THE EFFECTS OF THE HEME OXYGENASE-1/CARBON MONOXIDE SYSTEM ON CARDIORESPIRATORY CONTROL IN FISH

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ABSTRACT

Endogenously produced carbon monoxide (CO) is an important gaseous signalling molecule which regulates a variety of cardiorespiratory functions. CO is produced in cells by the heme oxygenase (HO) family of proteins by the breakdown of heme into equimolar amounts of CO, bilirubin and Fe^{2+}. My thesis focuses on the hypoxia- and hyperoxia-inducible HO-1/CO system exclusively and aims to provide the first evidence that the HO-1/CO system is involved in cardiorespiratory control in the zebrafish (*Danio rerio*) and goldfish (*Carassius auratus*). Overall, I hypothesise that the HO-1/CO system acts as a negative regulator of cardiorespiratory function in fish. Using immunohistochemistry, I was able to characterise the distribution of HO-1 and thus reveal the potential for endogenous CO production (from heme breakdown) in branchial and skin neuroepithelial cells (NECs; putative O_2 chemoreceptors) and associated innervation as well as the heart of the developing zebrafish larva. The presence of HO-1 in these structures suggests the likelihood of specific and localized production of CO in fish. To assess the functional significance of the HO-1/CO system in control of cardiorespiratory function, I used pharmacological and gene knock down approaches to diminish HO-1 activity, and presumably endogenous CO production, in adult and larval fish, respectively. The results from these experiments provided evidence that 1) CO has an inhibitory influence on ventilation in goldfish and zebrafish but that its function is temperature- and species-dependent and 2) showed that the HO-1/CO system tonically inhibits cardiac activity in larval zebrafish.

RÉSUMÉ

La production du monoxyde de carbone (MC) endogène est une importante molécule gazeuse de signalisation qui régule une variété de fonctions cardio-respiratoires. MC est produit dans les cellules par la famille de protéines hème oxygénase (HO) par la décomposition chimique de l'hème en quantité équimolaire de MC, bilirubine et Fe^{2+}. Ma thèse se concentre sur le système HO-1/MC inductible par l'hypoxie et l'hyperoxie exclusivement et vise à fournir la première preuve que le système HO-1/MC est impliqué dans le contrôle cardio-respiratoire chez le poisson-zèbre (*Danio rerio*) et le poisson rouge (*Carassius auratus*). Dans l'ensemble, mon hypothèse est que le système HO-1/MC agit en tant que régulateur négatif sur la fonction cardio-respiratoire chez les poissons. En utilisant l'immunohistochimie, j'ai été capable de caractériser la distribution de HO-1 et ainsi révéler la production potentielle du MC endogène dans les cellules neuro-épithéliales branchiales et de la peau (CNE; chémorécepteur d'oxygène putatif) et les innervations associées ainsi que le cœur de la larve du poisson-zèbre en développement. La présence de HO-1 dans ces structures suggère la probabilité d'une production spécifique et localisée de MC chez les poissons. Pour évaluer l'importance fonctionnelle du système HO-1/MC dans le contrôle de la fonction cardio-respiratoire, j'ai utilisé des approches de réductions géniques et pharmacologiques pour diminuer l'activité de HO-1 et probablement la production de MC endogène chez les poissons adultes et larvaires respectivement. Les résultats de ces expériences ont fourni des preuves 1) que MC possède une influence inhibitrice sur la ventilation des poissons rouges et poissons-zèbres mais cette fonction est dépendante de l'espèce et de la température et 2) qui ont montré que le système HO-1/MC inhibe de manière tonique l'activité cardiaque chez le poisson-zèbre larvaire.

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Table 3.1

LIST OF ABBREVIATIONS

5 – HT, serotonin

AD, adrenaline

ANOVA, analysis of variance

CO, carbon monoxide

CORM, carbon monoxide releasing molecule

CO_2, carbon dioxide

dpf, days post fertilization

f_H, cardiac frequency

f_{vent}, ventilation frequency

Q, cardiac output

HO, heme oxygenase

Isl1, islet 1

MO, morphant

N, number of animals

NEC, neuroepithelial cell

O_2, oxygen

PBS, phosphate buffered saline

PBST, Triton – X - 100 in phosphate – buffered saline

PFA, paraformaldehyde

PHZ, phenylhydrazine – HCl

PwO_2, partial pressure of oxygen in the water

RM, repeated measures

S. E. M., standard error of the mean

SV, stroke volume

MS-222, tricaine methanesulfonate

V_{amp}, ventilation amplitude

zn12, zebrafish-derived neuron-specific antigen

ZnPPIX, zinc protoporphyrin IX

CHAPTER 1.

General Introduction

1.1 INTRODUCTION

Fish may experience extreme changes in their environment which make them an excellent model for studying the environmental impact on physiological processes. Oxygen content and temperature of the water are two factors which can vary diurnally as well as seasonally (for review see Diaz, 2001). There are two well studied model teleost fish species that are able to thrive in such variable environments – namely, the goldfish (*Carassius auratus*) and the zebrafish (*Danio rerio*). For example, goldfish can withstand a temperature range from 5ºC to 35ºC which is met with an increase in their standard metabolic rate (Fry and Hart, 1948). Goldfish are one of the few fish species that have been studied that can tolerate extreme hypoxia (P_wO_2 < 30 mmHg) and even anoxia (no oxygen) for prolonged periods of time especially at the lower end of their temperature range (Shoubridge and Hochachka, 1980). They are able to survive such harsh conditions in part because of their high haemoglobin O_2 affinity (HbO_2 50% saturated with arterial PO_2 of ~2 mmHg at P_wO_2 = 30 mmHg; Burggren, 1982). Even more impressive is the goldfish's ability to withstand prolonged bouts of anoxia at low temperature owing to their ability to utilize liver glycogen stores for anaerobic glycolysis with ethanol as the end metabolic by-product coupled with metabolic depression (van den Thillart et al., 1980; Shoubridge and Hochachka, 1980; van den Thillart et al., 1983).

Zebrafish cannot withstand such wide temperature fluctuation as goldfish, but they are also hypoxia tolerant and can endure P_wO_2 as low as 30 mmHg (Vulesevic and Perry, 2006; Vulesevic et al., 2006). This hypoxia tolerance is in contrast to species inhabiting O_2 rich environments such as salmonids which have relatively low HbO_2 affinities (see reviews by Powers, 1980; Nikinmaa, 2001). Furthermore, the fully sequenced zebrafish genome and

translucent larvae have made this species a popular model for studying the development of physiological processes including cardiorespiratory function.

As mentioned above, goldfish and zebrafish are able to withstand prolonged intervals of hypoxia exposure but they are also well equipped to cope with higher than normal O_2 levels (hyperoxia) in the water. Hyperoxia in the water can occur during periods of high photosynthesis and the addition of supplementary oxygen in aquaculture (Dong et al., 2011; Kristensen et al., 2010). Both species respond to hyperoxia by decreasing ventilation frequency and amplitude (Vulesevic and Perry, 2006; Tzaneva and Perry, 2014). Presumably this response is aimed at preventing high O_2 levels in the blood which could lead to oxidative damage to tissues (Lushchak et al., 2005). Hypoventilation, however, can result in blood acidification due to the build-up of CO_2 (Heisler et al., 1988; reviewed in Perry and Gilmour, 2006).

Goldfish and zebrafish are well-suited for studying control of cardiorespiratory function in the face of hypoxia or hyperoxia because changes in ventilation can be easily observed and recorded in larvae and adults. In addition, there is an established collection of reverse genetics tools that can be used to study gene function in zebrafish - from development to adulthood. Goldfish on the other hand are unique in the way they deal with extreme hypoxia/anoxia. In this thesis I have focused on the role of the heme oxygenase-1 (HO-1)/carbon monoxide (CO) system in control of cardiorespiratory function in teleosts by using goldfish and zebrafish as model organisms.

1.2 CONTROL OF CARDIORESPIRATORY FUNCTION IN FISH

Control of ventilation: The neuroepithelial cells (NECs)

The fish gill is a multifunctional organ important for a number of physiological processes from gas exchange and gas sensing to ion and acid-base regulation and nitrogenous waste excretion (see special issue 184(3) in Resp. Physiol. Neurobiol. for manuscripts focusing on the structure/function relationships of the fish gill). Species from almost all taxa are able to sense and respond to changes in O_2 levels of their environments. For water-breathing teleost fish, the most common response to a decrease in the O_2 content of the water is hyperventilation which is reflected in an increase of ventilation amplitude and/or frequency (Randall and Shelton, 1963; Holeton, 1971; McDonald and McMahon, 1977; Randall, 1982; Smith and Jones, 1982; Boutilier et al., 1988; Sundin et al., 2000; Vulesevic et al., 2006; Tzaneva and Perry, 2010). These changes in the ventilation response to hypoxia are mediated by peripheral O_2 chemoreceptors found on the gills of adult fish and the skin of larvae (zebrafish being the most studied species at that stage; Coccimiglio and Jonz, 2012). Studying fish O_2 chemoreceptors (neuroepithelial cells; NECs) is of interest because of their phylogenetic proximity to the carotid body (the peripheral O_2 chemoreceptor organ in mammals) and thus such studies can provide insight on the evolution of O_2 sensing in vertebrates (Jonz et al., 2004; Milsom and Burleson, 2007; reviewed in Zachar and Jonz, 2012).

In adult goldfish and zebrafish, as well as other species that have been studied, the NECs are strategically located along the leading edge of the distal part of the gill filament within close proximity of the efferent filamental artery (eFA) as well as on the lamellae (Bailly et al., 1989; Jonz and Nurse, 2004; Saltys et al., 2006; Coolidge et al., 2008; Tzaneva and Perry, 2010; Tzaneva and Perry, 2014). The location of NECs along the gill filament and lamellae makes them an ideal candidate for sensing changes in the levels of respiratory gases both from the blood and the external environment (inspired water), respectively, and signalling for the appropriate

ventilatory response (Jonz and Nurse, 2003). In contrast to the carotid body, which contains a variety of neurotransmitters including serotonin (5-HT), catecholamines (CA) and acetylcholine (ACh), NECs are identified by the presence of a single neurotransmitter, 5-HT. The NECs of larval zebrafish are located exclusively on the skin prior to gill development (Coccimiglio and Jonz, 2012). The density of skin NECs in larvae gradually declines as the gill forms until eventually the gill becomes the principal location of NECs at 14 days post fertilisation (dpf). Although NECs are not found on the gill primordia until 5 dpf, physical responses to hypoxia are observed in zebrafish as early as 2 dpf and hyperventilation is observed at 4 dpf (Coccimigliio and Jonz, 2012; Jonz and Nurse, 2006). These findings suggest that the skin NECs function as early O_2 chemoreceptors prior to development and maturation of the gill (Coccimiglio and Jonz, 2012). Furthermore, both skin and gill NECs are extensively innervated (Jonz and Nurse, 2004; Coccimiglio and Jonz, 2012; Tzaneva and Perry, 2010) in keeping with their role as chemoreceptors.

Control of cardiac function: Pacemaker cells and cardiac innervation

Intrinsic heart rate in fish is determined by a small population of rhythm generating myocytes referred to as the cardiac pacemaker cells. These cells are located in a ring of tissue around the junction of the atrium and the sinus venosus creating the primary pacemaker region (Haverinen and Vornanen, 2007; Icardo, 2012). The pacemaker cells were recently characterized in the myocardium of zebrafish larvae (Tessadori et al., 2012). The pacemaker region of the teleost heart is heavily innervated by cholinergic and adrenergic neurons. Cardiac performance is tightly linked to heart rate which is governed by the pacemaker cells and the sympathetic and parasympathetic branches of the autonomic nervous system. In most adult fishes, hypoxia exposure is met with reflex bradycardia (a decrease in heart rate) mediated by an increase in

vagal cardioinhibitory tone (Wood and Shelton, 1980; Burleson and Milsom, 1995; Stecyk and Farrell, 2006).

Although bradycardia is the typical response to hypoxia in adult fish, the situation is much less clear in the developing larvae. Zebrafish larvae, in particular, exhibit various cardiac responses depending on the severity of hypoxia and/or the duration of exposure (acute *versus* chronic) (Barrionuevo and Burggren, 1999; Jacob et al., 2002; Yaqoob and Schwerte 2010; Bagatto, 2005; Steele et al., 2011). Tachycardia may be the initial response to progressive hypoxia but can develop into bradycardia as the severity or duration of the hypoxia is increased. Hypoxic tachycardia was observed in zebrafish larvae exposed to moderate levels of hypoxia (~75 mmHg O_2) whereas more severe levels of hypoxia (~20 mmHg) led to pronounced bradycardia (Jacob et al., 2002; Bagatto, 2005; Steele et al., 2011). Bradycardia was also observed in trout embryos (before hatching) exposed to chronic and severe hypoxia (Miller et al., 2011). Hypoxic tachycardia is also a typical response of larval Arctic char (*Salvelinus alpinus*) and trout (*Oncorhynchus mykiss*) (McDonald and McMahon, 1976; Holeton, 1971). In larval zebrafish, the onset of cardiac control via the parasympathetic and sympathetic nervous systems (or circulating catecholamines) is evident as early as 4 dpf (Steele et al., 2009; Steele et al., 2011). The development of bradycardia in the face of chronic hypoxia in zebrafish larvae is mediated by cardiac muscarinic receptors (Bagatto, 2005) of the M_2 sub-type (Steele et al., 2009) while the tachycardia response to acute hypoxia likely is controlled by a release of inhibitory vagal tone or stimulation of adrenergic receptors by elevated circulating catecholamine levels or catecholamines released from sympathetic neurons presumably acting on the pacemaker cells of the heart (Steele et al., 2009; Steele et al., 2011).

1.3 HO-1/CO SYSTEM

Carbon monoxide (CO) is classified as a gaseous signalling molecule (gasotransmitter) that is produced *in vivo* in vertebrates and is involved in controlling various physiological processes including cardiorespiratory function (reviewed in Prabhakar and Semenza, 2012). Endogenous CO is produced by the heme oxygenase (HO) family of enzymes and several lines of evidence suggest that it is through CO that HO exerts its effect on physiological functions. HO breaks down heme into equimolar amounts of bilirubin, Fe^{2+} and CO (Tenhunen et al., 1969). This mechanism of action is depicted in Figure 1.1 which has been adapted from Yoshida and Migita (2000). Of the three isozymes (HO-1, HO-2, HO-3) that have been identified in mammals, two have been found in teleost fish; the hypoxia inducible HO-1 and the constitutively expressed HO-2 (Scapagnini et al., 2002; Tzaneva and Perry, 2014; Voelker et al., 2008; Wang et al., 2008; Zhong et al., 2009). In mammals, endogenously formed CO is largely eliminated in exhaled air or sequestered intracellularly by heme proteins; a small portion may be converted to CO_2 (Wu and Wang, 2005). CO excretion and catabolism have not been examined in fish but it seems likely that CO diffuses easily across the gill epithelia. The function of CO in O_2 sensing has been well described in the carotid bodies of mammals where it acts as an endogenous inhibitory gaseous messenger (Prabhakar, 2012). Prabhakar and colleagues (Prabhakar et al., 1995) inhibited HO activity, and presumably endogenous production of CO, with zinc protoporphyrin IX (ZnPPIX) to assess the functional significance of HO on carotid body discharge. They found that inhibition of HO significantly augmented carotid body discharge suggesting that the HO/CO system has an inhibitory effect on carotid body activity (Prabhakar et al., 1995). Furthermore, reducing HO activity resulted in an enhanced respiratory response to hypoxia in rats further supporting the inhibitory actions of endogenously produced CO on control of breathing (Yang et al., 1998). The carotid body and in particular the glomus cells, are

presumed to be the initial site of sensory discharge in mammals and sensory transduction at the glomus cells relies entirely on Ca^{2+}-dependent neurotransmitter release. The source of the Ca^{2+} in the glomus cells may be entry through voltage dependent L-type Ca^{2+} channels (Overholt and Prabhakar, 1997; Wilkinson and Kemp, 2011). The notion that control of O_2 sensing in peripheral chemoreceptors by CO produced via HO involves intracellular Ca^{2+} is well described in the literature on mammals (Prabhakar, 1999; Peers and Wyatt, 2007; Kumar and Prabhakar, 2012). For example, Overholt et al. (1996) demonstrated that inhibition of HO, and thus reduction of endogenous CO production, resulted in significant increases of intracellular Ca^{2+} levels in glomus cells further confirming the inhibitory actions of CO on ventilation.

In the hypoxic mammalian heart, the HO-1/CO system maintains ventricular size by reducing cardiac apoptosis and blood pressure via CO mediated cGMP vasodilation (Chen et al., 2003; Vulapalli et al., 2002). CO has also been shown to induce relaxation of blood vessels through vasodilation (Stanford et al., 2003; Boissiere et al., 2006). HO-derived CO serves to regulate blood pressure and vascular contractility via the soluble guanylyl cyclase (sGC) and cyclic guanosine monophosphate (cGMP) pathways (Ndisang et al., 2004). In addition sGC acts to increase cGMP, which causes vasodilation and serves to lower blood pressure. Furthermore, several lines of evidence suggest that HO-1 exerts cytoprotective and anti-apoptotic effect on mammalian cardiomyocytes via CO (Abraham and Kappas, 2008).

Relatively few studies have investigated the role and expression of HO-1 in teleost species although zebrafish has received the most attention. HO-1 mRNA was detected in the red blood cells of wild type normoxic zebrafish embryos as early as 34 hours post fertilization (Craven et al., 2005). In addition, exposure to toxic substances such as 3,4-dichloroaniline caused an increase in whole larva HO-1 mRNA (Voelker et al., 2008). More recently, a

significant increase in HO-1 mRNA was reported in the hearts of adult zebrafish exposed to 9 h of hypoxia (Parente et al., 2013). HO-1 protein and mRNA were also detected in the gills of adult zebrafish and European sea bass (*Dicentrarchus labrax* L.) (Prevot-D'Alvise et al., 2013; Li et al., 2015). Although not yet investigated, these results suggest that the HO/CO system may be involved in the control of cardiorespiratory function in teleost fish in a similar fashion as in mammals.

1.4 THESIS OULINE AND RATIONALE

The role of the HO/CO system on cardiorespiratory function in fish in response to O_2 changes in the environment is unclear. While the HO/CO system is well-described in the mammalian literature, mammalian species, for the most part, are intolerant to change in environmental O_2 levels unlike many fish species. Therefore, to gain further insight into how the hypoxia-induced cardiorespiratory response is controlled, an examination of the HO/CO system in hypoxia tolerant species such as the goldfish and zebrafish is imperative. In this thesis, I specifically aim to link endogenously produced CO via HO-1 to control of breathing and heart function in fish. This thesis consists of three data chapters. Chapter 1 was published in the *Journal of Respiratory Physiology & Neurobiology*. Chapter 2 is in revision at the *Journal of Experimental Biology* after a first positive round of peer review. Finally, Chapter 3 is presented as a manuscript in preparation to be submitted to *American Journal of Physiology*. I have tried to avoid repetition where possible but some repetition is unavoidable because this thesis is composed of accepted and submitted manuscripts.

General hypothesis:

The HO-1/CO system acts as a negative regulator of cardiorespiratory function in fish.

Chapter 2 hypothesis:

HO-1 is present in the NECs on the gills of goldfish (*Carassius auratus*) and thus it mediates the respiratory response to acute hypoxia in these fish.

Chapter 3 hypothesis:

Chapter 4 hypothesis:

Endogenously produced carbon monoxide (CO), via HO-1 present in the NECs, regulates control of respiration in larval and adult zebrafish (*Danio rerio*) exposed to acute hypoxia. My thesis focuses on the HO-1/CO system and aims to provide the first evidence that this system is involved in cardiorespiratory control in two model teleost species, goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*). In Chapter 2, I demonstrate that HO-1 plays an inhibitory role in regulating ventilation frequency in 7^{o}C acclimated goldfish but not in goldfish acclimated to 25^{o}C. I attribute this difference to the lack of HO-1 in the neuroepithelial cells (NECs; putative O_2 chemoreceptors) of the 25^{o}C acclimated goldfish and to the increased activity of HO-1 in the gills, and likely the NECs, of 7^{o}C acclimated goldfish. Chapter 3 demonstrates the potential of presumptive cardiac pacemaker cells in 4 day old zebrafish larvae to produce CO. I also show that HO-1 inhibits resting heart rate presumably via CO. In Chapter 4, I used immunocytochemistry to localize HO-1 and address the interactive effects of developmental age and the HO-1/CO system in control of breathing. The results reveal the presence of HO-1 in the NECs found on the skin of larvae and the gills of adult zebrafish, and thus the potential for CO production within these cells. The data further demonstrate that the HO-1/CO system plays an inhibitory role in the control of breathing in *both* adult and larval zebrafish under normoxic but

not hypoxic conditions. Taken together, the results of this thesis provide the first evidence for a physiological role of the HO-1/CO system in regulating cardiorespiratory function in fish.

1.5 FIGURES

Figure 1.1: Proposed mechanism of the second step of HO-catalyzed conversion of α-meso-hydroxyhemin to verdoheme. This is the step which produces carbon monoxide CO. This scheme was adapted from Yoshida and Migita (2000). The reader is referred to this publication for a more detailed explanation of the complete mechanism.

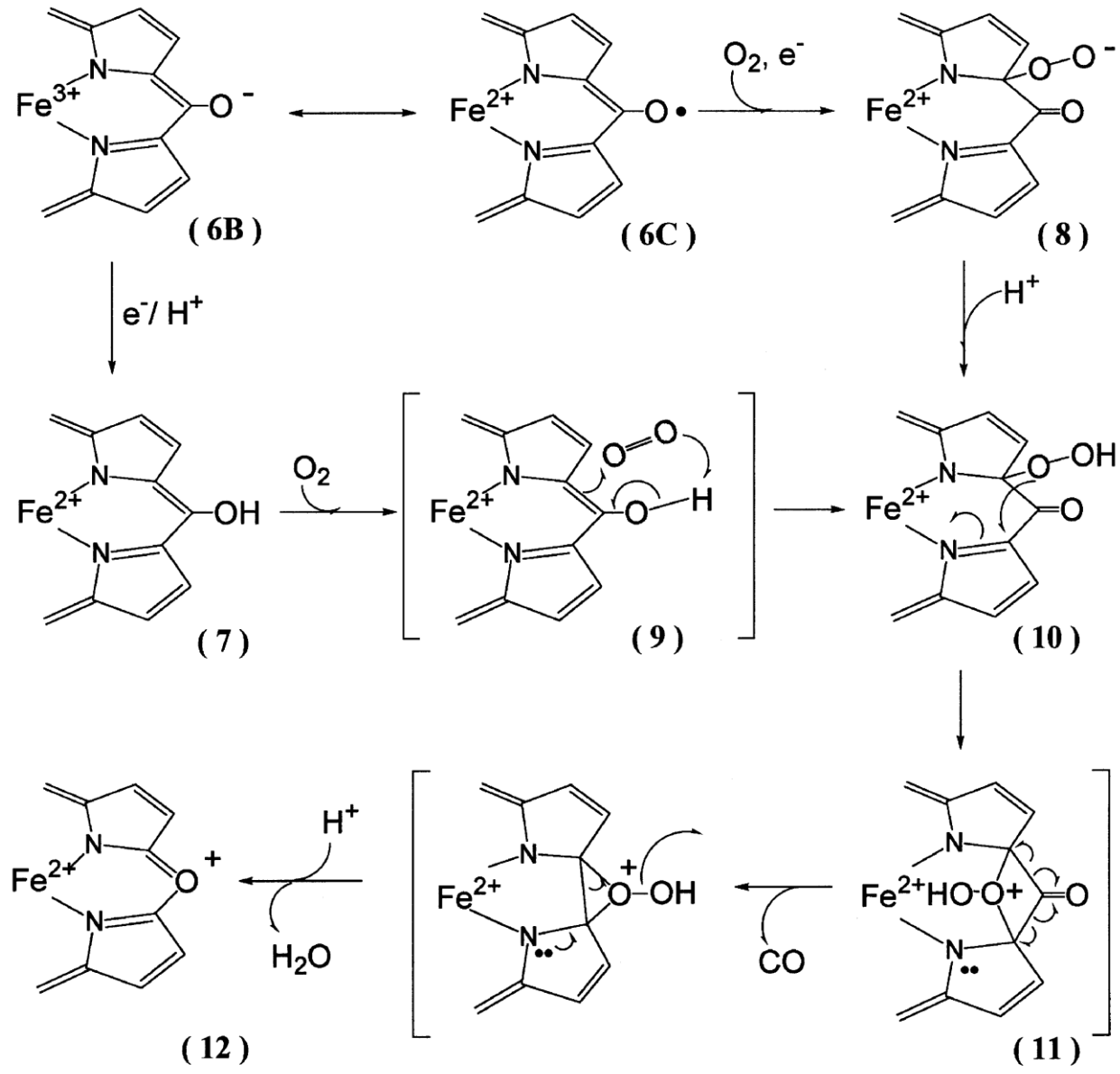
Fe3+
O -
(6B)
Fe2+
O •
(6C)
O2, e-
O -
O
(8)
e-/ H+
H+
Fe2+
OH
(7)
O2
O=O
O
H
(9)
O-OH
(10)
Fe2+
O
+
(12)
H+
H2O
Fe2+
+
O-OH
CO
Fe2+HO-O+
O
(11)

Figure 1.1

CHAPTER 2.

Heme oxygenase-1 (HO-1) mediated respiratory responses to hypoxia in the goldfish, *Carassius auratus*

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ABSTRACT

In this study we investigated the role of heme oxygenase-1 (HO-1) in modulating the hypoxic and hyperoxic ventilatory responses of goldfish (*Carassius auratus*) acclimated to 7 and 25^{o}C. HO- 1 was present in the neuroepithelial cells (NECs; putative branchial O_2 chemoreceptors) of fish acclimated to 7^{o}C only. Hypoxia exposure increased gill HO-1 activity in 7^{o}C fish (14.0 ± 1.4 to 42.5 ± 3.2 pmol bilirubin min^{-1} mg protein^{-1}). Inhibition of HO-1 activity with zinc protoporphyrin IX (ZnPPIX) increased the ventilation frequency response to acute hypoxia (30 mmHg); frequency increased from 48.3 ± 5.1 to 137.4 ± 16.0 breaths per min (BPM) in hypoxic 7^{o}C fish treated with ZnPPIX compared to 46.2 ± 4.2 to 77.9 ± 5.3 BPM in control fish. Unlike in the control (untreated) 7^{o}C fish exposed to hyperoxia, fish injected with ZnPPIX did not significantly decrease breathing frequency. Inhibiting HO-1 activity was without effect on the hypoxic or hyperoxic ventilatory responses of fish acclimated to 25^{o}C. Based on these observations, we suggest that HO-1 plays an inhibitory role in regulating breathing frequency but only in goldfish acclimated to 7^{o}C.

2.1 INTRODUCTION

Teleost fish possess peripheral O_2 chemoreceptors able to sense changes in the O_2 partial pressure and/or content of the water and/or blood and for initiating a cascade of cardiorespiratory responses. Recent studies have focused on the peripheral chemoreceptors found on the gills of adult fish. In zebrafish (*Danio rerio*), these chemoreceptors, termed neuroepithelial cells (NECs; Dunel-Erb et al., 1982) sense changes in O_2 and CO_2 (Jonz et al., 2004; Qin et al., 2010) and thus are functionally analogous to type I cells of the mammalian carotid body (Perry et al., 2009; Perry and Abdallah, 2012; Porteus et al., 2012). Although NECs have been identified in the gills of numerous species (Dunel-Erb et al., 1982; Jonz and Nurse, 2003; Saltys et al., 2006; Tzaneva and Perry, 2010), only the NECs of the zebrafish filament epithelium have been conclusively identified as being responsive to changes in O_2 or CO_2 status. In goldfish (*Carassius auratus*), the NECs are found distributed on the lamellar and filament epithelia of the gill (Jonz et al., 2004; Coolidge et al., 2008; Tzaneva and Perry, 2010). The filament NECs, situated on the leading edge, are ideally located to sense changes in inspired water and arterial PO_2.

Fish regulate their breathing to adjust branchial water flow in the face of changing metabolic rates or water oxygenation levels. Decreases in O_2 levels of the water (hypoxia) typically are met with a hyperventilatory response and depending on the species can arise from either an increase in the ventilation amplitude (A_{vent}) or ventilation frequency (f_{vent}) or both (Holeton, 1971; reviewed by Gilmour and Perry, 2007). Hyperventilation is an important strategy for maximizing O_2 uptake from the water and thus sustaining arterial partial pressure of O_2 (P_aO_2) at higher levels than otherwise would be possible (Holeton and Randall, 1967; Holeton, 1971). In contrast, acute increases in water O_2 levels (hyperoxia) elicit hypoventilatory responses in many fish species arising from a decrease in A_{vent} and/or f_{vent} (Takeda, 1990; Wood

and Jackson, 1980). Hypoventilation during ambient hyperoxia, by minimizing the extent to which P_aO_2 will increase, may protect against oxidative stress that otherwise might yield tissue damage and possibly death (Lushchak et al., 2005; Olsvik et al., 2005; reviewed in Lushchak, 2011).

