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Velislava Tzaneva
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

M.Sc. (Biology)
GRADE / DEGREE

Department of Biology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

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TITRE DE LA THÈSE / TITLE OF THESIS

Steve Perry
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

Patrick Walsh

Michael Jonz

Steven Cooke

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

CAUSES AND CONSEQUENCES OF GILL REMODELLING IN THE GOLDFISH, *CARASSIUS AURATUS*

By

Velislava Tzaneva

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ABSTRACT

Goldfish are able to drastically change their gill morphology by forming an interlamellar cell mass (ILCM) when acclimated to temperatures below 15 ° C. This morphological change is reversed when the fish is exposed to hypoxia or an increase in temperature. The main goal of this thesis was to investigate how the presence of the ILCM affects the transfer and sensing of respiratory gases as well as to identify the cues that trigger gill remodelling. Using an extracorporeal blood shunt to measure blood gases, I show that the presence of the ILCM does not impede O_2 uptake under normoxic or hypoxic conditions but presents an additional diffusion limitation to CO_2 excretion owing to chemical equilibrium constraints. Hypoxemia induced by carbon monoxide or phenylhydrazine elicited a decrease in the ILCM in cold (7° C) acclimated goldfish while exposure to hyperoxia partially prevented the reduction of the ILCM during a temperature increase.

RÉSUMÉ

Les cyprins dorées possèdent l'habilité de modifier leur morphologie branchiale en augmentant une masse de cellule inter-lamellaire (MCIL) quand ils sont acclimatés à des températures inférieures à 15° C. La présence de la MCIL est réduite quand ils sont exposé à l'hypoxie ou à des augmentations de température. Le but de cette thèse est d'examiner quels effets la MCIL pourrait avoir sur l'habilité de l'animal de transférer et de détecter ces gaz respiratoires en plus de percevoir des signaux potentiels pour le remodelage de la MCIL. En utilisant un shunt extra-corporel, nous avons mesuré le pH et les gaz dans le sang de l'animal. Nous avons démontré que la présence de la MCIL ne gêne pas le captage d'O_2 sous des conditions normoxiques ou hypoxique, mais qu'elle nuit à l'expulsion de CO_2 hors de l'animal sous des conditions normoxiques. Le monoxyde de carbone (CO) et la phénylhydrazine-HCL (PHZ) induisent l'hypoxémie et ils ont déclenché une baisse dans la MCIL dans les cyprins dorés acclimaté à des températures froides (7° C) tandis que l'exposition à l'hyperoxie a partiellement prévenu la réduction de la MCIL durant un changement de température dans l'environnement.

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Throughout my M.Sc. there have been several colleagues, professors and staff of the University of Ottawa who have helped me through the completion of my degree. First, I'd like to express my gratitude towards my supervisour Dr. Steve Perry. I came into the Perry lab with a background in respiratory ecology and a very limited knowledge of comparative physiology. I was plunged into the world of comparative physiology soon after I started my degree and haven't looked back. It has been a steep learning curve and I have learned a great deal over the past 2 years which would not have been possible without his guidance and mentorship.

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LIST OF ABBREVIATIONS

5 – HT, serotonin

ANOVA, analysis of variance

CA, carbonic anhydrase

CaO_2, total arterial oxygen content

CO, carbon monoxide

CO_2, carbon dioxide

ED_{50}, half maximal effective dose

EtOH, ethanol

[Hb], haemoglobin concentration

ILCM, interlamellar cell mass

$K_3Fe(CN)_6$, potassium ferricyanide

MRC, mitochondria rich cell

N, number of animals

NaCN, sodium cyanide

NEC, neuroepithelial cell

O_2, oxygen

$PaCO_2$, arterial partial pressure of carbon dioxide

PaO_2, arterial partial pressure of oxygen

PBS-T, Triton – X in phosphate – buffered saline

PFA, paraformaldehyde

pHa, arterial pH

pHw, pH of water

PHZ, phenylhydrazine – HCl

$PwCO_2$, partial pressure of carbon dioxide in the water

PwO_2, partial pressure of oxygen in the water

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

V_{AMP}, ventilation amplitude

V_f, ventilation frequency

ZN – 12, zebrafish-derived neuron-specific antigen

CHAPTER 1.

General Introduction

INTRODUCTION

Fish routinely experience extreme changes in their environment which make them an excellent model for studying the environmental impact on physiological processes. Oxygen content and temperature of the water are two factors which can vary diurnally as well as seasonally (for review see Diaz, 2001). Goldfish (*Carassius auratus*) are one of the few teleost fish species which are able to thrive in such variable environments. They can withstand a temperature range from as low as 5° C up to 35° C which is met with an increase in their standard metabolic rate (Fry and Hart, 1948). Not only are they able to survive such temperature fluctuations but they are also exceptionally well-adapted to living in hypoxic (low oxygen) waters especially at the lower end of their temperature range. Aquatic hypoxia persists as a problem today due to expanding human activity which exacerbates naturally occurring hypoxia (Diaz, 2001). Goldfish are one of the few fish species that can tolerate extreme hypoxia (water oxygen $PO_2 < 30$ mm Hg) and even anoxia (no oxygen) for prolonged periods of time (Shoubridge and Hochachka, 1980). They are able to survive such harsh conditions in part because of their high haemoglobin O_2 affinity (Burggren, 1982). Under severe hypoxic (water $PO_2 = 30$ mm Hg) conditions the haemoglobin of goldfish has been shown to be 50% saturated with O_2 at 25° C (Burggren, 1982). This is in contrast to species inhabiting O_2 rich environments such as salmonids which have relatively low haemoglobin O_2 affinities (see reviews by Powers, 1980; Nikinmaa, 2001).

The standard metabolic by product in teleosts exposed to hypoxia (if severe enough) is lactate. Increase in lactate levels in plasma signal a switch to increasing reliance on anaerobic metabolism (van den Thillart 1989; Chippari-Gomes et al., 2005, Mandic et al., 2008). Goldfish, and the closely related crucian carp, are able to withstand long bouts of anoxia at low

temperatures by utilizing liver glycogen stores for anaerobic glycolysis with ethanol as the end metabolic by product of anaerobic metabolism (van den Thillart et al., 1980; Shoubridge and Hochachka, 1980; van den Thillart et al., 1983) in addition to lowering whole-body metabolic rates (Van Waversveld et al., 1989).

Even more impressive is the goldfish's ability to structurally remodel its gills. At low temperatures (< 15° C) the space between adjacent lamellae is filled with a cell mass (interlamellar cell mass; ILCM) which leads to a covering of the lamellae (Sollid et al., 2005). This morphological change is completely reversible when the fish are exposed to hypoxia or the temperature of the water is increased (Sollid and Nilsson, 2006). Their unique method of dealing with extreme hypoxia/anoxia and ability to drastically change their gill morphology makes goldfish an excellent model organism to study the interactive effects of temperature and water O_2 levels on a variety of physiological processes; in this thesis I have focused on chemoreception (gas sensing), ventilatory control and gas transfer.

THE FISH GILL: A MULTIFUNCTIONAL ORGAN

The fish gill is a multifunctional organ important for a number of physiological processes from gas exchange and gas sensing to ion and acid-base regulation and nitrogenous waste excretion (see review by Evans et al., 2005). A gill with a larger surface area will have a higher respiratory capacity and can sustain higher metabolic rates, but will also, in freshwater, result in greater diffusive ion loss and osmotic water gain which may be energetically expensive to correct (Matey et al., 2008). There is also a decline in structural stability and a greater likelihood of exposure to pathogens, toxins and pollutants as the surface area of the gills increase (Nilsson, 2007). Thus, many species will develop an optimal gill size based on their behavioral and environmental needs.

Gas transfer

In goldfish possessing an ILCM, there is an additional barrier potentially impeding gas transfer. Branchial gas transfer has been extensively studied in teleosts and refers to the exchange of O_2 and CO_2 between the water and the blood of the fish via the gill. With respect to O_2 uptake, partially deoxygenated blood from the venous circulation arrives at the gill where O_2 crosses into the blood stream because of the large O_2 partial pressure gradient that exists between the water and the blood of the fish. Oxygen transfer across the gill epithelia is thought to behave as a perfusion limited system (Gilmour and Perry, 2002). This means that O_2 transfer efficiency remains constant even if the blood transit time through the respiratory organ decreases. In other words, any area of the gill which is both perfused by blood and ventilated is available for O_2 uptake. The extent to which arterial blood equilibrates with O_2 in the inspired water is a measure of O_2 uptake efficiency (Gilmour and Perry, 2002).

Even though the capacitance of water for CO_2 is higher than that for O_2, CO_2 excretion is theoretically constrained by diffusion limitations for a number of reasons. The molecular CO_2 entering the blood from the tissues is hydrated to HCO_3^- in the red blood cell (RBC) by carbonic anhydrase (CA) and is subsequently exchanged for plasma Cl^- via the band 3 anion exchange protein (Romano and Passow, 1984; Perry, 1986; Tufts et al., 1998). This reaction is reversed at the gill where HCO_3^- from the plasma is transported into the RBC via the same band 3 anion exchange protein. This reaction has been experimentally shown to be the rate limiting step in CO_2 excretion (Perry and Gilmour, 1993; Desforges et al., 2001) and its slow rate relative to the brief period of blood transit (1-3 sec; Cameron and Polhemus, 1974) is the basis for CO_2 excretion behaving as a diffusion-limited system. Once in the RBC the HCO_3^- is dehydrated to CO_2 and water by CA (Gilmour and Perry, 2009) and the CO_2 rapidly diffuses across the gill.

Previous studies have shown that alteration in the functional surface area of the gill or the thickness of the diffusion barrier can impede the transfer of either O_2 or CO_2, or even both (Bindon et al., 1994; Julio et al., 2000). In those studies the gill surface area was decreased dramatically by surgically removing gill arches or the thickness of the blood-to-water barrier was increased by inducing the proliferation of ionocytes on the lamellar epithelium. Such drastic morphological changes of the respiratory organ may occur naturally in goldfish because of their ability to remodel their gills (appearance or removal of the ILCM) depending on the environmental conditions (see above). The presence of an ILCM may impose two challenges for gas transfer; 1) a decrease in the functional surface area, and 2) an increase in the water-to-blood barrier thickness – each could potentially impede the transfer of O_2 and CO_2.

Gas sensing

Species from almost all taxa are able to sense and respond to changes in O_2 levels of their environments. For water-breathing teleost fish, the most common response to a decrease in the oxygen content of the water is hyperventilation which is brought about by an increase in ventilation amplitude and frequency (Holeton, 1971). These changes in ventilation response to hypoxia are mediated by peripheral O_2 chemoreceptors found on the gills (Jonz et al., 2004). Studying fish O_2 chemoreceptors is of interest because of their phylogenetic proximity to the carotid body (the primary O_2 sensing organ in mammals) which will provide a better insight on the evolution of O_2 sensing in vertebrates (Jonz et al., 2004). Only recently have O_2 sensing cells (neuroepithelial cells; NEC) been characterized in the gills of zebrafish (Jonz et al. 2004) and catfish (Burleson et al., 2006).

In goldfish and other species that have been studied, NECs are strategically located along the leading edge of the distal part of the gill filament as well as on the lamellae (Saltys et al., 2006, Coolidge et al., 2008). These locations are seen as optimal for gas sensing both the external and internal environments. NECs found along the filamental epithelium have been shown to be associated with the nerve bundle of the filament which runs along the filament and adjacent to the efferent filamental artery (EFA) which carries oxygenated blood through the gill filament (Jonz and Nurse, 2008). Furthermore, this filamental nerve bundle has been shown to branch into the lamellae to possibly innervate lamellar NECs at least in zebrafish and goldfish (Jonz and Nurse, 2003; Saltys et al., 2006).

MORPHOLOGICAL PLASTICITY OF FISH GILLS

Environmental change can be a lead cause in altering the gill morphology of teleost fish (Laurent and Perry, 1991). For example, trout acclimated to soft water (low Ca^{2+} content) causes the proliferation of lamellar chloride cells leading to a thickening of the blood-to-water diffusion barrier (Greco et al., 1996). Previous studies clearly show that cues such as hypoxia and temperature change can cause gill remodelling in goldfish towards decreasing the ILCM and thus increasing the functional surface area for gas exchange. While such morphological changes are expected to benefit gas transfer, they will likely compromise osmoregulation (Sollid et al., 2003; Sollid and Nilsson, 2006; Mitrovic et al., 2009; Mitrovic and Perry, 2009). When fish are placed back in normoxic water the lamellae become re-embedded in the ILCM (Sollid et al., 2003; Mitrovic et al., 2009).

The above environmental cues cause rapid and reversible morphological changes within the lifetime of the animal. Environmental pressures as well as natural selection may also play a role in triggering gill morphological changes but on a longer time scale. Chapman et al. (2000) and

Schaack and Chapman (2003) reported that a population of African cichlids raised in hypoxic habitats have larger respiratory surface area. This increase in gill size was due to an increase in filament length and lamellar size; morphological changes which are more permanent than the ones described in goldfish and carp. One caveat of increasing the gill size is compromising feeding structures associated with the branchial cavity such as the pharyngeal jaws which in some cyprinid fish have well developed molars and are used to crush food.

ADVANTAGES AND DISADVANTAGES OF GILL REMODELLING

Better gas transfer is one clear advantage of having larger gills. As mentioned above, goldfish, and carp, inhabiting hypoxic waters typically lack an ILCM and thus have larger gill surface area. Carp (and goldfish) in their natural habitat would experience prolonged bouts of hypoxia followed by anoxia at the transition between Fall and Winter when temperatures drop and ponds become covered with ice. In order to avoid switching to anaerobic metabolism and possibly using up their glycogen stores prematurely, carp and goldfish shed their ILCM during the hypoxic period in order to increase the gill surface area for gas transfer and rely on aerobic metabolism for as long as possible (Sollid et al., 2005; Nilsson, 2007).

Nilsson (2007) makes a compelling argument for the advantage of not having protruding lamellae in normoxic waters. During the spring when the O_2 levels are high and glycogen stores are being built up, a larger gill surface area may result in greater energy expenditure on osmoregulation. This in turn may reduce the final size of precious glycogen stores which are used as fuel for anaerobic glycolysis over the anoxic winter months. Although there are clear benefits to gill remodelling, one must wonder about the costs involved in growing, maintaining and then losing the ILCM over an annual cycle.

THESIS OUTLINE AND RATIONALE

Even though the ILCM in goldfish and crucian carp has been well described over the past decade (Sollid et al., 2003; Sollid et al., 2005; Sollid and Nilsson, 2006; Sollid et al., 2006; Mitrovic and Perry, 2009; Mitrovic et al., 2009) there are still unanswered questions as to the limitations that the ILCM imposes on gas sensing and gas transfer. Furthermore, internal cues for gill remodelling in goldfish have not yet been investigated. The data chapters (Chapters 2-4) in this thesis are presented as manuscripts in preparation. I have tried to avoid repetition where possible.

Hypotheses

1.) Goldfish acclimated to 7° C have a decreased capacity to sense O_2 because of the physical covering of the NECs by the ILCM.
2.) The presence of the ILCM in 7° C goldfish imposes further limitations on CO_2 excretion while O_2 uptake is impeded under hypoxic conditions only.
3.) Hypoxemia in 7° C goldfish causes a reduction in ILCM while hyperoxia exposure reduces the extent of gill remodelling during a temperature increase.

This thesis is composed of three data chapters (Chapters 2-4). Chapter 2 considers the effects of gill remodelling on gas sensing and control of breathing in the goldfish, *Carassius auratus*. Here I show how goldfish with different gill morphologies (ILCM present or no ILCM) respond to hypoxic stimuli. I relate the responses to the location and number of NECs, the putative O_2 chemoreceptors. In chapter 3 I investigate the consequences of gill remodelling on gas transfer across the goldfish gill. Through whole animal experiments I attempt to provide evidence that the presence of the ILCM disproportionately affects the transfer of O_2 and CO_2.

Finally, chapter 4 examines the cues for gill remodelling in goldfish. Taken together, the results of this thesis provide a comprehensive understanding of the physiological consequences of gill remodelling on gas sensing and gas transfer while providing insight into the specific mechanisms that trigger gill remodelling.

CHAPTER 2.

The control of breathing in goldfish (*Carassius auratus*) experiencing thermally induced gill remodelling

This chapter will be submitted for publication to the Journal of Experimental Biology. Authors:

Velislava Tzaneva and Steve F. Perry

ABSTRACT

At temperatures below 15° C the gill lamellae of goldfish (*Carassius auratus*) are largely covered by an interlamellar cell mass (ILCM) which decreases the functional surface area of the gill. The presence of the ILCM in goldfish acclimated to cold water conceivably could lead to a covering of the neuroepithelial cells (NECs) which are believed to be important for sensing ambient O_2 and CO_2 levels. In this study I tested the hypothesis that goldfish with covered lamellae (and presumably fewer NECs exposed to the water) exhibit a decreased capacity to hyperventilate in response to hypoxic stimuli. Measurements of ventilation amplitude and frequency were performed during exposure to acute hypoxia (PwO_2 = 30 mm Hg) or following injections of the O_2 chemoreceptor stimulant NaCN into the buccal cavity or caudal vein of fish acclimated to 25° C (uncovered lamellae) or 7° C (covered lamellae) to stimulate predominantly externally- or internally-oriented NECs, respectively. The results demonstrated no significant differences in the response to hypoxia with each group exhibiting similar percentage increases in ventilation amplitude (90 – 91%) and frequency (34 - 43%). Similarly, with the exception of a rightward shift of the ventilation frequency dose response in the fish acclimated to 7° C, there were no significant differences between the two groups of fish in the ED_{50} values for externally- or internally-injected NaCN. These findings suggest that goldfish with covered lamellae retain the capacity to sense external hypoxic stimuli. Using immunohistochemistry to identify serotonin enriched NECs, it was demonstrated that with lamellar covering, the NECs redistribute towards the distal regions of lamellae. In 25° C fish, the NECs were distributed evenly along the length of the lamella with 53 ± 3% of them localized to the distal half whereas in fish acclimated to 7° C, 83 ± 5% of the NECs were confined to the distal half. Using the neuronal marker antibody ZN-12, it was demonstrated that the NECs localised to the distal edges of the lamella are innervated by nerve fibres. Thus, it is hypothesised that the capacity to sense external hypoxic

stimuli in goldfish acclimated to cold water is maintained despite the increasing coverage of the gill epithelial surfaces because of a redistribution of innervated NECs to the exposed distal regions of the lamellae.

INTRODUCTION

In response to aquatic hypoxia, fish typically exhibit gill hyperventilation (Randall and Shelton, 1963; Holeton and Randall, 1967; for reviews see Shelton et al., 1986; Perry and Gilmour, 2002; Gilmour and Perry, 2007; Perry et al., 2009a; Perry et al., 2009b; Perry and Gilmour, 2010). Depending on species, the hyperventilatory response may reflect variable increases in breathing frequency (V_f) and/or amplitude (V_{AMP}; see Table 1 in Perry et al., 2009b). Regardless of whether it is achieved by increases in V_f or V_{AMP}, an increase in water flow over the gills during hypoxia is a crucial response aimed at maintaining O_2 uptake during periods of reduced O_2 availability. Essentially, hyperventilation allows arterial blood PO_2 (PaO_2), and hence O_2 content, to be maintained at higher levels than otherwise would be possible. The increased ventilation in response to hypoxia arises from the stimulation of externally- and/or internally-oriented O_2 chemoreceptors which respond to changes in O_2 partial pressure in the inspired water or the arterial blood, respectively (Saunders and Sutterlin, 1971; Milsom and Brill, 1986; Burleson and Smatresk, 1990; reviewed by Burleson et al. 1992; Perry et al., 2009b). Based on its structural similarity to the O_2 sensing glomus cell of the mammalian carotid body or pulmonary neuroepithelial bodies (Bailly et al., 1992), and some indirect evidence of its activation during severe hypoxia in trout (Dunel-Erb et al., 1982), the neuroepithelial cell (NEC) of the gill filament has long been suspected as the O_2 chemoreceptor of the fish gill (Dunel-Erb et al., 1982; Bailly et al., 1992). More recently, Jonz et al. (2004) and Burleson et al. (2006) provided direct electrophysiological evidence that NECs of the zebrafish (*Danio rerio*) and catfish (*Ictalurus punctatus*) gill filament behave as O_2 chemoreceptors. Like the glomus cells of the carotid body, at least a subset of zebrafish gill NECs act as dual O_2 and CO_2 chemoreceptors

(Qin et al., 2010) through a common pathway promoting membrane depolarization involving inhibition of potassium conductance (Perry et al., 2009b).

Gill NECs have been identified in several fish species (Zaccone et al., 1992; Jonz and Nurse, 2003; Zaccone et al., 2006; Vulesevic et al., 2006; Saltys et al., 2006; Coolidge et al., 2008) [see Table 5.7 in Perry et al. (2009b)] where they are localized to filament and lamellar (with the exception of rainbow trout) epithelia. Strictly speaking, only the NECs of the gill filament have been implicated in chemoreception because it was these cells which were isolated and subjected to patch clamp experiments (Jonz et al., 2004; Qin et al., 2010). In developing zebrafish, the NECs of the filament may be the exclusive sites of chemoreception until the lamellae fully mature (Jonz and Nurse, 2005). It has been suggested that the NECs localized within the filament epithelium in proximity to the efferent filament artery are ideally situated to monitor the status of the oxygenated blood exiting the gill lamellae (Jonz et al., 2004). The NECs of the filament may be less able, however, to detect changes in inspired water gas levels because of the significant physical barrier separating the NECs and the external environment. Thus, the NECs of the lamellae, especially in those species possessing lamellar NEC innervation, presumably are also critical in chemoreception and in particular the sensing of inspired water.

The goldfish (*Carassius auratus*) is an example of a species possessing innervated NECs which are localized to the filament and lamellar epithelia (Saltys et al., 2006; Coolidge et al., 2008). The dual location of NECs in goldfish raises an interesting question as to the possible impact of gill remodelling on the capacity of goldfish to sense their environment. In this context, gill remodelling refers to the reversible insertion or retraction of a cell mass (termed the interlamellar cell mass; ILCM) between adjacent lamellae with changes in external temperature or water O_2 levels (see review by Sollid and Nilsson, 2006). Similar to crucian carp (*Carassius*

carassius) (Sollid et al., 2003), the acclimation of goldfish to cold water (< 15° C) leads to the formation of the ILCM which reduces functional gill surface area owing to a physical covering of lamellae (Sollid et al., 2005). Upon exposure of cold acclimated goldfish to hypoxia (Mitrovic et al., 2009) or warm water (Mitrovic and Perry, 2009), the ILCM is removed and thus the lamellae are re-exposed. It is thought that the reduction in lamellar functional surface area at cold temperatures reduces the passive loss of salts and thus reduces the energetic costs associated with ionic regulation. An obvious potential negative impact of such gill remodelling is the impairment of gas transfer associated with increasing diffusion distances and decreasing surface areas. In this study, I assess another potential impact of gill remodelling in goldfish; the capacity to sense environmental hypoxic stimuli. It is hypothesised that the presence of the ILCM in goldfish acclimated to cold water will lead to a physical covering of the lamellar NECs and thus these fish will exhibit a reduced capacity to respond to hypoxic stimuli. This hypothesis was tested by examining the distribution of branchial NEC in fish acclimated to warm water (25° C; no ILCM present) or cold water (7° C; ILCM present) and assessing their capacity to hyperventilate in response to environmental or chemical (NaCN) hypoxia.

MATERIALS AND METHODS

Experimental Animals

Goldfish (*Carassius auratus*) were purchased from a commercial supplier [Aleong's International (Mississauga, Canada)] and were held in circular tanks supplied with dechloraminated City of Ottawa tap water at 18° C. After at least two weeks, the fish were acclimated (increase or decrease of 2° C per day) to either 7 or 25° C and were maintained at these temperatures under a 12/12-h light-dark photoperiod for at least two weeks prior to experimentation. Fish were fed a diet of commercial pellets once per day. All experiments were carried out at 7 or 25° C at the University of Ottawa Aquatic Care Facility according to Canadian Council of Animal Care (CCAC) guidelines and with the approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

Surgical procedures

Goldfish (average mass = 254.5 g; N = 28) were anaesthetised in 10 mg l^{-1} benzocaine (Sigma-Aldrich, Inc.) until ventilation had ceased. The fish were then placed on a surgery table where the gills were irrigated with aerated anaesthetic solution. To measure ventilation frequency and amplitude, impedance leads with 1 cm^2 brass plates were attached with sutures to the outer surface of each operculum. To allow injections into the inspired water, a cannula (Clay Adams PE 160) was inserted into the snout between the nostrils and into the buccal cavity. To access the caudal vein and artery, a 2 cm lateral incision was made at the caudal peduncle. Saline (0.9 % NaCl) filled cannulae (Clay Adams PE 50 with a ~ 2 cm piece of PE 10 attached at the end) were inserted into the caudal vein and artery in the anterior direction. The incision was closed

with silk sutures and the cannulae were secured to the skin with ligatures. The fish were then placed in transparent plastic boxes (3.8 l) provided with flowing aerated water at the appropriate acclimation temperature (7 or 25° C) to recover for 24 h before experiments were performed.

