OXYGEN SENSITIVITY OF SKIN NEUROEPITHELIAL CELLS IN DEVELOPING ZEBRAFISH, *DANIO RERIO*

by

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Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
University of Ottawa
in partial fulfillment of the requirements for the
MSc Degree in the
Ottawa-Carleton Institute of Biology

November 15, 2011

ABSTRACT

In zebrafish, the ventilatory response to hypoxia first develops at 3 days post-fertilization (d.p.f.) before O_2-chemoreceptive neuroepithelial cells (NECs) of the gill appear at 7 d.p.f. This indicates the presence of extrabranchial chemoreceptors in embryos and a developmental transition to primarily gill O_2 sensing. This thesis examined the skin NECs, which reach peak density in embryos but decline as gill NECs appear. Exposure of embryos and larvae to chronic hypoxia prevented the loss of skin NECs, shifted peak basal ventilation to a later developmental stage, and induced a hypoventilatory response to acute hypoxia. Chronic exposure to hyperoxia rapidly diminished skin NECs, shifted peak ventilation to earlier stages and eliminated the response to acute hypoxia. Administration of the neurotoxin 6-hydroxydopamine degraded nerve terminals that contact skin NECs and reduced both basal ventilation frequency and the hypoxic ventilatory response. Thus, skin NECs are candidates for extrabranchial O_2 chemoreceptors in developing zebrafish.

RÉSUMÉ

Chez le poisson-zèbre, la réponse ventilatoire à l'hypoxie se développe à partir de 3 jours post-fécondation (d.p.f.), avant l'apparition, à 7 dpf, des cellules neuro-épithéliales (CNE) chémoreceptives d'O_2 des branchies. Ceci suggère la présence de chémorécepteurs extra-branchiaux chez les embryons et une transition développementale vers une détection de l'O_2 principalement par les branchies. Cette thèse porte sur les CNE de la peau, qui atteignent leur densité maximale chez les embryons, mais diminuent en nombre à mesure que les CNE des branchies apparaissent. L'exposition d'embryons et de larves à une hypoxie chronique empêche la perte des CNE de la peau, décale le pic de ventilation basale à un stade ultérieur de développement, et induit une réponse hyperventilatoire à l'hypoxie aiguë. L'exposition chronique à une hyperoxie résulte en une diminution rapide du nombre de CNE de la peau, déplace le pic de ventilation vers des stades plus précoces de développement, et élimine la réponse à l'hypoxie aiguë. L'administration de la neurotoxine 6-hydroxydopamine provoque la dégradation des terminaisons nerveuses en contact avec les CNE de la peau et réduit à la fois la fréquence de ventilation basale et la réponse hypoxique ventilatoire. Cette étude suggère un rôle pour les CNE de la peau en tant que chémorécepteurs d'O_2 extrabranchiaux chez le poisson-zèbre au cours du développement.

ACKNOWLEDGEMENTS

First I would like to thank my thesis supervisor Dr. Michael Jonz for giving me the opportunity to continue working in his lab and to complete my project. Starting off as an undergraduate student in your lab, and being able to continue my project as a graduate student has been such a wonderful experience and you have continually inspired and encouraged me throughout this entire process. Thank you for everything!

To my advisory committee, Drs. Marc Ekker and Steve Perry, thank you for taking the time to be on my committee and providing me with valuable input. All of your questions and suggestions helped me to make my project that much better.

To all the past and present Jonz lab members, all of you have helped me along my journey. I especially would like to thank SA and PZ for your constant encouragement, kind words, and introducing me to some great music (although I still can't stand country). I wish you both the best of luck during the rest of your studies. I also need to thank all of my close friends who have provided me with wonderful support over my entire university career. VC, CB, BM, KM and DP: you all have contributed to the person I am today, and I am proud to call you my friends. To CR, who has always encouraged me to do my best and stay positive, thank you!

Finally I need to thank my family for encouraging me to pursue my dreams and to never give up. To my mother and late father, thank you for instilling in me the importance of education, for your unfaltering love and great care packages. To my sister, who has been a great roommate here in Ottawa and a constant source of inspiration as she continues her journey towards a PhD, and to my brother and sister-in-law for giving me encouragement to stay in school and for always making me laugh. Also to my zio and zia here in Ottawa for the great Italian home cooked meals.

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5-HT, 5-hydroxytryptamine or serotonin

6-OHDA, 6-hydroxydopamine

AMCs, adrenomedullary chromaffin cells

ANOVA, analysis of variance

ASR, aquatic surface respiration

CB, carotid body

CNS, central nervous system

d.p.f., days post-fertilization

FITC, fluorescein isothiocyanate

h.p.f., hours post-fertilization

NEBs, neuroepithelial bodies

NECs, neuroepithelial cells

PBS, phosphate buffered solution

PO_2, partial pressure of oxygen

SCCs, solitary chemosensory cells

SEM, standard error of mean

TBs, taste buds

zn-12, zebrafish specific neuronal antibody

1. LITERATURE REVIEW

1.1 Introduction

The focus of this thesis has been to determine if skin neuroepithelial cells (NECs) of developing zebrafish are oxygen sensitive and if they may play a role in mediating the ventilatory response to low O_2 (hypoxia). This section will review the pertinent literature on respiratory chemoreceptors in vertebrates and give examples of these cells in both the mammalian and fish models. The development of O_2 sensing in zebrafish embryos and larvae is then outlined, and the importance of extrabranchial O_2 chemoreceptors in ventilatory control is discussed. Finally, the skin is then discussed as playing an important respiratory role during development, and details about its innervation and known populations of chemoreceptors are included to provide context of this organ as an established site of chemosensing.

The zebrafish, *Danio rerio*, is an excellent model that has been used for explanations pertaining to many elements such as vertebrate development and gene expression. The ease of breeding and maintenance in the laboratory make this an attractive animal model to use in developmental studies. Zebrafish embryos and larvae are amenable to imaging, and much is known about the ontogenesis of oxygen sensing of this species. Additionally, this animal represents the only non-mammalian species for which O_2 chemoreceptors have been characterized in larvae and adults (Jonz and Nurse, 2006). Through the current study, the zebrafish will be further developed as a model of respiratory research and oxygen sensing. As well, this study will improve the overall understanding of the development of oxygen sensing in vertebrates.

1.2 Oxygen sensing and respiratory chemoreceptors

The ability to sense and respond to levels of oxygen is vital for the survival of many organisms, including vertebrates. Respiratory chemoreceptors are specialized cells that detect changes in the level of environmental or arterial O_2 or CO_2 and initiate compensatory changes in heart rate, ventilation and release of catecholamines (Burlson and Milsom, 2003; Lopez-Barneo *et al*., 2008; Perry *et al*., 2009). Because of their functionality, respiratory chemoreceptors have been morphologically identified according to the following general criteria: access to a sampling environment (external or arterial), retention of neurotransmitter-containing synaptic vesicles for neurosecretion, and sensory innervation (Gonzàlez *et al*., 1994; Jonz and Nurse, 2005).

Respiratory chemoreceptors are well-described in mammals, such as the pulmonary neuroepithelial bodies (NEBs) of the lungs, carotid body (CB) type I cells and neonatal adrenomedullary chromaffin cells (AMCs; Cutz and Jackson, 1999; Lopez-Barneo *et al*., 2008; Nurse *et al*., 2009). These cells are biochemically and morphologically similar to the NECs of the fish gill, a population of cells first described by Dunel-Erb *et al*. (1982) that were suspected of having O_2 chemosensory capabilities. More recently, NECs of the adult zebrafish gill were shown to display morphological properties (i.e. retention of serotonin, synaptic vesicles and innervation) and physiological responses to hypoxia, such as ion channel inhibition and membrane depolarization, that are typical of O_2 chemoreceptors (Jonz and Nurse, 2003; Jonz *et al*., 2004). Furthermore, exposure to chronic hypoxia induces hypertrophy, and proliferation in NECs (Jonz *et al*., 2004), which are typically observed in mammalian O_2 chemoreceptors (Nurse and Vollmer, 1997; Wang and Bisgard, 2002).

1.2.1 Proposed sensors, mechanisms and the hypoxic ventilatory response

Oxygen sensing is very important in order for vertebrates to maintain homeostasis in environments which experience oxygen fluxes. Fish are regularly affected by changes in oxygen saturation in water. In fact, hypoxia is a natural component of many freshwater habitats (Diaz and Breitburg, 2009). Depending on temperature and salinity, water contains 20-40 times less O_2 by volume and O_2 diffuses about 10000 times more slowly through water than air (Diaz and Breitburg, 2009). In order to respond to O_2 flux, there must be sensors in place to recognize this change, a mechanism to transmit this information to the central nervous system (CNS) and a compensatory response can then occur.

There has been much research on the subject of how oxygen is sensed in respiratory chemoreceptors. Thus, two hypotheses have emerged for the site of the molecular O_2 sensor: the mitochondrial model and the membrane model (Lahiri *et al*., 2006; López-Barneo *et al*., 2008). The mitochondrion uses oxygen as the final electron acceptor, and, similarly to hypoxia, inhibitors of the electron transport chain increase the afferent activity of the CB sinus nerve (Mills *et al*., 1972). However, this model has lost much support due to other findings. For example, inhibitors of the mitochondrial electron transport chain do not block the hypoxia-induced release of transmitter (Ortega-Sáenz *et al*., 2003). Therefore, it is generally accepted that mitochondria do not act as O_2 sensors but changes in mitochondrial function may contribute to the hypoxia-induced rise of cytosolic Ca^{2+} concentration necessary to trigger respiratory chemoreceptor secretion, although the details for this action are not known (Lahiri *et al*., 2006).

The membrane hypothesis, originally proposed for the CB (López-Barneo *et al*., 1988), predicts that O_2 is sensed at the plasma membrane. While a membrane-delimited O_2 sensor is dependent on species and is controversial as a general mechanism, there is at least a consensus in

the literature that transduction of the hypoxic stimulus is mediated by inhibition of plasma membrane K^+ channels, and this produces the necessary depolarization for neurosecretion (Perry *et al*., 2009). The current view of O_2 sensing in vertebrate chemoreceptors suggests that a variety of molecular sensors may be involved, and that regulation of plasma membrane K^+ channels may be linked to mitochondrial respiration (Perry *et al*., 2009).

Regardless of the molecular mechanism of O_2 sensing in chemoreceptors, an accepted view of the downstream effects of hypoxia on chemoreceptors has been established for both mammals and fish, in which reduced PO_2 leads to the following sequence of events: (1) inhibition (closure) of membrane-bound K^+ channels; (2) membrane depolarization; (3) activation (opening) of voltage-gated Ca^{2+} channels; (4) increase in cytosolic Ca^{2+} concentration; (5) neurotransmitter release from cytoplasmic synaptic vesicles; and (6) activation of afferent nerve fibres (López-Barneo *et al*., 2008; Perry *et al*., 2009).

Once hypoxia is sensed by the respiratory chemoreceptors and the signal is transmitted, a number of physiological, anatomical and behavioural adaptations can occur. Manners in which fish can adapt to hypoxia are by reducing metabolic rates, accessing highly oxygenated environments, aquatic surface respiration (ASR) and hyperventilation (Diaz *et al*., 2009; Perry *et al*., 2009). The hypoxic ventilatory response (i.e. hyperventilation) has been identified as the most important physiological response of fish upon exposure to hypoxia (Perry *et al*., 2009). The majority of sole water-breathing fish respond to acute hypoxia by increasing the volume of water ventilated (see Table 5.1, Perry *et al*., 2009). In adult zebrafish, acute hypoxia exposure led to an increase in breathing frequency and no change in breathing amplitude (Vulesevic *et al*., 2006).

The hypoxic ventilatory response serves to maintain oxygen uptake when oxygen availability declines. Gas transfer across the gills involves the net direction of oxygen inward, as oxygen is extracted from the water and travels into the bloodstream and the net direction of carbon dioxide to be outward, as CO_2 is excreted by the blood and goes into the water (Perry and Gilmour, 2002). Hyperventilation leads to the increase of PO_2 and decrease of PCO_2 by reducing the carbon dioxide concentration in the blood to below its normal level, as more CO_2 is being excreted than is being produced, leading to the alteration of the blood-to-water diffusion gradient. Through the increase of water flow across the gills, the inspired-expired PO_2 difference decreases and serves to minimize the extent of the reduction in arterial PO_2 (Perry *et al*., 2009).

1.2.2 The carotid body and adrenomedullary chromaffin cells

Since CB type I cells are relatively insensitive to hypoxia during the first few weeks of life, AMCs have been identified as the oxygen chemoreceptors during the perinatal period in mammals (Thompson *et al*., 1997). These cells, located in the adrenal glands, are important for survival of the newborn during birth as a high level of hypoxic stress is experienced at this time (López-Barneo *et al*., 2010). As demonstrated in rats, AMCs possess a developmentally regulated O_2 sensing mechanism (Thompson *et al*., 1997). AMCs of neonatal rats responded to hypoxia through an increase in cytosolic Ca^{2+} concentration, resulting in membrane depolarization as a result of suppressed voltage-gated K^+ channels, and a release of catecholamines (Thompson *et al*., 1997; Rico *et al*., 2005). About 2-3 weeks postnatal, the sensitivity to hypoxia is lost following pre-ganglionic cholinergic innervation (Thompson *et al*., 1997; Nurse *et al*., 2009). Coincidentally at this time, CB type I cells mature and become sensitive to hypoxia (Slotkin and Seidler, 1988).

The CB is a small, paired, neural-crest derived organ located at the carotid bifurcation of adult mammals and is the principal acute O_2-sensing organ (López-Barneo *et al*., 2010). Upon exposure to chronic hypoxia, the CB undergoes a number of morphological and physiological changes: enlargement and mitosis of the type I cells, growth of new blood vessels and vasodilation, all leading to CB hypertrophy (Stea *et al*., 1992; Mills and Nurse, 1993; Wang and Bisgard, 2002). These changes serve to increase the sensitivity of the CB chemoreceptors to hypoxia in adults (Barnard *et al*., 1987; Bisgard, 2000). However, it has been demonstrated that humans native to high altitudes and developing neonates exposed to chronic hypoxia may have a blunted ventilatory response to acute hypoxia due to a decreased O_2-sensitivity of the carotid body (Bisgard, 2000; Lahiri *et al*., 2000). Similarly, chronic hyperoxia exposure during development alters ventilation in adult life due to blunted afferent inputs from the carotid body and neurons in the carotid sinus nerve (Bisgard *et al*., 2005).

