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RESEARCH ARTICLE

The contribution of air breathing to aerobic scope and exercise performance in the banded knifefish *Gymnotus carapo* L.

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SUMMARY

The contribution of air breathing to aerobic metabolic scope and exercise performance was investigated in a teleost with bimodal respiration, the banded knifefish, submitted to a critical swimming speed (U_{crit}) protocol at 30°C. Seven individuals (mean ± s.e.m. mass 89±7 g, total length 230±4 mm) achieved a U_{crit} of 2.1±1 body lengths (BL) s^{-1} and an active metabolic rate (AMR) of 350±21 mg kg^{-1} h^{-1}, with 38±6% derived from air breathing. All of the knifefish exhibited a significant increase in air-breathing frequency (f_{AB}) with swimming speed. If denied access to air in normoxia, these individuals achieved a U_{crit} of 2.0±0.2 BL s^{-1} and an AMR of 368±24 mg kg^{-1} h^{-1} by gill ventilation alone. In normoxia, therefore, the contribution of air breathing to scope and exercise was entirely facultative. In aquatic hypoxia (P_{O_2}=4 kPa) with access to normoxic air, the knifefish achieved a U_{crit} of 2.0±0.1 BL s^{-1} and an AMR of 338±29 mg kg^{-1} h^{-1}, similar to aquatic normoxia, but with 55±5% of AMR derived from air breathing. Indeed, f_{AB} was higher than in normoxia at all swimming speeds, with a profound exponential increase during exercise. If the knifefish were denied access to air in hypoxia, U_{crit} declined to 1.2±0.1 BL s^{-1} and AMR declined to 199±29 mg kg^{-1} h^{-1}. Therefore, air breathing allowed the knifefish to avoid limitations to aerobic scope and exercise performance in aquatic hypoxia.

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Key words: critical swimming speed, hypoxia, metabolic rate, oxygen uptake, respirometry.

INTRODUCTION

Physiological adaptations for air breathing have evolved multiple times in bony fishes, particularly in tropical freshwater habitats (Randall et al., 1981a; Graham, 1997). Freshwater airbreathing fishes are all bimodal breathers; they possess gills but can also rise to the surface and gulp air, which is stored in a well-vascularised air-breathing organ, which can be a true lung, a modified swimbladder, diverticula of the buccal, opercular or pharyngeal cavities, or the gut (Graham, 1997). Aquatic hypoxia in tropical habitats is believed to have been the main selective pressure leading to the evolution of air breathing in freshwater fishes. All air-breathing fishes studied to date respond to aquatic hypoxia by increasing air-breathing frequency and the proportion of their oxygen uptake derived from the air (Johansen, 1971; Johansen et al., 1971; Randall et al., 1981b; Graham, 1997; Chapman and McKenzie, 2009).

It is also true, however, that fishes with bimodal respiration increase air breathing in response to factors that raise their metabolic demand (Graham, 1997; Graham, 2006), such as increased water temperature (Johansen et al., 1971) or increases in activity (Grigg, 1965; Farmer and Jackson, 1998; Seymour et al., 2004; Seymour et al., 2007). Air-breathing fishes are often classified as either obligate or facultative, the former indicating that, even in aquatic normoxia, they cannot survive without access to air, the latter indicating that they can, if necessary, meet all of their normoxic oxygen requirements from the water by gill ventilation alone (Johansen, 1971; Graham, 1997). These classifications are based on experiments under routine 'resting' conditions, so it is not yet clear whether species that are facultative air breathers under resting conditions in normoxia might in fact have to air breathe to achieve their full aerobic metabolic scope and perform activities such as aerobic exercise (Graham, 1997; Graham, 2006).

When submitted to controlled increases in swimming speed, facultative air breathers such as *Amia calva* and *Megalops cyprinoides*, and obligate breathers such as *Lepisosteus oculatus*, show pronounced increases in oxygen uptake from air (Farmer and Jackson, 1998; Seymour et al., 2004; Seymour et al., 2007). In *A. calva*, incremental exercise to fatigue in a swim flume elicited what appeared to be an exponential increase in air-breathing frequency, although there was variation in the intensity of this response among individual fish (Farmer and Jackson, 1998). The contribution that air-breathing makes to aerobic metabolic scope, and its potential role in sustaining maximum aerobic exercise performance remains, however, to be demonstrated explicitly for a fish with bimodal respiration (Graham, 2006).

In water-breathing fishes, aquatic hypoxia limits their ability to raise metabolic rate (Fry, 1971) and perform aerobic exercise (Dahlberg et al., 1968; Bushnell et al., 1984; Jourdan-Pinet et al., 2010). Various studies have demonstrated that, in deep aquatic hypoxia, fish with bimodal respiration can maintain their metabolic rate at routine 'resting' levels independently of oxygen availability in the water by breathing air (Graham, 1997; Johansen et al., 1971; Randall et al., 1981b; McKenzie et al., 2007a). There is some

evidence that fish with bimodal respiration may be able to avoid limitations to aerobic scope and exercise performance in aquatic hypoxia by increasing their reliance on aerial oxygen uptake. In *M. cyprinoides*, a facultative air breather, a combination of aerobic exercise and aquatic hypoxia stimulated aerial respiration more than either factor on its own (Seymour et al., 2004; Seymour et al., 2007). In an obligate air breather, *L. oculatus*, submitted to relatively intense exercise in a swim flume, there was no difference in endurance (time to exhaustion) of fish that were in aquatic normoxia *versus* a degree of deep aquatic hypoxia that effectively eliminated the gills as a site of oxygen uptake (Burleson et al., 1998). Nonetheless, whether air breathing allows a bimodal species to avoid limitations to aerobic metabolic scope and maximal aerobic exercise performance in aquatic hypoxia has not yet been explicitly demonstrated.

The present study investigated patterns of respiratory partitioning between water and air during aerobic exercise in the banded knifefish *Gymnotus carapo* L., a neotropical teleost that swims by means of an elongated ventral anal fin ['gymnotiform locomotion' (*sensu* Breder, 1926)] and uses a modified swimbladder for facultative air breathing (Liem et al., 1984). To elicit maximum aerobic exercise performance, the knifefish were submitted to an incremental critical swimming speed (U_{crit}) test (Brett, 1964) in a swim tunnel respirometer that was modified to allow measurement of rates of oxygen uptake from both water and air (Farmer and Jackson, 1998). The specific objectives of this study were to examine: (1) whether air breathing is obligatory in order for *G. carapo* to achieve maximum aerobic scope and swimming performance in aquatic normoxia and (2) whether air breathing allows the knifefish to avoid limitations to aerobic scope and swimming performance in aquatic hypoxia.

