

Chemoreceptor plasticity and respiratory acclimation in the zebrafish *Danio rerio*

B. Vulesevic, B. McNeill and S. F. Perry*

Department of Biology, University of Ottawa, 10 Marie Curie, Ottawa, ON K1N 6N5, Canada

*Author for correspondence (e-mail: sfperry@science.uottawa.ca)

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Summary

The goals of this study were to assess the respiratory consequences of exposing adult zebrafish *Danio rerio* to chronic changes in water gas composition (hypoxia, hyperoxia or hypercapnia) and to determine if any ensuing effects could be related to morphological changes in branchial chemoreceptors. To accomplish these goals, we first modified and validated an established non-invasive technique for continuous monitoring of breathing frequency and relative breathing amplitude in adult fish. Under normal conditions 20% of zebrafish exhibited an episodic breathing pattern that was composed of breathing and non-breathing (pausing/apneic) periods. The pausing frequency was reduced by acute hypoxia ($P_{wO_2} < 130$ mmHg) and increased by acute hyperoxia ($P_{wO_2} > 300$ mmHg), but was unaltered by acute hypercapnia.

Fish were exposed for 28 days to hyperoxia ($P_{wO_2} > 350$ mmHg), or hypoxia ($P_{wO_2} = 30$ mmHg) or hypercapnia ($P_{wCO_2} = 9$ mmHg). Their responses to acute hypoxia or hypercapnia were then compared to the response of control fish kept for 28 days in normoxic and normocapnic water. In control fish, the ventilatory response to acute hypoxia consisted of an increase in breathing frequency while the response to acute hypercapnia was an increase in relative breathing amplitude. The stimulus promoting the hyperventilation during hypercapnia was increased P_{wCO_2} rather than decreased pH. Exposure to prolonged hyperoxia decreased the capacity of fish to increase breathing frequency during hypoxia and prevented the usual increase in breathing amplitude during acute hypercapnia. In fish previously

exposed to hyperoxia, episodic breathing continued during acute hypoxia until P_{wO_2} had fallen below 70 mmHg. In fish chronically exposed to hypoxia, resting breathing frequency was significantly reduced (from 191 ± 12 to 165 ± 16 min⁻¹); however, the ventilatory responses to hypoxia and hypercapnia were unaffected. Long-term exposure of fish to hypercapnic water did not markedly modify the breathing response to acute hypoxia and modestly blunted the response to hypercapnia.

To determine whether branchial chemoreceptors were being influenced by long-term acclimation, all four groups of fish were acutely exposed to increasing doses of the O₂ chemoreceptor stimulant, sodium cyanide, dissolved in inspired water. Consistent with the blunting of the ventilatory response to hypoxia, the fish pre-exposed to hyperoxia also exhibited a blunted response to NaCN. Pre-exposure to hypoxia was without effect whereas prior exposure to hypercapnia increased the ventilatory responses to cyanide.

To assess the impact of acclimation to varying gas levels on branchial O₂ chemoreceptors, the numbers of neuroepithelial cells (NECs) of the gill filament were quantified using confocal immunofluorescence microscopy. Consistent with the blunting of reflex ventilatory responses, fish exposed to chronic hyperoxia exhibited a significant decrease in the density of NECs from 36.8 ± 2.8 to 22.7 ± 2.3 filament⁻¹.

Key words: zebrafish, *Danio rerio*, hypoxia, hyperoxia, hypercapnia, neuroepithelial cell, serotonin, breathing.

Introduction

In water-breathing fish, gill ventilation is affected by several factors including the status of dissolved gases in the environment and metabolic demand. Chemoreceptors on, and within the gill, sense changes in ambient and intravascular gas levels. These chemoreceptors are either oriented externally to sense environmental changes or internally to sense changes in

the blood (see reviews by Bursleson and Smatresk, 2000; Sundin and Nilsson, 2002). The chemoreceptors, when stimulated by changes in O₂ and/or CO₂/pH, initiate a variety of cardiorespiratory and hormonal responses, including changes in breathing rate and/or amplitude, heart rate and systemic resistance and plasma catecholamine levels (Satchell, 1959; Randall and Smith, 1967; Holeton and Randall, 1967)

(see reviews by Fritsche and Nilsson, 1993; Smatresk, 1990; Burlleson et al., 1992; Perry and Gilmour, 2002; Sundin and Nilsson, 2002; Reid and Perry, 2003). The chemoreceptor cells share many histological and cytochemical similarities with mammalian carotid body glomus cells (Fritsche and Nilsson, 1993) and indeed they are likely to be the evolutionary precursors of the mammalian carotid body, the main site for sensing changes in blood gases in mammals (Gonzalez et al., 1992).

Like other components of the nervous system, respiratory control systems exhibit marked plasticity. This plasticity can be morphological and/or functional and is based on prior experience (Baker et al., 2001; Mitchell and Johnson, 2003). There are several potential sites of respiratory neuroplasticity, including the sensory chemoreceptors themselves, signal transmission pathways, central rhythm generation or pattern formation control (Powell et al., 2000). Together with genotype, age and gender, the partial pressure of respiratory gases can influence plasticity (Mitchell and Johnson, 2003). In the wild, fish can be exposed to fluctuations in environmental O₂ and CO₂ levels both diurnally and spatially (Crocker et al., 2000). Consequently, fish have developed behavioural, physiological and morphological mechanisms of acclimating to fluctuating environments, a reflection of their respiratory plasticity (Perry and Gilmour, 2002; Burlleson et al., 2002; Sollid et al., 2003; Jonz et al., 2004).

Ventilatory acclimation to hypoxia (VAH) is one of several examples of hypoxia-induced respiratory plasticity in mammals. VAH may manifest itself as an increase in breathing response to subsequent hypoxia owing to heightened chemoreceptor and central nervous system (CNS) sensitivity (Forster et al., 1971; Sato et al., 1992; Aaron and Powell, 1993; Bisgard and Neubauer, 1995; Soulier et al., 1997; Dwinell and Powell, 1999; Bisgard, 2000; Powell et al., 2000; Baker et al., 2001). Only a single study (Burlleson et al., 2002) has assessed VAH in fish; that study demonstrated that catfish *Ictalurus punctatus* exposed to chronic moderate hypoxia (75 mmHg; 1 mmHg≡1 Torr≡133.3 Pa) exhibited a heightened ventilatory sensitivity to acute hypoxia.

Hyperoxia-induced respiratory plasticity has been reported in mammals (Lahiri et al., 1987; Liberzon et al., 1989; Torbati et al., 1989) and is manifested by a reversible blunting of the ventilatory response to hypoxia. To our knowledge, no studies have yet addressed the potential for respiratory plasticity in fish exposed to hyperoxia.

In mammals, pre-exposure to hypercapnia does not alter the response to acute hypoxia or hypercapnia (Remmers and Lahiri, 1998; Kondo et al., 2000). Despite the accruing evidence that environmental CO₂ is a potent and specific ventilatory stimulant in fish (Heisler et al., 1988; Graham et al., 1990; Milsom, 1995; Perry et al., 1999; Burlleson and Smatresk, 2000; McKendry et al., 2001; Perry and Reid, 2002; McKenzie et al., 2003) (reviewed by Gilmour, 2001), we are unaware of any studies that have examined respiratory acclimation to hypercapnia in fish.

The present study focused on evaluating respiratory

plasticity in zebrafish *Danio rerio* following exposure to chronic hypoxic, hyperoxic or hypercapnic conditions. This was accomplished using a non-invasive recording method that registers the change in voltage that is transferred through the water during opercular movements (Almitras and Larsen, 2000). To implicate morphological changes of branchial chemoreceptors (Jonz and Nurse, 2003; Jonz and Nurse, 2005; Jonz et al., 2004) as a mechanism of plasticity, gills were analyzed by confocal immunofluorescence microscopy.

Materials and methods

Animals

Adult zebrafish *Danio rerio* Hamilton were obtained from a commercial supplier (MIRDO, Montreal, Canada) and transported to the University of Ottawa Aquatic Care Facility where they were maintained in acrylic tanks (4 l) supplied with well-aerated, dechloraminated City of Ottawa tapwater at 28.0°C. Fish were subjected to a constant 10 h:14 h L:D photoperiod. All procedures for animal use were carried out according to institutional guidelines and in accordance with those of the Canadian Council on Animal Care (CCAC).

