The Role of Size in Synchronous Air Breathing of *Hoplosternum littorale*

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ABSTRACT

Synchronized air breathing may have evolved as a way of minimizing the predation risk known to be associated with air breathing in fish. Little is known about how the size of individuals affects synchronized air breathing and whether some individuals are required to surface earlier than necessary in support of conspecifics, while others delay air intake. Here, the air-breathing behavior of *Hoplosternum littorale* held in groups or in isolation was investigated in relation to body mass, oxygen tensions, and a variety of other physiological parameters (plasma lactate, hepatic glycogen, hematocrit, hemoglobin, and size of heart, branchial basket, liver, and air-breathing organ [ABO]). A mass-specific relationship with oxygen tension of first surfacing was seen when fish were held in isolation; smaller individuals surfaced at higher oxygen tensions. However, this relationship was lost when the same individuals were held in social groups of four, where synchronous air breathing was observed. In isolation, 62% of fish first surfaced at an oxygen tension lower than the calculated P_{crit} (8.13 kPa), but in the group environment this was reduced to 38% of individuals. Higher oxygen tensions at first surfacing in the group environment were related to higher levels of activity rather than any of the physiological parameters measured. In fish held in isolation but denied access to the water surface for 12 h before behavioral testing, there was no mass-specific relationship with oxygen tension at first surfacing. Larger individuals with a greater capacity to store air in their ABOs may, therefore, remain in hypoxic waters for longer periods than smaller individuals when held in isolation unless prior access to the air is prevented. This study highlights how social interaction can affect air-breathing behaviors and the importance of considering both behavioral and physiological responses of fish to hypoxia to understand the survival mechanisms they employ.

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Introduction

One of the many challenges that fish encounter is the need to extract sufficient oxygen from the water to meet their metabolic demands. Fish that inhabit waters that frequently become hypoxic have to possess a suite of behavioral, physiological, biochemical, and molecular attributes that will allow survival under oxygen-deficient conditions. While many freshwater fish from temperate waters respond poorly to hypoxia, the Amazon boasts an amazing diversity of fish that are able to survive in hypoxic or even anoxic conditions for long periods of time (Almeida-Val et al. 1993). Floodplain lakes of the Amazon may see oxygen levels falling to less than 1.5 kPa at night and rising to supersaturation during the day. Given that more than 3,000 species of fish are believed to inhabit the Amazon, it is not surprising that the Amazon has been described as an "under-explored biological goldmine," particularly for fish biologists interested in studying hypoxia tolerance (Almeida-Val et al. 1993).

In contrast to air-breathing marine fish that generally utilize the skin, gills, and buccal cavities as gas-exchange epithelia, freshwater air-breathing fish have typically evolved air-breathing organs (ABOs), including areas of the gastrointestinal tract, modified gas bladders, and labyrinth organs (Gans 1970; Graham 1997). However, for many fish, surviving hypoxia requires integrated behavioral and physiological attributes. An example of a behavioral response to hypoxia that may have been a precursor to air breathing (Perry et al. 2001) is aquatic surface respiration (ASR), where fish choose to move to surface waters in which oxygen diffusion from the air results in a thin layer of well-oxygenated water (Kramer and Mehegan 1981: Kramer and McClure 1982; Shingles et al. 2005). Air breathing also relies on migrating to the water surface, but to gulp air. Unfortunately, moving to the water surface comes with the

trade-off of increased susceptibility to predation from aerial predators or other larger aquatic predators that have also migrated to the water surface (Wolf 1985; McIntyre and McCollum 2000; Robb and Abrahams 2003).

It is likely that the increased predation threat associated with traveling to the water surface has driven the evolution of synchronized air breathing in some fish species where individuals come to the surface in social groups (Chapman and Chapman 1994). Thus, a dilution effect, similar to that associated with shoaling fish, has been suggested (Kramer and Graham 1976). In fish isolated from their social group, surfacing may be delayed for longer periods of time (Randle and Chapman 2005), increasing the need to physiologically cope with hypoxia exposure. In the catfish *Clarias liocephalus*, Chapman and Chapman (1994) found that body size had no effect on synchronous air breathing, with synchronous bouts being initiated by both large and small fish and with no difference in air-breathing rates. This is perhaps surprising given that many species show a relationship between size and hypoxia tolerance (Nilsson et al. 2007), and smaller fish are more likely to be susceptible to predation. In fish known to perform ASR, both size and predation risk modulate this behavioral response. Small oscar *Astronotus ocellatus* will choose to remain in hypoxic waters to lower oxygen tensions in order to reduce predation risk (Sloman et al. 2006) even though they are less physiologically tolerant of hypoxia when compared with larger individuals. Tide pool sculpins *Oligocottus maculosus* will also delay ASR and remain in hypoxic waters longer if predation risk is high (Sloman et al. 2008). While synchronized air breathing has been documented in a variety of fish species, little is known about how individual variation in size or other physiological parameters affect air-breathing behavior and whether synchronized air breathing requires some individuals to surface earlier than necessary in altruistic support of conspecifics while others are required to delay air intake. In the cascudo *Hypostomus regani*, a large amount of individual variability in air-breathing behaviors has been demonstrated, with a potential link to an individual's surfacing tachycardia, suggesting that between-individual variation in physiological parameters may well affect individual surfacing behaviors (Nelson et al. 2007).

