OXYGEN-SENSING IN THE FISH GILL: A COMPARATIVE AND IMMUNOHISTOCHEMICAL STUDY OF PUTATIVE OXYGEN-SENSING CELLS

by

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ABSTRACT

Numerous studies have determined approximate locations of chemoreceptive cells in freshwater fish, with the majority suggesting the gills as the primary oxygen-sensing site. Using immunohistochemistry, I investigated the distribution of neurotransmitters and markers for neuroepithelial cells (NECs), the putative oxygen sensing cells, in gills of freshwater fish with varying degrees of hypoxia tolerance. I compared the patterns of immunoreactivity of NEC markers (anti-serotonin and anti-synaptic vesicle marker) in two distantly related species with different hypoxia tolerances (*Onchoryncus mykiss* and *Carassuis auratus*) as well as two species from the same genus *(Hoplias)* that possess distinct differences in habitat and hypoxia tolerance. To speculate on the role of other potential chemosensory neurotransmitters in the gill, I also examined the distribution of catecholamines, acetylcholine, and adenosine. I found that all fish exhibited a cluster of innervated, serotonergic NECs at the filament tip acting as putative chemoreceptors, consistent with the ability of all fish to respond to aquatic hypoxia, while unique distributions of serotonergic NECs associated with the efferent filament artery correlated with physiological responses to internal hypoxia. These differences in serotonergic NEC distribution appeared to be adaptations related to hypoxia tolerance. Catecholaminergic cells were distinctly different from serotonergic cells, but often colocalized with acetylcholine. Cells containing both acetylcholine and catecholamine were located neighboring serotonergic cells. I propose that the non-innervated serotonergic cells of the lamellae, which are unique to hypoxia tolerant fish, play a chemosensory role in oxygen-sensing and function as neuromodulators acting directly on local vasculature or neighboring acetylcholine-catecholamine-containing cells.

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CHAPTER 1:

General Introduction

Fish can be exposed to a variety of hypoxic environments, ranging from deeply stratified lakes to tide pools, areas of high vegetative turnover, or iced-over winter lakes. As carbon dioxide is 30 times more soluble in water than in air, fish are primarily faced with a difficulty in extracting O_2 from their environment rather than a difficulty in releasing CO_2. Thus, it is imperative for water-breathing aquatic vertebrates to monitor the level of oxygen via chemoreceptors in either the internal or external environment. However, descriptions of the specific location, distribution, and innervation of O_2 chemoreceptors in aquatic vertebrates have not been conclusive. This is largely the result of too little data from too few species studied in too few environments. Therefore, it is necessary to take a comparative approach that incorporates both environmental and physiological factors.

In general, fish exposed to acute hypoxia exhibit bradycardia, hyperventilation, increased ventilatory amplitude, and elevated gill vascular resistance. Physiological adaptations such as lowered metabolic rates, higher anaerobic capacities, increased respiratory surface area, and increased oxygen affinity of the blood are also found in fish living in chronically hypoxic waters.

1.1 Possible chemoreceptor locations

Numerous sites have been proposed for the location of oxygen chemoreceptors in fish including the brain (Smatresk et al., 1986), arterial/venous vasculature (Randall, 1982), orobranchial cavity (Milsom et al., 2002), pseudobranch (Laurent and Dunel, 1980), and the gills (Burleson and Milsom, 1993; Reid and Perry, 2003; Sundin et al., 2000). As the

respiratory structures of water-breathing fish, the gills are an ideal site at which to sense oxygen in either the incident flow of water or within the gill vasculature itself. The gill is highly vascularized, with afferent and efferent branchial, filamental, and lamellar arteries. As well, a venous system provides the nutritive supply to each filament (Olson, 2002). Initial studies suggested that internally-oriented receptors sensing P_aO_2 were involved in producing the ventilatory response to hypoxia, while externally-oriented receptors sensing P_wO_2 also contributed to both the ventilatory and the cardiovascular reflexes (Randall and Smith, 1967; Smatresk et al., 1986; McKenzie et al., 1991; Burleson and Milsom, 1993). Additionally, while all fish have been shown to respond to external hypoxia (decreased P_wO_2), not all fish respond to internal hypoxia (decreased P_aO_2) (Lumsden, 1996).

1.2 Peripheral chemoreceptors: Lessons from Mammals

It may be that chemoreceptors evolved from multiple sites in fish to more localized sites in mammals. Mammals have three peripheral chemoreceptive sites that mediate the hypoxic response—the neuroepithelial bodies (NEBs) of the lung, the aortic bodies, and the carotid bodies. NEBs act as intrapulmonary receptors in the mammalian lung sensing only airway hypoxia (not hypoxemia) and contain 5-HT that was initially thought to have only local secretory effects on pulmonary vasoconstriction (Lauweryns and Cokelaere, 1973). More recent studies, however, have proposed that 5-HT also acts as an excitatory neurotransmitter acting on vagal afferent nerves (Fu et al., 2002). The aortic bodies, innervated by the vagus (CN X) nerve, respond to hypoxia in a similar fashion as the carotid bodies, but have a blunted and weaker response (Lahiri et al., 1981). The primary peripheral chemoreceptive sites in mammals are the carotid bodies, which sit at the bifurcation of the internal and external carotid arteries (Heymans et al., 1930). Additionally, the common

carotid artery is believed to be a homologous structure to the vasculature of the first gill arch of fish (Milsom, 1998).

The highly vascularized carotid body has been studied extensively at the cellular level, detailing the presence of type I chemoreceptive cells and type II supporting cells (Gonzalez et al., 1994). The type I glomus cells contain multiple neurotransmitters (Prabhakar, 2006; Nurse, 2005), and are sensitive to changes in arterial PO_2 (P_aO_2), arterial PCO_2 (P_aCO_2), and pH. These cells reflexly control the hypoxic ventilatory response by hypoxia-induced inhibition of K^+ channels resulting in membrane depolarization and activation of voltage-gated Ca^{2+} channels leading to transmitter release onto the carotid sinus nerve, however this mechanism is still debated (Gonzalez et al., 1994). The first gill arch of fish is similar to the mammalian carotid body in embryonic origin (Milsom, 1998), pattern and level of afferent nerve discharge (Milsom and Brill, 1986; Burleson and Milsom, 1993) and innervation by the glossopharyngeal and vagus nerves (cranial nerves, CN IX and X) (Sundin and Nilsson, 2002). This supports the hypothesis that the gills are the primary peripheral chemoreceptive sites in fish, and therefore are the focus of this study.

1.3 General gill anatomy

Teleosts have four gill arches composed of numerous gill filaments aligned in parallel along two hemibranchs, and each gill filament has a multitude of perpendicular lamellae from the proximal to distal end. The plate-like lamellae protrude from the filament and create a high surface area for gas exchange, but also an avenue for ion loss thus an osmoregulatory compromise to lamellar surface area exists. The first gill arch is innervated by the post-trematic branch of the glossopharyngeal (CN IX) nerve as well as the pre-trematic branch of the vagus nerve (CN X) (Sundin and Nilsson, 2002). The remaining gill arches are innervated

by the vagus (CN X) nerve (Sundin and Nilsson, 2002). Each filament has an efferent and afferent filament artery (eFA and aFA) running along the central axis that further branch into efferent and afferent lamellar arterioles (eLA and aLA). Additionally, the gill has a secondary circulation that provides a nutritive blood source to the gill via anastomoses and the central venous sinus (Olson, 2002). Thus, the complex vascularization and anatomy of the fish gill lends to its multifunctional roles in respiration, osmoregulation, acid/base balance, and waste excretion.

1.4 Neuroepithelial cells of the fish gill

Neuroendocrine cells have been characterized in the respiratory epithelia of all classes of vertebrates. First described in the airway epithelia of mammals (Feyrter, 1938) as neuroepithelial bodies (NEBs), these cells belong to the group of amine precursor uptake and decarboxylation (APUD) endocrine cells. This general group of amine containing cells is characterized by granulated vesicles, proximity to efferent and afferent nerve fibres, fluorescence when treated with formaldehyde vapor, and staining with silver salts (Pearse, 1969). Glomus cells, which are an example of APUD endocrine cells, are present in the carotid and aortic bodies of mammals and the carotid labyrinth of amphibians. The first report of similar neuroepithelial cells (NECs) in fish gills used formaldehyde-induced fluorescence to reveal both isolated and clustered populations of serotonin-containing cells in the primary epithelium of the fish gill, characterized by the presence of dense-cored vesicles (DCV, 80-100 nm in diameter) containing monoamines and were located near nerves (Dunel-Erb et al., 1982).

NECs have been located in every fish studied to date with the majority of studies focusing exclusively on the first gill arch (Bailly et al., 1992; Zaccone et al., 1992; Zaccone

et al., 1997). Using antibodies for serotonin (5-HT) and synaptic vesicle markers (SV2), immunohistochemical techniques have made it easier to locate and quantify the presence of NECs across the fish gill (Jonz and Nurse, 2003). Jonz and Nurse (2003) colocalized the immunoreactivity of these antibodies and mapped the innervation of the cells containing them using a zebrafish neuronal marker, zn-12. They showed a strong relationship between NEC location and both intrinsic and extrinsic nerve fibres. NECs of zebrafish have been identified along the central axis of the filament and the trailing edges of the distal lamellae, locations ideal for sensing both the oxygen partial pressure of the ambient water and the blood, P_wO_2 and P_aO_2, respectively (Jonz and Nurse, 2003). In addition, severe hypoxia induced degranulation of NECs, suggesting neurotransmitter release from vesicles (Dunel-Erb et al., 1982), while a chronic hypoxic exposure of zebrafish showed a 15 percent increase in NEC size (area) and extension of cytoplasmic processes to nerve fibres (Jonz et al., 2004). Direct recording of isolated NECs, detailed a hypoxia-induced inhibition of K^+ channels similar to that seen in glomus cells of the carotid body, further supporting their role in oxygen-sensing (Jonz et al., 2004).

1.5 Proposed experiments

Previous studies have designated the neuroepithelial cells as the O_2 chemoreceptors of the fish gill, and numerous comparisons of NECs have been made to type I chemoreceptive glomus cells of the mammalian carotid body. However, there have not been any studies to date that correlate the distribution of NECs with the specific physiological responses of fish species. As well, serotonin, the primary identifying monoamine of NECs, does not consistently simulate gill afferent nerve discharge (Burleson and Milsom, 1995a). Therefore, there remain unanswered questions concerning the distribution of NECs among

species with different physiological responses to hypoxia, and the role of serotonin as the primary putative neurotransmitter in the gill.

The first objective of my study, therefore, was to compare the distribution and abundance of putative neuroepithelial cells among fish of varying hypoxia tolerances. To delineate whether NEC distributions differed among the species due to environmental adaptation or phylogeny, I examined two closely related species of fish (*Hoplias lacerdae* and *Hoplias malabaricus*), as well as two distantly related fish (*Onchoryncus mykiss* and *Carassius auratus*) of varying hypoxia tolerances. Hypoxia intolerant species generally inhabit well-oxygenated waters and have a higher metabolic rate than the hypoxia tolerant species, which compensate for surviving in more hypoxic waters with a high Hb-O_2 affinity, lower metabolic rate, and a higher anaerobic capacity (Fernandes et al., 1994). One standing hypothesis is that internal chemoreceptors (sensing P_aO_2) are prevalent in more active fish, while more sluggish fish, often inhabiting hypoxic waters, have evolved chemoreceptors that sense the external environment (P_wO_2) (Butler et al., 1979; Randall, 1982).

The rainbow trout (*O. mykiss*) is the white rat of fish biology, yet few studies have documented NECs in this species (Dunel-Erb et al., 1982; Saltys et al., 2006). The trout is an example of a hypoxia intolerant fish, occupying well-aerated waters, and having a high metabolic rate. The carp, on the other hand, has an amazing hypoxia tolerance and can even withstand anoxia. For example, the crucian carp (*Carassius carassius*) is able to sustain long bouts of anoxia by using anaerobic metabolism and creating ethanol as a byproduct (Shoubridge and Hochachka, 1990; Johnston and Bernard, 1983; Stecyk et al., 2004), which easily diffuses across the gills. As well, previous studies have documented the response of the rainbow trout to both internal and external hypoxia (Burleson and Milsom, 1990;

Burleson and Milsom, 1993); however, the carp was shown to respond only to external hypoxia (Lumsden, 1996). Therefore, these two species show a difference in the hypoxic stimuli that produce physiological responses. Comparing trout and carp NEC distributions and the effects of hypoxia on the NECs will provide an idea of the role of these cells in O_2-sensing in teleosts of such different hypoxia tolerances.

Cyclic flooding and high vegetative turnover of the Amazon River create a highly hypoxic environment that fluctuates on a daily and seasonal basis. Traira (*Hoplias malabaricus*) and trairão (*Hoplias lacerdae*) are closely related yet ecologically distinct water-breathing Erythrinids. While the traira is well-adapted to life in shallow, stagnant, and hypoxic lakes, the trairão inhabits well-aerated waters of central and southern Brazil (Rantin et al., 1993). By examining the distribution of NECs in two species of fish from the same genus *(Hoplias)*, I hope to determine whether any differences seen between trout and carp were due to phylogeny or were physiological adaptations.

In investigating the distribution of putative O_2-sensing cells among four species of fish in Chapter 2, I hypothesized that (1) differences between physiological responses to hypoxia would be associated with different distributions of putative O_2-sensing cells in the gills, and (2) the distribution of these cells would be related to each species' hypoxia tolerance. Based on my hypotheses, I predicted that the hypoxia intolerant fish (trout and trairão) would have NEC distributions that favoured sensing arterial oxygen levels, whereas hypoxia tolerant species (goldfish, *Carassius auratus*, and traira) would have distributions that favoured sensing environmental oxygen levels.

The second objective of my study was to examine the presence of multiple neurochemicals in the fish gill. Dunel-Erb et al. (1982) showed that serotonin was the major

monoamine in the NECs of fish gills and 5-HT has become the cornerstone of NEC identification. Arguably, secretion of serotonin from the dense-cored vesicles of the NECs increases blood flow to the gills and enhances the respiratory surface area (Sundin et al., 1995; Sundin and Nilsson, 2002). However, Burleson and Milsom (1995a) experimented with a variety of monoamines and catecholamines in an isolated gill preparation, recording from the glossopharyngeal nerve, and found that serotonin actually had no consistent effect on the discharge frequency arising from the first gill arch of rainbow trout—either exogenously or internally administered. As well, the carotid body, which is consistently referred to as a homologue of the gill, is host to numerous neurotransmitters and neuromodulators (Gonzalez et al., 1994; Nurse, 2005). Therefore, the second objective of this study was to examine the presence of immunoreactivity to several neurotransmitters in the fish gill that have been shown to have chemosensory effects in the carotid body, specifically serotonin, catecholamines, acetylcholine, and adenosine. I hypothesized that multiple neurotransmitters would colocalize in the same cell, presumably the serotonergic NECs, as they do in other vertebrate peripheral chemoreceptive structures.

REFERENCES

Bailly, Y., Dunel-Erb, S. and Laurent, P. (1992). The neuroepithelial cells of the fish gill filament: Indolamine-immunocytochemistry and innervation. *Anat. Rec.* **233**, 143-161.

Burleson, M. L. and Milsom, W. K. (1990). Propranolol inhibits O_2-sensitive chemoreceptor activity in trout gills. *Am. J. Physiol.* **258**, 1089-1091.

Burleson, M. L. and Milsom, W. K. (1993). Sensory receptors in the first gill arch of rainbow trout. *Respir. Physiol.* **93**, 97-110.

Burleson, M.L. and Milsom, W.K. (1995a). Cardio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. *Respir. Physiol.* **100(3)**, 231-238.

Butler, P.J., Taylor, E.W., and Davison, W. (1979). The effect of long term, moderate hypoxia on acid-base balance, plasma catecholamines and possible anaerobic end products in the unrestrained dogfish *Scyliorhinus canicula. J. Comp. Physiol.* **132,** 297-303.

Dunel-Erb, S., Bailly, Y. and Laurent, P. (1982). Neuroepithelial cells in fish gill primary lamellae. *J. App. Physiol.* **53**, 1342-1353.

Fernandes, M. N., Rantin, F. T., Kalinin, A. L. and Van Moron, S. E. (1994). Comparative study of gill dimensions of three erythrinid species in relation to their respiratory function. *Can. J. Zool.* **72**, 160-165.

Feyrter, F. (1938). Uber diffuse endocrine epitheliale Organe. Leipzig, East Germany: Barth.

Fu, X. W., Nurse, C. A., Wong, V. and Cutz, E. (2002). Hypoxia-induced secretion of serotonin from intact pulmonary neuroepithelial bodies in neonatal rabbit. *J. Physiol. (Lond)* **539**, 503-510.

Gonzalez, C., Almarez, L., Obeso., A. and Rigual, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol. Rev.* **74(4)**, 829-896.

Heymans, C., Bouckaert, J.J., Farber, S., and Hus, F.Y. (1936). *Arch Int. Pharmacodyn.* **54**, 129.

Johnston, I.A. and Bernard, L.M. (1983). Utilization of the ethanol pathway in carp following exposure to anoxia. *J. Exp. Biol.* **104**, 73-78.

Jonz, M. G. and Nurse, C. A. (2003). Neuroepithelial cells and associated innervation of the zebrafish gill: a confocal immunofluorescence study. *J. Comp. Neurol.* **461**, 1-17.

Jonz, M. G., Fearon, I. M. and Nurse, C. A. (2004). Neuroepithelial oxygen

chemoreceptors of the zebrafish gill. *J. Physiol. (Lond.)* **560**, 737-752.

Lahiri, S., Mokashi, A., Mulligan, E., and Nishino, T. (1981). Comparison of aortic and carotid chemoreceptor responses to hypercapnia and hypoxia. *J. Appl. Physiol.* **51,** 55-61.

Laurent, P. and Dunel, S. (1980). Morphology of gill epithelia in fish. *Am. J. Physiol.* **238**, R147-159.

Lauweryns, J. M. and Cokelaere, M. (1973). Intrapulmonary neuro-epithelial bodies: hypoxia-sensitive neuro(chemo-)receptors. *Separatum Experientia* **29**, 1384-1386.

Lumsden, A.L. (1996) Oxygen-sensitive chemoreceptors and cardioventilatory control in carp. MSc Thesis. University of British Columbia. 97 pp.

