CHAPTER

10

Environmental Influences on the Respiratory Physiology and Gut Chemistry of a Facultatively Air-breathing, Tropical Herbivorous Fish *Hypostomus regani* (Ihering, 1905)

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INTRODUCTION

Dissolved oxygen is an unpredictable resource for fishes in tropical freshwater ecosystems. Fish occupying these environments have evolved a suite of strategies to either avoid or exploit hypoxic waters (reviewed in

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Val, 1996). Facultative air breathing is one hypoxia-avoidance strategy that has attracted substantial interest from both physiologists and ecologists. Physiologists are primarily interested in the mechanisms, metabolic cost and physiological consequences associated with switching between the respiratory modes of breathing water or air (Graham, 1997). Ecologists have primarily studied how interspecific interactions such as predation change when animals switch between these respiratory modes (e.g., Kramer et al., 1983). Despite this multidisciplinary interest, there is a lack of information on how environmental factors other than oxygen levels influence facultative air breathing.

Aquatic hypoxia can develop in tropical habitats due to various combinations of: (1) aquatic respiratory rates exceeding photosynthetic rates, (2) poor mixing at the aerial-aquatic interface, (3) poor light penetration due to shading or turbidity, and/or (4) isolation of water bodies during tropical dry seasons (Junk, 1984). The Neotropical catfish family Loricariidae is the most diverse siluriform family and occupies most hypoxia-prone habitats in the Neotropics. This family contains at least 108 genera, more than 692 described species (Isbrücker, 2002) and many more species awaiting description (Donald Stewart, SUNY-Syracuse USA personal communication). Although air-breathing is not synapomorphic in this family (Armbruster, 1998), most loricariids examined to date will facultatively breath air upon exposure to hypoxia using their gut as an air-breathing organ (ABO; Graham, 1997), or show morphological evidence of air-breathing capabilities (Armbruster, 1998). Cascudo (*Hypostomus regani*) are usually found on rocky substrates in well-oxygenated environments, but follow the predominant loricariid behavioral pattern and facultatively breath air when they are exposed to aquatic hypoxia (Mattias et al., 1998). While loricariids were among the first subjects of facultative air-breathing studies (Carter and Beadle, 1931) and studies of environmental influences on facultative air breathing in loricariids have continued sporadically (e.g., Graham and Baird, 1982; MacCormack et al., 2003), there is still limited knowledge about facultative air breathing in this speciose group and how the environment influences it.

Environmental temperature interfaces with air-breathing physiology of aquatic ectotherms because it influences the rates of metabolic processes as well as environmental oxygen availability and diffusivity. Environmental temperature has been studied with respect to how it influences facultative air breathing (e.g., Graham and Baird, 1982), but little attention has been given to the time course of temperature change. Animals may encounter waters of disparate temperature and dissolved

oxygen content suddenly as they move between habitats or when flooding occurs. Water temperature and dissolved oxygen content also change more slowly with seasonal and climatic cycles. Thus, one aim of this study was to examine how temperature changes of different time course influence the physiological responses of cascudo to hypoxia.

The use of the gut as an ABO potentially compromises digestive function in herbivorous fishes. Many loricariids are herbivorous and presumably require an anaerobic gut to facilitate energy extraction from fermentative processes (Choat and Clements, 1998). Thus, the use of the gut as an ABO may oxygenate portions of the gut and diminish digestive performance. If this is the case, it may be manifest by a reluctance of fish with fibrous material in their guts to breath air under hypoxia. Conversely, the additional metabolic demand created by having food in the gut (specific dynamic action—SDA) could require a greater amount of facultative air breathing under hypoxic conditions. This would be manifest as increased surfacing behavior in well-fed fish under hypoxic conditions.

Finally, although hints are pervasive throughout the literature (e.g., Graham, 1997; MacCormack et al., 2003), to our knowledge, the physiology corresponding to individual variance in air-breathing behavior at a given level of water oxygenation has not been analyzed. Thus, we will pay particular attention to whether variability in air-breathing behavior is related to individual variation in physiology under hypoxic water conditions.

MATERIALS AND METHODS

Experimental Animals

Adult specimens of *Hypostomus regani* (20.7 ± 2.0 SD cm total length; 202.2 ± 53.8 g for the dietary experiment; 243 ± 64.2 g SD for the temperature experiment) were collected in the Mogi Guaçu River Basin near Pirassununga, São Paulo State, Brazil by cast net. A group of eight animals of similar size from the same collection had 8.25 ± 2.32 SD growth rings on their sagittae otoliths (Nonogaki et al., 2004). Since there is only a single rainy and dry season per year at this locale, each otolith ring is thought to be an annulus, suggesting that these animals are about 8 years old. In the laboratory, fish were maintained in 1000 L tanks supplied with dechlorinated, normoxic water (~140 mmHg) at 25 ± 1°C (acclimation temperature) for at least 3 weeks prior to experimentation.

Fish were fed *ad libitum* with commercial food pellets during acclimation. Food was withheld 2-3 days before an experimental trial in the temperature experiment. For the dietary manipulation experiment, animals were randomly assigned to either a fed or starved tank at least two weeks prior to experimentation. The "fed" tank contained natural algal growth that the animals foraged on and was supplemented every second day with summer squash (zucchini; *Cucurbita* sp.) and cucumbers (*Cucumis sativus*). The 'starved' tank was covered with black plastic to prevent algal growth and no additional food was provided.

Animal Preparation

Before surgery, fish were anaesthetized by immersion in a solution of benzocaine (0.1%) previously dissolved in 95% ethanol. Subsequently, animals were fixed to a surgical table with a continuous flow of a 0.08% benzocaine solution over the gills. Animals for the temperature experiment had electrocardiogram (ECG) electrodes, buccal and opercular cavity catheters inserted. Fish used in the diet experiment had ECG and a buccal cavity catheter installed. ECG electrodes were fashioned from 22 gauge syringe needles and inserted into the cleithra bones on either side of the heart. The electrodes were secured with atraumatic sutures on the ventral surface of the fish and again around the first dorsal fin spine. Sutures were further secured with acrylic cement. The buccal and opercular cavities were catheterized by inserting flared open-ended polyethylene tubing (PE90) across the tissue surrounding the cavity and gluing them into place (Hughes et al., 1983). All wounds were dusted with antibiotic prior to placing the fish in the experimental chamber.