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

In a separate experimental series, zebrafish were placed in a breathing recording chamber constructed at University of Ottawa. The chamber was a cylindrical transparent plastic tube (length=3 cm, diameter=1 cm). Two electrodes (standard copper–tin wires) were submerged in the water inside the chamber and separated by a distance of approximately 2 cm. Coarse mesh was inserted into each end of the chamber to prevent the fish from making contact with the electrodes. Each chamber was provided with continuous water flow (~10 ml min⁻¹). The experiments were filmed using a Canon digital movie camera (model NTSC ZR70mc) and the images were transferred to a personal computer using Digital Video Camcorder software (Ulead Video Studio SE Basic Version 6.0). A scale bar was included in the visual field to allow measurement of opercular displacement (in mm, the measure of ventilation amplitude used in the present study). The analog voltage signals associated with opercular movements were amplified using an amplifier made at University of Ottawa, converted to digital data and stored on computer by interfacing with a data acquisition system (Biopac Systems Inc., Goleta, CA, USA) using AcqKnowledge™ data acquisition software (sampling rate set at 50 000 Hz) and a Pentium™ PC. A high pass filter (0.5 Hz) and two low-pass filters (each 21 Hz) were built into the amplifier. The amplifier had a gain of 40 and the Biopac system was set to a gain of 50 for a total gain of 2000.

Breathing frequencies (f_R) and amplitude were measured independently by analyzing video recordings and AcqKnowledge™ files. To assess the suitability of the electronic recording technique to reliably provide accurate measurements of breathing frequency and relative amplitude, data obtained from the two techniques were compared and

subjected to correlation analysis. Opercular breathing movements produced oscillating voltage changes, with each breathing cycle producing a distinct minimum and maximum voltage. Thus, f_R was determined by counting the number of voltage peaks over a set time interval. On the basis of video analysis, it was determined that opercular displacement during normal breathing was 1–3 mm (the sum of both operculae). Thus, for simplicity, the data acquisition was calibrated assuming that the voltage fluctuations at rest represented a 1 mm total opercular deflection. Thus, in all experiments, relative breathing amplitude was calculated as the difference between minimum and maximum values of voltage changes that were continuously recorded for each breathing cycle. Fish were exposed to hypercapnia (3.5 mmHg, see below) to induce changes in breathing amplitude that could be quantified independently by video analysis and electronic recording. To ensure a wide range of breathing frequencies to analyse by each technique, data were obtained from fish allowed to recover for varying periods of time after transfer to the respirometer.

To further prove that the measured voltage oscillations were exclusively related to breathing movements, four 28 day control fish (see below) were anaesthetized *in situ* with 0.1 ml ml⁻¹ benzocaine. Breathing was absent in the anaesthetized fish but the heart continued to beat and some involuntary body movements remained. The signal obtained during anaesthesia was then compared to the signal obtained before and after the fish was in anaesthesia. These fish were not used for any other experiments.

Pre-exposure of fish to hypercapnia, hypoxia or hyperoxia

Zebrafish were exposed to hypercapnia (P_{wCO_2} =7–9 mmHg), hypoxia (P_{wO_2} =30–40 mmHg), or hyperoxia (P_{wO_2} =350–450 mmHg) at 28°C in a 2 l tank for 28 days. Control fish were kept under similar conditions for 28 days but were provided with normoxic and normocapnic water. For each treatment, at least two groups of fish were pre-exposed at different times. Hypercapnia was achieved by pumping mixtures of CO₂ in air (1–2%) through a gas equilibration column provided with aerated water. For hypoxia, the water equilibration column was supplied with a mixture of 95% nitrogen (N₂) and 5% air. The gas mixtures were supplied by a Cameron Gas Mixer (model GF-3/MP, Port Aransas, TX, USA). To achieve hyperoxia, pure O₂ (100%) was bubbled directly into fish tanks being supplied with minimal volumes of normoxic-dechloraminated water (30 ml h⁻¹). Water P_{CO_2} was measured using a CO₂ electrode (Cameron Instrument Company, model E201) connected to a Cameron BGM 200 blood gas meter. Measurements of O₂ were made using a fiber optic oxygen electrode (Ocean Optics Foxy AL300, Dunedin, FL, USA) and associated hardware and software (Ocean Optics SD 2000). After 28 days, the fish were tested for their ventilatory responses to acute hypoxia, hypercapnia or external cyanide. They were then euthanized by overdose of anaesthetic (1 mg ml⁻¹ ethyl 3-aminobenzoate methanesulfonate salt; MS 222), and the

gills were removed and prepared for immunocytochemistry (see below).

Ventilatory responses to acute hypoxia

A group of fish from each pre-exposure group was randomly chosen for this experiment and were not used in any other experiment. Fish were placed in the breathing recording chamber for 1–3 h prior to beginning of experiments, to allow their breathing to become uniform. For each fish, resting breathing amplitude (V_{AMP}) was assumed to be 1 mm and the system was calibrated accordingly. P_{wO_2} within the fish chamber was continuously measured by using a P_{O_2} electrode calibrated with solutions of sodium sulphite (20 mg ml⁻¹; P_{O_2} =0 mmHg) and air-saturated dechlorinated Ottawa tapwater (P_{O_2} =153 mmHg). After taking the breathing measurements for normoxic water, fish were exposed to hypoxia in seven equal steps ranging from 130 to 20 mmHg. Hypoxic conditions were achieved by bubbling N₂, progressively increasing flow rates, through a water–gas equilibration column that provided flowing water to the fish. Continuous data recordings were obtained for ventilation frequency and V_{AMP} after the P_{wO_2} had reached the target value (~10 min later). In each case, at least 5 min of breathing data were analyzed to obtain mean values. Typically, fish were exposed to each step of hypoxia for 15–20 min.

Ventilatory responses to acute hypercapnia

In a separate series of experiments, groups of fish from each pre-exposure group were randomly chosen to assess the ventilatory responses to hypercapnia and were not used in any other experiment. P_{wCO_2} within the fish chamber was continuously measured by a P_{CO_2} electrode (Cameron Instrument Company, model E201) connected to a Cameron BGM 200 blood gas meter calibrated using mixtures of 0.25 and 1.0% CO₂ in water provided by a gas-mixer (Cameron Instrument Company GF-3/MP). After a 1–3 h stabilisation period in normocapnic water (P_{wCO_2} <0.5 mmHg; pH=7.4–7.5), measurements of ventilation were initiated. Fish were then exposed to hypercapnia in three steps: 1 mmHg, 2.5 mmHg and 3.5 mmHg, and then returned to normocapnia for a final set of breathing measurements. Fish were exposed to each level of hypercapnia for 15–20 min. Hypercapnia was achieved by bubbling different percentages of CO₂ through a water–gas equilibration column providing flowing water to the fish. Breathing data were obtained as described above.

To confirm that hypercapnia (elevated P_{wCO_2}) rather than the reduction in water pH was initiating the observed breathing changes, ventilation of eight control fish was monitored in acidified (pH=6.3) normocapnic water. A pH of 6.3 was chosen because it corresponded to the pH reached at the highest degree of hypercapnia (P_{wCO_2} =3.5 mmHg). Approximately 15 l of water was titrated with 1 mol l⁻¹ HCl until the pH was lowered to 6.3. The water was then bubbled overnight with air having passed through a 10 mol l⁻¹ solution of KOH (which reduces the amount of CO₂ in air) and if necessary, the pH was adjusted to 6.3 the next day.

Ventilatory responses to acute hyperoxia

Ten fish were randomly selected from the 28-day control group and were not used in any other experiment. Four fish exhibiting episodic breathing and six fish displaying continuous breathing were subjected to acute hyperoxia. P_{O_2} within the fish chamber was continuously measured by a fibre optic O_2 electrode (see above). After breathing was assessed in normoxic water, the water supplying the chambers was bubbled with O_2 through a water–gas equilibration column using a gas mixer (Cameron Instrument Company GF-3/MP). Breathing was again analyzed once the water P_{O_2} had reached at least 300 mmHg.

