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Cardiac responses to hypercapnia in larval zebrafish (*Danio rerio*): The links between CO_2 chemoreception, catecholamines and carbonic anhydrase

Scott Miller, Jacob Pollack, Julia Bradshaw[2], Yusuke Kumai[3] and Steve F. Perry[*]

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, Ontario, Canada K1N 6N5

[1]Department of Fisheries and Oceans, Pacific Biological Station, 3190 Hammond Bay road, Nanaimo, BC, V9T 6N7

[2]Department of Physiology and Biophysics, Case Western Reserve University School of Medicine, 10900 Euclid Avenue, Cleveland, OH

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*Corresponding author:

Steve F. Perry

Department of Biology, University of Ottawa

30 Marie Curie, Ottawa, Ontario, Canada K1N 6N5

Tel. 1-613-562-5985

E-mail: sfperry@uottawa.ca

Running title: Cardiac responses to CO_2 in developing zebrafish

Abstract

The ontogeny of carbon dioxide (CO_2) sensing in zebrafish (*Danio rerio*) has not been examined. In this study, CO_2-mediated increases in heart rate were used to gauge the capacity of zebrafish larvae to sense CO_2. CO_2 is thought to be detected via neuroepithelial cells (NECs), which are homologous to mammalian carotid body glomus cells. Larvae at 5 days post-fertilization (dpf) exhibited tachycardia when exposed for 30 min to 0.75% CO_2 (~ 5.63 mm Hg); at 7 dpf, tachycardia was elicited by 0.5% CO_2 (~ 3.75 mm Hg). Based on pharmacological evidence using β-adrenergic receptor (ß-AR) antagonists, and confirmed by β_1-AR translational gene knockdown using morpholinos, the reflex tachycardia accompanying hypercapnia was likely mediated by the interaction of catecholamines with cardiac β_1 receptors. Because the cardiac response to hypercapnia was abolished by the ganglionic blocker, hexamethonium, it is probable that the reflex cardio-acceleration was mediated by catecholamines derived from sympathetic adrenergic neurons.

Owing to its likely role in facilitating intracellular acidification during exposure to hypercapnia, it was hypothesized that carbonic anhydrase (CA) is involved in CO_2 sensing, and that inhibition of CA activity would blunt the downstream responses. Indeed, the cardiac response to hypercapnia (0.75% CO_2) was reduced in fish at 5 dpf exposed to acetazolamide, a CA inhibitor, and in fish experiencing zCAc (CA2-like a) knockdown. Successful knockdown of zCAc was confirmed by CA activity measurements, western blotting and immunocytochemistry. Co-injection of embryos with zCAc morpholino and mRNA modified at the morpholino binding site, restored normal levels of CA activity and protein levels, and restored (rescued) the usual cardiac responses to hypercapnia. These data, combined with the finding that zCAc is expressed in NEC's located on the skin, suggest that the afferent limb of the

CO_2-induced cardiac reflex in zebrafish larvae is initiated by coetaneous CO_2-sensing neuroepithelial cells.

Key Words: Hypercapnia, chemoreception, neuroepithelial cell, tachycardia cardiorespiratory control

Introduction

Adult fish including zebrafish (*Danio rerio*) exhibit well-defined cardiorespiratory responses to elevated environmental CO_2 (hypercapnia). Typically, most species that have been examined exhibit hyperventilation that is mediated by increases in breathing frequency and/or ventilatory stroke volume (Dejours, 1973; Janssen and Randall, 1975; Randall et al., 1976; Smatresk and Cameron, 1982; Perry and Gilmour, 1996; Crocker et al., 2000; Vulesevic et al., 2006; Perry et al., 2009b) (see reviews by (Shelton et al., 1986; Perry and Wood, 1989; Milsom, 1995; Gilmour, 2001; Perry and Gilmour, 2002; Gilmour and Perry, 2007; Perry et al., 2009a; Perry and Abdallah, 2012). The cardiovascular responses to hypercapnia have received less attention but in those few species that have been examined, a conserved response appears to be bradycardia (Perry et al., 1999; Sundin et al., 2000; Reid et al., 2000; McKendry et al., 2001; Perry and McKendry, 2001; Gilmour et al., 2005) which may, or may not, be associated with an increase in arterial blood pressure (reviewed by Gilmour and Perry, 2007). Bradycardia, however, is not a universal response to hypercapnia; two species were shown to exhibit tachycardia [*Acipenser transmontanus* (Crocker et al., 2000) and *Tinca tinca* (Randall and Shelton, 1963)] and in others, cardiac frequency is unaltered (Gilmour and Perry, 2007).

Because cardiorespiratory adjustments in fish are principally keyed to O_2 status (Dejours, 1973; Randall and Jones, 1973; Smith and Jones, 1982; reviewed by Perry and Wood, 1989), the hyperventilatory responses to hypercapnia originally were thought to reflect the indirect effects of respiratory acidosis on lowering blood O_2 content (hypoxaemia) via Bohr and Root shifts (Smith and Jones, 1982). The striking inverse correlation between blood oxygenation status and ventilation in rainbow trout (*Oncorhynchus mykiss*) led to the novel, yet still unproven concept of a blood O_2 content receptor (Randall, 1982). Although hypoxaemia may contribute indirectly to the reflex responses to hypercapnia, it is now well-established that fish possess specific

peripheral CO_2 chemoreceptors that are directly involved in the initiation of downstream responses (Kinkead and Perry, 1991; Perry and McKendry, 2001; McKendry and Perry, 2001; McKendry et al., 2001; Gilmour et al., 2005) and which function independently of O_2 (Heisler et al., 1988; Perry and Gilmour, 1996). In adult fish, the CO_2 chemoreceptors are localized to the gills (McKendry et al., 2001; Perry and Reid, 2002) or buccal cavity (Reid et al., 2000) where they typically respond to external (but not internal) CO_2 (McKendry and Perry, 2001; Perry and McKendry, 2001) and exhibit little, if any, reactivity to external $[H^+]$ (Reid et al., 2000; Perry and McKendry, 2001; Gilmour et al., 2005). Despite the weight of evidence supporting the existence of externally oriented CO_2 receptors (reviewed by Gilmour and Perry, 2007), several studies have indeed provided evidence supporting a role for internally oriented CO_2/H^+ receptors (Wood et al., 1990; Wood and Munger, 1994).

Regardless of their modality (CO_2 *versus* H^+) or orientation (external *versus* internal), there is compelling *in vitro* evidence that neuroepithelial cells (NECs; Dunel-Erb et al., 1982) of the adult gill filament are dual O_2/CO_2 chemoreceptors (Jonz et al., 2004; Burleson et al., 2006; Olson et al., 2008; Qin et al., 2010; Abdallah et al., 2012; Abdallah et al., 2014) and thus functionally analogous to the Type I (glomus) cells of the mammalian carotid body (Milsom and Burleson, 2007). Similar to O_2 sensing (Jonz et al., 2004), CO_2 chemoreception by zebrafish NECs is associated with membrane depolarization following inhibition of background potassium conductance (Qin et al., 2010). Ultimately, the membrane depolarization initiates an elevation of intracellular $[Ca^{2+}]$ owing to mobilization of intracellular Ca^{2+} stores (Abdallah et al., 2014) which is believed to trigger neurotransmitter release that promotes the downstream reflex responses. Although these is no direct *in vivo* evidence implicating the NECs in reflex cardiorespiratory responses to changing ambient O_2 levels, alterations in NEC morphology or

abundance during hypoxia (Jonz et al., 2004) or hyperoxia (Vulesevic et al., 2006) are consistent with their presumed role in O_2 sensing. In contrast, the density of NECs on the adult gill of zebrafish is unaltered by chronic elevation of ambient CO_2 (Vulesevic et al., 2006).

In mammals, CO_2 chemoreception by Type 1 cells is facilitated by the enzyme carbonic anhydrase (CA) because it promotes rapid intracellular acidification via the catalysed hydration of molecular CO_2 (Lahiri and Forster, 2003). Neuroepithelial cells isolated from adult zebrafish gill exhibit CA immunoreactivity and inhibition of CA with acetazolamide, while slowing the rate of intracellular acidification (Abdallah et al., 2014), delayed and blunted the membrane depolarization in isolated NECs exposed to hypercapnia (Qin et al., 2010). Interestingly, however, the CO_2-mediated rise in intracellular [Ca^{2+}] was unaffected by CA inhibition (Abdallah et al., 2014). Thus, given the conflicting *in vitro* data and in the absence of *in vivo* data, it is difficult to assign a role to CA in the facilitation of the cardiorespiratory reflex responses to hypercapnia.