Following surgery, animals that weren't already ventilating were ventilated by hand until spontaneous breathing resumed and placed into either a flow-through respirometer (temperature experiment) or experimental chambers that allowed the animals to either breath water or air (diet experiment; Fig. 10.1). Animals were allowed a minimum of 16 h to recover from surgery. For the temperature experiment, oxygen consumption ($\dot{V}O_2$), respiratory frequency (f_R), heart rate (f_H) and the oxygen tension of inspired and expired water (P_IO_2, P_EO_2, respectively) were monitored during an experiment. For the diet experiment, f_R, f_H, air-breathing frequency (f_{AIR}) and air-breathing duration (t_{AIR}) were recorded. Water pressure was recorded with either a Telos® 4-327-I or a

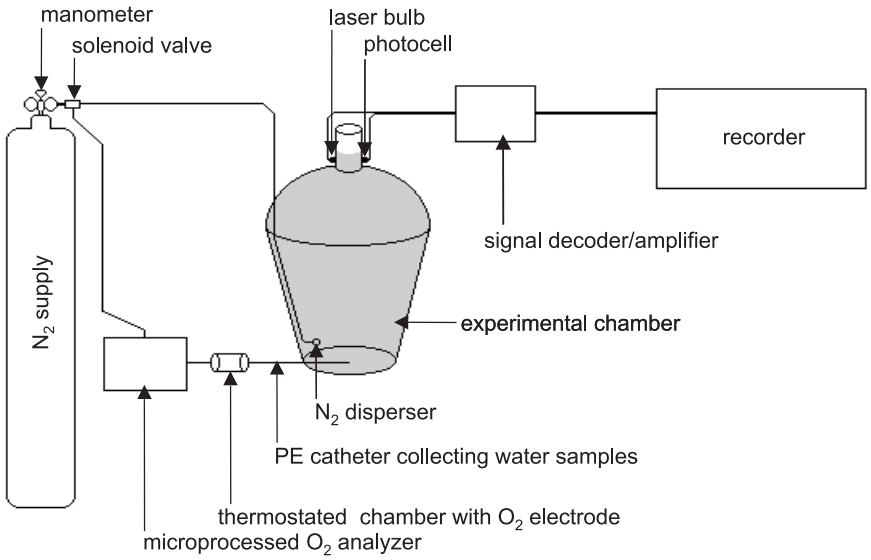

Fig. 10.1 Diet experiment chamber

Narco® P-1000B pressure transducer. Electrical signals were appropriately amplified and either recorded on a Narco® Narcotrace 40 (Narco Bio-Systems, Houston, TX, USA) physiograph or digitized and captured with MacLab® hardware utilizing Chart™ data acquisition software running on a MacIntosh I-book computer.

Exposure to Hypoxia

Dietary experiment

The experimental design was 2 × 2 with two levels of water oxygen and either fed or starved fish as the experimental treatments and 25°C as the experimental temperature. Data were either recorded during 3 h of normoxia (air saturated) or for 3 h at a nominal P_wO_2 level of 20 mmHg, below the air-breathing threshold of 60 mmHg and P_cO_2 of 27 mmHg for this species at 25°C (Fernandes et al., 1999). Hypoxia was induced by bubbling nitrogen into the water supplying the experimental chamber (Fig. 10.1). Fish in the hypoxia treatments were also recorded for 50 min under normoxia and during a 20 min reduction in P_wO_2 to the nominal level of 20 mmHg. At the end of the recording period, animals were anaesthetized until unresponsive (Stage III anesthesia) by introducing a solution of 1%

benzocaine retrograde through the breathing cannula. As soon as the animal was unresponsive, it was removed from the chamber for blood and gut content sampling.

Temperature Experiment

There were five separate temperature treatments: (1) 4 weeks of acclimation to 20°C (20°C), (2) 4 weeks of acclimation to 25°C (25°C), (3) 4 weeks of acclimation to 30°C (30°C), (4) 4 weeks of acclimation to 25°C with a rapid transfer (~ 1°C/10 min) to 20°C (20° C-T), and (5) 4 weeks of acclimation to 25°C with a rapid transfer (~ 1°C/10 min) to 30°C (30°C-T). For each treatment, (n) was equal to 8 fish for each treatment except the 20°C acclimation where n was equal to 7. Animals were exposed to graded aquatic hypoxia by bubbling N_2 gas into the water supply of the respirometer. Measurements were made at nominal P_wO_2 levels of 130, 90, 70, 50, 40, 30, 20 and 10 mmHg). The PO_2 of the inflowing water was monitored by a computerized feed back system as described by Rantin et al. (1998), and each nominal exposure level was maintained for at least 1 h.

Blood and Gut Chemistry

Fish from the diet experiment were sampled for blood and gut chemistry immediately following the 3 h hypoxic (PwO_2 = 20 mmHg) or a 3 h normoxic period. Animals in Stage III anesthesia were removed from the tank and rapidly killed by a blow to the head. The heart was exposed within seconds and blood withdrawn via cardiac puncture into "gas-tight" syringes. Immediately after the blood sample was taken, a small incision was made with artery scissors and a PE 190 cannula coated with mineral oil was advanced in one of three pre-determined gut regions in a randomly determined order as the gut contents were aspirated into a 'gas-tight' syringe. The three gut regions were: (1) foregut, immediately posterior to the stomach, (2) midgut, in the region of the ducting from an accessory liver lobe, and (3) hindgut, proceeding anterior from the gut's entrance to the cloaca. The PE 190 cannula approximated the diameter of the gut lumen such that surface tension and smooth muscle constriction formed a seal around the advancing cannula. Since one-half of the animals were starved, one-half were breathing air, and the gut length of this species is approximately twenty times the standard length of the animal (average 4 m for the animals in this study), there was some deviation from this

prescribed protocol. If there was no fluid material or the material was too viscous, no sample could be taken. If the prescribed region was entirely filled with air, the sample was taken from the next adjacent region. As soon as enough material for a pH and PO_2 determination and a perchloric acid extract was removed, sampling moved to the next gut region.

Venous blood and gut content PO_2 were measured by injecting the fresh samples anaerobically into a thermostated cuvette housing an O_2 electrode (FAC 001—O_2, FAC—São Carlos, SP, Brazil), connected to an FAC-204A O_2 analyzer. The exponential decay of PO_2 was recorded on the PowerLab®/Chart™ data acquisition system. Extrapolation of the linear portion of the PO_2 decay curve back to the time of injection was used as the measure of the *in situ* PO_2. Venous blood and gut content pH were measured by injecting the sample anaerobically into a sealed cuvette containing the pH sensing portion of a Mettler Toledo # 405-M3-S7/60 pH electrode connected to a Quimis 400A pH meter (Quimis, Brazil). Care was taken not to use the portion of the sample at the air interface for either measurement.

A portion of the blood and gut samples were deproteinized by placing 0.1 ml of sample into 0.9 ml of 0.6N $HClO_4$ and subsequently neutralized with KOH and centrifuged. The extracted supernatant was divided into two fractions: for short chain fatty acid analysis (SCFA), deproteinized supernatants of blood and gut contents were diluted 1:1 and brought to pH 1.0 with HCl to keep all acids in the protonated form; the second fraction was frozen at neutral pH. Both supernatant fractions were immediately frozen at −80°C for later analysis.

Hematocrit (Ht) was determined by centrifugation at 3000 × g in microhematocrit capillary tubes. The red blood cell count (RBC#) was determined optically with a Neubauer chamber. The mean of seven replicate counts of 1 mm^2 was calculated. The hemoglobin concentration ([Hb]) was determined by a cyanomethemoglobin method that made the blood react with Drabkin's reagent and then the optical density was recorded at 540 nm. Mean cell volume (MCV), cell hemoglobin (MCH), and cell hemoglobin concentration (MCHC) were computed from the Ht, [Hb] and RBC# by standard methods.

In order to determine SCFA levels, acidified extracts of blood and gut contents were extracted with an equal volume of chromatography grade methanol and run on a Supelco SPB-1000 capillary column on a Hewlett-Packard 5973 helium flow gas chromatograph coupled to a Hewlett-

Packard 5973 mass spectrometer. Hewlett-Packard "Chemstation®" software (version B.02.00) controlled operations and coordination of the gas chromatograph with the mass spectrometer; peak detection and identification utilized Hewlett-Packard NIST98® spectral search software. Prepared standards of formic acid, acetic acid, propanoic acid, butyric acid, isobutyric acid, isovaleric acid, pentanoic acid, hexanoic acid and heptanoic acid were cleanly separated and identified by this system. The detection limit for non-polar compounds in aqueous media has been reported to be in the parts per billion range for this system (Li and Fingas, 2003).