McKenzie, D. J., Burleson, M. L. and Randall, D. J. (1991). The effects of branchial denervation and pseudobranch ablation on cardioventilatory control in an air-breathing fish. *J. Exp. Biol.* **161**, 347-365.

Milsom, W. K. and Brill, R. W. (1986). Oxygen sensitive afferent information arising from the first gill arch of yellowfin tuna. *Respir. Physiol.* **66**, 193-203.

Milsom, W. K., Reid, S. G., Rantin, F. T. and Sundin, L. (2002). Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish. *J. Exp. Biol.* **205**, 1765-1774.

Milsom, W.K. (1998). Phylogeny of respiratory chemoreceptor function in vertebrates. *Zoology*. **101,** 316-332.

Nurse, C. A. (2005). Neurotransmission and neuromodulation in the chemosensory carotid body. *Auton. Neurosci.* **120**, 1-9.

Olson, K. R. (2002). Vascular anatomy of the fish gill. *J. Exp. Zool.* **293**, 214-231.

Pearse, A. G. E. (1969). The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. *J. Histochem. Cytochem.* **17**, 303.

Prabhakar, N. R. (2006). O_2 sensing at the mammalian carotid body: why multiple O2 sensors and multiple transmitters? *Exp. Physiol.* **91**, 17-23.

Randall, D. J. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.* **100**, 275-288.

Randall, D. J. and Smith, J. C. (1967). The regulation of cardiac activity in fish in a hypoxic environment. *Physiol. Zool.* **40**, 104-113.

Rantin, F. T., Glass, M. L., Kalinin, A. L., Verzola, R. M. M. and Fernandes, M. N. (1993). Cardio-respiratory responses in two ecologically distinct erythrinids (*Hoplias malabaricus* and *Hoplias lacerdae*) exposed to graded environmental hypoxia. *Environ. Biol. Fishes.* **36**, 93-97.

Reid, S. G. and Perry, S. F. (2003). Peripheral O_2 chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am. J. Physiol.* **284**, R990-R999.

Saltys, H. A., Jonz, M. G. and Nurse, C. A. (2006). Comparative study of gill neuroepithelial cells and their innervation in teleosts and *Xenopus* tadpoles. *Cell Tissue Res.* **323**, 1-10.

Shoubridge, E. A. and Hochachka, P.W. (1980). Ethanol: novel end product in vertebrate anaerobic metabolism. *Science.* **209,** 308-309.

Smatresk, N. J., Burleson, M. L. and Azizi, S. Q. (1986). Chemoreflexive responses to hypoxia and NaCN in longnose gar: evidence for two chemoreceptor loci. *Am. J. Physiol.* **251**, R116-R125.

Stecyk, J. A. W., Stenslokken, K.-O., Farrell, A. P. and Nilsson, G. E. (2004). Maintained cardiac pumping in anoxic crucian carp. *Science.* **306**, 77.

Sundin, L. and Nilsson, S. (2002). Branchial Innervation. *J. Exp. Zool.* **293**, 232-248.

Sundin, L., Nilsson, G. E., Block, M. and Lofman, C. O. (1995). Control of gill filament blood flow by serotonin in the rainbow trout, Onchoryncus mykiss. *Am. J. Physiol.* **268**, R1224-R1229.

Sundin, L., Reid, S.G., Rantin, F.T., and Milsom, W.K. (2000). Branchial receptors and cardiorespiratory reflexes in a neotropical fish, the tambaqui (*Colossoma macropomum*). *J. Exp. Biol.* **203**, 1225-1239.

Zaccone, G., Fausulo, S., Ainis, L. and Licata, A. (1997). Paraneurons in the gills and airways of fishes. *Microsc. Res. Tech.* **37**, 4-12.

Zaccone, G., Lauweryns, J. M., Fasulo, S., Tagliafierro, G., Ainis, L. and Licata, A. (1992). Immunocytochemical localization of serotonin and neuropeptides in the neuroendocrine paraneurons of teleost and lungfish gills. *Acta Zoologica* **73**, 177-183.

CHAPTER 2:

A comparative analysis of putative O_2-sensing cells in the fish gill[1]

INTRODUCTION

When fish are exposed to acute hypoxia they exhibit bradycardia, elevated gill vascular resistance, hyperventilation, and increased ventilatory amplitude (Randall, 1982). These acute hypoxic responses are reflexly initiated by oxygen chemoreceptors, but the sites of these receptors have not been definitively established. Previous researchers have proposed that oxygen chemoreceptors in fish could be located in the brain (Smatresk et al., 1986), the arterial/venous vasculature (Randall, 1982), the orobranchial cavity (Milsom et al., 2002), the pseudobranch (Laurent and Dunel, 1980), or the gills (Burleson and Milsom, 1993; Reid and Perry, 2003; Sundin et al., 2000). It has also been proposed that the receptors in the gills are situated in locations that sense either external oxygen tension (PO_2) in the incident flow of water (P_wO_2), internal PO_2 within the gill vasculature itself (P_aO_2 or P_vO_2), or both (Randall, 1982).

Of note is that the arterial blood supply to the first gill arch of fish is homologous to the carotid artery where the carotid body, the primary peripheral chemoreceptive organ in mammals, is found. Furthermore, putative O_2 chemosensing cells in the first gill arch and carotid body have similar ultrastructure (Bailly et al., 1992; Jonz and Nurse, 2003), patterns

[1] A version of this chapter will be submitted for publication.
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and levels of afferent nerve discharge (Milsom and Brill, 1986; Burleson and Milsom, 1993), embryonic origin (Milsom, 1998), innervation by the glossopharnygeal (CN IX) nerve (Sundin and Nilsson, 2002; Gonzalez et al., 1994), and chemoreceptive mechanisms (Jonz et al., 2004).

The putative oxygen chemosensing cells in fish gills are neuroendocrine cells (NECs). Neuroendocrine cells have been characterized in the respiratory epithelia of all classes of vertebrates. First described in the airway epithelia of mammals (Feyrter, 1938) as neuroepithelial bodies (NEBs), this general group of amine containing cells is characterized by granulated vesicles, proximity to efferent and afferent nerve fibres, and fluorescence when treated with formaldehyde vapor (Pearse, 1969). The first report of NECs in fish gills used formaldehyde-induced fluorescence to reveal both isolated and clustered populations of cells in the primary epithelium of the fish gill, characterized by the presence of dense-cored vesicles (DCV, 80-100 nm in diameter) containing monoamines (serotonin) in proximity to neurons (Dunel-Erb et al., 1982). Since this first report, NECs have been located in every fish studied to date with the majority of studies focusing exclusively on the first gill arch.

Using antibodies for serotonin (5-hydroxytryptamine, 5-HT), a transmembrane synaptic vesicle marker (SV2), and a general neuronal marker raised in zebrafish (zn12), Jonz and Nurse (2003) showed a strong association between NECs and both intrinsic and extrinsic nerve fibres in zebrafish gills. NECs of zebrafish were located along the central axis of the gill filament as well as the trailing edges of the lamellae, locations that would be ideal for sensing both P_aO_2 and P_wO_2, respectively. NECs have been previously shown to degranulate during acute hypoxia (Dunel-Erb et al., 1982), suggesting that neurotransmitter release occurs, and subsequent studies found that this was likely mediated by inhibition of

background K^+ channels leading to membrane depolarization (Jonz et al., 2004). Chronic hypoxia, on the other hand, resulted in NEC hypertrophy and extension of cell processes (Jonz and Nurse, 2003) revealing time domain effects of hypoxia on NECs and further supporting their role as chemoreceptors.

Rainbow trout (*Onchoryncus mykiss*) respond more vigorously to arterial than aquatic hypoxia and have relatively poor hypoxia tolerance (Tetans and Lykkeboe, 1981; Burleson and Milsom, 1990; Burleson and Milsom, 1993). Carp, on the other hand, respond only to aquatic hypoxia and are extremely hypoxia tolerant (Hughes et al., 1983; Lumsden, 1996). Given the data presented above, we hypothesized (1) that such differences between physiological responses to hypoxia in these two species would be associated with different distributions of putative O_2-sensing cells in the gills, and (2) that the distribution of these cells would be related to each species' hypoxia tolerance. Initial studies suggested that internally-oriented receptors sensing P_aO_2 were involved in producing the ventilatory response, while externally-oriented receptors sensing P_wO_2 also contributed to the ventilatory response but mediated the cardiovascular reflexes as well (Randall and Smith, 1967; Smatresk et al., 1986; McKenzie et al., 1991; Burleson and Milsom, 1993). More recent results have been inconclusive, showing no difference in distribution of putative chemoreceptors among fish species (Saltys et al., 2006), which does not agree with physiological data. The objective of the present study was to further test the above hypotheses in several species with varying hypoxia tolerances, and to comprehensively analyze all putative O_2-sensing regions within the gills. Thus, as well as examining NEC distributions in trout and carp, species which have been the subject of previous investigations, we collected data from two previously unstudied species of closely related

Amazonian fish that differ in their hypoxia tolerance (traira, *Hoplias malabaricus*, and trairão, *Hoplias lacerdae*). Based on our hypotheses, we predicted that the hypoxia intolerant fish (trout and trairão) would have NEC distributions that favour sensing arterial oxygen levels, whereas hypoxia tolerant species (goldfish, *Carassius auratus*, and traira) would have distributions that favour sensing environmental oxygen levels.

METHODS

Animals

Adult rainbow trout (*Onchoryncus mykiss* (Walbaum), n=6, 520-1190 g) and goldfish (*Carassius auratus* (Linnaeus), n=6, 20-100 g) were kept in outdoor, flow-through, and aerated tanks in the Zoology Aquatic Facility at the University of British Columbia. Trairão (*Hoplias lacerdae* (Ribeiro), n=6, 90-200 g) and traira *(Hoplias malabaricus* (Bloch), n=6,140-260 g) were maintained in 250 L outdoor tanks at 25 ± 1°C at the Federal University of São Carlos (SP, Brazil) and supplied with aerated water. Fish used for tissue analysis were randomly selected and killed by an overdose of MS-222 (3-aminobenzoic acid ethyl ester) followed by a sharp blow to the head.

Tissue preparation

The first and second gill arches were excised from each fish and washed in ice-cold phosphate-buffered solution (PBS) containing (in mM): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; buffered to pH 7.8 with 1 M NaOH (Jonz and Nurse, 2003). Individual gill arches were syringe-perfused with heparinized PBS (1000 iu/ml) until filaments appeared clear and fixed by immersion in 4% paraformaldehyde in PBS at 4°C overnight. Tissues were then rinsed in PBS and cryoprotected in a 30% sucrose solution. Segments of the first and second gill arches were then frozen in Tissue-Tek® (Sakura Finetek, Fisher Scientific) at -80°C until the blocks were either sectioned longitudinal to the gill filament at 10-30 μm or transverse to the gill filament at 10-14 μm using a cryostat (Leica CM3050 S, Leica Microsystems, Germany) and mounted on Superfrost® plus slides (VWR International) for immunohistochemistry.

Immunohistochemistry

Slides were washed in PBS and blocked in 10% Normal Goat Serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, Ontario) for one hour. Primary antibodies (used individually or in combination) were diluted in a permeabilizing solution (PBS/0.2% Triton X-100/0.1% sodium azide) according to the optimal dilutions detailed in Table 2.1, and set on the slides to incubate overnight at room temperature. Following incubation of the primary antibodies, the slides were again washed in PBS. The slides were then treated with fluorescently labeled secondary antibodies diluted in PBS (Table 2.1) 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 (Vector Laboratories, Burlington, Ontario) to prevent photobleaching. Slide boxes were stored at 4°C until viewed under a fluorescent light microscope. 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 by a reaction of the antibodies' primary hosts. The results of these control experiments showed only negligible staining with no specific immunolabelling of the gill structures (data not shown). Immunolabeled slides were observed under a light microscope (Axioplan 2, Zeiss, Jena, Germany) and digitally captured using a Q-Imaging (Burnaby, British Columbia, Canada) CCD camera and analyzed using image analysis software (Northern Eclipse, Empix Imaging, Ontario). A few representative slides cut at 30 μm were further examined using a confocal scanning system (Pascal 2, Axioskop X, Zeiss, Jena, Germany) equipped with argon (Ar) and helium-neon (He-Ne) lasers with peak outputs of

488 nm and 543 nm, respectively. Images obtained were subsequently used for quantification of staining intensity and cell counts.

Quantification

Distribution patterns of 5-HT-immunoreactivity (5-HT-IR) were obtained through analysis of average staining intensity from the tips of the lamellae to the midline of the filament after subtracting background (threshold) intensity. This distance was expressed relative to lamellar length, where 0 represents the lamellar tip and 100 represents the filament midline, to standardize the distance measured in each individual fish for varying gill sizes and across all species examined. Intensity thresholds were created for each individual fish, and line intensity measurements were made along the relative lamellar length of 140 individual lamella from six individual fish per species. This method of quantification displays the pattern of immunoreactivity analyzed pixel by pixel from numerous microscope images. Threshold and intensity analysis were conducted using SigmaScan Pro 5.0 (SPSS, Chicago, IL, USA). Additionally, the numbers of 5-HT-IR cells were counted per mm of filament (McCormick et al., 2003) from six representative images per individual fish.

Statistics

Data are presented as mean average intensity with 95% confidence limits and as mean ± s.e.m. values for 5-HT-IR cell number. Differences between filament and lamellae cell numbers within each species were compared using a Student's paired t-test. One-way analysis of variance (ANOVA) followed by a Tukey-Kramer *post hoc* test was used to test differences in filament and lamellar cell number among the four species. $P<0.05$ was used to reject the null hypothesis. Statistical analyses were performed using the software package JMP 5.1.2 (SAS Institute Inc., Cary, NC, USA).

RESULTS

There were no observable differences in morphology or immunoreactive staining between the first and second gill arches; therefore, the results from analysis of both first and second gill arches were pooled together. There were distinct differences in the distribution of serotonergic cells along the filament among the four species examined in this study. In trout, 5-HT-IR cells were found exclusively along the filament, frequently near filamental blood vessels (Fig. 2.1A). This contrasted sharply with what was seen in the goldfish, where serotonergic cells were located exclusively in the lamellae (Fig. 2.1B). In both trairão (Fig. 2.1C) and traira (Fig. 2.1D), 5-HT-IR cells were found both along the filament and in the lamellae. 5-HT-IR cells of the lamellae in goldfish, trairão, and traira were primarily located at lamellar tips. Quantification of staining intensity for 5-HT from lamellar tips to the central axis of the filament confirmed this difference in distribution (Fig. 2.2). Trout showed no staining along the lamellae, but had a strong peak of intense staining beginning at the filament epithelium (Fig. 2.2A). The average intensity profile of the goldfish revealed the opposite pattern, with staining intensity highest near the lamellar tip (Fig. 2.2D). The two species of *Hoplias*, trairão and traira, had similar staining profiles, with stronger staining intensity towards the tip of the lamellae and gradually less intense staining towards the filament (Fig. 2.2B, 2.2C).

Cell counts of 5-HT-IR cells produced results similar to the average intensity profiles. The number of cells on the filament versus the lamellae within each species were significantly different in all fish ($p<0.001$) (Fig. 2.3). As well, significant differences were found between species (Fig. 2.3). Rainbow trout had a higher number of immunoreactive

cells on the filament compared with the three more hypoxia tolerant species. Additionally, there was a significant difference in the cell counts of 5-HT-IR cells in the lamellae between the species of *Hoplias*.

Double immunolabeling of the gills with antibodies for a synaptic vesicle membrane protein (SV2) and serotonin, produced a strong colocalization in all four species (Fig. 2.4). Synaptic vesicle containing cells were located along the central filament and on the lamellae in all four species. The majority of cells immunoreactive for SV2 were also 5-HT-positive; however, not all SV2-IR cells contained 5-HT (arrowheads, Fig. 2.4B, 2.4C). Serotonergic cells of the lamellae in goldfish, trairão, and traira always colocalized with SV2, which is especially apparent in the goldfish (Fig. 2.4B), but while the SV2 marker did appear in the lamellae of trout, 5-HT did not (Fig. 2.4A). Furthermore, serotonin in cells was not always found in synaptic vesicles (arrow, Fig. 2.4D).

Confocal images of filaments doubly-labeled with anti-5-HT and a general neuronal marker (anti-zn12) traced nerve fibres solely down the filament in all four species (Fig. 2.5). There did not appear to be any nerve fibres extending out of the primary epithelium into the lamellae. Thus, while the 5-HT-IR cells along the central filament were associated with nerve fibres, the 5-HT-IR cells of the lamellae were not innervated.

A closer examination of NECs in the filament of rainbow trout (Fig. 2.6A) and the lamellae of goldfish (Fig. 2.6B) using transmission electron microscopy revealed NECs characterized by dense-cored vesicles (insets) that were near to red blood cells (RBC) and pillar cells (pc). NECs in the filament of rainbow trout were associated with nerves (N), near vasculature (RBC), and were located within the filament epithelium, as denoted by the basal lamina (BL) (Fig. 2.6A). NECs of the goldfish lamellae were in close proximity to the

ambient environment (H_2O), red blood cells (RBC) and pillar cells (pc), but not in association with nerves (Fig. 2.6B).

To further define the location of the serotonergic cells located within the central filament, cross-sections of individual gill filaments were immunolabeled and examined. Trout filament cross-sections showed 5-HT-SV2-IR cells primarily located around the efferent filament artery (eFA), with a few cells located near the afferent filament artery (aFA) and central venous sinous (cvs) (Fig. 2.7A). Cross-sections of goldfish filaments supported the observation of serotonergic distribution at the lamellae tips, with no cells located in the central filament (Fig. 2.7B). Results from both trairão and traira also supported the findings from longitudinal sections (Fig. 2.4), showing 5-HT-SV2-IR cells both in the lamellae and less frequently in the filament (Fig. 2.7C-D). Cross-sectional nerve tracings (zn12) further corroborated our failure to find evidence of innervation of the lamellae in any of the species examined. 5-HT-positive cells within the central filament again showed a strong association with neurons, especially around the eFA.

Although there were distinct differences in the distribution of 5-HT-IR cells along the lamellae and filament, the distribution of 5-HT-IR cells at the tips of the gill filaments was consistent among the four species examined (Fig. 2.8). In all four species, clusters of 5-HT-SV2-positive cells were found at the tip of every filament, where the lamellae gradually decreased in size and the filament was directly exposed to the ambient environment (Fig. 2.8). A general neuronal marker (zn12) traced nerve profiles into the tip of the filament, and higher magnification images using confocal microscopy show a strong association between the 5-HT-IR cells and nerves (Fig. 2.9). This finding was characteristic of all fully intact filaments in all four species.