A separate group of 7° C goldfish (average mass = 21.7 g; N = 6) were subjected to sham surgeries. The fish were anesthetised and placed on the surgery table while irrigating the gills for 30 min. The muscle of the caudal peduncle region was exposed and the incision was stitched as described above; the fish were not cannulated. The purpose of the sham surgery was to account for any effect that anaesthesia, surgery and subsequent confinement might have on the ILCM. The fish were allowed to recover for 24 h in dark plastic poxes (~1 l) after which they were euthanized by an overdose of benzocaine and the first right gill arch was collected for sectioning and ILCM analysis. The gill tissue was fixed in 4% paraformaldehyde (PFA) overnight after which it was transferred to 30% sucrose (w/v) for 24 h before sectioning. Prior to sectioning the gill tissue was placed in OCT cryomatrix for 1 h. The tissue was then frozen in the OCT cryomatrix at -30° C and was sectioned into 12 – 14 μm slices using a Leica CM 3050 cryostat. The ILCM was measured as a percentage of the area of ILCM present between the lamellae over the total interlamellar area using the right first gill arch of 6 fish.

Experimental protocols

External O_2 chemoreceptors were stimulated by injecting 2 ml kg^{-1} of increasing concentrations of sodium cyanide (NaCN; 0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1.0 and 2.0 mg ml^{-1}) into the buccal cavity through the snout PE160 cannula over a 10 - 15 sec period followed by 2 ml water flush, again over a 10 - 15 sec period. The doses of externally administered NaCN were determined based on a previous study (Reid and Perry, 2003) on rainbow trout (*Oncorhynchus mykiss*) as

well as preliminary experiments. Another set of 1 ml kg^{-1} NaCN solutions (0.02, 0.04, 0.08, 0.1, 0.15 and 0.2 mg ml^{-1}) were injected sequentially into the caudal vein to preferentially stimulate internally oriented O_2 chemoreceptors. The NaCN solutions injected into the buccal cavity were prepared using aquarium water at either 7 or 25° C; NaCN solutions for internal injections were prepared using 0.9% NaCl. The concentrations listed above in mg ml^{-1} correspond to a range of doses of 0.02 to 4 mg kg^{-1} and 0.01 to 0.02 mg kg^{-1} for external and internal injections, respectively. The fish were allowed to recover for 3 - 5 min after each injection of NaCN. The frequency and amplitude of opercular displacements (Figure 2A) were assessed as indices of ventilation using a custom-built impedance converter (University of Ottawa Electronics Workshop) that detected and quantified the changes in impedance between the brass plates attached to the opercula (Peyraud and Piquemal, 1962). Ventilation amplitude (V_{AMP}) was determined and averaged over 10 sec intervals after conversion of the impedance data to linear opercular deflections (in cm) through appropriate calibration. Ventilation frequency (V_f) was determined from the impedance traces using an automatic rate function within the data acquisition software (see below). Goldfish were allowed 30 – 60 min to recover from the NaCN injections. After the recovery period, the fish were gradually exposed to hypoxia (final P_wO_2 ~ 30 mm Hg achieved within ~30 min). The ventilation responses were recorded continually but only the values of V_{AMP} and V_f before and at the lowest P_wO_2 are reported here. Hypoxia was achieved by bubbling N_2 through the water – air equilibration column to displace the air until the water PO_2 reached approximately 30 mm Hg.

Water PO_2 was monitored continuously by pumping using a peristaltic pump (Fisher Scientific, Canada; 0.8 ml min^{-1}) water from the holding box through the sample compartment of a PO_2 electrode (Cameron Instruments) connected to a gas meter (Cameron Instruments, BGM

200). The PO_2 electrode was housed in a thermostatted cuvette and was calibrated prior to each experiment using a two point calibration method. A solution with zero PO_2 (0.02 g ml^{-1} sodium sulphite) was used to set the low point of the calibration; air-saturated water was used to set the high point. An extracorporeal blood shunt was used to monitor arterial gases (PaO_2 and $PaCO_2$) and pHa. Blood was pumped (< 1 ml) at a rate of 0.4-0.5 ml min^{-1} from the caudal artery through three microelectrodes (Microelectrodes Inc.; PO_2, PCO_2 and pH) connected in series and returned to the fish via the caudal vein cannula. The O_2 electrode was calibrated as described above. The CO_2 electrode was calibrated using water equilibrated with 0.5% CO_2 (low point) and 1% CO_2 (high point). Precision buffer solutions (pH = 7.00 for the low point and pH = 8.00 for the high point, Fisher Scientific) were used to calibrate the pH electrode. The microelectrodes were connected to a blood gas meter (Cameron Instruments, BGM 200). Analog outputs from the impedance converter and gas meters were interfaced with a data acquisition system (BioPac); the resultant digital data were graphically presented and stored using AcKnowledge data-acquisition software.

Immunohistochemistry

Using separate groups of fish that were euthanized by anaesthetic overdose and that were not subjected to any prior experimentation, the right first gill arch was removed and fixed overnight in 4% paraformaldehyde. The rakers were removed and the remaining tissue was placed in a permeabilizing solution containing 5% Triton-X in phosphate-buffered saline (PBS-T; pH = 7.4) overnight. NECs and nerve fibres were identified by incubating the gill tissue with antibodies against serotonin (5-HT; Sigma Aldrich) and zebrafish-derived neuron-specific antigen (ZN-12; Developmental Studies Hybridoma Bank, University of Iowa), respectively, for 24 to 48 h at

room temperature. Secondary antibodies [Alexa 488 (green) and Alexa 594 (red)] conjugated with fluorescent markers were used to visualize the structures (for more details on the antibodies used in this study refer to Table 1 in Jonz and Nurse, 2003). The whole mount gills were placed on a well slide containing mounting media (Vectashield; Vector Laboratories, Ltd.) to reduce photo bleaching. A confocal microscope (Zeiss LSM 510 META) equipped with argon (peak output = 488 nm) and helium-neon (peak output = 543 nm) lasers was used to examine the gill tissue. Optical slices (1 - 3 μm thickness) were taken to produce a composite 3D image. Image processing was performed using LSM Image Browser and Image-J software (http://rsbweb.nih.gov/ij/index.html).

NEC distribution and quantification

Composite confocal images of the distal half of a single filament of (3 - 6 filaments per fish; N = 5 for goldfish acclimated to 7° C and N = 6 for goldfish acclimated to 25° C) were used to quantify the distribution and the number of NECs. Typically, each image depicted 9 - 10 lamellae per side per gill filament. To quantify the distribution of NECs along the lamellae, the length of the lamellae on one side of the filament (determined by coin flip) was measured and the NECs were counted on both the distal and proximal halves (with respect to the filament) of the lamellae along the filament. NECs on each side of the lamellae were then divided by the total number of lamellar NECs to represent the distribution of the NECs. The total number of lamellar NECs was counted for each filament, averaged over the number of fish per temperature treatment and represented as NECs per lamella. The NECs of the filament were also counted and averaged over the number of fish per temperature treatment. The same images were used to count both NECs of the filament and of the lamellae.

ILCM analysis

Statistical analysis

The data are presented as means ± 1 S.E.M. Unpaired two-tailed Student's t-test and One Way Analysis of Variance (ANOVA) with Tukey post-hoc test were used to calculate the statistical differences between means using commercial software (SigmaStat 3.5, SPSS). Statistical significances for the NaCN ED_{50} values were determined by plotting 95% confidence intervals for the dose response curves in Figure 3; ED_{50} values were considered to be statistically different if they were found in non-overlapping regions.

RESULTS

The effects of surgical procedures on gill morphology

The experimental protocols required that fish be subjected to anaesthesia, surgery and post-surgery recovery prior to monitoring ventilatory responses to NaCN or hypoxia. This raised the question as to whether the ILCM in the 7° C goldfish gills was still intact prior to performing experiments. To determine if there was a significant reduction in ILCM caused by anaesthesia and handling stress, smaller goldfish (20 – 30 g; N = 6) were anaesthetized, subjected to sham surgery and allowed to recover for 24 h. The gill tissue of these goldfish was collected and the extent of the ILCM was quantified and compared to gills removed from a separate group of control fish (N = 6) at 7° C. The results confirmed that there was no effect (P = 0.76) of anaesthesia and fish handling on the ILCM area as determined by the percentage of total interlamellar area (ILCM area = 79.3 ± 3.19 and 78.1 ± 2.36 % in the sham-treated and control fish, respectively).

Baseline values of ventilation amplitude (V_{AMP}) and frequency (V_f)

The baseline values of V_{AMP} and V_f were measured at the start of the experiment prior to any treatment (Table 2.1). Goldfish acclimated to 25° C exhibited significantly higher V_f than goldfish acclimated to 7° C. Although V_{AMP} was not significantly different between the two groups of fish (P = 0.05), there was an obvious trend for higher values of V_{AMP} in the fish acclimated to 25° C (Table 2.1).

Ventilatory responses to acute hypoxia or external and internal injections of NaCN

The hyperventilatory responses to acute hypoxia are summarized in Figure 2.1. Both groups of fish exhibited obvious increases in V_{AMP} and V_f [although the V_{AMP} response in the fish acclimated to 25° C was not statistically significant (P = 0.104)]. At 25° C, V_{AMP} and V_f were increased by 91 and 43%, respectively whereas at 7° C, they were increased by 90 and 34%, respectively. Clearly, the presence of the ILCM did not impair the capacity of 7° C fish to mount a hyperventilatory response during acute hypoxia.

Goldfish were injected with NaCN in the buccal cavity and in the caudal vein to preferentially stimulate externally- and internally-oriented O_2 chemoreceptors, respectively. Figure 2.2 depicts a particularly striking set of representative traces obtained from a single fish receiving four consecutive external injections of NaCN. As well as demonstrating obvious NaCN-evoked increases in V_{AMP} and V_f (Figure 2.2A-C), the accompanying continuous measurements of blood gases demonstrated pronounced effects of each episode of hyperventilation consisting of a rise in PaO_2, a fall in $PaCO_2$ and a corresponding increase in pHa (Figure 2.2D-F).

The ventilation data were quantified and compiled as dose response curves (Figures 2.3-2.4).Figures 2.3 and 2.4 illustrate the responses of goldfish acclimated to either 7 or 25°C to external or internal injections of NaCN, respectively. Hill plots were generated from the dose response curves to calculate the effective NaCN dose at which 50% of the response was elicited (ED_{50}); these values are summarized in Table 2.2. 95% confidence intervals (omitted on figures for clarity) were drawn for each Hill plot regression to determine if the differences between the two temperature treatments were significantly different. Although the ED_{50} for the V_f response to external NaCN was significantly higher in the 7° C fish (Table 2.2; Figure 2.3C), the V_{AMP} response (Figure 2.3A) was unaffected. Moreover, while the baseline values for V_{AMP} and V_f

tended to be lower in the 7° C fish, their capacity to increase ventilation (on a percentage basis) was equivalent to the fish acclimated to 25° C (Table 2.1). External injections of NaCN elicited a 63.1 ± 13.1% and a 67.6 ± 4.9% increase in the V_{AMP} of 7 and 25° C goldfish, respectively. V_f also increased by 92.4 ± 26.4% in 7° C goldfish and 45.9 ± 5.8% in 25° C goldfish (these two values did not differ statistically; P = 0.204). With internal NaCN injections, the increase in V_{AMP} was 75.5 ± 17.0% and 80.6 ± 19.6% for 7 and 25° C goldfish, respectively; V_f was increased by 46.0 ± 13.9% and 42.5 ± 8.9% at 7 and 25° C. The increase in V_{AMP} and V_f caused by internal injections was not significantly different between the two groups of fish (P = 0.86 and 0.83, respectively).

Neuroepithelial cells: Location, innervation and quantification

The branchial distribution and innervation patterns of NECs in fish acclimated to 25 or 7° C are illustrated by a series of representative confocal micrographs (Figures 2.5 – 2.7); the data are quantified in Figure 2.8. Reconstruction of optical sections revealed that the NECs in the 25° C fish (no ILCM) were more-or-less evenly distributed along the length of the uncovered lamellae, being present in apparently equal proportions in the proximal (lower half relative to the filament) and distal (upper half relative to the filament) lamellar regions (Figure 2.5A and B). In the fish acclimated to 7° C (ILCM present), there was an obvious redistribution of NECs to the distal regions of the lamellae that remained uncovered by the ILCM (Figure 2.5C and D; Figure 2.6). A similar distribution pattern was observed when examining cross sections through the gill filament which allowed individual lamellae to be exposed (Figure 2.7). Figure 2.7 clearly illustrates that the NECs are concentrated on the uncovered regions of the lamellae, which are reduced in the 7° C fish owing to the presence of the ILCM. Based on the distribution pattern of ZN-12

immunoreactive nerve fibres, it would appear that at least a portion of the lamellar NECs are innervated regardless of their proximity to the filament (Figures 2.6 – 2.8). The ILCM itself and in particular the outer regions appear to be devoid of ZN-12 immunoreactive nerve fibres (Figures 2.5 and 2.6).

To quantify the redistribution of NECs, the ratio of NECs found along the distal half of the lamellae to the total number of NECs was calculated (Figure 2.8). In the 25° C fish, 53.1 ± 3.4% of the NECs were found on the distal half (Figure 2.8A). In the fish acclimated to 7° C, 82.6 ± 5.2% of the NECs were confined to the distal half of the lamellae (Figure 2.8A). The redistribution to the distal regions of the lamella in the 7° C fish coupled with an absolute increase in the numbers of NECs per lamella (Figure 2.8B) resulted in an increased density of NECs in the distal regions of lamellae. The number of filament NECs was constant regardless of acclimation temperature (Figure 2.8C).

FIGURES AND TABLES

Figure 2.1. (A) Ventilation amplitude (V_{AMP}) and (B) frequency (V_f) of goldfish (*Carassius auratus*) acclimated to 25° C (filled bars; N = 4 - 6) or 7° C (unfilled bars; N = 6) as measured during normoxia (N) and during exposure to acute hypoxia (H). Data are shown as means ± 1 SEM; significant differences from normoxic values are indicated by asterisks ($P < 0.05$; unpaired two-tailed Student's t-test).

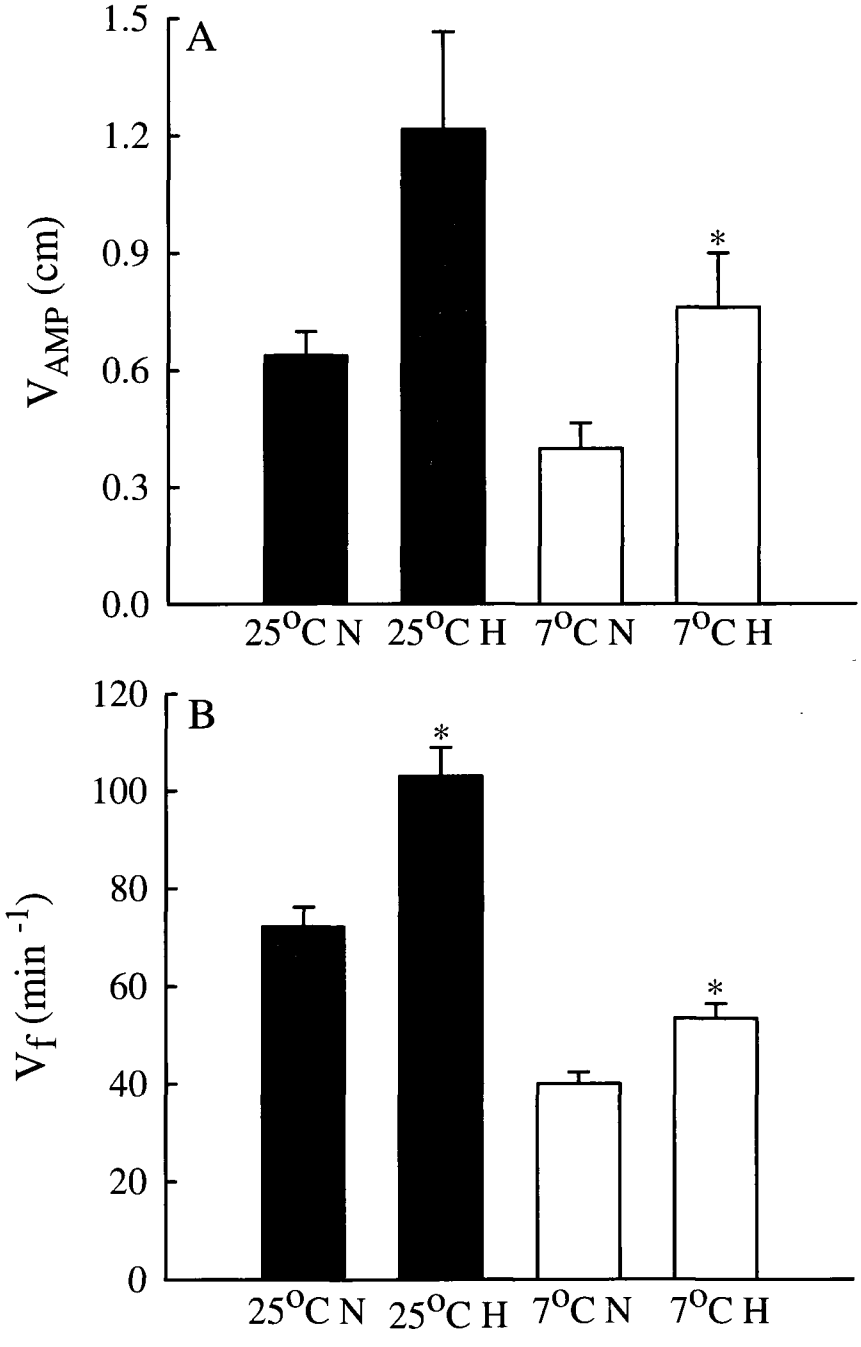
A
1.5
1.2
0.9
0.6
0.3
0.0
V_{AMP} (cm)
*
25°C N
25°C H
7°C N
7°C H
B
120
100
80
60
40
20
0
V_f (min $^{-1}$)
*
*
25°C N
25°C H
7°C N
7°C H

Figure 2.1

Figure 2.2. Representative recordings illustrating the effects of four consecutive NaCN injections (indicated by the dotted lines) into the buccal cavity of a goldfish (*Carassius auratus*) on (A-C) ventilation parameters and (D-F), arterial blood gases. The measured ventilation parameters are A) linear deflections of the opercular flaps as measured by impedance changes, B) ventilation frequency (V_f) and C) ventilation amplitude (V_{AMP}) as determined from the impedance trace. Arterial blood was continuously monitored for D) PO_2 (PaO_2), E) PCO_2 ($PaCO_2$) and F) pH (pHa). Note that each bout of hyperventilation associated with NaCN injection caused transient increases in PaO_2 and pHa and decreases in $PaCO_2$. To synchronize the ventilation and blood gas data, the lag time associated with the blood arriving at the electrodes (approximately 1 min) was subtracted from the blood gas data.

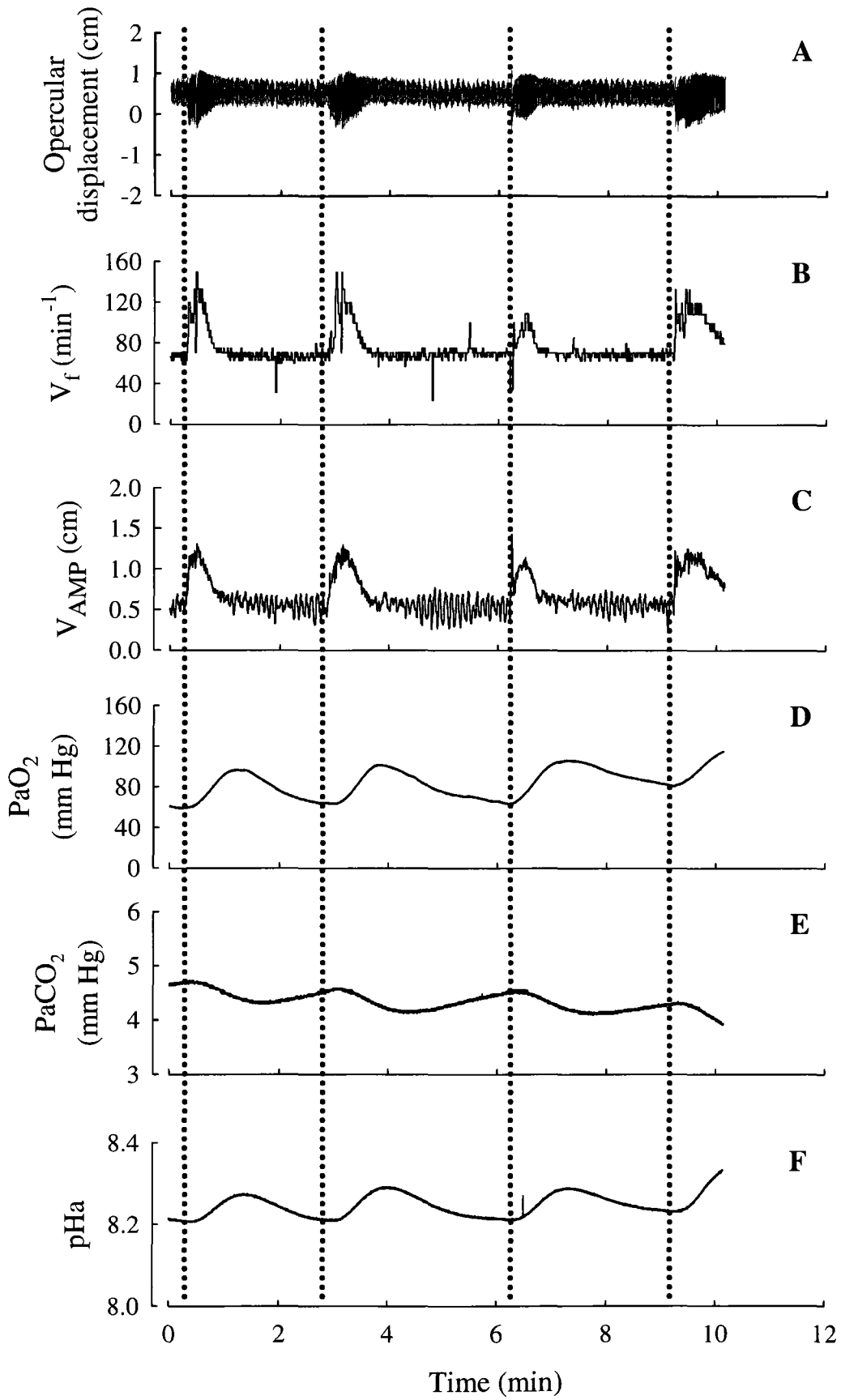
Opercular displacement (cm)
2
1
0
-1
-2
A
V_f (min^{-1})
160
120
80
40
0
B
V_{AMP} (cm)
2.0
1.5
1.0
0.5
0.0
C
PaO_2 (mm Hg)
160
120
80
40
0
D
$PaCO_2$ (mm Hg)
6
5
4
3
E
pHa
8.4
8.2
8.0
F
0
2
4
6
8
10
12
Time (min)

Figure 2.2

Figure 2.3. Dose response curves for hyperventilation responses including A, B) ventilation amplitude (V_{AMP}) and C, D) ventilation frequency (V_f) in goldfish (*Carassius auratus*) acclimated to 7° C (unfilled circles; N = 10 - 13) or 25° C (filled circles; N = 5 - 7) and injected externally with NaCN (0.02 – 4.00 mg kg^{-1}). Dose-response curves were fit by iteration using a sigmoidal 4-parameter equation and linearized using Hill plots.