Physiological Measurements

Oxygen uptake

Oxygen uptake ($\dot{V}O_2$ - $mLO_2 \cdot kg^{-1} \cdot h^{-1}$) was measured by flow-through respirometry according to Rantin et al. (1992). The oxygen tension of incoming ($P_{IN}O_2$) and outgoing ($P_{OUT}O_2$) water was continuously monitored. This was accomplished by siphoning water samples via polyethylene tubing to O_2 electrodes (FAC 001—O_2, FAC—São Carlos, SP, Brazil) that were housed within temperature controlled cuvettes and connected to a FAC-204A O_2 analyzer. Oxygen uptake was calculated as:

$$\dot{V}O_2 = V_R \cdot \alpha \cdot (P_{IN}O_2 - P_{OUT}O_2) \cdot Wt^{-1},$$

where V_R represents the constant water flow rate through the respirometer ($L \cdot h^{-1}$) α denotes the solubility coefficient for O_2 in water ($mLO_2 \cdot L^{-1} \cdot mmHg^{-1}$) and Wt the body mass (kg). Flow rate was 300 $ml \cdot min^{-1}$ and measured manually. Critical oxygen tension was calculated by curve-fitting according to Yeager and Ultsch (1989).

Gill ventilation

Gill ventilation ($\dot{V}_G$ - $mLH_2O \cdot kg^{-1} \cdot min^{-1}$) was measured and calculated according to Hughes et al. (1983). Permanently implanted PE catheters allowed continuous measurement of inspired (P_IO_2—buccal catheter) and expired (P_EO_2—opercular catheter) water O_2 tensions. Gill ventilation was calculated according to:

$$\dot{V}_G = V_R \cdot [(P_{IN}O_2 - P_{OUT}O_2)/(PIO_2 - PEO_2)] \cdot Wt^{-1}.$$

Respiratory frequency

Respiratory frequency (f_R — breaths · min^{-1}) was obtained from buccal pressure variations and calculated manually from physiograph (Narco, Narcotrace 40, Houston TX, USA) traces or automatically via the ratemeter function of the Chart™ software.

Ventilatory tidal volume

Tidal volume (V_T — mLH_2O·kg^{-1}·breath^{-1}) was calculated by dividing gill ventilation by the respiratory frequency ($\dot{V}_G \cdot f_R^{-1}$).

Oxygen extraction from the ventilatory current

The oxygen extraction by the gills from the ventilatory water current (EO_2 —%) was estimated according to the following equation (Dejours, 1981):

$$EO_2\ (\%) = 100 \cdot (P_IO_2 - P_EO_2) \cdot P_IO_2^{-1}$$

Heart rate

Heart rate (f_H — beats·min^{-1}) was obtained from ECG recordings and was either calculated manually from physiograph traces or automatically computed by the ratemeter function of the Chart™ software.

Air-breathing frequency and duration

Air-breathing frequency (f_{AIR}) and air breathing duration (t_{AIR}) were recorded automatically in the dietary experiment. A laser light was shone across the air portion of the air/water interface of the experimental tank (Fig. 10.1) activating photocells on the opposite side of the tank. Any surfacing by the fish (fish are benthic and only surface to breath air) was recorded as disruption of the signal from the photocell by the PowerLab®/Chart™ data acquisition system (Fig. 10.1). At 1 h and 2 h of hypoxic exposure, the files were saved and recording re-initiated to void filling the computer's memory buffer. This process required 0.5–1 min, during which time any surfacing activity by the fish would not have been recorded. This

may have resulted in slight underestimates of reported air-breathing frequencies.

Statistical Analysis

Significant variance among the various treatments was detected with repeated measures multiple analysis of variance (MANOVA). When significant variance was detected, differences between groups were analyzed with Scheffe's test. Relationships between variables were evaluated by least-squares linear regression and F-test. All statistical analyses were performed with Statistica® for microcomputers. ANOVA was used to determine the levels of significance of the physiological data, and the Tukey test with 95% confidence limits was applied to compare the means whenever there was a significant difference (GraphPad InStat software, San Diego, CA).

RESULTS

Dietary Experiment

Blood and gut chemistry

Animals in the dietary experiment were statistically uniform for all measured variables except venous oxygen tension. Venous PO_2 was significantly lower in the animals exposed to three hours of hypoxia ($P<0.05$; Table 10.1). Venous blood pH was approximately 8 for all treatments and venous blood PO_2 was about 10 mmHg in hypoxic fish and 30 mmHg in normoxic animals (Table 10.1). All digestive tract pH values were near neutral and the gut was oxygenated under both air and water breathing conditions.

No SCFAs were detected in any blood or gut samples. The test had a conservative detection limit of 10 μM, so if free fatty acids were present in the blood or gut samples, they were present in nanomolar amounts, well below the levels found in fish suspected of having fermenting microorganisms present in their gut (Clements, 1997).

Erythrocyte data were pooled by environmental oxygen treatment (Table 10.2). This was because only a few normoxic animals were analyzed and there were no statistical differences between hypoxic animals based upon gut fullness. Hypoxic animals were characterized by having more,

Table 10.1 Animal sizes, blood and gut chemistry after either a three-hour normoxic or hypoxic recording period in fed and starved cascudo. Treatments significantly different from each other are distinguished by superscripted letters. Means ± 1 standard deviation are reported; 'nm' means "not measured".

	Treatment			
Parameter	*Normoxia Fed*	*Normoxia Starved*	*Hypoxia Fed*	*Hypoxia Starved*
n	5	4	6	6
Standard Length	19.0 ± 1.3	21.2 ± 1.0	21.6 ± 1.0	20.9 ± 3.0
Mass	166.6 ± 29.9	214.6 ± 32.2	217.1 ± 33.9	208.7 ± 86.1
Venous blood pH	8.032 ± 0.133	8.058 ± 0.336	8.134 ± 0.436	7.824 ± 0.631
Venous blood PO_2	24.8 ± 6.2[a]	29.1 ± 7.8[a]	12.5 ± 5.9[b]	8.61 ± 5.2[b]
Foregut pH	7.94 ± 0.20	7.48 ± 0.03	7.49 ± 0.17	7.53 ± 0.59
Foregut PO_2	18.2 ± 14.4	19.5 ± 8.0	33.3 ± 40.2	22.4 ± 19.5
mid/hindgut pH	8.00 ± 1.2	nm	7.30 ± 0.21	7.38 ± 0.37
mid/hindgut PO_2	35.3 ± 34.1	42.8 ± 24.7	57.8 ± 72.5	41.33

Table 10.2 Erythrocytic characteristics of cascudo after either a three-hour normoxic or hypoxic recording period. Treatments significantly different from each other are distinguished by superscripted letters. Means ± 1 standard deviation are reported.

	Treatment	
Parameter	*Normoxia*	*Hypoxia*
n	4	10
Hematocrit (%)	27.4 ± 5.0	27.1 ± 6.7
Hemoglobin (g/dL)	5.7 ± 1.8	6.7 ± 1.5
Erythrocyte count (#/$mm^3 \times 10^6$)	0.89 ± 0.043	0.93 ± 0.21
Mean cell volume (μm^3)	308.6 ± 68.6	290.4 ± 25.0
Mean cell hemoglobin concentration (%)	19.8 ± 2.6[a]	24.8 ± 1.8[b]

smaller erythrocytes that contained proportionally more hemoglobin than normoxic animals; the only statistically distinct inter-group difference was the proportion of the cell taken up by hemoglobin ($P < 0.05$; Table 10.2).

