, RA. 2000. Transcription of eukaryotic protein-coding genes. *Annu Rev Genet.* 34, 77–137.
- Lehtonen, J., Shen, B., Vihinen, M., Casini, A., Scozzafava, A., Supuran, CT., Parkkila, AK., Saarnio, J., Kivelä, AJ., Waheed, A., Sly, WS., Parkkila, S. 2004. Characterization of CAXIII, a novel member of the carbonic anhydrase isozyme family. *J Biol Chem.* 279, 2719–2727.
- Lin, TY., Liao, BK., Horng, JL., Yah, JJ., Hsiao, CD., Hwang, PP. 2008. Carbonic anhydrase 2-like a and 15a are involved in acid-base regulation and Na⁺ uptake in zebrafish H⁺-ATPase-rich cells. *Am J Physiol.* 294, C1250–C1260.
- Lin, H., Randall, DJ. 1993. H⁺-ATPase activity in crude homogenates of fish gill tissue: inhibitor sensitivity and environmental and hormonal regulation. *J Exp Biol.* 180, 163–174.
- Lindskog, S., 1997. Structure and mechanism of carbonic anhydrase. *Pharmacol. Ther.* 74, 1–20.
- Lindskog, S., Silverman, DN. 2000. The catalytic mechanism of mammalian carbonic

- anhydrases. In: Chegwiddden, W.R., Carter, N.D., Edwards, Y.H. (Eds.), *The Carbonic Anhydrases: New Horizons*. Birkhäuser Verlag, Basel, Switzerland, pp. 175–195.
- Lineweaver, H., Burk, D. 1934. The Determination of Enzyme Dissociation Constants. *Journal of the American Chemical Society*. 56, 658–666.
- Liu, L., Simon, M. 2004. Regulation of transcription and translation by hypoxia. *Cancer Biol Ther*. 6, 492-7.
- McCormick, SD. 1995. Hormonal control of gill Na⁺,K⁺-ATPase and chloride cell function. In *Cellular and Molecular Approaches to Fish Ionic Regulation (Fish Physiology, XIV)*, Wood, CM.; and Shuttleworth, TJ. (Eds), Academic Press, San Diego, CA, pp. 285–315.
- Madsen, SS., Jensen, MK., Nohr, J., and Kristiansen, K. 1995. Expression of Na/K ATPase in the brown trout, *Salmo trutta*: *in vivo* modulation by hormones and seawater. *Am J Physiol*. 269, R1339–R1345.
- Maetz, J. 1971. Fish gills: mechanisms of salt transfer in fresh water and sea water. *Philosophical Transactions of the Royal Society of London Series B: Biological Sciences*. 262, 209–249.
- Maffia, M., Rizzello, A., Acierno, R., Rollo, M., Chiloiro, R., and Storelli, C. 2001. *J Exp Biol*. 204, 3983–3992.
- Mallis, R.J., Hamann, M.J., Zhao, W., Zhang, T., Hendrich, S., and Thomas, JA. 2002. *Biol Chem*. 383, 649–662.
- Mallis, R.J., Poland, BW., Chatterjee, TK., Fisher, RA., Darmawan, S., Honzatko, RB., and Thomas, JA. 2000. *FEBS Lett*. 482, 237–241.

- Mangelsdorf, DJ., Thummel, C., Beato, M., Herrlich, P., Schutz, G., Umesono, K., Blumberg, B., Kastner, P., Mark, M., Chambon, P., and Evans, RM. 1995. The nuclear receptor superfamily: The second decade. *Cell*. 83, 835–839.
- Manning, G., Plowman, G. D., Hunter, T., Sudarsanam, S. 2002. Evolution of protein kinase signaling from yeast to man. *Trends Biochem Sci*. 27, 514–520.
- Maren, TH. 1967. Carbonic anhydrase: chemistry, physiology, and inhibition. *Physiological Reviews*. 47, 595–781.
- Maren, TH., Friedland, BR., Rittmaster, RS. 1980. Kinetic properties of primitive vertebrate carbonic anhydrases. *Comp Biochem Physiol*. 67, B69–B74.
- Maren, TH., Wynns, GC., Wistrand, PJ. 1993. Chemical properties of carbonic anhydrase IV, the membrane-bound enzyme. *Mol Pharmacol*. 44, 901–905.
- Marshall, WS. 2002. Na^+ , Cl^- , Ca^{2+} and Zn^{2+} transport by fish gills: Retrospective review and prospective synthesis. *J Exp Zool*. 293, 264–283.
- Maynard, JR., and Coleman, JE. 1971. Elasmobranch carbonic anhydrase: Purification and properties of the enzyme from two species of shark. *J Biol Chem*. 246, 4455–4464.
- McMurtrie, HL., Cleary, HJ., Alvarez, BV., Loiselle, FB., Sterling, D., Morgan, PE., Johnson, DE., Casey, JR. 2004. The bicarbonate transport metabolon. *J Enzyme Inhib Med Chem*. 19, 231–236.
- Mommsen, TP., Vijayan M.M., Moon, TW. 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. *Reviews in Fish Biology and Fisheries*. 9, 211–268.
- Mori, K., Ogawa, Y., Ebihara, K., Tamura, N., Tashiro, K., Kuwahara, T., Mukoyama, M., Sugawara, A., Ozaki, S., Tanaka, I., Nakao, K. 1999. Isolation and characterization of

