

## Gill chemoreceptors and cardio-respiratory reflexes in the neotropical teleost pacu, *Piaractus mesopotamicus*

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**Abstract** This study examined the location and distribution of O<sub>2</sub> chemoreceptors involved in cardio-respiratory responses to hypoxia in the neotropical teleost, the pacu (*Piaractus mesopotamicus*). Intact fish and fish experiencing progressive gill denervation by selective transection of cranial nerves IX and X were exposed to gradual hypoxia and submitted to intrabuccal and intravenous injections of NaCN while their heart rate, ventilation rate and ventilation amplitude were measured. The chemoreceptors producing reflex bradycardia were confined to, but distributed along all gill arches, and were sensitive to O<sub>2</sub> levels in the water and the blood. Ventilatory responses to all stimuli, though modified, continued following gill denervation, however, indicating the presence of internally and externally oriented receptors along all gill arches and either in the pseudo-branch or at extra-branchial sites. Chemoreceptors located on the first pair of gill arches and innervated by the glosso-pharyngeal nerve appeared to attenuate the cardiac and respiratory responses to hypoxia. The data indicate that the location and distribution of cardio-respiratory O<sub>2</sub> receptors are not identical to those in tambaqui (*Colossoma*

*macropomum*) despite their similar habitats and close phylogenetic lineage, although the differences between the two species could reduce to nothing more than the presence or absence of the pseudo-branch.

**Keywords** Cardio-respiratory control · Chemoreceptors · Hypoxia · NaCN · Pacu · *Piaractus mesopotamicus*

### Abbreviations

$f_H$	Heart rate
$f_R$	Ventilatory rate
$V_{AMP}$	Amplitude of ventilation
$V_{TOT}$	Total ventilation
Pa	Arterial blood pressure
$P_{buccal}$	Intrabuccal pressure
$P_{wO_2}$	Water oxygen tension

### Introduction

Most teleosts respond to hypoxia with a substantial decrease in heart rate. The receptors involved in eliciting this bradycardia are located within the gills but there is variability both in the distribution of the receptors amongst the different gill arches, in different species, as well as their location on each gill arch, i.e., whether they are positioned to sense changes in O<sub>2</sub> in the water flowing over, or the blood perfusing the gills (Randall and Smith 1967; Saunders and Sutterlin 1971; Smith and Jones 1978; Smatresk et al. 1986; Bursleson and Smatresk 1990b; McKenzie et al. 1991; Bursleson and Milsom 1993; Sundin et al. 1999, 2000; Milsom et al. 2002, Gilmour et al. 2005). The ventilatory response to hypoxia in teleosts consists of an increase in breathing frequency and/or amplitude

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(Shelton et al. 1986). The results from fish species studied to date suggest that ventilatory O<sub>2</sub> chemoreceptors also do not share common locations and distribution among species, just as the O<sub>2</sub>-sensitive chemoreceptors eliciting cardiac reflexes do not (Saunders and Sutterlin 1971; Smatresk et al. 1986; Burlison and Smatresk 1990b; McKenzie et al. 1991; Burlison and Milsom 1993; Sundin et al. 1999, 2000; Milsom et al. 2002). To date, no clear picture emerges that can link O<sub>2</sub> receptor distribution to phylogenetic or life history traits. These previous results suggest that studies of the distribution of cardio-respiratory chemoreceptor populations for a variety of species, adapted to different habitats, are still needed before testable hypothesis concerning the evolution, phylogeny and adaptive significance of these receptors and their distribution can be formulated.

Recently, there have been a series of studies focusing on the cardio-respiratory responses to hypoxia of a hypoxia-tolerant neotropical fish that is an aquatic surface breather, the tambaqui (*Colossoma macropomum*) (Sundin et al. 2000; Milsom et al. 2002; Reid et al. 2003, 2005; Florindo et al. 2004, 2006). Under conditions of environmental hypoxia, this fish will come to the surface and siphon the well-oxygenated surface layer (Carter and Beadle 1931; Kramer and McClure 1982; Rantin and Kalinin 1996). To facilitate this, the inferior lip swells to form a funnel that can direct the surface water into the mouth and over the gills. The lower lip is not involved in gas exchange but serves purely as a mechanical structure enhancing skimming of the surface water (Kramer and McClure 1982; Saint-Paul and Bernardinho 1988; Val and Almeida-Val 1995).