MATERIALS AND METHODS

Experimental animals

Banded knifefish of unknown sex with a body mass of approximately 90 g and a total length of approximately 23 cm were obtained from a commercial supplier and transported to the Universidade Estadual Paulista (UNESP) campus in Rio Claro, São Paulo state, Brazil. They were maintained in indoor 1.5 m^3 tanks provided with a recirculating flow of biofiltered freshwater at 30°C, under a natural photoperiod, for at least 2 weeks prior to use in experiments. They were fed each day with commercial pellets, but individuals were fasted for at least 36 h prior to use in experiments. Experiments were performed in accordance with United Kingdom Home Office regulations for animal experimentation, which also complied with the guidelines for animal experimentation at UNESP, Rio Claro, Brazil. All experiments were performed at 30±0.1°C.

Swimming respirometry

Swimming respirometry was performed with a Steffensen-type swim-tunnel respirometer constructed of Plexiglas® (volume 13.4 l), designed to exercise fish in a non-turbulent water flow with a uniform velocity profile. The swim tunnel has been described in detail previously (McKenzie et al., 2007b); however, for the present study it was modified to permit the fish to breathe air during exercise (Farmer and Jackson, 1998). The modification comprised a 150 ml hermetically sealed air space in the lid of the respirometer, positioned at the anterior end of the swimming chamber. This air space had a port for an oxygen probe (see below), and two further ports to allow a gentle flow of humidified air to be passed through the chamber when required (see below).

Individual knifefish were gently wrapped in a moist cloth and measured for mass (to the nearest 0.1 g) and total length (to the nearest 1 mm), and placed in the respirometer in the evening. They were left to recover from the handling overnight, in a water current at 10 cm s^{-1}. At this speed they rested on the bottom, with occasional gentle swimming movements, in particular when they rose through the water column to access the air space (supplementary material Movie 1). The following morning they were exposed to stepwise increments in swimming speed, 10 cm s^{-1} every 30 min, until they fatigued. All swimming speeds were corrected for the blocking effect of the fish (Bell and Terhune, 1970). U_{crit} (cm s^{-1}) was calculated as described previously (Brett, 1964) using an equation that adds the velocity of the most recently completed increment to the product of the incremental increase in velocity and the proportion of the final increment completed before fatigue. The knifefish were then recovered for at least 4 h while swimming gently at a speed of 10 cm s^{-1}, prior to being submitted to a second swim test, as described below. It is well established that teleost fishes can repeat a U_{crit} protocol with no decline in aerobic metabolic scope or swimming performance after as little as 45 min recovery (Jain et al., 1998; McKenzie et al., 2007b).

Four different experimental conditions were tested, with any two being completed on a single day in random order. These were: (1) normoxic water [partial pressure of O_2 (P_{O_2})~19 kPa] with access to the air space; (2) normoxic water but no access to air (the normal lid being placed on the swim chamber); (3) hypoxic water (P_{O_2}=4.0±0.2 kPa) with access to the air space; and (4) hypoxic water without access to air. Air was normoxic under all conditions. Water P_{O_2} was monitored with an OxyGuard mini-electrode (OxyGuard International, www.oxyguard.dk) attached to an oxygen regulator system based on a PR5714 Programmable Indicator (PR Electronics, www.prelectronics.dk), which regulated P_{O_2} at the hypoxic setpoint by controlling a flow of 100% nitrogen into the column *via* a solenoid valve (Jourdan-Pinet et al., 2010). Hypoxia exposure began at 1 h prior to the swim trail, with the fish at a swimming speed of 10 cm s^{-1}, and was then maintained throughout the trial.

Measurements of O_2 uptake from the water ($\dot{M}_{wO_2}$; mg kg^{-1} h^{-1}) were made at each swimming speed by intermittent stopped-flow respirometry (Steffensen et al., 1984; Steffensen, 1989) over a 15 min cycle, providing two measures of $\dot{M}_{wO_2}$ for each swimming speed during the swim test. Details of this method have been provided previously (Chatelier et al., 2005; McKenzie et al., 2007b; Jourdan-Pinet et al., 2010). Water oxygen concentration was recorded continuously using an optical oxygen probe and meter (Fibox, PreSens GmbH, www.presens.de) and $\dot{M}_{wO_2}$ was calculated automatically with Loliresp software (Loligo Systems, www.loligosystems.com), considering the rate of decline in oxygen concentration, the water volume in the swim tunnel and the mass of the fish (Steffensen, 1989). Oxygen uptake from the air ($\dot{M}_{aO_2}$) was measured as described previously (McKenzie et al., 2007a), using an optical fibre optode (Microx, PreSens GmbH) and associated software (PreSens Oxyview) continuously to monitor air P_{O_2}. The $\dot{M}_{aO_2}$ was calculated considering the rate of decline in oxygen concentration in the air space, the air volume and the mass of the fish (Randall et al., 1981b). The air space could be flushed with a gentle flow of water-saturated air at 30°C; this was performed manually as required, and air $P_{O_2}$was never allowed to decline below 95% of air saturation.

For swim tests where the fish had access to air breathing, total rates of oxygen uptake ($\dot{M}_{tO_2}$) were calculated for each swimming speed by summing $\dot{M}_{wO_2}$ and $\dot{M}_{aO_2}$. The percentages of $\dot{M}_{tO_2}$ taken up from water or air were then calculated. In aquatic hypoxia, there was a net diffusion of oxygen from the air space into the water (McKenzie et al., 2007a). To correct for this, tests were run at each

swimming speed with no fish, and rates of oxygen decline from the air, and increase in the water, were calculated. Suitable corrections were then applied to $\dot{M}_{wO_2}$ and $\dot{M}_{aO_2}$ for each fish. Blank tests were also run at the end of each day to correct for the contribution of bacterial metabolism to $\dot{M}_{wO_2}$. This was never greater than 10% of $\dot{M}_{wO_2}$ by the fish.