DISCUSSION

Denervation of the glossopharyngeal and vagus nerves innervating the gills has provided indirect evidence for a role of the gills in O_2-sensing (Burleson and Smatresk, 1990; McKenzie et al., 1991; Sundin et al., 2000; Florindo et al., 2006). Additionally, electrophysiological recording of chemoreceptive nerve fibres in the first gill arch (Milsom and Brill, 1986; Burleson and Milsom, 1993) and patch-clamp recording of isolated neuroepithelial cells (NECs) (Jonz et al., 2004) provided direct evidence of the chemoreceptive responses of the gill and NECs to hypoxia. Since their first identification by Dunel-Erb et al. (1982), gill NECs have been documented in every fish species studied to date, and their putative role in oxygen-sensing and chemoreception has been supported by histological and electrophysiological examination. Characterized by the presence of serotonin stored in vesicles, NECs degranulate and depolarize in acute hypoxia (Dunel-Erb et al., 1982; Jonz et al., 2004), presumably releasing vesicular serotonin either on target cells, vasculature, or afferent nerves.

Within the fish gill, there are numerous potential sites for oxygen sensing. Sites capable of monitoring arterial or venous oxygen levels include the efferent filament artery (eFA), the central venous sinus (cvs), or the afferent filament artery (aFA), while aquatic hypoxia (P_wO_2) may be detected where the lamellar or filament epithelium is exposed to the ambient water. Oxygen-sensing cells at these different sites could also be either chemoreceptive or chemosensory. A respiratory chemoreceptive cell releases a neurotransmitter directly onto an afferent nerve thereby transmitting information to the central nervous system to produce hypoxic reflexes, as in the case of the glomus cells of the

carotid body (Krammer, 1978). A chemosensory cell, on the other hand, is not innervated but plays a paracrine role, releasing neurotransmitter directly onto a nearby target cell, such as vascular smooth muscle (Krammer, 1978). In our comparative analysis of potential O_2-sensing sites, we observed NECs that could serve both chemoreceptive and chemosensory roles based on their location and innervation. Furthermore, our results indicated a striking difference in the distribution of putative O_2-sensing cells (chemoreceptive and chemosensory), as identified by colocalization of serotonin (5-HT) and synaptic vesicles (SV2), among the four species examined.

2.1 Chemoreceptive NECs

In all species examined in this study, we observed a cluster of innervated cells containing serotonin in vesicles at the filament tips (Fig. 2.8, 2.9). NECs at the filament tips have also been described in cod (Sundin et al., 1998a), the Antarctic borch (*Pagothenia borchgrevinki)* (Sundin et al., 1998b) and zebrafish (Jonz and Nurse, 2003), and thus this appears to be a consistent location for chemoreceptive neuroepithelial cells across species. NECs at the filament tip are in an ideal location to sense changes in P_wO_2, releasing serotonin directly onto an afferent nerve, transmitting the signal to the central nervous system (CNS) and producing a reflex hypoxic response. The presence of such a mechanism is supported by physiological data, indicating that all fish included in this study reflexly respond (increase ventilation) to aquatic hypoxia (Burleson and Milsom, 1993; Sundin et al., 1999; Lumsden, 1996). More specifically, denervation of the glossopharyngeal and vagus nerves in rainbow trout (Reid and Perry, 2003) and traira (Sundin et al., 1999) abolished the hyperventilation induced by application of exogenous (aquatic) NaCN, thereby localizing the external chemoreceptors to the gills. Subsequent studies supported this finding in other fish species

(Milsom et al., 2002; Florindo et al., 2006), indicating that external NaCN stimulates hyperventilation by chemoreceptors located in the gills monitoring the aquatic environment.

Although innervated NECs were consistently present at the filament tip, we found species differences in the distribution of innervated NECs along the center of the filament. Innervated cells containing serotonergic vesicles were present in high number in the filament core of rainbow trout, less so in trairão and traira, but not at all in goldfish (Fig. 2.4, 2.5). The filament is flanked by an efferent filament artery (eFA) and afferent filament artery (aFA), with a central venous sinuous running down the middle of the filament, making it an ideal location to sense internal oxygen levels (P_aO_2 or P_vO_2). Examination of cross-sections of the filament in trout, traira, and trairão (Fig. 2.7) revealed that most NECs along the central filament were associated with the eFA, a location that would sense the oxygenated blood returning to the systemic circulation. NECs in the center of the filament may play a chemoreceptive role, sensing internal (P_aO_2) hypoxia and producing a reflex response. Examination of cross-sections of goldfish filaments failed to identify any NECs in the filament (Fig. 2.7), and this is supported by the lack of response of carp to internal hypoxia (Lumsden, 1996). In addition to chemoreceptive responses, the NECs of the filament may act in a paracrine manner. *In vivo* examination of rainbow trout gill vasculature exposed to hypoxia demonstrated constriction of the eFA resulting in overflow to the cvs and a continuous flow through the lamellae (Sundin and Nilsson, 1997).

The finding of NECs in the filament that are innervated suggests that they release neurotransmitter (serotonin) across a synapse, transmitting a signal to the CNS. Thus, these cells may serve a chemoreceptive role, and physiological data supports the presence of hypoxic reflexes stimulated by internal hypoxia in these species. Altering internal O_2 levels

by hypoxemia, injection of hypoxic blood, or reduced blood flow to the gills stimulates ventilation in the rainbow trout (Holeton, 1971; Smith and Jones, 1982). In traira, ventilatory amplitude increased with both internal hypoxia (P_aO_2) and bolus injections of NaCN (Sundin et al., 1999). As well, single fibre nerve recordings of the trout gills displayed chemoreceptive afferent transmission in response to both internal and external hypoxia (Burleson and Milsom, 1993). On the other hand, carp (*Cyprinus carpio*) did not respond to internal hypoxia or injection of NaCN (Lumsden, 1996), which is consistent with our failure to find innervated filamental NECs in a location able to sense blood oxygen levels.

2.2 Chemosensory NECs

The cells with serotonin-containing vesicles in the lamellae of goldfish, traira, and trairão can not play a role in afferent signaling to the CNS, as there were no nerve fibres extending out of the primary epithelium and into the lamellae (Fig. 2.5-2.7). Saltys et al. (2006), using the same neuronal marker (zn12), also did not see nerves extending into the lamellae of juvenile trout using whole-mount fixations examined with confocal microscopy. Jonz and Nurse (2003), however, did see innverated lamellae in the zebrafish, and Saltys et al. (2006) found that goldfish appeared to have innervated lamellae. However, re-examination of our data with confocal microscopy (Fig. 2.5) and electron microscopy of trout and goldfish gills (Fig. 2.6) still did not reveal innervated lamellae in any of the species examined. Furthermore, detailed reviews of gill morphology and branchial innervation do not describe any innervation extending deep into the lamellae (Laurent and Dunel, 1980; Wilson and Laurent, 2002; Sundin and Nilsson, 2002).

Thus, our data suggest that in addition to the chemoreceptors monitoring the P_wO_2 at the filament tip, and P_aO_2 in the efferent filament artery (trout, traira, and trairão), goldfish,

traira, and trairão also have chemosensory cells potentially monitoring P_wO_2 in the lamellae (Fig. 2.4). Acting in a paracrine fashion, these chemosensory cells may release serotonin, a potent vasoconstrictor, directly onto the contractile pillar cells of the lamellae, thereby optimizing respiratory surface area. Under resting conditions, fish decrease the loss of ions across the gill by perfusing only two-thirds of the lamellae (Booth, 1978; Farrell et al., 1979); however, hypoxic conditions require microcirculatory alterations to increase the respiratory surface area to its maximum. Pillar cell contraction increases lamellar blood spaces and drives blood across the full lamellar sheet, which has a significant effect on matching the respiratory surface area with the oxygen demands of the fish (Smith and Johnson, 1977; Stenslokken et al., 2006). In this way, these lamellar chemosensory cells found in the goldfish, traira, and trairão potentially monitor the hypoxic waters they frequently inhabit, functioning to increase the respiratory surface area in response to lowered P_wO_2. Lamellar NECs were not present in the trout, which indicates that this fish species may not require additional monitoring of the external environment, as rainbow trout rarely encounter hypoxic waters. Additionally, in-vivo microcirculation of the rainbow trout gill did not show direct vasodilation via pillar cell contraction localized in the lamellae in rainbow trout (Sundin and Nilsson, 1997), which is consistent with our failure to find NECs in the trout lamellae (Fig. 2.1, 2.4).

Furthermore, aquatic hypoxia caused lamellae to protrude in the crucian carp (*Carassius carassius*), increasing the respiratory surface area as an adaptive and reversible morphological change (Sollid et al., 2003). A similar gross morphological alteration was seen in both crucian carp and goldfish as a response to temperature (Sollid et al., 2005), and it is likely that this morphological change due to environmental factors may be characteristic of

Carassius. Therefore, it would be especially advantageous for the O_2-sensing cells of carp to be located at the tips of the lamellae as opposed to the middle of the lamellae (Fig. 2.4), where they are continually exposed to the ambient water regardless of increases in intra-lamellar cell mass associated with hypoxia- and temperature-induced changes.

2.3 NECs in relation to hypoxia tolerance

There is a significant difference in the distribution of serotonergic cells among the species examined. This difference is most apparent when comparing the rainbow trout with the three more hypoxia tolerant species, goldfish, traira, and trairão. Trout have an abundance of serotonergic-vesicular (5-HT-SV2-IR) cells in the filament and lack immunoreactive cells in the lamellae (Fig. 2.1, 2.3). Thus, the rainbow trout, which does not often encounter hypoxic environments, does not appear to possess lamellar chemosensory cells, but may solely rely on the ability to monitor changes in aquatic oxygen via the chemoreceptive NECs at the filament tip (Fig. 2.9). In the more hypoxia tolerant goldfish, traira, and trairão, however, there are numerous 5-HT-SV2-IR cells in the lamellae, especially at the lamellar tips. Thus, the more hypoxia tolerant species that frequently encounter hypoxic waters appear to have evolved a second location for monitoring P_wO_2, which acts in a paracrine manner to produce direct, local effects of enhance lamellar blood flow. The difference in distribution of serotonergic cells in the filament versus lamellae may be related to the hypoxia tolerance or activity of the fish species.

Hypoxia tolerant fish are well known to have a higher hemoglobin-oxygen (Hb-O_2) affinity than hypoxia intolerant fish, and thus P_{50} is a good indicator of relative hypoxia tolerance. This is true of the species in the present study, whose P_{50} values are 23 torr for trout (Tetens and Lykkeboe, 1981), 2.6 torr for goldfish (Burggren, 1982), and 8.7 torr for

traira (Perry et al, 2004). There is no available data on the P_{50} value for trairão, but an approximation of 13 torr can be made based on the critical oxygen tensions (P_cO_2) of traira and trairão (Rantin et al., 1992) and P_{50} values of other Erythrinids (Perry et al., 2004). In general, there was a positive correlation between the number of 5-HT-IR cells in the filament and the P_{50}, and a negative correlation with the number of 5-HT-IR cells in the lamellae (Fig. 3).

Active fish inhabiting well-aerated waters, such as the rainbow trout, maintain a high arterial PO_2 and have a low Hb-O_2 affinity, which assists in oxygen delivery to the tissues during sustained activity. The consequence is a relatively hypoxia intolerant species. Hypoxia tolerant species, on the other hand, often have a lowered metabolic rate and a low arterial PO_2 and are characterized by a high Hb-O_2 affinity, promoting oxygen uptake from a hypoxic environment. The goldfish (*Carassius auratus*, P_{50} = 2.6 torr) is a champion of hypoxia tolerance, and *Carassius* sp. can even survive long bouts of anoxia (Shoubridge and Hochachka, 1980; Hughes et al., 1983; Stecyk et al., 2004). Correspondingly, the goldfish has a significantly higher number of putative NECs in the lamellae compared with trout and traira (Fig. 2.3). In general, it appeared that active hypoxia intolerant fish, such as rainbow trout, had a significantly higher number of serotonergic cells in the filament compared to the lamellae. The opposite was true in less active, hypoxia tolerant fish (traira, trairão, and carp), which had more serotonergic cells in the lamellae (Fig. 2.3). Single fibre nerve recording from tuna gills revealed more fibres responsive to alterations in internal hypoxia than external hypoxia, suggesting that active, hypoxia intolerant fish are more sensitive to a drop in P_aO_2 than P_wO_2 (Milsom and Brill, 1986). However, the rainbow trout gill exhibited an

equal number of fibres responsive to internal or external hypoxia (Burleson and Milsom, 1993), making this generalization speculative at best.

Although there was a large difference in the neuroepithelial cell distribution in the rainbow trout compared to the goldfish, traira, and trairão, we did not observe the hypothesized difference in cell distribution related to hypoxia tolerance between the two *Hoplias* species. While the traira is well adapted to life in shallow, stagnant, hypoxic lakes, the trairão inhabits well-aerated waters of central and southern Brazil (Rantin et al., 1993). Consequently, the traira has a higher hypoxia tolerance (P_cO_2 = 20 torr), higher Hb-O_2 affinity (P_{50} = 8.7 torr), larger respiratory surface area, lower metabolic rate and higher anaerobic capacity compared to the trairão (P_cO_2 = 35 torr) (Fernandes et al., 1994; Rantin et al., 1993; Perry et al., 2004). Filament and lamellar cell counts show a difference between the two *Hoplias* species, but the specific cell numbers did not produce the expected correlation to hypoxia tolerance (Fig. 2.3). We would have expected the more hypoxia tolerant traira to have more putative NECs than trairão in the lamellae compared to the filament due to differences in hypoxia tolerances, which was not the case. However, the difference in hypoxia tolerances of these two *Hoplias* species is slight compared to the larger difference in P_{50} between trout and goldfish. We can not firmly establish whether the striking difference we see in NEC distribution between trout and carp is due to differences in phylogeny. Regardless, there is an obvious difference in the number of filamental versus lamellar serotonergic cells in the hypoxia intolerant trout compared to the hypoxia tolerant traira, trairão, and goldfish, which may be instead a cause of adaptive variation or an alternative factor. While speculative, we attribute this difference in distribution to differences in

physiological responses to internal versus external hypoxia, and different actions of the putative O_2-sensing cells.

In conclusion, we describe innervated NECs at the filament tips that were present in all fish in a prime location to sense P_wO_2, in agreement with physiological data, indicating that all fish studied to date respond to aquatic hypoxia. It also appeared that there were putative chemoreceptors monitoring P_aO_2 surrounding the eFA, but the presence of these internal chemoreceptors were species specific and correlated with the ability to respond to internal (arterial) hypoxia. As well, we propose a chemosensory role for the non-innervated NECs we found in the lamellae with a paracrine role, acting directly on the pillar cells to enhance respiratory surface area when exposed to aquatic hypoxia. Finally, we speculate that differences in NEC distribution are not due to phylogeny, but instead appear to be adaptations related to hypoxia tolerance.

Table 2.1: Details of primary and secondary antibodies used for immunohistochemistry.

Antisera	Dilution	Antigen	Host	Source	Secondary antisera[1]
Primary					
5-HT	1:400	serotonin	rabbit	Sigma-Aldrich	Alexa Fluor® 488
SV2[2]	1:200	synaptic vesicles, neuronal and endocrine	mouse	DSHB[3]	Alexa Fluor® 594
zn-12[2,4]	1:25	neuron, surface	mouse	DSHB[3]	Alexa Fluor® 594
Secondary[1]					
Alexa Fluor® 488	1:500	rabbit IgG (H+L)	goat	Molecular Probes, Invitrogen	--
Alexa Fluor® 594	1:300	mouse 1gG (H+L)	goat	Molecular Probes, Invitrogen	--

[1]Secondary antisera were conjugated with a fluorescent marker.
[2]Monoclonal antibody.
[3]Developmental Studies Hybridoma Bank, University of Iowa.
[4]Zebrafish specific antibody.

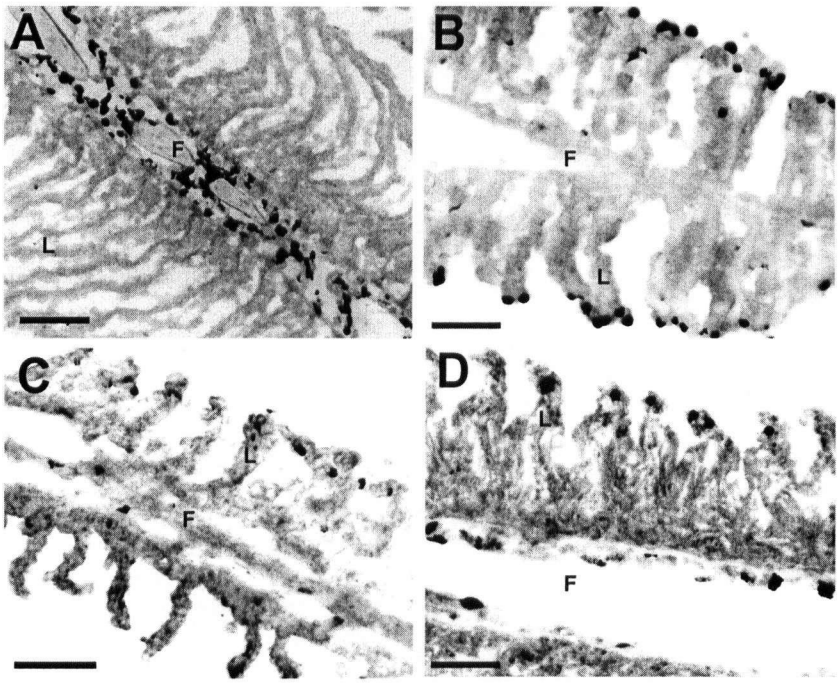


Figure 2.1: Distribution of serotonergic immunoreactive cells (darkly stained cells) along the filament (**F**) and lamellae (**L**) in (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae*, and (D) *H. malabaricus* (scale bar A 200 μm, B-D 100 μm).

