TIME DOMAINS OF THE HYPOXIC CARDIO-RESPIRATORY RESPONSE IN BOWFIN (*AMIA CALVA*) AND IMPLICATIONS FOR CARDIO-RESPIRATORY CONTROL IN FISH

by

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Abstract

Time domains of the hypoxic ventilatory response (HVR) are time dependent changes in ventilation that take place without changes in metabolic rate or arterial PCO$_2$. Time domains of the HVR have been described in mammals along with their underlying mechanisms. Each time domain reflects a different change in the various steps in the control of breathing. Peripheral chemoreceptors are necessary for the expression of all time domains in mammals and changes in their sensitivity (reflected in release of different neurotransmitters) are involved in several of the time domains.

I demonstrated that neuroepithelial cells (NECs) in the gills of trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) contain serotonin, but not acetylcholine or catecholamines. Acetylcholine was expressed in bipolar neurons in the filaments, and nerve bundles near the efferent filament arteries (eFA) in trout and goldfish and evidence suggests that they may play a key role in producing the HVR in these species.

In bowfin (*Amia calva*), I found five different types of serotonin containing NECs in the gills (identified as Type I to V). Three of these cell types (I - III) had the potential to be oxygen chemoreceptors. Time domains were evident in the HVR of bowfin during moderate exposure to hypoxia but only when they did not have access to air. In these fish, all three putative chemoreceptors increased in size during sustained hypoxia. The NECs found in the basal lamina near the eFA responded exclusively to hypoxaemia (by changing cell size and shape) while the other two NEC types responded to aquatic hypoxia as well.

Exposure to sustained hypoxia also caused changes within the oxygen transport cascade: the gill ventilatory sensitivity to hypoxia increased and the Hb-O$_2$ binding affinity decreased suggesting that acclimation to hypoxia in *Amia* elicits changes that both increase oxygen uptake and enhance oxygen delivery to the tissues.

This thesis focused primarily on a time domain in bowfin similar to the ventilatory acclimatization to hypoxia seen in mammals and highlights the importance of changes in both the peripheral oxygen chemoreceptors as well as in the oxygen transport cascade in establishing this time dependent change.
Preface

Part of chapter 1 has been published. Porteus, C.S., Hedrick M.S., Hicks J.W., Wang T., and Milsom, W.K. (2010) Time domains of the hypoxic ventilatory response in ectothermic vertebrates. J. Comp. Physiol. 181B: 311-333. I coordinated the review and wrote the section on fish (included in chapter 1) of the manuscript. M.S. Hedrick wrote the section on amphibians. J.W. Hicks and T. Wang wrote the sections on reptiles. W.K. Milsom wrote the introduction and conclusion. All authors contributed revisions to this manuscript.

A version of chapter 2 has been accepted for publication. Porteus, C.S., Brink, D., Coolidge, E.H., Fong, A.Y., and Milsom, W.K. The localization of multiple neurotransmitters and neuromodulators in fish gills. Acta Histochem. (In Press). I performed all of the experiments and wrote most of the manuscript. E.H. Coolidge and W.K. Milsom conceived the project and I developed the experiments further. E.H. Coolidge performed preliminary experiments and wrote the original introduction of the manuscript. Drs. D. Brink, A.Y. Fong, and W.K. Milsom provided advice on protocols, analyses and all authors contributed revisions to this manuscript.

All experiments in this dissertation were approved by the UBC animal care committee (A06-1510). All experiments performed at the University of Guelph were also approved by the UoG animal care committee (10R068).
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Figure 5.7 Hill plots for bowfin before (solid) and after exposure to sustained hypoxia (SH) either with (dotted) or without (dashed) access to air during exposure to sustained hypoxia at 0.5 (black), 1.0 (red), and 1.5 (blue) % CO2. Data points are shown only for bowfin before exposure to sustained hypoxia. Values are means ± s.e.m.

Figure 6.1 (A) Projected surface area (cell size, μm$^2$) and (B) cell shape in Type I, II, and III cells in bowfin exposed to normoxia or to sustained hypoxia (SH, 45 mm Hg for 7 days) with or without access to air. Type I cell size were large in bowfin exposed to hypoxia without access to air. Type II cell size was larger in both groups exposed to hypoxia than in normoxia. Type III cell size was larger in both groups exposed to hypoxia than in normoxia. For cell shape 1.0 would represent a perfect circle. There were no significant differences in cell shape between the groups for all three cell types. Bar
graphs not sharing the same letters are significantly different from one another (P<0.05). Values are presented as mean ± SEM. Figure 6.2 (A) Cell density (cells mm\(^{-1}\) filament), (B) cell volume (μm\(^3\)), (C) skeletal length (μm), and (D) skeletal width (μm) in Type I cells in various locations in bowfin exposed to normoxia or to sustained hypoxia (SH, 45 mm Hg for 7 days) with or without access to air. There were no significant differences between cell density, cell volume, and skeletal width of bowfin exposed to normoxia or sustained hypoxia at the different locations. Bar graphs not sharing the same letters are significantly different than one another within a certain location (P<0.05). eFA epi, efferent filament artery in the top layer of the epithelium; eFA bas, efferent filament artery near basal lamina; aFA epi, efferent filament artery in the top layer of the epithelium; aFA bas, efferent filament artery near basal lamina. Values are presented as mean ± SEM.
List of symbols and abbreviations

Δ: delta, change (e.g. ΔpH)
Δ $V_{tot}/ΔP_{O_2}$: change in ventilatory sensitivity (calculated by dividing the change in ventilation by the change in partial pressure of oxygen)
5-HT: 5-hydroxytryptophan (serotonin) or serotonin cellular marker
ABO: air breathing organ
ACh: acetylcholine
aFA: afferent filament artery
ANOVA: analysis of variance
APUD: amine precursor uptake and decarboxylation
AR: acute response
ATP: adenosine triphosphate
°C: degrees Celsius
CAM: contact adhesion molecules
Cl$: chloride
CO$_2$: carbon dioxide
cvs: central venous sinus
DA: dorsal aorta
DAPI: 4',6-diamidino-2-phenylindole (cellular marker that labels cell nuclei)
ECG: electro-olfactogram
EDTA: ethylenediaminetetraacetic acid
eFA: efferent filament artery
F: filament
f$_H$ : heart rate
GABA: gamma-aminobutyric acid
GTP: guanosine triphosphate
Hb: haemoglobin
Hct: hematocrit
HNK-1: human natural killer-1 (labels a subset of cells derived from the neural crest)
HVD: hypoxic ventilatory decline
HVD: hypoxic ventilatory response
IgG: immunoglobulin G
K⁺: potassium
KH₂PO₄: potassium phosphate monobasic
kPa: kilopascals, where 1 kPa is 0.0099 atm or 7.50 mm Hg
L: lamellae
mm Hg: millimeters of mercury, where 1 mm Hg is 0.1333 kPa or 0.0013 atm
MS-222: tricaine methanesulphonate (anaesthetic)
Na⁺: sodium
NaCN: sodium cyanide
Na₂HPO₄: sodium phosphate dibasic
NaOH: sodium hydroxide
NB: nerve bundle
NEBs: neuroepithelial bodies
NECs: neuroepithelial cells
n_H: Hill coefficient
NKA: Na⁺, K⁺, ATPase
NTS: nucleus tractus solitarius (or the nucleus of the solitary tract)
O₂: oxygen
OEC: oxygen equilibrium curve
P₅₀: partial pressure of oxygen where 50% of haemoglobin is saturated with oxygen
PBS: phosphate buffer solution
PCO₂: partial pressure of CO₂
PO₂: partial pressure of O₂
Pₖₙ₉: critical oxygen tension
N₂: nitrogen
RBC: red blood cell
S.E.M.: standard error around a mean value
SO₂: saturation of blood with oxygen
SR101: sulforhodamine 101 (activity dependent dye)
STD: short term depression
STP: short term potentiation
SV2: synaptic vesicles (synaptic vesicle marker)
t: time
TH: tyrosine hydroxylase
TXR: Texas Red hydrazide (activity dependent dye)
VAChT: vesicular acetylcholine transporter (marker for transporter of acetylcholine into storage or synaptic vesicles)
VAH: ventilatory acclimatization to hypoxia
\( V_{\text{amp}} \): gill ventilatory amplitude
\( V_f \): gill ventilatory frequency
\( V_{\text{tot}} \): total gill ventilation or total gill ventilatory product (\( V_f \times V_{\text{amp}} \))
zn-12: zebrafish neuronal marker
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Dedication
In loving memory of my aunt, Lucia Olariu, who shared my passion for science.
Chapter 1. Introduction

It is unlikely that terrestrial animals encounter environmental hypoxia unless they sojourn or live at high altitude or in deep burrows. However, hypoxia is quite common in aquatic environments as water contains about 20-40 times less oxygen than air and the diffusion of oxygen through water is 10,000 times slower than in air (Graham, 1990). A depletion of oxygen in water, or hypoxia, occurs when the oxygen consumed exceeds oxygen supply. Oxygen is replenished by convection and diffusion from the surface or by photosynthesis and is used up by the organisms (both plants and animals) present in the water as well as by decaying matter. Water bodies can also become stratified due to differences in the density of water at various temperatures and this phenomenon can intensify the hypoxia, essentially locking the hypoxic layer in place for days to months. Therefore a variety of factors can give rise to differences in the intensity and duration of hypoxia encountered in aquatic environments.

Fish can encounter brief exposures to hypoxia either spatially (in the middle of large schools of fish (McFarland and Moss, 1967)), or temporally (seasonal change in eutrophication) (Val and Almeida-Val, 1995). Intermittent hypoxia occurs in tidal pools where $P_O^2$ drops (sometime to almost anoxic levels) over a few hours and remains low until the next tide (Truchot and Duhamel-Jouve, 1980). Seasonal variations in oxygen availability are seen in shallow temperate lakes (Val and Almeida-Val, 1995) or in hypoxic upwelling events in coastal marine systems where hypoxia can persist for months (Chan et al., 2008). Moreover, the frequency and severity of hypoxic events have been increasing in both fresh and seawater due to anthropogenic factors such as nutrient loading from agricultural run off and changes in climate pattern (reviewed by Diaz and Breitburg, 2009). Although the response of fish to hypoxia has been thoroughly studied (as reviewed below), much less is known about how their response varies over time (time domains) and about the role of peripheral chemoreceptors in the control of breathing, particularly during sustained exposures to hypoxia.

The main aim of my thesis was to characterize the time domains of the hypoxic ventilatory response (HVR) in bowfin (Amia calva) and to determine some of the mechanisms that underlie ventilatory acclimatization to hypoxia. In this thesis I also attempt
to determine if time domains are evident in the heart rate response to hypoxia. The ventilatory and cardiac responses are referred together as the cardio-ventilatory response to hypoxia. My thesis addresses mechanisms related to both changes in the delivery of the oxygen through the system (changes in the oxygen transport cascade) and the importance of peripheral chemoreceptors in the hypoxic cardio-respiratory response (changes in the respiratory control system). Additionally, I assessed the similarities and differences between peripheral oxygen chemoreceptors in fish and other vertebrate classes, particularly mammals, in order to draw conclusions about the evolution of peripheral oxygen chemoreceptors in vertebrates.

1.1 Peripheral oxygen chemoreceptors

The carotid body of mammals is a small organ found at the bifurcation between the internal and external carotid arteries and is capable of detecting changes in blood PO$_2$ (Kumar et al., 2009a). The glomus cells of the carotid body are the primary oxygen sensing cells in mammals. They depolarize in response to a drop in blood PO$_2$, releasing neurotransmitters onto afferent nerves that project to the central nervous system where this response is processed. In fish, neuroepithelial cells (NECs) are believed to be the putative oxygen chemoreceptors, and are found as single cells on all four gill arches of all the fish studied to date (see Porteus et al., 2012 for a recent review). Many parallels have been drawn between the glomus cells and the NECs and it has been suggested that NECs are phylogenetic precursors of glomus cells (Milsom and Burleson, 2007). Both glomus cells and NECs respond to hypoxia by depolarizing due to closing of background K$^+$ channels (Buckler, 2007; Jonz et al., 2004). Both glomus cells and NECs have similar innervation, contain many mitochondria and dense core vesicles (reviewed by Jonz and Nurse, 2009). Glomus cells contain as many neurochemicals as the brain (Kumar et al., 2009a). Of these neurochemicals, acetylcholine and ATP are thought to be the main neurochemicals involved in oxygen chemoreception during the acute response to hypoxia (Zhang et al., 2000). In fish, recent studies have focused on serotonin and this has been used as a marker for identifying NECs in fish. However, nerve recording experiments in trout suggest that acetylcholine plays a major role in oxygen chemoreception (Burleson and Milsom, 1995), but whether NECs contain other neurochemicals remains unknown.
1.2 What are time domains of the hypoxic cardio-respiratory response and why do they need to be considered in fish?

Time domains of the HVR were first described in mammals and they refer to time dependent changes in ventilation during exposure to hypoxia and they take place independent of changes in metabolic rate or arterial PCO$_2$ (reviewed by Powell et al., 1998). Time domains of the HVR exist due to changes in ventilatory control. The specific changes in the control mechanisms underlying time domains vary with the severity and time course of the exposure of the animal. In mammals, time domains of the HVR have been defined from short exposures to hypoxia (seconds to minutes) to longer time scales (minutes to months) including adaptations associated with living at high altitude (over many generations) (reviewed by Powell et al., 1998).

In mammals the described time domains of the HVR include the acute response (AR) (Fig. 1.1A), which is the immediate increase in ventilation that takes place within seconds of the hypoxic stimulus being applied (Powell et al., 1998). This is followed by a short term potentiation (STP), which is a further increase in total ventilation or breathing amplitude. At the same time, short term depression (STD) can take place, which is a decrease in breathing frequency. These immediate responses take place over a period of seconds to a minute and are followed by time domains associated with prolonged exposure to hypoxia (Fig. 1.1 B). In mammals, the acute response is followed by hypoxic ventilatory decline (HVD), which is a decline in total ventilation associated with exposure to hypoxia over a period of minutes to hours. This is followed by a secondary increase in total ventilation termed ventilatory acclimatization to hypoxia (VAH) that takes place over a period of hours to days. Exposure of mammals to hypoxia over a period of months to years gives rise to a secondary decrease in total ventilation called hypoxic deacclimatization (HD) associated with a decreased sensitivity ($\Delta V_{\text{tot}} / \Delta P_{O_2}$) to hypoxia. Time domains associated with exposures to intermittent hypoxia have also been described (Fig 1.1C). Progressive augmentation (PA) is seen in mammals exposed to brief (5 min) bouts of intermittent hypoxia and it refers to a progressive increase in the HVR in subsequent bouts of hypoxia. Additionally, once these animals are no longer exposed to hypoxia resting ventilation slowly increases, and this has
been termed long term facilitation (LTF). Each of these time domains have particular mechanisms associated with them and these are described in the following sections.

In fish, the hypoxic ventilatory response is arguably the first, and most important, response to aquatic hypoxia as it serves to improve oxygen supply to match oxygen demand. However, in fish, the hypoxic ventilatory response has mostly been studied from the perspective of gas exchange and blood gas transport, while the control of breathing has received far less attention (Perry et al., 2009). What data there are, however, suggest that chemoreflex pathways have been conserved over evolutionary time (from fish to mammals). In the next sections I summarize what is known about these pathways and their role in time domains of the HVR in mammals and review the scientific literature in fish and present what data there are to suggest time domains of the HVR are also evident in fish.

1.3 Responses to acute hypoxia in fish

The initial response of fish to hypoxia has been reviewed recently (Perry et al., 2009), and while there is much interspecific and intraspecific variation in the response, in general most species increase total gill ventilation (hyperventilate) by increasing ventilation amplitude with no or small increases in ventilatory frequency (Perry et al., 2009). There is large inter- and intra-species variability in this regard, however, with species that normally breathe episodically increasing ventilation frequency as they switch to continuous ventilation (Glass and Soncini, 1999; Leite et al., 2007; Lomholt and Johansen, 1979; Reid et al., 2003; Vulesevic et al., 2006) and other species increasing ventilation frequency in moderate hypoxia but ventilation amplitude in more severe hypoxia (Kinkead et al., 1991). The magnitude of the response is generally a hyperbolic increase in ventilation with decreasing levels of $P_{O_2}$ (Fritsche and Nilsson, 1993; Perry and Gilmour, 2002; Perry and Wood, 1989; Shelton et al., 1986). The shape of the response generally reflects the position of the Hb-$O_2$ equilibrium curve as well as the discharge characteristics of the peripheral $O_2$ chemoreceptors (Burleson and Millsom, 1995). If the aquatic hypoxia is moderate or severe the HVR is also associated with a decrease in heart rate (bradycardia) as well as changes in gill vascular resistance that serve to increase oxygen uptake from the water (Perry et al., 2009).
In mammals the primary mechanism responsible for the AR is the increase in afferent discharge due to neurotransmitter release from peripheral chemoreceptors (the glomus cells of the carotid body). As mentioned above, in fish the peripheral chemoreceptors appear to be NECs within the gills and orobranchial cavity. A subset of these have been shown to depolarize in response to hypoxia (Burleson et al., 2006; Jonz et al., 2004) and have been found in all species of fish studied to date (Coolidge et al. 2008; Saltys et al. 2006; for a more detailed review see Table 5.7 in Perry et al. 2009). In mammals the synaptic input from the chemoreceptors is “gated” in the nucleus of the solitary tract (NTS) in the medulla such that the immediate response differs depending on the phase of the ongoing respiratory cycle during which the afferent input arrives (Eldridge and Millhorn, 1986). Whether this also occurs in fish is unknown although afferent nerve discharge from the peripheral chemoreceptors projects to a central sensory area homologous to the mammalian NTS (Sundin et al., 2003b; Turesson and Sundin, 2003).

While the ventilatory responses of fish to hypoxia may be initiated very rapidly (within a breath or two) (Bamford, 1974; Kinkead et al., 1991), typically in most studies, the time course over which aquatic hypoxia was produced was sufficiently slow that makes it hard to distinguish whether all of the short term time domains (seconds to minutes) that have been described in mammals (AR, STP and STD) also occur in fish. Although scarce, data for fish do suggest that some fish exhibit an AR and STP. For instance, in *Amia calva* rapidly exposed to moderate hypoxia (47 mmHg) at 20ºC (McKenzie et al., 1991) there was an immediate (within 1 min) increase in both respiration amplitude and frequency, followed by a plateau in the response (1-2 min), followed by a progressive increase in ventilation amplitude and frequency that developed more gradually (2-15 min). Taking into account a $Q_{10}$ of 2 to 3, the time course of this increase in ventilation in *Amia* is likely analogous to the STP observed in mammals. Until the mechanisms underlying these changes in fish are examined, however, this must remain speculative. STP is also manifest as a slow return of ventilation to original levels following return to normoxia but no studies yet have investigated the off response (the response that occurs on the return to normoxia) of fish exposed to hypoxia in any detail.
1.4 Responses to intermittent hypoxia in fish

In mammals, short episodic hypoxic exposures (1-5 min) can produce PA (increasingly greater responses to the same stimulus) and LTF (a slowly developing post-stimulus increase in breathing) (Fig. 1.1 C). Surprisingly, only a small number of studies have looked at the HVR of fish under fluctuating oxygen conditions. The length of the hypoxic exposures during each bout was generally much greater in these studies than those conducted in mammals (in order to more accurately reflect situations in the wild). None of the studies measured the changes in all components of the breathing pattern (breathing amplitude and breathing frequency), precluding any detailed assessment of time domains (Taylor and Miller, 2001). Thus, no conclusions on the responses of fish to intermittent hypoxia can be drawn at this time.

1.5 Responses to prolonged hypoxia in fish

Studies investigating changes in breathing frequency and amplitude over time periods greater than a couple of hours in fish are few, and for those that have, often the data are not presented at a fine enough scale to determine the presence of time domains of the HVR. Overall, the acute response appears to be sustained for hours to days and suggests that the acute ventilatory response to hypoxia is independent of the length of exposure (Borch et al., 1993; Florindo et al., 2006; Forgue et al., 1989; Glass et al., 1990; Thomas and Hughes, 1982a; Thomas and Hughes, 1982b). That said, there is some indication that time domains of the HVR exist in adult fish and their apparent absence in many studies may reflect a lack of resolution (see next section).

1.5.1 Hypoxic ventilatory decline (HVD) in fish

It has been proposed that the HVD that occurs in mammals with sustained hypoxia serves to conserve energy as breathing is energetically costly (Neubauer et al., 1990) and this strategy might also be beneficial for fish. The proposed mechanisms behind this decline in total ventilation (independent of changes in metabolic rate) in mammals are thought to be changes in ventilatory sensitivity and/or in central ventilatory drive. In fish, most studies show a sustained HVR, however, in a few studies ventilation decreases following a few hours of exposure to sustained hypoxia. For example, tambaqui (*Colossoma macropomum*)
exposed to severe hypoxia (10 - 20 mmHg) initially exhibited the typical acute response, but ventilation amplitude then started to decrease slowly over the next 6 h of exposure (Florindo et al., 2006; Rantin and Kalinin, 1996). Carp exposed to anoxia for 24 h at 6°C exhibited an initial five-fold rise in ventilation frequency over the first 2 h and then a ventilatory “roll-off” to initial levels over the next 14 h (Stecyk and Farrell, 2002). A similar response occurred at higher temperatures however over a shorter time course. As in the studies on tambaqui, the level of hypoxia was severe and the decrease in ventilation frequency was probably at least in part due to a metabolic depression as well as to time dependent changes in the ventilatory response. These studies are the best evidence for the presence of HVD in fish but if this is HVD, then this phenomenon that occurs within 5 to 30 minutes of hypoxic exposure in mammals takes several hours to develop in fish, a time course that could be explained by Q<sub>10</sub> effects.

Changes with similar time domains have been seen at the cellular level providing a possible explanation for the mechanisms behind these time dependent changes in ventilation. AMP-activated protein kinase (AMPK) serves as a cellular regulator in order to match ATP supply and demand within the cell. During low oxygen availability, an increase in free AMP activates AMPK which in turn inhibits anabolic processes and activates catabolic processes in the cell maintaining [ATP] stable. In goldfish, AMPK in the liver was activated after 30 min of exposure to hypoxia, then decreased partially after about 3 h and increased again after 8 h of hypoxic exposure (Jibb and Richards, 2008). AMPK activity returned to pre-exposure levels after 12 h of exposure. Therefore, it is possible that the partial decrease in total ventilation of fish exposed to hypoxia for several hours represents a reduction in energy requirement at the cellular level regulated by AMPK and this in turn translates into a reduction in ventilation.

1.5.2 Ventilatory acclimatization to hypoxia (VAH) in fish

The impact of sustained hypoxia on the HVR is variable in fish. In flounder (Platichthys flesus) gill ventilation increased two-fold after a 3 week acclimatization to hypoxia (30 mmHg) compared to fish acutely exposed to the same level of hypoxia (Kerstens et al., 1979). Similarly, channel catfish (Ictalurus punctatus) exposed to moderate hypoxia for 7 days increased resting breathing frequency and ventilatory sensitivity (Δ V<sub>tot</sub> / Δ PO<sub>2</sub>) to
hypoxia (Burleson et al., 2002). In other species, however, no changes or reduced ventilation have also been reported (Lomholt and Johansen, 1979; Nikinmaa and Weber, 1984; Vulesevic et al., 2006). These differences might reflect differences in coping strategies between species or simply could be due to differences in the time and severity of the hypoxic acclimation. No studies to date have looked at the off response (the change in ventilation on return to normoxia) of fish exposed to sustained hypoxia.

The mechanisms behind VAH in mammals involve changes in plasticity at both the chemoreceptor level and the level of the central nervous system. In zebrafish (Danio rerio) exposed to sustained hypoxia (35 mmHg, 60 days) exhibited hypertrophy and changes in morphology (Jonz et al., 2004), but not proliferation of serotonin containing NECs (Jonz et al., 2004; Vulesevic et al., 2006). These morphological changes could reflect changes in chemoreceptor plasticity in fish. In mammals, changes in plasticity of the central nervous system include changes in gene expression mediated by hypoxia inducible factor 1α (HIF-1α) (Powell and Fu, 2008). Similar changes in gene expression have also been shown in fish exposed to 10% oxygen saturation (~15 mm Hg) for up to 6 days and they likely involve regulation by HIF-1α (Gracey et al., 2001). This suggests that changes in gene expression and their regulatory mechanisms are conserved between vertebrate classes and are critical for acclimatization to hypoxia. In fish these changes reflect tissue specific and time dependent decreases in the expression of genes involved in protein synthesis, cell growth, proliferation and muscle contractility and increases in expression of genes involved in ATP metabolism and glycolysis (Gracey et al., 2001).

1.6 The hypoxic ventilatory response in fish exhibiting aquatic surface respiration or air breathing

Most studies discussed so far have been performed on obligate water breathers; that is fish that use only gills to obtain oxygen from the surrounding water. However, some fish have evolved ways to increase their oxygen supply when faced with aquatic hypoxia by exhibiting aquatic surface respiration or air breathing. Air breathing has evolved 67 times independently giving rise to a variety of air breathing organs, including modified gas bladders, suprabrachial chambers, or modifications of the digestive system (Graham, 1997). Fish employing aquatic surface respiration can use the oxygen enriched layer at the surface
of the water-air interface to obtain more oxygen during gill breathing. Facultative air breathing fish normally use their gills to supply their oxygen needs, but when water becomes hypoxic, they have the ability to come to the surface and supplement their oxygen needs by gulping air. On the other hand, obligate air breathing fish rely almost exclusively on coming to the water surface to breathe air and cannot survive extended periods of time without air breathing.

The typical response to aquatic hypoxia of both fish employing aquatic surface respiration and facultative air breathing is an initial increase in gill ventilation. However, if the increase in gill ventilation is not sufficient to meet oxygen needs then aquatic surface respiration or air breathing increases (Hedrick et al., 1994; Rantin and Kalinin, 1996). In the latter case, gill breathing is also inhibited, which is thought to prevent loss of oxygen to the hypoxic aquatic environment (Johansen, 1970). It is unclear whether obligate air breathers respond to aquatic hypoxia; in some studies these fish show an increase in air breathing (Babiker, 1979; Glass et al., 1986; Jesse et al., 1967; Johansen and Lenfant, 1967) while in other studies on the same species they show no response (Johansen and Lenfant, 1968; Perry et al., 2005; Sanchez et al., 2001). In all genera of lungfish, aerial hypoxia stimulates air breathing (Babiker, 1979; Jesse et al., 1967; Johansen and Lenfant, 1968; Pack et al., 1990; Perry et al., 2008; Perry et al., 2005; Sanchez et al., 2001) and this was also the case in terrestrialized (cacooned) *Protopterus dolloi* (Perry et al., 2008). There are, however, no studies of the time domains of the air breathing response to aquatic or aerial hypoxia in air breathing fish.