Many species of catfish are known to perform synchronized air breathing. Here, we utilized the armored catfish *Hoplosternum littorale* (Callichthyidae; Hancock 1828) to study synchronized air-breathing behavior. *Hoplosternum littorale* is a facultative air breather, well adapted to coping with hypoxia (Jucá-Chagas 2004) and, in addition, tolerant of both acidic and hydrogen sulfide–rich waters (Brauner et al. 1995). Unlike many other fish species of the Amazon, *H. littorale* is able to survive a seasonal phenomenon known as a "friagem," where water-column mixing results in hypoxic (0–1 mg L^{-1}), acidic (pH 5.4), and hydrogen sulfide–rich (500–900 μM) waters (Brauner et al. 1995; Val and Almeida-Val 1995). Synchronized air breathing has been noted in the closely related species *Hoplosternum thoracatum* (Kramer and Graham 1976). *Hoplosternum littorale* uses its posterior intestine as an accessory air-breathing organ, where approximately 63% of the gut is modified for oxygen extraction (Persaud et al. 2006*b*). Although it is a facultative air breather, *H. littorale* is known to take air from the water surface during normoxia (Affonso and Rantin 2005), and it is hypothesized that air in the intestine is also a requirement for buoyancy and passage of food through the digestive tract (Persaud et al. 2006*b*). Additionally, *H. littorale* has a high aquatic P_{crit} (the environmental oxygen tension at which oxygen uptake of the animals switches from being independent of to being dependent on environmental oxygen [Hughes 1981]) relative to other facultative air breathers, suggesting a higher dependency on atmospheric oxygen (Jucá-Chagas and Boccardo 2006). Nothing is known about the ability of different sizes of *H. littorale* to tolerate hypoxia. Therefore, the aim of our study was first to confirm the synchronous air-breathing behavior of *H. littorale* and then to investigate the hypothesis that surfacing behavior would be affected by between-individual variation in both size and physiology, but that any relationships found would be altered by social environment.

Material and Methods

Hoplosternum littorale ranging in mass from 36 to 140 g were obtained from their natural habitats, close to the confluence of the Rio Negro and Rio Solimões, near Manaus, and transferred to the Ecophysiology and Molecular Evolution laboratory at the Instituto Nacional de Pesquisas da Amazônia (INPA; Manaus, Amazonas, Brazil), 3 wk before the start of the experiment. Stock fish were held outside in 500-L tanks at 28° ± 3°C under a natural light-dark cycle. The tanks were continually aerated, and 50% of the water was replaced every 2 d. Fish used in experiments were individually tagged at least 24 h before the experiment was initiated. To achieve this, fish were carefully netted from the stock tank and lightly anesthetized (buffered MS222, 4 g L^{-1}; Sigma), and a combination of small colored beads were sutured to the skin at the base of the dorsal fin. They were then placed into a bucket of aerated tank water until they had fully recovered from the anesthesia, and then they were returned to the stock tank. Fish were not fed for 48 h before experimentation but were fed to satiation with commercial fish food between experiments. Three fish developed a fungal infection during the experimental period and were not included in any analyses.

Experiment 1: Individual Variation in Behavior and Physiology of Hoplosternum littorale

Hoplosternum littorale ($n = 24$) were tested for their surfacing behavior during gradual exposure to hypoxia both when held in isolation and when held in groups. The P_{crit} of each fish was also calculated. After these three measurements had been made, fish were killed and sampled for a variety of physiological parameters (see below). The combination of these measurements allowed an individual profile of behavior and physiology to be acquired for each fish. The order of testing for nonlethal measurements—that is, group or isolated exposure to hypoxia and calculation of P_{crit}—was randomly selected for each individual.

As a test of whether experiment order or repeated hypoxic exposure could effect experimental results, the relationship between the threshold Po_2, which initiated surfacing in fish held individually, and the order of sampling was tested. No significant effect of experimental order was found ($P = 0.977$).

Surfacing Behaviors in Isolation. Hoplosternum littorale were carefully netted from the stock tank, placed in a bucket of tank water, and individually transferred to a 74-L glass tank shielded on three sides with black plastic to minimize disturbance to the fish. The water was continuously aerated with an air stone, and the fish were allowed to acclimate to the tank for 1 h. Preliminary behavioral observations found that the fish remained motionless on introduction to the tank, but that within an hour, they had started to move around and explore the tank with their barbels. Longer acclimation periods did not alter this behavioral pattern. Before each experiment, the observer sat for 20 min in front of the tank to allow the fish to acclimate to the observer's presence. Initial appearance of the observer resulted in the fish becoming motionless, but slow exploratory movement was resumed within 20 min. Following the acclimation period, oxygen tensions were steadily reduced (Fig. 1) by switching the air supply to nitrogen. Oxygen tensions were continuously monitored using a WTW Oxi 235 oxygen meter (Oxi325, WTW, Weilheim, Germany). Activity was recorded during this time by dividing the tank horizontally in half (with a line drawn on the outside of the tank). Each time the snout of a fish crossed into the other section of the tank was counted as one horizontal movement. Horizontal movements of the fish were then totaled to give an "activity" score. The activity of the fish was continuously monitored as the oxygen tensions fell, until the point at which the fish went to the water surface and took a gulp of air. At this point the experiment was terminated and the oxygen tension was noted. The oxygen tension in the tank was then gradually raised back to normoxia, and the fish were carefully netted, placed in a bucket of tank water, and returned to the stock tank.

Group Surfacing Behaviors. Groups of four *H. littorale* (chosen for maximum size disparity; $n = 6$) were transferred to 500-L plastic (nontransparent) tanks as above. Preliminary studies showed that a maximum of four fish within a group allowed for accurate identification of individuals during surfacing. Water within the tanks was continually aerated by recirculation through a gas-exchange column supplied with air. Fish were allowed to acclimate to the tank for 2 h before the experiment was started. Preliminary recordings of the groups in the tank illustrated that fish remained motionless for up to 2 h when placed into the experimental tank, but then they started periods of slow exploratory behavior. Fish were then filmed from above using a Panasonic PV G585 digital video camera for 2 h as the oxygen tension in the tank was gradually reduced (Fig. 1) at the same rate as in the isolation experiments by switching the air supply in the gas-exchange column to nitrogen. Oxygen tensions were continuously monitored as before. Video tapes were then analyzed, and the time and oxygen tension at which each individual fish came to the water surface to take a breath were recorded. The order in which fish surfaced within a group could be determined and, therefore, a rank order of surfacing. The total amount of time an individual spent swimming versus remaining still at the bottom of the tank was also calculated as seconds spent swimming (activity). At the end of the experiment, the oxygen tension was gradually raised back to normoxia and the fish were returned to the stock tank as before.

