The Journal of Experimental Biology 213, 3666-3675

doi:10.1242/jeb.047431

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

Velislava Tzaneva and Steve F. Perry*

Department of Biology, University of Ottawa, 30 Marie Curie, Ottawa, ON K1N 6N5, Canada

*Author for correspondence (sfperry@uottawa.ca)

Accepted 3 August 2010

SUMMARY

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 we 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 ($P_{w_{O_2}}$=30 mmHg) 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 the 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. 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 the presence of the ILCM results in the NECs being redistributed towards the distal regions of the lamellae. In 25°C-acclimated fish, the NECs were distributed evenly along the length of the lamellae with 53±3% of them in 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 at the distal edges of the lamellae 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.

Key words: neuroepithelial cell, gill, hypoxia, ventilation, chemoreception.

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 (f_V) and/or amplitude [V_{AMP}; see table 1 in Perry et al. (Perry et al., 2009b)]. Regardless of whether it is achieved by increases in f_V 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 the arterial blood partial pressure of oxygen ($P_{a_{O_2}}$), and hence O_2 content, to be maintained at higher levels than would otherwise 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). 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), 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). More recently, Jonz et al. (Jonz et al., 2004) and Burleson et al. (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 depolarisation 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. (Perry et al., 2009b)] in the 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 that 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 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 crucial in chemoreception, and in particular the sensing of inspired water.

The goldfish (*Carassius auratus*) is an example of a species possessing innervated NECs in 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 (reviewed 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 associated impairment of gas transfer associated with increasing diffusion distances and decreasing surface areas. In this study, we 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 NECs 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* Linnaeus 1758) were purchased from a commercial supplier [Aleong's International (Mississauga, ON, Canada)] and were held in circular tanks supplied with dechloraminated City of Ottawa tap water at 18°C. After at least 2 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 h:12 h light:dark photoperiod for at least 2 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., Oakville, ON, Canada) 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 litres) 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-acclimated goldfish (hereafter referred to as 7°C fish; mean 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 litre), after which they were killed 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 six 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 s period followed by 2 ml water flush, again over a 10–15 s 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 (Fig. 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 Ferret-Bouin, 1960). Ventilation amplitude (V_{AMP}) was determined and averaged over 10-s intervals after conversion of the impedance data to linear opercular deflections (in·cm) through appropriate calibration. Ventilation frequency (f_V) 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 $P_{W_{O_2}}$ ~30 mmHg (1 mmHg=133 Pa) achieved within ~30 min]. The ventilation responses were recorded continually but only the values of V_{AMP} and f_V before and at the lowest $P_{W_{O_2}}$ are reported here. Hypoxia was achieved by bubbling N_2 through the water–air equilibration column to displace the air until the water P_{O_2} reached approximately 30 mmHg.

Water P_{O_2} was monitored continuously by pumping using a peristaltic pump (Fisher Scientific, Ottawa, ON, Canada; 0.8 ml min^{-1}) water from the holding box through the sample compartment of a P_{O_2} electrode (Cameron Instruments, Port Aransas, TX, USA) connected to a gas meter (Cameron Instruments, BGM 200). The P_{O_2} electrode was housed in a thermostatically controlled cuvette and was calibrated prior to each experiment using a two point calibration method. A solution with zero P_{O_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 ($P_{a_{O_2}}$ and $P_{a_{CO_2}}$) 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.; P_{O_2}, P_{CO_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). Analogue outputs from the impedance converter and gas meters were interfaced with a data acquisition system (BioPac, Montreal, QC, Canada); the resultant digital data were graphically presented and stored using AcKnowledge data-acquisition software.

Immunohistochemistry

Using separate groups of fish that were killed 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 permeabilising solution containing 5% Triton X-100 in phosphate-buffered saline (PBST; pH 7.4) overnight. NECs and nerve fibres were identified by incubating the gill tissue with antibodies against serotonin (5-HT) and zebrafish-derived neuron-specific antigen (ZN-12), respectively, for 24–48 h at room temperature. Secondary antibodies [Alexa Fluor 488 (green) and Alexa Fluor 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 (Jonz and Nurse, 2003)]. The whole-mount gills were placed on a well slide containing mounting medium (Vectashield; Vector Laboratories, Ltd, Burlington, ON, Canada) 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 three-dimensional image. Image processing was performed using LSM Image Browser and ImageJ software (http://rsbweb.nih.gov/ij/index.html).

NEC distribution and quantification

Composite confocal images of the distal half of a single filament of (three to six 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 nine to ten 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 NECs of both the filament and of the lamellae.

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's *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 Fig. 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 anaesthetised, 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 with 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 (f_V)

The baseline values of V_{AMP} and f_V were measured at the start of the experiment prior to any treatment (Table 1). Goldfish acclimated to 25°C exhibited significantly higher f_V 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 1).