### 1.7 Aquatic hypoxia and the oxygen transport cascade

Most studies assessing the response of fish to hypoxia have focused on the effect of hypoxia on the oxygen transport system (depicted in the left panel of Fig. 1.2). As mentioned above, fish respond to hypoxia by increasing ventilation, lamellar perfusion and decreasing heart rate (bradycardia) in order to increase the supply of oxygen. However, if the hypoxia is maintained, a slew of secondary adjustments can take place in order to achieve a long term balance between ATP supply and demand. These responses include increasing cardiac output, increasing blood hemoglobin concentration, increasing Hb-O₂ binding affinity, increasing tissue extraction, increasing lamellar surface area, and decreasing the
thickness of the lamellar epithelium to name a few (reviewed by Farrell and Richards, 2009). The secondary responses mentioned above can lead to an improvement in the flow of oxygen through the system which could in turn lead to a decrease in gill ventilation. Therefore, the oxygen transport cascade cannot be ignored when considering time domains of the HVR. Additionally, fish tend to reduce metabolic rate in response to hypoxia, much more than mammals, and the fish that do this most extensively are those that have exceptional hypoxia tolerance (Hochachka and Somero, 2002). However, in mammals time domains of the HVR are independent of changes in metabolic rate and are solely due to changes in the respiratory control as described below. Therefore, in order to compare mechanisms behind time domains of the HVR in fish to those in mammals the intensity of the hypoxic exposure must not be so severe to cause metabolic depression.

1.8 Study species

The first part of my thesis focuses on the role of NECs in the acute response to hypoxia and on the role of different neurochemicals in this response. I used trout (*Onchorhynchus mykiss*) and goldfish (*Carassius auratus*) for the first part of my thesis because these fish are readily available and one is hypoxia tolerant (goldfish) while the other is hypoxia intolerant (trout). The distribution of NECs is also quite different between these species: trout only have NECs in the filament of their gill arches while goldfish have NECs in both the filament and the lamellae (Coolidge et al., 2008; Saltys et al., 2006). Initial experiments in our lab have suggested that the degree in hypoxia tolerance is related to the distribution of NECs in fish gills (Coolidge et al., 2008), but recent studies on more fish species have revealed that this might not necessarily be the case. Despite these recent findings, these two species remain the most widely studied fish and have been used here to represent different distributions of NECs in fish.

The bowfin, *Amia calva*, is the only extant species in the order Amiiformes. It has evolved over the past 100 my, but is also relatively closely related to the modern day teleost fishes. They are considered to be a “primitive” fish. Interestingly, air bladders evolved early, in the stem group after the chondrichthys but before all the osteichthys. It is still debated whether they arose for buoyancy control or air breathing although these two uses are not
mutually exclusive. Most of the primitive fishes, however, do air breathe suggesting that balancing water and aerial gas exchange is also a primitive trait.

Bowfin are found in eastern North America from southern Canada to Florida and Texas. They live in slow-moving back waters or in shallow lakes and these waters are warm in the summer and likely hypoxic. Bowfin are well suited to live in this habitat as they are facultative air breathers, which use well developed gills to obtain oxygen from the water, but when this becomes depleted or if metabolic demands rise they can come to the surface and breathe air using their well vascularized gas bladder. Furthermore, at cold temperatures these fish rarely air breathe and instead use their well developed gills essentially becoming more like obligate water breathers; at higher temperatures they increase air breathing frequency and become more reliant on air breathing. This makes bowfin ideal not only for studying time domains of the HVR but also for determining how air breathing affects time domains of the HVR. Additionally, as a facultative air breather with well-developed gills, the bowfin presents a natural study system for differentiating between internal and external chemoreceptors (see Chapter 6). The acute hypoxic response of these fish is well described (Randall et al., 1981), as is their air breathing response (Hedrick and Jones, 1993) and some aspects of the control of breathing during hypoxic exposures (Hedrick et al., 1991; Hedrick and Jones, 1999; McKenzie et al., 1991). These studies have provided the backdrop for studying time domains and aspects of the control of breathing in these fascinating animals.

1.9 Research objectives and organization of this thesis

The overall objectives of my thesis were to define the time domains of the hypoxic cardio-respiratory response in bowfin and determine the role of putative peripheral oxygen chemoreceptors, the NECs of the gill, in this response (Fig. 1.2). First I established the extent to which an exclusive focus on serotonin containing cells for analyzing changes in peripheral oxygen chemoreceptor form and function was justified in fish. As many similarities exist between the glomus cells of mammals and the NECs of fish, I hypothesized that fish NECs contain multiple neurochemicals as observed in the glomus cells of mammals. To this end I used immunohistochemical markers to label for acetylcholine and catecholamines (the two other commonly occurring neurochemicals in mammalian carotid
bodies) in trout and goldfish (Chapter 2). In this chapter, I also assessed the role of the NECs and these neurochemicals in the acute response (AR) of trout to hypoxia (Fig. 1.2).

The remainder of my work concentrated on the hypoxic cardio-respiratory response of bowfin and the role NECs play in modulating this response. Although the NECs of bowfin have been described previously, my preliminary findings indicated that more variation existed in these cells than previously described; therefore, first I had to characterize these chemoreceptors before I could determine their role in the hypoxic cardio-respiratory response (Chapter 3). Despite this discrepancy in cell types, I hypothesized that all NEC types would be innervated and would contain synaptic vesicles just like in other fishes. Next, I characterized the hypoxic cardio-respiratory response of bowfin with and without access to air in both acute and sustained moderate hypoxia (Chapter 4) (Fig. 1.2). I hypothesized that time domains would be evident in bowfin when denied access to air and that they would be similar to those seen in mammals. I also hypothesized that bowfin with access to air would not exhibit time domains because their oxygen supply would not be limited (i.e. they would not become sufficiently hypoxaemic). Additionally, I hypothesized that the heart rate response would match these ventilatory responses in both groups as cardiorespiratory variables are tightly matched in fish.

In subsequent chapters I determined some of the mechanisms behind VAH in fish. I hypothesized that VAH could arise either due to changes in the flow of oxygen through the system (changes in oxygen transport cascade) or due to changes in the respiratory control system or both (Fig. 1.2). In Chapter 5 I determined whether changes in the oxygen transport cascade took place in bowfin exposed to moderate sustained hypoxia with and without access to air. In this chapter I determined whether sustained moderate hypoxia increased the ventilatory and heart rate response of bowfin to acute progressive hypoxia. I also measured the Hb-O₂ binding affinity in bowfin blood before and after exposure to sustained moderate hypoxia. In Chapter 6 I examined the possibility that changes in the peripheral oxygen chemoreceptors themselves (i.e. changes in respiratory control) could be involved in producing VAH in bowfin. In Chapter 3 I identified three populations (types) of cells that could be involved in the reflex response to hypoxia using immunohistochemistry; therefore, in Chapter 6 I examined whether any of these cell types could be involved in the response to sustained moderate hypoxia. Additionally, I set out to determine whether I could detect
specific internal versus external chemoreceptors in bowfin, something that has yet to be done in any species of fish (Chapter 6).

**Figure 1.1** Schematic diagram of ventilatory responses of mammals to A) brief (sec to min) hypoxic exposure (AR = acute response; STP = short term potentiation; STD = short term depression); B) prolonged (min to years) hypoxic exposure (HVD = hypoxic ventilatory decline; VAH = ventilatory acclimatization to hypoxia; HD = hypoxic desensitization) and C) intermittent hypoxic exposure (PA = progressive augmentation; LTF = long term facilitation) (modified after Powell et al. 1998) ($V_{\text{amp}}$, tidal volume; $V_f$, breathing frequency, $V_{\text{tot}}$, total ventilation)
Figure 1.2 Mechanisms behind time domains of the hypoxic cardio-respiratory response involve either changes in the oxygen transport system or changes in the control of breathing. Chapter 2 assesses the role of neuroepithelial cells (NECs) in the acute response to hypoxia in trout (*Oncorhynchus mykiss*). Chapter 3 characterizes the NECs of bowfin (*Amia calva*). Chapter 4 identifies the presence of time domains in the cardio-respiratory response of bowfin. Chapter 5 determines if exposure to sustained hypoxia gives rise to changes in the ventilatory response to acute progressive hypoxia, and assesses the Hb-O$_2$ oxygen binding affinity and metabolic rate in bowfin. Chapter 6 assesses the role of sustained hypoxia on chemoreceptor morphology (size and shape) in bowfin.
Chapter 2. Distribution of acetylcholine and catecholamines in fish gills and their potential roles in the hypoxic ventilatory response

2.1 Summary

Carotid body glomus cells in mammals contain a plethora of different neurochemicals. Several hypotheses exist to explain their roles in oxygen-chemosensing. In the present study we assessed the distribution of serotonin, acetylcholine and catecholamines in the gills of trout (*Oncorhynchus mykiss*) and goldfish (*Carassius auratus*) using immunohistochemistry, and an activity-dependent dye, Texas Red hydrazide (TXR). In fish the putative oxygen sensing cells are neuroepithelial cells (NECs) and the focus in recent studies has been on the role of serotonin in oxygen chemoreception. The NECs of trout and goldfish contain serotonin, but, in contrast to the glomus cells of mammals, not acetylcholine or catecholamines. I found that acetylcholine was expressed in chain and proximal neurons and in extrinsic nerve bundles in the filaments. The serotonergic NECs did not label with the Human Natural Killer 1 antibody suggesting that if they are derived from the neural crest, they are no longer proliferative or migrating. Furthermore, I predicted that if serotonergic NECs were chemosensory, they would increase their activity during hypoxia (endocytose TXR), but following 30 min of hypoxic exposure (45 mm Hg), serotonergic NECs did not take up TXR. Based on these and previous findings I propose several possible models outlining the ways in which serotonin and acetylcholine could participate in oxygen chemoreception in completing the afferent sensory pathway.

2.2 Introduction

The acute hypoxic ventilatory response in teleost fish (Randall, 1982), can be initiated by changes in the level of external (water) or internal (blood) oxygen. These reflex responses likely arise primarily from the stimulation of chemoreceptors in the gills (Dunel-Erb et al., 1982; Sundin et al., 1995; Wilson and Laurent, 2002). Evidence suggests that the chemoreceptors involved in these reflexes are neuroepithelial cells (NECs) containing dense core vesicles in which serotonin (5-HT) is the major monoamine (Bailly et al., 1992; Coolidge et al., 2008; Jonz and Nurse, 2003; Sundin et al., 1998; Zaccone et al., 1992). Serotonergic NECs are situated where they are exposed to both efferent branchial blood flow
and water flow, and are innervated by neurons that project to the nucleus tractus solitarius (NTS) in the medulla (Bailly et al., 1992; Dunel-Erb et al., 1982; Jonz and Nurse, 2003). Patch clamp recordings from isolated serotonergic NECs reveal that they express O₂ sensitive background K⁺ channels similar to glomus cells from mammalian carotid bodies (Jonz et al., 2004). These data suggest that hypoxia triggers serotonin release from NECs onto afferent nerves that project to the central nervous system to produce the hypoxic cardio-respiratory responses (Jonz et al., 2004).

The NECs of fish and the oxygen-sensitive glomus cells of the carotid body of terrestrial vertebrates are comparable in many respects. The vascular supply to the first gill arch in fish and the carotid artery on which the carotid body sits share the same embryonic origin (Milsom, 1998). Both the first gill arch and the carotid body are innervated by the glossopharyngeal nerve (CN IX) (and vagus nerve (CN X) in some species) (Gonzalez et al., 1994; Sundin and Nilsson, 2002). Furthermore, both fish gill NECs and carotid body glomus cells share a common chemoreceptive mechanism involving an O₂-sensitive background K⁺ channel (Jonz et al., 2004). The hypoxic response of gill NECs and carotid body glomus cells, are similar in that they depolarize. Likewise, hypoxia results in increasing afferent nerve discharge (Burleson and Milsom, 1993; Jonz et al., 2004; Milsom and Brill, 1986). In addition, recent experiments suggest that, like the carotid body glomus cells, some of the NECs in fish gills are multimodal sensors (Garcia-Fernandez et al., 2007; Gonzalez et al., 1994). Carotid body glomus cells are sensitive to changes in O₂ partial pressure (PO₂), CO₂ partial pressure (PCO₂) and/or pH (Gonzalez et al., 1994). In comparison, in vitro some serotonergic NECs respond to changes in both PO₂ and PCO₂ (Qin et al., 2010), while others respond to changes in ammonia (Zhang et al., 2011).

One striking difference between the fish gill NECs and carotid body glomus cells is that all the glomus cells are host to many neurotransmitters, such as dopamine, adrenaline, noradrenaline, acetylcholine, substance P, GABA, serotonin, and adenosine (Prabhakar, 2000). Current evidence suggests that acetylcholine and ATP are co-released as the cooperative neurotransmitters, and are the primary neurotransmitters released during the acute hypoxic response (Nurse, 2005; Zhang et al., 2000), while the other neurochemicals, such as catecholamines, act as inhibitory and excitatory neuromodulators (Nurse, 2005;
Prabhakar, 2006; Schweitzer and Wright, 1938). In contrast, current evidence suggests that fish gill NECs only synthesize serotonin.

Although serotoninergic NECs appear to be the putative chemosensory cells in fish gills (Bailly et al., 1992; Coolidge et al., 2008; Jonz and Nurse, 2003; Sundin et al., 1998; Zaccone et al., 1992), the physiological evidence supporting this is equivocal (Burleson and Milsom, 1995). In trout (Onchorhynchus mykiss) gill nerve recordings from single afferent nerve fibres of the glossopharyngeal nerve (IX) sensitive to hypoxia, serotonin evoked a relatively weak excitation followed by an inhibition (Burleson and Milsom, 1995). In contrast, acetylcholine acting via nicotinic receptors, consistently produced strong chemoreceptive nerve discharge akin to stimulation by hypoxia or NaCN (Burleson and Milsom, 1995). These data suggested that acetylcholine was either the primary chemoreceptive neurotransmitter present in the first gill arch of trout or cholinergic synapses were strongly recruited in response to hypoxic stimuli. Furthermore, cells expressing a synaptic vesicle protein SV2, but not serotonin, have been identified in the gills of zebrafish (Jonz et al., 2004), as well as other fish species (Coolidge et al., 2008; Saltys et al., 2006). Moreover, SV2 positive cells proliferated (increased in density) after exposure to sustained hypoxia (Jonz et al., 2004) suggesting that some O₂ responsive sensory cells express neurochemicals other than 5-HT.

Given the strong electrophysiological and functional evidence that neurotransmitters other than serotonin may act as primary neurotransmitter in the HVR in fish gills, I hypothesized that fish NECs contain multiple neurochemicals as observed in the glomus cells of mammals. I examined the expression pattern of specific markers of acetylcholine, catecholamines (tyrosine hydroxylase) and serotonin in the gills of rainbow trout (O. mykiss) and goldfish (Carassius auratus), two species for which the hypoxic ventilatory response has been well described. Furthermore, carotid body glomus cells are derived from the neural crest (Pearse et al., 1973), while the developmental origin of NECs remains unknown. Therefore, I also aimed to test if NECs are derived from the neural crest by using a human natural killer (HNK-1) antibody. In addition, the involvement of NECs in the acute response (AR) to hypoxia was examined using incorporation of activity-dependent dyes during acute exposure to hypoxia. Turtle brain neurons that were more active labelled more strongly with Sulforhodamine 101 (SR101) because the dye was taken up pre-synaptically in an activity
dependent manner (Keifer et al., 1992). SR101 is a highly charged fluorescent molecule that cannot cross cell membranes but is incorporated into cells via endocytosis in proportion to neurotransmitter recycling (Keifer et al., 1992). SR101 also accumulates in lysosomes (Wang and Goren, 1987). Unlike other cells that reduce their activity during hypoxia, chemosensory cells become more active during hypoxia, increasing the amount of neurotransmitter recycling (Kumar et al., 2009b). Therefore, we predicted that if NECs are indeed chemosensory, they would increase their activity during hypoxia and would take up more SR101 while other cells in the gills would decrease their activity in hypoxia. In our experiments we used both SR101 and Texas Red hydrazide (TXR), a fixation stable version of the SR101 and was therefore compatible with immunohistochemical identification of serotonin.

2.3 Materials and methods

2.3.1 Animals

Adult rainbow trout (Oncorhynchus mykiss (Walbaum), 4-380g) and goldfish (Carassius auratus (Linnaeus), 2-54 g) were kept in outdoor, freshwater flow-through, aerated tanks in the Zoology Aquatic Facility at the University of British Columbia.

2.3.2 Tissue preparation

Fish used for tissue analysis were randomly selected and killed by an overdose of benzocaine followed by a sharp blow to the head. At least 6 trout (4-380g) and 6 goldfish (2-54 g) were used for each combination of immunohistochemistry labels. Trout were perfused with heparinized (100 IU/ml), ice-cold phosphate-buffered solution (PBS) containing (in mM): NaCl, 137; Na2HPO4, 15.2; KCl, 2.7; KH2PO4, 1.5; buffered to pH 7.8 with 1M NaOH (Jonz and Nurse, 2003). Trout were perfused via the bulbous arteriosus using a blunt 25 gauge needle and a 20cc syringe until the gill filaments appeared clear. The 1st gill arch, head kidney, pseudobranch and nodose ganglion were removed and fixed in 4% paraformaldehyde in PBS overnight at 4°C. In small trout (<40g) and all the goldfish the first gill arches were excised without first perfusing the whole animal (due to their small size) and washed in ice-cold PBS and individual gill arches were syringe-perfused through the brachial arteries and
veins with heparinized PBS (200 IU/ml) until filaments appeared clear, and subsequently fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight. Tissues were then rinsed in PBS, cryoprotected in a 30% sucrose solution, and frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific) at -80°C. Blocks were sectioned longitudinal to the gill filament at 10-12 μm thick sections using a cryostat (Leica CM3050 S, Leica Microsystems, Germany) and mounted on Superfrost® plus slides (VWR International) for immunohistochemistry. Longitudinal serial sections were cut and every section was inspected throughout the length of individual filaments. Cross sections along the length of the filament in 6 trout and 6 goldfish were also examined to supplement findings from longitudinal sections. Slide-mounted sections were used right away or stored at -80°C until further processing.

2.3.3 Immunohistochemistry

Slide-mounted gills were washed in PBS and blocked in either 10% normal goat or donkey serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, Ontario), depending on the host-species of the secondary antibody, for one hour. Primary antibodies (either individually or in combination) were diluted in a permeabilizing solution (PBS/0.1% Triton X-100/ 3% normal goat or donkey serum) according to the optimal dilutions detailed in Table 2.1, and set on the slides to incubate overnight at room temperature, in a humidified chamber. Following incubation with primary antibodies, the slides were again washed in PBS. The slides were then incubated with fluorescently labeled secondary antibodies diluted in PBS containing 0.1% Triton-X and 3% normal goat or donkey serum (Table 2.1) and left to incubate at room temperature for 2 hours in darkness, in a humidified chamber. Following a final wash with PBS, a cover-slip was placed on each slide using Vectashield with DAPI (Vector Laboratories, Burlington, Ontario) to prevent photobleaching and to label cell nuclei. Slide mounted tissue was stored in darkness at 4°C until viewed under a fluorescent light microscope. Control labeling experiments excluded incubation with primary antibodies which demonstrated no non-specific labeling of any of the secondary antibodies. In addition, normal serum (IgG) from primary antibody host species was used (at an equivalent protein concentration) to test for non-specific labeling due to reaction with the primary host serum. Positive controls were performed for tyrosine
hydroxylase (TH) primary antibody using the head kidney (for chromaffin cells) (Bernier and Perry, 1997) (Fig. 2.1), and the nodose ganglion for acetylcholine labeling (Gauda et al., 2004) (Fig. A1 in Appendix).

2.3.4 Microscopy

Some immunolabelled tissue sections were observed using an epifluorescence microscope (Axioplan 2, Zeiss, Jena, Germany) using a G365, HQ470 or BP546/12 excitation filter and a LP420, HQ525/50 or LP590 emission filter to detect DAPI, Alexa 488 and Alexa 594 respectively. Images were digitally captured using a QImaging camera (Model Retiga 1300) and QCapture image capture software (Version 2.95.0). All other images were captured using a confocal scanning system (Pascal 2, Axioskop X, Zeiss, Jena, Germany) equipped with argon (Ar) and helium-neon (He-Ne) lasers emitting at 488 nm and 543 nm, respectively; and using LP560 and BP 505-530 filters for detection of Alexa 488 and Alexa 594. Z-stacks of 9-33 optical sections and 0.32-0.88 µm apart were captured using EC Plan-Neofluar 40x/1.30 NA Oil DIC M27 and Plan-Apochromat 63x/1.4 NA Oil DIC objectives. Images were adjusted for brightness and contrast using ImageJ. Additional images were captured using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with solid state lasers emitting at 405 nm, 473 nm and 559 nm. Z-stacks of 6-13 optical sections and 0.68-0.82 µm apart were captured using the 60x oil immersion objective of this confocal microscope.

2.3.5 Sulforhodamine 101/Texas Red hydrazide experiments

In order to determine the activity of candidate NECs during hypoxia, we used two activity dependent dyes: Sulforhodamine 101 (SR101, Sigma) and Texas Red hydrazide (TXR, Molecular Probes), a paraformaldehyde fixable analog of the SR101 activity dye (Nimmerjahn et al., 2004). Dye incorporation via synaptic vesicle recycling in gill tissue was assessed from fish that were exposed to acute (30min) normoxia and hypoxia. SR101 and TXR (a derivative of Rhodamine) are activity-dependent dyes that are differentially taken up by cells actively engaged in secretion and recycling of neurotransmitters (Nimmerjahn et al., 2004; Reichel et al., 2008). These experiments were only done in trout as goldfish were too small to cannulate. Trout were anesthetized with benzocaine (0.2 mmol l⁻¹ final
concentrations of p-aminobenzoate) and instrumented with a dorsal aorta cannula (PE90) via the mouth (Soivio et al., 1980). Fish recovered overnight (~20 h) in a black box, in an aerated recirculating system. The next day the fish were assigned to either a normoxic or hypoxic group (n=5 per group, 180-380g). Normoxic fish were injected via the DA cannula with sulphorhodamine 101 (SR101, Sigma) at a dose of 5-10 mg kg\(^{-1}\) (Keifer et al., 1992) diluted to 1 mg ml\(^{-1}\) in Cortland’s saline (modified from Wolf, 1963) while exposed to normoxic water. Hypoxic fish were exposed to 30% O\(_2\) saturated water (~45 mm Hg) and SR101 was injected when water oxygen levels reached 50% saturation (~75 mm Hg). In preliminary experiments the amount of SR101 incorporated into trout gills following exposure to this level of hypoxia for 30 to 120 min showed no qualitative differences in dye uptake. This value is consistent with previous studies in fish that indicated that 30 min was the ideal time for maximal dye incorporation (Reichel et al., 2008). Consequently, 30 min after SR101 was injected the fish were sacrificed by an overdose of benzocaine followed by a sharp blow to the head. These experiments were repeated in two trout per group (300-500g) using Texas Red hydrazide (TXR, 100µM) as described for SR101.

Tissue preparation (1\(^{st}\) gill arch only) and immunohistochemistry were performed as above with the following modification to the protocol. For the SR101 experiments, the tissue blocks were immediately sectioned and mounted on Superfrost® plus slides, the slides were rinsed 2 x 5 min in PBS and slides cover-slipped using Vectashield with DAPI. This was done quickly to minimize dye loss. The tissue was imaged in two steps using a widefield epifluorescence microscope (Axioplan 2, Zeiss, Jena, Germany) and camera (details above) and the location of the images on the slides was logged in order to take pictures of the same location after labeling with antibodies. At least 6 pictures of SR101 labeled cells were taken per individual fish from filaments where NECs and innervation were expected to occur. After the first round of imaging, coverslips were carefully removed, and the slides were rinsed 2 x 5 min in PBS. To achieve double immunolabeling of the SR101 treated sections, immunolabeling with primary antibodies was then performed as described above. Slides were imaged again in the same location and the images were aligned and superimposed using Adobe Photoshop 7.0.1. For the TXR experiment, tissue preparation and immunohistochemistry were performed as above except all imaging was performed.
simultaneously, after immunolabeling, using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with solid state lasers emitting at 473 nm and 559 nm.

2.4 Results

2.4.1 Control experiments for immunohistochemical procedures
In trout, chromaffin cells in the head kidney were clearly labeled for tyrosine hydroxylase (TH, Fig. 2.1A), and neurons in the nodose ganglion were also labeled for vesicular acetylcholine transporter (VACHT) (Fig. A1 in Appendix). VACHT consistently labeled the large neuronal cell bodies in the nodose ganglion in both species of fish (data not shown). In all immunohistochemical procedures, omission of the primary antibody resulted in no discernible labeling. Single labeled sections showed no qualitative differences from multiple labeled sections for any studied antigen. These results confirm that the antibodies and techniques used were highly specific and effective in these species. There were no visible differences in the labeling and distribution between large and small trout.

2.4.2 Catecholamine expression patterns in fish gills
Immunohistochemical labeling for tyrosine hydroxylase (TH) did not reveal any structures in the first gill arch of either trout or goldfish. In contrast, TH-immunoreactive fibres were found sparsely distributed in the filament in the pseudobranch of trout (Fig. 2.1B).

2.4.3 Distribution of serotonin in fish gills
In both trout and goldfish, relatively large serotonergic NECs were distributed along the length of the filaments and an example is shown in a filament from the ventral edge of gill arch (Fig. 2.2 and Fig. 2.3 bottom panels). In trout, the serotonergic NECs were relatively large (~15 µm) and some had one or two small projections (Fig. 2.2 bottom right) and were more concentrated towards the filament tips. The serotonergic NECs in goldfish were also large (~12 µm) and concentrated toward the distal end of the filament (Fig. 2.3 bottom right). In goldfish, but not in trout, the tips of the lamellae contained small NECs about half the size (6-7 µm) of those found in the filament (Fig. 2.3 middle and top panel).
These cells are distributed along the entire lateral edges of the lamellae and also appear in sections taken at the level of the central venous sinus (Fig. 2.3 top panel).

A few bipolar serotonergic neurons, previously described as chain neurons (Jonz and Nurse, 2003), were found in a deeper plane of section, on the medial side of the efferent filament artery of both trout and goldfish (Fig. 2.2 top panel and Fig. 2.3 middle panel). In both species, these chain neurons were of similar size to serotonergic NECs in the filament and evenly distributed along the entire length of the filament. Their projections extended exclusively along the filament, and never into the lamellae.

### 2.4.4 Immunohistochemistry of NECs

Contrary to expectations, NECs containing 5-HT never colabelled with VACChT in either the filament of trout (Fig. 2.4A) or the filament and lamellae of goldfish (Fig. 2.4C and D). However, VACChT consistently labelled bipolar neurones running along the length of the filament in both trout (Fig. 2.4B) and goldfish (Fig. 2.4E) (see next section). Notably, serotonergic NECs found in the filament of trout and goldfish and the lamellae of goldfish did not colabel with the HNK-1 antibody (Fig. 2.5 A, E and F), however nerve fibres in their vicinity often did (Fig. 2.5 A and E).

### 2.4.5 Immunohistochemistry of neuronal structures

VACChT immunoreactivity was found in nerve bundles in the gill filaments of both trout and goldfish. The HNK-1 antibody also labelled the outside of the extrinsic nerve bundle expressing VACChT in both trout (Fig. 2.5C and D) and goldfish (Fig. 2.5H and I). The extrinsic VAChT-immunoreactive nerve bundles were also co-localized with the neuronal marker (zn-12), but only formed a subpopulation of the extrinsic nerve bundle (Fig. 2.6A and B). These fibres were found primarily running on the medial side of the efferent filament artery with some fibres running within the central venous sinus as seen in cross section in both species (Fig. 2.6C and D). These nerve bundles arose extrinsic to the gill, as no VACChT+ cell bodies were present within the gills, suggesting they were part of the extrinsic innervation of the gill (Fig. 2.7A).