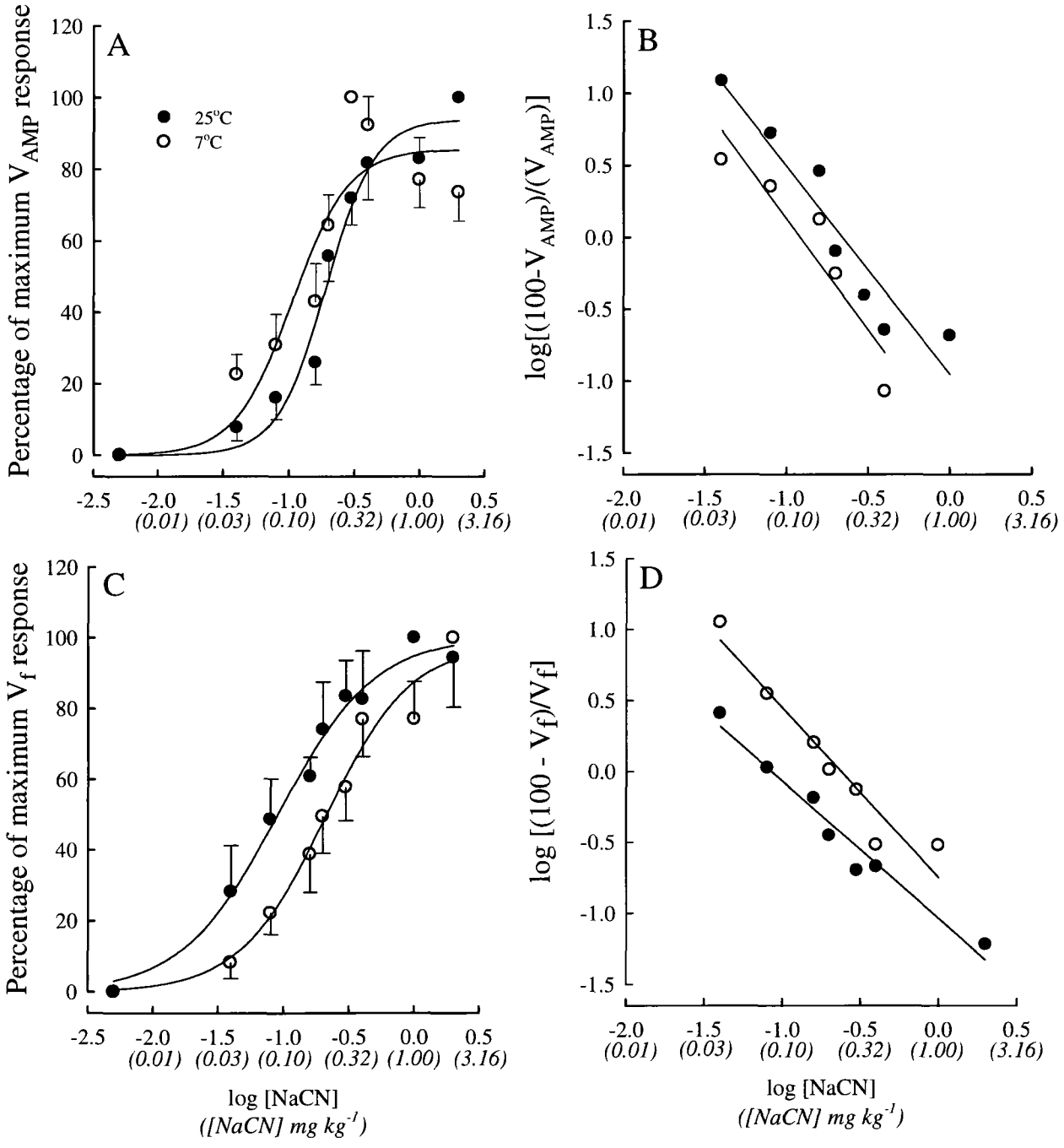
A
B
C
D
25°C
7°C
Percentage of maximum V_{AMP} response
$\log[(100-V_{AMP})/(V_{AMP})]$
Percentage of maximum V_f response
$\log[(100-V_f)/V_f]$
120
100
80
60
40
20
0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5
(0.01)
(0.03)
(0.10)
(0.32)
(1.00)
(3.16)
log [NaCN]
([NaCN] mg kg^{-1})

Figure 2.3

Figure 2.4. Dose response curves for hyperventilation responses including A, B) ventilation amplitude (V_{AMP}) and C, D) ventilation frequency (V_f) in goldfish (*Carassius auratus*) acclimated to 7° C (unfilled circles; N = 9 - 13) or 25° C (filled circles; N = 10 - 16) injected internally (via the caudal vein) with NaCN (0.01 – 0.20 mg kg^{-1}). Dose-response curves were fit by iteration using a sigmoidal 4-parameter equation and linearized using Hill plots.

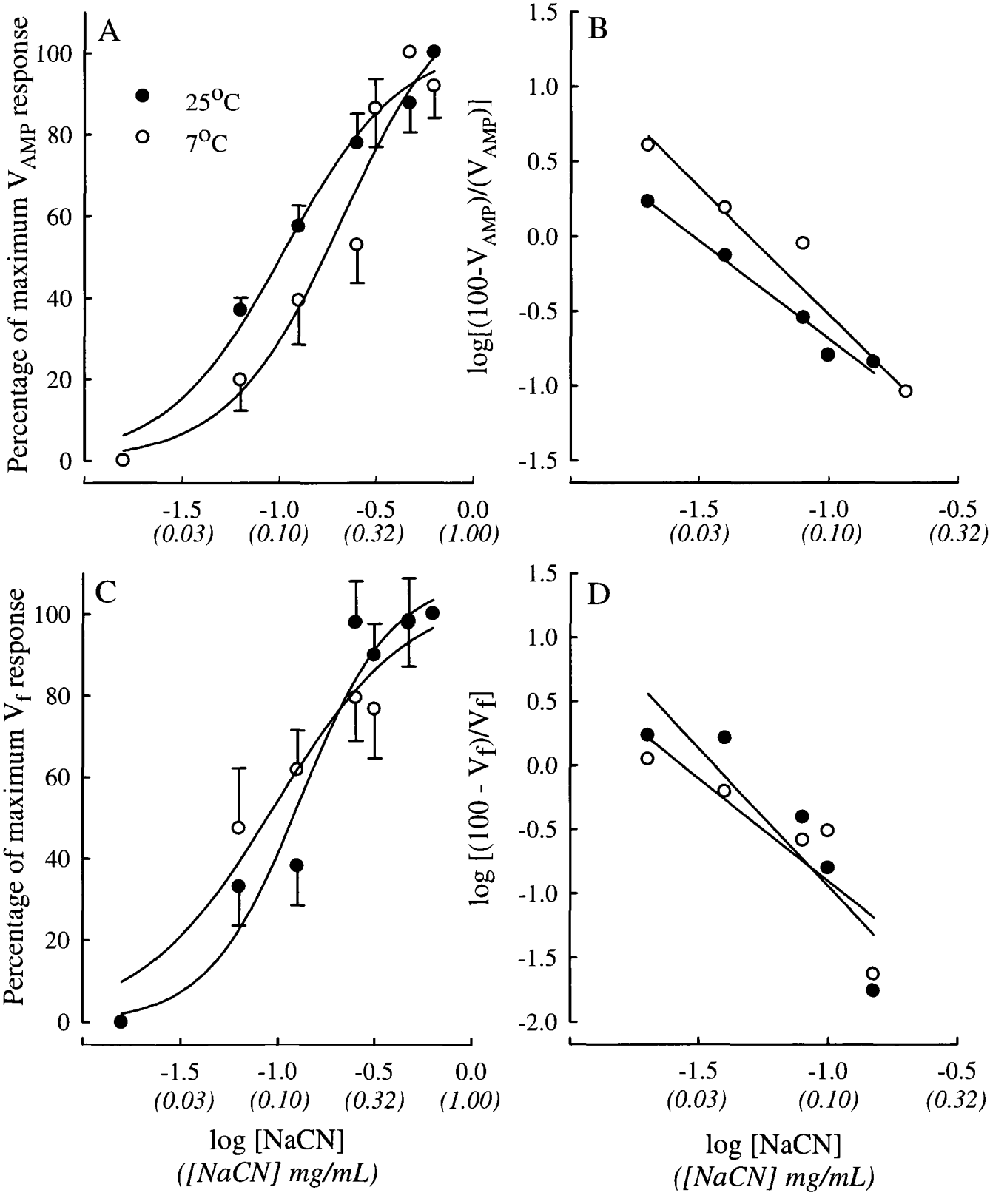
A
B
C
D
25°C
7°C
Percentage of maximum V_{AMP} response
$\log[(100-V_{AMP})/(V_{AMP})]$
Percentage of maximum V_f response
$\log[(100-V_f)/V_f]$
0
20
40
60
80
100
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
-2.0
-1.5 (0.03)
-1.0 (0.10)
-0.5 (0.32)
0.0 (1.00)
log [NaCN]
([NaCN] mg/mL)

Figure 2.4

Figure 2.5. Representative confocal micrographs showing serotonin-immunoreactive neuroepithelial cells (NEC; green fluorescence) and ZN-12-immunoreactive nerve fibres (red fluorescence) within the gill filament (F) and on the lamella (L) of goldfish (*Carassius auratus*) acclimated to 25° C (A, B) or 7° C (C, D). The dotted line represents the edge of the interlamellar cell mass (ILCM). NECs of the filament (F) are indicated by arrows; NECs of the lamella (L) are indicated by arrow heads. The numbers above the scale bars are in μm.

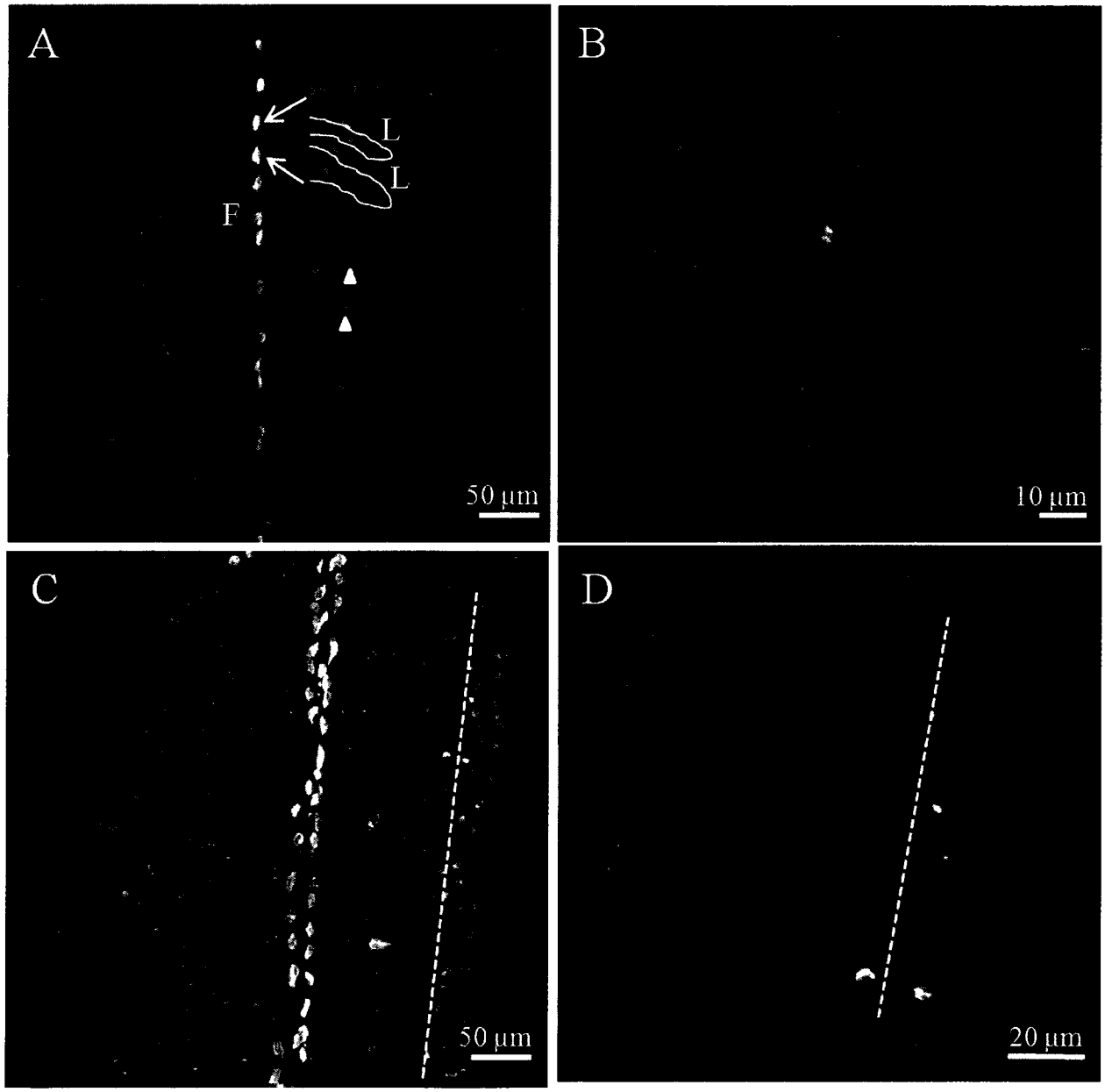

Figure 2.5

Figure 2.6. Representative confocal micrographs showing serotonin-immunoreactive neuroepithelial cells (NEC; green fluorescence) and ZN-12-immunoreactive nerve fibres (red fluorescence) within the gill filament (F) and on the lamella (L) of goldfish (*Carassius auratus*) acclimated to 7° C. The dotted line represents the edge of the interlamellar cell mass (ILCM). To more clearly demonstrate the distribution pattern of nerve fibres, the green colour was removed from panel A; panel B is the identical image with the green colour added. NECs are indicated by arrows; arrow heads indicate nerve fibres apparently innervating NECs. The numbers above the scale bars are in μm.

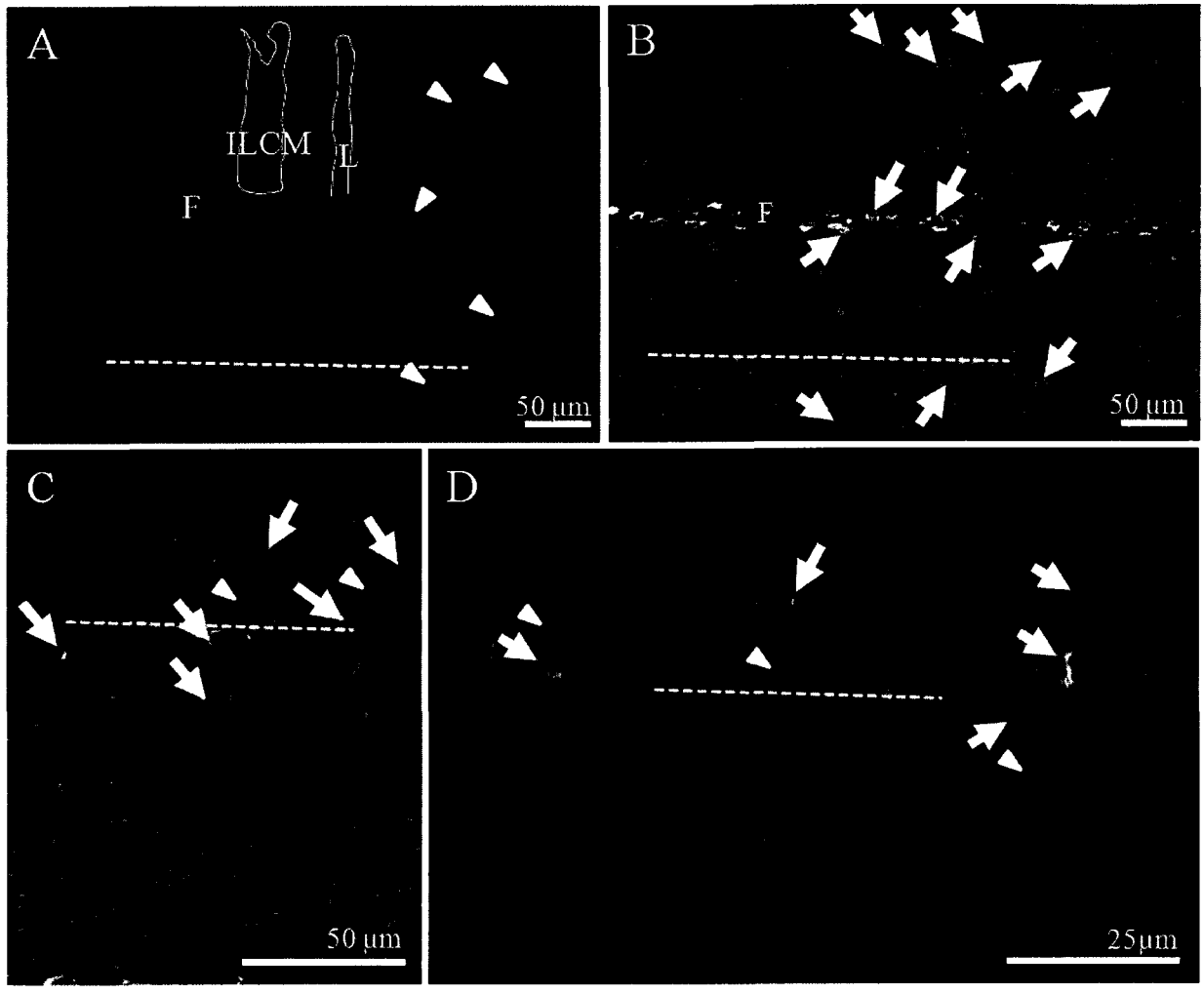

Figure 2.6

Figure 2.7. Serotonin-immunoreactive neuroepithelial cells (NEC; arrows) and ZN-12-immunoreactive nerve fibres (red fluorescence in insets) on the lamellae of goldfish (*Carrasius auratus*) acclimated to (A) 25° C or (B) 7° C. The solid lines represent the edge of the ILCM; the numbers above the scale bars are in μm; AFA = afferent filament artery, EFA = efferent filament artery.

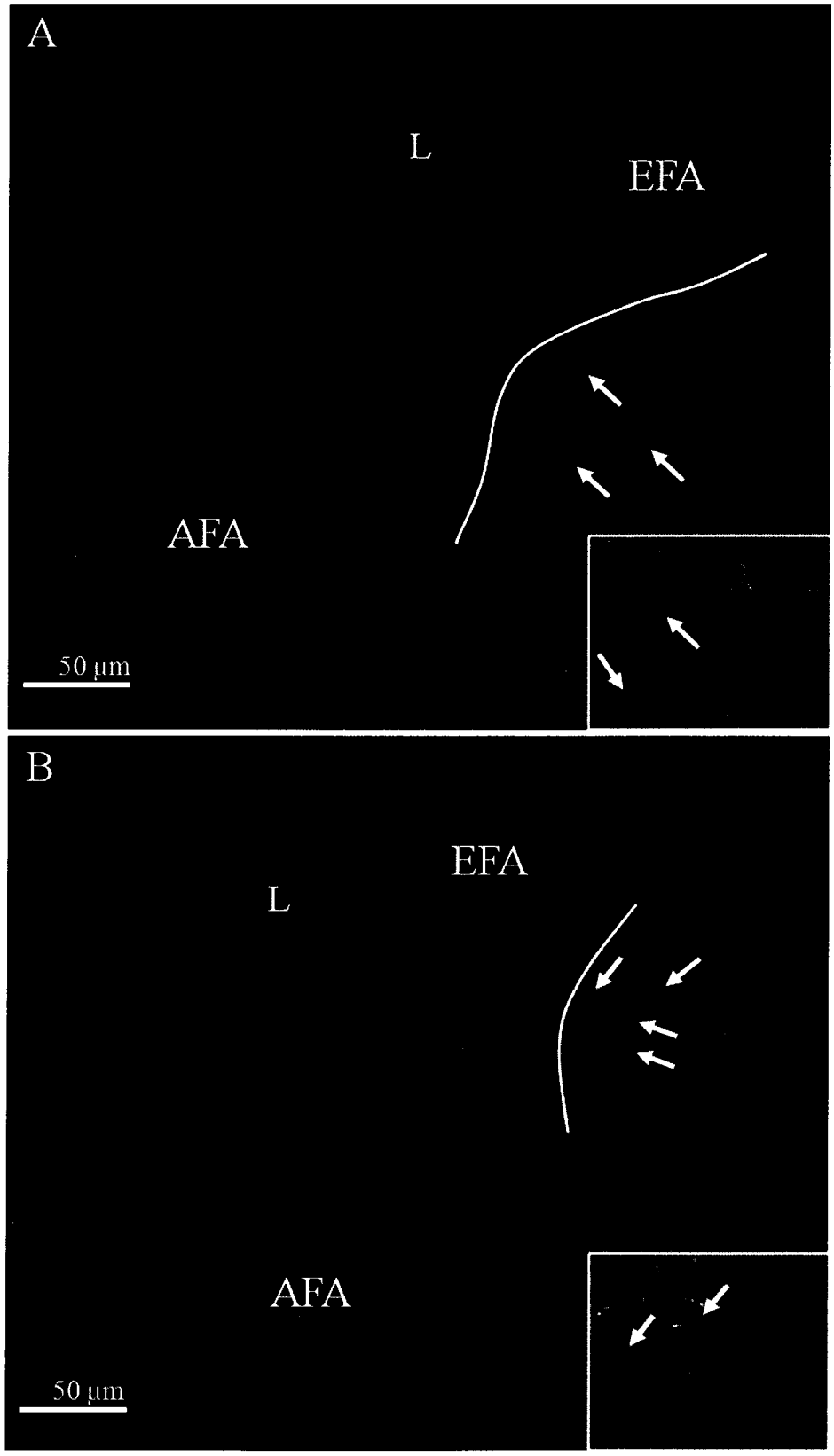

Figure 2.7

Figure2. 8. A) Distribution of the neuroepithelial cells (NECs) on the lamellae of goldfish (*Carassius auratus*) acclimated to 7 or 25° C; B) The number of lamellar NECs per lamella; C) the number of filament NECs per mm of filament length. Data are shown as means ± 1 SEM; significant differences from values in the 25° C fish are indicated by asterisks ($P < 0.05$; unpaired two-tailed Student's t-test).

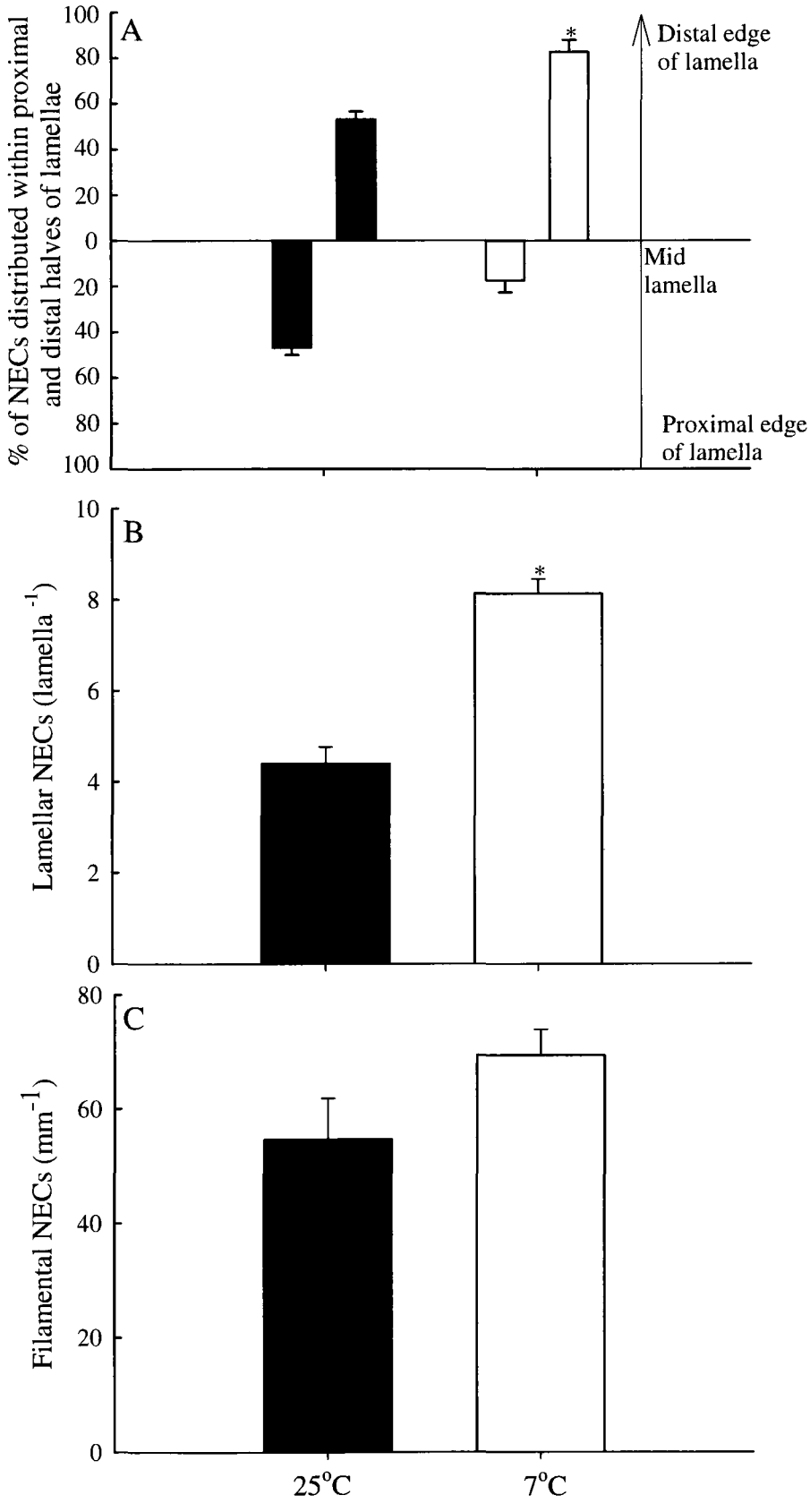
A
% of NECs distributed within proximal and distal halves of lamellae
100
80
60
40
20
0
20
40
60
80
100
*
Distal edge of lamella
Mid lamella
Proximal edge of lamella
B
Lamellar NECs (lamella -1)
10
8
6
4
2
0
*
C
Filamental NECs (mm-1)
80
60
40
20
0
25°C
7°C

Figure 2.8

Table 2.1. The effects of bolus injections NaCN into the buccal cavity (4 mg kg^{-1}) or caudal vein (0.2 mg kg^{-1}) of goldfish acclimated to 7° C (ILCM present) and 25° C (no ILCM) on ventilation amplitude (V_{AMP}) and frequency (V_f). Data shown are means ± 1 S.E.M; N numbers are indicated in parentheses.

Temp. (° C)	V_{AMP} (cm)			V_f (min^{-1})		
	Pre NaCN	**NaCN**	**% increase**	**Pre NaCN**	**NaCN**	**% increase**
			External Injections			
7 (6)	0.39 ± 0.09	0.72 ± 0.1	63.1 ± 13.1	41.5 ± 2.5	98.6 ± 10.2	92.4 ± 26.4
25 (12)	0.55 ± 0.04	0.92 ± 0.1	67.6 ± 14.9	73.1 ± 4.9	102.9 ± 4.1	45.9 ± 5.9
			Internal Injections			
7 (11)	0.39 ± 0.04	0.75 ± 0.1	75.5 ± 17.0	36.6 ± 3.4	4.0 ± 11.6	46.0 ± 13.9
25 (13)	0.51 ± 0.04	0.93 ± 0.1	80.6 ± 19.6	68.9 ± 3.8	108.9 ± 7.4	42.5 ± 8.9

Table 2.2. Effective dose (ED_{50}; mg kg^{-1}) of NaCN at which 50% of the maximal ventilation response was elicited for external or internal injections of NaCN in goldfish acclimated to 7 (ILCM present) or 25° (no ILCM). The ED_{50} values were determined from dose response curves expressed as Hill plots (Figures 2.3 and 2.4). V_{AMP}, ventilation amplitude; V_f, ventilation frequency. N numbers are indicated in parentheses.