Little is known about the ontogeny of O_2 sensing in fish and there are no data concerning CO_2 chemoreception in developing larvae. With respect to O_2 sensing, it is thought that the NECs of the skin are critical in promoting the ventilatory responses to hypoxia prior to development and innervation of gill NECs (Jonz and Nurse, 2005; Coccimiglio et al., 2012). Given that gill NECs (or more likely a subset of gill NECs) are dual O_2/CO_2 chemoreceptors, it seems likely that the responses to CO_2 in larval zebrafish (if present) also are initiated by the coetaneous NECs. Thus, the initial objective of the present study was simply to determine whether zebrafish larvae display cardiorespiratory responses to elevated external CO_2. Initial experiments demonstrated a robust and reproducible elevation in cardiac frequency to hypercapnia while the ventilatory responses were more variable. Thus, throughout this study, a

change in heart rate was used as the physiological determinant of CO_2 sensing. After establishing the neurohumoral mechanisms underlying hypercapnic tachycardia, the principal goal of this study was to test the hypothesis that CA is involved in CO_2 sensing and ultimately in promoting the ensuing downstream physiological reflex responses. The sensing of CO_2 and the pathways associated with it were assessed using standard pharmacological methods, coupled with translational gene knockdown.

Results

At 4 dpf, none of the concentrations of CO_2 that were tested affected heart rate (f_H; Fig. 1A). Note, however, that the resting f_H in the fish at 4 dpf was somewhat higher than in the other groups and thus it is possible that the fish at this age lacked the scope to increase f_H. At 5 dpf, only the highest level of CO_2 (0.75%) produced a significant increase in f_H when compared to the normocapnic group ($P = 0.002$; Fig. 1B). At 7 dpf, f_H was increased, at 0.5% and 0.75% CO_2 ($P < 0.001$; Fig. 1C). Because 5 dpf was the earliest stage of development when fish exhibited CO_2 sensitivity, this stage was chosen for all subsequent experiments, except for those involving CA rescue (performed at 4 dpf) inhibition of CA by acetazolamide (ACTZ) for which experiments were conducted at 5 and 7 dpf. The more robust f_H response to CO_2 at 7 dpf made it easier to detect potential inhibitory effects following CA inhibition.

At 0.75% CO_2 (the highest concentration used in the initial phase of 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 f_H ($P = 0.037$), thereby demonstrating that the tachycardia observed during exposure to hypercapnic acidosis was likely the result of CO_2 and not the associated reduction in pH.

The addition of hexamethonium, a nicotinic receptor antagonist and ganglionic blocker within the parasympathetic and sympathetic divisions of the autonomic nervous system, prevented a significant increase in f_H in fish exposed to 0.75% CO_2 (Fig. 3). The heart rate of the control fish exposed to 0.75% CO_2 was significantly higher than either the air-exposed (normocapnic) controls or the CO_2-exposed fish treated with hexamethonium. Therefore, it can be concluded that the downstream responses to CO_2 at 5 dpf are 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 bpm) and prevented the increase in f_H that was observed in the hypercapnic control fish (Fig. 4). Unlike propranolol, the specific β_1 adrenergic receptor antagonist, atenolol, did not affect f_H in normocapnic fish; however, atenolol did prevent the usual increase in f_H accompanying 0.75% CO_2 (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 f_H when exposed to 0.75% CO_2 ($P = 0.003$), the group of fish experiencing β_1-AR knockdown did not increase its f_H during hypercapnia (157.3 +/- 6.8 in normocapnia *versus* 151.7 +/- 3.9 bpm ($N = 6$). At 0.75% CO_2, the fish experiencing β_1-AR knockdown had a significantly lower f_H than the sham- injected fish ($P < 0.001$).

To determine whether CA was involved in CO_2 sensing, cardiac responses to hypercapnia were assessed with and without ACTZ, a membrane permeable CA inhibitor. At 5 dpf, the ACTZ-treated fish failed to increase f_H at all levels of CO_2 (data for exposure to 0.75% CO_2 are shown in Fig. 6A). When 7 dpf zebrafish (which exhibited a more robust response to CO_2 in

comparison to fish at 5 dpf) were tested, ACTZ treatment again prevented a significant increase in f_H (Fig. 6B).

To ensure that the ACTZ-treated fish were still capable of increasing f_H, fish at 5 and 7 dpf were exposed to the cardiac stimulant noradrenaline (Fig. 7A & B). In both sets of ACTZ-treated fish, the addition of noradrenaline caused a significant increase in f_H. This showed that ACTZ was not preventing the heart from responding normally to β-adrenergic receptor stimulation.

Because acetazolamide is likely to inhibit all isoforms of zebrafish CA, an alternate approach employing selective gene knockdown was used to specifically assess the role of the zCAc isoform. The data summarized in Fig. 8 clearly demonstrate that knockdown of zCAc prevented the increase in f_H that was observed in the fish injected with the control morpholino. The effectiveness of the CA knockdown and its rescue using zCAc mRNA (slightly modified to prevent binding of the morpholino) are depicted in Figures 9 and 10. Preliminary experiments revealed that although CA knockdown was effective until 5 dpf (CA activity was reduced from 425.9 to 141.2 pmol/min/μg), rescue of CA activity could not be reliably sustained longer than 4 dpf. At 4 dpf, CA activity in morphants was reduced by 84% (from 305.5 to4 9.6 pmol/min/μg); Fig. 9A) and protein levels were undetectable by western blotting (Fig. 9B). Additionally, CA localized by ICC to cells of the yolk sac in sham fish was not detectable in the zCAc morphants (Fig. 10 A – D). Co-injection of the zCAc mRNA with morpholino resulted in normal levels of CA activity and protein levels (Fig. 9A, B). The elevation in heart rate during hypercapnia exposure, normally absent in the larvae experiencing zCAc knockdown, was restored after zCAc rescue (Fig. 9C). However, the rescue was only partial because the increase in heart rate, although significant, was less pronounced than in the sham fish (Fig. 9C).

192 The NEC's on the skin were readily identified by immunocytochemical detection of 5-
193 HT (Fig 10E). A subset of the 5-HT-containing NEC's also expressed zCAc (Fig. 10F, G). The
194 majority of cells expressing zCAc, however, did not co-express 5-HT; these cells were most
195 likely H^+-ATPase enriched ionocytes (data not shown).

Discussion

The purpose of this study was to examine CO_2 sensing in developing zebrafish and to establish the mechanisms of the ensuing downstream effects on cardiac function. The major findings emerging from this research are that i) zebrafish larvae respond to hypercapnia with tachycardia that likely is mediated by a neuronal reflex involving adrenergic activation of cardiac β-adrenergic receptors and ii) the CO_2-mediated tachycardia relies on the activity of CA presumably owing to its role in CO_2 chemoreception.

Recently, it was observed that NECs of the skin are innervated after 1 dpf (Coccimiglio and Jonz, 2012) and appear to function in O_2 sensing well before gill NECs become innervated at approximately 7 dpf (Jonz and Nurse, 2005). Because at least a portion of the NECs also act as CO_2 chemoreceptors (Qin et al., 2010), an underlying assumption of the present study was that any effects of CO_2 on cardiac function would presumably be initiated by the NECs either on the skin (at 4 and 5 dpf) and/or gill (at 7 dpf). Despite the likelihood that NECs function as CO_2 sensors *in vivo*, strictly speaking and similar to O_2 sensing, there are as yet no **direct** *in vivo* data implicating NECs in CO_2 chemoreception.

The cardiac response to hypercapnia

The basic response to elevated CO_2 at 5-7 dpf was an elevation 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). Similar to the bradycardia in adult fish (e.g. Perry et al., 1999), the physiological significance of the hypercapnia-mediated tachycardia in larvae is unknown. Given that the results of a recent study (Gilmour et al., 2009) suggested that the red blood cells of larval zebrafish may be involved in

CO_2 excretion, it is conceivable that internal convection might selectively promote CO_2 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.

Zebrafish at 5 dpf increased their heart rate when exposed to elevated PCO_2 (0.75% CO_2 or 5.6 mm Hg); the response was more robust at 7 dpf with heart rate being increased at CO_2 levels of 0.5% or 3.75 mm Hg (Fig. 1). The CO_2 levels required to elicit cardiac responses in larvae far exceeded those needed to promote hyperventilation in adults (1 mm Hg; Vulesevic et al., 2006). Without directly comparing the response characteristics of larval and adult zebrafish NECs to CO_2, it is not possible to determine whether these differences in responsiveness reflect age-dependent differences in NEC sensitivity thresholds, lack of maturation of the efferent limb of the CO_2-mediated reflex in larvae (see below) or simply intrinsic variability in the CO_2 thresholds required to elicit cardiac *versus* ventilation responses.