As mentioned above, all serotonergic chain neurons in both trout and goldfish colabelled for 5-HT and VACChT (Fig. 2.4B and E respectively, and Fig. 2.7B). These
neurons also labelled with the HNK-1 antibody (Fig. 2.5B, C, G, and H) and projected to the base of filaments, more specifically the efferent filament artery (Fig. 2.7C). Additionally, neurons at the base of efferent filament artery previously described as proximal neurons also labelled for both 5-HT and VAChT (Fig. 2.7D).

2.4.6 Cell activity during acute hypoxia

Both SR101 and TXR were used to evaluate the activity of candidate NECs during exposure to hypoxia and similar results were found using both compounds. As predicted, in general, there were more TXR/SR101 positive cells in normoxia than in hypoxia, indicative of cell activity being suppressed during hypoxia. In trout exposed to normoxia, the activity dependent dye TXR was taken up by many types of cells including the mitochondrion rich cells (labelled with NAK121) (Fig. 2.8D). Surprisingly, serotonin-immunoreactive NECs never took up TXR (or SR101) in either normoxia (Fig. 2.8A) or hypoxia (Fig. 2.8E). However, in one trout exposed to hypoxia a few serotonergic NEC positive cells were closely associated with nerve fibres that took up SR101 in hypoxia but not normoxia (data not shown). Neither bipolar neurons nor the extrinsic nerve bundle took up the activity-dependent dye (Fig. 2.8). As expected, mitochondrion rich cells reduced their activity in hypoxia and did not take up TXR (Fig. 2.8H).

2.5 Discussion

The aim of this study was to determine and compare the distribution of serotonin (5-HT), catecholamines, and vesicular acetylcholine transporter (VAChT) in putative chemosensing cells in the gills of trout and goldfish and, notably, to determine if neuroepithelial cells (NECs) contained multiple neurochemicals, as do glomus cells in mammals. One major and unequivocal result of our study was that no NECs colocalized the markers for 5-HT, catecholamines and ACh. More surprisingly, serotonin containing NECs that have been proposed as putative oxygen chemoreceptors did not take up the activity dependent dye TXR in trout when exposed to acute hypoxia. We also note that the serotonergic NECs did not label with the HNK-1 antibody. Taking this and previous results into consideration we propose different scenarios for oxygen chemoreception in fish gills.
2.5.1 Critique of methods

The monoclonal antibodies for HNK-1 and zn-12 both bind to one, highly conserved epitope on multiple contact adhesion molecules (CAM) that are present in cells that grow throughout the animal's life, and in regenerating neurons (Peterson et al., 2001; Schmidt and Schachner, 1998). HNK-1 antibodies have been previously used as a marker for migrating neural crest cells in fish (Sadaghiani and Vielkind, 1990) and other vertebrates (Reyes et al., 2010). The HNK-1 antibody also labels peripheral nerve fibres in fish which are derived from the neural crest as are all peripheral neurons (Metcalfe et al., 1990). However, HNK-1 does not label all neural crest cells as has been recently shown in humans (Betters et al., 2010); and some cells no longer express this epitope on the surface once differentiated (Metcalfe et al., 1990). In the present study the HNK-1 antibody labeled peripheral neurons in adult fish, in a pattern highly reminiscent of zn-12 labeling. Since the zn-12/HNK-1 epitope is conserved, one explanation for this observation is that the HNK-1 antibody is simply binding to cells previously described as zn-12 immunoreactive cells. Our salient observation is that HNK-1 did not label NECs and this indicates that either these cells are not derived from the neural crest, or they are no longer proliferating and therefore not expressing the cell surface marker labeled by the HNK-1 antibody. It has been previously suggested that NECs are not derived from neural crest cells but perhaps from epithelial cells just like the neuroepithelial bodies found in the lungs of mammals (Dunel-Erb et al., 1994).

Sulforhodamine 101 (SR101) is a small, highly-charged molecule that cannot cross cell membranes, but can be taken up via endocytosis by cells that are neuroactive (i.e. undergoing neurotransmitter recycling). Because chemosensory cells increase their activity during hypoxia (Kumar et al., 2009b), we expected NECs to take up this dye in fish exposed to 30 min of hypoxia. Contrary to expectations NECs did not take up the dye suggesting that these cells were not highly active during hypoxia. We initially used a non-fixable version of the dye and quickly fixed, froze, sliced and imaged the tissue to minimize dye loss. We further confirmed our results using a fixable version of the dye, Texas Red hydrazide (TXR). Similarly, we failed to find NECs labeled with TXR, although it is possible that the sulforhodamine dyes fail to label NECs regardless of their synaptic activity (Nimmerjahn et al., 2004).
2.5.2 Putative neuroepithelial cells (NECs)

One question that must be addressed in interpreting these findings is “how does one identify NECs in fish gills?”. In general, NECs are derived from APUD (amine precursor uptake and decarboxylation) cells, a diffuse group of cells, originating in the neural crest, that share certain cytochemical and ultra-structural characteristics (Pearse, 1969). APUD cells produce substances such as epinephrine, norepinephrine, dopamine, serotonin, enkephalin, somatostatin, neurotensin, and substance P and are characterized by the presence of large, electron dense, and cytoplasmic vesicles (Cutz, 1982). One would expect, therefore, that NECs should contain at least one amine precursor neurochemical (i.e. serotonin), synaptic vesicles and label with a neural crest marker. In previous studies on fish, labeling for SV2 and serotonin were used as identifying markers for NECs. SV2 is a membrane glycoprotein found only in the secretory vesicles of neural and endocrine cells in vertebrates (Bindra et al., 1993). SV2 is required for normal calcium-regulated secretion of hormones and neurotransmitters. Our data suggest, however, that serotonergic NECs may not be derived from the neural crest or that if they are, these cells have differentiated and have down-regulated this cell surface marker.

Given what is known of the reflex responses of fish to hypoxia, we would expect to find putative O₂-chemosensing cells in locations that detect changes in PO₂ in blood, water, or both. Our data are consistent with those of previous studies showing that serotonin containing NECs are present near the efferent filament artery (Dunel-Erb et al., 1982; Jonz et al., 2004; Jonz and Nurse, 2003) as well as on the lamellae of most fish species (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al., 2006), positions consistent with this prediction.

Based on this evidence, serotonin containing cells that fit the description of NECs remain the primary candidate for the chemosensing cells involved in cardio-respiratory reflexes. In our study, as in several previous studies, 5-HT containing cells were abundant in the first gill arch located on gill filaments, as described in the literature (Coolidge et al., 2008; Jonz et al., 2004; Jonz and Nurse, 2003; Saltys et al., 2006). The serotonergic NECs in the present study could be divided into two groups: larger NECs (10-15 μm) located within the filament, usually more concentrated towards the distal ends that have been found in all species examined to date (Coolidge et al., 2008; Dunel-Erb et al., 1982; Jonz et al., 2004;
Saltys et al., 2006), and small lamellar NECs (6-7 µm) that have been found in the lamellae of most species such as zebrafish (Danio rerio), traira (Hoplias malabaricus), and trairaõ (Hoplias lacerdae) but not in trout and the mangrove rivulus (Kryptolebias marmoratus) (Coolidge et al., 2008; Dunel-Erb et al., 1982; Jonz et al., 2004; Regan et al., 2011; Saltys et al., 2006).

The large serotonin containing NECs are situated where they could be exposed to both efferent branchial blood flow as well as water flow (Fig. 2.2 and Fig. 2.3). This was true in both trout and goldfish in our study, as it was for various species in previous studies (Bailly et al., 1992; Dunel-Erb et al., 1982; Jonz and Nurse, 2003). In culture, some of these larger filament NECs (~60%) responded to hypoxia by depolarization involving a background K⁺ conductance just as do glomus cells from mammalian carotid bodies (Burleson et al., 2006; Jonz et al., 2004). These cells are also innervated by both intrinsic and extrinsic neural processes (Fig. 2.4, Fig. 2.5, Fig. 2.6, and Fig. 2.7) (Jonz and Nurse, 2003), thus, implicating them as primary oxygen sensors. If NECs were primary oxygen sensors it was predicted that these cells would increase activity in hypoxia just as other oxygen chemosensing cells do (Kumar et al., 2009b). Interestingly, however, while it has been shown that serotonergic NECs undergo hypertrophy in sustained hypoxia in zebrafish (Jonz et al., 2004). In the present study, these cells in trout did not take up the activity-dependent dye TXR during acute hypoxia suggesting that they are not O₂ chemosensory. As expected of non-chemosensory cells, the activity-dependent dye was taken up by the mitochondrion rich cells in normoxia but not during hypoxia showing that 30min was sufficient for dye incorporation and that the activity of these cells was reduced in acute hypoxia. Furthermore, the 5HT-immunoreactive NECs did not co-label for the zn-12/HNK-1 shared epitope. While this would suggest that 5 HT-immunoreactive cells are not active in hypoxia and are not neural crest derived, this may also simply reflect the lack of specificity of our markers (see Critique of methods section). The small serotonergic NECs found in the lamellae of most fish species (Fig. 2.3 middle and top panels) are also innervated by the extrinsic nerve bundle (Jonz and Nurse, 2003). Due to their smaller size, these cells have never been recorded from in patch clamp experiments (Jonz et al., 2004), therefore, it is currently not known if they respond to hypoxia by depolarizing or releasing 5-HT. In goldfish, however, during gill remodeling events after acclimatization to cold water (7ºC),
these cells relocated to the outer edges of the lamellae and remained in contact with the environment consistent with them playing a primary chemosensory role (Tzaneva and Perry, 2010). However, in the current study these cells also did not take up the activity dependent dye in hypoxia and did not label with the HNK-1 antibody, again raising the question of the origin of NECs.

While there has been much recent focus on the possible role of the 5-HT containing NECs as chemosensors involved in cardio-respiratory reflexes in fish, it should be remembered that these cells may also play a paracrine or neuro-endocrine role as chemosensors involved in vascular control. In trout, injections of 5-HT cause vasoconstriction of the efferent filament artery and stop blood flow to the distal part of the filament, just as does hypoxia (Sundin et al., 1995). The effect of hypoxia could be due to a direct effect of hypoxia acting on the NECs, causing release of 5-HT onto neighbouring vascular muscle cells (paracrine effect), or due to reflex stimulation of the NECs to release 5-HT onto neighbouring vascular muscle cells by an efferent pathway (neuroendocrine effect).

A subset of NECs (featuring synaptic vesicles detected with anti-SV2) that are 5-HT negative have also been reported in most species of fish studied to date (Coolidge et al., 2008; Jonz et al., 2004). These cells are innervated (Jonz and Nurse, 2003) and increase in number after exposure to sustained hypoxia (Jonz et al., 2004), however, the neurochemical phenotype remain unknown. I anticipated that these NECs would contain catecholamines and/or ACh but found no evidence for either within the gill filaments and lamellae of either trout or goldfish. I did, however find evidence of bipolar neurons immunoreactive for VAChT in both trout and goldfish (see below). The chemical phenotype of the non-serotonergic, synaptic vesicle containing cells in fish gills remains to be determined.

2.5.3 Gill innervation

Although neither the marker for catecholamine synthesis (TH) nor that for VAChT were found in serotonergic NECs, they both labelled nerve fibres in the gill, as did the marker for 5-HT, as previously described (Jonz and Nurse, 2003) (Fig. 1 and Fig. 7). TH was found in nerve fibres in the pseudobranch of trout, but not in the 1st gill arches of either trout or goldfish. In the Indian catfish (*Heteropneustes fossilis*) an absence of tyrosine hydroxylase immunostaining in the gills has also been previously reported (Zaccone et al.,
This was surprising given that sympathetic innervation of the gill vasculature supplying the efferent filament artery sphincter and the nutritive vasculature has been well documented in studies of most teleost species studied to date (Nilsson and Sundin, 1998). This most likely reflects the scarcity of adrenergic nerves (Nilsson and Sundin, 1998) and these nerves likely function together with circulating catecholamines released from the head kidney, in response to hypoxia (arterial PO$_2$ below the P$_{50}$ of arterial blood) (Reid, 1999). The net result is an $\alpha$-adrenoceptor mediated vasoconstriction of efferent filament artery and a concomitant $\beta$-adrenoceptor mediated vasodilation of afferent filament artery resulting in no change in arterio-arterial blood pressure (Sundin and Nilsson, 2002). Thus these responses are believed to regulate blood flow and improve oxygen uptake at the gills (Reid, 1999).

The intrinsic neurons make up one type of nerve fibres, so named because their cell bodies are found within the filament. As reported previously (Jonz and Nurse, 2003), intrinsic 5-HT-immunoreactive neurons were found in the filament of both trout and goldfish (Fig. 2.3, Fig. 2.5 and Fig. 2.7) and these were of 2 kinds: chain neurons, and proximal neurons with cell bodies near the base of the efferent filament artery. Chain neurons are a chain of bipolar neurons found along most of the filament in the central venous sinus that extend processes towards the proximal neurons at the base of the filament artery, suggesting a vasomotor role (Jonz and Nurse, 2003). Chain neurons double labelled with VAChT and 5-HT (Fig. 2.4) or VAChT and the HNK-1 antibodies (Fig. 2.5) suggesting they are derived from the neural crest or that the zn-12/HNK-1 conserved epitope is binding the immunohistochemical probe. Intrinsic chain and proximal neurons also double labelled with 5-HT and VAChT (Fig. 2.7). Intrinsic proximal neurons labeling for 5HT have previously been shown to be present in the gills of zebrafish innervating 5HT positive NECs in the filaments, but not the lamellae (Jonz and Nurse, 2003).

The extrinsic nerve fibres are a second type of nerve fibres that have cell bodies outside the gill arch. In the present study, we also found the marker for VAChT present in what we believe to be the extrinsic nerve bundle feeding the gill filament (as determined by the absence of cell bodies along the fibres) and also expressing the zn-12/HNK-1 conserved epitope (Fig. 2.5, Fig. 2.6 and Fig. 2.7A). These findings are consistent with reports of cholinergic nerve fibres in perch (*Perca fluviatilis*) coursing along the efferent filament artery (including the sphincter region) and the efferent lamellar arterioles (Dunel-Erb et al., 1989).
In perch, denervation of the pre and meta-trematic nerve decreased acetylcholinesterase (ACHE) staining indicating that these cholinergic fibres were extrinsic to the gill (Bailly and Dunel-Erb, 1986). More recently, the serotonergic NECs of zebrafish (in the filament core and in the lamellae) were also shown to receive extrinsic innervation that degenerated in gill explants (Jonz and Nurse, 2003). While the neurotransmitters present in this extrinsic innervation were not identified previously, their location is consistent with the labeling pattern of extrinsic VACHT positive nerve fibres in our study.

2.5.4 Projections from chemosensors to the CNS

Single fibre from afferent nerve fibres in isolated fish gills in trout that responded to hypoxia and cyanide (NaCN) (indicating these fibres innervated oxygen chemoreceptors in the gill) by an increase frequency of nerve discharge when ACh, 5-HT and dopamine were administered to the gill vasculature (Burleson and Milsom, 1995). The strongest response was to ACh. ACh along with ATP is postulated to be the main neurotransmitters released during the hypoxic ventilatory response in mammalian glomus cells (Zhang et al., 2000), and electrophysiological recording indicate that ACh is an important signaling molecule in the hypoxic ventilatory response of trout as well (Burleson and Milsom, 1995). Given that our study did not find any NECs that expressed the cholinergic marker VACHT, this would support the idea that serotonergic NECs are likely the primary chemosensing cells in the gills, with VACHT positive neurons completing the afferent pathway. To explain how single chemosensitive fibres arising from isolated gills respond to perfusion/superfusion with ACh and nicotine (Burleson and Milsom, 1995), there must be a cholinergic synapse somewhere in the afferent pathway inside the gill.

There are a number of possible scenarios that could explain this observation, and these possibilities are illustrated in Fig. 2.9. In the first scenario, serotonergic NECs act as the primary oxygen sensors. Here, the primary sensory cells release serotonin onto a primary afferent neuron projecting to the nodose or petrosal ganglion. ACh is subsequently released at a reciprocal synapse between the NEC and its afferent nerve. In the second scenario, serotonergic NECs still act as the primary oxygen sensors but in this scenario, the primary sensory cells would release serotonin onto a primary afferent neuron that would then in turn release ACh onto a secondary neuron within the gill that would then project to the nodose or
petrosal ganglion. In the third scenario, cholinergic bipolar neurons act as the primary sensory cells in fish gills, with chemosensory dendrites and cell bodies within the filament. With this scenario, upon detection of hypoxia, ACh would be released onto a secondary neuron that would project to the nodose or petrosal ganglion. In the fourth scenario, an extrinsic sensory neuron acts as the primary sensory cell and projects centrally to the NTS, where it would release either 5-HT or ACh. This scenario would not explain the response of neurons in isolated gills to ACh however. Interestingly, scenarios one, two and three have also been suggested to occur in the neuroepithelial bodies in the airways of mammals (Brouns et al., 2009). Note that only scenarios three and four are consistent with our finding that the serotonergic NECs did not actively take up the activity dependent dye during hypoxia.

2.6 Conclusions

Unlike mammalian glomus cells, fish NECs do not contain markers for acetylcholine and catecholamines, therefore, serotonin remains a suitable marker for NECs in fish. Markers for acetylcholine were found in nerve fibres in the gill filaments. My findings suggest that if acetylcholine plays an important role in the hypoxic response in fish as suggested by previous studies, it must do so either by being released from a relay neuron in the transduction pathway or at a reciprocal synapse between serotonergic NECs and their afferent innervation. Finally, serotonergic NECs did not label with the HNK-1 antibody suggesting either that these cells are derived from epithelial precursor cells (not neural crest cells) or that they are differentiated mature neural crest cells that do not express the zn-12/ HNK-1 on the cell surface. Serotonergic NECs did not take up activity dependent dyes suggesting that these cells do increase activity (vesicle turnover) during hypoxia.

2.7 Acknowledgements

The zn-12 and SV-2 antibodies developed by Bill Trevarrow and Kathleen M. Buckley respectively were obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242. The authors would like to thank the University
of British Columbia BioImaging Facility staff for technical assistance in obtaining the images and the NSERC of Canada for funding to WKM.

Figure 2.1 A) Wide-field image of double immunolabeling for tyrosine hydroxylase (TH, green) and cell bodies (DAPI, blue) in *O. mykiss* kidney. The upper left panel (green) shows a cluster of TH containing cells while the lower left panel (blue) shows the cluster of TH containing cells sitting amongst a dense population of smaller cells. B) Double immunolabeling of tyrosine hydroxylase (TH, green) and cell nuclei (DAPI, blue) in the pseudobranch of *O. mykiss*. The only evidence of TH labeling in the gills was in small, sparse nerve fibres in the pseudobranch. The enlargement in the right panel shows nerve terminals interspersed amongst epithelial cells of the pseudobranch. Scale bar, 50 µm.
Figure 2.2 Gill schematic and Z-stack compressions of sections of gill arch showing single immunolabeling of serotonin (5-HT) positive cells in the filament of trout (*O. mykiss*). Shallow sections along the efferent filament artery (eFA) reveal a row of 5-HT positive neuroepithelial cells (bottom right). A deeper section into the filament through the central venous sinus (cvs) near the eFA (top right) shows the presence of serotonin positive bipolar neurons. Scale bars, 20 µm. The gill schematic is reprinted from (Jonz et al., 2004) with permission.
Figure 2.3 Gill schematic and Z-stack compressions of sections of gill arch showing single immunolabeling of serotonin (5-HT) positive cells in the filament of goldfish (*C. auratus*). Neuroepithelial cells (NECs) lie along the efferent filament artery (eFA) and are clearly visible in some sections (bottom right) but are not present in sections through the central venous sinus (cvs) of the filament (top right). The NECs are concentrated towards the tips of the filament (bottom right, the distal end of the filament is near the top of the image). The middle panel shows serotonin positive bipolar neurons (arrowhead) that lie on the medial side of the eFA and usually not in the same plane of section as NECs. Scale bars, 20 µm. eFA, efferent filament artery; aFA, afferent filament artery. The gill schematic is reprinted from (Jonz et al., 2004) with permission.
Figure 2.4 Z-stack compressions of sections of gill arch showing double immunolabeling of vesicular acetylcholine transporter (VACHT, green) and serotonin (5-HT, red) markers in trout (*O. mykiss*, A and B) and goldfish (*C. auratus*, C-E). Serotonin labels neuroepithelial within the filament in trout (A) and goldfish (D) and at the tip of the lamellae in goldfish (C). Serotonin also labels bipolar chain neurons in both trout (B) and goldfish (E). The lamellar NECs of goldfish did not colabel with VACHT and neither did NECs in the filament of either trout (A) or goldfish (D). Bipolar chain neurons colabeled with 5-HT and VACHT in both species (B and E). In each case the images from the green and red channels are shown separately and as a merged image with DAPI (blue) showing cell nuclei. The arrow shows a cholinergic nerve fiber in the proximity of a NEC in goldfish. Scale bars, 10 µm.
Figure 2.5 Z-stack compressions of sections of the first gill arch of rainbow trout (*O. mykiss*) (A-D) and goldfish (*C. auratus*) (E-I) with double immunolabeling of the human natural killer (NHK-1, red) antibody and either a serotonin marker (5-HT, green) or a vesicular acetylcholine transporter (VAChT, green). Serotonin positive NECs from the filament of trout and goldfish (A, F) and the lamellae of goldfish (E) do not colabel with the NHK-1 antibody but a nearby nerve fibre does. Chain neurons labeling with either 5-HT or VAChT colabel with the NHK-1 antibody in both trout (B and C respectively) and goldfish (G and H respectively). The intrinsic extrinsic nerve fibres colabel for VAChT and the NHK-1 antibody in both trout (D) and goldfish (I). Scale bars, 10 μm.
Figure 2.6 Z-stack compressions of sections of gill arch showing double immunolabeling of vesicular acetylcholine transporter (VAClT, green) and a neuronal marker (zn-12, red) along a single filament (F) with multiple lamellae (L) in longitudinal (A and B) and cross section (C and D) in O. mykiss (A and C) and C. auratus (B and D) respectively. Coincidence between the red and green labels appears in yellow or orange. Note that most but not all zn-12 nerve fibres label for VAClT. * indicates location of the efferent filament artery. Scale bars, 50 µm.
Figure 2.7 Localization of the vesicular acetylcholine transporter (VACHT) near the efferent filament artery of goldfish (*C. auratus*). (A) Z-stack compression of double immunolabeling of VACHT (green) and a neuronal marker (zn-12, red) at the base of a filament showing the nerve bundle in the gill arch. (B) Z-stack compression of triple immunolabeling of chain neurons within the filament of trout labelled with VACHT (green), serotonin (5-HT, red) and a nuclear stain (DAPI, blue). (C) Cross section in the vicinity of the efferent filament artery (eFA) showing triple immunolabeling of VACHT (green), a synaptic vesicle marker (SV2, red) and a nuclear stain (DAPI, blue). (D) Z-stack compression of triple immunolabeling of a proximal neuron within the filament of trout labelled with VACHT (green), serotonin (5-HT, red) and a nuclear stain (DAPI, blue). The white arrows indicate proximal neurons, yellow arrows indicate chain neurons, and the arrowheads indicates neuroepithelial cells in the lamellae. Scale bare, 50 µm in A and C and 20 µm in B,D.
Figure 2.8 Z-stack compressions of sections of gill arch of trout (O. mykiss) showing double immunolabeling with an activity dependent dye with a Texas Red hydrazide (TXR) and either serotonin (5-HT) (A, B, E, F), vesicular acetylcholine transporter (VACHT) (C, G) or sodium potassium ATPase (NAK121) (D, H) from trout exposed to 30 min of normoxia (A, B, C, D) or hypoxia (E, F, G, H). 5-HT positive neuroepithelial cells did not pick up the activity dependent dye TXR in either trout exposed to normoxia (A) or hypoxia (E). Bipolar neurons labeled for either 5-HT or VACHT also did not pick up the activity depended dye in normoxia (B and C respectively) or hypoxia (F and G respectively). Mitochondrion rich cells did pick up the activity depended dye in normoxia (D) but not hypoxia (H). Scale bars, 10 µm.
Figure 2.9 Proposed models for oxygen chemoreception in fish. In the first scenario, the primary oxygen sensing is a neuroepithelial cell likely releasing serotonin (5-HT) on an extrinsic neuron (cell body in the nodose/petrosal ganglion) with reciprocal synapses containing acetylcholine (ACh). The second scenario involves a neuroepithelial cell as the primary sensor likely releasing 5-HT on an intrinsic cholinergic neuron, with a cell body in the gill, releases ACh onto a second order neuron. In the third scenario, the primary oxygen sensor is an intrinsic neuron (bipolar neuron) that releases ACh or both 5-HT and ACh onto a synapse with another neuron that projects to the NTS. In a fourth scenario, the primary oxygen sensor is an extrinsic sensory neuron that projects directly to the nucleus tractus solitarius (NTS) (or equivalent in fish) releasing 5-HT or ACh or both.
Table 2.1 Details of primary and secondary antibodies used for immunohistochemistry.

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<th>Antisera</th>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Dilution</th>
<th>Cat. No.</th>
<th>Secondary antisera¹</th>
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<tr>
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<td>Alexa Fluor® 488⁶</td>
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<tr>
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<td>ImmunoStar</td>
<td>goat</td>
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<tr>
<td>TH</td>
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<td>mouse</td>
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</tr>
<tr>
<td>VACH T</td>
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<tr>
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<tr>
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<td></td>
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<tr>
<td>NAK121</td>
<td>Na⁺/K⁺-ATPase α-subunit</td>
<td>(Uchida et al., 2000)</td>
<td>rabbit</td>
<td>1:1000</td>
<td>n/a</td>
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<tr>
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<td>559048</td>
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¹Secondary antisera were conjugated with a fluorescent marker.
²–⁵Secondary antisera antigen corresponds with primary antibody host.
Chapter 3. Characterization of putative oxygen chemoreceptors in bowfin

(*Amia calva*)

3.1 Summary

Although neuroepithelial cells (NECs) have been previously described in bowfin (*Amia calva*) preliminary results using immunohistochemistry suggested that more types of NECs exist that previously described. Five cell types have been identified using immunohistochemical markers for serotonin, a synaptic vesicle marker, a neuronal marker and a neural crest marker. Type I cells were found near the eFA contained synaptic vesicles and were innervated, but did not label with the neural crest marker. Type II cells resembled intrinsic neurons previously described in other fish species. As bipolar neurons they labelled with the neuronal marker, the neural crest marker and contained synaptic vesicles. Type III cells were found in the filament at the level of the central venous sinus, were innervated and weakly labelled with the neural crest marker, but did not contain synaptic vesicles. These cells had never been described in any fish species before. Type IV cells were found in the lamellae and did not label for any marker except serotonin. Type V cells were found in the interfilamental support bars and also did not label for any marker except serotonin. Based on innervation cell Type I, II and III are consistent with these cells being putative oxygen chemoreceptors involved in a reflex response to hypoxia. Cell Types IV and V could be putative oxygen chemoreceptors acting in a paracrine fashion to regulate blood flow during exposure to hypoxia.