The whole respirometer was shielded from view to avoid any visual disturbance to the fish (Shingles et al., 2005; McKenzie et al., 2007a), which was filmed from within the shielded area with a webcam (Hercules Dualpix HD, www.hercules.com), and the images were displayed and recorded on a PC with H264WebCam software (Timhillone Software Co., Ltd, www.h264soft.com). The fish were filmed from slightly below, such that air-breathing behaviour was clearly visible when the fish broke the surface of the air space, and then released small bubbles from the buccal and/or opercular cavities as they descended back into the water column. Air-breathing frequency (f_{AB}; breaths h^{-1}) was calculated for each swim speed as appropriate.

Each fish could be identified by their body mass and total length. Therefore, after each fish had swum a first set of two trials, they were returned to a second holding tank, where they were allowed at least 7 days recovery, after which they were submitted to a further day of experimentation to complete the second pair of the four experimental trials. To avoid any effects of fasting on exercise performance, all fish were fed each evening with pellets in their holding tank, except for a single individual that was set aside in a small separate tank, to be placed in the respirometer the following evening for use on the subsequent day of experimentation.

Data analysis and statistics

For each fish, the maxima for $\dot{M}_{tO_2}$, $\dot{M}_{aO_2}$ and $\dot{M}_{wO_2}$ were identified under each experimental condition, as appropriate. These always occurred simultaneously, at the highest swimming speed achieved prior to fatigue, when $\dot{M}_{tO_2}$ was considered an estimate of active metabolic rate (AMR) (Chatelier et al., 2005; Dupont-Prinet et al., 2009). The f_{AB} and percentages of M_{O_2} taken from water and air were also calculated for AMR, for experimental conditions with access to air.

For each fish, an exponential equation was fitted to the relationships between swimming speed and $\dot{M}_{tO_2}$, $\dot{M}_{aO_2}$ and $\dot{M}_{wO_2}$ (as appropriate for the swim test in question), to derive a value for the *y*-intercept, and thereby an estimate for these variables at a notional swimming speed of zero (Brett, 1964). The derived value of $\dot{M}_{tO_2}$ was termed the 'immobile metabolic rate' (IMR) (Chatelier et al., 2005; Dupont-Prinet et al., 2009). Aerobic metabolic scope (AS) during exercise was calculated as the difference between AMR and IMR (Chatelier et al., 2005; Dupont-Prinet et al., 2009). The contributions of $\dot{M}_{wO_2}$ and $\dot{M}_{aO_2}$ to AS were calculated from their relative values at IMR and AMR. A value for f_{AB} at IMR was also estimated for each fish by fitting an exponential equation to the relationships between swimming speed and that variable, and deriving a value for the *y*-intercept. The amount of oxygen consumed per breath was calculated by dividing $\dot{M}_{aO_2}$ by f_{AB} at a given measurement interval.

All statistics were performed with SigmaPlot 11.0 (Systat Software, www.systat.com). Single variables, such as U_{crit}, AMR, IMR or AS, were compared across the four experimental conditions with a one-way ANOVA for repeated measures, with each fish as a subject.

For all of the respiratory variables measured in the swim tests in normoxia and hypoxia with access to air, a two-way ANOVA for repeated measures was used in all cases, with data compared for any given variable at IMR, at a swimming speed of 10 cm s^{-1}, and at AMR, with each fish a subject. The measured data for 10 cm s^{-1} were included in this analysis in addition to IMR and AMR, because IMR was a derived variable. The ANOVA factors differed according to the variable being considered. For the measures of oxygen uptake, the main factor was $\dot{M}_{aO_2}$ *versus* $\dot{M}_{wO_2}$ and the repeated factor was a composite variable of measurement interval and oxygen level. That is, IMR, a swimming speed of 10 cm s^{-1} and AMR were each flagged as either being measured in normoxia or hypoxia. For the contributions of $\dot{M}_{aO_2}$ and $\dot{M}_{wO_2}$ to AS in normoxia and hypoxia, the main factor was air *versus* water and the repeated factor was the oxygen level (normoxia *versus* hypoxia). For the percentages of $\dot{M}_{O_2}$ derived from air breathing, f_{AB} and O_2 uptake per breath, the main factor was oxygen level and the repeated factor was the measurement interval. The percentage data were arc-sine transformed prior to analysis.

In all cases, where a significant effect was found, Holm–Sidak *post hoc* tests were used to identify significant differences between means. Critical significance levels for multiple testing were corrected following the sequential Bonferroni procedure. Therefore, $\alpha<0.05$ was taken as the minimal requirement for statistical significance. Where a significant interaction was found in any two-way ANOVA, this was interpreted in the Results section but further separate tests were not performed.

RESULTS

Experiments were completed on seven knifefish, with a mean (±s.e.m.) mass of 89.2±7.3 g and a mean total length of 230±4 mm. The knifefish adapted well to the swim tunnel, immediately orienting and swimming into the current at 10 cm s^{-1}, with a rigid body and undulations of their anal fin, taking occasional air breaths (supplementary material Movie 1). When the knifefish were denied access to air and then exposed to hypoxia, they initially attempted to air breathe, searching for an air space, but then calmed down and oriented into the water current.

In all experimental conditions, fatigue was unequivocal in the knifefish, which fell against the back screen of the swim chamber until current speed was reduced. Incremental speeds were associated with increased ventral fin undulation frequency; the body was always held rigid. Prior to fatigue there was no evidence of a gait transition from use of the ventral fin to undulations of the whole body, which might have indicated recruitment of fast-twitch glycolytic (FG) muscle and anaerobic 'burst-and-coast' swimming (Webb, 1998; Marras et al., 2010). The 4 h recovery between swim tests on the same day was sufficient for the fish to return to the stable rates of M_{tO_2} that were measured prior to the first test in the morning.