- CA XIV, a novel membrane-bound carbonic anhydrase from mouse kidney. *J Biol Chem.* 274, 15701–15705.
- Morrison, JF. 1969. Kinetics of the reversible inhibition on enzyme-catalyzed reactions by tight binding inhibitors. *Biochim Biophys Acta.* 185, 269–286.
- Moriyama, T., Srere, PA. 1971. Purification of rat heart and rat liver citrate synthases. Physical, kinetic, and immunological studies. *J Biol Chem.* 246, 3217-23.
- Nawata CM., Hung CC., Tsui TK., Wilson JM., Wright PA., Wood CM. 2007. Ammonia excretion in rainbow trout (*Oncorhynchus mykiss*): evidence for Rh glycoprotein and H⁺-ATPase involvement. *Physiol Genomics.* 31, 463–474.
- Narumi, S., and Miyamoto, E. 1974. Activation and phosphorylation of carbonic anhydrase by adenosine 3',5'-monophosphate-dependent protein kinases. *Biochim Biophys Acta.* 350, 215-224.
- Nesan, D., Kamkar, M., Burrows, J., Scott, IC., Marsden, M., Vijayan, MM. 2012. Glucocorticoid receptor signaling is essential for mesoderm formation and muscle development in zebrafish. *Endocrinology.* 153(3), 1288–1300.
- Nilsson, S. 1984. *Innervation and Pharmacology of the Gills*, In: Hoar, W.; Randall, DJ. (Eds.), *Fish Physiology*, vol. 10, Part A. Academic Press, pp. 185–227.
- Nishi, H., Hashimoto, K., and Panchenko AR. 2011. Phosphorylation in Protein-Protein Binding: Effecton Stability and Function. *Structure.* 19, 1807–1815.
- O'Connor, CM., and Clarke, S. 1984. Carboxyl methylation of cytosolic proteins in intact human erythrocytes. Identification of numerous methyl-accepting proteins including hemoglobin and carbonic anhydrase. *J Biol Chem.* 259, 2570-2578.

- Oakley, RH., Sar, M., Cidlowski, JA. 1996. The human glucocorticoid receptor β isoform. Expression, biochemical properties, and putative function. *J Biol Chem.* 271, 9550–9559.
- Oakley, RH., Jewell, CM., Yudit, MR., Bofetiado, DM., Cidlowski, JA. 1999. The dominant negative activity of the human glucocorticoid receptor β isoform. Specificity and mechanisms of action. *J Biol Chem.* 274, 27857–27866.
- Oh, K., Han, H., Kim, M., Koo, S. 2013. Transcriptional regulators of hepatic gluconeogenesis. *Arch Pharm Res.* 36, 189–200.
- Okamoto, N., Fujikawa-Adachi, K., Nishimori, I., Taniuchi, K., Onishi, S. 2001. cDNA sequence of human carbonic anhydrase-related protein, CA-RP X: mRNA expressions of CA-RP X and XI in human brain. *Biochim Biophys Acta.* 1518, 311–316.
- Oliveira, AP., Sauer, U. 2012. The importance of post-translational modifications in regulating *Saccharomyces cerevisiae* metabolism. *FEMS Yeast Research.* 12, 104–117.
- Opavský, R., Pastoreková, S., Zelník, V., Gibadulinová, A., Stanbridge, EJ., Závada, J., Kettmann, R., Pastorek, J. 1996. Human MN/CA9 gene, a novel member of the carbonic anhydrase family: structure and exon to protein domain relationships. *Genomics.* 33, 480–487.
- Parkkila, S. 2000. An overview of the distribution and function of carbonic anhydrase in mammals. *EXS* 90, 79–93.
- Patton, EE., Zon, LI. 2001. The art and design of genetic screens: zebrafish. *Nat Rev Genet.* 2, 956–966.
- Pawson, T., Nash, P. 2000. Protein–protein interactions define specificity in signal transduction. *Genes Dev.* 14, 1027–1047.