Fluctuating O_2 levels can also lead to altered transcription of genes or post-translational stabilization of proteins involved in cellular homeostasis, including hypoxia inducible factors (Sutter et al., 2000) which induce the transcription of the heme oxygenase group of enzymes such as heme oxygenase -1 (HO-1) (Dunn et al., 2013; Lee et al., 1997). Heme oxygenase (HO) breaks down heme into equimolar amounts of carbon monoxide (CO), biliverdin and iron (reviewed in Maines and Gibbs, 2005; Neubauer and Sunderram, 2012). Two structurally similar isoforms of HO, HO-1 and HO-2, well-described in mammals, have more recently been characterized in zebrafish, Takifugu (*Takifugu rubripens*) and goldfish (*C. auratus*) (Shi and Zhou, 2010; Wang et al., 2008; Zhong et al., 2009). HO-1 can be induced in response to oxidative stress (hyperoxia or hypoxia) whereas HO-2 is constitutively expressed and not inducible (Gozzelino et al., 2010). Several lines of evidence indicate that endogenous CO produced via heme breakdown acts as a signalling molecule in mammalian peripheral O_2 chemoreceptors suggesting that it is involved in modulating the ventilatory response to altered O_2 levels (Prabhakar et al., 1995; Prabhakar and Semenza, 2012).

Although heme oxygenase is considered as a potential modulator of breathing in mammals, the available data appear to be contradictory. For example, Ortega-Saenz et al. (2006) did not observe any difference in the neurosecretory response to hypoxia of type I cells between wild-type and HO-2 knockout mice. In contrast, Adachi et al. (2004) reported a blunted hypoxic ventilatory response in HO-2 knockout mice. To date, no studies have examined the role of HO

in the ventilatory responses of lower vertebrates to hypoxia. Here we examine the potential involvement of HO-1 in modulating the hypoxic ventilatory response in goldfish. The goals were to determine whether (i) inhibition of HO-1 activity affects the ventilatory response of goldfish to hypoxia or hyperoxia, and (ii) HO-1 is present in the presumptive branchial chemoreceptors (NECs). These experiments were performed on fish acclimated to two different temperatures (7 and 25°C) so as to compare the effects of HO-1 on breathing in fish experiencing widely different metabolic/O_2 requirements.

2.2 MATERIALS AND METHODS

2.2.1 Experimental animals and surgical procedures

Goldfish (157.3±7.7g, N =26) were purchased from Aleong's International (Mississauga, ON). The fish were held in circular tanks supplied with recirculating dechloraminated City of Ottawa water under a 12h:12h light:dark cycle at either 25 or 7°C for at least 2 weeks prior to experimentation. Experiments were performed at water temperatures of 25 or 7°C at the University of Ottawa Aquatic Care Facility with the approval of the University of Ottawa Animal Care Committee (Protocol BL-226) and according to the guidelines of the Canadian Council of Animal Care (CCAC).

Goldfish were anesthetized with 10 mg l^{-1} benzocaine (Sigma Aldrich, Inc., Oakville, ON) and when breathing ceased, they were placed on a surgery table where the gills were irrigated with aerated anaesthetic solution. To measure ventilation amplitude (A_{vent}) and frequency (f_{vent}), fish were equipped with impedance leads terminating in 1 cm^2 brass plates that were attached with sutures to the end of each operculum. A polyethylene (PE50) cannula terminating in a PE10 attachment was inserted into either the caudal vein or artery to allow injections of zinc protophorphyrin IX (ZnPPIX; HO-1 inhibitor) or saline (controls). The aim of the cannulation was to access a blood vessel to introduce the saline or ZnPPIX into the blood stream without specifically targeting either the vein or artery. Briefly, a ~2 cm lateral incision was made in the caudal peduncle slightly ventral to the lateral line to access both blood vessels and the cannula was inserted in the anterior direction. The incision was closed with silk sutures and the cannula was secured to the skin with ligatures. The fish were placed into a 3.8 L

transparent plastic box provided with flowing aerated water and were allowed to recover 24 h before experiments commenced.

2.2.2 Experimental protocol

The partial pressure of O_2 in the water (P_wO_2) was decreased or increased by replacing air flowing through a water–gas equilibrium column with N_2 or O_2, respectively. Fish were exposed to hypoxia (~30 mmHg) or hyperoxia (~500 mmHg) for 1h followed by a 30 min period of normoxic recovery. Injections of 0.4 mg kg^{-1} ZnPPIX or saline were administered after recording baseline conditions and 1h before hypoxia or hyperoxia commenced. Ventilation parameters were measured in real time using a BioPac Systems (Montreal, QC) data acquisition system and were recorded and stored using AcqKnowledge data acquisition software.

After experiments were completed, the fish were killed by a sharp blow to the head and the left and right first gill arches were removed and either fixed in 4% paraformaldehyde (PFA; right gill arch) for immunohistochemistry or flash frozen for later determination of HO-1 activity and protein levels (left gill arch).

2.2.3 HO-1 activity assay

The HO-1 assay was performed as described in Chapter 9 of Current Protocols in Toxicology (McCoubrey, 1999). Briefly, frozen tissue was thawed and homogenized in ice cold homogenization buffer (1 M Tris·Cl, 250 mM sucrose, pH 7.4). The homogenized tissue was centrifuged at 12000× *g* for 30 min at 4°C and the crude supernatant was collected from the lysates. The supernatant was incubated in the dark with heme (18 μM), 30 μL of 30 U ml^{-1} biliverdin reductase (BVR; Abcam Inc., Toronto, ON) and NADPH (2.75 mM) for 30 min at 37°C. The reaction was stopped on ice and the concentration of bilirubin was calculated by the

difference of absorbance between 470 and 530 nm using an extinction coefficient of 40 mM^{-1} cm^{-1}. Heme oxygenase activity was expressed as pmol bilirubin min^{-1} mg protein $^{-1}$.

2.2.4 Western blot analysis and protein quantification

Frozen tissue was thawed on ice and homogenized in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris–HCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride) with a protease inhibitor cocktail tablet (Roche, USA). Protein concentration was determined using a bicinchoninic acid (BCA) assay and approximately 50 μg of protein was used for Western blotting. Protein samples were separated using a 12% SDS-PAGE gel and were then transferred to a PVDF membrane (Bio-Rad, USA). The membrane was blocked in 5% milk for 2 h at room temperature after which it was incubated overnight at 4°C with a custom made primary zebrafish anti-HO-1 antibody (immunogen peptide MDSTKSKAAENTGSC) raised in rabbits [Gen Script, USA; 64% amino acid identity to putative goldfish HO-1 N-terminus (Wang et al., 2008)] at 1:4000 dilution. After washing, the membrane was probed with a goat anti-rabbit secondary antibody (1:15000; Pierce, USA) for 2 h at room temperature. The membrane was washed and bands were detected using Clarity Western ECL substrate (Bio-Rad, Mississauga, ON) using a ChemiDoc system (Bio-Rad, USA). The membrane was stripped (ReBlot Plus kit, Millipore, Billerica, MA) and re-probed with an anti-rabbit β-actin antibody (1:4000; Sigma–Aldrich, USA). To determine the specificity of the antibody, the primary HO-1 antibody was incubated with excess peptide (1:2000) for 2 h and applied to the membrane. After washing, the membrane was probed with the secondary antibody (1:15000). Heme oxygenase-1 protein expression was normalized to that of β-actin using Image J software (http://rsb.info.nih.gov.proxy.bib.uottawa.ca/ij/) to estimate the intensity of the bands.

2.2.5 Immunohistochemistry

Gill tissue was embedded in OCT Cryomatrix (Thermo Scientific, USA) and sectioned longitudinally and transversely into 20 μm slices using a Leica CM3050 cryostat at −25°C (Leica Microsystems Inc.). The sections were incubated with 2% Triton X-100 in phosphate buffered saline (PBS; pH=7.4) for 2h. Neuroepithelial cells were identified by incubating the slices with an antibody against serotonin (5-HT; Sigma–Aldrich, USA) and HO-1 positive cells were identified with a custom made zebrafish HO-1 antibody (described above). The primary antibodies were added to 1X PBS to yield final dilutions of 1:200 and 1:250 for 5-HT and HO-1, respectively. The sections were incubated with the primary antibodies at room temperature overnight. Secondary antibodies [Alexa Fluor 488 (green) and Alexa Fluor 594 (red)] were applied and sections were incubated in the dark for 2 h at RT. The tissue was imaged using an upright confocal microscope equipped with argon (peak output = 488 nm) and helium-neon (peak output = 543 nm) lasers. Optical slices of 1 μm were taken to produce a 3 dimensional image using Fluoview Viewer (Olympus, PA).

2.2.6 Statistical analysis

Data are presented as means ± 1 SEM. One way analysis of variance (ANOVA), one-way repeated measures ANOVA (RM ANOVA), two-way RM ANOVA or one-way ANOVA on ranks (when assumptions for parametric analysis were not met) were used to test for significant differences between the means. A Holm–Sidak post hoc test for multiple comparisons was used when significant differences were revealed by ANOVA. The limit of significance was 0.05 for all analyses. SigmaPlot v. 11.0 (SPSS Inc.) was used to perform all the statistical analyses.

2.3 RESULTS

2.3.1 Heme oxygenase-1 in gill neuroepithelial cells

Two or three sections from four different fish (at each acclimation temperature) were examined using confocal imaging to determine the presence of HO-1 in the gills of goldfish acclimated to 7 and 25°C. The presence of HO-1 in the lamellar and filament NECs is illustrated in a series of representative confocal images in Figs. 2.1–2.3. In fish acclimated to 7°C, HO-1 was expressed in the majority of the lamellar NECs as well as in the filament NECs (Figs. 2.1 and 2.3 A). HO-1 positive NEC's were never observed in goldfish acclimated to 25°C (Figs. 2.2 and 2.3 B).

2.3.2 The effects of HO-1 on ventilation (A_{vent} and f_{vent}) during hypoxia or hyperoxia

To examine the effects of HO-1 on breathing, A_{vent} and f_{vent} were measured in saline- (control) and ZnPPIX- (HO-1 inhibitor) injected fish acclimated to either 7 or 25°C during normoxic or acute hypoxic (30 mmHg) or hyperoxic (~500 mmHg) conditions. Acute hypoxia exposure resulted in a significant increase in the A_{vent} of both saline- and ZnPPIX-injected fish acclimated to either temperature. In the 25°C acclimated fish, A_{vent} increased from 0.45 ± 0.11 cm during normoxia to a maximum of 0.99 ± 0.19 cm when exposed to hypoxia. In fish acclimated to 7°C, A_{vent} increased from 0.16 ± 0.02 to 0.62 ± 0.16 cm (two-way RM ANOVA $P < 0.001$; Fig. 2.4 A and B). Inhibiting HO-1 with ZnPPIX did not impair the capacity of either group to elevate breathing during hypoxia; A_{vent} rose from 0.48 ± 0.06 cm during normoxia to a maximum of 0.86 ± 0.11cm during hypoxia in the 25°C acclimated fish and from 0.21 ± 0.04 to 0.47 ± 0.05 cm in the goldfish acclimated to 7°C (two-way RM ANOVA $P < 0.001$; Fig. 2.4 A and B). Control fish at both temperatures significantly increased f_{vent} when exposed to hypoxia

(Fig. 2.4 C and D). At 25°C, f_{vent} increased from 105.8 ± 8.8 breaths per minute (BPM) to a peak during hypoxia of 142.9 ± 6.3 BPM and from 46.2 ± 4.2 to 77.9 ± 5.3 BPM for goldfish kept at 7°C (two-way RM ANOVA $P < 0.001$; Fig. 2.4 C and D). The response of 25°C fish injected with ZnPPIX and exposed to hypoxia was similar to that of the controls (Fig. 2.4 C). In contrast however, inhibiting HO-1 in 7°C goldfish caused an even greater increase in f_{vent} during hypoxia in comparison to the control fish. The increase from 48.3 ± 5.1 to 137.4 ± 16.0 BPM was significantly greater than the increase exhibited by the controls (two-way ANOVA $P < 0.001$, Fig. 2.4 D).

The ventilatory responses to hyperoxia (P_wO_2 ~500 mmHg) of control and ZnPPIX-injected goldfish acclimated to either 7 or 25°C are summarized in Fig. 2.5. Hyperoxia evoked a hypoventilatory response in control fish at both temperatures. During hyperoxia A_{vent} was reduced from 0.48 ± 0.10 to 0.12 ± 0.05cm for fish at 25°C and from 0.41 ± 0.06 to 0.15 ± 0.04 cm for fish at 7°C (Fig. 2.5 A and B). Ventilation frequency also was significantly reduced by acute hyperoxia from 84.4 ± 5.1 to 17.9 ± 4.7 BPM in 25°C fish and from 54.7 ± 5.2 to 36.5 ±3 .1 BPM in 7°C fish. Inhibiting HO-1 activity with ZnPPIX did not alter the f_{vent} response to hyperoxia in 25°C fish but goldfish acclimated to 7°C did not significantly decrease f_{vent} when exposed to hyperoxia (Fig. 2.5 C and D).

2.3.3 HO-1 enzyme activity

In fish acclimated to 7°C, heme oxygenase activity increased significantly during hypoxia from 14.0 ± 1.4 to 42.5 ± 3.2 pmol bilirubin min^{-1} mg $protein^{-1}$ (one-way ANOVA $P < 0.001$; Fig. 2.6 B). There was no significant change in gill HO-1 activity during hypoxia at 25°C (Fig. 2.6 B). Fig. 2.6 also illustrates the HO-1 activity in the gills of goldfish that had

experienced hyperoxia. During hyperoxia, gill HO-1 activity increased significantly yielding 27.3 ± 2.9 and 26.9 ± 1.2 pmol bilirubin min^{-1} mg protein $^{-1}$ for 25 and 7°C fish, respectively (one-way ANOVA; $P < 0.001$; Fig.2. 6). Zinc protoporphyrin IX injections significantly inhibited HO-1 activity in all fish exposed to either hypoxia or hyperoxia (Fig. 2.6).

2.3.4 Western blot analysis and quantification of gill HO-1 protein

Western blots from protein extracted from the gills of normoxic fish acclimated to 7 or 25°C showed a clear band at ~32 kDa (insert in Fig. 2.1 B). Additional bands also were present; however, pre-absorption with the immunizing peptide eliminated only the band of interest (Fig. 2.3 C). Hypoxia or hyperoxia did not cause a significant change in the protein content in fish acclimated to either temperature although there was a trend for an increase in HO-1 protein during hypoxia at 25°C (two-way ANOVA, $P = 0.153$). Treatment with ZnPPIX did not significantly affect HO-1 protein levels (Fig. 2.7).

2.4 FIGURES

Figure 2.1. Representative confocal micrographs depicting the presence of heme oxygenase-1 (HO-1; B) and serotonin (5-HT; C) immuno-reactive cells on the lamellae of normoxic goldfish (*Carassius auratus*) acclimated to 7°C. Nuclei were stained with DAPI to visualize individual nuclei (A). Arrows indicate neuroepithelial cells (NECs) and arrow heads indicate cells expressing HO-1. The HO-1 containing NECs are circled in the merged image (D) and a single representative outlined region indicates the interlamellar cell mass (ILCM). The insert in D shows a magnified view of a cell co-expressing 5-HT and HO-1. Total protein was extracted from whole gills and analyzed for HO-1 protein by western blot (insert in B). Scale bar = 50 μm

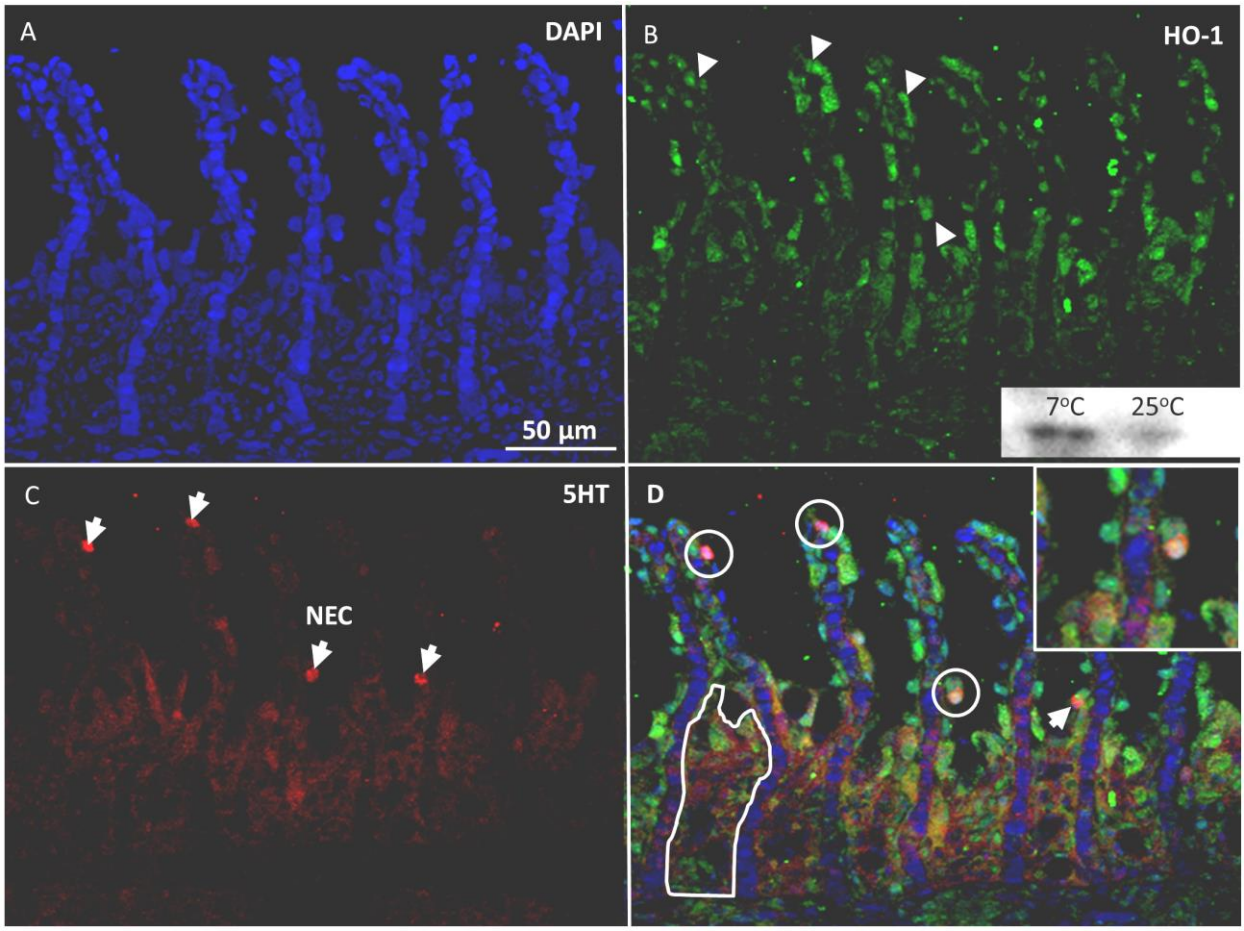

Figure 2.1

Figure 2.2. Representative confocal micrographs depicting the presence of heme oxygenase-1 (HO-1; B) and serotonin (5-HT; C) immuno-reactive cells on the lamellae of normoxic goldfish (*Carassius auratus*) acclimated to 25°C. Nuclei were stained with DAPI to visualize individual cells (A). 5-HT-positive cells did not co-express HO-1 (merged image; D). Arrows indicate neuroepithelial cells (NECs) and arrow heads indicate cells expressing HO-1. The insert in panel D shows a magnified view of a 5-HT-positive cell apparently lacking HO-1 and a single representative outlined region indicates the interlamellar cell mass (ILCM). Scale bar = 50 μm

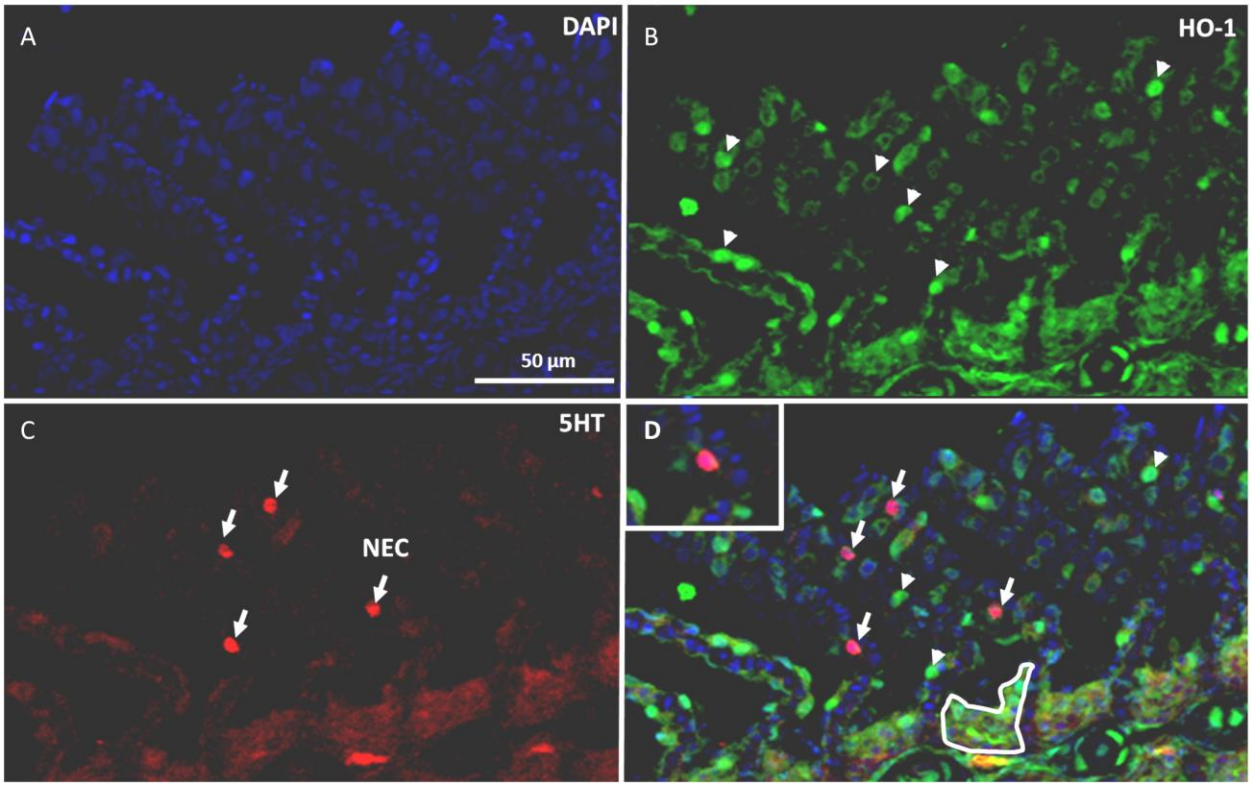

Figure 2.2

Figure 2.3. Representative confocal micrographs depicting the presence of heme oxygenase-1 (HO-1) and serotonin (5-HT) immuno-reactive cells on the gill filament in proximity to the efferent filament artery (EFA) of normoxic 7°C (A) and 25°C (B) acclimated goldfish (*Carassius auratus*). Nuclei were stained with DAPI to visualize individual cells (A). Arrows indicate neuroepithelial cells (NECs) expressing HO-1 and arrow heads indicate NECs lacking HO-1. The inserts show magnified views of a gill cell co-expressing 5-HT and HO-1 in A and a 5-HT positive cell lacking HO-1 in B. Panel C depicts representative control (ctr) and pre-absorbed (pre-abd) western blots of gill protein from normoxic goldfish. A rectangle has been drawn around the band of interest (HO-1). Scale bar = 30 µm

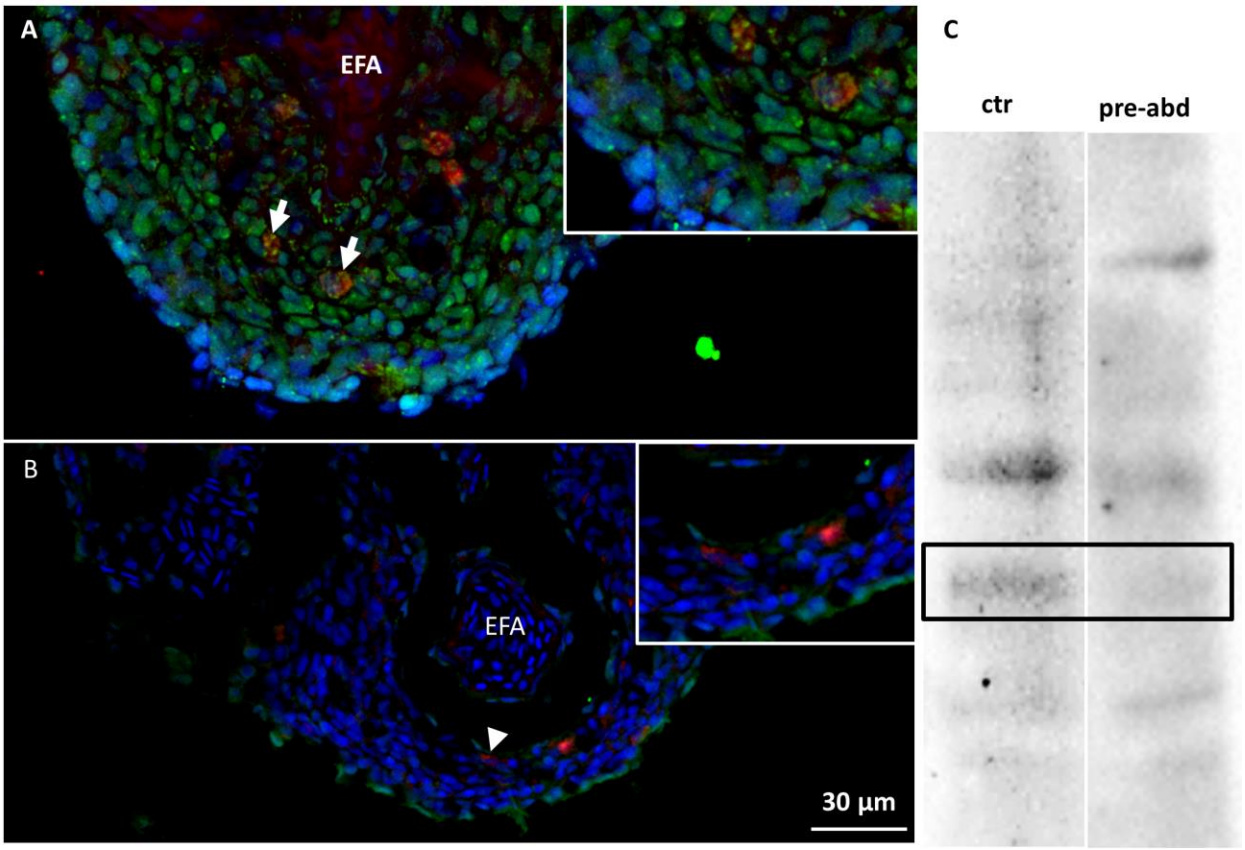

Figure 2.3

Figure 2.4. The effects of acute hypoxia on ventilation amplitude (A_{vent}; A and B) and frequency (f_{vent}; C and D) in control (saline-injected; filled bars) or zinc protoporphyrin IX- (ZnPPIX; unfilled bars) injected goldfish (*Carassius auratus*) acclimated to 25°C (A and C) or 7°C (B and D). Measurements were performed during normoxia (N), acute hypoxia (H) and normoxic recovery (R). Different letters represent statistically significant differences within a group. An asterisk denotes a statistically significant difference between groups of the same oxygen treatment. N = 4–6; values are means ± SEM (two-way ANOVA; $P<0.05$).

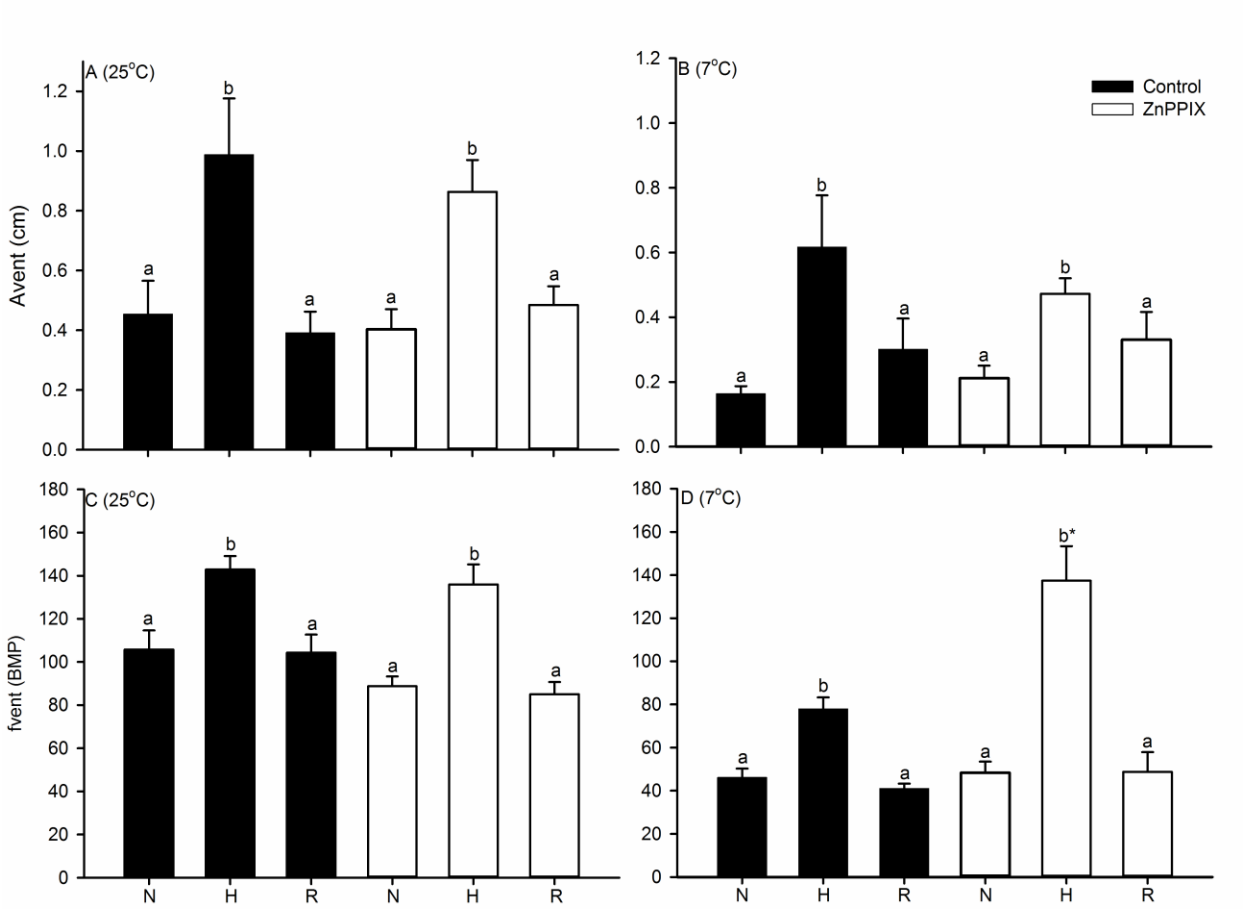

Figure 2.4

Figure 2.5. The effects of acute hyperoxia on ventilation amplitude (A_{vent}; A and B) and frequency (f_{vent}; C and D) in control (saline-injected; filled bars) or zinc protoporphyrin IX- (ZnPPIX; unfilled bars) injected goldfish (*Carassius auratus*) acclimated to 25°C (A and C) or 7°C (B and D). Measurements were performed during normoxia (N), acute hyperoxia (O_2) and normoxic recovery (R). Different letters represent statistically significant differences within a group. N = 4–6. Values are means ± SEM (two-way ANOVA; $P < 0.05$).