	External NaCN		**Internal NaCN**	
Temperature (° C)	**V_{AMP} ED_{50}**	**V_f ED_{50}**	**V_{AMP} ED_{50}**	**V_f ED_{50}**
7° C	0.12 (6)	0.24(6)	0.05 (11)	0.03 (11)
25° C	0.22 (12)	0.08(12)	0.03 (13)	0.04 (13)

DISCUSSION

This study examined the ability of goldfish acclimated to different temperatures and with different gill morphologies to sense and respond to hypoxic stimuli. The basic premise being tested was that the presence of an ILCM in fish acclimated to cold water (7° C) would lead to a covering of lamellar NECs and thus impede the capacity of fish to respond to external hypoxia. This idea was tested by stimulating the putative externally- and internally-oriented O_2 chemoreceptors with increasing concentrations of NaCN delivered to the buccal cavity or caudal vein, respectively or by exposing fish to acute hypoxia. Despite the presence of an ILCM in the fish acclimated to 7° C, their hyperventilatory responses to NaCN or hypoxia were largely similar in goldfish acclimated to 25° C (without an ILCM) or 7° C (with an ILCM). I suggest that the continued ability to respond to external hypoxic stimuli in the fish acclimated to cold water reflects a profound redistribution of the NECs whereby they are clustered on the distal surfaces of lamellae which tend to remain uncovered during gill remodelling. Hence, despite the presence of the ILCM, the NECs remain exposed to the water and presumably fully operational.

Resting ventilation in goldfish acclimated to 7 and 25° C

As shown previously for other fish species [e.g. carp, *Cyprinus carpio* and goby, *Gillichthys mirabilis* (Crawshaw, 1976; Courtois, 1976; Stecyk and Farrell, 2002)] and consistent with increasing rates of routine metabolism with rising temperature (Fry and Hart, 1948; Beamish and Mookherjii., 1964), V_{AMP} and V_f were significantly elevated with increasing acclimation temperature. These temperature related differences in resting ventilation values, while important in matching increasing metabolic demands by tissues to increased rates of branchial O_2 uptake,

can potentially confound the interpretation of experiments such as in the present study which were designed to measure the impact of hypoxic stimuli in fish with (7° C) and without (25° C) an ILCM. Thus, it is possible that regardless of the extent of ILCM coverage of NECs, equivalent hypoxic stimuli would have produced different breathing responses simply because of the different initial resting ventilation values or alternatively, as a result of differences in temperature, *per se*. For example, increased resting rates of V_f in fish acclimated to 25° C could conceivably constrain the extent of hyperventilation if there was an absolute ceiling to V_{AMP} or V_f beyond which further increases were no longer possible; if so, the fish acclimated to 7° C might possess a greater scope for increasing ventilation. On the other hand, colder temperature, in itself, could potentially reduce the sensitivity of O_2 chemoreceptors to hypoxic stimuli owing to Q_{10} effects. While several previous studies have compared ventilatory responses of fish to hypoxia at different temperatures (Glass et al., 1990; Stecyk and Farrell, 2002), I am unaware of any studies which have specifically investigated the effects of acclimation temperature on the sensitivity or maximal capacity of the piscine ventilatory response to hypoxic stimuli (e.g. as determined from NaCN dose response curves or stepwise decreases in ambient PO_2). However, given that the fish acclimated to 25° C are far from the upper ceiling (~30° C) of aerobic scope for this species (Fry and Hart, 1948), it seems unlikely that ventilatory responses were being constrained by the higher temperature. Finally, because the ventilatory responses of the 7° C fish were essentially identical to those of the 25° C fish (see below), it also seems unlikely that low temperature, in itself, was impeding ventilatory responses (see also Glass et al., 1990).

Ventilatory responses of goldfish to hypoxic stimuli

Surprisingly, and despite the wealth of studies related to their metabolism, this is the first published study to examine the ventilatory responses of goldfish to hypoxic stimuli. However, in keeping with traditional responses exhibited by many other species including common carp (Itazawa and Takeda, 1978; Lomholt and Johansen, 1979), goldfish exhibited robust hyperventilatory responses to injections of external/internal NaCN or acute hypoxia which consisted of increases in V_{AMP} and V_f. Similar to carp which respond to moderate (Glass et al., 1990) and even mild (Itazawa and Takeda, 1978) levels of hypoxia, goldfish also appear to exhibit marked sensitivity to hypoxia with hyperventilatory responses being initiated well above 100 mm Hg (V. Tzaneva, K.M. Gilmour and S.F. Perry; unpublished observations). Because this is the first study to employ dose response curves to characterize the sensitivity of the ventilatory response to the hypoxic stimulant NaCN, strict comparisons with other species are not possible. However, it would appear that the sensitivity of goldfish to NaCN is on par with (or exceeds) other species if one compares the ED_{50} values (Table 2.2) to the results obtained in prior experiments using single doses of NaCN (e.g. Sundin et al., 2000; Reid and Perry, 2003).

Despite the presence of an ILCM, goldfish acclimated to 7° C responded similarly (although the V_f response to external NaCN was blunted) to hypoxic stimuli (external and internal) as the fish acclimated to 25° C. A continued capacity to respond to internally injected NaCN and hypoxia can be readily explained by presence of filamental NECs in close proximity to the efferent filament artery (Saltys et al., 2006). Interestingly, Coolidge et al. (2008) were unable to locate NECs within the filamental epithelia of the goldfish gill but instead reported a clustering of innervated NECs at the distal tip of the filament. The lack of central filament NECs reported by Coolidge et al. (2008) implied that goldfish might lack responsiveness to internal hypoxic stimuli. The results of the present study, however, clearly demonstrated a

hyperventilatory response to internally administered NaCN which supports the presence of a population of internal O_2 chemoreceptors which are presumably the NECs within the filamental epithelia in proximity to the efferent filament artery (this study; Saltys et al., 2006). Currently, it is unclear as to why the goldfish gills examined by Coolidge et al. (2008) did not appear to contain NECs within the filamental epithelia.

Branchial NEC distribution in fish with or without an ILCM

This is the first study to compare the location of NECs on the gills of goldfish with and without an ILCM present. In the 25° C fish without an ILCM, the lamellar NECs were distributed evenly along the length of lamellae (i.e. ~50% in each of the proximal and distal regions). Although not specifically quantified, a similar random distribution pattern was also observed by Saltys et al. (2006). In contrast, Coolidge et al. (2008) reported that lamellar NECs in goldfish were preferentially distributed to the distal surfaces of lamellae and suggested that such a location might confer an advantage to fish experiencing a growth of the ILCM presumably by allowing continued O_2-sensing of the external environment. In the present study, the NECs were concentrated on the distal half of the lamella only in the fish acclimated to 7° C and exhibiting an ILCM. Thus, the formation of the ILCM was associated with a profound reorganization of lamellar NECs whereby they were redistributed to distal regions where they remained uncovered. Because the distally located NECs are innervated, they are presumed to be fully functional O_2 chemoreceptors. The nerve fibres innervating the lamellar NECs arise from branchial nerves which also innervate the filament (Jonz and Nurse, 2008). Interestingly, there appeared to be little, if any, nerve fibres present within the ILCM, itself, a finding which is consistent with the apparent absence of NECs on the outer edge of the ILCM.

The most parsimonious explanation for the redistribution of NECs to the distal regions of lamellae is to preserve the O_2-sensing capacity of the gill, a conclusion which is supported by the results obtained from the continued capacity of the 7° C fish to hyperventilate during exposure to hypoxia or external NaCN. The redistribution of NECs to the distal regions of lamellae mimics the rearrangement of ion-transporting mitochondria rich cells (MRCs) when the lamellae are covered by the ILCM (Mitrovic et al., 2009; Mitrovic and Perry, 2009). A major difference however, is that the MRCs not only become localised to distal zones of lamellae but also to the outer edge of the ILCM, itself. Like the redistribution of NECs which we believe confers continued O_2-sensing capacity, the rearrangement of MRCs to the distal lamellae and outer edge of the ILCM is thought to preserve branchial ion transport capacity (Mitrovic and Perry, 2009). Mitrovic and Perry (2009) used a "time-differential double fluorescent staining" technique (Katoh and Kaneko, 2003) to demonstrate that the majority of MRCs found at the edge of the ILCM after a reduction in water temperature resulted from the migration of pre-existing MRCs with a smaller proportion newly differentiated MRCs appearing. Currently, the mechanism underlying the redistribution of NECs during gill remodelling is unclear but presumably, like the MRCs, may involve migration of pre-existing cells as well as differentiation of progenitor cells. Certainly, the greater number of NECs per lamella in the fish acclimated to 7° C (Figure 2.8) would suggest that differentiation of new cells is a significant component of the overall redistribution. In the context of my original hypothesis that lamellar NECs would become covered with the appearance of an ILCM, we considered it possible that fish would respond with a compensatory increase in the numbers of filament NECs; clearly, this was not the case. The redistribution of lamellar NECs and their absolute increase in numbers with cold acclimation attest to the importance of this population of NECs in O_2 sensing and suggest that other potential

sites for detecting O_2 changes in the water such as NECs associated with gill rakers (Coolidge et al., 2008) are considerably less important. Although Coolidge et al. (2008) suggested that NECs associated with the tips of filaments might be an additional site of external O_2 sensing in goldfish, it is questionable as to whether the NECs localized to the filament tip are truly externally oriented. The limitations associated with examining thin tissue sections (rather than confocal microscopy of whole mount specimens), coupled with the previous reports of NECs within the filament of goldfish (this study; Saltys et al., 2006), suggest that the NECS reported to be on the filament tip (Coolidge et al., 2008) may simply be of the same population as the more proximally (relative to the gill arch) located NECs that are associated with the efferent filament artery.

ACKNOWLEDGEMENTS

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CHAPTER 3.

Respiratory responses to hypoxia or hypercapnia in goldfish (*Carassius auratus*) experiencing gill remodelling

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Authors: Velislava Tzaneva, Kathleen M. Gilmour and Steve F. Perry

ABSTRACT

The gill of goldfish (*Carassius auratus*), acclimated to 7° C is largely covered with an interlamellar cell mass (ILCM) that is absent in fish acclimated to 25° C. The presence of the ILCM significantly decreases the functional lamellar surface area and increases the diffusion distance for gas transfer and thus may impose a serious challenge for the transfer of respiratory gases (O_2 and CO_2). Here we tested the hypothesis that the presence of the ILCM in goldfish acclimated to 7° C impedes the uptake of O_2 and excretion of CO_2. This was accomplished by exposing fish to acute hypoxia or hypercapnia while measuring ventilation amplitude (V_{AMP}), ventilation frequency (V_f) and arterial blood gases (PaO_2, $PaCO_2$ and pHa) continuously in real time. While PaO_2 was unaltered, the baseline values of $PaCO_2$ were significantly higher in goldfish with covered gills (ILCM present) (5.55 ± 0.54 mm Hg) than in goldfish with uncovered gills (3.98 ± 0.18 mm Hg). These results suggest that under normoxic conditions the ILCM imposes an additional limitation on the transfer of CO_2 from the blood to the water. This apparent diffusion limitation on CO_2 transfer presumably caused by the ILCM was relieved when the fish were injected with bovine carbonic anhydrase (CA; 2 mg kg^{-1}). After CA injection, $PaCO_2$ fell significantly to 3.07 ± 0.32 mm Hg within 90 min. These results, coupled with the fact that the inward movement of gaseous CO_2 into the blood during acute hypercapnia was unaffected by increased lamellar covering, suggest that the impediment of the ILCM to CO_2 excretion reflects the slow rate of conversion of plasma HCO_3^- to CO_2 rather than the diffusion of CO_2, per se. Interestingly, the exposure of fish to acute hypoxia evoked similar changes in PaO_2 at the two acclimation temperatures, a result which may, in part, be explained by a rapid (partial) loss of the ILCM during the brief period of hypoxia exposure. Ethanol (EtOH) exposure

was also used as a tool to further investigate the potential effects of the ILCM on branchial solute transfer. The results showed that the ILCM does not impede EtOH uptake in 7° C goldfish. Overall, the results of this study demonstrate that the remodelling of the goldfish gill associated with acclimation to cold water, while increasing $PaCO_2$, has minimal impact on branchial O_2 transfer.

INTRODUCTION

Branchial gas transfer in teleost fish has been extensively studied over the past five decades (Holeton and Randall, 1967; Randall et al., 1967; Cameron and Davis, 1970; Randall et al., 1982; Randall and Daxboeck, 1984; Perry and Wood, 1989; Gilmour, 1997; Perry and Gilmour, 2002; Perry et al., 2009a; Perry and Gilmour, 2010). The existing theoretical (Piiper and Scheid, 1984; Malte and Weber, 1985; Piiper, 1989) and empirical evidence (Daxboeck et al., 1982; Part et al., 1984; Julio et al., 2000; Desforges et al., 2002) suggests that O_2 uptake in rainbow trout (and presumably in other teleost fish) is subjected to minimal diffusion limitations under resting conditions and therefore behaves as a perfusion-limited system (Perry and Gilmour, 2002). In a perfusion-limited system, the extent to which arterial blood equilibrates with the inspired water is independent of blood transit time through the gill (Perry and Gilmour, 2002). Thus, in a perfusion-limited system, arterial PO_2 (PaO_2) can remain constant over a broad range of cardiac outputs and consequently, O_2 uptake tends to increase linearly with increasing blood flow. The absence of diffusion limitations for O_2 transfer under normal conditions implies that arterial PO_2 should be relatively insensitive to increases in the water-to-blood diffusion distance or a reduction in lamellar surface area. Indeed, the results of previous studies that have monitored PaO_2 in trout experiencing reduced gill surface area (Julio et al., 2000) or a thickened diffusion barrier (Bindon et al., 1994; Greco et al., 1995) have shown that PaO_2 (at least during normoxia) can remain unaltered. However, if the thickening of the diffusion barrier is severe enough, reductions in PaO_2 may indeed occur even under normoxic conditions (Thomas et al., 1988; Perry et al., 1996; Perry, 1998). In accordance with theory, the diffusion limitations for O_2 transfer increase with external hypoxia as the water-to-blood PO_2 gradient (the driving force for diffusion) decreases. Thus, as demonstrated for rainbow trout (Bindon et al., 1994; Greco et al.,

1995), the negative impact of increasing diffusion distance caused by thickening of the lamellar epithelium is most readily observed during exposure of fish to hypoxia.

By contrast, the transfer of CO_2 across the gill of teleosts behaves as a diffusion-limited system (Desforges et al., 2002). Although the higher capacitance of water for CO_2 relative to O_2 should favour the diffusivity of molecular CO_2, the overall process of branchial CO_2 transfer is restricted by the need to convert plasma HCO_3^- to molecular CO_2 within the brief period of blood transit through the gill vasculature (1 – 3 sec; Cameron and Polhemus, 1974). In other words, the apparent diffusion limitations for CO_2 transfer reflect chemical equilibrium constraints imposed by the relatively slow conversion of plasma HCO_3^- to molecular CO_2. The dehydration of plasma HCO_3^- to CO_2, while occurring rapidly at the catalysed rate in the presence of red blood cell (RBC) carbonic anhydrase (CA), is constrained by the slow rate of the RBC Cl^-/HCO_3^- exchanger, the rate limiting step in overall CO_2 excretion (Perry et al., 1982; Perry, 1986; Perry and Gilmour, 1993; Gilmour et al., 2004). For these reasons, the addition of exogenous CA to the plasma in teleosts (Julio et al., 2000; Desforges et al., 2001; Desforges et al., 2002; Gilmour and MacNeill, 2003) or the presence of endogenous membrane-associated CA oriented to face the plasma in elasmobranchs (Wood et al., 1994; Gilmour et al., 1997; Henry et al., 1997; Gilmour et al., 2001; Gilmour and Perry, 2004; Gilmour et al., 2007) greatly facilitates CO_2 excretion (see review by Gilmour et al., 2010).

The goldfish (*Carrasius auratus*), like crucian carp (*Carrasius carassius*) (Sollid et al., 2003), exhibits a remarkable structural remodelling of the gills in response to changes in ambient temperature or O_2 levels (Sollid et al., 2005; Mitrovic et al., 2009; Mitrovic and Perry, 2009). With decreasing temperature (<15° C), functional lamellar surface area is decreased and water-to-blood diffusion distances are increased owing to the formation between adjacent lamellae of a

mass of cells termed the interlamellar cell mass (ILCM) (Sollid et al., 2003). This strategy, while presumably beneficial for reducing passive loss of salts across the gill and hence lowering the energetic costs of ionic regulation during periods of lowered metabolic rate, is likely to impede branchial gas transfer especially under conditions of acute hypoxia (Sollid et al., 2003) (see reviews by Sollid and Nilsson, 2006; Nilsson, 2007). Thus, the goldfish is an excellent model species for studying the consequences of naturally occurring reductions in lamellar surface area and increases in diffusion distances on branchial gas transfer. Given the presumed differences in the extent of apparent diffusion limitations for O_2 and CO_2 transfer, it seems plausible that the imposition of additional diffusion limitations associated with gill remodelling would disproportionately affect CO_2 relative to O_2 transfer. Specifically it is hypothesised that the presence of the ILCM in fish acclimated to cold water (7° C) would have a greater impact on CO_2 excretion and that any negative impacts on O_2 transfer would be magnified during exposure of fish to acute hypoxia. Because the apparent diffusion limitations for CO_2 transfer stem from the slow conversion of plasma HCO_3^- to CO_2, it was further hypothesized that the negative consequences of gill remodelling on CO_2 transfer might be partially relieved by the addition of exogenous CA to the plasma. These hypotheses were tested by measuring gill ventilation and arterial blood gases and pH in real time during periods of normoxia, hypoxia and hypercapnia using an extracorporeal circulation in goldfish with (7° C) or without (25° C) covered lamellae.

MATERIALS AND METHODS

Experimental animals and surgical procedures

Goldfish (*Carassius auratus*) were purchased from Aleong's International (Mississauga, Ontario) and were held in circular tanks supplied with flowing, aerated, dechloraminated City of Ottawa tap water at 18° C within the University of Ottawa Aquatic Care Facility. The goldfish were divided into two groups; one group was acclimated to 7° C and the other to 25° C. The final acclimation temperatures were achieved by increasing or decreasing the water temperature at an average rate of 2° C per day. Based on our previous studies (Mitrovic and Perry, 2009; Mitrovic et al., 2009; Tzaneva et al., 2010), the lamellae of the 25° C fish are largely devoid of an ILCM (occupying ~20% of the lamellar channels) while the fish acclimated to 7° C exhibit gills with extensive coverage of the lamellae by the ILCM (occupying ~80-90% of the lamellar channels). Goldfish were held at their respective temperatures on a 12/12-h light/dark photoperiod and fed commercial food pellets daily for at least two weeks prior to experimentation. All experiments were conducted at the University of Ottawa at these two temperatures in compliance with guidelines of the Canadian Council of Animal Care (CCAC) and after approval of the University of Ottawa Animal Care Committee (Protocol BL-226).

Goldfish (average mass = 236 g; N = 42) were anaesthetised in 10 mg l^{-1} benzocaine (Sigma-Aldrich, Inc.) until ventilation had ceased. The fish were then placed on a surgery table where the gills were irrigated with aerated anaesthetic solution. To measure ventilation frequency (V_f) and amplitude (V_{AMP}), impedance leads with 1 cm^2 brass plates were attached with sutures to the outer surface of each operculum. To allow sampling of the inspired water, a cannula (Clay Adams PE 160) was inserted into the snout between the nostrils and into the buccal cavity. For continuous measurements of blood respiratory variables *in vivo*, the caudal

vein and artery were cannulated. Please refer to the Materials and Methods section in Chapter 2 for more details on the surgical procedures.

Experimental protocols – extracorporeal circulation

Goldfish acclimated to 25° C (average mass = 260 ± 15 g; N = 17) were exposed to hypoxia followed by hypercapnia. The fish were allowed ~ 30 min to 1 h to recover after the hypoxia exposure and before the hypercapnia exposure began. Separate groups of goldfish acclimated to 7° C were used for hypoxia (average mass = 298 ± 14 g; N = 8) and hypercapnia exposure (average mass = 227 ± 10 g; N = 10). It should be noted that the data from all goldfish were analyzed but that not all goldfish yielded complete data sets.

Acute hypoxia was achieved gradually over 30 - 40 min by replacing the air bubbling through a water-air equilibration column with N_2 until a water PO_2 (PwO_2) of approximately 30 mm Hg was reached. At this point, normoxia was re-established. Arterial PO_2, PCO_2 and pH (PaO_2, $PaCO_2$ and pHa) and ventilation parameters (V_{AMP} and V_f) were monitored in real time during hypoxia exposure and recovery.

Acute hypercapnia was initiated by bubbling 1.5 - 3% CO_2 (in air) through the water equilibration column using a gas-mixing flow meter (Cameron Instrument Company model GF-3/MP, Port Aransas, TX, USA). Once a $PwCO_2$ of approximately 8 mm Hg was reached (~15 - 30 min), the fish was returned to normocapnia. Blood gases and ventilatory parameters were measured continuously before, during and after exposure to hypercapnia.

The effect of CA administration was tested using a separate group of goldfish acclimated to 7° C (average mass = 157 ± 8 g; N = 7). Fish were cannulated and the extracorporeal circulation was used to monitor arterial PO_2, PCO_2 and pH. After recording baseline conditions, saline (0.9% NaCl, 1 ml kg^{-1}) was injected into the caudal vein and blood gases and pH were monitored for 90

min. CA (1 mg kg^{-1} using a volume of 1 ml kg^{-1}) was then injected into the caudal vein and blood gases and pH were recorded for 90 min. Saline injection served to ensure that the fish was not responding to the injection procedure itself. Mean values of arterial PO_2, PCO_2, and pH were determined at the point following CA injection where the PCO_2 was lowest.

Experimental protocol – ethanol exposure

Two groups of goldfish acclimated to 7° C were used to assess the passive permeability of the gill by measuring ethanol accumulation in the blood during exposure to ethanol in the water. One group (average mass = 31 ± 2 g, N = 5) was kept at normoxia while the other group (average mass = 30 ± 1 g, N = 5) was exposed to hypoxia (P_wO_2 = 10 mm Hg) for a week to cause a retraction of the ILCM (Mitrovic et al., 2009). Goldfish exposed to hypoxia were allowed to recover in normoxic waters for 24 h before experimentation. Goldfish from both groups were fitted with a single cannula inserted into the caudal artery using the cannulation techniques described above, and were allowed 24 h to recover before exposure to 1% (vol/vol) ethanol (EtOH) for1.5 h. Blood samples (0.3 ml) were collected at 0 and 1.5 h. The volume of blood removed from the fish at 0 h was replaced by injecting an equivalent volume of saline (9% NaCl solution) at 7° C. The blood ethanol concentration was analyzed using an EnzyChrom™ Ethanol Assay Kit (Bioassay Systems, U.S.A). Goldfish in this experiment were held in individual 1 l dark boxes during recovery from surgery and for the duration of the experiment.

Analytical procedures

The frequency and amplitude of opercular displacements were assessed as indices of ventilation using a custom-built impedance converter (University of Ottawa Electronics Workshop) that detected and quantified the changes in impedance between the brass plates attached to the

opercula (Peyraud and Piquemal, 1962). Ventilation amplitude (V_{AMP}) was determined and averaged over 10 sec intervals after conversion of the impedance data to linear opercular deflections (in cm) through appropriate calibration. Ventilation frequency (V_f) was determined from the impedance traces using an automatic rate function within the data acquisition software (see below).

Water PO_2 and PCO_2 were monitored continuously by using a peristaltic pump (Fisher Scientific, Canada; 0.83 ml min^{-1}) to pass water from the holding box through sample compartments containing PO_2 and PCO_2 electrodes (Cameron Instruments) that were connected to a gas meter (Cameron Instruments, BGM 200). Both electrodes were housed in thermostatted cuvettes and were calibrated prior to each experiment using a two point calibration method. For PO_2, a "zero" solution of 0.02 g ml^{-1} sodium sulphite was used to set the low point of the calibration; air-saturated water was used to set the high point. The CO_2 electrode was calibrated using water equilibrated with 0.5% CO_2 (low point) and 1% CO_2 (high point).

PaO_2, $PaCO_2$ and pHa were monitored continuously using an extracorporeal blood shunt (Thomas and Le Ruz, 1982; Thomas, 1994). Blood was pumped (0.4 - 0.5 ml min^{-1}; low flow mini-pump, Control Company, USA) from the caudal artery through a series of three microelectrodes (Microelectrodes Inc.; PO_2, PCO_2 and pH) and then returned to the fish via the caudal vein cannula. The O_2 and CO_2 electrodes were calibrated prior to each experiment as described above. Precision buffer solutions (pH = 7.00 for the low point and pH = 8.00 for the high point; Fisher Scientific) were used to calibrate the pH electrode. The microelectrodes were connected to a blood gas meter (Cameron Instruments, BGM 200). Immediately prior to experimentation, the extracorporeal shunt was rinsed for 10 - 15 min with heparinised (540 i.u. ml^{-1}) saline to reduce blood clotting in the tubing and electrode chambers. After initiating the

extracorporeal blood shunt, ~ 20 min were required to obtain stable readings for blood gas variables and ventilation parameters. Upon stabilisation, experiments commenced with a 10 min period of baseline recording.