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

The hyperventilatory responses to acute hypoxia are summarised in Fig. 1. Both groups of fish exhibited obvious increases in V_{AMP} and f_V (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 f_V 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

Table 1. Effective dose of NaCN at which 50% of the maximal ventilation response was elicited for external or internal injections of NaCN in goldfish acclimated to 7°C (ILCM present) or 25°C (no ILCM)

	External NaCN		Internal NaCN	
Temperature (°C)	V_{AMP} ED_{50}	f_V ED_{50}	V_{AMP} ED_{50}	f_V ED_{50}
7°C	0.07 (6)	0.27(6)	0.05 (11)	0.03 (11)
25°C	0.17 (12)	0.08(12)*	0.03 (13)	0.04 (13)

ED_{50}, effective dose (mg kg^{-1}). The ED_{50} values were determined from dose response curves (see Fig. 3).
f_V, ventilation frequency; V_{AMP}, ventilation amplitude.
Numbers in parentheses are the number of fish in each treatment;
*significant differences between the ED_{50} values at the two temperatures.

oriented O_2 chemoreceptors, respectively. Fig. 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 f_V (Fig. 2A–C), the accompanying continuous measurements of blood gases demonstrated pronounced effects of each episode of hyperventilation consisting of a rise in Pa_{O_2}, a fall in Pa_{CO_2} and a corresponding increase in pHa (Fig. 2D–F).

The ventilation data were quantified and compiled as dose–response curves (Fig. 3). Fig. 3A–D illustrate the responses of goldfish acclimated to either 7 or 25°C to external (Fig. 3A,B) or internal injections of NaCN (Fig. 3C,D). The effective NaCN doses at which 50% of the response was elicited (ED_{50}) are summarised in Table 1. 95% confidence intervals (not shown on graphs) were constructed for each dose–response curve to determine if the differences between the two temperature treatments were significantly different. The similarity in ED_{50} values for NaCN between the two groups of fish (Table 1) suggests that the presence of an ILCM in the fish acclimated to 7°C did not impede their capacity to sense external or internal hypoxic stimuli. Although the ED_{50} for the f_V response to external NaCN was significantly higher in the 7°C fish (Table 1; Fig. 3B), the V_{AMP} response (Fig. 3A) was unaffected. Moreover, while the baseline values for V_{AMP} and f_V tended to be lower in the 7°C fish, their capacity to increase

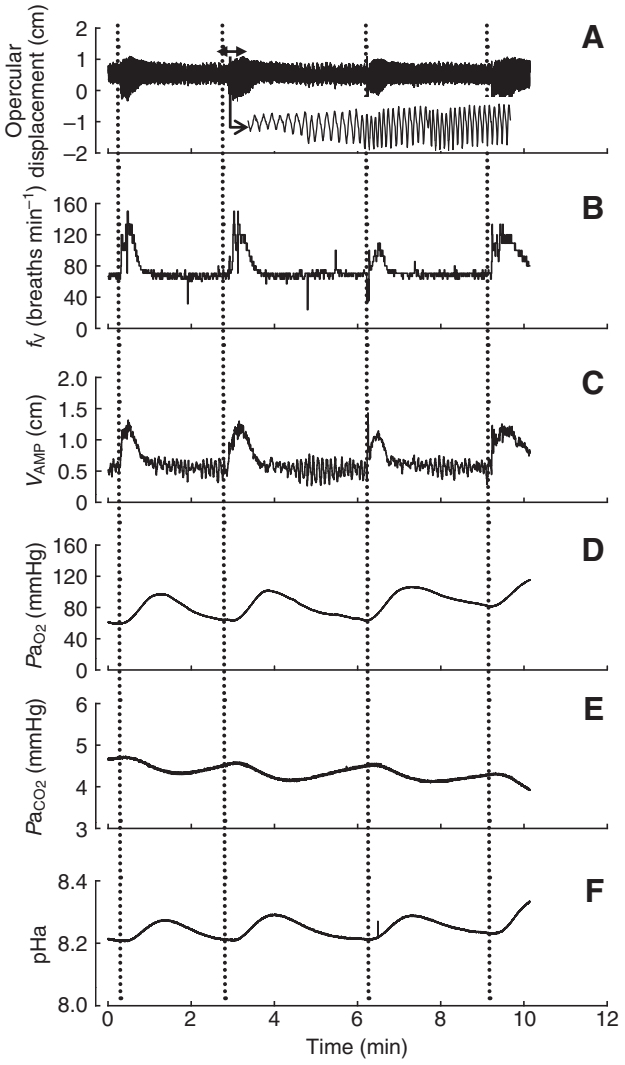

Fig. 2. Representative recordings illustrating the effects of four consecutive NaCN injections (indicated by the dotted lines) into the buccal cavity of a goldfish (*Carassius auratus*) on (A–C) ventilation parameters and (D–F) arterial blood gases. The measured ventilation parameters are (A) linear deflections of the opercular flaps as measured by impedance changes, (B) ventilation frequency (f_V) and (C) ventilation amplitude (V_{AMP}) as determined from the impedance trace. Arterial blood was continuously monitored for (D) P_{O_2} (Pa_{O_2}), (E) Pa_{O_2} (Pa_{CO_2}) and (F) pH (pHa). Note that each bout of hyperventilation associated with NaCN injection caused transient increases in Pa_{O_2} and pHa and decreases in Pa_{CO_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. 1 mmHg=133 Pa.