Externally administered cyanide

In a separate series of experiments, groups of fish from each pre-exposure group were randomly chosen for this experiment. Different concentrations (0.5–200 $\mu\text{g ml}^{-1}$) of sodium cyanide (NaCN) dissolved in water were introduced into the water supplying the flow-through recording chambers, and then flushed with cyanide-free water within 30 s. The NaCN flows across the gills and interacts with externally oriented (water-sensing) chemoreceptors and consequently stimulates the O_2 chemoreceptors on the gill arches. After each injection, respiratory values were recorded for 1–2 min. The next dose was administered at least 10 min later. The doses of NaCN were determined based on pilot experiments in which dose–response curves for respiratory responses to NaCN were studied.

Confocal immunofluorescence microscopy

The basic protocols for gill extraction, immunolabeling and confocal imaging were modified from a previous study (Jonz and Nurse, 2003). Zebrafish were killed by overdose with anaesthetic (MS 222). Gill baskets were rinsed in 1 mol l^{-1} phosphate-buffered saline (PBS; pH 7.4) and fixed by immersion in 4% paraformaldehyde (prepared in PBS) at 4°C for 4 h. Fixed gills were rinsed in PBS and permeabilized for 24–48 h at 4°C in PBS containing 1% fetal calf serum and 0.5% Triton X-100 (pH 7.8).

Neuroepithelial cells (NEC) of gill filaments were identified in whole-mount preparations using antibodies directed against serotonin (5-HT; Jonz and Nurse, 2003) and against synaptic vesicle protein (SV2; Jonz and Nurse, 2003), found in neuronal and endocrine cells. Neurons and nerve fibres of the gill arches and developing filaments were identified using antibodies against a zebrafish-derived neuron-specific antigen (ZN-12; Trevarrow et al., 1990; Jonz and Nurse, 2003). Polyclonal rabbit 5-HT antibodies (Sigma) were used at a dilution of 1:200 and localized with goat anti-rabbit secondary antibodies Alexa 488 (1:600, Molecular Probes, Eugene, OR, USA). Monoclonal mouse anti-ZN-12 (Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) was used at a dilution of 1:25. Monoclonal mouse anti-SV-2 (Developmental Studies Hybridoma Bank, The University of Iowa, Department of Biological Sciences, Iowa City, IA 52242) was used at a dilution of 1:100. Both anti-mouse antibodies were localized

with goat anti-mouse secondary antibodies conjugated with Alexa 546 (1:400, Molecular Probes). All antibodies were diluted with PBS-TX. Fixed gill filaments were incubated in primary antibodies for 4 days at 4°C and in secondary antibodies at room temperature (22–24°C) for 1 h in darkness. Gill filaments were prepared as whole mounts on glass microscope slides in Crystal Mount™ (Sigma) or Vectashield® (Vector Laboratories, Inc, Burlingame, CA, USA).

Whole-mount gill preparations were examined with a confocal scanning system (Olympus BX50WI, Melville, NY, USA) equipped with an argon (Ar) laser. Images were collected using confocal graphics software (Fluoview 2.1.39, Melville, NY, USA). Each image obtained using a confocal scanning system is presented as a composite projection of serial optical sections size 0.3 μm . Image processing and manipulation was performed using Paint Shop Pro.

Gill baskets were also viewed using a Zeiss Axiphot light microscope and a digital Hamamatsu C5985 chilled CCD camera (East Syracuse, NY, USA). Images were captured using the Metamorph imaging system (Version 4.01).

The assessment of the number of chemoreceptors per filament and the percentage of the area occupied by chemoreceptors was done using Scion Image Beta 4.02 software (Frederick, MD, USA). Each gill was examined for the number of chemoreceptors on the filaments that were captured in their full length, and then the relative area of the filament was compared to the relative area of the chemoreceptors, as seen on the computer.

Statistical analysis

Ventilation frequencies and relative amplitudes from all experiments are reported as means \pm 1 standard error of the mean (s.e.m.). All data sets were analyzed using two-way

Table 1. Average breathing frequencies, number of pauses after return to normoxia, and chemoreceptors immunoreactive to 5HT (5HT-IR) per gill filament, of fish pre-exposed to hyperoxia, hypoxia or hypercapnia, as well as control fish

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

Values are means \pm s.e.m.; *N* values for each treatment are given in parentheses.

Resting pause frequency was measured in normoxia 3 h post exposure to 28 days hypoxia, hyperoxia or hypercapnia, and in control fish.

NECs, neuroepithelial cells.

*Significant difference between groups (one-way RM ANOVA, $P < 0.05$).

repeated-measures analysis of variance (ANOVA). If a statistical difference was identified, a *post hoc* multiple ('all pair wise') comparison test (Bonferroni's *t*-test) was applied. Where appropriate, some data were analysed by one-way ANOVA followed by Bonferroni's *t*-test (Table 1) or by unpaired Student's *t*-test (Fig. 3 and Fig. 7B). All statistical tests were performed using a commercial statistical software package (SigmaStat version 3.0).

Results

Validation of the method for recording breathing frequency and amplitude

To demonstrate that the voltage oscillations recorded from the water were indeed exclusively derived from opercular movements, recordings were obtained from fish rapidly anaesthetised in their chambers. The voltage oscillations that were observed in breathing fish (Fig. 1A) were eliminated by anaesthesia indicating that the heartbeat was not contributing to the recorded voltages (Fig. 1B). On the other hand, spontaneous swimming activity or struggling produced large voltage changes that obscured the underlying smaller oscillations associated with breathing (data not shown).

Breathing frequencies as determined from video recordings of the filmed fish were essentially identical to those calculated from the voltage oscillations acquired by computer (Fig. 2A). A correlation of the f_R data obtained by the two independent

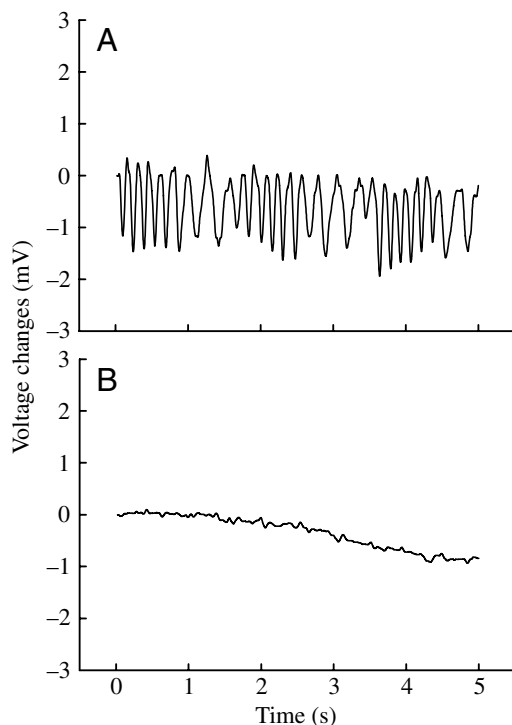


Fig. 1. Representative raw data acquisition recordings illustrating the voltage changes measured in the water of (A) a fish undergoing spontaneous breathing and (B) the same fish after *in situ* anaesthesia with benzocaine.

methods yielded a linear relationship ($r^2=0.99$) with a slope of 1 (Fig. 2A). To verify that the voltage changes were indicative of breathing amplitude, opercular displacement was measured from the video recordings and compared to the peak-to-peak differences in voltage acquired concurrently from the same fish. The data plotted in Fig. 2B demonstrate that the absolute values of ventilation amplitude varied markedly from those determined from the electronic tracings, generally being under-

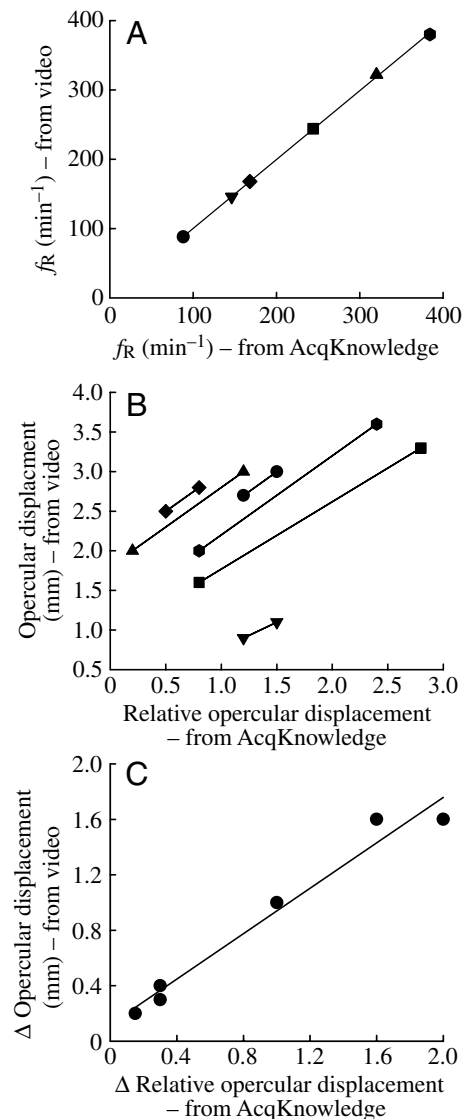


Fig. 2. The relationships between breathing parameters as measured by analysis of video recordings or from computerized data acquisition. (A) Correlation between breathing frequencies (f_R) determined by the two methods based on analysis of six different fish exhibiting a wide range of f_R ($r^2=0.999$, $y=0.986x+0.687$). (B) Correlation between opercular displacement (a measure of breathing amplitude) determined by the two methods during normocapnia and hypercapnia ($P_{wCO_2}=3.5$ mmHg). Each plot represents a change in amplitude of a single fish and each fish is represented by a different symbol ($N=6$). (C) Changes in ventilation amplitude during filming were analogous regardless of the method of measurement; data plotted are taken from B ($r^2=0.968$, $y=0.819x+0.119$); $N=6$.

estimated. Moreover, despite calibrating the set-up assuming that each fish, at rest, was exhibiting an opercular displacement of 1 mm, during actual experimentation, the ventilation amplitudes that were determined *via* computer for these same fish were highly variable, ranging from 0.2 to 1.2 mm (Fig. 2B). Nevertheless, a comparison of ventilation amplitudes obtained using the two methods demonstrated that ventilation amplitude was clearly related to the magnitude of the peak-to-peak voltage changes. Indeed, the correlation between changes in ventilation amplitude as measured by the two techniques was highly significant ($r^2=0.97$) yielding a slope of nearly 1 (0.82; Fig. 2C).

Pausing frequency

Episodic breathing during the 1–3 h pre-experimental period in normoxic/normocapnic water was exhibited by 20% of all zebrafish that were tested, and was composed of breathing and non-breathing (apnea or pause) periods. Episodic breathing in normal water was never observed in the fish that were pre-exposed to hypoxia or hypercapnia (Table 1); hyperoxia pre-exposure was without significant effect on the pattern of episodic breathing. The average number of pauses (apneic periods) in all control (seven out of 12 fish tested for acute

hypoxia) and hyperoxia pre-exposed fish was 19 and 12 min^{-1} , respectively ($P=0.095$). The total non-breathing period in control fish was $28.7\pm 5.2 \text{ s min}^{-1}$ compared to $35.5\pm 8.4 \text{ s min}^{-1}$ in the hyperoxia pre-exposed fish ($P=0.056$).

The pausing frequency in ten control fish exposed to acute hyperoxia was not increased significantly: average for the four fish exhibiting episodic breathing during normoxia = $12.0\pm 1.1 \text{ pauses min}^{-1}$ versus $13.0\pm 2.2 \text{ pauses min}^{-1}$ during hyperoxia (Fig. 3A) but the duration of time occupied by apnoeic periods was increased from 19.5 ± 2.22 to $43.1\pm 2.2 \text{ s min}^{-1}$ (Fig. 3B). During acute hyperoxia, 80% of fish exhibited episodic breathing. Representative original data recordings from fish during normoxia and acute hyperoxia are illustrated in Fig. 3C,D, respectively. In contrast to acute hyperoxia, acute hypoxia decreased the number of breathing pauses and the total duration of apnoeic periods in control and hyperoxia pre-exposed fish (Fig. 4). However, while pausing frequency and duration of apnea were decreased significantly in control fish at a P_{wO_2} of 130 mmHg and disappeared at 110 mmHg, the episodic breathing pattern in the hyperoxia pre-exposed fish disappeared only when P_{wO_2} had fallen below 70 mmHg (Fig. 4A,B). Unlike acute hyperoxia or hypoxia, acute hypercapnia (Fig. 4C,D) did not significantly alter episodic breathing.

Response to acute hypoxia and hypercapnia

Acute hypoxia and hypercapnia clearly influenced the breathing of zebrafish (Fig. 5). During progressively severe hypoxia, the breathing frequency increased, becoming statistically significant at a P_{wO_2} of 110 mmHg (Fig. 5A); ventilation amplitude was unchanged (Fig. 5B). Hypercapnia caused an increase in relative breathing amplitude at P_{wCO_2} levels as low as 1 mmHg (Fig. 5D); breathing frequency was unaffected (Fig. 5C). Because breathing amplitude did not change during acute hypoxia and frequency remained constant during acute hypercapnia (these responses were unaffected by pre-exposure), in subsequent experiments only a single index of breathing was presented (frequency during hypoxia and amplitude during hypercapnia).

To ascertain whether acidification of the water during hypercapnia was contributing to the increase in ventilation amplitude, experiments were performed on acidified water at constant P_{wCO_2} . Fish that were exposed to a change of pH from 7.4 to 6.3 (the reduction in pH associated with the highest level of

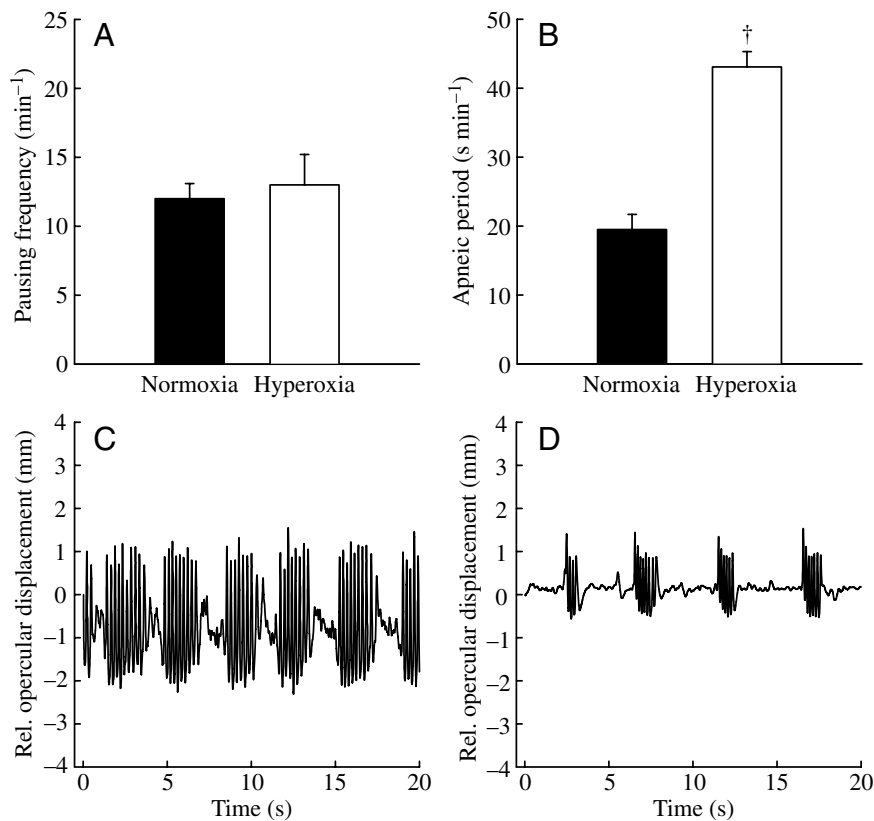


Fig. 3. (A) Frequency of breathing pauses and (B) proportion of total breathing occupied by apnea in s min^{-1} in zebrafish during normoxia (black bars; $N=4$ pausers out of 10 fish) and during hyperoxia (white bars; $N=8$ pausers out of 10 fish). (C,D) Representative original data recordings from normoxic (C) and hyperoxic fish (D). †Statistically significant difference ($P<0.05$) between the two groups.

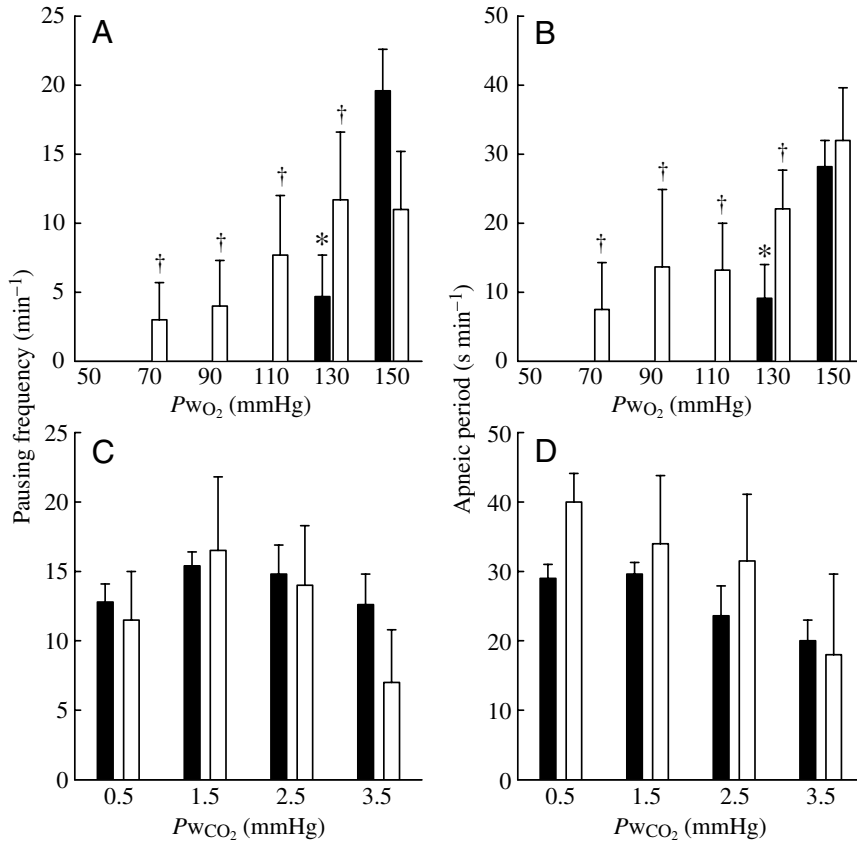


Fig. 4. The frequency of breathing pauses (A,C) and the proportion of total breathing occupied by apnea (B,D) in control (black bars, $N=7$) and hyperoxia pre-exposed (white bars, $N=8$) zebrafish *Danio rerio* exposed to acute hypoxia (A,B) or acute hypercapnia (C,D). Statistically significant differences ($P<0.05$) *within the groups; †between the two groups.

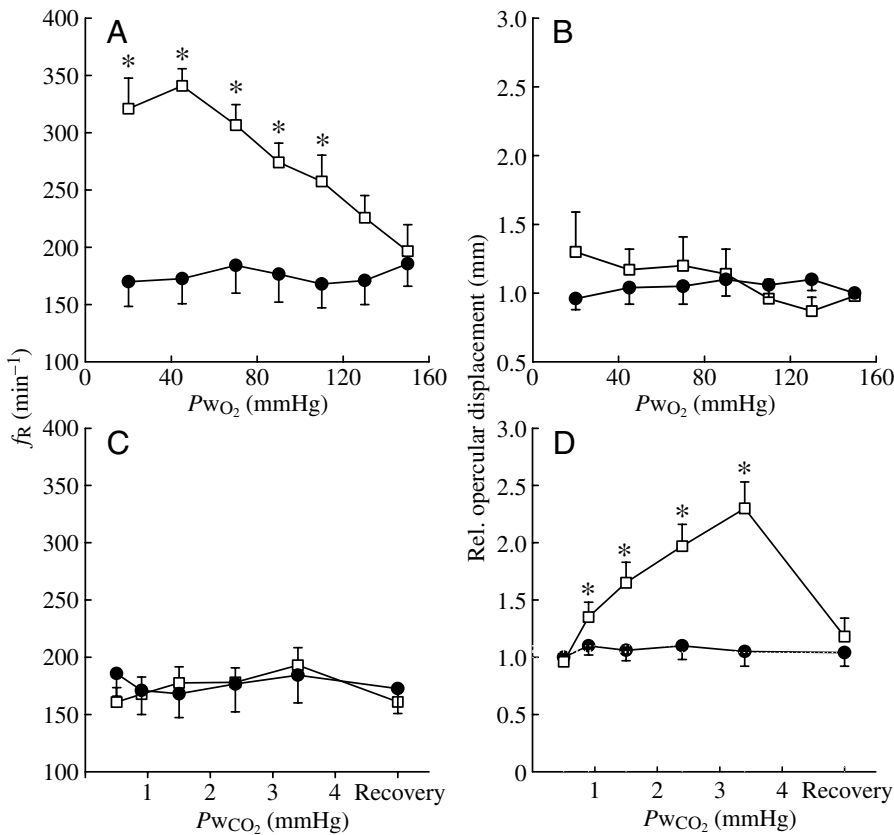
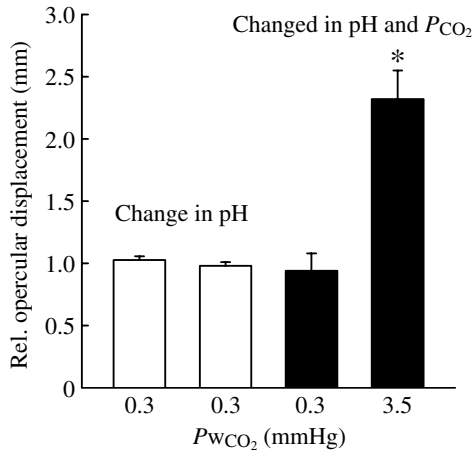


Fig. 5. Respiratory responses of zebrafish *Danio rerio* to acute hypoxia (A,B) or hypercapnia (C,D). Control fish were taken directly from the main zebrafish facility (filled circles, $N=12$) and were monitored in normal water for a similar period of time as the experimental fish (kept for 28 days in normoxic/normocapnic water) and acutely exposed to hypoxia or hypercapnia (unfilled circles, $N=9$ for hypoxia; $N=14$ for hypercapnia). (A,C) Changes in breathing frequency (f_R); (B,D) changes in relative breathing amplitude (opercular displacement). *Significant differences within the control or experimental groups; †significant differences between the control and experimental groups; two-way RM ANOVA ($P<0.05$).



hypercapnia) did not increase relative ventilation amplitude (Fig. 6).

Pre-exposure to hyperoxia

The respiratory responses of zebrafish that were pre-exposed to hyperoxia (≈ 350 mmHg for 28 days) to acute hypoxia, hypercapnia or external cyanide are depicted in Fig. 7. The breathing frequency response to hypoxia was significantly blunted. Indeed, a statistically significant increase in frequency was observed only when PwO_2 had fallen to 40 mmHg compared to 110 mmHg in the control fish. Furthermore, by re-plotting the data between PwO_2 values of 150 and 40 mmHg

Fig. 6. The effects on ventilation amplitude (relative opercular displacement) in zebrafish *Danio rerio* of changing water pH with, or without, accompanying hypercapnia. One group of fish (right) was exposed to an increase in $PwCO_2$ from 0.3 to 3.5 mmHg, causing pH to change from 7.4 to 6.3 (filled bars; $N=14$). Another group of fish (left; unfilled bars; $N=8$) was subjected to a change in water pH only from 7.4 to 6.3 at constant $PwCO_2$ of 0.3 mmHg. *A significant change in opercular displacement; one-way RM ANOVA ($P<0.05$).

as linear regressions and analyzing the slopes, it was possible to demonstrate a significant reduction in the rate at which ventilation frequency increased during hypoxia in the fish pre-exposed to hyperoxia (1.26 ± 0.19 versus 0.68 ± 0.16 breaths $\text{min}^{-1} \text{mmHg}^{-1}$ in control and hyperoxic fish, respectively, Fig. 7B). The breathing amplitude responses to hypercapnia were eliminated in fish pre-exposed to hyperoxia (Fig. 7C) and the response to external cyanide was blunted (Fig. 7D).

Immunocytochemistry results (representative pictures shown on Fig. 8) demonstrated that the numbers of 5HT-positive cells on the filament were significantly reduced (one-way ANOVA, $P=0.04$) in the fish pre-exposed to hyperoxia compared to other groups of fish (Table 1).

Pre-exposure to hypoxia

The respiratory responses of zebrafish pre-exposed to hypoxia (30 mmHg for 28 days) to acute hypoxia, hypercapnia or cyanide were essentially equivalent to the control fish (Fig. 9). Although the response to hypoxia appeared to be blunted (Fig. 9A), the lower breathing frequencies were likely related to a lower frequency at rest (although the resting values were not statistically significant for this particular group of fish; $P=0.052$). Re-plotting the data in Fig. 9A as linear regressions demonstrated that the rate of change of breathing frequency during hypoxia up to 40 mmHg was similar in both groups of fish (1.35 ± 0.22 for hypoxia pre-exposed

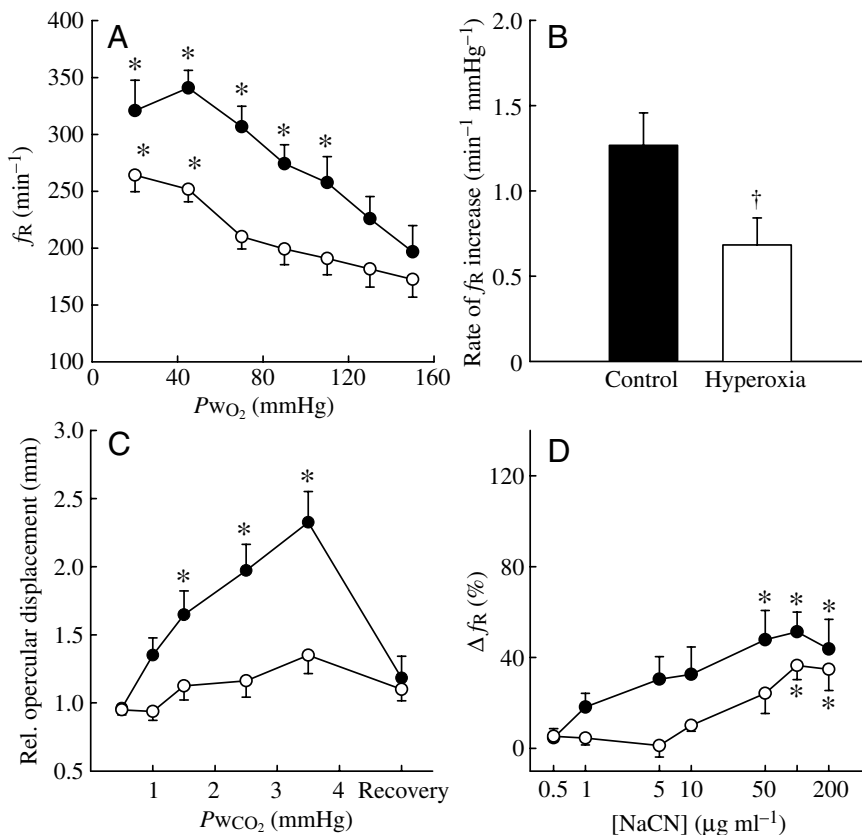


Fig. 7. (A,C,D) The respiratory responses of zebrafish *Danio rerio* pre-exposed to hyperoxia ($PwO_2 > 350$ mmHg) for 28 days (unfilled circles) to (A) acute hypoxia ($N=12$), (C) hypercapnia ($N=8$) or (D) sodium cyanide ($N=7$) compared to the responses of control fish (filled circles; $N=12$ different fish for each treatment). (B) Average rates of change of breathing frequency (f_R) between 40 (Control) and 155 mmHg (Hyperoxia) for the two groups. *Significant differences within the control or experimental groups; †significant differences between the control and experimental groups; two-way RM ANOVA ($P<0.05$).

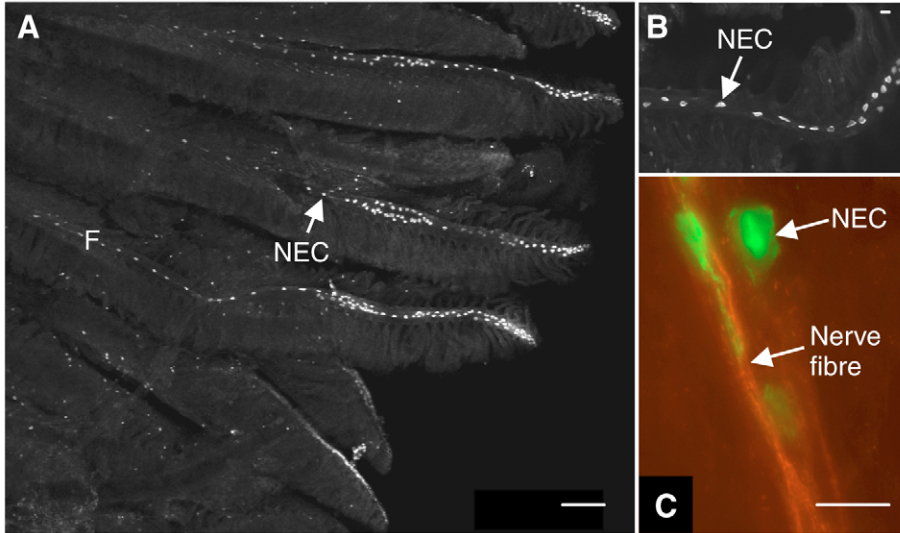
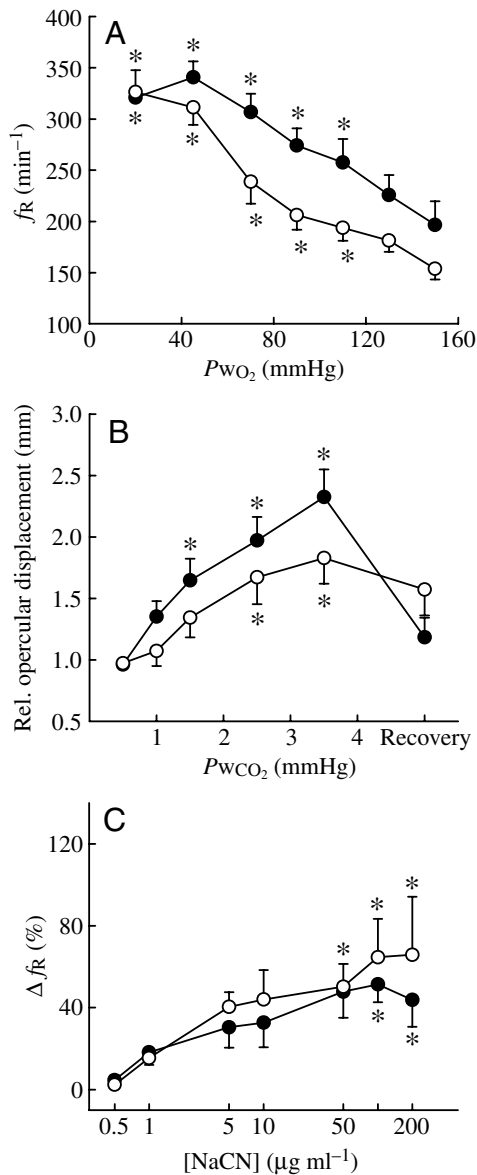


Fig. 8. Serotonin-immunoreactive (5-HT-IR) neuroepithelial cells (NECs) of the gill filament (F) in zebrafish *Danio rerio*. (A) 5-HT-IR NECs along the filament in a control fish; (B) 5-HT-IR NECs along the filament at higher magnification in a hyperoxia pre-exposed fish; (C) higher magnification of double labeled 5-HT-IR with associated nerve fibers (ZN-12-IR) of the proximal filament epithelium in a control fish. Scale bars, 100 μm (A); 10 μm (B,C).



compared to 1.26 ± 0.19 breaths $\text{min}^{-1} \text{mmHg}^{-1}$ in controls; data not shown). An analysis of all fish pre-exposed to hypoxia (Table 1) revealed a significant reduction in breathing frequency of 31 min^{-1} ($P=0.044$).

Pre-exposure to hypercapnia

Except for increased breathing frequency at a P_{wO_2} of 20 mmHg, the zebrafish pre-exposed to hypercapnia (9 mmHg for 28 days) displayed similar responses to acute hypoxia as the control fish (Fig. 10A). The ventilatory response to hypercapnia was blunted (Fig. 10B) if one considers that breathing amplitude was not significantly elevated until the final stage (3.5 mmHg) was reached. The response to cyanide was significantly increased in the fish pre-exposed to hypercapnia (Fig. 10C).

Discussion

Critique of the technique used to measure breathing

In the present study, the basic method of Altimiras and Larsen (Altimiras and Larsen, 2000) was exploited to record non-invasively the f_R and relative breathing amplitude in adult zebrafish. By comparing the computer-acquired data with video recordings taken of the same fish, it was demonstrated that the voltage oscillations measured in the water were an accurate and reproducible index of f_R . Although this procedure, when slightly modified, can be used to measure cardiac frequency (Altimiras and Larsen, 2000), there was no obvious contribution of the heart contraction to the measured voltage

Fig. 9. The respiratory responses of zebrafish *Danio rerio* pre-exposed to hypoxia ($P_{\text{wO}_2}=30 \text{ mmHg } P_{\text{O}_2}$) for 28 days (unfilled circles) to (A) acute hypoxia ($N=8$), (B) hypercapnia ($N=7$) or (C) sodium cyanide ($N=6$) compared to the responses of control fish (filled circles, $N=12$ different fish for each treatment). *Significant differences within the control or experimental groups; †significant differences between the control and experimental groups; two-way RM ANOVA ($P<0.05$).

changes based on a comparison of the data from breathing and non-breathing (anaesthetized) fish. The technique also proved to be a reliable indicator of breathing amplitude, assuming that opercular displacement reflects ventilatory stroke volume. However, because there is no simple method to measure the absolute magnitude of opercular displacement in resting fish, the equipment could not be calibrated to obtain absolute data for breathing amplitude. Thus, without further refinement, this method is only suited to monitor relative changes in breathing amplitude. In the present experiments, resting opercular

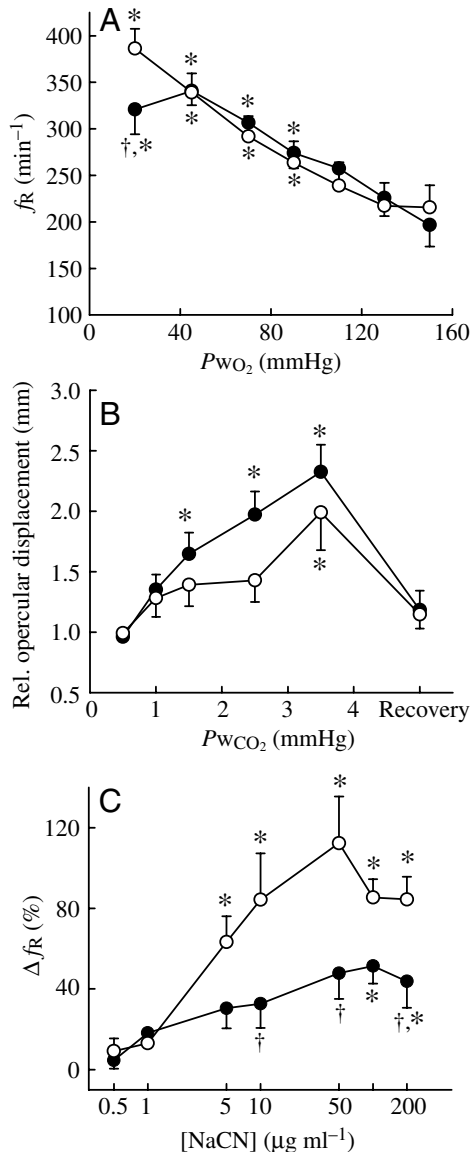


Fig. 10. The respiratory responses of zebrafish *Danio rerio* pre-exposed to hypercapnia ($PwCO_2=9$ mmHg) for 28 days (unfilled circles) to (A) acute hypoxia ($N=11$), (B) hypercapnia ($N=11$) or (C) sodium cyanide ($N=8$) compared to the responses of control fish (filled circles, $N=12$ different fish for each treatment). *Significant differences within the control or experimental groups; †significant differences between the control and experimental groups; two-way RM ANOVA ($P<0.05$).

displacement was assumed to be 1 mm in all fish regardless of body mass and breathing frequency and thus 1 mm was selected as the calibration factor for converting the peak-to-peak voltage oscillations to measures of linear displacement. However, as the data in Fig. 2B show, there can be a marked difference in the absolute values of opercular displacement from those calculated using the online recording system. The other significant limitation with this technique is that any swimming movements beyond those required for the fish to remain stationary often obscure the signals associated with breathing. Thus, reliable data could only be obtained from fish that were stationary within their chambers. In our experience, similar problems are also encountered when attempting to analyze breathing motions from video recordings when zebrafish are swimming or struggling.

Having validated the use of the non-invasive recording technique to monitor f_R and relative opercular displacement, subsequent experiments were designed to evaluate the pattern of breathing in resting zebrafish and the impact of long-term acclimation to environments of altered gas composition.

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

Under resting conditions, 20% of the fish examined in this study exhibited episodic breathing, characterized by periods of regular breathing interspersed with periods of apnea or breathing pauses. Episodic (or periodic) breathing has been described under resting conditions in all vertebrates (Milsom, 1991), including water-breathing fish (Smith et al., 1983; Nonnotte et al., 1993; Reid et al., 2003). Most water-breathing fish that have been examined, however, exhibit continuous breathing during normoxia, although under conditions of lowered respiratory drive (e.g. hyperoxia), episodic breathing may occur (see review by Milsom, 1991). In the present study, pausing frequency was increased by hyperoxia and decreased by hypoxia. These findings reinforce previous studies (e.g. Reid et al., 2003) and are consistent with the view that episodic breathing is shaped, at least in part, by afferent input from peripheral chemoreceptors (see review by Smatresk, 1990). The original findings of the present study were that (i) long-term acclimation to hypoxia or hypercapnia abolished episodic breathing (51 fish were assessed in normal water) and (ii) acclimation to hyperoxia postponed the disappearance of episodic breathing in fish exposed to acute hypoxia. Because all studies were performed at least 3 h after acclimated fish had been returned to normal water, the changes in breathing patterns presumably reflect a continuing effect of the prior acclimation. Thus, if driven by changes in afferent sensory input from peripheral chemoreceptors, the effects appear to endure for at least 3 h after the chemoreceptors are once again experiencing normoxic and normocapnic conditions. Further support for a long-term effect on breathing patterns was the fact that episodic breathing continued in fish acclimated to hyperoxia even under conditions of increased respiratory drive (hypoxia). It would be interesting to determine the length of time required to re-establish normal breathing patterns.

Acute respiratory responses to hypoxia or hypercapnia

As reported for other fish species, zebrafish displayed an increase in f_R in response to acute hypoxia (see table 1 in Gilmour, 2001). Interestingly, hypoxia did not elicit an accompanying increase in ventilation amplitude. This pattern of response to hypoxia is different from that observed in most fish that have been studied in which both f_R and amplitude increase (Shelton et al., 1986). Although the common carp *Cyprinus carpio* also does not display an increase in ventilation amplitude during hypoxia (Soncini and Glass, 2000), this response does not appear to be shared by all Cyprinids because the tench *Tinca tinca* increases both f_R and opercular amplitude during hypoxia (Hughes and Shelton, 1962). Furthermore, the absence of a ventilation amplitude response to hypoxia in zebrafish cannot be attributed to the high resting f_R because a different group of fish from the same population fish exhibited a pronounced increase in opercular displacement during hypercapnia (see below).

External injections of NaCN (to pharmacologically stimulate O_2 chemoreceptors) also caused an increase in f_R without altering breathing amplitude. This confirms that, in this species, at least part of the hyperventilatory response to hypoxia is being mediated by branchial (most likely external) O_2 chemoreceptors (see reviews by Burlinson and Milsom, 1995; Fritsche and Nilsson, 1993). Recently, Jonz et al. (Jonz et al., 2004) provided direct evidence that the neuroepithelial cells of the zebrafish gill respond to hypoxia in a similar fashion as the glomus cells of the mammalian carotid body and thus are likely to be the O_2 sensors of the fish gill.

This is the first study to examine the respiratory response of zebrafish to hypercapnia. As documented for other species (see table 2 in Gilmour, 2001), zebrafish responded to hypercapnia by increasing ventilation amplitude. However, unlike in the majority of species previously examined, zebrafish did not display a concomitant increase in f_R (Gilmour, 2001). Recent evidence suggests that the cardiorespiratory responses of fish to elevated CO_2 are initiated largely by external branchial receptors that respond to changes in ambient P_{CO_2} rather than pH (Burlinson and Smatresk, 2000; McKendry et al., 2001; McKendry and Perry, 2001; Perry and Reid, 2002; Gilmour et al., 2005). The results of the present study provided additional evidence that it is the change of P_{wCO_2} and not pH that is responsible for increasing breathing amplitude during hypercapnia.

It is believed that the glomus cells of the mammalian carotid body sense changes in both P_{O_2} and P_{CO_2} and thus act as combined O_2/CO_2 chemoreceptors (Gonzales et al., 1994; Zhang and Nurse, 2004; Prabhakar and Jacono, 2005). It is not known whether the O_2 -sensing neuroepithelial cells of the fish gill are also able to detect changes in P_{CO_2} . Although the ventilatory responses to hypoxia and hypercapnia in zebrafish were markedly different (increased f_R during hypoxia; increased amplitude during hypercapnia), this does not exclude the presence of a single receptor type sensing both O_2 and CO_2 . Indeed, it is plausible that stimulation of single cell type could be linked to varied responses, given that the nature of the

response is likely to be dictated by downstream signal transduction pathways.

Acclimation to hyperoxia

This is the first study to assess the impact of long-term acclimation to hyperoxia on ventilatory reflexes in fish. The results demonstrated that exposure to hyperoxia for 28 days blunted the subsequent ventilatory response to hypoxia, external cyanide and hypercapnia. Long-term hyperoxia is known to cause a similar attenuation of the carotid body chemosensitivity to hypoxia and cyanide in cats (Lahiri et al., 1987; Lahiri et al., 1990) and rats (Arieli et al., 1988) but is apparently without effect on humans (Gelfand et al., 1998), even when the levels of hyperoxia approach the limits of toxicity. In those mammals exhibiting a hyperoxic blunting of the ventilatory response to hypoxia, the response to hypercapnia is sustained (Torbaty et al., 1989), reduced (Lahiri et al., 1990) or even enhanced (Lahiri et al., 1987). The continuance of CO_2 sensitivity in the face of a severe blunting or abolishment of the response to hypoxia has led to the idea that hyperoxia in mammals specifically targets O_2 -sensing mechanisms of the carotid body. The carotid body also retains its usual responsiveness to nicotine or dopamine following hyperoxia (Lahiri et al., 1987; Lahiri et al., 1990), further suggesting that the blunting effects of hyperoxia are not caused by general cellular damage. In the present study, the reflex hyperventilatory response to hypercapnia was eliminated (at least statistically; Fig. 7C) by prior exposure to hyperoxia. The coincident inhibitory effects of hyperoxia on O_2 - and CO_2 -mediated reflexes suggest that a common element of chemoreception is being affected. The simplest explanation is that the NECs, functioning as dual O_2 and CO_2 sensors, are being influenced by hyperoxia. In support of this idea, the density of gill filament NECs was significantly reduced after 28 days of hyperoxia. Thus, we speculate that the sensitivity of the ventilatory response to hypoxia or hypercapnia is controlled, at least partially, by the numbers of NECs exposed to the inspired water. This theory does not exclude the possibility that other levels of levels of respiratory control are being impacted by hyperoxia.

Acclimation to hypoxia

Previous experiments evaluating respiratory acclimation to hypoxia have focused on mammals. The results have demonstrated that the hypoxic ventilatory response (HVR) is either increased after continuous chronic hypoxia (Weil, 1986; Bisgard and Forster, 1996; Dwinell and Powell, 1999) or unaffected (Powell et al., 2000). To date, only a single study has investigated the respiratory consequences of chronic hypoxia in fish (Burlinson et al., 2002). The results of that study on catfish *Ictalurus punctatus* showed that 7 days of acclimation to moderate hypoxia caused an increase in f_R and increased sensitivity to hypoxia. In contrast, the results of the present study using zebrafish revealed a significant reduction of resting f_R without any effect on the ventilatory responsiveness to hypoxia or cyanide. The lack of an effect

of hypoxia acclimation on the acute responses to hypoxia or cyanide is consistent with the finding that the density of gill filament NECs was unaltered by chronic hypoxia. In a previous study, Jonz et al. (Jonz et al., 2004) also showed that the numbers of 5-HT positive NECs were unchanged by 60 days of hypoxic exposure (35 mmHg) although their size was increased by 15%. However, by combining a marker for synaptic vesicle protein (SV-2), it was demonstrated that the total number of NECs (5-HT-positive and 5-HT-negative) was increased by chronic hypoxia (Jonz et al., 2004). Whether or not the 5-HT-negative NECs are also capable of sensing O₂ is unclear. Several explanations for the reduced breathing frequency in the fish exposed to chronic hypoxia can be offered. First, an enhancement of O₂ transfer and blood O₂ transport (Perry and Wood, 1989; Nikinmaa, 2001) may result in a lowering of the ventilatory convection requirement. Second, the return of the fish to normoxia after 28 days in hypoxic water may result in the perception of a state of relative hyperoxia by the branchial O₂ chemoreceptors.

Acclimation to hypercapnia

In mammals, studies suggest that pre-exposure to hypercapnia does not alter the response to acute hypoxia or hypercapnia (Bisgard and Forster, 1996; Remmers and Lahiri, 1998; Kondo et al., 2000). Like in mammals, the ventilatory response of zebrafish to hypoxia was unaltered by chronic hypercapnia although the response to external cyanide was increased and the response to hypercapnia was modestly blunted. The attenuation of the breathing response to cyanide without affecting the hypoxic response is particularly interesting and suggests that hypercapnia might be influencing the responsiveness of external branchial O₂ chemoreceptors (thus explaining an enhanced response to cyanide) without affecting, or even possibly reducing, the responsiveness of internal O₂ receptors. Although the numbers of branchial filament 5-HT-positive NECs were unaffected by chronic hypercapnia, we cannot exclude the possibility that 5-HT-negative NECs were being targeted. Unfortunately, the single laser confocal microscope used for this study did not allow us to co-localize 5-HT and SV-2 and thus we were unable to identify the 5-HT-negative/SV-2-positive cells.

Perspectives

Currently, it is unknown whether the gill NECs, demonstrated to be responsive to hypoxia (Jonz et al., 2004) and sharing similar properties with the glomus cells of the carotid body, are also able to sense changes in ambient P_{CO₂}. Although no direct evidence was provided in this study, the finding that hyperoxia blunted the breathing responses to hypercapnia (as well as hypoxia), together with the result that hypercapnia enhanced the response to cyanide, suggests a certain degree of interaction between O₂- and CO₂-sensing and suggest that the NECs may be acting as both O₂ and CO₂ chemoreceptors. Clearly this is an area that warrants further investigation.

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