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Balancing the competing requirements of air-breathing and display behaviour during male–male interactions in Siamese fighting fish *Betta splendens*

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ABSTRACT

Air-breathing fish of the Anabantoidei group meet their metabolic requirements for oxygen through both aerial and aquatic gas exchange. Siamese fighting fish *Betta splendens* are anabantoids that frequently engage in aggressive male–male interactions which cause significant increases in metabolic rate and oxygen requirements. These interactions involve opercular flaring behaviour that is thought to limit aquatic oxygen uptake, and combines with the increase in metabolic rate to cause an increase in air-breathing behaviour. Air-breathing events interrupt display behaviour and increase risk of predation, raising the question of how Siamese fighting fish manage their oxygen requirements during agonistic encounters. Using open-flow respirometry, we measured rate of oxygen consumption in displaying fish to determine if males increase oxygen uptake per breath to minimise visits to the surface, or increase their reliance on aquatic oxygen uptake. We found that the increased oxygen requirements of Siamese fighting fish during display behaviour were met by increased oxygen uptake from the air with no significant changes in aquatic oxygen uptake. The increased aerial oxygen uptake was achieved almost entirely by an increase in air-breathing frequency. We conclude that limitations imposed by the reduced gill surface area of air-breathing fish restrict the ability of Siamese fighting fish to increase aquatic uptake, and limitations of the air-breathing organ of anabantoids largely restrict their capacity to increase oxygen uptake per breath. The resulting need to increase surfacing frequency during metabolically demanding agonistic encounters has presumably contributed to the evolution of the stereotyped surfacing behaviour seen during male–male interactions, during which one of the fish will lead the other to the surface, and each will take a breath of air.

1. Introduction

Animals use display behaviour for many different purposes, including courtship and territory defence. These display behaviours are influenced by physiological indicators of fitness, and are necessary for beneficial rewards such as access to territory, resources and mates. Animals exhibiting such display behaviours must balance the time spent displaying against time spent foraging and their own energetic requirements. Siamese fighting fish *Betta splendens* are a facultatively air-breathing freshwater fish belonging to the Anabantoidei group (Graham, 1997; Mendez Sanchez and Burggren, 2012), possessing an air breathing labyrinth organ that enables them to obtain oxygen from the air to supplement aquatic oxygen uptake via the gills and skin (Peters, 1978). During both aggressive territorial encounters and courtship displays, male Siamese fighting fish engage in fin and opercular flare behaviour (Simpson, 1968). Such displays are intense, causing significant changes in muscle metabolites (Haller, 1991a,b) and resulting in significantly increased rates of oxygen uptake (Castro et al., 2006). The extent to which display behaviour is supported by anaerobic metabolism has not been determined, but Haller (1992) suggests that glycolysis is the major energy source during aggressive behaviour in Siamese fighting fish, and the observation that rates of oxygen uptake remain elevated following an agonistic encounter (Castro et al., 2006) supports the view that anaerobic metabolism is likely to be important. Indeed, opercular flaring is also hypothesised to decrease gill ventilation because it inhibits action of the opercular pump (Abrahams et al., 2005), which acts to move water over the gills (Hughes and Shelton, 1958; Hughes, 1960). Thus, while holding the opercular spread during agonistic encounters, Siamese fighting fish are unable to rise to the surface to breathe air, and are unable to effectively exchange respiratory gases with water (Abrahams et al., 2005).

The duration of opercular flare displays is a significant predictor of success in agonistic encounters (Evans, 1985), rather than morphological features such as fin size (Allen and Nicoletto, 1997). Moreover, exposure to hypoxia causes individuals to reduce opercular displays (Abrahams et al., 2005), suggesting that opercular displays are a reliable signal of body condition. Individuals in better physical condition

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are physiologically more able to cope with the reduction in respiratory gas exchange that occurs during opercular flaring, and thus are able to continue to display more vigorously, for longer periods of time, under challenging conditions.