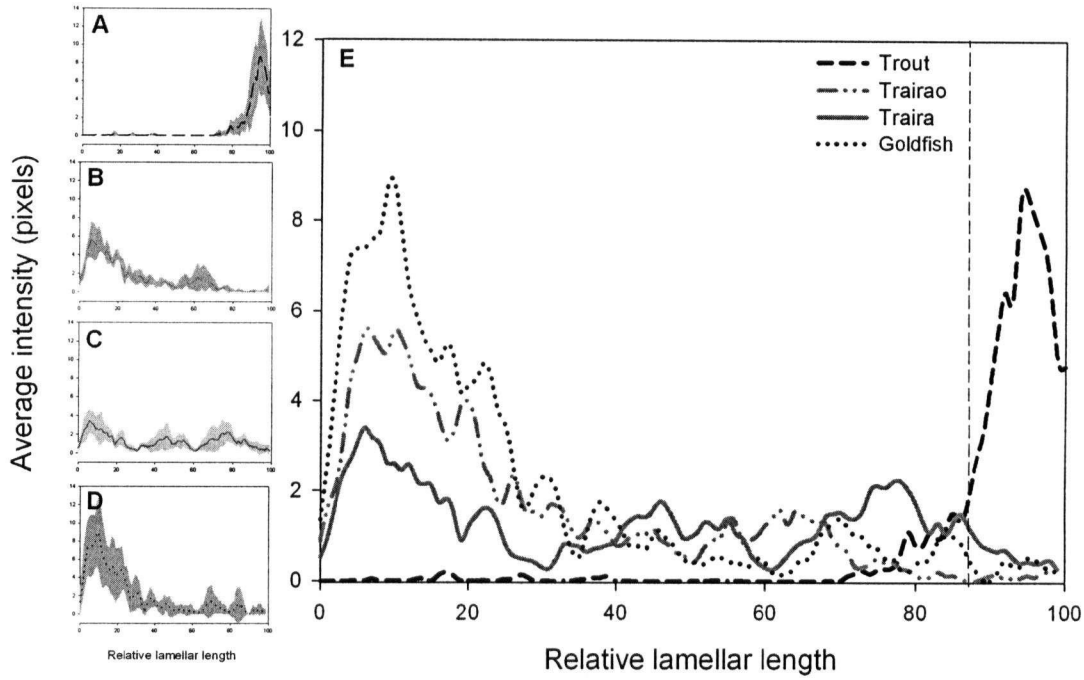


Figure 2.2: Distribution of 5-HT-IR cells expressed as the average staining intensity (pixels) from lamellar tips (0) to the central axis of the filament (100) in (A) *O. mykiss*, (B) *H. lacerdae*, (C) *H. malabaricus*, and (D) *C. auratus*. Shaded area represents the 95% confidence interval associated with the mean distribution (solid line) for each fish species. (E) Comparison of the mean distribution of 5-HT-IR cells among the four species. Dashed-line represents the division between lamellae and filament.

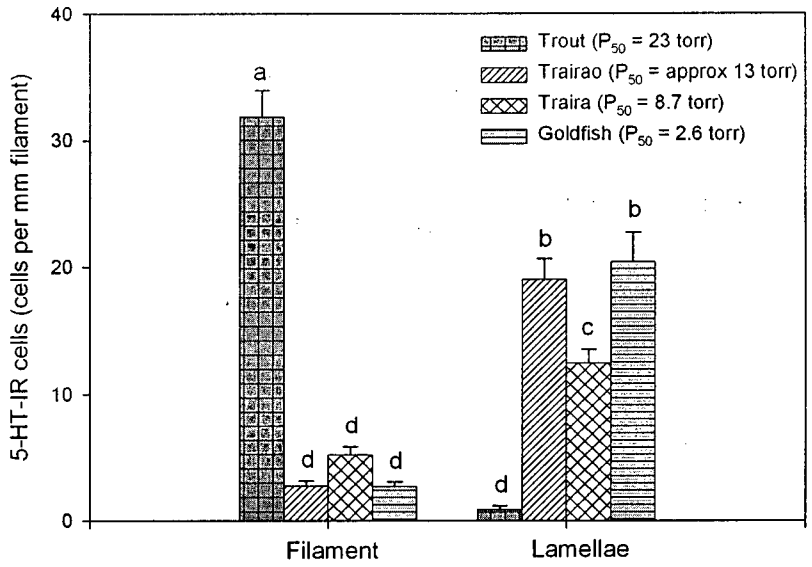


Figure 2.3: Number of 5-HT-IR cells on the filament and lamellae in rainbow trout (*O. mykiss*), trairão (*H. lacerdae*), traira (*H. malabaricus)*, and goldfish (*C. auratus*). Values are expressed as means ± s.e.m. At least 50 cells per individual and six individuals per species were measured. Different letters (a-d) indicate cell counts significantly different from each other.

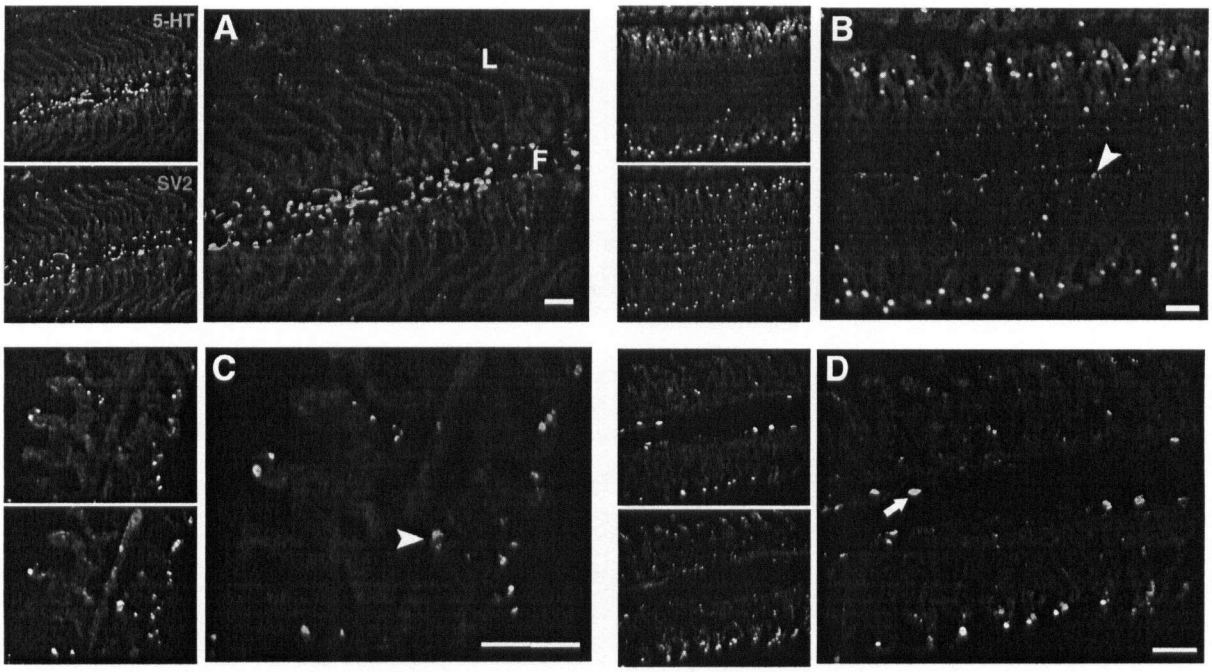


Figure 2.4: Double immunolabeling along a single gill filament with multiple lamellae of serotonin (5-HT, green) and synaptic vesicles (SV2, red) in (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae*, and (D) *H. malabaricus* (scale bars 100 μm). Colocalization appears in yellow. Arrowheads indicate non-5-HT-IR but SV2-IR cells and arrows specify non-SV2-IR but 5-HT-IR cells.

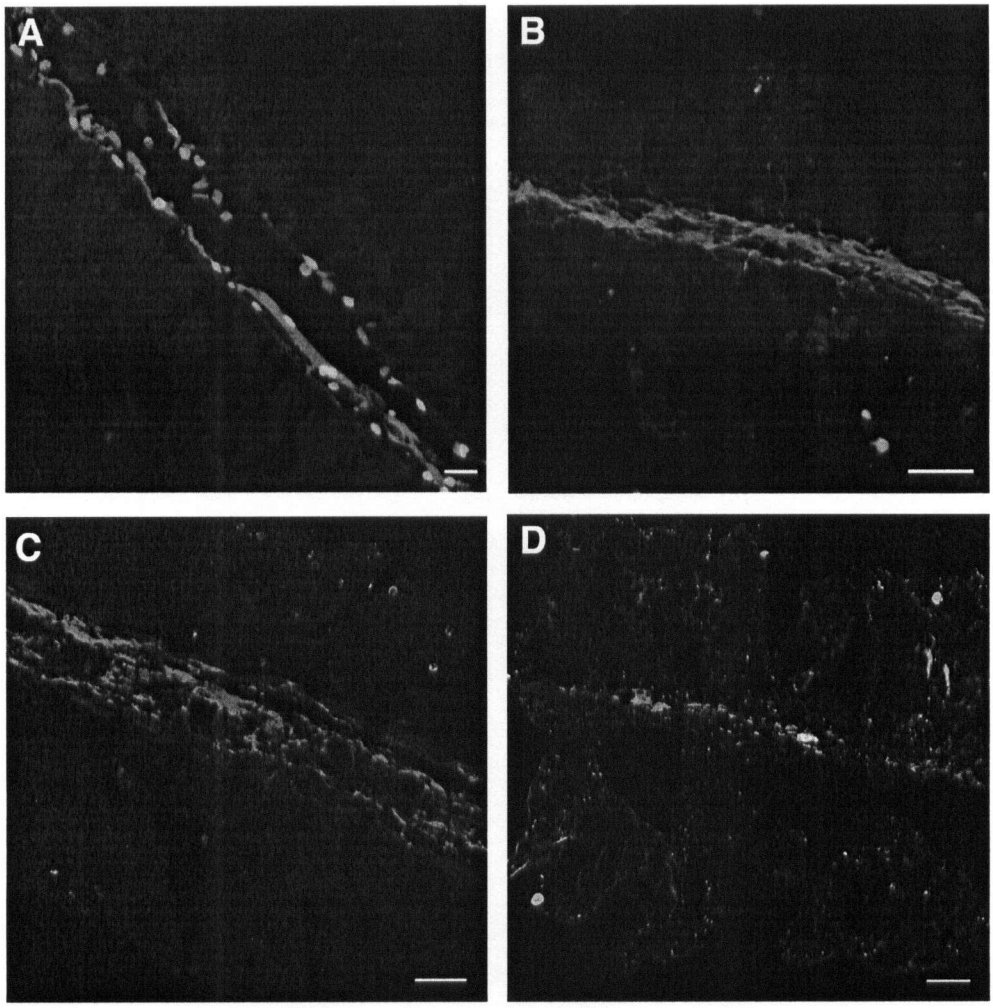


Figure 2.5: Double immunolabeling of serotonin (5-HT, green) and a neuronal marker (zn12, red) along the gill filament and lamellae of (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae*, and (D) *H. malabaricus*. Images were captured using a confocal scanning system from longitudinal gill sections 30 μm thick.

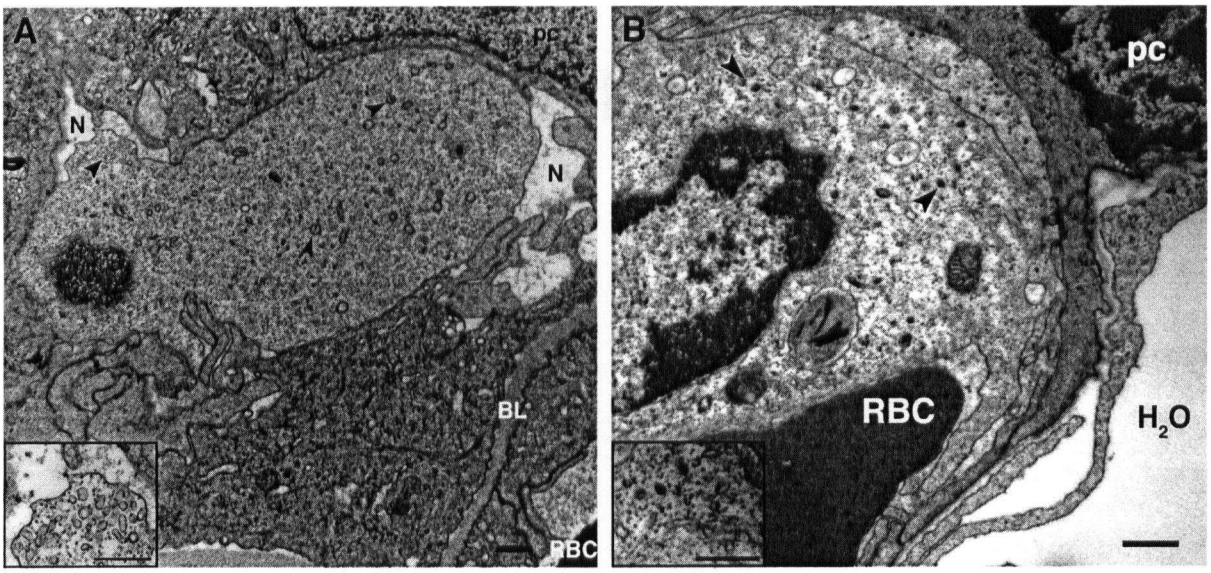
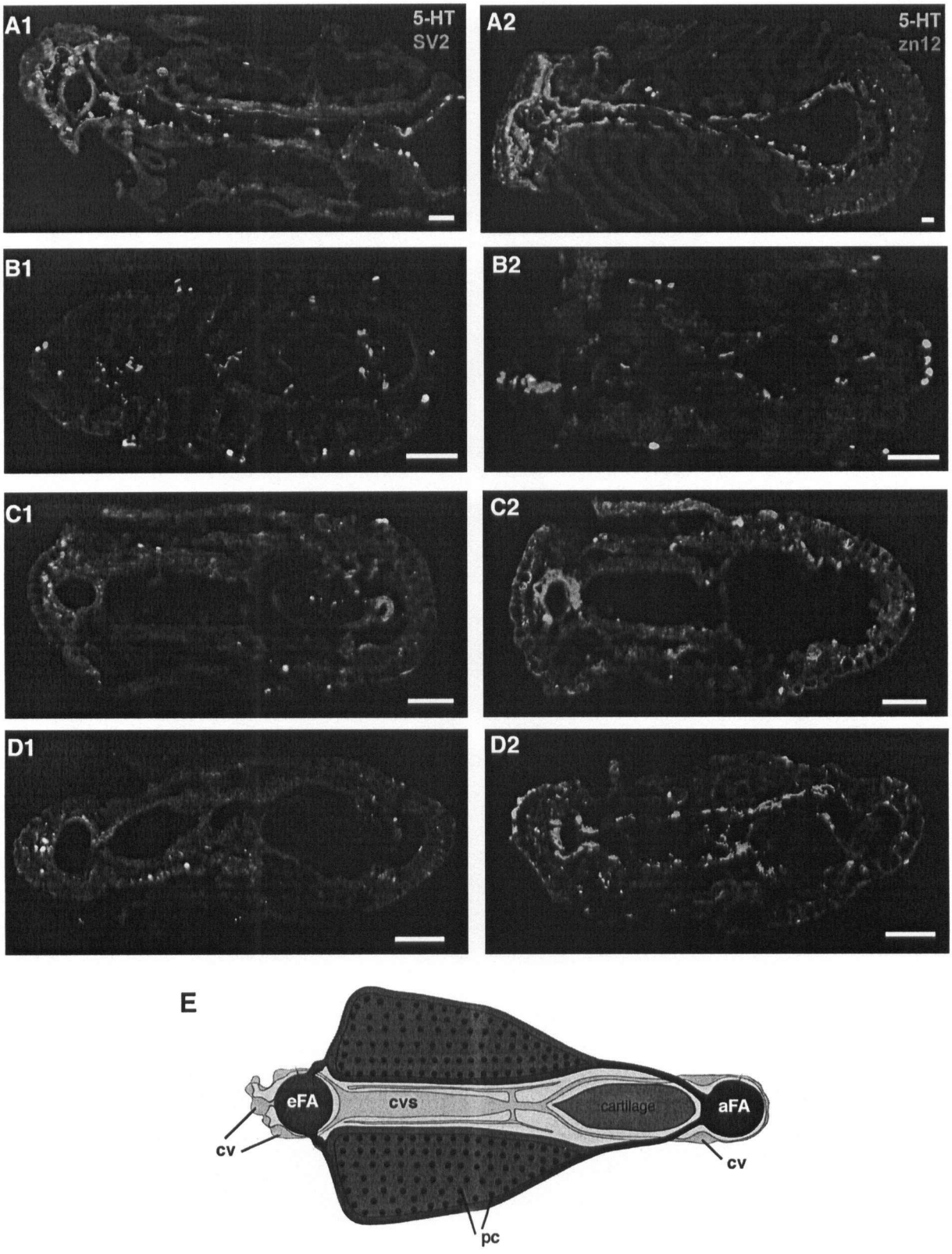


Figure 2.6: TEM images of neuroepithelial cells (NECs) in (A) the filament of *O. mykiss* and (B) the lamellae of *C. auratus*. Lower left-hand insets detail the presence of dense-cored vesicles (arrowheads). Note the proximity of NECs in both (A) and (B) to a red blood cell (RBC), as well as a nerve (N) and the basal lamina (BL) in (A). NEC of the goldfish lamellae (B) is also juxtaposed to a pillar cell (pc) and near the ambient environment (H_2O). Scale bars are 500 nm.

Figure 2.7: Cross-sections of gill filaments from (A) *O. mykiss*, (B) *C. auratus*, (C) *H. lacerdae*, and (D) *H. malabaricus* doubly immunolabeled with 5-HT (green) and SV2 (red) (left hand column, 1) and 5-HT (green) and zn12 (red) (right hand column, 2). A schematic of a filament cross-section (E) details the location of the efferent filament artery (eFA), central venous sinus (cvs), afferent filament artery (aFA), lamellar pillar cells (pc), and companion vessels (cv) (adapted from Farrell, 1979). Note that lamellae are rarely sectioned in a straight plane. Scale bars are 100 μm.