3.2 Introduction

Bowfin respond to acute hypoxia by increasing air breathing frequency and gill ventilation and reducing heart rate (bradycardia). Branchial denervation and pseudobranch ablation eliminate the air breathing response and the bradycardia and diminish the gill ventilatory response (McKenzie et al., 1991). These observations are consistent with the finding that bowfin do not possess central oxygen chemoreceptors (Hedrick et al., 1991) and indicate that the gills are the main location for oxygen sensing chemoreceptors in bowfin.
Neuroepithelial cells (NECs) containing serotonin have been described in both the filament and lamellae of bowfin using immunohistochemistry and electron microscopy (Goniakowska-Witalinska et al., 1995). In this study, the filament NECs were not found to be in direct contact with the water, while in the lamellae the NECs were. In contrast to the NECs of other fish species, which contain many small dense cored vesicles (80-100 nm), the NECs of bowfin contained only a few very large dense cored vesicles (100-560 nm), but the significance of this observation remains unclear. Consecutive sections of these NECs also revealed that the vesicles contained leu-enkephalin, met-enkephaline, and endothelin, neumodulator substances found in NECs lining the lungs of other vertebrate species (Dunel-Erb et al., 1982; Goniakowska-Witalinska et al., 1995).

Preliminary immunohistochemistry labeling of bowfin gills revealed that bowfin gills contain more putative chemoreceptor cell types (Ciuhandu et al., 2008) than previously described (Goniakowska-Witalinska et al., 1995). To determine the effects of sustained hypoxia on NEC form, distribution and abundance (Chapter 6), it is essential to have a quantitative description of all the types of NECs present in the bowfin gill. Therefore, the aim of this chapter was to thoroughly describe putative oxygen chemoreceptors in bowfin gills. I hypothesized that all the NEC types would be innervated and would contain synaptic vesicles just like the NECs found in other fish species. Immunohistochemistry was first used to identify potential oxygen chemoreceptors by double labeling with serotonin and other markers previously used to characterize oxygen chemoreceptors in fish and mammals. For oxygen chemoreceptors to be involved in a reflex response to hypoxia they must be innervated in order for the information to be relayed to the central nervous system for processing, and they must release their neurotransmitters from vesicles into a synapse onto these nerves. A synaptic vesicle marker, SV2, and a neuronal marker, zn-12 have been previously used to label NECs in fish and their nearby innervation (Coolidge et al., 2008; Jonz et al., 2004; Jonz and Nurse, 2003; Regan et al., 2011; Tzaneva and Perry, 2010). Additionally, glomus cells from the carotid bodies of birds and mammals are derived from the neural crest (Pearse et al., 1973). I also wanted to determine if this was the case for the NECs of bowfin. A mammalian human natural killer (HNK-1) antibody labels a subset of
proliferative neural crest cells in fish (Chapter 2) and we used this to determine whether the NECs in bowfin gills were proliferative and of neural crest origin.

3.3 Materials and methods

3.3.1 Animals

Adult bowfin (340 - 2500g), *Amia calva*, were wild caught by a local fisherman from the Bay of Quinte, Ontario and were transported to the University of Guelph in aerated tanks in a pickup truck. They were kept in tanks with recirculated water at the Hagen Aqualab, University of Guelph. Bowfin were acclimated to 22°C for at least 3 weeks before the start of any experiments. The bowfin were kept on a 12/12-h light/dark photoperiod and fed live goldfish once a week.

3.3.2 Immunohistochemistry

At the end of the acclimation period bowfin were killed by an overdose of benzocaine followed by a sharp blow to the head. Bowfin were perfused with heparinized (100 iu/ml), ice-cold phosphate-buffered solution (PBS) containing (in mM): NaCl, 137; Na₂HPO₄, 15.2; KCl, 2.7; KH₂PO₄, 1.5; buffered to pH 7.8 with 1M NaOH (Jonz and Nurse, 2003). The fish was perfused via the bulbous arteriosus using a blunt 25 gage needle and a 20cc syringe until the gill filaments appeared clear. The 1st gill arch was removed and fixed in 4% paraformaldehyde in PBS overnight. Tissues were then rinsed in PBS, cryoprotected in a 30% sucrose solution, and frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific) at -80°C. Blocks were sectioned longitudinal to the gill filament at 10-12 μm increments using a cryostat (Leica CM3050 S, Leica Microsystems, Germany) and mounted on Superfrost® plus slides (VWR International) for immunohistochemistry. Longitudinal serial sections were cut and every section was inspected. Cross sections 12 μm thick were cut every 700-1000 μm along the length of the filament.

Slides containing frozen tissue were washed in PBS and blocked in 10% Normal Goat Serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, Ontario) for one hour. The primary antibodies (Table 3.1) were diluted in a permeabilizing solution
(PBS/0.1% Triton X-100/ 3% normal goat serum) and set on the slides to incubate overnight at room temperature. Following incubation with primary antibodies, the slides were again washed in PBS. The slides were then incubated with fluorescently labelled secondary antibody (Table 3.1) diluted in PBS containing 0.1% Triton-X and 3% normal goat and left to incubate at room temperature for 2 hours in darkness. Following a final washing with PBS, slides were mounted with coverslips and Vectashield with DAPI (Vector Laboratories, Burlington, Ontario) to prevent photobleaching and to label cell nuclei. Slide boxes were stored at 4°C until viewed under a fluorescent light microscope. Control experiments were performed in which the primary antibodies were excluded to control for effects of the secondary antibody, and normal serum (IgG) from primary antibody host species was used (at an equivalent protein concentration) to test for adverse staining due to reaction with the primary hosts. The results of these control experiments showed only negligible staining with no specific immunolabeling of the gill structures present (data not shown).

3.3.3 Microscopy

Images were captured using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with solid state lasers emitting at 405 nm, 473 nm and 559 nm. Z-stacks of 5-12 optical sections and 1.0 µm apart were captured using the 60x objective of this microscope. Images were pseudo-coloured and adjusted for brightness and contrast using ImageJ.

3.4 Results

In longitudinal sections, I identified five types of cells by labeling for serotonin that I classified based on morphology, innervation, and location in the 1st fill arch (Fig. 3.1). Type I cells did not have cell projections and were found near the filament arteries. Type II cells were large bipolar neurons found in the middle of the filament, in the lining of the central venous sinus (cvs). Type III cells had no cell projections and were found in the vicinity of the bipolar neurons in the cvs. Type IV cells also did not have any projections and were
found in the lamellae. Type V cells had a similar morphology to Type IV cells but were found in the interfilamental support bars.

Triple labeling with DAPI, a marker for serotonin (5-HT) and a synaptic vesicle marker, (SV2) revealed that Type I and II cells contained synaptic vesicles (Fig. 3.2 A, B). Type III cells did not themselves contain synaptic vesicles, but nearby nerve fibres innervating them did (Fig. 3.2 C). Type IV and V cells did not contain synaptic vesicles (Fig. 3.2 D, E). All cell types labelled for serotonin. Triple labeling with DAPI, a marker for serotonin (5-HT) and a neuronal marker, (zn-12) revealed that Type I cells were found in close proximity to large nerve bundles (Fig. 3.3 A). Type II cells labelled with zn-12, confirming they were neurons (Fig. 3.3 B). Type III cells did not label with the neuronal marker but were also found in close proximity to nerve fibres (Fig. 3.3 C). Type IV and V cells also did not label with the neuronal marker and were not found near nerve fibres (Fig. 3.3 D, E). Triple immunolabeling with DAPI, a marker for serotonin (5-HT) and the HNK-1 antibody revealed that Type I cells did not label with this antibody (Fig. 3.4 A). Type II cells did label with the HNK-1 antibody (Fig. 3.4 B), while Type III cells labeled with HNK-1 but more weakly than Type II cells or other neurons (Fig. 3.4 C). Type IV and V cells did not label with the HNK-1 antibody (Fig. 3.4 D, E).

Triple labeling for the vesicular acetylcholine transporter, serotonin and with DAPI showed VACChT labeling in the vicinity of Type I cells (Fig. 3.5 A). A rotation of 3D projections of these images indicates that labeling was likely the innervation of these cells and not the cells themselves (data not shown). Type II cells contained VACChT in both the cell body and its projections (Fig. 3.5 B). The other cell types did not show labeling with VACChT (data not shown).

Cross sections revealed that Type I cells were found in four locations: near the basal lamina of the epithelium on the efferent side of the efferent filament artery (eFA), within the first cell layer of the epithelium near the eFA and similarly on the afferent filament artery (aFA) (Fig 3.6 A). The cells near the aFA contained synaptic vesicles and were found within the top layer of the epithelial layer, often at the base of mucous cells, and most were not in direct contact with the water (Fig. 3.6 A, B). Cells near the basal lamina on the aFA were
innervated and contained synaptic vesicles but were rare. The cells near the basal filament artery were less round and often had short projections that contacted the large nerve bundle nearby (Fig. 3.6 A, C). These cells also contained synaptic vesicles (Fig. 3.6 C).

3.5 Discussion

In this chapter I fully describe serotonin containing cells (the putative oxygen chemoreceptors) found in the gills of bowfin, (A. calva), and their association with other markers for neuroepithelial cells (NECs) (the synaptic vesicle marker SV2, the neuronal marker zn-12, and the neural crest cell marker HNK-1) (Table 3.2). Contrary to my hypothesis, not all NEC cell types were innervated and contained synaptic vesicles. Based on the results I also describe the possible role of these cell types in oxygen chemoreception.

Type I cells found near the eFA in bowfin contained synaptic vesicles (Fig. 3.2, 6 B) and were innervated by a nerve bundle running in close proximity to these cells (Fig. 3.3, 3.6 A, 3.6 C). These cells were similar in morphology and location to those previously described in the filament of all other species of fish studied to date (Coolidge et al., 2008; Jonz et al., 2004; Regan et al., 2011; Saltys et al., 2006; Tzaneva and Perry, 2010). In zebrafish (D. rerio) and catfish (Ictalurus punctatus), these cells grown in culture have been shown to depolarize in response to hypoxia (Burleson et al., 2006; Jonz et al., 2004; Qin et al., 2010). Additionally, just like in goldfish (C. auratus) and trout (O. mykiss), Type I cells did not label with the HNK-1 antibody, suggesting they are either not derived from the neural crest, or more likely, not proliferative (Chapter 2). These cells are likely to be mature oxygen chemoreceptors derived from precursor epithelial cells just like the neuroepithelial body cells in mammals (Dunel-Erb et al., 1994). VAChT labelled synaptic clefts in the proximity of these cells suggesting that acetylcholine is also released at this synapse but most likely post-synaptically. Acetylcholine is found in clear synapses and these have been found in the nerves synapsing with NECs in catfish (Ictalurus melas) (Dunel-Erb et al., 1982). This explanation is consistent with the 1st scenario proposed in the previous chapter in which NECs are the main oxygen chemoreceptors and are innervated by nerve fibres that contain
ACh. These nerve fibres could be either intrinsic or extrinsic neurons, as both of these contained VACHT in trout and goldfish (Chapter 2).

Type I cells found near the outer layer of the epithelium on the aFA side of the filament were similar in morphology and had similar immunolabeling to cells near the outer layer of the epithelium of the eFA side of the epithelium and we suggest these are the same cell type. These cells contained synaptic vesicles and were innervated by small nerve fibres (Fig. 3.6), indicating a possible chemosensory role. These cells have not been described in any other fish species to date in this location. Their location close to the water suggests that these cells could be external oxygen chemoreceptors, sensing changes in water PO2, with afferent nerves completing the chemosensory pathway. It is also possible that these are Merkel-like cells associated with gustation, as previously described in the orobranchial cavity of zebrafish (*D. rerio*) (Zachar and Jonz, 2012). In zebrafish, Merkel-like cells have been described as containing both serotonin and synaptic vesicles (Zachar and Jonz, 2012) as in the present study. These Merkel-like cells also had small projections of 2-3 μm, however, which were not observed in the present study. Therefore, although these cells possess some similarities to the Merkel-like cells of zebrafish (Zachar and Jonz, 2012), there are also some differences.

As in previous studies on zebrafish (*D. rerio*), trout (*O. mykiss*) and goldfish (*Carassius auratus*), intrinsic bipolar neurons (Type II cells) labeling for serotonin were found in bowfin gills (Jonz and Nurse, 2003; Porteus et al., 2012). Just like in trout and goldfish (Chapter 2), these intrinsic neurons double labelled with 5-HT and VACHT. These intrinsic bipolar neurons innervate the eFA sphincter, indicating they are involved in regulating blood flow through the gill (Jonz and Nurse, 2003). Gill blood flow is modulated during hypoxia by the contraction of the eFA sphincter, which causes an increase in branchial blood pressure, which in turn causes an increase in lamellar recruitment increasing the functional surface area for gas exchange (reviewed by Sundin and Nilsson, 2002). They also innervate filamental NECs (Type I cells) in zebrafish suggesting they may be the second step in the chemoreceptor reflex pathway. Alternatively they could themselves be the
primary oxygen sensors with other neurons completing the reflex pathway (3rd scenario in Chapter 2).

Type III cells have never been described in any fish species studied to date. These cells were located in the filament in the area of the central venous sinus, were innervated, contained serotonin, but did not contain synaptic vesicles (Fig. 3.2C, 3.3C). It is possible that these cells are involved in the hypoxic ventilatory response acting as effector cells, releasing serotonin on nearby vasculature in response to hypoxia, using vesicles that did not label with the synaptic vesicle marker. Serotonin causes a dilation of the central venous sinus (cvs) in Atlantic cod (*Gadus morhua*) (Sundin, 1995), increasing blood flow to the mitochondrion rich cells. It has also been suggested that dilation of the cvs could divert plasma from the arterio-arterial pathway increasing the hematocrit in the lamellae and improving oxygen uptake (Sundin and Nilsson, 2002), but no anastomoses between these two circulations have been found in bowfin (Olson, 1981). Alternatively, Type III cells could be precursor NECs that will differentiate into mature NECs and this explanation is consistent with the weak labeling of these cells with the HNK-1 antibody. This antibody labels a subset of proliferative neural crest cells. In this scenario, these cells may have started synthesizing serotonin and transforming into NECs but have not yet begun to package it into synaptic vesicles. The density of these cells was not determined due to difficulty in obtaining full longitudinal sections of the gills, but future studies using bromodeoxyuridine (BrdU) would reveal if these cells are indeed replicating.

Type IV cells found in the lamellae have been described in most species of fish studied to date (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al., 2006; Vulesevic et al., 2006), but not in trout or mangrove rivulus (Coolidge et al., 2008; Regan et al., 2011; Saltys et al., 2006). In all other species of fish in which these cells are present, they contained synaptic vesicles and most were innervated. This was not the case for the lamellar NECs of bowfin (Fig. 3.2 D, 3.3 D). It is unclear why this difference exists, but this might either reflect species differences or represent an ancestral phenotype. If these cells are oxygen chemoreceptors they could release serotonin in a paracrine fashion via non-synaptic vesicles on nearby pillar cells. Serotonin causes pillar cells of the lamellae to contract which serves...
to redistribute blood flow through the lamellae and thus, improve oxygen uptake (Stensløkken et al., 2006). Goldfish remodel their gills increasing surface area in warm water or hypoxia, and reducing surface area in cold water or normoxia to reduce osmoregulatory costs (Sollid et al., 2003). Lamellar NECs also move during this gill remodeling, remaining in close proximity to the water. These changes are consistent with these cells being involved in hypoxic cardiorespiratory responses (Tzaneva and Perry, 2010). However, no electrophysiological recordings have been made from these cells demonstrating whether they depolarize in response to hypoxia, due to their small size.

Type V cells were similar in morphology and immunolabeling to Type IV (lamellar) cells (Fig. 3.2 E, 3.3 E, 3.4 E) and are likely from the same cell population despite their different location. The inter-lamellar bar of tissue where they are found is to our knowledge unique to bowfin and its function remains unknown, but it likely serves to prevent gill collapse and support the gill curtain during air breathing. This tissue is supplied by the outer vascular margins of the lamellae which are imbedded in it (the outer 1-3 lamellar channels), reducing oxygen uptake capacity in this area of the lamellae (Olson, 1981). Additionally, unlike other fish, bowfin do not have connections (anastomoses) between their nutritive and respiratory circulation (Olson, 1981), therefore, all of the cardiac output must perfuse the lamellae, which, during air breathing in hypoxia, could lead to a loss of oxygen from the blood to the water. Serotonin released from these cells could act to modify blood flow through the respiratory lamellae to either enhance oxygen uptake during water breathing, or reduce oxygen loss, during air breathing. In normoxia, air breathing frequency is minimal and the gills are used to obtain oxygen from the water. In this case a moderate amount of perfusion of the lamellae would ensure adequate gas exchange. Some lamellae would not be perfused to reduce ion loss. In aquatic hypoxia, when the animal has access to air, the perfusion of blood over lamellae could be reduced, and blood would be diverted to the outer lamellar channels by release of serotonin in the lamellae to vasoconstrict the middle lamellar channels.
3.6 Conclusions

Type I cells were found in the filament, in close proximity to the eFA, contained synaptic vesicles, and were innervated. Even though these cells did not label with the HNK-1 antibody, these cells are consistent with them being putative oxygen chemoreceptors involved in a reflex response to hypoxia. Intrinsic bipolar neurons (Type II) cells contained synaptic vesicles, labelled with the zn-12 and HNK-1 antibodies. These cells could either be chemosensitive themselves or part of a chemo-reflex arc. In this chapter I also described a new type of cell (Type III) never before described in fish gills. Type III cells did not contain synaptic vesicles, were innervated and weakly labelled for the HNK-1 antibody suggesting that that they are either precursor NECs or involved in the efferent regulation of the oxygen chemoreception pathway. Furthermore, NECs found in the lamellae and the interfilamental support bars (Type IV and V cells) did not contain synaptic vesicles and were not innervated, indicating that these cells may have a paracrine role if involved in oxygen chemoreception. Because Type I, II, and III cells were innervated, they could be involved in reflex cardiorespiratory responses to hypoxia and thus their involvement in the responses to sustained hypoxia was assessed and this information is presented in Chapter 6.
Figure 3.1 Gill schematic and Z-stack compressions showing double immunolabeling with serotonin (5-HT, green) and cell nuclei (DAPI, blue) indicating the location of serotonergic cells in the gills of bowfin (*Amia calva*). Type I cells are located near the filament arteries. Type II cells are bipolar neurons found in the central venous sinus of the filament. Type III cells are round cells without any projections in the central venous sinus of the filament. Type IV cells are round cells found in the lamellae and Type V cells are found in the lamellar bars. Scale bars = 200 μm for top panel and 100 μm for bottom panel. F, filament; L, lamellae; S, interfilamental support bars; eFa, efferent lamellar artery; aFA, afferent lamellar artery; cvs, central venous sinus. The gill schematic is reprinted from (Jonz et al., 2004) with permission.
Figure 3.2 Z-stack compressions of triple immunolabeling of different cell types in the gills of bowfin (Amia calva) with antibodies for serotonin (5-HT, green), a synaptic vesicle marker (SV2, magenta), and cell nuclei label (DAPI, blue). Type I (A) and II (B) cells labelled for synaptic vesicles. Type III cells (C) did not contain synaptic vesicles but were found in close proximity to nerve fibres that did. Type IV (D) and V (E) cells did not label with synaptic vesicles. Coincidence in labeling between magenta and green appears in white. Scale bars = 10 μm.
Figure 3.3 Z-stack compressions of triple immunolabeling of different cell types in the gills of bowfin (Amia calva) with antibodies for serotonin (5-HT, green), a neuronal marker (zn-12, magenta), and a cell nuclei label (DAPI, blue). Type I (A) cells are found in close proximity to nerve fibres. Type II (B) cells labelled with synaptic vesicles confirming they are bipolar neurons. Type III cells (C) were found in close proximity to nerve fibres. Type IV (D) and V (E) cells were never located near nerve fibres, therefore were not innervated. Scale bars = 10 μm.
Figure 3.4 Z-stack compressions of triple immunolabeling of different cell types in the gills of bowfin (*Amia calva*) with antibodies for serotonin (5-HT, green), an HNK-1 antibody (HNK-1, magenta), and cell nuclei label (DAPI, blue). Type I (A) cells did not label with the HNK-1 antibody. Type II (B) cells strongly labelled with the HNK-1 antibody. Type III cells (C) weakly labelled with the HNK-1 antibody. Type IV (D) and V (E) cells did not label with the HNK-1 antibody. Scale bars = 10 μm.
Figure 3.5 Z-stack compressions of triple immunolabeling of different cell types in the gills of bowfin (*Amia calva*) with antibodies for vesicular acetylcholine transporter (VAChT, green), serotonin (5-HT, magenta), and cell nuclei label (DAPI, blue). VAChT labeling was found in the vicinity of some Type I cells (A) likely in synapses of nearby innervation. Type II cells contained VAChT (B). Scale bars = 10 μm.
Figure 3.6 Type I cells shown in cross section in a filament of bowfin (*Amia calva*). (A) Z-stack compression of triple immunolabeling for antibodies of serotonin (5-HT, green), a neuronal marker (zn-12, magenta) and cell nuclei (DAPI, blue). B) Close up of the area around the efferent filament artery and (C) the afferent filament artery using triple immunolabeling with serotonin (5-HT, green), a synaptic vesicle marker (SV2, magenta) and cell nuclei (DAPI, blue). Arrowheads indicate nerve fibres innervating nearby cells. Coincidence in labeling between magenta and green appears in white. Scale bars are 100 μm in A and 10 μm in B and C. aFA, afferent filament artery; eFA, efferent filament artery; NB, nerve bundle; cvs, central venous sinus.
Table 3.1 Details of primary and secondary antibodies used for immunohistochemistry.

<table>
<thead>
<tr>
<th>Antisera</th>
<th>Antigen</th>
<th>Manufacturer</th>
<th>Host</th>
<th>Dilution</th>
<th>Cat. No.</th>
<th>Secondary antisera&lt;br&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primary</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5-HT</td>
<td>serotonin</td>
<td>Sigma-Aldrich</td>
<td>rabbit</td>
<td>1:500</td>
<td>S5545</td>
<td>Alexa Fluor® 488&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>SV2</td>
<td>synaptic vesicles, neuronal and endocrine neuron, surface</td>
<td>DSHB</td>
<td>mouse</td>
<td>1:200</td>
<td>n/a</td>
<td>Alexa Fluor® 594&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>zn-12</td>
<td>CD-57</td>
<td>DSHB</td>
<td>mouse</td>
<td>1:50</td>
<td>n/a</td>
<td>Alexa Fluor® 594&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HNK-1</td>
<td></td>
<td>BD Pharmigen</td>
<td>mouse</td>
<td>1:1000</td>
<td>559048</td>
<td>Alexa Fluor® 594&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>Secondary</strong>&lt;sup&gt;1&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alexa Fluor® 488</td>
<td>rabbit IgG (H+L)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Molecular Probes, Invitrogen</td>
<td>goat</td>
<td>1:500</td>
<td>A11008</td>
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<tr>
<td>Alexa Fluor® 594</td>
<td>mouse IgG (H+L)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Molecular Probes, Invitrogen</td>
<td>goat</td>
<td>1:300</td>
<td>A11005</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>1</sup>Secondary antisera were conjugated with a fluorescent marker.
<sup>a,b</sup>Secondary antisera antigen corresponds with primary antibody host.
Table 3.2 Summary of immunoreactivity of 5 types of putative chemoreceptive cells with tested antisera and their location in the bowfin gill.

<table>
<thead>
<tr>
<th>Structure</th>
<th>Antibody</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type I</td>
<td>5-HT</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SV2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>zn-12</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HNK-1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VAChT</td>
<td>*</td>
</tr>
<tr>
<td>Type II</td>
<td>5-HT</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SV2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>zn-12</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HNK-1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VAChT</td>
<td>+</td>
</tr>
<tr>
<td>Type III</td>
<td>5-HT</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>SV2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>zn-12</td>
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</tr>
<tr>
<td></td>
<td>HNK-1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>VAChT</td>
<td>-</td>
</tr>
<tr>
<td>Type IV</td>
<td>5-HT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SV2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>zn-12</td>
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</tr>
<tr>
<td></td>
<td>HNK-1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VAChT</td>
<td>-</td>
</tr>
<tr>
<td>Type V</td>
<td>5-HT</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SV2</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>zn-12</td>
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</tr>
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<td></td>
<td>HNK-1</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>VAChT</td>
<td>-</td>
</tr>
</tbody>
</table>

(+) indicates positive immunoreaction; (-) indicates no positive immunoreaction; eFA, efferent filament artery; cvs, central venous sinus; S, interfilamental support bars

*cell Type I did not contain VAChT, but some contained neuronal clefts that did
Chapter 4. Time domains of the hypoxic cardio-respiratory response in bowfin (*Amia calva*)

4.1 Summary

Time domains of the hypoxic ventilatory response (HVR) and their mechanisms have been characterized in mammals. The aim of this study was to determine if time domains of the HVR exist in bowfin (*Amia calva*) exposed to sustained hypoxia (45 mm Hg for 7 days). It was hypothesized that time domains of the HVR would be evident in bowfin when denied access to air, similar to those seen in mammals. However, in bowfin with access to air and an unlimited oxygen supply, time domains of the HVR would be absent. Bowfin without access to air exhibited some time domains similar to those described in mammals (acute response, short term potentiation, hypoxic ventilatory decline, and ventilatory acclimatization to hypoxia) but not others (short term depression). As hypothesized, bowfin with access to air did not exhibit time domains in either the gill or air breathing response to hypoxia. These findings indicate that chemoreflex pathways have been conserved over evolutionary time.

4.2 Introduction

When faced with an environment that is low in oxygen most animals will try to escape it; however, if this is not an option they must either increase oxygen supply to meet oxygen demand, or reduce oxygen demand to match oxygen supply. The first step in increasing oxygen supply is achieved by increasing ventilation, an energetically costly response. In mammals the cost of breathing can be as much as 5.5% of total metabolism during increased ventilation (Frappell et al., 1998). In fish it has been estimated to be 3-10% of resting metabolic rate while the costs associated with increased ventilation during exposure to hypoxia are unknown but would be expected to be even higher (Kramer, 1983). Thus, in these vertebrate groups it is important for ventilation to be tightly regulated to minimize unnecessary use of what little oxygen exists.

Mammals exposed to a constant level of hypoxia exhibit time dependent changes in the level of ventilation and these fluctuations in ventilation along with their underlying
mechanisms have been termed time domains of the hypoxic ventilatory response (HVR) (Powell et al., 1998). These time dependent changes in ventilation can take place without changes in metabolic rate or reductions in arterial $\text{PCO}_2$ and each one is associated with specific adjustments along the ventilatory control circuit (Powell et al., 1998). Many time domains are evident in mammals and they have been categorized based on the time course over which they take place: brief hypoxic exposures (acute response, short term potentiation, short term depression), episodic/intermittent hypoxic exposures (progressive augmentation, long term facilitation), and sustained hypoxic exposures (hypoxic ventilatory decline, ventilatory acclimatization to hypoxia, ventilatory deacclimatization to hypoxia, hypoxic desensitization) (Powell et al., 1998).

In fish, time domains are also evident in some studies but it is unclear whether the fluctuations in total ventilation seen are independent of changes in metabolic rate as the hypoxic levels studied were sometimes severe (Florindo et al., 2006; Kerstens et al., 1979; McKenzie et al., 1991; Stecyk and Farrell, 2002). The variation in the intensity of hypoxic exposures used in various studies is further complicated by the variety of hypoxic protocols used: some used sustained hypoxia, while others used acute progressive hypoxia. No study to date has fully characterized time domains of the HVR in fish and they are often ignored when considering the responses of fish to aquatic hypoxia. Therefore, the purpose of this study was to determine if time domains exist, and to identify and characterize the time scale over which they occur, in a facultative air breather, the bowfin ($Amia\ calva$) using a sustained moderate hypoxic exposure.