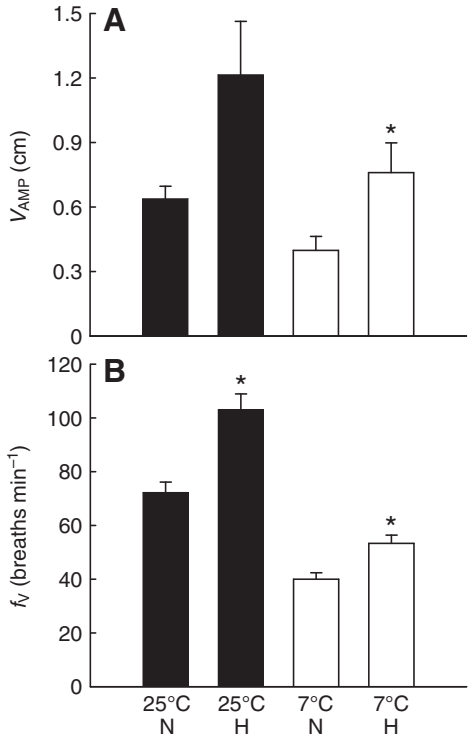

Fig. 1. (A) Ventilation amplitude (V_{AMP}) and (B) frequency (f_V) of goldfish (*Carassius auratus*) acclimated to 25°C (filled bars; N=4–6) or 7°C (unfilled bars; N=6) as measured during normoxia (N) and during exposure to acute hypoxia (H). Data are shown as means ± 1 s.e.m.; *significant differences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

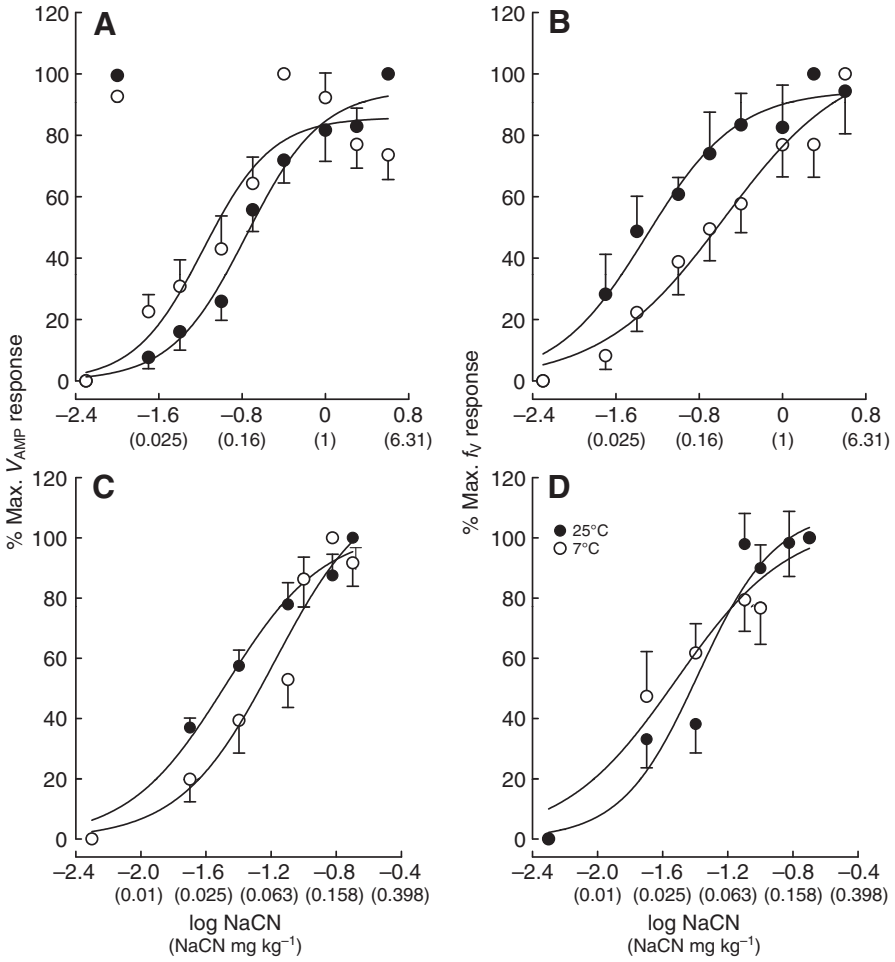

Fig. 3. Dose–response curves for hyperventilation responses including (A,B) ventilation amplitude (V_{AMP}) and frequency (f_V), respectively, for goldfish (*Carassius auratus*) injected externally with NaCN (0.02–4.00 mg kg^{-1}) and (C,D) ventilation frequency (f_V) and ventilation amplitude (V_{AMP}), respectively, for goldfish injected internally (*via* the caudal vein) with NaCN (0.01–0.20 mg kg^{-1}). Goldfish were acclimated either to 7°C (unfilled circles; *N*=9–13) or 25°C (filled circles; *N*=5–16). Dose–response curves were fit by iteration using a sigmoidal three-parameter equation (Sigmaplot version 11.0).