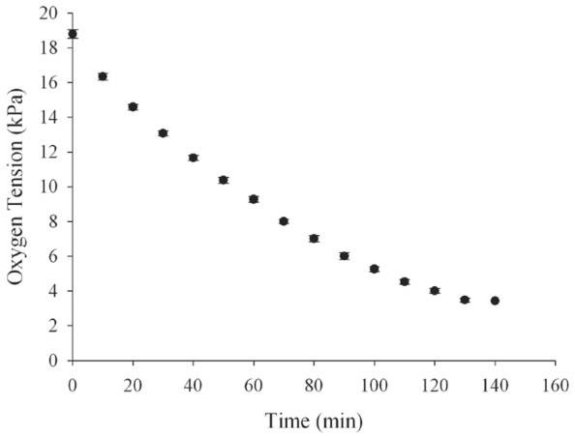

Figure 1. Rate of decline in Po_2 utilized in behavior experiments. Data are expressed as means ± SEM.

Individual P_{crit} Measurements. Hoplosternum littorale were carefully netted from the stock tank, placed in a bucket of tank water, and transferred to sealable Nalgene containers (1,750 mL) and allowed to settle overnight for a 12-h period. The containers were placed in a water bath to control temperature (26.9° ± 0.2°C) and were covered with black plastic to minimize visual disturbance. The containers were vigorously aerated to maintain air-saturated conditions during the settling period and were supplied with a continuous water flow. The following morning, water flow and aeration were stopped and the containers were sealed with an oxygen electrode inside (WTW Oxi 235 oxygen meter). Containers were gently mixed to dissipate boundary layers. The time course of the fall in Po_2 was then monitored as the fish consumed oxygen, until the time that the fish lost equilibrium, a process that took 2–4 h. Oxygen consumption was calculated over sequential 5-min periods, changes in oxygen uptake were plotted against the mean water oxygen tension, and P_{crit} was calculated using the algorithm described by Yeager and Ultsch (1989). Rates of oxygen consumption at the beginning of the experiment were used to calculate the resting oxygen consumption rates (Mo_2) of the animal. Measurements from three individuals were discarded due to problems determining a precise P_{crit} value. At the end of the experiment, the boxes were carefully lifted and opened, and the fish were gently returned to a bucket of water and then to the stock tank.

Physiological Measurements. At the end of the four measurements described above, *H. littorale* were placed in sealable Nalgene containers (1,750 mL) as before and allowed to settle

overnight. The containers were placed in a water bath to control temperature (26.9° ± 0.2°C) and were covered with black plastic to minimize visual disturbance. The containers were vigorously aerated to maintain air-saturated conditions during the settling period and were supplied with a continuous water flow. The following morning, fish were exposed to a 1-h hypoxic challenge where the oxygen tension was reduced to 6.3 kPa. This represented an oxygen tension above which all fish had surfaced when it was held in a group environment. Water flow was stopped, the boxes were sealed, the air supply was switched to nitrogen, and the fall in oxygen tension in the boxes was measured as before. The oxygen tension was gradually dropped until it reached 6.3 kPa, where it was held for 1 h. After 1 h at 6.3 kPa, the boxes were carefully lifted and opened, and the fish were tipped into a net that was placed immediately into a solution of lethal anesthetic (buffered MS222, 10 g L^{-1}; Sigma). Transfer of the fish from the box to the anesthetic took no more than 60 s. A blood sample was withdrawn by caudal venipuncture. An aliquot of blood was used for the determination of hematocrit and hemoglobin by Drabkin's method. The remaining blood sample was then centrifuged and the plasma was decanted, frozen in liquid nitrogen, and stored at −80°C for later analysis of lactate using a lactate meter (Accutrend-Lactate, TYP3012522) calibrated with solutions of known lactate concentration. The mass and the length of each fish were recorded. The whole left branchial basket, the liver, and the heart were removed from each individual and weighed. The mass of the left branchial basket was multiplied by 2 to represent the mass of the whole branchial basket. The liver was frozen in liquid nitrogen and stored at −80°C for later analysis of hepatic glycogen by the anthrone method (Wedemeyer and Yasutake 1977). The intestine was carefully removed and kept intact without any gas escaping. The volume of the lower, gas-absorbing part was then calculated by submerging it in a known volume of water in a measuring cylinder. The intestine was then carefully opened and placed flat on graph paper. The area of the opened intestine was traced onto the graph paper and the surface area was calculated.

Experiment 2: Forced Submersion Effects on Subsequent Surfacing Behavior and P_{crit} of Individuals

During the acclimation periods for experiment 1, fish had access to the water surface and were potentially able to store air in their intestines. They had also been taken directly from a stock tank open to the air. To investigate whether this could have affected surfacing oxygen tensions and P_{crit}, two additional experiments were performed in which fish were confined below the water surface in normoxia for 12 h before either being subjected to declining oxygen tensions for behavioral observations or for the calculation of P_{crit}.