During fights, air breathing frequency is positively correlated with the frequency and duration of opercular flaring and with tail beating (Dore et al., 1978; Meliska et al., 1980). This suggests that these activities increase metabolic rate or compromise aquatic gas exchange, or both, thereby necessitating an increased reliance on aerial gas exchange to support aerobic metabolism. Given that opercular display rate is a significant predictor of success in male–male interactions (Simpson, 1968; Evans, 1985), and that air breathing during an agonistic encounter could potentially compromise display behaviour, it seems reasonable to suggest that individuals would benefit from minimising the time engaged in air-breathing behaviour in order to maximise the time engaged in display behaviour. However, empirical data are lacking for males during fights where gas exchange and air breathing behaviour are measured simultaneously. Therefore, through continuous measuring of aerial and aquatic gas exchange during male–male interactions, we tested two non-exclusive hypotheses for how male Siamese fighting fish balance the need for metabolically demanding aggressive displaying against the need to obtain oxygen from air and water: (1) during display behaviour, Siamese fighting fish will increase the their oxygen uptake per breath relative to routine activity in order to minimise the time engaged in air-breathing behaviour, or (2) during display behaviour, Siamese fighting fish will increase their reliance on aquatic oxygen uptake, in order to minimise air-breathing behaviour.

2. Materials and methods

2.1. Animals and experimental protocol

Fish were purchased from a commercial supplier and maintained in a constant temperature room at 25 ± 1 °C. Fish were held individually in 4 L aquaria containing dechlorinated tap water (Brisbane, QLD, Australia) treated with Prime® (Seachem Laboratories, Madison, GA, USA), and fed 4 *Betta* pellets (Betta Bites®, Springville, UT, USA) 3–4 times a day.

For measurements of gas exchange and air-breathing behaviour at rest and during displays, fish were transferred to individual square-sided glass bottles (21 820 545, Schott Glas, Mainz, Germany) filled with 1080 mL of aquarium water. The fish were accustomed to being handled in this way, and all individuals resumed apparently normal air-breathing behaviour within a few minutes of being transferred to the bottle. Bottles containing fish were wrapped in black plastic and placed in a 25 ± 1 °C water bath regulated using an immersion heater (F12-ED, Julabo GmbH, Seelbach, Germany). The water within the bottles was gently aerated and fish were held within these bottles overnight for a minimum of 12 h and a maximum of 48 h before measurements commenced. At the commencement of measurements the bottle containing the fish was sealed leaving a 50 mL air-space into which the fish could breathe, and through which air was passed for measurement of aerial gas exchange (see below). Gas exchange of resting and inactive fish was measured at night for 1–2 h, while gas exchange of active displaying fish was measured during the day for 1–1.5 h.

For measurements of display behaviour two identical measurement systems ran in parallel and display behaviour was encouraged by putting the two bottles containing fish next to each other within the water bath and removing the black plastic from around the bottles to allow fish to see each other and interact through the glass. A mirror was also placed next to the bottles to encourage fish to display to themselves if the adjacent fish was not sufficient to promote display behaviour. The fish typically commenced display behaviour almost immediately (e.g. Castro et al., 2006). Measurements of resting and display behaviour were separated by at least 24 h.

2.2. Respirometry

Aerial rates of oxygen consumption ($\dot{V}_{O_2}$, μL h^{-1}) and carbon dioxide production ($\dot{V}_{CO_2}$, μL h^{-1}) were measured using positive-pressure open-flow respirometry (Withers, 2001; Lighton, 2008) and a system similar to that used previously for other air-breathing fish (Alton et al., 2007). Air was drawn from outside using a pump (SS-3 subsampler, Sable) and scrubbed of water vapour (Drierite) and CO_2 (soda lime) before passing through a mass flow controller (Aalborg GFC17, Stanton Scientific), the air-space in the respirometry chamber, a CO_2 analyser (Li-7000, John Morris Scientific), and an oxygen analyser (Oxzilla II, Sable Systems). Incurrent flow rate ($\dot{V}_I$) through the air-space was regulated at 105 mL min^{-1} STPD. The CO_2 and O_2 analysers were interfaced with a PowerLab 8/30 A/D convertor (ADInstruments, Bella Vista, NSW, Australia), which recorded the concentrations of CO_2 and O_2 in the excurrent air at a frequency of 10 Hz. High-frequency noise in the CO_2 and O_2 data was removed by applying 0.5 Hz and 0.1 Hz low-pass filters using LabChart v7.0 (ADInstruments). The O_2 analyser was calibrated using dry CO_2-free air, and the CO_2 analysers were calibrated using N_2 and a certified gas mix (0.386 ± 0.008% CO_2 in N_2, BOC Gases, Wetherill Park, Australia). Each individual breath produced a conspicuous inverted spike in the trace of excurrent fractional oxygen concentration (F_{eO_2}) and a corresponding positive spike in the trace of excurrent fractional CO_2 concentration (F_{eCO_2}). $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ were calculated by integrating the product of $\dot{V}_I$ and F_{eO_2} or F_{eCO_2} on a breath-by-breath basis using the Data Pad function of LabChart.