The O_2-sensitive type I cells of the CB are innervated by sensory nerve fibres of the carotid sinus nerve and by postganglionic sympathetic nerve fibres from the superior cervical ganglion (Gonzalez *et al*., 1994; Wang and Bisgard, 2002). The CB type I cells have been shown to exhibit catecholaminergic efferent and afferent innervation (Katz *et al*., 1983; Finley *et al*., 1992). It has also been demonstrated that carotid body afferent fibres may release neurotransmitter (dopamine) onto type I cells and is supported by observations that these fibres contain synaptic vesicles and presynaptic membrane specializations (McDonald and Mitchell, 1975; Smith and Mills, 1976).

1.2.3 Gill neuroepithelial cells

The NECs found on the gills of zebrafish have been recently identified as bimodal O_2 and CO_2 chemoreceptors (Jonz *et al*., 2004; Qin *et al*., 2010). A diverse number of NECs have been described in the zebrafish gill, but the serotonergic NECs of the gill filament have been identified as the O_2-sensitive cells due to their exposure to the external environment, synaptic vesicle retention, innervation and electrophysiological recordings during hypoxic exposure (Jonz and Nurse, 2003; Jonz *et al*., 2004).

Similarly to the CB, chronic hypoxia induces morphological change to gill NECs. After *in vivo* exposure of zebrafish to chronic hypoxia, the gill NECs increased in size by approximately 15% compared to controls (Jonz *et al*., 2004). More NECs had neuron-like processes after chronic hypoxia, and these extensions were longer in exposed fish versus controls (Jonz *et al*., 2004). No change in density of serotonergic gill NECs has been reported after chronic hypoxia exposure, although the density of non-serotonergic NECs did increase under these conditions (Jonz *et al*., 2004; Vulesevic *et al*., 2006). Upon exposure of fish to chronic hyperoxia, the density of serotonergic gill NECs was significantly reduced (Vulesevic *et al*., 2005). Chronic exposure of adult zebrafish to hypoxia had no effect on their ventilatory response to acute hypoxia, whereas chronic exposure to hyperoxia significantly blunted breathing frequency upon acute hypoxia exposure (Vulesevic *et al*., 2005). Zebrafish reared under hypoxic conditions showed no change in their response to acute hypoxia as adults, whereas zebrafish reared under hyperoxic conditions showed a blunted response to acute hypoxia as adults (Vulesevic and Perry, 2006).

In teleost fish, there are generally at least three types of nerve fibres with which gill NECs establish synapses. The first type is characterized by catecholaminergic nerve endings,

suggesting that NECs are modulated by spinal autonomic nerves (Bailly *et al*., 1992; Jonz and Zaccone, 2009). The second type is made of serotonergic neurons, which are not degenerated after treatment with 5- or 6-hydroxydopamine (5- or 6-OHDA; Bailly *et al*., 1992). The third type is nitrergic nerve fibres that may be of cranial origin (Zaccone *et al*., 2003). The NECs may also receive efferent innervation, as many nerve fibres associated with gill NECs contain synaptic vesicles representative of presynaptic nerve endings (Jonz and Nurse, 2003; Jonz and Zaccone, 2009).

1.3 Ontogenesis of oxygen sensing in zebrafish

Zebrafish go through many stages of development before gill NECs and associated sensory pathways, which are necessary for oxygen sensing in adults, are functional. Prior to the development of gill NECs, zebrafish are able to sense oxygen levels (Jonz and Nurse, 2005). These pathways, however, are not fully developed until 7 d.p.f. (Jonz and Nurse, 2006). In order to enable the developing zebrafish to respond to hypoxic challenges, there must be alternative sites of O_2 chemoreceptors.

When subjected to 24 hours of anoxia, zebrafish embryos 25 h.p.f. and younger survived by entering a state of metabolic suppression where all cell division, cardiac function, and movement ceased (Padilla and Roth, 2001). Upon re-oxygenation, embryos were able to develop normally and produce offspring that were indistinguishable from fish raised under normal conditions. This type of anoxia tolerance has been observed in other teleost fish such as the annual killifish, *Austrofundulus limnaeus* (Podrabsky *et al*., 2007) and several mammalian species (Renfree and Shaw, 2000; Kamei *et al*., 2011). Tolerance to anoxia can be seen as a protective measure to

enhance survival, increase reproductive fitness or as an evolutionary adaptation to natural environmental fluctuations (Padilla and Roth, 2001; Podrabsky *et al*., 2007).

By 2 d.p.f., zebrafish embryos lose their ability to tolerate anoxia, and mortality is induced (Padilla and Roth, 2001). The decrease in duration of anoxic viability has been shown to correlate with the rate of anoxic lactate accumulation (Mendelsohn *et al*., 2008). During the first few days of development, there is a significant increase in O_2 consumption (Barrionuevo and Burggren, 1999), which may be related to this decreased anoxia tolerance. Also at this stage of development, zebrafish embryos responded to hypoxia by increasing pectoral fin and whole body movements (Jonz and Nurse, 2005), improving the diffusion gradient for respiratory gas exchange across the skin (Rombough, 1988). Whole body and pectoral fin movements have been shown to be coordinated with gill ventilation, and can be used to identify a response to hypoxia before the hyperventilatory response develops (Jonz and Nurse, 2005). At 2 d.p.f., there appears to be no morphological basis for gas exchange to occur across the gills, because gill NECs and respiratory lamellae are not present (Rombough 2002; Jonz and Nurse, 2006). Zebrafish predominately respire cutaneously up to 14 d.p.f., and respiratory gas exchange eventually shifts to the gills (Rombough, 2002).

By 3 d.p.f. gill development begins with the appearance of filament primordia, innervation of gill arches and gill arch NECs, and the gills are ventilated (Jonz and Nurse, 2005; Higashijima *et al*., 2000; Rombough, 2002). Despite the apparent lack of gas exchange across the gills, the hyperventilatory response to hypoxia also begins at this stage (Jonz and Nurse, 2005). At 5 d.p.f. the gill NECs, which have been demonstrated to be responsible for O_2-chemorecepton in adults (Jonz and Nurse, 2006), are present and their innervation begins and is completed by 7 d.p.f. (Jonz and Nurse, 2005). As the gills mature, the contribution of cutaneous gas exchange

decreases, and the gills become the dominant site for respiration by 21 d.p.f. (Rombough, 2002). Since the NECs responsible for O_2 sensing are neither innervated nor functional until 7 d.p.f., they are unlikely to serve as oxygen chemoreceptors until this time. This phenomenon suggests that embryonic and larval O_2 sensing must occur via chemoreceptors that occupy extrabranchial sites.

1.4 Extrabranchial O_2 chemoreceptors

The gill NECs of fish have been well documented to be the oxygen-sensitive cells of the adult teleost fish (Dunel-Erb *et al*., 1982; Bailly *et al*., 1992; Jonz *et al*., 2004; Jonz and Nurse, 2006). However, sensitivity to hypoxia appears early in zebrafish development, well before the gill NECs are functional (see section 1.3), indicating that extrabranchial sites of O_2 sensing exist during these early stages, similarly to mammalian foetuses (Schwerte, 2009). Several suggestions have been made as to where extrabranchial chemoreceptors are located in adult fish, such as the pseudobranch, the central nervous system (brain), and orobranchial cavity (Perry *et al*., 2009; Schwerte, 2009). One study has proposed that extrabranchial O_2 sensing is a developmental phenomenon, and may occur in the skin (Jonz and Nurse, 2006).

The pseudobranch is a reduced, gill-like organ that resides within the cranial portion of the opercular epithelium, and is not exposed to the external environment. It has been proposed to be involved in O_2 sensing on the basis of its morphology and responsiveness to hypoxic perfusate (Laurent and Rouzeau, 1972). However, additional studies on the pseudobranch were unable to elicit the same response characteristic, and removal of the pseudobranch did not change the ventilatory response to hypoxia (Parry and Holliday, 1960; Bamford, 1974). As well, many species that do not have a pseudobranch (e.g. channel catfish) still possess robust responses to

hypoxia (Perry *et al*., 2009). Therefore, the role of the pseudobranch in oxygen sensing is inconclusive.

The central nervous system (brain) has been implicated as a site of oxygen chemosensing. Experiments demonstrated that infusion of hypoxic blood into the dorsal aorta of a normoxic sea raven elicited a hyperventilatory response (Saunders and Sutterlin, 1971). Conversely, a study performed on the neotropical fish *Colossoma macropomum* (tambaqui) demonstrated that the brain is not the site of the oxygen chemoreceptors, as superfusion of hypoxic saline did not elicit the hyperventilatory response (Milsom *et al*., 2002). Therefore, central O_2 sensing may be species-specific. It has been demonstrated in adult fish that increased ventilation frequency in response to hypoxia is reliant on central ionotropic glutamate receptors (NMDA receptors; Turesson and Sundin, 2003). In zebrafish, these receptors are necessary for mediating the hypoxic ventilatory response beginning at 8 d.p.f. and this response is completely dependent on NMDA receptors by 13 d.p.f. (Turesson *et al*., 2006). This study also found that the NMDA receptor subunits NMDAR1 and NMDAR2A are not present in fish as young as 3 d.p.f., suggesting that the hyperventilatory response is not dependent on NMDA receptors during early developmental stages, and other mechanisms may be responsible (Turesson *et al*., 2006).

The orobranchial cavity has been suggested as a site of extrabranchial O_2 chemoreceptors. In a neotropical fish, the tambaqui (*Colossoma macropomum*), complete denervation of the gill did not eliminate the hypoxic response (Milsom *et al*., 2002). This same study provided evidence to support the orobranchial cavity, innervated by cranial nerves V and VII, as possessing O_2-sensitive chemoreceptors. It was shown that the chemoreceptors in this area were involved in reflex increases in breathing amplitude (Milsom *et al*., 2002). Therefore, it remains a possibility that such chemoreceptors of the buccal cavity may be present in zebrafish.

To date, the only tissue that has been considered as a site for extrabranchial O_2 chemoreception in developing fish is the skin (Jonz and Nurse, 2006). There is evidence of many types of chemosensory cells in the skin which direct information to the brain (see section 1.5), therefore it is reasonable to propose that chemoreceptors which detect O_2 may also be present in the skin. The skin has been identified as an accessory respiratory surface, and the upper layer of the dermis is most strongly vascularised (Le Guellec *et al*., 2004). Only one study has described the presence of serotonergic (5-HT) positive cells on the skin of 3 d.p.f. zebrafish that resemble O_2 chemoreceptors of the gills (Jonz and Nurse, 2006). These cells are distributed all over the surface of the zebrafish and appear to be associated with nerve fibres. A similar population of cells has also been observed in *Xenopus* larvae (Jonz and Nurse, 2006).

1.5 Skin

The skin of fish provides a protective barrier between the animal and its aquatic environment. Several important functions are encompassed by the skin including maintaining body shape, protecting the fish from shocks and various attacks, improving hydrodynamics and housing sensory functions that are essential to survival (Le Guellec *et al*., 2004). The skin can be divided into three compartments: the epidermis, dermis and hypodermis (Le Guellec *et al*., 2004). The epidermis is composed of several regions, and is penetrated by nerve fibres which are either free nerve endings or associated with epidermal sensory cells (Whitear, 1971). Before 24 h.p.f., the skin of zebrafish is comprised of only a two cell layer (about 4 μm thick) of epithelial cells which surround the body, and the skin remains thin (>10 μm) throughout embryonic and larval stages (Le Guellec *et al*., 2004). Even during these early stages, the skin remains a highly functioning organ as it has been implicated to be the main site of gas exchange, and contains

numerous chemosensory cells necessary for survival (Kotrschal *et al*., 1997; Rombough, 2002; Hansen *et al*., 2002; Døving and Kasumyan, 2008).

1.5.1 Innervation

The head epidermis of teleosts receives dual sensory innervation by trigeminal and facial fibres, while the trunk receives facial and spinal innervation (Kotrschal *et al*., 1997). Single areas of skin have been found to be innervated by multiple nerve fibres, similarly to mammalian skin (Whitear, 1952). As described by Whitear (1971), there are four sensory components of fish skin innervation: (1) somatic, which innervates the organs of the lateral line system (neuromasts); (2) visceral, which innervates taste buds; (3) general cutaneous, which innervates the outer skin surface with receptor fibres for tactile, temperature and pain senses, and also produce free nerve endings; and (4) general visceral, which produces free nerve endings in the oral and pharyngeal epithelia.

1.5.2 Surface chemoreceptors

There are three chemosensory systems in fish: (1) the olfactory system, the sense of smell, can detect substances of minute quantities and is localized to the snout in a peripheral organ; (2) the gustatory system, the sense of taste, detects chemicals at a higher concentration than the olfactory system. The taste receptor cells (taste buds; TBs) can be found in the oral cavity and over the entire skin surface; (3) the common chemical sense, the function of which has not been determined, is presented by free nerve endings, and solitary chemosensory cells (SCCs) which are located on the entire skin surface (Døving and Kasumyan, 2008). Only the TBs and SCCs

will be discussed here, as they are distributed over the entire skin surface, similarly to the previously identified 5-HT cells (see section 1.4).

Mature fish TBs are ovoid in shape, about 80-100 μm high and 40-60 μm wide, and found in the epithelium, sitting on a small dermal papilla (Hansen and Reutter, 2004). They can be found in many areas of the zebrafish, including the epithelia of the lips and oropharyngeal cavity, and also on the skin of the barbels, head and whole body surface (Hansen *et al*., 2002). Innervation of TBs is derived from the facial (VII), glossopharyngeal (IX), or vagal (X) cranial nerves (Døving and Kasumyan, 2008). TB primordia do not appear on the zebrafish until 3-4 days post-fertilization (d.p.f.)., and are isolated to the skin of the lips and gill arches. TBs of the mouth and oropharyngeal cavity appear one day later (4-5 d.p.f.) and around 12 d.p.f. is when the TBs appear on the surface of the head (Hansen *et al*., 2002).

All groups of fish have been shown to possess SCCs, which are solitary, spindle-shaped, and have single or multiple microvilli (Whitear, 1992; Døving and Kasumyan, 2008). SCCs appear in the zebrafish after hatching, approximately 3 d.p.f., and continue to increase in density after metamorphosis (25 d.p.f.), remaining relatively constant thereafter (Kotrschal *et al*., 1997). They are found all over the surface of the fish, with higher densities occurring in the head, rather than the trunk (Kotrschal *et al*., 1997). Innervation of SCCs depends on location: the head and oropharyngeal SCCs can receive facial or spinal innervation, while SCCs on the body surface may receive innervation from a recurrent facial nerve (Whitear, 1992). The role of SCCs has not been elucidated, however these cells have been proposed to play a role in predator avoidance or to be the evolutionary precursors of TBs (Kotrschal, 1991).