Physiological Responses to Hypoxia

Dietary experiment (access to air)

Figure 10.2 demonstrates the heart rate response of fed and unfed cascudo to 3 hours of hypoxic exposure to a nominal P_wO_2 level of 20 mmHg or exposure to normoxic water. Hypoxia exposure immediately initiated an approximate 50% reduction in heart rate that was gradually ameliorated over time. Mean heart rate was significantly lower in the hypoxia-exposed animals throughout the exposure period (MANOVA $P<0.001$), although individual animals would briefly elevate their heart rate back to control levels or even higher when surfacing to breath air (see below). Fed animals tended to have a slightly higher heart rate in both treatments than unfed animals (significant interaction term MANOVA $P < 0.05$).

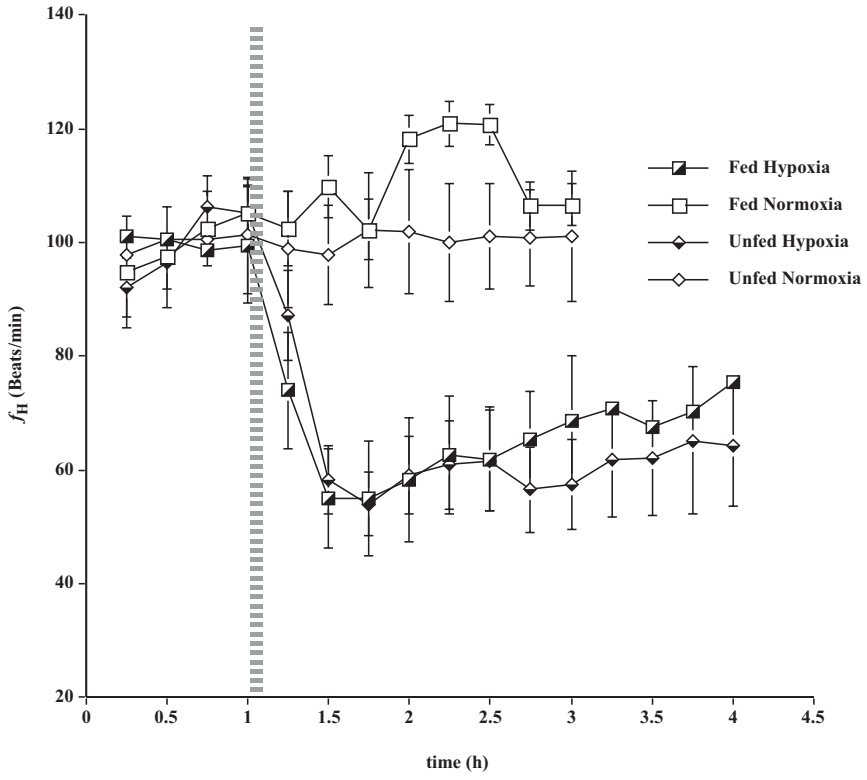

Fig. 10.2 Heart rate f_H in *Hypostomus regani* exposed to 20 mmHg PO_2 for three hours (half-closed symbols) or under normoxic conditions for three hours (open symbols). Each symbol represents the mean for that particular group ± 1 SE. Fed animals are represented by squares and unfed animals by diamonds. The striped bar designates hypoxia initiation.

Aquatic respiratory rate was quite variable among individuals (Fig. 10.3), but there was no discernible effect of hypoxia exposure on f_R, nor were there any apparent differences between the two dietary treatments (Fig. 10.3).

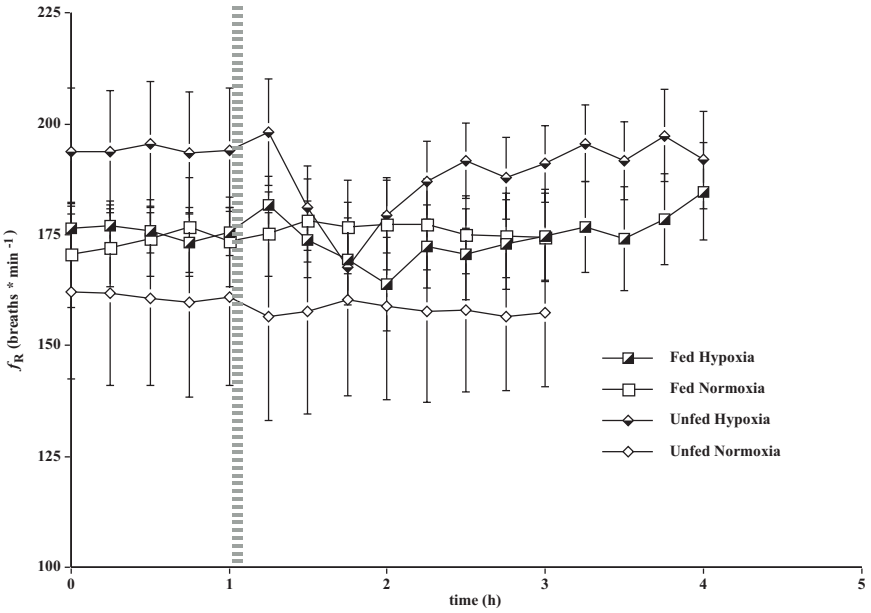

Fig. 10.3 Aquatic respiratory rate f_R in *Hypostomus regani* exposed to 20 mmHg PO_2 for three hours (half-closed symbols) or under normoxic conditions for three hours (open symbols). Each symbol represents the mean for that group ± 1 SE. Fed animals are represented by squares and unfed animals by diamonds. The striped bar designates hypoxia initiation.

Air-breathing behavior and physiology were remarkably consistent between groups of *Hypostomus regani* differing in their dietary status (Table 10.3); fed and unfed fish had virtually identical responses to hypoxia.

Individual analysis of air-breathing

Although dietary treatment did not affect mean air-breathing behavior and physiology, coincident monitoring of air-breathing activity and heart rate revealed substantial inter-individual variation in both behavior and physiology, and a connection between the two. Figure 10.4 plots the change in heart rate after an air-breathing episode as a function of the

Table 10.3 Air-breathing behavior and physiology of cascudo exposed to hypoxia (3 h at a nominal P_wO_2 of 20 mmHg); reported by dietary regime. Means ± 1 standard deviation are reported.

Parameter	*Fed*	*Unfed*
n	6	7
Total # of air breaths	10.8 ± 9.4	9.2 ± 4.5
Average P_wO_2 at air breath (mmHg)	20.5 ± 2.6	20.6 ± 3.7
Average duration of air breath (s)	1.7 ± 1.4	1.9 ± 1.8
Average f_H before surfacing (beat · min^{-1})	60.2 ± 13.9	56.8 ± 19.7
Average f_H after surfacing	79.0 ± 22.8	77.5 ± 29.3
Average f_R before surfacing (breaths · min^{-1})	175.7 ± 19.7	176.9 ± 28.1
Average f_R after surfacing	174.9 ± 20.7	178.1 ± 33.4

individual air breath. These data are split into two groups: those four animals that breathed air the most and the four animals that most favored remaining submerged. The former were characterized by more than ten surfacing events over a three-hour period, a more frequent and dramatic "surfacing tachycardia" and a surfacing tachycardia that almost oscillated between subsequent breaths (Fig. 10.4). The latter were distinguished by less than four surfacing events in a three-hour period, an initially non-existent surfacing tachycardia and a surfacing tachycardia that gradually increased if the animal took subsequent air breaths (Fig. 10.4). All of the 'frequent air-breathing' fish had an initial surfacing tachycardia of at least 20 bpm, whereas the infrequent air-breathing fish had virtually no surfacing tachycardia at their first air breath.

There was virtually no change in aquatic ventilatory rate (f_R) with air-breathing activity (Table 10.3). The mean change in f_R was –0.36 ± 11.6 SD breaths · min^{-1} for 123 total air-breathing episodes. With a mean f_R around 176 breaths · min^{-1}, this is strong evidence that individual cascudo do not adjust aquatic ventilation when breathing air. No trend was observed for f_R to change as air breaths accumulated; nor was there a discernible difference in aquatic respiration between frequent air breathers and infrequent air breathers.

The periodicity of air breathing was highly dependent on the individual and no effects due to diet or the propensity for air breathing were found. One of the most regular air breathers took only 3 breaths at intervals of 63.6 ± 0.2 SD min (56.7 min would have been perfectly

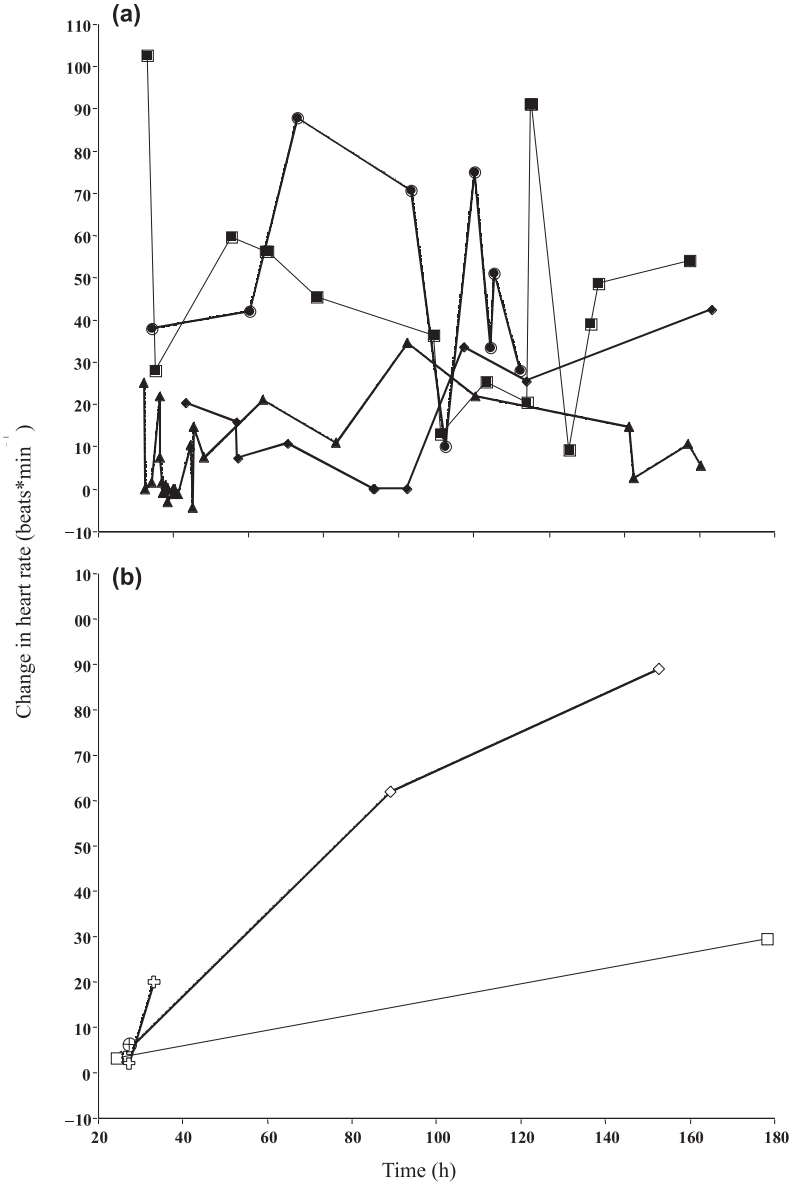

Fig. 10.4 Heart rate changes in individual *Hypostomus regani* exposed to 20 mmHg PO_2 for 3 h. Each symbol represents the time post-hypoxia initiation of an individual air-breathing episode for an individual plotted as the difference in heart rate immediately after the surfacing event minus the heart rate immediately before the surfacing event. (a) Upper panel (closed symbols) contains the four animals that most frequently surfaced (>10 times) (b) bottom panel (open symbols) those four animals that surfaced least (<4 times).

periodic), whereas two of the least regular air breathers took only two breaths: one fish taking both breaths right at the beginning of the hypoxic period and the other taking one at the beginning and the second near the end (Fig. 10.4b). Similarly, fish that breathed air regularly could be almost perfectly periodic (one fish that took 16 breaths had a mean breath interval of 9.6 ± 9.4 SD min; 10.6 min expected for perfectly periodic) or not at all periodic (a fish that took 10 breaths had a mean breath interval of 3.5 ± 1.7 SD min; 17 min expected for perfectly periodic).

Temperature experiment (no access to air)

As reported earlier (Fernandes et al., 1999), when cascudo are denied access to air, they cease oxygen regulation at a point called the critical oxygen tension (P_cO_2) and metabolism drops with further declines in environmental PO_2 (oxygen conforming; Fig. 10.5). When chronically acclimated to temperatures between 20°C and 30°C, (P_cO_2) was largely independent of environmental temperature (Fig. 10.5). The critical oxygen tension was determined to be 27 mmHg for both the 25°C and the 30°C treatments and 33 mmHg for the 20°C treatment. However, when fish were acclimated to 25°C and then acutely transferred to 20°C or 30°C, the (P_cO_2) tended to increase irrespective of whether the transfer was made to colder or warmer temperature water (Fig. 10.5). This elevation of (P_cO_2) was 13 mmHg when rapid transfer to 30°C is compared with acclimation to that temperature, but 55 mmHg when the same comparison is made for the 20°C treatments. The metabolic rate of animals chronically acclimated to temperature followed the usual ectothermic pattern and varied directly with temperature with a Q_{10} of approximately 2.0 at PO_2s above the (P_cO_2). Interestingly, above the (P_cO_2), metabolic rates of acutely transferred fish were highly dependent upon which temperature the transfer was made to. Fish rapidly transferred to 30°C had a metabolic rate that was statistically indistinguishable from fish chronically acclimated to 30°C. In contrast, animals acutely transferred to 20°C had a metabolic rate that was statistically indistinguishable from the animals at the temperature they came from (25°C) but significantly higher than fish chronically acclimated to 20°C (Fig 10.5). The significant PO_2 × treatment interaction (MANOVA; $P<0.01$) shows that the change in metabolism with change in PO_2 was different from the corresponding chronic acclimation temperature for both 20°C and 30°C.

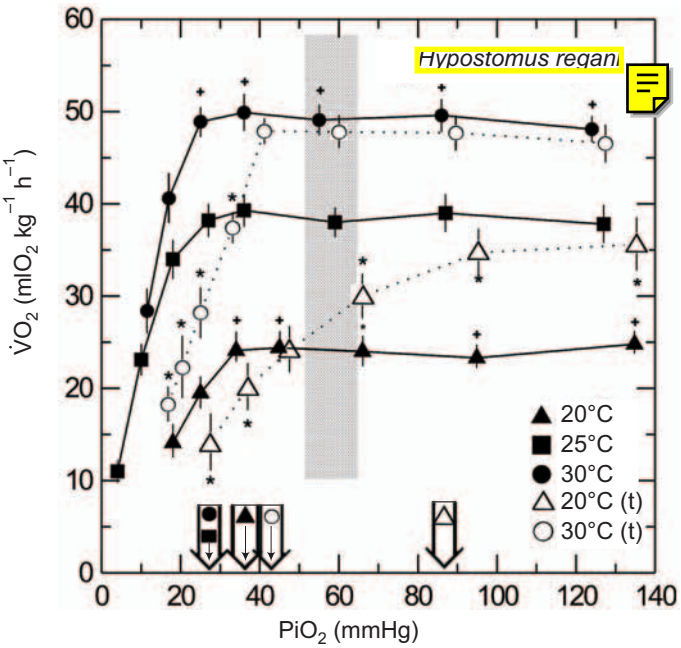

Fig. 10.5 Oxygen consumption of *Hypostomus regani* exposed to progressive hypoxia over a period of 9 hours and denied access to the surface. Closed symbols represent animals chronically acclimated to a temperature, whereas the opened symbols represent animals acutely transferred to the experimental temperature. Each symbol represents the mean for that specific group ± 1 SE. The stippled bar designates the range of PO_2s over which air breathing would normally commence. The arrows designate the calculated P_cO_2 for a treatment.

Analysis of gill ventilation in cascudo revealed a strong uncoupling effect due to rapid temperature change when exposed to hypoxia but denied access to surface (Fig. 10.6).

In animals chronically acclimated to 20°, 25° and 30°C, gill ventilation was temperature insensitive. Although metabolic rate was unaffected by rapid transfer to 30°C, fish rapidly transferred to 30°C had significantly elevated gill ventilation to achieve the same metabolic rate as animals that had acclimated to that temperature (Fig. 10.6). The significant elevation in metabolic rate in fish rapidly transferred to 20°C was also accompanied by an increase in gill ventilation. Greater gill ventilation in rapidly transferred fish was largely accomplished through increases in tidal volume V_T (Fig. 10.7); Aquatic ventilatory rate (f_R) was relatively unaffected by whether transfer to temperature was acute or chronic at

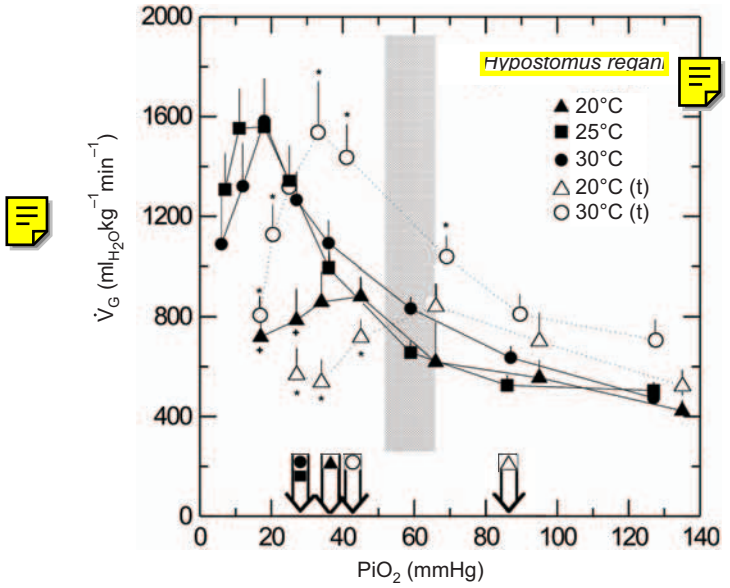

Fig. 10.6 Gill ventilation of *Hypostomus regani* exposed to progressive hypoxia over a period of 9 hours and denied access to the surface. Closed symbols represent animals chronically acclimated to a temperature, whereas the opened symbols represent animals acutely transferred to the experimental temperature. Each symbol represents the mean for that specific group ± 1 SE. The stippled bar designates the range of PO_2s over which air breathing would normally commence. The arrows designate the calculated P_cO_2 for a treatment.

normoxic PO_2s and was temperature-specific. However, upon exposure to hypoxia, both acute exposure groups saw an earlier decrease in both tidal volume and respiratory frequency compared to their chronically exposed counterparts as hypoxia progressed (Fig. 10.7). This is reflected in the higher P_cO_2 for these treatments (Fig. 10.5). Thus, acute change of temperature produced an earlier and more dramatic decline in gill ventilation during progressive hypoxia when compared to chronically acclimated fish (Fig. 10.6). Only two groups had tidal volumes (V_T) that differed significantly from the 25°C control temperature: (1) those acclimated to 30°C, and (2) those acutely transferred to 20°C.

Aquatic respiratory frequency (f_R) mirrored the oxygen consumption results and varied directly with temperature with a Q_{10} of 2.0 at PO_2s above the (P_cO_2). As in the dietary experiment, hypoxia had little effect upon f_R, particularly if the animals were chronically acclimated to temperature. Tidal volumes were substantially increased in both acutely

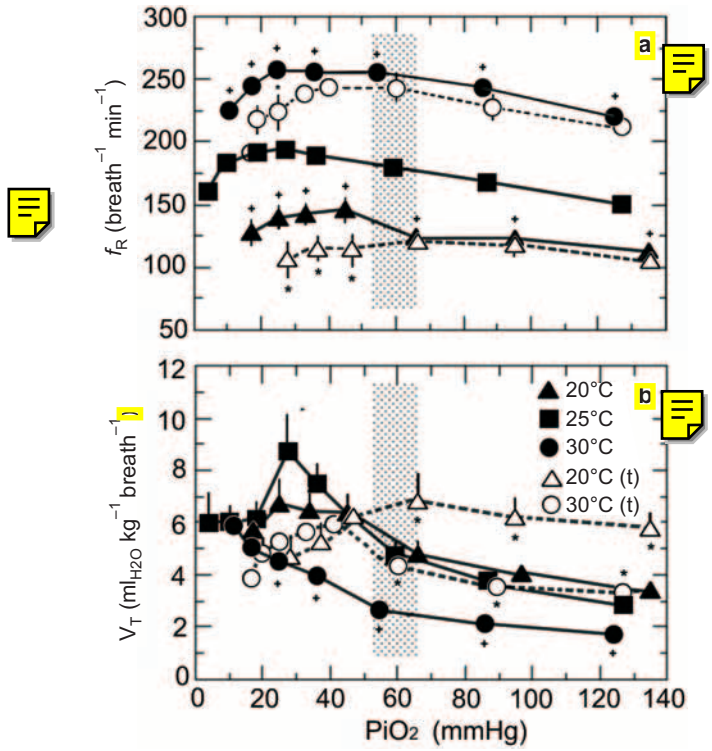

Fig. 10.7 Respiratory frequency (f_R; top panel) and tidal volume (V_T; bottom panel) of *Hypostomus regani* exposed to progressive hypoxia over a period of 9 hours and denied access to the surface. Closed symbols represent animals chronically acclimated to a temperature whereas the opened symbols represent animals acutely transferred to the experimental temperature. Each symbol represents the mean for that group ± 1 SE. The stippled bar designates the range of PO_2 over which air breathing would normally commence.

transferred groups of fish when compared with chronically acclimated animals at the same temperature ($P<0.01$; Fig. 10.7).

Heart rate in chronically acclimated animals generally increased with temperature at PO_2s above the (P_cO_2), but with a substantially larger Q_{10} (2.6) between 25°C and 30°C than between 20°C and 25°C ($Q_{10}=1.2$; Fig. 10.8). Heart rate of acutely transferred fish was highly dependent upon which temperature the transfer was made to, above the P_cO_2. Acute transfer to 30°C caused a significant elevation of heart rate compared to animals acclimated to that temperature, whereas transfer to 20°C caused a significant depression of f_H when compared to acclimated animals (Fig. 10.8). There was also a significant interaction between temperature and

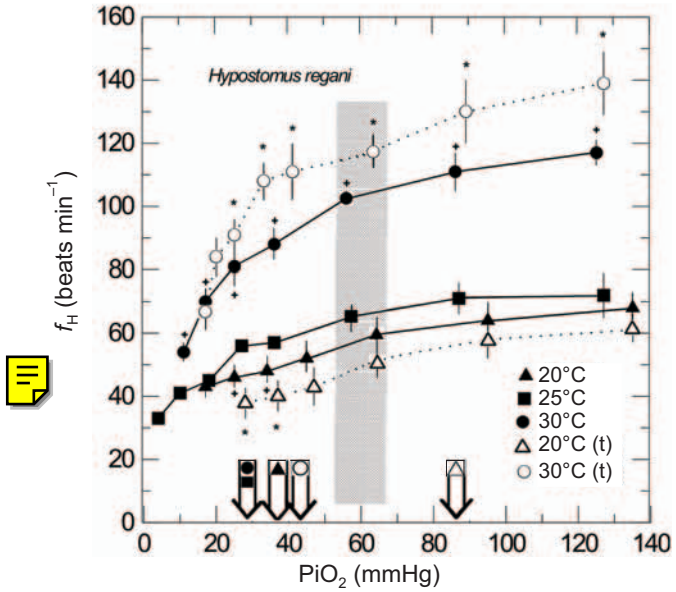

Fig. 10.8 Heart rate (f_H) of *Hypostomus regani* exposed to progressive hypoxia over a period of 9 hours and denied access to the surface. Closed symbols represent animals chronically acclimated to a temperature whereas the opened symbols represent animals acutely transferred to the experimental temperature. Each symbol represents the mean for that group ± 1 SE. The stippled bar designates the range of PO_2s over which air breathing would normally commence. The arrows designate the calculated P_cO_2 for a treatment.

f_H with respect to the bradycardia that developed with progressive hypoxia. Animals acutely transferred to 30°C developed a more substantial bradycardia than chronically acclimated animals at that temperature so that animals from the two treatments at 30°C had identical heart rates at 20 mmHg PO_2. In contrast, animals acutely transferred to 20°C developed a similar bradycardia to animals chronically acclimated to that temperature (Fig. 10.8). The hypoxic bradycardia was significantly more established at 30°C than at either 20°C or 25°C.

DISCUSSION

Individual Analysis

The results from the dietary experiment show that air-breathing behavior and its physiological support varied substantially between individuals in the laboratory. The results from the temperature experiment indicated that cascudo are similar to other facultative air breathers capable of

oxygen conforming when faced with hypoxic conditions and only breathing water (Graham, 1997; Fig. 10.5). When fish are given access to air, as in the dietary experiment, the "choice" to keep breathing water and oxygen conform or to breath air and oxygen regulate varies considerably among individuals. Air-breathing frequency among hypoxia-exposed individuals varied from only 1 breath to at least 27 breaths over the same 3 h hypoxic period. These variable breath numbers corresponded to total surface times of less than a second in the least frequently surfacing fish to almost a minute in the fish most likely to surface. Some of this large variance in surfacing behavior may be due to a differential response to the cannulae or the experimental chamber. Graham (1997) discussed the likelihood that air-breathing behavior changed due to cannulation in the obligate air-breathing species *Electrophorus* and *Arapaima*. A second possible explanation for these results is that the frequently surfacing animals had explored the tank during the acclimation period (16 h) and were aware of the air access, whereas the infrequently surfacing animals may have been first discovering the air access during the hypoxic exposure. Several other authors have commented on the large individual variability in air-breathing frequency among conspecific facultative air-breathers exposed to hypoxia (e.g., Gee, 1976; Graham and Baird, 1982; Graham, 1997; MacCormack et al., 2003) suggesting that this phenomenon may be widespread. Considering the additional exposure to predation surfacing entails (Kramer et al., 1983), one can easily envision opposing selection pressures creating the diversity of air-breathing behavior found in cascudo.

The behavioral difference in surfacing behavior between individual *Hypostomus regani* was somewhat connected to the pattern of surfacing tachycardia (Fig. 10.4). Animals that surfaced frequently had a large initial surfacing tachycardia that appeared to oscillate over subsequent surfacing episodes (Fig. 10.4a). Conversely, infrequently surfacing animals had no initial surfacing tachycardia, but then exhibited an increasing surfacing tachycardia with subsequent breaths, if taken (Fig. 10.4b). Surfacing tachycardia, although not universal, has been observed frequently in air-breathing fishes (Table 6.5 in Graham, 1997). In this study, an average 34% increase in heart rate was observed with surfacing in cascudo (Table 10.3); this result is similar to the 36% surfacing tachycardia reported by Graham (1983) for the facultatively air-breathing cofamiliar *Ancistrus*. To our knowledge, ours is the first study to show such

large individual variance in surfacing tachycardia. Individual tachycardia varied from 4.4 ± 8.8 SD beats·min^{-1} (post-air-breath cardiac rate–pre-air-breath cardiac rate) from 8 breaths for the animal that exhibited the least tachycardia to three animals that had a mean surfacing tachycardia of around 50 beats·min^{-1} for as many as 16 breaths. Surfacing tachycardia was not dependent upon the total number of air breaths taken, the dietary treatment or f_H immediately preceding the air breath. Thus the oscillatory nature of the tachycardia exhibited by frequently air-breathing fish (Fig. 10.4a) was not merely a result of cardiac rate being relatively high from a previous air breath. This large variation in a physiological measurement, purportedly under just autonomic control (Taylor, 1992), in animals exposed to identical environmental conditions, clearly merits further study. The parsimonious explanation for these results is that a differential stress response to the experimental conditions (cannulation, human presence, foreign tank upon arousal from anaesthesia, etc.) modulated the physiological response to surfacing in *Hypostomus regani*. However, from these data, we cannot exclude the possibility of higher brain center coordination between cardiac regulation and air-breathing behavior in this species.

Our conclusion that air breathing during hypoxia is an individualized event in cascudo was also supported by the periodicity of air breathing. Some animals exhibited highly periodic air breathing whereas other animals breathed air in a manner that could only be described as chaotic (Fig. 10.4). Air breathing in fish has been described by most investigators as arrhythmic, so it was not surprising to find that for these 13 fish, air breathing was not significantly periodic. Interestingly, four of the thirteen animals exhibited very periodic breathing for substantial portions of the three-hour exposure period. Periodicity of air breathing has been demonstrated in *Amia*, but only after long-term recordings and sophisticated statistics were employed by Hedrick et al. (1994). We conjecture from the behavior of these four animals that longer-term studies under more natural conditions may have evoked rhythmic air-breathing in cascudo. MacCormack et al. (2003) studied air-breathing behavior of *Glyptoperichthyes gibbceps,* another air-breathing loricariid, under simulated natural conditions. They found this species more likely to surface at night-time. Although the chamber in the present experiment was darkened, it is possible that less variant surfacing behavior might have been observed had the experiments been run at night.

Chemistry of Hypoxia Exposure

Despite dramatic differences in air-breathing behavior, blood and gut oxygen and pH levels were maintained at relatively constant levels across treatments in the dietary experiment. The only significant difference between the hypoxic and normoxic treatments was the lower venous oxygen tension in the hypoxic group. Whether this was due to greater oxygen extraction or diminished oxygen uptake by these animals is unknown because arterial blood samples were not taken. The hypoxic group generally had higher gut oxygen tensions, but not significantly so. Since these animals had each taken at least one air breath, finding the gut to be well oxygenated was not surprising. Indeed, fish that breathed air frequently during the hypoxic period had air bubbles throughout their four-meter digestive tracts. Finding well-oxygenated gut contents in the control fish, coupled with the lack of volatile short chain fatty acids (SCFAs), if confirmed, is suggestive of novel digestive mechanisms in loricariids. The gut and blood samples were stored at –80°C for 1 year in sealed containers as acidified perchloric acid extracts, so decomposition should have been minimal. However, the possibility exists that some SCFAs were originally present but were later lost in handling. Oxygenated guts at neutral pH levels, although consistent with the flora culturable from loricariid guts (Nelson et al., 1999), are not suitable for any currently known mechanism of recalcitrant carbon bond breakage in fishes (Choat and Clements, 1998); future studies of digestive mechanisms in loricariids and interactions with air-breathing could prove very interesting.

Hypoxic cascudo were characterized by having more, smaller erythrocytes that contained statistically more hemoglobin per erythrocyte than normoxic animals (Table 10.2). This result is most likely due to the hypoxic animals releasing immature erythrocytes from the spleen to enhance oxygen transport. These results are very similar to those reported by Fernandes et al. (1999) for cascudo exposed to graded hypoxia at 25°C for a longer period and Val et al. (1990) also reported a higher cell hemoglobin concentration in a loricariid exposed to hypoxia. However, Weber et al. (1979) reported cell swelling and decreased cell hemoglobin concentrations in loricariids exposed to hypoxia for 4-7 days, suggesting that the cascudo response is not a generalized loricariid one.

Physiology of Hypoxia Exposure

Metabolic and ventilatory responses of *Hypostomus regani* to hypoxia and temperature have been described previously by Fernandes et al. (1999)

and Mattias et al. (1998) and will not be repeated here. The focus of this study has been to differentiate between rapid transfer to temperature and acclimation to that temperature in determining the response to hypoxia.

Rapid transfer to a new temperature dramatically altered an animal's metabolic response to progressive hypoxia when compared to animals chronically held at a temperature (Fig. 10.5). Fish rapidly transferred to a 5°C cooler temperature, despite having a respiratory rate entirely appropriate for the temperature they were at (Fig. 10.7a), had a significant elevation of metabolic rate compared with conspecifics acclimated to 20°C. This higher oxygen consumption was accomplished through a significant elevation of aquatic tidal volume (Fig. 10.7b). Fish rapidly transferred to a 5°C warmer temperature also had a significant elevation of gill ventilation when compared to chronically acclimated animals (Fig. 10.6), that was also accomplished entirely through changes in V_T (Fig. 10.7b). As these changes in ventilatory parameters did not translate into elevated metabolic rates at 30°C, this suggests that either O_2 uptake or utilization is compromised by rapid transfer to a 5°C warmer temperature. Graham and Baird (1982) studied the air-breathing response to hypoxia in another facultatively air-breathing loricariid (*Ancistrus chagresi*) and found that at acclimation times intermediate to the chronic and acute times used here, air-breathing frequency changed little between 20°C and 25°C but dramatically increased in animals at 30°C. Graham and Baird (1982) also found that P_cO_2 changed little between 20°C and 30°C when animals are acclimated, supporting our findings for *Hypostomus*. The mechanism behind the elevation of P_cO_2 in both groups of rapidly transferred animals is unknown, but we speculate that a rapid change of temperature is stressful to cascudo, more so when the change is towards colder temperatures. Furthermore, changes in ventilation immediately adjacent to air breaths, common in other air breathers (Graham, 1997) were not apparent in cascudo (Table 10.3). An interesting future experiment would be to determine if the rapid transfer to a different temperature further altered the already variable air-breathing behavior.

Bradycardia is a universal response to hypoxia in *Hypostomus regani* regardless of temperature or dietary treatment (Figs. 10.2 and 10.8). Comparison of fish from the dietary experiment (Fig. 10.2) with the 25°C treatment of the temperature experiment (Fig. 10.8) suggests that the sudden onset of hypoxia elicits a more severe bradycardia than does progressive hypoxia. The development of a significant bradycardia during progressive hypoxia, when metabolic rate remained unchanged, suggests

that the cardiac response of cascudo to hypoxia may be somewhat similar to that for carp (*Cyprinus carpio*) at 15°C (Stecyk and Farrell, 2002), where falling f_H was somewhat compensated for by increases in stroke volume. The hypoxic bradycardia observed in cascudo is presumably the same generalized, although not universal, vagally mediated reflex bradycardia response to hypoxia (Taylor, 1992). Interestingly, some fish that do not exhibit hypoxic bradycardia are also loricariids (MacCormack et al., 2002). Although f_H generally tracked metabolic rate in animals chronically acclimated to temperature (Figs. 10.5 and 10.8), a rapid transfer of 5°C had an interesting effect upon f_H. Rapid transfer to 30°C caused animals to have a higher f_H than conspecifics chronically acclimated to that temperature, whereas rapid transfer to 20°C produced a lower cardiac rate than in the comparison group. Rapid transfer did not alter the development of hypoxic bradycardia from that observed in acclimated fish at the same temperature. Bradycardia development was, however, temperature dependent and mirrored metabolic demand. Bradycardia was greatest in fish at 30°C followed by fish at 25°C and was least in animals at 20°C. Presumably the significantly higher cardiac rate in food supplemented fish when compared with starved fish (Fig. 10.2) was due to a higher metabolic rate from specific dynamic action, although SDA is difficult to detect in herbivorous catfishes (Nelson, 2002) and without actual $\dot{V}O_2$ measurements, this is speculative.

In conclusion, dietary treatment had little influence on the air-breathing behavior or physiology of cascudo except for a modest tachycardia in fed animals under both normoxia and hypoxia when compared with starved conspecifics. In contrast, rapid transfer of animals to a 5°C different temperature dramatically altered their response to progressive hypoxia, suggesting that rapid exposure to hypoxic water of different temperature may be the most significant environmental physiological challenge faced by tropical loricariids. The partitioning of respiratory demand between air and water in animals challenged with hypoxia in the laboratory appears to be a highly individualized phenomenon, and may be connected to individual variance in physiology. Presumably the increased susceptibility to avian predation experienced by surfacing loricariids has produced this large variance in air-breathing behavior, but that hypothesis needs to be tested in the field.

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