Analog outputs from the impedance converter and gas meters were collected with a data acquisition system (BioPac) that interfaced with a PC; the resultant digital data were graphically presented and stored using AcKnowledge data-acquisition software.

At completion of an experiment, the right first gill arch was dissected and fixed in 4% paraformaldehyde (PFA) overnight. The rakers were removed and the remaining tissue was placed in 30% (w/v) sucrose overnight. Prior to sectioning, the tissue was embedded in OCT cryomatrix (Thermo Scientific, Inc) for 1 h before freezing at -30° C. The tissue sample was sectioned in 12 to 14 μm thick slices using a Leica CM3050 cryostat and stained with haematoxylin (Harris Hematoxylin with Glacial Acetic Acid, Poly Scientific) to visualize the tissue. For each fish, 6 gill sections were examined using light microscopy. Photos were taken for 6 gill filaments chosen at random from each section using an Axiophot (Zeiss, Germany) microscope equipped with an Olympus DP70 digital camera and Image Pro software version 6.0 (Media Cybernetics, Bethesa, MD). The relative cross sectional area of the ILCM was determined using ImageJ software (http://rsbweb.nih.gov/ij/index.html).

Data and statistical analysis

Data are presented as means ± 1 SEM. The raw traces for water and blood gases as well as V_{AMP} and V_f were analyzed by measuring 4 - 5 s of the trace in the middle of every 10 s segment to obtain a single measurement. The baseline values were obtained by averaging the 4 - 5 s measurements over 5 min before the experiment commenced. All of the 4 – 5 s measurements were averaged for every 10 mm Hg (for P_wO_2) or 0.5 mm Hg (for P_wCO_2) segment to produce a

data point. . Unpaired Student's *t*-tests were used to identify significant differences in the baseline values of blood gases or ventilation parameters between temperature treatments; this approach was also used to examine blood EtOH levels for significant differences upon EtOH exposure. A Mann-Whitney Rank Sum test was used when the data failed the equality of variance test. One-way repeated measures (RM) analysis of variance (ANOVA), or one-way RM ANOVA on ranks (when the equality of variance test was failed), with Tukey post-hoc tests for multiple comparisons were used to detect statistically significant differences due to CA/saline injection. Two-way RM ANOVA with Tukey post-hoc multiple comparison tests were used to detect statistically significant differences in blood gases and ventilation parameters during hypoxia or hypercapnia exposures within and between acclimation temperatures. SigmaStat v 3.5 (SPSS Inc.) was used to perform the statistical analyses, and the fiducial limit of significance in all analyses was 0.05.

RESULTS

Baseline values in goldfish acclimated to 7 or 25° C

Table 3.1 summarizes the baseline measurements of arterial blood PO_2, PCO_2 and pH and ventilation (V_{AMP} and V_f) in goldfish at 7° or 25° C. Regardless of acclimation temperature and hence the extent of lamellar coverage by the ILCM, goldfish exhibited similar baseline values of PaO_2 (Student's *t*-test; P = 0.652) and pH (Student's *t*-test; P = 0.085). However, the goldfish acclimated to cold water (with the ILCM present) exhibited significantly higher $PaCO_2$ than warm acclimated goldfish (Student's *t*-test; P = 0.011). Injection of CA into fish acclimated to 7° C (Table 3.2) caused $PaCO_2$ to decline to a level that was even lower than that in fish acclimated to 25° C; injections of saline were without effect on $PaCO_2$ (Table 3.2). Ventilation amplitude (Student's *t*-test; P = 0.05) and frequency (Student's *t*-test; P < 0.001) were both higher in fish acclimated to 25° C than in those acclimated to 7° C.

Exposure of goldfish to acute hypoxia

Exposure of goldfish to acute, severe hypoxia (final PwO_2 = 30 mm Hg) elicited pronounced hyperventilatory responses in animals acclimated to either temperature (Figure 3.1). Figure 3.1 demonstrates that goldfish responded to hypoxia by increasing both V_f and V_{AMP} (albeit the apparent increase in V_{AMP} in the 7° C fish was not statistically significant). Although the increases in V_f and V_{AMP} (25° C fish only) were statistically significant only during the final stages of the graded hypoxia exposure, it was nevertheless apparent that fish were exhibiting ventilatory responses to relatively mild levels of hypoxia (Figure 3.1).

Predictably, with a decrease in PwO_2, there was a significant fall in the PaO_2 in goldfish at both acclimation temperatures (Figure 3.2A). Interestingly, PaO_2 in the goldfish at 7° C, and thus experiencing greater lamellar coverage, was significantly higher than that in the 25° C fish during graded hypoxia exposure and for part of the recovery period (Figure 3.2A). $PaCO_2$ fell significantly during graded hypoxia and then gradually increased toward baseline levels during the recovery period (Figure 3.2B). Although both acclimation groups exhibited these patterns of change in $PaCO_2$, $PaCO_2$ of goldfish acclimated to 7° C remained significantly higher than that of goldfish acclimated to 25° C throughout the hypoxic exposure period but not during recovery (Figure 3.2B). The lowering of $PaCO_2$ during hypoxia was associated with significant increases in blood pH in both acclimation groups (Figure 3.2C).

Morphological analysis (Figure 3.3) of the gills of goldfish acclimated to 7° C revealed that there was a significant reduction in the size of ILCM after hypoxia exposure compared to controls (no surgery or confinement) or sham-treated fish (surgery and confinement but no exposure to hypoxia).

Exposure of goldfish to acute hypercapnia

In this series of experiments, goldfish acclimated to 7° C (covered gills) and 25° C (uncovered gills) were gradually (over ~ 15 - 30 min) exposed to hypercapnia until the $PwCO_2$ reached approximately 8 mm Hg. In both groups of fish, hypercapnia was without effect on V_f or V_{AMP} (Figure 3.4). Similarly, hypercapnia did not affect PaO_2 in either group (Figure 3.5A). $PaCO_2$ rose with increasing $PwCO_2$ (Figure 3.5B) with the 25° C fish exhibiting the larger increase. Consequently, during hypercapnia, the difference between $PaCO_2$ and $PwCO_2$ ($PaCO_2$ - $PwCO_2$) increased from 2.50 ± 0.72 to 5.68 ± 1.39 mm Hg in the 25° C fish (N = 6) while actually

decreasing (from 5.69 ± 1.47 to 3.63 ± 2.05 mm Hg, N = 6) in the 7° C fish. Arterial pH decreased in both acclimation groups in keeping with the increases in $PaCO_2$ (Figure 3.5C).

Exposure of goldfish to 1% EtOH

There was a substantial increase in the EtOH concentration in the blood of goldfish exposed to 1% EtOH. Goldfish previously kept in hypoxia for one week and thus without a significant ILCM, exhibited an increase in blood EtOH concentration from 65 ± 10.4 $\mu g\ ml^{-1}$ at 0 h to 4178 ± 1205.5 $\mu g\ ml^{-1}$ at 1.5 h. Goldfish maintained under normoxic condition and thus possessing an ILCM showed an increase from 87.7 ± 13.1 $\mu g\ ml^{-1}$ to 4701.7 ± 431.9 $\mu g\ ml^{-1}$ at 1.5 h (Figure 3.6). The increase in goldfish previously kept in hypoxia (no ILCM) did not differ significantly from that of goldfish maintained under normoxic conditions and thus possessing an ILCM (Student's *t*-test, P = 0.693).

FIGURES AND TABLES

Figure 3.1. The effects of acute hypoxia (final water PO_2 (PwO_2) ~30 mmHg) exposure on A) ventilation frequency (V_f) and B) ventilation amplitude (V_{AMP}) in goldfish (*Carassius auratus*) acclimated to 7° C (unfilled symbols; N = 4 – 6) or 25° C (filled symbols; N = 4 – 6). The shaded area represents the period of exposure to hypoxia; note that the break in the x axis represents the point of deepest hypoxia after which PwO_2 increased as the recovery period commenced. Data were analyzed by two-way RM ANOVA with PwO_2 and acclimation temperature as factors; significant interactions between the two factors were detected only for V_f ($P < 0.001$ and $P = 0.853$, respectively). Thus, asterisks represent significant differences ($P < 0.05$) from pre-exposure (normoxia) values within each temperature group; horizontal lines represent significant differences ($P < 0.05$) between the two temperature groups at a given PwO_2.

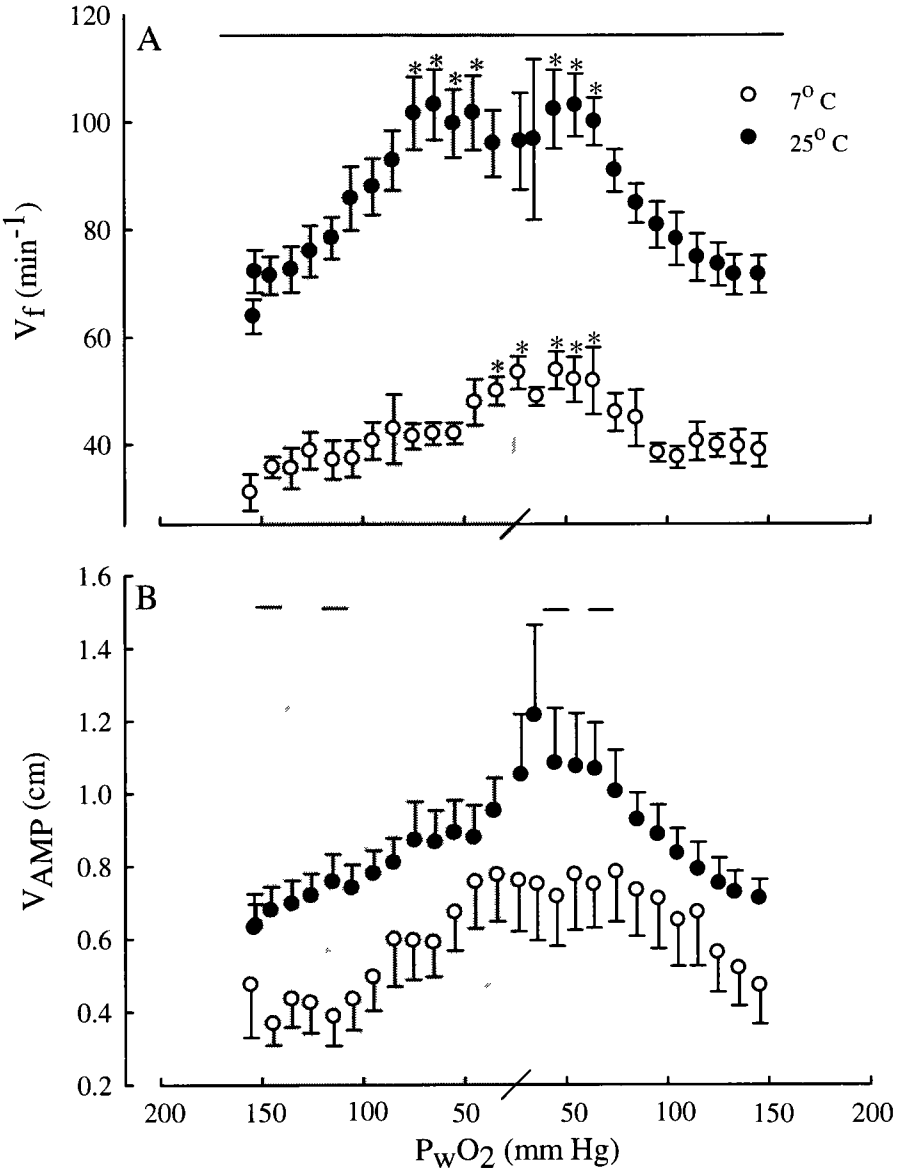
A
B
7° C
25° C
V_f (min^{-1})
V_{AMP} (cm)
P_wO_2 (mm Hg)
120
100
80
60
40
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
200
150
100
50
50
100
150
200

Figure 3.1

Figure 3.2. The effects of acute hypoxia (final water PO_2 (PwO_2) ~30 mmHg) exposure on arterial blood partial pressure of A) O_2 (PaO_2), B) CO_2 ($PaCO_2$) and C) arterial blood pH (pHa) in goldfish (*Carassius auratus*) acclimated to 7° C (unfilled symbols; N = 6) or 25° C (filled symbols; N = 6). The shaded area represents the period of exposure to hypoxia; note that the break in the x axis represents the point of deepest hypoxia after which PwO_2 increased as the recovery period commenced. Data were analyzed by two-way RM ANOVA with PwO_2 and acclimation temperature as factors; for all three variables, significant interactions between the two factors were detected ($P < 0.001$, < 0.001 and < 0.001 for PaO_2, $PaCO_2$ and pHa, respectively). Thus, asterisks represent significant differences ($P < 0.05$) from pre-exposure (normoxia) values within each temperature group; horizontal lines represent significant differences ($P < 0.05$) between the two temperature groups at a given PwO_2.

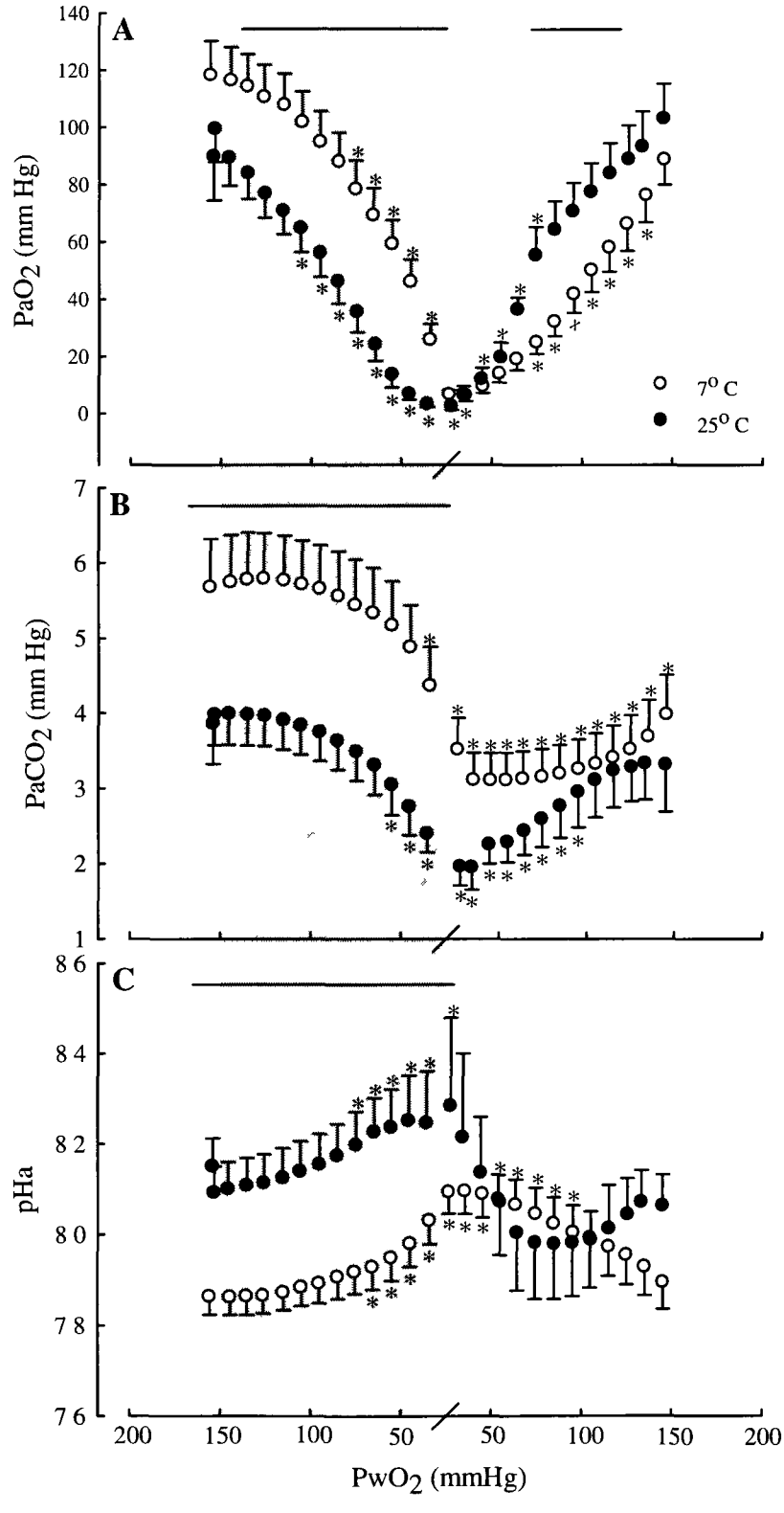
A
B
C
PaO2 (mm Hg)
PaCO2 (mm Hg)
pHa
PwO2 (mmHg)
7° C
25° C
140
120
100
80
60
40
20
0
7
6
5
4
3
2
1
8 6
8 4
8 2
8 0
7 8
7 6
200
150
100
50
50
100
150
200

Figure 3.2

Figure 3.3. The effects of acute hypoxia (final water PO_2 (PwO_2) ~30 mmHg; unfilled bar; N = 5) exposure on the relative interlamellar cell mass (ILCM) area in goldfish (*Carassius auratus*) in comparison to controls (filled black bar; N = 6) and fish which underwent a sham surgery (filled grey bar; N = 6); all fish were acclimated to 7°C. An asterisk indicates a significant difference from the control group (one-way ANOVA, $P < 0.001$).

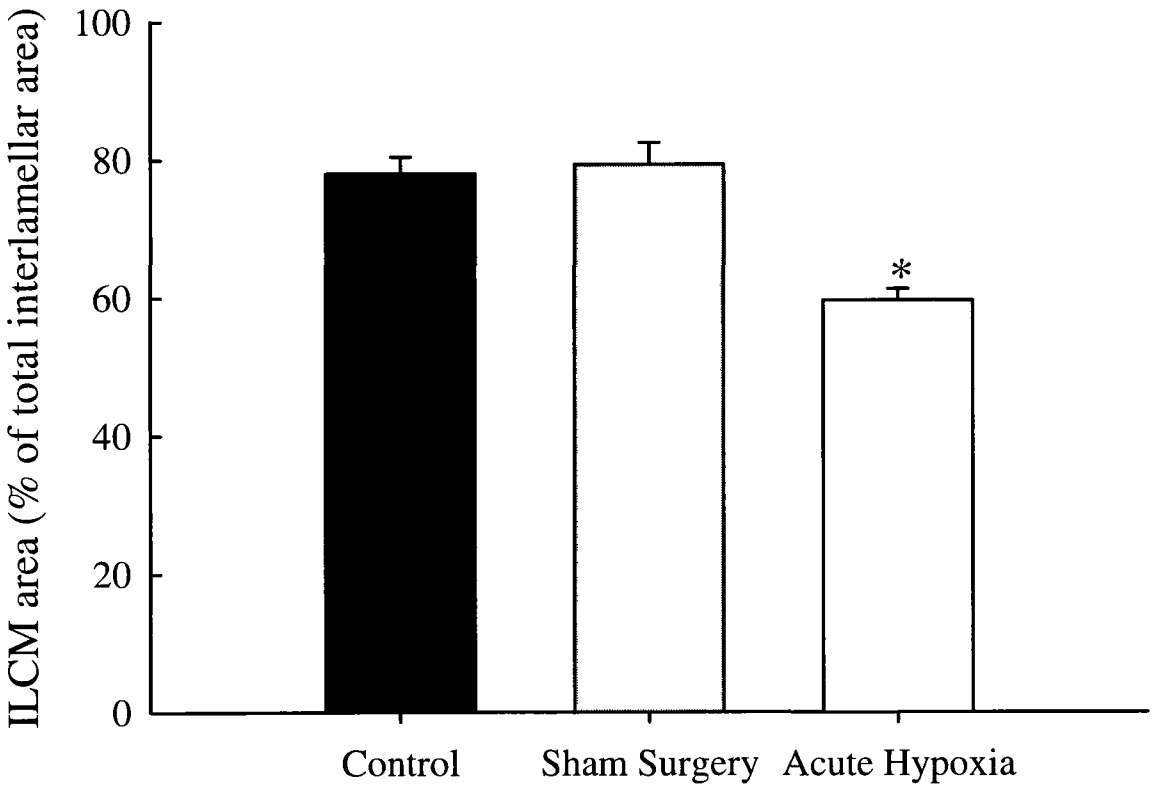
ILCM area (% of total interlamellar area)
100
80
60
40
20
0
*
Control
Sham Surgery
Acute Hypoxia

Figure 3.3

Figure 3.4. The effects of acute hypercapnia (final water PCO_2 ($PwCO_2$) ~8 mmHg) exposure on A) ventilation frequency (V_f) and B) ventilation amplitude (V_{AMP}) in goldfish (*Carassius auratus*) acclimated to 7° C (unfilled symbols; N = 4 – 6) or 25° C (filled symbols; N = 4 – 6). The shaded area represents the period of exposure to hypercapnia; note that the break in the x axis represents the point of most severe hypercapnia after which $PwCO_2$ decreased as the recovery period commenced. Data were analyzed by two-way RM ANOVA with PwO_2 and acclimation temperature as factor. There were no significant interactions detected between the two factors for V_f and V_{AMP} ($P = 0.186$ and $P = 0.625$, respectively). Horizontal lines represent significant differences ($P < 0.05$) between the two temperature groups at a given $PwCO_2$.

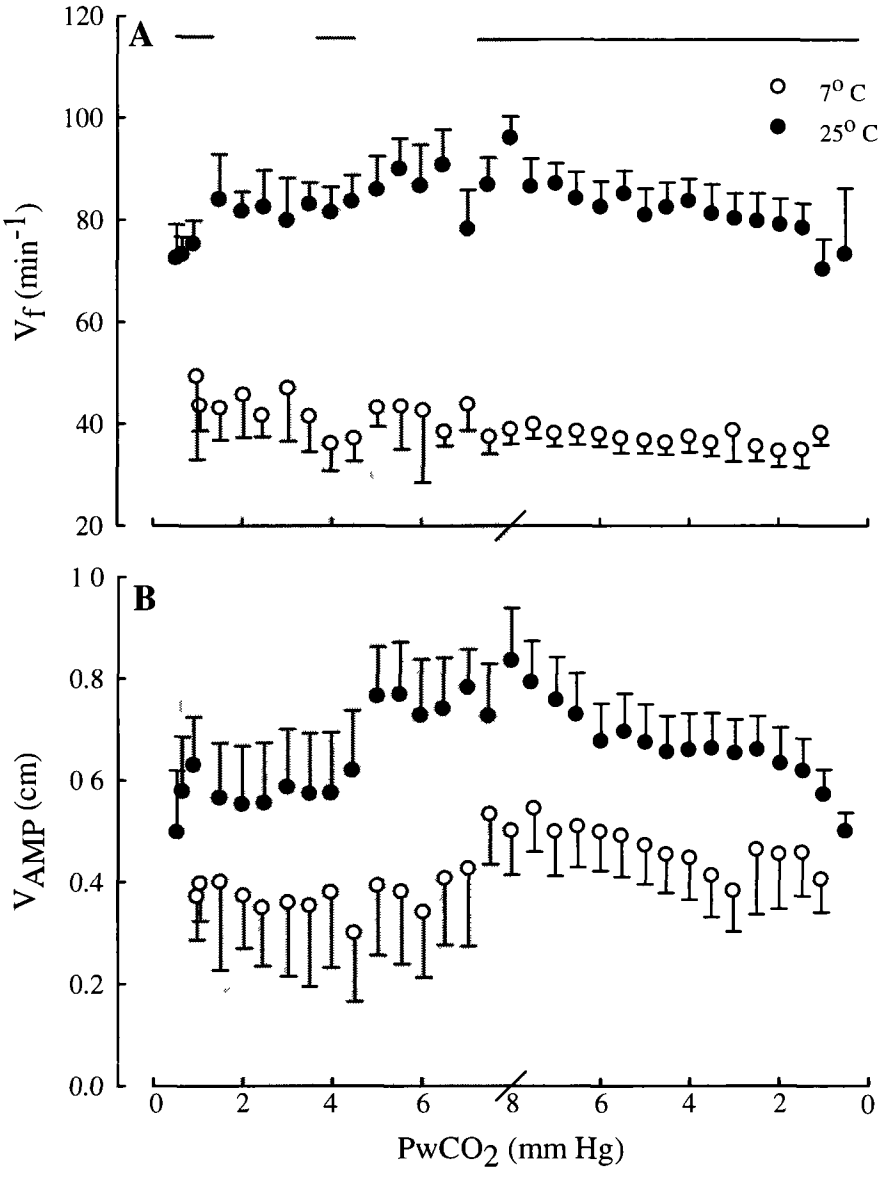
A
B
7° C
25° C
V_f (min^{-1})
V_{AMP} (cm)
PwCO$_2$ (mm Hg)
120
100
80
60
40
20
1.0
0.8
0.6
0.4
0.2
0.0
0
2
4
6
8
6
4
2
0

Figure 3.4

Figure 3.5. The effects of acute hypercapnia (final water PCO_2 ($PwCO_2$) ~8 mmHg) exposure on arterial blood partial pressure of A) O_2 (PaO_2), B) CO_2 ($PaCO_2$) and C) arterial blood pH (pHa) in goldfish (*Carassius auratus*) acclimated to 7° C (unfilled symbols; N = 4 – 6) or 25° C (filled symbols; N = 4 – 6). The shaded area represents the period of exposure to hypercapnia; note that the break in the x axis represents the point of most severe hypercapnia after which $PwCO_2$ decreased as the recovery period commenced. Data were analyzed by two-way RM ANOVA with $PwCO_2$ and acclimation temperature as factors. Significant interactions between the two factors were only detected for $PaCO_2$ ($P = 0.986$, $P < 0.001$ and $P = 0.590$ for PaO_2, $PaCO_2$ and pHa, respectively) Thus, asterisks represent significant differences ($P < 0.05$) from pre-exposure (normocapnia) values within each temperature group; horizontal lines represent significant differences ($P < 0.05$) between the two temperature groups at a given $PwCO_2$.