A1
5-HT
SV2
A2
5-HT
zn12
B1
B2
C1
C2
D1
D2
E
eFA
cvs
cartilage
aFA
cv
cv
pc

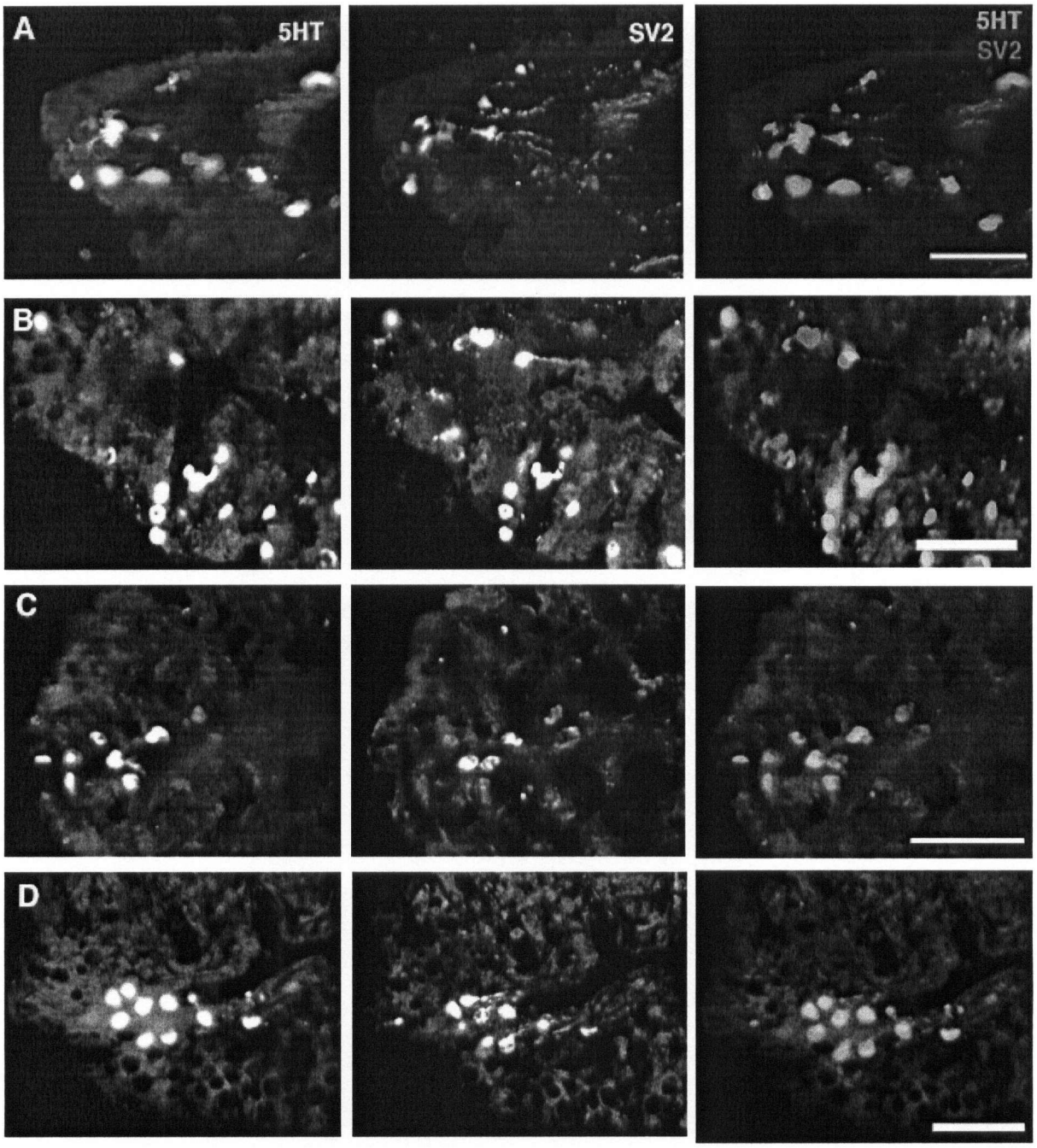


Figure 2.8: Four species comparison of the tip of the filament using antibody immunolabeling of serotonin (5-HT) and synaptic vesicle marker (SV2) in (A) *O. mykiss*, (B) *C. auratus,* (C) *H. lacerdae*, and (D) *H. malabaricus.* Colocalization appears in yellow. Scale bars are 100 μm.

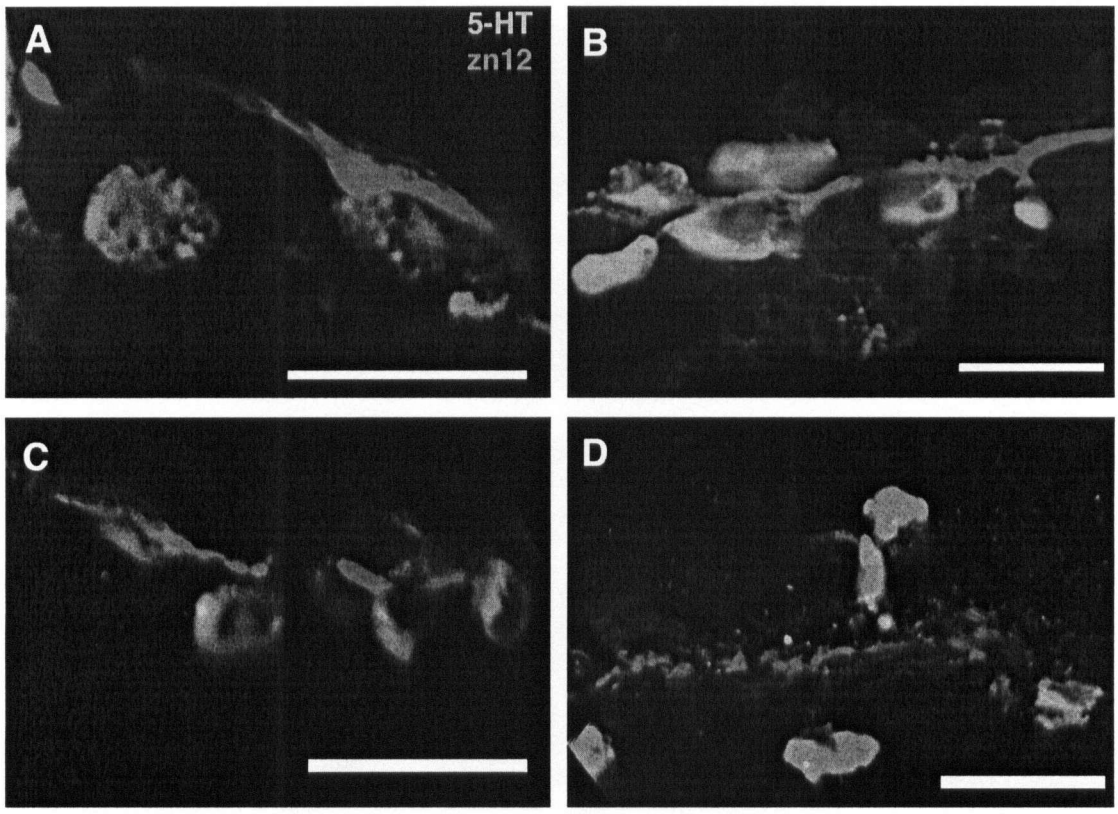


Figure 2.9: Innervated serotonergic cells located at the filament tips in (A) *O. mykiss*, (B) *C. auratus,* (C) *H. lacerdae*, and (D) *H. malabaricus* immunolabeled with serotonin (5-HT, green) and a neuronal marker (zn12, red). Scale bars are 25 μm.

REFERENCES

Bailly, Y., Dunel-Erb, S. and Laurent, P. (1992). The neuroepithelial cells of the fish gill filament: Indolamine-immunocytochemistry and innervation. *Anat. Rec.* **233**, 143-161.

Booth, J.H. (1978). The distribution of blood flow in gills of fish: application of a new technique to rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **73**, 119-129.

Burggren, W. W. (1982). "Air gulping" improves blood oxygen transport during aquatic hypoxia in the goldfish *Carassius auratus*. *Physiol. Zool.* **55**, 327-334.

Burleson, M. L. and Milsom, W. K. (1990). Propranolol inhibits O_2-sensitive chemoreceptor activity in trout gills. *Am. J. Physiol.* **258**, 1089-1091.

Burleson, M. L. and Milsom, W. K. (1993). Sensory receptors in the first gill arch of rainbow trout. *Respir. Physiol.* **93**, 97-110.

Burleson, M.L. and Milsom, W.K. (1995a). Cardio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. *Respir. Physiol.* **100(3)**, 231-238.

Burleson, M. L. and Smatresk, N. J. (1990). Effects of sectioning cranial nerves IX and X on cardiovascular and ventilatory reflex responses to hypoxia and NaCN in channel catfish. *J. Exp. Biol.* **154**, 407-420.

Butler, P.J., Taylor, E.W., and Davison, W. (1979). The effect of long term, moderate hypoxia on acid-base balance, plasma catecholamines and possible anaerobic end products in the unrestrained dogfish *Scyliorhinus canicula*. *J. Comp. Physiol.* **132,** 297-303.

Dunel-Erb, S., Bailly, Y. and Laurent, P. (1982). Neuroepithelial cells in fish gill primary lamellae. *J. App. Physiol.* **53**, 1342-1353.

Farrell, A. P., Daxboeck, C. and Randall, D. J. (1979). The effect of input pressure and flow on the pattern and resistance to flow in the isolated perfused gill of a teleost fish. *J. Comp. Physiol.* **133**, 233-240.

Farrell, A.P. (1979). Gill blood flow in teletosts. PhD Thesis, University of British Columbia, 255 pp.

Fernandes, M. N., Rantin, F. T., Kalinin, A. L. and Van Moron, S. E. (1994). Comparative study of gill dimensions of three erythrinid species in relation to their respiratory function. *Can. J. Zool.* **72**, 160-165.

Feyrter, F. (1938). Uber diffuse endocrine epitheliale Organe. Leipzig, East Germany: Barth.

Florindo, L. H., Leite, C. A. C., Kalinin, A. L., Reid, S. G., Milsom, W. K. and Rantin, F. T. (2006). The role of branchial and orobranchial O_2 chemoreceptors in the control of aquatic surface respiration in the neotropical fish tambaqui *(Colossoma macropomum)*: progressive responses to prolonged hypoxia. *J. Exp. Biol.* **209**, 1709-1715.

Fu, X. W., Nurse, C. A., Wong, V. and Cutz, E. (2002). Hypoxia-induced secretion of serotonin from intact pulmonary neuroepithelial bodies in neonatal rabbit. *J. Physiol. (Lond)* **539**, 503-510.

Gonzalez, C., Almarez, L., Obeso., A. and Rigual, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol. Rev.* **74(4)**, 829-896.

Heymans, C., Bouckaert, J.J., Farber, S., and Hus, F.Y. (1936). *Arch Int. Pharmacodyn.* **54**, 129.

Holeton, G.F. (1971). Oxygen uptake and transport by the rainbow trout during exposure to carbon monoxide. *J. Exp. Biol.* **54**, 239-254.

Hughes, G. M., Albers, C., Muster, D. and Gotz, K. H. (1983). Respiration of the carp, *Cyprinus carpio* L., at 10 and 20°C and the effects of hypoxia. *J. Fish Biol.* **22**, 613-628.

Johnston, I.A. and Bernard, L.M. (1983). Utilization of the ethanol pathway in carp following exposure to anoxia. *J. Exp. Biol.* **104**, 73-78.

Jonz, M. G. and Nurse, C. A. (2003). Neuroepithelial cells and associated innervation of the zebrafish gill: a confocal immunofluorescence study. *J. Comp. Neurol.* **461**, 1-17.

Jonz, M. G., Fearon, I. M. and Nurse, C. A. (2004). Neuroepithelial oxygen chemoreceptors of the zebrafish gill. *J. Physiol. (Lond.)* **560**, 737-752.

Krammer, E.B. (1978). Carotid body chemoreceptor function: hypothesis based on a new circuit model. *Proc. Nat. Acad. Sci.* **75(5)**, 2507-2511.

Lahiri, S., Mokashi, A., Mulligan, E., and Nishino, T. (1981). Comparison of aortic and carotid chemoreceptor responses to hypercapnia and hypoxia. *J. Appl. Physiol.* **51,** 55-61.

Laurent, P. and Dunel, S. (1980). Morphology of gill epithelia in fish. *Am. J. Physiol.* **238**, R147-159.

Lauweryns, J. M. and Cokelaere, M. (1973). Intrapulmonary neuro-epithelial bodies: hypoxia-sensitive neuro(chemo-)receptors. *Separatum Experientia* **29**, 1384-1386.

Lumsden, A.L. (1996) Oxygen-sensitive chemoreceptors and cardioventilatory control in carp. MSc Thesis. University of British Columbia. 97 pp.

McCormick, S. D., Sundell, K., Bjornsson, B. T., Brown, C. L. and Hiroi, J. (2003).

Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). *J. Exp. Biol.* **206**, 4575-4583.

McKenzie, D. J., Burleson, M. L. and Randall, D. J. (1991). The effects of branchial denervation and pseudobranch ablation on cardioventilatory control in an air-breathing fish. *J. Exp. Biol.* **161**, 347-365.

Milsom, W. K. and Brill, R. W. (1986). Oxygen sensitive afferent information arising from the first gill arch of yellowfin tuna. *Respir. Physiol.* **66**, 193-203.

Milsom, W. K., Reid, S. G., Rantin, F. T. and Sundin, L. (2002). Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish. *J. Exp. Biol.* **205**, 1765-1774.

Milsom, W.K. (1998). Phylogeny of respiratory chemoreceptor function in vertebrates. *Zoology.* **101,** 316-332.

Nurse, C. A. (2005). Neurotransmission and neuromodulation in the chemosensory carotid body. *Auton. Neurosci.* **120**, 1-9.

Olson, K. R. (2002). Vascular anatomy of the fish gill. *J. Exp. Zool.* **293**, 214-231.

Pearse, A. G. E. (1969). The cytochemistry and ultrastructure of polypeptide hormone-producing cells of the APUD series and the embryologic, physiologic and pathologic implications of the concept. *J. Histochem. Cytochem.* **17**, 303.

Perry, S. F., Reid, S. G., Gilmour, K. M., Bojink, C. L., Lopes, J. M., Milsom, W. K. and Rantin, F. T. (2004). A comparison of adrenergic stress responses in three tropical teleosts exposed to acute hypoxia. *Am. J. Physiol.* **287**, R188-R197.

Prabhakar, N. R. (2006). O_2 sensing at the mammalian carotid body: why multiple O2 sensors and multiple transmitters? *Exp. Physiol.* **91**, 17-23.

Randall, D. J. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.* **100**, 275-288.

Randall, D. J. and Smith, J. C. (1967). The regulation of cardiac activity in fish in a hypoxic environment. *Physiol. Zool.* **40**, 104-113.

Rantin, F. T., Glass, M. L., Kalinin, A. L., Verzola, R. M. M. and Fernandes, M. N. (1993). Cardio-respiratory responses in two ecologically distinct erythrinids (*Hoplias malabaricus* and *Hoplias lacerdae*) exposed to graded environmental hypoxia. *Environ. Biol. Fishes.* **36**, 93-97.

Rantin, F. T., Kalinin, A. L., Glass, M. L. and Fernandes, M. N. (1992). Respiratory

responses to hypoxia in relation to mode of life in two erythrinid species (*Hoplias malabaricus* and *Hoplias lacerdae*). *J. Fish Biol.* **41**, 805-812.

Reid, S. G. and Perry, S. F. (2003). Peripheral O_2 chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am. J. Physiol.* **284**, R990-R999.

Saltys, H. A., Jonz, M. G. and Nurse, C. A. (2006). Comparative study of gill neuroepithelial cells and their innervation in teleosts and *Xenopus* tadpoles. *Cell Tissue Res.* **323**, 1-10.

Shoubridge, E. A. and Hochachka, P.W. (1980). Ethanol: novel end product in vertebrate anaerobic metabolism. *Science.* **209,** 308-309.

Smatresk, N. J., Burleson, M. L. and Azizi, S. Q. (1986). Chemoreflexive responses to hypoxia and NaCN in longnose gar: evidence for two chemoreceptor loci. *Am. J. Physiol.* **251**, R116-R125.

Smith, D. G. and Johnson, D. W. (1977). Oxygen exchange in a simulated trout gill secondary lamellae. *Am. J. Physiol.* **233**, R145-R161.

Smith, F.M. and Jones, D.R. (1982). The effect of changed in blood oxygen-carrying capacity on ventilation volume in the rainbow trout (*Salmo gairdneri*). *J. Exp. Biol.* **97**, 325-334.

Sollid, J., De Angelis, P., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, 3667-3673.

Sollid, J., Weber, R. E. and Nilsson, G. E. (2005). Temperature alters the respiratory surface area of crucian carp, *Carassius carassius,* and goldfish, *Carassius auratus*. *J. Exp. Biol.* **208**, 1109-1116.

Stecyk, J. A. W., Stenslokken, K.-O., Farrell, A. P. and Nilsson, G. E. (2004). Maintained cardiac pumping in anoxic crucian carp. *Science.* **306**, 77.

Stenslokken, K.-O., Sundin, L. and Nilsson, G. E. (2006). Endothelin receptors in teleost fishes: cardiovascular effects and branchial distribution. *Am. J. Physiol.* **290**, R852-860.

Sundin, L. and Nilsson, G. E. (1997). Neurochemical mechanisms behind gill microcirculatory responses to hypoxia in trout: *in vivo* microscope study. *Am. J. Physiol.* **272**, R576-R585.

Sundin, L. and Nilsson, S. (2002). Branchial Innervation. *J. Exp. Zool.* **293**, 232-248.

Sundin, L., Davison, W., Forster, M. and Axelsson, M. (1998b). A role of 5-HT_2 receptors in the gill vasculature of the Antarctic fish, *Pagothenia borchgrevinki*. *J. Exp. Biol.* **201**,

2129-2138.

Sundin, L., Holmgren, S. and Nilsson, S. (1998a). The oxygen receptor of the teleost gill? *Acta Zool.* **79**, 207-214.

Sundin, L., Nilsson, G. E., Block, M. and Lofman, C. O. (1995). Control of gill filament blood flow by serotonin in the rainbow trout, Onchoryncus mykiss. *Am. J. Physiol.* **268**, R1224-R1229.

Sundin, L., Reid, S. G., Kalinin, A. L., Rantin, F. T. and Milsom, W. K. (1999). Cardiovascular and respiratory reflexes: the tropical fish, traira (*Hoplias malabaricus*) O_2 chemoresponses. *Respir. Physiol.* **116**, 181-199.

Sundin, L., Reid, S.G., Rantin, F.T., and Milsom, W.K. (2000). Branchial receptors and cardiorespiratory reflexes in a neotropical fish, the tambaqui (*Colossoma macropomum*). *J. Exp. Biol.* **203**, 1225-1239.