ventilation (on a percentage basis) was equivalent to the fish acclimated to 25°C (Table 2). 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. f_V 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; f_V was increased by 46.0±13.9% and 42.5±8.9% at 7 and 25°C. The increase in V_{AMP} and f_V 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 (Figs 4–6); the data are quantified in Fig. 7. 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 (the half nearest to the filament) and distal (the half nearest to the tip) lamellar regions (Fig. 4A,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

Table 2. The effects of external or internal bolus injections of NaCN into goldfish acclimated to 7°C (ILCM present) and 25°C (no ILCM) on ventilation amplitude and frequency

	V_{AMP} (cm)			f_V (breaths min^{-1})		
Temp (°C)	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	74.0±11.6	46.0±13.9
25 (13)	0.51±0.04	0.93±0.1	80.6±19.6	68.9±3.8	1108.9±7.4	42.5±8.9

*Injections into the buccal cavity (4 mg kg^{-1}).
†Injections into the caudal vein (0.2 mg kg^{-1}).
V_{AMP}, ventilation amplitude; f_V, ventilation frequency.
Data shown are means ± 1 s.e.m.; numbers in parentheses are the number of fish in each treatment.

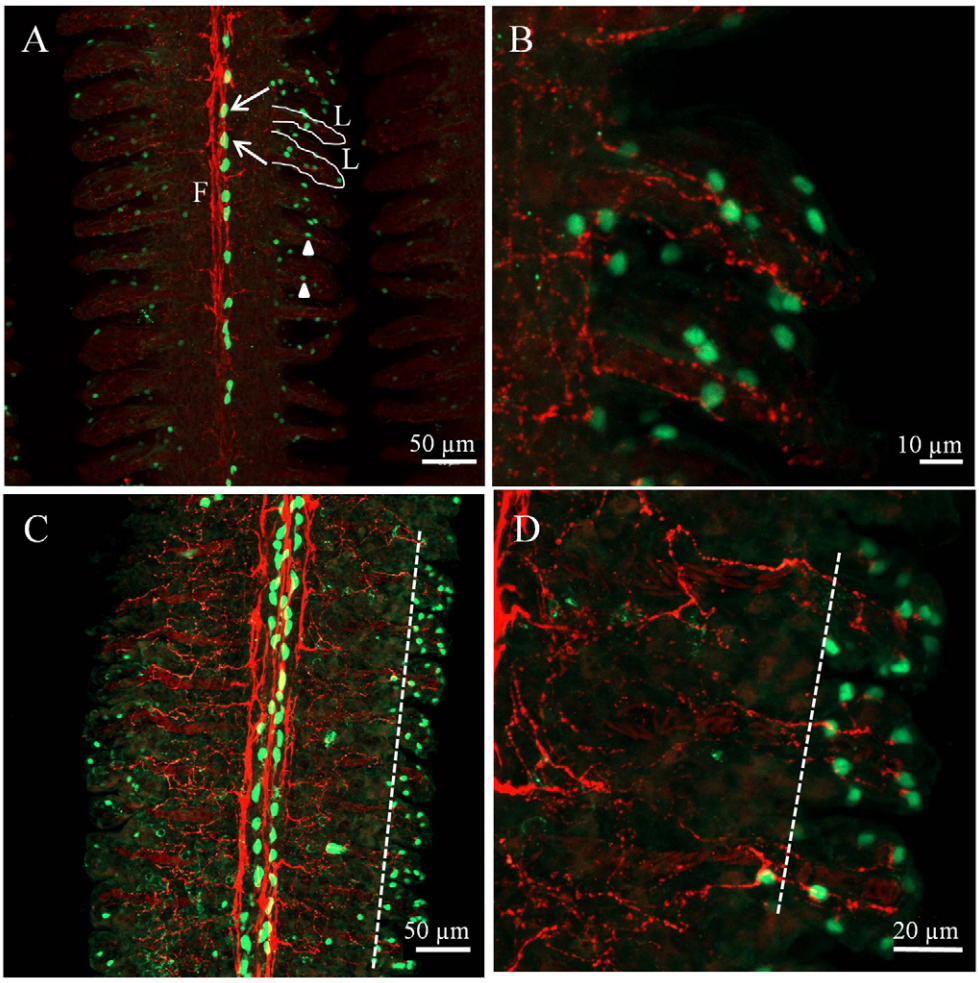

Fig. 4. Representative confocal micrographs showing serotonin-immunoreactive neuroepithelial cells (NEC; green fluorescence) and ZN-12-immunoreactive nerve fibres (red fluorescence) within the gill filament (F) and on the lamellae (L) of goldfish (*Carassius auratus*) acclimated to 25°C (A,B) or 7°C (C,D). The dashed line indicates the edge of the interlamellar cell mass (ILCM). NECs of the filament are indicated by arrows; NECs of the lamellae are indicated by arrowheads.

remained uncovered by the ILCM (Fig. 4C,D; Fig. 5). A similar distribution pattern was observed when examining cross sections through the gill filament, which allowed individual lamellae to be examined (Fig. 6). Fig. 6 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 (Figs 5–7). The ILCM itself and in particular the outer regions appear to be devoid of ZN-12 immunoreactive nerve fibres (Figs 4 and 5).