Bowfin have evolved for over 100 million years and they are actinopterygian fish closely related to the modern day teleosts (Graham, 1997). Additionally, they are facultative air breathers, using well developed gills to obtain oxygen from the water, able to supplement oxygen needs by coming to the surface of the water to breath air using their gas bladder. By denying bowfin access to air, the time dependent changes in their HVR were determined and used as an indication of their hypoxic response when obliged to water breathe. By allowing bowfin access to air, the air breathing response to hypoxia was also monitored. It was hypothesized that time domains would be evident in bowfin when denied access to air and
that they would be similar to those seen in mammals. It was also hypothesized that bowfin with access to air would not exhibit time domains because their oxygen supply would not be limited (they would not be sufficiently hypoxaemic). Additionally, it was hypothesized that the heart rate response would match these ventilatory responses in both groups as cardiorespiratory variables are tightly matched in fish. In the first series of experiments the time domains of the HVR were determined in bowfin acclimated to 8°C without access to air over a period of 4d, with a focus on the response over the first 24 h. These were compared with time matched controls maintained in normoxia. At this temperature air breathing is minimal, therefore, their HVR can be compared to the HVR of other obligate water breathing fish (those that only breathe water). In the second series of experiments bowfin were acclimatized to 22°C to increase air breathing frequency and the response of bowfin with or without access to air was measured over a period of 7d to determine if time domains associated with a longer time course were evident. In this series of experiments the fish were also monitored during recovery as most time domains are also evident in reverse during recovery from hypoxia (Powell et al., 1998).

4.3 Materials and methods

4.3.1 Series I: time domains over 4d at 8°C

4.3.1.1 Animals

Bowfin (720 - 2860g), *Amia calva*, were wild caught by a local fisherman from the Bay of Quinte, Ontario and were transported in aerated tanks in a pickup truck to Toronto from where they were transported in aerated water by airplane to Vancouver. They were kept in flow-through water tanks at the University of British Columbia. These bowfin were kept on a natural photoperiod and fed live goldfish once a week. For this series bowfin were kept at 8°C for at least a month and then experiments were run at this temperature (7.9 ± 0.5°C).
4.3.1.2 Sustained hypoxia

Fish were anesthetized with benzocaine (0.75ml/L of 400mM Benzocaine in 75% Ethanol) by immersion and then instrumented with a buccal and opercular cannula. During the surgery, the gills were flushed with aerated water containing benzocaine (0.75 – 1.0 ml/L of 400mM Benzocaine in 75% Ethanol). The fish were allowed to recover for at least 24h in a black box supplied with aerated flow-through water. Animals were randomly assigned to either of two groups both without access to the surface of the water to breath air: normoxia or hypoxia. Sustained hypoxia was achieved by bubbling nitrogen using a Parker Balston nitrogen generator (Model N2-04, Parker Hannifin Corp., Haverhill, MA, USA) into an exchange column filled with glass beads and by controlling the water flow from the exchange column into the experimental tank (a black box). This design allowed the oxygen level in the experimental tank to be kept within a narrow O$_2$ range (155±0.8 mm Hg for normoxia and 25.7±3.9 mm Hg for hypoxia). This level of hypoxia was estimated to be just below the critical oxygen tension of bowfin without access to air (Chapter 5), a level that would stimulate ventilation but not depress metabolism. Ventilation was measured with a disposable pressure transducer connected to the buccal cannula, filled with water, and calibrated with water using a glass column. The pressure transducer was connected to an amplifier (Electronics Workshop, University of British Columbia) and this was connected to a second amplifier (Universal amplifier, Gould Instruments). This amplifier was connected to DI-720 interface (DataQ) and the data were recorded on a PC using WindaQ Version 2.19 software (DataQ, Akron, OH, USA). These variables were measured for 30 min at each time point in both groups: 0, 1, 3, 6, 12, 18, 24, 48 and 72 h. The last 10 min of data from each time point were used to calculate the average for each variable in an individual fish. A data analysis script for Spike 2 (Version 5.08, Cambridge Electronic Design, Cambridge, England) was used to measure the breathing amplitude by measuring the amplitude on a pressure trace deflection. These fish did not have access to air and did not air breathe.
4.3.2 Series II: time domains over 7d at 22°C

4.3.2.1 Animals

Bowfin (340 - 2500g), *Amia calva*, were wild caught by a local fisherman from Bay of Quinte, Ontario and were transported to the University of Guelph in aerated tanks in a pickup truck. They were kept in re-circulated water tanks at the Hagen Aqualab, University of Guelph. For this series bowfin were first kept at 9ºC for a month, after which the temperature was increased by 1ºC/day to 22ºC, and the animals were acclimated at that temperature for at least 3 weeks before the start of any experiments. All bowfin were kept on a 12/12-h light/dark photoperiod and fed live goldfish once a week.

4.3.2.2 Surgery

Procedures were as described above except these fish were also instrumented with 1 ECG electrode placed midventrally between pectoral fins and secured in place with 3 sutures on the belly and 1 at the anterior edge of the dorsal fin. Sometimes a second electrode was placed mid-ventrally between the pelvic fins and also secured with 3 sutures on the belly and 1 at the anterior edge of the dorsal fin.

4.3.2.3 Sustained hypoxia

Animals were randomly assigned to either of two groups: with or without access to air. They were put in 2 m diameter tanks and separated using perforated pvc (up to 4 animals per tank at once) that had perforated pvc covering either 2cm below surface of water (in the group without access to air) or 10cm above surface of water (in the group with access to air). Sustained hypoxia was achieved by bubbling nitrogen using a Parker Balston nitrogen generator (Model N2-04, Parker Hannifin Corp., Haverhill, MA, USA) into a large header tank (~100 l) and into the experimental tank and by controlling the water flow from the header tank into the experimental tank. This flow-through design allowed the oxygen level in the experimental tank to be kept within a narrow O₂ range (45.4 ± 6.9 mm Hg). This level of hypoxia was chosen as it was a level that would stimulate ventilation but not significantly depress metabolism in bowfin without access to air (Chapter 5). The animals were exposed
to sustained hypoxia for 7 days and their response to hypoxia was monitored over this time period.

Ventilation was measured as in Series 1 but only one amplifier (Electronics Workshop, University of British Columbia) was used. Heart rate was recorded using a differential amplifier (World Precision Instruments, Dam 50). Both amplifiers were connected to a DI-720 interface (DataQ) and the data were recorded on a PC using Windaq Version 2.19 software (DataQ, Akron, OH, USA). These variables were measured for 30 min to 1h each day of the 7d sustained hypoxia exposure, as well as before in normoxia (control). The last 10 min of data from each time point were used to calculate the average for each variable in an individual fish. Breathing traces were analyzed as described above using Spike 2.

4.3.2.4 Acute and recovery responses

The acute (3h) and recovery (5h) responses from hypoxia were measured in a plexiglass experimental chamber as described previously (Rantin and Kalinin, 1996). In brief, the experimental chamber was 75 l recirculation unit divided into two compartments: one for conditioning the water and one where the fish was maintained. The two were connected by a pump (Rule Bilge Pump 500gph) and standpipe. Both compartments could be drained and filled individually allowing water to be changed with minimum disturbance to the fish. Water was changed every 5h or less in order to prevent nitrogenous waste from building up. The experimental chamber had 3 opaque walls and one clear wall facing away from the experimenters. An angled mirror was used to monitor the fish. During the acute response the level of hypoxia was controlled manually by turning the nitrogen regulator on and off and using a Hach 30d meter oxygen probe (Hach, Loveland, CO, USA) that measured oxygen tension every 30s. During recovery from hypoxia air was bubbled into the experimental tank.

For measuring the acute response, animals were put in the experimental tank and left to get used to the experimental chamber for 3-12h before the experiment was started. Nitrogen was then started and the experimental tank reached the desired oxygen level (45
mm Hg) within 30min. The variables mentioned above were measured continuously for at least 30min in normoxia and for up to 3h in hypoxia, after which the bowfin were transferred to the sustained hypoxia experimental tank (described above) for 7d. Fish that did not have access to air during sustained hypoxia also did not have access to air during the acute response and fish with access to air during sustained hypoxia also had access to air during the acute response. After 7d the bowfin were transferred from the sustained hypoxia experimental tank back to the acute response experimental tank and were kept hypoxic for a further 2-3h. Fish that did not have access to air during sustained hypoxia also did not have access to air during recovery and fish with access to air during sustained hypoxia also had access to air during recovery. The acute exposure tank was switched from being bubbled with nitrogen to air and the oxygen level was raised to normoxia over a period of 30-45min. Heart rate, breathing frequency and breathing amplitude were measured continuously from the time of the transfer and up to 5h in normoxia.

4.3.3 Statistical analysis

Data are routinely expressed as mean ± SEM. In Series I data were normalized as relative values to control (time 0h). Data transformations (log) did not result in marked differences in statistical interpretation therefore they were analyzed without transformation. In Series I a 2-way RM ANOVA followed by Holm-Sidak test was used to compare differences from time 0h and between treatments. A 1-way RM ANOVA followed by Holm-Sidak test all pairwise comparison was used to determine differences exclusive of actual time point. In Series II the data were not normalized and a 2-way RM ANOVA followed by Holm-Sidak test was used to compare differences from time 0h and between treatment in each cardiorespiratory variable. To determine the time of the recovery a 1-way RM ANOVA was used in each treatment for each cardiorespiratory variable, followed by a Holm-Sidak test to compare differences from normoxia (time 0h). To determine differences before and after an air breath a 2-way RM ANOVA was used. To determine if the differences were larger than zero, one-tailed paired t-tests were performed at each time point. All statistical analysis was done using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA), except the
t-tests which were done using Microsoft Excel. A significance level of P<0.05 was used throughout.

4.4 Results
4.4.1 Series I: time domains over 4d at 8°C

Bowfin exposed to hypoxia without access to air increased gill ventilation compare to time matched control animals exposed to normoxia without access to air (Fig. 4.1). Hypoxic bowfin had higher relative breathing frequency than control animals (F$_{1,78}$=10.08, P<0.01) but there were no significant differences between different time points (F$_{8,78}$=1.421, P=0.20), however the time treatment interaction term was significant (F$_{8,78}$=2.921, P<0.01). Bowfin exposed to hypoxia also had higher relative buccal pressures than bowfin exposed to normoxia (F$_{1,78}$=6.11, P<0.05), but there were no differences between different time points (F$_{8,78}$=0.81, P=0.60) and the interaction term was not significant (F$_{8,78}$=0.80, P=0.60). Bowfin exposed to hypoxia also had higher relative index of ventilation product than bowfin exposed to normoxia (F$_{1,78}$=27.17, P<0.001), but there were no differences between different time points (F$_{8,78}$=1.25, P=0.28) and the interaction term was not significant (F$_{8,78}$=1.37, P=0.22).

Time domains of the HVR are evident when data of individual fish are plotted independently, with an initial increase in respiratory variables followed by a decrease and a secondary increase, however the timing of these events varied considerably between fish (Fig. 4.2 A-G). When these responses are averaged independent of time points, time domains became evident (Fig. 4.2 H) and significant differences were seen in relative breathing frequency, relative buccal pressure and relative index of ventilation product (F$_{3,18}$=4.61, P<0.05; F$_{3,18}$=5.45, P<0.01; and F$_{3,18}$=10.5, P<0.001 respectively). On average, relative breathing frequency reached a maximum response 3.7 ± 0.9 h after beginning of exposure to hypoxia, then decreased and reached a minimum at 18 ± 5.8 h, followed by a second increase reaching a maximum response at 43.7 ± 9.2 h. On average, relative buccal pressure reached a maximum response 2.0 ± 0.8 h after beginning of exposure to hypoxia, then decreased and reached a minimum at 12 ± 3.4 h, followed by a second increase reaching
a maximum response at 36.0 ± 8.3 h. Relative index of ventilation product reached a maximum response 2.3 ± 0.8 h after the beginning of exposure to hypoxia, then decreased and reached a minimum at 12 ± 3.4 h, followed by a second increase reaching a maximum response at 48.0 ± 8.0 h.

4.4.2 Series II: time domains over 7 d at 22°C

At 22°C, bowfin exposed to hypoxia without access to air increased gill breathing frequency compare to bowfin with access to air (F_{1, 138}=109.6, P<0.001). Time was a significant factor (F_{13, 138}=4.54, P<0.001), and there was a significant interaction between time and treatment (F_{13, 138}=6.76, P<0.001). In bowfin without access to air there was a 2x increase in gill breathing frequency at all time points when compared to initial resting values (normoxia) (Holm-Sidak, P<0.05) and these values were significantly different than those of bowfin with access to air at all time points except initial resting values (Holm-Sidak, initial P=0.96, P<0.0001 for all others) (Fig. 4.3 A). Gill breathing frequency in bowfin with access to air was not significantly different from resting values (normoxia) (Holm-Sidak, P= 0.059 – 0.96). Bowfin exposed to hypoxia without access to air tended to have higher buccal pressure than bowfin with access to air, however these differences were not statistically significant (F_{1, 106}=3.07, P=0.11) and these values were not significantly different from initial resting values (F_{13, 106}=1.55, P=0.11) (Fig. 4.3 B). Bowfin exposed to hypoxia without access to air had increased gill ventilation product (total ventilation) compare to bowfin with access to air (F_{1, 106}=18.5, P<0.01), time was a significant factor (F_{13, 106}=2.40, P<0.01), and there was a significant interaction between time and treatment (F_{13, 106}=2.22, P<0.05). In bowfin without access to air there was a 2.5 - 3x increase in gill ventilation product at all time points when compared to initial resting values (normoxia) (Holm-Sidak, P<0.05) and these values were significantly different from those of bowfin with access to air at all time points except initial resting values and the first 1.5h of hypoxia (Holm-Sidak, initial to 1.5h P=0.06 – 0.852, P<0.05 for all others) (Fig. 4.3 C).

Bowfin exposed to hypoxia without access to air had significantly lower heart rates compare to bowfin with access to air (F_{1, 126}=7.47, P<0.05). Time was a significant factor
(F_{13, 126}=3.77, P<0.001), and there was a significant interaction between time and treatment (F_{13, 126}=4.37, P<0.001) (Fig. 4.3 E). Bowfin without access to air responded to hypoxia by decreasing heart rate by 20% within 0.5h but this immediately recovered. In these bowfin heart rate was significantly lower at 24h but then recovered to initial resting values. Bowfin with access to air had an acute tachycardia from 1.5 to 3h of hypoxia but this also recovered within 24h of exposure to hypoxia.

Bowfin with access to air did not display any significant changes in cardiorespiratory variables during recovery from hypoxia since all variables were already at initial levels at the end of the 7d of hypoxia (Fig. 4.4 A-E). Bowfin without access to air maintained an elevated gill breathing frequency, which was significant 5h after return to normoxia (F_{7, 31}=18.2, P<0.001) (Fig. 4.4 A). In these fish buccal pressure was significantly higher at the end of hypoxia than initial values (F_{7, 24}=3.92, P<0.01), but recovered within 0.5h (Fig. 4.4 B). Similarly, the index of ventilation product was significantly higher at the end of the hypoxic exposure than initial values (F_{7, 24}=8.07, P<0.001), but recovered to within initial values within 0.5h of return to normoxia (Fig. 4.4 C). In bowfin with access to air, air breathing frequency increased from 1.5 ± 1.3 to 7.0 ± 2.2 breaths h^{-1} (Fig. 3D) and then slowly decreased back to 2.8 ± 1.2 breaths h^{-1} (Fig. 4.4 D). These changes were not significant. Interestingly, in bowfin without access to air, heart rate was significantly higher during recovery than both resting values and at the end of hypoxic exposure (F_{7, 27}=15.4, P<0.001) (Fig. 4.4 E). Furthermore, heart rate remained significantly higher for the remainder of the 5 h recovery period.

In bowfin with access to air differences between cardiorespiratory variables before and after an air breath were observed (Fig. 4.5). These observations were quantified to see if time domains occurred during this response. Generally, there was a trend for gill breathing frequency to be larger before an air breath than after (F_{1, 7}=7.54, P=0.07) (Fig. 4.6 A). One-tail t-tests revealed that the difference in gill breathing frequency was higher before an air breath at 1, 2, and 6d of exposure to hypoxia (Fig. 4.6 B). Heart rate was significantly lower before an air breath than after (F_{1, 7}=219.7, P<0.05) (Fig. 4.6 C) and the difference in heart
rate was significantly lower before an air breath at 2, 4, 6, and 7d of hypoxia than after the air breath (Fig. 4.6 D).

4.5 Discussion

4.5.1 Are time domains associated with brief hypoxic exposures evident in bowfin?

As hypothesized, time domains of the hypoxic ventilatory response were apparent in bowfin without access to air. Bowfin exposed to hypoxia increased gill breathing frequency and amplitude but the response was much slower than anticipated. In mammals the acute response takes place during the first couple of breaths of inhaled hypoxic air and this response is activated by the peripheral chemoreceptors; the glomus cells in the carotid bodies (Bisgard and Neubauer, 1995). In contrast, the response in bowfin took as much as 1-2h to reach a maximum at both 8 (Fig. 4.1, 4.2) and 22°C (Fig. 4.3). This could be due to the gradual onset of hypoxia which took ~30 min to reach the desired level in the experimental chamber during both series of experiments. However, in a very similar experiment in which bowfin were instantly exposed to a similar level of hypoxia (47 mm Hg at 20°C), there was also a gradual increase in both opercular pressure and gill ventilation frequency over the first 15 min of exposure to hypoxia (McKenzie et al., 1991), a much longer time scale than the response in mammals (seconds). It is likely that, just as in mammals, peripheral oxygen chemoreceptors, (the neuroepithelial cells (NECs) found in fish gills) are responsible for the acute hypoxic ventilatory response. Hypoxia causes the depolarization of gill NECs in zebrafish (Danio rerio) and channel catfish (Ictalurus punctatus) grown in culture (Burleson et al., 2006; Jonz et al., 2004) and increases the firing rate of afferent nerves arising from the gills in trout (Oncorhynchus mykiss) (Burleson and Milsom, 1995).

Although the resolution required for making inferences related to time domains related to brief exposure to hypoxia was not present in the current experiments, conclusions can be drawn by also considering findings from a previous study on bowfin. Bowfin without access to air respond to acute hypoxia with a gradual increase in gill breathing frequency and buccal pressure (amplitude) (McKenzie et al., 1991) indicating that a progressive increase in ventilation following the acute response is taking place. In mammals this gradual increase
has been called short term potentiation (STP). The mechanisms behind this time domain in mammals are still largely unknown but might involve central integration of respiratory information. In mammals, the afferent neuron innervating the carotid body, the carotid sinus nerve, projects to the medulla in an area called the nucleus tractus solitarius (NTS), where afferent information is processed (Chitravanshi and Sapru, 1995).

The response of neurons in the NTS to carotid sinus nerve stimulation increased two minutes after the cessation of stimulation of the carotid sinus nerve indicating they are likely involved in STP (Mifflin, 1997). It has also been suggested that STP increases respiratory stability by reducing variability in the hypoxic response (a smoothing effect) (Lee et al., 2009). Although, not much is known about the integration of respiratory information in fish, preliminary studies indicate that the integration of respiratory information takes place in the medulla in the general visceral nucleus in channel catfish (Ictalurus punctatus) (Sundin et al., 2003a), a similar location to the NTS of mammals. This is suggesting that the respiratory reflex pathways are similar in fish and mammals and perhaps similar mechanisms underlie this hypoxic ventilatory time domain.

In mammals short term depression (STD) has been described as the fall in respiratory frequency following the initial overshoot that takes place a few seconds to a min after the beginning of the hypoxic exposure. The reverse is often seen on return to normoxia (recovery). In vertebrates, neuronal circuits within the medulla are capable of producing a respiratory rhythm endogenously, without chemosensory feedback. The purpose of the sensory feedback is to alter this endogenous respiratory rhythm in order to match ventilation to the animal’s needs. Because STD is strictly associated with respiratory frequency in mammals, the mechanism behind this time domain is believed to be associated with the rhythm generator sites in the brain (Powell et al., 1998). STD was not evident in either the current study or a previous one (McKenzie et al., 1991) which might have to do with the relative slow development of HVR in bowfin and other fish.
4.5.2 Are time domains associated with sustained hypoxic exposures evident in bowfin?

When looking at the average response of bowfin to sustained hypoxia (Figs. 4.1, 4.3), the interspecific variation in the response was striking (Fig. 4.2). Despite this, a decrease in gill buccal pressure during the first 24h was apparent in all bowfin (Series I) resulting in a decrease in the index of ventilation product (indicative of total gill ventilation). Hypoxic ventilatory decline (HVD) has been defined as a decrease in ventilation compared to the acute response which serves to conserve energy during hypoxia (Neubauer et al., 1990). It is still uncertain whether this response is due to a decrease in peripheral chemosensory activity or central neural activity but recent research has suggested that the carotid bodies of rats respond to hypoxia in a biphasic manner suggestive of HVD (Pandit et al., 2010). Similar recordings have not been performed in the NECs of fish but similar changes would explain the HVD in this vertebrate group.

Ventilatory acclimatization to hypoxia (VAH) was defined as a time-dependent increase in ventilation that occurs over a period of hours to months of exposure to hypoxia (Powell et al., 1998). The mechanisms behind this increase in ventilation are increases in peripheral chemosensitivity and altered central nervous system processing as a result of changes in gene expression (Powell, 2007) modulated by hypoxia inducible factor 1α (HIF-1α) (Powell and Fu, 2008). In the current study a secondary increase in ventilation took place around 48h of exposure to hypoxia at 8°C (Fig. 4.2). Gene expression was not measured in the present study, but in a previous study of Gillichthys mirabilis exposed to 10% air saturated water (~ 15mm Hg) at 15°C, changes in gene expression, likely modulated by HIF-1α, were observed in muscle and liver (Gracey et al., 2001). Unfortunately, although gill and brain tissues were analyzed in the Gracey et al. (2001) study, the data was omitted from the results, and it was stated that only 4 genes were differentially expressed in the brain, with no indication of what these genes were. Again, this would indicate similar mechanisms behind the hypoxic response between vertebrate classes, but more studies are needed to further assess this.
4.5.3 Are time domains apparent in bowfin during recovery from hypoxia?

It has been shown that the time domains evident during acute exposure to hypoxia are also seen in reverse during recovery from hypoxia (Powell et al., 1998). Thus the slow progressive increase in breathing frequency on acute exposure to hypoxia in bowfin was mirrored by a gradual decrease during recovery. Just as there was no overshoot and subsequent reduction of gill breathing frequency on acute hypoxic exposure in bowfin without access to air, there was no undershoot during recovery. Total ventilation recovered relatively quickly in bowfin without access to air (over a period of 1 h) compared to the period taken for gill frequency to recover fully (>5h). Bowfin with access to air did not have elevated gill ventilation (Figs. 4.1, 4.3) and respiratory variables did not change from baseline values (Fig. 4.4 A-C). The trend for air breathing frequency to increase in hypoxia was gradually reversed over 5 h of aquatic hypoxia (Fig. 4.4 D).

4.5.4 Are time domains evident in the heart rate response to hypoxia?

In all primitive fishes heart rate is intrinsically low compared to teleosts at the same temperature, but the reason behind this phenomenon remains unknown (Farrell, 2007a). In the present study, bowfin without access to air had a resting heart rate of 31.2 ± 1.7 beats min\(^{-1}\) and it decreased by 20% at 0.5 and 24 h of exposure to hypoxia (Fig. 4.3 E), indicating that time domains are evident in bowfin without access to air. Interestingly, in this group of fish heart rate was ~50% higher during the 5h recovery period in this group of fish (Fig. 4.4 E). Very few studies have measured cardio-respiratory variables during recovery from hypoxia in fish, but a similar tachycardia was found in channel catfish (\textit{I. punctatus}) after exposure to mild hypoxia (75mm Hg) for 6d (Burleson et al., 2002).

In fish, heart rate is controlled by autonomic input and consists of the balance between adrenergic stimulation and cholinergic inhibition and the hypoxic bradycardia is caused by increased cholinergic tone (Olson and Farrell, 2006). In zebrafish (\textit{D. rerio}) larva \textit{M}_2 muscarinic receptors have a cardio-inhibitory role and have been shown to be responsible for the hypoxic bradycardia seen during sustained hypoxia (Steele et al., 2009). In bowfin without access to air heart rate returned to pre-exposure levels. Similarly, in the obligate
water breather the tambaqui (*Colossoma macropomum*) exposed to hypoxia (10 mm Hg) the bradycardia recovered within 6h (Florindo et al., 2006) and in carp (*Cyprinus carpio*) exposed to severe hypoxia (~6 mm Hg) the hypoxic bradycardia also partially recovered (Stecyk and Farrell, 2002). The mechanism behind this recovery in heart rate could be either a decrease in chemoreceptor input, adaptation of the central response, or changes in receptor densities of the heart. The significantly higher heart rate during recovery from hypoxia suggests that the likely mechanisms is a decrease in M2 muscarinic receptors, which would result in a larger adrenergic stimulation during hypoxia leading to a recovery in heart rate and then an increase in heart rate as cholinergic tone is released upon return to normoxia. Another potential explanation is that the increase in heart rate is due to an increase in circulating catecholamines. In trout (*O. mykiss*) moderate hypoxia (60 mm Hg) induced an increase in circulating catecholamines (Montpetit and Perry, 1998). Plasma catecholamines were not measured in the current study therefore this remains a possible explanation.

In bowfin with access to air, there was a tachycardia during the first 3 h of exposure to hypoxia, which returned to pre-hypoxic values within 24h (Fig. 4.3 E), but remained at resting levels during recovery (Fig. 4.4 E). To our knowledge no other studies have monitored heart rate in facultative air breathing fish exposed to sustained hypoxia with access to air. The initial tachycardia in bowfin with access to air is likely due to the increase activity related to air breathing, followed by a recovery as the fish settled down. In this group of fish changes in receptor density seems unlikely as the heart rate did not change during recovery from hypoxia.

### 4.5.5 The influence of air breathing on cardio-respiratory responses to hypoxia

Time domains were not evident in bowfin with access to air in either gill ventilation (Fig. 4.3 A-C) or air breathing (Fig. 4.3 D). This is not entirely surprising as these animals are probably not experiencing hypoxemia as shown in a previous study in bowfin that did not find significant differences in arterial PO2 when these fish had access to air (Randall et al., 1981). However, arterial PO2 has never been monitored during an entire air breathing cycle, thus these values are likely not static but fluctuating to low O2 values before an air
breath, that would serve to alleviate the hypoxemia (Hedrick et al., 1994). Air breathing behaviour has been shown to be modulated mainly by peripheral chemoreceptors, as complete denervation of bowfin gills and pseudobranch abolished the air breathing response (McKenzie et al., 1991). The peripheral chemoreceptors are believed to be able to sense both internal (blood) and external (water) \( \text{PO}_2 \), and relay this information via afferent nerves that project to the medulla and produce the air breathing behaviour.