Aquatic $\dot{V}_{O_2}$ was measured by circulating water through a T-piece containing a fibre-optic oxygen sensor connected to a connected to an oxygen meter (TX3, PreSens GmbH, Germany) and recording the oxygen saturation at 5 s intervals. Controls were run following each measurement by recording the decline in aquatic oxygen saturation without a fish in the chamber. Aquatic $\dot{V}_{O_2}$ was calculated from the slope of the line relating oxygen saturation and time for chambers containing fish (m_f, % h^{-1}), the equivalent slope for control chambers containing no fish (m_c), the oxygen capacitance of air-saturated water (β_{O_2}, 5.77 mL L^{-1} at 25 °C (Riley and Chester, 1971)) and water volume (V, L):

$$\dot{V}_{O_2} = -1\left[\left(m_f - m_c\right)/100\right]V\beta_{O_2}$$

Measurement of aerial O_2, aerial CO_2 and aquatic O_2 was made for fish resting during their inactive circadian phase, and for fish displaying to a mirror and conspecific; from these data a range of gas exchange parameters were calculated: aerial and aquatic $\dot{V}_{O_2}$, total $\dot{V}_{O_2}$ (the sum of aerial and aquatic $\dot{V}_{O_2}$; a proxy for aerobic metabolic rate), aerial $\dot{V}_{CO_2}$, aerial respiratory exchange ratio (RER = aerial $\dot{V}_{CO_2}$ divided by aerial $\dot{V}_{O_2}$), air breathing frequency (f_{ab}, breaths h^{-1}), O_2 uptake and CO_2 release per breath (μL).

2.3. Statistical analysis

Comparisons between resting and displaying fish were initially made using linear mixed models including mass, behaviour (resting or displaying), and their interaction as fixed effects, and individual identity as a random effect. However, because testing the significance of fixed effects in linear mixed models is problematic due to difficulties in computing the appropriate degrees of freedom (Pinheiro and Bates, 2000; Bolker et al., 2009), we examined the significance of individual identity using likelihood ratio tests of models with and without individual identity. Models with individual identity as a random effect never provided significantly better fits to the data than equivalent models with only fixed effects ($P > 0.56$ in all cases), so data were analysed using linear models with mass, behaviour, and their interaction as fixed effects with α set at 0.05 for all tests. In all models the interaction term was not significant and was removed. To maintain statistical power, Bonferroni corrections were not employed (Quinn and Keough, 2002; Nakagawa, 2004). Data were analysed using R v2.15.0 (R Development Core Team, 2012); linear mixed models were analysed using the 'nlme' package in R (Pinheiro et al., 2012).