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

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 cover 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). We 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 f_V were significantly elevated with increasing acclimation temperature. These temperature-related differences in resting ventilation values, although important in matching increasing metabolic demands by tissues to increased rates of branchial O_2 uptake, can potentially

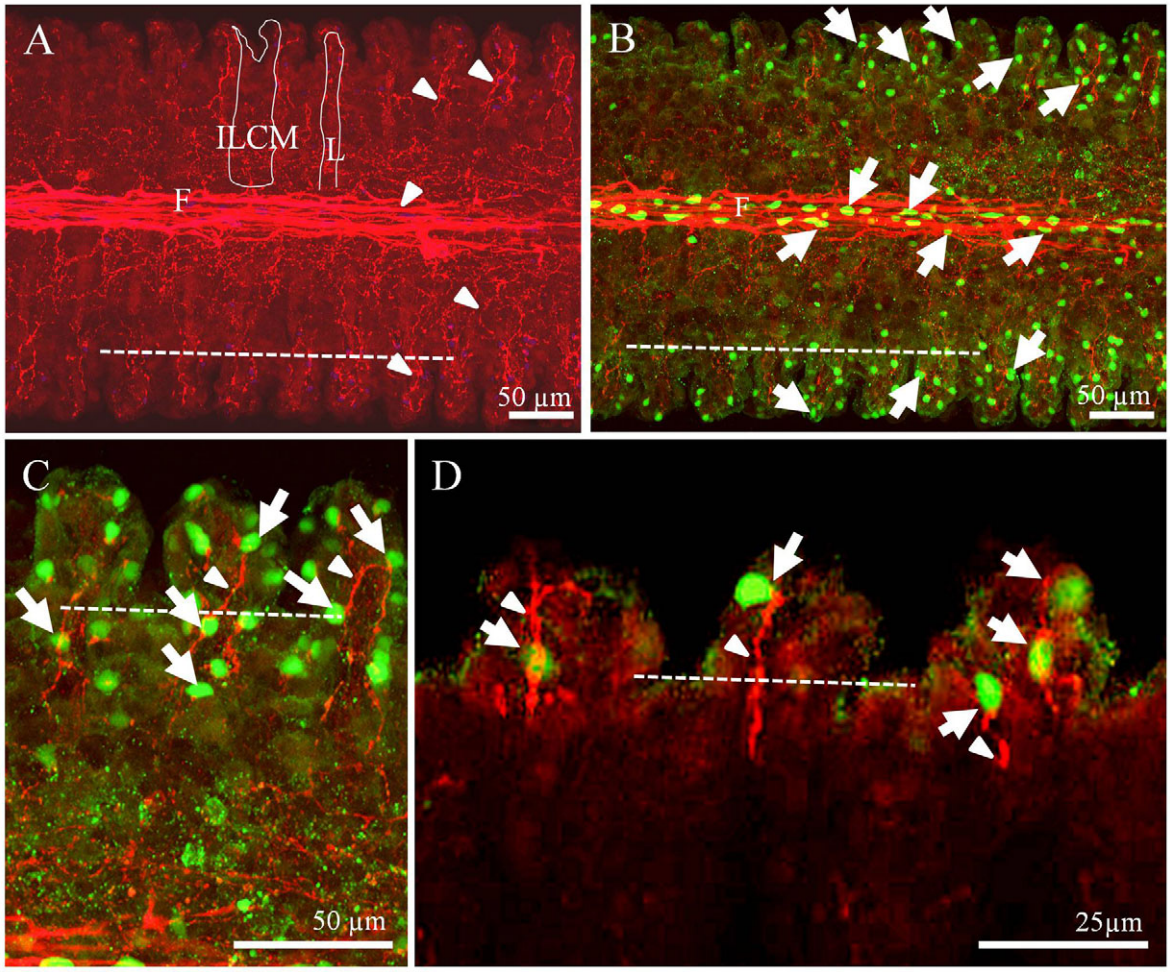

Fig. 5. Representative confocal micrographs showing serotonin-immunoreactive neuroepithelial cells (NEC; green fluorescence) and ZN-12-immunoreactive nerve fibres (red fluorescence) within the gill filament (F) and on the lamellae (L) of goldfish (*Carassius auratus*) acclimated to 7°C. The dashed line indicates the edge of the interlamellar cell mass (ILCM). To more clearly demonstrate the distribution pattern of nerve fibres (arrowheads), the green colour was removed from A; B is the identical image with the green colour added. NECs are indicated by arrows; arrowheads in C and D indicate nerve fibres apparently innervating NECs.

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 f_V in fish acclimated to 25°C could conceivably constrain the extent of hyperventilation if there was an absolute ceiling to V_{AMP} or f_V beyond which further increases were no longer possible; if so, the fish acclimated to 7°C might possess a greater scope for increasing ventilation. By contrast, colder temperature, in itself, could potentially reduce the sensitivity of O_2 chemoreceptors to hypoxic stimuli owing to Q_{10} effects. Although several previous studies have compared ventilatory responses of fish to hypoxia at different temperatures (Glass et al., 1990; Stecyk and Farrell, 2002), we are unaware of any studies that 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 P_{O_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 or internal NaCN or acute hypoxia, which consisted of increases in V_{AMP} and f_V. Similar to carp that 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 mmHg (V.T., K. M.