Air breathing frequencies in this study were similar with those reported from previous studies in bowfin (Hedrick and Jones, 1993; Hedrick and Jones, 1999; Randall et al., 1981) [but see (Johansen et al., 1970)]. Unlike African catfish (**Clarias gariepinus**) that increase gill breathing frequency after an air breath (Belao et al., 2011), bowfin tended to have a decrease gill breathing frequency after an air breath or showed no significant difference (Fig. 6). It has been suggested that inhibition of gill breathing might prevent oxygen loss to the water via the gills (Johansen et al., 1970) but no shunts have been found in **Amia** to prevent oxygenated blood from the air breathing organ going back to the gills and some oxygen is lost to the water at the gills (Randall et al., 1981).

Unlike many other facultative air breathers which increase heart rate by >50% after an air breath (Belao et al., 2011; Farrell, 1978; McKenzie et al., 2007), bowfin show only a small (10-20%) change (this study) or no increase in heart rate during air breathing (Johansen et al., 1970). Similarly other air breathing fish such as the pirarucu (**Arapaima gigas**) (Farrell, 1978) and the African lungfish (**Protopterus aethiopicus**) [see table 3 in (Graham et al., 1995) for references] also do not increase heart rate after air breathing. The increase in heart rate after an air breath is associated with increased blood flow to the air breathing organ to ensure proper ventilation:perfusion matching (Farrell, 2007b). In bowfin however, this was achieved by an increase in gas bladder blood flow with an increase in total blood flow (Randall et al., 1981) perhaps only partly due to changes in heart rate but mostly due to an increase in stroke volume.
4.6 Conclusions

Just as in mammals, time domains are evident in the hypoxic ventilatory response of bowfin during exposure to moderate hypoxia but only when these animals do not have access to air, likely because hypoxaemia develops. However, the time domains were much slower in fish than in mammals. This may be partly due to procedural differences in the ability to induce a rapid environmental hypoxia, and partly due to differences in body temperature and could be related to \( Q_{10} \) effects as previously suggested (Porteus et al., 2011). Given that, changes in heart rate and ventilation do occur over time in response to hypoxia, however, comparison of data between studies should be made with this in mind as should the design of future studies. Additionally, the mechanisms underlying time dependent changes in responses are still largely unknown providing an area ripe for future research.
Figure 4.1 Ventilatory responses of bowfin (*Amia calva*) exposed to normoxia (155±0.8 mm Hg, 8°C) or hypoxia (25.7±3.9 mm Hg, 9°C) for 72h without access to air. A) relative breathing frequency (Vf, % change); B) relative buccal pressure (Vampl, % change); C) index of ventilation product (Vtot, % change). # indicate significant differences from time 0h (P<0.05). † indicate significant difference between treatments at a particular time point (P<0.05). Bar graphs not sharing the same letters are significantly different from one another (P<0.05). Values are mean ± SEM.
Figure 4.2 The response of individual bowfin (*Amia calva*) (A-F) to hypoxia (25.7±3.9 mm Hg, 8°C) for 72h without access to air. Note that each fish had an initial increase in ventilatory variables, followed by an initial decrease and often a secondary increase in relative breathing frequency (*V_t*, dashed light grey line), relative buccal pressure (*V_{ampl}*), dashed dark grey) or relative index of ventilation product (*V_{tot}* solid black line). G) Average + SEM relative breathing frequency (*V_t*, % change), relative buccal pressure (*V_{ampl}* % change) and relative index of ventilation product (*V_{tot}* % change) during normoxic conditions, acute response (AR), hypoxic ventilatory decline (HVD), and ventilatory acclimatization to hypoxia (HVD) (averages independent of the time they occur at). Responses not sharing the same letters are significantly different from one another (P<0.05).
Figure 4.3 Cardio-respiratory responses of bowfin (*Amia calva*) in response to sustained aquatic hypoxia (45.4 ± 6.9 mm Hg, 22°C) with (grey lines) and without (black lines) access to air: A) gill breathing frequency (*V*_f_*, breaths min^{-1}); B) buccal pressure (*V*_amp*, cm H₂O); C) index of ventilation product (*V*_tot*, cm H₂O min^{-1}); D) air breathing frequency (*V*_f_, breaths h^{-1}) in bowfin with access to air; E) heart rate (beats min^{-1}). * indicate significant differences from time 0h (normoxia). Values are mean ± SEM.
Figure 4.4 Cardio-respiratory responses of bowfin (*Amia calva*) during 5h of recovery from sustained aquatic hypoxia (45.4 ± 6.9 mm Hg, 22°C) with (grey lines) and without (black lines) access to air. A) gill breathing frequency ($V_f$, breaths min$^{-1}$); B) buccal pressure ($V_{ampl}$, cm H$_2$O); C) index of ventilation product ($V_{tot}$, cm H$_2$O min$^{-1}$); D) air breathing frequency ($V_f$, breaths h$^{-1}$) in bowfin with access to air; E) heart rate (beats min$^{-1}$). Bowfin that had access to air during hypoxia also had access to air during recovery, and animals without access to air during hypoxia did not have access to air during recovery. Solid grey line and dashed lines represents normoxic values and their SEM. * indicate significant differences from time 0h (hypoxia). Values are mean ± SEM.
Figure 4.5 Sample traces from the buccal pressure trace (A) and the ECG signal trace (B) of a bowfin (*Amia calva*) at 22°C during an air breathing event (arrow) which caused a large deflection in the buccal pressure trace. The small up and down reflections in the buccal pressure trace (A) represent gill breathing movements and they were used to calculate breathing frequency and changes in buccal pressure before and after an air breathing event. Similarly each spike on the ECG trace represents a heartbeat and these were used to calculate heart rate.
Figure 4.6 Cardiorespiratory changes during an air breathing event in bowfin (*Amia calva*) exposed to hypoxia (45.4 ± 6.9 mm Hg, 22°C) with access to air before (light grey line) and after (black line) an air breath (AB). Values were calculated using traces similar to the ones shown in the previous figure. A) Average gill breathing frequency ± SEM; B) Average difference + SEM between before and after an air breath; C) Average heart rate ± SEM; D) Average difference + SEM between before and after an air breath. Note positive values indicate the rate was lower before the air breath and negative values indicate the frequency was higher before the air breath. * indicate statistically significant difference from zero (no difference) at individual time points (P<0.05).
Chapter 5. The effect of sustained hypoxia on the cardio-respiratory response of bowfin (*Amia calva*): implications for changes in the oxygen transport system

5.1 Summary

Time domains of the hypoxic ventilatory response (HVR) have been identified in bowfin (*Amia calva*) exposed to sustained hypoxia without access to air but not in those with access to air. The purpose of this study was to examine mechanisms underlying cardio-respiratory acclimation to moderate sustained hypoxia (45 mm Hg for 7 days at 22°C) in the facultative air-breathing bowfin *Amia calva*. This level of hypoxia is slightly below the critical oxygen tension of bowfin denied access to air (70.0 ± 8.8 mm Hg). Before exposure to sustained hypoxia, bowfin with access to air increased air breathing frequency on exposure to acute progressive hypoxia while bowfin without access to air increased gill ventilation frequency. Exposure to sustained hypoxia increased the gill ventilation response to acute progressive hypoxia in *Amia* that lacked access to air, regardless of whether they had access to air or not during the sustained hypoxia. Additionally, there was a decrease in Hb-O$_2$ binding affinity in these fish. This suggests that acclimation in *Amia* elicits changes that increase oxygen delivery to the gas exchange surface for oxygen uptake, and that reduce haemoglobin affinity to enhance oxygen delivery to the tissues. These data suggest that VAH in bowfin could be partially due to changes in the oxygen transport system.

5.2 Introduction

Previously, I showed that time domains are evident in the hypoxic ventilatory response of bowfin (*Amia calva*) to moderate hypoxia (Chapter 4). Such changes over time in the hypoxic ventilatory response could reflect changes in other steps that match ATP supply and demand by optimizing the flow of oxygen from the gills to the tissues (Weibel, 1984). Alternatively, time domains in the hypoxic ventilatory response could reflect changes in elements of the respiratory control system as has been shown in mammals (Powell et al.,
A third possibility is that time domains reflect both changes in the oxygen transport cascade and changes in the control of breathing.

In some obligate water breathers it has been shown that oxygen uptake and transport to the tissues is enhanced following sustained exposure to moderate hypoxia. This was due to an increase in Hb-O$_2$ binding affinity in trout *Oncorhynchus mykiss* (Walbaum 1792) (Bushnell et al., 1984; Rutjes et al., 2007; Soivio et al., 1980; Tetens and Lykkeboe, 1981) and an increase in oxygen carrying capacity of the blood in trout and cod *Gadus morhua* (Linnaeus 1758) (Petersen and Gamperl, 2011; Rutjes et al., 2007; Tetens and Lykkeboe, 1981). The responses of some facultative air breathers to sustained hypoxia have also been examined, but usually while the fish had access to air, and the results have been variable. For example, in one study of the facultative air breather *Synbranchus marmoratus* (Bloch 1795) sensitivity to hypoxia increased after exposure to sustained hypoxia (6 weeks at 20-25 mm Hg with access to air at 25°C) (Eduardo et al., 1979), while in another study the same species no changes were observed in sensitivity to hypoxia after a similar exposure to sustained hypoxia (6-10 weeks at <20 mm Hg with access to air at 25-27°C) (Graham and Baird, 1984). The reasons for such differences are not clear but may include differences in behaviour, such as the use of air breathing. The bowfin *Ampia calva* (Linnaeus 1766), a facultative air breathing fish with well developed gills, when given access to the surface to breath air during exposure to severe aquatic hypoxia (14 mm Hg) is reported to either remain normoxic (Randall et al., 1981) or become transiently hypoxaemic only just before an air breath (Hedrick et al., 1994). This raises the possibility that changes in the hypoxic cardio-ventilatory response following sustained hypoxia arise from hypoxaemia and not hypoxia per se.

The purpose of this study was to test this hypothesis; that exposure of bowfin to sustained moderate hypoxia would lead to long term changes (acclimation) in the oxygen transport pathway only in fish experiencing sustained hypoxaemia when denied access to air, but not in fish experiencing transient hypoxaemia when they have access to air. We first selected a level of moderate hypoxia just below the level that begins to cause metabolic depression in bowfin. The cardio-respiratory responses of bowfin to acute progressive
hypoxia with and without access to air were also measured, before and after exposure to sustained moderate hypoxia (7 days, just below the critical oxygen tension) with and without access to air. The Hb-O₂ binding properties of bowfin before and after exposure to sustained hypoxia were also determined.

### 5.3 Materials and methods

#### 5.3.1 Animals

Adult bowfin (340 - 2500g), *Amia calva*, were caught by a local fisherman from the Bay of Quinte, Ontario and were transported to the University of Guelph where they were kept in recirculating water tanks at the Hagen Aqualab. Bowfin were acclimated to 22°C before the start of any experiments, and were kept on a 12/12-h light/dark photoperiod and fed live goldfish once a week.

#### 5.3.2 Series I: metabolic rate

The metabolic rate was measured as previously described (Scott et al., 2008). Briefly, bowfin were transferred from their holding tank to a black box (volume 7.5 l) supplied with flow-through aerated water and were left overnight before the start of any measurement. A small submersible pump (ExoTerra® Repti Flo 200) inside the black box ensured that the water was well mixed. The bowfin did not have access to air during this series. A Hach 30d Oxygen probe (Hach, Loveland, CO, USA) placed in the box was used to measure oxygen partial pressure. The water flow to the black box was stopped and the oxygen was measured every 30s until it reached ~ 17 mm Hg. The flow of water was then restarted.

#### 5.3.3 Series II: acute progressive hypoxic exposure prior to and following exposure to sustained hypoxia

Fish were anesthetized with benzocaine (0.3 mM Benzocaine) by immersion. They were then instrumented with buccal and opercular cannulae. One ECG electrode was placed midventrally between pectoral fins and secured in place with 3 sutures on the belly and 1 at
the anterior edge of the dorsal fin. Sometimes to improve the signal quality a second electrode was placed midventrally between the pelvic fins and also secured with 3 sutures on the belly and 1 at the anterior edge of the dorsal fin. During the surgery, the gills were flushed with aerated water containing benzocaine (0.3 mM Benzocaine). The fish were allowed to recover for at least 24h in a black box supplied with aerated flow-through water.

For acute progressive hypoxic exposure a closed 75 l experimental chamber was used consisting of 2 compartments: one chamber was used to control the O$_2$ level by introducing air or N$_2$ gas and the other contained the fish. The two were connected by a pump (Rule Bilge Pump 500gph) and standpipe. Both compartments could be drained and filled individually, therefore water could be changed with minimum disturbance to the fish (see Rantin and Kalinin, 1996 for details). The fish were placed in the experimental tank and left to adjust for 3-12 h. Water was changed every 4-5 h in order to prevent nitrogenous waste from building up. The experimental chamber had 3 opaque walls and one clear wall that faced away from the experimenters to avoid unnecessary stress to the fish. An angled mirror facing the clear side was used to monitor the fish in order to confirm air breaths on the buccal pressure trace.

Fish with access to air were exposed to normoxia (140 mm Hg) and 4 levels of hypoxia (112, 84, 63, and 35 mm Hg) for 30 min each to characterize their cardio-respiratory responses to hypoxia. The level of hypoxia was controlled manually by turning the nitrogen regulator on and off while using a Hach 30d oxygen probe (Hach, Loveland, CO, USA) to measure oxygen tension. Fish were then allowed to recover for 2 - 3 h. They were then denied access to air by placing a piece of perforated pvc just below the water surface. The experiment was then repeated to characterize their cardio-respiratory responses without access to air. The bowfin were then exposed to sustained hypoxia for 7 days (described below) and their response to acute hypoxia with and without access to air was re-measured.

After the initial progressive hypoxia exposure, bowfin were randomly assigned to either of two groups: one with and one without access to air. They were placed in 2 m diameter tanks (up to 4 animals per tank at once) and individually separated using perforated pvc partitions. Coverings were placed either 2 cm below the surface of the water (denying
fish access to air) or 10cm above the surface of the water (allowing fish access to air). Sustained hypoxia was achieved by bubbling nitrogen using a Parker Balston nitrogen generator (Model N2-04, Parker Hannifin Corp., Haverhill, MA, USA) into a large header tank (~100 l) and into the experimental tank. This design allowed the oxygen level in the experimental tank to be kept within a narrow O$_2$ range of 45.4 ± 6.9 mm Hg. Ventilation was measured with a disposable pressure transducer (Utah Medical Products Inc., USA) connected to the buccal cannula, filled with water, and connected to an amplifier (Electronics Workshop, University of British Columbia). Heart rate was recorded using a differential amplifier (World Precision Instruments, Dam 50). Both amplifiers were connected to a DI-720 interface (DataQ) and the data were recorded to computer using WindaQ Version 2.19 software (DataQ, Akron, OH, USA) at a sampling rate of 500 Hz. Buccal pressure and heart rate were measured continuously during each progressive hypoxia trial. The last 10 min of data recorded at each oxygen level were used to calculate the average for each variable in an individual fish. A data analysis script for Spike 2 (Version 5.08, Cambridge Electronic Design, Cambridge, England) was used to measure the amplitude of the pressure trace deflection associated with gill movements, and the gill breathing frequency.

5.3.4 Series III: blood haemoglobin oxygen binding affinity

Bowfin were lightly anaesthetized using benzocaine (0.3mM Benzocaine) by immersion and immediately a 100-200 µl blood sample was taken by caudal puncture using a 1.5 inch 22 gage needle attached to a 1cc syringe that had been pre-rinsed with 0.5M EDTA to prevent blood clotting. Bowfin did not show any signs of struggle during this procedure. Blood samples were collected both before exposure to sustained hypoxia and after overnight recovery from exposure to sustained hypoxia. Blood was stored in pre-rinsed (0.5M EDTA) 1.5 ml tubes at 4 °C for 6-12 h before analysis. Blood (1 µl) was analyzed for Hb-O$_2$ binding affinity using a custom built P$_{wee}$ 50 (La Trobe University, Bundoora, Australia, model 1.1 oxygen mixer and analyzer). The absorbance at 396 nm (the approximate isosbestic point between oxy- and deoxy-Hb) and 425 nm (approximate peak
absorption for deoxy-Hb) was measured at 0, 1, 2, 3, 4, 5, 6, 12, 18, and 22 % O₂ at 3 different levels of CO₂ (0.5, 1, and 1.5%) the balance being nitrogen gas. The measurements were taken at 22°C. The P₅₀ values were calculated using the Microsoft Excel calculation file of the P_wee system using Excel macros. The Root effect (the reduction in Hb-O₂ binding affinity due to a decrease in pH) of each blood sample was calculated as a decrease in its saturation relative to 20% O₂ and 0.5% CO₂ gas conditions. nH was determined from the oxygen dissociation curves generated by the P_wee system.

5.3.5 Statistical analysis

Water oxygen content was calculated from partial pressure measurements based on the solubility of oxygen in water at 22°C. Metabolic rate (µm kg⁻¹ min⁻¹) was calculated over sequential 5 min periods using the slope of the oxygen trace (water O₂ content over time) and the volume of water in the box. The P_crit was determined by using the BASIC program designed by (Yeager and Ultsch, 1989) to determine the inflection point at which metabolic rate transitions from being independent to being dependent on environmental PO₂ (Pörtner and Grieshaber, 1993).

An ANCOVA was used to test for differences between non mass-corrected metabolic rates using oxygen level as a factor and weight as a co-factor as suggested by Packard and Boardman (Packard and Boardman, 1999). A Holm-Sidak post-hoc test was used to test for differences from control (metabolic rate in normoxia). In Series II a False Discovery Rate test (Benjamini and Hochberg, 2000) was used to test for differences from control (normoxia or before exposure to sustained hypoxia). To determine if there were differences between gill breathing frequency and heart rate before and after an air breath at the different oxygen tensions and between groups, a RM ANOVA was performed. This analysis did not reveal any statistical differences between groups or oxygen tensions, therefore, the data for each variable were pooled and tested for differences before and after an air breath by a t-test. RM ANOVA was not used for testing for differences in P₅₀ and Hill coefficients (n_H) because experimental constraints negated the use of data from the same animal before and after exposure to sustained hypoxia, therefore a 2-way ANOVA (instead of a 2-way RM ANOVA)
was performed on these data to look for differences between groups. Where necessary, a Holm-Sidak post-hoc test was used to test for differences between treatment groups and among levels of CO₂. All statistical analyses were done using SigmaStat 3.5 (Systat Software Inc., San Jose, CA, USA), except those from Series II where R (R Development Core Team, 2012) was used. A significance level of P < 0.05 was used throughout.

5.4 Results

5.4.1 Series I: metabolic rate

The average resting metabolic rate in bowfin in normoxic water without access to air at 22°C was 33.5 ± 3.6 µmol kg⁻¹ min⁻¹ (Fig. 1). This level of metabolism was sustained initially as the water PO₂ began to drop and became significantly reduced at 43 mm Hg (Fig. 1). Average Pₖᵣᵢₜ in bowfin without access to air was 70.0 ± 8.8 mm Hg and ranged between 45 and 115 mmHg.

5.4.2 Series II: acute progressive hypoxic exposure prior to and following exposure to sustained hypoxia

Bowfin with access to air during acute progressive hypoxia:

Bowfin with access to air during acute progressive hypoxia maintained gill breathing frequency (Fig. 2A), amplitude (Fig. 2B), ventilation product (Fig. 2C) and heart rate (Fig. 2C) at normoxic levels (P > 0.05) and increased air breathing frequency (Fig. 2E) as the level of oxygen in the water decreased (P < 0.0001). Following exposure to sustained hypoxia, bowfin that had had access to air during the sustained hypoxia had a significantly lower air breathing frequency at the most severe level of acute hypoxia compared to their response before exposure to sustained hypoxia (P = 0.003, Fig. 2E). This was not the case for bowfin that did not have access to air during the sustained hypoxia. The heart rate of all bowfin was significantly greater after an air breath than before an air breath (P = 0.021); this occurred irregardless of the level of water PO₂ (P = 0.26) or treatment (P = 0.05) (Fig. 3 A). Gill breathing frequency was not statistically different before and after an air breath (P=0.11) (Fig. 3 B).
Bowfin without access to air during acute progressive hypoxia:

Bowfin without access to air during acute progressive hypoxia significantly increased gill breathing frequency at 63 and 35 mmHg (P = 0.048 and P = 0.009, respectively, Fig. 4A) but not amplitude. Following exposure to sustained hypoxia, both the bowfin with and without access to air during the sustained hypoxia had higher resting breathing frequencies; however, the bowfin exposed to sustained hypoxia without access to air did not increase gill breathing frequency as much during acute progressive hypoxia while the bowfin with access to air during sustained hypoxia increased gill breathing frequency more resulting in significantly different responses from before exposure to sustained hypoxia in both groups (P < 0.00001 and P< 0.00001 respectively).

Furthermore, following exposure to sustained hypoxia, both the bowfin with and without access to air during the sustained hypoxia increased gill breathing amplitude with a decrease in oxygen tension (P=0.022, P=0.025 respectively) (Fig. 4B). Bowfin with and without access to air during exposure to sustained hypoxia increased ventilation product (total ventilation) more at the most severe level of acute hypoxia than fish that had not been exposed to sustained hypoxia (P = 0.005, and P = 0.049 respectively) (Fig. 4C).

As expected, bowfin without access to air during acute progressive hypoxia significantly decreased heart rate at 112, 84, 63 and 35 mmHg (P < 0.05 for all oxygen tensions) (Fig. 4 D) compared to normoxic bowfin. The heart rate of bowfin with access to air during sustained hypoxia was significantly higher at 35 mmHg than before exposure to sustained hypoxia (P = 0.021).

5.4.3 Series III: blood haemoglobin oxygen binding affinity

In blood taken from fish before exposure to sustained hypoxia at air saturated oxygen tensions (~160 mm Hg) the Root effect reduced the Hb-O₂ saturation from 100% to 82.3±2.6% and 83.9±4.7% with an increase in CO₂ from 0.5% to 1.0 and 1.5% (Fig. 5). After exposure to sustained hypoxia in animals with access to air, at air saturated oxygen tensions (~160 mm Hg) the Root effect reduced the Hb-O₂ saturation from 100% to 89.1±3.4% and 89.7±3.2% with an increase in CO₂ from 0.5% to 1.0 and 1.5% (Fig. 5). After exposure to
sustained hypoxia in fish without access to air, at air saturated oxygen tensions (~160 mm Hg) the Root effect reduced the Hb-O₂ saturation from 100% to 77.2±10.7% and 80.7±11.2% with an increase in CO₂ from 0.5% to 1.0 and 1.5% (Fig. 5).

The P₅₀ values at different CO₂ tensions were significantly different from one another (F₂, s₄=12.2, P<0.001) (Fig. 6). The P₅₀ values were also significantly different between treatment groups (F₂, s₄=9.24, P<0.001). The P₅₀ values before exposure to sustained hypoxia were 13.0±0.9, 15.7±0.9, and 19.5±1.6 mm Hg at 0.5, 1.0, and 1.5% CO₂ respectively. These values were significantly lower than in bowfin after exposure to sustained hypoxia with and without access to air (P<0.001, P=0.001 respectively), however there were no significant differences between P₅₀ values of fish with or without access to sustained hypoxia after sustained hypoxia (P=0.75).

The cooperativity (nₜ) was low in bowfin before exposure to hypoxia (1.47±0.03, 1.51±0.06, and 1.47±0.08 at 0.5, 1.0, 1.5 % CO₂ respectively) (Fig. 7). nₜ was not significantly different between the three CO₂ tensions (P=0.683) and it did not change significantly in bowfin exposed to sustained hypoxia with or without access to air (P=0.541).

5.5 Discussion

This study demonstrates that sustained exposure to moderate hypoxia is sufficient to induce changes in the oxygen transport cascade of bowfin, *Amia calva*. The critical oxygen tension of *Amia* without access to air was determined for the first time (Pₕ₅₀ = 78.8 ± 8.3 mmHg) and exposure to sustained hypoxia at levels just below this increased the gill ventilation response to acute progressive hypoxia (increased aquatic ventilatory sensitivity) when the *Amia* lacked access to air, regardless of whether they had access to air or not during the sustained hypoxia. Additionally, there was a decrease in Hb-O₂ binding affinity in these fish. This suggests that acclimation in these fish elicits changes that increase oxygen delivery to the gas exchange surface for oxygen uptake, and that reduce haemoglobin affinity to enhance oxygen delivery to the tissues.
5.5.1 Critical oxygen tension

Bowfin without access to air had resting metabolic rates of 33.5 ± 3.6 µmol O₂ kg⁻¹ min⁻¹ (~64 mg O₂ kg⁻¹ hr⁻¹) at 22°C, which were similar to previously reported values in resting bowfin with access to air at similar temperatures (Farmer, 1998; McKenzie et al., 1991; Randall et al., 1981). 43 mm Hg was the first point at which oxygen consumption rate was significantly depressed below normoxic values, therefore, the Pcrit (critical oxygen tension) of bowfin without access to air is between 43 and 60 mm Hg (Fig 5.1). These data indicate that bowfin without access to air are only moderately hypoxia tolerant. This Pcrit was similar to other previous reported values (33 - 70 mm Hg) for facultative air breathing fish without access to air (Affonso and Rantin, 2005; Belao et al., 2011; Hughes and Singh, 1971; Lefevre et al., 2011; Mattias et al., 1998; Oliveira et al., 2004; Perna and Fernandes, 1996).

5.5.2 Acute progressive hypoxic exposure prior to sustained hypoxia

Bowfin with access to air during acute progressive hypoxia responded by increasing air breathing frequency without any changes in gill breathing or heart rate (Fig. 2). Unlike other facultative air breathers (Geiger et al., 2000; Lefevre et al., 2011), bowfin did not decrease gill breathing frequency or amplitude in hypoxia, a response that is thought to prevent oxygen loss from the blood to the water. However, despite this, there are reports that oxygen loss via the gills is minimal in bowfin even in severe hypoxia (14 mm Hg) (Randall et al., 1981). The reasons for this are unclear but may reflect changes in blood flow through the gills rather than in water flow over the gills.

Two types of air breaths have been identified in bowfin: type I breaths that consist of an exhalation and an inhalation important for gas exchange, and type II breaths that consist of an inhalation phase only, important for buoyancy control (Hedrick and Jones, 1993). In the present study it was impossible to distinguish between the two types of breaths from the buccal pressure traces. In bowfin exposed to mild hypoxia (6-7 kPa or 45-53 mm Hg), air breathing frequency was reported to increase (Hedrick and Jones, 1993; McKenzie et al., 1991) and similar results were found in the present study.
Another defining characteristic of most facultative air breathers are changes in gill breathing frequency and heart rate in association with an air breath: gill breathing frequency tends to be higher and heart rate lower before an air breath than after. In the current study there was a modest but significant increase in heart rate of 4.7 ± 0.65 beats min\(^{-1}\) after an air breath compared to before an air breath, but no significant change in gill breathing frequency (Fig. 3). This increase in heart rate was not as dramatic as that seen in other facultative air breathers where the difference can be as large as 22-37 beats min\(^{-1}\) (Belao et al., 2011; McKenzie et al., 2007; Singh and Hughes, 1973). This increase in heart rate following an air breath, however, has been shown to be affected by the oxygen content of the air breathing organ (ABO) probably stimulating chemoreceptors, as well as by feedback from mechanoreceptors (volume changes) in the ABO in *S. marmoratus* and *Monopterus albus* (Zuiew 1793) (Graham et al., 1995). Such mechanoreceptors (slowly adapting pulmonary stretch receptors) have been identified in the ABO of *Amia* (Milsom and Jones, 1985). Neuroepithelial cells in the air breathing organ of *Amia* have also been described (Goniakowska-Witalińska, 2009) and as in other facultative air breathers they are thought to be involved in oxygen chemoreception and to generate cardio-respiratory reflex responses to hypoxia (Coolidge et al., 2007).