3. Results

Resting metabolic rate measurements were obtained for 13 fish (mean mass 1.5 ± 0.3 [s.d.] g); one fish would not display, therefore only 12 fish were measured during display behaviour. During display behaviour there was a significant 2.1-fold increase in total V_{O_2} (Fig. 1A, Table 1), which was met by a significant 2.3-fold increase in air-breathing frequency (Fig. 1B, Table 1) and 3.2-fold increase in aerial V_{O_2} (Fig. 1C, Table 1), but no significant increase in aquatic V_{O_2} (Fig. 1D, Table 1). Behaviour had no significant effect on O_2 uptake per breath (Fig. 1E, Table 1), but during display behaviour CO_2 release per breath increased 1.4-fold (Fig. 1F, Table 1). Aerial V_{CO_2} showed a 2.9-fold increase during display behaviour (Fig. 1G, Table 1), and aerial RER was not significantly altered during display behaviour (Fig. 1G, Table 1), being comparable to other studies of air-breathing fish (Fig. 3). Total V_{O_2}, aerial V_{O_2}, O_2 uptake per breath, CO_2 release per breath, and aerial V_{CO_2} were significantly positively associated with mass, but air-breathing frequency, aquatic V_{O_2} and RER were not associated with mass (Table 1).

4. Discussion

The present study shows that the rate of oxygen uptake (V_{O_2}) of displaying male Siamese fighting fish is significantly higher than that of non-displaying Siamese fighting fish (Fig. 1A), as has been shown previously (Castro et al., 2006). However, the 2.1-fold increase in V_{O_2} we found for fish displaying to both conspecific males and mirrors is greater than the 1.4-fold increase previously reported during displays to mirrors alone (Castro et al., 2006), which are generally more vigorous than those to conspecifics (Dore et al., 1978). The increased oxygen requirements of Siamese fighting fish during activity were met primarily by increased oxygen uptake from the air (Fig. 1C), with a non-significant increase in aquatic oxygen uptake (Fig. 1C), contrary to hypothesis (2). The increased aerial oxygen uptake was achieved by an increase in air-breathing frequency (Fig. 1B), as has been shown previously (Dore et al., 1978), and not by an increase in oxygen uptake per breath, contrary to hypothesis (1) (Fig. 1E). In contrast to O_2 uptake per breath, CO_2 release per breath was significantly higher for displaying individuals than resting ones (Fig. 1F), and was significantly related to body mass for both resting and displaying individuals (Table 1, Fig. 2B). The size-dependent effects of activity on breath-by-breath O_2 uptake and CO_2 release largely balance one another, such that the aerial respiratory exchange ratio does not change significantly with activity or size (Table 1, Fig. 2C) and is broadly comparable with other air-breathing fish respiring in normoxic water (Fig. 3).

Similar responses to the increased metabolic demands of activity have been observed in other air-breathing fish. Bowfin *Amia calva* breathe water almost exclusively when inactive at low temperatures, but increase their reliance on air-breathing at higher temperatures and when active (Johansen et al., 1970). Gar *Lepisosteus oculatus* also have a limited reliance on aerial oxygen uptake when resting, and increase aerial oxygen uptake during activity (Farmer and Jackson, 1998), as do lungfish *Neoceratodus forsteri* (Grigg, 1965), Senegal birchir *Polypterus senegalus* (Magid, 1966), and Atlantic tarpon *Megalops atlanticus* (Shlaifer and Breder, 1940). In Pacific tarpon *Megalops cyprinoides*, air breathing frequency increases with exercise