Given that gross motor responses to hypoxia were observed in zebrafish as early as 2 dpf (Jonz and Nurse, 2005) and ventilatory responses to hypoxia were apparent at 3 dpf (Coccimiglio et al., 2012), it is tempting to speculate that the skin NECs may be less responsive to changes in ambient CO_2 (cardiac responses observed only at 4 dpf) in comparison to O_2. However, for similar reasons as discussed above, these differences may represent varying rates of maturation of the efferent branches of the cardiac and ventilation control systems as well as the severe levels of hypoxia used to elicit responses in larvae (e.g. 25 mm Hg PO_2 in larvae (Coccimiglio et al., 2012) compared to 100 mmHg in adults (Vulesevic et al., 2006). The delayed onset of sensitivity to CO_2 (relative to O_2) may reflect the relatively benign effects of hypercapnia on

zebrafish development compared to the marked negative effects on growth in fish exposed to hypoxia (Pelster, 2003; Vulesevic and Perry, 2006).

Typically adult fish exhibit similar cardiorespiratory responses to hypoxia and hypercapnia, the conserved responses being hyperventilation and bradycardia (see Introduction). There are insufficient data to draw similar conclusions for larvae and those few studies that have been performed on hypoxic zebrafish have yielded widely conflicting results. For example, although hypoxic bradycardia was observed in 4 dpf larvae in at least two studies (Bagatto, 2005; Steele et al., 2009), there are also reports of the zebrafish heart being unresponsive to hypoxia until 30 dpf at which time bradycardia develops (Barrionuevo and Burggren, 1999) or actually exhibiting tachycardia (Jacob et al., 2002). The underlying basis for this variability in the cardiac responses to hypoxia in larval zebrafish is unclear but may reflect, at least in part, the different levels of hypoxia used [e.g. Jacob et al. (2002) exposed fish to a much milder hypoxia (75 mm Hg)] or the different regimes of exposure [e.g. Barrionuevo and Burggren (1999) exposed fish acutely to hypoxia whereas in the other studies, embryos/larvae were reared in hypoxic water]. Thus, until additional experiments are performed that directly compare hypoxic and hypercapnic responses under similar conditions, it is not yet possible to conclude that the tachycardia observed in the present study in response to hypercapnia truly differs from the larval hypoxic response.

CO_2 versus pH

As CO_2 concentrations in water increase, the external pH is decreased, a condition known as acidic hypercapnia. To assess the effects of decreasing water pH in the absence of elevated CO_2, the pH of the water was decreased from 7.2 to 6.6 (representing the change in pH

accompanying the exposure to 0.75% CO_2). This isocapnic acidosis resulted in a *drop* in heart rate, showing that the *increase* in heart rate caused by acidic hypercapnia was related specifically to CO_2 (Fig. 2). Similar results were obtained in previous studies using adult fish (Burleson and Smatresk, 2000; McKendry et al., 2001; McKendry and Perry, 2001; Perry and McKendry, 2001; Perry and Reid, 2002; Gilmour et al., 2005; Abdallah et al., 2014). Thus, the bulk of available evidence now suggests (although see Introduction) that the cardiorespiratory reflexes associated with acidic hypercapnia reflect the increase in PCO_2 rather than the decrease in pH (Gilmour and Perry, 1997). Given the apparent opposite effects of external isocapnic acidosis and acidic hypercapnia, it is conceivable that exposure to isohydric hypercapnia (increase in CO_2 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.

Hypercapnic tachcycardia is an adrenergic reflex

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. Therefore, it can be concluded that the tachycardia induced by CO_2 is a neural reflex and that the increase in heart rate is not caused by a local effect of CO_2 on the heart. Because hexamethonium acts on both the parasympathetic and sympathetic divisions of the autonomic nervous system, the CO_2-mediated increase in heart rate could potentially reflect decreased activity of the inhibitory parasympathetic pathways or increased activity of stimulatory adrenergic pathways, assuming that both of these pathways are tonically active (see below). The muscarinic receptor blocker, atropine, had no effect on resting heart rate or the cardiac response to CO_2 (results not shown). Therefore, it would appear that the

parasympathetic pathway is not involved. Previous studies have also reported a lack of an effect of atropine on resting heart rate until about 12 dpf (Steele et al., 2009; Schwerte et al., 2006) suggesting that parasympathetic cholinergic tone is absent until ~12 dpf. However, Hsieh and Liao (2002) reported a significant reduction in heart rate in 3 dpf larvae experiencing muscarinic (M_2) receptor knockdown and Steele et al. (2009) demonstrated that hypoxic bradycardia at 3 dpf was prevented by M_2 receptor knockdown. The latter two findings provide evidence for parasympathetic tone in larvae of the age used in the present study but nevertheless it would appear to play no role in regulating heart rate during hypercapnia. The present study, like others that have assessed cardiac function in zebrafish larvae, utilized animals lightly anaesthetised with MS-222. Thus, it is conceivable that the anaesthesia may have somehow interfered with the normal parasympathetic control of the heart.

Blockade of β-adrenergic receptors using the non-specific β-AR antagonist propranolol, or the β_1-AR antagonist atenolol, prevented the increase in heart rate during hypercapnia. To resolve potential problems associated with non-specific effects of pharmacological blockade of β-receptors, a gene knockdown approach additionally was employed to specifically target the β_1 receptor (Steele et al., 2011). In contrast to the results of Steele et al. (2011), β_1-AR knockdown did not lower resting heart rate. However, similar to β-AR receptor pharmacological blockade, the tachycardia induced by hypercapnia was prevented by β_1-AR knockdown. Therefore, the increase in heart rate during hypercapnia is certainly related to adrenergic activation of β_1-adrenergic receptors.

Although the present results and those of previous studies provide evidence for adrenergic tone in larvae at 4 – 7 dpf (Bagatto, 2005; Schwerte et al., 2006; Wang et al., 2009; Steele et al., 2011), it is not straightforward to distinguish between tone arising from a functional

sympathetic innervation of the heart and tone associated with circulating catecholamines. Control of catecholamine secretion, although well-characterized in adults (Reid et al., 1998; Perry and Bernier, 1999; Perry and Capaldo, 2011), has not been studied in larval fish. Thus, it remains unknown whether the larvae investigated in the present study had yet developed an acute humoral adrenergic stress response capable of raising circulating catecholamine levels to stimulate cardiac β_1-ARs. Although it has been suggested (Schwerte et al., 2006) that the zebrafish heart is not yet innervated at 4 – 7 dpf, the prevention of the cardiac response to hypercapnia by hexamethonium in the current study (Fig. 3) does support a role for sympathetic cardiac innervation. Alternatively, it is conceivable that hexamethonium exerted its effects by interacting with nicotinic receptors (if innervated in larvae at 5 dpf) on the catecholamine-secreting chromaffin cells (Montpetit and Perry, 1999). Thus, the results of the present study can be interpreted in at least two ways; activation of cardiac sympathetic nerve fibres or activation of pre-ganglionic sympathetic nerve fibres innervating chromaffin tissue. In both cases, elevated catecholamines produced locally via sympathetic nerves or arriving via the circulatory system would activate cardiac adrenergic receptors. We propose that regardless of the precise mechanism(s) underlying cardiac β-AR activation, the response is initiated by the CO_2- sensing NECs of the skin or gill (in older larvae). Future experiments presumably incorporating new techniques will be required to firmly establish a direct link between the NECs and downstream cardiorespiratory responses.

A role for carbonic anhydrase

Qin et al. (Qin et al., 2010) demonstrated that CA was present in NECs isolated from adult zebrafish gills where it played an important role in setting the magnitude and speed of

membrane depolarization during exposure of these cells to hypercapnia. In the current study, CA was localized to the NEC's of the skin of larvae and its role in CO_2 transduction was assessed using pharmacological inhibition (ACTZ) and selective gene knockdowns. When ACTZ was added to the water, the magnitude of the tachycardia response to elevated CO_2 was reduced. In carotid body type 1 cells, inhibition of CA delayed the onset of, and reduced the magnitude of chemosensory responses to CO_2 (Iturriaga, 1993). Similarly, CA inhibition reduced the discharge rate from laryngeal receptors (Coates et al., 1996) and blunted central chemosensory responses to elevated CO_2 in cat (Coates et al., 1991).

Acetazolamide inhibits all isoforms of CA including the red cell specific isoform zCAb and the more general cytosolic form zCAc. It is the zCAc paralog that is believed to be present in the zebrafish NEC, a notion that was confirmed in the present study by using a zCAc specific antibody. 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 5 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 noradrenaline. Thus, ACTZ in itself does not appear to interfere with the efferent arm of the CO_2-mediated cardiac reflex.

To more selectively inhibit specific CA isoforms, a gene knockdown strategy was employed to specifically lower zCAc activity. The results of a previous study (Gilmour et al.,

2009) demonstrated the specificity of the zCAc knockdown and most importantly showed that zCAb was not a target for the zCAc morpholino. Thus, we are confident that the similar knockdown approach used in this study also specifically targeted zCAc. Thus, while a decrease in whole body CA activity was anticipated after zCAc knockdown (Fig. 9), the large magnitude of the response was unexpected given that zCAb activity should have remained. The most likely explanation for this finding is that red blood cell CA activity accounts for only a small fraction of total CA activity in larvae at 4 dpf.