- Perry, SF., Shahsavarani, A., Georgalis, T., Bayaa, M., Furimsky, M. and Thomas, SL. 2003. Channels, pumps, and exchangers in the gill and kidney of freshwater fishes: their role in ionic and acid-base regulation. *J Exp Zool.* 300, A53- A62.
- Peterson, RE., Tu, C., Linser, PJ. 1997. Isolation and characterization of a carbonic anhydrase homologue from the zebrafish (*Danio rerio*). *J Mol Evol.* 44, 432–439.
- Pineda-Molina, E., Klatt, P., Vazquez, J., Marina, A., Garcia de Lacoba, M., Perez-Sala, D., and Lamas, S. 2001. Glutathionylation of the p50 subunit of NF- κ B: a mechanism for redox-induced inhibition of DNA binding. *Biochemistry.* 40, 14134–14142.
- Potter C, Harris AL. 2004. Hypoxia inducible carbonic anhydrase IX, marker of tumour hypoxia, survival pathway and therapy target. *Cell Cycle.* 3, 164–167.
- Potter, CP., Harris, AL. 2003. Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer. *Br J Cancer.* 89, 2–7.
- Purkerson, JM., Schwartz, GJ. 2007. The role of carbonic anhydrases in renal physiology. *Kidney Int.* 71, 103–115.
- Rahim, SM., Delaunoy, JP., Laurent, P. 1988. Identification and immunocytochemical localization of two different carbonic anhydrase isoenzymes in teleostean fish erythrocytes and gill epithelia. *Histochem.* 89, 451–459.
- Rodela, TM., Esbaugh, AJ., McDonald, MD., Gilmour, KM., and Walsh, PJ. 2011. Evidence for transcriptional regulation of the urea transporter in the gill of the Gulf toadfish, *Opsanus beta*. *Comp Biochem Physiol.* 160, B72-B80.
- Ryan, K., Bauer, DLV. 2008. Finishing Touches: Post-translational modification of protein factors involved in mammalian pre-mRNA 3' end formation *Int J Biochem Cell Biol.* 40, 2384–2396.

- Schaaf, MJ., Champagne, D., van Laanen, IH., van Wijk, DC., Meijer, AH., Meijer, OC., Spink, HP., Richardson, MK. 2008. Discovery of a functional glucocorticoid receptor beta-isoform in zebrafish. *Endocrinol.* 149, 1591–1599.
- Schlessinger, J. 2000. Cell signaling by receptor tyrosine kinases. *Cell.* 103, 211–225.
- Scholz, RW., Graham, KS., Gumprich, E., Reddy, CC. 1989. Mechanism of interaction of vitamin E and glutathione in the protection against membrane lipid peroxidation. *Ann NY Acad Sci.* 570, 514-7.
- Schulte, PM., Glemet, HC., Fiebig, AA., Powers, DA. 2000. Adaptive variation in lactate dehydrogenase-B gene expression: role of a stress-responsive regulatory element. *Proc Natl Acad Sci USA.* 97, 6597–6602.
- Schwartz, GJ., Kittelberger, AM., Watkins, RH., O'Reilly, MA. 2001. Carbonic anhydrase XII mRNA encodes a hydratase that is differentially expressed along the rabbit nephron. *Am J Physiol.* 284, F399–F410.
- Scott, DK., Stromstedt, P., Wang, J., and Granner, DK. 1998. Further characterization of the glucocorticoid response unit in the phosphoenolpyruvate carboxykinase gene. The role of the glucocorticoid receptor-binding sites. *Mol Endo.* 12, 482-491.
- Seet, BT., Dikic, I., Zhou, MM., Pawson, T. 2006. Reading protein modifications with interaction domains. *Nat Rev Mol Cell Biol.* 7, 473–483.
- Segard-Maurel, IK., Rajkowski, N., Jibard, G., Schweizer-Groyer, EE., Baulieu, F. Cadepond, A. 1996. Glucocorticosteroid receptor dimerization investigated by analysis of receptor binding to glucocorticosteroid responsive elements using a monomer–dimer equilibrium model, *Biochemistry.* 35, 1634.