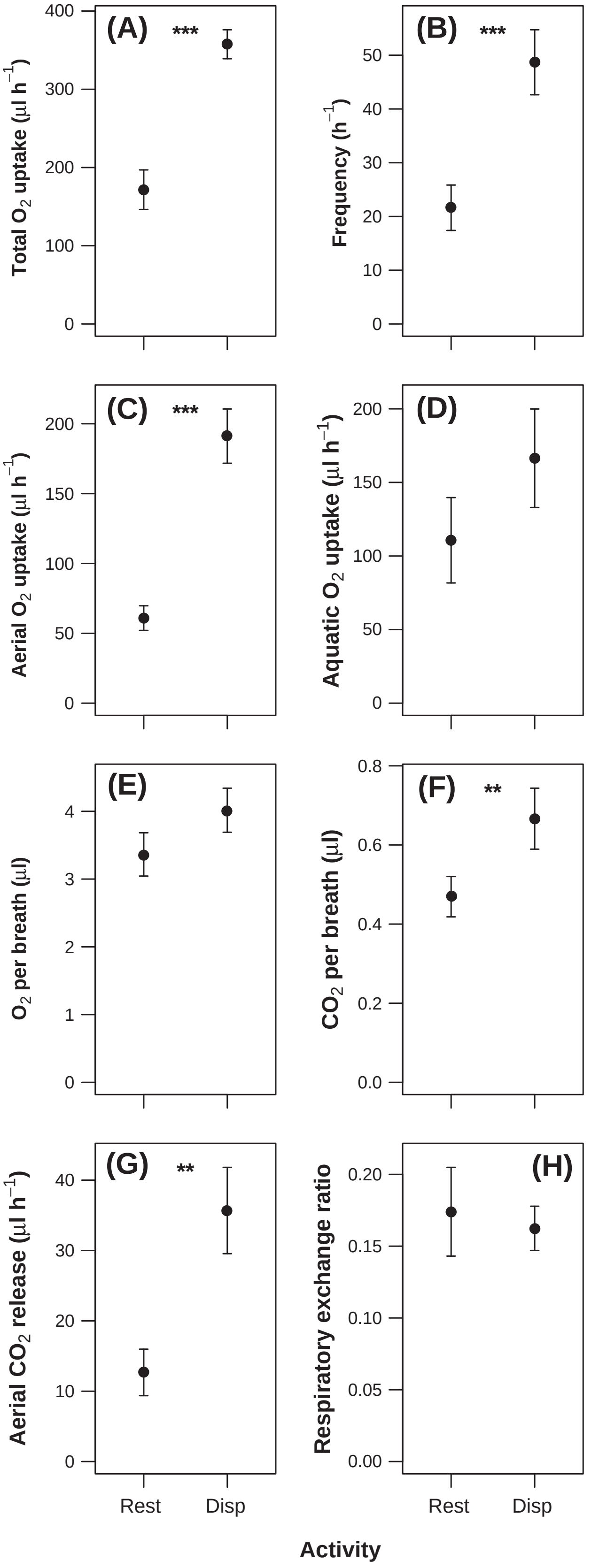

Fig. 1. The effect of display behaviour on (A) total rate of oxygen uptake, (B) air-breathing frequency, (C) aerial rate of oxygen uptake, (D) aquatic rate of oxygen uptake, (E) oxygen uptake per breath, (F) CO_2 release per breath, (G) aerial rate of CO_2 release, and (H) aerial respiratory exchange ratio (= CO_2 release divided by O_2 uptake). Data are shown as mean ± SE (n = 13 for rest, 12 for display); traits that are significantly associated with mass are presented adjusted to a mean mass of 1.5 g using the parameter estimates from Table 1. Total rate of oxygen uptake, air-breathing frequency, aerial rate of oxygen uptake, CO_2 release per breath, and rate of aerial CO_2 release all vary significantly between resting (Rest) and displaying (Disp) fish (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Table 1

Parameter estimates (shown ± SE) for the effects of mass (g) and behaviour (resting or displaying) on total rate of oxygen uptake (V_{O_2}, µL h^{-1}), air-breathing frequency (f_{ab}, h^{-1}), aerial V_{O_2}, aquatic V_{O_2}, oxygen uptake per breath (µL), CO_2 release per breath (µL), aerial rate of CO_2 release (V_{CO_2}, µL h^{-1}), and aerial respiratory exchange ratio (RER = aerial V_{CO_2} divided by aerial V_{O_2}). Degrees of freedom are 22 for all models.