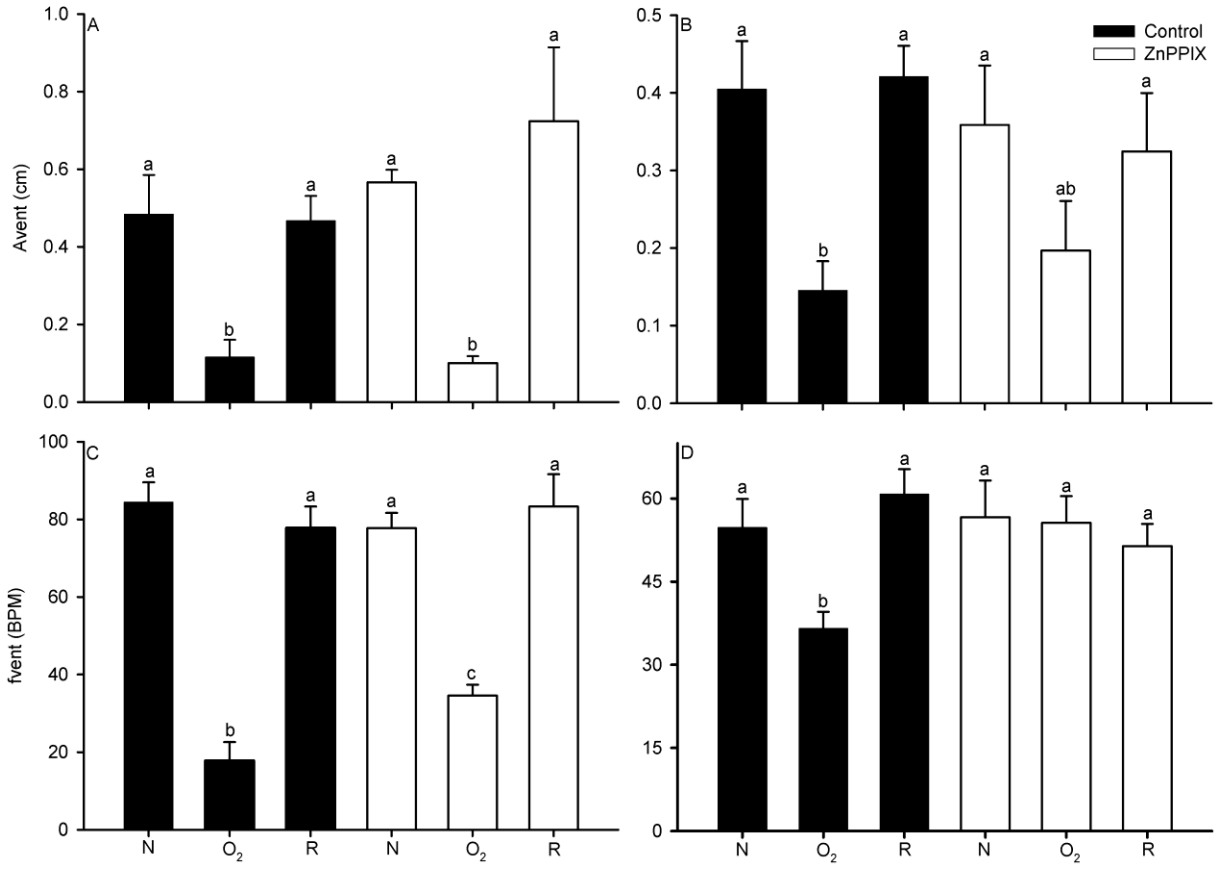

Figure 2.5

Figure 2.6. Heme oxygenase activity in the gills of 25°C (A) and 7°C (B) acclimated goldfish during normoxia (N), acute hypoxia (H) or acute hyperoxia (O_2). Different letters represent statistically significant differences. N = 4–6. Values are means ± SEM (one-way ANOVA; $P < 0.05$).

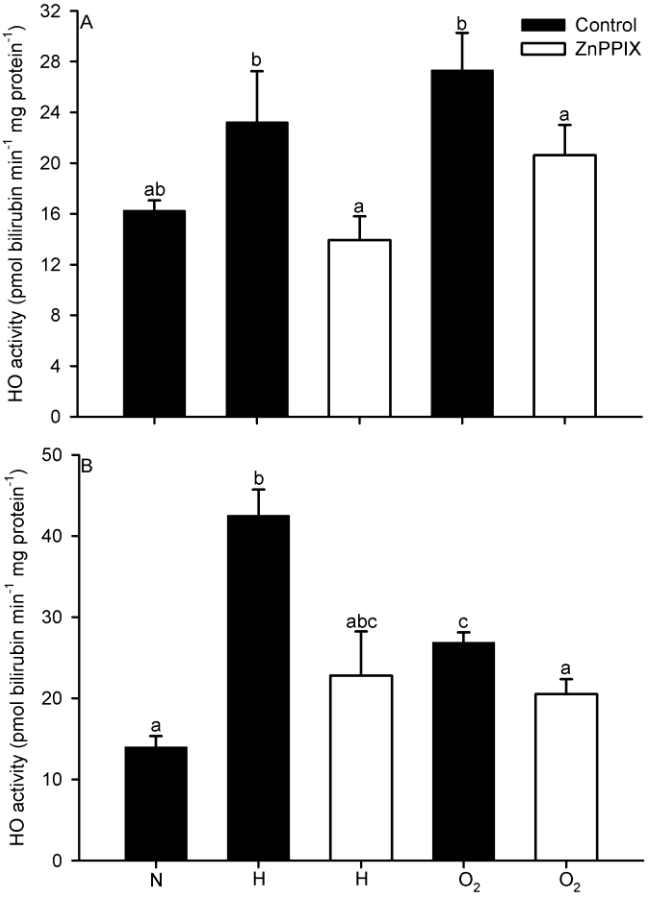
A
B
Control
ZnPPIX
HO activity (pmol bilirubin min-1 mg protein-1)
HO activity (pmol bilirubin min-1 mg protein-1)
0
4
8
12
16
20
24
28
32
0
10
20
30
40
50
ab
b
a
b
a
a
b
abc
c
a
N
H
H
O2
O2

Figure 2.6

Figure 2.7. Heme oxygenase-1 relative protein expression in the gills of 25°C (A) and 7°C (B) acclimated goldfish during normoxia (N), acute hypoxia (H) or acute hyperoxia (O_2). N = 4–6. Values are means ± SEM (one-way RM ANOVA; $P > 0.05$).

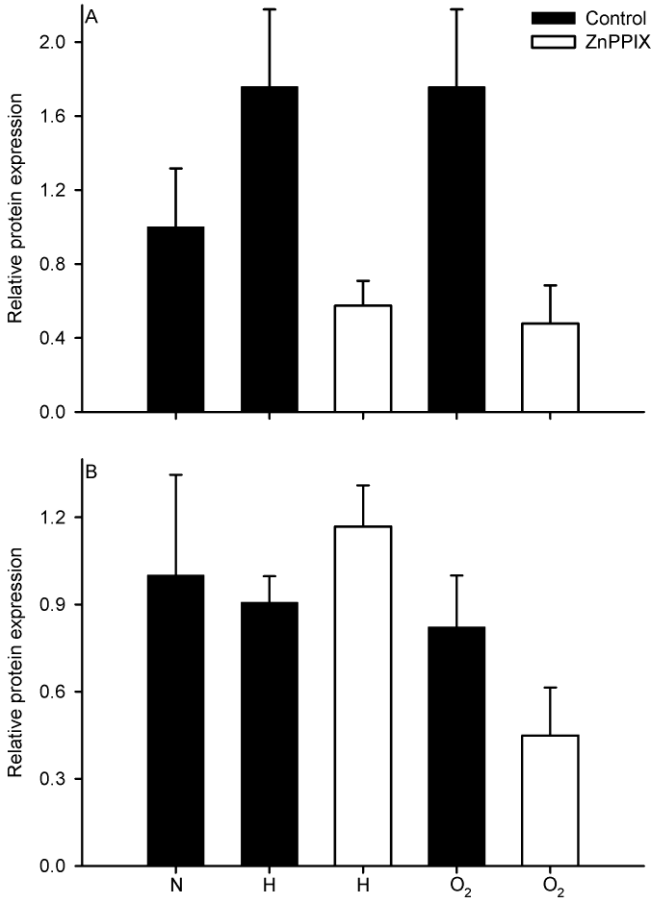
A
Control
ZnPPIX
Relative protein expression
2.0
1.6
1.2
0.8
0.4
0.0
B
Relative protein expression
1.2
0.9
0.6
0.3
0.0
N
H
H
O2
O2

Figure 2.7

2.5 DISCUSSION

The overall aim of this study was to investigate the role of HO-1 in the control of breathing in normoxic, hypoxic or hyperoxic goldfish experiencing widely different metabolic rates resulting from acclimation to either 25°C or 7°C water. Although metabolic rates were not determined, the results of previous studies on this species suggest that O_2 consumption was likely increased by ~7-fold as temperature increased from 7 to 25°C (Fry and Hart, 1948). Three major findings emerged from this study; (i) HO-1, while expressed in the branchial NECs in 7°C-acclimated goldfish, was undetectable in the NECs of fish acclimated to 25°C, (ii) HO-1 appeared to attenuate the f_{vent} response to hypoxia in 7°C fish but not 25°C fish without affecting the A_{vent} response, and (iii) HO-1 enzyme activity increased significantly during hypoxia in 7°C fish only, while hyperoxic exposure caused significant increases of activity in both groups of fish without any significant changes in protein content.

2.5.1 Heme oxygenase-1 in the neuroepithelial cells of goldfish

This is the first study to investigate the potential role of HO-1 in the presumptive peripheral O_2 chemoreceptors (NECs) on, and within, the fish gill. The results demonstrate the presence of HO-1 in the lamellar and filament NECs of 7°C (but not 25°C) goldfish. Lower temperatures, by increasing the solubility of O_2 in water, create relatively hyperoxic conditions. HO-1 is induced during hypoxic as well as hyperoxic conditions and thus the presence of HO-1 in the NECs of cold- but not warm-acclimated goldfish may be due to a relative increase in oxygen content rather than lower temperature per se (Lee et al., 1996, 2000). Although not known in lower vertebrates, evidence from literature on mammals suggests that the transcription factors involved in hypoxic and hyperoxic HO-1 induction are the hypoxia inducible factor-1 (HIF-1) and activator protein-1 (AP-1) coupled with the signal transducer and activator of

transcription (STAT), respectively (Lee et al., 1997, 2000). There have been conflicting reports, however, on the presence of basal levels of HO-1 in the peripheral chemoreceptors of mammals with one study by Di Giulio et al. (2009) providing evidence for its existence and induction by hypoxia in the carotid body of rats while others report HO-2, but not HO-1, immunoreactivity in mammalian carotid body (Prabhakar et al., 1995; Prabhakar, 2013). In addition, we observed that a smaller population of lamellar NECs appeared to lack the HO-1 enzyme (arrow in Fig. 2.1 D). Furthermore, we also see a population of HO-1 positive, 5-HT negative gill cells which could also include 5-HT negative NECs. The role of 5-HT negative NECs in the gill is unknown, however, it is possible that these cells may be a newly formed, not yet differentiated and thus not fully functional, population of NECs which have not yet synthesized 5-HT (Saltys et al., 2006). Therefore it is most likely that these cells, even if they were HO-1 positive, are not yet involved in contributing to the ventilatory response in goldfish during fluctuations of water PO_2. HO-1 positive, 5-HT-negative cells found at the base of the lamella and on the filament epithelium may represent other cell types such as ionocytes. Unpublished results from our lab have shown that Na^+/K^+ ATPase positive cells (i.e. ionocytes) also contain HO-1.

Although not investigated in this study, the most likely mechanism through which HO-1 exerts its effects on breathing is through CO. Given its high diffusivity across cell membranes, CO may not only act in an autocrine manner but may influence nearby cells and tissues (neurons, blood vessels) in a paracrine manner (reviewed in Prabhakar and Semenza, 2012). Endogenous CO production in the carotid body leads to inhibition of Ca^{2+} channels preventing Ca^{2+} influx which reduces sensory discharge (Overholt and Prabhakar, 1997; reviewed in Prabhakar, 1999). Thus, the inhibitory effects of HO-1 on f_{vent} in goldfish acclimated to 7°C may reflect the HO-1-mediated production of CO within NECs.

2.5.2 Role of HO-1 in goldfish acclimated to 7°C versus 25°C

The evidence from this study suggests that HO-1 has an inhibitory influence on the f_{vent} but not A_{vent} response in 7°C goldfish exposed to acute hypoxia or hyperoxia. In vertebrates the net respiratory response to altered ambient O_2 is controlled by numerous substances acting at different levels of integration (Doi and Ramirez, 2008). It is possible that the differential effects of HO-1 on f_{vent} and A_{vent} may arise from the stimulation of NEC efferent nerves involved in modulating only the frequency component of respiratory activity in 7°C fish. The absence of HO-1 in the NECs coupled with the lack of alteration in f_{vent} during changes in water PO_2 in 25°C acclimated fish injected with ZnPPIX further associates HO-1 in the NECs in the inhibitory control of f_{vent} in goldfish.

A mechanism for inhibiting ventilation during acute hypoxia may not be appropriate in warm acclimated goldfish. Fish acclimated to 25°C must exert a large hyperventilatory response (both A_{vent} and f_{vent}) during hypoxia to maintain the high demand for oxygen imposed by their increased metabolic rates (Fry and Hart, 1948). In contrast, cold acclimated goldfish have lower metabolic rates and thus an inhibitory mechanism may be in place to fine-tune the ventilation response to hypoxia and preventing fish from excessive increases in breathing when not necessary. To the best of our knowledge there are no comparable studies published to date investigating the effects of the HO system on the carotid bodies of mammals with lower metabolic rates. However, the acute hypoxic ventilatory response of 7°C goldfish to HO-1 inhibition resembles that of mammals. In mammals, inhibition of the HO system with ZnPPIX caused increased excitation of the carotid body when exposed to hypoxia (Prabhakar et al., 1995). Exposure of the hypoxic carotid body to CO reversed the excitatory effects of ZnPPIX. This suggests that endogenously produced CO via heme breakdown due to the HO system is

inhibitory to the carotid body (Prabhakar et al., 1995; reviewed in Prabhakar and Peers, 2014). In addition, the respiratory responses of rats experiencing HO inhibition were increased when the animals were exposed to acute hypoxia (Yang et al., 1998). In a more recent study, Yang et al. (2007) demonstrated that inhibition of HO with ZnPPIX in the respiratory rhythm generating regions of the central nervous system, namely the medulla oblongata, lead to an increased discharge frequency as recorded from the hypoglossal rootlets. The authors reported little or no change in the discharge duration or amplitude and thus concluded that HO is involved in regulating rhythmic respiration at the level of the medulla oblongata (Yang et al., 2007). Although, we only investigated the presence of HO-1 in peripheral chemoreceptors of goldfish we cannot rule out that HO-1, as in mammals, could also be acting on regions of the medulla responsible for generating the breathing frequency (central rhythm generators; CRG) in fish. Future studies should aim to distinguish the degree of contribution of HO-1 between the peripheral O_2 chemoreceptors (NECs) and the central network of rhythm generating cells involved in setting the breathing frequency response of fish.

In this study the effects of HO-1 on ventilation were associated with an increase in enzyme activity without any accompanying change in HO-1 protein content (Figs. 2.6 and 2.7). Because HO-1 exhibits cyto-protective properties against oxidative stress via bilirubin (Llesuy and Tomaro, 1994; Tomaro and Batlle, 2002), the trend towards an increase in HO-1 activity during hyperoxia in the gills of goldfish acclimated to 25°C was possibly related to maintaining cell viability rather than control of ventilation. The transcriptional induction of HO-1 and subsequent long-term protection against chronic oxidative stress is well characterized (Neubauer and Sunderram, 2012). It is possible, however, that for immediate protection, as might be required when fish are exposed to acute bouts of O_2 fluctuations, may depend on post-

translational modifications of basal HO-1 levels in the gills to increase protein activity. For example, the protein kinase, Akt, was shown to phosphorylate recombinant human HO-1 increasing its activity when compared with the non-phosphorylated form (Salinas et al., 2004; reviewed in Dunn et al., 2013). This post-translational phosphorylation also increases HO-1 binding affinity for biliverdin reductase and cytochrome P450 reductase (Salinas et al., 2004) presumably increasing production of the cyto-protective by-product, bilirubin.

2.5.3 Possible mechanism of action of HO-1in the NECs of the goldfish gill

HO-1 likely reduces sensory discharge by inhibiting Ca^{2+} channels (Overholt and Prabhakar, 1997; reviewed in Prabhakar, 1999). A recent study (P. Zachar and M. Jonz, unpublished data) showed that hypoxia activates L-type voltage gated Ca^{2+} channels on the NECs of goldfish acclimated to 18°C resulting in Ca^{2+} influx into the cell. We propose that the degree of activation of the Ca^{2+} channels during hypoxia is in part controlled by CO generated by HO-1. Thus, the reduction of f_{vent} associated with HO-1in the 7°C acclimated goldfish observed in this study may have been caused by decreased sensory activity of the NECs via inhibition of the L-type voltage gated Ca^{2+} channels by endogenous CO produced by HO-1. In contrast, the absence of CO production (due to lack of HO-1) in warm acclimated goldfish presumably augments the opening of Ca^{2+} channels leading to a greater increase in intracellular [Ca^{2+}] thereby potentiating the NECs' sensory activity. The notion that CO is responsible for regulating intracellular Ca^{2+} in peripheral chemoreceptors is supported by the mammalian literature; inhibition of HO with ZnPPIX resulted in marked elevations in intracellular [Ca^{2+}] in glomus cells (Overholt et al., 1996). This response was eliminated in the absence of extracellular calcium suggesting that the increase in intracellular [Ca^{2+}] was caused by the influx of extracellular Ca^{2+} via plasma membrane Ca^{2+} channels (Overholt et al., 1996; Prabhakar, 1998).

2.5.4 Conclusion

The results of this study provide the first evidence that HO-1 is involved in the control of breathing in fish. Moreover, the study demonstrates differential control of f_{vent} by HO-1 in fish experiencing different metabolic requirements. Thus, we observed that HO-1 plays an inhibitory role in regulating f_{vent} during hypoxia in 7°C acclimated goldfish but not in goldfish acclimated to 25°C. We attribute this difference partially to the lack of HO-1 in the NECs of 25°C acclimated goldfish and to the increased activity of HO-1 in the gills, and likely the NECs, of 7°C acclimated goldfish during both hypoxia and hyperoxia.

2.6 ACKNOWLEDGEMENTS

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CHAPTER 3.

Evidence for a role of heme oxygenase-1 in the control of cardiac function in zebrafish (*Danio rerio*) larvae exposed to hypoxia.

Tzaneva, V. and Perry, S. F. (2015). Evidence for a role of heme oxygenase-1 in the control of cardiac function in zebrafish (*Danio rerio*) larvae exposed to hypoxia. *J. Exp. Biol.* 219:10. 1563-1571.

ABSTRACT

Carbon monoxide (CO) is a gaseous neurotransmitter produced from the breakdown of heme via heme oxygenase-1 (HO-1; hypoxia inducible isoform) and 2 (HO-2; constitutively expressed isoform). In mammals, CO is involved in modulating cardiac function. The role of the HO-1/CO system in the control of heart function in fish, however, is unknown and investigating its physiological function in lower vertebrates will provide a better understanding of the evolution of this regulatory mechanism. We explored the role of the HO-1/CO system in larval zebrafish (*Danio rerio*) *in vivo* by investigating the impact of translational gene knockdown of HO-1 on cardiac function. Immunohistochemistry revealed the presence of HO-1 in the pacemaker cells of the heart at 4 days post fertilization and thus the potential for CO production at these sites. Sham zebrafish larvae (experiencing normal levels of HO-1) significantly increased heart rate (f_H) when exposed to hypoxia (P_wO_2 = 30 mmHg). Zebrafish larvae lacking HO-1 expression after morpholino knockdown (morphants) exhibited significantly higher f_H under normoxic (but not hypoxic) conditions when compared to shams. The increased f_H in HO-1 morphants was rescued (f_H was restored to control levels) after treatment of larvae with a CO releasing molecule (40 μM CORM). The HO-1 deficient larvae developed significantly larger ventricles and when exposed to hypoxia they displayed higher cardiac outputs (Q) and stroke volumes (SV). These results suggest that under hypoxic conditions, HO-1 regulates Q and SV presumably via the production of CO. Overall, this study provides a better understanding into the role of the HO-1/CO system in controlling heart function in lower vertebrates. We demonstrate for the first time the ability for CO to be produced in presumptive pacemaker cells of the heart where it plays an inhibitory role in setting the resting cardiac frequency.

3.1 INTRODUCTION

Low environmental oxygen (O_2) levels, also known as hypoxia, are common occurrences in a variety of aquatic environments (Diaz and Breitburg, 2009). Adult water-breathing fish exposed to hypoxia exhibit well-defined cardiorespiratory responses consisting of reflex hyperventilation (Dejours, 1973; Randall et al., 1976; Perry and Gilmour, 1996; Vulesevic et al., 2006; Tzaneva et al., 2011) and bradycardia (Randall and Shelton, 1963; Randall and Smith, 1967; reviewed in Farrell, 2007 and Gamperl and Shields, 2014). Hypoxic bradycardia usually is accompanied by an increase in stroke volume (SV) such that cardiac output (Q) is maintained (Holeton and Randall, 1967; Short et al., 1979; Wood and Shelton, 1980; Fritsche and Nilsson, 1989). Although bradycardia is the typical response to hypoxia in adult water-breathing fish, the situation is much less clear in developing larvae. Zebrafish (*Danio rerio*) larvae, in particular, exhibit various cardiac responses depending on the severity of hypoxia and/or the duration of exposure (acute *versus* chronic) (Barrionuevo and Burggren, 1999; Jacob et al., 2002; Yaqoob and Schwerte 2010; Baggato 2005; Steele et al., 2011). Tachycardia may be the initial response to progressive hypoxia but can develop into bradycardia as the severity or duration of the hypoxia is increased. For example, Jacob et al. (2002) reported significant tachycardia in 5 day old zebrafish larvae exposed to moderate levels of hypoxia (~75 mm Hg or roughly 4 mg l^{-1} dissolved O_2). In a similar experiment, however, Baggato (2005) demonstrated that zebrafish larvae developed a pronounced bradycardia in response to severe hypoxia (the reported level of dissolved O_2 was 0.8 mg l^{-1}). Steele and colleagues (2011) also observed a decrease in cardiac frequency (f_H) of zebrafish larvae exposed to severe hypoxia (~20 mm Hg or roughly 1.0 mg l^{-1} dissolved O_2) at 5-7 days post fertilisation (dpf). In one specific instance, hypoxic bradycardia in larval zebrafish (6 dpf) was accompanied by an elevation of Q owing to disproportionally

increased SV (Yaqoob and Schwerte, 2010). Hypoxic tachycardia also is a typical response of larval Arctic char (*Salvelinus alpinus*) (McDonald and McMahon, 1976) and trout (*Oncorhynchus mykiss*) (Holeton, 1971; McDonald and McMahon, 1976).

Previous studies on hypoxic modulation of cardiac function in adult teleost fish have focused on the involvement of the autonomic nervous system (Wood and Shelton, 1980; Burleson and Milsom, 1995; Stecyk and Farrell, 2006). The results of these studies have demonstrated that low O_2 levels result in cholinergically mediated bradycardia (Wood and Shelton, 1980; Burleson and Milsom, 1995; Stecyk and Farrell, 2006). In larval zebrafish, the onset of cardiac control via the parasympathetic and sympathetic nervous systems (or circulating catecholamines) is evident as early as 4 dpf (Steele et al., 2009; Steele et al., 2011). The development of bradycardia in the face of chronic hypoxia in zebrafish larvae is mediated by cardiac muscarinic receptors (Baggato, 2005) of the M_2 sub-type (Steele et al., 2009) while the tachycardia response to acute hypoxia likely is controlled by a release of inhibitory vagal tone or stimulation of adrenergic receptors by elevated circulating catecholamine levels or catecholamines released from sympathetic neurons (Steele et al., 2009; Steele et al., 2011).

Although there is evidence of autonomic nervous system control of heart function in fish, more recent models of cardiac modulation during acute hypoxia incorporate the involvement of gasotransmitters such as carbon monoxide (CO) (Durante et al., 2006). In fish, endogenous CO is involved in controlling several physiological functions including ventilation (Tzaneva and Perry, 2014) and f_H (reviewed in Olson and Donald, 2009; Perry et al., 2016). Endogenous CO is produced from the breakdown of heme into biliverdin, Fe^{2+} and CO by the heme oxygenase (HO) family of enzymes. Biliverdin is further reduced to bilirubin by biliverdin reductase (reviewed in Abraham and Kappas, 2008). Both biliverdin and bilirubin are reducing molecules

and act as scavengers for reactive oxygen species (ROS) thereby reducing oxidant-mediated cell death (Kushida et al., 2002; reviewed in Abraham and Kappas, 2008). Fe^{2+} generated from the HO catalyzed degradation of heme can result in the formation of ROS which can damage various cellular components. Thus, it is sequestered by ferritin induced by Fe^{2+} release and is eventually recycled as a substrate for hemoglobin synthesis (Sassa, 2006). Two isoforms of the HO enzyme have been identified in zebrafish – the hypoxia inducible heme-oxygenase-1 (HO-1) and the constitutively expressed heme oxygenase-2 (HO-2) (Thisse and Thisse, 2004; Voelker et al., 2008). CO cannot be stored in vesicles and thus upon synthesis, it diffuses readily across membranes to evoke appropriate physiological responses in an autocrine/paracrine manner (Levitt and Levitt, 2015).

Relatively few studies have investigated the expression of HO-1 and its activity in teleost species although zebrafish has received the most attention. HO-1 mRNA was detected in the red blood cells of wild type zebrafish larvae as early as 34 hours post fertilization (Craven et al., 2005) and expression in whole larvae was shown to increase with exposure to the toxicant 3, 4-dichloroaniline (Voelker et al., 2008). More recently, a significant increase in HO-1 mRNA was reported in the hearts of adult zebrafish exposed to 9 h of hypoxia (Parente et al., 2013). Although HO-1 is known to increase in zebrafish under toxic (e.g. exposure to 3, 4-dichloroaniline; Voelker et al., 2008) or hypoxic conditions, little is known about its role in regulating cardiac function at such times. In the mammalian heart, the HO-1/CO system maintains ventricular size (volume and muscle mass) and blood pressure during acute hypoxia (Chen et al., 2003; Heartsfield et al., 2004). Furthermore, several lines of evidence suggest that HO-1 exerts cytoprotective and anti-apoptotic effects on mammalian cardiomyocytes via CO (Abraham and Kappas, 2008). In contrast to acute hypoxia where increases in HO-1 activity but

not protein levels are seen, chronic hypoxia causes a progressive increase of HO-1 in both activity and protein levels within the first 14 days of exposure in the hearts of rats (Grilli et al., 2003). Furthermore, the highest activity of the non-inducible HO-2 was detected in the brain. CO produced via HO-2 functions as a signalling molecule in the central nervous system and in the circulatory system (reviewed in Olas, 2014)

The present work focuses on zebrafish, a cyprinid species that has been used widely as a model organism to study cardiac function (reviewed in Bakkers, 2011). Here, we specifically aim to link endogenously produced CO via HO-1 to control of heart function in developing larvae. We hypothesize that the hypoxia inducible HO-1 is found in pacemaker cells of the larval zebrafish heart where it functions to lower heart rate via CO in during acute hypoxia exposure. Measurements of f_H, SV and Q will be complemented with immunohistochemistry and HO-1 enzyme activity measurements to determine the location of HO-1 and the potential for endogenous CO production in the heart of zebrafish larvae.