1.6 Hypotheses and objectives

The question which drove this research was: are skin NECs oxygen sensitive chemoreceptors during early development in zebrafish? To address this question, a number of hypotheses were tested:

1) It was hypothesized that if skin NECs are oxygen chemoreceptors in embryos and larvae, they will share similar morphological characteristics with oxygen chemoreceptors of the gills in adults, such as innervation by sensory nerve fibres. It is also predicted that skin NECs will be observed primarily during the embryonic and early larval stages, before gill NECs develop. These hypotheses will be addressed using immunohistochemistry and fluorescence microscopy.

2) Chronic exposure to hypoxia or hyperoxia early in development will induce morphological changes in skin NECs, such as changes in number, density and size, which have been observed in O_2 chemoreceptors of both fish and mammals. Furthermore, since embryos and early larvae exhibit behavioural responses to changes in water PO_2, the above acclimation to chronic hypoxia or hyperoxia will also lead to changes in the way the larvae respond to acute hypoxia. These hypotheses will be addressed using morphometric analysis of immunolabelled skin NECs and behavioural assays.

3) Innervation of skin NECs is required for the preservation of the embryonic and larval response to acute hypoxia. This hypothesis will be tested by pre-exposing developing zebrafish to a sublethal concentration of a neurotoxin that selectively degrades catecholaminergic nerve endings. These embryos and larvae will then be subjected to behavioural assays to determine changes in the acute response to hypoxia.

2. MATERIALS AND METHODS

2.1 Animals

Animals used in this study were developing zebrafish (*Danio rerio*) ranging from 24 hours post-fertilization (h.p.f.) to 9 days post-fertilization (d.p.f.). Adult zebrafish, used for breeding, were obtained from a commercial supplier (MIRDO, Montreal, Canada) and transported to the University of Ottawa Aquatic Care Facility and maintained in 9 litre acrylic tanks supplied with aerated, dechloraminated City of Ottawa tap water at 28°C. Fish were maintained on a constant 10:14 light-dark photoperiod. Embryos were obtained using the standard zebrafish breeding technique (Westerfield, 2000). Briefly, plastic traps were placed in tanks with mixed males and females. Embryos were collected through a fine mesh the following morning and placed in embryo medium in an incubator equilibrated with room air and set at 28.5°C. Embryo medium (E3) contained the following chemicals (in mM): NaCl, 5; KCl, 0.17; $CaCl_2 \cdot 2H_2O$, 0.33; $MgSO_4 \cdot 7H_2O$, 0.33; 0.0001% (w/v) methylene blue; pH 7.78. After 2 days, embryos were transferred to 2 litre plastic tanks filled with dechlorinated water maintained at 28.5°C in a water bath. Embryos and larvae were not fed during experiments. Care and handling of animals was done according to guidelines set out by the University of Ottawa Veterinary Service and carried out in accordance with those of the Canadian Council on Animal Care (CCAC).

2.2 Immunohistochemistry

Once the larvae reached the desired chronological age, their chorion was removed (if necessary; Westerfield, 2000). Tissue was fixed in a cold phosphate buffered solution (PBS) containing 4% para-formaldehyde and kept at 4°C overnight. PBS contained the following chemicals (in mM): NaCl, 137; Na_2HPO_4, 15.2; KCl, 2.7; KH_2PO_4, 1.5; pH 7.7-7.8 (Bradford *et*

al., 1994; Jonz and Nurse, 2003). The larvae were then rinsed in cold PBS. The tissue was placed overnight at 4°C in a permeabilizing solution, containing 2% Triton X-100 in PBS. This concentration of Triton X-100 was chosen because preliminary experiments using a lower concentration (0.5%) resulted in poor labelling. The higher concentration improved labelling without any adverse effects (i.e. no non-specific labelling or background, etc.).

For experiments in which skin was isolated for immunohistochemistry, pieces of skin were collected from 3 month old adult zebrafish after a sharp blow to the head and decapitation. The tissue was pinned down into a Sylgard (184 Silicone Elastomer Kit, Dow Corning Corporation, Midland, MI) coated petri dish and kept in PBS. Under a dissecting microscope (Leica MZ6), skin was separated from underlying connective tissue using fine forceps and kept on ice until fixation.

Morphological characteristics of serotonergic skin cells were identified using an array of commercially available antibodies (Table 1) and previously developed procedures (Jonz and Nurse, 2003). Identification of gill NECs and serotonergic skin cells have previously been performed using anti-5-HT, diluted 1:250 (Jonz and Nurse, 2003; Coccimiglio, 2008). Innervation of the serotonergic skin cells was detected using the primary antibody zn-12 (neuron-specific surface antigen; Trevarrow *et al*., 1990). Monoclonal zn-12 was raised in mouse against membrane fractions from adult zebrafish CNS and recognizes an HNK-1-like (human natural killer-1, a carbohydrate expressed on certain neural cell adhesion molecules) epitope (manufacturer specifications). Western blot analysis has indicated that both zn-12 and HNK-1 antibodies label similar bands ranging in molecular weight from 60-248 kDa (see Metcalfe *et al*., 1990). Permeabilized tissue was bathed in the desired primary antibodies for 24 hours at 4°C and then exposed to the appropriate secondary antibody after rinsing with

permeabilizing solution. Positive immunofluorescence was identified using secondary antibodies conjugated with fluorophores (e.g. FITC and Alexa 594). The tissue was exposed to the secondary antibodies for 50-60 minutes in a dark, room temperature environment. Prior to mounting on slides, the tissue was rinsed with PBS, placed in a mounting medium (Vectashield, Vector Laboratories Inc., Birlingame, CA) and then viewed using a confocal microscope (Fluoview 200, Olympus), or microscope equipped with epifluorescence (Axiophot (Zeiss or Nikon). The confocal microscope was equipped with argon (Ar) and krypton (Kr) lasers with peak outputs of 488 nm and 568 nm respectively. A photomultiplier tube detected images and photographs were captured using confocal graphics software (Fluoview 2.1, Olympus). Tissue was scanned using the appropriate laser for each fluorophore. When conventional epifluorescence microscopy was used, images were collected with Northern Eclipse software (Empix Imaging Inc., Mississauga, ON, Canada). All image processing and manipulation was done with ImageJ software (v. 1.42q; National Institutes of Health).

Table 1. Primary and Secondary Antibodies Used for Immunohistochemistry.

Antisera	Dilution	Antigen	Host	Source	Secondary
Primary					
5-HT	1:250	serotonin	rabbit	Sigma	FITC
zn-12[2,4]	1:100	neuron, surface	mouse	DSHB[3]	Alexa 594
Secondary[1]					
Alexa 594	1:100	mouse (IgG)	goat	Molecular Probes	
FITC[5]	1:50	rabbit (IgG)	goat	Jackson Laboratories	

[1]Secondary antisera was conjugated with a fluorescent marker.
[2]Monoclonal antibody.
[3]Developmental Studies Hybridoma Bank, University of Iowa.
[4]Zebrafish derived antibody.
[5]Fluorescein isothiocyanate.

2.3 Acclimation to hypoxia and hyperoxia

To study the impact of water PO_2 on zebrafish development, embryos and larvae were acclimated to one of four different levels of water PO_2: hyperoxia (300 Torr), normoxia or control (150 Torr), mild hypoxia (80 Torr), or severe hypoxia (30 Torr). For mild hypoxia acclimation experiments, fresh embryos were placed in an incubator equipped with an O_2 sensor and pumped with a mixture of air and nitrogen gas to gradually acclimate the embryos to the desired level of hypoxia. Embryo medium was replaced daily to minimize mortality. When larvae reached 2 d.p.f. they were placed in dechlorinated water bubbled with compressed air and nitrogen gas using a Pegas 4000 gas mixer (Columbus Instruments, Columbus, OH) to maintain the required level of hypoxia. An oxygen meter (YSI model 55, YSI Inc., Yellow Springs, OH) was used to test the level of dissolved oxygen in the water every day. Control embryos were held in normoxia in which water was bubbled with compressed air only.

For more severe hypoxic treatment, embryos were housed in an incubator until 6 h.p.f. and then placed in dechlorinated water and bubbled with the above combination of gases to achieve the desired hypoxia level. The earlier transfer of embryos exposed to 30 Torr hypoxia promotes survival (Vulesevic and Perry, 2006). Chronic hyperoxia exposures were performed in the same manner as above, except water was bubbled with 100% oxygen to achieve the desired oxygen level (300 Torr). Control embryos were also transferred to dechlorinated water at 6 h.p.f. and were bubbled with compressed air only.

2.4 Exposure to neurotoxin, 6-hydroxydopamine (6-OHDA)

6-OHDA has been shown to selectively degrade catecholaminergic nerve endings in mammalian models and the zebrafish (Blum *et al*., 2001; Parng *et al*., 2007). 6-OHDA, a

hydroxylated analog of the natural dopamine neurotransmitter, shows a high specificity to catecholaminergic cells because of their specific uptake transporters (Blum *et al*., 2001). Broadly speaking, 6-OHDA exerts its deleterious effects through inducing oxidative stress and decreasing cellular adenosine triphosphate (ATP) levels. Cell death is said to occur by three main mechanisms: 1) the creation of reactive oxygen species (ROS); 2) production of hydrogen peroxide induced by monoamine oxidase activity; and 3) direct inhibition of the mitochondrial respiratory chain (Blum *et al*., 2001).

For 6-OHDA (Sigma) treatment, previously determined procedures were employed (Parng *et al*., 2007) with some modifications. 2 d.p.f. larvae from the same pool of embryos were split into separate 50 ml beakers and administered 6-OHDA or left untreated (for negative control). 6-OHDA was applied directly to the fish water in 250 μM or 500 μM concentrations. Varicosity distance and number were observed 3 and 5 days post-treatment (5 and 7 d.p.f.; see section 2.6). Fish were tested in ventilation assays (see section 2.5) everyday from 1-5 days post-treatment (3-7 d.p.f.). Negative controls were also tested at these same time points. Fish water containing 6-OHDA was replaced after 3 days to minimize mortality.

Ventilation frequency measurements of fish acutely exposed to sodium cyanide (NaCN, Sigma) were completed in the same manner as described in section 2.5, with some modifications. Control and 6-OHDA treated fish were raised until 5 d.p.f. Normoxia measurements were taken 3 minutes after transfer using control solution. Fish were then exposed to a 1 mM solution of NaCN dissolved in dechlorinated water for 1 minute before measurements were recorded.

2.5 Ventilation frequency measurements

Previously determined procedures for measuring ventilation frequency were employed (Jonz and Nurse, 2005). Once the embryos (or larvae) achieved the desired stage of development (1 d.p.f. to 9 d.p.f.), the zebrafish was placed into a glass bottomed culture dish (MatTeck Corporation, Ashland, MA) which has a 14 mm well, and continuously perfused at 4 ml/min. Larvae ≥ 3 d.p.f. were lightly anaesthetized with 0.05 mg/ml MS222 (tricane methanesulfonate; Syndel Laboratories, Vancouver BC) dissolved in dechlorinated water. Zebrafish were transferred to the culture dish using a Pasteur pipette containing a small volume of dechlorinated water. A piece of nylon mesh was placed over the bottom of the dish to confine the zebrafish to the glass bottomed well. A moulded Sylgard ring was placed over the mesh to keep it in place during perfusion. Observations were made using a dissecting microscope (Leica MZ6).

Behavioural response to hypoxia was determined by observing the rate of whole body/pectoral fin movements in embryos aged 1 d.p.f. to 2 d.p.f., while movement of the gills/operculae, and buccal pumping was monitored for older larvae (≥ 3 d.p.f.). Activity of gill ventilation has been observed to be coordinated with pectoral fin and whole body movements in larvae, and the latter behaviours can be used to identify a response to hypoxia in embryos which have yet to develop the hyperventilatory response (Jonz and Nurse, 2005). Hypoxia (23 Torr) was produced by bubbling the solution in 500 ml Nalgene bottles with nitrogen gas and verified using an oxygen meter. Control solution (150 Torr) was kept in a separate plastic bottle. The tubing to connect the perfusate to the perfusion chamber was gas impermeable (Tygon, Saint-Gobain Performance Plastics Corporation, Pittsburgh PA). Ventilation frequency measurements were conducted about 3 minutes after the fish were placed in the observation chamber to allow the fish to recover from transfer. Fish were observed for 30 seconds and the frequency of

movement was recorded manually using a hand-held counter and stopwatch (Fisher Scientific). Values were expressed as the number of gill beats or body movements per minute. Ventilation frequency measurements of fish treated with 6-OHDA and those acclimated to hypoxia and hyperoxia, as described above, were completed in the same manner.

2.6 Morphometric analysis

To determine whether the abundance or morphology of serotonergic skin NECs changed during early development, or following acclimation to various levels of water PO_2 (section 2.3), parameters such as cell number, number per tissue area (density), and size (two-dimensional area) were recorded by hand through the use of the software ImageJ (National Institutes of Health). Once tissue was labelled with the 5-HT antibody (see section 2.2), images of the head, trunk and tail were collected for each individual sample, using a microscope equipped with epifluorescence (Axiophot (Zeiss or Nikon). Cells were identified on each sample area (i.e. head, trunk, and tail), quantified using the "cell counter plugin" to keep track of the number of cells, and presented as an average of all samples for each developmental stage. Density of cells was calculated as the number of cells per unit area (e.g. 200 μm^2) for each sample area, and then presented as the average density per mm^2.

The two-dimensional area of cells through development was also calculated using ImageJ. Once images were collected, the clearest picture (usually the tail region) for each individual was converted into a black-and-white ("threshold") image, and area was automatically calculated by the software ($A = \pi r^2$). At random, five cells were selected per individual and presented as the average of all samples for each developmental stage.

Distance between varicosities and points of contact for the 6-OHDA treatments (section 2.4) was also conducted using the above software. Varicosities are swellings along a nerve axon that represent contact with another cell or fibre. The distance between varicosities, therefore is a measure of varicosity density. Points of contact reflect putative synapses with NECs. Oil immersion images of skin NECs and zn-12-positive nerve fibres were collected using a confocal microscope (see section 2.2). Points of contact between a NEC and nerve fibres were determined from images as any apparent overlap of a nerve fibre varicosity on the surface of the cell. The distance between varicosities was determined from images of zn-12 positive nerve fibres. A grid was placed over each image, so that the measurements could be taken from the same grid regions on each image. A line was hand drawn between two varicosities in any given region, and the length of the line was determined by the software.

2.7 Statistics

Data are presented as mean ± S.E.M. for skin NEC number, density, area, and ventilation frequencies. Multiple comparisons within one treatment group were performed using ANOVA followed by Bonferroni test. Comparisons between different treatment groups were performed using two-way ANOVA followed by Bonferroni test. Varicosity distance and points of contact for 6-OHDA treated zebrafish were compared to control values using a paired t-test. Statistical analyses were performed using commercial software (GraphPad Prism v.5.03; GraphPad Software Inc., San Diego, CA).