Response	Parameter	Estimate	*t*	*P*
Total V_{O_2}	Intercept	−63 ± 91	−0.69	0.50
	Displaying	186 ± 33	5.72	<0.001
	Mass	156 ± 59	2.65	0.01
f_{ab}	Intercept	−19 ± 21	−0.94	0.36
	Displaying	27 ± 7	3.64	0.001
	Mass	27 ± 13	2.03	0.05
Aerial V_{O_2}	Intercept	−161 ± 59	−2.71	0.01
	Displaying	130 ± 21	6.13	<0.001
	Mass	148 ± 39	3.84	<0.001
Aquatic V_{O_2}	Intercept	98 ± 126	0.78	0.44
	Displaying	56 ± 45	1.24	0.23
	Mass	8 ± 82	0.10	0.92
O_2 breath^{-1}	Intercept	0.5 ± 1.1	0.46	0.65
	Displaying	0.6 ± 0.4	1.56	0.13
	Mass	1.9 ± 0.7	2.55	0.02
CO_2 breath^{-1}	Intercept	−0.5 ± 0.2	−3.04	0.006
	Displaying	0.2 ± 0.1	3.43	0.002
	Mass	0.6 ± 0.1	6.23	<0.001
Aerial V_{CO_2}	Intercept	−36 ± 19	−1.86	0.08
	Displaying	23 ± 7	3.30	0.003
	Mass	33 ± 13	2.58	0.02
RER	Intercept	0.02 ± 0.10	0.16	0.88
	Displaying	−0.01 ± 0.03	−0.37	0.72
	Mass	0.11 ± 0.06	1.73	0.10

intensity (Clark et al., 2007; Seymour et al., 2007), but the amount of oxygen extracted per breath is not significantly affected by aquatic hypoxia or exercise (Seymour et al., 2004). Similarly, suprabranchial tidal volume does not vary with aerial or aquatic oxygen concentration in blue gouramis *Trichogaster trichopterus* (Burggren, 1979). To date, oxygen uptake per breath has been shown to vary only with aerial oxygen concentration in pearl gouramis *Trichogaster leeri* (Alton et al., 2007). The association between oxygen uptake per breath and aerial oxygen concentration in pearl gouramis arises because suprabranchial tidal volume is likely to be independent of aerial oxygen content, so oxygen extraction per breath is related to the concentration of oxygen in the suprabranchial chambers at the start of an apnoea. Taken together, these findings suggest that, in general, anabantoids are unable to increase tidal volume in response to increased oxygen requirements, meaning that Siamese fighting fish can increase aerial oxygen uptake during activity only by increasing air-breathing frequency. There is, however, a significant increase in CO_2 release per breath during display behaviour, which presumably arises because opercular flaring during display behaviour limits aquatic gas exchange, and therefore reduces CO_2 loss to the water.

The finding that Siamese fighting fish rely more on aerial than aquatic oxygen uptake to meet the increased oxygen demands of display behaviour suggests that Siamese fighting fish have little capacity to increase oxygen uptake by the gills beyond that which occurs at rest. We therefore suggest that the constraints imposed by the reduced gill surface area of air-breathing fish (Palzenberger and Pohla, 1992) and the reduced gill ventilation during opercular flaring necessitate increased air-breathing frequency during agonistic encounters. The association between activity and air-breathing was first noted several decades ago (Grigg, 1965), and has recently regained prominence as a putative advantage of air-breathing (Farmer and Jackson, 1998; Clark et al., 2007; Wells et al., 2007).

The need to breathe air during male–male interactions is potentially problematic for Siamese fighting fish. Air-breathing is associated with increased predation risk (Kramer et al., 1983; Wolf and Kramer, 1987), and could cause breaks from display behaviour that increase the probability of losing an encounter. However, many species of air-breathing fish breathe synchronously (Kramer and Graham, 1976; Graham and Baird, 1982; Chapman and Chapman, 1994) and Siamese