3.2 MATERIALS AND METHODS

3.2.1 Zebrafish husbandry

Adult zebrafish (*Danio rerio*) were bred and raised at the University of Ottawa aquatic care facility where they were kept in 10 l acrylic tanks in multi-rack flow-through aquatic housing systems (Aquatic Habitats, Apopka, FL, USA). The tanks were supplied with aerated dechloraminated City of Ottawa tap water maintained at 28^{o}C under a 12:12h light:dark cycle. To obtain embryos, one male was paired with two females in a 2 l tank with a separator dividing males and females into separate compartments. After an overnight acclimation period, the water was changed and the separator was lifted allowing spawning to proceed for at least 15 min.

3.2.2 Injection of HO-1 morpholino

The embryos were collected and injected with an antisense morpholino oligonucleotide (5'-AGTCCATCTTTGTGCTGTAGATGTC-3') intended to bind to the translation start site of zebrafish HO-1 (UniProt accession number B0UXS0-1) and prevent translation of the HO-1 protein. Embryos were injected at the one cell developmental stage which lasts 15-30 min post-fertilization (Kimmel et al., 1995). The working stock morpholino was diluted prior to injection in 1X Danieu buffer [58 mmol l^{-1} NaCl, 0.7 mmol l^{-1} KCl, 0.4 mmol l^{-1} $Ca(NO_3)_2$, 5.0 mmol l^{-1} Hepes (pH 7.6)] and 0.05% Phenol Red. The working concentration of the morpholino was 4 ng nl^{-1}. Approximately 1 nl was injected at the border between the yolk sack and the cell of each embryo to facilitate entry of the morpholino into the cell. The morpholino dose was validated by calibrating the microinjection needles used to disperse a constant droplet volume. The volume of the droplet was set to 1 nl by adjusting the microinjector pressure and time settings for each microinjection needle. To control for any effects of injecting nucleotide, matching concentrations

of a control morpholino (5′-CCTCTTACCTCAGTTACAATTTATA-3′; Gene Tools, LLC. Philomath, OR, USA) were also administered to sham embryos; there are no known targets for this morpholino in zebrafish. Injected embryos were transferred to 300 ml Petri dishes containing system water with 0.03% methylene blue and incubated at 28^{o}C for four days at which time experiments were performed.

3.2.3 Heart rate (f_H) and size measurements

Morphant and sham larvae were placed individually for the duration of the experiments in temperature controlled perfusion chambers with maximum volumes of 200 µl. All treatments contained 0.05 mg ml^{-1} Tris-buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma Aldrich Inc.) and all experiments were performed at 28^{o}C. The dose of 0.05 mg ml^{-1} of MS-222 was chosen because it was the lowest dose able to keep the larvae immobilized. Other studies have shown that high doses of MS-222 decrease heart rate in zebrafish larvae, however, the concentration used in this study is lower than that used in other zebrafish studies (Steele et al., 2009; Steele et al., 2011). Furthermore, in our own experiments only a dose of MS-222 above 0.12 mg ml^{-1} caused a decrease in heart rate after 10-15 min exposure of 4 dpf larvae. Therefore, we are confident that the concentration of MS-222 used in the current study did not cause any non-specific effects on heart rate during the experiments. Each larva was exposed to normoxia for 3 min to obtain a baseline f_H after which the water flow was switched for 15 min to normoxic water containing either 40 µM carbon monoxide releasing molecule (CORM; Catalog number: SML0496; Sigma Aldrich, Oakville ON) or 10^{-4} mol l^{-1} adrenaline (general adrenergic receptor agonist; (±)-Epinephrine hydrochloride; Catalog number: E4250; Sigma Aldrich, Oakville ON). A separate group of larvae were exposed to normoxia for 3 min to obtain a baseline f_H after which the water flow was switched to hypoxic (partial pressure of O_2 in the water (P_wO_2) = 30

mmHg) water for 15 min. Hypoxia was achieved by bubbling a gas mixture of 85% N_2 and 15% air continuously into a water reservoir maintained at 28°C to maintain a stable O_2 partial pressure of approximately 30 mmHg. Following the hypoxic treatment, larvae were exposed to hypoxic water containing either 40 µM CORM or 10^{-4} mol l^{-1} adrenaline for an additional 15 min. The adrenaline concentration used was based on a previous study (Steele et al., 2011) and preliminary trials which demonstrated that it was the lowest concentration to reliably produce a positive cardiac chronotropic effect. The CORM concentration was chosen based on dose response curves performed with concentrations used previously in mammals (Clark et al., 2003). One group of control (no injections) larvae was exposed to the same hypoxia treatment to ensure that there was no effect of injection on f_H in sham larvae under normoxic conditions and that they did not exhibit an altered response to hypoxia (Supplementary material Fig. 1).

To measure f_H, SV and Q, each trial (one larva per trial) was recorded continuously using a high speed video camera (3CCD camera, Dage-MTI, USA) mounted on a dissecting microscope (CMZ1500, Nikon, USA) and connected to a personal computer. Videos were captured using CyberLink Power Director Software (Santa Clara, CA, USA) at 720 x 480 pixels and 30 frames s^{-1}. Heart beats were counted for 30 s and heart size was measured by taking a single photo of the heart and using the formula for an ellipsoid to determine heart volume. SV and Q were measured/calculated from the captured images as per Kopp et al. (2007).

3.2.4 HO-1 activity assay

The HO-1 activity assay was performed as described in Chapter 9 of *Current Protocols in Toxicology* (McCoubrey, 1999). Briefly, tissue samples (~30 larvae per sample) underwent 2 freeze-thaw cycles before homogenization in ice-cold homogenization buffer (1M Tris•Cl, 250

mM sucrose). The homogenized tissue was centrifuged at 12 000 x *g* for 30 min at 4^{o}C and the crude supernatant was collected from the lysates. The supernatant was incubated in the dark in a solution containing heme (18 µM), 30 µL of 30 U ml^{-1} biliverdin reductase (BVR; Abcam Inc., Toronto, ON) and NADPH (2.75 mM) for 30 min at 37^{o}C on a shaker. The reaction was stopped on ice and the concentration of bilirubin was calculated using the difference of absorbance between 470 and 530 nm using an extinction coefficient of 40 mM^{-1} cm^{-1}. Heme oxygenase activity was calculated as pmol bilirubin min^{-1} mg protein^{-1}.

3.2.5 Western blots and protein quantification

Frozen tissue samples (~30 larvae per sample) were thawed on ice and homogenized in 200 µL RIPA buffer (150 mM NaCl, 1% Triton-X 100, 0.5 sodium deoxycholate, 0.1% SDS, 50 mM Tris HCl, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride) with a protease inhibitor cocktail tablet (1 tablet per 10 mL RIPA buffer; Roche, USA). A bicinchoninic acid (BCA) assay was used to determine protein content and approximately 50 µg of total protein was used for western blotting. Protein samples were separated using a 12% TGX Stain-Free FastCast Acrylamide gel (Bio-Rad, USA). After separation the gel was activated for 1 min under UV light to visualize total protein before transfer. The separated samples were then transferred to a PVDF membrane (Bio-Rad, USA) and total protein on the membrane was visualized. The membrane was then blocked in 5% milk for 2 h at room temperature after which it was incubated overnight at 4^{o}C with a primary zebrafish anti-HO-1 antibody (immunogen peptide MDSTKSKAAENTGSC) raised in rabbit (GenScript, USA) targeting the N-terminus of the HO-1 protein at 1:4000 dilution. After washing, the membrane was probed with goat anti-rabbit secondary antibody (1:5000; Pierce, USA) for 2 h at room temperature. Protein amount was represented as fold change by measuring the intensity of the detected HO-1 band and total

protein for each sample using ImageLab software (Bio-Rad, USA) and normalizing it against a pooled protein sample.

3.2.6 Immunohistochemistry

Morphant and sham larvae were euthanised using MS-222 and fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature. Larvae were immersed for 24 h in 30% sucrose then embedded in OCT Cryomatrix (Thermo Scientific, USA) after which they were sectioned longitudinally into 50 µm slices using a Leica CM3050 cryostat at -25ºC (Leica Microsystems Inc.). The sections were incubated with 0.8% Triton X-100 in phosphate buffered saline (PBS; pH = 7.4) for 2 h. HO-1 immunoreactive cells were identified with an antibody against HO-1 (described above) and pacemaker cells of the heart were identified using an anti-mouse islet 1 (Isl1) antibody (Developmental Studies Hybridoma Bank, University of Iowa, Iowa; Tessadori et al., 2012). The primary antibodies were added to 1X PBS to yield final dilutions of 1:200 each. The sections were incubated with the primary antibodies overnight at room temperature. Secondary antibodies [Alexa 488 (green) and Alexa 594 (red)] were applied and sections were incubated in the dark for 2 h at room temperature. Sections were imaged using an upright confocal microscope (A1R MP+ Multiphoton Nikon Instruments Inc., USA) equipped with an argon (peak output – 488 nm) and helium-neon (peak output = 543 nm) lasers. Optical slices of 1 µm were taken to produce a three dimensional image using NIS Elements AR Viewer software (Nikon Instruments Inc., USA).

3.2.7 Statistical analyses

Data are represented as means ± SEM. One way and two way repeated analysis of variance (ANOVA) was used to reveal significant differences between means with a Tukey post-hoc test

when significant differences were detected. The limit of significance was 0.05 for all analyses. Sigma Plot v. 12.0 (SPSS Inc.) was used to perform all statistical analyses.

3.3 RESULTS

3.3.1 Heme oxygenase-1 in the heart and pacemaker cells of zebrafish larvae

Three or four sections from each of 5 larvae at 4 dpf were examined to determine the presence of HO-1 in the heart of sham and morphant fish. HO-1 immunoreactive cells were found in the atrium and ventricle of sham fish (Fig. 3.1B) but were apparently absent (unobserved) in HO-1 morphant fish (Fig. 3.2B). Furthermore, HO-1 appeared to be present within the presumptive pacemaker (Isl-positive) cells of the ventricle and atrium of sham, but not morphant fish (Figs. 3.1C, 3.2C). The pacemaker cells are found at the junction of the atrium and sinus venosus (Thessadori et al., 2012; Stoyek et al., 2015). We have described the Isl-1 positive cells only as presumptive pacemaker cells because another antibody such as HCN 4 (labels a voltage gated potassium channel) is needed in combination with Isl-1 to definitively indicate the pacemaker cells. Thus, we report that Isl-1 positive cells as presumptive pacemaker cells but it is also possible that a subpopulation of the Isl-1 positive cells are cardiac progenitors and/or neuronal progenitors of the heart of zebrafish. Two sections of 4-5 sham larvae at 4 dpf were used as negative controls on which the primary HO-1 antibody was omitted; no HO-1 immunoreactivity was detected (data not presented).

3.3.2 Heme oxygenase-1 activity assay

In sham 4 dpf larvae exposed to acute hypoxia (15 min of P_wO_2 = 30 mmHg), heme oxygenase activity increased significantly from 27.3 ± 1.8 to 41.9 ± 5.3 pmol bilirubin min^{-1} mg $protein^{-1}$ (one-way ANOVA; P = 0.006; Fig. 3.3A). There was no significant difference detected in the HO activity between normoxic morphant and sham larvae (one-way ANOVA; P = 0.433; Fig. 3.3A). Zebrafish morphant larvae failed to exhibit a significant increase in HO activity when

exposed to acute hypoxia (one-way ANOVA; P = 0.93; Fig. 3.3B). Any residual HO activity is due to heme breakdown via HO-2 which may be compensating for the lack of HO-1 in morphant larvae and thus the lack of decrease in HO activity in normoxic morphants.

3.3.3 HO-1 knockdown confirmation: Western blots and protein quantification

Western blots using protein extracted from 4 dpf normoxic larvae showed a clear band at ~32 kDa which was absent in normoxic HO-1 morphant larvae (insert in Fig. 3.3B). Hypoxia exposure did not cause a significant increase in HO-1 protein expression in either sham or HO-1 morphant larvae although there was a trend for an increase in HO-1 protein content during hypoxia in sham larvae (one-way ANOVA $P_{O2\ treatment} = 0.07$; Fig. 3B). HO-1 morpholino injections significantly diminished HO-1 protein content by ~22-fold in normoxic morphant larvae when compared to shams and in hypoxic morphant larvae by ~19 fold, thus confirming knockdown of the HO-1 protein by the morpholino injections (Fig. 3.3B).

3.3.4 The effect of HO-1 on f_H, heart size, SV and Q in zebrafish larvae

The f_H of sham fish did not differ significantly from control fish under normoxic conditions and in their response to acute hypoxia (Supplementary material Fig. 3.1). Based on this evidence we are confident that the sham procedure did not affect baseline f_H and the ability of sham fish to respond to hypoxia. Therefore, all subsequent experiments compared results from sham- and morpholino-injected experiments.

Baseline f_H of HO-1 morphant fish was significantly higher than in shams (Fig. 3.4A). Hypoxia significantly increased the f_H of sham fish from 144.1 ± 3.9 beats per minute (BPM) to 155.0 ± 6.0 BPM but was without effect in morphant fish (Fig. 3.4A). Because f_H already was elevated in the normoxic HO-1 morphant fish, it raised the question as to whether HO-1

morphant fish have the capacity to increase their f_H further during hypoxia (i.e. f_H may have reached its maximal level). To test this idea, fish were exposed to the cardiac stimulant adrenaline. Adrenaline exposure caused an increase in f_H in sham (158.7 ± 3.4 to 197.0 ± 4.0 BPM) and morphant fish (180.0 ± 4.1 to 196.0 ± 6.7 BPM) fish exposed to normoxia (Fig. 3.4B). Exogenous carbon monoxide (CO) significantly decreased the f_H in both groups of fish under normoxic or hypoxic conditions (Fig. 3.4C, D).

Ventricular size is another indicator of heart function because it determines, at least in part, the amount of blood that is pumped from the heart during systole. A double blind design was used to measure the ventricular volume of larvae. The measurements of ventricular volume (Fig. 3.5A) were performed on terminally anesthetised (MS-222), fixed 4 dpf zebrafish larvae. MS-222 causes depression of cardiac contractility at higher doses in larval fish and thus the ventricles of these fish were most likely arrested and measured in a diastolic state (Denvir et al., 2008). HO-1 morphant fish exhibited a significantly larger diastolic ventricular volume (1.1 ± 0.23 nl) compared to the shams (0.5 ± 0.06 nl) (Fig. 3.5A). Hypoxia caused a significant decrease in diastolic volume but not systolic volume in sham fish only (Table 3.1). Systolic volume did not change significantly in either sham or morphant fish when exposed to hypoxia (Table 3.1). There was no significant difference in the SV or *Q* between sham and HO-1 morphant fish under normoxic conditions, but when exposed to hypoxia, morphant fish exhibited a significantly higher SV and *Q* (Fig. 3.5B, C).

3.4 FIGURES AND TABLES

Figure 3.1. Micrographs depicting 50 µm cross section of the heart of a 4 day old sham zebrafish larva. The ventricle is indicated with a white outline and cells have been treated with DAPI to stain nuclei (A). The insert in A is a light micrograph of the ventricle structure (outlined with a black dotted line) of a 4 day old sham zebrafish larva. Cells positive for heme oxygenase-1 (HO-1; B) and islet 1 (Isl1; C) are indicated with arrowheads. Arrowheads in D indicate co-localization of HO-1 and Isl1. Insert in D represents a magnified view of HO-1 and Isl1 positive cells. Anterior, a; dorsal, d; posterior, p; ventral, v. Scale bar = 20 µm

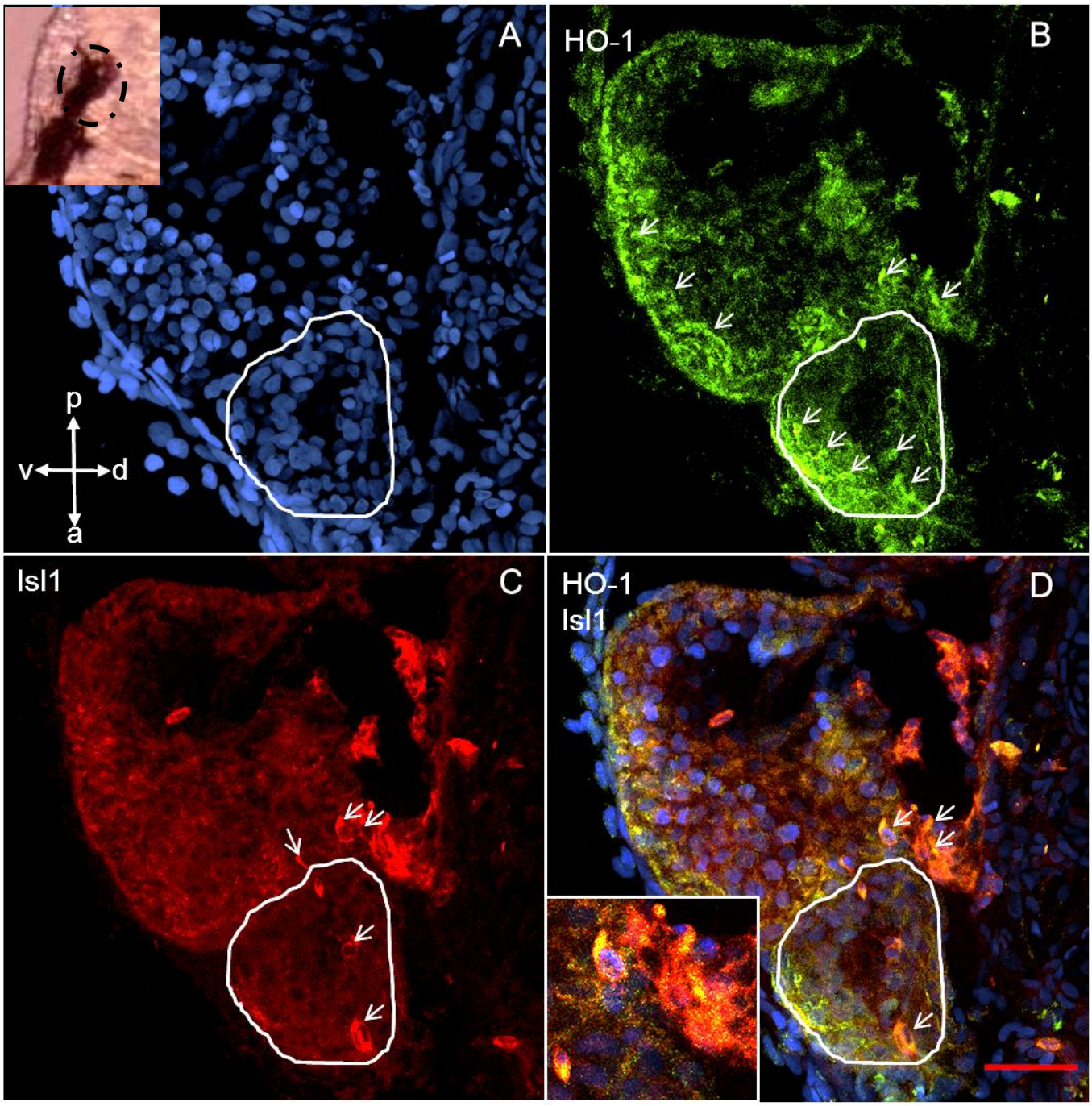

Figure 3.1

Figure 3.2. Micrographs depicting 50 µm cross section of the heart 4 day old heart of heme oxygenase-1 (HO-1) morphant zebrafish. The ventricle is indicated with a white outline and cells have been treated with DAPI to stain nuclei (A). The insert in A is a light micrograph of the ventricle structure (outlined with a black dotted line) of a 4 day old HO-1 morphant zebrafish larva. HO-1 immunoreactivity was not detected in the hearts of morphant zebrafish (B). Cells positive for islet 1 (Isl1; C) are indicated with arrowheads. Arrowheads in panel D indicate the lack of co-localization of HO-1 and Isl1. The fish is in the same orientation as in Figure 1. Scale bar = 20 µm

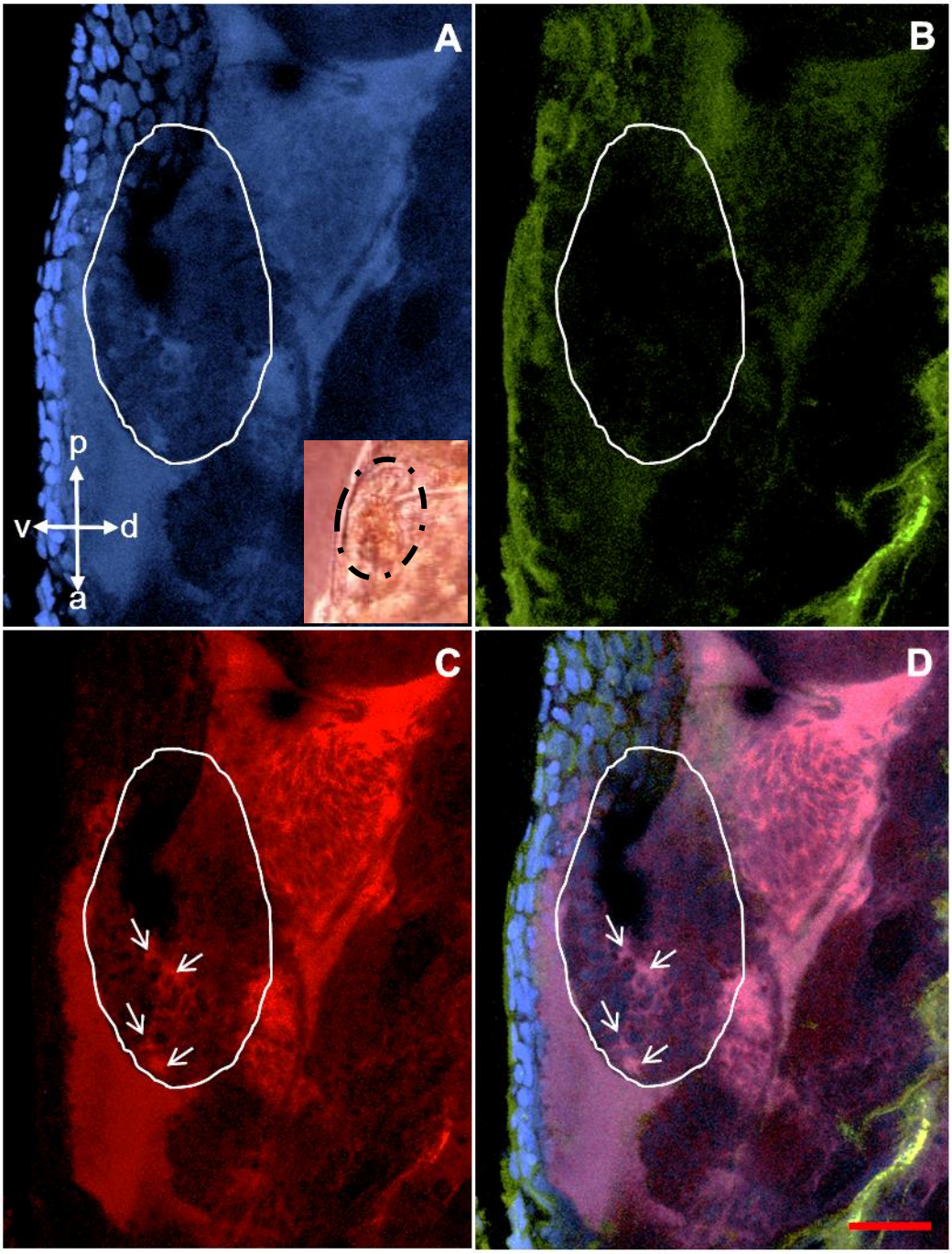

Figure 3.2

Figure 3.3. Heme oxygenase (HO; A) activity and protein content (B) in sham and HO-1 morphant (MO) 4 day old zebrafish larvae. The insert in (B) shows representative western blot bands confirming the knock down of HO-1 protein in the morphant larvae. Different letters indicate significant differences within a group. An asterisk indicates a significant difference between groups within a treatment. Normoxia, N; Hypoxia, H. Two Way ANOVA, $P < 0.05$. N = 6-8.

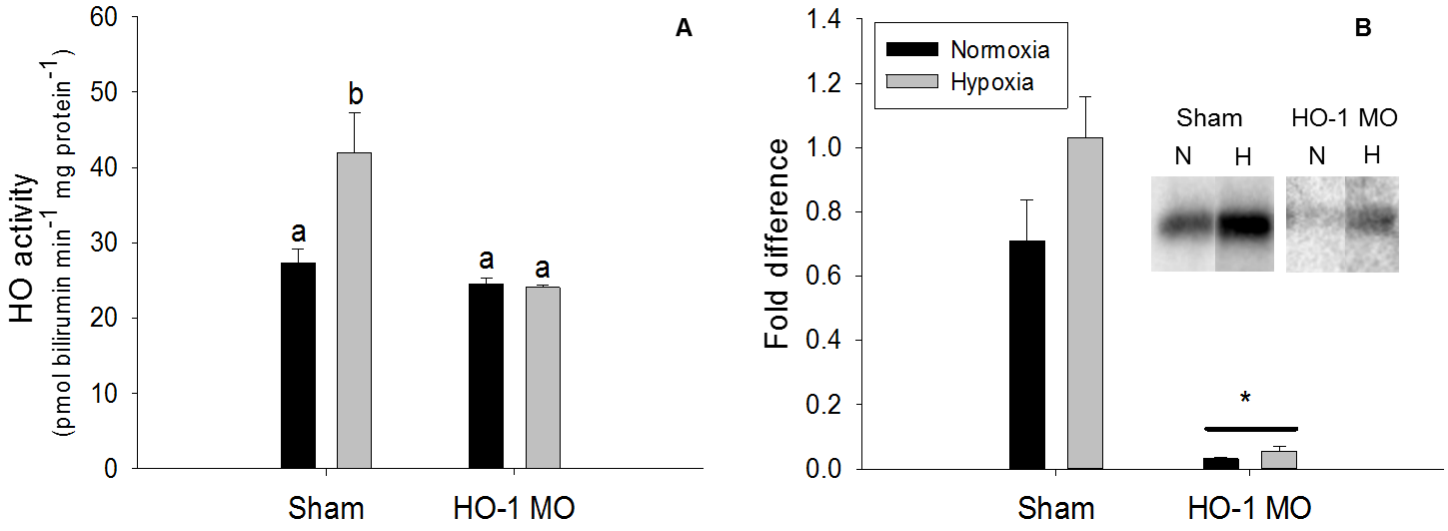
A
HO activity
(pmol bilirumin min-1 mg protein-1)
60
50
40
30
20
10
0
a
b
a
a
Sham
HO-1 MO
B
Fold difference
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Normoxia
Hypoxia
Sham
HO-1 MO
N
H
N
H
*
Sham
HO-1 MO

Figure 3.3

Figure 3.4. Average heart rate (f_H) of 4 dpf sham-treated and HO-1 morphant zebrafish larvae. (A) Normoxia (N) and hypoxia (H). (B) Normoxia and normoxia+adrenaline (A). (C) Normoxia and normoxia+carbon monoxide (CO, 40 μmol l^{-1}, via a CO-releasing molecule). (D) Normoxia, hypoxia and hypoxia+CO (CORM, 40 μmol l^{-1} CO-releasing molecule). Different letters indicate significant differences between normoxia and hypoxia within either sham-treated or HO-1 morphant fish. An asterisk indicates a significant difference between sham treated and HO-1 morphant fish within either normoxia or hypoxia. Two-way repeated measures ANOVA, $P<0.05$, N=8–14.

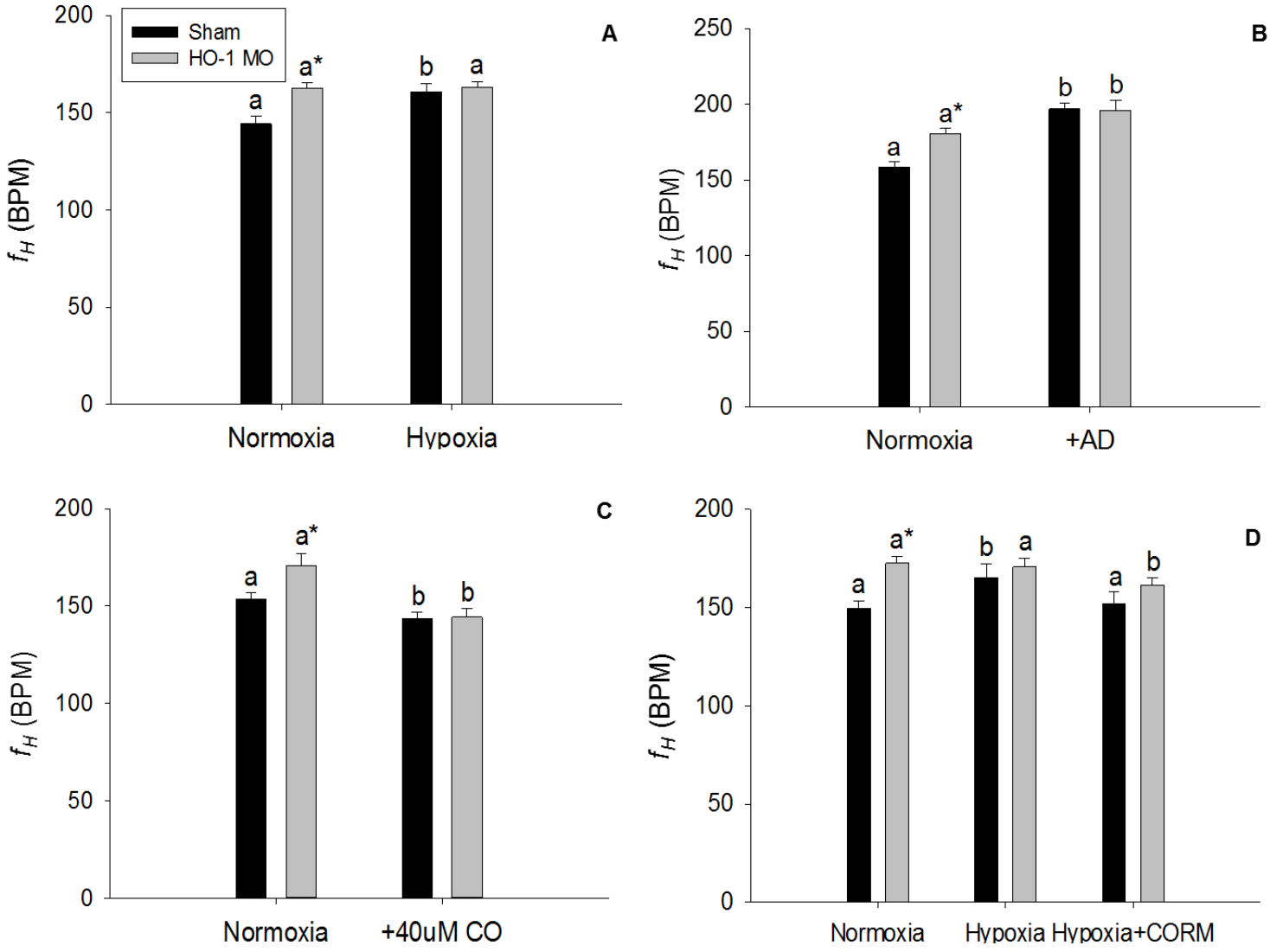
A
Sham
HO-1 MO
f_H (BPM)
200
150
100
50
0
a
a*
b
a
Normoxia
Hypoxia
B
250
200
150
100
50
0
a
a*
b
b
Normoxia
+AD
C
200
150
100
50
0
a
a*
b
b
Normoxia
+40uM CO
D
200
150
100
50
0
a
a*
b
a
a
b
Normoxia
Hypoxia
Hypoxia+CORM

Figure 3.4

Figure 3.5. Ventricle volume (A), stroke volume (SV) (B), and cardiac output (*Q*) (C) of 4 day old sham and heme oxygenase-1 (HO-1) morphant (MO) zebrafish larvae. An asterisk indicates a significant difference between groups within an oxygen treatment. Two way RM ANOVA; $P < 0.05$. N = 5-9.