Overall exercise performance in normoxia and aquatic hypoxia

Fig. 1 shows $\dot{M}_{tO_2}$ as a function of swimming speed for the seven knifefish under the four experimental conditions. Each individual showed a clear exponential rise in $\dot{M}_{tO_2}$ with swimming speed under all experimental conditions (see individual regression coefficients in supplementary material Table S1), and their estimated IMR is visible for each condition. Their AMR is also visible as the highest $\dot{M}_{tO_2}$ value achieved under any given condition. Although there was variation among individuals for their responses in any given trial, the graphs reveal that the general pattern of $\dot{M}_{tO_2}$ during exercise was similar in aquatic normoxia with (Fig. 1A) or without (Fig. 1B) access to air, and in hypoxia with access to air (Fig. 1C), but was markedly different, with limited AMR, in aquatic hypoxia without access to air (Fig. 1D). This is reflected in the mean values for U_{crit},

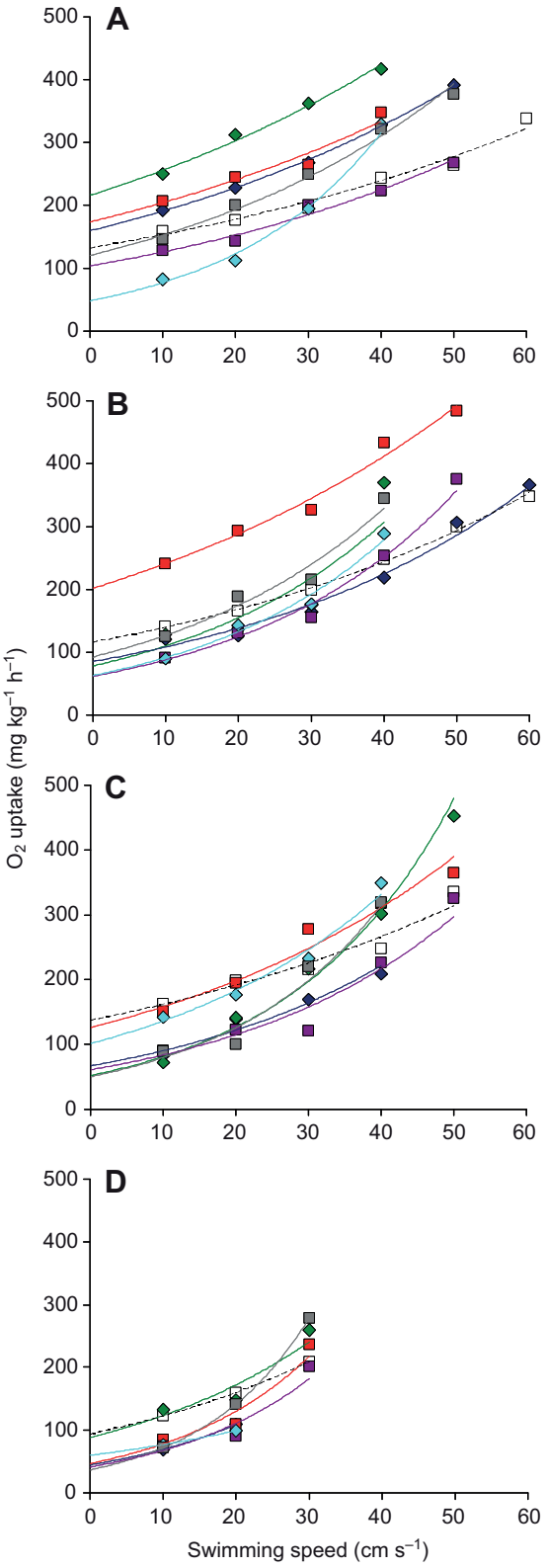

Fig. 1. Relationship between swimming speed and rate of oxygen uptake in seven *Gymnotus carapo* submitted to a critical swimming speed protocol in aquatic normoxia (~18 kPa P_{O_2}) with (A) or without (B) access to atmospheric air, or in aquatic hypoxia (4 kPa P_{O_2}) with (C) or without (D) access to air. On each panel, each symbol represents the same individual fish; the matching coloured (or dotted) line is a least-squares exponential regression fitted to that individual's data points. Individual regression coefficients are given in supplementary material Table S1.

AMR and AS (Table 1). These were not significantly different in aquatic normoxia (with or without air access), or in aquatic hypoxia with access to air, but were significantly reduced in aquatic hypoxia without air access, by comparison with all other conditions (Table 1).

The estimates of IMR tended to be higher and more variable in aquatic normoxia with air access, and low and much less variable in the other conditions, particularly in aquatic hypoxia without access to air (Fig. 1). In fact, mean IMR in normoxia with air access was significantly higher than in the two aquatic hypoxia conditions, whereas IMR in hypoxia without access was lower than all other conditions (Table 1). This was not an artefact of the order in which the four trials were performed, because this was randomised over two separate days for each individual.

Although it is evident from Fig. 1 that individual knifefish performed somewhat differently among the three conditions where U_{crit}, AMR and AS did not differ (Fig. 1A–C), the mean ratio of U_{crit} between any two of these trials (i.e. calculated for each individual and then averaged) did not differ significantly from unity (Jain et al., 1998; McKenzie et al., 2007b). By contrast, the mean ratio of U_{crit} for the trial in aquatic hypoxia *versus* all other conditions was significantly lower than unity, being approximately 0.65 in all cases.

Patterns of aerial and aquatic respiration during exercise

When allowed access to air, all of the knifefish air-breathed during exercise, in both normoxia and hypoxia. Fig. 2 shows the relative contribution of $\dot{M}_{aO_2}$ and $\dot{M}_{wO_2}$ to $\dot{M}_{tO_2}$ in normoxia and hypoxia.

In normoxia, $\dot{M}_{aO_2}$ varied amongst individuals but nonetheless represented a significant proportion of $\dot{M}_{tO_2}$ at all swimming speeds and showed a clear exponential increase with swimming speed in all of the knifefish (Fig. 2A, supplementary material Table S1). Nonetheless, $\dot{M}_{wO_2}$ was greater than $\dot{M}_{aO_2}$ in all but one individual at all speeds, showed an exponential increase with speed, and reached higher maximum values than $\dot{M}_{aO_2}$ at AMR (Fig. 2B, supplementary material Table S1). It is also evident that the reason for the elevated and highly variable IMR in normoxia with access to air (Fig. 1A) was largely attributable to variation in $\dot{M}_{wO_2}$ at IMR in normoxia (Fig. 2B), because $\dot{M}_{aO_2}$ tended to be less variable at IMR (Fig. 2A). The exponential increase in $\dot{M}_{aO_2}$ with swimming speed in normoxia was associated with a parallel exponential increase in f_{AB} in all individuals (Fig. 3A, supplementary material Table S1). That is, as exercise progressed, each fish made increasingly frequent trips to the surface to take single air breaths (supplementary material Movie 2).