Bowfin without access to air during acute progressive hypoxia responded by increasing gill breathing frequency but not breathing amplitude (Fig. 4A,B) and also exhibited a moderate bradycardia (Fig. 4D). Only one previous study has looked at the response of a facultative air breather to hypoxia without access to air. In that study, channel catfish *Ictalurus punctatus* (Rafinesque 1818) responded to acute progressive hypoxia by increasing gill ventilation and reducing heart rate (Burleson et al., 2002). Thus the responses to acute progressive hypoxia seen in both studies of facultative air-breathers when denied access to air are similar to those of obligate water breathers.

**5.5.3 Acute progressive hypoxic exposure with access to air after sustained hypoxia**

Before exposure to sustained hypoxia, bowfin with access to air increased air breathing frequency and showed no changes in gill ventilation or heart rate. Following
exposure to sustained hypoxia (with or without access to air) these fish still did not exhibit any changes in gill breathing or heart rate in response to acute progressive hypoxia. Their primary response to the acute hypoxia was still to increase air breathing. This suggests that the initial response to aquatic hypoxia is to air-breathe, and that an increase in gill ventilation and accompanying bradycardia are only elicited if air breathing is prevented and/or the arterial blood becomes sufficiently hypoxaemic. This suggests that O$_2$ chemoreceptors sensing aquatic PO$_2$ may set the level of respiratory drive but that those sensing blood PO$_2$ determine whether that is expressed as an increase in ABO versus gill ventilation (Hedrick et al., 1994). Furthermore it suggests that the internal threshold for triggering air breaths is higher than that which triggers increases in gill ventilation and this is similar to what has been found in the jeju *Hoplerythrinus unitaeniatus* (Spix and Agassiz 1829) (Lopes et al., 2010).

Interestingly, in the fish that had access to air during the sustained hypoxia, this air breathing response was reduced (Fig. 2E). This was surprising but may not necessarily reflect an overall reduction in pulmonary gas exchange. The response we are describing here is the increase in air-breathing frequency, not in total air breathing ventilation. In the present study we did not measure the tidal volume of each air breath. An increase in air breathing amplitude would allow a reduction in air breathing frequency, resulting in conservation of energy by reducing trips to the surface to air breathe. Additionally, a decrease in air breathing frequency could decrease the risk of predation. It is also possible that exposure to sustained hypoxia in this group could have led to an increase in the efficiency of O$_2$ transfer from the gas bladder to the blood reducing air breathing frequency.

### 5.5.4 Acute progressive hypoxic exposure without access to air after sustained hypoxia

Before exposure to sustained hypoxia bowfin without access to air increased gill ventilation frequency but not gill breathing amplitude. Following exposure to sustained hypoxia, with or without access to air, *Amia* also increased gill breathing amplitude with a decrease in oxygen tension (Fig. 4B). It has been proposed that increasing breathing...
amplitude rather than frequency is a more efficient way of breathing (Shelton et al., 1986) suggesting that bowfin exposed to sustained hypoxia adopted this strategy as a way of reducing the oxidative cost of the increased ventilation. The net result was that bowfin exposed to sustained hypoxia with and without access to air had an enhanced ventilatory response to acute progressive hypoxia. An increased ventilatory sensitivity to hypoxia after exposure to sustained hypoxia (7 days acclimation at 75 mm Hg) has also been reported in the facultative air breathing channel catfish, *I. punctatus* (Burleson et al., 2002) and the water breathing flounder, *Platichthys flesus* (Linnaeus 1758) following 3 weeks at 30 mm Hg (Kerstens et al., 1979). However, exposure of carp *Cyprinus carpio* (Linnaeus 1758) to 4 weeks at 30 mm Hg showed a trend towards a decrease in ventilatory sensitivity during acute hypoxia (Lomholt and Johansen, 1979). The extent to which the severity of hypoxia, the duration of the acclimation period and species difference play a role in the magnitude and direction of the ventilatory responses to hypoxia remains unknown.

Primitive fishes generally have a lower resting heart rate compared to teleosts at the same temperature and this was the case in the current study in bowfin. The reasons behind the low heart rate in primitive fishes are unknown, but it has been suggested that a potential benefit could be lower myocardial oxygen demand and likely results from a lower intrinsic cardiac pacemaker frequency (Farrell, 2007a).

During acute progressive hypoxia bowfin without access to air developed a mild bradycardia with a decrease in heart rate of about 25% at 35 mmHg (Fig. 4D). Bowfin exposed to sustained hypoxia without access to air had significantly higher heart rates at 35 mmHg. Similarly channel catfish acclimated to moderate hypoxia had higher heart rates during exposure to acute hypoxia than non-acclimated catfish (Burleson et al., 2002) but the mechanism behind this has not been determined. There has been much debate about the significance of the hypoxic bradycardia in fish (Farrell, 2007b) but the higher heart rates seen in the acclimated bowfin will certainly increase oxygen delivery to the tissues in this group.
5.5.5 **Blood haemoglobin oxygen binding affinity**

Bowfin showed a small Root effect; a roughly 11% fall in O₂ saturation in the presence of 1-1.5% CO₂ (Fig. 5). This small Root effect was similar to a previously published value of an 8% fall in O₂ saturation at a CO₂ tension of 26 mmHg in the whole blood of *Amia* (Black and Irving, 1938). Values of P₅₀ were also similar to those previously reported in bowfin (15 mm Hg at 1% CO₂, 15°C Black, 1940; 24 mm Hg at 27°C pH 7.6 Johansen et al., 1970). Surprisingly, P₅₀ increased significantly in bowfin exposed to sustained hypoxia regardless of whether they had access to air or not (Fig. 6). This is not consistent with previous findings for other fish acclimated to moderate hypoxia in which there was either no change in P₅₀ in cod *G. morhua* (Petersen and Gamperl, 2011) or a decrease in P₅₀ in trout *O. mykiss* (Bushnell et al., 1984; Soivio et al., 1980; Tetens and Lykkeboe, 1981). It has been suggested that an increase in P₅₀ would enhance oxygen delivery to the tissues by increasing the diffusion gradient for oxygen (Brauner and Wang, 1997) and would be beneficial as long as the decreased Hb-O₂ binding affinity did not reduce O₂ loading at the gas exchange surface. In the case of the bowfin, the increase in gill ventilation, coupled with a small Root effect would definitely help sustain O₂ uptake at the gills. A likely mechanism behind the increase in P₅₀ after exposure to hypoxia would be an increase in organophosphate concentration (GTP). Although [GTP] was not measured in the present study, bowfin hemoglobins are very sensitive to changes in organophosphate concentrations, which cause a right shift in the Hb-O₂ dissociation curve, and thus, an increase in P₅₀ (Regan and Brauner, 2010).

5.6 **Conclusions**

Unlike the longnose gar (*Lepisosteus osseus*) and other fishes, bowfin do not have central chemoreceptors (Hedrick et al., 1991; Remmers et al., 2001), thus, peripheral chemoreceptors must be responsible for initiating cardio-respiratory responses to hypoxia. Just like all other fish species studied to date (recently reviewed by Jonz and Nurse, 2009) bowfin possess NECs in their gills (Goniakowska-Witalinska et al., 1995; Chapter 3), the putative oxygen chemoreceptors thought to be responsible for cardio-respiratory reflex
responses in fish. Furthermore, bowfin in which the nerves to all four gill arches and the pseudobranch have been cut (denervated) no longer exhibited a hypoxic bradycardia compared to sham operated animals and they had diminished ventilatory responses (McKenzie et al., 1991). This supports the idea that NECs in the gill are important in the cardiorespiratory reflex response in bowfin, but it also indicates that there are extrabranchial receptors found somewhere else other than the brain responsible for part of the ventilatory response to hypoxia.

Overall this study shows that exposure to sustained hypoxia causes changes along the oxygen transport cascade, specifically an increase in the ventilatory sensitivity to hypoxia in fish without access to air, an increase in tissue perfusion, and changes in Hb-O$_2$ binding affinity that would promote unloading of oxygen at the tissues. The changes in oxygen transport system could partially explain the VAH observed in bowfin without access to air (Chapter 4) as the increase in total ventilation acts to compensate for the decrease Hb-O$_2$ binding affinity in order to maintain oxygen supply.
Figure 5.1 Mean oxygen consumption rate (µmol kg⁻¹ min⁻¹) of bowfin (*Amia calva*) without access to air during hypoxia decreased significantly at water O₂ tensions below 43 mm Hg and the critical oxygen tension was determined to be 70.0 ± 8.8 mm Hg. * indicates significant difference from normoxia (P < 0.001). Values are means ± s.e.m, n=10.
Figure 5.2 Cardio-respiratory responses of bowfin (*Amia calva*) with access to air during acute progressive hypoxia before (○) and after exposure to sustained hypoxia in fish with (▼) and without (●) access to air during exposure to sustained hypoxia (SH): A) gill breathing frequency (\(V_f\), breaths min\(^{-1}\)); B) gill ventilatory amplitude (\(V_{amp}\), cm H\(_2\)O); C) gill ventilation product (total ventilation) (\(V_{tot}\), cm H\(_2\)O min\(^{-1}\)); D) heart rate (\(f_H\), beats min\(^{-1}\)); and E) air breathing frequency (\(f_{AB}\)). All variables were maintained constant during exposure to acute progressive hypoxia, except \(f_{AB}\) which increased 4 fold. * indicates significant difference from normoxia. † indicates significant difference from before exposure to sustained hypoxia. Values are means ± s.e.m, n= 4-6 per group.
Figure 5.3 Effects of an air breath on A) heart rate ($f_H$, beats min$^{-1}$) and B) gill breathing frequency ($V_f$, breaths min$^{-1}$) in bowfin (*Amia calva*) with access to air during acute progressive hypoxia ($P_O_2$, mm Hg) either before (black bars) or after exposure to sustained hypoxia (45 mm Hg for 7 days) with (dark grey bars) or without (light grey bars) access to air. Values represent the differences ($\Delta$) in measurements after vs before the air breath. Note: positive values indicate the heart rate was lower before the air breath and negative values indicate the gill breathing frequency was higher before the air breath. † indicates that the pooled values were significantly higher after an air breath than before an air breath ($P=0.021$). Values are means ± s.e.m.
Figure 5.4 Cardio-respiratory responses of bowfin (*Amia calva*) without access to air during acute progressive hypoxia before (○) and after exposure to sustained hypoxia (SH) in fish with (▼) and without (●) access to air during exposure to sustained hypoxia: A) gill breathing frequency ($V_f$, breaths min$^{-1}$); B) gill ventilatory amplitude ($V_{amp}$, cm H$_2$O); C) gill ventilation product (total ventilation) ($V_{tot}$, cm H$_2$O min$^{-1}$); D) heart rate ($f_H$, beats min$^{-1}$).

When bowfin did not have access to air they responded with an increase in $V_{tot}$ by increasing $f_G$, and exhibiting a moderate hypoxic bradycardia. Bowfin responded to acute progressive hypoxia without access to air by increasing $V_{amp}$ only after exposure to sustained hypoxia with and without access to air. * indicates significant differences from normoxia. † indicates significant difference from before exposure to sustained hypoxia. Values are means ± s.e.m, n= 4-6 per group.
Figure 5.5 Oxygen equilibrium curves of bowfin (*Amia calva*) whole blood measured at 3 different CO$_2$ tensions: A) 0.5%; B) 1.0%; C) 1.5%. Blood was taken from bowfin before and after exposure to sustained hypoxia (SH) in fish with and without access to air during exposure to sustained hypoxia. Dashed gray lines represent 100% O$_2$ saturation. Values are means ± s.e.m, n=6 per group.
Figure 5.6 Mean partial pressure of oxygen at which the haemoglobin was 50% saturated with oxygen (P$_{50}$) for bowfin (*Amia calva*) before and after exposure to sustained hypoxia (SH) in fish with and without access to air during exposure to sustained hypoxia. Bars not sharing the same letters indicate significant differences between oxygen tensions within a group and † indicate significant differences from before exposure to sustained hypoxia. Values are means ± s.e.m, n=6 per group.
Figure 5.7 Hill plots for bowfin before (solid) and after exposure to sustained hypoxia (SH) either with (dotted) or without (dashed) access to air during exposure to sustained hypoxia at 0.5 (black), 1.0 (red), and 1.5 (blue) % CO₂. Data points are shown only for bowfin before exposure to sustained hypoxia. Values are means ± s.e.m.
Chapter 6. The effect of sustained hypoxia on putative oxygen chemoreceptors in bowfin (*Amia calva*): implications for changes in the respiratory control system

6.1 Summary

Time domains of the HVR similar to those described in mammals have been identified in bowfin (*Amia calva*) without access to air. The aim of this study was to determine the extent to which these time domains were associated with changes in oxygen chemoreceptor morphology. The size and density of three cell types (I – III) previously identified as putative oxygen chemoreceptors involved in a reflex response to hypoxia were measured in bowfin exposed to normoxia or to hypoxia (45 mm Hg for 7 days) either with or without access to air. Type I cells near the efferent filament artery were larger in bowfin without access to air than in bowfin without access to air or bowfin exposed to hypoxia indicating these cells were specific internal chemoreceptors. Type II and III cells found near the central venous sinus were larger in bowfin exposed to hypoxia with or without access to air than in bowfin exposed to normoxia indicating these cells were also likely involved in the hypoxic ventilatory response. Changes in cell size could reflect changes in peripheral chemoreceptor sensitivity to sustained hypoxia in bowfin without access to air and partially explain the VAH observed in bowfin without access to air.

6.2 Introduction

Time domains of the HVR are evident in bowfin (*Amia calva*) exposed to sustained hypoxia (Chapter 4). In Chapter 5, I suggest that changes over time in the hypoxic ventilatory response could reflect changes in other steps that match ATP supply and demand by optimizing the flow of oxygen from the gills to the tissues (Weibel, 1984) or, alternatively, time domains in the hypoxic ventilatory response could reflect changes in elements of the respiratory control system as has been shown in mammals (Powell et al., 1998). A third possibility is that time domains reflect both changes in the oxygen transport cascade and changes in the control of breathing. In Chapter 5 I show that moderate sustained exposure to
hypoxia is sufficient to induce changes in the oxygen transport cascade of bowfin and explain the VAH observed in bowfin. In this chapter I explore the possibility that VAH involves changes in steps of the respiratory control system.

In mammals peripheral chemoreceptors are not only necessary for the initiation of all hypoxic responses, changes in their sensitivity also contribute to many of the time domains in the hypoxic response described to date. For example, the glomus cells of the carotid body are not only responsible for initiating the acute hypoxic ventilatory response, but following sustained hypoxia, changes in their sensitivity contribute to ventilatory acclimatization to hypoxia arising from changes in the calcium and potassium channel composition of their cell membranes (Hempleman, 1995; Hempleman, 1996). Peripheral chemoreceptors have been found in the gills of all fish species studied to date (see Porteus et al., 2012 for review) and evidence suggests that these are also involved in both initiating cardiorespiratory responses to acute hypoxia and in the changes that occur over time with exposure to sustained hypoxia (Jonz et al., 2004).

Unlike most cells that decrease energy demands during exposure to hypoxia, oxygen chemoreceptors become more metabolically active (Kumar et al., 2009a). Just like in mammals (Wang et al., 2008), putative oxygen chemoreceptors found in zebrafish (Danio rerio) and mangrove rivulus (Kryptolebias marmoratus) gills increase in size during exposure to sustained hypoxia (Jonz et al., 2004; Regan et al., 2011). Although the exact mechanism behind this hypertrophy is unknown it is likely related to the increased turnover and synthesis of neurotransmitters associated with the increased activity of the chemoreceptors.

Unlike longnose gar (Lepisosteus osseus) (Smatresk, 1986), bowfin do not possess central chemoreceptors (Hedrick et al., 1991), thus the gills are thought to be the main, if not exclusive location for oxygen chemoreception. Bowfin respond to both internal (changes in blood PO₂) and external (changes in water PO₂) hypoxia (McKenzie et al., 1991). This suggests that bowfin have gill chemoreceptors that are oriented such that they can sense internal (blood) or external (water) changes in PO₂ or both.
In Chapter 3, three Types of cells were identified as putative oxygen chemoreceptors involved in a reflex response to hypoxia: Type I cells found near the filament arteries; Type II cells (large bipolar neurons) found in the middle of the filament, in the central venous sinus (cvs); and Type III cells found in the vicinity of the bipolar neurons in the cvs. The first aim of this chapter was to identify which of the innervated cells types identified in Chapter 3 might be involved in the response to hypoxia. I hypothesized that all these three cell types would be involved in the hypoxic response. The second aim of this study was to identify changes in putative internal versus external chemoreceptors resulting from exposure to sustained aquatic hypoxia. These two groups of receptors have never been specifically identified in fish. The approach for this aim was to compare the changes in abundance, size and shape of various putative oxygen chemoreceptors in three groups of fish: those exposed to either normoxia, sustained hypoxia with access to air, or sustained hypoxia without access to air. The level of hypoxia chosen was 45 mm Hg, just above the $P_{\text{crit}}$ of this species (Chapter 5). In trout (*Oncorhynchus mykiss*), this level of hypoxia has been shown to maximally stimulate gill oxygen chemoreceptors (Burleson and Milsom, 1993). Bowfin exposed to normoxia rarely air breathe (~ 1 breath h$^{-1}$) and utilize their well-developed gills for extracting oxygen from the water. These fish were assumed to be neither hypoxic nor hypoxaemic and their oxygen chemoreceptors to be minimally stimulated under these conditions. Bowfin exposed to aquatic hypoxia without access to air would be both hypoxic and hypoxaemic and both internal and external chemoreceptors would be stimulated. Bowfin exposed to hypoxia with access to air show a moderate increase in air breathing frequency to ~7 breaths h$^{-1}$ (Chapter 4). The reason for this moderate increase was proposed to be a fall in internal $P_{\text{O}_2}$ to an threshold that triggers air breathing, which in turn increases blood $P_{\text{O}_2}$ to normoxic levels (Hedrick et al., 1994). Therefore, bowfin exposed to aquatic hypoxia would experience hypoxia but only intermittent hypoxaemia, and therefore external chemoreceptors would be stimulated far more than internal chemoreceptors. It was my hope that the bowfin, with its well-developed gills, would be an ideal organism for differentiating between the effects of hypoxia and hypoxaemia on internal and external chemoreceptors. Type I cells are located near the eFA as well as in relatively close proximity to the water, therefore I
hypothesized that Type I cells are both internal and external chemoreceptors. Type II and III cells are located near the central venous sinus, far away from the surrounding water, therefore, I hypothesized that both cell types would be internal chemoreceptors.

6.3 Material and methods
6.3.1 Animals

Bowfin (340 - 2500g), *Amia calva*, were caught by a local fisherman from the Bay of Quinte, Ontario and were transported to the University of Guelph in aerated tanks in a pickup truck. They were kept in recirculated water tanks at the Hagen Aqualab, University of Guelph. Animals were first kept at 8°C for a month, after which the temperature was increased by 1°C/day to 22°C, and the animals were acclimated at that temperature for at least 3 weeks before the start of any experiments. The bowfin were kept on a 12/12-h light/dark photoperiod and fed live goldfish once a week.

6.3.2 Sustained hypoxia

Bowfin were randomly assigned to either of two groups: one group with and one group without access to air. They were put in 2 m diameter tanks and separated using perforated pvc (up to 4 animals per tank at once) that had perforated pvc covering either 2cm below surface of water (in the group without access to air) or 10cm above surface of water (in the group with access to air). Sustained hypoxia was achieved by bubbling nitrogen using a Parker Balston nitrogen generator (Model N2-04, Parker Hannifin Corp., Haverhill, MA, USA) into a large header tank (~100 l) and into the experimental tank and by controlling the water flow from the header tank into the experimental tank. This design allowed the oxygen level in the experimental tank to be kept within a narrow O₂ range (45.4 ± 6.9 mm Hg). The animals were exposed to sustained hypoxia for 7 days.

6.3.3 Immunohistochemistry

At the end of the experiment bowfin were killed by an overdose of benzocaine followed by a sharp blow to the head. Bowfin were perfused with heparinized (100 iu/ml),
ice-cold phosphate-buffered solution (PBS) containing (in mM): NaCl, 137; Na$_2$HPO$_4$, 15.2; KCl, 2.7; KH$_2$PO$_4$, 1.5; buffered to pH 7.8 with 1M NaOH (Jonz and Nurse, 2003). Bowfin were perfused via the bulbous arteriosus using a blunt 25 gage needle and a 20cc syringe until the gill filaments appeared clear. The 1$^{st}$ gill arch was removed and fixed in 4% paraformaldehyde in PBS overnight. Tissues were then rinsed in PBS, cryoprotected in a 30% sucrose solution, and frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific) at -80°C. Blocks were sectioned longitudinal to the gill filament at 10-12 μm increments using a cryostat (Leica CM3050 S, Leica Microsystems, Germany) and mounted on Superfrost® plus slides (VWR International) for immunohistochemistry. Longitudinal serial sections were cut and every section was inspected. Cross sections 12 μm thick were cut every 700-1000 μm along the length of the filament.

Slides containing frozen tissue were washed in PBS and blocked in 10% Normal Goat Serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, Ontario) for one hour. The primary antibody (anti-serotonin raised in rabbit, Sigma Aldrich, Cat # S5545) was diluted (1:500) in a permeabilizing solution (PBS/0.1% Triton X-100/ 3% normal goat serum) and set on the slides to incubate overnight at room temperature. Following incubation with primary antibodies, the slides were again washed in PBS. The slides were then incubated with fluorescently labelled secondary antibody (goat anti-rabbit Alexa Fluor® 488, Molecular Probes, Cat # 11008) diluted (1:500) in PBS containing 0.1% Triton-X and 3% normal goat serum and left to incubate at room temperature for 2 hours in darkness. Following a final washing with PBS, slides were mounted with coverslips and Vectashield with DAPI (Vector Laboratories, Burlington, Ontario) to prevent photobleaching and to label cell nuclei. Slide boxes were stored at 4°C until viewed under a fluorescent light microscope. Control experiments were performed in which the primary antibodies were excluded to control for effects of the secondary antibody, and normal serum (IgG) from primary antibody host species was used (at an equivalent protein concentration) to test for adverse staining due to reaction with the primary hosts. The results of these control experiments showed only negligible staining with no specific immunolabeling of the gill structures present (data not shown).
6.3.4 Microscopy and cell size quantification for longitudinal sections

Some images were captured using a confocal scanning system (Pascal 2, Axioskop X, Zeiss, Jena, Germany) equipped with argon (Ar) and helium-neon (He-Ne) lasers emitting at 488 nm and using a BP 505-530 filter to detect Alexa 488. Z-stacks of 11-35 optical sections and 0.22-0.39 µm apart were captured using EC Plan-Neofluar 40x/1.30 Oil DIC M27. Additional images were captured using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with a solid state laser emitting at 473 nm. Z-stacks of 5-12 optical sections and 1.0 µm apart were captured using the 60x objective of this microscope. Compressed Z-stacks of the green channel (serotonin) were used to measure projected surface area (surface area of a 2D projection of a cell) using the measurement function in Volocity imaging software Version 5.5.1 (Perkin Elmer Inc., Waltham, MA, USA). For each animal 15-20 images, containing about 200 cells, were used to measure cell size of three different cell types: serotonin containing neuroepithelial cells near the filament arteries, serotonin containing bipolar neurons and cells in the filament.

6.3.5 Density and cell size quantification using cross sections

Images were captured using a confocal microscope (Olympus Fluoview FV10i, Tokyo, Japan) equipped with solid state lasers emitting at 473 nm. Z-stacks of 5-12 optical sections and 1.0 µm apart were captured using the 60x objective of this microscope. 3D projections of the acquired stats were used to measure cell volume using the measurement function in Volocity imaging software Version 6.1.2 (Perkin Elmer Inc., Waltham, MA, USA). Cell counts were done in cross sections only because it was impossible to get perfect longitudinal sections of the entire filament in the same area (near the efferent filament artery for example) and this would have made it impossible to get comparable cell density counts. Cell densities were measured for Type I cells at four different locations: near the basal lamina of the eFA (eFA bas), in the outer epithelial layer of the eFA (eFA epi), in the outer epithelial layer of the aFA (aFA epi), and near the basal lamina of the aFA (aFA bas). For each animal 40 images, containing about 20-30 cells, were used to measure cell size of Type I cells at three different locations: near the basal lamina of the eFA (eFA bas), in the outer
epithelial layer of the eFA (eFA epi), in the outer epithelial layer of the aFA (aFA epi). There were too few cells near the basal lamina of the aFA to be able to get reliable measurements; therefore, the cells in this location were not measured.

6.3.6 Statistical analysis

Data are routinely expressed as mean ± SEM. For cell Type I projected cell surface area a Kruskal-Wallis one-way ANOVA on ranks was performed because the treatment groups had unequal variances. For cell Types II and III a one-way ANOVA using treatment group as a factor followed by a Tukey test was used to test for differences between projected surface area and cell shape. A one-way ANOVA using treatment group as a factor was used to test for differences between Type I densities, cell volume, skeletal length and skeletal width at the different locations. A significance level of P<0.05 was used throughout.

6.4 Results

6.4.1 Longitudinal sections

Type I cells in bowfin exposed to sustained hypoxia without access to air had 20% larger cell surface area than Type I cells in bowfin either exposed to sustained hypoxia with access to air or bowfin exposed to normoxia (H$_{2, 15}$= 8.98, P = 0.01) (Fig. 6.1 A). Type II cells in bowfin exposed to sustained hypoxia with and without access to air had 25% larger projected surface areas than bowfin exposed to normoxia (F$_{2, 15}$= 4.31, P = 0.03) (Fig. 6.1 A). Type III cells in bowfin exposed to sustained hypoxia with and without access to air had 20% larger projected surface areas than bowfin exposed to normoxia (F$_{2, 15}$= 7.35, P = 0.006) (Fig. 6.1 A).

A shape factor of 1.0 indicated a projected area of a perfect circle. Type I cells were generally round scoring 0.74 in shape factor and there were no differences between treatment groups (F$_{2, 15}$ = 0.30, P= 0.743) (Fig. 6.1B). Type II cells had cell projections and an average shape factor of 0.49 in bowfin exposed to normoxia or sustained hypoxia with access to air. There was a trend for a decrease in shape factor (more irregular shape or more cell projections) in bowfin exposed to sustained hypoxia without access to air (F$_{2, 15}$ = 2.65, P=
Type III cells were also generally round scoring 0.67 in shape factor and there were no differences in shape factor between treatment groups ($F_{2, 15} = 1.66, P= 0.22$) (Fig. 6.1 B).