Tetens, V. and Lykkeboe, G. (1981). Blood respiratory properties of rainbow trout, *Salmo gairdneri*: responses to hypoxia acclimation and anoxic incubation of blood in vitro. *J. Comp. Physiol.* **145**, 117-125.

Wilson, J. M. and Laurent, P. (2002). Fish gill morphology: inside out. *J. Exp. Zool.* **293**, 192-213.

Zaccone, G., Fausulo, S., Ainis, L. and Licata, A. (1997). Paraneurons in the gills and airways of fishes. *Microsc. Res. Tech.* **37**, 4-12.

Zaccone, G., Lauweryns, J. M., Fasulo, S., Tagliafierro, G., Ainis, L. and Licata, A. (1992). Immunocytochemical localization of serotonin and neuropeptides in the neuroendocrine paraneurons of teleost and lungfish gills. *Acta Zoologica* **73**, 177-183.

CHAPTER 3:

The localization of multiple neurotransmitters and neuromodulators in fish gills

INTRODUCTION

Production of the acute hypoxic response—bradycardia, increased gill resistance, and hyperventilation (Randall, 1982)—may be due to changes in the level of external or internal oxygen that must be monitored by chemoreceptors, and the neuroepithelial cells (NECs) are the proposed chemoreceptive cells in the gills (Dunel-Erb et al., 1982; Sundin et al., 1995; Wilson and Laurent, 2002). The location and innervation of NECs (Dunel-Erb et al., 1982; Bailly et al., 1992; Jonz and Nurse, 2003), along with direct evidence from patch-clamp recordings of isolated NECs (Jonz et al., 2004), support a role for these cells in O_2 chemoreception. Dunel-Erb et al. (1982) showed that serotonin (5-HT) was the major monoamine in the neuroepithelial cells (NECs) of fish gills and since then, 5-HT has been prolifically used to identify NECs (Bailly et al., 1992; Zaccone et al., 1997; Sundin et al., 1998a; Jonz and Nurse, 2003). As such, serotonin is presumably released synaptically onto afferent nerves to produce the hypoxic cardio-respiratory responses (Jonz et al., 2004). Secretion of serotonin in a paracrine fashion may also lead to vasoconstriction of the efferent sphincters or lamellar arterioles, thus increasing blood flow to the gills and enhancing respiratory surface area (Sundin et al., 1995; Sundin and Nilsson, 2002).

The primary peripheral chemoreceptive structure in mammals, the carotid body, reflexly controls respiration by sensing arterial PO_2 (PaO_2), arterial PCO_2 ($PaCO_2$), and pH, and responds by releasing neurotransmitter onto the carotid sinus nerve, resulting in hyperventilation (Gonzalez et al., 1994). Numerous comparisons have been made between

and the oxygen-sensitive carotid body. The vascular supply to the first gill arch in fish and the carotid artery, where the carotid body sits, share the same embryonic origin (Milsom, 1998), and both the carotid body and first gill arch are innervated by the glossopharnygeal (CN IX) nerve (Sundin and Nilsson, 2002; Gonzalez et al., 1994). As well, the first gill arch and carotid body have similar levels and patterns of chemoreceptive afferent nerve discharge (Milsom and Brill, 1986; Burleson and Milsom, 1993). Furthermore, they appear to share a common and chemoreceptive mechanism involving an O_2-sensitive K^+ channel (Jonz et al., 2004). The carotid body is host to multiple neurotransmitters, such as dopamine, adrenaline, noradrenaline, acetylcholine, substance P, GABA, serotonin, and adenosine; however, only serotonin is assumed to be the primary chemoreceptive neurotransmitter released from putative O_2-sensitive neuroepithelial cells of the gill.

When exposed to hypoxia, carotid body glomus cells release the neurotransmitters and neuromodulators just described which act to set the level of afferent nerve discharge of the carotid sinus nerve (Gonzalez et al., 1994; Zhang et al., 2000). These multiple neurochemicals have both inhibitory and excitatory roles; however, the identity of the primary excitatory neurotransmitter responsible for afferent nerve activation by hypoxia is still under debate (Nurse, 2005; Prabhakar, 2006). In the 1930s, acetylcholine was shown to produce reflex respiratory responses via the carotid sinus nerve (Heymans et al., 1936; Schweitzer and Wright, 1938), thereby giving rise to the cholinergic theory for carotid body neurotransmission. An adrenergic theory arose when histological examination of the carotid body revealed a strong presence of catecholamines (Lever and Boyd, 1957), and hypoxia was shown to elicit catecholamine release (Almaraz and Fidone, 1986). Current research favours the co-release of acetylcholine and ATP as cooperative neurtransmitters primarily mediating

the hypoxic response and setting the level of respiratory drive (Zhang et al., 2000; Nurse, 2005).

Although its role in chemoreception is frequently assumed, the physiological effects of serotonin are uncertain, especially in fish (Burleson and Milsom, 1995a). In trout gill chemosensory recordings, serotonin produced only a mild excitation followed by an inhibition of chemoreceptor-like activity (Burleson and Milsom, 1995a). In contrast, acetylcholine, specifically acting on nicotinic receptors, was the only chemical administered to the gill preparation that consistently produced chemoreceptive nerve discharge akin to stimulation by hypoxia or NaCN (Burleson and Milsom, 1995a). This suggested that acetylcholine was the primary chemoreceptive neurotransmitter present in the first gill arch of fish.

Given the strong presence of serotonin containing NECs in fish gills but the weak reflex and electrophysiological evidence for serotonin as the primary neurotransmitter involved in hypoxic reflexes, the objective of the current study was to determine the distribution of other neurotransmitters and neuromodulators in the fish gill similar to those shown to play a chemosensory role in the carotid body of mammals—notably serotonin, catecholamines, acetylcholine, and adenosine. Using immunohistochemistry for specific markers or receptors of these neurochemicals, we examined their presence and location in the gills of rainbow trout (*Onchoryncus mykiss*) and goldfish (*Carassius auratus*). By investigating the distribution of multiple neuromodulators and neurochemicals within the gills of two different species, we hoped to (1) confirm a potential role for acetylcholine, catecholamines, and adenosine in the gill, and (2) determine whether they are found in serotonergic neuroepithelial cells, and (3) ascertain the degree of homology between carotid

body glomus cells and NECs in the gills of fish. We predicted that multiple neurotransmitters would colocalize to the same cells, as they do in other vertebrate peripheral chemoreceptive structures.

METHODS

Animals

Adult rainbow trout (*Onchoryncus mykiss* (Walbaum), n=4, 750-1190 g) and goldfish (*Carassius auratus* (Linnaeus), n=4, 65-95 g) were kept in outdoor, flow-through, and aerated tanks in the Zoology Aquatic Facility at the University of British Columbia. Fish used for tissue analysis were randomly selected and killed by an overdose of buffered MS-222 (3-aminobenzoic acid ethyl ester) followed by a sharp blow to the head.

Tissue preparation

The first and second gill arches were excised from each fish and washed in ice-cold phosphate-buffered solution (PBS) containing (in mM): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; buffered to pH 7.8 with 1M NaOH (Jonz and Nurse, 2003). Individual gill arches were syringe-perfused with heparinized PBS (1000 iu/ml) until filaments appeared clear and 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 10-14 μm using a cryostat (Leica CM3050 S, Leica Microsystems, Germany) and mounted on Superfrost® plus slides (VWR International) for immunohistochemistry.

Immunohistochemistry

Slides were washed in PBS and blocked in 10% Normal Goat Serum (Jackson Laboratories, distributed by Cedarlane Laboratories, Hornby, Ontario) for one hour. Primary antibodies (used individually or in combination) were diluted in a permeabilizing solution (PBS/0.2% Triton X-100/0.1% Sodium azide) according to the optimal dilutions detailed in Table 1, and set on the slides to incubate overnight at room temperature. Following

incubation of the primary antibodies, the slides were again washed in PBS. The slides were then treated with fluorescently labeled secondary antibodies diluted in PBS (Table 1) 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 (Vector Laboratories, Burlington, Ontario) to prevent photobleaching. 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 immunolabelling of the gill structures present (data not shown) most of the time; however exceptions to this are addressed in the discussion. Immunolabelled slides were observed under a light microscope (Axioplan 2, Zeiss, Jena, Germany) and digitally captured using a Q-Imaging (Burnaby, BC, Canada) CCD camera and image analysis software (Northern Eclipse, Empix Imaging, Ontario).

RESULTS

There were no observable differences in morphology or immunoreactive staining between first and second gill arches. For all results, colocalization of neurochemicals was assessed based on double staining with pairs of antibodies due to the small range of primary and secondary hosts for the antibodies used in this study and the difficulty of triple-immunostaining.

1. Serotonin and catecholamines

Serotonin (5-HT) and tyrosine hydroxylase (TH), the biosynthetic enzyme for general catecholamines, did not colocalize to the same cells in either trout or goldfish. Serotoninergic (5-HT-IR) cells in the trout were found along the filament, while catecholaminergic (TH-IR) cells were present in the filament epithelium between lamellae as well as diffusely located throughout the lamellar epithelium (Fig. 3.1). Serotonergic cells in the goldfish were located in the lamellae, especially at the lamellar tips, where they did not colocalize with catecholaminergic cells (Fig. 3.1). As well, the cluster of 5-HT-IR cells seen at the filament tips in both trout and goldfish did not colocalize for tyrosine hydroxylase, indicating that serotonin and catecholamines are stored in different cells, even at the filament tips (Fig. 3.2). Thus, there was no indication that serotonin and catecholamines were stored in the same cells, either in the filament tip, central filament, or lamellae. Furthermore, there were no visible cellular projections between the 5-HT-IR and TH-IR cells.

2.1 Serotonin and the vesicular acetylcholine transporter

Vesicular acetylcholine transporter (vACht), the protein responsible for moving cytoplasmic acetylcholine into vesicles to be released by exocytosis (Eiden et al., 2004), was found in the filament and lamellar epithelium in both trout and goldfish (Fig. 3.3). However, vACht was not localized to serotonergic cells in either trout or goldfish (Fig. 3.3A, C). Rather, it appeared that 5-HT and vACht were sometimes present in neighboring cells (arrowheads, Fig. 3.3A, C). This same association of neighboring 5-HT-IR and vACht-IR cells was present at the filament tips (arrowheads, Fig. 3.4A, C).

2.2 Catecholamines and the vesicular acetylcholine transporter

Catecholaminergic cells of the lamellae and filament epithelia between the lamellae also stained for the vesicular acetylcholine transporter in both trout and goldfish (arrow, Fig. 3.3B, D). However, not all TH-IR cells were associated with vACht; likewise, not all vACht-IR cells colocalized with catecholaminergic cells (arrow, Fig. 3.3B, D). At the filament tip, catecholaminergic cells were less abundant than serotonergic cells, but nevertheless, they often contained the vesicular acetylcholine transporter (arrow, Fig. 3.4B, D).

3.1 Serotonin and the purinoreceptor, $P2X_3$

$P2X_3$ is one of the heteromeric subunits for purinergic receptors, which are a family of ligand-gated ion channels found in peripheral neurons and activated by extracellular ATP to generate action potentials in primary afferent neurons (Dunn et al., 2001). Immunoreactivity of $P2X_3$ occurred down the filament in both trout and goldfish (Fig. 3.5A, C). Serotonin and $P2X_3$ do not colocalize (Fig. 3.5), however, they are present in associating cells in the filament of rainbow trout (Fig. 3.5A). $P2X_3$ staining did not extend into the

lamellae in either trout or goldfish, therefore the serotonergic cells of the goldfish lamellae were not associated with $P2X_3$ (Fig. 3.5C).

3.2 Catecholamines and the purinoreceptor, $P2X_3$

Unfortunately, due to low sample sizes, inconsistent sectioning, and lack of dependable immunoreactivity patterns, it was difficult to conclusively determine whether TH-IR and $P2X_3$-IR occurred in the same cells in the gills. Based on our evidence, catecholaminergic (TH-IR) cells in the lamellae of trout did not colocalize or associate with cells containing purinergic receptors ($P2X_3$-IR) (Fig. 3.5B, D). However, it appeared that TH-IR cells in the filament of goldfish were located near $P2X_3$-IR cells (Fig. 3.5D).

DISCUSSION

Recent studies of oxygen-sensing sites in the fish gill have focused on the neuroepithelial cells (NECs) as putative O_2 chemoreceptors and have used the presence of serotonin as a marker for NECs (Bailly et al., 1992; Zaccone et al., 1997; Jonz and Nurse, 2003). Given the similarities between the structure and response to hypoxia of the neuroepithelial cell with the type I glomus cell of the carotid body (Dunel-Erb et al., 1982; Jonz et al., 2004), the proposal that serotonergic NECs are chemoreceptive is very plausible. However, serotonin applied to the isolated first gill arch of trout did not produce a profound chemoreceptive response in the glossopharyngeal nerve (CN IX) (Burleson and Milsom, 1995a). Rather, the only neurotransmitter to have a consistent effect of chemoreceptor-like discharge was acetylcholine, specifically acting on nicotinic receptors (Burleson and Milsom, 1995a). Consistent with these observations, our data suggest that while catecholamines and acetylcholine are contained in the same cells in fish gills, these are distinct from serotonin-containing cells and raise questions about the relative roles of both cell groups in O_2 chemoreception.

3.1. Critique of methods

Immunohistochemistry is a valuable tool for locating antigens that react with specific antibodies. However, the results presented in this study should be treated as a preliminary qualitative assessment of the location of neurochemicals in the fish gill. All the antibodies used in this study were raised in mammalian species, and we are assuming that the antigens

they bind to the fish gill share the same specificity. Furthermore, our sample sizes were low (n=4 for each species) and we did not achieve consistent positive staining for all the antibodies. Finally, the control experiments, both excluding primary antibody and adding host IgG serum, were consistent most of the time, however, there were a few exceptions of unexplained arbitrary staining that questioned the specificity of the anti-vAChT and anti-$P2X_3$ antibodies. As a result, more exclusive control experiments, using specific blocking peptides for each antibody are required to validate our results.

3.2. Potential physiological effects of neurochemicals in the filament

The innervated serotonergic, vesicular NECs in the filament of rainbow trout are the proposed chemoreceptors monitoring the internal oxygen (P_aO_2) in trout at the level of the efferent filament artery (Chapter 2). As noted above, however, this role is not completely consistent with physiological data that suggest a primary role for cells releasing acetylcholine (Burleson and Milsom, 1995a). The presence of vAChT in cells neighboring serotonergic cells in the filament epithelium of trout (Fig. 3.3A), where there is rich innervation (Chapter 2), suggests that the release of 5-HT may regulate the release of acetylcholine across a synapse. Recent studies on glomus cells provided evidence for a neuromodulatory role of serotonin in the carotid body (Jacono et al., 2005). As a neuromodulator in the carotid body, 5-HT was found to act as a positive regulator of type 1 glomus cell function in an autocrine-paracrine manner (Jacono et al., 2005). Our observations raise the possibility that serotonin serves a similar function in the gill, regulating the release of acetylcholine. Serotonin has been shown to have a direct impact on adrenaline release in trout chromaffin cells (Fritsche et al., 1993). Therefore, the juxtaposition of cells containing these neurochemicals in the filament may implicate the release of serotonin from neuroepithelial cells as a

neuromodulator acting to positively regulate the release of acetylcholine as a neurotransmitter onto afferent nerves.

Additionally, 5-HT NECs of the filament in trout may have local vasoactive effects on the efferent filament artery (Chapter 2). Both acetylcholine and serotonin function as vasoconstrictors. Release of acetylcholine in the gill resulted in vasoconstriction of the efferent filament artery (eFA) (Smith, 1977). Exogenous 5-HT had a direct vasoconstrictive effect on gill vasculature in the distal portion of the efferent filament artery and adjacent efferent lamellar arterioles (Fritsche et al., 1992; Sundin et al., 1995). Vasoconstriction of the efferent filament artery increases perfusion pressure and produces more rigid lamellae, thereby enhancing the area for oxygen transfer and the oxygen-diffusing capacity of the gills (Smith, 1977; Olson, 2002). Thus, in addition to producing chemoreceptive reflex responses, 5-HT and ACh may function as direct vasoconstrictors on the eFA to increase perfusion pressure and distribute the lamellar blood flow across the entire lamellar sheet.

Our results indicated that 5-HT-IR and TH-IR cells were in distinctly different cells in both trout and goldfish (Fig. 3.1). Blood catecholamines are known to rise in severe hypoxia (Butler et al., 1979; Perry and Reid, 1992). Stimulation of internal O_2 chemoreceptors in rainbow trout gills mediated catecholamine release from chromaffin cells located in the head kidney or posterior cardinal vein (Reid and Perry, 2003). Therefore, it may be that serotonergic NECs of the filament in trout (Fig. 3.1), the putative internal O_2 chemoreceptors, play a reflexogenic role by detecting the blood oxygen level (P_aO_2), and initiating catecholamine release from gill chromaffin cells, which results in either a direct (vasoactive) or indirect (adrenergic blood pressure control) increase in gill diffusing capacity.

Immunoreactivity of the purinoreceptor subunit, $P2X_3$, was localized to the densely innervated central core of the filament in both trout and goldfish (Fig. 3.5). Receptors for ATP (purinoreceptors) have been located on the afferent nerve terminals apposed to rat type I glomus cells (Zhang et al., 2000), and a similar pattern was present in our data, although this result is inconclusive as we did not have direct evidence of colocalization of the ACh and a neuronal marker. Many now believe that the co-release of ATP and ACh is the primary mediator of the hypoxic response in glomus cells in the carotid body (Zhang et al., 2000; Nurse, 2005). The presence of $P2X_3$ at highly innervated sites may reflect a similar co-release mechanism in fish gills, and provide preliminary qualitative evidence to suggest that ATP is involved as a co-transporter of putative chemoreceptive cells. Further investigations using specific co-staining for acetylcholine and ATP will be necessary to support this speculation.

3.3. Potential physiological effects of neurochemicals in the lamellae

Our results indicated populations of catecholaminergic and cholinergic (TH-vAChT-IT) cells present in the lamellae of trout and goldfish, and a separate population of serotonergic (5-HT-IR) cells in goldfish lamellae. Since cells of the lamellae were not innervated (Chapter 2), these cells presumably act in a paracrine fashion on target cells including vascular endothelial cells, pillar cells, or each other. In addition their neurochemical roles, serotonin and acetylcholine resulted in branchial vasoconstriction, while catecholamines produced an overall vasodilatation (Wood, 1974). Vasodilatation of the lamellar arterioles or constriction of the pillar cells expands the area for blood flow, allowing blood to pass through the entire lamellar sheet, rather than only the basal and exterior marginal channels, as is the case at rest (Randall, 1982). Lamellar arteriole vasoconstriction

may help to create a more rigid lamellae, thereby enhancing blood flow through the lamellar sheet (Wood, 1974; Farrell, 1979; Sundin et al., 1995). A hypoxic stimulus could cause the release of any of these neurochemicals directly onto target cells; alternatively, any of these neurochemicals could act on other cells containing neurochemicals to modulate its release.