In aquatic hypoxia, $\dot{M}_{aO_2}$ was higher than $\dot{M}_{wO_2}$ in most individuals at most swimming speeds (Fig. 2C,D), and all knifefish showed an exponential increase in $\dot{M}_{aO_2}$ with speed (Fig. 2C, supplementary material Table S1) and achieved higher $\dot{M}_{aO_2}$ at AMR than they did in normoxia (Fig. 2A,C). Indeed, some individuals had extremely low $\dot{M}_{wO_2}$ at low swimming speeds in aquatic hypoxia and, although all showed an exponential increase in $\dot{M}_{wO_2}$ as swimming speed increased (Fig. 2D), they still all had lower values of $\dot{M}_{wO_2}$ at AMR than they did in normoxia (Fig. 2B,D).

The elevated $\dot{M}_{aO_2}$ in hypoxia was linked to a significantly higher f_{AB} than in normoxia for all individuals at all swimming speeds (Fig. 3). Indeed, f_{AB} showed a much more pronounced exponential increase with speed than was observed in normoxia for all individuals (Fig. 3B, supplementary material Table S1). The high frequencies at AMR were achieved by a change in behaviour, whereby the fish swam very close to the water surface and took repeated bouts of air breaths in quick succession (supplementary material Movie 3).

Table 2 summarises elements of aquatic and aerial respiration during aerobic exercise, allowing a comparison between normoxia

Table 1. Elements of performance and respiratory metabolism of *Gymnotus carapo* submitted to a critical swimming speed (U_{crit}) protocol in aquatic normoxia (~18 kPa P_{O_2}) or hypoxia (4 kPa P_{O_2}), with or without access to air to breathe

	Normoxia		Hypoxia	
	Air access	No air access	Air access	No air access
U_{crit} (cm s^{-1})	49±3^{a}	47±4^{a}	47±3^{a}	29±3^{b}
U_{crit} (BL s^{-1})	2.1±0.1^{a}	2.0±0.2^{a}	2.0±0.1^{a}	1.3±0.1^{b}
AMR (mg kg^{-1} h^{-1})	350±21^{a}	368±24^{a}	336±29^{a}	199±29^{b}
IMR (mg kg^{-1} h^{-1})	132±21^{a}	107±19^{a,b}	90±14^{b}	63±10^{c}
AS (mg kg^{-1} h^{-1})	218±18^{a}	261±13^{a}	245±32^{a}	136±28^{b}

Data show mean (±s.e.m.) U_{crit}, active metabolic rate (AMR) during U_{crit}, derived immobile metabolic rate (IMR) and aerobic metabolic scope (AS). N=7 in all cases. A common superscript indicates no significant difference between the means for that variable (P>0.05).

and aquatic hypoxia. There was a significant interaction between the two factors in the ANOVA, indicating that the magnitude of the increase in oxygen uptake from IMR to 10 cm s^{-1} to AMR in normoxia and hypoxia depended on whether this was $\dot{M}_{aO_2}$ or $\dot{M}_{wO_2}$. This reflected the large changes in respiratory partitioning between normoxia and hypoxia. In normoxia, aquatic respiration dominated, and mean $\dot{M}_{wO_2}$ was higher than $\dot{M}_{aO_2}$ at all measurement intervals (Table 2). The increased reliance on aerial respiration in hypoxia meant that mean $\dot{M}_{wO_2}$ was lower than in normoxia at all measurement intervals (Table 2), and mean $\dot{M}_{aO_2}$ was higher than in normoxia at 10 cm s^{-1} and AMR (Table 2). Furthermore, mean $\dot{M}_{aO_2}$ was greater than $\dot{M}_{wO_2}$ at IMR and 10 cm s^{-1} in hypoxia, although not significantly at AMR (Table 2).

The mean contribution of $\dot{M}_{wO_2}$ or $\dot{M}_{aO_2}$ to AS did not differ significantly between normoxia and hypoxia. Note, however, that because mean $\dot{M}_{wO_2}$ was very low at IMR in hypoxia, and also significantly depressed at AMR, the contribution to scope was over a lower range of absolute rates of oxygen uptake than in normoxia (Table 2).

For the mean percentage of aerobic metabolism that was derived from air breathing, there was a significant interaction between oxygen level and measurement interval, because the effects of exercise depended upon whether it occurred during normoxia or hypoxia. Aerial respiration met the majority of metabolic demand during exercise in hypoxia; the overall mean percentage (IMR, 10 cm s^{-1} and AMR considered together) was 76±3% in hypoxia *versus* 36±3% in normoxia (least-squares mean ± s.e.m.). The overall mean percentage of metabolism derived from air breathing decreased from 61±3% (IMR) to 46±3% (AMR) when both hypoxic and normoxic data were considered together. When, however, normoxia and hypoxia were considered separately, the mean percentage of metabolism from air breathing remained constant at approximately 35% in normoxia (Table 2), but decreased from over 85% at IMR to 56% at AMR in hypoxia (Table 2).

Table 3 shows mean f_{AB} and O_2 uptake per air breath at IMR, 10 cm s^{-1} and AMR in normoxia and hypoxia. For f_{AB}, the magnitude of the effects of exercise depended upon whether it occurred during normoxia or hypoxia, and the ANOVA revealed a significant interaction between oxygen level and measurement interval. The overall mean f_{AB} was higher in hypoxia than in normoxia, being 95±7 *versus* 43±7 breaths h^{-1}, respectively. Furthermore, the overall mean f_{AB} (normoxia and hypoxia considered together) increased significantly with exercise intensity, from 30±8 to 43±8 to 134±8 breaths h^{-1} at IMR, 10 cm s^{-1} and AMR, respectively. When normoxia and hypoxia were considered separately, the transition from IMR to 10 cm s^{-1} and then AMR elicited a significant increase in mean f_{AB} in both conditions, but in hypoxia, mean f_{AB} was significantly higher than in normoxia at all measurement intervals (Table 3). Indeed, some individuals showed frequencies in excess of 200 h^{-1} at AMR in hypoxia (Fig. 4B), such that there was an almost fivefold increase in f_{AB} from the value estimated at IMR and a more than threefold increase from that measured at a swimming speed of 10 cm s^{-1} (Table 3).