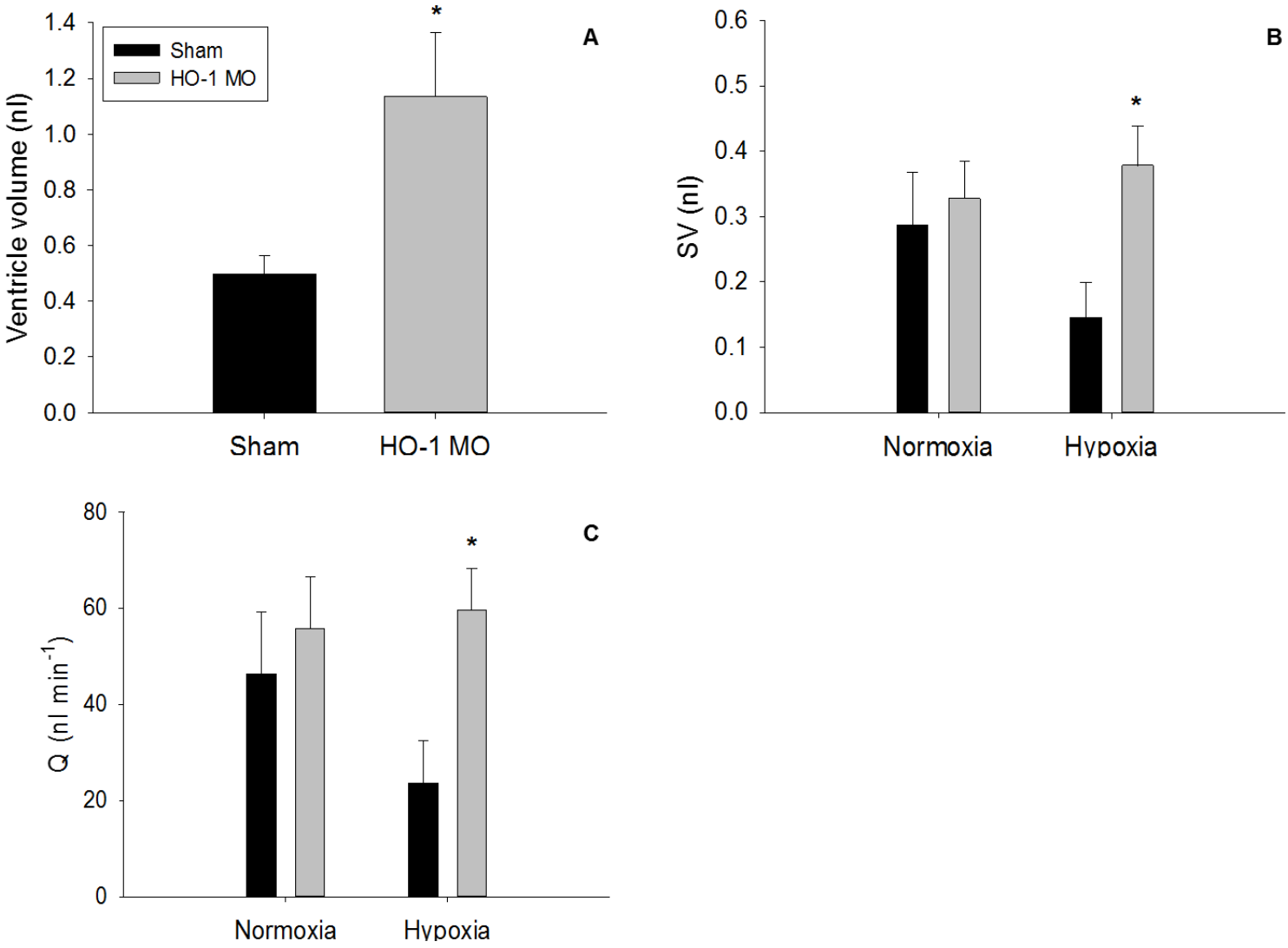
A
Sham
HO-1 MO
Ventricle volume (nl)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
Sham
HO-1 MO
B
SV (nl)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
*
Normoxia
Hypoxia
C
Q (nl min-1)
80
60
40
20
0
*
Normoxia
Hypoxia

Figure 3.5

Table 3.1: Diastolic and systolic ventricle volumes of sham and heme oxygenase-1 (HO-1) morphant (MO) fish exposed to hypoxia (P_wO_2 = 30 mmHg)

Oxygen treatment	Ventricle volume (nl)			
	Sham		HO-1 MO	
	Diastolic	Systolic	Diastolic	Systolic
Normoxia	0.86 ± 0.09	0.57 ± 0.1	1.15 ± 0.198	0.80 ± 0.23*
Hypoxia	0.52 ± 0.1[a]	0.37 ± 0.08	1.09 ± 0.204	0.71 ± 0.15*

An asterisk represents significant differences between diastolic and systolic volume within the same oxygen treatment for either sham or HO-1 morphant (MO) fish.
A letter represents a significant difference from normoxic values within either diastole or systole for either sham or HO-1 morphant (MO) fish.
Two Way ANOVA; $P < 0.05$; N = 5-9.

3.5 DISCUSSION

The overall aim of this study was to investigate the role of the HO-1/CO system on control of heart function in the developing zebrafish larva. Three major findings emerged; (i) HO-1 was found to be present in the pacemaker cells of the heart; (ii) lack of HO-1 resulted in significantly higher f_H under normoxic but not hypoxic conditions; and (iii) HO-1 deficient fish exhibited significantly larger ventricles and when exposed to hypoxia they displayed higher *Q* and SV. Thus, we show the HO-1/CO system had a negative chronotropic effect under normoxic conditions *only*, presumably by acting on the pacemaker cells of the heart, and under hypoxic conditions, it regulates *Q* and SV.

3.5.1 Presence of HO-1 in the hearts of fish

Previous studies on mammals have localised HO-1 in cardiac fibroblasts, myocytes, endothelial cells and vascular smooth muscle (reviewed in Banerjee et al., 2007; Harvey and Rosenthal, 1999). However, there is no direct evidence as yet for the presence of HO-1 in the pacemaker cells of the mammalian heart. In zebrafish, the cardiac pacemaker cells are located at the sinoatrial boundary (Tessadori et al., 2012; Stoyek et al., 2015). In this study, we present evidence that HO-1 is located in the presumptive pacemaker cells of the developing zebrafish heart. This finding suggests indirectly that the HO-1/CO system may be involved in setting cardiac rhythm in fish. Further immunohistochemistry studies using antibodies against other pacemaker proteins such as hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels coupled with Isl1 are needed to definitively state that HO-1 is indeed present in pacemaker cells of the heart. In addition, Isl1 is found in cardiac progenitor cells of mice (Cai et

al., 2003) and thus it may also label myocyte and/or neuronal progenitors in the heart of the developing zebrafish which may explain its distribution pattern.

Previous studies demonstrated that exogenous application of CO to mouse sinoatrial node preparations caused an accelerated sinus rhythm demonstrating a direct effect of CO on f_H (Abramochkin, 2013). A positive chronotropic effect of CO also was observed in the working myocardium of mouse, providing further evidence that CO is involved in controlling cardiac frequency (Abramochkin et al., 2015). The constitutively expressed form of HO, HO-2, also is present in interstitial cells of Cajal (ICC) which act as pacemaker cells in the gastrointestinal tracts of mammals (Farrugia and Szurszewski, 1999). CO synthesized from ICCs targets nearby gastrointestinal smooth muscle cells causing the activation of a potassium current and hyperpolarization of the cell plasma membrane (Farrugia et al., 1993; Farrugia et al., 1998). However, there are no studies reporting on the presence of either isoform in the pacemaker cells of the heart.

3.5.2 *HO-1/CO system in control of heart function of the zebrafish larva*

The cardiotropic effects of the HO-1/CO system deserve significant attention owing to the known potency of CO as a gaseous signalling molecule or gasotransmitter. Control of f_H is particularly important under stressful conditions, such as hypoxia, when regulating cardiac rhythm is crucial for the delivery of O_2 to vital organs such as the brain. Under normoxic conditions, the developing zebrafish larva relies on diffusive cutaneous O_2 uptake and delivery of O_2 to tissues is accomplished without the requirement of hemoglobin-mediated O_2 transport (Pelster and Burggren, 1996). Indeed, because zebrafish without a beating heart develop more-or-less normally for the first few days of development, it is thought that internal convection

(circulation) may not be required to sustain normal rates of metabolism in early life stages of zebrafish (Pelster and Burggren, 1996; Burggren, 2004; Kopp et al., 2010). However, under hypoxic conditions, the carriage of O_2 by hemoglobin is critical to sustain normal rates of O_2 uptake in zebrafish larvae (Rombough and Drader, 2009). Similarly, convective O_2 delivery to larval tissues may become more important during acute hypoxia. At such times, an ability to regulate *Q* via control of heart frequency would be highly beneficial. Our results suggest that the HO-1-CO system may be an important determinant of cardiac frequency in larvae.

The knockdown of HO-1 caused an increase in resting f_H in normoxic larvae that was rescued by the addition of exogenous CO. These results clearly indicate a tonic inhibitory role of the HO-1/CO system on cardiac frequency in developing zebrafish. These findings are in contrast to the positive chronotropic effects reported in mammals (Abramochkin, 2013, Abramochkin et al., 2015). Based on the present study, the inhibitory effects of CO do not appear to persist under hypoxic conditions because there was no further increase in f_H when HO-1 deficient larvae were exposed to hypoxia. Thus, it is possible that the prevention of CO production results in a maximal f_H under normoxic conditions and therefore leaves no scope for further increases when fish are challenged subsequently with hypoxia. Exposure to adrenaline under normoxic conditions, however, caused a significant increase of f_H in both sham and HO-1 deficient larvae indicating that larvae retain the capacity to increase f_H even when endogenous CO production is low. Similarly, inhibition of the HO/CO system in trout using the enzyme inhibitor zinc protoporphyrin-IX resulted in a significant increase in smooth muscle tension of isolated arteries but did not affect the magnitude of subsequent noradrenaline induced contractions (Dombkowski et al., 2009). These results suggest that under normoxic conditions, at least, the effects of endogenously produced CO on regulation of cardiovascular function are

acting separately from that of catecholamines. Under hypoxic conditions, however, the HO/CO system has been shown to regulate cardiovascular function by modulating sympathetic tone in mammals (Hirakawa and Hayashida, 2006; Ding et al., 2007). In addition, it has been reported that the sympathetic nervous system is responsible for evoking the tachycardic response observed in larval zebrafish exposed to high environmental CO_2 (Miller et al., 2014) and therefore this may also be the mechanism responsible for the hypoxic tachycardia reported in this study. This notion was supported by the further increase of f_H observed in hypoxic sham larvae in the presence of adrenaline (from 155.0 ± 5.7 to 187.0 ± 5.7 BPM; data not shown). Furthermore, the origins of hypoxic tachycardia in zebrafish larvae, may reflect an active sympathetic nervous system regulated by the HO-1/CO system under hypoxic conditions because hypoxic HO-1 deficient larval hearts were insensitive to adrenaline (from 161.0 ± 3.4 to 181.0 ± 4.0 BPM; Two way ANOVA P = 0.091; data not shown). Thus, the inhibitory effects of CO observed under normoxia implicates an active HO-1/CO pathway on resting f_H in the developing zebrafish. This tonic inhibition of cardiac activity via the HO-1/CO system may allow fish a greater capacity to increase f_H when experiencing hypoxic conditions. Moreover, the HO-1/CO system seems to be a necessary component, in coordination with the sympathetic nervous system, in regulating f_H under hypoxia because lack of CO prevented adrenaline from exerting its effects on f_H under hypoxic conditions.

Ventricle size is another indicator of heart function because it sets, in part, the volume of blood that can be pumped per heart beat (SV). In this study, we observed significantly larger ventricular volumes in HO-1 deficient larvae. Consequently, the increase in ventricular volume in HO-1 deficient larvae resulted in higher SV and *Q* in these fish when exposed to hypoxia. The tendency in sham larvae during acute hypoxia was to increase f_H while maintaining SV and *Q*. In

sham zebrafish larvae exposed to hypoxia, diastolic volume was decreased without a change in systolic volume which, when coupled with an increase in f_H, resulted in sustained cardiac performance. Sustained cardiac performance during hypoxia also was reported in tilapia (*Oreochromis* hybrid) which compensated for a decrease in f_H induced by severe hypoxia by elevating SV and thus maintained *Q* (Lague et al., 2012). Based on our results, HO-1 deficient larvae also were able to maintain normoxic SV and *Q* under hypoxic conditions. A high f_H coupled with a larger ventricle volume may have contributed to the routine normoxic levels of SV and *Q* observed in HO-1 deficient larvae under hypoxic conditions. The increased ventricle size observed in HO-1-deficient larvae is in contrast to a previous report on mouse heart. Yet et al. (1999) reported that the myocardium of HO-$1^{-/-}$ mice appeared normal under normoxic conditions but under hypoxic conditions the mice developed severe right ventricular dilatation which was not observed in wild type mice.

3.5.3 Possible mechanisms of HO-1/CO system in control of heart function in fish

The molecular mechanisms through which the HO/CO system exerts its effects on cardiovascular function have been well described in the mammalian literature with only a single previous study conducted on fish. Because it is so readily diffusible across membranes, CO may act quickly in either an autocrine or paracrine manner to affect cells within the vicinity of the cell from which it was synthesized. CO readily binds to heme-containing proteins and among them, soluble guanylyl cyclase is one of the most well studied and is essential for the vasodilatory effects of CO (Wilkinson and Kemp, 2011; Abramochkin et al., 2015). In fish, the HO-1/CO pathway plays a vasodilatory role in blood vessels via a CO-mediated increase in cGMP (Dombkowski et al., 2009). Other CO targets may include ion channels such as voltage-gated Ca^{2+} (T-type Ca^{2+} channels; reviewed in Capel and Terrar, 2015) and K^+ channels (HCN and

ERG K^+ channels) important for establishing the robust T-type Ca^{2+} current coupled with a "funny" current (I_f) and a rapid delayed rectifier K^+ current (I_{Kr}) that are important for the diastolic depolarization rate of the developing zebrafish heart (Nemtsas et al., 2010; Alday et al., 2014). Studies from mammals have shown that endogenous CO inhibits all isoforms of T-type Ca^{2+} channels as well as ERG K^+ channels (responsible for I_{Kr}) under normoxic conditions (reviewed in Peers et al., 2015) but there is no information on the effect of endogenous CO on HCN channels (responsible for I_f). The effects of endogenous CO on the cardiac pacemaker currents have not been studied in teleost fish thus far, therefore the following interpretation of the mechanisms of action is an extrapolation from studies on mammals. In the present study, it is possible that the HO-1/CO system acted to control resting f_H by inhibiting Ca^{2+} current via T-type Ca^{2+} channels subsequently reducing I_f in the pacemaker cells resulting in a decreased f_H which could explain the increased baseline f_H in HO-1 morphant larvae. The lack of a further increase in f_H in hypoxic HO-1 morphants may be related to the possibility that in zebrafish larvae cardiac T-type Ca^{2+} channels are activated by acute hypoxia exposure in a CO-dependent manner in a similar fashion as L-type Ca^{2+} channels (Movafagh and Morad, 2010) and if already in their active state under normoxia because of low CO production, f_H, SV and Q remained unchanged in hypoxic HO-1 morphant larvae.

3.5.4 HO-1/CO system in other vertebrates

The above discussion uses the mammalian HO-1/CO system as a point of comparison to the one found in zebrafish. The HO-1/CO system, however, has been well conserved throughout all vertebrates. The amino acid protein sequence of zebrafish (*Danio rerio*) HO-1 bears an 86% identity with goldfish (*Carassius auratus*; Wang et al., 2008), 60% with *Xenopus* (Shi et al., 2008) and approximately 50% when compared to mammalian and avian protein sequences

(reviewed in Olson et al., 2012). Furthermore, the amino acid sequence of the "HO signature motif", believed to be important for heme binding and catabolism, shares 92 - 96% similarity between all vertebrate species investigated to date (Want et al., 2008). Physiological control by endogenously produced CO is not restricted to mammalian vertebrates but is also involved in control of vascular tone and breathing in fish (Dombkowski et al., 2009; Tzaneva and Perry, 2014), retinal pathways and neuromuscular transmission in amphibians (Pong and Eldred, 2009; Sidtikova et al., 2007; reviewed in Sidtikova et al., 2012), and learning and vascular control in birds (Cutjar et al., 2005; Van der Sterren et al., 2011). Therefore, the HO-1/CO system appears to be a well conserved signalling pathway throughout all extant vertebrates. The reader is referred to a comprehensive review of the HO/CO system by Olson et al. (2012) for an in depth comparison of the function of the HO/CO system in vertebrates.

3.5.5.Conclusion

This study implicates the HO-1/CO system in control of cardiac function in the developing zebrafish larva. We demonstrate for the first time the presence of HO-1, and presumably the endogenous production of CO, in the pacemaker cells of the developing heart. We also show that HO-1 plays an inhibitory role in regulating the resting f_H.

3.6 ACKNOWLEDGEMENTS

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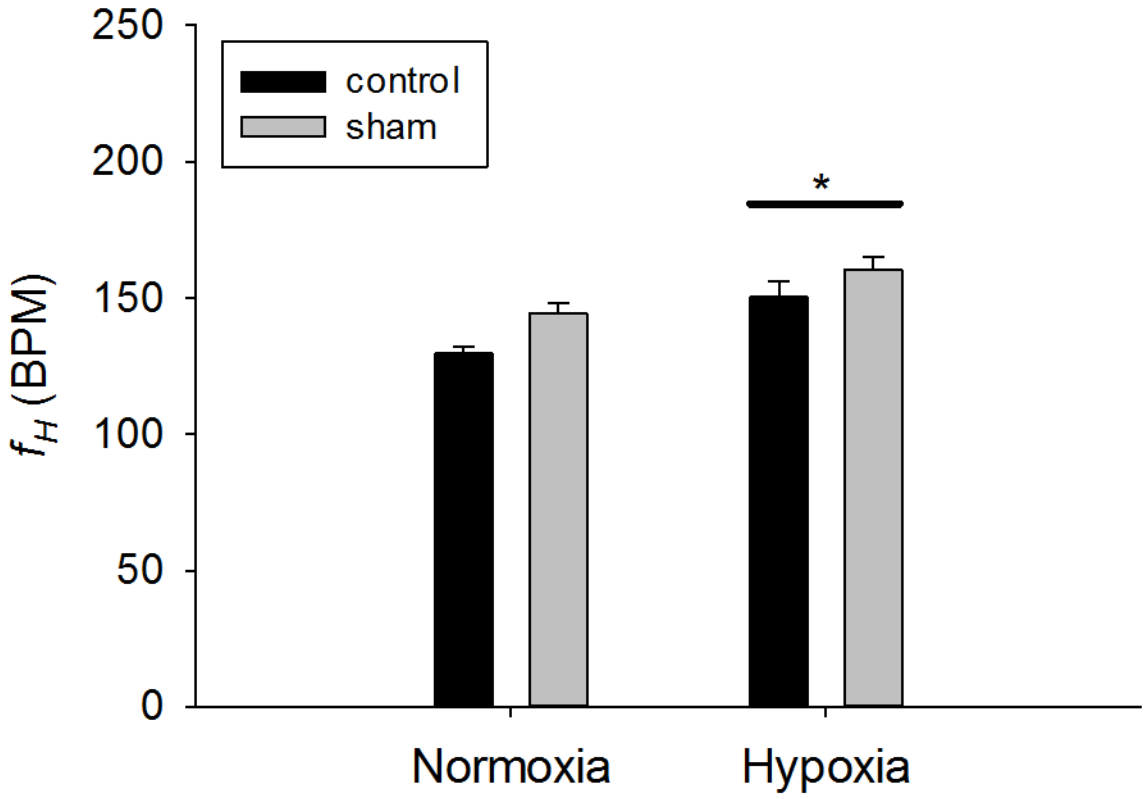
3.7 SUPPLEMENTARY MATERIAL

Supplementary material Figure 3.1. Average heart rate (f_H) of control and sham 4 day old zebrafish larvae exposed to normoxia and hypoxia. The line with an asterisk above represents a statistical difference from normoxic values. Beats per minute, BPM. Two way RM ANOVA; $P_{O2} < 0.05$. N = 10 - 14.



Sup. mat. Figure 3.1

CHAPTER 4.

The role of endogenous carbon monoxide (CO) in the control of breathing in zebrafish (*Danio rerio*)

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ABSTRACT

Carbon monoxide (CO) is a gaseous signalling molecule that is produced *in vivo* from the intracellular breakdown of heme via the heme oxygenase (HO) family of enzymes. In this study we investigated the role of the HO-1/CO system in the control of ventilation in zebrafish, *Danio rerio.* Immunohistochemistry revealed the presence of HO-1 in the chemoreceptive neuroepithelial cells (NECs) of larvae (4 days post fertilization) and adults indicating the potential for endogenous CO production in the NECs. Hypoxia (20 min, water PO_2 of 30 mm Hg) caused a significant increase in HO-1 activity in whole larvae and in the gills of adult fish. Zebrafish with reduced HO-1 activity (via HO-1 knockdown in larvae or zinc protoporphyrin IX (ZnPPIX) treatment in adults) exhibited increased ventilation frequency (f_{vent}) under normoxic but not hypoxic conditions. The addition of exogenous CO restored resting f_{vent} in fish with diminished CO production and in some cases (e.g. hypoxic sham larvae) CO modestly reduced f_{vent} below resting levels. Larval fish were treated with phenylhydrazine (PHZ) to eliminate the potential confounding effects of CO-haemoglobin interactions which might influence ventilation. PHZ treatment did not cause changes in f_{vent} of normoxic larvae and the addition of CO to PHZ-exposed larvae resulted in a significant decrease in sham and HO-1 deficient fish under normoxic conditions. This study, demonstrates for the first time that CO plays an inhibitory role in control of breathing in larva and adult zebrafish.

4.1 INTRODUCTION

Teleost fish respond to low ambient oxygen (O_2) levels (hypoxia) with reflex hyperventilation. Depending on the species, the hyperventilatory response can arise from increases in breathing frequency (f_{vent}) and/or ventilation amplitude (V_{amp}) (Randall and Shelton, 1963; Holeton, 1971; McDonald and McMahon, 1977; Randall, 1982; Smith and Jones, 1982; Boutilier et al., 1988; Sundin et al., 2000; Vulesevic et al., 2006; Tzaneva and Perry, 2010). Hyperventilation serves to increase water flow over the gills and to maximise O_2 uptake from the water to sustain arterial PO_2 which may delay the transition from aerobic to anaerobic metabolism in the face of hypoxia (Holeton and Randall, 1967; Burggren and Cameron, 1980; Glass et al., 1990; Soncini and Glass, 2000; Tzaneva et al., 2011).

The ventilatory responses to hypoxia in adult teleost fish are initiated by branchial neuroepithelial cells (NECs; Dunel-Erb et al., 1982) which are chemoreceptors enriched with the neurotransmitter serotonin (5-HT). The NECs sense changes in O_2 and CO_2 levels of the water and/or blood and thus are functionally analogous to type I cells of the mammalian carotid body (Zaccone et al., 1989; González et al., 1994; Cutz and Jackson, 1999). Although NECs have been identified in several species (Bailly et al., 1992; Jonz and Nurse, 2003; Saltys et al., 2006; Coolidge et al., 2008; Tzaneva and Perry, 2010; Regan et al., 2011), only the NECs of channel catfish (*Ictalurus punctatus*; Burleson et al., 2006) and zebrafish (*Danio rerio*; Jonz and Nurse, 2003; Abdallah et al., 2012; Qin et al., 2010) have been shown to be responsive to changes in O_2 or CO_2. In adult zebrafish, the NECs are distributed along the leading edge of the gill filament and the lamellar epithelium (Jonz and Nurse, 2003; Vulesevic et al., 2006). In larval zebrafish, however, the NECs are located exclusively on the skin prior to gill development (Coccimiglio

and Jonz, 2012). Therefore, the NECs are ideally located to sense changes in O_2 or CO_2 of inspired water in adult zebrafish and water flow over the skin in larvae.

In the few fish species (zebrafish, *Danio rerio*; goldfish, *Carassius auratus*; rainbow trout, *Onchorhynchus mykiss*) that have been examined, the respiratory responses to hypoxia are modulated by a suite of gaseous neurotransmitters that include hydrogen sulphide (H_2S; Porteus et al., 2014), nitric oxide (NO; Porteus et al., 2015) and carbon monoxide (CO; Tzaneva and Perry, 2014), acting on the NECs and associated innervation (see Perry et al., 2016 for review). CO is a signalling molecule that is produced *in vivo* in vertebrates (reviewed in Olson and Donald, 2009; Prabhakar and Semenza, 2012) by the heme oxygenase (HO) family of enzymes via the breakdown of heme into equimolar amounts of CO, Fe^{2+} and biliverdin (Tenhunen et al., 1969). Two HO isoforms have been identified in zebrafish; the hypoxia inducible HO-1 and the constitutively expressed HO-2. In mammals, endogenously produced CO inhibits carotid body sensory activity (Prabhakar et al., 1995) whereas blocking endogenous CO production leads to augmented hypoxic ventilatory responses (Yang et al., 1998; reviewed in Prabhakar, 1999). There is emerging evidence that CO also reduces ventilation in fish because inhibition of HO-1 activity and thus endogenous CO production in goldfish, resulted in an increased response to hypoxia in animals acclimated to 7 but not 25^{o}C (Tzaneva and Perry, 2014). The authors attributed the temperature dependency of CO in the hypoxic ventilatory response to the presence of HO-1 in the NECs of goldfish acclimated to 7^{o}C but its absence in fish acclimated to 25^{o}C (Tzaneva and Perry, 2014).

This study aims to investigate the function of the HO-1/CO system in control of ventilation in zebrafish - a well-established animal model for studying control of breathing in fish (Jonz and Nurse, 2006; Vulesevic et al., 2006). First, changes in ventilation are readily

observed and recorded in larvae and adults. Second, a comprehensive collection of reverse genetics tools has been developed to study gene function in this species (reviewed in Huang et al., 2012). Third, commercially available pharmacological agents such as zinc protoporphyrin IX (ZnPPIX; an inhibitor of HO-1 activity) are readily absorbed from the water in adult zebrafish. Therefore, using the reverse genetic technique of morpholino knockdown (Nasevicius and Ekker, 2000) to specifically reduce HO-1 activity in larvae and comparing these effects with pharmacological inhibition of HO-1 activity in adults allowed us to functionally evaluate the effects of CO in the control of breathing in zebrafish at two different developmental stages; larvae and adults. Based on the previous literature (see above), we hypothesize that the HO-1/CO system plays an inhibitory role in the control of ventilation in larvae and that this role is maintained through development into adults. To test this hypothesis, f_{vent} was monitored in normoxic or hypoxic fish exhibiting normal or reduced HO-1/HO activity and thus normal or reduced rates of endogenous CO production. A morpholino gene knockdown technique was used to lower HO-1 activity in larvae and a pharmacological technique (ZnPPIX administration) was used to inhibit total HO activity in adults. In addition, immunohistochemistry was used to localize HO-1 in the NECs of larvae and adults.