Although CA activity was previously implicated in the speed and magnitude of membrane depolarization in isolated zebrafish NECs exposed to CO_2 (Qin et al., 2010) and the results of the current study clearly identify CA as a key component of the CO_2 transduction pathway *in vivo*, Abdallah et al. (Abdallah et al., 2014) demonstrated that the CO_2-mediated rise in NEC intracellular [Ca^{2+}] was unaffected by CA inhibition although it slowed the rate of intracellular acidification. The uncoupling of intracellular acidification and intracellular Ca^{2+} levels ultimately was attributed to the exclusive dependency of Ca^{2+} mobilisation in zebrafish NECs on extracellular rather than intracellular acidification (Abdallah et al., 2014), an observation which is consistent with the presence of extracellular pH-sensing TASK channels in zebrafish (Pena-Munzenmayer et al., 2013). Clearly, further research should be directed at elucidating how CA inhibition impedes NEC membrane depolarization (Qin et al., 2010) and blunts CO_2-mediated cardiac reflexes (present study) without influencing intracellular Ca^{2+} levels.

Materials and Methods

Animals

Adult zebrafish, *Danio rerio*, were purchased from a commercial supplier (Big Al's, Ottawa, Canada) and maintained at the University of Ottawa's Aquatic Care Facility. They were

kept in 10 litre (L) tanks that were supplied with well-aerated, dechloraminated tap water at 28°C. The fish were kept at a constant photoperiod of 14 hours light and 10 hours dark and fed daily until satiation with No. 1 crumble-Zeigler™ (Aquatic Habitats, Apopka, FL, USA). Embryos were collected and reared in 50 ml Petri dishes with E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 0.1% methylene blue). The Petri dishes were kept in incubators set at 28.5° C (in the dark) until they were removed for experimentation (4 - 7 days post fertilization; dpf). Dead embryos were removed and E3 medium was changed daily.

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 embryos for micro-injections (see below), 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 (University of Ottawa Animal Care and Veterinary Service protocol BL-226) that conform to the guidelines of the Canadian Council on Animal Care (CCAC).

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. Individual larvae at 4 – 7 dpf were placed in a rectangular depression well (14 mm) inside a superfusion chamber made from a glass-bottomed Petri dish (MatTek, Ashland, MA, USA) and Sylgard (Dow Corning, Midland, MI, USA). Two holes were carved out of the Sylgard on opposite sides of the depression well to enable inflow and outflow

of water. These holes were covered with nylon mesh to retain the fish in the well. Using a gravity-fed system, dechloraminated water containing 0.05 mg/mL MS-222 was continuously provided to the chamber at 4 ml/min through gas-impermeable tubing (Tygon, Saint-Gobain Performance Plastics, Pittsburgh, PA, USA). Solutions were removed from the chamber using a peristaltic pump (ThermoFisher Scientific). To visualize heart rate, the chamber was placed under a stereomicroscope fitted with a CCD video camera with output to a computer.

Series 1- the effect of CO_2 on heart rate

Baseline measurements were taken on fish for 1 min; CO_2 levels were increased from air-saturated water to 0.75% CO_2 in 0.25% increments with heart rate measurements taken after 30 min for each level of CO_2. CO_2 levels in the perfusate reservoirs were controlled using a Cameron 2- or 3-channel gas mixer which provided mixed gas to the reservoirs at a rate of 2000 ml per min.

To distinguish between the effects of CO_2 and H^+ on cardiac function during hypercapnia, fish were exposed to isocapnic acidosis where the pH of the water was lowered to 6.6 (matching the acidosis associated with 0.75% CO_2) using HCl.

A separate group of fish was exposed to the CA inhibitor acetazolamide (10^{-4} M; Sigma-Aldrich Inc., St. Louis MO, USA), dissolved in water for 20 min prior to measurements and for the course of the treatment. To dissolve the acetazolamide, the pH of the water was raised to ~11.0 using NaOH and then titrated back down to 7.2 using HCl (Gilmour et al., 2009).

Series 2- the effects of ganglionic and β-adrenergic receptor blockade on the cardiac responses to hypercapnia

Fish were anaesthetized as described above and placed in the flow-through system for baseline measurements. However, instead of first increasing the CO_2, one of the following drugs (prepared in water; 10^{-4} M) was added to the air-equilibrated water; hexamethonium (nicotinic receptor antagonist and ganglionic blocker), propranolol (non-specific ß-receptor antagonist) or, atenolol (β_1 adrenergic receptor antagonist). Another measurement was taken after 30 min of exposure to one of the three drugs. The CO_2 was then increased to 0.75%, and a final measurement was taken.

Series 3 – translational gene knockdown using morpholinos

Morpholino Injection

Morpholinos (tagged with 3'-carboxyfluorescein) for zebrafish cytosolic CA (zCAc; NM_199215, also referred to as CA2-like a) (Gilmour et al., 2009), the β_1 adrenergic receptor (β_1-AR; AB294717) (Steele et al., 2011) and a control morpholino were purchased from Gene Tools (Philomath, OR, USA) with the following sequences, respectively; 5'-AGTGGTCAGCCATTCCGCCAGCTGT-3', 5'-ACGGTAGCCCGTCTCCCATTG-3' and 5'-CCTCTTACCTCAGTTACAATTTATA-3'. All morpholinos were prepared to a final concentration of 4 ng nl^{-1} in Danieau buffer (58 mM NaCl, 0.7 mM KCl, 0.4 mM $MgSO_4$, 0.6 mM $Ca(NO_3)_2$, and 5 mM HEPES with a final pH of 7.6), and phenol red was used as an indicator of positive injection. Injections into one- or two-cell embryos were performed using a Narishige IM300 microinjector (Narishige International USA Inc., Long Island, NY, USA) with needles made from 1.0 mm borosilicate glass (Sutter Instrument, Novato, CA, USA). Dosage and sequence of zCA isoforms are based on Gilmour et al. (2009) and β_1-AR from Steele et al. (2011). Neither study reported adverse effects when using a dosage of 4 ng $embryo^{-1}$. After

injections, embryos were placed in petri dishes containing E3 medium and placed in an incubator at 28°C. On the following day, embryos were screened for positive expression of fluorescein using a Nikon SMZ 150 stereo-microscope (Nikon Instrument Inc., Melville, NY, USA).

Series 4 - carbonic anhydrase rescue

For rescue experiments, zCAc mRNA was synthesized *in vitro* with 5 neutral mismatches in the morpholino-binding 5' region and included in the injection solution at a concentration of 100 or 200 pg/nL, or 100 - 200 pg per embryo. To control for toxic effects of the morpholino, a separate group of embryos were injected with 4 ng/embryo of nonsense morpholino (standard control morpholino oligonucleotide; Gene Tools LLC). Because the effects of rescue on CA activity were declining by 5 dpf, the effects of hypercapnia on f_H were assessed in larvae at 4 dpf using a higher level of CO_2 (1%; 7.5 mm Hg).

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 zCAc rescue were: 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 purified by phenol:chloroform extraction following the transcription and then run on a denaturing gel to confirm the size and integrity of the mRNA. 100 or 200 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.

Western Blotting for zCAc and CA Activity Measurements

To confirm knockdown and exogenous rescue of cytosolic zCAc, western blotting was carried out on larvae at 4 dpf and CA activity measurements were performed at 3-5 dpf. For western blotting, larvae were collected, pooled into groups of 20 (N = 1) and homogenized on ice with a protease inhibitor cocktail (Roche, USA) and RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % sodium deoxycholate, 50 mM Tris–HCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 0.1 % SDS). The samples were incubated for 30 min and centrifuged at 15,000 *g* for 20 min at 4˚C. The protein-containing supernatant was stored at -20˚C until use. Protein samples were size-separated using10% SDS-PAGE and then transferred to a PVDF membrane (Bio-Rad, USA). The membrane was blocked for 1 h in TBST (137 mM NaCl, 19 mM Tris base, 2.7 mM KCl, 0.01% Tween 20) with 5% milk, and then incubated overnight on a shaker at 4˚C with a 1:3000 dilution of zCAc antibody in TBST containing 2% milk (peptide sequence: WGYDKHNGPDKWGC; Genscript, NJ) in 2% milk (TBST). The membrane was washed 3 times for 15 min in TBST and subsequently incubated for 2 h with 1:15000 goat anti-rabbit secondary antibody (Pierce, USA). Luminata Western HRP Substrates (Millipore, USA) was used to visualize immunoreactive bands. To verify equal protein loading, the membrane was stripped with Re-Blot Plus solution (Millipore, USA) and incubated with 1:4000 β-actin antibody (A2066, Sigma-Aldrich) at room temperature for 2 h. Band intensity was estimated using ImageJ (http://imagej.nih.gov/ij/) and compared to that of β-actin.