Overall mean O_2 uptake per breath did not differ between normoxia and hypoxia (2.12±0.22 *versus* 1.91±0.22 mg O_2 kg^{-1}, respectively), but dropped significantly between IMR and AMR

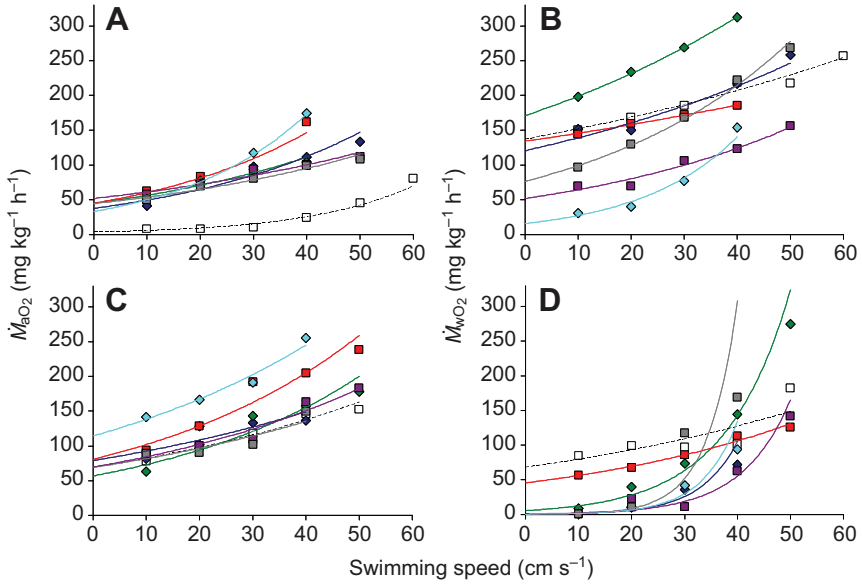

Fig. 2. Relationship between swimming speed and rate of oxygen uptake from air ($\dot{M}_{aO_2}$) or water ($\dot{M}_{wO_2}$) in seven *G. carapo* submitted to a critical swimming speed protocol in aquatic normoxia (~18 kPa P_{O_2}) or hypoxia (4 kPa P_{O_2}). (A) $\dot{M}_{aO_2}$ in normoxia; (B) $\dot{M}_{wO_2}$in normoxia; (C) $\dot{M}_{aO_2}$ in hypoxia; (D) $\dot{M}_{wO_2}$ in hypoxia. On each panel, each symbol represents the same individual fish; the matching coloured (or dotted) line is a least-squares exponential regression fitted to that individual's data points. Individual regression coefficients are given in supplementary material Table S1.

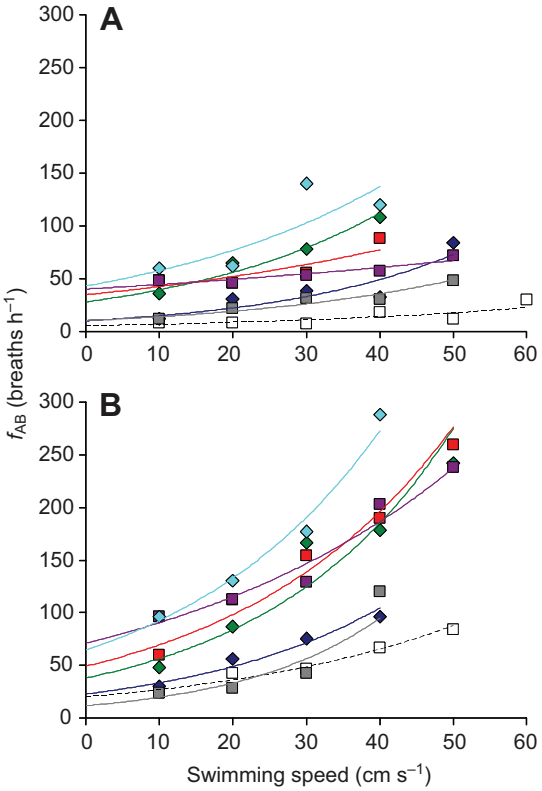

Fig. 3. Relationship between swimming speed and air-breathing frequency (f_{AB}) in seven *G. carapo* submitted to a critical swimming speed protocol in (A) aquatic normoxia (~18 kPa P_{O_2}) or (B) hypoxia (4 kPa P_{O_2}). On each panel, each symbol represents the same individual fish; the matching coloured (or dotted) line is a least-squares exponential regression fitted to that individual's data points. Individual regression coefficients are given in supplementary material Table S1.

(2.59±0.27 *versus* 1.44±0.27 mg O_2 kg^{-1}, respectively). When normoxia and hypoxia were considered separately, mean O_2 uptake per breath did not change with exercise normoxia, but decreased significantly between IMR and AMR in hypoxia. Therefore, the very high mean f_{AB} at AMR in hypoxia was associated with a significant decline in the amount of O_2 consumed per breath.

DISCUSSION

The results demonstrate that air breathing makes a significant contribution to AS in the banded knifefish, which used aerial respiration to meet the metabolic demands of exercise up to maximum aerobic performance in both normoxia and hypoxia. However, in normoxia, the contribution of air breathing appears to be entirely facultative, because the fish achieved the same AS and U_{crit} when denied access to air. By contrast, this study provides the first demonstration that access to the surface allows a fish with facultative bimodal respiration to perform sustained aerobic exercise as well in aquatic hypoxia as it does in normoxia, by a significantly increased reliance on air breathing.

Comparative exercise performance and metabolism in *G. carapo*

The knifefish exercised relatively well, given their unusual gymnotiform locomotor mode where the body is held rigid and they are propelled by undulations of the modified anal fin (Breder, 1926; Lindsay, 1978; Blake, 1983). The data from the present study comprise the first report of U_{crit} performance in a fish with bimodal respiration, but *A. calva*, *L. oculatus* and *M. cyprinoides* are all capable of sustained exercise at speeds greater than 2 BL s^{-1} (Farmer and Jackson, 1998; Seymour et al., 2007), which was the U_{crit} measured for *G. carapo*. The normoxic U_{crit} of *G. carapo* is similar to that of unimodal water-breathing species with 'anguilliform' locomotion, such as *Anguilla anguilla* (McKenzie et al., 2003), but *G. carapo* is significantly less athletic than 'subcarangiform' swimmers of similar size at similar temperatures, such as *Leuciscus cephalus*, *Cyprinus carpio* or *Dicentrarchus labrax* (Claireaux et al., 2006; McKenzie et al., 2007b). The relatively limited aerobic swimming performance of the knifefish is consistent with the species' ecology: it inhabits slow-moving turbid waters, where it uses its electrical sense to hunt for prey (Graham, 1997) and it is reported to bury in the mud if it is exposed to sudden rapid currents (Graham, 1997).