### 6.4.2 Cross sections

Cross sections revealed that Type I cells were found in four locations: near the basal lamina of the eFA (eFA bas), in the outer epithelial layer of the eFA (eFA epi), in the outer epithelial layer of the aFA (aFA epi), and near the basal lamina of the aFA (aFA bas) (Chapter 3). There were about 198 ± 54 cells per mm filament in the outer epithelial layer of the eFA, 424 ± 47 cells per mm filament near the basal lamina of the eFA, 21 ± 4 cells per mm filament near the basal lamina of the aFA, and 609 ± 43 cells per mm filament in the outer epithelial layer of the aFA (Fig. 6.2 A). There were no differences in cell density between treatment groups ($P>0.05$). Cells in the outer epithelial layer of the eFA were 183 ± 8 $\mu$m$^3$, 261 ± 11 $\mu$m$^3$ near the basal lamina of the eFA, and 208 ± 10 $\mu$m$^3$ in the outer epithelial layer of the aFA (Fig. 6.2 B). There were no differences in cell density between treatment groups ($P>0.05$). The length of cells in the outer epithelial layer of the eFA was 12 ± 0.6 $\mu$m, 14.6 ± 0.9 $\mu$m near the basal lamina of the eFA, and 14.9 ± 10 $\mu$m in the outer epithelial layer of the aFA (Fig. 6.2 C). Cells near the basal lamina of the eFA were larger in bowfin exposed hypoxia without access to air than in bowfin exposed to normoxia ($P<0.05$), but not larger than bowfin exposed to hypoxia with access to air ($P>0.05$). There were no significant differences in skeletal length between treatment groups at the other locations. The width of cells near the basal lamina of the eFA was 4.5 ± 0.1 $\mu$m, 4.9 ± 0.1 $\mu$m in the outer epithelial layer of the eFA, and 4.4 ± 0.2 $\mu$m in the outer epithelial layer of the aFA (Fig. 6.2 D). There were no differences in cell width between treatment groups ($P>0.05$). Cells near the basal lamina of the aFA were too few to get any reliable cell measurements.

### 6.5 Discussion

Exposure to moderate sustained hypoxia led to changes in cell morphology indicating that, as hypothesized, all three cell types identified in Chapter 3 as putative oxygen
chemoreceptors could be involved in the hypoxic response. Additionally, Type I cells near the basal lamina of the eFA were identified as specific putative internal oxygen chemoreceptors in bowfin. Surprisingly, no cell types potentially acting exclusively as external chemoreceptors were identified in this study.

6.5.1 Type I cells

Type I cells were similar to NECs previously described at similar locations in the filaments of all other species of fish (Coolidge et al., 2008; Jonz et al., 2004; Regan et al., 2011; Saltys et al., 2006; Tzaneva and Perry, 2010). When grown in culture these cells responded to acute hypoxia by depolarizing due to the closure of a background K$^+$ channel (Jonz et al., 2004). These cells were larger in zebrafish exposed to sustained hypoxia (35 mm Hg for 60 days) than in control fish, but there was no change in density, providing further evidence that these cells were most likely oxygen chemoreceptors (Jonz et al., 2004). The same was also true of the Type I cells in bowfin. Longitudinal measurements on the projected cell surface areas of these cells revealed that they were larger in bowfin exposed to hypoxia without access to air than in bowfin with access to air or those exposed to normoxia (Fig. 6.1 A). Cross sections further revealed that this was due primarily to an increase in length of the Type I cells near the basal lamina of the eFA (the most abundant of the Type I cells seen in the longitudinal sections) (Fig. 6.2 C). Additionally, Type I cells near the basal lamina of the eFA tended to have a larger cell volume in bowfin exposed to hypoxia without access to air, while Type I cells at other locations tended to be smaller (Fig. 6.2 B). This selective response suggests that Type I cells near the basal lamina of the eFA respond to internal hypoxia (hypoxaemia) only; i.e. these are internal chemoreceptors, and not external chemoreceptors.

These results also indicate that these cells were changing in shape, becoming longer either due to elongation of the cell itself or due to growth of cell projections. Although, cell shape factor was not different in Type I cells (Fig. 6.1 A), this might simply reflect the mixed population of cells measured longitudinally. Previously, it has been shown that zebrafish exposed to sustained hypoxia (35 mm Hg for 60 days) had larger NECs and more NECs with
cell processes contacting the nerve bundle than fish exposed to normoxia suggesting these cells increased neurotransmitter release (Jonz et al., 2004).

Type I cells were also found in close proximity to the water, near the outer layer of the epithelium on both the aFA and eFA side of the filament (i.e. eFA epi and aFA epi). While these cells contained synaptic vesicles and were innervated by small nerve fibres (Chapter 3), indicating they could serve a chemosensory role, they did not change in size in bowfin exposed to sustained hypoxia suggesting it is unlikely they are involved in oxygen sensing. In fact, there was a trend for these cells to be smaller in bowfin exposed to hypoxia without access to air. These results are consistent with the idea that these are Merkel-like cells associated with taste buds as previously described in the orobranchial cavity of zebrafish (Zachar and Jonz, 2012). These cells are found in an ideal location to sense chemical stimuli from food being in close proximity to the water in the buccal cavity.

Type I cells near the basal lamina of the aFA were scarce and too few to categorize.

6.5.2 Type II cells

In the present study Type II cells (intrinsic bipolar neurons) were larger in bowfin exposed to hypoxia with and without access to air (Fig. 6.1 A), indicating that these cells may be involved in both the response to internal and external hypoxia. Due to their location deep in the filament, not in close proximity to the external water, these cells are unlikely to be external chemoreceptors and are more likely involved in transduction in the oxygen sensing pathway. The overall lower shape factor score in these cells reflects their irregular shape. Additionally, there was a trend for an even smaller Type II cell shape factor (more irregular shape) in bowfin exposed to hypoxia without access to air than in the other two groups (Fig. 6.1 B), indicating that these cells had more processes in bowfin exposed to hypoxia. An increase in cell projections in bipolar neurons would indicate an increase in interconnection between them and nearby neurons and/or NECs and possibly enhanced sensitivity (neurotransmission), but this idea is highly speculative.
6.5.3 Type III cells

Type III cells were significantly larger in bowfin exposed to hypoxia both with and without access to air than in bowfin exposed to normoxia (Fig. 6.1 A), indicating that they too may be involved in the response to hypoxia. As mentioned in Chapter 3, these cells are innervated but do not contain synaptic vesicles and have been suggested to have two possible roles. First, they could be involved in the vasomotor regulation of blood flow through the gill vasculature. The larger size of these cells in bowfin exposed to hypoxia could mean an increase production and storage of serotonin during hypoxia. Alternatively, these cells could be precursor NECs, and the increase in their size might reflect that these cells are differentiating in response to hypoxia. These two possibilities are not mutually exclusive.

6.6 Conclusions

Type I cells found near the basal lamina of the eFA, identified as NECs in other fish species, may be exclusively internal oxygen chemoreceptors in bowfin. These cells responded to sustained hypoxaemia by changing cell size and shape. This study also shows that bipolar neurons (Type II cells) may be involved in the hypoxic response; responding to both hypoxia and hypoxaemia. Additionally, bowfin have a cell Type (III) previously not described in other fish, that do not contain synaptic vesicles, but that are innervated and also respond to both internal and external hypoxia by increasing in size. In this chapter I showed that putative oxygen chemoreceptors change in cell size and shape, indicative of changes in the sensitivity of these chemoreceptors to sustained hypoxia.
Figure 6.1 (A) Projected surface area (cell size, μm$^2$) and (B) cell shape in Type I, II, and III cells in bowfin exposed to normoxia or to sustained hypoxia (SH, 45 mm Hg for 7 days) with or without access to air. Type I cell size were large in bowfin exposed to hypoxia without access to air. Type II cell size was larger in both groups exposed to hypoxia than in normoxia. Type III cell size was larger in both groups exposed to hypoxia than in normoxia. For cell shape 1.0 would represent a perfect circle. There were no significant differences in cell shape between the groups for all three cell types. Bar graphs not sharing the same letters are significantly different from one another (P<0.05). Values are presented as mean ± SEM.
Figure 6.2 (A) Cell density (cells mm\(^{-1}\) filament), (B) cell volume (μm\(^3\)), (C) skeletal length (μm), and (D) skeletal width (μm) in Type I cells in various locations in bowfin exposed to normoxia or to sustained hypoxia (SH, 45 mm Hg for 7 days) with or without access to air. There were no significant differences between cell density, cell volume, and skeletal width of bowfin exposed to normoxia or sustained hypoxia at the different locations. Bar graphs not sharing the same letters are significantly different than one another within a certain location (P<0.05). eFA epi, efferent filament artery in the top layer of the epithelium; eFA bas, efferent filament artery near basal lamina; aFA epi, efferent filament artery in the top layer of the epithelium; aFA bas, efferent filament artery near basal lamina. Values are presented as mean ± SEM.
Chapter 7. General discussion and conclusions

7.1 Major findings and implications

The main objectives of this thesis were to determine whether time domains exist in the hypoxic ventilatory response (HVR) of bowfin Amia calva and to determine the role of the neuroepithelial cells (NECs) in the cardio-respiratory response to acute and sustained hypoxia. I hypothesized that, just like the glomus cells of mammals, the NECs in fish gills would contain multiple neurochemicals. I found that, unlike mammalian glomus cells, NECs in fish gills did not contain catecholamines and acetylcholine. These findings led me to reject my hypothesis and propose different scenarios for the involvement of ACh in the response to acute hypoxia. This study also justified using serotonin as cellular marker for NECs in fish and used it as a marker to identify NECs in bowfin.

I also hypothesized that time domains exist in the hypoxic ventilatory response of fish and reflect changes in both the oxygen transport system and changes in the respiratory control system. In support of this hypothesis, I found that similar time domains exist in fish as they do in mammals: acute response (AR), short term potentiation (STP), hypoxic ventilatory decline (HVD), and ventilatory acclimatization to hypoxia (VAH); while I did not find evidence of short term depression (STD). I also found that VAH was associated with a decrease in Hb-O₂ binding affinity and increased ventilatory sensitivity of bowfin to acute progressive hypoxia regardless of whether these fish had access to air during sustained hypoxia. These findings suggest that changes along the oxygen transport pathway as a potential explanation for the VAH observed in bowfin. Furthermore, VAH to hypoxia was associated with changes in chemoreceptor size and morphology. Together these findings reflect the complexity of changes that take place in fish with bimodal respiration exposed to hypoxia and highlight the importance of considering changes in both oxygen transport and the control of breathing that take place during sustained hypoxic stimulation in future studies.
7.1.1 The role of various neurochemicals in oxygen sensing

The glomus cells of the carotid body are the primary peripheral oxygen sensing cells in mammals and they contain a myriad of neurochemicals, many of which are involved in oxygen sensing (reviewed by Nurse, 2010). In fish, the NECs in the gills are the proposed oxygen sensors and many similarities exist between these NECs and glomus cells. A previous study of the response of the afferent nerve fibres innervating oxygen sensing cells in the gills indicated that acetylcholine elicited a similar response to hypoxia (Burleson and Milsom, 1995), and was likely to be the primary neurotransmitter involved in eliciting this response. By labeling gill tissue with markers for acetylcholine and catecholamines I showed that the NECs of trout and goldfish do not contain these neurochemicals. I found acetylcholine in intrinsic bipolar neurons in trout, goldfish, and bowfin, neurons that have also been shown to innervate NECs in zebrafish (Jonz and Nurse, 2003). Therefore in Chapter 2 I proposed various alternate scenarios for oxygen chemoreception. Subsequently, in bowfin I found acetylcholine in close proximity to NECs near the efferent filament artery. I then proposed that the ACh is found in synaptic clefts and it is likely these are reciprocal synapses between afferent nerve terminals and the NECs. This finding is consistent with the first and second scenarios presented in Chapter 2 in which serotonin containing NECs are the oxygen chemoreceptors and ACh acts as a neuromodulator of the response by release from afferent nerves.

NECs of trout did not take up activity dependent dyes and this result remains puzzling. This indicates that NECs were not active during hypoxia, but should have been if they were oxygen sensing. As mentioned in Chapter 2 it seems unlikely that the severity or time of exposure to hypoxia was inadequate since the dye was taken up by the mitochondria rich cells, amongst others. Despite this finding, strong evidence still points at NECs near the eFA being the primary oxygen chemoreceptors: in culture they depolarize during hypoxia (Burleson et al., 2006; Jonz et al., 2004), they increase in size during exposure to hypoxia (Jonz et al., 2004; Regan et al., 2011) and in Chapter 6, I showed that bowfin exposed to sustained hypoxia had larger Type I cells near the basal lamina of the eFA than bowfin exposed to normoxia. Additionally, I showed that bowfin exposed to sustained hypoxia with
and without access to air had larger Type II cells (intrinsic bipolar neurons) than bowfin exposed to normoxia. These data are consistent with the second scenario proposed in Chapter 2 (Fig. 2.9) in which NECs are the primary oxygen sensor, and are innervated by intrinsic bipolar neurons, with ACh acting at a reciprocal synapse between them.

In goldfish and zebrafish lamellar NECs contain serotonin, synaptic vesicles and are innervated (Coolidge et al., 2008; Jonz et al., 2004; Saltys et al., 2006; Tzaneva and Perry, 2010). Interestingly, they are not present in the lamellae of trout (Coolidge et al., 2008; Saltys et al., 2006) or mangrove rivulus (K. marmoratus) (Regan et al., 2011). Previous studies have shown that in goldfish these cells migrate during hypoxia or decreasing temperature accompanying gill remodelling to remain in contact with the water, indicating they are important in the response to hypoxia (Tzaneva and Perry, 2010), but their response to hypoxia has never been measured directly (using patch clamp) due to their small size. In bowfin Type IV cells (lamellar NECs) containing serotonin were not innervated and did not contain synaptic vesicles (Chapter 3). If these cells are indeed hypoxia sensing, these data suggest that the initial function of lamellar NECs might have been to regulate blood flow through the lamellae by releasing serotonin on nearby pillar cells. Additionally, this also indicates that these cells were likely present in the ancestors of teleosts and they have been secondarily lost in trout, mangrove rivulus and perhaps other fish species. At the present their function remains speculative.

My findings also give rise to an interesting evolutionary question: “Are NECs phylogenetic precursors of glomus cells or are they homologous to the neuroepithelial bodies of vertebrate lungs?” The carotid body in mammals sits at the bifurcation between the common carotid artery into the internal and external carotid arteries. The common carotid artery in mammals is homologous to the 1st afferent branchial artery (supplying blood to the first gill arch) in fish (Milsom and Burleson, 2007). Additionally, the carotid body is innervated by the IX cranial nerve (glossopharyngeal), while the 1st gill arch of fish is innervated by the IX and X (vagus) cranial nerves. Due to the similarity in the location and innervation between these carotid bodies and the 1st gill arch in fish it has been proposed that the carotid bodies of mammals have been derived from the chemosensory cells of the gill
(the NECs) (Milsom and Burleson, 2007). My results indicated that it is possible that NECs are the precursors of glomus cells but they do not yet express the neurochemical complexity of the glomus cells that are more derived.

There is another possibility, however. In trout, goldfish, and bowfin the NECs near the filament artery and the lamellae (in goldfish and bowfin) did not label with the HNK-1 antibody (Chapters 2 and 3). Although this might reflect that these cells have differentiated and lost this specific neural crest marker, it is also possible that they are not derived from the neural crest but are derived from precursor epithelial cells, as are neuroepithelial bodies (NEBs) (Dunel-Erb et al., 1994). Therefore, it is also possible that NECs are homologous to the pulmonary neuroendocrine cells found in neuroepithelial bodies of vertebrate lungs. The pulmonary artery of mammals is homologous to the afferent branchial artery of the 4th gill arch (Milsom and Burleson, 2007). Additionally, both NECs and NEBs contain serotonin as the main neurotransmitter, are innervated by both intrinsic (with cell bodies within the airway ganglion) and extrinsic (cell bodies in the vagal or nodose ganglion) nerves and some of the nerve fibres label for acetylcholine (VACHT) (Adriaensen et al., 2003; Brouns et al., 2009). In bowfin NECs also contain enkephalin, and endothelin (Goniakowska-Witalinska et al., 1995) just like the NEBs of mammals (Scheuermann et al., 1992). What is more striking is the variety of innervation patterns observed in both of these cell types; not all NECs in bowfin were innervated by fibres that labeled with VACHT and similar observations have been made in rat NEBs (Brouns et al., 2009). Although many hypoxia related functions have been proposed for NEBs during various developmental stages the function of NEBs remains unclear (Adriaensen et al., 2006).

It is also possible that both of these occurred. Although, most of the research to date has focused on the function of NECs found in the first gill arch, NECs are found in all four gill arches (Dunel-Erb et al., 1994; Zhang et al., 2011). The evolutionary trend has been from many diffuse oxygen sensing sites in the gills, orobranchial cavity and skin of fish to a few concentrated oxygen sensing sited in mammals (Milsom and Burleson, 2007). Thus, it is also possible that the NECs found in the four gill arches have experienced different evolutionary pressures that gave rise to both the carotid and neuroepithelial bodies in
mammals. The NECs from the 1st gill arch of fish likely started expressing other neurochemicals involved in modulating and fine tuning the response of these cells to hypoxia. Additionally, they became more concentrated in an area of the carotid body without a respiratory surface area, perhaps by evolutionary pressures on their developmental trajectory (perhaps by expressing different adhesion proteins over different developmental times). The NECs in the 4th gill continued to express the same neurochemicals, but evolutionary pressures caused them to change their organization from diffuse cells to distinct bodies with a common innervation. These different evolutionary pressures probably came about with the transition from obligate water breathing in most fish to bimodal breathing in some fish and amphibians to air breathing alone in reptiles, birds and mammals and more research is underway right now in our lab to determine more about the oxygen sensing sites in amphibians and reptiles to provide a clearer picture of the evolution oxygen sensing sites in vertebrates.

7.1.2 Time domains of the HVR in fish and underlying mechanisms

I hypothesized that just like in mammals time domains exist in the HVR of bowfin. I measured the HVR of bowfin exposed to moderate sustained hypoxia at both cold and warm temperatures and time domains during acute exposures to hypoxia were assessed from a previous study in bowfin at a similar temperature (McKenzie et al., 1991). I found that HVD and VAH are evident in the HVR of bowfin without access to air but not in bowfin with access to air. These time domains in bowfin were similar in pattern to those found in mammals, however, they developed over much longer time periods. The initial AR to hypoxia was slow to develop (taking up to 1h), unlike in mammals in which it takes place within one breath. Data from a previous study indicated that the initial response in breathing amplitude was followed by a gradual further increase in breathing amplitude, termed STP in mammals. Although the exact mechanisms still remain unknown in mammals, STP is thought to smooth out the response to hypoxia and prevent an overshoot in breathing amplitude, acting as a way of energy preservation. In mammals, breathing frequency falls after the acute response (short term depression), but in bowfin breathing frequency continued
to rise reaching a maximum between 30 min and 2 hours and remained high for the remainder of the hypoxic exposure indicating this time domain did not take place.

In bowfin, the AR was followed by a decrease in total gill ventilation similar to the HVD observed in mammals. While in mammals this time domain takes place over a period of a few minutes to hours in bowfin this response started at 30 min at 9°C at 25 mmHg and 2h at 22°C at 45 mmHg, which could simply reflect differences in severity of hypoxia and $Q_{10}$ effects. In bowfin, this HVD was followed by a secondary increase in gill ventilation similar to the VAH observed in mammals. In mammals, this time domain is associated with changes in gene expression in peripheral chemoreceptors and altered central sensitivity. In bowfin, sustained exposure to hypoxia was associated with increase in cell size in NECs near the basal lamina of the epithelium in the vicinity of the eFA (Chapter 6). Gene expression of these cells would have been ideal to address this question, but these cells are sparse and difficult to identify and separate without immunolabeling. Although gene expression or sensitivity of these chemoreceptors was not directly measured my results would indicate that this was perhaps the case. An increase in cells size is likely due to an increase in cell activity due to neurotransmitter recycling.

I also wanted to examine the physiological mechanisms behind these observed time domains. I hypothesized that the VAH might be due to changes in the oxygen transport system as shown in previous studies (Bushnell et al., 1984; Soivio et al., 1980; Tetens and Lykkeboe, 1981). Although arterial oxygen content was not measure in the study the hypoxic ventilatory decline observed during the first few hours of exposure to hypoxia could have been caused by a release of RBCs from the spleen, increasing oxygen carrying capacity of the blood and reducing the need to ventilate the gills as much. To determine if VAH was associated with changes in the oxygen transport cascade, I measured whole blood $O_2$-Hb binding affinity before and after exposure to sustained hypoxia in bowfin. Contrary to previous studies in trout (O. mykiss) in which $O_2$-Hb binding affinity increased (Bushnell et al., 1984; Soivio et al., 1980; Tetens and Lykkeboe, 1981) or in cod (G. morhua) in which it did not change (Petersen and Gamperl, 2011), I found that the whole blood $O_2$-Hb binding affinity of bowfin decreased after exposure to moderate sustained hypoxia. A decrease in
O$_2$-Hb binding affinity would benefit unloading of oxygen at the tissues, but hinder uptake at the gills. However, VAH was also associated with an increase in gill ventilatory sensitivity after exposure to sustained hypoxia of bowfin without access to air. This enhanced sensitivity to hypoxia likely served to maintain oxygen uptake at the gills despite the reduced Hb-O$_2$ binding affinity. These results could explain VAH in bowfin without access to air.

As time domains of the HVR were evident in bowfin with access to air but not in bowfin without access to air this indicated that time dependent changes were driven by the stimulation of internal oxygen chemoreceptors or by stimulation of both chemoreceptors that were both internally and externally oriented. Next, I wanted to determine the role of the peripheral oxygen chemoreceptors themselves in VAH. I first described the NECs of bowfin using immunohistochemistry. I found three different types of cells that contained serotonin and were innervated, indicating these cells were candidates for oxygen chemoreceptors involved in a reflex response. I then wanted to determine which of these cells might be oxygen sensing during exposure to sustained hypoxia. For the first time ever, I found that NECs near the efferent filament artery appeared to be exclusively sensitive to changes in internal oxygen concentration. Bipolar neurons were also more active in the response to both internal and external hypoxia but it remains unclear if they are oxygen chemosensing or only part of the oxygen reflex pathway. Similarly, cells found in the central venous sinus were larger in bowfin exposed to sustained hypoxia with and without access to air than in normoxic bowfin. These cells have never been described previously in fish and it is unclear what their role is in the response to hypoxia. These combined results support my hypothesis that VAH in bowfin without access to air was associated with both changes in the oxygen transport system and in the control of breathing.

My thesis describes time domains of the HVR in bowfin by drawing upon knowledge from mammals. It highlights the similarities and differences between these based on the timing and magnitude of the response, and made inferences about the mechanisms of time domains in bowfin wherever possible. Furthermore, I found that although changes in the oxygen transport system take place during exposure to sustained hypoxia, changes in the mechanisms controlling breathing cannot be ignored and should be considered further as
relatively little is known especially about the peripheral and central control of breathing in fish.

7.2 Future directions

One of the main findings of this thesis was that the NECs of fish do not contain multiple neurotransmitters, only serotonin. As previous studies have highlighted the importance of acetylcholine in oxygen chemoreception, I proposed several possible scenarios for the function of acetylcholine in oxygen chemoreception (Fig 2.9). In the first scenario serotonin is released from NECs onto afferent nerves and ACh is found in the afferent nerve terminals in reciprocal synapses with the NECs, acting as a modulator. In the second scenario ACh is found in an afferent neuron and ACh is released on a second neuron acting as a neurotransmitter in this pathway. The third and fourth scenarios involve bipolar neurons as the oxygen chemoreceptors themselves, with ACh being released either at a synapse in the gill or the neuron projecting directly to the central nervous system. Although there is some support for these scenarios, they need to be tested further using various techniques including immunohistochemistry and nerve recording. Previous work in trout has suggested that the nicotinic acetylcholine chemoreceptor is important in oxygen sensing (Burleson and Milsom, 1995). One way of elucidating how and where acetylcholine acts in fish gills is to double label gill tissues with acetylcholine (either its synthesizing enzymes or vesicular transporters) and nicotinic acetylcholine receptors. If reciprocal synapses are involved in the oxygen sensing response, I would predict that NECs would contain nicotinic acetylcholine receptors. In a previous study in trout, the response to various neurochemicals was measured, showing a strong increase in afferent nerve discharge of nerve fibres sensitive to hypoxia to acetylcholine and nicotine (Burleson and Milsom, 1995). Although this study has shown the response to ACh it did not show whether this response would be abolished if ACh (or its nicotinic receptors were blocked). In vitro gill nerve recording could be used to determine whether blocking specific receptors for either serotonin or acetylcholine would prevent or attenuate afferent nerve discharge during hypoxia providing further insight into oxygen
chemoreception in fish. Together these experiments would show if ACh is necessary for the response or where the nicotinic receptors were located.

Although this thesis provides a general assessment of time domains during sustained exposures to hypoxia, only one study to date has looked at the effect of intermittent hypoxia on cardiorespiratory responses even though many fish are exposed to regular fluctuations in oxygen levels in many aquatic environments, tide pools being a prime example. Therefore, the response to intermittent hypoxia should be examined from both a control of breathing perspective as well as from the perspective of changes in the oxygen transport system. In mammals, short 5 minute bouts of moderate hypoxia lead to a progressive increase in total ventilation upon return to hypoxia and the release of serotonin from raphe neurons in the central nervous system is thought to play an important role in this response.

This thesis focused on the role of peripheral oxygen chemoreceptors in the HVR, with a focus on bowfin. However, the role of changes in the central nervous system with respect to the control of breathing in contributing to the time domains seen in this study still remains unknown. But before determining the role of the central nervous system, we first must identify the location of the respiratory centres in fish, which is a crucial first step to learning more about the control of breathing in this vertebrate group. Again, drawing upon the vast knowledge of mammalian respiratory physiology would be a good starting point. One approach to identifying these respiratory centres would be to combine immunohistochemical labeling for specific respiratory centre markers such as μ-opioid receptors and neuropeptide-1 (a substance P receptor), with nerve recording of respiratory centres.

One interesting finding from chapter 4 was an elevated heart rate up to 24h after the return to normoxia and this response has also previously been reported in channel catfish exposed to moderate sustained hypoxia (Burleson et al., 2002). As I mentioned in chapter 4, there could be several explanations for this, such an increase in circulating catecholamines or perhaps a change in muscarinic receptors in the heart and these hypothesis should be explored further. Spectral analysis of heart rate patterns combined with pharmacological blockade could be used to compare the responses in fish before and after exposure to sustained hypoxia. This could be combined with direct measurement of receptors in the heart.
of fish exposed to normoxia and sustained hypoxia. Overall, given the increase in hypoxic events in the recent decades, more studies focused on the recovery of fish from sustained hypoxia should be undertaken to better understand how fish are affected by exposure to hypoxia after the stress has subsided and how this affects future exposures to hypoxia.

7.3 Concluding remarks

With the increasing frequency and severity of hypoxia in both freshwater and saltwater aquatic environments (Diaz and Breitburg, 2009) the need to better understand the response of fish to hypoxia becomes more and more important. We currently heavily rely on fish for food and it is imperative that we understand best practices for sustaining fish stocks. Although many previous studies have focused on changes in gas transport and transfer in fish, a better understanding of these processes from a control of breathing standpoint is necessary. This thesis provides a first step towards this end, characterizing time domains in the hypoxic ventilatory response in bowfin, a facultative air breather and highlighting the importance of peripheral oxygen chemoreceptors in establishing these domains.
References


Appendix
Supplementary Data for Chapter 2

Figure A1. Wide-field image of double immunolabeling for vesicular acetylcholine transporter (VAChT, green) and for cell bodies (DAPI, blue) in the nodose ganglion of *O. mykiss*. The upper left panel (green) shows the axons of neurones within the ganglion while the lower left panel (blue) shows the nuclei of many of these neurones. Scale bars, 50 μm.