Surfacing Behaviors in Isolation. Twelve *H. littorale* ranging in mass from 65 to 140 g were analyzed. Approximately 12 h before the experiment, the fish were placed in sealable Nalgene containers (1,750 mL) as before and allowed to settle overnight. The containers were placed in a water bath to control temperature (26.9° ± 0.2°C) and were covered with black plastic to minimize visual disturbance. The containers were vigorously aerated to maintain air-saturated conditions during the settling period and were supplied with a continuous water flow. Fish had no access to the water surface. The fish were then tested for their surfacing behavior by placing them individually into a 74-L glass tank shielded on three sides with black plastic to minimize disturbance to the fish. The Nalgene containers were disconnected from the experimental setup, sealed to prevent water loss, and carefully submerged in the glass tank. The seals were then removed. No access to air occurred during transport to the glass tank, and the fish were left in the submerged containers for 20 min, with a flow of water between the tank and the containers. The water in the tank was continuously aerated with an air stone. The fish were then carefully released from the containers and allowed an additional 20 min to acclimate to the tank. No surfacing behaviors were observed during this time. Before each experiment, the observer sat for 20 min in front of the tank to allow the fish to acclimate to the observer's presence. Oxygen tensions were then steadily reduced at the same rate as in experiment 1 and continuously monitored. At the point when the fish first surfaced to take a gulp of air, the experiment was terminated and the oxygen tension was noted. The oxygen tension in the tank was then gradually raised back to normoxia, and the fish were returned to the stock tank as before.

Individual P_{crit} Measurements. Ten *H. littorale* ranging in mass from 38 to 157 g were placed in sealable Nalgene containers (1,750 mL) as before, with no access to the water surface, and were allowed to settle overnight for a 12-h period. This differed from the P_{crit} values measured in experiment 1, where fish could access the water surface during the acclimation period before the boxes were sealed. P_{crit} and Mo_2 values were then calculated as in experiment 1, and the fish were returned to the stock tank as before.

Statistical Analysis

Synchrony of air breathing was calculated by quantifying the temporal frequency distribution of breaths averaged across all oxygen tensions (Kramer and Graham 1976; Chapman and Chapman 1994). The observation period for each group was divided into 10-min intervals, and the number of breaths per interval was calculated (breathing rate). The frequency distribution of breaths per interval was then analyzed for clumping by calculating the coefficient of dispersion (CD), s^2/x (Chapman and Chapman 1994), where s is the variance and x is the mean. A CD value >1.0 indicates a clumped distribution, a value of 1.0 indicates random distribution, and a value <1.0 indicates an even distribution. The difference between 1.0 and the observed values was tested for significance using a one-sample t-test. The synchrony of air breathing was then considered at varying oxygen tensions by averaging across groups in the same way.

Values for the heart, branchial basket, and liver masses were

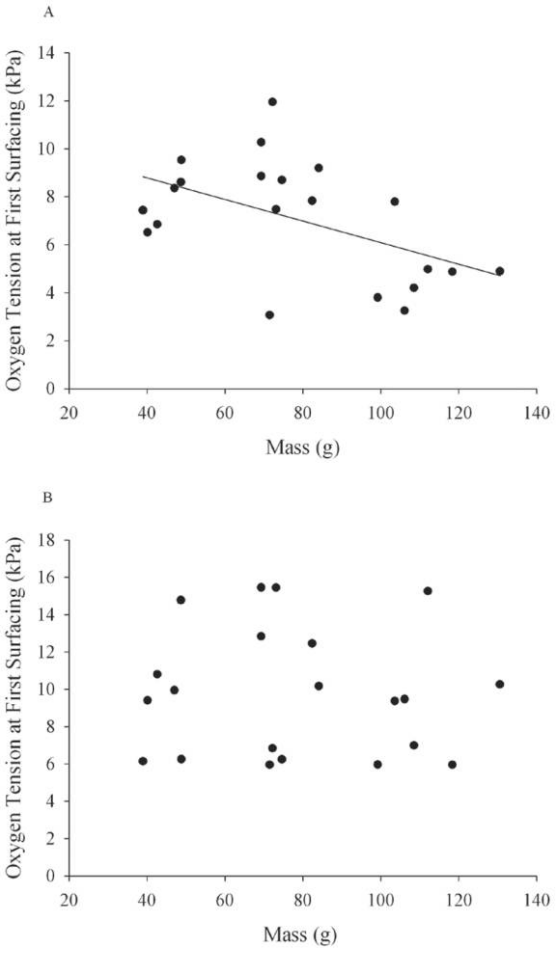

Figure 2. Relationship between mass of fish and the Po_2 at which it first came to the surface to breathe air, during a gradual decline in Po_2 (seen in Fig. 1) when held in isolation (*A*; Pearson correlation = −0.512, $P < 0.05$, $n = 21$) or in a group environment (*B*; Pearson correlation = −0.048, $P = 0.837$, $n = 21$).

regressed against body mass and residual values (i.e., with the effect of body mass removed) used to look for relationships with other behavioral and physiological parameters. The scaling coefficients (b, where log organ mass = $b \times$ (log body mass) + a) for the heart, branchial basket, and liver masses were 0.907, 0.579, and 0.951, respectively. The two intestinal measurements (gas volume and surface area) were also regressed against body mass, and the scaling coefficients were 1.35 and 0.881, respectively. Residual values were then combined using a principal components analysis to produce an intestinal index taken to represent the potential of the fish to absorb oxygen across the intestine. Residual values for surface area accounted for 70.43% of the axis variability, and residual values for gas volume accounted for 29.57%. A high intestinal index was taken to represent a fish with a greater capacity to use the lower intestine as an ABO. Mo_2 (μmol h^{-1}) was regressed against body mass in experiments 1 and 2 (see "Results" for regression equations).

Behavioral and physiological data were tested for normality using a Kolmogorov-Smirnov test, and homogeneity of variance was tested using a Levene's test before using the appropriate parametric (Pearson correlation; *t*-tests: paired, unpaired, and one-sample) or nonparametric (Spearman's rank) statistic. Where necessary, data were transformed before statistical analysis. Data are presented as mean ± SEM.

Results

Experiment 1: Individual Variation in Behavior and Physiology of Hoplosternum littorale

Surfacing Behaviors in Isolation. When fish were held in isolation with prior access to the water surface, smaller fish surfaced first at a higher oxygen tension (Pearson correlation = −0.512, $P < 0.05$, $n = 21$; Fig. 2*A*). There was no relationship between total amount of activity during exposure to hypoxia and first-

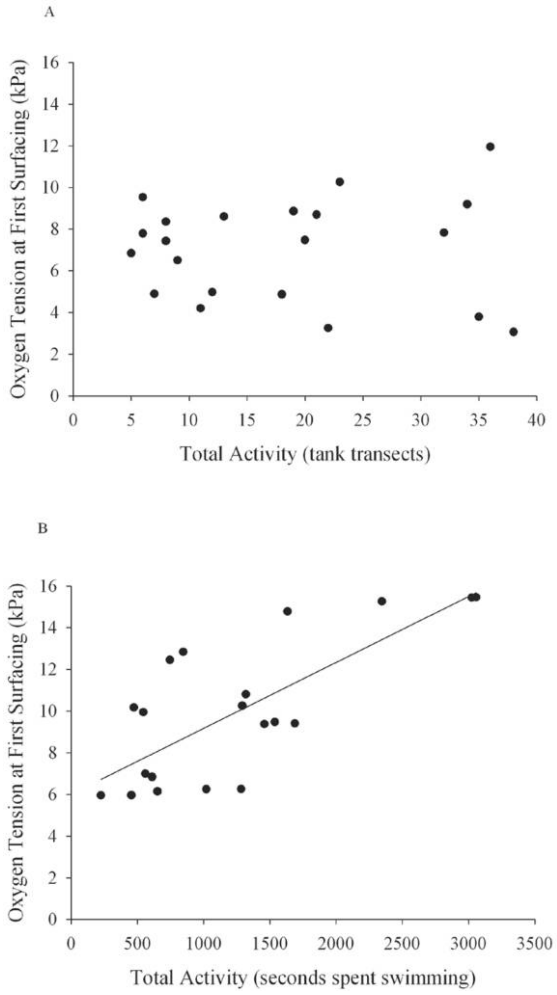

Figure 3. Relationship between total amount of activity (measured as number of tank transects in fish held in isolation and as seconds spent swimming in fish held in groups) and the Po_2 at which each individual fish came to the surface to breathe air during a gradual decline in Po_2 (seen in Fig. 1) when held in (*A*) isolation (Pearson correlation = 0.013, $P = 0.955$, $n = 21$) or (*B*) a group environment (Pearson correlation = 0.748, $P < 0.001$, $n = 21$).

Table 1: Mean rates of air breathing per 10-min interval (B/I) for groups of four *Hoplosternum littorale* and the coefficient of dispersion (CD)

Group No.	B/I	CD[a]
1	1.75	4.06
2	5.25	3.71
3	1	2.55
4	1	2
5	1.42	5.06
6	1	3.09

Note. Number of intervals for each group = 12.
[a] s^2/x, where s = variance and x = mean.

surfacing Po_2 (Pearson correlation = 0.013, $P = 0.955$, $n = 21$; Fig. 3*A*).

Group Surfacing Behaviors. There was a significantly clumped frequency of surfacing (both first surfacing and subsequent surfacing; i.e., CDs of >1.0) of *Hoplosternum littorale* individuals when held in groups averaged across all oxygen tensions (one-sample *t*-test: $t = 5.364$, df = 5, $P < 0.005$; Table 1), confirming that this species shows synchronous air breathing in a group environment. Synchronicity of air breathing was also compared at different oxygen tensions, and a positive correlation between coefficient of dispersion and oxygen tension at the end of each 10-min interval was found (Pearson correlation = 0.656, $P < 0.05$, $n = 12$; Fig. 4); therefore, it appears that synchronicity becomes less pronounced at lower oxygen tensions. However, air breathing still remained significantly synchronous across different oxygen tensions (one-sample *t*-test: $t = 3.693$, df = 11, $P < 0.005$). Unlike when fish were held in isolation, there was no significant effect of mass on the oxygen tension at which fish first surfaced within a group environment (Pearson correlation = −0.048, $P = 0.837$, $n = 21$; Fig. 2*B*). However, when the rank order of oxygen tensions at which fish first surfaced in groups was calculated, small fish surfaced first (Spearman's rank correlation coefficient = 0.516, $P < 0.05$, $n = 21$). Interestingly, in the group environment, the oxygen tension at which fish first surfaced was significantly correlated with total activity (Pearson correlation = 0.748, $P < 0.001$, $n = 21$; Fig. 3*B*). There was no relationship between the oxygen tension at which individual fish first surfaced when held in a group environment and when that same fish was held in isolation (Pearson correlation = 0.233, $P = 0.310$, $n = 21$; Fig. 5). However, on average, individuals first surfaced at a lower oxygen tension when held in isolation compared with when they were in a group environment (paired *t*-test: $t = 3.376$, df = 20, $P < 0.01$; Fig. 5).

The change in first-surfacing oxygen tensions seen in individuals from an isolated to a group environment was not related to the mass of the fish (Pearson correlation = −0.586, $n = 21$, $P = 0.199$; Fig. 6*A*). Four individuals surfaced at a lower oxygen tension when held in groups than when they were held in isolation; the remainder surfaced at higher oxygen tensions. Different measures of activity were used for fish held in groups and in isolation because of differences in experimental setup. Therefore, for comparison, fish were ranked according to their level of activity in each environment. The change in activity rank of an individual from being held in isolation to a group environment was significantly correlated with change in first-surfacing oxygen tensions (Pearson correlation = 0.438, $n = 21$, $P = 0.047$; Fig. 6*B*). Individuals that were more active in group environments compared with their rank activity in isolation showed the biggest change in first-surfacing oxygen tensions. Those individuals that were more active in a group environment showed the biggest increase in first-surfacing oxygen tensions from an isolated to a group environment.

Individual P_{crit} Measurements. There was no significant effect of mass on P_{crit} (Pearson correlation = 0.377, $P = 0.171$, $n = 18$), and there was no relationship between P_{crit} of an individual fish and the oxygen tension at which it first surfaced, either in a group environment (Pearson correlation = −0.025, $P = 0.931$, $n = 18$) or in isolation (Pearson correlation = −0.009, $P = 0.975$, $n = 18$). The average P_{crit} across all sizes of fish when access to the water surface was allowed during the acclimation period was 4.67 ± 0.41 kPa. There was no relationship between mass and resting Mo_2 calculated when fish had prior access to the water surface (μmol h^{-1}; Pearson correlation = −0.171, $P = 0.497$, $n = 18$; Fig. 7).

Physiological Measurements. To understand the physiological factors that may be influencing surface time when fish were held either in groups or alone, the first-surfacing oxygen tensions in these environments were compared with each of the physiological parameters measured after the fish had been exposed to 6.3 kPa for 1 h. Values for heart, branchial basket, and liver mass were regressed against body size, and residual values were used for comparisons. A summary of these results is given in Table 2. No statistical correlations between surfacing

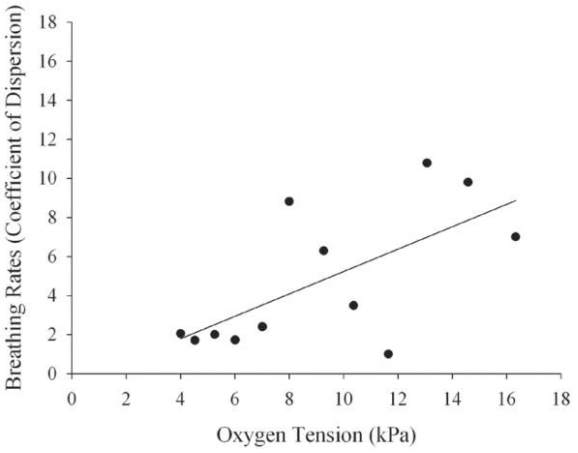

Figure 4. Relationship between oxygen tension at the end of each 10-min interval and the coefficient of dispersion (CD; s^2/x, where s = variance and x = mean) for air-breathing rates. Synchronicity of air breathing decreases with decreasing oxygen tension (Pearson correlation = 0.656, $P < 0.05$, $n = 12$).

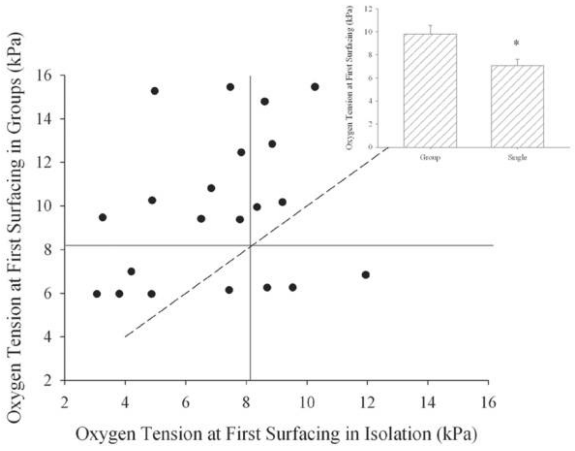

Figure 5. Relationship between oxygen tension at which an individual fish first surfaced when held in groups of four and when held in isolation. (Pearson correlation = 0.233, $P = 0.310$, $n = 21$). The solid vertical and horizontal lines represent P_{crit} as calculated for the species in this study when fish are denied access to the water surface (8.13 kPa). The dashed line represents the predicted relationship if an individual surfaced at the same oxygen tension in both environments. The inset shows mean oxygen surfacing tensions for all fish held in groups or isolation. Data are expressed as mean + SEM (paired *t*-test: $t = 3.376$, $n = 21$, $P < 0.05$).

oxygen tensions, either in groups or isolation, and these physiological parameters were found.

Experiment 2: Forced Submersion Effects on Subsequent Surfacing Behavior and P_{crit} of Individuals

Surfacing Behaviors in Isolation. Fish were held for 12 h under the water surface before being exposed to declining Po_2 in isolated conditions. When this occurred, there was no relationship between mass of fish and Po_2 at first surfacing (Pearson correlation = 0.118, $P = 0.714$, $n = 12$).

Individual P_{crit} Measurements. As in experiment 1, there was no significant effect of mass on P_{crit} (Pearson correlation = −0.042, $P = 0.908$, $n = 10$), and the average P_{crit} was 8.13 ± 0.77 kPa. However, P_{crit} values were significantly higher in these fish that were denied access to the water surface for 12 h before determination of P_{crit} than in those of experiment 1 (4.67 ± 0.41 kPa), which had access to the water surface during the acclimation period (unpaired *t*-test: $t = -4.39$, df = 27, $P < 0.001$). In contrast to experiment 1, there was a significant correlation between mass of an individual and its resting Mo_2 (μmol h^{-1}; Pearson correlation = 0.815, $P < 0.004$, $n = 10$; Fig. 7).

Discussion

When *Hoplosternum littorale* were held in an isolated environment, there was a correlation between oxygen tension at first surfacing and mass of fish, with smaller fish surfacing at higher oxygen tensions. This mass-specific relationship was not present when fish were held in a group environment (Fig. 2). The elimination of a relationship between mass and oxygen tension of first surfacing in social groups is perhaps not surprising, because the opportunity to participate in synchronous air breathing and the influence of conspecifics will modulate the behavior of each individual. Mattias et al. (1998) also found no relationship between surfacing oxygen tension and mass when the facultatively air-breathing fish *Hypostomus regani* was held in groups. This loss of mass-specific relationship with surfacing oxygen tension does not appear to be attributable to a certain size class; there was no relationship between the change in surfacing oxygen tensions in different social environments and the mass of the fish (Fig. 6*A*). However, the change in activity of an individual between the two social environments did affect surfacing behavior. Individuals with the biggest rank increase in activity from isolated to group environments showed the biggest increase in surfacing oxygen

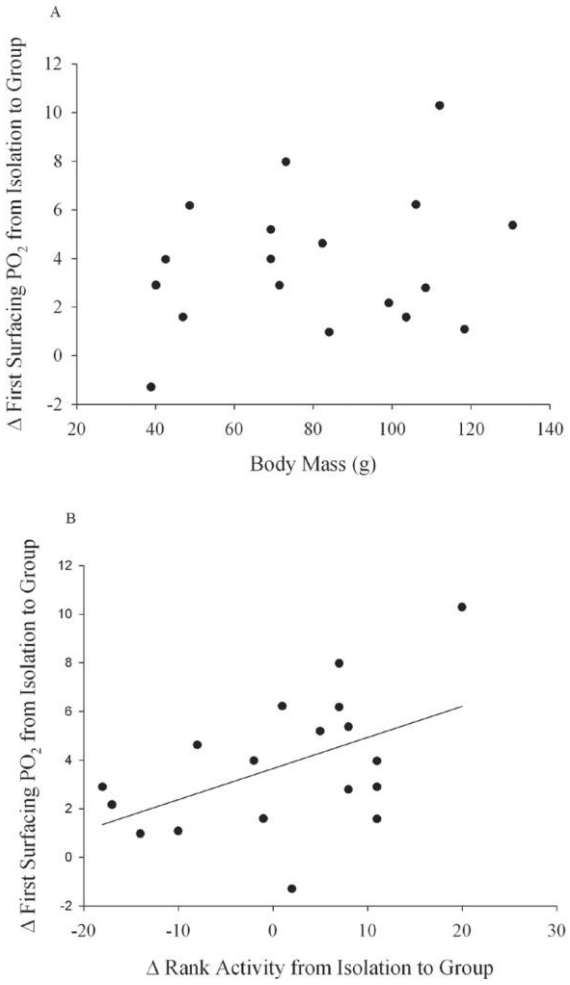

Figure 6. *A*, Relationship between body mass and change in oxygen tensions (kPa) at which the fish surfaced in isolation or in a group of four (Pearson correlation = 0.292, $P = 0.199$, $n = 21$). *B*, Relationship between change in activity rank and change in oxygen tensions (kPa) at which the fish first surfaced when fish where held in isolation or in a group environment (Pearson correlation = 0.438, $n = 21$, $P = 0.047$).

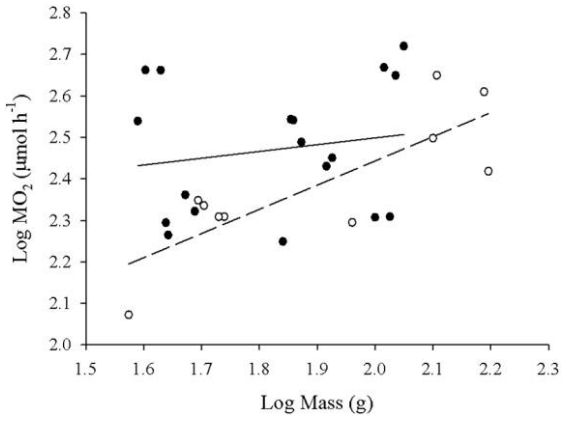

Figure 7. Relationship between log mass (g) and log Mo_2 (μmol h^{-1}) in fish allowed (*filled circles*; $y = 0.163x + 2.1728$, *solid line*) or denied (*unfilled circles*; $y = 0.5835x + 1.2763$, *dashed line*) access to the water surface during the acclimation period before respirometry trials (allowed: Pearson correlation = 0.171, $P = 0.497$, $n = 18$; denied: Pearson correlation = 0.815, $P = 0.004$, $n = 10$).

thresholds (Fig. 6*B*). Fish displaying high activity in a social environment may experience increased oxygen demands associated with the cost of increased swimming. Therefore, these fish may drive synchronous excursions to the water surface under social situations. Gee and Graham (1978) also reported that more active *Hoplosterum thoracatum* surfaced at higher oxygen tensions.

Two average P_{crit} values for this species were calculated in this study, where fish were either allowed or denied access to the water surface during acclimation to the respirometry boxes. P_{crit} values were 4.67 kPa (35 mmHg) when fish were allowed access to the water surface and 8.13 kPa (61 mmHg) when they were denied access. It is likely that, when fish were allowed access to the water surface during acclimation, they could store residual air reserves in their ABOs, allowing them to maintain total Mo_2 independent of aquatic oxygen tensions for a longer period of time. Therefore, P_{crit} values calculated in individuals that did not have access to the water surface during acclimation are more likely to reflect "actual" P_{crit} values. Indeed, similar values (50 mmHg) have been reported in *H. littorale* by Affonso and Rantin (2005). The relationship between resting water Mo_2 levels and mass also differed between those fish with and without access to the water surface during acclimation (Fig. 7). Only when fish were denied access to the water surface during the acclimation period was the expected allometric relationship between mass and water Mo_2 observed, supporting the idea of interference of residual air in the ABO if fish were not acclimated without access to air. Mo_2 levels calculated in experiment 2 were similar to those reported in other Neotropical catfish species (Nelson 2002) and somewhat higher than those reported in 100-g *H. littorale* (Brauner et al. 1995).

Interestingly, although the size of an individual affected its surfacing oxygen tension in isolation, there was no relationship between P_{crit} of an individual and mass, either when a fish was allowed or denied access to the water surface. The relationship between size and P_{crit} in fish has been subject to some controversy; Nilsson et al. (2007) suggest that the degree of hypoxia tolerance of individuals is likely to reflect their need for survival under hypoxic conditions rather than being a factor that passively follows scaling. In the Amazonian oscar, a positive relationship between size and hypoxia tolerance has been demonstrated (Sloman et al. 2006), but in other species, a negative relationship has been suggested (Robb and Abrahams 2003). Here, no relationship between P_{crit} and mass was found.

It is likely that in an isolated environment, *H. littorale* will delay surfacing for as long as physiologically possible (Randle and Chapman 2005). In isolation, the predation threat that could be associated with traveling to the water surface may potentially restrain individuals in hypoxic waters. In contrast, synchronized air breathing of groups of fish may allow "safety in numbers" (Kramer and Graham 1976). Taking 8.13 kPa as the average P_{crit} value for individuals used in our study, it was found that only five individuals surfaced below P_{crit} in both the group environment and when fish were held in isolation (Fig. 5). When held in isolation, 62% of fish surfaced below the average P_{crit} value, while only 38% of fish in the group environment remained in hypoxia to below the P_{crit} value before surfacing (Fig. 5). Overall, individuals surfaced at higher oxygen tensions when held in groups than when held in isolation. Additionally, synchronicity of air breathing was reduced with declining oxygen tensions (Fig. 4), and it is possible that, as the physiological need to surface increases, the need for air intake takes priority over predation risk.

Isolation from conspecifics produced a mass-dependent air-breathing response, with smaller fish migrating to the water surface at higher oxygen tensions than larger individuals. However, when individuals were prevented from replenishing the air in their intestine for 12 h before behavioral observations, this relationship no longer existed among fish held in isolation. As body mass does not appear to affect P_{crit} in *H. littorale* during aquatic respiration, it is possible that the size of the ABO in *H. littorale* could account for the differences in time of surfacing in isolation between large and small individuals when prior access to air is allowed. Jucá-Chagas (2004) demonstrated that, when compared with *Lepidosiren paradoxa* and *Hoplerythrinus unitaeniatus*, there is a much stronger correlation between the amount of oxygen extracted per breath and body mass in *H. littorale*. Additionally, changes in gulping frequency in larval *H. littorale* during development suggest that larger fish are more efficient extractors of oxygen via the ABO (Persaud et al. 2006*a*). Larger individuals with a greater capacity to store air in their ABOs may, therefore, be able to remain in hypoxic waters for longer periods than smaller individuals. However, it appears that activity within a social group, rather than the size of the fish or its ABO, explains the difference in air-breathing behaviors between fish held in isolation and in a group environment.

Surfacing of the majority of individuals in a group environment above the P_{crit} value suggests that air breathing may be important in factors other than oxygen uptake. If the point of surfacing in *H. littorale* represents the oxygen tension at which the cost of remaining in hypoxic water outweighs the risk of

Table 2: Correlation results (*P* values) between a variety of physiological parameters measured in individual *Hoplosternum littorale* and the oxygen tensions (kPa) at which they first surfaced during a gradual decline in oxygen with fish held either in groups of four or individually

Physiological Parameter	Actual Physiological Value (mean ± SEM)	Group Environment (*P*)	Isolation (*P*)
Mass (g)	78.15 ± 6.09	.837	**<.05**
Heart mass (g)	.074 ± .005	.132	.200
Branchial basket mass (g)	1.215 ± .075	.309	.743
Liver mass (g)	.615 ± .059	.540	.217
Intestinal index		.612	.240
Surface area (cm^2)	12.59 ± 1.39		
Volume of gas (mL)	1.64 ± .28		
Plasma lactate (mmol L^{-1})	2.35 ± .82	.989	.836
Liver glycogen (mg g^{-1})	32.08 ± 9.08	.051	.871
Hematocrit (%)	29.51 ± 1.44	.796	.939
Hemoglobin (g dL^{-1})	10.24 ± .69	.917	.081

Note. Residual values for heart mass, branchial basket mass, and liver mass when regressed against body mass were used in correlations. The average value for each of the physiological parameters measured is also given where the mass of organs are actual rather than residual values.

traveling to the water surface, then requirements other than oxygen may contribute to the need to surface. As continuous facultative air breathers, *H. littorale* are believed to require gas in their intestine for buoyancy and to aid passage of food through the digestive tract as well as for air breathing (Persaud et al. 2006*b*). In *H. thoracatum*, Gee and Graham (1978) demonstrated a 7.8% decline in the volume of the ABO during the period between breaths (~22.5 min) in normoxic water, creating a need to continually replace air in the intestine. In *H. littorale* held in isolation without a decline in Po_2, it was rare to see any air-breathing attempts, and so perhaps in this species, declining Po_2 triggers air breathing for air that is then stored in a stochastic manner for a variety of purposes rather than being utilized immediately. Storage of air in the ABO would certainly be advantageous in an environment such as the Amazon, where oxygen availability is often unpredictable.

In conclusion, *H. littorale* display a mass-specific relationship with surfacing oxygen tension when held in isolation, but this relationship is lost when fish are held in a group environment. Groups of *H. littorale* show synchronicity of air breathing, and activity appears to drive surfacing behavior. A greater percentage of fish refrained from air breathing when exposed to oxygen tensions lower than P_{crit} when held in isolation than when in groups; potentially, the risk of predation when traveling to the water surface is reduced in a group environment. Comparisons of the mass-specific surfacing relationship in isolated fish that were allowed and denied access to air for the 12 h before behavioral observations indicate that the capacity to store air in the ABO may influence this relationship. However, under more natural conditions, where access to the water surface is not prevented, it appears that surfacing behavior in groups is most closely related to activity. In this study, we used groups of four fish, and in the natural environment, it is likely that social groups of *H. littorale* will vary in size. However, we hypothesize that, in larger social groups, the influence of conspecifics will continue to modulate the behavior of individuals and eliminate any relationships between mass and oxygen tension of first surfacing. The effect of group size on synchronous air-breathing behavior is an interesting subject for future study, as are factors that influence the activity of individuals within social groups. Therefore, the integrated study of behavior and physiology of these animals is highlighted as a necessity in order to truly understand the mechanisms of adaptation to the hypoxic waters of the Amazon. Future research will need to address the interactions between a suite of ecologically relevant parameters—such as feeding, predator avoidance, and reproduction—that combine to determine the behavior of these animals within social groups and the subsequent effects of hypoxia.

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