

Cardiac responses to carbon dioxide in developing zebrafish (*Danio rerio*)

Scott Miller

Thesis submitted to the
School of Graduate Studies and Research
University of Ottawa
In partial fulfillment of the requirements for the
Masters of Science in Biology

Ottawa-Carleton Institute of Biology

Thèse soumise à
L'École des études supérieures et de la recherche
Université d'Ottawa
En vue de l'obtention de la maîtrise ès sciences biologie

L'Institut de biologie d'Ottawa-Carleton

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Acknowledgements

There are a number of people who deserve recognition and thanks during my time as a master's student at the University of Ottawa.

Firstly, there is my supervisor, Dr. Steve F. Perry, for his guidance and patience during this process. This project would not have been possible without his knowledge and guidance. I would also like to thank my committee members, Drs Kathleen Gilmour, Michael Jonz, and Pat Walsh, for always having an open door for assistance with any issues that I was encountering and offering suggestions.

Secondly, I thank my friends and colleagues in the lab. I am especially grateful for SS for teaching me about the zebrafish room and my measurement set-up; YK for his help with micro-injections; and JB and VT for their help with immuno/in-situ hybridization and confocal microscope use.

I also thank Aquatic Services, the Biology Department and the Biology Graduates Students Association (BGSA). This research was funded by a research grant from the Natural Science and Engineering Research Council (NSERC) to SFP.

Finally, I must thank my parents who without their support, this project would not have been possible. I would like to thank my fiancée, Lindsay, for her support as well.

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List of Abbreviations

Abbreviation	Full Name
5-HT	5-Hydroxytryptamine / serotonin
α -CA	Alpha carbonic anhydrase
ACTZ	Acetazolamide
Ca^{2+}	Calcium
$\text{Ca}(\text{NO}_3)_2$	Calcium nitrate
CA	Carbonic anhydrase
CA-I	Carbonic anhydrase isoform I
CA-II	Carbonic anhydrase isoform II
CA-III	Carbonic anhydrase isoform III
CA-IV	Carbonic anhydrase isoform IV
CA-V	Carbonic anhydrase isoform V
CA-VII	Carbonic anhydrase isoform VII
CA-VIII	Carbonic anhydrase isoform VIII
CA-IX	Carbonic anhydrase isoform IX
CA-XII	Carbonic anhydrase isoform XII
CA-X	Carbonic anhydrase isoform X
CA-XI	Carbonic anhydrase isoform XI
CA-XIII	Carbonic anhydrase isoform XIII
CA-XIV	Carbonic anhydrase isoform XIV
CA-XV	Carbonic anhydrase isoform XV
CA-RP	Carbonic anhydrase related protein
CAb	red blood cell carbonic anhydrase
Cac	Cytosolic carbonic anhydrase
CCAC	Canadian Council on Animal Care
CSF	Cerebrospinal fluid
CO_2	Carbon dioxide
dpf	days post fertilization
H^+	Proton
H_2CO_3	Carbonic acid
HCl	Hydrochloric acid
HCO_3^-	Bicarbonate ion
h	Hour
hpf	hours post fertilization
HR	Heart rate
K^+	Potassium
Kcat	Catalytic turnover constant
KCl	Potassium chloride
L	Liter
MgSO_4	Magnesium sulfate

min	Minute
mmHg	Millimeters of mercury
mM	Millimolar
MS-222	Ethyl 3-aminobenzoate methansulfonate salt
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NEC	Neuroepithelial cell
ng	Nanogram
nl	Nanoliter
O ₂	Oxygen
Pco ₂	Partial pressure of carbon dioxide
pg	Picogram
RBC	Red blood cell
SEM	Standard error of the mean

Abstract

The ontogeny of carbon dioxide (CO₂) sensing in zebrafish (*Danio rerio*) has not been studied. In this thesis, CO₂-mediated increases in heart rate were used to gauge the capacity of zebrafish larvae to sense CO₂. CO₂ is thought to be sensed through neuroepithelial cells (NECs), which are homologous to mammalian carotid body glomus cells. Owing to its role in facilitating intracellular acidification during exposure to hypercapnia, it was hypothesized that carbonic anhydrase (CA) is involved in CO₂ sensing, and that inhibition of CA would blunt the downstream responses. The cardiac response to hypercapnia (0.75% CO₂) was reduced in fish exposed to acetazolamide, a CA inhibitor, and in fish experiencing CA knockdown. Based on pharmacological evidence using β -adrenergic receptor (β -AR) antagonists, and confirmed by β 1AR gene knockdown, the efferent limb of the reflex tachycardia accompanying hypercapnia is probably mediated by sympathetic adrenergic neurons interacting with cardiac β 1 receptors.

Résumé

L'ontogenèse de senseur de dioxyde de carbone des poissons zébrés (*Danio rerio*) n'a pas été étudié chez les larves de poissons zébrés. Cette étude a utilisé une mesure de la fréquence cardiaque comme une réponse physiologique à CO₂. On pense que le CO₂ peut être sensé par les cellules neuroépithéliales (NEC), qui sont homologues les cellules du corps glomérulaire de la carotide des mammifères. En raison de son rôle dans la facilitation de l'acidification intracellulaire lors de l'exposition à une hypercapnie, on a énoncé l'hypothèse que l'anhydrase carbonique (CA) est impliqué dans le role senseur de CO₂, et inhibition du CA affaiblirait la réaction plus tard. La réponse à l'hypercapnie (CO₂ 0,75%) a été réduite dans les poissons exposés à l'acétazolamide, un inhibiteur de CA, et la perte de l'expression des deux formes de récepteur CA. Basé sur l'évidence pharmacologiques utilisant des antagonistes des récepteurs adrénrgiques β , et confirmé par le perte de l'expression β 1AR, le efférente de la tachycardie réflexe qui accompagne l'hypercapnie est médiée par les neurones adrénrgiques sympathiques qui interagissent avec les récepteurs β 1.

1 Introduction

1.1 Carbonic anhydrase

Carbonic anhydrase (CA) is an enzyme that is found in all living species, including vertebrates, plants and bacteria. CA is involved in numerous key functions such as bone reabsorption/calcification, acid-base balance, ionic regulation, and primarily metabolic processes such as gas transport. The existence of CA was first predicted because the uncatalyzed rate of bicarbonate dehydration is slower than the transit time through the respiratory surface (Faurholt 1924; Henriques 1928a, b). CA was first discovered 80 years ago in the red blood cell (RBC) (Brinkman et al., 1932; Meldrum and Roughform 1933) and subsequently identified in the stomach (Davenport 1939) and kidney (Davenport and Wilhelmi, 1941).

Early research on CA focused on how CA activity was related to the size of the animal. It was found that CA activity decreased as the size of the animal increased (Larmier and Schmidt-Nielsen, 1961), due to metabolic scaling, because mass-specific metabolic rates decrease as animal mass increases. The focus of CA research began to shift in 1961 when multiple isoforms of CA were first discovered (Nyman 1961).

1.1.1 CA molecular structure

For CA to function quickly and effectively, four specific sites must be present and functional. The most important of these is the zinc-binding site; it is in this pocket that the zinc is bound directly to the enzyme by three histidine amino acids (Christianson and Alexander, 1989). The zinc-binding pocket is conserved among all isoforms of CA except

for the carbonic anhydrase related-proteins (CA-RP) which has no activity (Tashian et al., 2000). In order for the zinc to bind with hydroxide (the mechanism of which will be explained below), an adjacent substrate-associated hydrophobic pocket formed by six amino acids must also be functional (Krebs et al., 1993). For the regeneration of the zinc bound to hydroxide, a proton shuttling mechanism is used to transfer the proton from a zinc bound water molecule to cytoplasmic buffers (Tu and Silverman, 1989). In high activity forms of CA, this is accomplished by His-64, the reaction of which can be slowed or eliminated with an amino acid substitution at 64 or an amino acid with a bulkier side chain at 65. This is the case in CA-I and V where the proton shuttling mechanism is slowed down and eliminated in CA-III (Tu et al., 1990; Lindskog and Silverman, 2000; Duda et al., 2005).

1.1.2 Mechanism of CA function

The general mode of action (for a detailed review see Lindskog and Silverman, 2000) of CA begins with CO₂ entering the active pocket and nucleophilic attack by the zinc bound hydroxide (Silverman and Lindskog, 1988; Christianson and Fierke, 1996). This directly forms a bicarbonate ion (HCO₃⁻), thus bypassing the much slower uncatalysed rate of first forming carbonic acid, followed by proton dissociation (CO₂ + H₂O ↔ H₂CO₃ ↔ H⁺ + HCO₃⁻). Regeneration of the zinc bound hydroxide is accomplished by the proton shuttling mechanism (Tu and Silverman, 1989). It has since been found that the catalytic turnover constant (K_{cat}) value of CA II is 1x10⁶1x10⁶ s⁻¹ and for; CA I is about 10% of that value (Chegwidden and Carter, 2000). CA III is even slower yet, displaying less than 1% the activity of CA II, with the CA-RP showing no activity at all. The very slow activity of CA III

has been attributed to changes in the amino acid sequence that cause a decrease in the proton shuttling mechanism, specifically when the histidine at position 64 is replaced with lysine (Jewell et al., 1991). In terms of the membrane bound CAs, CA IV has similar activity to CA II and the others are between 22-34% of CA II (Chegwidden and Carter 2000; Supuran 2008a).

1.1.3 CA in mammals

Of the five families of CA, only α -CA is found in mammals (Chegwidden and Carter 2000; Hewett-Emett 2000). In total, 16 isoforms of α -CA have been identified. Six are found in the cytoplasm (CAI-III, V, VII and XIII) of which CA-V is found only within the mitochondrion; five are located on the cell membrane (CA-IV, IX, XII, XIV, XV); and three others have no CA activity and have been named (CA-RP) (CA-VIII, X, XI) (Tashian et al., 2000). Of the cytosolic isoforms, the two most prevalent in the RBC of mammals are CA I (low activity) and CA II (high activity) (Chegwidden and Carter, 2000).