The separate populations of cells containing 5-HT and ACh, both potent vasoconstrictors, in the lamellae of goldfish (Fig. 3.3C), may function independently to locally affect lamellar vasculature. Additionally, serotonin may again function as a regulator of acetylcholine release in a positive feedback manner to increase vasoconstriction. Therefore, it is plausible that the chemosensory NECs of the goldfish lamellae, in addition to acting directly on lamellar arterioles, respond to aquatic hypoxia (P_wO_2) by releasing 5-HT in a likewise autocrine-paracrine fashion to modulate the release of acetylcholine.

At rest, nearly 40 percent of the lamellae are unperfused (Booth, 1978; Farrell et al., 1979). Oxygen-uptake at the gill is proportional to blood flow (Daxboeck et al., 1982) and fish increase the gill oxygen diffusing capacity in both exercise and hypoxia by recruiting more lamellae and creating a more even lamellar blood flow (Randall, 1982). Serotonin is a potent vasoconstrictor, while catecholamines result in an overall vasodilatation (Wood, 1974; Wahlqvist, 1980); consequently, the presence of both 5-HT and TH in the lamellae of goldfish (Fig. 3.1B) may reflect a push-pull mechanism for positive and negative regulation of lamellar blood flow. Additionally, adrenaline results in a thinning of the lamellar epithelium, thereby decreasing the diffusion distance between the water and blood (Isaia, 1984).

Cells containing both catecholamines (TH-IR) and acetylcholine (vAChT-IR) were situated throughout the lamellae as well as in the filament epithelia between successive

lamellae in both trout and goldfish (Fig. 3.3B, D), in locations indicative of mitochondrial rich cells (MRCs) (McCormick et al., 2003). Mitochondrial rich cells, or chloride cells, function in osmoregulation to regulate the uptake or excretion of sodium and chloride salts from the environment (Foskett and Scheffey, 1982). Both catecholamines and acetylcholine have been shown to have regulatory effects on chloride secretion in the gill. May and Degnan (1985) showed that both epinephrine acting on α2-adrenoreceptors and acetylcholine decreased chloride secretion. Therefore, it may be that TH-vACht-IR cells in the filament epithelium between the lamellae are juxtaposed to chloride cells and function to regulate salt secretion. Examination of these immunoreactive cells with an antibody for Na^+K^+-ATPase, a marker of chloride cells (McCormick et al., 2003), would be a first step to test this theory.

3.4. Potential physiological effects of neurochemicals in the filament tip

Innervated, serotonergic NECs at the filament tip have been proposed to sense the external oxygen level (P_wO_2), producing reflex responses via afferent signaling (Chapter 2). At the filament tip, most 5-HT-IR and TH-IR cells continued to be in distinctly different cells (Fig. 3.2). Given than serotonin, acetylcholine, and noradrenaline applied exogenously to gills stimulate ventilation (Burleson and Milsom, 1995b), it follows that these cell types may be able to produce reflex responses to hypoxia. Catecholamines have also been shown to increase blood pressure, resulting in an increase perfusion pressure of the gill and enhanced lamellar recruitment, creating a more even distribution of blood flow within the lamellar sheet (Farrell et al., 1979). Serotonergic and TH-vACht-IR cells were often neighbors at the filament tip in both trout and goldfish (Fig. 3.4), and again it is possible that either cell type could respond directly to hypoxia or indirectly through paracrine effects from its neighboring cells. Thus, our results indicate that there may be more than one population of innervated

chemoreceptors sensing P_wO_2 located at the filament tip that produce cardio-respiratory hypoxic responses.

In conclusion, we described the presence of multiple neurotransmitters in fish gill epithelia using immunohistochemistry. Our results indicate that most cells containing catecholamines also contain the vesicular acetylcholine transporter. However, serotonergic cells rarely colocalized with the vesicular acetylcholine transporter and never with catecholamines. Often however, the two cell types occurred as neighbors. The distribution of serotonin, catecholamines, and acetylcholine in the lamellae may reflect their roles as vasoactive substances. However, we can only speculate on the functionality of the distribution of these chemicals as neuromodulators or neurotransmitters in the fish gill. While our results are consistent with the hypothesis that NECs are the putative O_2 chemoreceptors, they are also consistent with the theory that serotonergic NECs participate in O_2 chemoreception by modulating the release of acetylcholine from neighboring cells, which may or may not themselves be chemoreceptive, in the fish gill. Finally, catecholaminergic cells containing acetylcholine are located at the base of the lamellae where they may regulate salt secretion via mitochondrial rich cells.

Table 3.1: Details of primary and secondary antibodies used for immunohistochemistry.

Antisera	Dilution	Antigen	Host	Source	Secondary antisera[1]
Primary					
5-HT	1:400	serotonin	rabbit	Sigma-Aldrich	Alexa Fluor® 488[a]
TH	1:200	tyrosine hydroxylase	mouse	Sigma-Aldrich	Alexa Fluor® 488[b] and 594[c]
vACht	1:200	vesicular acetylcholine transporter	guinea pig	Sigma-Aldrich	Alexa Fluor® 594[d]
$P2X_3$	1:200	purinoreceptor subunit	guinea pig, rabbit	Upstate Chemicon, Millipore	Alexa Fluor® 488[a] and 594[d]
Secondary[1]					
Alexa Fluor® 488	1:500	rabbit IgG (H+L)[a] mouse IgG (H+L)[b]	goat	Molecular Probes, Invitrogen	--
Alexa Fluor® 594	1:300	mouse 1gG (H+L)[c] guinea pig IgG (H+L)[d]	goat	Molecular Probes, Invitrogen	--

[1]Secondary antisera were conjugated with a fluorescent marker.
[a-d]Secondary antisera antigen corresponds with primary antibody host

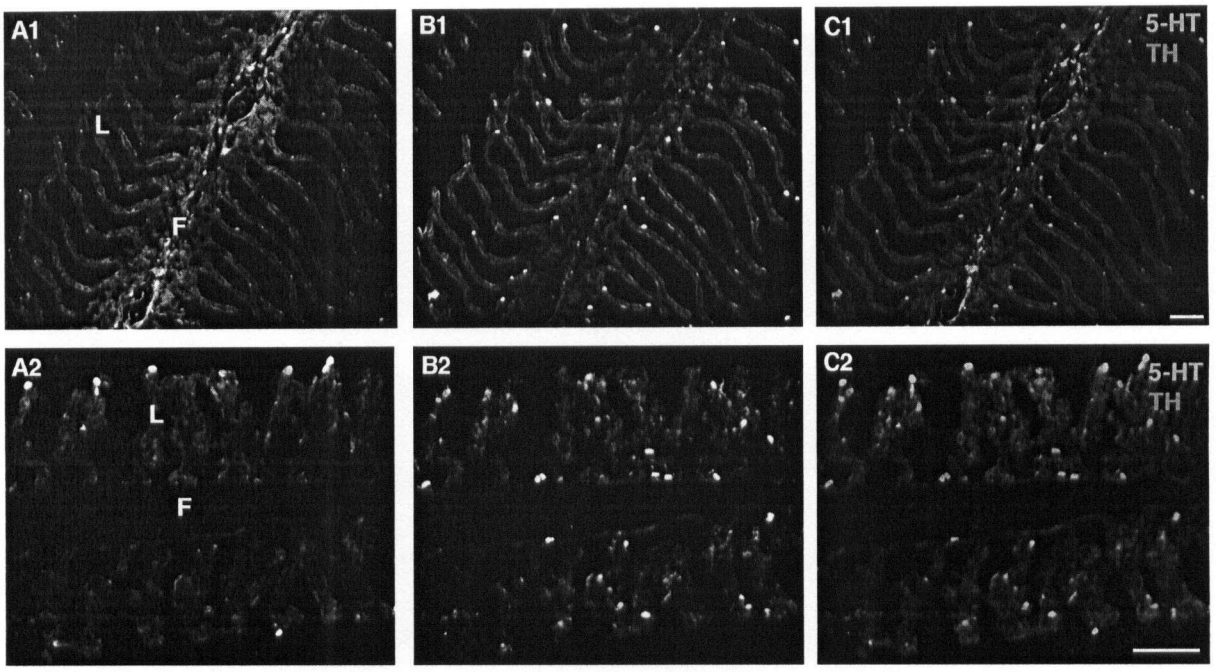


Figure 3.1. The presence of serotonin (5-HT, A, green) and tyrosine hydroxylase (TH, B, red) along the filament (F) and lamellae (L) of (1) *O. mykiss* and (2) *C. auratus*. Double immunolabeled micrographs appear in (C). Scale bars are 100 μm.

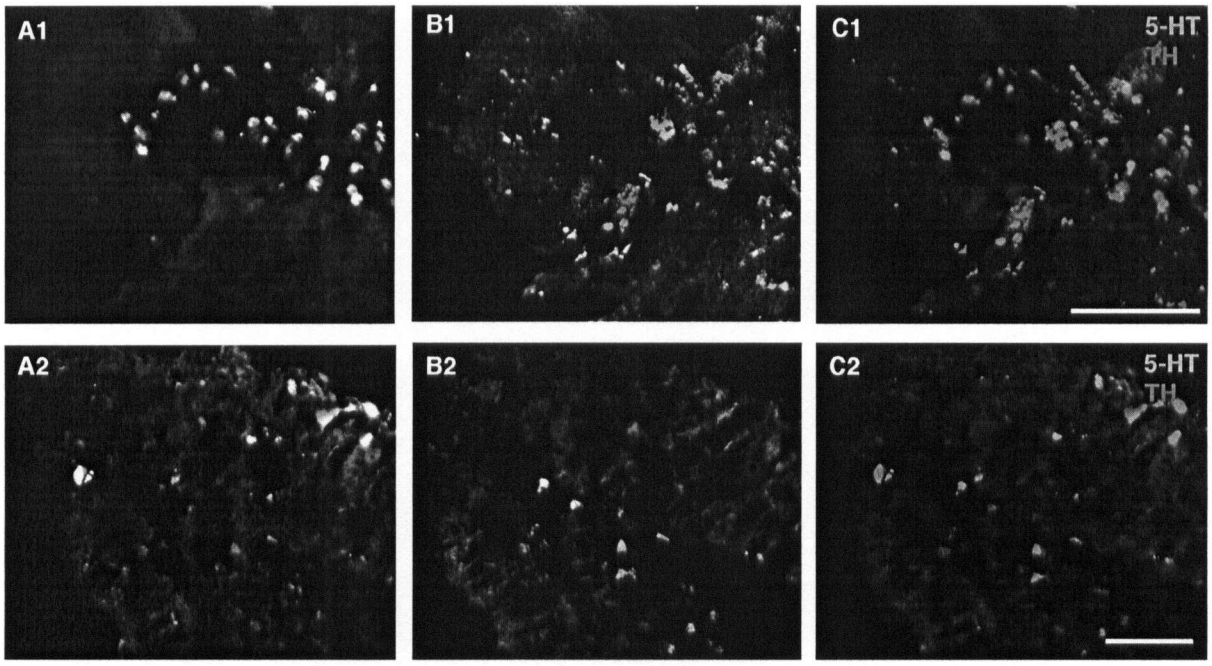


Figure 3.2. Serotonergic (5-HT-IR, A, green) and tyrosine hydroxylase (TH-IR, B, red) cells located at the filament tip in (1) *O. mykiss* and (2) *C. auratus*. Double immunolabeled micrographs appear in (C). Scale bars are 100 μm.

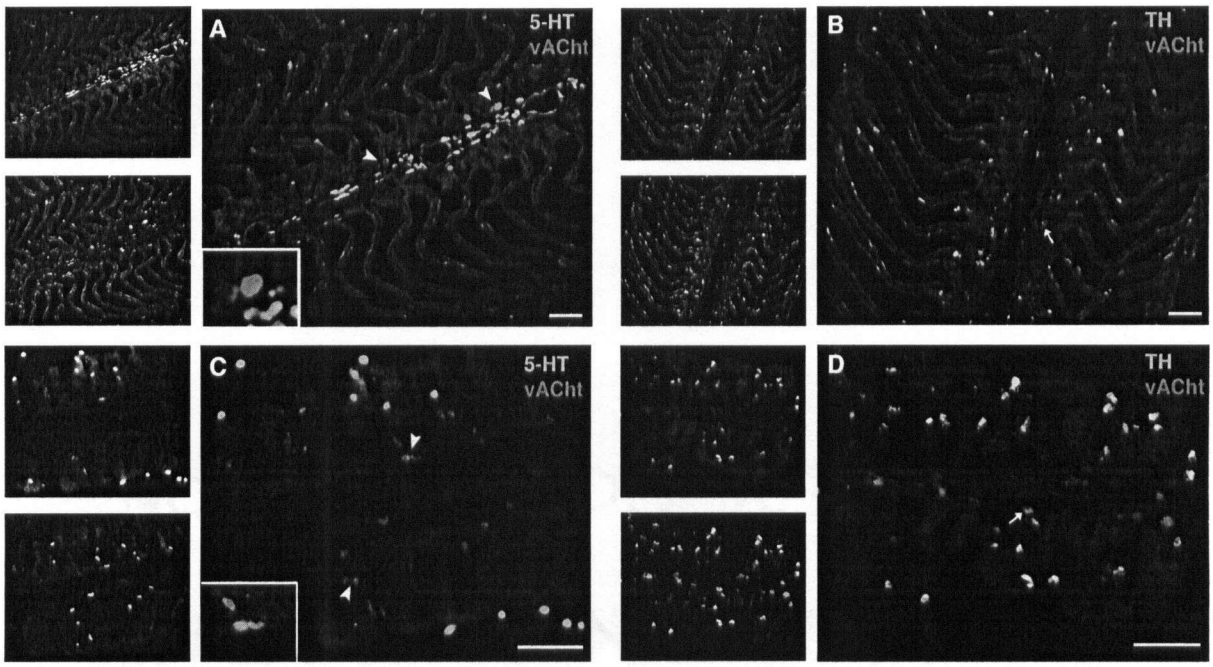


Figure 3.3. Immunoreactivity of serotonin (5-HT) and tyrosine hydroxylase (TH) paired with vesicular acetylcholine transporter (vACht) in the filament and lamellar epithelium in (A-B) *O. mykiss* and (C-D) *C. auratus*. Colocalization appears in yellow. Arrowheads indicate neighboring 5-HT-IR and vACht-IR cells (A, C), and inset pictures detail the two separate sets of cells. Arrows indicated non-vACht TH-IR cells (B) or non-TH-IR vACht-IR cells (D). Scale bars are 100 μm.

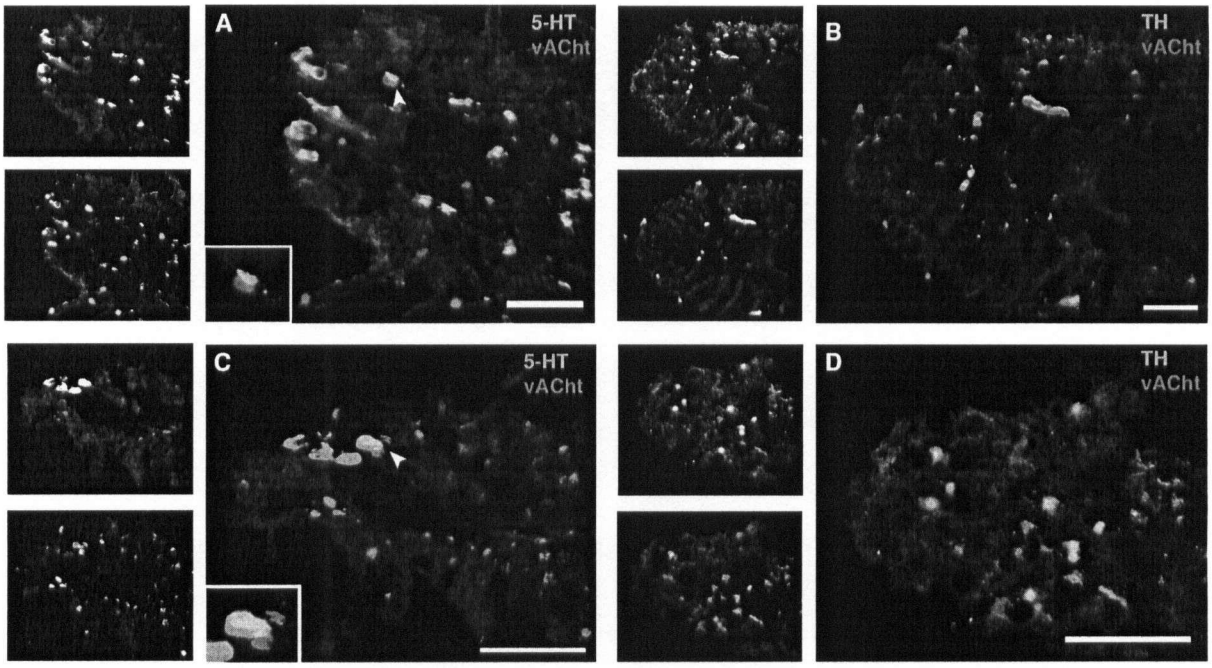


Figure 3.4. Immunoreactivity of serotonin (5-HT) and tyrosine hydroxylase (TH) paired with vesicular acetylcholine transporter (vACht) in the filament tips of (A-B) *O. mykiss* and (C-D) *C. auratus*. Colocalization appears in yellow. Arrowheads indicate neighboring 5-HT-IR and vACht-IR cells (A,C). Scale bars are 100 μm.