4.2 MATERIALS AND METHODS

4.2.1 Series I: Larval zebrafish

Animal husbandry

Adult zebrafish (*Danio rerio*) were bred and raised at the University of Ottawa aquatic care facility where they were kept in 10 l acrylic tanks in multi-rack flow through aquatic housing systems (Aquatic Habitats, Apopka, FL, USA). The tanks were supplied with aerated dechloraminated City of Ottawa tap water maintained at 28ºC under a 12:12h light:dark cycle. To obtain embryos, one male was paired with two females in a 2 l tank with a separator dividing males and females into separate compartments. After an overnight acclimation period, the water was changed and the separator was lifted and spawning was allowed to proceed for at least 15 min.

Injection of HO-1 morpholino

Embryos were collected and injected with an antisense morpholino oligonucleotide as described in Chapter 3.

Experimental design

Individual morphant and sham larvae were placed into temperature controlled (28ºC) perfusion chambers (maximum volumes of 200 µl) for the duration of the experiments. All water provided to the perfusion chamber contained 0.05 mg ml^{-1} Tris-buffered MS-222 (ethyl-3-aminobenzoate methanesulfonate salt, Sigma Aldrich Inc.). Each larva was exposed to normoxia for 3 min to obtain a baseline f_{vent} after which the water flow was switched for 20 min to water containing 40 µM carbon monoxide releasing molecule (CORM; Catalog number SML0496; Sigma Aldrich, Oakville ON). A separate group of larvae were exposed to normoxia for 3 min to obtain a baseline measurement of f_{vent} after which the water flow was switched to hypoxic

(partial pressure of O_2 in the water (P_wO_2) = 30 mmHg) water for 20 min. Following the hypoxic treatment, larvae were exposed to hypoxic water containing 40 µM CORM for an additional 20 min. The CORM concentration was chosen based on dose response curves performed with concentrations used previously in mammals (Clark et al., 2003).

A separate group of sham and morphant larvae at 1 dpf were exposed to a bath of 10 mg ml^{-1} phenylhydrazine (PHZ; Catalog number P26252, Sigma Aldrich, Oakville, ON) until they reached at 4 dpf. The larvae were kept in the dark and the water bath was changed daily before experiments commenced.

To measure f_{vent}, each trial (one larva per trial) was recorded continuously using a high speed video camera (3CCD camera, Dage-MTI, USA) mounted on a dissecting microscope (CMZ1500, Nikon, USA) and connected to a personal computer. Videos were captured using CyberLink Power Director Software (Santa Clara, CA, USA) at 720 x 480 pixels and 30 frames s^{-1}. f_{vent} was counted for 1 min every 5 min. The maximum response from baseline was reported for each treatment.

Larvae were killed by overdose of MS-222 and were either fixed in 4% paraformaldehyde (PFA) for immunohistochemistry or flash frozen for later analysis of HO-1 activity or protein content.

4.2.2 Series II: Adult zebrafish

Animal husbandry

Adult zebrafish were maintained as described above.

Experimental design

Adult zebrafish (average weight = 0.77 ± 0.11 g; N = 12) were held either in normoxic water or bathed in normoxic water containing 0.25 mg ml^{-1} of the HO-1 activity inhibitor zinc

protoporphyrin IX (ZnPPIX) for 1 h before experiments commenced. Fish were placed in ~5 ml flow through chambers supplied with gravity fed water at a flow of ~1 ml min^{-1}. The chambers (see Vulesevic et al., 2006) were equipped inside with two electrodes submerged in the water and a mesh was used to prevent the fish from making contact with the electrodes. Opercular displacement (an index of V_{amp}) was measured in real time by recording the analog signals from the electrodes which were amplified (amplifier was built at the Electronic Shop at University of Ottawa) and recorded on a computer as digital data using an A/D interfacing system and data acquisition software (AcqKnowledge, BioPac Systems Inc., Galeta, Ca, USA) at a sampling rate of 500 Hz. In this study we focused on f_{vent} because hypoxia does not affect V_{amp} in adult zebrafish (Vulesevic and Perry, 2006). The fish were allowed to adjust to the chambers for 30 – 60 min prior to the start of experiments. Baseline f_{vent} of both groups of fish (control and ZnPPIX-treated) was recorded under normoxic conditions for 15 min after which fish were exposed to normoxic water containing 40 µM of the CO releasing donor CORM for 20 min. In a separate experiment, fish were exposed to 20 min of hypoxia (P_wO_2 = 30 mmHg) followed for an additional 20 min by hypoxic water containing 40 µM CORM.

After experiments were completed, the fish were killed with an overdose of anaesthetic (MS-222). The gill baskets were removed and fixed in 4% paraformaldehyde (PFA) for immunohistochemistry (right gill basket) or flash frozen for later determination of HO-1 activity and protein levels (left gill basket).

4.2.3 HO-1 activity assay

The HO-1 activity assay was performed as described in Chapter 9 of Current Protocols in Toxicology (McCoubrey, 1999). Briefly, crude extract was obtained from thawed frozen gill

tissue or larva homogenized in ice cold homogenization buffer (1M Tris•Cl, 250 mM sucrose) and the HO activity assay was performed as described in Chapter 3.

4.2.4 Western blots and protein quantification

Frozen gill tissue and larvae were thawed on ice and protein extraction was performed as described in Chapters 2 and 3, respectively. The protein samples were separated using a 12% TGX Stain-Free FastCast Acrylamide gel (Bio-Rad, USA). Protein transfer and quantification were performed as described in Chapter 3.

4.2.4 Immunohistochemistry

Larvae

A sequential staining procedure was used for antibodies raised in the same host (rabbit). We used a sequential staining procedure for larvae because we did not have a working anti-5-HT primary antibody raised in a different host species at the time the immunohistochemistry was performed. This technique has been shown to successfully label cell types in previous studies performed on zebrafish larvae (Jonz and Nurse, 2006; Porteus et al., 2015). Zebrafish larvae were killed by an overdose of MS-222 and whole larvae were placed in 4% PFA prepared in phosphate buffered saline (PBS) containing (in mmol l^{-1}): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; buffered to pH 7.8 with 1 mol l^{-1} NaOH overnight at 4°C (Jonz and Nurse, 2003). The larvae were then rinsed with PBST (0.1% Tween) and gradually dehydrated to 100% MeOH and incubated at -20ºC for at least 1 h. After incubation, the larvae were rehydrated gradually by first replacing the 100% MeOH with 50% MeOH and incubating the larvae for 5 min followed by another 5 min incubation in100% PBST. The PBST was replaced with 1 ml Tris HCl (150 mM, pH 9) and left to incubate at room temperature for 10 min and then at 65ºC for 15 min. The larvae were washed 5x with PBST and placed in blocking solution (2% BSA, 2% goat serum, in

PBS with 0.8% Triton-X) for 1 h at room temperature. The blocking solution was removed and the larvae were incubated in an anti-5-HT primary antibody raised in rabbit to label the NECs (1:250; Catalog number S5545; Sigma Aldrich) on a shaker overnight at 4^{o}C. The anti-5-HT antibody was washed 3x with PBST (0.1% Tween) and the larvae were then incubated in the secondary antibody (anti rabbit Alexa 488, 1:500, fluoresces green, Sigma Aldrich) for 1 h. Five washes with PBST were performed to remove remaining antibodies. The larvae were then incubated with a rabbit anti-HO-1 primary antibody (see above; 1:500) diluted in PBS (0.8% Triton-X) overnight at 4^{o}C. The larvae were washed 3x with PBST and a secondary antibody fluorescing red (anti-rabbit Alexa 564, 1:500, Sigma Aldrich) was applied for 1 h. All antibodies were diluted in PBST. Final washes (5x with PBST) were performed before mounting the larvae in PBST on concave slides for imaging. The larvae were observed and images were captured using a confocal microscope (A1R MP Nikon, NY, USA) equipped with solid state lasers emitting at 405 nm, 473 nm and 559 nm. Z-stacks of 20-50 optical sections taken 1.5 µm apart were captured using the 25x objective of this microscope.

Gills

Control adult zebrafish were killed by an overdose of MS-222 and the right gill basket was removed and fixed in 4% PFA overnight at 4^{o}C. The PFA was removed and the gill basket was washed with 1x PBS 3x for 3 min each. The final wash was replaced with 1 ml of blocking solution (1% fetal calf serum, 5% Triton-X 100) and the tissue permeabilized for 48 h on a shaker at 4^{o}C. The gill basket was incubated with mouse anti-5-HT (1:100; Catalog number: ab16007, Abcam, Cambridge, MA), rabbit anti-HO-1 (1:500) and mouse anti-zn12 (1:200; Catalog number zn-12; Developmental Studies Hybridoma Bank, University of Iowa, Iowa, USA) primary antibodies diluted in fresh blocking solution for 48 h at 4^{o}C. The zn-12 antibody

was used to label and visualise any innervation associated with the gill. The primary antibody solution was removed and the gill basket was washed 3x for 5 min each with 1x PBS. Secondary antibodies fluorescing red (anti-rabbit Alexa 594; 1:500) or green (anti-mouse Alexa 488; 1:500) were applied for 1 h at room temperature. The secondary antibody solution was removed and the gill basket was washed 4x for 5 min each in 1x PBS. The gills were separated and mounted on a concave slide. Only the first gill arch was imaged using the protocol described above.

4.2.5 Statistical analysis

All statistical analyses were performed using SigmaPlot software (v 12.0 Systat Software). Data are presented as means ± standard error of the mean (SEM). One way and two way repeated measures analysis of variance (ANOVA) were used to reveal significant differences between means and followed by a Tukey post-hoc test when significant differences were detected. The limit of significance was 0.05 for all analyses.

4.3 RESULTS

4.3.1 Heme oxygenase-1 in the NECs of larval and adult zebrafish

Sham and HO-1 deficient larvae (4 dpf) and the gills of adults were examined to determine the presence of HO-1 in the NECs. Two populations of NECs were detected in larvae (Fig. 4.1 A-C) and adults (Fig. 4.2); NECs that were immunoreactive for 5-HT (5-HT positive; 5-HT^{+}) but not HO-1 (HO-1 negative; HO-1^{-}) and NECs that were immunoreactive for both 5-HT and HO-1 (HO-1^{+}). Larvae from HO-1 knockdown experiments qualitatively showed diminished overall HO-1 immunoreactivity (Fig. 4.1 D-F). The pattern of HO-1 immunoreactivity in the gills of ZnPPIX treated adult fish did not change (data not shown). The insert in Fig. 4.1C illustrates putative serotonergic innervation of NECs which also appears to be immunoreactive for HO-1 in larval zebrafish. Innervation of 5-HT^{+}/HO-1^{+} and 5-HT^{+}/HO-1^{-} NECs also was observed in the gills of adult fish (Fig. 4.2C insert). In addition, the gills of adult zebrafish contained innervated 5-HT^{-}/HO-1^{+} cells (Fig. 4.2C insert). We were limited to co-labelling the nerves and NECs with the same secondary antibody and therefore were not able to determine if any of the nerves in adult zebrafish gills were serotonergic. Furthermore, we did not observe any zn12 positive nerves that were immunoreactive for HO-1 in adult zebrafish gills. We did however notice a “beads on a string” HO-1 staining pattern of zn12 positive innervation in close proximity to a 5-HT^{+}/HO-1^{+} NEC in the eye region of 4 dpf zebrafish larvae (Appendix 1).

4.3.2 HO-1 knockdown confirmation: Western blots and protein content analysis

Hypoxia exposure did not cause a significant increase in the HO-1 protein content in either shams or larvae subjected to HO-1 knockdown (Fig. 4.3). HO-1 MO treated larvae expressed lower levels of HO-1 protein compared to sham conspecifics under both normoxic and hypoxic conditions (Fig. 4.3A). The reduced expression of HO-1 protein in HO-1 deficient

larvae is represented qualitatively in the insert of Fig. 4.3A. Similarly, hypoxia exposure did not cause an increase in the HO-1 protein content of gills from control or ZnPPIX-treated adult fish (Fig. 4.3B). ZnPPIX did not significantly change HO-1 protein content in the gills of adult zebrafish from that of control fish (Fig. 4.3B).

4.3.3 HO activity assay

HO activity was increased significantly from 27.3 ± 1.8 to 41.9 ± 5.3 pmol bilirubin mg protein^{-1} min^{-1} in sham larvae exposed to hypoxia (Fig. 4.4A). In contrast, hypoxia exposure did not cause a significant change in the HO activity in larvae experiencing HO-1 knockdown (normoxia = 24.5 ± 0.8 pmol bilirubin mg protein^{-1} min^{-1}; hypoxia = 24.1 ± 0.3 pmol bilirubin mg protein^{-1} min^{-1}; Fig. 4.4A). HO activity increased significantly in the gills of hypoxia-exposed adults (from 59.1 ± 6.7 to 115.9 ± 13.0 pmol bilirubin mg protein^{-1} min^{-1}) which was prevented by treating fish with ZnPPIX (Fig. 4.4B). ZnPPIX is an HO-1 inhibitor at the dose administered so it did not affect HO-2 activity. Therefore, any residual HO activity in fish treated with ZnPPIX is due to heme breakdown via HO-2.

4.3.4 Ventilation frequency

Series I: Larvae

Larvae deficient in HO-1 exhibited significantly higher f_{vent} (123.7 ± 11.5 breaths min^{-1}) than sham conspecifics (30.4 ± 8.8 breaths min^{-1}) under normoxic conditions (Fig. 4.5A). Subsequent exposure to CO did not affect breathing in normoxic sham larvae but caused a significant drop in the f_{vent} of HO-1 deficient larvae (to 55.0 ± 7.2 breaths min^{-1}) which nevertheless remained higher (by 22.4 ± 9.9 breaths min^{-1}) than in the CO-exposed shams. There was a significant rise in f_{vent} (from 61.3 ± 12.1 to 95.7 ± 9.2 breaths min^{-1}) in sham larvae exposed to hypoxia. This hypoxic hyperventilatory response was prevented by the addition of

CO and there was a trend toward a decrease in f_{vent} (Fig. 4.5B). HO-1 deficient larvae did not experience a change in f_{vent} when exposed to hypoxia (f_{vent} normoxia = 114.5 ± 10.5 breaths min^{-1}; f_{vent} hypoxia = 93.2 ± 17.4 breaths min^{-1}) but decreased f_{vent} significantly from hypoxic values with the addition of CORM to 59.0 ± 13.7 breaths min^{-1} (Fig. 4.5B). Larvae were exposed to PHZ to elicit anaemia and thus eliminate any effects that CO may have mediated via its interaction with hemoglobin. PHZ exposure did not cause a change in f_{vent} in either sham (54.4 ± 7.1 breaths min^{-1}) or HO-1 deficient larvae (93.5 ± 12.3 breaths min^{-1}; Fig. 4.5C) but subsequent addition of CO decreased f_{vent} in both sham (12.0 ± 7.8 breaths min^{-1}) and HO-1 deficient larvae (12.0 ± 4.2 breaths min^{-1}; Fig. 4.5C).

Series II: Adults

Inhibition of HO-1 with ZnPPIX caused a significant increase in f_{vent} in normoxic adults (Fig. 4.6) from 161.3 ± 15.2 to 249.8 ± 10.9 breaths min^{-1}). The addition of CO caused a significant decrease in the f_{vent} of ZnPPIX treated fish (from 249.8 ± 10.9 to 166.4 ± 17.0 breaths min^{-1}) but not in control fish (Fig. 4.6A). Thus, f_{vent} in CO-exposed ZnPPIX-treated adults was not significantly different from that of normoxic control or CO-exposed control adults. Hypoxia exposure caused an increase in the f_{vent} of control adults (from 214.0 ± 12.3 to 367.3 ± 10.1 breaths min^{-1}) but did not significantly change the f_{vent} of adults with inhibited HO-1 activity (from 300.7 ± 20.2 to 355.2 ± 20.5 breaths min^{-1}) (Figure 4.6 A and B). Exogenous CO addition significantly decreased f_{vent} in hypoxic control (to 290.6 ± 25.7 breaths min^{-1}) and hypoxic ZnPPIX treated adults (to 241.8 ± 12.8 breaths min^{-1}).

4.4 FIGURES AND TABLES

Figure 4.1. Confocal micrographs of the eye region of whole mount zebrafish (*Danio rerio*) larvae at 4 days post fertilization. Arrows represent cells immunoreactive for HO-1 (A) and 5-HT (labelling neuroepithelial cells, NECs; B and C) in sham larvae. Arrow heads represent co-localization of HO-1 and 5-HT in the NECs (C). Left insert in panel C depicts a magnified image of a NECs lacking HO-1. Right insert in panel C depicts a NEC containing HO-1. In panels D-F arrows represent cells immunoreactive for HO-1 (D) and 5-HT (labelling NECs; E and F) in HO-1 deficient larvae. Insert in panel F depicts a NEC lacking HO-1 in an HO-1 deficient (morphant) larvae. Scale bar = 50 µm and applies to all panels.

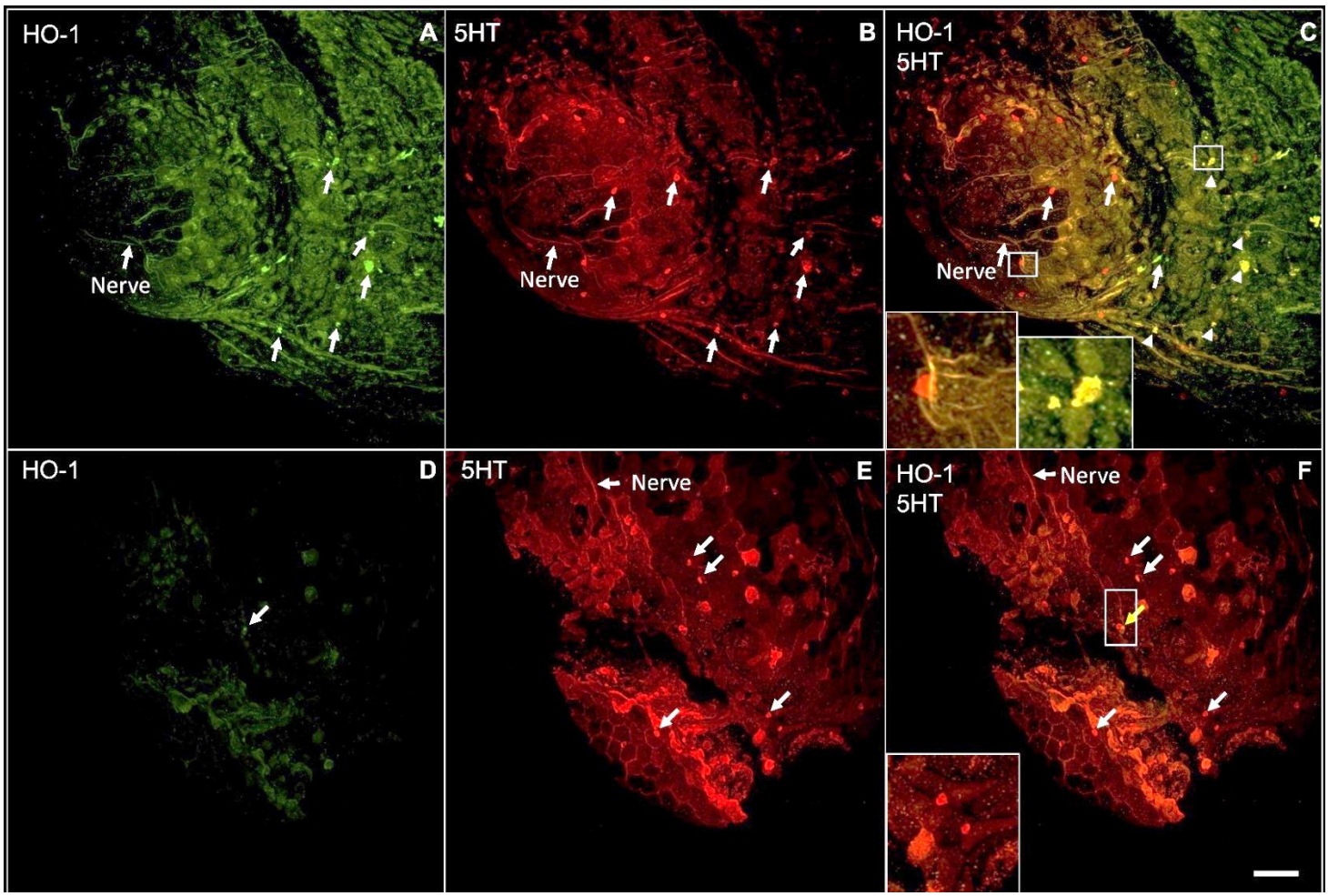

HO-1
A
Nerve
5HT
B
Nerve
HO-1
5HT
C
Nerve
HO-1
D
5HT
Nerve
E
HO-1
5HT
Nerve
F

Figure 4.1

Figure 4.2. Confocal micrographs of whole mount gill filament of an adult zebrafish (*Danio rerio*). Arrows represent cells immunoreactive for HO-1 (A), 5-HT (labelling neuroepithelial cells, NECs; B) and co-localization of HO-1 and 5-HT in the NECs (C) on the gills of adult zebrafish. Top insert in panel C depicts a magnified image of an innervated NECs immunoreactive for HO-1 (image is from a separate gill filament). Bottom insert in panel C depicts a NEC lacking HO-1 and a HO-1^{+} cell lacking 5-HT in close proximity of each other and nerve fibers. Scale bar = 25 μm and applies to all panels.

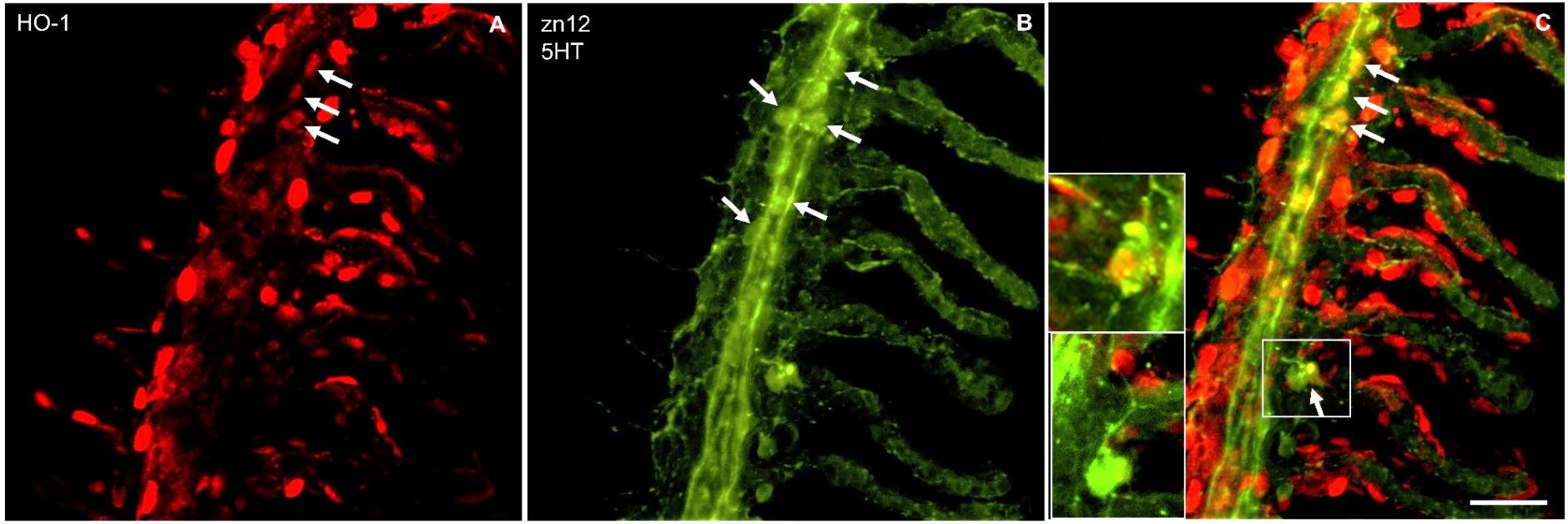


Figure 4.2

Figure 4.3. Heme oxygenase-1 (HO-1) protein content in sham and HO-1 morphant (HO-1 MO) larvae at 4 day post fertilization (A) and in control adult zebrafish and adult zebrafish treated with the HO-1 activity inhibitor zinc protoporphyrin IX (ZnPPIX) (B). An asterisk indicates a significant difference between groups within a treatment. Normoxia, N; Hypoxia, H. Two Way ANOVA, $P < 0.05$. N = 6-8.

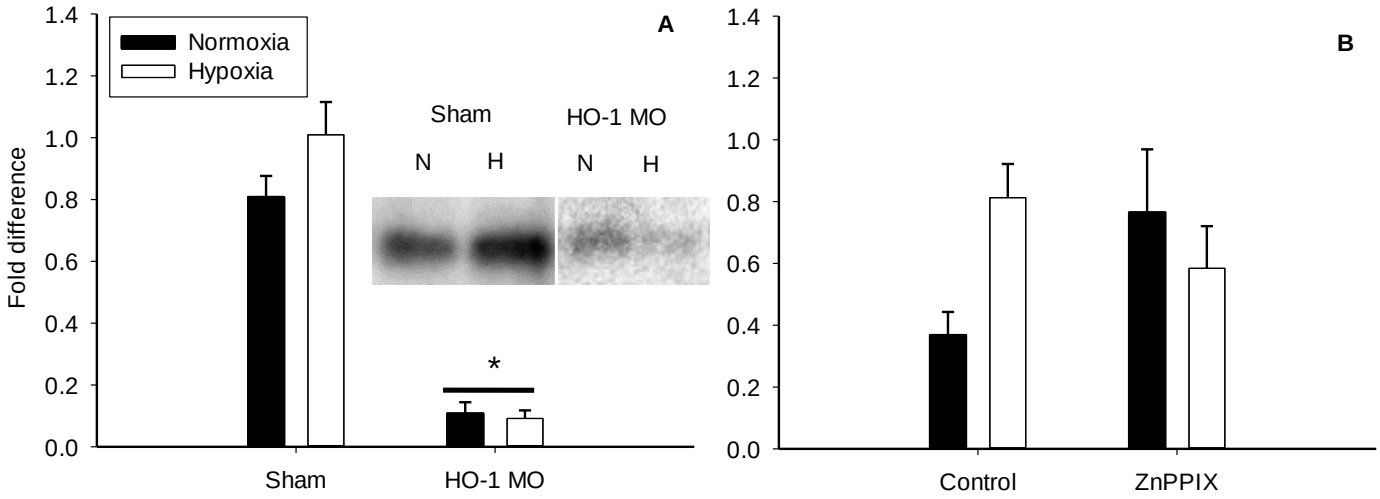

A
B
Normoxia
Hypoxia
Fold difference
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Sham
HO-1 MO
N
H
N
H
*
Sham
HO-1 MO
Control
ZnPPIX

Figure 4.3

Figure4.4. Heme oxygenase (HO) activity in sham and HO-1 morphant (HO-1 MO) larvae at 4 day post fertilization (A) and in control adult zebrafish and adult zebrafish treated with the HO-1 activity inhibitor zinc protoporphyrin IX (ZnPPIX) (B). Different letters indicate significant differences within a group. An asterisk indicates a significant difference between groups within a treatment. Two Way ANOVA, $P_{hypoxia} = 0.038$; $P_{control} = 0.029$. N = 6-7.

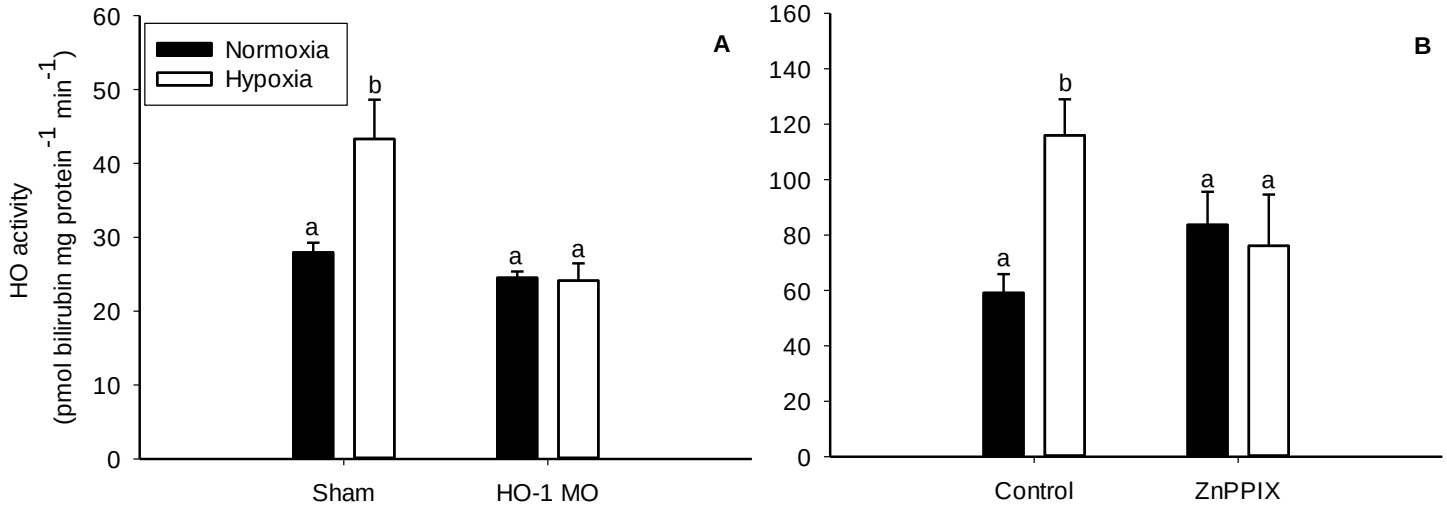

HO activity
(pmol bilirubin mg protein -1 min -1)
Normoxia
Hypoxia
A
B
Sham
HO-1 MO
Control
ZnPPIX
a
b

Figure 4.4

Figure 4.5. Ventilation frequency (f_{vent}) in breaths min^{-1} of sham and heme oxygenase-1 morphant (HO-1 MO) larvae exposed to normoxia (Nox) and 40 µM carbon monoxide releasing molecule (CORM; A), hypoxia and CORM (B), and phenylhydrazine (PHZ; C). Different letters indicate significant differences within a group. An asterisk indicates a significant difference between groups within a treatment. Two Way ANOVA, $P < 0.05$. N = 6-8.