In bony fishes, aerobic exercise is powered by slow-twitch oxidative (SO) muscle, which, in the majority of species, is mainly found in two discrete longitudinal strips along either side of the

Table 2. Elements of the partitioning of aerial and aquatic respiration by *G. carapo* during an incremental critical swimming speed protocol in aquatic normoxia (~18 kPa P_{O_2}) or hypoxia (4 kPa P_{O_2})

	Normoxia	Hypoxia
$\dot{M}_{aO_2}$ at IMR	41±9^{b,c}	73±8^{b,c,d}
$\dot{M}_{aO_2}$ at 10 cm s^{-1}	52±12^{b,c}	91±10^{d,e}
$\dot{M}_{aO_2}$ at AMR	125±14^{e}	185±19^{f,g}
$\dot{M}_{wO_2}$ at IMR	97±22^{d,e}	18±11^{a}
$\dot{M}_{wO_2}$ at 10 cm s^{-1}	117±24^{d,e}	21±14^{b}
$\dot{M}_{wO_2}$ at AMR	222±28^{g}	151±27^{f}
Net $\dot{M}_{aO_2}$ contribution to AS	84±14^{a}	112±14^{a}
Net $\dot{M}_{wO_2}$ contribution to AS	124±19^{a}	133±29^{a}
% Respiration from air at IMR	35±9^{a}	86±10^{b}
% Respiration from air at 10 cm s^{-1}	34±9^{a}	85±9^{b}
% Respiration from air at AMR	38±6^{a}	56±5^{c}

Data show mean (±s.e.m.) O_2 uptake rates ($\dot{M}_{O_2}$; mg kg^{-1} h^{-1}) from air ($\dot{M}_{aO_2}$) and water ($\dot{M}_{wO_2}$) as estimated at immobile metabolic rate (IMR), or as measured when swimming gently at a speed of 10 cm s^{-1} and at active metabolic rate (AMR), plus the resultant %$\dot{M}_{O_2}$ taken from air, and the contribution of $\dot{M}_{aO_2}$ and $\dot{M}_{wO_2}$ to aerobic scope (AS; mg kg^{-1} h^{-1}). N=7 in all cases. For a given variable (i.e. measures of $\dot{M}_{O_2}$, % from air, or net contribution to AS), a common superscript indicates no significant difference between the means for that variable (P>0.05).

Table 3. Air-breathing frequency (f_{AB}; breaths h^{-1}) and rates of oxygen uptake per air breath (O_2 uptake AB^{-1}; mg kg^{-1}) by *G. carapo*, as estimated at their immobile metabolic rate (IMR) or as measured when swimming gently at a speed of 10 cm s^{-1} and at active total metabolic rate (AMR) during an incremental critical swimming speed protocol in aquatic normoxia (~18 kPa P_{O_2}) or hypoxia (4 kPa P_{O_2})

	Normoxia	Hypoxia
f_{AB} at IMR	20±7^{a}	39±9^{b}
f_{AB} at 10 cm s^{-1}	31±9^{b}	52±14^{c}
f_{AB} at AMR	79±13^{d}	190±35^{e}
O_2 uptake AB^{-1} at IMR	2.69±0.75^{a}	2.48±0.65^{a}
O_2 uptake AB^{-1} at 10 cm s^{-1}	1.91±0.53^{a}	2.14±0.44^{a}
O_2 uptake AB^{-1} at AMR	1.77±0.23^{a}	1.14±0.16^{b}

N=7 in all cases. A common superscript indicates no significant difference between the means for that variable (P>0.05).

myotomal muscle mass at the midline, where they work antagonistically to beat the tail (Bone, 1978; Webb, 1998). Although *G. carapo* has been studied extensively for the anatomy of their electricity-generating and sensing organs, which are found associated with the anal fin below the ventral epaxial muscle (Perreira et al., 2007), such studies have not described the distribution of SO and FG muscles in the species. In the closely related green knifefish, *Eigenmannia virescens*, SO muscle appears only to occur in a ventral area in association with the modified anal fin, the remainder of the main body musculature being FG muscle (Behrend, 1986).

During an incremental U_{crit} protocol, axial SO muscle is typically used until a fish can no longer generate tailbeats fast enough to respond to a speed increment and, therefore, recruits the large myotomal blocks of FG muscle. This is visible as a transition from a gait with a steady tailbeat frequency to a 'burst-and-coast' gait comprising intermittent bursts of rapid tailbeats (Webb, 1998; Marras et al., 2010). It is not clear why this was never observed in the knifefish in the present study.

The IMR measured in the present study was similar to previous reports of 'resting' metabolic rate in *G. carapo* at a similar temperature (Liem et al., 1984). The relative partitioning of M_{tO_2} between water and air at IMR was also similar to a previous report, which found that approximately 35% of M_{tO_2} was derived from air in normoxia (Liem et al., 1984), very similar to the present study, rising to approximately 75% in hypoxia at ~10 kPa (Liem et al., 1984), compared with 85% at ~4 kPa in the present study. The AMR and net AS of the knifefish were similar to that of the anguilliform swimmer *A. anguilla* but less than that of subcarangiform swimmers (*L. cephalus*, *C. carpio* and *D. labrax*) of similar size at similar temperatures (McKenzie et al., 2003; McKenzie et al., 2007b; Claireaux et al., 2006).

It is not clear why $\dot{M}_{wO_2}$ at IMR was so elevated and variable among individuals in normoxia with access to air, by comparison with the other conditions. It was not an artefact of recovery from exercise, because the four exercise tests were randomised, and IMR did not differ among the remaining three tests. Graham (Graham, 1997) reviewed evidence that denial of air-access tended to reduce resting $\dot{M}_{tO_2}$ in facultative air-breathing fishes, which is consistent with the lower IMR observed when fish had no access to the air in the present study. Similar to the present study, aquatic hypoxia significantly reduced $\dot{M}_{tO_2}$ in *M. cyprinoides* swimming at low speeds, but not at higher speeds (Seymour et al., 2004). This unusual effect of aquatic hypoxia on $\dot{M}_{tO_2}$ in bimodal fishes deserves further study.