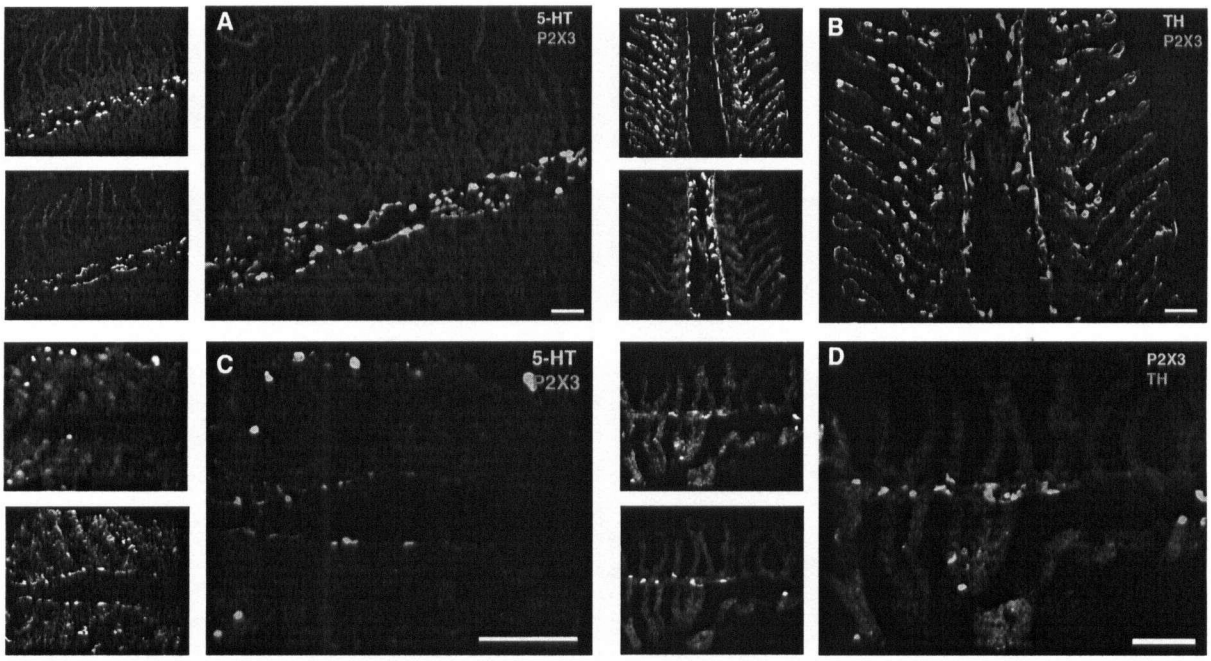


Figure 3.5. The distribution of immunoreactive cells containing serotonin (5-HT) and tyrosine hydroxylase (TH) with relation to the location of a purinoreceptor, $P2X_3$, within the gills of (A-B) *O. mykiss* and (C-D) *C. auratus*. Scale bars are 100 μm.

REFERENCES

Almaraz, L. and Fidone, S. (1986). Carotid sinus nerve C-fibers release catecholamines from the cat carotid body. *Neuorsci. Lett.* **67(2)**, 153-158.

Bailly, Y., Dunel-Erb, S. and Laurent, P. (1992). The neuroepithelial cells of the fish gill filament: Indolamine-immunocytochemistry and innervation. *Anat. Rec.* **233**, 143-161.

Burleson, M. L. and Milsom, W. K. (1993). Sensory receptors in the first gill arch of rainbow trout. *Respir. Physiol.* **93**, 97-110.

Burleson, M.L. and Milsom, W.K. (1995a). Cardio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. *Respir. Physiol.* **100(3)**, 231-238.

Burleson, M.L. and Milsom, W.K. (1995b). Cardio-ventilatory control in rainbow trout: II. Reflex effects of exogenous neurochemicals. *Respir. Physiol.* **100(3)**, 289-300.

Butler, P.J., Taylor, E.W., and Davison, W. (1979). The effect of long term, moderate hypoxia on acid-base balance, plasma catecholamines and possible anaerobic end products in the unrestrained dogfish *Scyliorhinus canicula. J. Comp. Physiol.* **132,** 297-303.

Daxboeck, C., Davie, P.S., Perry, S. F., and Randall, D.J. (1982). Oxygen uptake in a spontaneously ventilating, blood-perfused trout preparation. *J. Exp. Biol.* **101**, 35-45.

Dunel-Erb, S., Bailly, Y. and Laurent, P. (1982). Neuroepithelial cells in fish gill primary lamellae. *J. App. Physiol.* **53**, 1342-1353.

Dunn, P. M., Zhong, Y. and Burnstock, J. (2001). P2X receptors in peripheral neurons. *Prog. Neurobiol.* **65**, 107-134,

Eiden, L.E., Schäfer, M.K.-H., Weihe, E. and Schütz, B. (2004). The vesicular amine transporter family (SLC18): amine/proton antiporters required for vesicular accumulation and regulated exocytotic secretion of monoamines and acetylcholine. *Pflügers Archiv Euro. J. Physiol.,* **447(5),** 636-640.

Farrell, A. P., Daxboeck, C. and Randall, D. J. (1979). The effect of input pressure and flow on the pattern and resistance to flow in the isolated perfused gill of a teleost fish. *J. Comp. Physiol.* **133**, 233-240.

Farrell, A.P. (1979). Gill blood flow in teletosts. PhD Thesis, University of British Columbia, 255 pp.

Foskett, J.K. and Scheffey, C. (1982). The chloride cell: definitive identification as the salt-secretory cell in teleosts. *Science*, **215**, 164-166.

Fritsche, R., Reid, S. G., Thomas, S. and Perry, S. F. (1993). Serotonin-mediated release of catecholamines in the rainbow trout *Onchoryncus mykiss*. *J. Exp. Biol.* **178**, 191-204.

Fritsche, R., Thomas, S. and Perry, S. F. (1992). Effets of serotonin on circulation and respiration in the rainbow trout, *Onchoryncus mykiss*. *J. Exp. Biol.* **173**, 59-73.

Gonzalez, C., Almarez, L., Obeso., A. and Rigual, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol. Rev.* **74(4)**, 829-896.

Heymans, C., Bouckaert, J.J., Farber, S., and Hus, F.Y. (1936). *Arch Int. Pharmacodyn.* **54**, 129.

Jacono, F. J., Peng, Y.-J., Kumar, G. K. and Prabhakar, N. R. (2005). Modulation of the hypoxic sensory response of the carotid body by 5-hydroxytryptamine: role of the 5-HT_2 receptor. *Respir. Physiol. Neurobiol.* **145**, 135-142.

Jonz, M. G. and Nurse, C. A. (2003). Neuroepithelial cells and associated innervation of the zebrafish gill: a confocal immunofluorescence study. *J. Comp. Neurol.* **461**, 1-17.

Jonz, M. G., Fearon, I. M. and Nurse, C. A. (2004). Neuroepithelial oxygen chemoreceptors of the zebrafish gill. *J. Physiol. (Lond.)* **560**, 737-752.

May, S.A. and Degnan, K.J. (1985). Converging adrenergic and cholinergic mechanisms in the inhibition of Cl^- secretion in fish opercular epithelium. *J. Comp. Physiol. B.* **156(2)**, 183-189.

McCormick, S. D., Sundell, K., Bjornsson, B. T., Brown, C. L. and Hiroi, J. (2003). Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). *J. Exp. Biol.* **206**, 4575-4583.

Milsom, W. K. and Brill, R. W. (1986). Oxygen sensitive afferent information arising from the first gill arch of yellowfin tuna. *Respir. Physiol.* **66**, 193-203.

Milsom, W.K. (1998). Phylogeny of respiratory chemoreceptor function in vertebrates. *Zoology*. **101**, 316-332.

Nurse, C. A. (2005). Neurotransmission and neuromodulation in the chemosensory carotid body. *Auton. Neurosci.* **120**, 1-9.

Olson, K. R. (2002). Vascular anatomy of the fish gill. *J. Exp. Zool.* **293**, 214-231.

Perry, S.F. and Reid, S.G. (1992). Relationship between blood O_2 content and catecholamine levels during hypoxia in rainbow trout and American eel. *Am. J. Physiol.* **263**, R240-R249.

Prabhakar, N. R. (2006). O_2 sensing at the mammalian carotid body: why multiple O2 sensors and multiple transmitters? *Exp. Physiol.* **91**, 17-23.

Randall, D. J. (1982). The control of respiration and circulation in fish during exercise and hypoxia. *J. Exp. Biol.* **100**, 275-288.

Reid, S. G. and Perry, S. F. (2003). Peripheral O_2 chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am. J. Physiol.* **284**, R990-R999.

Schweitzer, A. and Wright, S. (1938). Action of prostigmine and acetylcholine on respiration. *Q. J. Exp. Physiol. Cogn. Med. Sci.,* **28**, 33-47.

Smith, D. G. (1977). Sites of cholinergic vasoconstriction in trout gills. *Am. J. Physiol.*, **233**, 222-229.

Sundin, L. and Nilsson, G. E. (1997). Neurochemical mechanisms behind gill microcirculatory responses to hypoxia in trout: *in vivo* microscope study. *Am. J. Physiol.* **272**, R576-R585.

Sundin, L. and Nilsson, S. (2002). Branchial Innervation. *J. Exp. Zool.* **293**, 232-248.

Sundin, L., Holmgren, S. and Nilsson, S. (1998a). The oxygen receptor of the teleost gill? *Acta Zool.* **79**, 207-214.

Sundin, L., Nilsson, G. E., Block, M. and Lofman, C. O. (1995). Control of gill filament blood flow by serotonin in the rainbow trout, Onchoryncus mykiss. *Am. J. Physiol.* **268**, R1224-R1229.

Wahlqvist, I. (1980). Effects of catecholamines on isolated systemic and branchial vascular beds of the cod, *Gadus morhua. J. Comp. Phys. B.* **137 (2)**, 139-143.

Wilson, J. M. and Laurent, P. (2002). Fish gill morphology: inside out. *J. Exp. Zool.* **293**, 192-213.

Wood, C. M. (1974). A critical examination of the physical and adrenergic factors affecting blood flow through the gill of the rainbow trout. *J. Exp. Biol.* **60**, 241-265.

Zaccone, G., Fausulo, S., Ainis, L. and Licata, A. (1997). Paraneurons in the gills and airways of fishes. *Microsc. Res. Tech.* **37**, 4-12.

Zhang, M., Zhong, H., Vollmer, C. and Nurse, C. A. (2000). Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. *J. Physiol.* **535**, 143-158.

CHAPTER 4:

General Conclusions

To summarize, I have described innervated NECs at the filament in all fish studied. All fish studied to date also respond to aquatic hypoxia and these cells are in a prime location to serve this role. This cluster of 5-HT-SV2-IR NECs was not immunoreactive to tyrosine hydroxylase or the vesicular acetylcholine transporter, implying that these cells do not contain catecholamines or acetylcholine. Rather, there was a second population of catecholamine and acetylcholine containing cells (TH-vACht-IR) also present at the filament tip. Although I did not use direct double-immunostaining of these specific cells with a neuronal marker, given the rich innervation of the filament it is highly likely that these TH-vACht-IR cells are also in close association with nerve fibres. I believe that these cells play a secondary role in the O_2 chemotransduction process. I propose that the serotonergic neuroepithelial cells at the filament tips are the primary sensors of aquatic hypoxia in all fish. This is supported by their location and by the hypoxic depolarization of isolated patched NECs (Jonz et al., 2004). Due to the lack of a strong chemoreceptive response of afferent nerve discharge to serotonin (Burleson and Milsom, 1995a), however, it does not appear that serotonin is the primary neurotransmitter acting on afferent nerve fibres. I suggest, therefore, that NECs at the tip of the filament may detect a drop in P_wO_2 and release 5-HT onto neighboring TH-vACht-IR cells. In this manner, 5-HT would have a neuromodulatory role on the release of catecholamines and acetylcholine onto afferent nerves. In several (but not all) ways, this is similar to the proposed mechanism of O_2 chemotransduction in the carotid body glomus cells (Jacono et al., 2005; Nurse, 2005). Afferent nerve activity stimulated by

catecholamines may be centrally integrated to produce increased blood pressure, which would indirectly increase lamellar recruitment, and respiratory surface area. Release of acetylcholine onto afferent nerves, on the other hand, may produce the reflex hypoxic ventilation (Burleson and Milsom, 1995a; Burleson and Milsom, 1995b).

However, this mechanism is merely speculative. The results presented in this study of chemoreceptive cells at the filament tip must be taken as a preliminary step in understanding the mechanism of aquatic hypoxia sensing, and the evolutionary steps leading to the sensing mechanism seen in carotid body glomus cells. Further investigation of the distribution of $P2X_3$ at the filament tip as well as of such putative O_2 sensors as heme-oxygenasese-2 (HO-2) and NADPH (Williams et al., 2004), would be an excellent follow-up to this study. If in fact neuroepithelial cells share the same transduction mechanism as glomus cells, then we would expect to see co-localization or a close association of an O_2 sensor to the serotonergic NECs at the filament tip.

In addition to putative external chemoreceptors at the filament tip, I have also described 5-HT-SV2-IR innervated chemoreceptors along the efferent filament artery. In my comparative analysis of species of varying hypoxia tolerances, I found that the presence of NECs on the eFA, presumably monitoring P_aO_2, was species-specific and correlated with the hypoxia tolerance of the fish. In contrast to previous studies, I found that the distribution of these proposed internal chemoreceptors correlated with the response of fish to arterial hypoxia (Lumsden, 1996; Sundin et al., 1999; Perry et al., 2004). Based on the weak chemoreceptive response to 5-HT (Burleson and Milsom, 1995a) and my examination of multiple neurochemicals in the filament epithelium, I again propose a neuromodulatory role of 5-HT release from P_aO_2-sensitive NECs on neighboring catecholamine/acetylcholine-

containing cells that function in a similar fashion to those just described in the filament tip. The presence of post-synaptic purinoreceptors in the same region suggests that ATP may be co-released with acetylcholine much as it appears to be in the carotid body glomus cells of mammals (Zhang et al., 2000).

The presence of TH-vACht-IR cells in the filament epithelium at the base between successive lamellae of goldfish, where we rarely found serotonergic NECs, suggests that these cells may serve a completely different function. The location of these TH-vACht-IR cells in both trout and goldfish was similar to the locations of osmoregulatory mitochondrial rich cells (McCormick et al., 2003). Thus, these cells may not serve a respiratory function, but instead may act directly on chloride cells to regulate salt excretion.

As well, I propose a chemosensory role for the non-innervated NECs found in the lamellae. These 5-HT-SV2-IR NECs presumably sense P_wO_2, and are present in species that frequently encounter hypoxic waters, where these fish may require external O_2-sensitive cells in addition to those located at the filament tip. Since these cells are not innervated, as detailed by both light and confocal microscopy, I suggest that they act in a paracrine manner directly on lamellar vasculature and pillar cells to enhance respiratory surface area when exposed to aquatic hypoxia. In addition to these putative chemosensory NECs in the lamellae, I also describe a second population of TH-vACht-IR cells present in the lamellae of both trout and carp. The presence of these cells in both a hypoxia tolerant and intolerant species may imply that the TH-vACht-IR cells are universal and have both vasoconstrictive and vasodilatory effects on the lamellar vasculature. This would be advantageous for all fish species, as control of the diameter of the lamellar arterioles is critical to the respiratory and osmoregulatory roles of the gill.

In final conclusion, this thesis details the distribution of putative oxygen-sensing neuroepithelial cells in four species, showing that differences in NEC may be adaptive and related to hypoxia tolerance. It also demonstrated a correlation in NEC distribution with physiological responses to internal and external hypoxia, and proposed both chemosensory and neuromodulatory roles for serotonergic NECs in the fish gill. Using a homlogous structure, the carotid body, as a tool for comparison, I proposed a model for an interactive role of the multiple neurochemicals in the fish gill. Therefore, this project in its entirety is a detailed and descriptive work of putative oxygen sensing in the fish gill.

REFERENCES

Burleson, M.L. and Milsom, W.K. (1995a). Cardio-ventilatory control in rainbow trout: I. Pharmacology of branchial, oxygen-sensitive chemoreceptors. *Respir. Physiol.* **100(3)**, 231-238.

Burleson, M.L. and Milsom, W.K. (1995b). Cardio-ventilatory control in rainbow trout: II. Reflex effects of exogenous neurochemicals. *Respir. Physiol.* **100(3)**, 289-300.

Jacono, F. J., Peng, Y.-J., Kumar, G. K. and Prabhakar, N. R. (2005). Modulation of the hypoxic sensory response of the carotid body by 5-hydroxytryptamine: role of the 5-HT_2 receptor. *Respir. Physiol. Neurobiol.* **145**, 135-142.

Jonz, M. G., Fearon, I. M. and Nurse, C. A. (2004). Neuroepithelial oxygen chemoreceptors of the zebrafish gill. *J. Physiol. (Lond.)* **560**, 737-752.

Lumsden, A.L. (1996) Oxygen-sensitive chemoreceptors and cardioventilatory control in carp. MSc Thesis. University of British Columbia. 97 pp.

May, S.A. and Degnan, K.J. (1985). Converging adrenergic and cholinergic mechanisms in the inhibition of Cl^- secretion in fish opercular epithelium. *J. Comp. Physiol. B.* **156(2)**, 183-189.

McCormick, S. D., Sundell, K., Bjornsson, B. T., Brown, C. L. and Hiroi, J. (2003). Influence of salinity on the localization of Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter (NKCC) and CFTR anion channel in chloride cells of the Hawaiian goby (*Stenogobius hawaiiensis*). *J. Exp. Biol.* **206**, 4575-4583.

Nurse, C. A. (2005). Neurotransmission and neuromodulation in the chemosensory carotid body. *Auton. Neurosci.* **120**, 1-9.

Perry, S. F., Reid, S. G., Gilmour, K. M., Bojink, C. L., Lopes, J. M., Milsom, W. K. and Rantin, F. T. (2004). A comparison of adrenergic stress responses in three tropical teleosts exposed to acute hypoxia. *Am. J. Physiol.* **287**, R188-R197.

Sundin, L., Reid, S. G., Kalinin, A. L., Rantin, F. T. and Milsom, W. K. (1999). Cardiovascular and respiratory reflexes: the tropical fish, traira (*Hoplias malabaricus*) O_2 chemoresponses. *Respir. Physiol.* **116**, 181-199.

Williams, S.E.J., Wooton, P., Mason, H.S., Bould, J., Iles, D.E., Riccardi, D., Peers, C., and Kemp, P.J. (2004). Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel. *Science.* **306,** 2093-2097.

Zhang, M., Zhong, H., Vollmer, C. and Nurse, C. A. (2000). Co-release of ATP and ACh mediates hypoxic signalling at rat carotid body chemoreceptors. *J. Physiol.* **535**, 143-158.