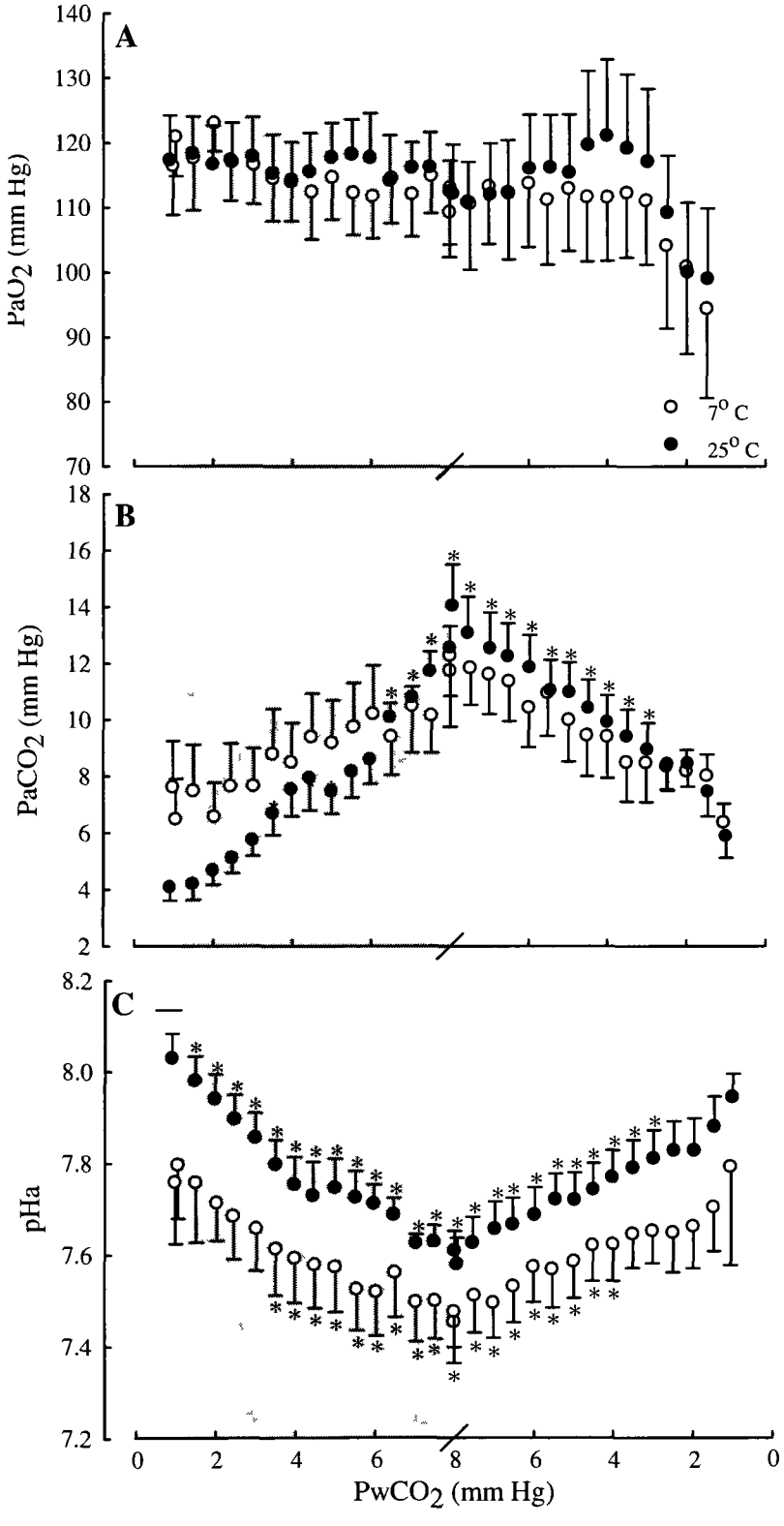
A
B
C
PaO2 (mm Hg)
PaCO2 (mm Hg)
pHa
PwCO2 (mm Hg)
7° C
25° C
140
130
120
110
100
90
80
70
18
16
14
12
10
8
6
4
2
8.2
8.0
7.8
7.6
7.4
7.2
0
2
4
6
8
6
4
2
0

Figure 3.5

Figure 3.6. The effects of 1% EtOH exposure on the blood EtOH concentrations of goldfish (*Carassius auratus*) at 7° C that had been exposed to hypoxia for one week to reduce the extent of the ILCM (filled bars; N = 5) or to normoxia for one week (covered gills; unfilled bars; N = 5). Blood EtOH concentrations were measured prior to (0 h) and after 1.5 h of EtOH exposure. An asterisk indicates a significant difference (paired Student's *t*-test; $P < 0.005$) from the pre-exposure value within a treatment group.

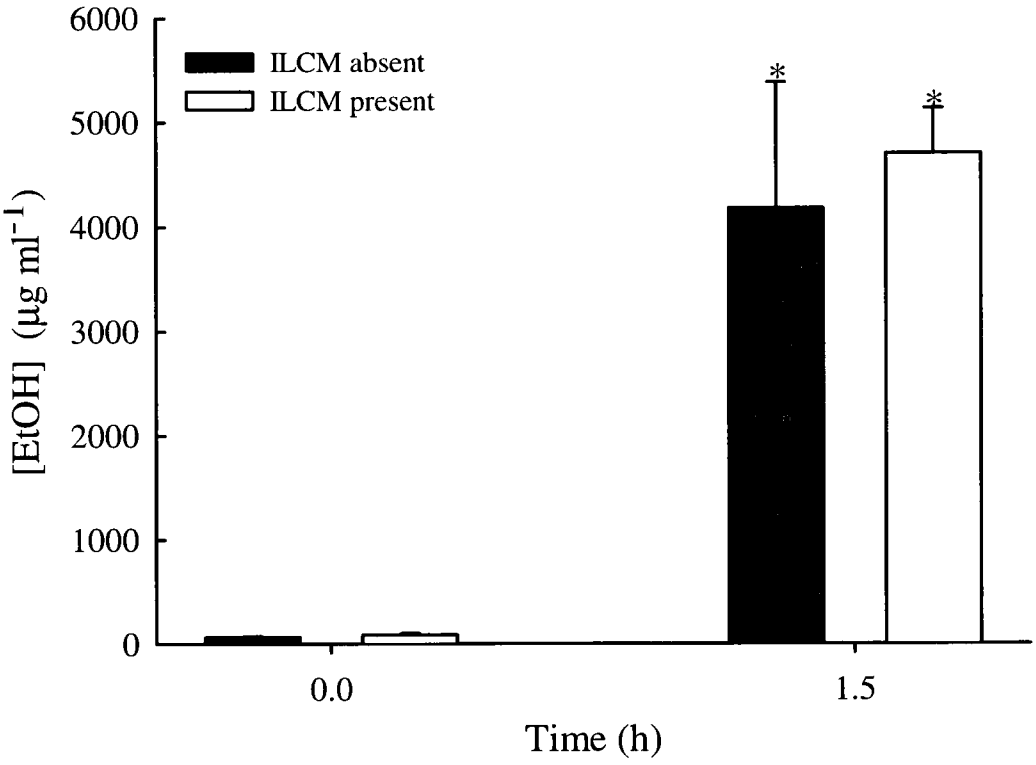
6000
5000
4000
3000
2000
1000
0
[EtOH] (μg ml-1)
ILCM absent
ILCM present
*
*
0.0
1.5
Time (h)

Figure 3.6

Table 3.1. Baseline values of arterial blood gases (PaO_2 and $PaCO_2$) and pH in goldfish (*Carassius auratus*) acclimated to 7 (ILCM present) or 25° C (no ILCM present). Data are presented as means ± 1 SEM; N numbers are indicated in parentheses. Significant differences are indicated by asterisks (Student's *t*-test; $P < 0.05$).

	Acclimation Temperature (° C)		***P* value**
	7	**25**	
PaO_2 (mm Hg)	87.2 ± 11.1 (7)	95.9 ± 12.0 (11)	0.652
$PaCO_2$ (mm Hg)	5.55 ± 0.54 (7)*	3.98 ± 0.18 (11)	0.011
pHa	7.91 ± 0.06 (7)	8.07 ± 0.04 (11)	0.085
V_{AMP} (cm)	0.4 ± 0.04 (6)	0.5 ± 0.04 (4)	0.05
V_f (min^{-1})	42.9 ± 2.9 (6)*	70.3 ± 2.8 (4)	<0.001

Table 3.2. The effects of carbonic anhydrase (CA) injections (2 mg kg^{-1}) on the arterial PO_2, PCO_2 and pH (PaO_2, $PaCO_2$ and pHa) of goldfish (*Carassius auratus*) acclimated to 7° C (ILCM present). Data are presented as means ± 1 SEM; N = 5. Significant differences are indicated by asterisks (one-way ANOVA; $P < 0.05$).

	Baseline	**Saline injection**	**CA injection**	***P* value**
PaO_2 (mm Hg)	122.4 ± 10.0	123 ± 13.0	124.2 ± 9.5	0.993
$PaCO_2$ (mm Hg)	4.66 ± 0.81	4.75 ± 0.49	3.07 ± 0.32*	0.019
pHa	8.00 ± 0.08	8.02 ± 0.08	8.19 ± 0.13	0.370

DISCUSSION

The goal of the present study was to assess the consequences for gas transfer of thermally induced gill remodelling in goldfish (which consists of the physical covering or uncovering of lamellae) and to specifically test the idea that additional diffusion limitations imposed by the lamellar covering would disproportionately impair CO_2 relative to O_2 transfer. Essentially, it was predicted that in keeping with theory, the presence of an ILCM in fish acclimated to colder water (7° C) would cause gas transfer efficiency to decrease. Moreover, the additional diffusion limitations would be predicted to have a greater impact on CO_2 transfer given that CO_2 transfer normally behaves as a diffusion-limited system in fish such as rainbow trout possessing thin water-to-blood diffusion distances and large lamellar surface areas. Strictly speaking, estimates of gas transfer efficiency require measurements of diffusing capacity (the rate of gas transfer for a given partial pressure gradient). Estimates of diffusing capacity necessitate simultaneous measurements of gas transfer, venous and arterial gas tensions, as well as inspired and expired gas tensions. Owing to the complexity of such measurements on goldfish, we relied on measurements of arterial gas tensions as proxies of diffusing capacity. To our knowledge, this is the first study to assess blood gases at two different temperatures in fish experiencing markedly different gill morphologies and only the second to provide blood gas data on goldfish.

An important underlying assumption of the current study was that the physical covering of the lamellae in fish acclimated to 7° C would persist after surgical procedures and subsequent confinement. For this reason, the effects of sham surgery and confinement were assessed. The results of this experiment (Figure 3.3) demonstrated no significant impact of these procedures on the extent of ILCM coverage. Thus, it seems likely that the current experimental design did indeed allow a comparison of fish exhibiting two markedly different gill phenotypes. However,

the imposition of severe hypoxia for ~30 min did cause a significant loss (of ~18%) of the ILCM, a response which complicates the interpretation of the blood gas data in hypoxic fish.

Baseline values in fish acclimated to 7 or 25° C

In the one other study that has reported blood gas data for goldfish (Burggren, 1982), fish were acclimated to 26° C and single blood samples were withdrawn from indwelling cannulae and analysed using conventional techniques. In the current study, blood gases were monitored continually using an extracorporeal blood circuit. Thus, differences in the measurement techniques may explain the markedly different blood gas and pH data obtained in the two studies. In particular, Burggren (1982) reported an average PaO_2 of 23.8 ± 4.5 mm Hg which is much lower than the mean value of 95.9 ± 12.0 mm Hg reported in Table 3.1 for goldfish acclimated to 25° C. The lower PaO_2 reported by Burggren (1982) is more in keeping with the very high O_2 affinity of goldfish haemoglobin [e.g. 2.6 mm Hg at 26° C (Burggren, 1982)] and similar to values reported for the related common carp (*Cyprinus carpio*) (Itazawa and Takeda, 1978). Although neither study measured absolute ventilation volumes, the lower breathing frequency (by approximately 15 breaths per min) in the fish studied by Burggren (1982) suggests reduced ventilation volumes in those fish. Thus, increased ventilation in the present study may, at least in part, explain the much higher PaO_2 values. The increased ventilation in the fish used in the present study likely reflected the presence of opercular impedance leads which were required to record opercular movements because it was recently shown that similar instrumentation of goldfish with opercular cannulae caused a marked increase in V_f when compared to uninstrumented fish (A. Watters and K.M. Gilmour; unpublished observations).

The current study demonstrated that goldfish with an ILCM have significantly higher $PaCO_2$ levels than goldfish lacking an ILCM (Table 3.1). These results support my original hypothesis which was that the presence of the ILCM would impede the transfer of CO_2 – a diffusion-limited gas (reviewed in Perry and Gilmour, 2002). PaO_2, on the other hand, appeared to be unaffected by the gill remodelling accompanying acclimation to different temperatures. This result suggests that the equilibration of O_2 between blood and water is unchanged despite the presence of the additional barrier imposed by the ILCM. The implication is that goldfish with covered gills are equally efficient at transferring O_2 as their conspecifics with uncovered gills, which is contrary to what I hypothesized. Because the average extent of ILCM coverage in fish acclimated to 7° C was 80% (relative to total interlamellar area), the most distal portions of the lamellae remained uncovered and exposed to the water. It is feasible that arterial blood oxygenation is sustained in the 7° C fish by these exposed distal lamellar regions.

In addition to causing gill remodelling, the acclimation of goldfish to different temperatures was obviously associated with large differences in V_f and V_{AMP}. Thus, the potential effects of ventilatory changes must be considered in addition to changing diffusion limitations on arterial blood gases. While the lower ventilation in fish acclimated to 7° C could potentially contribute to an increase in $PaCO_2$ (Iwama et al., 1987), it cannot explain the maintenance of PaO_2 that was observed in these fish because hypoventilation is expected to lead to a lowering of PaO_2. To differentiate possible ventilatory effects on $PaCO_2$ from those specifically related to gill remodelling, fish acclimated to 7° C were injected with CA so that CO_2 excretion could bypass the rate limiting step of HCO_3^- entry into the RBC, instead allowing HCO_3^- dehydration to CO_2 at the catalysed rate within the plasma. Injection of CA caused a significant drop in $PaCO_2$ in goldfish at 7° C with covered gills to levels that were similar to the

fish at 25° C with uncovered gills, suggesting that the additional diffusion limitations at 7° C were the underlying cause for elevated $PaCO_2$ in 7° C fish. A previous study demonstrated that trout experiencing a 30% reduction in total gill surface area also exhibited higher than normal $PaCO_2$ levels that were corrected by CA injection (Julio et al., 2000). The results of the current study are consistent with the view that CO_2 excretion in teleosts behaves as a diffusion-limited system owing to chemical equilibrium constraints, and thus any additional diffusion limitations result in an elevation of $PaCO_2$.

Exposure of fish to hypoxia

The negative consequences of decreasing diffusing capacity should be most readily observed during exposure of fish to hypoxia because of the falling PO_2 gradient across the gill. As shown for most teleosts studied (see Table 1 in Perry et al., 2009b), exposure of goldfish to acute hypoxia elicited the expected significant increase in V_f, although only goldfish with fully uncovered gills raised V_{AMP} significantly; proportionally, the increases in V_f were similar between the two groups of fish. As in other species, the hyperventilatory responses during hypoxia promoted respiratory alkalosis owing to a lowering of $PaCO_2$, a response which would favour O_2 uptake at the gill because of an increase in haemoglobin-O_2 binding affinity via the Bohr effect (Riggs, 1988; Jensen, 2004). In contrast to our predictions, the decline in PaO_2 was similar in both groups of fish and moreover, PaO_2 in the fish acclimated to 7° C was actually higher than that in the warm-acclimated fish for much of the hypoxic exposure (Figure 3.3). These results are difficult to reconcile, especially in light of a previous study on crucian carp which documented significantly lower critical PO_2 values in fish with an ILCM (Sollid et al., 2003). A possible explanation for the apparent lack of an effect of lamellar covering on the

oxygenation of arterial blood during hypoxia is that the exposure of fish to hypoxia, in itself, caused a significant retraction/removal of the ILCM during the brief period of exposure. Thus, diffusion limitations would have decreased in the 7° C fish as the extent of ILCM lamellar coverage fell from approximately 80% during normoxia to 60% during hypoxia (Figure 3.3). While not quantified, Sollid et al. (2003) also reported that the extent of lamellar coverage by the ILCM in crucian carp decreased during closed respirometry trials as the water became increasingly hypoxic. The results of the current study are in contrast to those of previous studies investigating the effects of O_2 transfer across fish gills exhibiting additional diffusion limitations. For example, trout with thickened gill epithelia caused by proliferation of chloride cells induced by exposure to soft water (Greco et al., 1995) or injections of cortisol/growth hormone (Bindon et al., 1994) had significantly lower PaO_2 levels than control fish when exposed to severe acute hypoxia. A possible reason for the differences among the studies is that unlike goldfish which can rapidly modify the extent of ILCM coverage (and hence blood-to-water diffusion distances) during hypoxia, trout are unable to acutely modify branchial diffusing capacity except through changes in lamellar blood flow. Another possible factor contributing to the higher than expected PaO_2 in fish acclimated to 7° C is a likely lower cardiac output (Stecyk and Farrell, 2006) which theoretically would increase the blood transit time in the lamellae, thereby increasing the time available for equilibration of gases between water and blood.

Exposure of fish to hypercapnia

The results of previous studies have demonstrated that acute exposure of fish to hypercapnia elicits a significant increase in ventilation (V_{AMP} and/or V_f) across a broad range of species including agnathans (Perry et al., 2009c), elasmobranchs (Randall et al., 1976) and teleosts (Janssen and Randall, 1975; reviewed by Gilmour 2001). The sensitivity of the ventilatory

response to elevated ambient PCO_2 is highly species specific. In the present study, goldfish showed no obvious hyperventilatory response to an external PCO_2 of 8 mm Hg which is consistent with the study of Dejours (1973b) who reported that goldfish exposed to $PwCO_2$ of ~24 mm Hg did not hyperventilate. Similarly, exposure of the closely related common carp (*Cyprinus carpio*) to a level of hypercapnia (7 mm Hg) comparable to that in the present study also failed to elicit hyperventilation (Soncini and Glass, 1999); ventilation was, however, increased at a $PwCO_2$ of 14 mm Hg. Given the apparent insensitivity of goldfish and carp to hypercapnia, it is perhaps surprising that another member of the Family Cyprinidae, the zebrafish (*Danio rerio*), exhibits robust hyperventilatory responses to very low levels ($PwCO_2 < 1$ mm Hg) of hypercapnia; (Vulesevic et al., 2006; Vulesevic and Perry, 2006).

The exposure of fish to hypercapnia was used as a tool to further probe the mechanism(s) underlying the disproportionate effects of gill remodelling on branchial O_2 and CO_2 transfer. During the initial phases of exposure to elevated ambient CO_2, a reversal of the PCO_2 gradient across the gill causes CO_2 to diffuse into the blood. However, unlike CO_2 excretion which relies on the rapid dehydration of plasma HCO_3^-, the step that gives rise to chemical equilibrium constraints, the inward movement of CO_2 across the gill during hypercapnia is driven strictly by diffusion. Therefore, the additional diffusion limitations imposed by gill remodelling would be less likely to affect inward CO_2 diffusion than CO_2 excretion. Indeed, the rate of increase of $PaCO_2$ during the initial phases of hypercapnia exposure was similar in fish with or without covered lamellae; the overall greater impact of hypercapnic exposure on $PaCO_2$ values in 25° C fish relative to 7° C fish simply reflected the lower baseline $PaCO_2$ values in the former. Thus, when considering gaseous diffusion alone, it would appear that both O_2 and CO_2 transfer are unaffected by the differences in lamellar coverage associated with thermally induced gill

remodelling. It should be emphasized that the hypercapnia experiments on fish acclimated to 7° C were performed on a separate group of fish that had not experienced prior hypoxia. Thus, the results cannot be explained by a loss of cell mass in the 7° C fish.

Exposure of fish to 1% EtOH

In this experiment, EtOH exposure served as a tool to further investigate the potential effects of the ILCM on the branchial transfer of gaseous molecules. EtOH was chosen for its high lipid solubility and its ready transfer across the gill by passive diffusion (Ryback et al., 1969). Thus, I assumed that the transfer of EtOH from water to blood mimics the transfer of O_2 and CO_2.

The increase in EtOH concentration in the blood after 1.5 h was consistent with the study of Ryback et al. (1969) which investigated blood EtOH concentrations in goldfish exposed to similar water EtOH concentrations. The results demonstrated that the average rates of EtOH uptake were similar in goldfish previously exposed to hypoxia (uncovered lamellae) and those kept in normoxic water (covered lamellae). This result is consistent with the findings from the hypoxia and hypercapnia experiments conducted on fish acclimated to different temperatures. Specifically, it would appear that the presence of an ILCM does not impose an additional barrier for the transfer of gases or EtOH across the gill. It should be mentioned that, although not quantified, visual assessment of gills from EtOH-exposed fish indicated that the ILCM remained intact in normoxic fish, an observation that excludes EtOH as a trigger for gill remodelling.

Conclusions and perspectives

Despite the marked covering of lamellar surfaces associated with gill remodelling in goldfish acclimated to 7° C, the overall impact on arterial blood gases and pH was relatively modest. The

apparent maintenance of gas transfer in these fish may reflect the sustained exposure of distal lamellar regions. Because gill remodelling only modestly impacts branchial gas transfer, it can be used as an effective strategy to minimize the diffusive movements of salt and water across the gill without major negative consequences for blood respiratory and acid-base status. Moreover, if exposed to hypoxic conditions, goldfish can rapidly reduce the size of the ILCM in the face of a diminished water-to-blood O_2 diffusion gradient.

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CHAPTER 4.

The effects of hypoxemia and hyperoxia on the gill morphology of goldfish (*Carassius auratus*)

This chapter is in preparation for publication in the American Journal of Physiology. Authors:

Velislava Tzaneva, Shawn Bailey and Steve Perry

ABSTRACT

The acclimation of crucian carp and goldfish to temperatures below 15° C causes gross morphological changes to the gill consisting of the physical covering of lamellae by a mass of cells termed the interlamellar cell mass (ILCM). Exposure of these cold acclimated fish to hypoxia or warmer water causes a loss of the ILCM and thus functional lamellar surface area is increased, presumably to enhance branchial O_2 uptake. These previous findings suggest that changes in O_2 demand may be a common signal for gill remodelling. Here I explore the cues underlying gill remodelling and specifically test the hypotheses that i) depletion of internal O_2 stores in the absence of any change in external O_2 status, can trigger the removal of the ILCM in goldfish acclimated to 7° C, and ii) exposing fish acclimated to 25° C to an abundance of O_2 (hyperoxia) can reverse the gill remodelling (i.e. cause the covering of lamellae by an expansion of the ILCM). Hypoxemia was induced by exposing the fish to carbon monoxide (5% CO) or phenylhydrazine while holding the external environment normoxic. Goldfish were also exposed to hyperoxia (>500 mm Hg) at 7 or 25° C and during a temperature change from 7 to 25° C. Blood samples and gill tissue were collected for total O_2 content, haemoglobin concentration, haematocrit, arterial PO_2 and ILCM analysis. Both CO and PHZ treatments caused a decrease in the size of the ILCM from approximately 80% of total interlamellar area to 35 and 23%, respectively. Exposure of 25° C fish to hyperoxia caused a marked increase in the size of the ILCM (to 67% of total interlamellar area) such that the gill morphology of these fish was indistinguishable from fish acclimated to 7° C. The gradual (10 day) transfer of fish from 7 to 25° C was associated with a smaller decrease in the size of the ILCM when performed under hyperoxic conditions. Thus, the two major conclusions of this study are i) that gill remodelling

can occur in the absence of any external cues during periods of internal hypoxemia, and ii) while not excluding a possible role of temperature sensing in the gill remodelling response, it would appear that O_2 demand may be a significant driving force shaping gill remodelling in goldfish.