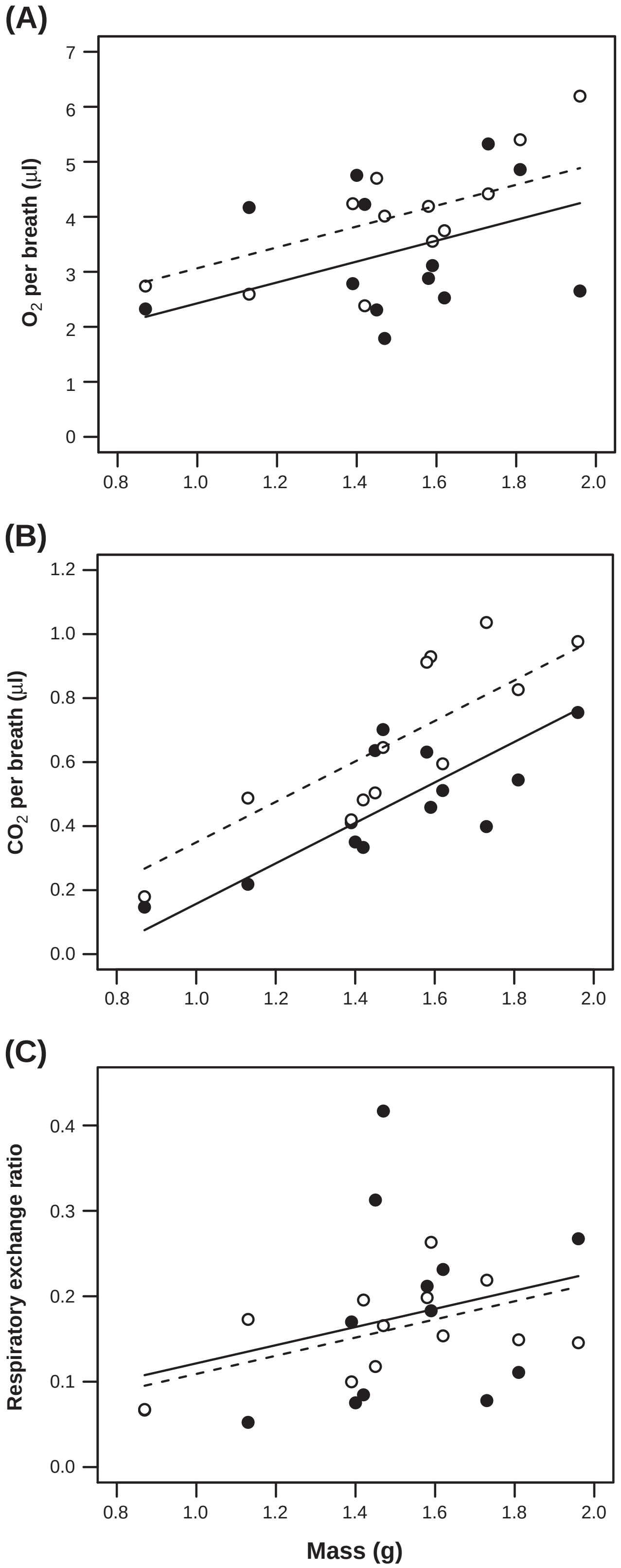

Fig. 2. Relationship between body mass and (A) aerial O_2 uptake per breath, (B) aerial CO_2 release per breath (C) aerial respiratory exchange ratio (RER) for resting (filled symbols, solid line) and displaying (unfilled symbols, dashed line) fish. The effect of behaviour is significant for CO_2 release but not O_2 uptake; the effect of mass is significant for O_2 uptake and CO_2 release, but not RER (Table 1); parameter estimates for the linear models including behaviour and mass are presented in Table 1.

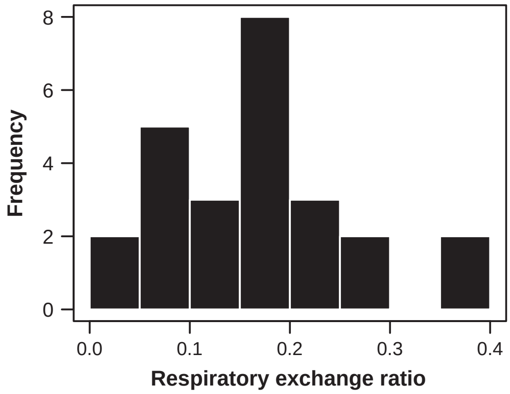

Fig. 3. Frequency distribution of aerial respiratory exchange ratios observed for a range of air-breathing fish respiring in approximately normoxic water.
Data from Graham (1997).

fighting fish have solved the problem of how to exchange gas with the atmosphere during display behaviour by engaging in simultaneous air breathing, such that one of the fish will lead the other to the surface, and each will take a breath of air (Simpson, 1968).

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