Air breathing contributes to aerobic metabolic scope during exercise

This is the first study to demonstrate that there is an exponential increase in M_{aO_2} during incremental aerobic exercise in a fish with bimodal respiration, and to quantify its contribution to AS. Nonetheless, previous studies have shown that, when $\dot{M}_{tO_2}$ increases in air-breathing fish submitted to sustained aerobic exercise, $\dot{M}_{aO_2}$ also increases. In *A. calva* and *L. oculatus* at 15–20°C, this was associated with an increase in the percentage of $\dot{M}_{tO_2}$ taken from air, which rose from 10 or 2%, respectively, at rest, to 53 or 66%, respectively, at 2 BL s^{-1} (Farmer and Jackson, 1998). At temperatures similar to those used in the present study, aerial respiration was essentially zero in *M. cyprinoides* swimming in aquatic normoxia at 0.4 BL s^{-1}, and only rose to approximately 4% of $\dot{M}_{tO_2}$ at 0.8 BL s^{-1}. By contrast, in aquatic hypoxia (P_{O_2}=8 kPa), M_{aO_2} accounted for 44% of $\dot{M}_{tO_2}$ at the lower speed and 47% at the higher speed (Seymour et al., 2004). When the same species was exposed to more severe hypoxia (below a P_{O_2} of 6 kPa), gill ventilation ceased completely, such that air breathing may have provided a very large proportion of oxygen uptake at low swimming speeds (0.65 BL s^{-1}) and also at high sustained speeds (2.4 BL s^{-1}), although a role for ram ventilation could not be discounted (Seymour et al., 2007). Thus, *M. cyprinoides* (Seymour et al., 2004) is similar to *G. carapo* (present study), in that exercise did not increase the percentage proportion of $\dot{M}_{tO_2}$ derived from air breathing.

The somewhat exponential increase in f_{AB} with swimming speed in normoxia was similar to that found in a previous study of another facultative air breather, *A. calva* (Farmer and Jackson, 1998). That study found quite wide individual variation in f_{AB} during exercise, which was also found in *G. carapo*. In *G. carapo*, the increase in f_{AB} was an entirely facultative response in normoxia: the knifefish achieved the same U_{crit}, AMR and AS when they were denied access to air in normoxia.

The present study also demonstrates, for the first time, that a fish with bimodal respiration can use air breathing to support maximum aerobic exercise performance in aquatic hypoxia. The contribution of $\dot{M}_{aO_2}$ to $\dot{M}_{tO_2}$ allowed the knifefish to maintain performance unchanged from their normoxic levels at a degree of aquatic hypoxia that significantly impaired their performance when they were denied access to air. This level of hypoxia would cause profound limitations to U_{crit} and AMR in unimodal water-breathing species (Dahlberg et al., 1968; Bushnell et al., 1984; Jourdan-Pineau et al., 2010). The increased reliance on aerial respiration during exercise in hypoxia was sustained by the pronounced increase in f_{AB}, with fish breathing on average once every 20 s. This very large increase in f_{AB} during exercise in hypoxia with access to air contrasts with results for *M. cyprinoides*, which did not show any significant change in f_{AB} between swimming speeds of 0.65 and 2.4 BL s^{-1}, remaining at approximately one breath every 85 s (Seymour et al., 2007).

It might be expected that the very high f_{AB} at AMR in hypoxia would have been associated with a decline in U_{crit} in the knifefish, if breaking the surface caused increased drag. Nonetheless, the fact that AS and U_{crit} were the same in hypoxia with access to air *versus* normoxia without access indicates that costs of swimming were also similar. The unusual anatomy and swimming mode of the knifefish, with an upturned mouth for gulping air while propelled by a ventral fin that never breaks the water surface, may also be significant, in combination with the fact that high frequencies were achieved by a change in behaviour, whereby the fish swam very close to the water surface and took repeated bouts of air breaths in quick succession. Nonetheless, it must be kept in mind that knifefish do not swim particularly well; maximal performance may be limited by their locomotor mode before surface drag becomes limiting. Farmer and Jackson (Farmer and Jackson, 1998) reported that some individual *A. calva* took air breaths once every 30 s at swimming speeds greater than 2 BL s^{-1} in normoxia. This is an interesting area for future research.

There was certainly a decline in the efficiency of O_2 uptake from the air-breathing organ at high swimming speeds, as revealed by the significant decline in mean O_2 uptake per breath at AMR. In *M. cyprinoides*, there was an inverse relationship between individual mean f_{AB} *versus* mean O_2 uptake per breath, although exercise did not have any effect on the latter variable in that species, which, at approximately 3.2 mg O_2 per breath (Seymour et al., 2007), was somewhat higher than observed in *G. carapo*. In *G. carapo*, this may be the factor that ultimately limits the potential contribution of aerial respiration to AS in hypoxia. Indeed, at the level of hypoxia tested here, aquatic respiration still made a significant contribution to AS, although it must be kept in mind that, at AMR in hypoxia, net $\dot{M}_{wO_2}$

was significantly limited compared with normoxia. It remains to be seen, nonetheless, whether the knifefish can meet its entire AS by air breathing alone at more extreme levels of aquatic hypoxia.

Why air breathe during exercise in normoxia, if it is not obligatory for performance?

Surfacing behaviours in air-breathing fishes put them at significantly increased risk of predation from above (Kramer et al., 1983; Smith and Kramer, 1986), and they can be inhibited by fear of predation (Shingles et al., 2005). The results of the present study therefore raise the question of why the knifefish would breathe air during exercise in normoxia if this was not necessary to sustain aerobic performance. It is conceivable that this is because the response is a chemoreflex, stimulated by information from peripheral chemoreceptors sensitive to oxygen levels in both water and blood (Smatresk et al., 1986; McKenzie et al., 1991). During exercise, the reflex may have been stimulated by a progressive decline in venous oxygen levels as exercise intensity increased (Stevens and Randall, 1967; Farrell and Clutterham, 2003). Whatever the mechanism that stimulated air-breathing during exercise in normoxia, the current results indicate that the advantages of air breathing, in terms of colonising hypoxic habitats, may come with significant ecological costs (Kramer, 1983; Chapman and McKenzie, 2009). Such costs may include not only increased risks of aerial predation, but also interference with behaviours that require aerobic exercise, such as predator–prey encounters or social interactions (Kramer, 1983). This may be one reason why, despite the prevalence of hypoxia in tropical freshwater habitats, there are only approximately 450 species with bimodal respiration amongst the more than 25,000 species of bony fishes (Graham, 1997).

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