INTRODUCTION

A variety of external and/or internal cues are known to alter the morphology of the gill epithelium in fish inhabiting freshwater. For example, exposing rainbow trout (*Oncorhynchus mykiss*) to soft (low [Ca^{2+}]) water, [23]) or ion-poor water (Perry and Laurent, 1989) induces the proliferation of mitochondrion rich cells (MRC) or ionocytes (also referred to as chloride cells; (Perry, 1997) which may result in a thickening of the blood-to-water diffusion distance (Greco et al., 1996; reviewed by Perry, 1998). Similar changes in gill morphology can be elicited by treatment of fish with hormones such as cortisol (Laurent and Perry, 1990) or growth hormone (Bindon et al., 1994) without any associated change in the external environment. Another example of gill remodelling occurs in at least two cyprinid species, crucian carp (*Carassius carassius*) and goldfish (*Carassius auratus*) which exhibit pronounced structural modification of the gill in response to changes in ambient temperature or O_2 levels (Nilsson 2007; Sollid et al., 2003; Sollid and Nilsson 2006; Sollid et al., 2005). For example, at temperatures above 15° C the goldfish gill contains protruding lamellae but upon acclimation to colder water (e.g. 7 - 8° C) the lamellae become largely embedded as they are covered by a mass of cells that has been termed the interlamellar cell mass (ILCM) (31). It is believed that the covering of the lamellae in fish acclimated to colder water serves to reduce the energetic costs of osmoregulation because passive salt and water fluxes across the gill can be minimized. However, when crucian carp (Sollid et al., 2003) or goldfish (Mitrovic et al., 2009; Mitrovic and Perry, 2009) that have been acclimated to cold water are exposed to hypoxia, the ILCM is removed thus exposing the previously embedded lamellae and increasing the functional surface area for gas (and ion

exchange). An increase in temperature also acts as a cue for the loss of the ILCM, which may be related to the higher demand for O_2 as the metabolic rate increases (Fry and Heart, 1948).

The specific cues for gill remodelling in crucian carp or goldfish have not been investigated. However, the fact that increasing temperature and hypoxia both lead to a loss of the ILCM and the uncovering of previously covered lamellae, suggests that increasing O_2 demand may be a common signal for gill remodelling. By analogy to the hypoxic hyperventilatory response (Perry et al., 2009), the removal of the ILCM associated with exposure of cold acclimated fish to hypoxia could be triggered by the stimulation of external O_2 chemoreceptors (Burleson et al., 2006; Jonz et al., 2004) or by activation of internally oriented receptors responding directly or indirectly to changing O_2 levels (for reviews see Burleson et al., 1992; Gilmour and Perry, 2007). Here I explore the cues underlying gill remodelling and specifically test the hypothesis that depletion of internal O_2 stores in the absence of any change in external O_2 status, can trigger the removal of the ILCM in goldfish acclimated to 7° C. To evoke hypoxemia under continuing conditions of external normoxia, fish were either treated with phenylhydrazine to induce anaemia (Houston et al., 1988) or carbon monoxide (CO) to prevent the binding of O_2 to haemoglobin (Holeton, 1971). In an attempt to dissociate the effects of temperature, per se, on gill remodelling from the associated changes in O_2 demand, fish were exposed to hyperoxic conditions. Specifically, I tested the hypothesis that exposing fish to hyperoxia would lead to the formation of an ILCM in warm acclimated fish and prevent/reduce the uncovering of gill lamellae that normally accompanies the gradual acclimation of goldfish from 7 to 25° C.

MATERIALS AND METHODS

Experimental animals and surgical procedures

Goldfish were purchased from a commercial supplier (Aleong's International, Inc, Mississauga, ON) and were held in circular tanks supplied with aerated dechloraminated City of Ottawa water at 18° C. One group of goldfish was acclimated to 7° C and another group was acclimated to 25° C by dropping or raising the water temperature by approximately 2° C per day. The fish were held at their respective temperatures on a 12/12 h light-dark photoperiod and were fed commercial pellets for at least two weeks prior to experimentation.

Large goldfish (average mass = 168 g; N = 8) acclimated to 7° C were fitted with caudal artery cannulae. The cannulation procedure is explained in detail in Chapter 2. Briefly, an approximately 2 cm incision was made in the caudal peduncle region and a PE50 cannula with a 2 cm PE10 extension was inserted into the caudal artery. The incision was closed with silk sutures and the fish were transferred to transparent tanks (2.75 l) provided with well aerated flow through water to recover for 24 h before experiments commenced.

Experimental protocol

1. Carbon monoxide (CO) exposure

Small goldfish acclimated to 7° C (average mass = 21.7 g; N= 24) were placed in 9 l transparent tanks receiving aerated flowing water. After 24 h, a commercial mixture of 5% CO in air (Linde Canada, Inc, Montreal, QC) was bubbled into the tanks. Water PO_2 remained at normoxic levels throughout the experimental period. Fish were euthanized by an overdose of benzocaine (1 g l^{-1}; Sigma-Aldrich, Inc) and gill tissue was collected for ILCM analysis at 1, 2, 3

and 7 days after beginning CO exposure. Large cannulated goldfish were kept in transparent tanks (2.75 l) for 24 h recovery prior to CO exposure. Those fish were exposed to CO for 24 h after which blood samples were taken to measure the arterial partial pressure of oxygen (PaO_2), the total O_2 content of the arterial blood (CaO_2) and haematocrit. After removing 0.5 -1 ml of blood, PaO_2 was measured by injecting a 0.2 – 0.3 ml sample of whole blood into the sample chamber of an O_2 microelectrode (Microelectrodes, Inc, NH, USA) attached to a blood gas meter (Cameron Instruments, Model BGM 200). The O_2 microelectrode was calibrated prior to each use using a two point calibration method. Zero PO_2 solution (0.02 g ml^{-1} sodium sulphite) was used to calibrate the low end calibration point and air saturated water was used to set the high point calibration. The gas meter was connected to a data integration system (BioPac) and the data were recorded by AcKnowledge software. CaO_2 was measured using a blood O_2 content analyzer (Oxycon, Cameron Instruments, Inc.). The instrument was calibrated by injecting a 20 μl of air into the sample chamber. A 50 μl blood sample was injected into the sample chamber containing a solution of 0.3 g saponin and 0.6 g of potassium ferricyanide ($K_3Fe(CN)_6$) per 100 ml of distilled water. Total haemoglobin content of the blood was determined using a microplate spectrophotometric assay. Briefly, haemoglobin standards ranging between 0 and 12 g dl^{-1} were prepared to construct a standard curve. A 20 µl sample of either haemoglobin standard or whole blood was added to 5 ml of Drabkin's solution (Sigma-Aldrich, Inc). After at least 15 min of incubation a 200 µl aliquot of the mix was added to microplate wells in triplicate and the samples were read at 540 nm wavelength using a SpectraMax 340PC and SOFTMax PRO software to record the data (Molecular Devices, Inc.).

2. *Phenylhydrazine (PHZ) exposure*

Small goldfish acclimated to 7° C (average mass = 32.1 g; N = 6) were placed in 9 l transparent tanks supplied with well aerated flowing water for 24 h prior to PHZ exposure. After the 24 h acclimation period, the water flow was stopped and the fish were exposed to PHZ (4 mg l^{-1}) for 48 h. Air was bubbled into the tanks to keep the water normoxic and the tank was kept in a 7° C water bath to maintain constant temperature during the period of exposure. After 48 h of exposure the water flow was returned to the tank and the fish were allowed to recover for 7 days. Blood samples (< 0.1 ml) were obtained by caudal puncture using a heparinised 0.5 ml syringe and a 23 guage needle after 7 days recovery to measure haemoglobin concentration ([Hb]) and haematocrit. Control fish (average mass = 28.5 g; N = 6) for both the CO and PHZ exposure fish were kept in 9 l tanks receiving aerated flowing water for 2 weeks after which the gill tissue and blood samples were collected for ILCM analysis and haematocrit and haemoglobin measurements.

3. Hyperoxia exposure

Six groups of small goldfish (average mass = 33 g; N = 36) were used for this experiment. Each group was held in a 9 l transparent tank supplied with aerated flow through water. Three groups of goldfish were exposed to hyperoxia (PwO_2 > 500 mm Hg). Two of the groups remained at their respective acclimation temperature of 7 or 25° C for the duration of hyperoxia exposure (2 weeks) while the temperature of the third group was increased from 7 to 25° C at a rate of approximately 2° C per day; fish remained at 25° C for a further 10 days. Control fish were either held at 7 or at 25° C for two weeks under normoxic conditions or subjected to a temperature change from 7 to 25° C under normoxic conditions. Hyperoxic conditions were achieved by bubbling the water flowing through a water-air equilibration column with O_2 using a mass flow controller (Mass Flow Smart-Trak, Sierra Instruments, Inc., USA) at a rate of 500 –

1000 ml min^{-1} until a PwO_2 > 500 mm Hg was reached. At the end of the exposure, gill tissue was collected and the area of the ILCM was measured.

ILCM analysis

The first right gill arch of each fish was removed and fixed in 4 % paraformaldehyde (PFA) until it was ready for sectioning. 24 h before sectioning, the gills were placed in 30% sucrose (w/v) solution to dehydrate and cryoprotect the tissue. Prior to sectioning they were placed in OCT Cryomatrix (Thermo Scientific, Inc.) for at least 1 h before being frozen at -30° C. The tissue sample was then sliced in 12 to 14 μm thick sections using a Leica CM3050 cryostat and stained with hematoxylin (Harris Hematoxylin with Glacial Acetic Acid, Poly Scientific) to visualize the tissue. Using light microscopy photos of 6 gill filaments were taken randomly per section (36 photos per fish). An Axiophot (Zeiss, Germany) microscope with an Olympus DP70 digital camera were used to obtain the images and the relative cross sectional area of the ILCM was determined using Image Pro version 6.0 (Media Cybernetics, Bethesda, MD) and Image-J software (http://rsbweb.nih.gov/ij/index.html).

Statistical analysis

Data are represented as means ± 1 S.E.M. Unpaired two-tailed Student's t-test was used to detect significant differences between goldfish acclimated to 7° C (ILCM present) and 25° C ILCM absent). Mann – Whitney U test was used to detect differences between means when groups did not exhibit equality of variance.

RESULTS

The effects of CO and PHZ exposure on the size of the ILCM

There was a marked decrease in the mass of the ILCM in fish exposed to 5% CO during the first 48 h of exposure after which there were no further decreases (Figure 4.1). In control fish, the ILCM occupied 78.1 ± 2.4% of the total interlamellar area; after 24 and 48 h, the ILCM had decreased to occupy 56.8 ± 5.9 and 35.4 ± 4.3% of the interlamellar area, respectively (Figure 4.1).

To ensure that the fish were indeed being rendered hypoxemic by the CO treatment, a separate group of cannulated goldfish was exposed to CO for 24 h after which arterial blood samples were taken for analysis of CaO_2, PaO_2 and haematocrit; the data are summarized in Table 4.1. While there was a pronounced decrease in the total O_2 levels of the blood after 24 h CO exposure, PaO_2 and haematocrit remained unchanged (Table 4.1).

A separate group of small goldfish was exposed to PHZ for 48 h and allowed to recover for 7 days. In these fish, the ILCM was reduced to a mere 23.1 ± 6.9% of total interlamellar area. [Hb] fell significantly from 8.9 ± 1.3 mg dl^{-1} in control fish to 1.6 ± 0.2 mg dl^{-1} in PHZ treated fish (Figure 4.2B) and haematocrit decreased from 25.0 ± 2.7 to 0.4 ± 0.3% (Figure 4.2C). These results confirm that the goldfish exposed to PHZ were indeed anaemic.

Figure 4.3 shows representative micrographs depicting the morphology of the gill in control goldfish (Figure 4.3A) and goldfish exposed to either PHZ (Figure 4.3B) or CO (Figure 4.3C and D). The images illustrate a noticeable decrease in the size of the ILCM after either CO or PHZ exposure. A single representative image of a goldfish gill is shown after 48 h of CO exposure only (Figure 4.3D) because the ILCM did not decrease further after that time.

Feeding intake (not quantified in this study) was decreased in goldfish with lowered O_2 content from both CO and PHZ treatments. The overall level of activity of the fish appeared to decrease after/during CO and PHZ exposures. While the ventilation was not quantified, it did appear that there was a slight hyperventilation in CO treated fish, at least initially; there was no observable change in ventilation in the PHZ treated fish.

The effects of hyperoxia exposure on the size of the ILCM

The exposure to hyperoxia of fish acclimated to 25° C caused a significant increase in the size of the ILCM from 36.0 ± 2.7 to 67.7 ± 2.8% of the total interlamellar area (Figure 4.4). The remodelling caused by hyperoxia was so extensive that there was no longer any difference in the average size of the ILCM between the fish at 7 and 25° C. The size of the ILCM in goldfish acclimated to 7° C was not significantly altered by exposure to hyperoxia (Figure 4.4). Goldfish experiencing a temperature increase from 7 to 25° C exhibited a decrease in the size of the ILCM from approximately 80% (assumed based on average values from normoxic fish acclimated to 7° C) to 33.4% of the total interlamellar area (Figure 4.4). Imposing an identical temperature change under hyperoxic conditions resulted in a final ILCM size of 45.9 ± 5.3% of the interlamellar area which was significantly greater than in the normoxic fish (i.e. the extent of gill remodelling with increasing temperature was decreased by hyperoxia treatment; Figure 4.4). Representative examples of gill morphology of goldfish exposed to different O_2 and temperature treatments are presented in Figure 4.5.

FIGURES AND TABLES

Figure 4.1. The effects of 5% carbon monoxide (CO) exposure (unfilled bars; N = 6 at each time point) on the interlamellar cell mass (ILCM) in goldfish (*Carassius auratus*) acclimated to 7° C; control fish (filled bar; N = 6) were maintained in air-saturated water. Data points not sharing identical letters are significantly different from each other ($P < 0.05$). Data are shown as means ± 1 SEM.

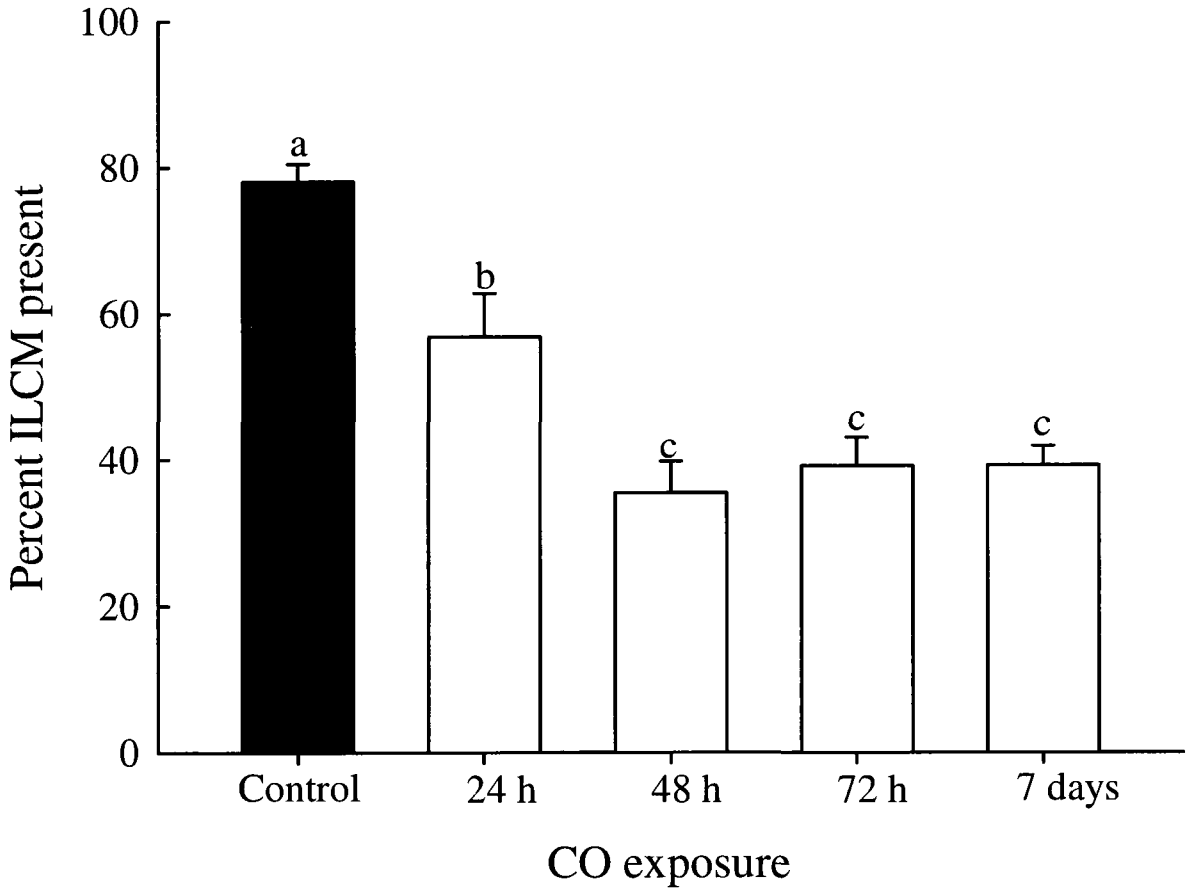
100
80
60
40
20
0
Percent ILCM present
a
b
c
c
c
Control
24 h
48 h
72 h
7 days
CO exposure

Figure 4.1

Figure 4.2. The effects of phenylhydrazine (PHZ; 4 mg l^{-1}) exposure (48 h) and 7 day recovery period (unfilled bar; N = 5) on (A) the interlamellar cell mass (ILCM), (B) haemoglobin concentration ([Hb]) and (C) haematocrit in goldfish (*Carassius auratus*) acclimated to 7° C; control fish (filled bar; N = 6) were maintained for 9 days in air-saturated water. Significant differences between control and PHZ exposed fish are indicated by asterisks ($P < 0.05$). Data are shown as means ± 1 SEM.

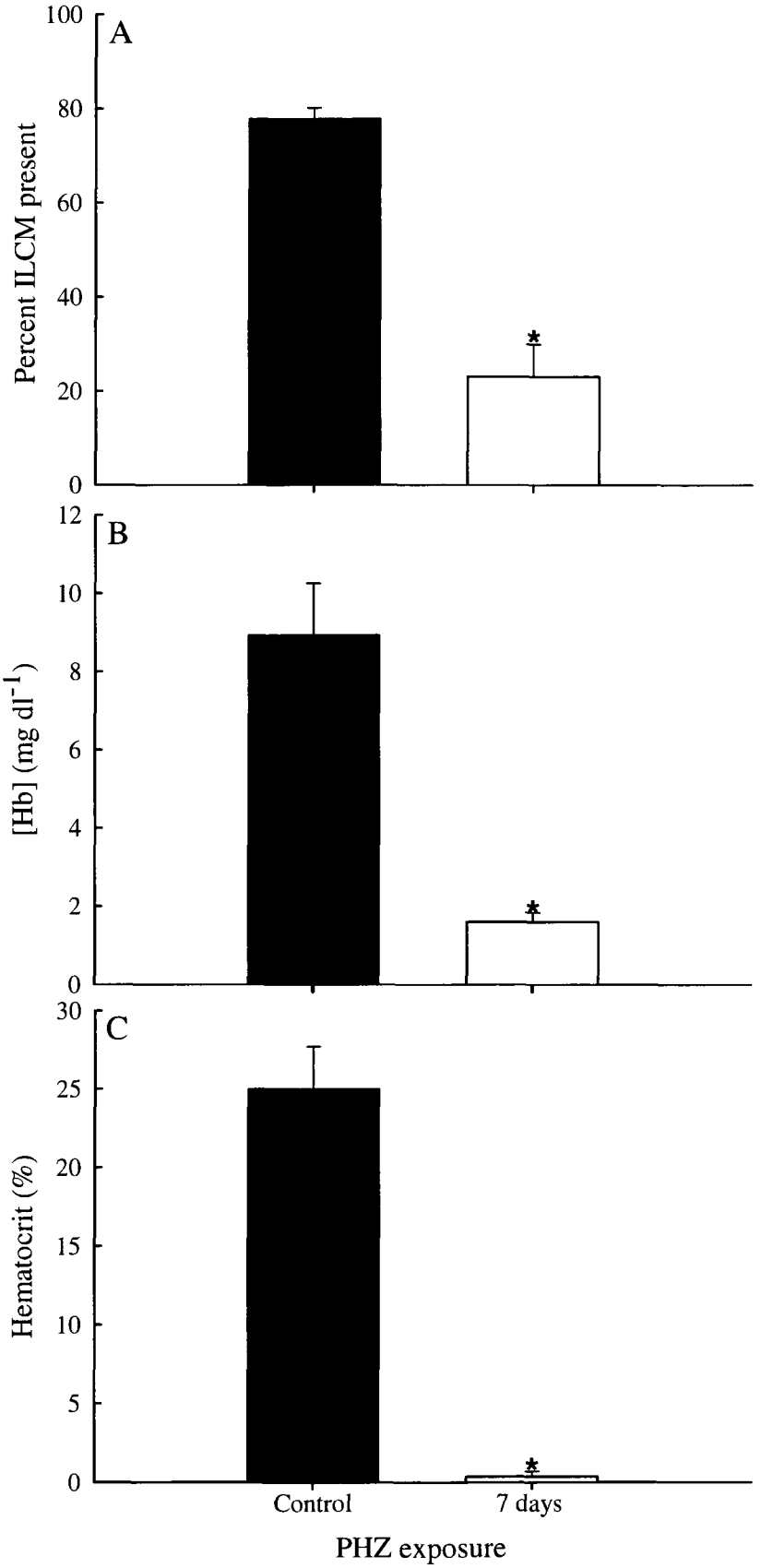
A
Percent ILCM present
100
80
60
40
20
0
*
B
[Hb] (mg dl^{-1})
12
10
8
6
4
2
0
*
C
Hematocrit (%)
30
25
20
15
10
5
0
*
Control
7 days
PHZ exposure

Figure 4.2

Figure 4.3. Representative photomicrographs illustrating the gill morphology of goldfish (*Carassius auratus*) acclimated to 7° C under control conditions (A) and following phenylhydrazine (PHZ) treatment (B) or after 24 or 48 h of carbon monoxide (CO) exposure (C and D, respectively) . The scale bars represent 50 μm. ILCM: interlamellar cell mass; L: lamella.

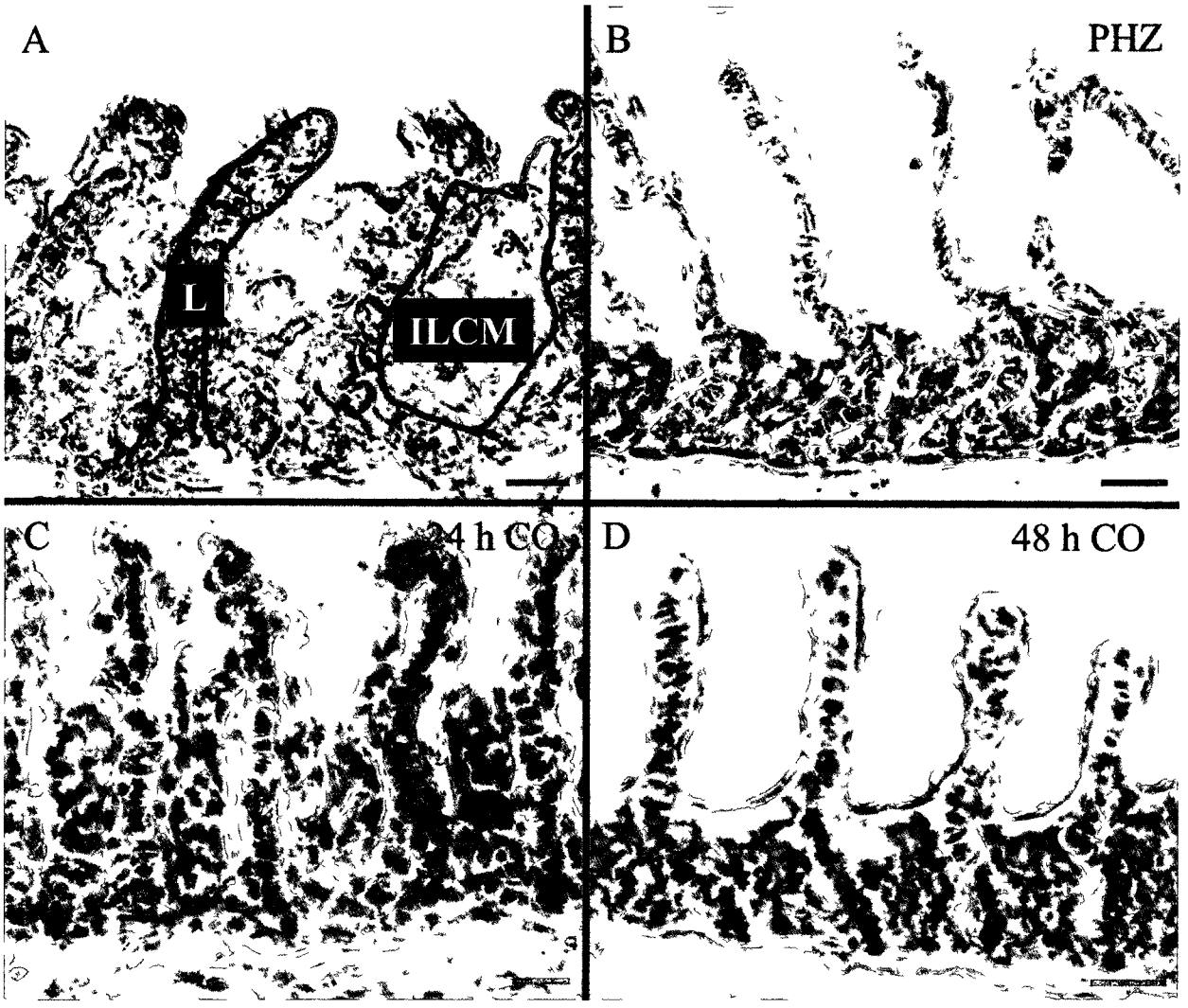

Figure 4.3

Figure4.4. The effect of hyperoxia exposure (unfilled bars) on the size of the ILCM in goldfish (*Carassius auratus*) acclimated to 25 or 7° C and in fish undergoing a temperature increase from 7 to 25° C under normoxic (filled bars) or hyperoxic conditions. Asterisks denote significant differences between hyperoxia and control groups within a temperature treatment ($P < 0.05$). N = 6 for each group. Data are shown as means ± 1 SEM.