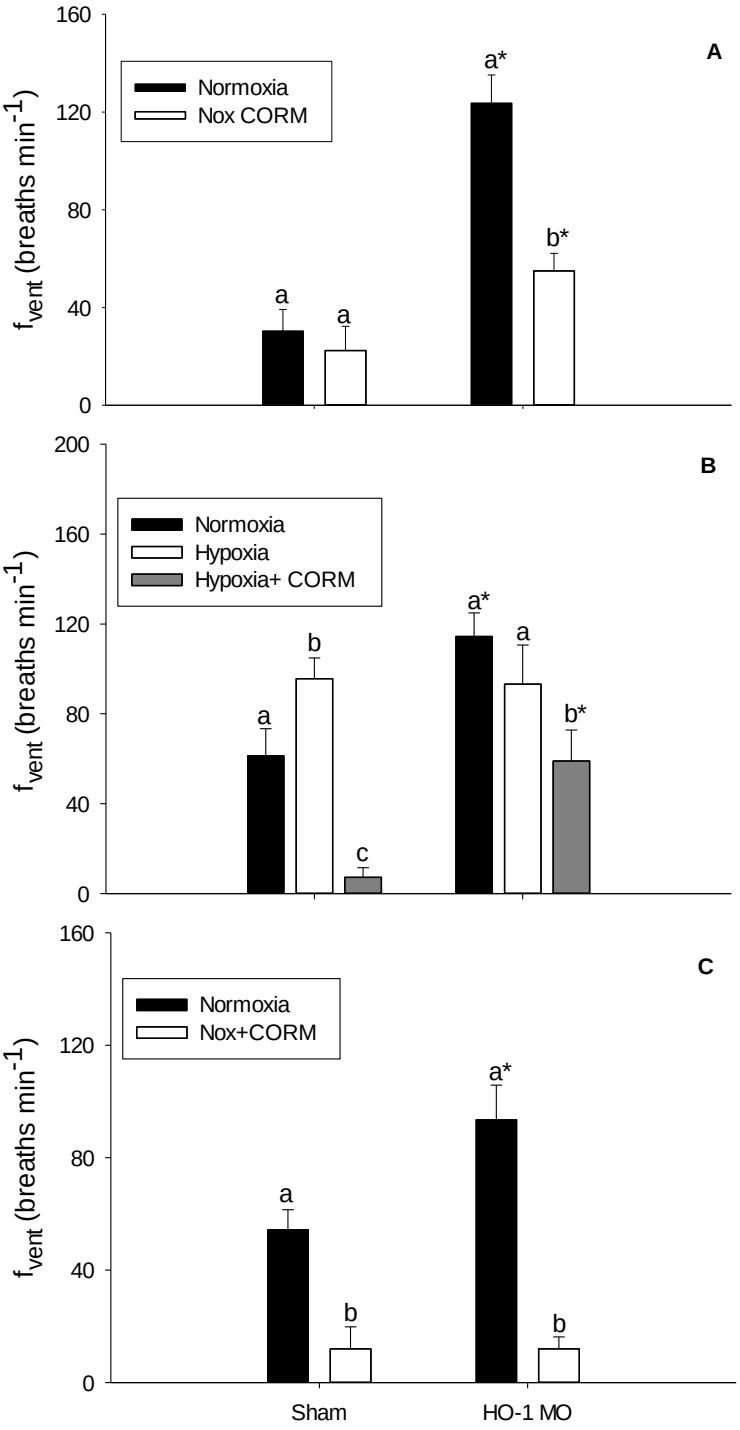
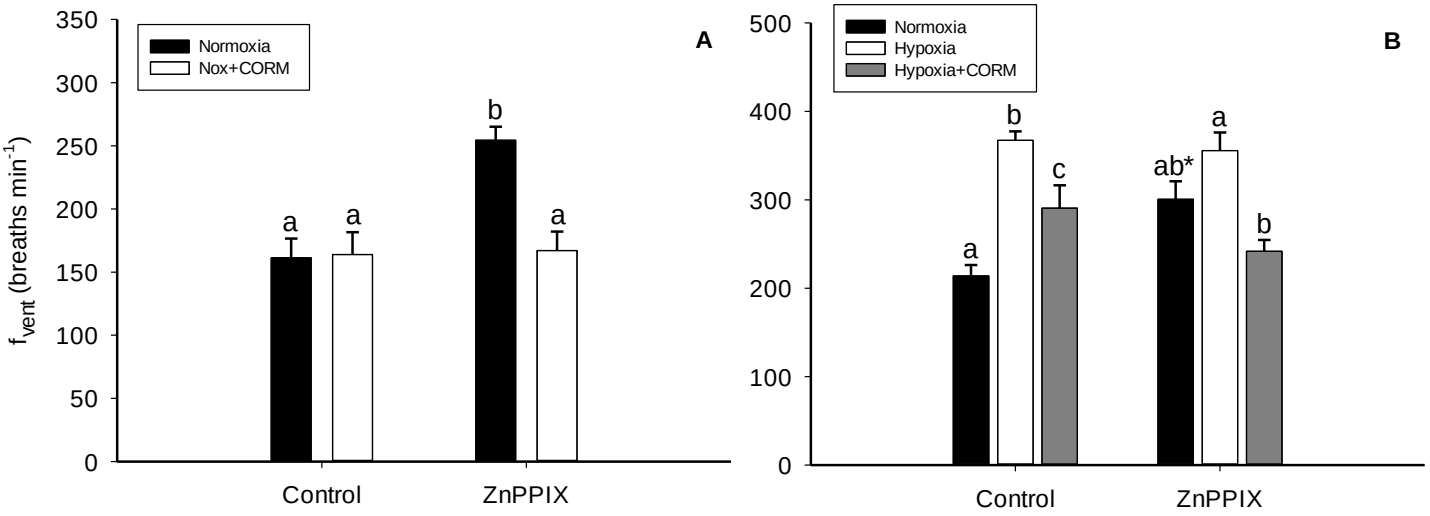

A
Normoxia
Nox CORM
f_{vent} (breaths min^{-1})
160
120
80
40
0
a
a
a*
b*
B
Normoxia
Hypoxia
Hypoxia+ CORM
200
160
120
80
40
0
a
b
c
a*
a
b*
C
Normoxia
Nox+CORM
160
120
80
40
0
a
b
a*
b
Sham
HO-1 MO

Figure 4.5

Figure 4.6. Ventilation frequency (f_{vent}) in breaths min^{-1} of control and zinc protoporphyrin IX (ZnPPIX; heme oxygenase-1 (HO-1) inhibitor) treated adult zebrafish exposed to normoxia and 40 μM carbon monoxide releasing molecule (CORM; A) and hypoxia and CORM (B). Different letters indicate significant differences within a group. An asterisk indicates a significant difference between groups within a treatment. Two Way Repeated Measures ANOVA, $P < 0.05$. N = 6-8.


A
Normoxia
Nox+CORM
f_{vent} (breaths min^{-1})
350
300
250
200
150
100
50
0
a
a
b
a
Control
ZnPPIX
B
Normoxia
Hypoxia
Hypoxia+CORM
500
400
300
200
100
0
a
b
c
ab*
a
b
Control
ZnPPIX

4.5 DISCUSSION

The main objective of this study was to investigate the role of the HO-1/CO system in the control of breathing in larval and adult zebrafish. Immunohistochemistry revealed the presence of HO-1, and thus the potential for endogenous CO production, in the NECs of both larvae and adults. We also report that zebrafish with reduced HO-1 activity (via HO-1 knockdown in larvae or ZnPPIX treatment in adults) exhibited increased f_{vent} under normoxic but not hypoxic conditions. The addition of exogenous CO was able to restore resting f_{vent} in fish with diminished CO production and in some cases (e.g. hypoxic sham larvae) CO reduced f_{vent} below resting levels (Fig. 4.5).

4.5.1 Significance of HO-1 in the NECs

As a first step in establishing a role for CO in the reflex control of breathing linked to peripheral chemoreception, it was necessary to demonstrate the presence of HO-1 in NECs, the peripheral O_2 chemoreceptors in zebrafish (Jonz et al., 2004). The results clearly demonstrated the presence of HO-1 in the NECs found on the skin of larvae at 4 dpf and in the NECs of the adult gill (Figs. 4.1 and 4.2). Thus, it is reasonable to assume that the NECs of zebrafish possess the capacity to produce endogenous CO. There are conflicting reports, however, on the presence of HO-1 in the peripheral chemoreceptors in mammals with one study providing evidence for its presence in the carotid body of rats (Di Giulio et al., 2009) while others reporting HO-2, but not HO-1, immunoreactivity in the rat carotid body (Prabhakar et al., 1995; Prabhakar, 2013). Nevertheless, the current results are consistent with a previous study on goldfish in which HO-1 immunoreactivity was demonstrated in the branchial NECs of goldfish acclimated to 7^{o}C (Tzaneva and Perry 2014). Thus, in the two fish species studied to date, basal expression of HO-1 was detected in NECs. It should be noted however, that HO-1 immunoreactivity was not

detected in branchial NECs of goldfish acclimated to 25^{o}C (Tzaneva and Perry, 2014; Chapter 2). Because the results of the goldfish study also indicated an inhibitory influence of endogenous CO on ventilation, it was suggested by Tzaneva and Perry (2014; Chapter 2) that expression of HO-1, and thus endogenous production of CO, would be inappropriate in goldfish acclimated to such high temperatures approaching the upper thermal limit of their preferred temperature range (~28^{o}C; Reynolds et al., 1978) where ventilation must be kept at elevated levels to promote high metabolic rates. Zebrafish in the present study were acclimated to 28^{o}C, a temperature which also approaches the upper thermal limit of their preferred temperature range (30^{o}C; Vergauwen et al., 2010). Thus, it is unclear as to why at such high temperatures, zebrafish NECs exhibit HO-1 immunoreactivity whereas goldfish NECs do not.

The presence of HO-1 in the NECs suggests the likelihood for specific and localized production of CO in the O_2-sensing chemoreceptors of zebrafish. In the current study, two distinct populations of NECs were detected; NECs that were HO-1^{+} and NECs that lacked HO-1 (HO-1^{-}). It is not uncommon for zebrafish to exhibit several sub-populations of a single class of epithelial cell type. For example, Porteus and colleagues (2014) identified 5 different types of NECs in the bowfin based on immunohistochemical findings. In addition, it is well documented that osmoregulation in zebrafish depends on at least five types of ionocytes based on their ion transport mechanisms and functional regulation (reviewed in Guh et al., 2015). Furthermore, we previously reported two distinct NEC populations in the gills of goldfish (Tzaneva and Perry, 2014). In addition, we also identified a population of HO-1^{+}/5-HT^{-} cells in both the larval and gill NECs. These could be newly formed NECs which have not yet synthesized or accumulated 5-HT (Jonz and Nurse, 2003). Therefore, it is plausible that these cells do not yet contribute to the ventilatory response to hypoxia. HO-1^{+}/5-HT^{-} cells found scattered on the surface of the skin

of larvae and at the base of the lamella may include other cell types such as ionocytes. Unpublished results from our lab have shown that Na^+/K^+ ATPase positive ionocytes in zebrafish also contain HO-1. Furthermore, we observed that NECs and unidentified cells that are HO-1^+/5-HT^- are innervated. Innervation of peripheral chemoreceptors obviously is an important feature enabling reflex control of ventilation in response to hypoxia in fish (Burleson and Smatresk, 1990; Sundin et al., 1999; Sundin et al., 2000). It is possible that both HO-1^+ NECs and HO-1^+/5-HT^- may be involved in O_2 sensing by releasing CO onto associated innervation. Interestingly, HO-1 was present in the nerves of larval zebrafish only, suggesting that expression of HO-1 becomes exclusive to non-neuronal cells in the gill epithelia as the larvae develop into adults. The HO-1 in nerves associated with larval NECs may play a role in the synthesis of CO and its subsequent delivery to NECs.

4.5.2 Ventilation in zebrafish exposed to hypoxia

Role of the HO-1/CO system

In this study, exogenous addition of CO caused a significant decrease in f_{vent} under both normoxic and hypoxic conditions in zebrafish larvae or adults suggesting an inhibitory role of CO in control of f_{vent} regardless of developmental age (Figs. 4.5 and 4.6). Zebrafish deficient in HO-1 activity exhibited a significant increase in f_{vent} under normoxic but not hypoxic conditions. This response was similar to the cardiac response in larval zebrafish (Tzaneva and Perry, 2016). To determine whether this increase of f_{vent} was caused by a lack of CO production, zebrafish deficient in HO-1 activity were exposed to 40 µM of a CO releasing molecule (CORM). Previous studies on fish have used 10x higher concentrations of CORM (Dombkowski et al., 2008). Although we attempted to more closely mimic endogenous levels of CO at least based on mammalian studies (Kajimura et al., 2010), the exact concentration of CO after CORM treatment

is unknown. Regardless, the dose of CORM used in the present study caused a significant decrease in f_{vent} under normoxic conditions. We exposed larval zebrafish to the hemolytic agent PHZ to eliminate the potential confounding effects of CO-haemoglobin interactions which might affect ventilation (Pelster and Burggren, 1996). In PHZ treated larvae, CO treatment continued to significantly reduce f_{vent} (Fig 4.5C). These results suggest that in larvae the effects of CO on f_{vent} are not mediated by interactions with hemoglobin. Adult zebrafish treated with PHZ under normoxic conditions exhibit increased ventilation (V. Tzaneva, personal observations) and thus it was not feasible to use PHZ treatment in adults to evaluate the contribution (if any) of the effects of CO on haemoglobin function on the f_{vent} response.

In this study, we observed an increase in HO activity in whole larvae and the gills of adult zebrafish when exposed to hypoxia (Fig. 4.4). This increase in activity cannot be attributed exclusively to NECs (or any other single cell type for that matter) because HO activity was measured in whole larvae and gill tissue in adults. Therefore, it is possible that HO-1 activity in the NECs did not actually increase with hypoxia exposure. Furthermore, given the depressant effect of CO on breathing and the fact that we did not observe a significant change in f_{vent} in hypoxic fish deficient in HO-1, the increase of HO-1 activity, and presumably CO production, may be more important for regulating ionic transport under hypoxic conditions. CO is known to regulate transport of ions such as Na^+, K^+, and Ca^{2+} in mammals (reviewed in Wilkinson and Kemp, 2011). Regardless, the rise in HO-1 activity was not caused by a significant increase in protein levels but may have resulted from post-translational modifications of pre-existing HO-1 protein. For example, protein kinase B (Akt) was shown to phosphorylate human recombinant HO-1 thereby increasing its activity (Salinas et al., 2004; reviewed in Dunn et al., 2013). This

modification also increases the affinity of HO-1 to cytochrome P450 reductase (Salinas et al., 2004) presumably increasing the production of CO from heme.

A possible reason for the lack of an increase in f_{vent} under hypoxic conditions in HO-1 deficient fish may be that fish lacking HO-1 have reached a maximum f_{vent} and thus do not have the capacity to further increase f_{vent}. This result is in contrast to a previous study where goldfish with diminished HO-1 activity via inhibition by ZnPPIX and acclimated to 7^{o}C did not respond with an increase in f_{vent} under normoxic conditions but showed a further increase in ventilation under hypoxic conditions when compared to their control conspecifics (Tzaneva and Perry, 2014; Chapter 2). Therefore there is an apparent tonic activity of the HO-1/CO system under normoxic conditions in zebrafish which is not present in goldfish. In zebrafish, the tonic inhibitory HO-1/CO system under normoxic conditions may provide a greater scope for increasing f_{vent} when the fish are faced with hypoxia at the high acclimation temperature of 28^{o}C. Furthermore, in contrast to zebrafish, cold-acclimated goldfish have lower metabolic rates and thus an inhibitory mechanism in the face of CO may be in place to fine tune the ventilation response during hypoxia by delaying the onset of the hypoxia-induced hyperventilatory response. These inter-specific differences may reflect temperature dependent variations in the onset of the hyperventilatory response to hypoxia between goldfish at 7^{o}C (onset of hypoxia-induced hyperventilation at P_wO_2 ~30 mmHg) and zebrafish at 28^{o}C (onset of hypoxia-induced hyperventilation at P_wO_2 ~90 mmHg) (Tzaneva et al., 2011; Vulesevic and Perry, 2006).

4.5.3 Importance of CO and developmental stage on ventilatory drive in zebrafish

As mentioned, CO plays an inhibitory role in larval and adult zebrafish under normoxic but not hypoxic conditions. Thus, in zebrafish the role of CO in the control of breathing does not change with developmental age, which is in contrast to the ventilatory effects of another

gasotransmitter, nitric oxide (NO). It was reported recently (Porteus et al., 2015) that NO affects f_{vent} differentially in larval and adult zebrafish, being excitatory during early development when the ventilatory drive is low and inhibitory in adults when the ventilatory drive is high. The authors attribute this difference to the notion that NO may be acting differentially on peripheral and central chemoreceptor sites and that the balance between the role of peripheral and central sites might be changing throughout development (Porteus et al., 2015). In mammals, endogenous CO production is necessary for the excitation of neurons in rhythm generating regions of the central nervous system (CNS) (e.g. the rostral ventrolateral medulla; RVLM) but is inhibitory in the carotid body (Neubauer and Sunderram, 2004). Interestingly, the HO-CO pathway in the CNS does not appear to be involved in the control of hypoxia-induced hyperventilation in conscious rats (Paro et al., 2002). This suggests that CO may be acting exclusively on peripheral chemoreceptors (i.e. the carotid body); a response which may be maintained through development. Therefore in zebrafish, the similarities in the ventilatory response to CO in larvae and adults may be dominated by the effect of CO on the NECs.

4.5.4 Conclusion

This study provides evidence for a role of the HO-1/CO system in control of breathing in zebrafish at different developmental stages. We demonstrate for the first time that CO plays an inhibitory role in control of breathing in larvae and adults. HO-1 is expressed in the NECs found on the skin of larvae and gills of adult fish indicating that both larval and adult NECs are able of producing endogenous CO. Therefore it is most likely through CO acting in an autocrine/paracrine manner on the NECs and associated innervation that HO-1 exerts its effects on breathing.

4.6 ACKNOWLEDGEMENTS

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CHAPTER 5.

General Discussion

5.1 SUMMARY AND SIGNIFICANCE OF THESIS

Fish can experience changes in ambient O_2 tensions (i.e. hypoxia or hyperoxia) and to meet the O_2 requirements imposed by metabolic demands, they must be able to compensate with the appropriate cardiorespiratory responses. Chemoreception in fish is critical for sensing changes in O_2 tensions of the water and the blood and is often the first step in a cascade of events leading to cardiorespiratory and metabolic adjustments. Studying the mechanisms leading to these complex adjustments in response to hypoxia ultimately aids us in understanding the limitations and variations of the cardiorespiratory responses to hypoxia in different fish species and is a key theme in the field of comparative physiology.

The overall objective of my doctoral thesis was to assess the role of the heme oxygenase-1/carbon monoxide (HO-1/CO) system in regulating cardiac and ventilatory function in fish exposed to changes in environmental O_2. My thesis also provides insight into developmental-, species- and temperature-specific modulation of control of breathing in fish via the HO-1/CO system.

Chapter 2 presented the novel finding that inhibition of HO-1, which prevents endogenous CO production, resulted in an augmented hypoxic ventilatory response in goldfish (*Carassius auratus)* acclimated to 7 but not 25^{o}C without affecting ventilation under normoxic conditions at either temperature. I attributed this difference to the presence of HO-1 in the neuroepithelial cells (putative branchial O_2 chemoreceptors; NECs) on the gills of goldfish acclimated to 7^{o}C but not in fish acclimated to 25^{o}C. Overall, the HO-1/CO system appears to attenuate the ventilation frequency response to hypoxia in 7^{o}C fish but not 25^{o}C fish without affecting the ventilation amplitude component of the response.

The role of the HO-1/CO system in the control of cardiac function in hypoxic zebrafish larvae was investigated in Chapter 3. The presence of HO-1 and thus the potential for CO production was found in the pacemaker cells of larvae at 4 days post fertilization. Inhibition of HO-1 via morpholino injections caused a significant increase in heart rate under normoxic but not hypoxic conditions. HO-1 deficient larvae also exhibited significantly larger ventricles and when exposed to hypoxia displayed higher cardiac output and stroke volume. Thus, it seems that the HO-1/CO system had a negative chronotropic effect under normoxic conditions only, presumably by acting on the pacemaker cells of the heart. Under hypoxic conditions, the HO-1/CO system is important for regulating cardiac output and stroke volume.

In Chapter 4, I elaborated on the role of the HO-1/CO system in the control of ventilation by incorporating a developmental component to the response. Here I showed that HO-1 is found in the NECs on the skin of larval (4 days post fertilization; dpf) and the gills of adult zebrafish (*Danio rerio*) acclimated to 28^{o}C. This study also indicated an apparent tonic activity of the HO-1/CO system under normoxic conditions. Interestingly, larvae and adult zebrafish deficient in HO-1 activity did not exhibit an augmented hyperventilatory response to acute hypoxia exposure. Therefore, the HO-1/CO system may be inhibitory under normoxic conditions to provide a greater range for increasing ventilation frequency when the fish are faced with hypoxia. In this chapter, I demonstrated for the first time that CO produced via HO-1 plays an inhibitory role in the control of ventilation in both larval and adult zebrafish.

5.2 INTEGRATING CONTROL OF VENTILATION AND CARDIAC FUNCTION IN RESPONSE TO HYPOXIA

Although control of ventilation and cardiac function in zebrafish are presented as two separate chapters, the link between the two is undeniable. In adult fish, the typical cardiovascular reflex response to hypoxia is bradycardia which is thought to be mediated by branchial O_2 chemoreceptors, namely the NECs (reviewed in Burleson, 2009). The results from Chapter 3, however, demonstrate a clear tachycardia in response to acute hypoxia in sham but not HO-1 deficient zebrafish larvae at 4 dpf. I attributed this response to the fact that the HO-1/CO system may be working in coordination with the sympathetic nervous system in regulating heart rate during hypoxia because lack of endogenous CO production prevented adrenaline from exerting its stimulatory effect on heart rate under hypoxic conditions. In adult fish, the gills (and by default the NECs) and heart are innervated by cranial nerves IX and X carrying both afferent and efferent neurons belonging to the sympathetic and parasympathetic branches of the autonomic nervous system (ANS) (Milsom et al., 2002; reviewed in Burleson, 2009; Bally-Cuif and Vernier, 2010; Stoyek et al., 2015). Sectioning the nerves to branchial O_2 chemoreceptors (NECs) abolishes the hyperventilatory and the bradycardic response to hypoxia in adult teleosts suggesting a clear link between branchial O_2 chemoreceptor and control of heart rate under hypoxic conditions (Sundin et al., 1999; Sundin et al., 2000; Milsom et al., 2002; Reid and Perry, 2003). However, zebrafish larvae at 4 dpf do not yet have developed gills and the majority of the NECs are dispersed on the skin surface with no known link to a nerve branch as is the case for branchial NECs (Jonz and Nurse, 2003; Tzaneva and Perry, 2010). Therefore it is difficult to predict whether or not the skin NECs and any associated innervation are involved in controlling cardiac function under hypoxic conditions in zebrafish larvae. In fact, it may be that zebrafish larvae respond with tachycardia in the early stages of hypoxia because there is no known neuronal link between peripheral O_2 chemoreceptors and the heart. Furthermore, it was reported

that the sympathetic nervous system was responsible for evoking the tachycardic response observed in larval zebrafish exposed to high environmental CO_2 (Miller et al., 2014) which may also be the case for acute hypoxia. Tachycardia may be the immediate response induced by acute hypoxia via an increase of circulating catecholamines in a CO dependent manner (Perry et al., 2004; Steele et al., 2011; Tzaneva and Perry, 2016). Chronic hypoxia, however, can evoke a pronounced bradycardia in larval fish that is mediated via increased parasympathetic activity. In zebrafish larvae experiencing bradycardia, there is about a 10% decrease in heart rate presumably via hypoxia induced vagal inhibition (Steele et al., 2009). Based on the data from this thesis, there is a similar increase of ~10% in HO-1 deficient (morphant) larvae under hypoxic conditions. Therefore it seems that the magnitude of inhibition is the same between the two systems.

5.3 OVERALL ROLE OF THE HO-1/CO SYSTEM IN CONTROL OF CARDIORESPIRATORY FUNCTION IN FISH

The aim of this section is to highlight the overall role of the HO-1/CO system in the control of cardiorespiratory function and to speculate on the mechanism of CO action in fish. The results from this thesis provide the first evidence that HO-1 is present in teleost fish in 1) branchial and skin NECs, 2) nerves associated with NECs, and 3) the heart of larval zebrafish. The presence of HO-1 in these structures suggests the likelihood for specific and localized production of CO in fish. As mentioned in Chapter 1 (General Introduction), CO is a gaseous molecule that cannot be stored in vesicles and thus upon synthesis, readily diffuses across membranes to act in an autocrine/paracrine manner to evoke the appropriate physiological responses (Levitt and Levitt, 2015).

To assess the functional significance of the HO-1/CO system in control of cardiorespiratory function in fish, I used both pharmacological and gene knock down approaches to diminish HO-1 activity in adult and larval fish, respectively. Similar approaches have been used in mammalian studies to show that endogenously produced CO hinders carotid body (peripheral O_2 chemoreceptors in mammals) sensory activity and thus augments respiration (Prabhakar et al., 1995). This response was rescued by administering exogenous CO (Prabhakar et al., 1995; Yang et al., 1998). Therefore, it was established that CO plays an inhibitory role in control of respiration in mammals by impeding carotid body activity and augmenting the respiratory response under both normoxic and hypoxic conditions. In addition, CO was shown to inhibit the mammalian heart because CO perfusion reduced the contractility of isolated rat papillary muscle (Liu et al., 2001). Based on the results of this thesis, the concept of an inhibitory role of CO on cardiorespiratory function can be extended to teleost fish. Figure 5.1 provides a diagrammatic overview of the regulatory inputs to the heart, gills and central nervous system (CNS) with respect to heart rate and ventilation frequency to include the proposed mode of action of CO and how the various nervous inputs interact to modulate cardioventilatory function in fish.

Although this thesis did not directly investigate the mechanisms through which CO exerts its effect on cardiorespiratory function in fish, I have extrapolated possible mechanisms of action from literature on mammals which are centered on regulation of intracellular Ca^{2+} concentrations. Indeed, the idea that control of ventilation via the HO/CO system involves intracellular Ca^{2+} is well described in the mammalian literature (Prabhakar, 1999; Peers and Wyatt, 1997; Kumar and Prabhakar, 2012). Glomus cells deficient in HO, and presumably experiencing a reduction of endogenous CO production, exhibited marked elevations of

intracellular Ca^{2+} levels further confirming the inhibitory actions of CO on ventilation (Overholt et al., 1996). Therefore, considering that NECs are thought to be analogous to carotid body glomus cells, it is possible that the mechanisms of CO action in NECs are similar to those described for the glomus cells of mammals. By analogy with the carotid body, the production of CO via HO in peripheral O_2 chemoreceptors in fish likely reduces sensory discharge by inhibiting Ca^{2+} channels and reducing neurotransmitter release from the NECs onto afferent innervation (Overholt and Prabhakar, 1997; reviewed in Prabhakar, 1999; Dallas et al., 2009; Tzaneva and Perry, 2014). Recently, Zachar and Jonz (unpublished data, 2014) showed that hypoxia activates L-type voltage gated Ca^{2+} channels on the NECs of goldfish acclimated to 18^{o}C resulting in Ca^{2+} influx into the cell. Therefore it is possible that, similar to mammals, CO generated by the HO family of enzymes regulates the Ca^{2+} channels responsible for modulating the function of peripheral O_2 chemoreceptors (e.g. NECs) in fish see Figure 5.2). The HO-1/CO pathway plays a vasodilatory role in blood vessels of fish via a CO-mediated increase in cGMP (Domkowski et al., 2009) with other potential targets including voltage-gated (T-type) Ca^{2+} channels (Nemtsas et al., 2010; Alday et al., 2014; reviewed in Peers et al., 2015) that may be responsible for the excitation-contraction coupling of the myocardium. In Chapter 3, it is possible that the HO-1/CO system acted on resting heart rate by inhibiting Ca^{2+} current via T-type Ca^{2+} channels in the pacemaker cells which could explain the increased baseline heart rate in HO-1 deficient larvae. The lack of a further increase in heart rate in HO-1 deficient larvae subjected to hypoxia may be related to the fact that cardiac T-type Ca^{2+} channels are activated by acute hypoxia exposure in a CO-dependant manner in a similar fashion as L-type Ca^{2+} channels (Movafagh and Morad, 2010). Overall, I speculate that as in mammals, the HO/CO system in fish acts to regulate cellular Ca^{2+} to modulate respiratory and cardiac rhythm. Unpublished

results from the Perry lab (E. Azzi and V. Tzaneva, unpublished data) partially support this notion because HO-1 inhibition (via morpholino knockdown) resulted in a significant increase in Ca^{2+} influx in zebrafish larvae at 4 days post fertilization under normoxic conditions (Appendix 3A). Follow up experiments with hypoxia exposure and application of exogenous CO are necessary to fully assess the role of the HO-1/CO system in the regulation of Ca^{2+} transport in fish.