Studies on CA I deficient mammals show they are able to maintain stable CO₂ tensions (Sly and Hu, 1995). Other studies have shown that certain species are able to maintain CO₂ transport without functional CA II, the higher activity isoform (Yang et al., 1998; Chegwidden and Carter, 2000). It has been suggested that only 20% of CA activity is needed to maintain proper gas exchange (Maren and Swenson, 1980; Swenson 2000).

1.1.4 CA in fish

It is important to note that most of the research on CA has been conducted on mammals; however, phylogenetic analysis has demonstrated that the typical nomenclature used for mammals may be inappropriate for fish. For example, in fish there are no orthologous genes matching the two blood CA isoforms, CA I and II. Instead, the ubiquitous cytosolic CA isoform has been termed cytosolic CA or CAC, while the RBC-specific isoform was named CAb (Esbaugh et al., 2005; Esbaugh and Tufts, 2006); this is the nomenclature applied throughout the remainder of this thesis.

In teleosts fish, CAb is found primarily in the blood and CAC is expressed mainly in the gills and has almost no expression within red blood cells (Rahim et al., 1988; Esbaugh et al., 2005; Lin et al. 2008). In trout, HCO_3^- dehydration was unaffected except at hematocrit levels below 5% of normal levels (Perry and Gilmour, 1993), suggesting that vertebrates have an excess of CA in order to maintain homeostasis as was seen in the mammals.

Early in zebrafish development, zCAb has a higher expression than zCAC for the first 120 hours post fertilization (hpf), while CO_2 excretion rates rose 15 times the basal amount, from 24 to 48 hpf. When the larvae are treated with acetazolamide (a CA inhibitor of both isoforms), CO_2 excretion rates dropped by 52%. Antisense morpholino oligonucleotides, gene knockdowns, of either or both genes, also caused a significant decrease in CO_2 excretion (Gilmour et al., 2009).

1.2 CO₂ transport

A brief review of carbon dioxide and oxygen transport will follow; for a diagrammatic representation refer to figure 2 in Esbaugh and Tufts (2006). For a more detailed review, see Brauner and Randall (1998). Starting in the tissue, the site of CO₂ production, CO₂ diffuses out of the tissue into the blood and then into the RBC along its partial pressure gradient. Once within the RBC, CA_b converts the carbon dioxide to a proton and a bicarbonate ion (CO₂ → H⁺ + HCO₃⁻). The bicarbonate ion exits the RBC via the band 3 ion transporter (Cameron 1978) and the proton is buffered by hemoglobin and other proton buffers within the RBC. The conversion of CO₂ to a proton and bicarbonate ion leads to an increase in the total amount of CO₂ that can be carried in the blood. At the respiratory surface, gills in fish/lungs in mammals, CA catalyzes the reverse reaction and CO₂ exits the RBC and blood into the water/air once again along a partial pressure gradient.

1.3 Chemoreceptors

Chemoreceptive cells are found in a large variety of animals including simple invertebrates, gastropods and crustaceans (Inoue et al., 2001; Kuange et al., 2002; Massabuau and Meyrand, 1996). The mammalian O₂ sensing chemoreceptors have been studied in the most detail.

1.3.1 In mammals

Development of peripheral chemosensitivity begins with the formation of afferent neurons, development of Ca²⁺ sensitivity and finally, the ability to release

neurotransmitters (Donnelly, 2000). After birth, in mammals, there is an increase in the innervation of principal peripheral sensing structure, the carotid body. This also marks the increase in sensitivity of hypoxia (Gonzales et al., 1994).

Central chemoreceptors in the brain respond to changes in the cerebrospinal fluid (CSF), caused by a drop in pH with the movement of CO₂ (Burlison and Smatresk, 2000; Lahiri and Forster, 2003). In mammals, 30-40% of the response to hypercapnia is caused by peripheral receptors, while the remainder is thought to be caused by central chemoreceptors.

1.3.2 In fish

Homeostasis and regulation of cardiorespiratory function are very important in fish, as they experience a great deal of fluctuation in their external environments. Fish neuroepithelial cells (NEC) have evolved to be efficient sensors within the gills for regulation of cardiorespiratory responses. Originally, researchers were trying to find a single model of gas transfer and sensing for all species of fish; however, a great deal of intra-specific variation exists.

1.4 Fish gill

In the wild, fish are often exposed to a wide variety of gas tensions that can occur diurnally, as well as spatially (Crocker et al., 2000). In general, the gill is thought to be the predominant location for chemoreception. The general response, among most species of fish, to hypercapnia and hypoxia, is hyperventilation, bradycardia and increased systemic

vascular resistance. Originally, it was not thought that CO₂ caused a cardio respiratory response in fish. It was thought that the response was indirectly caused by the Bohr and Root effects, with the changes in pH decreasing the oxygen carrying properties of hemoglobin (Randall 1982; Smith and Jones 1982).

The response after a stimulus such as hypoxia, hypercapnia, change in pH and application of a pharmacological agent is thought to result in the release of a neurotransmitter (5-HT), firing the carotid sinus nerve in mammals, and causing changes in respiratory or cardiac patterns. In teleost fish, hypoxia induces hyperventilation, bradycardia, and an increase in vascular resistance (Randall and Shelton, 1963). These responses are thought to originate from chemoreceptors located within the gill (Milsom and Brill, 1986; Burlison et al., 1992).

Teleost fish contain 8 gill arches (four on each side) which are innervated by cranial nerves. The first gill arch receives innervation from VII (facial) and the glossopharyngeal (IX). All eight arches are innervated from the vagus (X) cranial nerve. Electrical recordings from these nerves show that they are responsive to both internal (blood) and external (water) hypoxia (Milsom and Brill, 1986; Burlison and Milsom, 1993), suggesting that they innervate both internal and external chemoreceptors.

NECs were first identified by Dunel-Erb et al. (1982); it was noted that serotonin containing cells degranulated upon exposure to severe hypoxia. It has been established that fish contain peripheral CO₂ chemosensory cells, but it would appear that central CO₂ chemoreceptors are only present in air breathers (Milsom, 2002).

1.4.1 NEC

These chemoreceptors in fish are termed neuroepithelial cells (NECs) and have been identified in all fish species examined to date (Bailey et al., 1992; Zacccone et al., 1994; Perry et al., 2009). The distribution of chemosensing NECs are species dependent. In rainbow trout (*Oncorhynchus mykiss*), they are present on the first gill arch (Perry and Reid, 2002); in catfish (*Ictalurus punctatus*), they are present in the first 3 gill arches; and in zebrafish, they are located on all four gill arches (Jonz and Nurse, 2003).

NECs are positioned within the gill within the filament epithelium in such a way that they can monitor the partial pressure of both the blood (internal) and environmental water (external). The effect of hypoxia on fish NEC is similar to that of type 1 glomus cells in the carotid body of mammals which is homologous to the first and second gill arches (Fritsche and Nilsson, 1993; Milsom, 1998, 2002; Milsom and Burleson, 2007; Taylor et al., 1999; Burleson and Milsom, 2003), suggesting that they would be related to chemical sensing (Zacccone et al., 1997).

1.4.2 Development of gills in zebrafish

In zebrafish, gill primordia (lacking lamellae) do not develop until three days post fertilization (dpf). At this stage, the larvae are small enough that coetaneous respiration can meet the O₂ demands of the fish, and gills are not yet needed. NECs in the zebrafish gill first appear in the gill arch at 3 dpf, in the gill filament at 5 dpf but are not fully innervated until 7 dpf (Jonz and Nurse, 2005). The gills do not become fully functional or used as the primary means of respiration until 14 dpf (Kimmel et al., 2003; Rombough, 2002).

However, hyperventilation and changes in cardiac activity (Turesson et al., 2003; Jacob et al., 2002) have been recorded before 14 dpf. For example, zebrafish first exhibit a behavioral response to hypoxia at 2 dpf, as demonstrated by increased pectoral fin movement. However, this increase is not significant until 3 dpf, before the gills are innervated (Jonz and Nurse 2005). These results suggest the presence of extra-branchial chemoreceptors, which were recently identified on the skin (Coccimiglio and Jonz, 2012). The cutaneous NECs receive innervation prior to the gill NECs, and their number and density decrease as the number of NECs on the gill increases.

1.5 Development of the heart

The development of the zebrafish heart is similar to that of all vertebrates. The process begins with cardiac progenitor cells migrating medially from tubular epithelial primordial on either side of the midline and fusing to form the heart tube at 24 hpf (Stainier et al., 1993). When the zebrafish embryo is 30 hpf, the heart loop begins to move to the right hand side, as is conserved evolutionarily in all vertebrate. At 2 dpf, the chambers of the heart can be viewed; blood can be seen entering the heart through the sinus venous, flowing through the atrium and ventricle, and then leaving from the bulbus arteriosus. Finally, at 5 dpf, the heart has the same orientation as the adult heart, with the atrium sitting dorsally to the ventricle (Stainier and Fishman 1993).

1.6 Hypoxia

Most NECs are located within the gills, contain serotonin (5-HT) and a neurotransmitter that is proximally located to the nerve fibres (Dunel-Erb et al., 1982; Jonz and Nurse, 2003). Chronic hypoxia in zebrafish resulted in hypertrophy and proliferation of NECs containing both serotonin and synaptic vesicle (SV2) (Jonz et al., 2004). Cultured NECs respond to hypoxia under voltage clamp conditions with a decrease in K⁺ current that is quinidine sensitive (Jonz et al., 2004).

The first step in the sensory pathway of O₂ reception is membrane depolarization, associated with the decreased conductance of background K⁺ channels, which leads to opening of voltage-gated Ca²⁺ channels, an increase in cellular Ca²⁺, and neural secretion (Gonzalez et al., 1994; Lopez-Barneo et al., 2001; Fu et al., 2002; Jonz et al., 2004).

1.7 Hypercapnia

Using patch clamp electrophysiology, it was shown that a subset of NECs in zebrafish is responsive to both O₂ and CO₂. It was also shown that at least a subset of NECs in culture contains both carbonic anhydrase and 5-HT. The extent of membrane depolarization is reduced after CA inhibition that is independent of changes in pH (Qin et al. 2010). From the limited studies of CO₂ chemoreception in fish, the receptors appear to be externally oriented and respond to changes in Pco₂ instead of pH (Gilmour, 2001; Perry and Gilmour, 2002, 2006; Milsom, 2002).

As with hypoxia, there is a large amount of inter-specific variation in the hypercapnia response. Hypoxia and hypercapnia usually occur together and so it was

proposed that hypercapnia may serve as an early warning system for approaching hypoxia (Gilmour and Perry, 2006).

One of the biggest differences between air breathers and most fish is the difference in CO₂ levels within the blood. Mammals maintain ~40 mmHg, whereas fish have levels approaching 2-3 mmHg. This means that fish have a very low set point and an ability to detect very small changes in levels of CO₂. Zebrafish develop hyperventilation at 0.13% CO₂ corresponding to a change of ~1 mmHg (Vulesevic et al., 2005).

When exposed to hypercapnia, most fish examined hyperventilate (Perry and Wood 1989). In mammals, the stimulus within chemoreceptor cells is a drop in intracellular pH (Gonzalez et al., 1992). In fish gills, the stimulus that is generally accepted is a change in extracellular P_{CO₂} (Sundin et al., 2000; Reid et al., 2000; Perry and McKendry, 2001). Hyperventilation can be achieved with either an increase in the frequency or the amplitude of ventilatory movements; however, these adjustments are species-specific (Gilmour, 2001). The physiological benefit of hyperventilation during hypercapnia has been questioned, but the increased ventilation presumably will minimize the extent of acidosis associated with exposure to elevated CO₂ (Gilmour, 2001).

In developing zebrafish, tachycardia has been reported in larvae 4 dpf exposed to hypoxia (Jacob et al., 2002); hypoxic bradycardia does not develop until 20-30 dpf (Barrionuevo and Burggren, 1999). The cardiac responses of larval zebrafish to elevated CO₂ are, as yet, unknown.

1.8 Thesis goals and hypotheses

The goal of this thesis was to 1) study the effects of hypercapnia on heart rate in developing zebrafish and determine the point at which they become sensitive to increasing environmental CO₂; 2) determine whether the CO₂-mediated effects on heart function arise from the change in pH and/or the change in Pco₂; 3) determine if the responses are mediated through reflex neural pathways and characterize the nature of these pathways; and 4) evaluate the role of carbonic anhydrases in facilitating the cardiac responses to elevated CO₂.

With the recent discovery of extra brachial chemoreceptors on the skin and their similarity to NEC of the gill, the following hypotheses were formulated: 1) NEC of the gill or skin will need to be innervated for CO₂ sensing to occur at 5 dpf; 2) CO₂ will directly stimulate the heart rate response; 3) the CNS will be involved in CO₂ sensing; 4) both isoforms of CA will be involved in the response pathway to CO₂.

2 Materials and Methods

2.1 Animals

Adult zebrafish, *Danio rerio*, were purchased from a commercial supplier (Big Al's, Ottawa, Canada) and maintained in-house at the University of Ottawa's Aquatic Care Facility. They were kept in 10 litre (L) tanks that were supplied with well-aerated, dechlorinated tap water at 28°C. The fish were kept at a constant photoperiod of 14 hours light and 10 hours dark.

To obtain larvae, 1 L breeder traps were placed on the bottom of the tanks the night before breeding, and fish were allowed to spawn for 3 hours (h). To obtain the embryos for micro-injections, 2 L breeding traps were set up with 2 females and 1 male that were allowed to breed for 30 min the following morning. The experiments and handling of the animals were carried out in accordance with institutional guidelines (protocol BL-226) that conform to the guidelines of the Canadian Council on Animal Care (CCAC).

The zebrafish embryo (5mM NaCl, 0.17mM KCl, 0.33 CaCl₂, 0.33mM MgSO₄ and 0.1% Methylene Blue) medium was changed after hatching, and larvae were maintained at 28°C until they reached experimental time points (4-7 dpf).

2.2 Heart Rate Measurements

Larvae were anaesthetized in a small volume of 80 mg l⁻¹ of Tris-buffered MS-222 (ethyl-3-3aminobenzoate methanesulfonate salt, Sigma-Aldrich Inc., St. Louis MO, USA). After 5 min, larvae (N=6, for all trials unless noted otherwise) were transferred to a flow

through system for heart rate observations, under a dissection microscope, fitted with a CCD camera, and recorded on a computer.

Series 1- the effect of CO₂ on heart rate

Baseline measurements were taken on fish for 1 min; CO₂ levels were increased from air-saturated water to 0.75% CO₂ in 0.25% increments with heart rate measurements taken for each level of CO₂. CO₂ concentrations were changed using a Cameron 2 or 3 channel gas mixer, mixing the desired level of CO₂ with air, and bubbled through water at a rate of 2000cm² per min. Another group of fish was exposed to 10⁻⁴ M acetazolamide (Sigma-Aldrich Inc., St. Louis MO, USA), dissolved in water for 20 min prior to measurements and for the course of the treatment. In order to dissolve the acetazolamide, the pH was raised to 11 using NaOH until the acetazolamide was dissolved and titrated back down to 7.2 using HCl (Gilmour et al. 2009).

Series 2- the effect of nicotinic and β-adrenergic receptor blockade

Fish were anaesthetized as described above and placed in the flow-through system for baseline measurements; however, instead of first increasing the CO₂, one of the following drugs (10⁻⁴ M) was added to the air-equilibrated water: propranolol (non-specific β-receptor antagonist), atenolol (β₁ specific antagonist), hexamethonium (nicotinic receptor antagonist), (Sigma-Aldridge Inc.). Another measurement was taken after 30 min of exposure to the drug. The CO₂ was then increased to .75% CO₂, and a final measurement was taken.

Series 3- Microinjections/Rescue

Morpholino Injection

zCAb, zCAc, zebrafish β 1AR and control morpholino antisense oligos (Gene Tools, Philomath, OR, USA) have the following sequences respectively: 5'-CAAGCGTGGGCCATGATTATAAATG-3', 5'-AGTGGTCAGCCATTCCGCCAGCTGT-3', 5'-ACGGTAGCCCGTCTCCCATTG-3' and 5'-CCTCTTACCTCAGTTACAATTTATA-3', each with a 3'-cCarboxyfluorescein end modification. All morpholinos were prepared to a final concentration of 4 ng nl⁻¹ in *Danio* buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, and 5 mM HEPES with a final pH of 7.6), and phenol red was used as an indicator of positive injection. Injections were performed using a Narishige IM300 Microinjector (Narishige International USA Inc, Long Island, NY, USA). Dosage and sequence of zCA isoforms are based on Gilmour et al. (2009) and β 1AR from Steele et al. (2011). Neither study reported adverse effects based on a dosage of 4 ng embryo⁻¹. Injections were done up until the 4-cell stage. After injections, embryos were placed in petri dishes containing E3 medium (5mM NaCl, 0.17mM KCl, 0.33 CaCl₂, 0.33mM MgSO₄ and 0.1% Methylene Blue) and placed in an incubator at 28°C. The following day, embryos were screened for positive expression of 3'-Carboxyfluorescein using a Nikon SMZ 150 stereomicroscope (Nikon Instrument Inc., Melville, NY, USA).

All measurements for heart rate were made following the same procedure as in Series 1.

Carbonic Anhydrase Rescue

Zebrafish cDNA was made using revertAid primers (Fermentas Canada Inc., Burlington, ON), according to the manufacturer's instructions and primed with random hexamers. Primers used to make the mRNA for the CAc rescue were of the following sequence: forward: 5'-ACGGCAGGGCATGGCTGACCACT-3'; reverse: 5'-TTAAAAGATGCACGCACCAC-3'. The product was inserted into a p-drive (Qiagen Inc., Toronto, ON) for sequence confirmation and sub-cloned into a pCS2+ vector. The mRNA was then synthesized using a mMessage-mMachine kit (Ambion, Streetsville, ON), according to the manufacturer's instructions. The product was run through a gel to confirm the size and integrity of the mRNA. 100 pg of the mRNA was injected into the zebrafish embryos at the 1 or 2 cell stage, either alone or in conjunction with zCAc morpholino.

All measurements of heart rate were made following the same procedure as Series 1.

2.3 Statistical Analyses

All data are presented as means +/- SEM. Data were analyzed either by two-way repeated measures, ANOVA compared to controls or paired T-tests where appropriate. When needed, during ANOVA analysis, a Holmes-Sidak t-test *post hoc* analysis was conducted to determine statistical differences between data points within a series. All statistical analysis was completed using commercial software (SigmaPlot 9, SPSS Inc.). A P-value of <0.05 was set to determine statistical significance.

3 Results

At 4 days post fertilization (dpf), none of the concentrations of CO₂ that were tested affected heart rate (HR; Fig. 1A). At 5 dpf, only the highest level of CO₂ (0.75%) produced a significant increase in HR when compared to the normocapnic group (P = 0.002; Fig. 1B). At 7 dpf, HR was increased, at 0.5% and 0.75% CO₂ (P < 0.001; Fig. 1C). Because 5 dpf was the earliest stage of development when fish exhibited CO₂ sensitivity, this stage was chosen for all subsequent experiments, except for those involving inhibition of CA by acetazolamide (ACTZ) for which experiments were conducted at 5 and 7 dpf. The more robust HR response to CO₂ at 7 dpf made it easier to detect potential inhibitory effects of CA inhibition.

At 0.75% CO₂ (the highest concentration used in this study), the pH of the water decreased from 7.2 to 6.6. Exposing zebrafish to normocapnic water at pH 6.6 (Fig. 2) caused a significant decrease in HR (P = 0.037), thereby demonstrating that the tachycardia observed during exposure to hypercapnic acidosis was likely the result of CO₂ and not the acidity.

The addition of hexamethonium, a nicotinic ganglionic blocker, prevented a significant increase in HR in fish exposed to 0.75% CO₂ (Fig. 3). The heart rate of the control fish exposed to 0.75% CO₂ was significantly higher than either the air-exposed (normocapnic) controls or the CO₂-exposed fish treated with hexamethonium. Therefore, it can be concluded that the sensing of CO₂ at 5 dpf is under neural control.