3. RESULTS

3.1 General features of skin neuroepithelial cells (NECs)

In all zebrafish embryos and larvae observed, the presence of serotonergic skin NECs was determined by indirect immunohistochemistry using a primary antibody directed against serotonin (5-HT) and a secondary antibody conjugated with fluorescein isothiocyanate (FITC), causing the cells to appear green in colour. As shown in the representative images of Figure 1, NECs were distributed on the entire surface of the skin in larval zebrafish, including the head, eyes, trunk, yolk sac and tail. Cell bodies appeared round in shape and were approximately 10 μm in diameter. Cells were not clustered together, but instead found in isolation. Pieces of skin taken from adult (3 month old) zebrafish showed a drastic reduction in the number of observable cells using 5-HT immunolabelling (Figure 1D, E). Skin NECs of adults were observed primarily on the tail, with very few to none observed on the trunk.

Skin NECs were associated with nerve fibres in the skin of zebrafish. As Figure 2 demonstrates with cells located in the tail, skin NECs appeared to be closely associated with nerve fibres positively labelled with the zn-12 antibody. There was a high density of zn-12 positive nerve fibres in the skin (Fig. 2B, E) and these appeared to surround and make contact with the skin NECs (Fig. 2C, F). These same associations were present with all skin NECs regardless of location.

Figure 1. Distribution of serotonergic (5-HT)-positive skin NECs of larval and adult zebrafish as labelled with an antibody against 5-HT. **A-C:** Skin NECs of a 5 day post-fertilization (d.p.f.) zebrafish. Skin NECs were distributed all over the skin surface, including the head (**A**), trunk (**B**), and tail (**C**). **D-E:** Pieces of skin from a 3 month old adult zebrafish as labelled with anti-5-HT. Skin NECs were not found in trunk skin (**D**), but were sparsely seen in tail skin (**E**). A skin NEC is indicated with an arrow in each panel. **F:** Schematic diagram of larval zebrafish showing location of images in A-C. Scale bar = 50 μm in A-C; 100 μm in D-E.

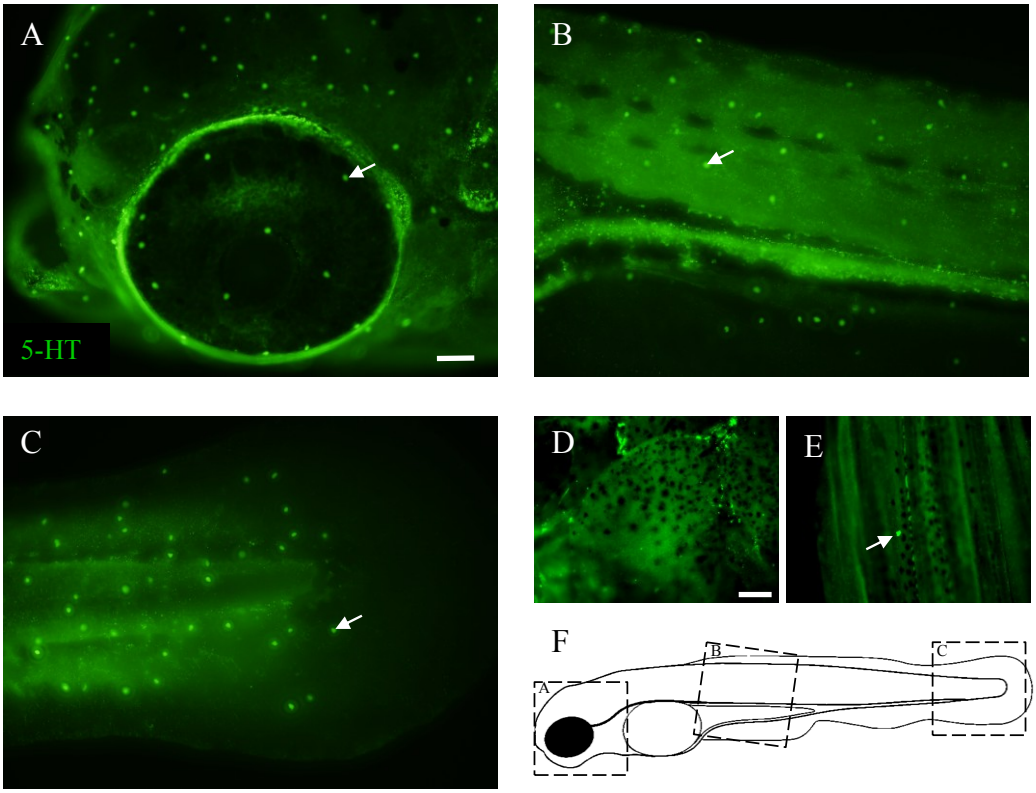
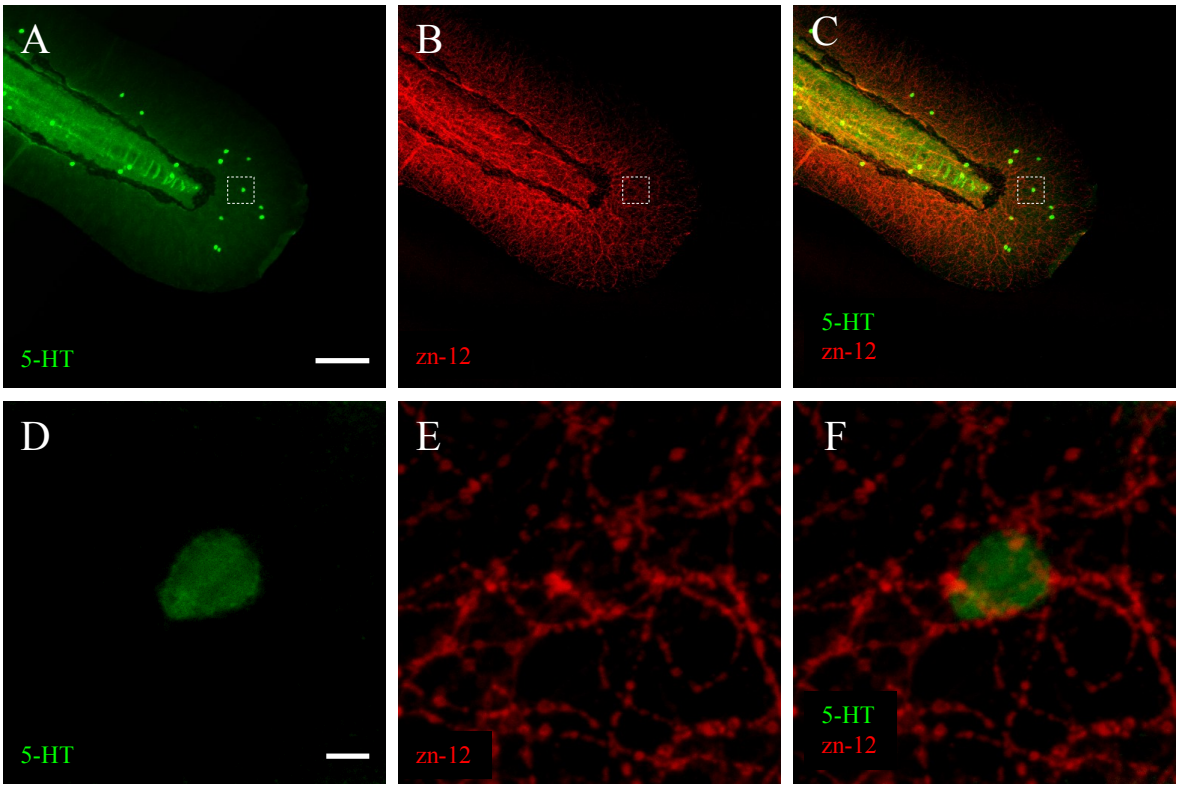

A
5-HT
B
C
D
E
F
A
B
C

Figure 2. Innervation of skin NECs with zn-12-positive nerve fibres. **A-C:** Distribution of 5-HT positive skin NECs (**A**) and zn-12 positive nerve fibres (**B**) of the tail in a 5 d.p.f. larva. **C:** 5-HT and zn-12 labelling in A and B shown together revealed that skin NECs were intimately associated with nerve fibres. **D-F:** Higher magnification of areas outlined in A-C of skin NECs and associated nerve fibres. **D:** One 5-HT positive skin NEC. **E:** zn-12 labelled nerve fibres. **F:** Panels D and E shown together show nerve associations with skin NEC. Scale bar = 100 μm in A-C; 5 μm in D-F.


A
5-HT
B
zn-12
C
5-HT
zn-12
D
5-HT
E
zn-12
F
5-HT
zn-12

3.2 Development of skin NECs and effects of water PO_2

To determine when skin NECs and their nervous innervation first appeared in developing zebrafish, embryos from 24-48 hours post-fertilization (h.p.f.) were examined after co-labelling with 5-HT and zn-12 antibodies. 5-HT immunolabelling indicated that NECs were not present at 24 h.p.f., although zn-12-immunoreactive nerve fibres had already begun to invade regions where NECs would later be found (Figure 3A, B). At 26-28 h.p.f., NECs were numerous in the skin and were predominately associated with nerve fibres (Figure 3C-F). NECs continued to increase in number until 48 h.p.f. Therefore, these observations indicated that nerve fibres appear in the skin first and NECs arise between 24 and 26 h.p.f.

Morphological parameters of skin NECs, such as number, density per tissue area and size (i.e. two-dimensional projection area) were obtained in larvae at different developmental stages and under different values of water PO_2. Figure 4 displays the change in number and density of NECs under control conditions (150 Torr), mild and severe hypoxia (80 and 30 Torr) and hyperoxia (300 Torr). Interestingly, under control conditions the number and density of NECs decreased over time, with the lowest number and density occurring at 7 d.p.f. (59±6 cells and 262±25 cells mm^{-2}, respectively, Fig. 4A). A similar decrease occurred when fish were reared under chronic mild hypoxia (80 Torr), with the lowest number of cells occurring at 9 d.p.f. (70±9 cells, Figure 4B). A more severe level of hypoxia (30 Torr) elicited no significant decrease of skin NECs occurring at any developmental stage. No significant changes in density were evident under chronic mild (Fig. 4B) and severe hypoxic (Fig. 4C) treatment. When reared under chronic hyperoxia (300 Torr), the number of NECs was markedly lower at 3 d.p.f. and were further reduced at 5 d.p.f. ($p<0.01$; paired t-test); whereas no change, with respect to density, was evident over development (Fig. 4D). As summarized in Figure 4E, these data collectively show

a significant effect of water PO_2 on NEC populations during early development, such that the developmental loss of skin NECs was reduced or delayed by chronic hypoxia (7 and 9 d.p.f., $p<0.01$; two-way ANOVA-Bonferroni test) and accelerated by chronic hyperoxia (3 and 5 d.p.f., $p<0.01$; two-way ANOVA-Bonferroni test), when compared to fish reared under control conditions. Compared to control conditions, as shown in Figure 4F the density of skin NECs was significantly higher during all stages of development during chronic, severe hypoxia exposure, including 3 d.p.f. ($p<0.001$; two-way ANOVA-Bonferroni test). This notable difference is likely related to an overall decrease in body size due to hypoxia, since absolute number remains stable and the fish is not increasing in length as normally observed in normoxia (see Appendix I). As with cell number, the density of NECs is significantly decreased when fish were chronically exposed to hyperoxic conditions (3 and 5 d.p.f., $p<0.001$; two-way ANOVA-Bonferroni test).

Two-dimensional area of skin NECs, a measure of cell size, was generally consistent throughout development in controls and larvae exposed to mild or severe hypoxia (Figure 5A-C). Cell area ranged between 31 and 33 μm^2 under these conditions. However, the area of skin NECs decreased significantly during chronic exposure of fish to chronic hyperoxia, producing areas as low as 6.2 ± 0.5 μm^2 ($p<0.01$; ANOVA-Bonferroni test; Fig. 5D). As summarized in Figure 5E, skin NECs were smaller at 3 d.p.f. during the 30 Torr exposure ($p<0.01$; ANOVA-Bonferroni test) and from 3-7 d.p.f. during 300 Torr exposure ($p<0.01$; ANOVA-Bonferroni test), compared to controls.

Figure 3. Skin-NECs and their innervation in embryonic zebrafish as labelled with 5-HT and zn-12 antibodies. **A:** 24 hours post-fertilization (h.p.f.) zebrafish tail with no observable skin-NECs. **B:** 24 h.p.f. zebrafish tail showing zn-12 nerve fibre labelling of image in A. **C:** 26 h.p.f. zebrafish tail with numerous skin-NECs (one indicated with arrow). **D:** zn-12 labelling of image in E showing association of nerve fibres with most skin-NECs (arrow). **E:** 28 h.p.f. zebrafish tail with numerous skin-NECs (arrow). **F:** image in G showing innervation of skin-NECs (arrow). Scale bar =100 μm and applies to panels A-F.