Carbonic anhydrase activity was measured using the electrometric delta pH method as described by Henry (1991). The assay used a temperature-controlled pH electrode (Radiometer GK2401C combination electrode) attached to a pH meter (Radiometer PHM64 research pH

meter) to measure the change in pH of a TRIS-buffered media (10 mM TRIS base, 225 mM mannitol, 75 mM sucrose; Sigma-Aldrich, pH adjusted to 7.4 with 30% phosphoric acid) in the presence of CO_2-saturated H_2O when a source of CA is added. To assess effectiveness of CA knockdown and efficiency of rescue, zebrafish were raised to 3, 4 and 5-dpf. Larvae were euthanized, pooled (~ 40 embryos to yield N = 1), weighed and homogenized in TRIS reaction medium (as described above). To calculate the CA activity (pmol/min/μg wet weight) in the larvae, the buffer capacity of the medium was first measured before each assay using 70 μL 0.1 M HCl to ensure a sufficiently high buffering capacity (between 4 and 5.5 μmol H^+/pH unit). The buffer capacity was used to calculate the uncatalysed rate of CO_2 hydration (without CA source added). The uncatalysed rate was subtracted from the measured rate in the reaction chamber after the addition of sample to determine the rate of CA activity in the larvae.

<u>Series 5 – localisation of cytosolic CA in zebrafish NECs by immunocytochemistry (ICC)</u>

To determine whether larval zebrafish NECs are enriched with cytosolic CA, 4 dpf larvae were immunostained with anti-5-HT rabbit polyclonal antibody (Sigma) and a custom-synthesized, homologous rabbit polyclonal antibody against zCAc (see above) conjugated with biotin. The same 5-HT antibody was used previously to label NECs in zebrafish larvae (e.g. Coccimiglio and Jonz, 2012). Larvae were killed by MS-222 overdose, fixed with 4% paraformaldehyde solution (prepared in PBS) for 2 h at room temperature (RT), briefly rinsed in PBS and stored in 100% ethanol at -20 °C until use. For whole mount ICC, larvae were re-hydrated in PBS and subjected to heat-induced antigen retrieval as described before (Inoue and Wittbrodt, 2011). After antigen retrieval, larvae were blocked with 10% natural donkey goat serum in 5% Triton-X/PBS (PBST) for 2 h at RT. Larvae were first incubated overnight with 5-

HT antibody (1:300 in PBS), rinsed with PBS and stained with a secondary antibody (Alexa-546 conjugated anti-rabbit IgG; Invitrogen; 1:400) in PBS for 2 h at RT. Subsequently, larvae were blocked for 2 h at RT with unconjugated streptavidn (30 µg/ml in PBS) to block endogenous biotin, incubated overnight at RT with anti-CA antibody (1:200 in PBS). After rinsing in PBS, samples were stained with Alexa-488 conjugated streptavidin (2.5 µg/ml in PBS) for 2 h at RT and washed in PBS. Samples were then mounted onto microscope slides and observed with an Olympus FV1000 BX61 confocal microscope with Olympus Fluoview software. To test whether the block of endogenous biotin was successful, experiments were performed in which the primary antibody was omitted; no staining was observed.

Statistical Analyses

All data are presented as means +/- SEM. Data were analyzed either by two-way repeated measures ANOVA, one-way ANOVA or paired Student's t-tests. When appropriate, ANOVA was followed by a Holmes-Sidak *post hoc* analysis to determine statistical differences between data points within a series. All statistical analyses were completed using commercial software (SigmaPlot 9, SPSS Inc.). A P-value of < 0.05 was set to determine statistical significance.

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719

720 **Figure Legends**

721

722 **Figure 1.** The effect of 30 min of hypercapnia (CO_2 = ~zero (air), 0.25, 0.5 or 0.75%) on heart
723 rate (f_H) in zebrafish (*Danio rerio*) larvae at (A) 4 days post fertilization (dpf), (B) 5 dpf and (C)
724 7 dpf. Values are expressed as means + 1 SEM; N = 6 different fish at each developmental
725 stage. Data points not sharing common letters are statistically different from each other ($P < 0.05$; one-way repeated measures ANOVA).

727 **Figure 2.** The effect of isocapnic acidosis (pH was lowered from 7.2 to 6.6 which corresponded
728 to the reduction in pH observed when fish were exposed to 0.75% CO_2) on heart rate (f_H) in
729 zebrafish (*Danio rerio*) larvae at 5 days post fertilization (dpf). Values are expressed as means +
730 1 SEM; N = 6. An asterisk denotes a statistically significant difference ($P < 0.05$) from the data
731 obtained at pH 7.2 (paired Student's t-test).

732 **Figure 3.** The effect of the ganglionic blocker hexamethonium (10^{-4} M) on the cardiac
733 frequency (f_H) response of zebrafish (*Danio rerio*) larvae at 5 days post fertilization (dpf) to
734 hypercapnia (30 min of 0.75% CO_2). Values are expressed as means + 1 SEM; N = 6. Letters
735 indicate differences among the group ($P < 0.05$; one-way repeated measures ANOVA).

736 **Figure 4.** The effects of β-adrenergic receptor (β-AR) blockade using (A) the non-specific β-
737 AR blocker propranolol (10^{-4} M) or (B) the β_1-AR antagonist atenolol (10^{-4} M) on the cardiac
738 frequency (f_H) response to hypercapnia (30 min of 0.75% CO_2) in zebrafish (*Danio rerio*) larvae
739 at 5 days post fertilization (dpf). Values are expressed as means + SE; an asterisk denotes a
740 significant difference ($P < 0.05$) between normocapnia (air) air and hypercapnia (0.75% CO_2); a

dagger indicates a significant difference ($P < 0.05$) between control (unfilled bars, N = 6) and propranolol-treated fish (filled bars, N = 6); two-way repeated measures ANOVA.

Figure 5. The effects of β_1-AR knockdown on the cardiac frequency (f_H) response to hypercapnia (30 min of 0.75% CO_2) in zebrafish (*Danio rerio*) larvae at 5 days post fertilization (dpf). Values are expressed as means + SE; an asterisk denotes a significant difference ($P < 0.05$) between normocapnia (air) and hypercapnia (0.75% CO_2); a dagger indicates a significant difference ($P < 0.05$) between sham (unfilled bars, N = 6) and fish experiencing β_1-AR knockdown (filled bars, N = 6); two-way repeated measures ANOVA.

Figure 6. The effects of the carbonic anhydrase inhibitor acetazolamide (ACTZ) on the cardiac frequency (f_H) response to hypercapnia (30 min of 0.75% CO_2) in zebrafish (*Danio rerio*) larvae at (A) 5 days post fertilization (dpf) and (B) 7 dpf. Values are expressed as means + SE; an asterisk denotes a significant difference ($P < 0.05$) between normocapnia (air) and hypercapnia (0.75% CO_2); a dagger indicates a significant difference ($P < 0.05$) between sham (unfilled bars, N = 6) and ACTZ treated fish (filled bars, N = 6); two-way repeated measures ANOVA.

Figure 7. The effects of noradrenaline (10^{-4} M) on cardiac frequency (f_H) in zebrafish (*Danio rerio*) larvae exposed to acetazolamide at (A) 5 days post fertilization (dpf; N = 6) and (B) 7 dpf (N = 6). Values are expressed as means + SE; an asterisk denotes a significant effect ($P < 0.05$; (paired Student's t-test) of noradrenaline (filled bars) on f_H.

Figure 8. The effect of zCAc knockdown on the cardiac frequency (f_H) response to hypercapnia (30 min of 0.75% CO_2) in zebrafish (*Danio rerio*) larvae at 5 days post fertilization (dpf). Values are expressed as means + SE; an asterisk denotes a significant difference ($P < 0.05$) between normocapnia (air) and hypercapnia (0.75% CO_2); a dagger indicates a significant

763 difference (P < 0.05) between sham (unfilled bars, N = 6) and fish experiencing zCAc
764 knockdown (filled bars, N = 6); two-way repeated measures ANOVA.

765 **Figure 9.** The effects of zCAc knockdown (filled bars; N = 4) and rescue with zCAc mRNA
766 (hatched bars; N = 3) on (A) whole body CA activity in zebrafish (*Danio rerio*) larvae at 4 dpf.
767 Values are expressed as means + SE; an asterisk denotes a significant difference (P < 0.05) from
768 the sham-injected (unfilled bars; N = 5) larvae (one-way ANOVA);

769 (B) zCAc protein levels as depicted in a representative western blot. Note that the
770 standard conditions used in all physiological trials involving knockdown and/or rescue were 4 ng
771 morpholino (MO) per embryo and 200 pg CAc mRNA per embryo;

772 (C) the cardiac frequency (f_H) response to hypercapnia (30 min of 1.0% CO_2) in larvae at
773 4 dpf. Values are expressed as means + SE; an asterisk denotes a significant difference (P <
774 0.05) between normocapnia (unfilled bars) and hypercapnia (filled bars; 1.0% CO_2); a dagger
775 indicates a significant difference (P < 0.05) between sham (N = 6) and fish experiencing zCAc
776 knockdown (N = 6) or zCAc knockdown/rescue (N = 6); two-way repeated measures ANOVA.