Addition of propranolol, a non-specific β-adrenergic receptor blocker (Fig. 4A), caused a significant decrease in heart rate in the normocapnic fish (from to 137.3 +/- 2.2 to

123.3 +/- 4.4) and prevented the increase in HR that was observed in the hypercapnic control fish (Fig. 4). Unlike propranolol, the specific β 1 receptor antagonist, atenolol, did not affect HR in normocapnic fish; however, atenolol did prevent the usual increase in HR accompanying 0.75% CO₂ (Fig. 4B).

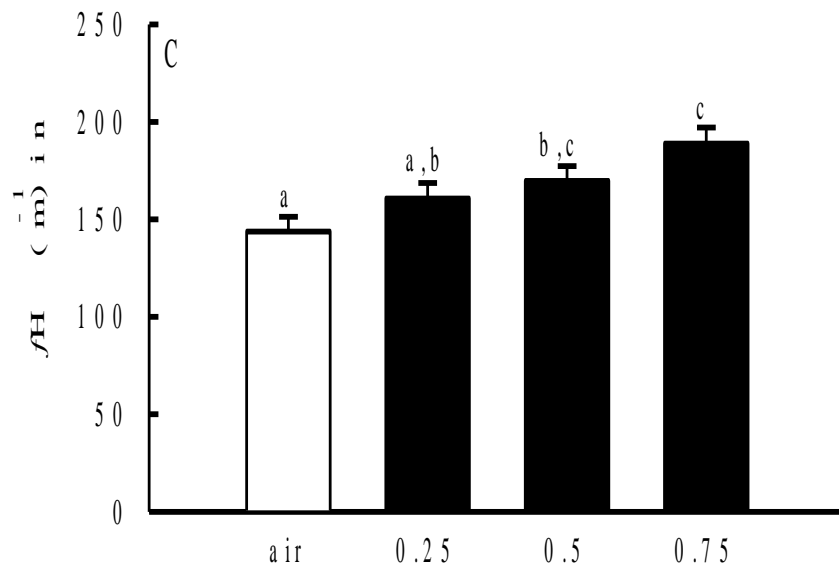
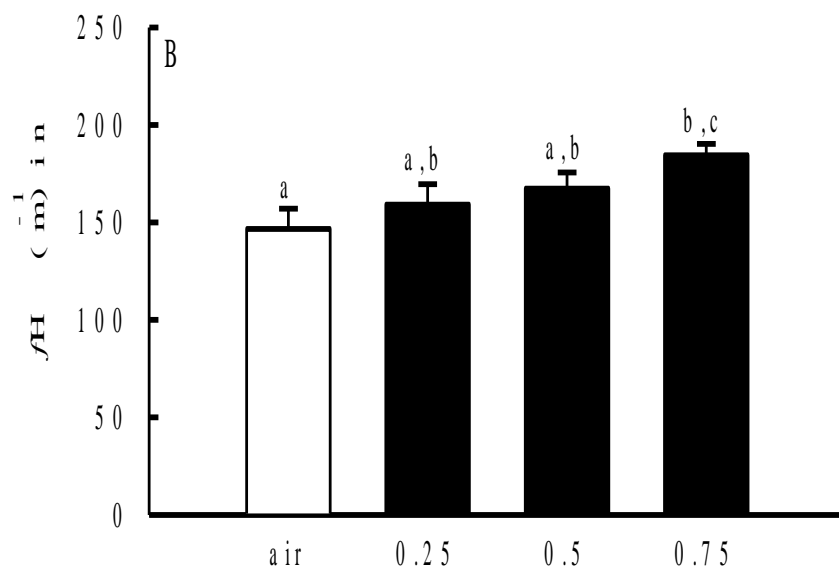
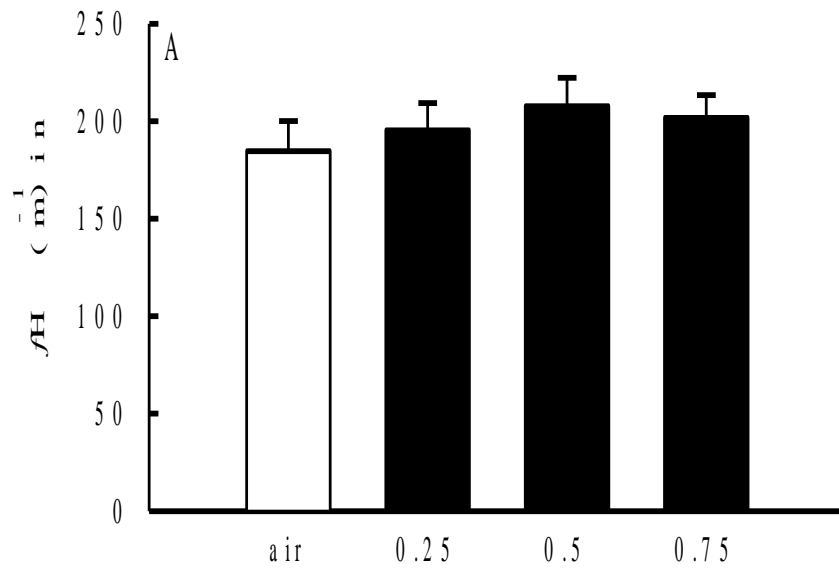
The studies using pharmacological blockade of β -adrenergic receptors were complemented by additional experiments employing translational gene knockdown of the β 1-AR (Fig. 5). Unlike the sham injected group that significantly increased their HR when exposed to 0.75% CO₂ ($P = 0.003$), the group of fish experiencing β 1-AR knockdown did not increase its HR during hypercapnia (157.3 +/- 6.8 in normocapnia versus 151.7 +/- 3.9 bpm ($N = 6$)). At 0.75% CO₂, the β 1-AR group had a significantly lower HR than the sham injected fish ($P < 0.001$).

To determine whether CA was involved in CO₂ sensing, cardiac responses to hypercapnia were assessed with and without acetazolamide, a membrane permeable CA inhibitor. At 5 dpf, the acetazolamide treated fish failed to increase HR at all levels of CO₂ (Fig. 6A). When 7dpf zebrafish (which exhibited a more robust response to CO₂ in comparison to fish at 5 dpf) were tested, acetazolamide treatment again prevented a significant increase in HR (Fig. 6B).

To ensure that the acetazolamide treated fish were still able to increase HR, fish at 5 and 7 dpf were exposed to the cardiac stimulant norepinephrine (Fig. 7 A & B). In both sets of acetazolamide treated fish, the addition of norepinephrine caused a significant increase in HR. This showed that acetazolamide was not preventing zebrafish from increasing their HR.

Because acetazolamide is likely to inhibit all isoforms of zebrafish CA, an alternate approach employing selective gene knockdown was used to specifically assess the roles of the RBC (zCAB) and the general cytosolic (zCAC) isoforms. The data summarized in Fig. 8 clearly demonstrate that knockdown of either zCAC or zCAB prevented the increase in HR that was observed in the fish injected with the control morpholino.

Figure 1. Effect of hypercapnia (P_{CO_2} = air, 0.25, 0.5, 0.75 %) on heart rate (HR) expressed as beats per minute (bpm) in (A) 4 dpf, (B) 5 dpf, (C) 7 dpf zebrafish. Values are expressed as means + SE; N=6. Letters indicate differences among the group ($P < 0.05$; one way repeated measure ANOVA).



CO₂ (%)

Figure 2. Effect of pH (7.2 and 6.6) on HR in 5 dpf zebrafish. Values are expressed as means + SE; N=6. (*P<0.05; Paired t-test).

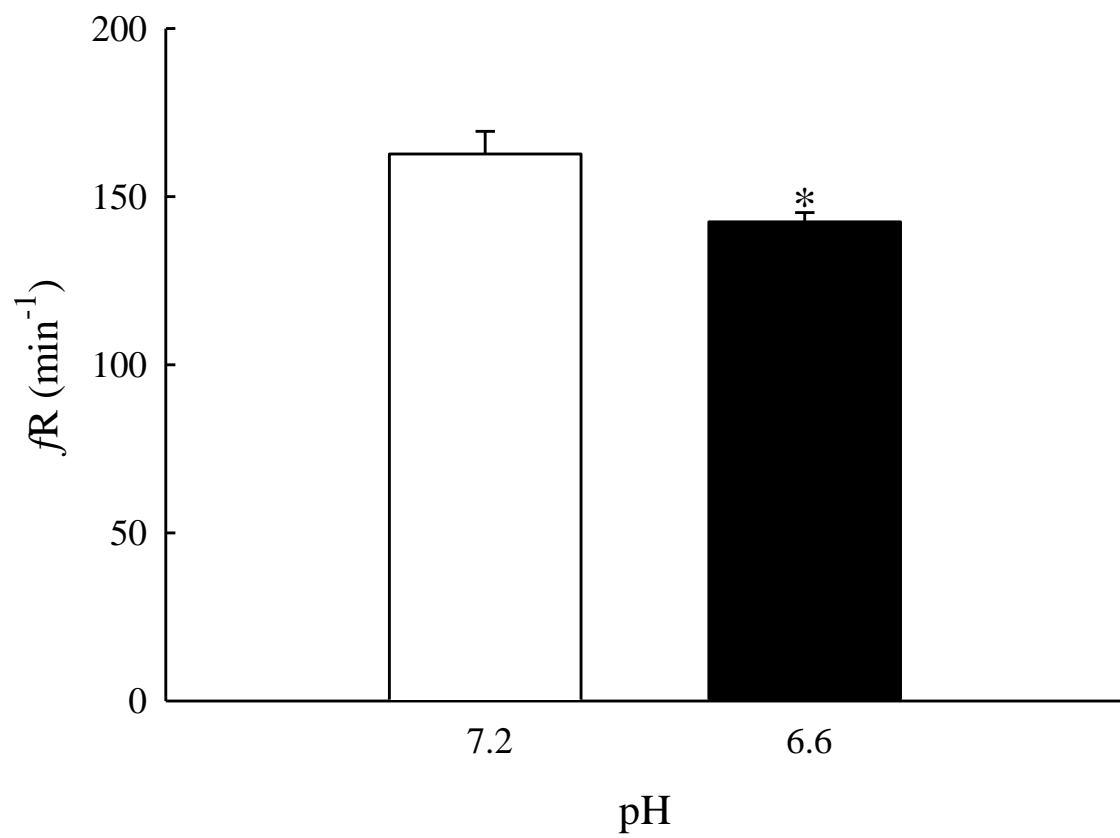


Figure 3. Effect of 10^{-4} M solution of hexamethonium on HR in 5 dpf zebrafish. Values are expressed as means + SE; N=6. Letters indicate differences among the group ($P < 0.05$; one way repeated measure ANOVA).