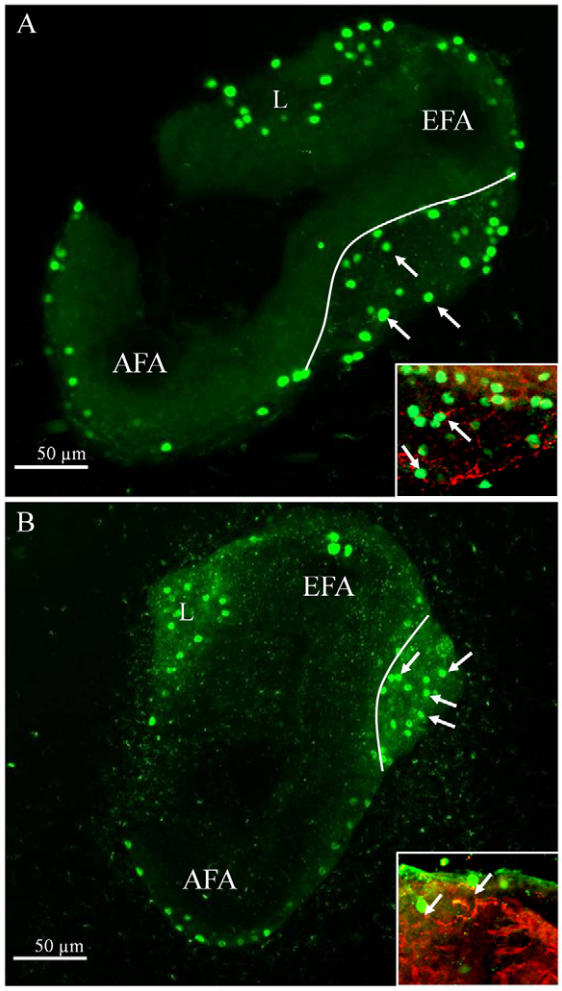

Fig. 6. Serotonin-immunoreactive neuroepithelial cells (NEC; arrows) and ZN-12-immunoreactive nerve fibres (red fluorescence in insets) on the lamellae of goldfish (*Carassius auratus*) acclimated to (A) 25°C or (B) 7°C. The solid lines indicate the edge of the ILCM; AFA, afferent filament artery; EFA, efferent filament artery.

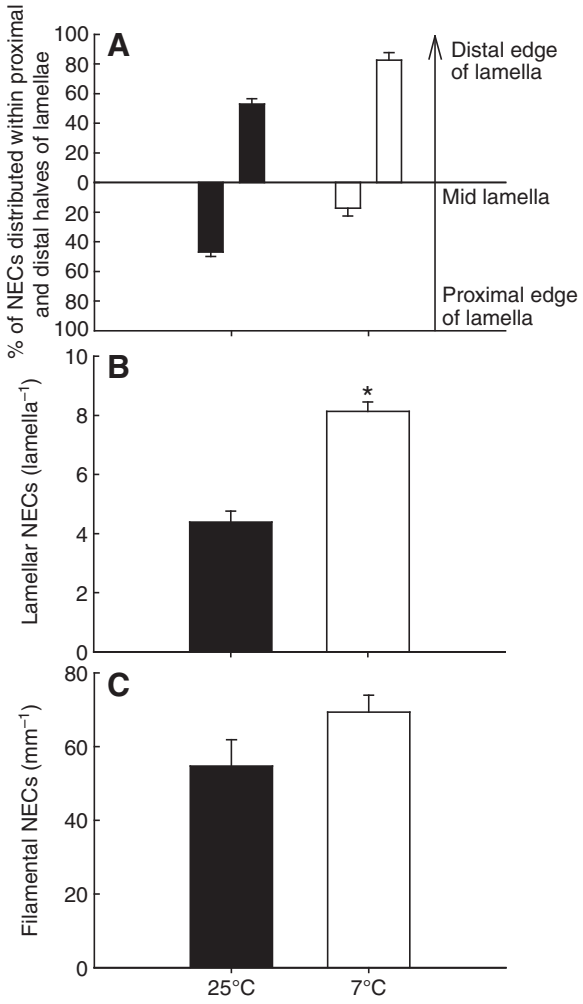

Fig. 7. (A) Distribution of the neuroepithelial cells (NECs) on the lamellae of goldfish (*Carassius auratus*) acclimated to 7°C or 25°C. (B) The number of lamellar NECs per lamella; (C) the number of filament NECs per mm of filament length. Data are shown as means ± 1 s.e.m.; significant differences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

Gilmour and S.F.P., 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) 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 f_V 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 the presence of filamental NECs in close proximity to the efferent filament artery (Saltys et al., 2006). Interestingly, Coolidge et al. (Coolidge et al., 2008) were unable to locate NECs within the central core of the goldfish gill filament 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. implied that goldfish might lack responsiveness to internal hypoxic stimuli (Coolidge et al., 2008). 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 that are presumably the NECs within the filament core in proximity to the efferent filament artery (this study) (Saltys et al., 2006). Currently, it is unclear why the goldfish gills examined by Coolidge et al. did not appear to contain NECs within the filament core (Coolidge et al., 2008).