777 **Figure 10.** Representative immunocytochemistry micrographs depicting (A) zCAc-expressing
778 cells (ionocytes) on the yolk sac epithelium and (B) their absence in larvae at 4 days post
779 fertilization (dpf) after zCAc knockdown. Panels C-E illustrate a subset of 5-HT positive NECs
780 expressing zCAc (arrows). Panel A is reproduced from Figure 2 in the review paper of Kwong
781 et al. (Kwong et al., 2014).

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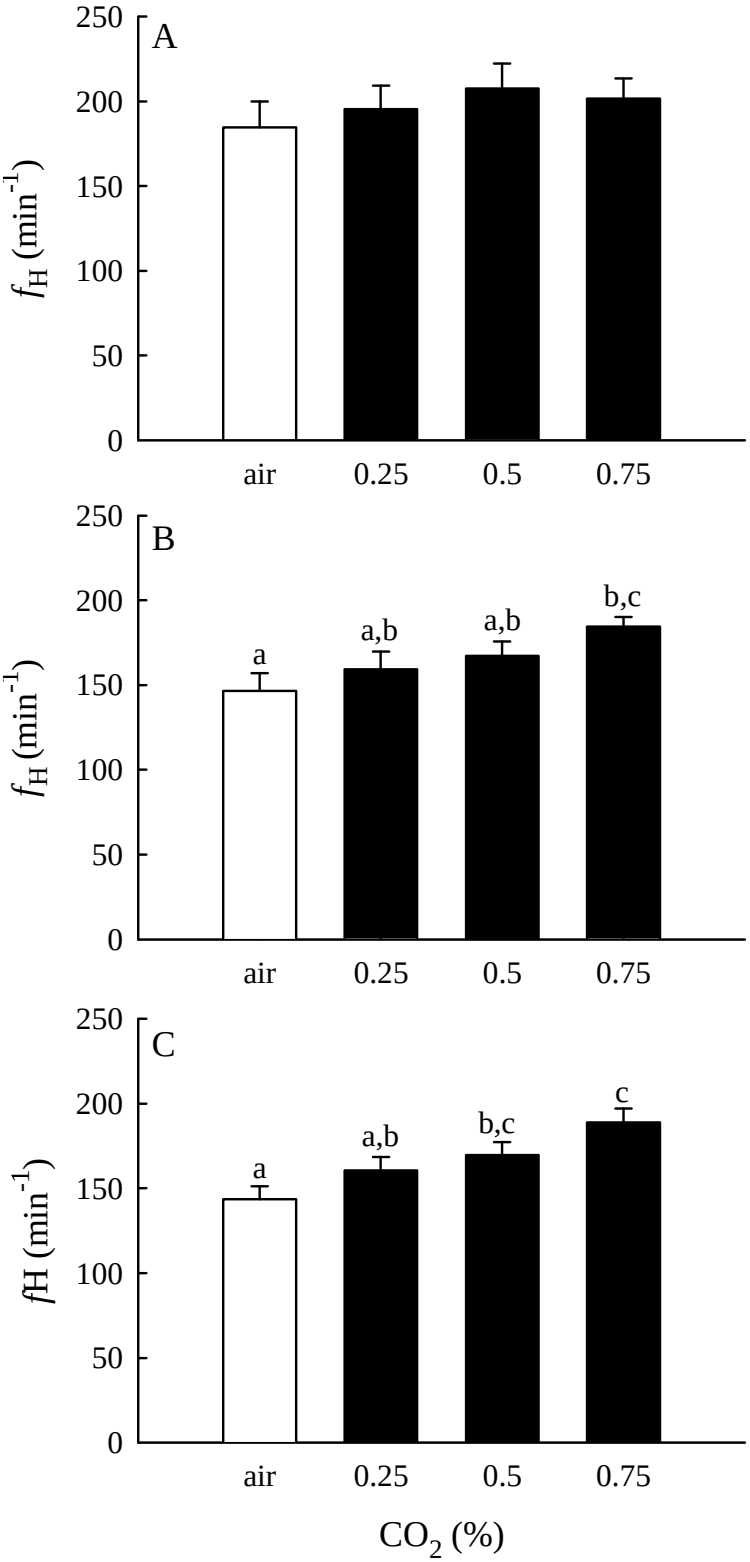
785 Figure 1



786 Figure 2

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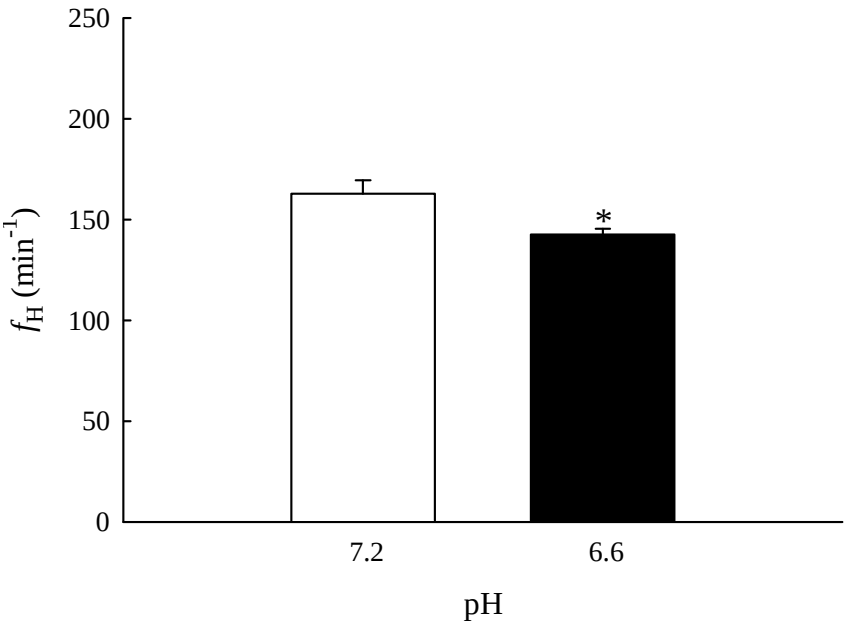

788 Figure 3

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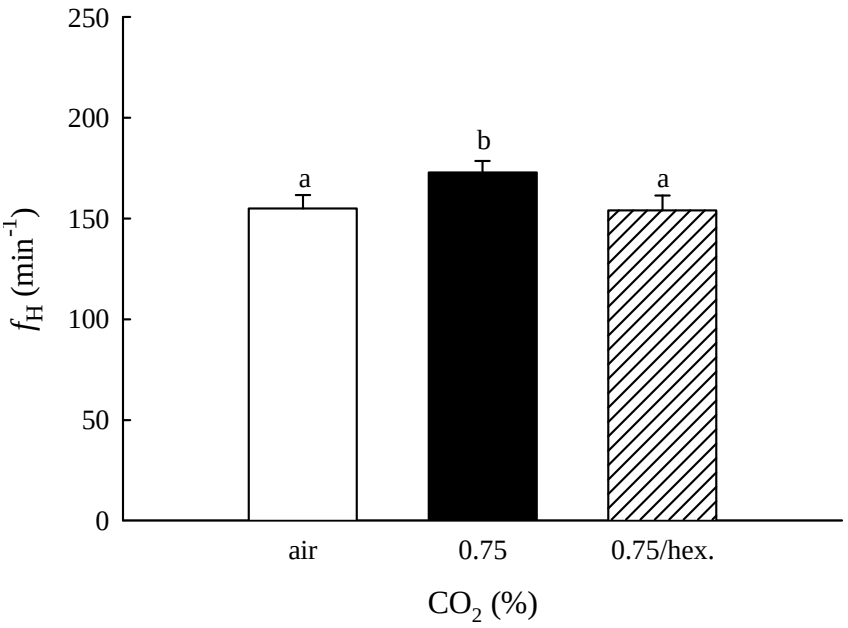