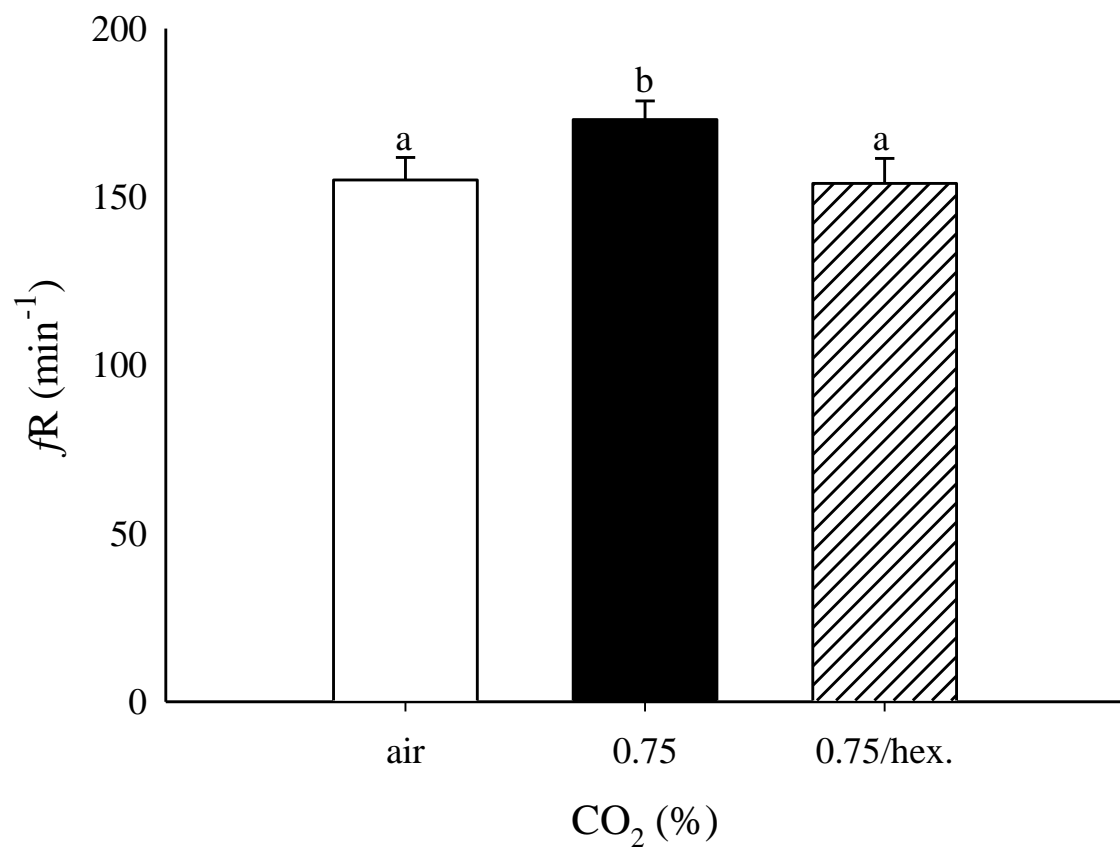


Figure 4. Effect of hypercapnia (P_{CO_2} = air and 0.75%) and 10^{-4} M solution of (A) propranolol or (B) atenolol on HR in 5 dpf zebrafish. Values are expressed as means + SE; N=6. (* $P < 0.05$ between air and 0.75% CO_2 ; T $P < 0.05$ between control and propranolol group; two way repeated measure ANOVA).

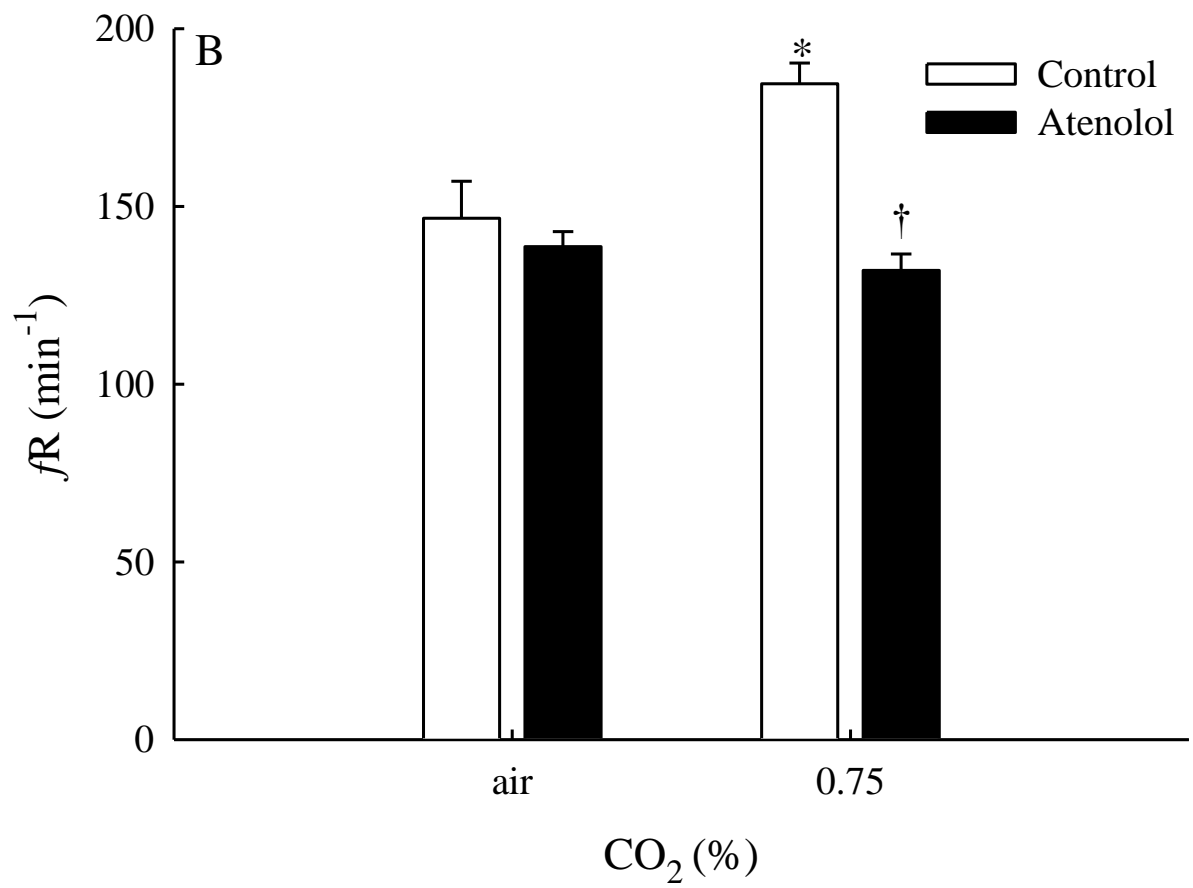
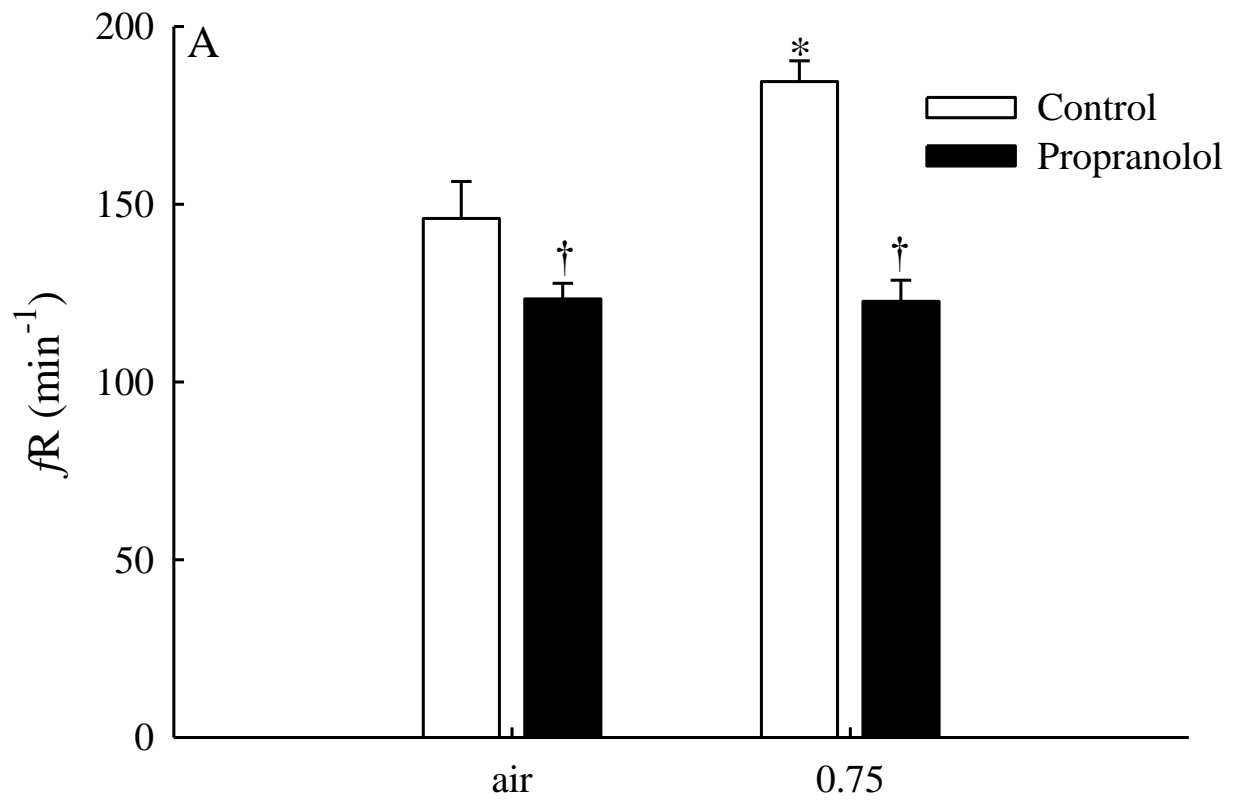


Figure 5. Effect of Hypercapnia (P_{CO_2} = air and 0.75%) on HR of 5 dpf zebrafish injected with control and β 1AR knockdown morpholino. Values are expressed as means + SE; N=6. (* $P < 0.05$; two way repeated measure ANOVA).