- Segel, IH. 1975. *Enzyme Kinetics: Behavior and Analysis of Rapid Equilibrium and Steady-State Enzyme Systems*. John Wiley & Sons Inc., New York.
- Sippel, K., Robbins, A, Domsic, J., Genis, C., Agbandje-McKenna, M., and McKenna, R. 2009. High-resolution structure of human carbonic anhydrase II complexed with acetazolamide reveals insights into inhibitor drug design. *Acta Cryst.* 65, F992–F995.
- Sly, WS. 2000. The membrane carbonic anhydrases: from CO₂ transport to tumor markers. *EXS* 90, 95–104.
- Sobotka, H., Kann, S., 1941. Carbonic anhydrase in fishes and invertebrates. *Journal of Cellular and Comparative Physiology.* 17, 341–348.
- Storey, KB. 1996. Metabolic adaptations supporting anoxia tolerance in reptiles: recent advances. *Comp Biochem Physiol.* 113, B23–B35.
- Storey, KB. 2007. Anoxia tolerance in turtles: metabolic regulation and gene expression. *Comp Biochem Physiol.* 147, A263–A276.
- Storey, KB., Storey, JM. 2004. Oxygen limitation and metabolic rate depression. In: *Functional Metabolism: Regulation and Adaptation* Storey, KB. (Ed), John Wiley & Sons, New York, pp. 415–442.
- Storey, KB., Storey, JM. 2007. Putting life on ‘pause’ — molecular regulation of hypometabolism. *J Exp Biol.* 210, 1700–1714.
- Supuran, CT., Scozzafava, A. 2004. Benzolamide is not a membrane-impermeant carbonic anhydrase inhibitor. *Journal of Enzyme Inhibition and Medicinal Chemistry* 19, 269–273.
- Supuran, CT. 2008a. Carbonic anhydrases — an overview. *Curr Pharm Des.* 14, 603–614.
- Supuran, CT. 2008b. Carbonic anhydrases: novel therapeutic applications for inhibitors and activators. *Nat Rev Drug Discov.* 7, 181.

- Takahashi, H., Sakamoto, T. 2013. The role of 'mineralocorticoids' in teleost fish: relative importance of glucocorticoid signaling in the osmoregulation and 'central' actions of mineralocorticoid receptor. *Gen Comp Endocrinol.* 181, 223-228.
- Tashian, RE., Hewett-Emmett, D., Carter, N., Bergenhem, NCH. 2000. Carbonic anhydrase (CA)-related proteins (CA-RPs), and transmembrane proteins with CA or CA-RP domains. In: *The Carbonic Anhydrases*, Chegwidde, WR.; Carter, N.; Edwards, YH. (Eds), New Horizons, Birkhäuser Verlag, Basel, Switzerland, pp. 105–120.
- Tarrant, MK., Cole, PA. 2009. The chemical biology of protein phosphorylation. *Annu Rev Biochem.* 78, 797–825.
- Thingholm, TE., Larsen, MR., Ingrell, CR., Kassem, M. 2008. TiO₂-based phosphoproteomic analysis of the plasma membrane and the effects of phosphatase inhibitor treatment. *J Proteome Res.* 7, 3304–3313.
- Tibell, L., Forsman, C., Simonsson, I., Lindskog, S. 1983. Inhibition of human carbonic anhydrase II by anions and some 'neutral' compounds. *Inorganica Chimica Acta.* 79, 122–123.
- Tresguerres, M., Katoh, F., Orr, E., Parks, S.K., Goss, G.G. 2006. Chloride uptake and base secretion in freshwater fish: a transepithelial ion-transport metabolon? *Physiological and Biochemical Zoology.* 79, 981–996.
- Türeci, Ö., Sahin, U., Vollmar, E., Siemer, S., Göttert, E., Seitz, G., Parkkila, AK., Shah, GN., Grubb, J., Pfreundschuh, M., Sly, WS. 1998. Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. *Proc Natl Acad Sci USA.* 95, 7608–7613.