793 Figure 4

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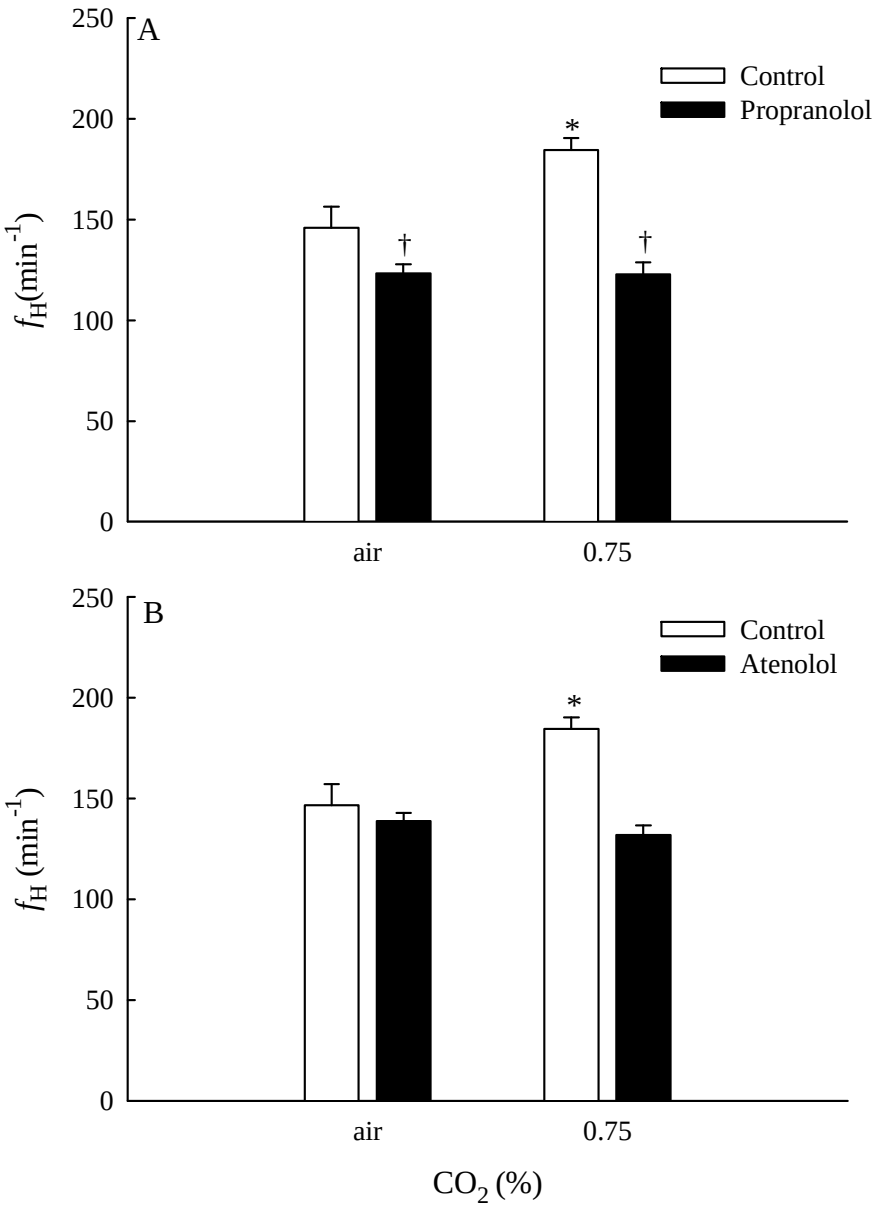

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797 Figure 5

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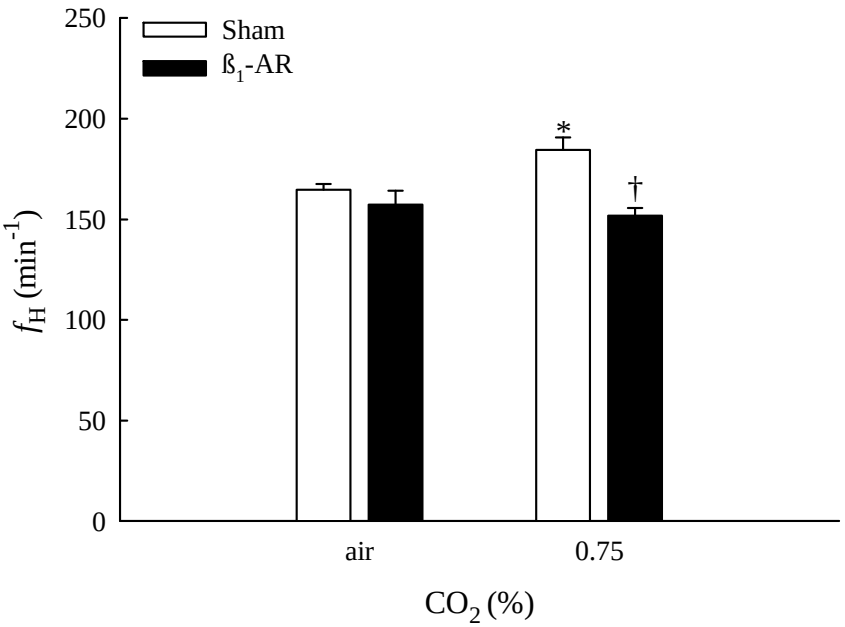

800 Figure 6

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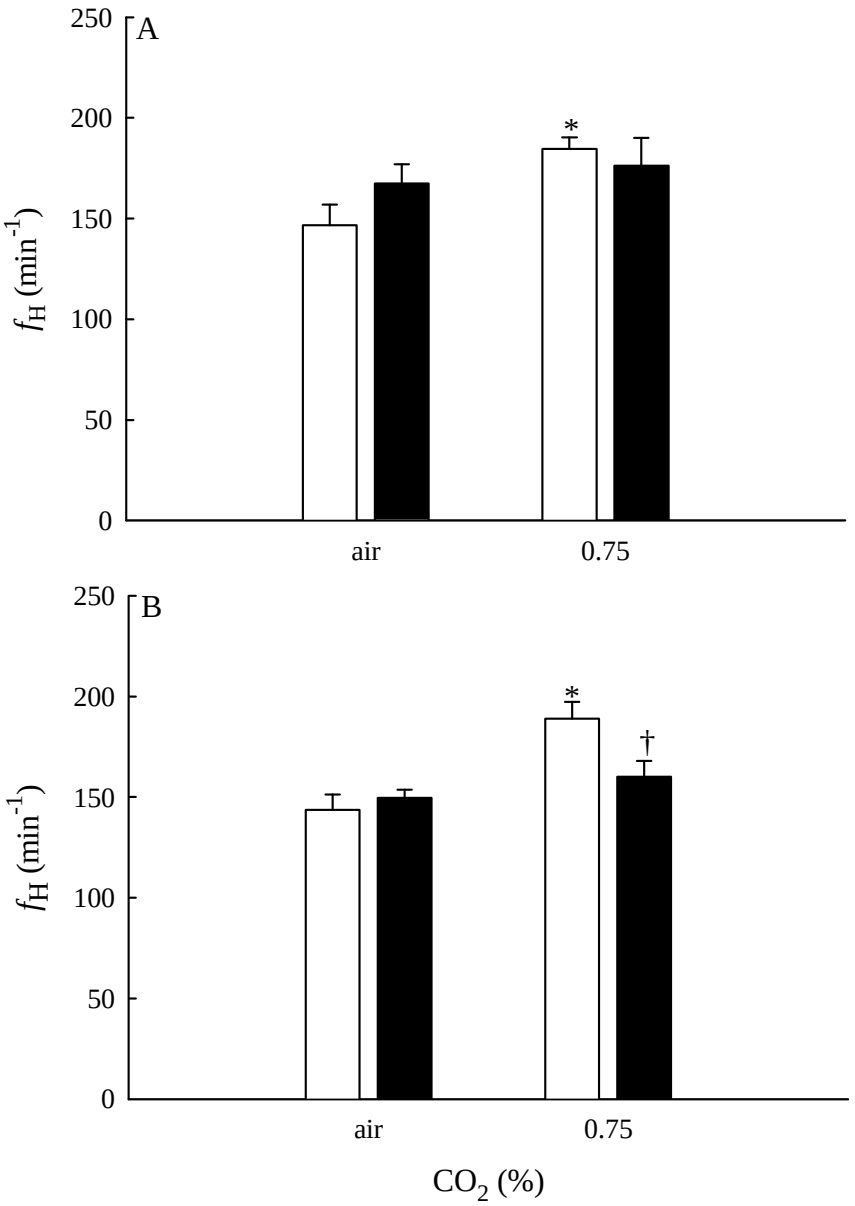