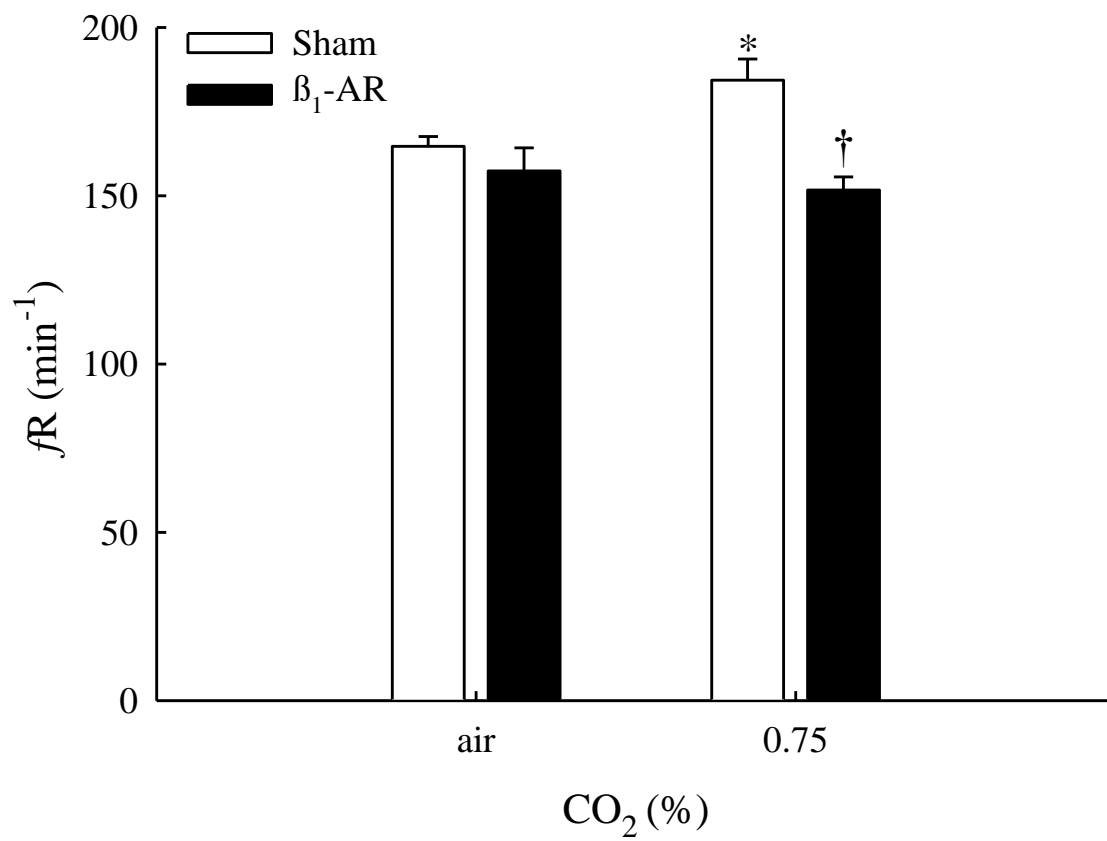


Figure 6. Effect of hypercapnia (P_{CO_2} = air, 0.25, 0.5 and 0.75%) and 10^{-4} M solution of acetazolamide on HR in (A) 5 dpf, (B) 7 dpf zebrafish. Values are expressed as means + SE; N=6. (* $P < 0.05$ between air and 0.75 CO_2 ; T $P < 0.05$ within between treatment and control; two way repeated measure ANOVA).

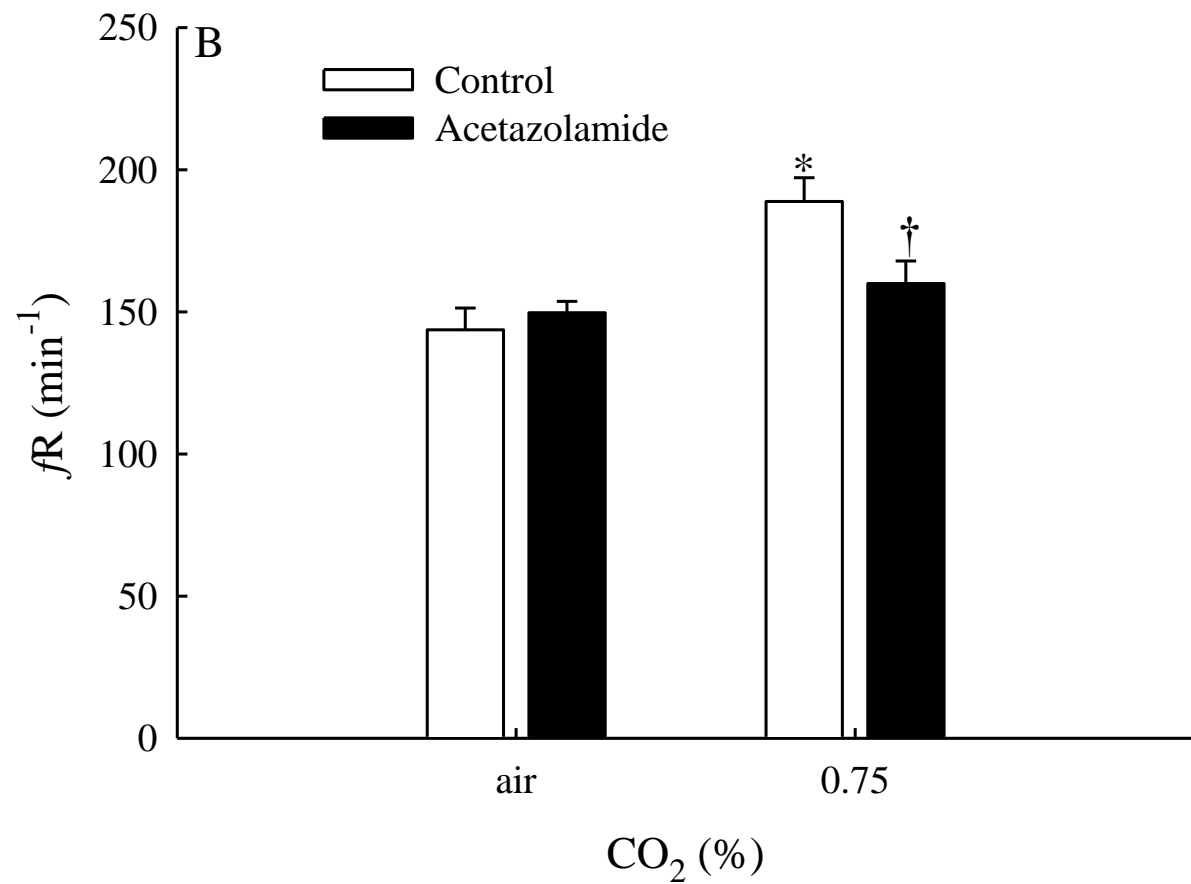
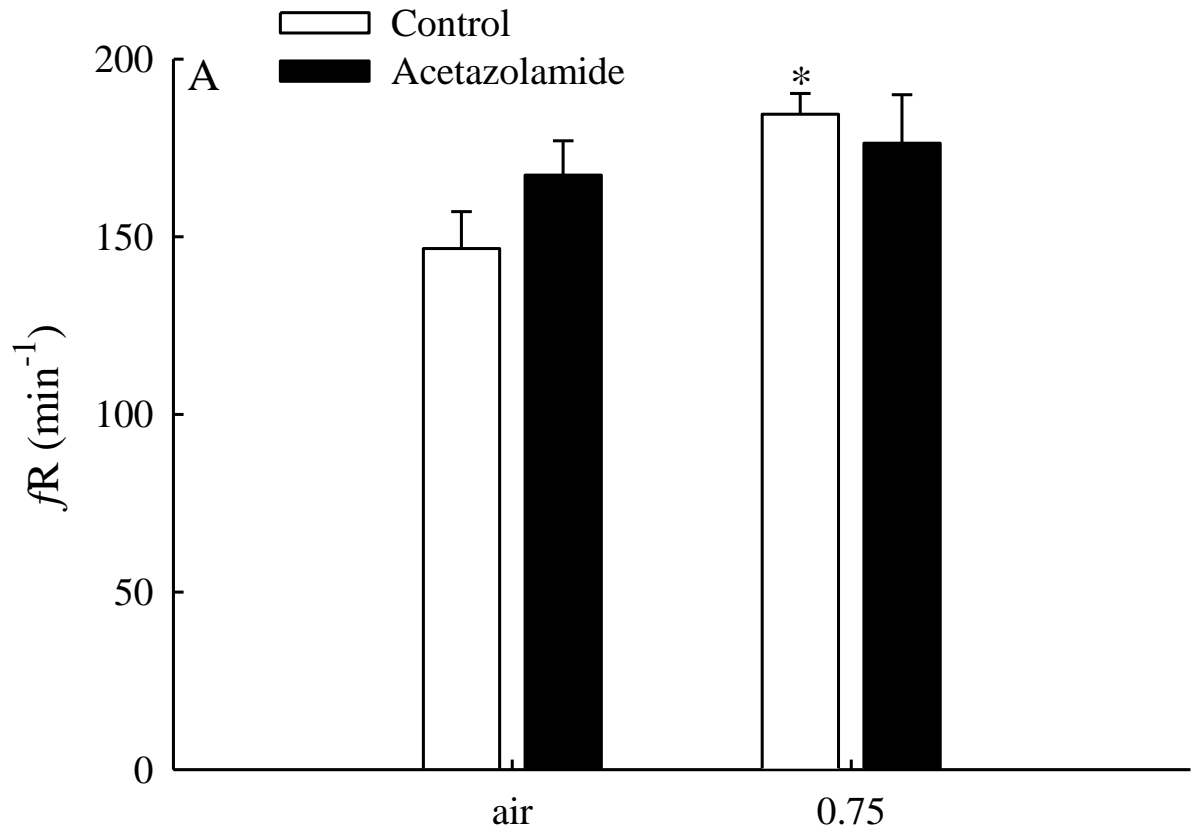


Figure 7. Effect of 10^{-4} M solution of acetazolamide and 10^{-4} M solution of acetazolamide and noradrenaline on HR in (A) 5 dpf, (B) 7 dpf zebrafish. Values are expressed as means + SE; N=6. (*P<0.05; paired t-test).