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. (Saltys et al., 2006). By contrast, Coolidge et al. 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 (Coolidge et al., 2008). In the present study, the NECs were concentrated on the distal half of the lamellae only in the fish acclimated to 7°C and exhibiting an ILCM. Thus, the formation of the ILCM was associated with a profound reorganisation 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 are protrusions of the branchial nerves which branch from bundles within the filament to innervate the lamellae (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 that 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 repositioning 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 (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 (Fig. 7) would suggest that differentiation of new cells is a significant component of the overall redistribution. In the context of our 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. (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 whether the NECs localised 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 a continuation of the more proximally (relative to the gill arch) located NECs that are associated with the efferent filament artery.

ACKNOWLEDGEMENTS

This study was supported by Natural Sciences and Engineering Research Council (NSERC) Discovery and NSERC Research Tools and Innovation (RTI) grants to S.F.P. We are grateful to Mr Bill Fletcher for his dedicated care of animals and Mr Andrew Ochalski for help with microscopy. Dr Katie Gilmour played a crucial role in the conception and development of these experiments. We would also like to thank the Ottawa Heart and Stroke Foundation Centre for Stroke Recovery for making available their confocal microscope.

REFERENCES

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

Beamish, F. W. H. and Mookherjii, P. S. (1964). Respiration of fishes with special emphasis on standard oxygen consumption. I. Influence of weight + temperature on respiration of goldfish *Carassius auratus* L. *Can. J. Zool.* **42**, 161-175.

Burleson, M. L. and Smatresk, N. J. (1990). Evidence for two oxygen-sensitive chemoreceptor loci in channel catfish, *Ictalurus punctatus*. *Physiol. Zool.* **63**, 208-221.

Burleson, M. L., Smatresk, N. J. and Milsom, W. K. (1992). Afferent inputs associated with cardioventilatory control in fish. In *The Cardiovascular System* (ed. W. S. Hoar, D. J. Randall and A. P. Farrell), pp. 389-423. San Diego: Academic Press.

Burleson, M. L., Mercer, S. E. and Wilk-Blaszczak, M. A. (2006). Isolation and characterization of putative O_2 chemoreceptor cells from the gills of channel catfish (*Ictalurus punctatus*). *Brain Res.* **1092**, 100-107.

Coolidge, E. H., Ciuhandu, C. S. and Milsom, W. K. (2008). A comparative analysis of putative oxygen-sensing cells in the fish gill. *J. Exp. Biol.* **211**, 1231-1242.

Courtois, L. A. (1976). Respiratory responses of *Gillichthys mirabilis* to changes in temperature, dissolved oxygen and salinity. *Comp. Biochem. Physiol.* **53A**, 7-10.

Crawshaw, L. I. (1976). Effect of rapid temperature-change on mean body-temperature and gill ventilation in carp. *Am. J. Physiol.* **231**, 837-841.

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

Fry, F. E. J. and Hart, J. S. (1948). The relation of temperature to oxygen consumption in the goldfish. *Biol. Bull.* **94**, 66-77.

Gilmour, K. M. and Perry, S. F. (2007). Branchial chemoreceptor regulation of cardiorespiratory function. In *Sensory Systems Neuroscience* (ed. T. J. Hara and B. Zielinski), pp. 97-151. London, Oxford, Boston, New York, San Diego: Academic Press.

Glass, M. L., Andersen, N. A., Kruhoffer, M., Williams, E. M. and Heisler, N. (1990). Combined effects of environmental P_{O_2} and temperature on ventilation and blood-gases in the carp *Cyprinus carpio* L. *J. Exp. Biol.* **148**, 1-17.

Holeton, G. F. and Randall, D. J. (1967). The effect of hypoxia upon the partial pressure of gases in the blood and water afferent and efferent to the gills of rainbow trout. *J. Exp. Biol.* **46**, 317-327.

Itazawa, Y. and Takeda, T. (1978). Gas exchange in the carp gills in normoxic and hypoxic conditions. *Respir. Physiol.* **35**, 263-269.

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

Jonz, M. G. and Nurse, C. A. (2005). Development of oxygen sensing in the gills of zebrafish. *J. Exp. Biol.* **208**, 1537-1549.

Jonz, M. G. and Nurse, C. A. (2008). New developments on gill innervation: insights from a model vertebrate. *J. Exp. Biol.* **211**, 2371-2378.

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

Katoh, F. and Kaneko, T. (2003). Short-term transformation and long-term replacement of branchial chloride cells in killifish transferred from seawater to freshwater, revealed by morphofunctional observations and a newly established 'time-differential double fluorescent staining' technique. *J. Exp. Biol.* **206**, 4113-4123.

Lomholt, J. P. and Johansen, K. (1979). Hypoxia acclimation in carp – how it affects O_2 uptake, ventilation, and O_2 extraction from water. *Physiol. Zool.* **52**, 38-49.