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803 Figure 7

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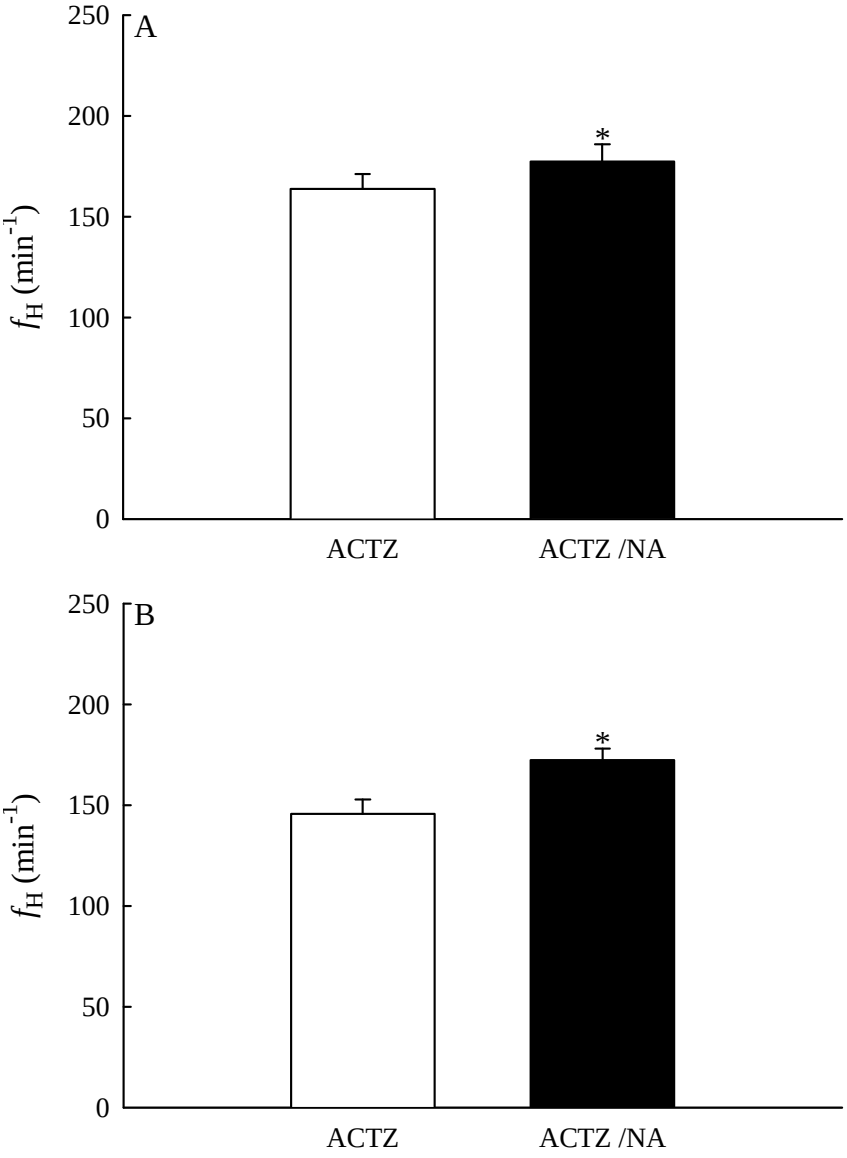
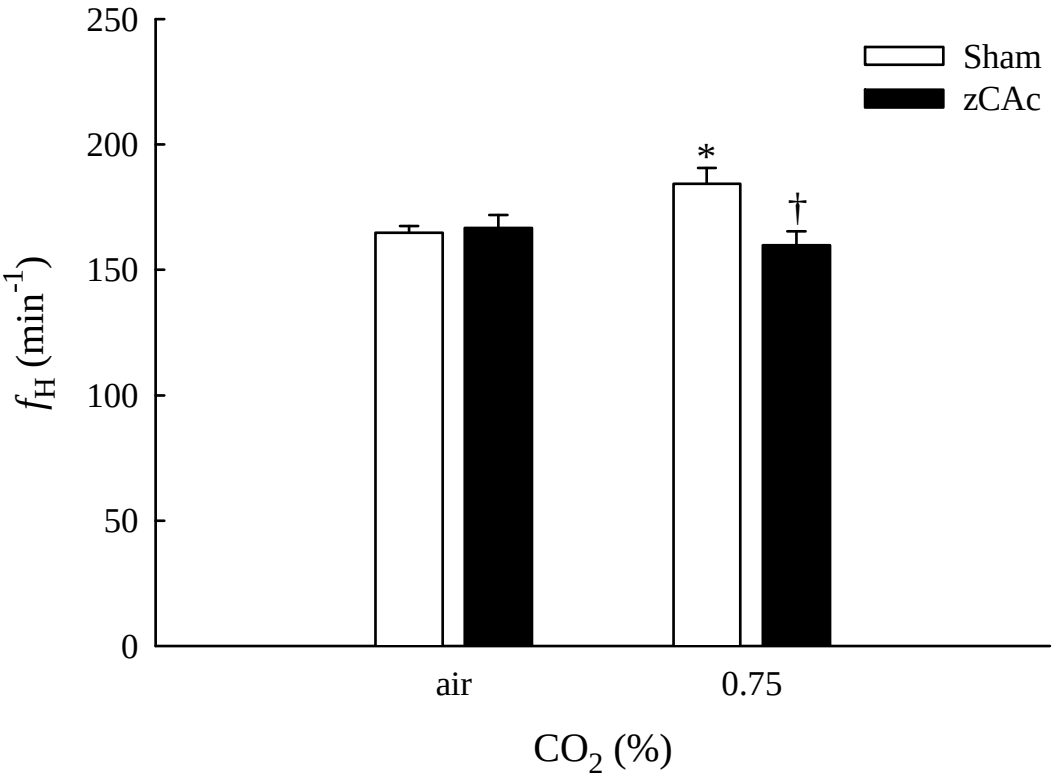
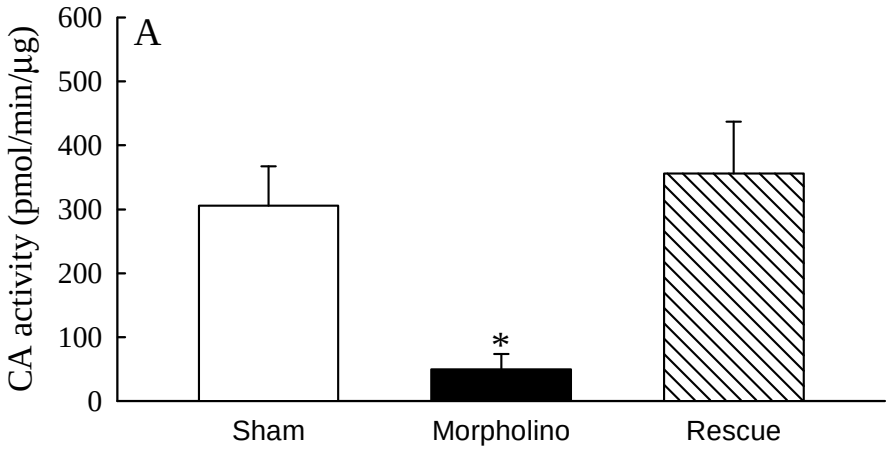
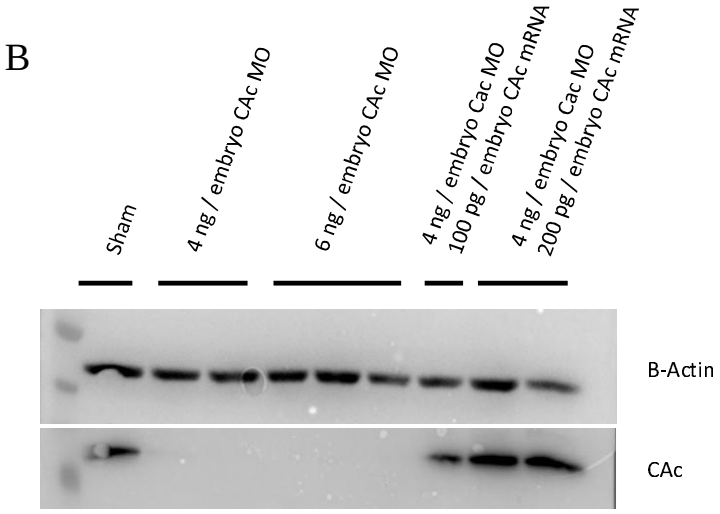


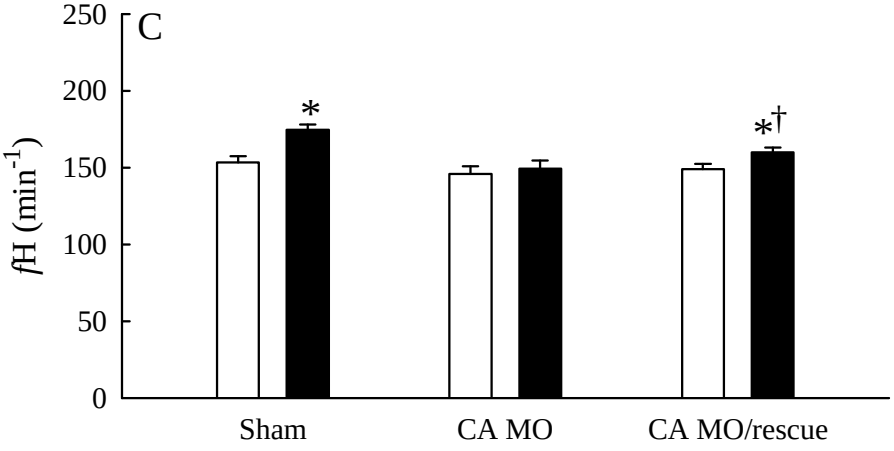
Figure 8



831 Figure 9







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833 Figure 10