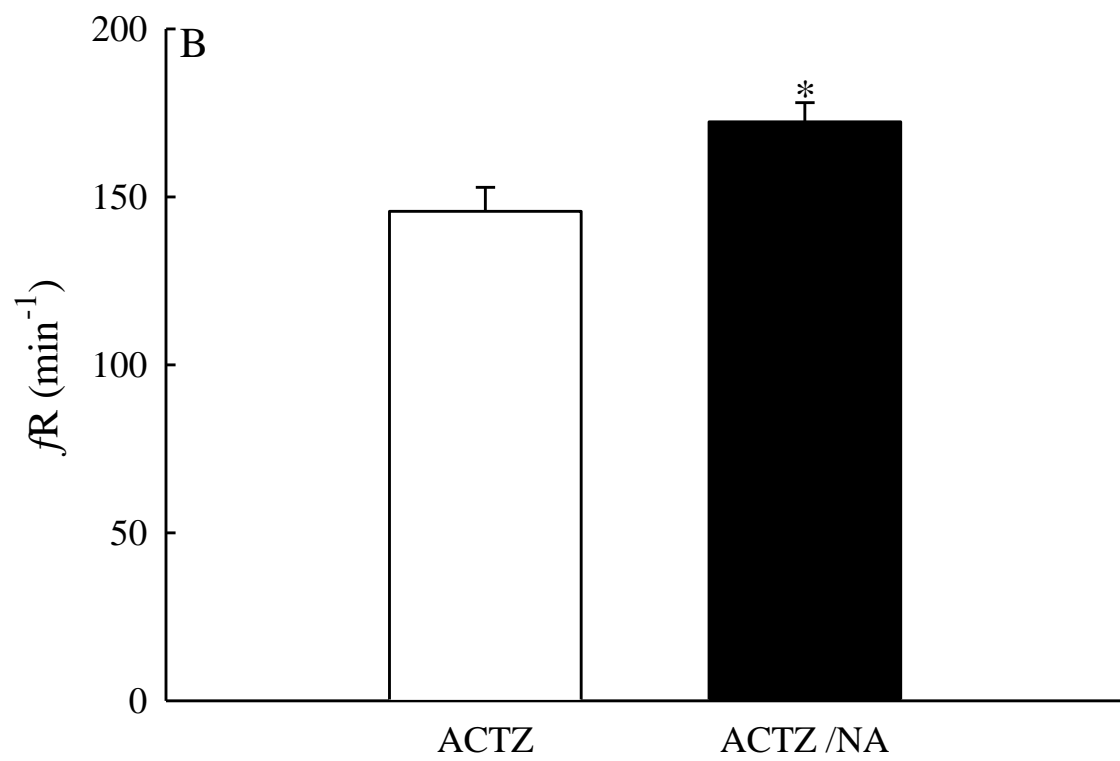
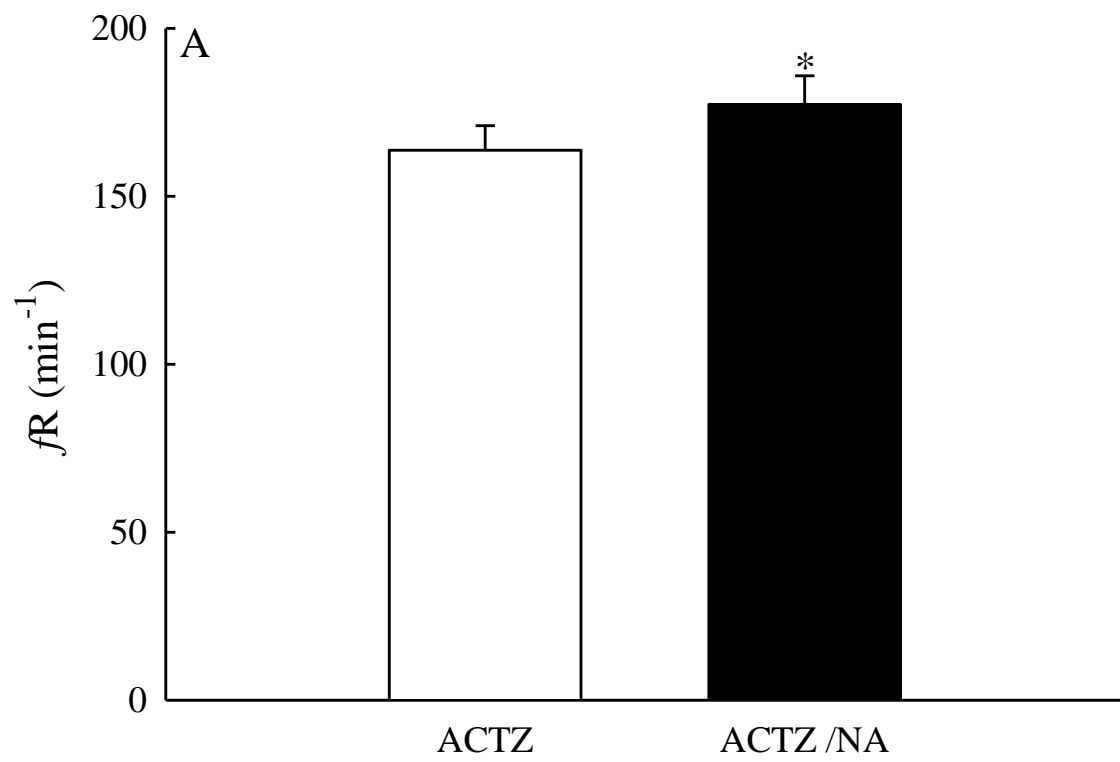
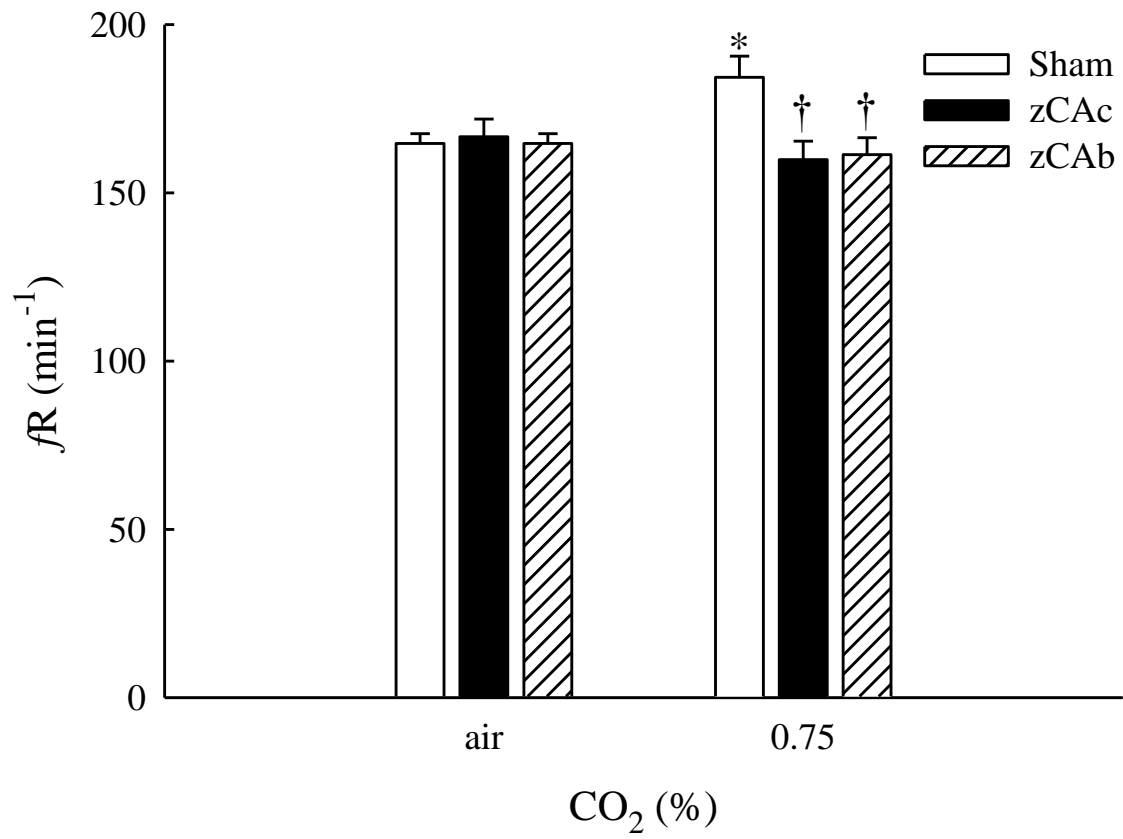


Figure 8. Effect of hypercapnia (P_{CO_2} = air and 0.75%) on HR of 5 dpf zebrafish injected with control, zCAC or zCAB morpholino. Values are expressed as means + SE; N=6. (* $P < 0.05$ between air and 0.75% CO_2 ; T $P < 0.05$ within treatment; two way repeated measure ANOVA).