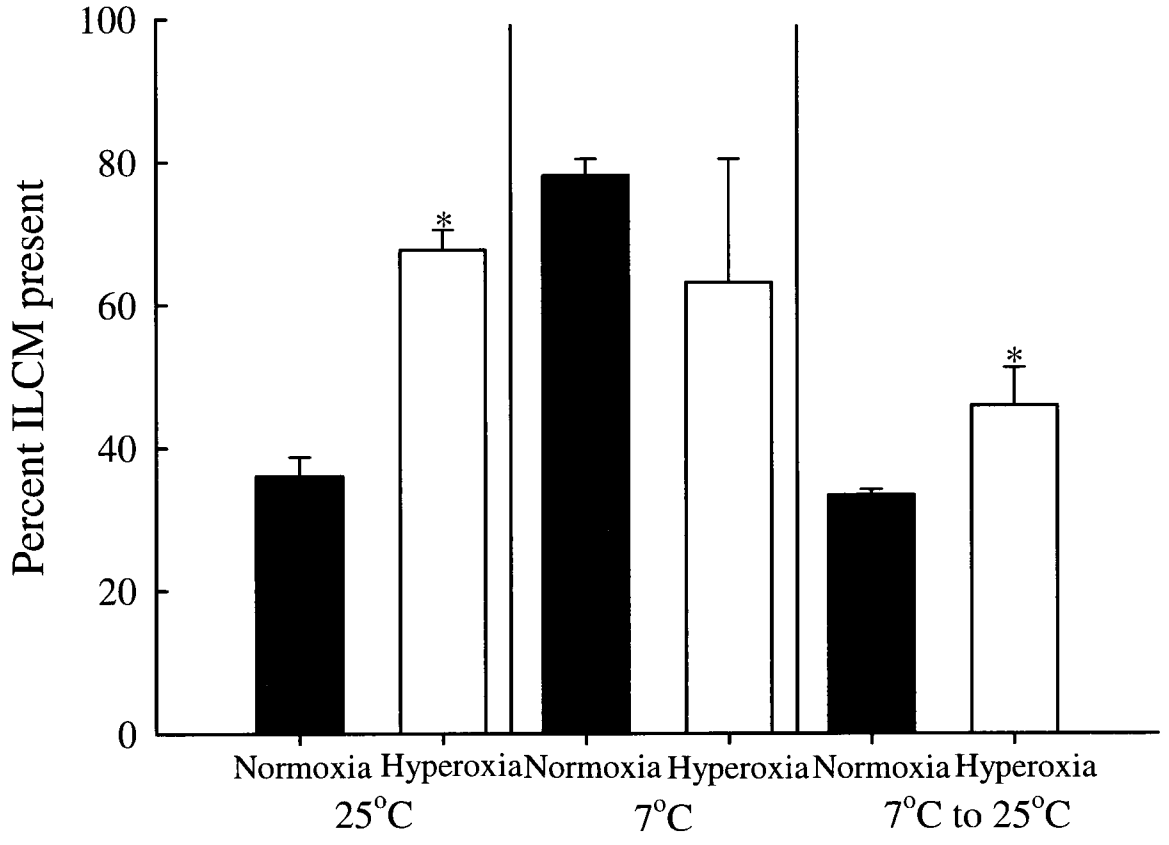
100
80
60
40
20
0
Percent ILCM present
*
*
Normoxia
Hyperoxia
Normoxia
Hyperoxia
Normoxia
Hyperoxia
25°C
7°C
7°C to 25°C

Figure 4.4

Figure 4.5. Representative photomicrographs illustrating the gill morphology of goldfish (*Carassius auratus*) exposed to hyperoxia acclimated to (B) 25° C or (D) 7° C or (F) undergoing a temperature increase from 7 to 25° C and in normoxic control fish acclimated to (A) 25° C or (C) 7° C or (E) undergoing a temperature increase from 7 to 25° C. Scale bars represent 50 μm; ILCM: interlamellar cell mass; L: lamella.

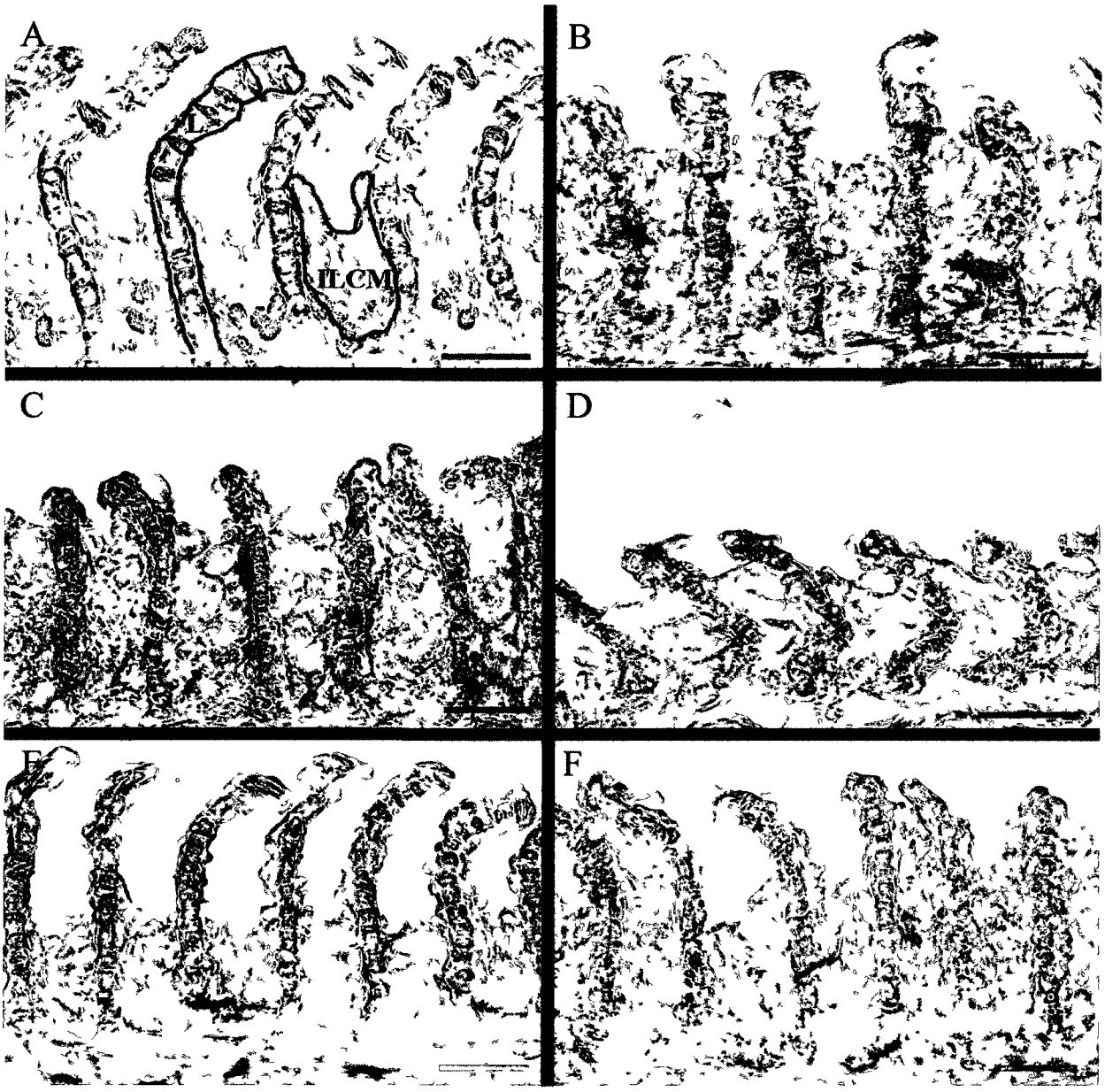

Figure 4 5

Table 4.1. The total O_2 content of arterial blood (CaO_2), arterial PO_2 (PaO_2) and haematocrit of goldfish acclimated to 7° C before and after 24 h exposure to 5% carbon monoxide (CO). Data are shown as means ± 1 S.E.M; N numbers are represented in parenthesis. Significant differences ($P < 0.05$) are denoted with asterisks.

	CO exposure	
	Before	After 24 h
CaO_2 (mmol l^{-1})	1.95 ± 0.39 (7)	0.04 ± 0.05 (6)*
PaO_2 (mm Hg)	125 ± 9.9 (8)	115 ± 5.7 (8)
Haematocrit (%)	18.2 ± 2.2 (7)	18.9 ± 1.7 (8)

DISCUSSION

To my knowledge this is the first study to specifically investigate whether a depletion of internal O_2 stores can act as a cue for gill remodelling in goldfish. The results clearly show that a decrease in arterial blood O_2 content induced either by CO exposure or anaemia is met with gill remodelling consisting of a marked reduction in the ILCM, a response which is presumably aimed at increasing the functional lamellar surface area available for O_2 transfer. Thus, gill remodelling can occur in the absence of any external cues. To assess whether gill remodelling (disappearance of the ILCM) associated with increasing acclimation temperature might be related to increasing O_2 demand as opposed to the temperature change, *per se*, fish were exposed to hyperoxia. In keeping with the idea that gill remodelling is specifically linked to O_2 demand, the exposure to hyperoxia of goldfish acclimated to 25° C caused a growth of the ILCM and increasing lamellar coverage such that the gill morphology of these fish was indistinguishable from fish acclimated to 7° C. Moreover, goldfish exposed to environmental hyperoxia and subjected to an increase in temperature from 7 to 25° C exhibited a smaller reduction of the ILCM in comparison to fish subjected to the same acclimation regimen but kept under normoxic conditions.

Hypoxemia as a cue for gill remodelling

Previous studies have shown that a temperature increase can act as a cue for the loss of the ILCM in goldfish possibly because of an increasing O_2 demand as metabolic rate rises (Sollid et al., 2005). Environmental hypoxia can also induce a decrease in the ILCM in crucian carp (Sollid et al., 2003) or goldfish (Mitrovic et al., 2009) acclimated to cold water. These cues for

remodelling, although external in origin, will also modify the internal environment of the fish by increasing the demand for O_2 (increasing temperature) or lowering blood O_2 content (hypoxia). Thus, from such studies it can be difficult to specifically identify the proximate stimulus (or stimuli) underlying the changes in gill morphology during environmental changes in temperature or O_2 levels. In this study I successfully manipulated the internal environment of the fish while keeping the external environment constant. By exposing the goldfish to PHZ or CO, it was possible to elicit profound hypoxemia without any changes in the O_2 levels of the external environment (Table 4.1 and Figure 4.2B and C). Because hypoxemia coincided with a decrease in ILCM with both treatments, it suggests that even though the fish are sensing normoxic levels of O_2 in the external environment, the lowering of blood O_2 carrying capacity can specifically act as a cue for gill remodelling. The specific mechanisms linking a decrease in arterial blood O_2 content to gill remodelling are unclear and were not specifically investigated in the current study. However, one factor which can be excluded as a contributing variable is PaO_2 because it was unchanged during CO exposure (Table 4.1). Although PaO_2 was not measured in the anaemic fish in the present study, previous studies on rainbow trout (Cameron and Davis, 1970; Gilmour and Perry, 1996) have demonstrated that anaemia is without effect on PaO_2. While there is some evidence that hypercapnia may elicit gill remodelling in goldfish (J. Bradshaw and S.F. Perry; unpublished observations), it is unlikely that changes in arterial blood CO_2 levels was a contributing factor to remodelling in the present study because $PaCO_2$, while likely increasing during anaemia (Gilmour and Perry, 1996), would presumably be unaffected by CO exposure (Holeton, 1971). Although Holeton (1977) reported an absence of any change in blood pH associated with acute (30 min) CO exposure in rainbow trout, it is conceivable that blood pH was

reduced by longer term CO (or PHZ) treatments in goldfish; the potential involvement of blood pH as a trigger for gill remodelling has yet to be investigated.

The potential role of internal *versus* external O_2 receptors in mediating ventilatory responses to ambient hypoxia has received considerable attention (see Perry et al., 2009 for review). Indeed, similar to the results of the present study which focused on gill remodelling, previous experiments designed to assess ventilatory responses demonstrated that rainbow trout exposed to CO (Holeton, 1977; Holeton, 1971) or rendered anaemic (Smith and Jones, 1982) exhibited hyperventilation. These findings led to the concept of internal O_2 content receptors linked to cardio-respiratory control (Randall, 1982). The notion of some type of internal O_2 content receptor was further supported by the results of a series of experiments which correlated arterial O_2 content with catecholamine release in fish experiencing acute hypoxia (Perry and Reid, 1992; Perry et al., 2004; Reid et al., 1998). Those experiments clearly demonstrated that catecholamine release was occurring at a critical PO_2 threshold which varied among fish depending on the haemoglobin O_2 affinity. Additional evidence to support hypoxemia as a proximate stimulus for catecholamine secretion was the finding that exposing rainbow trout to hyperoxia prevented catecholamine release normally associated with hypercapnic acidosis (Perry et al., 1989). In agreement with these previous studies, the results of the present investigation suggest that some type of internal receptor responding to blood O_2 content is able to initiate gill remodelling. While the theoretical basis for sensing of blood O_2 content is not known, it is conceivable that such receptors could be responding to the rate of O_2 delivery which in turn is influenced by blood O_2 content.

In the natural environment, gill remodelling might occur when goldfish (or crucian carp) inhabiting cold water encounter hypoxia. In such a situation where the external and internal

environments are changing simultaneously, there is the possibility that externally and internally oriented receptors could be promoting the morphological changes to the gill. While the current evidence implicates internal hypoxemia as a trigger for gill remodelling, there are no comparable data explicitly implicating external O_2 receptors. An obvious candidate for such a receptor is the gill neuroepithelial cell (NEC) (Bailly et al.,1992; Bindon et al.,1994; Jonz and Nurse, 2003) which is known to function as an O_2 sensor in zebrafish, *Danio rerio* (Jonz et al.,2004; Qin et al., 2010) and channel catfish, *Ictalurus punctatus* (Burleson et al., 2006).

Thermally induced gill remodelling is influenced by external O_2 levels

Changes in ambient water temperature while impacting on internal and external temperature receptors also cause predictable changes in metabolic rate (Fry and Heart, 1948). Thus, the loss of the ILCM in goldfish transferred from cold to warm water (e.g. 7 to 25° C in Mitrovic and Perry, 2009) could specifically reflect increasing water and/or internal temperatures or alternatively be related to increasing metabolic rate. To determine whether the increasing O_2 demand associated with increasing metabolism might be contributing to gill remodelling (in an attempt to increase O_2 uptake), fish acclimated to 25° C were exposed to hyperoxia. Remarkably, the results demonstrated that the gill morphology of 25° C goldfish exposed to hyperoxia closely resembled that of a 7° C normoxic goldfish with only the distal lamellar regions remaining uncovered and in contact with the water. These results, while not excluding a possible role of temperature sensing in the gill remodelling response, certainly indicate that O_2 demand may be an important driving force. Thus, while O_2 demand still remained high in the fish acclimated to warm water, the abundance of O_2 presumably negated the need to shed the ILCM and uncover the lamellae. This idea is consistent with the results of earlier studies which

demonstrated that hyperoxia was able to increase the thermal tolerance of goldfish (Weatherly, 1970; Weatherly 1973).

Another possible explanation for the increases in the size of the ILCM in fish exposed to hyperoxia is that it serves to protect tissues from oxidative damage produced by reactive oxygen species. It would be interesting to measure arterial PO_2 during exposure of goldfish with branchial ILCM coverage to determine whether the ILCM serves as a barrier to the entry of O_2 and thus might limit internal oxidative damage.

The increase in the ILCM in hyperoxic fish acclimated to 25° C presumably decreases the functional lamellar surface area (not quantified in this study) for gas transfer. Previous data (see Chapter 3) showed that 7° C normoxic goldfish (ILCM present) do not have a significantly different arterial PO_2 than goldfish with protruding lamellae at 25° C suggesting that the available functional surface area for O_2 uptake in 7° C normoxic goldfish (~20% exposed lamellae) is sufficient to maintain arterial PO_2 at normal levels. Presumably, this is also true for 25° C goldfish with an ILCM given that these fish were also exposed to hyperoxia. The increase in ILCM may be beneficial because it decreases the ionoregulatory costs associating with replenishing salts lost by diffusion across the gill. Normally, such a morphological adjustment while, benefitting osmoregulation, would be expected to compromise respiratory function especially during hypoxia as demonstrated by an increase in critical PO_2 in crucian carp with covered lamellae (Sollid et al., 2003) or sculpins exhibiting a thickening of the gill lamellae (Henriksson et al., 2008). However, my recent results suggest that respiratory function may in fact be unaltered during hypoxia by similar morphological changes in goldfish, perhaps owing to a rapid 20% loss of the ILCM as soon as hypoxia is encountered (see Chapter 3).

Although there was a significant decrease in the ILCM during a temperature change from 7 to 25° C under hyperoxic conditions, the extent of this remodelling was less than when performed under conditions of normoxia. Thus, while hyperoxia in fish acclimated to 25° C was able to promote a gill phenotype that was indistinguishable from fish at 7° C, it could only reduce (and not totally prevent) the gill remodelling in fish transferred from 7 to 25° C. At the present time there is no clear explanation for this intermediate state of the ILCM after a temperature change under hyperoxic conditions. It is possible that there is a population of temperature sensors which also trigger gill remodelling independently leading to a complex integrative system of temperature and O_2 sensing which acts together to set the final gill phenotype.

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CHAPTER 5.

General Discussion

OVERALL SUMMARY AND SIGNIFICANCE OF THESIS

The major objective of this thesis was to assess the impact of gill remodelling on the sensing and transfer of gases in the goldfish, *Carassius auratus* and to provide insight into the proximate cues that initiate gill remodelling.

Chapter 2 presents the novel findings that the extent of coverage of the gill lamellae by the ILCM does not affect the ability of goldfish to sense hypoxic stimuli (NaCN) and hypoxia. This was determined by measuring the ventilatory response (V_{AMP} and V_f) after external or internal NaCN injections as well as by the location of the NECs on covered and uncovered goldfish gills. The lack of a significant difference in the hyperventilatory response between the two groups of fish to external NaCN was attributed to a redistribution of the NECs to the uncovered distal regions of lamellae in fish possessing an ILCM compared to the more or less evenly distributed NECs on an uncovered goldfish gill.

In Chapter 3 I demonstrate that the presence of the ILCM does not impede the transfer of O_2 or CO_2 from the water to the blood. However, under normoxic conditions the ILCM imposes an additional diffusion limitation to CO_2 excretion from the blood into the water which is relieved by carbonic anhydrase injections.

The cues for gill remodelling are investigated in Chapter 4. Hypoxemia induced by CO or PHZ caused a drastic reduction in the ILCM while exposure to hyperoxia (PwO2 > 500 mm Hg) partially prevented the shedding of the ILCM during a temperature increase from 7 to 25° C and induced its growth in 25° C goldfish. Although previous studies (Sollid et al., 2003, Sollid et al., 2005) showed that environmental hypoxia acts as a cue for ILCM retraction, this is the first study to show that internal cues also play a role in causing gill remodelling.

INTEGRATING GAS SENSING, TRANSFER AND REMODELLING

Although gas sensing and gas transfer are presented as two separate chapters in this thesis, the link between the two is undeniable. Figure 2.1 shows representative traces of V_{AMP} and V_f and the respective transient changes in arterial PO_2, PCO_2 and pH under normoxic conditions when the fish is stimulated with external injections of NaCN. The raw trace was obtained from a goldfish acclimated to 25° C which normally have uncovered gills (no ILCM). We see the same pattern of transient blood gas changes during a short hyperventilation episode in goldfish with the ILCM (raw traces not shown). Thus, activation of external O_2 chemoreceptors (gas sensing) clearly led to hyperventilatory responses which served to enhance gas transfer.

When considering O_2 sensing and transfer, externally oriented O_2 chemoreceptors (NECs) located on the fish gill are likely the first in line to respond to a drop in the PwO_2 levels and signal for the proper behavioural responses associated with hypoxia to meet the increase in O_2 demand. The response must be quick. The fastest way for this to happen is for the NECs to be associated with nerve fibres (Jonz and Nurse, 2005). The results of Chapter 2 clearly show NECs in close proximity to nerve fibres in 7 and 25° C goldfish. Nerve fibres do not appear to be associated with the ILCM but instead are found in the center of the filament and branch into the lamellae keeping close to the lamellar surface. Among a series of well-characterized cardiorespiratory responses caused by hypoxia, the most common is hyperventilation which is caused by increases in V_{AMP} and/or V_f. This behaviour is an effective way to increase the transfer of O_2 across the gill to cause an increase in PaO_2. Hyperventilation is also beneficial for the excretion of CO_2 from the blood into the water. Although the presence of a well defined population of CO_2 chemoreceptors has not been reported in goldfish there is evidence suggesting that branchial CO_2 are present in the tambaqi (Gilmour et al., 2005). It is possible that NECs play a dual role as an O_2 and a CO_2 chemoreceptor (Qin et al., 2010).

The results of this thesis demonstrate that the presence of the ILCM does not impede the transfer of respiratory gases from the water into the blood. Moreover, when exposed to hypoxic stimuli goldfish with an ILCM are able to sense the change and respond with the same degree of hyperventilation as goldfish without the ILCM. Additionally, goldfish with covered lamellae do not appear to be at a disadvantage for O_2 uptake when exposed to acute hypoxia. In part, this may reflect a rapid dismantling of the ILCM with the onset of hypoxia. Thus I have shown that the presence of the ILCM does not impose a challenge for gas sensing nor gas transfer in the goldfish. The benefits of having an ILCM reflect decreasing energy expenditure on osmoregulatory processes which would otherwise be high in a fish possessing a large gill surface area. The cells of the ILCM are not innervated nor does it appear to be vascularized so it can be removed quickly as an emergency response in the event of hypoxia. Of course the profit of increased functional gill surface area allowing for better gas exchange will follow with possible increased costs of osmoregulation.

The CO_2 induced ventilatory drive seen in other cyprinids (e.g. zebrafish; Vulesevic and Perry, 2006) was not present in goldfish with either gill phenotype (Chapter 3). It is possible that the level of hypercapnia the goldfish were exposed to (~8 mm Hg) was not high enough to elicit a ventilatory response and there was no need for hyperventilation to correct arterial PO_2 levels since they remained unchanged.

Hypoxemia was induced in goldfish (Chapter 4) but the ventilation response was not measured. However, Holeton (1971) reported an increase in the breathing rate in rainbow trout during a 3 h exposure to 5% (in air) CO. Thus, any increase in ventilation (not reported in this thesis) associated with hypoxemia must be attributed solely to internally oriented O_2 chemoreceptors because the goldfish were kept under normoxic conditions.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS

This thesis has assessed the impact the ILCM on gas sensing, gas transfer while also investigating the nature of the cues that trigger gill remodelling. Although considerable progress has been made, there are also new questions emerging.

As mentioned in Chapter 2, there is the possibility that the increase in NECs on distal regions of lamellae in goldfish with covered lamellae may result from new NECs being generated as the ILCM invades the interlamellar space. If so, it would be interesting to determine the proportion of newly differentiated NECs to pre-existing migrating ones. Furthermore, would these new cells be innervated? Such experiments would require time series analysis.

Chapter 4 demonstrated that hypoxia and increasing temperature trigger gill remodelling. In the same chapter it was shown that hypoxemia and hyperoxia also can act as cues for gill remodelling. The signal transduction pathway(s) underlying this response is still unknown. Here I propose that the O_2-sensing NECs may initiate the reduction of the ILCM. To test this without exposing the fish to hypoxia, repeated NaCN injections into the buccal cavity will be performed to stimulate the O_2 chemoreceptors. The degree of gill remodelling as a result of this "chemical hypoxia" experiment will be assessed.

The results of this thesis also demonstrated that O_2 uptake from the water is not impeded by the ILCM. To further support this I exposed goldfish acclimated to 7° C with and without ILCM to 1% ethanol and assessed the accumulation of ethanol (EtOH) in the blood over a 1.5 h time period. These experiments were performed after the submission deadline for the first draft of my thesis and thus were not included in it. The data, however, is included in Chapter 3 in this version of my thesis.

CONCLUSION

This thesis is a body of three manuscripts in preparation investigating the effects of different gill phenotypes in goldfish on their respiratory function and gas transfer, and also what triggers such a quick morphological change of their respiratory organ. The novel results presented here are only a piece of the puzzle in understanding how the ILCM affects physiological processes not only in goldfish but in other species with the ability to remodel their gills (e.g. crucian carp, mangrove killifish). Thus this work sets the platform for further investigations aimed at understanding the challenges water-breathers face with drastic changes in gill phenotype.

In conclusion, this thesis 1) characterizes the distribution of NECs on the covered and uncovered gills and relates it to the fish's ability to respond to hypoxia and hypoxic stimuli, 2) shows that thickening of the gill epithelium by the ILCM does not impose an additional barrier for the uptake of respiratory gases but does impede CO_2 excretion under normoxic conditions, and 3) illustrates that there are clear internal cues to gill remodelling.

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