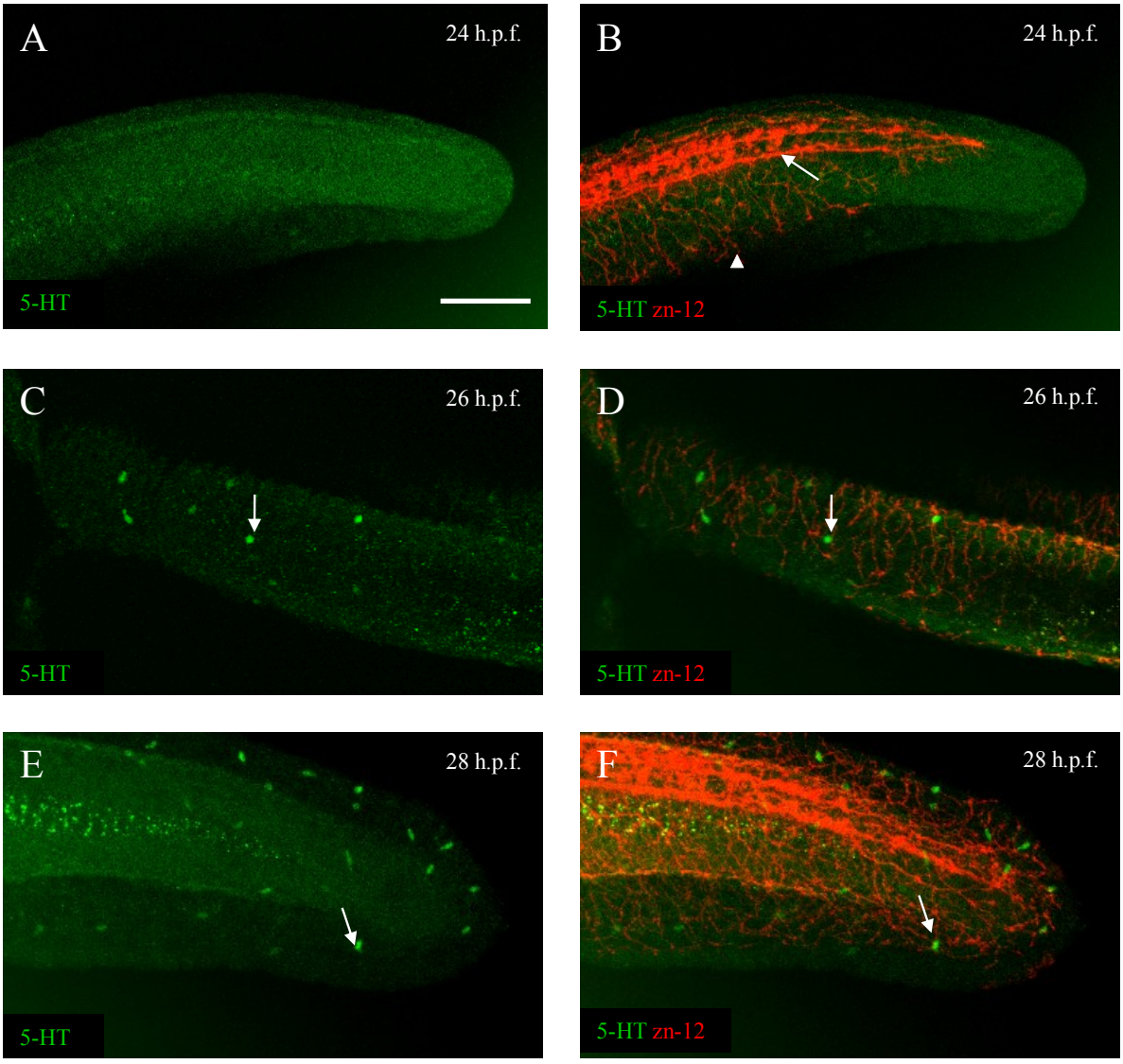

A
24 h.p.f.
5-HT
B
24 h.p.f.
5-HT zn-12
C
26 h.p.f.
5-HT
D
26 h.p.f.
5-HT zn-12
E
28 h.p.f.
5-HT
F
28 h.p.f.
5-HT zn-12

Figure 4. The number and density (mm^{-2}) of NECs found on the skin of developing zebrafish (3-9 d.p.f.) is dependent on developmental stage and water PO_2. **A:** Under control rearing conditions (PO_2 = 150 Torr) the number (closed circles) and density (open circles) of skin NECs decreased with time. **B:** Similarly to A, number and density decreased over time in zebrafish subject to chronic exposure to mild hypoxic (PO_2 = 80 Torr) rearing conditions. **C:** Severe hypoxic rearing conditions (PO_2 = 30 Torr) resulted in no change of NEC number and density during the stages tested. **D:** Chronic exposure to hyperoxia (PO_2 = 300 Torr) led to a more rapid decline in both number and density of skin NECs. Asterisks in panels A-D indicate significant difference from 3 d.p.f. (A-C: $p<0.01$; ANOVA-Bonferroni test, D: $p<0.01$; paired-t-test). Mean (± S.E.M) are indicated. **E:** Line graph summarizes data in panels A-D of skin NECs under hypoxic (30 Torr) and hyperoxic (300 Torr) rearing conditions, respectively, compared to control conditions. Asterisks indicate significant difference from control ($p<0.01$; two-way ANOVA-Bonferroni test). **F:** Line graph summarizes density data in panels A-D of skin NEC density under hypoxic (30 Torr) and hyperoxic (300 Torr) rearing conditions, respectively, compared to control conditions. Asterisks indicate significant difference from control ($p<0.001$; two-way ANOVA-Bonferroni test). Sample size ≥8 in all cases, except for hyperoxia at 7 d.p.f., where n=1.

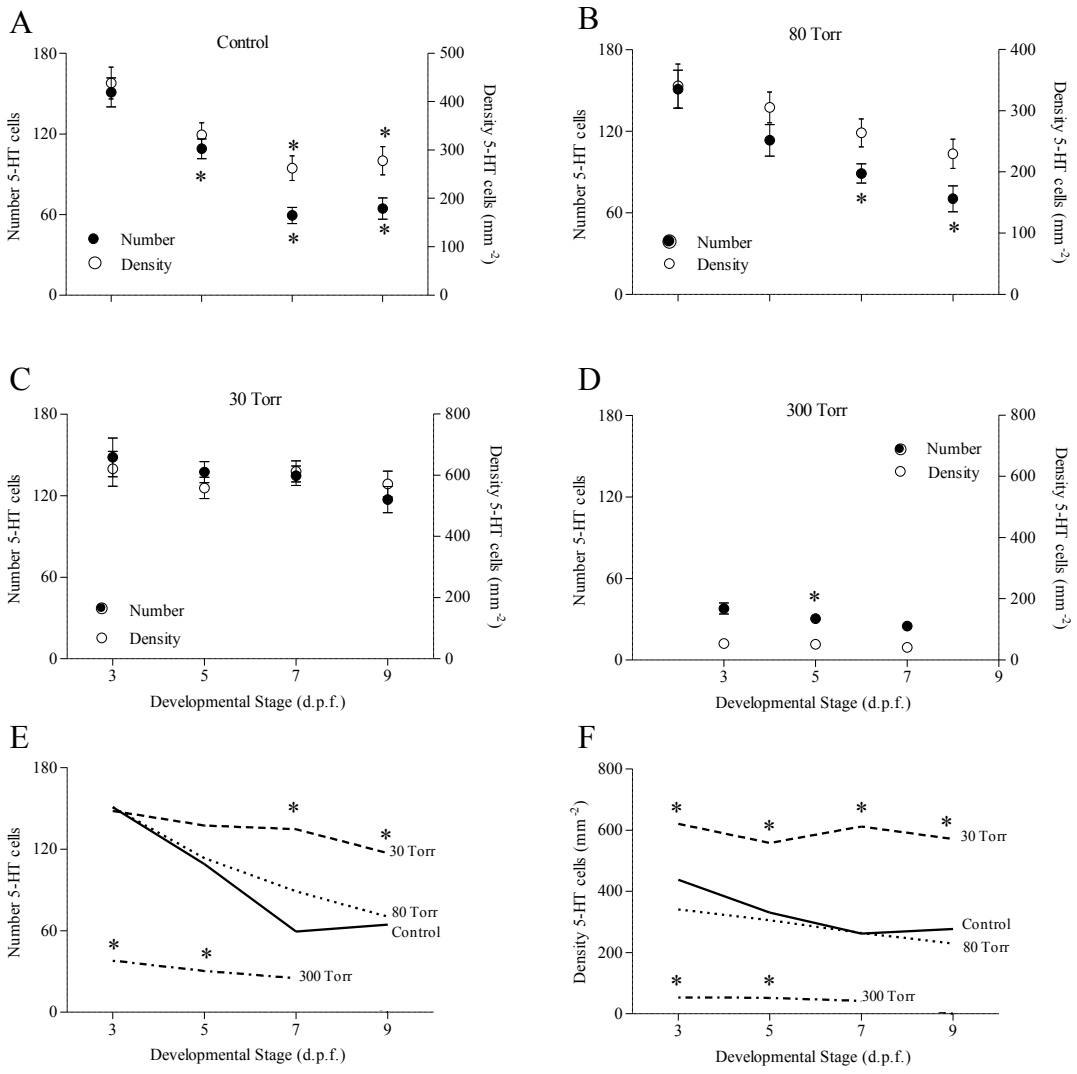
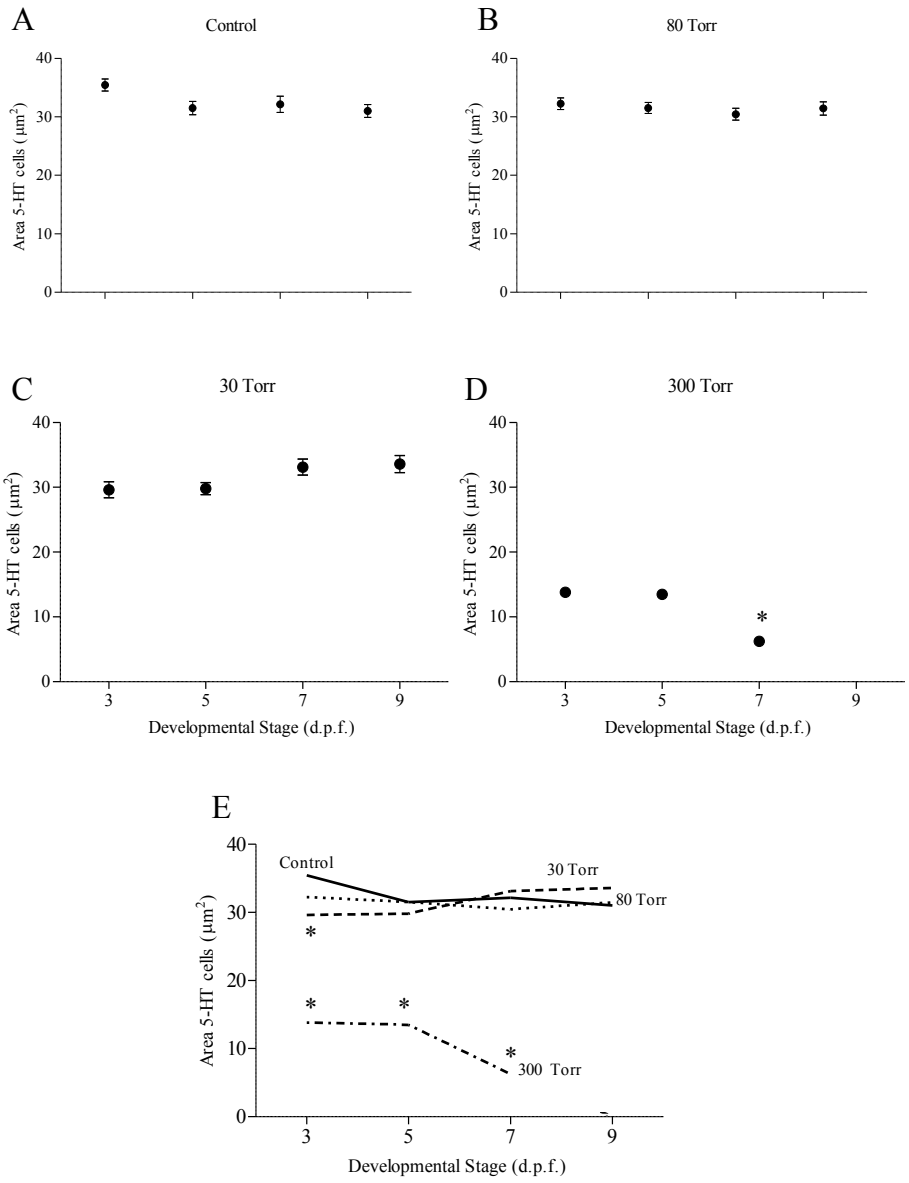

A
Control
Number 5-HT cells
Density 5-HT cells (mm-2)
Number
Density
B
80 Torr
C
30 Torr
Developmental Stage (d.p.f.)
D
300 Torr
E
30 Torr
80 Torr
Control
300 Torr
F
Control
80 Torr
300 Torr

Figure 5. The size of skin NECs is affected by chronic hyperoxia but not chronic hypoxia in 3-9 d.p.f. zebrafish. Projection area of skin NECs chronically exposed to (**A**) normoxia (PO_2 = 150 Torr), (**B**) mild hypoxia (PO_2 = 80 Torr), (**C**) severe hypoxia (PO_2 = 30 Torr) and (**D**) hyperoxia (PO_2 = 300 Torr). Mean (± S.E.M) are indicated. Area of cells decreased over time in hyperoxia (D; asterisk indicates significant difference from 3 d.p.f. ($p<0.01$; ANOVA-Bonferroni test). **E:** Line graph summarizing data from panels A-D showing significant difference from control with asterisks ($p<0.01$; two-way ANOVA-Bonferroni test). Sample size ≥ 25 in all cases, except for hyperoxia at 7 d.p.f., where n = 5.


A
Control
B
80 Torr
C
30 Torr
D
300 Torr
E
Area 5-HT cells (μm²)
Developmental Stage (d.p.f.)
Control
30 Torr
80 Torr
300 Torr
*

3.3 Development of the hypoxic response

Zebrafish embryos at 1 d.p.f. display spontaneous whole-body movements whose frequency was decreased from 14±2 min^{-1} to 1±0.4 min^{-1} at 2 d.p.f. (Figure 6A). During these early stages, the frequency of whole-body movement was increased by application of hypoxia at 2 d.p.f. to 8±2 min^{-1} but not at 1 d.p.f. (Fig. 6A). This behaviour has been discussed in previous work (Jonz and Nurse, 2005), and is thought to improve cutaneous gas exchange in developing embryos (Rombough, 1988). The response to hypoxia at 3 d.p.f. and later was measured as an increase of the frequency of buccal or opercular movements (hyperventilation) which were not observed at earlier stages. Hyperventilation was maintained for the rest of the developmental stages observed, as shown in Figure 6B, and reached its peak of 168±13 min^{-1} at 5 d.p.f.

While hyperventilation is a typical response to hypoxic stimuli in the zebrafish and many other species of fish (Perry *et al*., 2009), this response did not occur when zebrafish were raised under conditions in which water PO_2 was changed, as shown in Figure 7. Chronic exposure of zebrafish to either hyperoxia (300 Torr; Fig. 7B) or hypoxia (30 Torr; Fig. 7C) resulted in development of a hypoventilatory response (or decrease in ventilation frequency) when exposed to acute hypoxia. The most prominent changes in ventilation frequency observed were from 36±8 min^{-1} to 1.3±1.1 min^{-1} in larvae pre-exposed to hyperoxia (Fig. 7B) and from 40±10 min^{-1} to 10.5±4 min^{-1} in larvae pre-exposed to hypoxia (Fig. 7C). As well, a shift in peak basal ventilation was evident under these rearing conditions, as shown in Figure 7A-C and summarized in Figure 7D. Under control conditions, a peak basal ventilation frequency of 61±7 min^{-1} was first observed at 4 d.p.f., and maintained at this approximate level until 6 d.p.f. ($p>0.05$; ANOVA-Bonferroni test) before decreasing in magnitude. Under hyperoxic rearing conditions, peak basal ventilation frequency shifted to 4 d.p.f. and was reduced to 36±8 min^{-1} (Fig. 7B, D).

Conversely, under hypoxic rearing conditions, the peak basal ventilation frequency shifted to a later stage at 6 d.p.f. and was reduced to 40±10 min^{-1} (Fig. 7C, D). These shifts in peak basal ventilation frequency in acclimated larvae, and the onset of a hypoventilatory response to acute hypoxia (rather than a hyperventilatory response), may suggest underlying morphological changes of O_2 chemoreceptors. Interestingly, shifts in peak basal ventilation corresponded to changes in the number and density of skin NECs reared under these same conditions, where hypoxic-acclimated zebrafish displayed more skin NECs with a delayed development of peak basal ventilation frequency, while hyperoxic-acclimated zebrafish displayed a rapid loss of skin NECs and a rapid development of peak basal ventilation (recall section 3.2 and Fig. 4).

3.4 Innervation of skin NECs by catecholaminergic nerve fibres

It was hypothesized that if the skin NECs share a similar role in O_2 chemoreception as gill NECs, their innervation pattern would be similar. Gill NECs have been shown to be innervated by at least three different nerve fibre types, including those that are catecholaminergic and of spinal (sympathetic) origin (Jonz and Zaccone, 2009). Therefore the effects of the catecholaminergic nerve toxin, 6-hydroxydopamine (6-OHDA), were determined to see if this treatment would cause degeneration of nerves of the skin that make contact with serotonergic NECs (Figure 8). The nerve density of zn-12-positive fibres was greatly reduced in zebrafish treated with 250 μM 6-OHDA for 5 days (Fig. 8A, B). Upon closer inspection of nerve fibre varicosities (presumed regions of contact with other cells or axons), it was found that the distance between nerve varicosities (an inverse measure of the density of synaptic contacts along a single nerve fibre) was significantly increased with 6-OHDA treatment, and this effect was dependent on 6-OHDA concentration and exposure time (Fig. 8C, D, G). Moreover, compared

Figure 6. Development of behavioural responses to hypoxia in zebrafish from 1-9 d.p.f. **A:** Measurement of whole body movements indicated that fish first respond to hypoxia at 2 d.p.f. **B:** Increased ventilation in response to hypoxia is evident at 4 d.p.f. and continues for the rest of development. Acute hypoxia exposure PO_2 = 23 Torr. Sample sizes indicated in the figure. Asterisks indicate significant difference from control ($p<0.05$; two-way ANOVA-Bonferroni test).

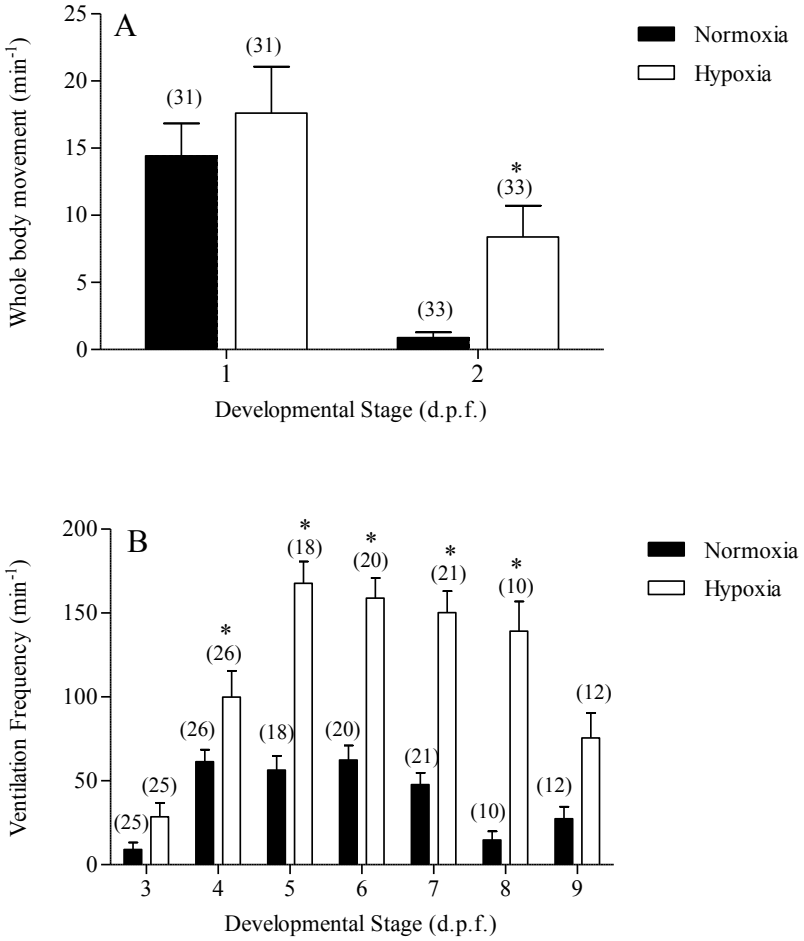
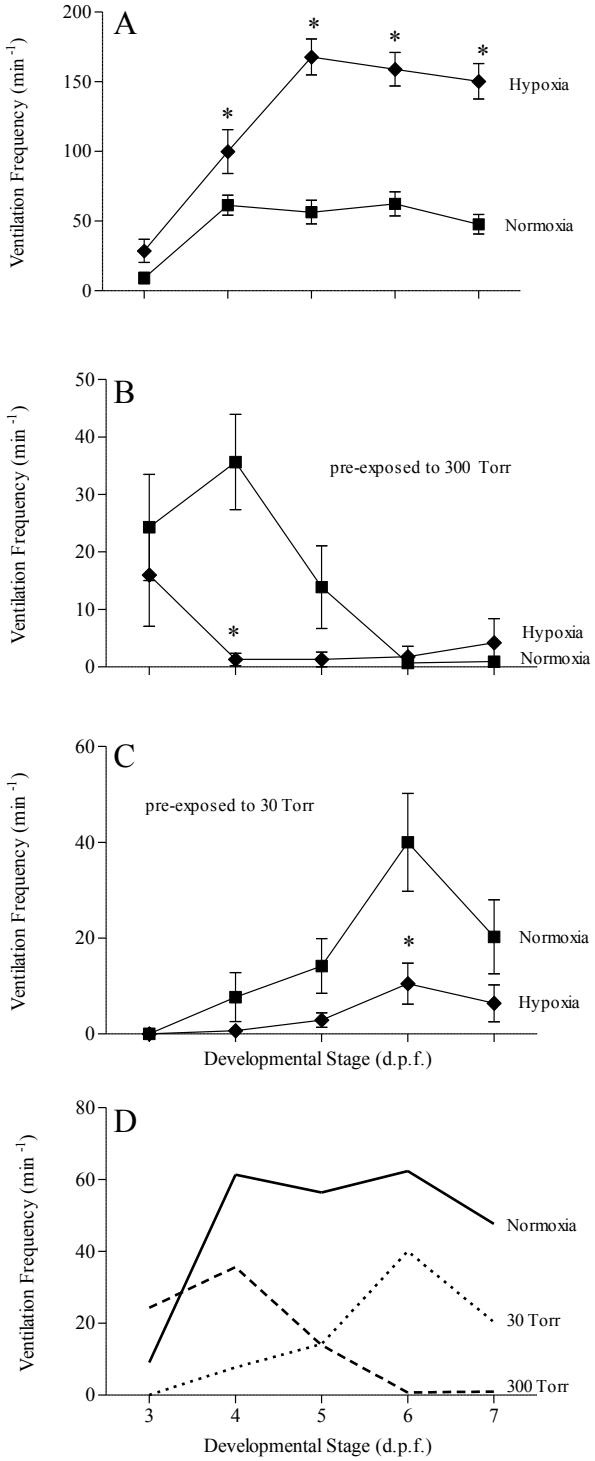

A
Normoxia
Hypoxia
Whole body movement (min-1)
25
20
15
10
5
0
(31)
(31)
(33)
*
(33)
1
2
Developmental Stage (d.p.f.)
B
Normoxia
Hypoxia
Ventilation Frequency (min-1)
200
150
100
50
0
(25)
(25)
(26)
*
(26)
(18)
*
(18)
(20)
*
(20)
(21)
*
(21)
(10)
*
(10)
(12)
(12)
3
4
5
6
7
8
9
Developmental Stage (d.p.f.)

Figure 7. Chronic exposure of zebrafish to hypoxia and hyperoxia caused a hypoventilatory response to hypoxia and a shift in peak basal ventilation frequency. **A:** Zebrafish raised in normoxic conditions (PO_2 = 150 Torr) hyperventilated when exposed to acute hypoxia (data from Fig. 6B). **B:** When raised in hyperoxic conditions (PO_2 = 300 Torr), zebrafish hypoventilated when exposed to acute hypoxia. Note also the shift in peak basal ventilation from 5 d.p.f (in A) to 4 d.p.f. **C:** Pre-exposure to hypoxic conditions (PO_2 = 30 Torr) resulted in a hypoventilatory response to acute hypoxia. Note also the shift in peak basal ventilation from 5 d.p.f. (in A) to 6 d.p.f. Acute hypoxia PO_2 = 23 Torr. Sample size $\geq$ 18 for all treatments and stages. Asterisks indicate significant difference from normoxia ($p<0.05$; two-way ANOVA – Bonferroni test). **D:** Summary of data from A-C illustrates that peak basal ventilation is shifted to a later developmental stage (6 d.p.f.) when fish were raised in hypoxic conditions, whereas chronic exposure to hyperoxia shifted the peak to an earlier developmental stage (4 d.p.f.), compared to normoxic rearing conditions.


A
Ventilation Frequency (min-1)
200
150
100
50
0
*
*
*
*
Hypoxia
Normoxia
B
Ventilation Frequency (min-1)
50
40
30
20
10
0
pre-exposed to 300 Torr
*
Hypoxia
Normoxia
C
Ventilation Frequency (min-1)
60
40
20
0
pre-exposed to 30 Torr
*
Normoxia
Hypoxia
Developmental Stage (d.p.f.)
D
Ventilation Frequency (min-1)
80
60
40
20
0
Normoxia
30 Torr
300 Torr
3
4
5
6
7
Developmental Stage (d.p.f.)

to controls, the number of synaptic contacts between NECs and zn-12-positive nerve fibre varicosities was diminished in zebrafish treated with 6-OHDA (Fig. 8E, F, H). These data indicate that the skin and, more specifically skin NECs, received catecholaminergic innervation. Since not all zn-12-positive nerve fibres and varicosities had degenerated following 6-OHDA treatment, this suggests that the serotonergic skin cells receive additional multiple innervation by non-catecholaminergic nerve terminals that are not sensitive to 6-OHDA, as do NECs of the gill.

Behavioural experiments conducted on zebrafish after prolonged treatment with 6-OHDA showed a change in both basal and hypoxia-induced ventilation frequency (Figure 9). When chronically exposed to 6-OHDA, basal ventilation frequency was significantly reduced compared to control from 4-6 d.p.f. (Fig. 9A; $p<0.05$; two-way ANOVA-Bonferroni test), suggesting that the ventilatory drive that may normally be provided by peripheral chemoreceptors was being suppressed by 6-OHDA-induced nerve terminal degradation. When further stimulated with acute hypoxia, 6-OHDA treated fish were unable to hyperventilate, as shown in Figure 9B, and the response to hypoxia was significantly reduced when compared to that of untreated controls ($p<0.001$; two-way ANOVA-Bonferroni test). These data suggest that the effects of 6-OHDA may be on innervation of peripheral chemoreceptors in the skin.

An alternative explanation to these data is that 6-OHDA had other negative effects directly on the animals' capacity to hyperventilate. To rule out the putative effects of 6-OHDA treatment on the motor ventilatory reflex, zebrafish were acutely exposed to NaCN, a potent ventilatory stimulant, to artificially induce hyperventilation. As Figure 9C demonstrates, both control and 6-OHDA treated zebrafish tested at 5 d.p.f. responded equally well to NaCN in the form of hyperventilation ($p<0.01$; two-way ANOVA-Bonferroni test). These results confirm that 6-OHDA treatment does not directly impair the ability of larvae to hyperventilate.

Figure 8. 6-hydroxydopamine (6-OHDA) exposure leads to neuronal loss in the skin of developing zebrafish and loss of contact between skin NECs and zn-12 positive nerve fibres. **A-B:** Compared to control, 6-OHDA treated fish had lost zn-12-positive nerve fibres, as shown in the tail of this 7 d.p.f. larva. Larvae were treated for 5 days with 6-OHDA at a concentration of 250 μM. **C-D:** Compared to control, 6-OHDA treated fish had a longer distance between varicosities, suggesting reduced varicosity density. **C:** 5 d.p.f. control. **D:** 7 d.p.f. treated for 5 days with 6-OHDA at a concentration of 250 μM. **E-F:** Images of skin NEC with associated nerve fibres. **E:** 5 d.p.f. control. **F:** 7 d.p.f. larva treated for 5 days with 6-OHDA at a concentration of 250 μM showed reduced points of contact between nerve fibres and NEC. **G:** Distance between varicosities was significantly increased by 3 day treatment of 500 μM 6-OHDA and 5 day treatment of 250 μM 6-OHDA. Sample sizes indicated in the figure. Asterisk indicates significant difference from control ($p<0.05$; paired t-test). **H:** Points of contact of nerve varicosities or terminals on skin NECs was significantly reduced only by application of 250 μM 6-OHDA for 3 days. Sample sizes indicated in the figure. Asterisk indicates significant difference from control ($p<0.05$; paired t-test). Scale bar = 100 μm in A and B; 5 μm in C and D; 5 μm in E and F.

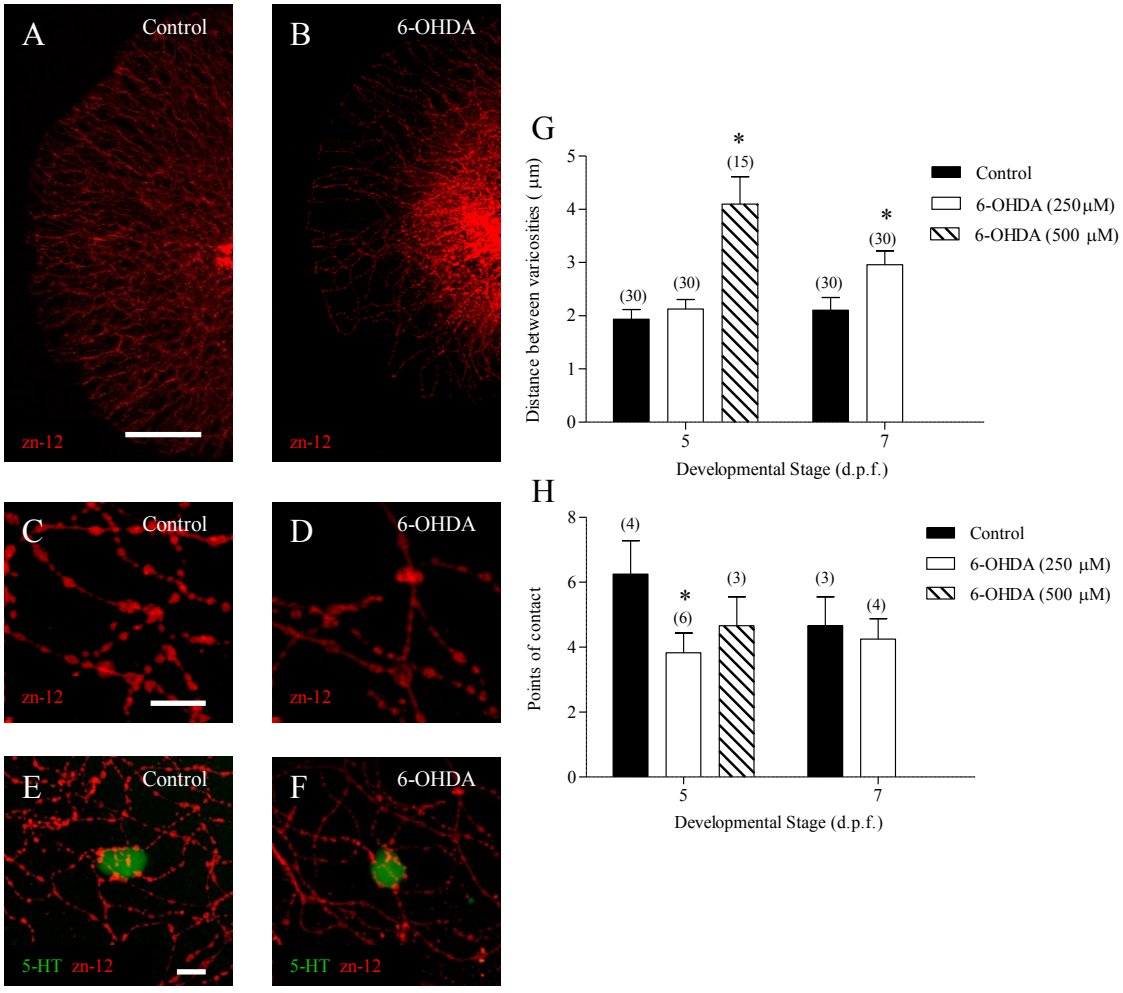
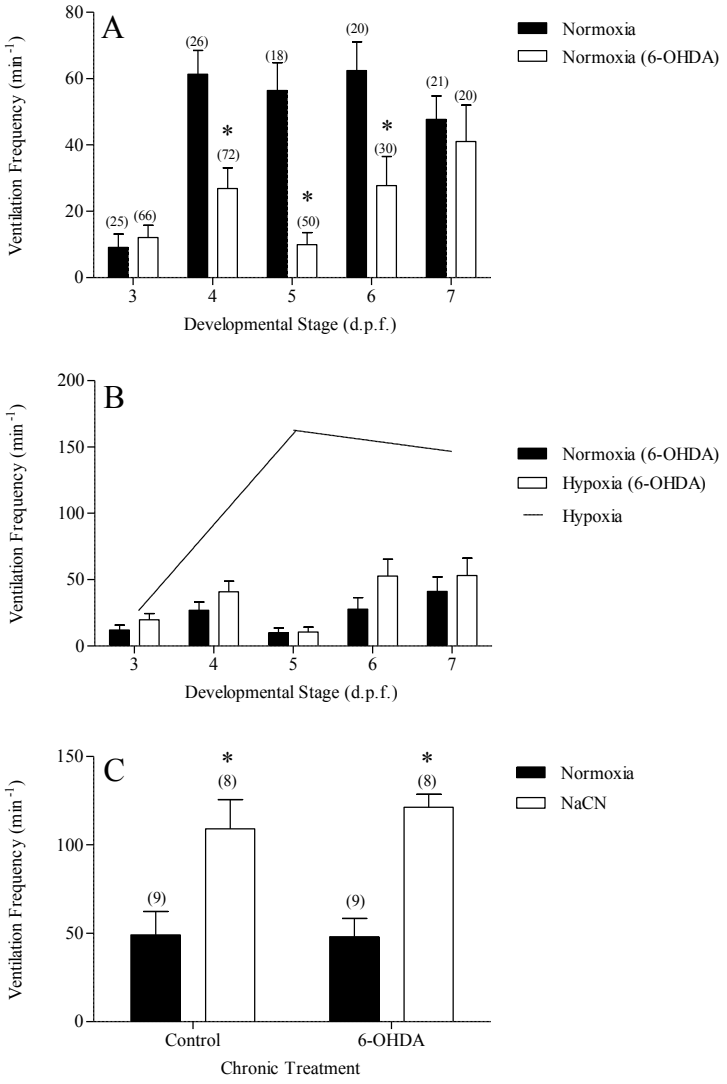

A
Control
zn-12
B
6-OHDA
zn-12
C
Control
zn-12
D
6-OHDA
zn-12
E
Control
5-HT zn-12
F
6-OHDA
5-HT zn-12
G
Distance between varicosities (µm)
Developmental Stage (d.p.f.)
Control
6-OHDA (250µM)
6-OHDA (500 µM)
(30)
(30)
(15)
(30)
(30)
H
Points of contact
Developmental Stage (d.p.f.)
Control
6-OHDA (250 µM)
6-OHDA (500 µM)
(4)
(6)
(3)
(3)
(4)

Figure 9. 6-OHDA treatment reduced basal ventilation frequency and eliminated response to acute hypoxia. **A:** Basal ventilation frequency was reduced in fish treated with 250 μM 6-OHDA compared to control (normoxia) from 4-6 d.p.f.. **B:** Zebrafish did not hyperventilate when exposed to acute hypoxia after 6-OHDA treatment. Hyperventilation in control fish (see Fig. 6B) when exposed to hypoxia is shown as a trend line for comparison. **C:** 6-OHDA treated zebrafish (5 d.p.f.) hyperventilated when exposed to sodium cyanide (NaCN). Sample sizes indicated in the figure. Asterisks in A and C indicate significant difference from control (A: $p<0.05$; C: $p<0.01$; two-way ANOVA-Bonferroni test).


A
Ventilation Frequency (min-1)
Developmental Stage (d.p.f.)
Normoxia
Normoxia (6-OHDA)
(25)
(66)
(26)
(72)
(18)
(50)
(20)
(30)
(21)
(20)
*
B
Ventilation Frequency (min-1)
Developmental Stage (d.p.f.)
Normoxia (6-OHDA)
Hypoxia (6-OHDA)
Hypoxia
C
Ventilation Frequency (min-1)
Chronic Treatment
Control
6-OHDA
Normoxia
NaCN
(9)
(8)

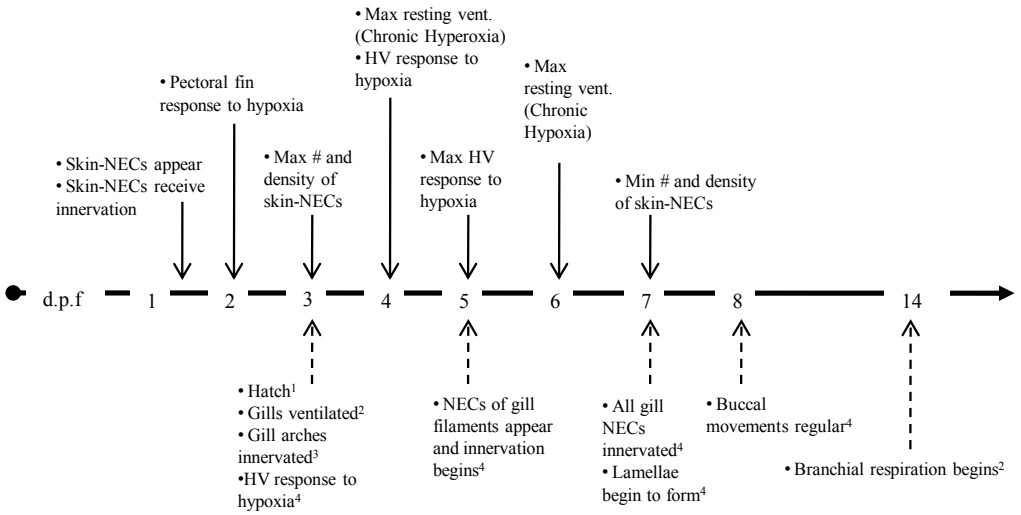
4. DISCUSSION

This study focused on characterizing neuroepithelial cells (NECs) found on the skin of developing zebrafish. Although skin NECs were briefly noted in one study as putative O_2 chemoreceptors (Jonz and Nurse, 2006), their detailed morphology and potential role in development have not been established. The purpose of the current thesis, therefore, was to further describe the morphology, distribution and development of skin NECs in embryos and larvae, and examine their putative role in O_2 sensing. Figure 10 summarizes the results of this study.

4.1 Skin NECs as oxygen chemoreceptors

There exists evidence of chemosensory cells in the skin of both developing and adult fish, which include taste buds (TBs) and solitary chemoreceptor cells (SCCs; Kotrschal *et al*., 1997; Hansen *et al*., 2002), so it is reasonable to propose that other chemosensory cells (i.e. O_2 sensing cells) exist in the skin. Compared to characteristics of TBs and SCCs, the skin NECs appear to be a distinct population of cells, not like the other surface chemoreceptors found on the zebrafish skin. All three cell types can be found on the entire body surface, with the distribution of TBs being somewhat limited to the head in most species. There is an even distribution of skin NECs and SCCs over the body surface; however, the cells in the present study have a higher density on the body and head compared to the tail, which differs from the density distribution of SCCs. While the skin NECs decrease in number and density as the zebrafish develops, SCCs and cells of the TBs increase with development (Kotrschal *et al*., 1997; Hansen *et al*., 2002). Therefore, these notable differences define the skin NECs as a newly described surface chemoreceptor.

Figure 10. Chronology of the development of ventilatory events and structures in zebrafish. Solid arrows correspond to results obtained from the present study and dashed arrows indicate work of other authors. [1]Kimmel *et al*., 1995; [2]Rombough, 2002; [3]Higashijima *et al*., 2000; [4]Jonz and Nurse, 2005. d.p.f., days post-fertilization; NECs, neuroepithelial cells; HV, hyperventilatory.


• Skin-NECs appear
• Skin-NECs receive innervation
• Pectoral fin response to hypoxia
• Max # and density of skin-NECs
• Max resting vent. (Chronic Hyperoxia)
• HV response to hypoxia
• Max HV response to hypoxia
• Max resting vent. (Chronic Hypoxia)
• Min # and density of skin-NECs
d.p.f
1
2
3
4
5
6
7
8
14
• Hatch[1]
• Gills ventilated[2]
• Gill arches innervated[3]
•HV response to hypoxia[4]
• NECs of gill filaments appear and innervation begins[4]
• All gill NECs innervated[4]
• Lamellae begin to form[4]
• Buccal movements regular[4]
• Branchial respiration begins[2]

Characterizing skin NECs as oxygen chemoreceptors requires a comparison between these cells and existing identifiable oxygen chemoreceptors, such as NECs of the fish gill, neuroepithelial bodies (NEBs) of the mammalian lung, carotid body (CB) type I cells, and adrenomedullary chromaffin cells (AMCs). NEBs, NECs, AMCs and type I cells function as oxygen chemoreceptors, releasing neurotransmitters to produce appropriate sensory responses, such as hyperventilation, to hypoxic challenges (Cutz and Jackson, 1999; Lopez-Barneo *et al*., 2008; Nurse *et al*., 2009; Perry *et al*., 2009). All of these O_2 chemoreceptors share commonalities with the currently studied skin NECs. The presence of the neurotransmitter serotonin is an important indicator of some oxygen chemoreceptors (e.g. Cutz and Jackson, 1999; Perry *et al*., 2009) and is present in the cells of the present study (Figure 1). Hypoxia evokes serotonin secretion in mammalian NEBs, which produces local vascular responses (Cutz and Jackson, 1999; Fu *et al*., 2002). In addition, serotonin release has also been shown to be important for vascular changes in the fish gill (Sundin *et al*., 1995). The role of serotonin as a neurotransmitter involved in oxygen sensing in the gill, however, has yet to be established.

Each type of peripheral O_2 chemoreceptor is also in contact with sensory nerve fibres in order to transmit nerve signals associated with hypoxic stimulation to the central nervous system, and skin NECs also appeared to make associations with nerve fibres (Figure 2). As previously discussed (see sections 1.2.2, 1.2.3), one type of sensory nerve fibre CB type I cells and gill NECs establish contact with is of the catecholaminergic type. Utilizing the neurotoxin 6-hydroxydopamine (6-OHDA), it was demonstrated that the skin of zebrafish contained catecholaminergic innervation, and skin NECs also established connections with these catecholaminergic nerve fibres (Figure 8). However, not all nerve terminals in contact with skin NECs were destroyed by 6-OHDA (Figure 8F), suggesting that these cells make contact with

other types of nerve fibres. Other types of nerve fibres which establish contact with other oxygen chemosensory cells, such as parasympathetic efferent nerves, afferent serotonergic nerves and efferent nitrergic nerves (Campanucci and Nurse, 2007; Jonz and Zaccone, 2009) may also establish contact with skin NECs. However, characterization of such additional innervation will require further study.

Skin NECs were present and appeared innervated well before 2 days post-fertilization (d.p.f.) in developing zebrafish (Figure 3). This phenomenon coincided with the approximate time when zebrafish were no longer capable of anoxia-induced metabolic supression, an indication of anoxia tolerance (>24 hours post-fertilization, h.p.f., Padilla and Roth, 2001), and when a hypoxic response was first observed in the form of increased pectoral fin and whole body movements (2 d.p.f., Jonz and Nurse, 2005; Figure 6A). Innervation of the skin NECs several hours before the embryo could actively respond to hypoxia may represent the first step in the development of a functional chemosensory pathway. The early embryo appears to be in a transition state from which it conforms to hypoxia (or anoxia) at 24 h.p.f. by metabolic suppression to one in which it will respond to hypoxia at 2 d.p.f. The small size of the embryo at these transitional stages may preclude any need for a response to hypoxia since the metabolic requirements of oxygen uptake are met through cutaneous respiration (Rombough, 2002).

Nerve fibres positively labelled with zn-12 antibody appeared in the skin before skin NECs were observed (Figure 3), suggesting that innervation may induce the development of skin NECs. The neural induction model has been proposed as the mechanism for TB development (Northcutt, 2004), although another study done in zebrafish found that innervation of TBs occurred after TB primordia, suggesting an induction model which is independent of innervation (Hansen *et al*., 2002).

The number and density of skin NECs gradually decreased over days 3-9 of development (Figure 4A), reaching their lowest values at 7 d.p.f.; the same day that gill NECs are identified as becoming functional because all of the cells receive sensory innervation (Jonz and Nurse, 2005). The decrease of skin NECs over development suggests that a functional role for these cells may be confined to early developmental stages (i.e. before 7 d.p.f.), especially since skin NEC populations are markedly decreased in 3 month old adults (Figure 1D, E), although whether the skin NECs are active or not in adults is unknown. As the gills become functional for both respiration and O_2 sensing later in larval development, zebrafish no longer rely solely on the skin for respiration (Rombough, 2002). Thus, the reduction in number and density of skin NECs during this time may be related to this shift in respiration from the skin to gills. As demonstrated in the mammalian model, the AMCs are highly sensitive to hypoxia during the first few weeks of life and become developmentally insensitive as the CB type I cells receive innervation and become mature (Nurse *et al*., 2009); thus, it is not unreasonable to propose the same mechanism could hold true for the zebrafish. The skin NECs may serve as the early oxygen sensors until gill NECs are mature and take over as the adult oxygen chemoreceptors.

Exposing zebrafish to chronic hypoxia and hyperoxia during the first 9 days of development affected the number and density of skin NECs (Figure 4B-F). Chronic hypoxia has been said to be a teratogen and negatively affect fish development (Shang and Wu, 2004; Wu, 2009). However, the number and density of skin NECs did not significantly change when zebrafish were raised in severe hypoxia (PO_2= 30 Torr), compared to the developmental loss of these cells in controls. This relative increase in both number and density with severe hypoxic treatment indicates that the number of skin NECs may be upregulated during chronic hypoxia, a response typical of O_2 chemoreceptors (Nurse and Vollmer, 1997; Wang and Bisgard, 2002; Jonz *et al*.,

2004). It is unknown whether this upregulation is due to a reduction in cell death, or an increase in cell division. Furthermore, it is interesting to note that chronic hypoxia appears to slow the development of gill NECs in zebrafish beyond 7 d.p.f. during early larval stages (Shakarchi and Jonz, unpublished observations), so the persistence of more skin NECs (with putative O_2 chemosensitivity) during this time may be beneficial. Chronic hypoxia appeared to slow body growth, and produced developmental tachycardia (see Appendix I), but it did not appear to impede the stability of the skin NEC population, thereby strengthening the conclusion that hypoxia was having a specific effect on these cells. The two-dimensional projection area of skin NECs during hypoxic exposure (Figure 5B, C) was unaffected, indicating that hypoxia does not induce hypertrophy in skin NECs. This is in contrast to acclimation studies of other oxygen chemoreceptors, as hypoxia usually led to an increase in area (Jonz *et al*., 2004; Nurse and Vollmer, 1997; Wang and Bisgard, 2002).

In mammals, chronic exposure to elevated oxygen leads to a reduction in size of the CB, fewer CB type I cells, and an attenuation of the CB's response to hypoxia (Bisgard *et al*., 2005). Hyperoxia has also been demonstrated to induce oxidative cell death (Horowitz, 1999). In adult zebrafish, the density of gill NECs decreased after chronic exposure to hyperoxic conditions (Vulesevic *et al*., 2006). Likewise, upon chronic hyperoxic exposure, the skin NEC population was severely reduced (Figure 4D) and many fish did not survive past 7 d.p.f. (N.B an n=1 at 7 d.p.f. and no values for 9 d.p.f. in Figure 4D). The reduction of skin NECs may be due to hyperoxia- induced oxidative cell death. As well, the two dimensional area of skin NECs was reduced after hyperoxic exposure (Figure 5D). Therefore, hyperoxia had a similar effect on skin NECs as it does on other oxygen chemoreceptors.

4.2 The hypoxic ventilatory response

Consistent with previous studies, zebrafish began to respond to hypoxia at 2 d.p.f. by increasing whole body and pectoral fin movements (Figure 6A; Jonz and Nurse, 2005). This early response to hypoxia has been observed in embryonic and larval fish and may facilitate gas exchange. Gill ventilation activity has been demonstrated to be coordinated with pectoral fin movements in larvae, and movement of the pectoral fin may improve gas exchange. This behaviour can, thus, be used to identify a response to hypoxia before the hyperventilatory response develops (Jonz and Nurse, 2005). Early zebrafish motor behaviour includes spontaneous side-to-side contractions beginning at 17 h.p.f., which peak in frequency by 19 h.p.f. and then decline progressively over the course of 6–7 hours (Drapeau *et al*., 2002). At hatching (2 d.p.f.) the swimming contraction frequency increases, but the larvae swim only infrequently and in bursts (Drapeau *et al*., 2002). Thus, the increased level of movement seen in the normoxic exposed fish at 1 d.p.f. (Figure 6A) may be explained by these frequent, spontaneous movements, which then decline by 2 d.p.f. Increased ventilation frequency in response to hypoxia begins at 3 d.p.f. and continues over the rest of development (Figure 6B). In contrast to a previous study, peak hyperventilation in response to hypoxia occurred at 5 d.p.f instead of 7 d.p.f. (Jonz and Nurse, 2005). This may be due in part to the decreased PO_2 used in this study compared to the other (23 Torr versus 25 Torr), or the chronological staging used in this study compared to the staging used in the other study following Kimmel *et al*. (1993).

While hyperventilation is the typical response to hypoxic stimuli in the zebrafish (Figure 6) and many other species of fish (Perry *et al*., 2009), this response can be reversed under different rearing conditions, as seen in Figure 7B, C. Chronic exposure of zebrafish to either hypoxic or hyperoxic conditions resulted in a hypoventilatory response (or decrease in ventilation

frequency) when exposed to acute hypoxia. Exposing zebrafish for the first 7 days of development to hyperoxia has been shown to alter adult reflexes to ventilatory stimuli including hypoxia, whereas chronic exposure to hypoxia does not alter these reflexes (Vulesevic and Perry, 2006). This study reasoned that there is a developmental window during maturation (i.e. the first 7 days of development) when the potential for respiratory plasticity is high. Blunting of ventilation to acute hypoxia in adults also occurs when developing mammals are exposed to chronic hypoxia or hyperoxia (Lahiri *et al*., 2000; Bisgard *et al*., 2005). Early insults to the fish (i.e. hypoxic or hyperoxic conditions) can disrupt the normal maturation of respiratory control, and these insults may be evident during this critical period of development. The fish may then be unable to hyperventilate when faced with acute hypoxia. This may be due to a reduced O_2 sensitivity of skin NECs, similarly to the reduced O_2 sensitivity of the CB in this same situation, or a change in innervation during these exposures, which has been reported in the CB (Carroll, 2003).

A shift in peak basal ventilation was evident under hypoxic and hyperoxic rearing conditions, as shown in Figure 7C. Under control conditions, the peak basal ventilation frequency was first observed at 4 d.p.f., and was maintained until 7 d.p.f. Under hyperoxic rearing conditions, the peak basal ventilation frequency shifted to 4 d.p.f. Conversely, under hypoxic rearing conditions, the peak basal ventilation frequency shifted to a later stage, 6 d.p.f. Interestingly, these shifts corresponded to changes seen in the population of skin NECs under these same rearing conditions (Figure 4). The number and density of cells were rapidly reduced, even at the earliest stages observed, when zebrafish were reared under hyperoxic conditions. During chronic hypoxia exposures, the skin NEC population did not significantly change over development and remained present in abundance at later developmental stages. This lends credence to the role of

skin NECs in sensing oxygen and playing a role in driving basal ventilation. In mammals, peripheral chemoreceptors have been shown to play an important role in maintaining the resting level of ventilation (Gonzalez *et al*., 1994). It has been found in fish, however, that peripheral chemoafferent input does not appear to be essential, but may provide some input, for resting rhythmic ventilation (Smatresk, 1990).

Upon treatment with 6-OHDA, basal ventilation frequency was decreased and the response to hypoxia was eliminated in developing zebrafish (Figure 9A, B). As shown in Figure 8F, there was a loss of nerve varicosities in contact with skin NECs, which may have contributed to the ventilation frequency changes. The CB has been shown to be important in maintaining the basic resting operation of breathing pattern generation in the brain stem, and denervation of the carotid sinus nerve led to changes in resting respiration patterns (Gonzalez *et al*., 1994). Thus, the loss of skin NEC catecholaminergic innervation may have suppressed resting ventilation frequency, and this may have contributed to the loss of the hypoxic ventilatory response. 6-OHDA, however, may not completely eliminate the ability of developing zebrafish to sense decreased O_2, as treatment with sodium cyanide (NaCN) induced hyperventilation (Figure 9C). NaCN halts cellular respiration by inhibiting cytochrome c oxidase in mitochondria, essentially mimicking tissue and cellular anoxia (Morocco, 2005). Thus, acute stimulation by NaCN may have induced a lower intracellular PO_2 in these experiments, possibly even anoxia, compared to acute application of hypoxic solution at a PO_2 of 23 Torr. These potent effects of NaCN may explain the hyperventilatory behaviour observed.

5. CONCLUSIONS AND FUTURE DIRECTIONS

The main objective of this thesis was to answer the question as to whether skin NECs are oxygen sensitive chemoreceptors during early development in zebrafish. The present results show that skin NECs are an attractive site for oxygen chemoreception as they share similar properties to other identified oxygen chemoreceptors, such as catecholaminergic innervation, retention of serotonin and display changes in cell number, and density when exposed to hypoxia and hyperoxia. Skin NECs were present and innervated before 2 d.p.f. when zebrafish first displayed behavioural responses to acute hypoxia, and the cells steadily declined over time, reaching a minimum at the time when gill NECs become functional. Pre-exposure of fish to hypoxic and hyperoxic conditions changed the way larvae reacted to acute hypoxia exposure. Skin NECs may also play a role in driving resting ventilation frequency. This thesis provides an in depth description of skin NECs as being extrabranchial oxygen chemoreceptors in early developing zebrafish.

Physiological examination during hypoxia exposures would elucidate the role of skin NECs as early oxygen chemoreceptors. As well, further investigation is needed to determine what other types of innervation are associated with these cells, and what is triggering developmental disappearance: the appearance of gill NECs, or another mechanism. It would also be of interest to investigate as to whether these cells are present in any other vertebrates. By creating a profile of serotonergic skin NECs as being oxygen chemoreceptors, this thesis contributes to the understanding of the evolutionary and developmental aspects of oxygen sensing in vertebrates in general. Given what is known about changes in O_2 sensitive sites in mammals with development, from AMCs in neonates to CB type I cells in adults, this research suggests that a

transition from one O_2 sensing site to another with development may be a phenomenon that arose early in vertebrate phylogenesis.

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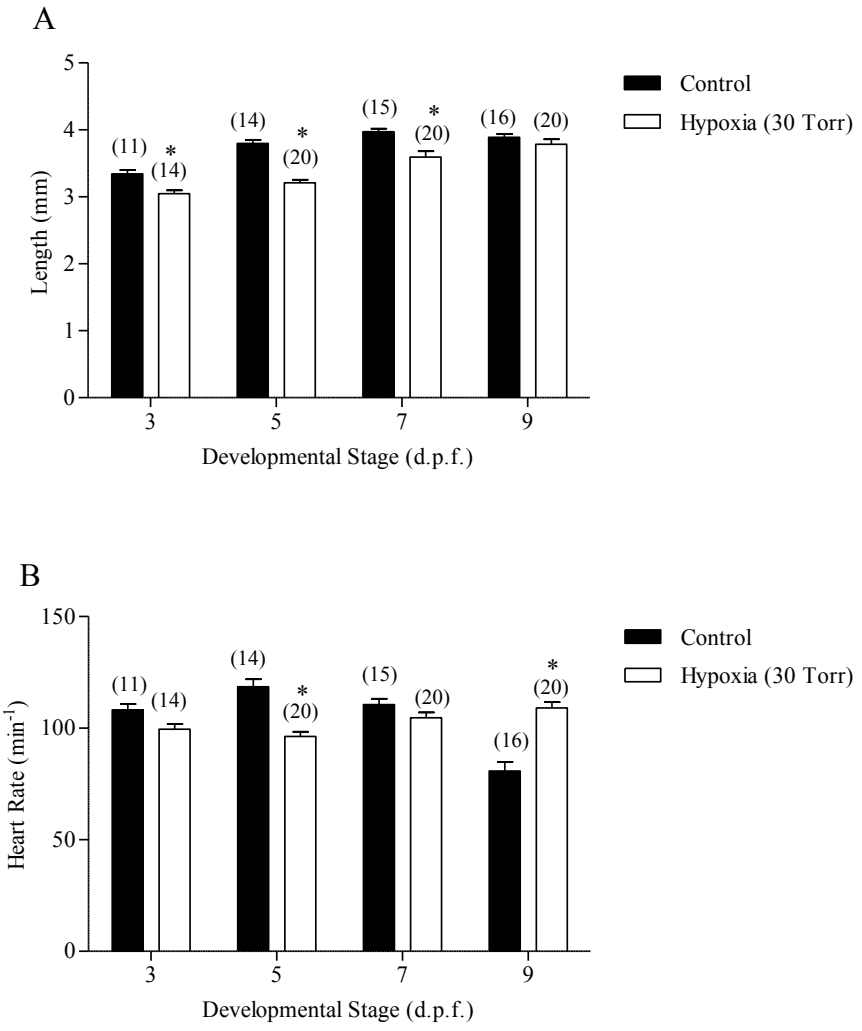
APPENDIX I

Measurements of heart rate frequency and body length were completed under a dissecting microscope (Leica MZ6). Body length (from snout to tail) was measured to the nearest 0.1 mm. Because early larvae are translucent, heart rate frequency was measured visually through the body wall. Heart rate was observed in individuals over one 30 second time interval (value expressed per minute) and were recorded using a hand-held counter and stopwatch (Fisher Scientific). Fish were transferred to a glass bottomed culture dish using a glass pipette and were lightly anaesthetized with 0.05 mg/ml MS222 (as described in section 2.5). Measurements were made 1-2 minutes after transfer.

Figure 1. Length and heart rate of developing zebrafish during chronic hypoxia exposure. (A) length of zebrafish is negatively affected up until 7 d.p.f. when chronically exposed to severe hypoxia (30 Torr). Sample sizes indicated in the figure. Asterisks indicate significant difference from control means ($p<0.05$; two-way ANOVA-Bonferroni test). (B) Heart rate of zebrafish when chronically exposed to severe hypoxia (30 Torr) shows a reduction at 5 d.p.f., and then an increase at 9 d.p.f. Sample sizes indicated in the figure. Asterisks indicate significant difference from control means ($p<0.001$; two-way ANOVA-Bonferroni test).


A
Length (mm)
Developmental Stage (d.p.f.)
Control
Hypoxia (30 Torr)
(11)
(14)
(14)
(20)
(15)
(20)
(16)
(20)
*
B
Heart Rate (min-1)
Developmental Stage (d.p.f.)
Control
Hypoxia (30 Torr)