4 Discussion

The purpose of this study was to examine CO₂ sensing in developing zebrafish and its downstream effects on cardiac function. NECs were shown to be O₂ chemoreceptors (Jonz and Nurse, 2006), as well as CO₂ chemoreceptors (Qin et al., 2010), similar to carotid body type 1 cells (Gonzales et al., 1994). Recently, it was observed that NECs of the skin are innervated after 1 dpf (Coccimiglio and Jonz, 2012) and appear to be functional well before gill NECs appear at approximately 5 dpf (Jonz and Nurse, 2005). In the present study, the sensing of CO₂ and the pathways associated with it were assessed using standard pharmacological methods, coupled with translational gene knockdown. The physiological variable measured in all cases was heart rate.

The basic response to elevated CO₂ at 5-7 dpf was an increase in heart rate. An increase in heart rate in zebrafish larvae during hypercapnia differs from the bradycardia that is usually observed in adult fish (note that no data are yet available for adult zebrafish) (see review by Jonz and Nurse, 2006). The physiological significance of the hypercapnia-mediated tachycardia is unknown. Given that a recent study (Gilmour et al. 2009) suggested that the red blood cells of larval zebrafish may be involved in CO₂ excretion, it is conceivable that internal convection might selectively promote CO₂ excretion and thereby be increased by any elevation of cardiac output associated with tachycardia. Thus, the tachycardia could serve to minimize the extent of the respiratory acidosis associated with exposure to hypercapnia.

4.1 NEC as CO₂ sensors

Gill NECs are in an ideal location to sense internal (blood) and external (water) gas tensions and pH. Skin NECs are also well situated due to the small size of zebrafish larvae and the fact that their skin is thin at this point in their development. It was found that zebrafish increase their heart rate in response to elevated PCO₂ at 5 dpf, and that the response is more robust at 7 dpf (Figure 1). These data suggest that the skin NECs may be less responsive to changes in ambient CO₂ in comparison to O₂ given that responses to hypoxia have been observed as early as 2 dpf (Jonz and Nurse, 2005). Alternatively, it is also conceivable that responses to CO₂ would have been observed in younger larvae had higher levels been used. Decreased sensitivity to CO₂ (relative to O₂) could reflect the relatively benign effects of hypercapnia on zebrafish development opposed to the negative effects on growth that are apparent in fish exposed to hypoxia (Vulesevic and Perry, 2006). The difference in sensitivity to O₂ and CO₂ is also the case in mammals (Rezzonico et al., 1990) and to a slightly lesser extent in birds (Bavi and Kilgoer, 2001).

The general responses of adult fish to hypercapnia and hypoxia are hyperventilation, bradycardia, and an increase in systemic vascular resistance (see reviews by Smatresk, 1990; Perry and Gilmour, 2002; Sundin and Nilsson, 2002; Reid and Perry, 2003). Depending on the developmental age of exposure and concentration of gases used, some studies have shown that zebrafish larvae increase their heart rate (Jacob et al., 2002). The decrease in heart rate when exposed to hypoxia has not been shown in zebrafish until 30 dpf when gills become adult like (Barrionuevo and Burggren, 1999). The increase in

heart rate could also be a result of the release of catecholamines that are released when adult fish are exposed to hypercapnia (Perry and Reid, 2002).

4.2 CO₂ versus pH

As CO₂ concentrations in water increase, the pH of the water is decreased, a condition known as acidic hypercapnia. In order to assess the effects of decreasing pH in the absence of elevated CO₂, the pH of the water was decreased from 7.2 to 6.6 (representing the change in pH accompanying the exposure to 0.75% CO₂). This metabolic acidosis resulted in a *drop* in heart rate, showing that the *increase* in heart rate caused by acidic hypercapnia was related specifically to CO₂ or bicarbonate (Figure 2). Similar results were obtained in previous studies on adult fish (Burleson and Smatresk, 2000; McKendry et al., 2001; McKendry and Perry, 2001; Perry and Reid, 2002). The bulk of available evidence now suggests that the cardiorespiratory reflexes associated with acidic hypercapnia reflect the increase in PCO₂ as opposed to the decrease in pH. Given the apparent opposite effects of external metabolic acidosis and acidic hypercapnia, it is conceivable that exposure to isohydric hypercapnia (increase in CO₂ with no change in pH) would lead to a greater increase in heart rate than with acidic hypercapnia, but that was not measured in this study.

4.3 Mechanism of pathway

It has been well established that the first step in chemoreception transmission to the central nervous system is the release of neurotransmitters, such as serotonin (Cutz and

Jackson, 1999; Perry et al., 2009). In the present study, the increase in heart rate associated with hypercapnia was blocked by the application of hexamethonium, showing that nicotinic acetylcholine receptors in the sympathetic and/or parasympathetic ganglia are involved (Figure 3). Therefore, it can be concluded that the tachycardia induced by CO₂ is a neural reflex and that the change in heart rate is not caused by a local effect of CO₂ on the heart.

Hexamethonium works on both parasympathetic and sympathetic divisions of the autonomic nervous system, the CO₂-mediated increase in heart rate could potentially reflect decreased activity of the inhibitory parasympathetic pathways or increased activity of stimulatory adrenergic pathways. The muscarinic receptor blocker, atropine, had no effect on the cardiac response to CO₂ (results not shown). Therefore, it would appear that the parasympathetic pathway is not involved. However, when β -adrenergic receptors were blocked with the non-specific antagonist propranolol, the increase in heart rate during hypercapnia was prevented (Figure 4A). Thus, the efferent arm of the reflex cardiac response to elevated CO₂ involves the adrenergic activation of cardiac β -receptors. Interestingly, the baseline cardiac frequency also was decreased by general β receptor blockade, as has been shown in other studies using propranolol (Finn et al., 2012) but not all before 5 dpf (Schwerte et al., 2006), likely due to the combined block of the β 1, β 2 (and possibly β 3) receptors. When atenolol was used to specifically block the β 1 receptor, the increase in heart rate during hypercapnia was also blocked, but baseline frequency was unaffected (Figure 4B). It is possible that the β 2 receptor is tonically active at a constant level and that the β 1 receptor is involved in the increase in heart rate.

To resolve potential problems associated with non-specific effects of pharmacological blockade of β -receptors, a gene knock down approach was employed to specifically target the β 1 receptor (Figure 5). These experiments yielded similar results as in the atenolol experiment. Therefore, the increase in heart rate is, at least in part, related to adrenergic activation of β 1-receptors.

4.4 Carbonic anhydrase and CO₂

Qin et al. (2011) demonstrated that carbonic anhydrase was present in NECs of adult zebrafish gills that are 5HT-positive and were involved in setting the magnitude and speed of membrane depolarization during exposure of these cells to hypercapnia. In the current study, the specific role of CA on CO₂ transduction was assessed using pharmacological toxins directed towards CA, suspected receptors within the pathways and selective gene knockdowns. When acetazolamide was added to the water, the magnitude of the tachycardia response to elevated CO₂ was reduced (Figure 6). As shown in carotid body type 1 cells, when CA is inhibited, there was an increase in time of response (Iturriaga, 1993) and a decrease in discharge rate (Coates et al., 1996). In cat ventral medulla neurons, the inhibition delayed the ventilatory response (Coates et al., 1991). In neonatal rat chromaffin cells, there was a decrease in catecholamine release (Munoz-Cabello et al., 2005).

Acetazolamide inhibits all isoforms of CA including the red cell specific zCA_b and the more general cytosolic form zCA_c; it is the latter paralog that is believed to be present in the zebrafish NEC. Given the previous results of Qin et al. (2010) demonstrating the

involvement of CA in promoting NEC membrane depolarization with elevated CO_2 , and the generally held view that CA in the Type I cells of the carotid body plays a role in CO_2 sensing (Iturriaga et al., 1991), the most parsimonious explanation for the effects of ACTZ observed in the current study is that they reflect inhibition of NEC CA activity. To ensure that fish were still able to respond to adrenergic stimulation with an increase in cardiac frequency (especially in light of the increased basal frequency in ACTZ-treated larvae at 4 dpf), noradrenaline was added to the bathing water; at 5 and 7 dpf, zebrafish were still able to increase their heart rates significantly in response to hypercapnia. Thus, ACTZ in itself, does not appear to interfere with the efferent arm of the CO_2 -mediated cardiac reflex.

In an attempt to more selectively inhibit specific CA isoforms, a gene knockdown strategy was employed to selectively lower zCac or zCab activity. While the inhibitory effects of zCac knockdown on the cardiac response to CO_2 were anticipated (see above), the similar inhibitory effects of zCab knockdown were not expected. At present, the mechanisms underlying the inhibitory effects of zCab knockdown are unclear. However, a possible explanation is the reduction in CO_2 excretion that accompanies zCab knockdown in zebrafish larvae (Gilmour et al. 2009). Assuming that the reduction in CO_2 excretion leads to elevated PCO_2 during the first 5 dpf, there may be ensuing acclimatization and desensitization of the NECs to further increases in PCO_2 .

5 General conclusions and future directions

In this thesis, I described the regulation of the CO₂ sensing pathway in zebrafish larvae. The results show that zebrafish first respond to increases in CO₂ beginning at 5 dpf, and that this response becomes more robust at 7 dpf. The pathway is under neuronal control by the sympathetic nervous system whereby activation of cardiac β -adrenergic receptors in the heart is responsible for increasing the heart rate. CA activity appears to be involved in the sensing of CO₂.

The results of this thesis provide the basis for a variety of future studies. For example, it would be pertinent to examine what effect pre-acclimation to hypoxia, hypercapnia, or hyperoxia may have on the development of CO₂ sensing. Measurements of RT-PCR of developing zebrafish exposed to hypercapnia would also be of value to increase our understanding of this field.

Other studies of the plasticity of gill chemoreceptors that are present in early stages of zebrafish development will lead to a better understanding of the molecular and cellular pathways within the zebrafish. This will foster a better understanding and of the cardiorespiratory system in zebrafish that can be used as a model organism, due to the similarity between CO₂ sensing in NEC and CO₂ sensing in carotid body type 1 glomus cells.

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