5.4 SPECIES AND TEMPERATURE-DEPENDENT DIFFERENCES IN THE ROLE OF THE HO-1/CO SYSTEM ON VENTILATION

In Chapters 2 and 4, I present results on the role of the HO-1/CO system in control of ventilation in adult goldfish and zebrafish as well as in larval zebrafish. Although the overall theme of the HO-1/CO system inhibiting ventilation in fish was retained across studies, there were two main differences that emerged - temperature and species differences.

There were clear temperature-specific differences within and between species in the role of HO-1/CO system on control of ventilation. In Chapter 2, I report an inhibitory influence of the HO-1/CO system on ventilation frequency response in goldfish acclimated to 7^{o}C and exposed to acute hypoxia and hyperoxia (higher than normal O_2 levels) but not in goldfish acclimated to 25^{o}C. I attributed this difference to the presence of HO-1, and thus the potential of localised endogenous CO production, in the NECs of 7^{o}C acclimated goldfish but not in 25^{o}C acclimated goldfish. A mechanism for inhibiting ventilation during acute hypoxia may be less appropriate in warm acclimated goldfish because of their need to exert a large hyperventilatory response to maintain the high demand for O_2 imposed by their increased metabolic rate (Fry and Hart, 1948).

Interestingly, HO-1 was found in a subpopulation of branchial and skin NECs of warm (28^{o}C) acclimated adult and larval zebrafish, respectively. Results from Chapter 4 revealed that the HO-1/CO system inhibited ventilation frequency in zebrafish but under normoxic conditions *only*. These data suggest a tonic activity of the HO-1/CO system in zebrafish under normoxic conditions which is not present in goldfish acclimated to either 7 or 25^{o}C. Therefore, it is possible that there are temperature- and species-specific differences in the ventilatory response to altered ambient O_2 levels that are mediated by CO. These differences in the control of ventilation are further reinforced by the result that goldfish (acclimated to 25^{o}C) and zebrafish respond differently to reduced endogenous CO production under normoxic conditions despite both species being acclimated to warm temperatures. I attribute this difference to a lack of tonic activity of the HO-1/CO system in the peripheral O_2 chemoreceptors of warm acclimated goldfish. Although HO-1 is present in the NECs of both cold acclimated goldfish (7^{o}C) and warm acclimated zebrafish (28^{o}C), it seems that the HO-1/CO system differentially inhibits ventilation frequency under different O_2 requirements. In cold acclimated goldfish there is no apparent tonic inhibition of ventilation via the HO-1/CO system under normoxic conditions but CO inhibition of ventilation frequency becomes more important under hypoxic and hyperoxic conditions (Figs 2.4 and 2.5). An inhibitory mechanism under hypoxic conditions may be in place in cold acclimated goldfish (low metabolic rates) to fine tune the ventilation response to changes in O_2 and prevent fish from excessive changes in breathing when not necessary. In contrast, tonic inhibition of ventilation frequency under normoxic conditions may be necessary in warm acclimated zebrafish (high O_2 demand) to provide a greater scope for increasing ventilation when exposed to hypoxia.

5.5 UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

This thesis assessed the impact of the HO-1/CO system on ventilation and cardiac function in two species of fish, goldfish (*Carassius auratus*) and zebrafish (*Danio rerio*) experiencing changes in ambient O_2 levels. Although considerable progress was achieved, there are also new questions emerging.

In this thesis, I have relied on information from studies on mammalian carotid body to speculate on the possible mechanism of action of the HO-1/CO system in the NECs. It is well established that CO regulates carotid body discharge in mammals via regulation of intracellular Ca^{2+} stores (Yang et al., 1998; reviewed in Prabhakar and Semenza, 2012). It is important to determine whether a similar mechanism underlies the effect of CO on NEC and pacemaker function in fish. Such experiments would require Ca^{2+} imaging of NECs and cells of the pacemaker region of the myocardium (individual pacemaker cells may be difficult to isolate) from control fish and fish deficient in HO-1 (diminished capacity to produce CO).

The presence of HO-1 protein and mRNA was reported in the gills of adult European sea bass (*Dicentrarchus labrax* L.; Prevot-D'Alvise et al., 2013; Li et al., 2015). Although not yet investigated, this result suggests that the HO-1/CO system may be involved in the control of ventilation in other fish species. The effects of CO on the control of ventilation in other species warrants investigation especially based on the emerging evidence from this thesis of species-specific differences in regulation of breathing via the HO-1/CO system. Furthermore, the HO-1/CO system may be more important in regulating branchial ion transport in species lacking HO-1 in their NECs (e.g. goldfish acclimated to 25^{o}C) as unpublished results have shown the presence of HO-1 in dissociated Na^+/K^+ ATPase positive ionocytes (Appendix 2). In addition, I have also shown that low doses of exogenous CO (~25 µM CORM) inhibit both Ca^{2+} and Na^+ whole body influx in zebrafish larvae at 4 days post fertilization (Appendix 3B).

5.6 CONCLUDING REMARKS

This thesis is a body of three manuscripts investigating the effects of the HO-1/CO system in the control of ventilation and cardiac function in two species of fish, goldfish and zebrafish. It 1) characterizes the distribution of HO-1, and thus the potential for endogenous CO production, in gill and skin NECs as well as the heart of the developing zebrafish larva; 2) provides evidence that CO has an inhibitory influence on ventilation in goldfish and zebrafish but that its specific effects are temperature- and species-dependent; and 3) shows that the HO-1/CO system inhibits heart rate in zebrafish.

As mentioned above, CO is a member of a class of gaseous signalling molecules (gasotransmitters) including nitric oxide (NO) and hydrogen sulphide (H_2S) which are involved in eliciting the reflex responses to altered levels of O_2. The data presented in this thesis suggest that overall CO has an inhibitory effect on heart rate and ventilation in fish. In contrast, H_2S has a stimulatory influence on ventilation in fish. It has been shown that hypoxic hyperventilation and bradycardia can be elicited by buccal injections of NaHS (H_2S donor) in adult trout (Olson et al., 2008). Further evidence for the involvement of H_2S in control of ventilation was provided from measurements on isolated NECs from zebrafish gills. These results demonstrated that application of 10 µM of NaHS resulted in membrane depolarization, a hallmark of the NEC response to hypoxia, which was accompanied by a significant rise in intracellular Ca^{2+} levels (Olson et al., 2008; Porteus et al., 2014). Nitric oxide possesses both the inhibitory properties of CO and stimulatory properties of H_2S depending on developmental age of the fish (Porteus et al., 2015). In adult zebrafish, NO inhibits ventilation and can cause vasodilation in adult trout which is coupled with a decrease in heart rate (Eddy et al., 1999; Porteus et al., 2015). NO has a stimulatory effect on ventilation in zebrafish larvae and has been shown to cause tachycardia

(increase in heart rate) in juvenile salmonid fish (Eddy and Tibbs, 2003; Porteus et al., 2015). NO may exert its effect on cardiorespiratory function by regulating cellular Ca^{2+} stores as reported for the Indian snakehead (*Channa punctatus*) and the goldfish (*Carassius auratus*) (Biswas et al., 2011; Chang et al., 2014). The common denominator for all three gasotransmitters in response to changes in O_2 levels is regulation of cellular Ca^{2+} in peripheral chemoreceptors (NECs) which then may relay the signal to elicit either a bradycardic or tachycardic response depending on the developmental stage and species. Therefore, all three gasotransmitters may work together to fine tune the cardiorespiratory responses to changes in O_2 levels in fish. The novel results presented in this thesis set the stage for additional studies aimed at understanding how CO and other gasotransmitters modulate physiological processes not only in goldfish and zebrafish but in other species fish species exhibiting different levels of tolerance to O_2 changes in the water (e.g., hypoxia intolerant trout). Thus, this work sets the platform for further investigations aimed at understanding the challenges associated with O_2 chemosensing and eliciting the optimal cardiorespiratory response in water-breathers faced with changes in ambient O_2.

5.7 FIGURES

Figure 5.1: Overview of proposed regulatory inputs to the heart, gills, and central nervous system (CNS) with respect to heart rate and ventilatory frequency. (1) The central pattern generator (CPG) neurons of the central nervous system (CNS) generate endogenous bursting activity which regulates respiratory motorneurons (RM). The RM innervate muscles involved in control of ventilation via the V, VI, IX and X cranial nerves. (2) The CPG is innervated by feedback sensory neurons (SN) that are linked to putative O_2 chemoreceptors (neuroepithelial cells, NECs) found on the gills which may be responsible for reflex ventilation (e.g. hyperventilation during hypoxia). (3) Heart rate is controlled by the vagus nerve (VN) which receives input from the CPG. The VN may be responsible for reflex changes in heart rate (e.g. hypoxic bradycardia) and/or may cause the heart to beat in phase with ventilation via inputs from the RVM and SN. (4) Carbon monoxide produced via HO-1 may be acting in an autocrine (onto the NEC or pacemaker cell), paracrine (onto other cells, not depicted) or neurocrine (onto associated innervation) manner to modulate cardioventilatory function in fish. This figure was odified from Taylor, 2011.

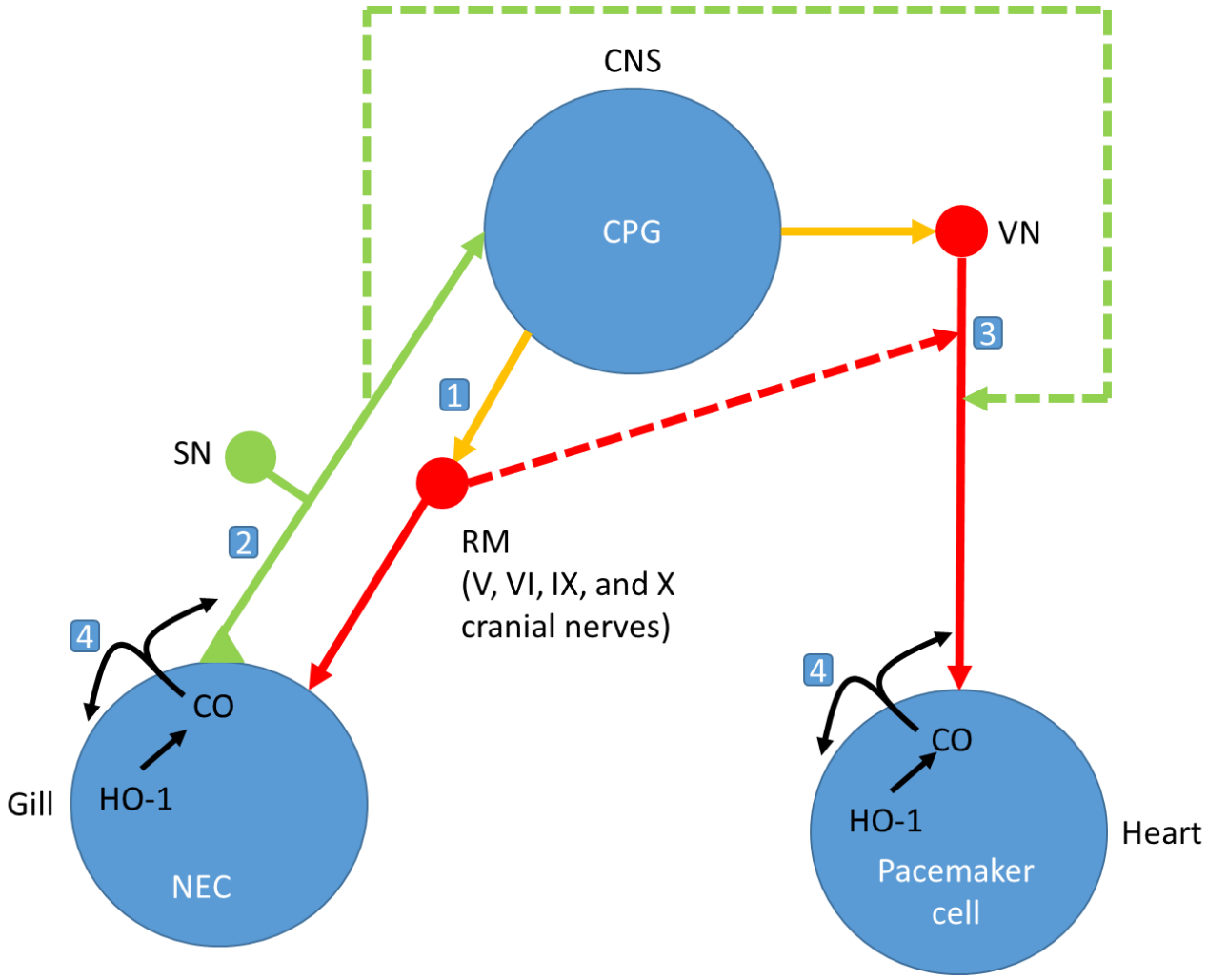
CNS
CPG
VN
3
1
SN
2
RM
(V, VI, IX, and X
cranial nerves)
4
CO
HO-1
Gill
NEC
4
CO
HO-1
Pacemaker
cell
Heart

Figure 5.1

Figure 5.2: Proposed model for O_2 sensing via the heme oxygenase-1 (HO-1)/carbon monoxide (CO) system in gill neuroepithelial cells (NECs) of a goldfish acclimated to 7ºC. (1) Hypoxia causes an increase in HO-1 activity and CO production. (2) CO inhibits L-type voltage gated Ca^{2+} channels (Ca_v) which prevents the increase in intracellular [Ca^{2+}] and (3) vesicle fusion and release of neurotransmitter (e.g. serotonin or 5-HT) onto associated afferent innervation inhibiting downstream chemotransduction and ventilation. (4) The lack of increase of intracellular [Ca^{2+}] may hinder the conductance of Ca^{2+}-activated K^+ (K_{Ca}) channels, modulating ΔV_m further contributing to the inhibition of ventilation. This figure was modified from Zachar and Jonz (2012) by incorporating information from Tzaneva and Perry (2014).

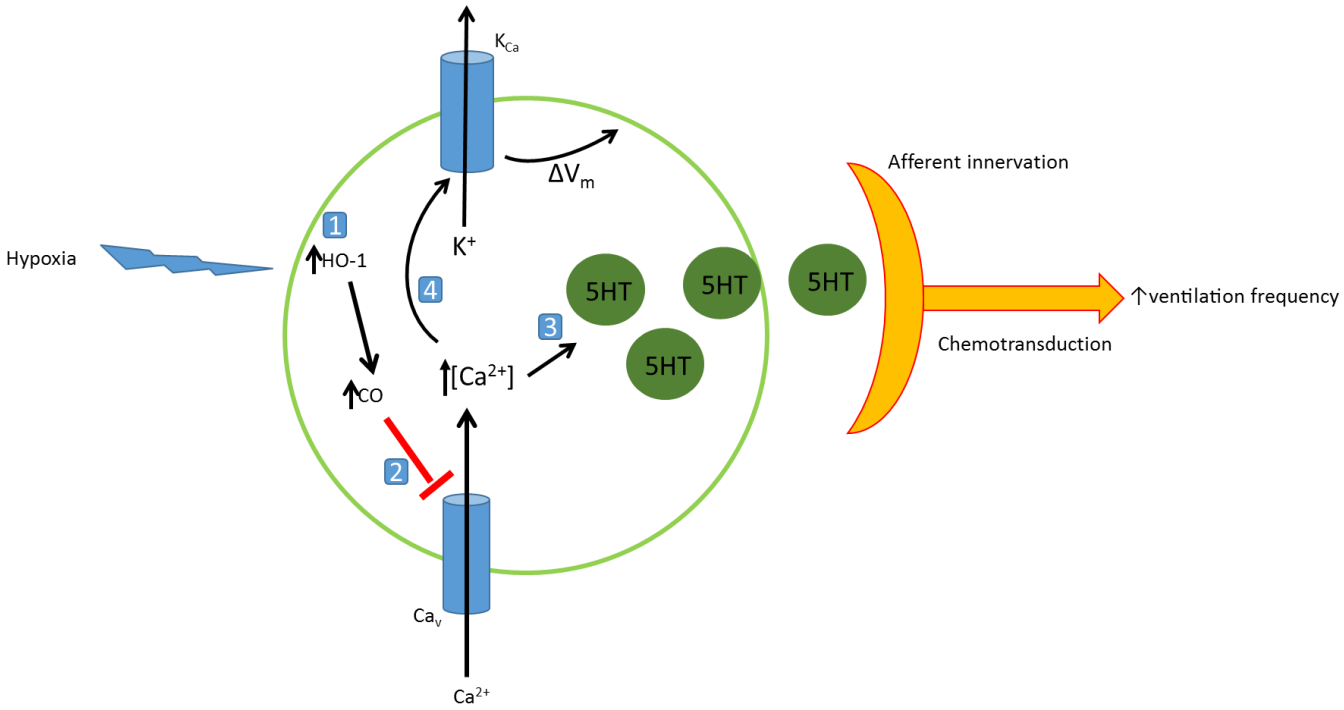
Hypoxia
1
HO-1
CO
2
4
K+
K_{Ca}
ΔV_m
3
$\uparrow[Ca^{2+}]$
Ca_v
Ca^{2+}
5HT
5HT
5HT
5HT
Afferent innervation
Chemotransduction
↑ventilation frequency

Figure 5.2

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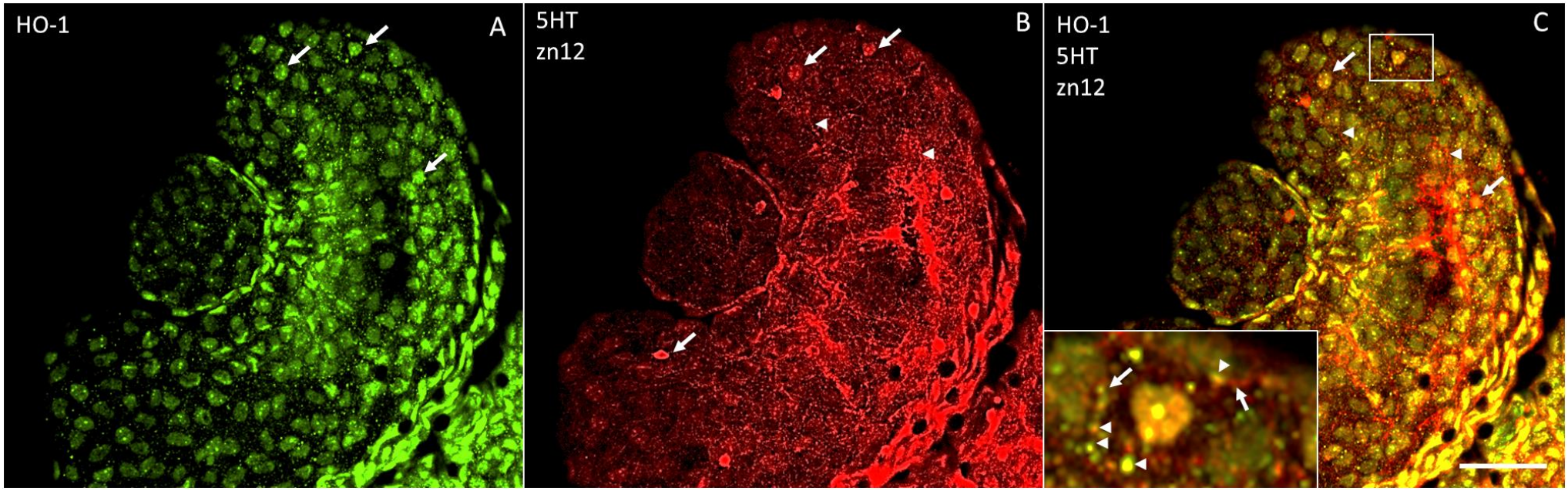
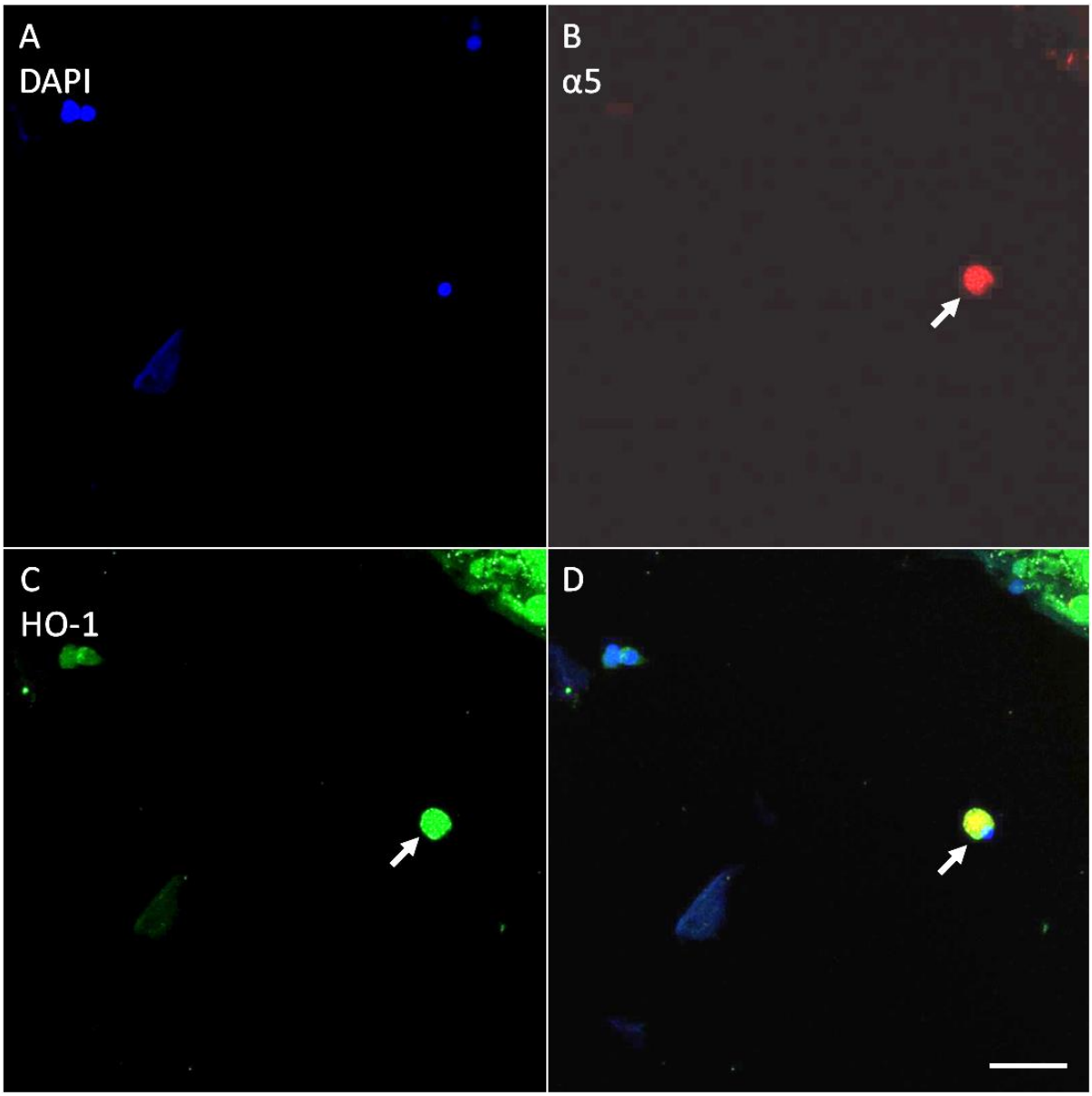
APPENDICES

Appendix 1. Confocal micrographs of the eye region of whole mount sham zebrafish (*Danio rerio*) larvae at 4 days post fertilization. Arrows represent cells immunoreactive for HO-1 (A) and 5-HT (labelling neuroepithelial cells, NECs; B and C) in sham larvae. Arrow heads in B and C indicate zn12 positive nerves. Insert in C depicts a $5\text{-HT}^{+}/\text{HO-1}^{+}$ NEC in close proximity to a zn12^{+} nerve (arrows) which is also HO-1 immunohreactive (arrow heads). Scale bar = 20 µm and applies to all panels.



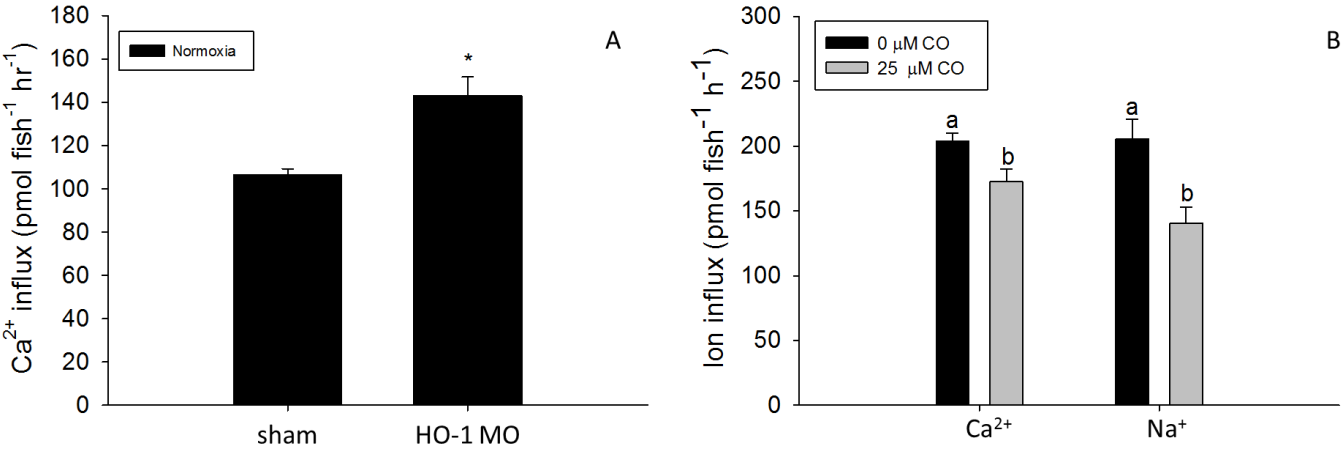
Appendix 1

Appendix 2. Confocal micrographs of dissociated cells from the gills of goldfish (*Carassius auratus*) acclimated to 25^{o}C. Panel A represents nuclei stained with DAPI. Arrows represent cells immunoreactive for α5 (Na^{+}/K^{+}ATPase containing ionocyte; B) and HO-1 (C) from the gills of control fish. Arrow in D shows colocalization of HO-1 and α5 indicating the potential for CO production in gill ionocytes. Scale bar = 10 μm and applies to all panels.



Appendix 2

Appendix 3. A: Consequence of heme oxygenase-1 (HO-1) knockdown (via morpholino injections; MO) on Ca^{2+} homeostasis in zebrafish larvae at 4 days post fertilization (N = 5-6, student's t-test $P < 0.001$). **B:** The effect of exogenously applied carbon monoxide (CO) by exposing 4 dpf sham zebrafish larvae to 0 or 25 µM carbon monoxide releasing molecule (CORM) (N = 6; One Way ANOVA $P = 0.008$).


A
Normoxia
Ca^{2+} influx (pmol $fish^{-1}$ hr^{-1})
180
160
140
120
100
80
60
40
20
0
*
sham
HO-1 MO
B
0 μM CO
25 μM CO
Ion influx (pmol $fish^{-1}$ h^{-1})
300
250
200
150
100
50
0
a
b
a
b
Ca^{2+}
Na^{+}

Appendix 4*: Validation of the HO-1 antibody*

I validated the custom made antibody used to label HO-1 in all chapters of the thesis by using immunohistochemistry, western blot analysis and HO activity assays in zebrafish. As a first validation step and to test antibody specificity, I used western blot analysis and observed a single band in zebrafish at the expected molecular weight for HO-1 (32kDa; Figs. 3.3, 4.4). Immunohistochemistry was also used to show the presence of HO-1 in branchial and skin NECs of fish (Figs. 2.1, 3.1, 4.1). Specificity of the HO-1 antibody was also checked by using a translational blocking morpholino in zebrafish larvae to block the translation of the HO-1 protein. The results clearly show the absence of the HO-1 protein on Western blots and the lack of immunofluorescence in the confocal images (Figs. 3.2, 3.3, 4.2, 4.4).

Appendix 5: Success of the HO-1 morpholino injections

The success of the HO-1 morpholino injections was measured by the disappearance of the HO-1 band on a western blot and the lack of HO-1 immunostaining in the morphant larvae. We observed a significantly diminished band at the expected size for HO-1 on a Western blot (Figs. 3.3 and 4.4) and lack of immunofluorescence in HO-1 morphant larvae (Figs. 3.2 and 4.2). We believe this is a strong indication that the HO-1 morpholino effect is specific for the targeted HO-1 gene (*hmox1*). In addition, the increase in ventilation frequency that is seen in larval fish lacking HO-1 activity is rescued by the addition of exogenous CO and lack of HO-1 activity under hypoxic conditions (Ch. 3 and 4) in the HO-1 morphants further indicates the specificity of the targeted phenotype by the HO-1 morpholino.

Appendix 6: Mechanism of action of the carbon monoxide releasing molecule-3 (CORM-3)

I specifically used carbon monoxide releasing molecule-3 (CORM-3) because it easily dissolves in water (stable at acidic pH) and produces carbon monoxide (CO) quickly (within 10 min) in comparison to other CORMs (CORM-1 and CORM-2). CORM-3 is a tricarbonylchloro(glycinato)ruthenium II molecule and can be obtained in a relatively pure form (>95%). This is a complex with ruthenium as a central atom surrounded by carbonyl groups as coordinated ligands. The release of CO is induced by ligand substitution in a water –gas shift reaction to yield 1 M CO from 1 M of CORM (reviewed in Motterlini et al., 2005). Below is the chemical structure of the CORM-3 molecule: