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Respiratory Plasticity in the Zebrafish (*Danio Rerio*)

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RESPIRATORY PLASTICITY IN THE ZEBRAFISH (*Danio Rerio)*

By

Branka Vulesevic

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Title: Respiratory plasticity in the zebrafish (*Danio Rerio)*

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Abstract

The aim of this thesis was to investigate respiratory plasticity in zebrafish (*Danio rerio*) confronted with long-term changes in water gas composition (hypoxia, hyperoxia or hypercapnia) either as embryos or adults. The ventilatory responses to acute changes in water gas composition (hypoxia, hypercapnia or cyanide) were assessed using a non-invasive technique (Altimiras and Larsen, 2000) to continuously monitor breathing frequency (f_R) and relative breathing amplitude. The ventilatory response to acute hypoxia consisted of an increase in f_R while the response to acute hypercapnia was an increase in relative breathing amplitude.

Plasticity in adults

Under normal conditions, 20% of adult zebrafish tested exhibited an episodic breathing pattern that was composed of breathing and non-breathing (pausing) periods. The pausing frequency was reduced by acute hypoxia ($PwO_2 < 130$ Torr), increased by acute hyperoxia ($PwO_2 > 350$ Torr) but was unaltered by acute hypercapnia ($PwCO_2 = 3.5$ Torr). Exposure of adult fish to 28 days of hyperoxia decreased the capacity of fish to increase f_R during acute hypoxia and external cyanide exposure as well as preventing the usual increase in breathing amplitude during acute hypercapnia.

In fish chronically exposed to hypoxia, resting f_R was significantly reduced (from 191 ± 12.5 to 165 ± 16.5 min^{-1}) however the ventilatory responses to acute hypoxia and hypercapnia were unaffected. Long-term exposure of fish to hypercapnic water did not markedly modify the breathing response to acute hypoxia, modestly blunted the response to hypercapnia and increased the response to external cyanide. To assess the impact of acclimatization to varying gas levels on branchial O_2 chemoreceptors in adult plasticity

experiments, the numbers of gill filament neuroepithelial cells (NECs) were quantified using confocal immunofluorescence microscopy. Consistent with the blunting of reflex ventilatory responses, fish exposed to chronic hyperoxia exhibited a significant decrease in the density of NECs (from 36.8 ± 2.8 to 22.7 ± 2.3 filament^{-1}).

Developmental plasticity

Embryos were exposed to hypoxia, hyperoxia or hypercapnia for the first seven days post fertilization (d.p.f.) and their acute reflex responses to ventilatory stimuli were assessed when they had reached maturity (3 months or older). The analysis of breathing patterns of fish exposed in hypoxia, hyperoxia or hypercapnia for the first seven d.p.f. demonstrated that fish reared under hypercapnic conditions ($PwCO_2$ = 7 - 9 Torr) were more likely to display episodic breathing. Zebrafish reared under hyperoxic conditions exhibited significantly higher f_R at rest (283 ± 27 *versus* 212 ± 16 min^{-1} in control fish); breathing frequency was unaffected in adult fish subjected to hyperoxia for 7 days. The respiratory response of fish reared under hypoxic conditions to acute hypoxia was slightly blunted while the ventilatory responses to hypercapnia or external cyanide were unaffected. The breathing responses of adult zebrafish reared in hypercapnia to the various ventilatory stimuli were similar to fish reared under normal conditions.

The data clearly demonstrated that there is flexibility in the design and functioning of the embryonic or larval respiratory system in fish, and it is not operating simply on the basis of a pre-set genetic program.

Résumé

Le but de cette thèse était d'étudier la plasticité respiratoire chez le poisson zèbre (*Danio rerio*) confronté avec des changements à long terme de la composition en gaz dans l'eau (hypoxie, hyperoxie ou hypercapnie) sur des embryons ou des adultes. La réponse aux changements aigus de la composition en gaz dans l'eau (hyperoxie, hypercapnie et cyanure) a été évaluée en utilisant une technique non envahissante (Altimiras et Larsen, 2000) qui mesure sans interruption la fréquence de respiration (f_R) et l'amplitude de respiration relative. La réponse ventilatoire à l'hypoxie aiguë s'est caractérisée par une augmentation de f_R tandis que la réponse à l'hypercapnie aiguë était composée d'une augmentation d'amplitude de respiration relative.

Plasticité chez les adultes

Dans des conditions normales, seuls 20% des poissons zèbre adultes examinés ont demontrés une respiration épisodique qui était composée de périodes de respiration et de périodes de non-respiration (de pause). La fréquence de pause fut réduite durant l'hypoxie aiguë ($PwO_2 < 130$ Torr), fut augmentée durant l'hyperoxie aiguë ($PwO_2 > 350$ Torr), mais resta inchangée durant l'hypercapnie aiguë ($PwCO_2 = 3.5$ torr). L'exposition de poissons adultes à 28 jours d'hyperoxie a diminué la capacité du poisson à augmenter sa f_R pendant l'hypoxie aiguë et pendant une exposition externe de cyanure, mais aussi a empêché une augmentation habituelle de l'amplitude respiratoire durant l'hypercapnie aiguë.

Chez les poissons chroniquement exposés à l'hypoxie, la f_R au repos a été sensiblement réduite (de 191 ± 12.5 à 165 ± 16.5 min^{-1}), cependant les réponses ventilatoires à l'hypoxie aiguë et à l'hypercapnie étaient inchangées.

L'exposition à long terme des poissons en eau hypercapnique n'a pas modifié la réponse respiratoire pendant une hypoxie aiguë, a modestement réduit la réponse en hypercapnie et a augmenté la réponse après addition de cyanure externe. Pour évaluer l'impact de l'acclimatation aux niveaux variables de gaz sur les chimiorécepteurs d'O_2 branchiaux, dans des expériences de la plasticité chez les adultes, les nombres de cellules neuroepitheliales de filaments de branchie (NECs) ont été mesurés en utilisant la microscopie confocale d'immunofluorescence. En accord avec la réduction des réponses réflexes ventilatoires, les poissons exposés à l'hyperoxie chronique ont exhibé une diminution significative de la densité de NECs de 36.8 ± 2.8 à 22.7 ± 2.3 filament^{-1}.

Plasticité au cours du développement

Les embryons ont été exposés à l'hypoxie, à l'hyperoxie ou à l'hypercapnie durant les premiers sept jours après la fertilisation, et leurs réponses aigues de réflexe respiration due aux stimulus ventilatoires (hypoxie, hypercapnie et cyanure externe) ont été évaluées quand ils avaient atteint la maturité (3 mois ou plus vieux). L'analyse des patrons de respiration des poissons exposés à l'hypoxie, à l'hyperoxie ou à l'hypercapnie durant les sept premiers jours après la fertilisation (j.a.f.) démontra que les poissons élevés dans des conditions hypercapniques ($PwCO_2$ = 7 - 9 Torr) ont eu une tendance accrue d'utiliser la respiration épisodique. Les poissons zèbre élevés dans des conditions hyperoxiques ont démontré des fréquences sensiblement plus hautes de respiration au repos (283 ± 27 contre 212 ± 16 min^{-1} chez les poissons contrôles) ; la fréquence de respiration était inchangée chez les poissons adultes soumis à l'hyperoxie pendant 7 jours. La réponse respiratoire des poissons élevés dans des conditions hypoxiques à l'hypoxie aiguë a été légèrement réduite tandis que les réponses ventilatoires à l'hypercapnie ou après

l'addition de cyanure externe étaient inchangées. Les réponses respiratoires des poissons zèbre adultes élevés en hypercapnie aux divers stimuli ventilatoires étaient semblables à celles des poissons élevés dans des conditions normales.

Les données ont clairement démontré qu'il y a une flexibilité dans la conception et le fonctionnement du système respiratoire embryonnaire ou larvaire chez les poissons, et qu'il ne fonctionne pas simplement sur la base d'un programme génétique préréglé.

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There are several people who I would like to thank for making my time at the University of Ottawa a pleasant experience. First and foremost I want to thank my supervisor Steve Perry for practicaly sharing his office with me, patiently listening to my bad English and tolerating my "balkan temperament" on more than one occasion.

I would like to thank Drs Tom Moon, Katie Gilmour and Chris Martin for serving on my advisory committee. Many thanks go to the students with whom I have had the pleasure of working and having lunch breaks with: Tina Georgalis, Mustafa Bayaa, Laura Kenney, Xi Chen (and his wife Gin who cooked for me), Arash Shahsavarani, Amira Mohamed, Rachel Euverman, Zhaohong Qin and all the students in the Perry and other labs who have made my experience in the department an enjoyable one. Special thanks goes to Brian McNeill, for being by my side when things did not work well, and letting me take it out on him, and for all the help and knowledge that he offers selflessly.

Finally I would like to thank my friend and biggest support, Louis Sherman, (who had to read this thesis several times, although it is not even remotely in his field, and who corrected all my bad spelling and grammar), for my thesis would not have been possible without his help throughout my university days.

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List of Abbreviations

ANOVA	Analysis of Variance
5-HT	Serotonin
Ca^{2+}	Calcium ion
CB	Carotid body
Cl^-	Chloride ion
CO_2	Carbon dioxide
d.p.f.	Days post fertilization
fR	Breathing frequency
H	Hour
Hb	Hemoglobin
HCO^{3+}	Bicarbonate ion
HVR	Hypoxic ventilatory response
Hz	Hertz
K^+	Potassium ion
min	Minute
mm	Millimeters
MS 222	1 mg ml–1 ethyl 3-aminobenzoate methanesulfonate salt
N_2	Nitrogen
Na^+	Sodium ion
NaCN	Sodium cyanide
NECs	Neuroepithelial cells
O_2	Oxygen
PBS	Phosphate-buffered saline
PBS-TX	PBS containing 1% fetal calf serum and 0.5% Triton X-100
PCO_2	Carbon dioxide partial pressure
PO_2	Oxygen partial pressure
$PwCO_2$	Carbon dioxide partial pressure in water
PwO_2	Oxygen partial pressure in water
RM	Repeated measurements
SEM	Standard Error of the Mean
SV2	Synaptic vesicle protein
V	Volts
VAH	Ventilatory acclimatization to hypoxia
Zn-12	Zebrafish-derived neuron-specific antigen

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CHAPTER 1

GENERAL INTRODUCTION

Animals possess respiratory control mechanisms that allow them to match their rates of O_2 uptake and CO_2 excretion to the rates of their metabolism and the availability of these gases in the environment. Although in many ways similar, the control of respiration differs in air- and water-breathers. Air-breathers typically inhabit an environment of stable gas composition. Aquatic environments, on the other hand, are markedly unstable with respect to gas composition and pH (Burleson and Smatresk, 2000; Crocker *et al.*, 2000; McKenzie *et al.*, 2002). The structural organization of gills in water-breathers allows for a higher efficiency of gas exchange owing to the countercurrent arrangement of water and blood flows. This higher efficiency compensates for the reduced availability of O_2 in water, a consequence of the much lower O_2 capacitance of water compared to air (Rombough, 1988).

Although it has long been recognized that change in aquatic gas composition influences breathing in fishes, the underlying mechanisms of chemoreception are not as well understood as in mammals. Determining the basic mechanism of chemoreception and acclimation in fish is still in progress. Clarifying the mechanisms that have evolved to allow fishes to survive with little or no oxygen, as well as in hypercapnic water, may offer new insight into the challenges created by hypoxia and could point to possible ways of counteracting hypoxic damage. Better knowledge of fish chemoreception and its similarities with mammalian chemoreception (as the pharyngeal arches give rise to carotid body in mammals and the gill in fish – Jonz *et al*, 2004) may provide insight into the pathophysiology of certain cardiorespiratory disorders.

Respiratory control systems

Mammalian and piscine respiratory control systems possess three basic components:

1. Central respiratory control: Regulates respiratory system according to information obtained from the peripheral sensors.
2. Sensors and their afferents nerves: Provide information on O_2, CO_2 and H^+ demand and supply.
3. Efferent nerves and effectors: Muscles that control breathing and cardiovascular function.

In mammals, the central respiratory centre consists of three groups of neurons located in the medulla oblongata, which are responsible for the basic control of respiratory rhythmicity (Feldman and Ellenberger, 1988). Similar neurons are also found in the medulla of fish, more diffusely arranged, but also possessing the capacity to initiate and coordinate rhythmic breathing movements (Fritsche & Nilsson, 1993). It is believed that all vertebrate groups share common mechanisms of central nervous and reflex control of cardiorespiratory functions (Taylor *et al.*, 1999).

Chemoreceptors

Two categories of chemoreceptors are described in mammalian respiratory systems:

- Central chemoreceptors: Receptive to arterial PCO_2 owing to changes in H^+ concentration in cerebrospinal fluid (CSF) (Putnam *et al.*, 2004).
- Peripheral chemoreceptors: Receptive to arterial PO_2, PCO_2 and H^+ concentration, located in the carotid body (CB) at the bifurcation of the ascending aorta and pulmonary artery, it consists of clusters of sensory cells (type I glomus cells) innervated by an array of afferent nerve fibers linked to

the central respiratory centre, aortic bodies and possibly slowly adapting stretch receptors in bronchial airways.

Clusters of innervated O_2-sensitive neurosecretory cells exist also in the neuroepithelial bodies of the lung where they detect PO_2 changes in the inspired air (Montoro et al., 1996). Because same embryonic arches give rise to the gills and the carotid and aortic bodies in mammals (Bailly *et al.,* 1992; Fritsche & Nilsson, 1993), a similar distribution of peripheral chemoreceptors is expected in fish. To date, peripheral chemoreceptors have been shown to exist on the gills of teleost fish (Burleson and Smatresk, 2000; Burleson and Milsom, 1993; Jonz *et al.*, 2003; Sollid *et al.*, 2003; Reid and Perry, 2003) and the response characteristics of these receptors suggest that they are homologous to the oxygen receptors of the mammalian carotid and aortic bodies (Sundin & Nilsson, 2002; Bailly *et al.*, 1992). In fish, the receptors are internally and externally oriented (Burleson and Milsom, 1993; Milsom and Brill, 1986; Jonz *et al.*, 2003), sensing the change in O_2 and CO_2 in water or in blood. Branchial O_2-sensitive chemoreceptors are innervated by branches of cranial nerves IX and X, probably also VII in fish with pseudobranchs (Burleson and Smatresk, 2000; Burleson & Milsom, 1993; Taylor *et al.*, 1999; Sundin & Nilsson, 2002). The distribution and orientation of these receptors, however, is not uniform among species; specific receptors in trout are located on the first gill arch (Perry & Reid, 2002), whereas in catfish they appear to be located on the first three gill arches (Burleson and Smatresk, 2000) and in zebrafish on all four gill arches (Jonz *et al.*, 2004). Some studies suggest extra-branchial sites of chemoreception also exist including the oral and pharyngeal areas and the vasculature (Burleson and Smatresk, 2000; Sundin & Nilsson, 2002, Milsom *et al*, 2002).

CO_2-sensitive chemoreceptors in mammals are found in the brain (central chemoreceptors - where the response to changes in cerebrospinal fluid pH caused by CO_2

crossing the blood-brain barrier), carotid body and intrapulmonary neuroepithelial cells (Nattie, 1999; Burleson & Smatresk, 2000). The CO_2/H^+ sensing involves carbonic anhydrase that mediates rapid equilibration of CO_2, H^+ and HCO_3^- (Milsom et al., 2004; Lahiri & Forster, 2003). In fish the response to change in $PwCO_2$ has been described in several studies (Perry *et al*, 1999; reviewed by Gilmour 2001), but CO_2 chemoreceptors on the gills have not yet been described morphologically, and their presence is still under investigation.

Responses to hypoxia

The cardiorespiratory responses to hypoxia are relatively well understood. Acute hypoxia requires fast respiratory and cardiovascular adjustments to ensure O_2 delivery to the most critical organs such as the brain or the heart. The human response to acute hypoxia involves increased respiration rate, bradycardia, vasoconstriction that reduces blood flow to peripheral capillaries and increased sympathetic outflow to the periphery (Foster & Sheel, 2005). Exposure to long-term hypoxia elicits changes in the carotid body as the result of alterations in the expression of genes that regulate glomus cell growth, excitability, and sensitivity to low PO_2 (Lopez-Barneo *et al.*, 2001).

Fish typically respond to hypoxia with hyperventilation, bradycardia and hypertension (caused by increased systemic vascular resistance) initiated by branchial chemoreceptors with both an internal and external orientation (Burleson & Milsom, 1993; Burleson & Smatresk, 2000; Burleson *et al*, 2002; Perry and Gilmour, 2002; Jonz & Nurse, 2005). The external receptors are thought to trigger the cardiovascular and ventilatory responses, whereas the internal receptors are believed to be involved solely in mediating the ventilatory responses (Burleson *et al.*, 1992, McKendry *et al*, 2001).

Responses to hypercapnia

In most air-breathing vertebrates, an acute increase in PCO_2 is associated with hypercapnic acidosis and increased tidal volume (Milsom *et al.*, 2004). It is now recognized that CO_2 also alters ventilation in fish (Perry *et al*, 1999; Burleson & Smatresk, 2000; Gilmour, 2001; McKendry *et al*, 2001; McKendry & Perry, 2001; Perry & Reid, 2002; McKenzie et al., 2003). However, the morphology of specific CO_2 chemoreceptors and afferent pathways involved in the ventilatory effects of hypercapnic acidosis has not been identified. It has been largely accepted that elevated CO_2 pressures, independently of blood O_2 status affects the chemoreceptors and cardioventilatory response of fish to hypercapnia through Root and Bohr effect present in fish (Milsom, 1995a; 1995b; Gilmour, 2001; Perry & Reid, 2002)

Mechanisms of chemoreception

From studies of mammalian NECs (neuroepithelial cells) and glomus cells, there is accumulating support for O_2 sensing mechanisms involving a potassium channel as the trigger of O_2 sensory reflexes (see Appendix 1). Hypoxia may affect chemoreceptor cells directly by inhibiting potassium (K^+) channels. The inhibition of K^+ channels causes membrane depolarization and calcium ion (Ca^{2+}) entry through voltage dependent Ca^{2+} channels (Gonzalez *et al.*, 1992) thereby initiating the release of excitatory neurotransmitter (e.g. ATP) from glomus cells in the carotid body (Gonzalez *et al.*, 1992; Prabhakar, 2000). These O_2-sensitive K^+ channels, initially discovered in glomus (type I cells) of the rabbit carotid body, are believed to be present in all hypoxia-responsive

responsive neurosecretory cells (Montoro *et al.*, 1996) including the branchial NECs of the zebrafish (Jonz 2004).

CO_2/H^+ sensing occurs both in the carotid body and in the central nervous system and carbonic anhydrase mediates the fast response in both (Lahiri & Forster, 2003) Changes of pH play a major role in intracellular responses to increased CO_2/H^+ levels. It appears that there is more than a single chemosensitive ion channel; but, rather, numerous ion channels (including K^+, Ca^{2+}, Na^+ and perhaps Cl^- and HCO_3^- channels) involved in CO_2 chemoreception (Gonzales *et al.*, 1992; Putnam *et al.*, 2004).

Plasticity

In their review of neuroplasticity, Mitchell and Johnson (2003) defined plasticity as a persistent change in the neural control system (morphology and/or function) based on prior experience including neural activity, hypoxia, injury, disease, or aging. It can be evoked at different levels of respiratory control (see Appendix 2) and at different periods of development. If the respiratory control network is affected by certain insults during its development, these changes can affect the respiratory responses of an animal in adult life. These persistent changes are recognized as developmental plasticity (Carroll, 2003).

The carotid body enlarges in human patients with diseases characterized by hypoxia. Enlargement of the carotid body in humans and animals exposed to chronic hypoxia may be caused by hyperplasia of type I cells (Bisgard, 2000). In several assays, intermittent hypoxia produced more potent changes in the carotid body than continuous hypoxia (Prabhakar and Jacono, 2005). Over time, hypoxia changes the overall activity of the peripheral chemoreflex (when measured as an isocapnic hypoxic ventilatory response), by decreasing the peripheral chemoreflex threshold (response to hypoxia starts

at a lower levels of hypoxia) and increasing the ventilation response to hypoxia at a given PCO_2 (Duffin and Mahamed, 2003).

Hyperoxia-induced respiratory plasticity has been described in animals (Lahiri *et al.*, 1987; Arieli *et al* 1988; Liberzon *et al.*, 1989. Torbati *et al.*, 1989) and humans (Gelfand *et al.*, 1998) giving contrary results that animals have an attenuated respiratory response to hypoxia after hyperoxia exposure, unlike humans. Studies of developmental hyperoxia are more intriguing as they may have some clinical relevance for hyperoxia experienced in a neonatal intensive care unit (Carroll, 2003).

Unlike for hypoxia, continuous hypercapnia appears to have more of an effect on long-term respiratory plasticity than episodic hypercapnia (Baker et al, 2001). Once the hypercapnia has ended, both sustained and intermittent hypercapnia ellicit long-term depression of phrenic nerve in anaesthetized rats (Mitchell & Johnson, 2003). In birds, in addition to the intrapulmonary chemoreceptors, the arterial and central CO_2 chemoreceptors are responsible for increases in ventilation during even mild hypercapnia (Fedee *et al.*, 2002). In acclimatized ducks, the mean intrapulmonary chemoreceptor PCO_2 response slope is increased, unlike in the burrowing owl, an animal genetically adapted to subterranean life and high inspired CO_2 levels whose ventilatory CO_2 response is blunted, thus showing a difference between genetic adaptation and physiological acclimatization to hypercapnia (Bebout & Hempleman, 1999). Until now, I am unaware of a study that dealt with acclimatization to chronic hypercapnia in fish.

Understanding the basic mechanisms of respiratory plasticity may help to explain natural compensatory mechanisms during diseases that cause hypoxia and provide ideas for possible therapies. The lack of O_2 is crucial in the pathogenesis of stroke, myocardial infarction, chronic lung disease or sudden infant death syndrome (Remmers& Lahiri, 1998). It has also been recognized that intermittent hypoxia occurring during recurrent

apnoeas may result in cardiovascular morbidity (Prabhakar *et al.*, 2005). Different studies are looking at the response to increases in CO_2/H^+ that can be caused by cerebral ischemia, anoxia, metabolic acidosis and environmental hypercapnia.

An important piece of the puzzle is to identify specific factors that regulate the expression of plasticity. It is also important to understand the biological significance of respiratory plasticity in everyday life in individuals exposed to diseases and injuries (for example lung disease, sleep-disordered breathing, and central nervous system injury) that affect normal respiration and in individuals with maladaptive plasticity caused by abnormal development.

Zebrafish as an animal model

This is the first time that zebrafish are used as a model for studying respiratory plasticity. Their small size facilitates the exposure of large numbers of individuals to changed environments simultaneously. Furthermore, O_2-sensitive chemoreceptors have been described and localized in zebrafish (Jonz et al., 2005) giving the possibility to examine the role of chemoreceptors in respiratory plasticity of adults. The embryos of zebrafish can be readily obtained in large numbers, and raised to adult size in only 3 months offering the opportunity to look at the respiratory response of adult fish raised in different gas environments and the effect of possible developmental plasticity.

Hypothesis

Two basic hypotheses are examined by monitoring respiratory control in zebrafish:

(1) Long lasting changes in gas composition in water will affect the acute respiratory response of the fish exposed to them

(2) Exposure of developing zebrafish to gas changes in water during early development will permanently alter their breathing response to acute changes in water later in life

Predictions

Adult plasticity:

- Based on hypoxia acclimatization in mammals and catfish, chronic water hypoxia should increase the sensitivity to hypoxia and hypercapnia in pre-exposed zebrafish
- Based on studies done on birds chronic hypercapnia should blunt respiratory response to acute hypoxia or hypercapnia
- Chronic hyperoxia will attenuate respiratory response to hypoxia and hypercapnia as was shown in studies done on mammals

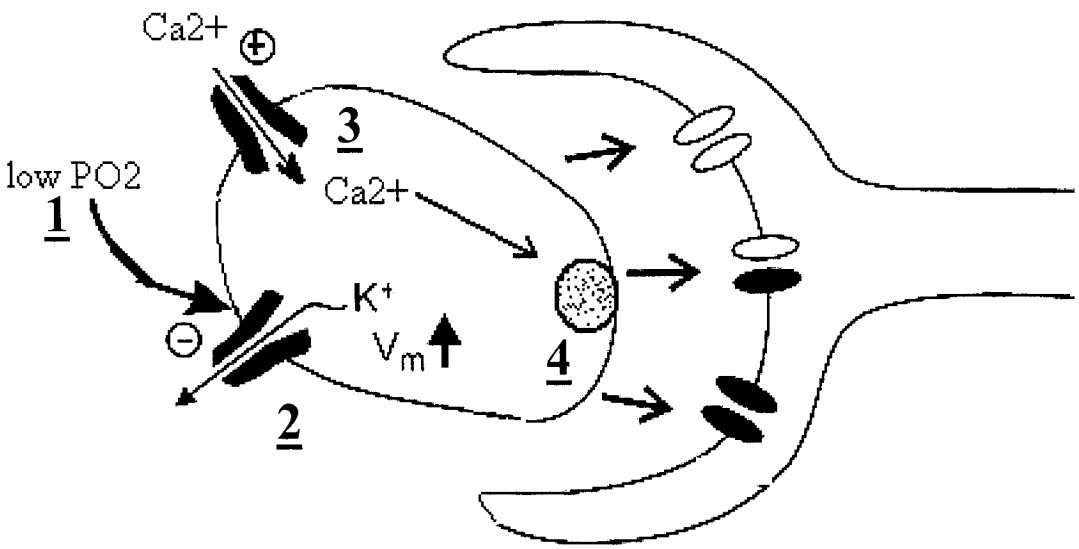
Developmental plasticity:

- Hypoxia in early developmental stages blunts ventilatory responses to acute hypoxia and hypercapnia
- Hyperoxia exposure will induce long-lasting reductions in the response to hypoxia and hypercapnia
- Exposure to CO_2 in early developmental stage will decrease the adult ventilatory response to hypoxia and hypercapnia

Appendix 1

Models for sensory transduction in chemoreceptor cells of carotid bodies

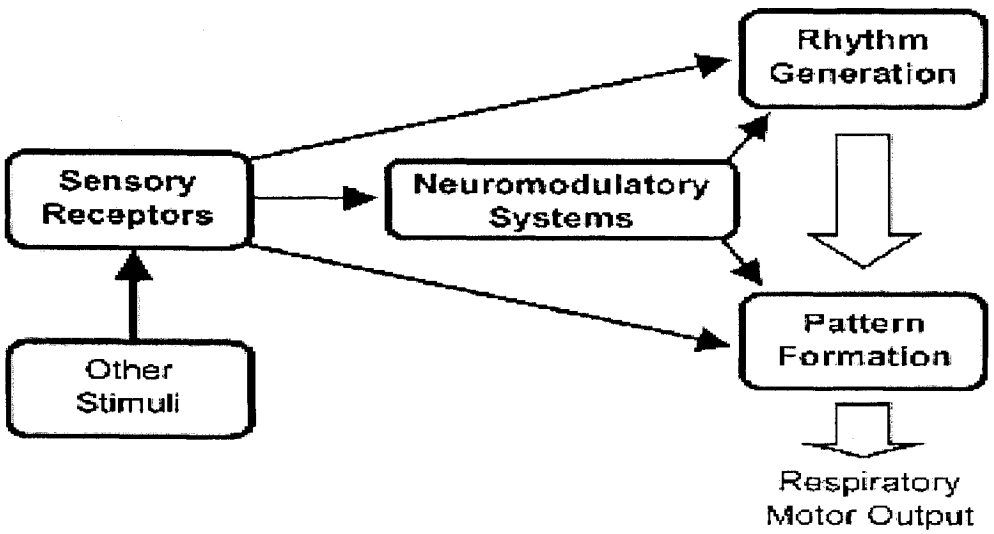
Based on the model proposed by Gonzalez, C., Almaraz, L., Obeso, A., Rigual, R., Trends in neurosciences (1992)



The proposed cascade for low PO_2 transduction. On lowering PO_2 (1) a signal originates at the O_2 sensor, inhibiting the K^+ current (2). A drop in membrane potential follows the inhibition of the K^+ current, which in turn activates voltage-dependent Ca^{2+} channels leading to Ca^{2+} influx and an increase in the intracellular concentration of Ca^{2+} (3). Increased $[Ca^{2+}]$ results in the activation of the exocytotic mechanism (4).

Appendix 2

POTENTIAL SITES OF RESPIRATORY NEUROPLASTICITY



Gordon S. Mitchell & Stephen M. Johnson, J App. Physiol (2003)

Respiratory rhythm is generated by neurons in the ventral medulla (rhythm generation) and transmitted to brain stem and spinal cord neurons that shape the detailed spatiotemporal pattern of respiratory motor output (pattern formation). Rhythm generation and pattern formation are continually influenced by sensory receptors (i.e., chemoreceptors and mechanoreceptors) and neuromodulatory systems (e.g., monoamines). Other inputs also influence the respiratory control system such as inputs from the cortex or the direct effects of oxygen, carbon dioxide, and pH on respiratory neurons (arrows). Plasticity may occur in one or multiple sites, and it is often initiated by sensory or neuromodulatory influences.

CHAPTER 2

Chemoreceptor plasticity and respiratory acclimatization in the zebrafish, *Danio rerio*

This chapter is based on a manuscript accepted in Journal of Experimental Biology:

Chemoreceptor plasticity and respiratory acclimatization in the zebrafish, *Danio rerio*

B.Vulesevic, B. McNeill and S.F. Perry[1]

Abstract

The respiratory consequences of exposing adult zebrafish (*Danio rerio*) to chronic changes in water gas composition (hypoxia, hyperoxia or hypercapnia) were assessed using a non-invasive technique (Altimiras and Larsen 2000) to continuously monitor breathing frequency (fR) and relative breathing amplitude. Under normal conditions 20% of zebrafish tested exhibited an episodic breathing pattern that was composed of breathing and non-breathing (pausing) periods. The pausing frequency was reduced by acute hypoxia (PwO2 < 130 Torr), and increased by acute hyperoxia (PwO2 > 350 Torr), but was unaltered by acute hypercapnia (PwCO2 = 3.5 Torr).

In control fish, the ventilatory response to acute hypoxia consisted of an increase in fR while the response to acute hypercapnia was an increase in relative breathing amplitude. The stimulus promoting the hyperventilation during hypercapnia was increased water PCO_2 ($PwCO_2$) rather than decreased water pH. Exposure to 28 days of hyperoxia decreased the capacity of fish to increase fR during acute hypoxia and external cyanide exposure as well as prevented the usual increase in breathing amplitude during acute hypercapnia. In fish previously exposed to hyperoxia, episodic breathing continued until PwO_2 had fallen below 70 Torr. In fish chronically exposed to hypoxia, resting breathing frequency was significantly reduced (from 191 ± 12.5 to 165 ± 16.5 min-1) however the ventilatory responses to hypoxia and hypercapnia were unaffected. Long-term exposure of fish to hypercapnic water did not markedly modify the breathing response to acute hypoxia, modestly blunted the response to hypercapnia, and increased the response to external cyanide.

To assess the impact of acclimatization to varying gas levels on branchial O_2

chemoreceptors, the numbers of gill filament neuroepithelial cells (NECs) were quantified using confocal immunofluorescence microscopy. Consistent with the blunting of reflex ventilatory responses, fish exposed to chronic hyperoxia exhibited a significant decrease in the density of NECs from 36.8 ± 2.8 to 22.7 ± 2.3 filament^{-1}.

Introduction

In water-breathing fish, gill ventilation is affected by several factors including the status of dissolved gases in the environment and metabolic demand. Chemoreceptors on, and within the gill, sense changes in ambient and intravascular gas levels. These chemoreceptors are either oriented externally to sense environmental changes or internally to sense changes in the blood (see reviews by Burleson and Smatresk, 2000; Sundin and Nilsson, 2002). The chemoreceptors, when stimulated by changes in O_2 and/or CO_2/pH, initiate a variety of cardiorespiratory and hormonal responses including changes in breathing rate and/or amplitude, heart rate, systemic resistance and plasma catecholamine levels (Satchell, 1959; Randall and Smith, 1967; Holeton and Randall, 1967; see reviews by Smatresk, 1990; Fritsche and Nilsson, 1983; Burleson *et al* 1992; Perry and Gilmour, 2002; Sundin and Nilsson, 2002, Reid and Perry, 2003). The chemoreceptor cells share many histological and cytochemical similarities with mammalian carotid body glomus cells (Fritsche and Nilsson 1983) and indeed they are likely to be the evolutionary homologous of the mammalian carotid body (glomus cells), the main site for sensing changes in blood gases in mammals (Gonzalez *et al* 1992).

Like other components of the nervous system, respiratory control systems exhibit marked plasticity. This plasticity can be morphological and/or functional and is based on prior experience (Baker *et al.*, 2001; Mitchell and Johnson, 2003). There are several potential sites of respiratory neuroplasticity including the sensory chemoreceptors themselves, signal transmission pathways, central rhythm generation or pattern formation control (Powell *et al.*, 2000). Together with genotype, age and gender, the partial pressure of respiratory gases can influence plasticity (Mitchell and Johnson, 2003). In the wild, fish can be exposed to fluctuations in environmental O_2 and CO_2 levels both

diurnally and spatially (Crocker *et al.*, 2000). Consequently, fish have developed behavioral, physiological and morphological mechanisms of acclimating to fluctuating environments, a reflection of the respiratory plasticity (Perry and Gilmour, 2002; Burleson *et al.*, 2002; Sollid *et al.*, 2003; Jonz *et al.*, 2004). Hypoxia-induced respiratory plasticity in mammals (known as ventilatory acclimatization to hypoxia or VAH) manifests itself as an increase in breathing response to subsequent hypoxia owing to heightened chemoreceptor and central nervous system (CNS) sensitivity (Forster *et al.*, 1971; Sato *et al.*, 1992; Aaron *et al.*, 1993; Bisgard and Neubauer 1995; Soulier *et al.*, 1997; Dwinell and Powell 1999; Bisgard 2000; Powell *et al.*, 2000; Baker *et al.*, 2001). Only a single study (Burleson *et al.*, 2002) has assessed VAH in fish; that study demonstrated that catfish (*Ictalurus punctatus*) exposed to chronic moderate hypoxia (75 Torr) exhibited a heightened ventilatory sensitivity to acute hypoxia.

Hyperoxia-induced respiratory plasticity has been reported in mammals (Lahiri *et al.*, 1987; Liberzon *et al.*, 1989. Torbati *et al.*, 1989) and is manifested by a reversible blunting of the ventilatory response to hypoxia. To our knowledge, no studies have yet addressed the potential for respiratory plasticity in fish exposed to hyperoxia.
In mammals, pre-exposure to hypercapnia does not alter the response to acute hypoxia or hypercapnia (Remmers and Lahiri, 1998; Kondo *et al.*, 2000). Despite the accruing evidence that environmental CO_2 is a potent and specific ventilatory stimulant in fish (Heisler *et al.*, 1988; Graham *et al.*, 1990; Perry *et al.*, 1999; Burleson and Smatresk, 2000; McKendry *et al.*, 2001; Perry and Reid, 2002; McKenzie *et al.*, 2003; reviewed by Milsom 1995a, b; Gilmour 2001), we are unaware of any studies that have examined respiratory acclimatization to hypercapnia in fish.

The present study focused on evaluating respiratory plasticity in zebrafish (*Danio rerio*) following exposure to chronic hypoxic, hyperoxic or hypercapnic conditions. This

was accomplished using a non-invasive recording method that registers the change in voltage that is transferred through the water during opercular movements (Altimiras and Larsen, 2000). To implicate morphological changes of branchial chemoreceptors (Jonz and Nurse 2003, 2005; Jonz *et al.,* 2004) as a mechanism of plasticity, chemoreceptors on gill filaments were analyzed by confocal immunofluorescence microscopy.

Materials and methods

Animals

Adult zebrafish (*Danio rerio*) were obtained from a commercial supplier (MIRDO, Montreal) and transported to the University of Ottawa Aquatic Care Facility where they were maintained in acrylic tanks (4 L) supplied with well-aerated, dechloraminated City of Ottawa tap water at 28°C. Fish were subjected to a constant 10L: 14D photoperiod. All procedures for animal use were carried out according to institutional guidelines and in accordance with those of the Canadian Council on Animal Care (CCAC).

Verification of the method for continuous monitoring of breathing frequency and relative breathing amplitude

Zebrafish were placed in a breathing recording chamber constructed at the University of Ottawa. The chamber was a cylindrical transparent plastic tube (length = 3 cm, diameter = 1 cm). Two electrodes (standard copper – tin wires) were submerged in the water inside the chamber and separated by a distance of approximately 2 cm. Coarse mesh was inserted into each end of the chamber to prevent the fish from making contact with the electrodes. Each chamber was provided with continuous water flow (~10 ml min^{-1}). The experiments were filmed with a Canon digital movie camera (model NTSC ZR70mc) and the images were transferred to a personal computer using Digital Video Camcorder software (Ulead Video Studio SE Basic Version 6.0). A scale bar was included in the visual field to allow measurement of opercular displacement (in mm, the measure of ventilation amplitude used in the present study). The analog voltage signals

associated with opercular movements were amplified using an amplifier (constructed at the University of Ottawa), converted to digital data and stored on computer by interfacing with a data acquisition system (Biopac Systems Inc.) using AcqKnowledge™ data acquisition software (sampling rate set at 50 000 Hz) and a Pentium™ PC. Breathing frequencies (fR) and amplitude were measured independently by analyzing video recordings and AcqKnowledge™ files. To assess the suitability of the electronic recording technique to reliably provide accurate measurements of breathing frequency and relative amplitude, data obtained from the two techniques were compared and subjected to correlation analysis. Opercular breathing movements produced oscillating voltage changes, with each breathing cycle producing a distinct minimum and maximum voltage. Thus, fR was determined by counting the number of voltage peaks over a set time interval. On the basis of video analysis, it was determined that opercular displacement during normal breathing was 1 – 3 mm (the sum of both operculae). Thus, for simplicity, the data acquisition was calibrated assuming that the voltage fluctuations at rest represented a 1 mm total opercular deflection. Thus, in all experiments, relative breathing amplitude was calculated as the difference between minimum and maximum values being continuously recorded for each breathing cycle.

To further prove that the measured voltage oscillations were exclusively related to breathing movements, 4 fish were anaesthetized *in situ* by flushing the fish chamber with 3 ml of benzocaine (0.05 g ml^{-1}). The anaesthetized fish stopped breathing but the heart continued to beat and some involuntary body movements remained. The signal obtained during anaesthesia was then compared to the signal obtained before and after the fish was anaesthetized.

Pre-exposure of fish to hypercapnia, hypoxia or hyperoxia

Zebrafish were exposed *in vivo* to hypercapnia ($PwCO_2$ = 7 - 9 Torr), hypoxia (PwO_2 =30 - 40 Torr), or hyperoxia (PwO_2 = 350 - 450 Torr) at 28°C in a 2 L tank for 28 days. Control fish were kept under similar conditions for 28 days but were provided with normoxic and normocapnic water. For each treatment, at least two groups of fish were pre-exposed at different times. Hypercapnia was achieved by pumping mixtures of CO_2 in air (1 - 2%) through a gas equilibration column provided with aerated water. For hypoxia, the water equilibration column was supplied with a mixture of nitrogen (N_2) in air (95% N_2/5% air).

The gas mixtures were supplied by a Cameron Gas Mixer (model GF-3/MP). To achieve hyperoxia, O_2 (100%) was bubbled directly into fish tanks being supplied with minimal volumes of normoxic-dechloraminated water (30 ml h^{-1}). Water PCO_2 was measured using a CO_2 electrode (Cameron Instrument Company, model E201) connected to a Cameron BGM 200 blood gas meter. Measurements of O_2 were made using a fiber optic oxygen electrode (Ocean Optics Foxy AL300) and associated hardware and software (Ocean Optics SD 2000). After 28 days, the fish were tested for their ventilatory responses to acute hypoxia, hypercapnia or external cyanide. They were then euthanized by overdose of anaesthetic (1 mg ml^{-1} ethyl 3-aminobenzoate methanesulfonate salt; MS 222), and the gills were removed and prepared for immunocytochemistry (see below).

Ventilatory responses to acute hypoxia

Fish were placed in the breathing recording chamber at least three h prior to beginning an experiment which allowed their breathing to become uniform. For each fish, resting breathing amplitude was assumed to be 1 mm and the system was calibrated

accordingly. PwO_2 within the fish chamber was continuously measured by using a PO_2 electrode calibrated with solutions of sodium sulphite (20 mg ml^{-1}; PO_2 = 0 Torr) and air-saturated dechloraminated Ottawa tap water (PO_2 = 153 Torr). After the measurements were taken for normoxic water, fish were exposed to hypoxia in 7 equal steps ranging from 130 to 20 Torr. Hypoxic conditions were achieved by bubbling N_2, at progressively increasing flow rates, through a water-gas equilibration column that provided flowing water to the fish. Continuous data recordings were obtained for fR and breathing amplitude after the PwO_2 had reached the target value (~10 min later). In each case, at least 5 min of breathing data were analyzed to obtain mean values.

Ventilatory responses to acute hypercapnia

$PwCO_2$ within the fish chamber was continuously measured by a PCO_2 electrode (Cameron Instrument Company, model E201) connected to a Cameron BGM 200 blood gas meter calibrated using mixtures of 0.25 and 1.0% CO_2 in water provided by a gas-mixer (Cameron Instrument Company GF-3/MP). After the measurements were taken in normocapnic water ($PwCO_2$ < 0.5 Torr; pH = 7.4 – 7.5), fish were exposed to hypercapnia in 3 steps: 1 Torr, 2.5 Torr and 3.5 Torr and then returned to normocapnia for a final set of breathing measurements. Hypercapnia was achieved by bubbling different percentages of CO_2 through a water-gas equilibration column providing flowing water to the fish. Breathing data were obtained as described above.

To confirm that hypercapnia (elevated $PwCO_2$) rather than the reduction in water pH was initiating the observed breathing changes, ventilation was monitored in acidified (pH = 6.3) normocapnic water. A pH of 6.3 was chosen because it corresponded to the pH reached at the highest degree of hypercapnia ($PwCO_2$ = 3.5 Torr). Approximately 15 L of water was titrated with 1 mol l^{-1} HCl until the pH was lowered to 6.3. The water was

then bubbled overnight with air having passed through a 10 M mol l^{-1} solution of KOH (which reduces the amount of CO_2 in air) and if necessary, the pH was adjusted to 6.3 the next day. Ph values were taken using pH glass electrode (Metrohom AG 9101 Herisau).

Ventilatory responses to acute hyperoxia

Four fish exhibiting episodic breathing and 6 fish displaying continuous breathing were subjected to acute hyperoxia. PO_2 within the fish chamber was continuously measured by a fiber optic O_2 electrode (see above). After breathing was assessed in normoxic water, the water supplying the chambers was bubbled with O_2 through a water-gas equilibration column using a gas mixer (Cameron Instrument Company GF-3/MP). Breathing was again analyzed once the water PO_2 had reached at least 300 Torr.

Externally administered cyanide

In this series of experiments, different concentrations (0.5 -200 μg ml^{-1}) of sodium cyanide (NaCN) dissolved in water were introduced into the water supplying the recording chambers, and then flushed away with cyanide-free water within 30 sec. The goal was for the NaCN to flow across the gills and interact rapidly with externally oriented (water-sensing) O_2 chemoreceptors. After each injection, respiratory values were recorded for 1 to 2 min. The next dose was administered at least 10 min later. The doses of NaCN were determined based on pilot experiments in which dose-response curves for respiratory responses to NaCN were studied.

Confocal immunofluorescence microscopy

The basic protocols for gill extraction, immunolabeling and confocal imaging were modified from a previous study (Jonz and Nurse, 2003). Zebrafish were killed by

overdose with anaesthetic (MS 222). Gill baskets were rinsed in 1 mol l^{-1} phosphate-buffered saline (PBS; pH 7.4) and fixed by immersion in 4% paraformaldehyde (prepared in PBS) at 4°C for 4 h. Fixed gills were rinsed in PBS and permeabilized for 24 - 48 h at 4°C in PBS containing 1% fetal calf serum and 0.5% Triton X-100 (PBS – TX) (pH 7.8). Neuroepithelial cells (NECs) of gill filaments were identified in whole-mount preparations using antibodies directed against serotonin (5-HT; Jonz and Nurse, 2003) and against synaptic vesicle protein (SV2, Jonz and Nurse, 2003), found in neuronal and endocrine cells. Neurons and nerve fibres of the gill arches and developing filaments were identified using antibodies against a zebrafish-derived neuron-specific antigen (ZN-12; Trevarrow et al., 1990; Jonz and Nurse, 2003). Polyclonal rabbit 5-HT antibodies (Sigma) were used at a dilution of 1:200 and localized with goat anti-rabbit secondary antibodies Alexa 488 (1:600, Molecular Probes, Eugene, OR, USA). Monoclonal mouse anti-ZN-12 (Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) was used at a dilution of 1:25. Monoclonal mouse anti SV-2 (Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) was used at a dilution of 1:100. Both anti-mouse antibodies were localized with goat anti-mouse secondary antibodies conjugated with Alexa 546 (1:400, Molecular Probes, Eugene, OR, USA). All antibodies were diluted with PBS-TX. Fixed gill filaments were incubated in primary antibodies for 4 days at 4°C and in secondary antibodies at room temperature (22–24°C) for 1 h in darkness. Gill filaments were prepared as whole mounts on glass microscope slides in Crystal Mount ™ (Sigma) or Vectashield ® (Vector Laboratories, Inc). Whole-mount gill preparations were examined with a confocal scanning system (OLYMPUS BX50WI) equipped with an argon laser. Images were collected using confocal graphics software (Fluoview 2.1.39). Each image obtained using the confocal

scanning system is presented as a composite projection of serial optical sections 0.3 μm in size. Image processing and manipulation was performed using Paint Shop Pro. Gill baskets were also viewed using a Zeiss Axiophot light microscope and a digital Hamamatsu C5985 chilled CCD camera. Images were captured using the Metamorph imaging system (Version 4.01). The number of NECs per filament was quantified using Scion Image Beta 4.02 software (http://www.scioncorp.com/). One whole mount preparation per fish was examined. The number of viewable filaments varied from 4 to 10 among the preparations that were examined. An average value for NEC density was then calculated for each whole mount preparation using the data from all viewable filaments. The two-dimensional surface area of each filament was also calculated to ensure filaments were of equal size among the various treatment groups.

Statistical analysis

Ventilation frequencies and relative amplitudes from all experiments are reported as means ± 1 standard error of the mean. In most cases data sets were analyzed using two-way repeated measures analysis of variance (ANOVA). If a statistical difference was identified, a post-hoc multiple ("all pair wise") comparison test (Bonferroni's t-test) was applied. Where appropriate, some data were analysed by one-way ANOVA followed by Bonferroni's t-test (Table 1) or by unpaired Student's t-test (Figures 1.3 and 1.7B). All statistical tests were performed using a commercial statistical software package (SigmaStat version 3.0).

Results

Validation of the method for recording breathing frequency and amplitude

To demonstrate that the voltage oscillations recorded from the water were indeed exclusively derived from opercular movements, recordings were obtained from fish rapidly anaesthetized in their chambers. The voltage oscillations that were observed in breathing fish (Figure 2.1A) were eliminated by anaesthesia indicating that the heartbeat was not contributing to the recorded signals (Figure 2.1B). On the other hand, spontaneous swimming activity or struggling produced large voltage changes that obscured the underlying smaller oscillations associated with breathing (data not shown). Breathing frequencies as determined from video recordings of the filmed fish were essentially identical to those calculated from the voltage oscillations acquired by computer (Figure 2.2A). A correlation of the fR data obtained by the two independent methods done on 6 fish yielded a linear relationship ($r^2 = 0.99$) with a slope of 1 (Figure 2.2A). To verify that the voltage changes were indicative of breathing amplitude, opercular displacement was measured from the video recordings and compared to the peak-to-peak differences in voltage acquired concurrently from the same fish. The data plotted in Figure 2.2B demonstrate that the absolute values of ventilation amplitude varied markedly from those determined from the electronic tracings, either being under- or over-estimated. Moreover, despite calibrating the set-up assuming that each fish, at rest, was exhibiting an opercular displacement of 1 mm, during actual experimentation, the ventilation amplitudes that were determined via computer for these same fish were highly variable, ranging from 0.2 to 1.2 mm (Figure 2.2B). Nevertheless, a comparison of ventilation amplitudes obtained using the two methods demonstrated that ventilation

amplitude was clearly related to the magnitude of the peak-to peak voltage changes. Indeed, the correlation between *changes* in ventilation amplitude as measured by the two techniques was highly significant ($r^2 = 0.97$) yielding a slope of nearly 1 (0.82; Figure 2.2C).

Pausing frequency

Twenty percent of all zebrafish that were tested in normal water exhibited episodic breathing that was composed of breathing and non-breathing (apnea or pause) periods. Episodic breathing in normal water was never observed in the fish that were pre-exposed to hypoxia or hypercapnia (Table 2.1); hyperoxia pre-exposure was without significant effect on the pattern of episodic breathing. The average number of pauses in all control and hyperoxia pre-exposed fish was 19 and 12 min^{-1}, respectively (Table 2.1; $P = 0.095$). The total non-breathing period in control fish was 28.7 sec min^{-1} compared to 35.5 sec min^{-1} in the hyperoxia pre-exposed fish (Figure 2.4; $P = 0.056$). The pausing frequency in all fish exposed to acute hyperoxia was increased significantly (from 4.9 ± 3.1 to 13.0 ± 2.2 pauses min^{-1}; Figure 2.3A) and the duration of time occupied by apnoeic periods was increased from 7.9 ± 4.9 to 43.1 ± 2.2 sec min^{-1} (Figure 2.3B). During acute hyperoxia, 60% of fish exhibited episodic breathing. Representative original data recordings from normoxia and hyperoxia fish are illustrated in Figure 2.3C and D, respectively.

In contrast to hyperoxia, acute hypoxia decreased the number of breathing pauses and the total duration of apnoeic periods in control and hyperoxia pre-exposed fish (Figure 2.4). However, while pausing frequency and duration of apnea were decreased significantly in control fish at a PwO_2 of 130 Torr and had disappeared at 110 Torr, the episodic breathing pattern in the hyperoxia pre-exposed fish disappeared only when

PwO_2 had fallen below 70 Torr (Figure 2.4A, B). Unlike acute hyperoxia or hypoxia, hypercapnia (Figure 2.4C, D) did not significantly alter episodic breathing.

Response to acute hypoxia and hypercapnia

Acute hypoxia and hypercapnia clearly influenced the breathing pattern of zebrafish (Figure 2.5). During progressively severe hypoxia, the breathing frequency increased, becoming statistically significant at a PwO_2 of 110 Torr (Figure 2.5A); ventilation amplitude was unchanged (Figure 2.5B). Hypercapnia caused an increase in relative breathing amplitude at $PwCO_2$ levels as low as 1 Torr (Figure 2.5D); breathing frequency was unaffected (Figure 2.5C). Because breathing amplitude did not change during hypoxia and frequency remained constant during hypercapnia (these responses were unaffected by pre-exposure), in subsequent experiments only a single index of breathing was presented (frequency during hypoxia and amplitude during hypercapnia). To ascertain whether acidification of the water during hypercapnia was contributing to the increase in ventilation amplitude, experiments were performed on acidified water at constant $PwCO_2$. Fish that were exposed to a change of pH from 7.4 to 6.3 (the reduction in pH associated with the highest level of hypercapnia) did not increase relative ventilation amplitude (Figure 2.6). External injections of NaCN (to pharmacologically stimulate O_2 chemoreceptors) also caused an increase in fR without altering breathing amplitude.

Pre-exposure to hyperoxia

The respiratory responses of zebrafish that were pre-exposed to hyperoxia ($\approx$350 Torr for 28 days) to acute hypoxia, hypercapnia or external cyanide are depicted in Figure 2.7. The breathing frequency response to hypoxia was significantly blunted.

Indeed, a statistically significant increase in frequency was observed only when PwO_2 had fallen to 40 Torr compared to 110 Torr in the control fish. Furthermore, by re-plotting the data between PwO_2s of 150 and 40 Torr as linear regressions and analyzing the slopes, it was possible to demonstrate a significant reduction in the rate at which ventilation frequency increased during hypoxia in the fish pre-exposed to hyperoxia (1.26 ± 0.19 versus 0.68 ± 0.16 breaths min^{-1} $Torr^{-1}$ in control and hyperoxic fish, respectively, Figure 2.7B). The breathing amplitude responses to hypercapnia were eliminated in fish pre-exposed to hyperoxia (Figure 2.7C) and the response to external cyanide was blunted (Figure 2.7D). The density of gill filament NECs was significantly decreased from 36.0 ± 2.8 to 22.7 ± 2.3 cells $filament^{-1}$ ($P = 0.04$; Table 2.1) as determined by counting 5-HT immuno positive cells on the gill filament (Figure 2.8).

Pre-exposure to hypoxia

The respiratory responses of zebrafish pre-exposed to hypoxia (30 Torr for 28 days) to acute hypoxia, hypercapnia or cyanide were essentially equivalent to the control fish (Figure 2.9). Although the response to hypoxia appeared to be blunted (Figure 2.9A), the lower breathing frequencies were likely related to a lower frequency at rest (although the resting values were not statistically significant for this particular group of fish ($P = 0.052$). An analysis of all fish pre-exposed to hypoxia (Table 2.1) revealed a significant reduction in resting normoxic breathing frequency of 31 min^{-1} ($P = 0.044$). Re-plotting the data in Figure 2.9A as linear regressions demonstrated that the rate of change of breathing frequency during hypoxia up to 40 Torr was similar in both groups of fish (1.35 ± 0.22 versus 1.26 ± 0.19 breaths min^{-1} $Torr^{-1}$ in control and hypoxic fish, respectively, data not shown). The density of gill filament NECs was unaffected by hypoxia pre-exposure (Table 2.1).

Pre-exposure to hypercapnia

Except for increased breathing frequency at a PwO_2 of 20 Torr, the zebrafish pre-exposed to hypercapnia (7 - 9 Torr for 28 days) displayed similar responses to acute hypoxia as the control fish (Figure 2.10A). The ventilatory response to hypercapnia was blunted (Figure 2.10B) if one considers that breathing amplitude was not significantly elevated until the final stage of hypercapnia (3.5 Torr) was reached. The response to cyanide was significantly increased at the higher dose levels in the fish pre-exposed to hypercapnia (Figure 2.10C). The density of gill filament NECs was unaffected by hypercapnia pre-exposure (Table 2.1).

Discussion

Critique of the technique used to measure breathing

In the present study, the method of Altimiras and Larsen (2000) was exploited to record non-invasively the fR and relative breathing amplitude in adult zebrafish. By comparing the computer-acquired data with video recordings taken of the same fish, it was demonstrated that the voltage oscillations measured in the water were an accurate and reproducible index of fR. Although this procedure, when slightly modified, can be used to measure cardiac frequency (Altimiras and Larsen, 2000), there was no obvious contribution of the heart contraction to the measured voltage changes based on a comparison of the data from breathing and non-breathing (anaesthetized) fish. The technique also proved to be a reliable indicator of breathing amplitude assuming that opercular displacement reflects ventilatory stroke volume. However, because there is no simple method to measure the absolute magnitude of opercular displacement in resting fish, the equipment could not be calibrated to obtain absolute data for breathing amplitude. Thus, without further refinement, this method is suited only to monitor ingrelative changes in breathing amplitude. In the present experiments, resting opercular displacement was assumed to be 1 mm in all fish regardless of body mass and breathing frequency and thus 1 mm was selected as the calibration factor for converting the peak-to-peak voltage oscillations to measures of linear displacement. However, as the data in Figure 2.2B show, there can be a marked difference in the absolute values of opercular displacement from those calculated using the online recording system. The other significant limitation with this technique is that any swimming movements beyond those required for the fish to remain stationary often obscure the signals associated with

breathing. Thus, reliable data could only be obtained from fish that were stationary within their chambers. In our experience, similar problems are also encountered when attempting to analyze breathing motions from video recordings when zebrafish are swimming or struggling. Having validated the use of the non-invasive recording technique to monitor fR and relative opercular displacement, subsequent experiments were designed to evaluate the pattern of breathing in resting zebrafish and the impact of long-term acclimation to environments of altered gas composition.

Episodic breathing in zebrafish – the influence of acclimation or acute environmental change

Under resting conditions, 20% of the fish examined in this study exhibited episodic breathing characterized by periods of regular breathing interspersed with periods of apnea or breathing pauses. Episodic (or periodic) breathing has been described under resting conditions in all vertebrates (Milsom, 1991) including water-breathing fish (Smith *et al.,* 1983; Nonnotte *et al.,* 1993; Reid *et al.,* 2003). Most water-breathing fish that have been examined however, exhibit continuous breathing during normoxia although under conditions of lowered respiratory drive (e.g. hyperoxia), episodic breathing may occur (see review by Milsom 1991). In the present study, pausing frequency was increased by hyperoxia and decreased by hypoxia. These findings reinforce previous studies (e.g. Reid *et al.,* 2003) and are consistent with the view that episodic breathing is shaped, at least in part, by afferent input from peripheral chemoreceptors (see review by Smatresk, 1990). The original findings of the current study were that i) long-term acclimation to hypoxia or hypercapnia abolished episodic breathing (51 fish were assessed in normal water) and ii) acclimation to hyperoxia postponed the disappearance of episodic breathing in fish exposed to graded acute hypoxia. Because all studies were

performed at least 3 h after acclimated fish had been returned to normal water, the changes in breathing patterns presumably reflect a continuing effect of the prior acclimation. Thus, if driven by changes in afferent sensory input from peripheral chemoreceptors, the effects appear to endure for at least 3 h after the chemoreceptors are once again experiencing normoxic and normocapnic conditions. Further support for a long-term effect on breathing patterns was the fact that episodic breathing continued in fish acclimated to hyperoxia even under conditions of increased respiratory drive (hypoxia). It would be interesting to determine the length of time required to re-establish normal breathing patterns, and discover if these changes in ventilation pattern are irreversible, long or short term.

Acute respiratory responses to hypoxia or hypercapnia

As reported for other fish species (see Table 1 in Gilmour, 2001), zebrafish displayed an increase in fR in response to acute hypoxia. Interestingly, hypoxia did not elicit an accompanying increase in ventilation amplitude. This pattern of response to hypoxia is different from that observed in most fish that have been studied in which both fR and amplitude increase (Shelton *et al* 1986). Although the common carp (*Cyprinus carpio*) also does not display an increase in ventilation amplitude during hypoxia (Soncini and Glass, 2000), this response does not appear to be shared by all Cyprinids because the tench (*Tinca tinca*) increases both fR and opercular amplitude during hypoxia (Hughes and Shelton, 1962). The absence of a ventilation amplitude response to hypoxia in zebrafish also cannot be attributed to the high resting fR because these same fish exhibited a pronounced increase in opercular displacement during hypercapnia (see below). External injections of NaCN (to pharmacologically stimulate O_2 chemoreceptors) also caused an increase in fR without altering breathing amplitude. This

confirms that, in this species, at least part of the hyperventilatory response to hypoxia is being mediated by branchial (most likely external) O_2 chemoreceptors (Fritsche and Nilsson, 1983; see review by Burleson and Milsom, 1995). Recently, Jonz *et al.* (2004) provided direct evidence that the neuroepithelial cells of the zebrafish gill respond to hypoxia in a similar fashion to the glomus cells of the mammalian carotid body and thus are likely to be the O_2 sensors of the fish gill.

This is the first study to examine the respiratory response of zebrafish to hypercapnia. As documented for other species (see Table 2 in Gilmour 2001), zebrafish responded to hypercapnia by increasing ventilation amplitude. However, unlike in the majority of species previously examined (see Gilmour 2001), zebrafish did not display a concomitant increase in fR. Recent evidence suggests that the cardiorespiratory responses of fish to elevated CO_2 are initiated largely by externally oriented branchial receptors that respond to changes in ambient PCO_2 rather than pH (Burleson and Smatresk, 2000; McKendry *et al.*, 2001; McKendry and Perry, 2001; Perry and Reid, 2002; Gilmour *et al.* 2005). The results of the present study provided additional evidence that it is the change of $PwCO_2$ and not pH that is responsible for increasing breathing amplitude during hypercapnia. It is believed that the glomus cells of the mammalian carotid body sense changes in both PO_2 and PCO_2 and thus act as combined O_2/CO_2 chemoreceptors (Gonzales et al., 1994; Zhang and Nurse, 2004; Prabhakar and Jacono, 2005). It is not known whether the O_2-sensing neuroepithelial cells of the fish gill are also able to detect changes in PCO_2. Although the ventilatory responses to hypoxia and hypercapnia in zebrafish were markedly different (increased fR during hypoxia; increased amplitude during hypercapnia), this does not exclude the presence of a single receptor type sensing both O_2 and CO_2. Indeed, it is plausible that stimulation of single cell type could be

linked to varied responses given that the nature of the response is likely to be dictated by downstream signal transduction pathways.

Acclimatization to hyperoxia

This is the first study to assess the impact of long-term acclimatization to hyperoxia on ventilatory reflexes in fish. The results demonstrated that exposure to hyperoxia for 28 days blunted the subsequent ventilatory responses to hypoxia, external cyanide and hypercapnia. Long-term hyperoxia is known to cause a similar attenuation of the carotid body chemosensitivity to hypoxia and cyanide in cats (Lahiri *et al.,* 1987; 1990) and rats (Arieli *et al.,* 1988) but is apparently without effect on humans (Gelfand *et al.,* 1998) even when the levels of hyperoxia approach the limits of toxicity. In those mammals exhibiting a hyperoxic blunting of the ventilatory response to hypoxia, the response to hypercapnia is sustained (Torbati *et al.,* 1989), reduced (Lahiri *et al.,* 1990) or even enhanced (Lahiri *et al.,* 1987). The sustained CO_2 sensitivity in the face of a severe blunting or abolishment of the response to hypoxia has led to the idea that hyperoxia in mammals specifically targets O_2 sensing mechanisms of the carotid body. The carotid body also retains its usual responsiveness to nicotine or dopamine following hyperoxia (Lahiri *et al.,* 1987; 1990) further suggesting that the blunting effects of hyperoxia are not caused by general cellular damage.

In the present study, the reflex hyperventilatory response to hypercapnia was eliminated (at least statistically; Figure 2.7C) by prior exposure to hyperoxia. The coincident inhibitory effects of hyperoxia on O_2- and CO_2-mediated reflexes suggest that a common element of chemoreception is being affected. The simplest explanation is that the NECs, functioning as dual O_2 and CO_2 sensors, are being influenced by hyperoxia. In support of this idea, the density of gill filament NECs was significantly reduced after 28

days of hyperoxia. Thus, we speculate that the sensitivity of the ventilatory response to hypoxia or hypercapnia is controlled, at least partially, by the numbers of NECs exposed to the inspired water. This theory does not exclude the possibility that other levels of respiratory control are being impacted by hyperoxia.

Acclimatization to hypoxia

Previous experiments evaluating respiratory acclimatization to hypoxia have focused on mammals. The results have demonstrated that the hypoxic ventilatory response (HVR) is either increased after continuous chronic hypoxia (Weil, 1986; Bisgard and Forster, 1996; Dwinell and Powell, 1999) or unaffected (Powell *et al.*, 2000). To date, only a single study has investigated the respiratory consequences of chronic hypoxia in fish (Burleson *et al*, 2002). The results of that study on catfish (*Ictalurus punctatus*) showed that 7 days of acclimatization to moderate hypoxia caused an increase in fR and increased sensitivity to hypoxia. In contrast, the results of the present study using zebrafish revealed a significant reduction of resting fR without any effect on the ventilatory responsiveness to hypoxia or cyanide. The lack of an effect of hypoxia acclimatization on the acute responses to hypoxia or cyanide is consistent with the finding that the density of gill filament NECs was unaltered by chronic hypoxia. In a previous study, Jonz et al (2004) also showed that the numbers of 5-HT positive NECs were unchanged by 60 days of hypoxic exposure (35 Torr) although their size was increased by 15%. However, by combining a marker for synaptic vesicle protein (SV-2) with the serotonin antibody (5HT), it was demonstrated that the total number of NECs (5-HT-positive and 5-HT-negative) was increased by chronic hypoxia (Jonz et al, 2004). Whether or not the 5-HT-negative NECs are also capable of sensing O_2 is unclear. Several explanations for the reduced breathing frequency in the fish exposed to chronic

hypoxia can be offered. First, an enhancement of O_2 transfer and blood O_2 transport (Perry and Wood, 1989; Nikinmaa, 2001) may result in a lowering of the ventilatory convection requirement. Second, the return of the fish to normoxia after 28 days in hypoxic water may result in the perception of a state of relative hyperoxia by the branchial O_2 chemoreceptors (Prabhakar and Jacono 2005).

Acclimatization to hypercapnia

In mammals, studies suggest that pre-exposure to hypercapnia does not alter the response to acute hypoxia or hypercapnia (Bisgard and Forster, 1996; Remmers and Lahiri, 1998; Kondo *et al.*, 2000). As in mammals, the ventilatory response of zebrafish to hypoxia was unaltered by chronic hypercapnia although the response to external cyanide was increased and the response to hypercapnia was modestly blunted. The attenuation of the breathing response to cyanide without affecting the hypoxic response is particularly interesting and suggests that hypercapnia might be influencing the responsiveness of external branchial O_2 chemoreceptors (thus explaining an enhanced response to cyanide) without affecting, or even possibly reducing, the responsiveness of internal O_2 receptors.

Although the numbers of branchial filament 5-HT-positive NECs were unaffected by chronic hypercapnia, we cannot exclude the possibility that 5-HT-negative NECs were being targeted. Unfortunately, the single laser confocal microscope used for this study did not allow us to co-localize 5-HT and SV-2 and thus we were unable to identify the 5-HT-negative/SV-2-positive cells.

Conclusions

Currently, it is unknown whether the gill NECs demonstrated to be responsive to hypoxia (Jonz et al, 2004) and sharing similar properties with the glomus cells of the carotid body, are also able to sense changes in ambient PCO_2. Although no direct evidence was provided in this study, the finding that hyperoxia blunted the breathing responses to hypercapnia (as well as hypoxia) together with the result that hypercapnia enhanced the response to cyanide, suggests a certain degree of interaction between O_2- and CO_2-sensing and suggests that the NECs may be acting as both O_2 and CO_2 chemoreceptors. Clearly this is an area that warrants further investigation.

Acknowledgements

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Table 2.1 Average breathing frequencies (breath min^{-1}) of fish pre-exposed to hyperoxia (N=27), hypoxia (N=21) or hypercapnia (N=30) as well as control fish (N=36). Average number of pauses in normoxia 3 h post pre-exposure to hyperoxia (N=5), hypoxia (N=21) or hypercapnia (N=30) as well control fish (N=4). Average number of chemoreceptors immunoreactive to 5HT (5HT-IR) per gill filament. Asterisk (*) presents a significant difference between groups (one way RM ANOVA, $P<0.05$).

Group	**Resting f_R (min^{-1})**	**Resting pausing f_R (min^{-1})**	**Density of 5HT-IR NECs ($filament^{-1}$)**
Control	191 ± 12 (36)	19 ± 3.8 (4)	36.8 ± 2.8 (8)
28 days hyperoxia	199 ± 14 (27)	12 ± 3.6 (5)	22.6 ± 2.3* (8)
28 days hypoxia	165 ± 16* (21)	0* (21)	32.9 ± 1.8 (4)
28 days hypercapnia	213 ± 13 (30)	0* (30)	37.5 ± 5.8 (3)

Figure 2.1 Representative raw data acquisition recordings illustrating the voltage changes measured in the water of (A) a fish undergoing spontaneous breathing and (B) the same fish after *in situ* anaesthesia with benzocaine.

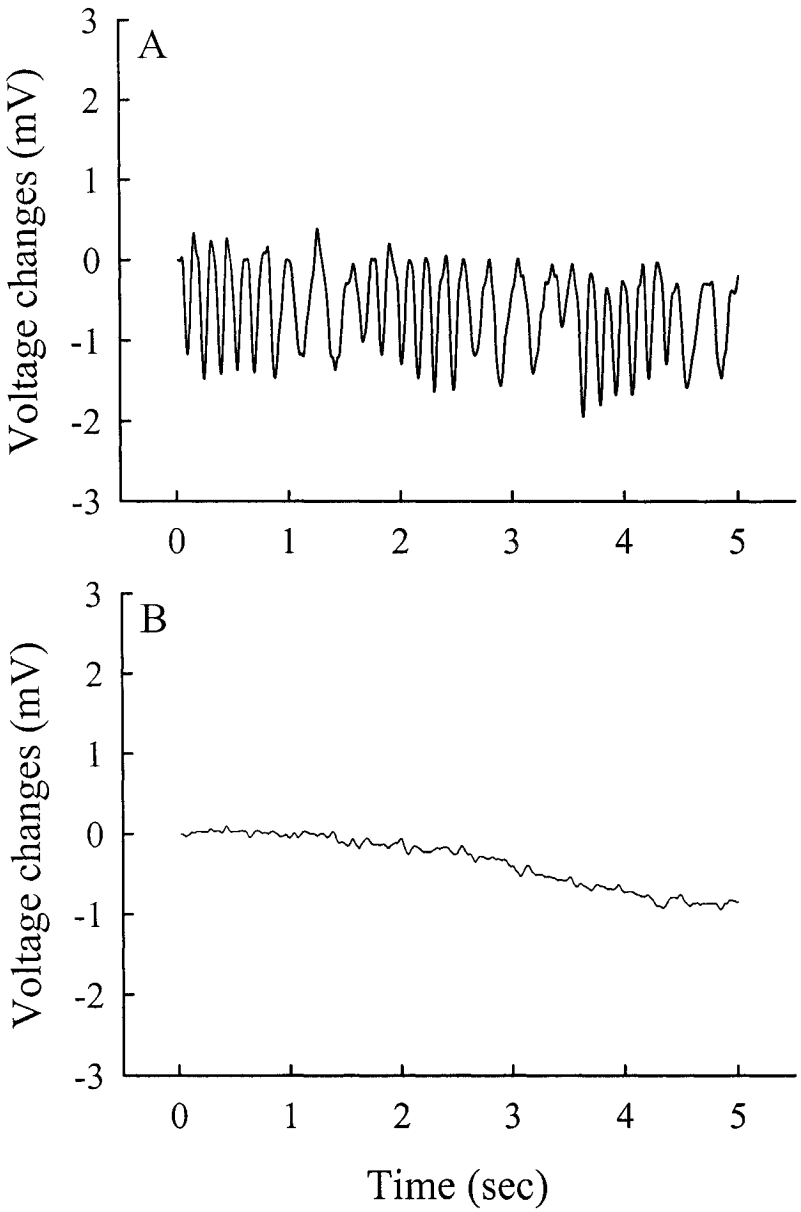

A
B
Voltage changes (mV)
Voltage changes (mV)
3
2
1
0
-1
-2
-3
0
1
2
3
4
5
Time (sec)

Figure 2.2. The relationships between breathing parameters as measured by analysis of video recordings or from computerized data acquisition (N=6). (A) The correlation between breathing frequencies (fR) determined by the two methods ($r^2 = 0.999$, $y = 0.99x + 0.69$), (B) the correlation between opercular displacement (a measure of breathing amplitude) determined by the two methods during hypoxia ($PO_2 = 40$ Torr) (N=3) and hypercapnia ($PCO_2 = 3.5$Torr) (N=3). The correlation in panel C demonstrates that the increases in ventilation amplitude induced by hypercapnia were analogous regardless of the method of measurement ($r^2 = 0.968$, $y = 0.82x + 0.12$); N = 6.

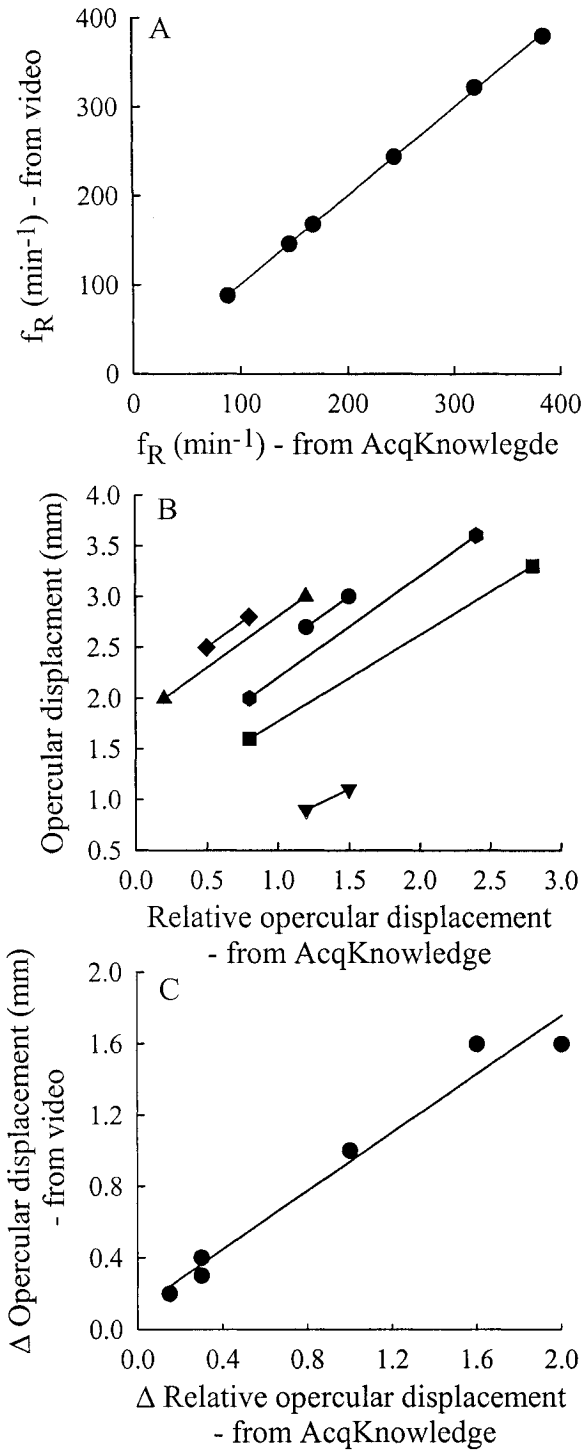

A
400
300
200
100
0
0
100
200
300
400
f_R (min^{-1}) - from video
f_R (min^{-1}) - from AcqKnowlegde
B
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
Opercular displacment (mm)
Relative opercular displacement
- from AcqKnowledge
C
2.0
1.6
1.2
0.8
0.4
0.0
0.0
0.4
0.8
1.2
1.6
2.0
Δ Opercular displacement (mm)
- from video
Δ Relative opercular displacement
- from AcqKnowledge

Figure 2.3. The frequency of breathing pauses (A) and the time occupied by apnea in seconds per minute (B) in zebrafish (N = 10) during normoxia (black bars) and during hyperoxia (white bars). Representative original data recordings from the fish in normoxia and hyperoxia are shown in panels C and D, respectively. A statistical difference ($P < 0.05$ – two way ANOVA RM) between the two groups is indicated by a dagger (†).

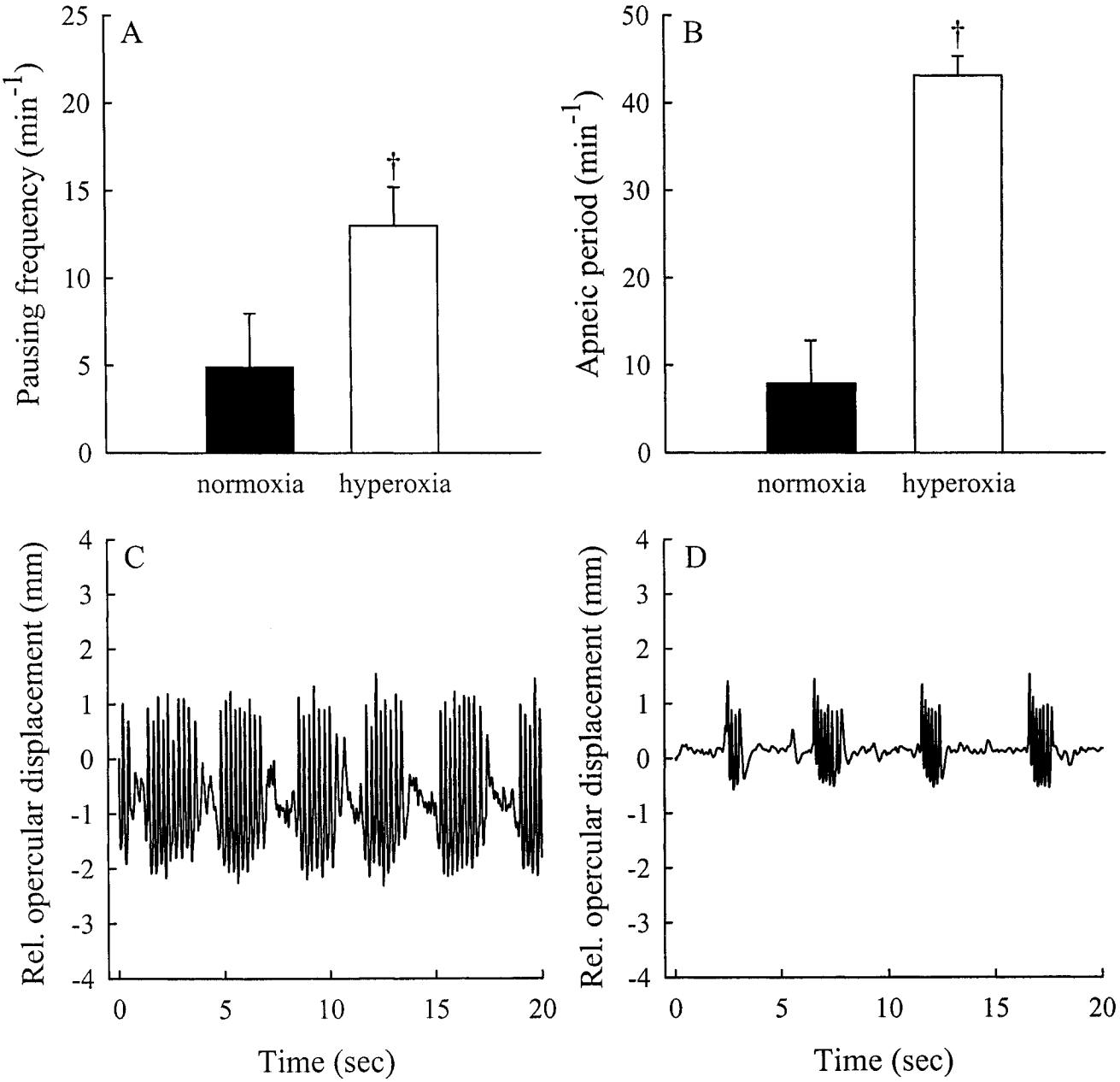

A
Pausing frequency (min-1)
25
20
15
10
5
0
†
normoxia
hyperoxia
B
Apneic period (min-1)
50
40
30
20
10
0
†
normoxia
hyperoxia
C
Rel. opercular displacement (mm)
4
3
2
1
0
-1
-2
-3
-4
0
5
10
15
20
Time (sec)
D
Rel. opercular displacement (mm)
4
3
2
1
0
-1
-2
-3
-4
0
5
10
15
20
Time (sec)

Figure 2.4. The frequency of breathing pauses (A, C) and the proportion of total breathing occupied by apnea (B, D) in control (black bars, N = 7) and hyperoxia pre-exposed (white bars, N = 8) zebrafish (*Danio rerio*) exposed to acute hypoxia (A, B) or acute hypercapnia (C, D). A statistical difference ($P < 0.05$ – two way ANOVA RM) between the two groups is indicated by a dagger (†).

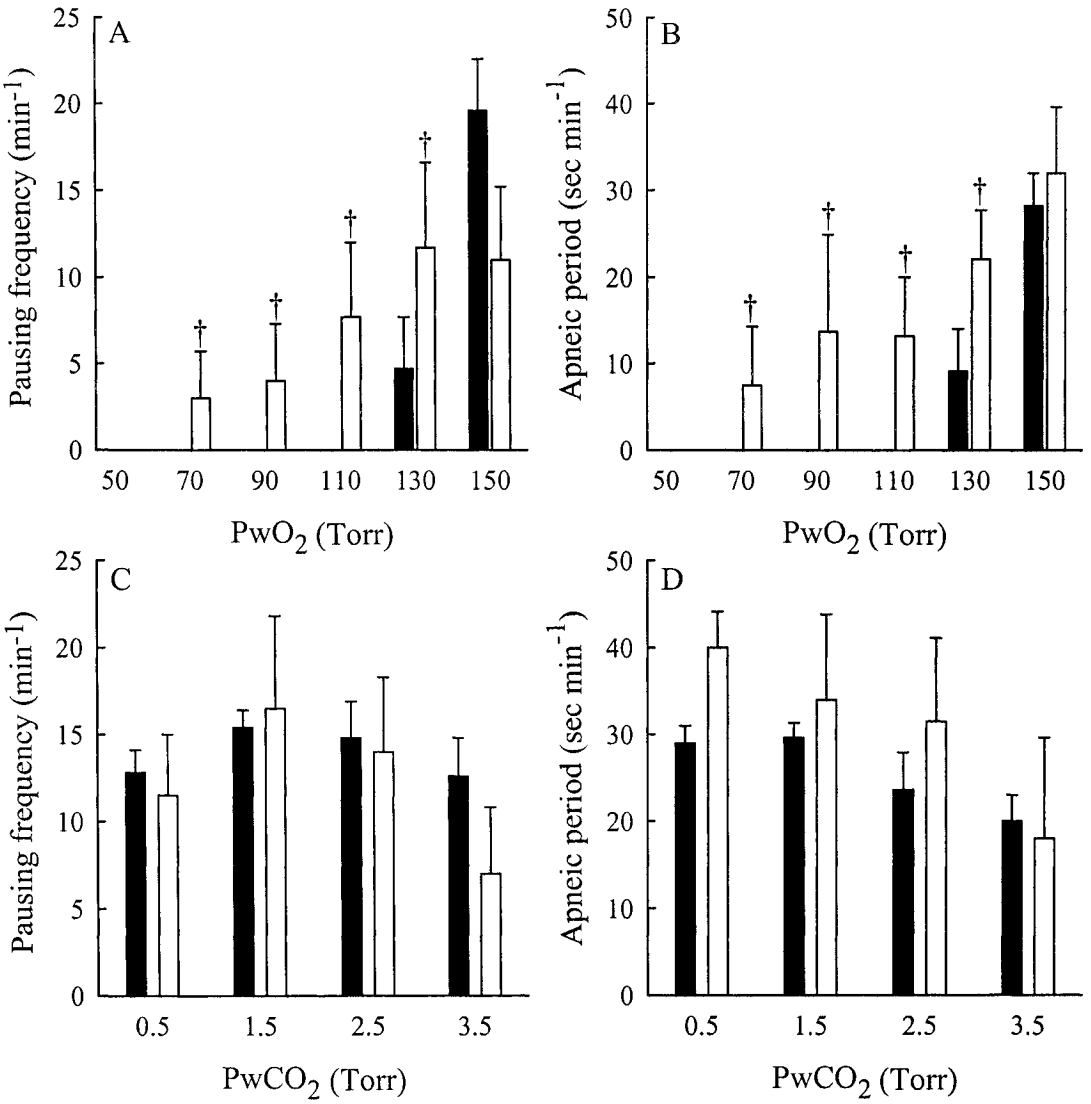

A
Pausing frequency (min-1)
25
20
15
10
5
0
50
70
90
110
130
150
PwO2 (Torr)
B
Apneic period (sec min-1)
50
40
30
20
10
0
50
70
90
110
130
150
PwO2 (Torr)
C
Pausing frequency (min-1)
25
20
15
10
5
0
0.5
1.5
2.5
3.5
PwCO2 (Torr)
D
Apneic period (sec min-1)
50
40
30
20
10
0
0.5
1.5
2.5
3.5
PwCO2 (Torr)

Figure 2.5. Respiratory responses of zebrafish (*Danio rerio*) to acute hypoxia (A, B) or hypercapnia (C, D). Control fish (filled circles, N = 12) were monitored in normal water for a similar period of time as the experimental fish (unfilled squares, N = 9 for hypoxia; N = 14 for hypercapnia). Panels A and C illustrate the breathing frequency (fR) changes, and panels B and D change show the changes in relative breathing amplitude (opercular displacement). Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).

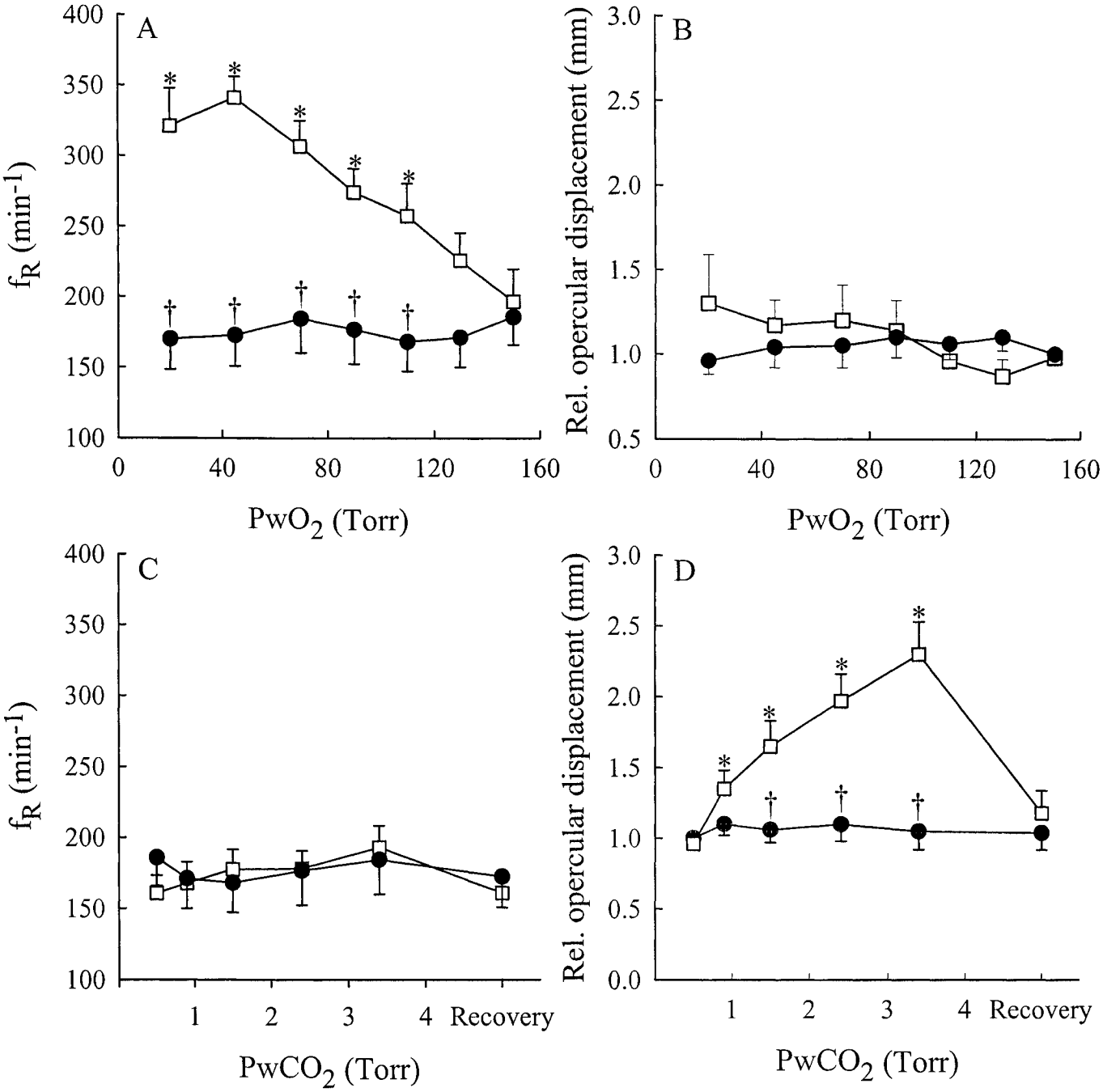

A
B
C
D
f_R (min^{-1})
Rel. opercular displacement (mm)
PwO$_2$ (Torr)
PwCO$_2$ (Torr)
Recovery
*
†

Figure 2.6. The effects on ventilation amplitude (relative opercular displacement) in zebrafish (*Danio rerio*) of changing water pH with, or without, accompanying hypercapnia. One group of fish was exposed to an increase in PwCO2 from 0.3 to 3.5 Torr causing pH to change from 7.4 to 6.3 (filled bars; N = 14). Another group of fish (unfilled bars; N = 8) was subjected to a change in water pH only from 7.4 to 6.3 at constant PwCO2 of 0.3 Torr. A significant change in opercular displacement is denoted by an asterisk (*); one-way RM ANOVA ($P < 0.05$).

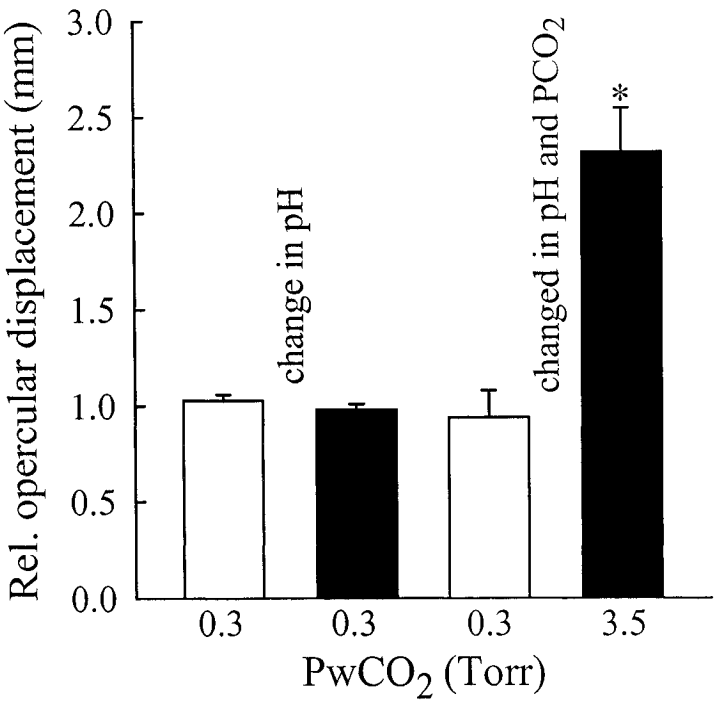

Rel. opercular displacement (mm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
change in pH
changed in pH and PCO2
*
0.3
0.3
0.3
3.5
PwCO2 (Torr)

Figure 2.7. The respiratory responses of zebrafish (*Danio rerio*) pre-exposed to hyperoxia (PwO_2 > 350 Torr) for 28 days (unfilled circles) to (A) acute hypoxia (N =12), (C) hypercapnia (N = 8) or (D) sodium cyanide (N = 7) compared to the responses of control fish (filled circles; N = 12 different fish for each treatment). In panel B, the average rates of change of breathing frequency (fR) between 40- 155 Torr for the two groups are presented. Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).

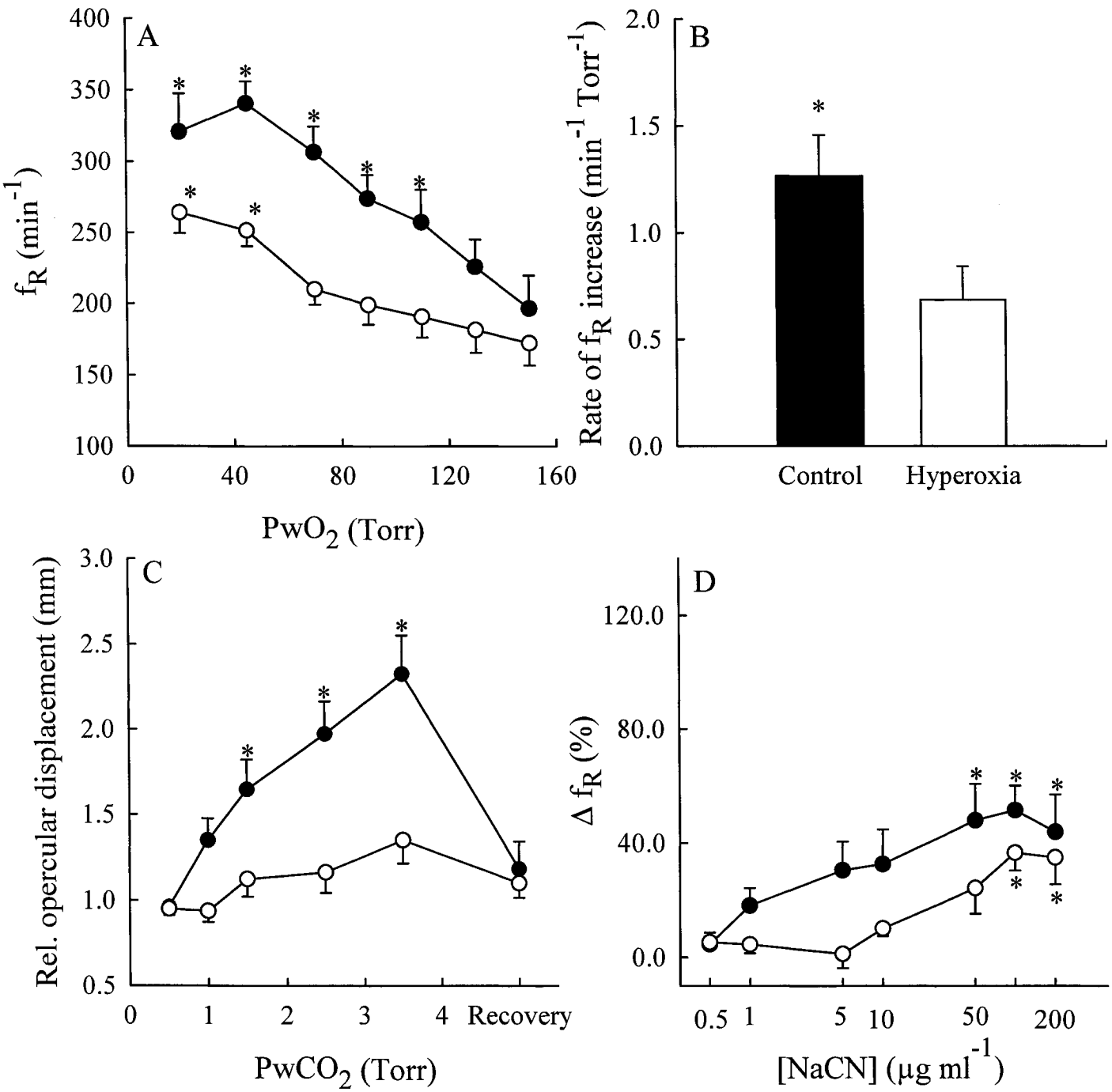

A
400
350
300
250
200
150
100
f_R (min^{-1})
0
40
80
120
160
PwO$_2$ (Torr)
B
2.0
1.5
1.0
0.5
0.0
Rate of f_R increase (min^{-1} Torr^{-1})
Control
Hyperoxia
C
3.0
2.5
2.0
1.5
1.0
0.5
Rel. opercular displacement (mm)
0
1
2
3
4
Recovery
PwCO$_2$ (Torr)
D
120.0
80.0
40.0
0.0
Δ f_R (%)
0.5
1
5
10
50
200
[NaCN] (μg ml^{-1})

Figure 2.8. Serotonin-immunoreactive (5-HT-IR) neuroepithelial cells (NECs) of the gill filament (F) in zebrafish (*Danio rerio*). (A) 5-HT-IR NECs along the filament in a control fish; (B) 5-HT-IR NECs along the filament at higher magnification in a hyperoxia pre-exposed fish; (**C**) higher magnification of double labeled 5-HT-IR and SV-2 – IR NEC with associated nerve fibers (ZN-12-IR) of the proximal filament epithelium in a control fish. Scale bars; **A** 100 μm; **B** and **C**: 10 μm.

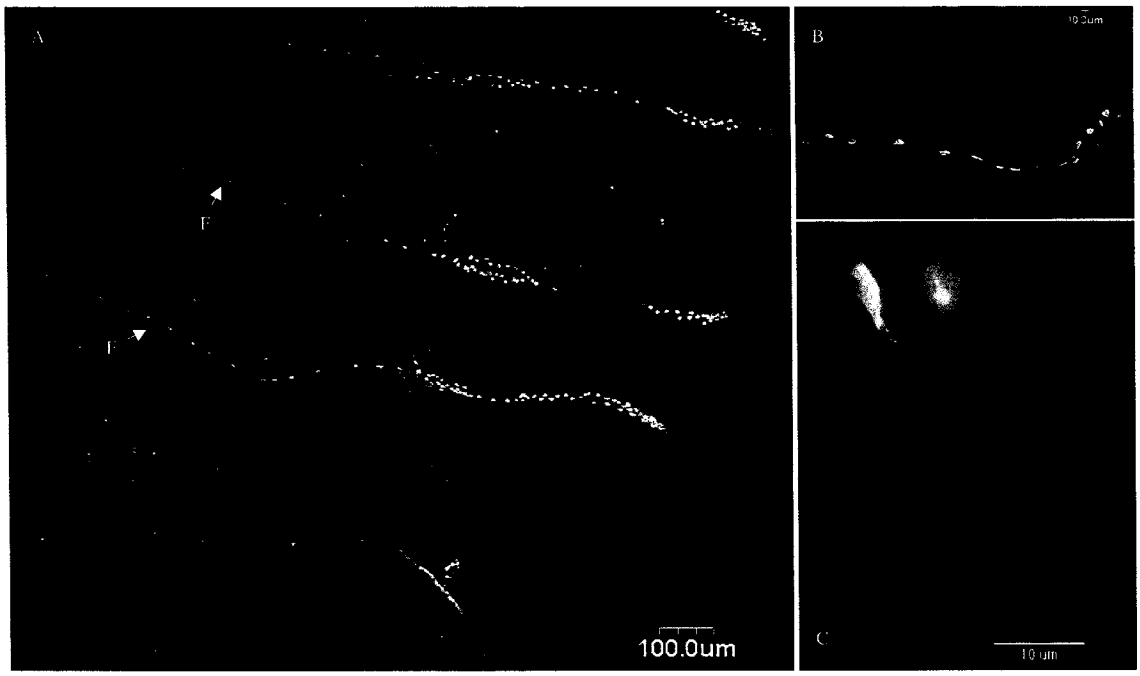

A
F
F
100.0um
B
10.0um
C
10 um

Figure 2.9. The respiratory responses of zebrafish (*Danio rerio*) pre-exposed to hypoxia (PwO2 = 30 Torr PO2) for 28 days (unfilled circles) to (A) acute hypoxia (N = 8), (B) hypercapnia (N = 7) or (C) sodium cyanide (N = 6) compared to the responses of control fish (filled circle, N = 12 different fish for each treatment). Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).

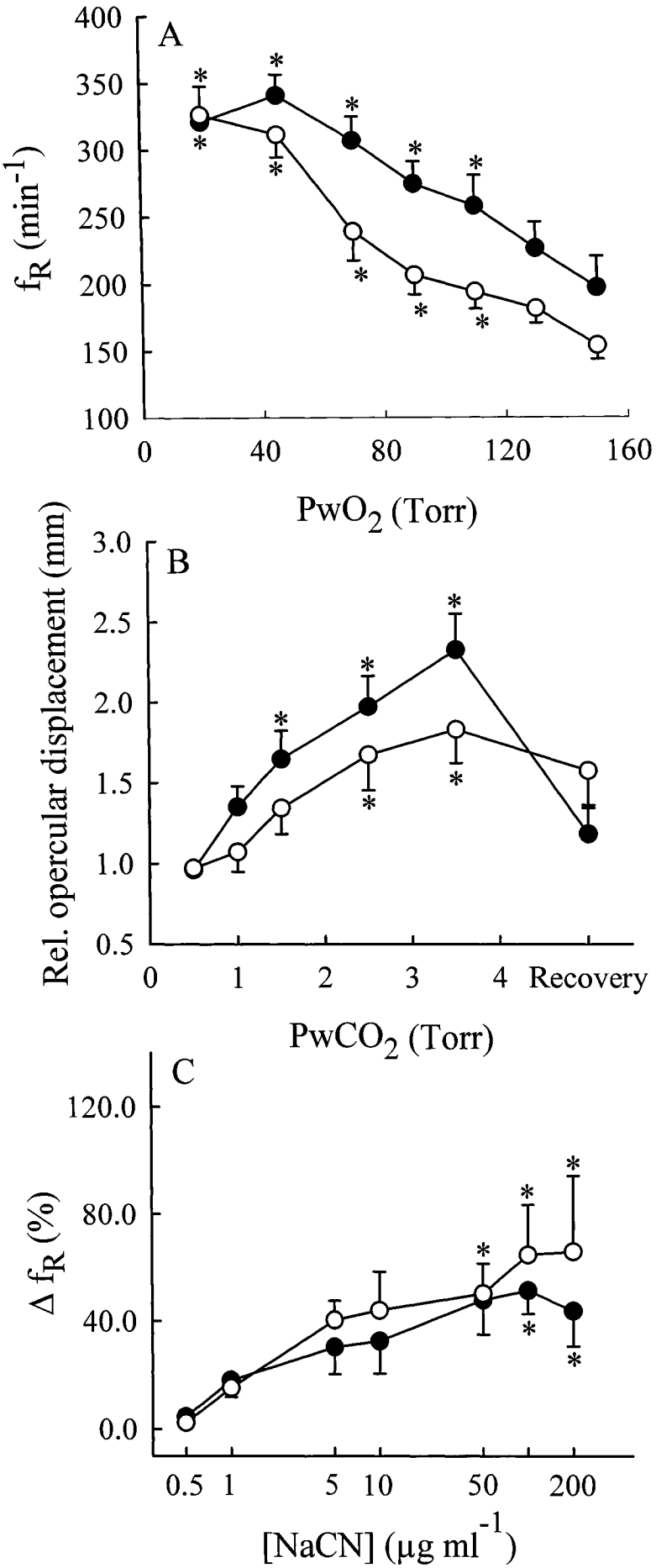
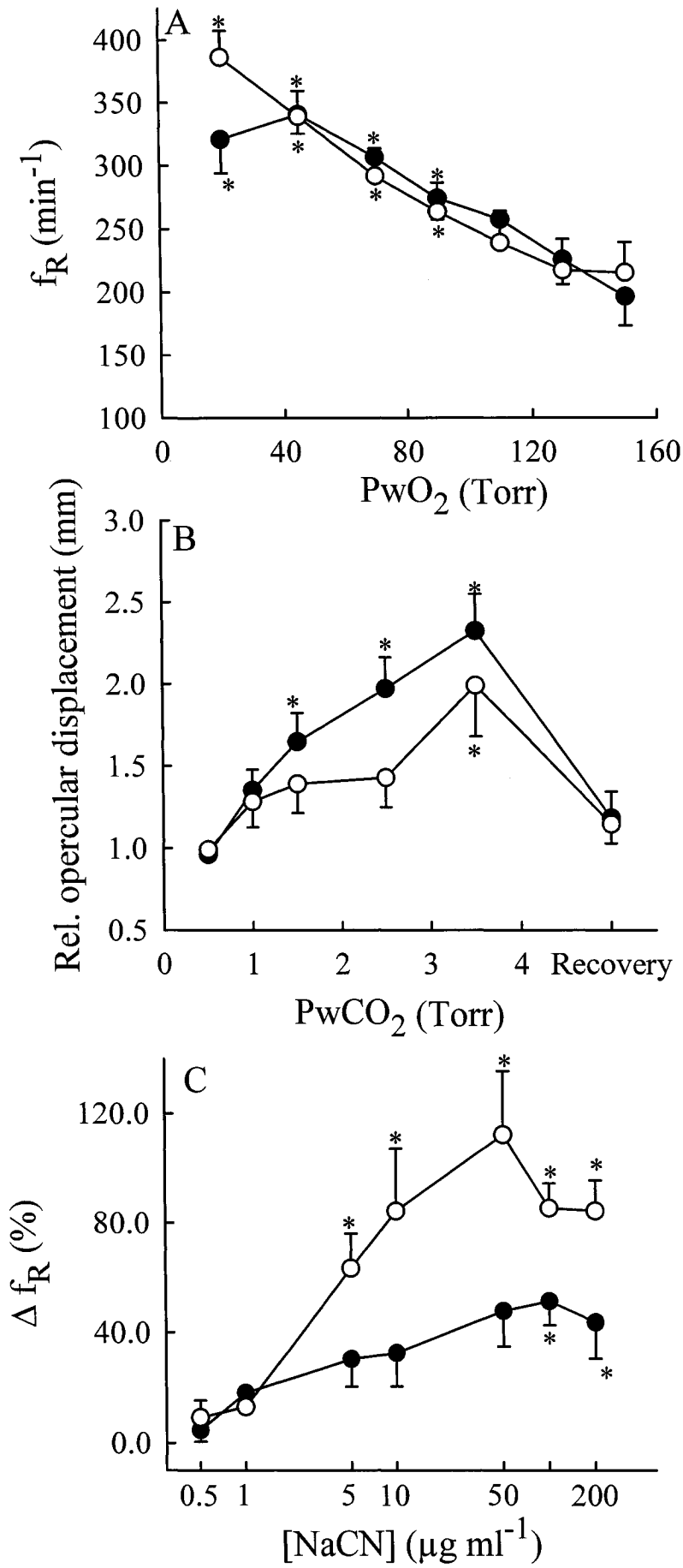

A
400
350
300
250
200
150
100
f_R (min^{-1})
0
40
80
120
160
PwO$_2$ (Torr)
B
3.0
2.5
2.0
1.5
1.0
0.5
Rel. opercular displacement (mm)
0
1
2
3
4
Recovery
PwCO$_2$ (Torr)
C
120.0
80.0
40.0
0.0
Δ f_R (%)
0.5
1
5
10
50
200
[NaCN] (μg ml^{-1})

Figure 2.10. The respiratory responses of zebrafish (*Danio rerio*) pre-exposed to hypercapnia (PwCO2 = 9 Torr) for 28 days (unfilled circles) to (A) acute hypoxia (N = 11), (B) hypercapnia (N = 11) or (C) sodium cyanide (N = 8) compared to the responses of control fish (filled circles, N = 12 different fish for each treatment). Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).


A
400
350
300
250
200
150
100
f_R (min^{-1})
0
40
80
120
160
PwO_2 (Torr)
B
3.0
2.5
2.0
1.5
1.0
0.5
Rel. opercular displacement (mm)
0
1
2
3
4
Recovery
$PwCO_2$ (Torr)
C
120.0
80.0
40.0
0.0
Δf_R (%)
0.5
1
5
10
50
200
[NaCN] (µg ml^{-1})

CHAPTER 3

Developmental plasticity of ventilatory control in zebrafish, (*Danio rerio)*

This chapter is based on a paper accepted in Journal Respiratory Physiology and Neurobiology:

Developmental plasticity of ventilatory control in zebrafish, *Danio rerio*

B. Vulesevic and S.F. Perry

Abstract

To determine whether development of ventilatory control in zebrafish (*Danio rerio*) exhibits plasticity, embryos were exposed to hypoxia, hyperoxia or hypercapnia for the first seven days post fertilization. Their acute reflex breathing responses to ventilatory stimuli (hypoxia, hypercapnia and external cyanide) were assessed when they had reached maturity (3 months or older). Zebrafish reared under hyperoxic conditions exhibited significantly higher breathing frequencies at rest (283 ± 27 *versus* 212 ± 16 min^{-1} in control fish); breathing frequency was unaffected in adult fish subjected to hyperoxia for 7 days. The respiratory responses of fish reared in hyperoxic water to acute hypoxia, hypercapnia or external cyanide were blunted (hypoxia, cyanide) or eliminated (hypercapnia). Adult fish exposed for seven days to hyperoxia showed no change in acute responses to these stimuli. The respiratory response to acute hypoxia of fish reared under hypoxic conditions was slightly blunted while the ventilatory responses to hypercapnia or external cyanide were unaffected. The breathing responses to the various ventilatory stimuli of adult zebrafish reared in hypercapnia were similar to those in fish reared under normal conditions.

A subset of all fish examined exhibited episodic breathing; an analysis of breathing patterns demonstrated that fish reared under hypercapnic conditions had an increased tendency to display episodic breathing. The results of this study reveal that there is flexibility in the design and functioning of the embryonic or larval respiratory system in zebrafish.

Introduction

Development of respiratory control is a complex and dynamic process that is affected by various factors that may alter the pattern of respiration in the adult. Developmental respiratory plasticity, defined as permanent alterations in mature respiratory control, can be induced by prior experience but only during "critical" periods of development (Carroll, 2003). The maturation of peripheral chemosensitivity, which in mammals is apparently triggered by the increase of PO_2 at birth, occurs at several levels. It begins pre natal with the formation of afferent neurons and the development of glomus cell Ca^{2+} sensitivity and ends with the capacity to release neurotransmitters (Donnelly, 2000). During postnatal life there is an increase in the number of sensory nerve fibres innervating the carotid body, and this corresponds to the rise of its sensitivity to hypoxia (Gonzales *et al.*, 1994). The carotid body, and possibly other peripheral chemoreceptors, mature after birth as they undergo structural and functional changes as a response to environmental changes in PO_2 (Lopez-Barneo *et al.*, 2001).

In mammals, exposure to hyperoxia during development alters ventilation in adult life (Mitchell and Johnson, 2003). For example, when imposed during critical periods of development, it impairs the maturation of carotid body chemoreceptors and may cause long-lasting reductions in carotid sinus nerve size (Bamford *et al.*, 1999). The consequence of such changes is a diminished response to hypoxia owing to blunted afferent inputs from the carotid body and neurons in the carotid sinus nerve (Bisgard *et al.*, 2005). Exposure of rats and kittens for 2 weeks preceding birth to 30% O_2 eliminated the response of carotid chemoreceptors to hypoxia in the short term; long-term effects

were not studied (Eden and Hanson, 1986; Hanson *et al.*, 1989). However, the long-term effects of developmental hyperoxia were established by Ling *et al.* (1996) who demonstrated that exposure of rats to 60% O_2 for the first month of life caused a marked impairment of ventilatory responses to hypoxia in adults.

Interestingly, chronic hypoxia (like hyperoxia) in developing neonates may also blunt ventilatory responses to acute hypoxia, probably as a result of decreased carotid body O_2 sensitivity (Donnelly and Doyle, 1994; Bisgard, 2000). In contrast, rats born after exposure to environmental hypoxia between embryonic days 5 and 20 exhibited marked augmentation of the ventilatory response to hypoxia, which by 9 weeks of age had normalized (Peyronnet *et al.*, 2000). Thus, depending on the stage of development, hypoxia may have differing effects on the ventilatory response in adult life (Carroll, 2003).

Exposure of rats to hypercapnia (7% CO_2) for the first week of life blunted the ventilatory response to acute hypercapnia at 45 – 50 days but did not induce longer-lasting effects in adults (Rezzonico *et al.*, 1990). In Japanese quail (*Coturnix japonica*), exposure of embryos to 2% CO_2 decreased the adult ventilatory response, but this alteration was observed primarily in females (Bavis and Kilgoer, 2001).

In the present study, we have investigated the developmental plasticity of respiratory control in the zebrafish (*Danio rerio*). This was accomplished by exposing fish to hypoxia, hyperoxia or hypercapnia for the first seven days post fertilization (d.p.f.) and then assessing normal breathing patterns as well as acute responses to hypoxia, external cyanide and hypercapnia in adults. The changes observed in the breathing pattern of these fish were compared to those of adult fish exposed to the same gas changes for seven days in adult life.

Materials and methods

Animals

Adult zebrafish (*Danio rerio*) were obtained from a commercial supplier (MIRDO, Montreal) and transported to the University of Ottawa Aquatic Care Facility where they were maintained in fiberglass tanks (4 L) supplied with aerated, dechloraminated City of Ottawa tap water at 28°C. Fish were subjected to a constant 10L: 14D photoperiod. Embryos were obtained using standard techniques for zebrafish breeding (ZFIN Fish book – Sprague *et al.*, 2001). Equal numbers of males (longer, slimmer, and more yellow especially on the belly) and females (plumper and more silvery) were placed into a breeding tank. Newly spawned eggs were transferred from the breeding tanks through a fine mesh. They were then placed in rearing tanks at 28°C and exposed to the particular gas treatment required for that group. The embryos normally hatched between 2 - 3 days of development but were not fed until 4 d.p.f. At 4 d.p.f., the embryos were fed live brine shrimp three times per day. After day 7 they were moved from the treatment tanks into tanks supplied with normal water, where they were fed brine shrimp until they were large enough to eat adult food (No. 1 crumble – Zeigler ™). For all treatment groups including the control group, the embryos were collected from the same group of parents thus reducing the genetic variation between the different experimental groups.

All procedures for animal use were carried out according to institutional guidelines and in accordance with those of the Canadian Council on Animal Care (CCAC).

Raising fish in hypercapnic, hypoxic or hyperoxic water

Different groups (N = 3 groups raised in hyperoxia, N = 4 groups raised in hypoxia, and N= 4 raised in hypercapnia) of zebrafish embryos were exposed for 7 days to hyperoxia (PwO_2 = 350 - 400 Torr), hypoxia (PwO_2 = 30 - 40 Torr) or hypercapnia ($PwCO_2$ = 7 - 9 Torr) in 2 L tanks starting at 6 h post fertilization. Five groups of control embryos were reared under similar conditions but were supplied with normoxic and normocapnic water. The gas composition of the water was adjusted by pumping through a gas equilibration column mixtures of CO_2 (1 - 2%) and air for hypercapnia or mixtures of N_2 (95%) and air for hypoxia. The gas mixtures were supplied by a Cameron gas mixer (model GF-3/MP). To achieve hyperoxia, O_2 (100%) was bubbled into a tank that was being supplied with normoxic water (30 ml h^{-1}). Water PCO_2 was monitored using a CO_2 electrode (Cameron Instrument Company, model E201) connected to a Cameron BGM 200 blood gas meter. Water PO_2 was measured using a fibre optics oxygen electrode (Ocean Optics FOXY AL300) and associated hardware and software (Ocean Optics SD 2000 and OOI sensors version 1.00.08). After 7 days, the fish embryos were transferred to normal water and reared for 3 – 4 months to adult size.

Pre-exposure of adult fish to hypercapnia, hypoxia or hyperoxia

Adult zebrafish were exposed to hypercapnia ($PwCO_2$ = 7 - 9 Torr), hypoxia (Pw_{O2} = 30 - 40 Torr), or hyperoxia (PwO_2 = 350 - 450 Torr) at 28°C in a 2 L tank for 7 days. Control fish were kept under similar conditions for 7 days but were provided with normoxic and normocapnic water. For each treatment, at least two groups of fish were pre-exposed at different times. Hypercapnia, hypoxia, and hyperoxia were achieved as described above.

Ventilation measurements, responses to acute hypoxia, acute hypercapnia and externally administered cyanide

Please see Materials and methods in chapter 2.

Statistical analysis

Ventilation frequencies and relative amplitudes from all experiments are reported as means ± 1 standard error of the mean. All data sets were analyzed using two-way repeated measures analysis of variance (ANOVA) or one way ANOVA. If a statistical difference was identified ($P < 0.05$), a post-hoc multiple ("all pair wise") comparison test (Bonferroni's t-test) was applied. All statistical tests were performed using a commercial statistical software package (SigmaStat version 3.0). For comparison of expected and observed number of fish expressing episodic breathing, *Chi*-square analysis was used ($P< 0.05$).

Results

Breathing patterns in fish reared in different environments

A subset of all fish examined exhibited episodic breathing that was characterized by cycles of breathing interspersed with periods of apnea. The percentage of fish displaying episodic breathing ranged between 12 and 23% (Table 3.1). The rearing of fish under hypercapnic conditions led to a significantly greater percentage of fish demonstrating episodic breathing (*Chi* square, $P < 0.05$; Table 3.1). In contrast, simply exposing adult fish for 7 days to similar conditions of hypercapnia did not alter the numbers of fish displaying episodic breathing (Table 3.1).

Zebrafish reared under hyperoxic conditions exhibited significantly higher resting breathing frequencies as adults (283 ± 27 *versus* 212 ± 16 in the control group, $P = 0.013$; Table 3.2). However, the number of pauses per min was similar to control fish (13 ± 1.0 *versus*14 ± 1.8 in the control group; Table 3.2). Breathing frequency was unaffected in adult fish subjected to hyperoxia for 7 days ($P = 0.739$, Table2).

Reflex responses to ventilatory stimuli

The respiratory responses to acute hypoxia, hypercapnia or external cyanide of adult zebrafish reared as larvae under hyperoxic conditions are depicted in Figure 3.1. The ventilatory response to hypoxia was blunted when comparing the rates of change of breathing frequencies with increasing hypoxia (Figure 3.1A, B). The rise in breathing amplitude in response to hypercapnia was eliminated in fish reared under hyperoxic conditions (Figure 3.1C). The effect of external cyanide on raising breathing frequency was attenuated (Figure 3.1D). The breathing responses of adult zebrafish pre-exposed to

hyperoxia for seven days to acute hypoxia, hypercapnia or NaCN were similar to control fish (data not shown).

The respiratory response of adult zebrafish to acute hypoxia was slightly blunted in fish reared under hypoxic conditions (30 Torr for 7 days). Thus, during mild hypoxia (PwO_2 = 100 to 140 Torr), breathing frequency was lower than in the control fish (Figure 3.2A). The ventilatory responses to hypercapnia (Figure 2.2B) or external cyanide (Figure 3.2C) were unaffected in fish reared under hypoxic conditions.

The breathing responses of adult zebrafish reared in hypercapnia (7 - 9 Torr for 7 days) to acute hypoxia, hypercapnia or NaCN (Figure 3.3) was similar to fish reared under normal conditions.

Discussion

The focus of this study was to assess the potential for developmental ventilatory plasticity in zebrafish (*Danio rerio*). This was accomplished by monitoring the ventilatory reflex responses of adults that had been reared for the first seven days of life in variable environments. The gills of zebrafish do not become fully functional until 14 days post fertilisation (d.p.f.) because the required respiratory lamellae are not yet developed. However, the pharyngeal arch from which the nerves innervating the gill filaments originate is present at 3 d.p.f. (Rombough, 2002). The hyperventilatory response to hypoxia appears to develop before this time, suggesting the presence of an O_2-sensing mechanism before complete development of the gills (Jacob *et al.*, 2002; Jonz *et al*, 2005). Furthermore, gill neuroepithelial cells (NEC), the presumed O_2 chemoreceptors of the fish gill, are consistently innervated by 7 d.p.f. (Jonz et al., 2005). Thus, we believe that the first 7 days of development was an appropriate period to target for potential developmental respiratory plasticity.

Our findings demonstrate that hyperoxia (unlike hypercapnia or hypoxia) during early development can markedly influence the resting breathing pattern in adults as well as acute responses to hypoxia or hypercapnia. In agreement with studies on mammals showing that hyperoxia exposure during a critical prenatal period may permanently impair the phrenic response to hypoxia (Ling *et al.*, 1996; 1997; Donnelly, 2000; Carroll, 2003), zebrafish reared under hyperoxic conditions displayed a blunted ventilatory response to acute aquatic gas changes (hypoxia or hypercapnia) as well as to the O_2 chemoreceptor stimulant, cyanide. The mechanisms of O_2 chemoreception in fish are not particularly well understood but are thought to be initiated by the stimulation of neuroepithelial cells (NEC) associated with the filament epithelium (Dunel-Erb and

Laurent, 1980; Bailly *et al.*, 2005; Jonz *et al.*, 2004). Peripheral arterial chemoreceptors, as well as central chemoreceptors, can be a site for modulation of respiratory rhythm generation during early neonatal development in mammals (Gauda and Lawson, 2000; Gauda *et al.*, 2004). Other studies on mammals demonstrated that the effects of developmental hyperoxia on the postnatal level of neuronal activity might be largely determined by changes to afferent neurons rather than to the chemoreceptor themselves. For example, rats reared in 60% O_2 for 4 weeks suffered a 41% loss of unmyelinated sinus nerve axons and a reduction in carotid body volume when compared with control rats (Donnelly, 2000). In our study, hyperoxia for an indefinite period changed the resting breathing frequency of zebrafish, thereby establishing a link between environmental O_2 and the development of the central rhythm generator. With this in mind, the blunted responses to hypoxia and cyanide may simply reflect the higher starting frequencies. Yet, the obvious attenuation of the hypercapnia response observed in this animal, suggests that there is an impairment of chemoreception or chemoreception pathways. This study provides additional evidence that developmental changes in functional O_2-sensing pathways are not unique to air-breathing mammals, but may have appeared earlier in vertebrate evolution.

In mammals, chronic hypoxia in developing neonates markedly blunts ventilatory responses to acute hypoxia, largely because of decreased carotid body O_2 sensitivity (Sterni *et al.*, 1999). Neonatal hypoxia in rats resulted in smaller body weights, especially in females and impaired both ventilatory and phrenic responses to hypoxia (Bavis *et al.*, 2004). In the present study, fish exposed to hypoxia for 7 days post-fertilization clearly showed a retarded development; their developmental age at day seven was approximately 5 days. Of all the experimental groups their adult size was significantly reduced (0.22 g compared to 0.50 g in control fish, $P = 0.032$). There was

no delay in development in the other experimental groups (hyperoxia or hypercapnia). Results from the present study suggest that an influence of hypoxia in early development is present (reduced growth) but that the effects do not include permanent changes to O_2 or CO_2 sensing. It is possible that changes in gas sensing did occur but that they were transitory.

Some mammals and birds exhibit a blunted ventilatory response to inspired CO_2, probably as a consequence of their developmental environment rather than a genetic adaptation (Bavis and Kilgoer, 2001). The hypercapnic ventilatory response in mammals, similar to the hypoxic ventilatory response, is determined at least in part by the respiratory environment experienced during development (Boggs *et al*, 1984). Fish reared under hypercapnic conditions for 7 days post-fertilization showed no change in body phenotype, survival rate or reflex responses to ventilatory stimuli. Thus, the results of this study exclude developmental hypercapnia (at least during the first seven days post-fertilization) as a contributor to chemoreception plasticity. On the other hand, this group had the highest number of fish within the group that expressed episodic breathing. Episodic breathing patterns arise at the level of the central respiratory rhythm (Richter, 1982) and are modified by various afferent inputs derived from chemoreceptors, mechanoreceptors and higher brain centers (Ballintijn and Juch, 1984; Feldman and Ellenberger, 1988; Reid *et al*, 2003). Our findings suggest that rhythm generation and/or its inputs can be influenced during development leaving lasting consequences on breathing patterns.

The existence of CO_2 chemoreception in fish is well established (reviewed by Gilmour, 2001; Perry and Gilmour 2002; Perry and Reid, 2002) but the specific cell type responsible for sensing CO_2 has yet to be identified. The glomus cells of the mammalian carotid body are believed to sense both O_2 and CO_2 (see review by Gonzalez *et al.*,

1994). It is unknown whether a similar situation exists for the NECs of the fish gill. The finding that raising fish under hyperoxic conditions blunted the ventilatory response to both hypercapnia and hypoxia, does suggest an interaction between O_2 receptors and CO_2 sensing. Clearly, further experiments are required to specifically assess the role of the fish gill NEC in CO_2 chemoreception.

Conclusion

The results of this study demonstrated that in zebrafish, exposure to hyperoxia during the first seven days of development causes long-lasting effects in adults on resting breathing frequency and reflex responses to ventilatory stimuli including hypoxia, cyanide and hypercapnia. The data clearly demonstrated that there is flexibility in the design and functioning of the embryonic or larval respiratory system in fish, and it is not operating simply on the basis of a pre-set genetic program.

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Table 3.1. The percentage of fish within the various treatment groups exhibiting episodic breathing. Data are presented as means ± 1 SEM; N numbers are indicated in parentheses. An asterisk (*) denotes a significant difference among the groups (*Chi* square test, $P < 0.05$).

Group	Exposed as embryos	Exposed as adults
Control	12.1 (33)	18.2 (22)
Hyperoxia	13.3 (30)	19.2 (26)
Hypoxia	16.0 (30)	23.1 (26)
Hypercapnia	19.4 (36)*	22.6 (31)

Table 3.2. Resting breathing variables including ventilation frequency (f_R) and pausing frequency in control fish or fish reared under (or exposed to as adults) conditions of altered water gas composition. Data are presented as means ± 1 SEM; N numbers are indicated in parentheses. An asterisk (*) denotes a significant difference among the groups (one way RM ANOVA, $P < 0.05$).

Group	Resting f_R (min^{-1})		Pausing frequency (min^{-1})
	Exposed as embryos	Exposed as adults	Exposed as embryos
Control	212 ± 16 (33)	197 ± 23 (19)	14 ± 1.8 (4)
Hyperoxia	283 ± 27 (30)*	218 ± 10 (19)	13 ± 1.0 (4)
Hypoxia	184 ± 13 (30)	176 ± 31 (12)	19 ± 4.2 (5)
Hypercapnia	197 ± 17 (36)	181 ± 18 (14)	23 ± 2.1 (7)

Figure 3.1. The respiratory responses of zebrafish (*Danio rerio*) reared in hyperoxia for the first seven days of life ($PwO_2 > 350$ Torr) (unfilled circles) to (A) acute hypoxia (N = 9), (C) hypercapnia (N = 7) or (D) sodium cyanide (N = 6) compared to the responses of control fish (filled circles; N = 10 different fish for each treatment). In panel B, the average rates of change of breathing frequency (f_R) between 20 - 155 Torr for the two groups are presented. Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).

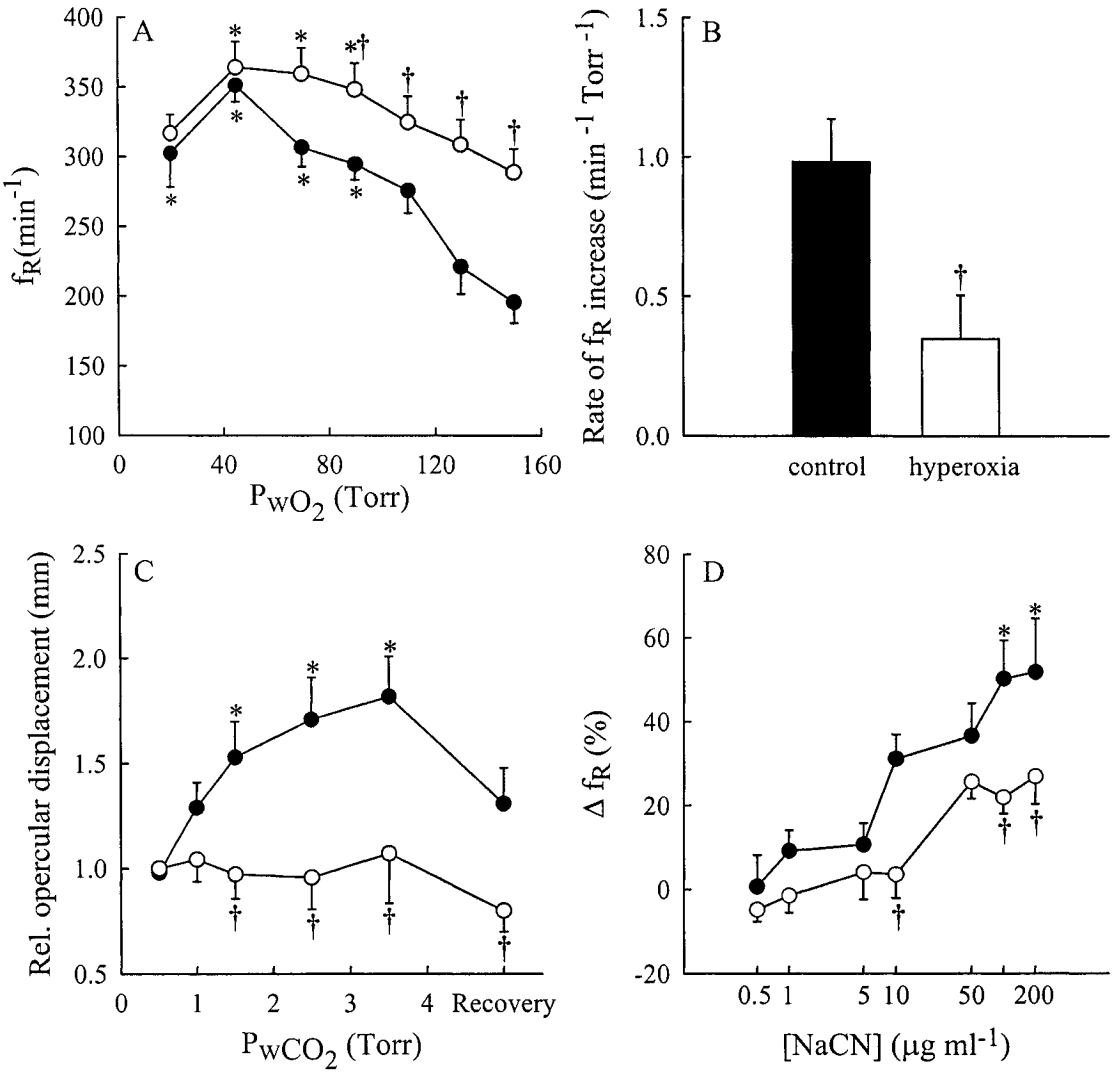

A
B
C
D
f_R(min^{-1})
P_{wO_2} (Torr)
Rate of f_R increase (min^{-1} Torr^{-1})
control
hyperoxia
Rel. opercular displacement (mm)
P_{wCO_2} (Torr)
Recovery
Δ f_R (%)
[NaCN] (μg ml^{-1})

Figure 3.2 The respiratory responses of zebrafish (*Danio rerio*) reared in hypoxia for the first seven days of life (PwO_2 = 30 Torr) (unfilled circles) to (A) acute hypoxia (N = 6), (B) hypercapnia (N = 7) or (C) sodium cyanide (N = 6) compared to the responses of control fish (filled circle, N = 10 different fish for each treatment). Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).

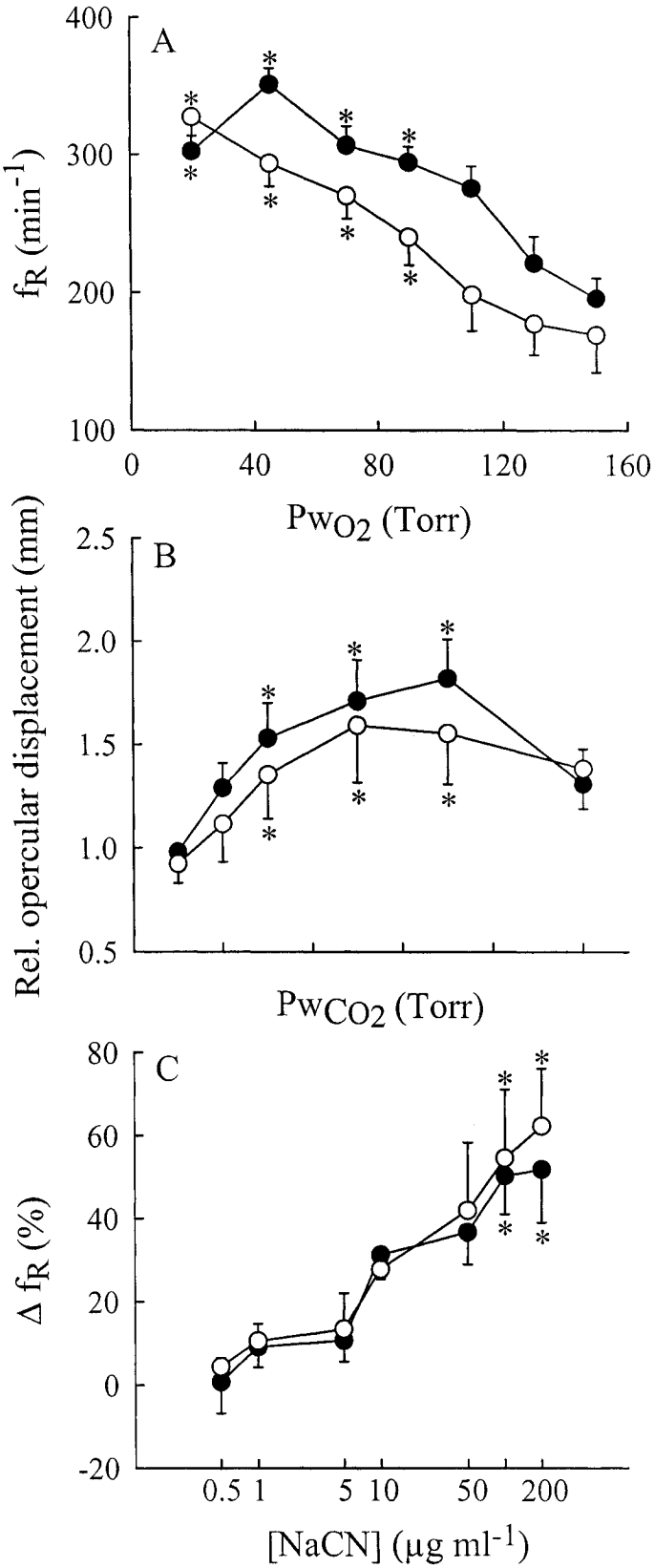
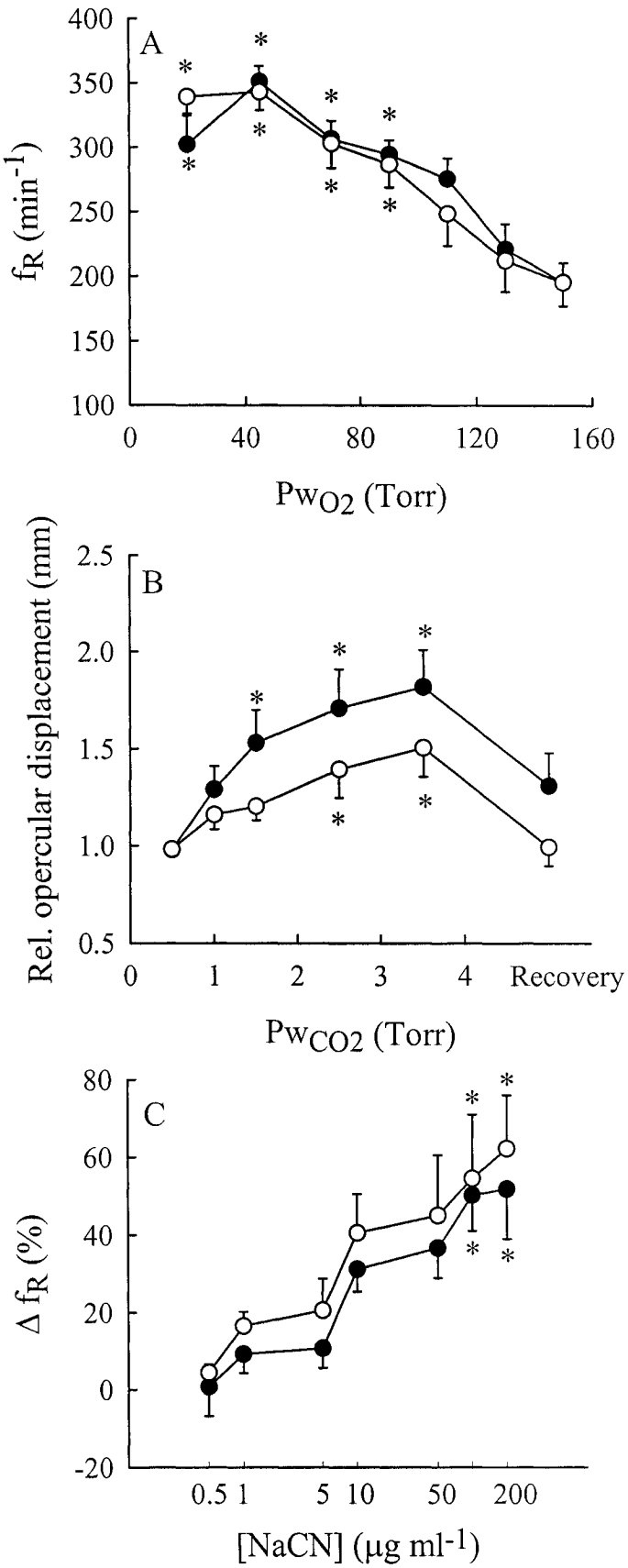

A
400
300
200
100
f_R (min^{-1})
0
40
80
120
160
Pw_{O2} (Torr)
B
2.5
2.0
1.5
1.0
0.5
Rel. opercular displacement (mm)
Pw_{CO2} (Torr)
C
80
60
40
20
0
-20
Δ f_R (%)
0.5 1
5 10
50
200
[NaCN] (μg ml^{-1})

Figure 3.3 The respiratory responses of zebrafish (*Danio rerio*) reared in hypercapnia for the first seven days of life ($PwCO_2$ = 9 Torr) (unfilled circles) to (A) acute hypoxia (N = 12), (B) hypercapnia (N = 11) or (C) sodium cyanide (N = 8) compared to the responses of control fish (filled circles, N = 10 different fish for each treatment). Significant differences within the control or experimental groups are denoted by asterisks (*) while significant differences between the control and experimental groups are denoted by daggers (†); two way RM ANOVA ($P < 0.05$).


A
400
350
300
250
200
150
100
f_R (min^{-1})
0
40
80
120
160
Pw_{O2} (Torr)
B
2.5
2.0
1.5
1.0
0.5
Rel. opercular displacement (mm)
0
1
2
3
4
Recovery
Pw_{CO2} (Torr)
C
80
60
40
20
0
-20
Δf_R (%)
0.5
1
5
10
50
200
[NaCN] (μg ml^{-1})

CHAPTER 4

GENERAL DISCUSSION

- **Zebrafish as a model organism for respiratory studies**

Although a very popular model organism in developmental and genetic studies, the zebrafish has only recently emerged as an important animal for physiological research (Sprague *et al.*, 2001; Jacob *et al.*, 2002; Rombough, 2002; Jonz and Nurse, 2003; 2005; Pelster, 2003; *Jonz et al.*, 2004). The breathing recording method developed for this thesis proved to be accurate and precise for measuring breathing frequency (f_R) and relatively useful in detecting the amplitude of operculum movements. Using this method, I was able to investigate the changes in respiration (f_R and relative change in amplitude) in a large number of fish and the results demonstrate that zebrafish is a useful model to study environmental influences on the control of breathing.

The small size of zebrafish allows for the easy manipulation of large numbers of animals but at the same time, limits the ability to assess cardiovascular function and to sample blood. For this particular study, it would have been useful to examine the blood chemistry of the fish acclimatized to the various environments but blood sampling from resting zebrafish is still impossible. For the developmental component of this thesis, zebrafish provided a great source of numerous and easily obtainable eggs, and was a relatively easy animal to rear following the provided guidelines (ZFIN - Fish book – Sprague et al, 2001).

- **Responses of zebrafish to acute changes in water gas composition**

Results presented in this thesis confirm those of previous studies using other species by showing that zebrafish displayed an increase in ventilation in response to acute hypoxia or hypercapnia. Interestingly, hypoxia did not elicit an accompanying increase

in ventilation amplitude, and hypercapnia did not affect fR. Two possible explanations for this phenomenon are I) two different chemoreceptors (O_2 and CO_2) each with different response patterns, or II) the same chemoreceptor cell responding to both stimuli, but evoking a different response depending on the stimulus. My findings are in accordance with others (Burleson and Smatresk, 2000; McKendry *et al.*, 2001; Perry and Reid, 2002) showing that fish detect changes in water PCO_2 rather than pH and respond by the increase in ventilation amplitude.

- **Effects of chronic exposure to changes in water gas composition**

This study is the first to assess the impact of long-term acclimatization to hyperoxia or hypercapnia on ventilatory reflexes in fish. Two other studies have investigated the consequences of chronic hypoxia in fish: one focusing on the cardiorespiratory effects of 7 days of hypoxia on the catfish (Burleson *et al*, 2002), and the other on the morphological changes in neuroepithelial cells (showing also that they are the sites of O_2 chemoreception) of zebrafish after 60 days of hypoxia (Jonz *et al*, 2004). The goal of this thesis was to assess the change in ventilatory response of zebrafish after 28 days of pre-exposure to different environments and correlate any changes with morphological changes of the chemoreceptors. Pilot experiments done prior to this showed no change in the acclimation of ventilatory response after day 28 (fish exposed to 60 days hypoxia, based on Jonz et al 2004, had similar values in their ventilatory response to hypoxia and hypercapnia as 28 days exposed fish). Seven days pre-exposure of adult fish to different gas changes did not result in change of ventilatory response companied to the control fish as well as fish exposed to acute hypoxia or hypercapnia for the first time. Exposure to hyperoxia for 28 days blunted the subsequent ventilatory response to hypoxia, external cyanide and hypercapnia, and the density of gill

filament neuroepithelial cells (NEC) was significantly reduced. Hypoxia pre-exposure caused a significant reduction of resting f_R without any effect on the ventilatory responsiveness to hypoxia, hypercapnia or cyanide. This decrease of f_R may be related to the conservation of energy (due to lack of oxygen) by the lowering of the branchial convention requirement and a shift toward anaerobic metabolism. Alternatively, it is possible that the branchial O_2 chemoreceptors were re-set in the pre-exposed fish resulting in the perception of normoxia as a state of relative hyperoxia.

Because water-breathing fish lack central chemoreceptors involved in promoting cardiovascular and ventilatory reflexes (Burleson and Smatresk, 2000), and because the effects of hypoxia and hypercapnia shown in this study were in accordance with findings on density of gill filament NECs, I speculate that the sensitivity of the ventilatory response to hypoxia or hypercapnia is controlled, at least partially, by the numbers of NECs exposed to the inspired water. This speculation does not rule out compensatory mechanisms on other levels (cardiovascular, metabolic, neurological) in the zebrafish.

- **Pausing frequency**

When examining the resting f_R of all (control and treated) zebrafish used in this study (including control raised fish and 7 and 28 days control adult fish), 16.5 percent expressed periodic breathing (periods of breathing broken up by periods of apnea), while the other 83.5 percent breathed continuously. The occurrence of breathing pauses was independent of gender, age or size of the fish. It was, though, influenced by acclimatization, as the effect of chronic hypoxia or hypercapnia was a complete disappearance of this breathing pattern in acclimatized fish. Findings in the development study (Chapter 3) suggested that episodic breathing can be influenced during development, as the groups of fish raised for 7 days in hypercapnia had a significantly

higher number of fish that expressed episodic breathing than control fish raised under similar conditions but in normocapnic and normoxic water. These data confirm the view that episodic breathing is shaped, at least in part, by afferent input from peripheral chemoreceptors (see review by Smatresk, 1990), possibly already during development of the respiratory control system.

- **Development study**

The findings of this thesis demonstrate that hyperoxia (unlike hypercapnia or hypoxia) during early development can markedly influence the resting breathing pattern in adults as well as their responses to hypoxia or hypercapnia. The findings that hyperoxia for an indefinite period changed the resting breathing frequency of zebrafish establishes a link between environmental O_2 and the development of the central rhythm generator. This study provides additional evidence that developmental changes in functional O_2-sensing pathways are not unique to air-breathing mammals, but may have appeared earlier in vertebrate evolution. Although fish exposed to hypoxia for 7 days post-fertilization did not show change in ventilatory response in adult life, the influence of hypoxia on their complete development was obvious; their developmental age at day 7 was approximately 5 days and of all the experimental groups their adult size was significantly reduced, results in accordance with a study showing that hypoxia may have a teratogenic effect on fish and delay fish embryonic development (Shang and Wu, 2004). The finding that developmental hypercapnia did not contribute to chemoreception plasticity but did increase the numbers of fish exhibiting episodic breathing confirms that different factors involved in developmental plasticity yield different effects (Carroll, 2003). Clearly, that there is flexibility in the design and functioning of the embryonic or

larval respiratory system in zebrafish, opening doors for new studies on developmental plasticity using zebrafish as a model species.

- **Chemoreceptors**

An interesting finding of this thesis was a different ventilatory response to hypoxia *versus* hypercapnia. While hypoxia caused an increase in f_R, hypercapnia influenced only ventilation amplitude. These types of different response have been described in some other fish species (Smith and Jones, 1982). Because it is known that the same carotid body chemoreceptors respond to alterations in both PO_2 and PCO_2 (Gonzales *et al.*, 1994; Zhang and Nurse, 2004), and that the chemoreceptor cells share many histological and cytochemical similarities with mammalian carotid body glomus cells (Fritsche and Nilsson, 1993), a possible interpretation of the different responses to CO_2 and O_2 may be the existence of different neurotransmitters produced according to the nature of the ventilatory stimulus. The results of this study suggest, though, that the numbers of NECs exposed to the inspired water controls the sensitivity of the ventilatory response to hypoxia or hypercapnia, at least partially.

- **Future studies**

Results from the present thesis raise interesting questions that can be considered for future studies:

- Possible difference between O_2 and CO_2 receptors (supported by difference in acute response and some acclimatization results)
- Presence of breathing pauses in zebrafish and the influence certain gas changes have on this phenomenon
- Possible recovery from changes in ventilatory control after chronic exposure to different environments.

- Oxygen consumption and metabolic rate in zebrafish as a response to acclimatization to chronic O_2 or CO_2 changes
- Finding a window for developmental plasticity of respiratory control in the early development of zebrafish and following the development of chemoreceptors and correlating nerves under different developmental conditions

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