- Sobotka, H., Kann, S. 1941. Carbonic anhydrase in fishes and invertebrates. *Journal of Cellular and Comparative Physiology* 17, 341–348.
- Rizzello, A., Ciardiello, A., Acierno, R., Carratore, V., Verri, T., di Prisco, G., Storelli, C., and Maffia, M. 2007. Biochemical characterization of a S-glutathionylated carbonic anhydrase isolated from gills of the antarctic icefish *Chionoraco hamatus*. *The Protein Journal*. 26, 335-348.
- Uyss, R. and Wold, F. 1977. Posttranslational covalent modification of proteins. *Science*. 198, 890–896.
- Vijayan, MM., Pereira, C. and Moon, TW. 1994a. Hormonal stimulation of hepatocyte metabolism in rainbow trout following an acute handling stress. *Comp Biochem Physiol*. 108, C321–C329.
- Vijayan, MM., Reddy, PK., Leatherland, JF. and Moon, TW. 1994b. The effects of cortisol on hepatocyte metabolism in rainbow trout: a study using the steroid analogue RU486. *Gen Comp Endocrinol*. 96, 75–84.
- Vijayan, MM., Mommsen, TP., Glémet, HC. and Moon, TW. 1996a. Metabolic effects of cortisol in a marine teleost, the sea raven. *J Exp Biol*. 199, 1509–1514.
- Vijayan, MM., Morgan, JD., Sakamoto, T., Grau, EG. and Iwama, GK. 1996b. Food-deprivation affects seawater acclimation in tilapia: hormonal and metabolic changes. *J Exp Biol*. 199, 2467–2475.
- Vijayan, MM., Pereira, C., Grau, EG. and Iwama, GK. 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. *Comp Biochem Physiol*. 116, C89–C95.

- Voon, DC., Subrata, LS., Baltic, S., Leu, MP., Whiteway, JM., Wong, A., Knight, SA., Christiansen, FT., Daly, JM. 2005. Use of mRNA- and protein-destabilizing elements to develop a highly responsive reporter system. *Nucleic Acids Res.* 33, e27
- Warren, DE., Matsumoto, S., Roessig, JM., Cech, JJ. Jr. 2004. Cortisol response of green sturgeon to acid-infusion stress. *Comp Biochem Physiol.* 137, A611–A618.
- Wehling, M. 1997. Specific, nongenomic actions of steroid hormones. *Annu Rev Physiol.* 59, 365–393.
- Wendelaar Bonga, SE. 1997. The stress response in fish. *Physiol Rev.* 77, 591–625.
- Whitney, PL. 1974. Affinity chromatography of carbonic anhydrase. *Anal Biochem.* 57, 467–476.
- Wilson, JM., and Laurent, P. 2002. Fish gill morphology: inside out. *J Exp Zool.* 293, 192–213.
- Winum, J., Rami, M., Scozzafava, A., Montero, J., Supuran, C. 2008. *MedRes Rev.* 28, 445–463.
- Wold, F. 1981. In vivo chemical modification of proteins (post-translational modification). *Annual Review of Biochemistry.* 50, 783–814.
- Wood, CM., LeMoigne, J. 1991. Intracellular acid–base responses to environmental hyperoxia and normoxic recovery in rainbow trout. *Respir Physiol.* 86, 91–113
- Wood, CM., Milligan, CL., Walsh, PJ. 1999. Renal responses of trout to chronic respiratory and metabolic acidoses and metabolic alkalosis. *Am J Physiol.* 277, R482–R492.
- Xiong, Z., Storey, K. 2012. Regulation of liver lactate dehydrogenase by reversible phosphorylation in response to anoxia in a freshwater turtle. *Comp Biochem Physiol.* 163, B221-B228.
- Xu, Y., Feng, L., Jeffrey, P.D., Shi, Y., Morel, F.M. 2008. Structure and metal exchange in the cadmium carbonic anhydrase of marine diatoms. *Nature.* 452, 56–61.

- Yan, JJ., Chou, M. Y., Kaneko, T. and Hwang, P. P. 2007. Gene expression of Na⁺/H⁺ exchanger in zebrafish H⁺-ATPase-rich cells during acclimation to low-Na⁺ and acidic environments. *Am J Physiol.* 293, C1814-C1823.
- Yang, XJ. 2005. Multisite protein modification and intramolecular signaling. *Oncogene.* 24, 1653–1662.
- Zaccolo, M., Movsesian, M. 2007. cAMP and cGMP Signaling Cross-Talk: Role of phosphodiesterases and implications for cardiac pathophysiology. *Circ Res.* 100, 1569-1578.
- Zimmermann, PL., Pierreux, CE., Rigaud, G., Rousseau, GG., Lemaigre, FP. 1997. *In vivo* protein–DNA interactions on a glucocorticoid response unit of a liver-specific gene: hormone-induced transcription factor binding to constitutively open chromatin. *DNA Cell Biol.* 16, 713.

APPENDIX

Figure A-1. Representative kinetic data for the effect of incubation conditions that modulated the phosphorylation status of rainbow trout, *Oncorhynchus mykiss*, gill CA. Conditions stimulated endogenous protein kinases ('kinase') or phosphatases ('phosphatase') relative to incubation conditions that inhibited both kinases and phosphatases to preserve the endogenous phosphorylation status ('endogenous') of gill CA.