Data from these studies suggested that the O<sub>2</sub> receptors eliciting reflex bradycardia in response to acute hypoxia were situated on all gill arches and sensed changes in both the blood and respiratory water (Sundin et al. 2000) while the O<sub>2</sub> receptors triggering the elevation in systemic vascular resistance during hypoxia were extrabranchial (Sundin et al. 2000). The data further suggested that the O<sub>2</sub> receptors eliciting reflex increases in breathing frequency were situated on all gill arches and sensed changes in both the blood and respiratory water (Sundin et al. 2000). The O<sub>2</sub> receptors triggering the elevation in breathing amplitude included receptors in the orobranchial cavity innervated by mandibular branches of cranial nerve V and the opercular and palatine branches of cranial nerve VII (Milsom et al. 2002). ASR behavior arose from stimulation of orobranchial receptors innervated by cranial nerve V (Florindo et al. 2006). Finally, inferior lip swelling did not arise from receptors at any of these sites but most likely arose from a direct effect of hypoxia on the lip tissue (Florindo et al. 2006; Saint-Paul and Bernardinho 1988).

These results do not reveal a simple picture of cardio-respiratory chemoreceptor control in tambaqui. Since the basis for the intraspecies variability in the location and

distribution of branchial and extrabranchial chemoreceptors in fish that have been studied remains elusive, in the present study we wished to examine cardio-respiratory responses to acute hypoxia in another, related species that partakes in ASR, the pacu, *Piaractus mesopotamicus*.

Both tambaqui and pacu belong to the order Characiformes, subfamily Myleinae. In Brazil, tambaqui are found primarily in the north, in the Amazon River drainage, while pacu are found further south within the drainages of the Paraguay and Parana rivers. Both species are found in areas prone to flooding and the formation of swamps and flooded areas that undergo large seasonal and daily fluctuations in dissolved oxygen concentration (Carter and Beadle 1931; Kramer et al. 1978). If receptor locations and distribution reflect phylogeny and/or ecological niche, then given their similar habitats and relatively close phylogenetic lineage, we hypothesized that the location and distribution of cardio-respiratory O<sub>2</sub> receptors should be identical in these two species.

## Materials and methods

### Collection and maintenance

Pacu, *Piaractus mesopotamicus* (Wt. = 557 ± 82 g), were obtained from commercial breeders near São Carlos, SP. They were maintained in the Laboratory of Zoophysiology and Comparative Biochemistry of the Department of Physiological Sciences of the Federal University of São Carlos, SP, Brazil, in 1,000 l tanks supplied with a continuous flow of normoxic (P<sub>w</sub>O<sub>2</sub> = 140 mmHg), de-chlorinated water at 25°C. Fish were fed “ad libitum” with commercial ration. Before each experiment fish were left without food for 48 h.

### Animal surgery

For surgical procedures, fish were first anesthetized by immersion in a solution of benzocaine (0.1 g l<sup>-1</sup>) until righting responses were gone. They were then transferred to a surgical table where the fish were held in air while their gills were irrigated with a second solution of benzocaine (0.05 g l<sup>-1</sup>) that was constantly aerated to maintain adequate levels of O<sub>2</sub> in the anesthetic solution.

Two polyethylene cannulae (PE 100 and PE 60) were introduced into the buccal cavity through a hole in the dorsal palate. One was used to measure changes in buccal pressure (P<sub>buccal</sub>) from which we could obtain measures of both ventilatory frequency (f<sub>R</sub>) and amplitude (V<sub>AMP</sub>). The other was used as a route for the intrabuccal administration of water and NaCN. In preliminary trials the responses to

NaCN injections were fast and so we cannulated both caudal vein and artery (PE 50) (see Gilmour et al. 2005 for details) to allow intravenous injections of saline and NaCN, without interrupting continuous measurements of arterial pressure (Pa) and heart rate ( $f_H$ ).

In order to determine which gill arches possess  $O_2$  chemoreceptors in *P. mesopotamicus*, the cardio-respiratory changes caused by NaCN injections and exposure to progressive hypoxia were recorded in groups of intact fish (i.e., nerves left intact) and in fish after selective denervation of the gills. Denervation was performed via two small incisions (<1 cm) behind the opening of the opercular aperture. A dorsal incision was made through the skin at the point where the first and second gill arches join the roof of the opercular cavity. The incision exposed the branchial branches of the glossopharyngeal (IX) and vagus (X) cranial nerves. The second incision was made along the caudal edge of the opercular compartment where the fourth branchial branch of the vagus separates from the cardiac branch. The branchial nerve branches were carefully dissected from their connective tissue sheaths and sectioned bilaterally to make up four experimental groups: The intact group (I) consisted of non-operated ( $n = 7$ ) and sham-operated ( $n = 3$ ) animals. The sham-operated animals had the nerves exposed but not sectioned. Since the results obtained from these animals were not different from those obtained from non-operated animals they were subsequently included in the intact group. The IX group ( $n = 10$ ) consisted of animals having the branchial branches of the IX cranial nerve sectioned; the G1 group ( $n = 10$ ) consisted of animals having the branches of cranial nerves IX and X supplying the first gill arch sectioned; and the G4 group ( $n = 8$ ) consisted of animals having all branchial branches of cranial nerves IX and X sectioned. The visceral and cardiac branches of the vagus were kept intact in all animals. All denervations were confirmed post mortem. It should be noted that pacu possess a pseudobranch innervated by cranial nerves VII and IX and that our transections did not denervate this structure or other regions of the oro-branchial chamber. To do so would have required a more invasive surgery.

After the implantation of cannulae and gill denervation, animals were ventilated with aerated water until they resumed spontaneous ventilation indicating recovery from anesthesia. Each fish was then placed in an experimental chamber where they did not have access to air and where they remained for a minimum of 24 h before the beginning of the experimental protocol.

#### Experimental protocol

Following recovery from surgery (24 h) the buccal and arterial cannulae were connected to pressure transducers to

monitor  $P_{\text{buccal}}$  and Pa and evaluate  $f_R$ ,  $V_{\text{AMP}}$  and  $f_H$ . The pressure transducers were connected to pre-amplifiers and the outputs were monitored and recorded on a computerized data acquisition system (Dataq DI-194) recording at 120 Hz per channel. The transducers were calibrated against a water column. The water  $O_2$  tension ( $P_{\text{W}O_2}$ ) was constantly monitored with an  $O_2$  electrode (FAC 001) attached to an  $O_2$  analyzer (FAC 204) and calibrated with a borax solution (0.01 N) saturated with sodium sulphite (zero  $O_2$  solution) and aerated water ( $P_{\text{W}O_2} = 140$  mmHg).

Once the catheters were connected and all equipment was calibrated we waited a minimum of 30 min to ensure the return of all cardio-respiratory variables to consistent resting levels. We then began a series of injections in the following sequence: intravenous injections of (a) 0.5 ml of saline (0.9%), (b) 0.5 ml of NaCN (1 mg NaCN/ml saline), followed by intrabuccal injections of (c) 1.0 ml of water and (d) 1.0 ml of NaCN (1 mg NaCN/ml water). Following the injections the cannulae were flushed with either 0.3 ml of saline or 0.5 ml of water, for the venous and buccal cannulae, respectively. Cardio-respiratory variables were recorded continuously from 1 min before until 4 min after the control or NaCN injections or until the return of the cardio-respiratory variables to starting levels. NaCN is a metabolic poison and bolus (discrete, rapid) injections of this compound have been shown to stimulate  $O_2$  chemoreceptors at these concentrations without disrupting normal mitochondrial function (Krylov and Anichkov 1968; Sundin et al. 2000).

Subsequently, the animals were exposed to progressive hypoxia produced by bubbling  $N_2$  into the water in the experimental chamber. The  $P_{\text{W}O_2}$  in the chamber was reduced from 140 mmHg to 100, 70, 50, 30, 20 and 10 mmHg in a step-wise fashion with animals spending 10 min at each step.

#### Data analysis

All cardiovascular and respiratory variables were averaged over the 30 s before each injection and over 10 s intervals at 10, 20, 30, 40, 50, 60, 120, 180 and 240 s after each injection. During exposure to progressive hypoxia we analyzed all cardio-respiratory variables for 1 min in normoxia ( $P_{\text{W}O_2} = 140$  mmHg) and for the final minute of exposure to each  $O_2$  tension.

Measures of  $f_R$  and  $f_H$  are expressed as breaths·min<sup>-1</sup> and beats min<sup>-1</sup>, respectively. Total ventilation ( $V_{\text{TOT}}$ ) was determined as  $V_{\text{TOT}} = V_{\text{AMP}} \cdot f_R$ .  $V_{\text{AMP}}$ , and  $V_{\text{TOT}}$  are expressed as percentage values relative to the resting values obtained under control conditions.

The occurrence of  $O_2$ -receptors in the denervated areas was detected by the comparison of the cardiorespiratory

responses among the experimental groups. Besides measuring the magnitude of the parameters on each step of the treatments, we also observed the time course of each response with respect to its starting point, peak and duration.

Data are presented as mean values  $\pm$  standard error. Differences between resting values and values obtained either post-injection, or at lower levels of  $O_2$  tension in each group were tested for significance with a one way repeated measures ANOVA and Tukey post hoc tests or with non-parametric Kruskal–Wallis and Wilcoxon tests. To evaluate the effect of selective denervations on the responses to the different treatments, the two-way repeated measures ANOVA was used. A Cochran test was used to evaluate differences between the episodic and continuous respiratory patterns. A significance level of 5% was assigned for all cases.

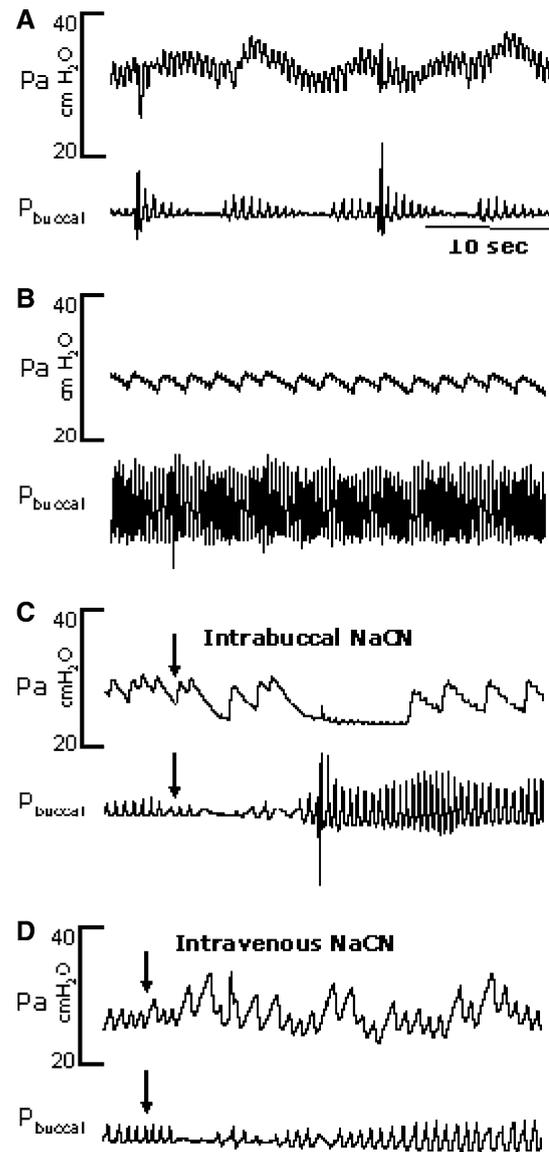
## Results

The cardio-respiratory pattern of the pacu was episodic at rest under normoxic conditions consisting of series of sequential respiratory cycles of varying amplitude interrupted by periods of apnea (Fig. 1a) during which heart rate slowed (not shown).

Progressive bilateral denervation of the branchial nerve branches tended to elevate the resting heart rate. The increase, however, was only significant in the G4 group. There was also a progressive decrease in  $f_R$  with progressive gill denervation (Table 1).

Hypoxia, as well as internal and external injections of NaCN, provoked bradycardia in the intact group (Figs. 1, 2, 3). While there was a direct correlation between the degree of bradycardia and the decrease in the  $P_{wO_2}$ , the fall in  $f_H$  was only significant at values of  $P_{wO_2}$  below 30 mmHg, i.e., below the critical  $O_2$  tension at which  $O_2$  supply is no longer sufficient to meet  $O_2$  demands and metabolic rate begins to fall, for this species ( $P_{cO_2} = 35$  mmHg; Rantin et al. 1998). In the IX, G1 and G4 groups,  $f_H$  was elevated at 70–50 mmHg and only fell significantly below the normoxic values at the lowest tension of 10 mmHg. Nevertheless, the  $f_H$  was significantly lower, below 30 mmHg compared to the values at 50 mmHg in the intact and IX groups but not in the G1 and G4 groups.

In intact fish, both internal and external injections of NaCN produced a bradycardia followed by a modest tachycardia (Fig. 3). In the IX and G1 groups the bradycardia produced in response to external NaCN injections was reduced. In the G4 group there was not any response to NaCN injections. Therefore, complete gill denervation abolished the cardiac response to both internal and external NaCN injections (Fig. 3).



**Fig. 1** Arterial pressure ( $P_a$ ) and intrabuccal pressure ( $P_{buccal}$ ) of intact *P. mesopotamicus* in **a** normoxia; **b** hypoxia ( $P_{wO_2} = 30$  mmHg); **c** after an intra-buccal injection of NaCN (1.0 ml of 1 mg/ml NaCN); and **d** after an intravenous injection of NaCN (0.5 ml of 1 mg/ml NaCN). The small deflections seen in the blood pressure signal were caused by the ventilatory movements

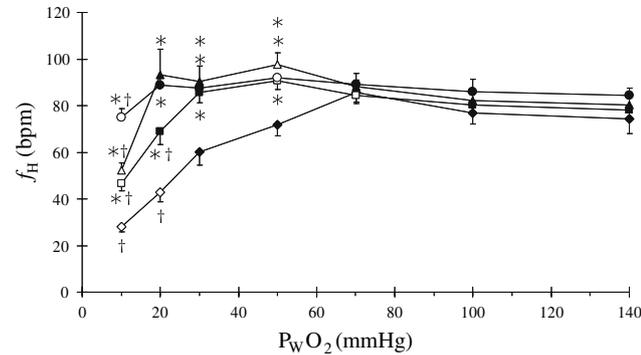
Hypoxia as well as internal and external injections of NaCN increased the ventilation in all experimental groups (Figs. 1, 4, 5, 6). In all groups, decreasing  $O_2$  tensions led to a progressive increase in  $f_R$ ,  $V_{AMP}$  and, consequently,  $V_{TOT}$  (Fig. 4). The  $O_2$  tensions at which the increases in  $f_R$ ,  $V_{AMP}$  and  $V_{TOT}$  became significant varied between groups;  $f_R$  rose a little slower in the G1 group, and  $V_{AMP}$  rose a little slower in the G1 and G4 groups. The denervated fish also had a less pronounced increase in  $f_R$  compared to the animals of the intact group with the increase in  $f_R$  diminishing progressively with the degree of denervation. The

**Table 1** Resting, normoxic values of cardio-respiratory variables in the intact and denervated groups (IX, G1 and G4) of *P. mesopotamicus*

Variable	Intact (n = 10)	IX (n = 10)	G1 (n = 10)	G4 (n = 8)
$f_H$ (bpm)	63 ± 5.6	61 ± 4.5	75 ± 1.9	82 ± 2.6 <sup>a</sup>
$f_R$ (breaths min <sup>-1</sup> )	74 ± 7	69 ± 7	58 ± 7.4 <sup>a</sup>	44 ± 5.1 <sup>a</sup>

Values are means ± SEM

<sup>a</sup> Indicates values that are significantly different from those recorded in the intact group ( $P < 0.05$ )

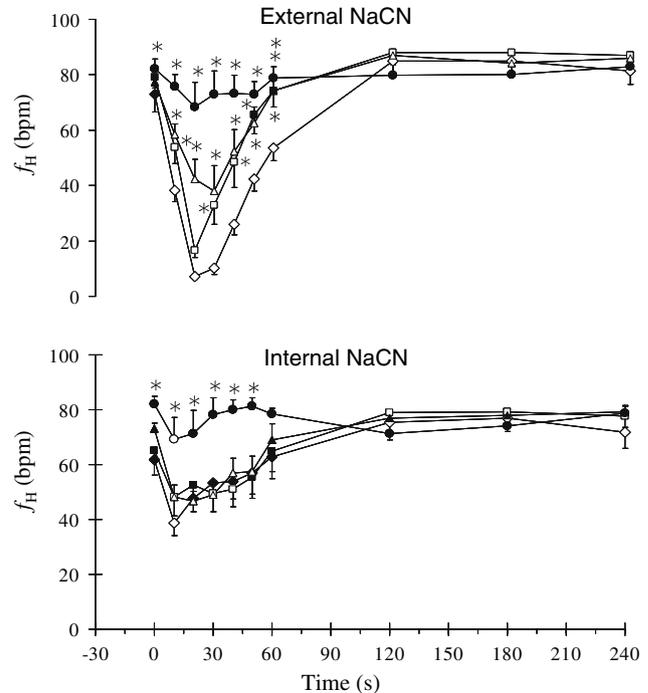


**Fig. 2** Changes in heart rate in *P. mesopotamicus* during exposure to gradual hypoxia. Filled diamond intact group (n = 10); filled square IX group (n = 10); filled triangle G1 group (n = 10); filled circle G4 group (n = 8). Open symbols indicate values that are significantly different from initial (normoxic) values for that group. The asterisk indicates values that are significantly different from those of the intact group at that level of  $P_{W}O_2$ ; and dagger indicates values that are significantly different from the value at  $P_{W}O_2$  of 50 mmHg, in the same group. Values are means ± one standard error

increases in  $V_{AMP}$  and  $V_{TOT}$ , on the other hand, were not altered in the IX and G1 groups but were reduced in the G4 group except at the lowest  $O_2$  tension (Fig. 4).  $f_R$ ,  $V_{AMP}$  and  $V_{TOT}$  had all peaked in the intact, and denervated groups by the time  $O_2$  tensions had fallen to 20 mmHg and tended to decline with further falls in  $P_{W}O_2$ . At such low  $P_{W}O_2$  the values for all groups were statistically similar.

In all groups the respiratory pattern, which was characteristically episodic in normoxia, became continuous once the  $P_{W}O_2$  fell to 100 mmHg and remained this way at all lower  $O_2$  tensions (Figs. 1, 4).

When applied internally, the NaCN provoked an initial fall in  $f_R$  and  $V_{AMP}$ . This also occurred with injection of saline and represents an injection artifact. Following this there was a rapid increase in  $f_R$  and  $V_{AMP}$  in the intact group, with values returning to initial levels within 2–3 min after injection. The responses of the IX group were very similar to those of the intact group although the increases in  $V_{AMP}$  were larger and lasted longer (took longer to return to initial values). In the G1 and G4 groups the maximum values of  $f_R$  reached were less than those observed in the intact and IX groups and there was also a delay in the initiation of the  $f_R$  increasing. The same delay

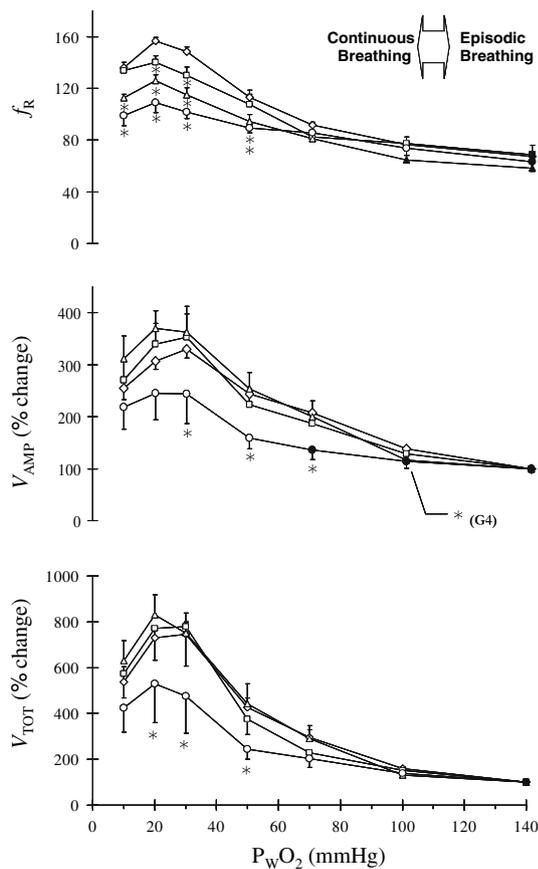


**Fig. 3** Changes in heart rate in *P. mesopotamicus* following external or internal injections of NaCN (1.0 and 0.5 ml of 1.0 mg/ml NaCN, respectively). Filled diamond intact group (n = 10); filled square IX group (n = 10); filled triangle G1 group (n = 10); filled circle G4 group (n = 8). Open symbols indicate values that are significantly different from initial (normoxic) values for that group and an asterisk indicates values that are significantly different from those of the intact group at that level of  $P_{W}O_2$ . Values are means ± one standard error

was observed with the increase in  $V_{AMP}$  in the G4 group. Total gill denervation, however, did not abolish the ventilatory response (Fig. 5).

All groups switched from an episodic to a continuous breathing pattern immediately following the internal injection of NaCN and only in the intact group had breathing returned to an episodic pattern within 4 min (Fig. 5).

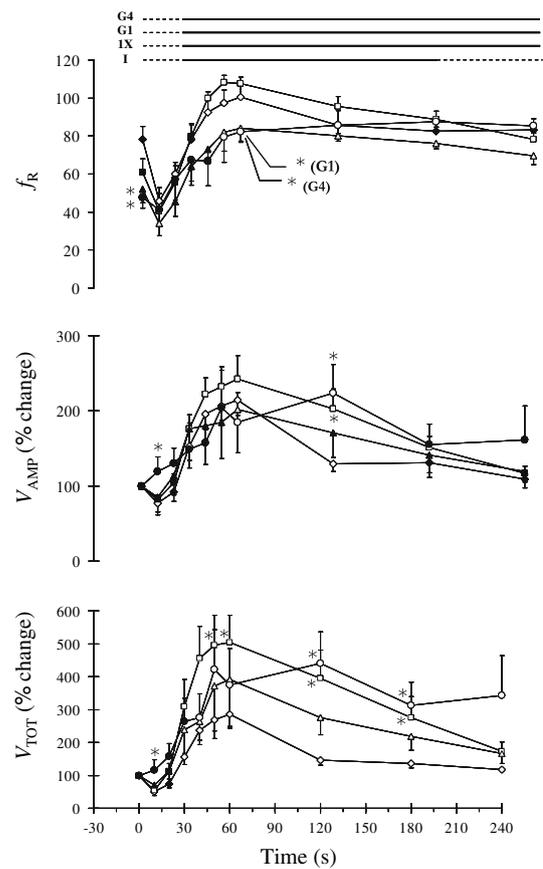
External injections of NaCN into the water flowing over the gills also provoked a fast and brief decrease in breathing followed by an increase in  $V_{AMP}$  and  $f_R$  in the intact group. In this group,  $V_{AMP}$  returned to resting values after 2 min and  $f_R$  did not achieved resting values until



**Fig. 4** Changes in  $f_R$ ,  $V_{AMP}$ ,  $V_{TOT}$  and breathing pattern in *P. mesopotamicus* during exposure to gradual hypoxia. Filled diamond intact group ( $n = 10$ ); filled square IX group ( $n = 10$ ); filled triangle G1 group ( $n = 10$ ); filled circle G4 group ( $n = 8$ ). Open symbols indicate values that are significantly different from initial (normoxic) values for that group and an asterisk indicates values that are significantly different from those of the intact group at that level of  $P_{wO_2}$ . Values are means  $\pm$  one standard error. The right and left arrows indicate the range of  $P_{wO_2}$  at which animals switched from breathing episodically to breathing continuously

4 min post-injection. The response of the IX group was similar with  $f_R$  returning to normal values after 4 min. In the G1 and G4 groups there was a delay of 10 and 20 s, respectively, before  $f_R$  increased significantly in comparison to the intact group. The initial increase in  $V_{AMP}$  seen in the IX and G1 groups in response to the external injections of NaCN were similar to those observed in the intact group and there was also no delay in the return of  $V_{AMP}$  to resting values. In the G4 group, however, there was a delay before increases were seen in  $V_{AMP}$  and  $V_{TOT}$  (Fig. 6).

As with the response to internal injections of NaCN, all groups switched the respiratory pattern from episodic to continuous just after the external injection of NaCN (Figs. 1, 6). Both the initiation of continuous breathing and the return to episodic breathing were delayed in the denervated groups relative to the intact group. Only the G4



**Fig. 5** Changes in  $f_R$ ,  $V_{AMP}$ ,  $V_{TOT}$  and breathing pattern in *P. mesopotamicus* following internal injections of NaCN (0.5 ml of 1.0 mg/ml NaCN). Filled diamond intact group ( $n = 10$ ); filled square IX group ( $n = 10$ ); filled triangle G1 group ( $n = 10$ ); filled circle G4 group ( $n = 8$ ). Open symbols indicate values that are significantly different from initial (normoxic) values for that group and an asterisk indicates significant differences from the intact group at any given time point following the NaCN injection. Values are means  $\pm$  one standard error. The lines at the top of the graph indicate the times during which animals were breathing episodically (dotted line) or continuously (solid line) for each group

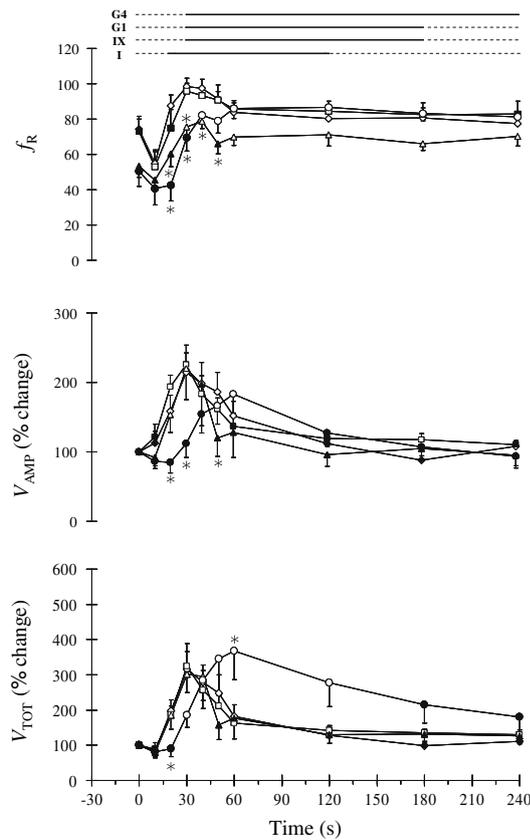
group, however, had not returned to episodic breathing within 4 min.

Despite the low hypoxic level reached during the progressive hypoxia, all intact and denervated fish survived the experiments and recovered the resting values of the cardio-respiratory variables after about 1 h of exposure to normoxia.

## