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Effect of aerial O_2 partial pressure on bimodal gas exchange and air-breathing behaviour in *Trichogaster leeri*

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Summary

The effects of experimental alterations of aerial O_2 partial pressure ($P_{O2,air}$) on bimodal gas exchange and air-breathing behaviour were investigated in the aquatic air-breathing fish *Trichogaster leeri* in normoxic water. Fish responded to increasing $P_{O2,air}$ by decreasing air-breathing frequency, increasing aerial O_2 consumption rate ($\dot{V}_{O2}$), increasing mean O_2 uptake per breath ($\dot{V}_{O2}$/breath) and decreasing aquatic $\dot{V}_{O2}$ to maintain a constant total $\dot{V}_{O2}$. The rate of oxygen uptake from the air-breathing organ (ABO) during apnoea ($\dot{V}_{O2,ap}$) was derived on a breath-by-breath basis from V_{O2}/breath and apnoea duration. $\dot{V}_{O2,ap}$ and estimates of ABO volume were used to calculate the P_{O2} in the ABO at the end of apnoea. This increased with increasing $P_{O2,air}$, suggesting that ABO-P_{O2} is not regulated at a constant level by internal chemoreceptors. Furthermore, mean $\dot{V}_{O2,ap}$ increased with increasing $P_{O2,air}$, indicating that the observed increase in $\dot{V}_{O2}$/breath with increasing $P_{O2,air}$ was facilitated not only by an increase in apnoea duration but also by an increase in the air–blood P_{O2} gradient.

Key words: fish, respiration, air-breathing, bimodal gas exchange, aerial O_2, air-breathing organ, O_2 chemoreceptor.

Introduction

Ventilation in bimodal fish (i.e. those that respire simultaneously in air and water) is characterised by variable branchial ventilation patterns and intermittent and arrhythmic air breathing (Shelton et al., 1986). It has been suggested therefore that air breathing in these animals is an on-demand phenomenon that is stimulated by afferent feedback from one or more peripheral receptors (Shelton et al., 1986). Studies on the air-breathing lungfish *Protopterus* and gar *Lepisosteus* show that O_2 chemoreceptors are located diffusely throughout the gills, as is the case for most fish, and evidence points to the existence of both internally and externally oriented O_2 chemoreceptors that respond to changes in blood O_2 partial pressure (P_{O_2}) and aquatic P_{O_2}, respectively (Johansen and Lenfant, 1968; Lahiri et al., 1970; Smatresk, 1986).

Since there is evidence to suggest that chemoreceptors are located at the site of aquatic respiration in air-breathing fish, it is reasonable to contemplate the existence of chemoreceptors at the site of aerial respiration, i.e. within the air-breathing organ (ABO). A common method used to identify sites of chemoreceptors in conscious air-breathing fish is to observe behavioural responses to independent changes in aquatic and aerial gas contents (e.g. Burggren, 1979; Graham et al., 1995; Hedrick and Jones, 1993; Hughes and Singh, 1970; Johansen and Lenfant, 1968; Sanchez et al., 2001). Studies involving manipulations of aquatic O_2 content by far exceed studies involving manipulations of aerial O_2 content. However, experimental manipulations of aerial O_2 content have the potential to offer insight into the presence and role of ABO-O_2 chemoreceptors. Air-breathing fish generally respond to aerial hypoxia by reducing apnoea (i.e. breath-hold) duration, whereas hyperoxia often lengthens it (Shelton et al., 1986). However, conclusions arising from experiments involving aerial O_2 manipulations are limited in that the activity of chemoreceptors within the ABO cannot be discriminated from those located remotely, in the efferent vasculature (Graham, 1997). Simulated breathing by injection of either hypoxic or hyperoxic gas into the ABO produces the same results as changes in aerial gas composition (e.g. Johansen and Lenfant, 1968). However, gas injection also increases the volume of the ABO, and such experiments may therefore be confounded by activation of ABO mechanoreceptors that transmit information about the rate or extent of organ wall deformation (Milsom, 1990; Pack et al., 1990).

Evidence for ABO-O_2 chemoreceptors was found for the swamp eel, *Monopterus albus*, by Graham et al. (Graham et al., 1995). *M. albus* was observed to expel severely hypoxic or anoxic breaths within a few seconds of inspiration. The rapidity

of the gas-voiding reflex suggested the presence of an ABO chemoreceptor, because it occurred about two to four times faster than would be expected if O_2 levels in the ABO had to be conveyed by blood flow to remote vascular receptors located somewhere in the systemic circulation. By contrast, the response of the blue gourami, *Trichogaster trichopterus*, to changes in aerial P_{O_2} was not immediate, suggesting that chemoreceptors were more centrally located (Burggren, 1979).

The present study investigates the effect of aerial O_2 partial pressure ($P_{O_2,air}$) on bimodal gas exchange and air-breathing behaviour in an air-breathing fish, the pearl gourami, *Trichogaster leeri* Bleeker 1852 (sub-order Anabantoidei, family Belontiidae). *T. leeri* is a freshwater pelagic fish that has a pair of suprabranchial chambers serving as its ABO (Peters, 1978). Changes in air-breathing frequency (f_{ab}), aerial and aquatic O_2 consumption rate ($\dot{V}_{O_2}$) and mean O_2 uptake per breath (V_{O_2}/breath) are analysed. Using a novel approach, apnoeic $\dot{V}_{O_2}$ ($\dot{V}_{O_2,ap}$=rate of O_2 uptake from the air within the ABO while the fish is submerged) is derived by a breath-by-breath assessment of O_2 uptake and apnoea duration. $\dot{V}_{O_2,ap}$ is used to address two questions: (1) is the response of *T. leeri* to changes in $P_{O_2,air}$ regulated by ABO-O_2 chemoreceptors and (2) is a change in mean V_{O_2}/breath with changing $P_{O_2,air}$ facilitated by a change in the air–blood P_{O_2} gradient as well as a change in apnoea duration?

To address the first question, $\dot{V}_{O_2,ap}$ and estimated measurements of ABO volume (V_{ABO}) are used to calculate the P_{O_2} in the ABO at the end of apnoea. It is hypothesised that if the P_{O_2} in the ABO is regulated by ABO-O_2 chemoreceptors, then the P_{O_2} in the ABO at the end of apnoea should not be significantly different across treatments of varying $P_{O_2,air}$. This would suggest the existence of an ABO-P_{O_2} threshold, which, once reached, triggers fish to renew the gas in their ABO.

The second question arises because O_2 uptake during apnoea may change simply as a result of changes in apnoea duration, i.e. V_{O_2}/breath may increase during aerial hyperoxia because *T. leeri* holds its breath for longer. However, a change in the air–blood P_{O_2} gradient may also facilitate O_2 uptake during apnoea. Thus, it is hypothesised that if a change in apnoea duration is solely responsible for a change in mean V_{O_2}/breath then $P_{O_2,air}$ will not have an effect on mean $\dot{V}_{O_2,ap}$. This is because mean $\dot{V}_{O_2,ap}$ accounts for variations in apnoea duration between treatments, and thus if mean $\dot{V}_{O_2,ap}$ changes with changing $P_{O_2,air}$ then a change in the air–blood P_{O_2} gradient must also be responsible. To our knowledge, this is the first study to examine how changes in the air–blood P_{O_2} gradient associated with aerial hypoxia and hyperoxia influence O_2 consumption in an air-breathing fish.

Materials and methods

Experimental animals

Experiments were performed on seven adult pearl gourami *Trichogaster leeri* Bleeker 1852 (wet mass 5.15±0.97 g; length 59.1±1.2 mm; means ± s.d.) that were obtained from a local aquarium supplier. Fish were maintained in aquaria filled with dechlorinated Adelaide tap water at 25°C. All fish were maintained under these conditions for at least one year prior to experimentation.

Aerial respirometry

Prior to experimentation, food was withheld for 24 h and fish were individually placed in 1 litre bottles (Schott Duran, Germany) that were covered in black plastic to minimise stress. Bottles containing fish were held in a constant-temperature water bath maintained at 25±1°C by a heater (Thermomix 1419; B. Braun, Melsungen, Germany). Fish were allowed 12 h to acclimate to the chambers and to recover from handling prior to each experiment. An air stone gently aerated the water during the acclimation period.

Respiratory studies were conducted with an open-flow respirometer system. O_2 and N_2 were supplied from compressed cylinders (BOC Gases, Adelaide, Australia) and delivered to the respirometry chamber at a desired O_2 content ($F_{I_{O_2}}$) and flow rate ($\dot{V}$I; 100, 150 or 200 ml min^{-1}). The flow rate for each trial was selected to minimise the washout time while providing reliably detectable changes in the excurrent O_2 content ($F_{E_{O_2}}$). O_2 content and flow rate were controlled by mass flow controllers (model GFC171; Aalborg Instruments and Controls, New York, NY, USA; 0–1 l min^{-1}, rated accuracy ±15 ml min^{-1}) using a PC running digital–analogue control software and hardware (PowerDAQ™ PD2-AO and ProfessorDAQ™; United Electronic Industries, Canton, MA, USA). Flow controllers were calibrated to each gas with a 3.5 litre calibrator (model 1057A Vol-U-Meter Calibrator; Brooks Instruments, Hatfield, PA, USA; accuracy of calibrated controllers was better than 5% of reading, usually 1–2% of reading). To ensure uniform mixing of O_2 and N_2, these gases were passed through a 1 litre, rigid mixing chamber before entering the respirometer. Nevertheless, the O_2 level was not as constant as expected in normal open-flow respirometry with atmospheric air, so the variability had to be accounted for in the analysis (see below).

Upon exiting the respirometry chamber, gases passed through a U-tube containing Drierite™ (Hammond Drierite Co. Ltd, Xenia, OH, USA) to remove water vapour and into a differential O_2 analyser (FC-2 Oxzilla I; Sable Systems International, Las Vegas, NV, USA). The analyser was calibrated using dried (Drierite™), CO_2-free (Ascarite™; Arthur H. Thomas Company, Swedesboro, NJ, USA) atmospheric air (0.2095 O_2). The analyser measured $F_{E_{O_2}}$ approximately every 1.25 s and used a running five-sample average for each data point. The data output from the O_2 analyser was received simultaneously in an analogue and digital format. The analogue output was recorded at 2 s intervals with a digital multimeter (model TX3 True RMS and WaveStar™ Version 2.2; Tektronix, Beaverton, OR, USA) interfaced with a PC *via* the RS232 port. Depending on the analogue output scaling chosen, these voltage values were converted to $F_{E_{O_2}}$ using relationships provided by Sable Systems International. The Oxzilla digital output was received by a DOS terminal program (SERIN; Sable Systems International) and recorded at intervals of approximately 1.1 s

on a second PC. For analyses, WaveStar data were preferred due to SERIN's unstable timing interval, but SERIN data were used in two cases where high-frequency noise in the analogue data signal prevented reliable detection of breaths.

Experimental protocol

Aerial P_{O_2} ($P_{O_2,air}$) in the respirometry chamber was manipulated to nominal levels of 5, 10, 21 (control), 40 or 60 kPa (actual $P_{O_2,air}$ range: 5.4–5.5, 9.6–10.1, 19.9–20.7, 38.4–39.0 and 57.5–57.9 kPa, respectively). Treatments were executed in random order and an arbitrary exposure time of 1 h was set so as to reduce the effect of declining aquatic P_{O_2}. However, due to the instability of $F_{E_{O_2}}$, some fish were exposed for longer than 1 h (maximum exposure time was 3 h), in order to accumulate 1 h worth of interpretable data. During trials, aquatic P_{O_2} dropped 2.3, 1.8, 1.5, 1.2 and 0.6 kPa h^{-1} in the 5, 10, 21, 40 and 60 kPa treatments, respectively. To determine whether declining aquatic P_{O_2} had an effect on aerial respiration, f_{ab}, aerial $\dot{V}_{O_2}$ and $\dot{V}_{O_2}$/breath in the first and final 15 min of each trial were compared. In each case, there was no significant difference between the first and final 15 min (P=0.22, 0.94 and 0.56, respectively). Following exposure to each treatment, fish were given at least 24 h rest before the next treatment. During this time, either the water in the chamber was aerated with an air stone or the fish were returned to their aquarium.

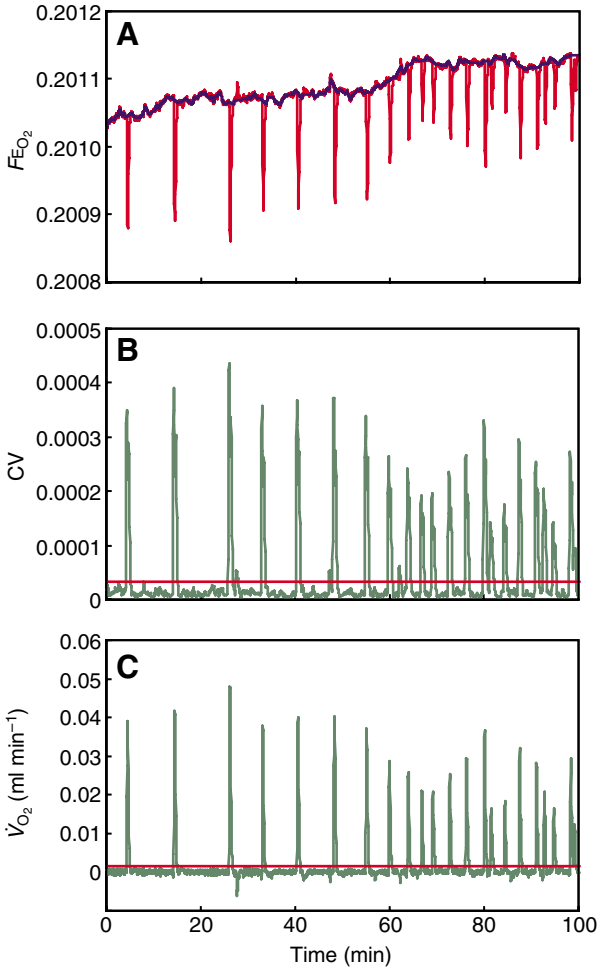

Fig. 1. (A) An example of the recorded fractional O_2 content of the excurrent air from the respirometer ($F_{E_{O_2}}$) (solid red line) over time. (B) The calculated coefficient of variation (CV=standard deviation divided by mean) (solid green line). A chosen CV threshold (red line in B) was used to derive the $F_{I_{O_2}}$ baseline (blue line in A). (C) Calculated rate of aerial O_2 consumption ($\dot{V}_{O_2}$) (green line) for each pair of recorded $F_{E_{O_2}}$ and derived $F_{I_{O_2}}$ values. A $\dot{V}_{O_2}$ threshold (red line) was chosen to separate breaths from noise.

Data processing

Calculation of aerial O_2 consumption rate (aerial $\dot{V}_{O_2}$; ml h^{-1}) was by integration of $F_{E_{O_2}}$ inverted spikes. Each spike represented one exhalation, confirmed by visual observation. The duration of the spike depended on the washout characteristics of the respirometry system. Because these occurred on an unstable baseline (Fig. 1), a spreadsheet method was devised to isolate the spikes from the baseline. To select data representing periods of apnoea, a coefficient of variation (CV=s.d./mean) was calculated for 15 consecutive measurements, and a CV threshold value (e.g. 3.3×10^{-5}) was manually set at a level that separated high-frequency, low-amplitude $F_{E_{O_2}}$ baseline noise from air-breathing events that were low frequency, high amplitude (Fig. 1B). Values below this threshold were considered to be baseline values, and, for every value above the threshold, a new baseline was linearly interpolated between previous and subsequent sub-threshold values. Where breaths were too frequent for the program to isolate values representing baseline $F_{E_{O_2}}$, a polynomial regression was fitted to the baseline trace and entered in place of the CV threshold criteria. The final baseline was then produced by completing two rounds of 9-point nearest-neighbour averaging to remove high-frequency noise (Keller et al., 1994). The resulting baseline was considered to represent incurrent oxygen content ($F_{I_{O_2}}$) and accounts for any exchange between the air and water in the system (Fig. 1A). Aerial $\dot{V}_{O_2}$ was then calculated for each data point (Fig. 1C) from the air flow rate through the chamber ($\dot{V}_I$; ml min^{-1}) and the respiratory quotient (RQ) according to Depocas and Hart (Depocas and Hart, 1957):

$$\dot{V}_{O_2} = [\dot{V}_I (F_{I_{O_2}} - F_{E_{O_2}})] / [1 - F_{E_{O_2}} (1 - RQ)] . \quad (1)$$

We assumed a respiratory quotient of 0.25, as measured in the closely related blue gourami, *Trichogaster trichopterus*, under normoxic conditions (Burggren, 1979).

Each breath was integrated individually to arrive at a breath-by-breath estimate of O_2 uptake from the ABO during the apnoeic period (V_{O_2}/breath; ml). This was summed across the trial and divided by trial duration to calculate aerial $\dot{V}_{O_2}$. Additionally, this approach measured f_{ab} (breaths h^{-1}) by tallying the number of breaths during a trial and dividing by trial duration.

Aquatic respirometry

To evaluate the partitioning of aerial and aquatic respiration, aquatic O_2 consumption (aquatic $\dot{V}_{O_2}$; ml h^{-1}) was measured.

For technical reasons, this was measured separately from aerial respirometry studies. Prior to experimentation, fish were treated in the same manner as they were for aerial respirometry. The aerial gas mix was produced as previously described but was vented to the atmosphere rather than fed through the O_2 analyser. A fibre-optic O_2 sensor (Implantable Oxygen Microoptode; Presens, Regensburg, Germany) encased within a Pasteur pipette was mounted through the respirometer lid to measure aquatic O_2 content (% air-saturation). The O_2 sensor was connected to a single-channel, temperature-compensated O_2 meter and software (Microx TX3, OxyView TX3-V5.20; Presens) that recorded at 1 min intervals. A trial without a fish (control) was conducted for each $P_{O_2,air}$ treatment to account for aquatic O_2 depletion not related to fish respiration. A linear regression was fitted to the data of O_2 content (% air-saturation) on time, and aquatic $\dot{V}_{O_2}$ was calculated using the equation:

$$\dot{V}_{O_2} = -1 \times [(m_f - m_c) / 100] \times V \times \beta_{O_2} , \quad (2)$$

where m_f is the slope derived from the trial with a fish (% air-saturation h^{-1}), m_c is the slope derived from the control (% air-saturation h^{-1}), V is the water volume in the respirometry chamber (=1.088 litres) and β_{O_2} is the O_2 capacitance of air-saturated freshwater at 25°C (=5.77 ml l^{-1}) (Riley and Chester, 1971). Note that the difference between m_f and m_c is divided by 100 to convert the percentage of O_2 in the water to a fraction.

Apnoeic $\dot{V}_{O_2}$ calculation

To calculate oxygen uptake from the ABO during apnoea ($\dot{V}_{O_2,ap}$; ml h^{-1}), V_{O_2}/breath (ml) was plotted against the apnoea duration (h) for each breath of each fish under each treatment (e.g. Fig. 2). A linear regression was fitted to the data, with the derived slope of the regression representing $\dot{V}_{O_2,ap}$ (ml h^{-1}) (e.g. Fig. 2).

End-apnoea ABO-P_{O_2} calculation

It would be possible to estimate end-apnoea P_{O_2} in the ABO from the data if tidal volume or ABO volume were known. Because there are no measurements of either for *T. leeri*, V_{ABO} was calculated for each fish according to relationships derived for the dwarf gourami, *Colisa lalia* (Schuster, 1989). He analysed the time-course of air volume changes in the ABO during apnoea at different temperatures, finding that the relationship between V_{ABO} (μl) and fish length (l; mm) was well described by the function:

$$V_{ABO} = (2.65 \times 10^{-4}) \times l^{3.1} . \quad (3)$$

This function is a rough estimate of a single suprabranchial chamber volume at ~26°C after an apnoea duration of approximately 120 s [$V_{ABO}(120)$].

Schuster stated that if one measurement of V_{ABO} is known, the V_{ABO} at any instant time during apnoea (t; s) at 25°C is given by:

$$V_{ABO}(t) = (0.897 + 0.166e^{-0.0046t}) \times [V_{ABO}(t_1) / (0.897 + 0.166e^{-0.0046t_1})] , \quad (4)$$

where $V_{ABO}(t_1)$ is the known V_{ABO} (μl) after an apnoea duration of t_1 (s). Since Eqn 3 can be used to calculate $V_{ABO}(120)$, this can be substituted as $V_{ABO}(t_1)$ in Eqn 4, and therefore V_{ABO} at the beginning of the apnoea period [$V_{ABO}(t_0)$] can be calculated.

$V_{ABO}(t_0)$ and the P_{O_2} in the ABO at the beginning of apnoea (i.e. initial P_{O_2}) are needed to calculate the initial volume of O_2 [$V_{O_2}(t_0)$; ml] in the ABO (Eqn 5). The initial P_{O_2} was assumed to be equivalent to $P_{O_2,air}$. This is reasonable because during expiration practically all of the gas in the ABO is displaced out of the mouth with water from the opercular cavity (Peters, 1978):

$$V_{O_2}(t_0) = P_{O_2,air} \times \beta_{O_2} \times V_{ABO}(t_0) , \quad (5)$$

where β_{O_2} is the O_2 capacitance of air at 25°C (=9.09 ml l^{-1} kPa^{-1}) (Dejours, 1981), and $V_{ABO}(t_0)$ is the initial volume of one suprabranchial chamber (litres).

The volume of O_2 consumed during apnoea is calculated using $\dot{V}_{O_2,ap}$ (ml h^{-1}) and mean apnoea duration (t_{ap}, h) for each fish under each treatment, and this is subtracted from $\dot{V}_{O_2}(t_0)$ to arrive at the volume of O_2 in the ABO at the end of apnoea ($V_{O_2,end}$; ml):

$$V_{O_2,end} = V_{O_2}(t_0) - [(\dot{V}_{O_2,ap} / 2) t_{ap}] . \quad (6)$$

In Eqn 6, $\dot{V}_{O_2,ap}$ is halved because it represents the rate of O_2 uptake across the surface area of the entire ABO (i.e. both suprabranchial chambers) and $V_{O_2,end}$ is calculated for a single suprabranchial chamber.

Although Schuster found that the ABO of *C. lalia* decreased in volume as O_2 was consumed (Schuster, 1989), the ABO of *T. leeri* is a bony structure (Graham, 1997; Peters, 1978) (L.A.A., personal observation) and the rate of change in V_{ABO} with declining ABO-O_2 may be different from that in *C. lalia*, or may not occur at all. Therefore, end-apnoea ABO-P_{O_2} was calculated with two assumptions that bracket reality (note that the following calculations make the simplifying assumption that CO_2 is not present within the ABO): (1) V_{ABO} remained constant as O_2 was consumed

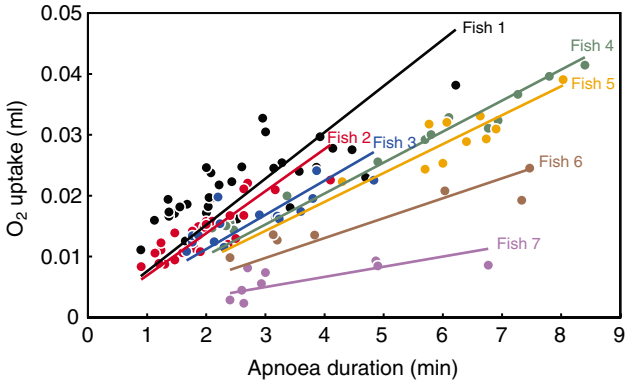

Fig. 2. An example of the relationship between O_2 uptake per breath and preceding apnoea duration for each fish at an aerial O_2 partial pressure of 40 kPa. The slope represents aerial O_2 consumption rate (ml min^{-1}) during apnoea: Fish 1=0.0076; Fish 2=0.0069; Fish 3=0.0056; Fish 4=0.0051; Fish 5=0.0047; Fish 6=0.0033; Fish 7=0.0017.

(Eqn 7) and (2) V_{ABO} decreased as if the ABO was totally compliant [i.e. $V_{O_2,end}$ (ml) is subtracted from $V_{ABO}(t_0)$ (litres); Eqn 8]:

$$P_{O_2} = V_{O_2,end} / [V_{ABO}(t_0) \times \beta_{O_2}] , \quad (7)$$

$$P_{O_2} = V_{O_2,end} / \{[V_{ABO}(t_0) - (\dot{V}_{O_2,end} / 1000)] \beta_{O_2}\} . \quad (8)$$

Although a totally compliant ABO is unlikely, these assumptions represent opposite, extreme situations and thus allow consideration of all possibilities and a broader interpretation of results.

End-apnoea ABO-P_{O_2} assuming both a constant and totally compliant ABO volume was determined for each fish and compared between treatments.

Air–blood P_{O_2} gradient

To ascertain whether a change in apnoea duration is solely responsible for a change in mean V_{O_2}/breath with changing $P_{O_2,air}$, or whether a change in the air–blood P_{O_2} gradient is also a contributing factor, mean $\dot{V}_{O_2,ap}$ was calculated for each treatment and plotted against $P_{O_2,air}$.

Statistical analysis

Where appropriate, mass-specific values were used to account for the variation attributed to mass differences between fish (McNab, 1999). Although this procedure does not completely remove mass effects (Packard and Boardman, 1999) because $\dot{V}_{O_2}$ scales allometrically in fish (White et al., 2006), the body size range was too small to determine the intraspecific allometric exponent for *T. leeri* accurately and arrive at mass-independent values.

A repeated-measures analysis of variance (ANOVA) was performed in JMP Version 5.1 (SAS Institute, Cory, NC, USA) to determine the effect of $P_{O_2,air}$ on all variables. To fulfil assumptions of normality and homogeneity of variance, $P_{O_2,air}$ was log transformed for the analysis of f_{ab}, which was not transformed; aerial $\dot{V}_{O_2}$, aquatic $\dot{V}_{O_2}$, $\dot{V}_{O_2}$/breath, end-apnoea ABO-P_{O_2} (assuming constant ABO volume) and end-apnoea ABO-P_{O_2} (assuming a totally compliant ABO) were log transformed together with $P_{O_2,air}$; and $\dot{V}_{O_2,ap}$ and total $\dot{V}_{O_2}$ were log transformed without $P_{O_2,air}$ being transformed. A Tukey's HSD test was used in *post-hoc* analyses where repeated-measures ANOVA revealed significant treatment effects. Statistical significance for all tests was determined with α=0.05.

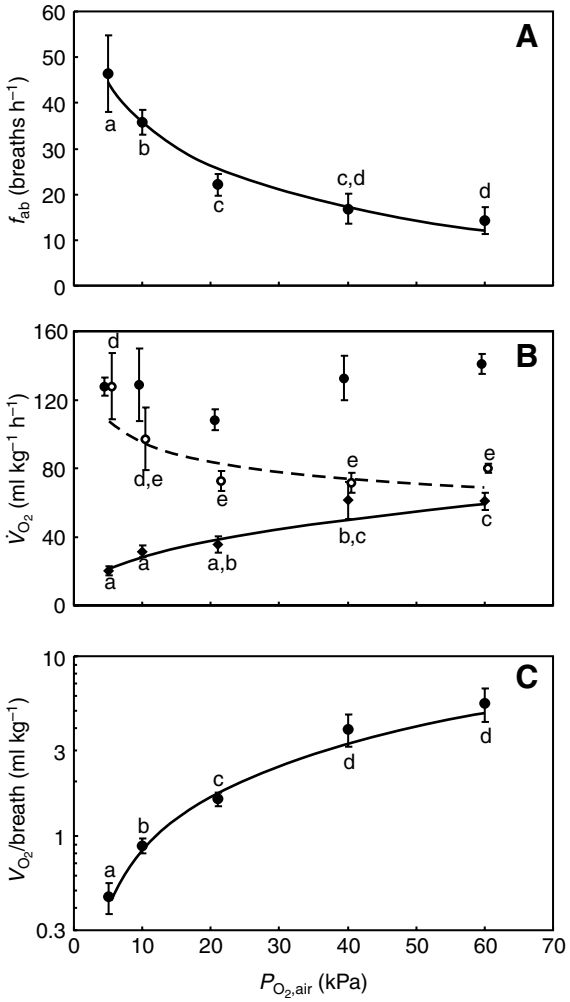

Fig. 3. Effect of changes in aerial O_2 partial pressure ($P_{O_2,air}$) on (A) air-breathing frequency (f_{ab}), (B) O_2 consumption rate ($\dot{V}_{O_2}$; total, ●; aquatic, ○; aerial, ◆) and (C) mean O_2 uptake per breath (V_{O_2}/breath) of *Trichogaster leeri*. (For f_{ab}, total $\dot{V}_{O_2}$, aerial $\dot{V}_{O_2}$ and V_{O_2}/breath, N=3 for 5 kPa treatment as individual breaths were invisible on the others and N=7 for remaining treatments; for aquatic $\dot{V}_{O_2}$ N=7 for all treatments.) Equations of regression lines: (A) f_{ab}= 65.8–30.3log($P_{O_2,air}$); (B) log(aquatic $\dot{V}_{O_2}$)=2.16–0.18log($P_{O_2,air}$); log(aerial $\dot{V}_{O_2}$)=1.04+0.413log($P_{O_2,air}$); (C) log(V_{O_2}/breath)= –1.01+0.98log($P_{O_2,air}$). Treatments not denoted by the same letter are significantly different (in B, a–c denote aerial $\dot{V}_{O_2}$, d,e denote aquatic $\dot{V}_{O_2}$). Measurements were made at 5, 10, 21, 40 and 60 kPa; some symbols are offset for presentation. All data are shown as means ± s.e.m.

Results

Aerial respiration

There was a significant negative effect of $P_{O_2,air}$ on f_{ab} ($F_{1,23}$=120.4, P<0.0001), with f_{ab} decreasing from 46.3 breaths h^{-1} at 5 kPa to 14.3 breaths h^{-1} at 60 kPa (Fig. 3A). There was a significant positive effect of $P_{O_2,air}$ on aerial $\dot{V}_{O_2}$ ($F_{1,23}$=37.2, P<0.0001), with aerial $\dot{V}_{O_2}$ increasing from 20.1 ml kg^{-1} h^{-1} at 5 kPa to 60.8 ml kg^{-1} h^{-1} at 60 kPa (Fig. 3B). Complementary to this, there was a significant positive effect of $P_{O_2,air}$ on V_{O_2}/breath ($F_{1,23}$=221.5, P<0.0001), with V_{O_2}/breath increasing from 0.5 ml kg^{-1} at 5 kPa to 5.5 ml kg^{-1} at 60 kPa (Fig. 3C).

Aquatic and total respiration

There was a significant negative effect of $P_{O_2,air}$ on aquatic $\dot{V}_{O_2}$ ($F_{1,27}$=17.8, P=0.0003), with a significant increase in aquatic $\dot{V}_{O_2}$ under the 5 kPa treatment (Fig. 3B). There was no significant effect of $P_{O_2,air}$ on total $\dot{V}_{O_2}$ ($F_{1,23}$=2.49, P=0.13) (Fig. 3B).

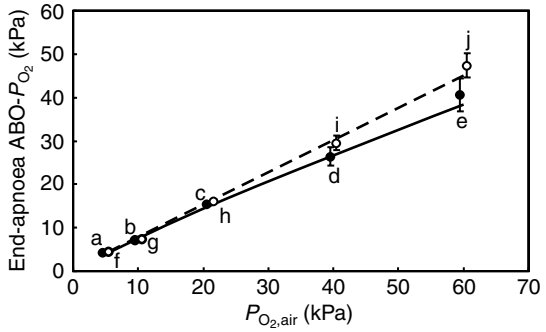

Fig. 4. Effect of changing aerial O_2 partial pressure ($P_{O_2,air}$) on the O_2 partial pressure (P_{O_2}) in the air-breathing organ (ABO) of *Trichogaster leeri* at the end of apnoea assuming ABO volume is constant (●) and totally compliant (○) (N=2 for 5 kPa and N=6 for 10 kPa as individual breaths were invisible on the others; and N=7 for remaining treatments). Equations of regression lines: (constant ABO volume) log(end-apnoea ABO-P_{O_2})=–0.0023+0.892log($P_{O_2,air}$); (totally compliant ABO volume) log(end-apnoea ABO-P_{O_2})= –0.084+0.977log($P_{O_2,air}$). Treatments not denoted by the same letter are significantly different (a–e, constant ABO volume; f–j, compliant ABO). Measurements were made at 5, 10, 21, 40 and 60 kPa; symbols are offset for presentation. All data are shown as means ± s.e.m., but error bars are concealed by symbols at low $P_{O_2,air}$.

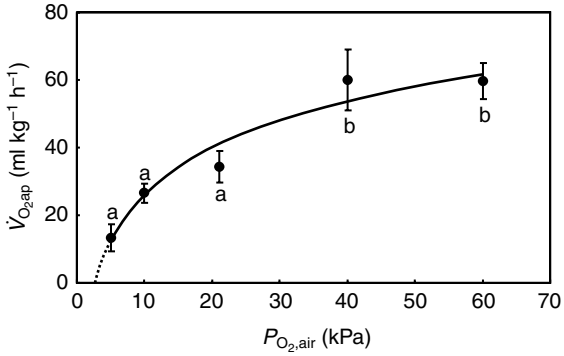

Fig. 5. Effect of changes in aerial O_2 partial pressure ($P_{O_2,air}$) on the mean apnoeic O_2 consumption rate ($\dot{V}_{O_2,ap}$) of *Trichogaster leeri*. The relationship is described by the logarithmic curve: $\dot{V}_{O_2,ap}$=–19.2+45.8log($P_{O_2,air}$) (r^2=0.63, N=2 for 5 kPa and N=6 for 10 kPa as individual breaths were invisible on the others; and N=7 for remaining treatments). Treatments not denoted by the same letter are significantly different. All data are shown as means ± s.e.m.

End-apnoea ABO-P_{O_2}

Regardless of whether it was assumed that the ABO volume was constant or that the ABO was totally compliant, there was a significant positive effect of $P_{O_2,air}$ on end-apnoea ABO-P_{O_2} ($F_{1,21}$=544, P<0.0001, and $F_{1,21}$=1091, P<0.0001, respectively) (Fig. 4). When ABO volume was assumed to be constant, end-apnoea ABO-P_{O_2} increased from 4.3 kPa at $P_{O_2,air}$=5 kPa to 40.6 kPa at $P_{O_2,air}$=60 kPa, and when ABO volume was assumed to be totally compliant, end-apnoea ABO-P_{O_2} increased from 4.4 to 47.3 kPa with the same change in $P_{O_2,air}$.

Air–blood P_{O_2} gradient

A repeated-measures ANOVA revealed a significant positive correlation between mean $\dot{V}_{O_2,ap}$ and $P_{O_2,air}$ ($F_{1,21}$=34.7, P<0.0001), with mean $\dot{V}_{O_2,ap}$ increasing from 13.2 ml kg^{-1} h^{-1} at 5 kPa to 59.8 ml kg^{-1} h^{-1} at 60 kPa. The relationship between mean $\dot{V}_{O_2,ap}$ and $P_{O_2,air}$ was described by the logarithmic curve: $\dot{V}_{O_2,ap}$=45.8log($P_{O_2,air}$)–19.2 (r^2=0.63) (Fig. 5).

Discussion

Aerial normoxia

Under normoxic conditions at 25°C, the mass-specific total (air + water) $\dot{V}_{O_2}$ of *T. leeri* was 108.2 ml kg^{-1} h^{-1}. This value is lower than that found for the closely related *T. trichopterus* (156.2 ml kg^{-1} h^{-1}) (Burggren and Haswell, 1979) and *T. pectoralis* (126.9 ml kg^{-1} h^{-1}) (Natarajan and Rajulu, 1982), all normalised to a common body temperature of 25°C with a Q_{10} of 1.65 (White et al., 2006). When viewed in comparison to an allometric relationship between $\dot{V}_{O_2}$ and body mass, the data obtained here for *T. leeri* are well within the 95% prediction intervals (31.8–290 ml kg^{-1} h^{-1}) for new data (White et al., 2006), indicating that the data obtained in this study are reliable.

Aerial hypoxia

The breathing frequency (f_{ab}) of *T. leeri* under normoxic conditions was 21.8±5.9 breaths h^{-1} (mean ± s.d.), which is greater than that observed for *T. trichopterus* (12.8± 4.1 breaths h^{-1}) (Burggren, 1979). However, like *T. trichopterus*, f_{ab} of *T. leeri* increased as the gas phase became increasingly hypoxic (Fig. 3A). At a $P_{O_2,air}$ of 5 kPa, f_{ab} was twofold higher than control (normoxic) levels, whereas *T. trichopterus* increased f_{ab} almost threefold. However, f_{ab} calculated for *T. leeri* at 5 kPa may be an underestimate, because only three fish yielded results where some individual breaths could be isolated. For the remaining fish, no or few breathing events were apparent. This suggests that the air–blood P_{O_2} gradient was insufficient to promote O_2 uptake. The air–blood P_{O_2} gradient may have in fact been reversed, resulting in fish losing O_2 gained from the water to the air. This futile behaviour would be expected, however, because severe aerial hypoxia is not encountered in the natural environment, and fish are unlikely to have evolved an appropriate response to such conditions.

An increase in f_{ab} in response to aerial hypoxia has been observed in species that have a reduced gill surface area and are therefore heavily reliant on aerial respiration. For example, *M. albus* showed a significant reduction in apnoea duration when inspiring gas mixtures containing 16 kPa O_2 or less, and breaths containing 1.5 kPa O_2 were exhaled almost immediately (Graham et al., 1995). *Monopterus cuchia* increased tidal volume as well as f_{ab} during hypoxic breathing (Lomholt and Johansen, 1974), but no such change in ABO tidal volume occurred in *T. trichopterus* (Burggren, 1979). *Protopterus aethiopicus* increased f_{ab} when N_2 gas was

injected into the ABO, and, interestingly, air breathing was stimulated by N_2 injection even when arterial P_{O_2} was higher than that towards the end of a normal apnoea period (Johansen and Lenfant, 1968). *Amia calva*, a fish less reliant on aerial respiration, increased f_{ab} twofold when air containing only 8 kPa O_2 was inspired (Hedrick and Jones, 1993). In contrast to *Amia*, other fish with efficient aquatic gas exchange do not show a change in f_{ab} when N_2 gas is injected into the lung (*Neoceratodus forsteri*) (Johansen et al., 1967) or air bladder (*Lepisosteus oculatus*) (Smatresk and Cameron, 1982b). The first and second gill arches of *T. leeri* are large and fully developed (Munshi, 1968), and *T. leeri* is sensitive to aquatic hypoxia (Miller, 2003), unlike *Protopterus* (Johansen and Lenfant, 1968) and *Monopterus* (Lomholt and Johansen, 1974). It therefore seems likely that, although *T. leeri* is not as reliant on aerial respiration as these species, aquatic respiration may not be sufficient to meet its metabolic demands, making it an obligate air breather (Graham, 1997). *T. trichopterus* is considered an obligate air breather at temperatures above 20–25°C, as it shows signs of distress if denied access to air (Burggren, 1979).

Despite an increase in f_{ab} during aerial hypoxic exposure, *T. leeri* was unable to sustain aerial $\dot{V}_{O_2}$ equal to that under normoxic conditions (Fig. 3B). Complementary to the observed decrease in aerial $\dot{V}_{O_2}$ with decreasing $P_{O_2,air}$, V_{O_2}/breath also showed a decline (Fig. 3C).

The contribution of aerial $\dot{V}_{O_2}$ to total $\dot{V}_{O_2}$ decreased from 33% in normoxia to 25% when $P_{O_2,air}$ was 10 kPa, and to 16% at 5 kPa. Similarly, under normoxic conditions at 27°C, the ABO of *T. trichopterus* accounted for 42% of the total $\dot{V}_{O_2}$ and less than 15% when $P_{O_2,air}$ was reduced to 7.2 kPa (Burggren, 1979). Both species showed an increase in aquatic $\dot{V}_{O_2}$ to compensate for their reduced ability to extract O_2 from the air (Fig. 3B). It is reasonable to assume that this increase in aquatic $\dot{V}_{O_2}$ arises almost entirely from gas exchange *via* the gills, because cutaneous gas exchange in air-exposed *T. trichopterus* accounts for only ~10% of the total gas exchange (Burggren and Haswell, 1979). Aquatic $\dot{V}_{O_2}$ may be increased *via* an increase in branchial ventilation frequency, branchial tidal volume or both. Branchial ventilation is known to increase initially in most air-breathing fish as aquatic P_{O_2} falls (Hughes and Singh, 1971; Johansen et al., 1970; Pettit and Beitinger, 1985; Smatresk and Cameron, 1982a), but the effect of aerial hypoxia on branchial ventilation appears not to have been investigated.

Aerial hyperoxia

At a $P_{O_2,air}$ of 60 kPa, f_{ab} significantly decreased in *T. leeri* (Fig. 3A). Similarly, *T. trichopterus* decreased f_{ab} when $P_{O_2,air}$ was increased to 80 kPa (Burggren, 1979). A decrease in f_{ab} has been observed in almost all species exposed to aerial hyperoxia: *P. ethiopicus* (Lahiri et al., 1970), *L. oculatus* (Smatresk and Cameron, 1982b), *Electrophorus electricus* (Johansen et al., 1968b) and *M. albus* (Graham et al., 1995).

Despite a decrease in f_{ab} in hyperoxic air, $\dot{V}_{O_2,ap}$ increased by almost twofold when $P_{O_2,air}$ was 40 kPa; however, no significant increase occurred when $P_{O_2,air}$ was increased to 60 kPa (Fig. 3B).

Although aerial $\dot{V}_{O_2}$ increased significantly in aerial hyperoxia compared with normoxia, there was no corresponding decrease in aquatic $\dot{V}_{O_2}$, and total $\dot{V}_{O_2}$ was unchanged (Fig. 3B). However, because aerial and aquatic respiration were measured separately, inherent variability may have obscured the expected correlations.

ABO-O_2 chemoreceptors

The responsiveness of *T. leeri* to changes in aerial O_2 content lends insight to the question of the existence of ABO-O_2 chemoreceptors. Graham et al. give support for the existence of ABO-O_2 chemoreceptors in some air-breathing species (Graham et al., 1995). They found that the rapidity of the gas-voiding reflex of *M. albus* to changed aerial O_2 content was indicative of ABO-O_2 chemoreceptors, not chemoreceptors in the systemic circulation. They suggested that an ABO-O_2 chemoreceptor would be advantageous in the regulation of cardiac responses to an air-breathing event. A common pattern of cardiac arrhythmia found in fishes during an air-breathing event is inspiration-induced tachycardia followed by the gradual onset of bradycardia as the ABO-O_2 content falls, leading to pronounced bradycardia with exhalation (Farrell, 1978; Johansen et al., 1968a; Singh and Hughes, 1973; Smatresk, 1988; Smatresk, 1990). Therefore, an ABO-O_2 chemoreceptor may be important in the modulation of mechanoreceptor and other stimuli affecting air-breathing tachycardia, in attenuating tachycardia as ABO-O_2 declines and in terminating the breath when ABO-P_{O_2} becomes too low to promote O_2 uptake (Graham and Baird, 1984). This would result in the effective matching of ventilation and perfusion (Johansen, 1966; Johansen, 1970).

However, the findings of Graham et al. (Graham et al., 1995) contrast with Burggren's indication of more centrally located chemoreceptors (i.e. in the brain) in *T. trichopterus*, which was based on the lag in ventilation response time (several seconds) to stepwise changes in aerial O_2 content (Burggren, 1979). Burggren argued that because aerial hypoxia is rarely, if ever, encountered in the natural environment, selection pressures should not be strong for the evolution of a peripheral chemoreceptor control system able to differentiate between reduced systemic blood O_2 resulting from gill ventilation with hypoxic water and that resulting from ABO ventilation with hypoxic gas.

More substantial evidence for ABO-O_2 chemoreceptors in *T. leeri* would have been an end-apnoea ABO-P_{O_2} that was independent of $P_{O_2,air}$. This would have suggested the existence of an ABO-P_{O_2} threshold that, once reached, triggered *T. leeri* to renew the gas in its ABO. However, this was not the case in this study; end-apnoea P_{O_2} increased with increasing $P_{O_2,air}$ (Fig. 4). This lack of correlation between ABO-P_{O_2} and renewal of ABO gas has also been recognised in lungfish (Johansen and Lenfant, 1968) and in Pacific tarpon that uses

the swimbladder as an ABO (Seymour et al., 2007). It has also been shown that apnoea termination occurs at high ABO-P_{O_2} levels when the rate of decline in ABO-P_{O_2} is rapid (Shelton et al., 1986). This corresponds with the findings in this study; mean apnoeic $\dot{V}_{O_2}$ ($\dot{V}_{O_2,ap}$) increased under hyperoxic conditions and end-apnoea ABO-P_{O_2} was higher than that under normoxic conditions. These findings indicate that if ABO-O_2 chemoreceptors are present, then their regulation of bimodal control in air-breathing fish is partial, and that respiration is mainly affected by chemoreceptors elsewhere in the central and peripheral nervous system and possibly by mechanoreceptors in the ABO.

Air–blood P_{O_2} gradient

Mean $\dot{V}_{O_2,ap}$ increased with increasing $P_{O_2,air}$ (Fig. 5), supporting the hypothesis that the observed increase in V_{O_2}/breath with increasing $P_{O_2,air}$ (Fig. 3C) was facilitated not only by an increase in apnoea duration but also by an increase in the air–blood P_{O_2} gradient. The logarithmic function between $\dot{V}_{O_2,ap}$ and $P_{O_2,air}$ suggests that when there is no O_2 uptake occurring in the ABO (i.e. $\dot{V}_{O_2,ap}$ is equal to zero), $P_{O_2,air}$ is equal to 2.62 kPa. Therefore, the air and blood are in equilibrium and the efferent blood from the gills can be assumed to have a P_{O_2} approximating this value. The relationship also indicates that in hyperoxia $\dot{V}_{O_2,ap}$ reaches a maximum where it becomes independent of $P_{O_2,air}$ (Fig. 5). Because the haemoglobin would be expected to be completely saturated in the hyperoxic ABO (Herbert and Wells, 2001), aerial $\dot{V}_{O_2}$ plateaus because the blood reaches a point where it can take up no more than can be dissolved in the plasma.

List of symbols and abbreviations

ABO	air-breathing organ
β_{O_2}	oxygen capacitance
f_{ab}	air-breathing frequency
$F_{E_{O_2}}$	excurrent oxygen content
$F_{I_{O_2}}$	incurrent oxygen content
P_{O_2}	oxygen partial pressure
$P_{O_2,air}$	aerial oxygen partial pressure
t_{ap}	apnoea duration
V_{ABO}	volume of the air-breathing organ
$V_{ABO}(t_0)$	ABO volume at the beginning of the apnoea period
$\dot{V}_I$	oxygen flow rate
$\dot{V}_{O_2}$	oxygen consumption rate
$\dot{V}_{O_2,ap}$	apnoeic $\dot{V}_{O_2}$
$V_{O_2,end}$	volume of oxygen in the ABO at the end of apnoea
V_{O_2}/breath	oxygen uptake per breath

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