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

Mitrovic, D. and Perry, S. F. (2009). The effects of thermally induced gill remodelling on ionocyte distribution and branchial chloride fluxes in goldfish (*Carassius auratus*). *J. Exp. Biol.* **212**, 843-852.

Mitrovic, D., Dymowska, A., Nilsson, G. E. and Perry, S. F. (2009). Physiological consequences of gill re-modeling in goldfish (*Carassius auratus*) during exposure to long-term hypoxia. *Am. J. Physiol.* **297**, R224-R334.

Perry, S. F. and Gilmour, K. M. (2002). Sensing and transfer of respiratory gases at the fish gill. *J. Exp. Zool.* **293**, 249-263.

Perry, S. F. and Gilmour, K. M. (2010). Oxygen uptake and transport in water breathers. In *Respiratory Physiology of Vertebrates: Life with and without Oxygen* (ed. G. E. Nilsson), pp. 49-94. Cambridge: Cambridge University Press.

Perry, S. F., Esbaugh, A., Braun, M. and Gilmour, K. M. (2009a). Gas transport and gill function in water-breathing fish. In *Cardio-Respiratory Control in Vertebrates: Comparative and Evolutionary Aspects* (ed. M. L. Glass and S. C. Wood), pp. 5-42. Heidelberg: Springer.

Perry, S. F., Jonz, M. G. and Gilmour, K. M. (2009b). Oxygen sensing and the hypoxic ventilatory response. In *Hypoxia* (ed. J. G. Richards, C. J. Brauner and A. P. Farrell), pp. 193-251. Amsterdam: Academic Press.

Peyraud, C. and Ferret-Bouin, P. (1960). Methode electronique pour l'analyse de la mechanique respiratoire des poissons. *Bull. Soc. Hist. Nat. Toulouse* **95**, 385-390.

Qin, Z., Lewis, J. and Perry, S. F. (2010). Zebrafish (*Danio rerio*) gill neuroepithelial cells are sensitive chemoreceptors for environmental CO_2. *J. Physiol.* **588**, 861-872.
Randall, D. J. and Shelton, G. (1963). The effects of changes in environmental gas concentrations on the breathing and heart rate of a teleost fish. *Comp. Biochem. Physiol.* **9**, 229-239.
Reid, S. G. and Perry, S. F. (2003). Peripheral O_2 chemoreceptors mediate humoral catecholamine secretion from fish chromaffin cells. *Am. J. Physiol.* **284**, R990-R999.
Saltys, H. A., Jonz, M. G. and Nurse, C. A. (2006). Comparative study of gill neuroepithelial cells and their innervation in teleosts and Xenopus tadpoles. *Cell Tissue Res.* **323**, 1-10.
Saunders, R. L. and Sutterlin, A. M. (1971). Cardiac and respiratiory responses to hypoxia in the sea raven, *Hemitripterus americanus*, and an investigation of possible control mechanismd. *J. Fish Res. Bd. Canada* **28**, 491-503.
Shelton, G., Jones, D. R. and Milsom, W. K. (1986). Control of breathing in ectothermic vertebrates. In *Handbook of Physiology, section 3. The Respiratory System, vol. 2, Control of Breathing* (ed. N. S. Cherniak and J. G. Widdicombe), pp. 857-909. Bethesda: American Physiological Society.
Sollid, J. and Nilsson, G. E. (2006). Plasticity of respiratory structures-adaptive remodeling of fish gills induced by ambient oxygen and temperature. *Respir. Physiol. Neurobiol.* **154**, 241-251.
Sollid, J., De Angelis, P., Gundersen, K. and Nilsson, G. E. (2003). Hypoxia induces adaptive and reversible gross morphological changes in crucian carp gills. *J. Exp. Biol.* **206**, 3667-3673.
Sollid, J., Weber, R. E. and Nilsson, G. E. (2005). Temperature alters the respiratory surface area of crucian carp *Carassius carassius* and goldfish *Carassius auratus*. *J. Exp. Biol.* **208**, 1109-1116.
Stecyk, J. A. W. and Farrell, A. P. (2002). Cardiorespiratory responses of the common carp (*Cyprinus carpio*) to severe hypoxia at three acclimation temperatures. *J. Exp. Biol.* **205**, 759-768.
Sundin, L., Reid, S. G., Rantin, F. T. and Milsom, W. K. (2000). Branchial receptors and cardiorespiratory reflexes in the neotropical fish, Tambaqui (*Colossoma macropomum*). *J. Exp. Biol.* **203**, 1225-1239.
Vulesevic, B., McNeill, B. and Perry, S. F. (2006). Chemoreceptor plasticity and respiratory acclimation in the zebrafish, *Danio rerio*. *J. Exp. Biol.* **209**, 1261-1273.
Zaccone, G., Lauweryns, J. M., Fasulo, S., Tagliafierro, G., Ainis, L. and Licata, A. (1992). Immunocytochemical localization of serotonin and neuropeptides in the neuroendocrine paraneurons of teleost and lungfish gills. *Acta Zool.* **73**, 177-183.
Zaccone, G., Mauceri, A. and Fasulo, S. (2006). Neuropeptides and nitric oxide synthase in the gill and the air-breathing organs of fishes. *J. Exp. Zool.* **305**, 428-439.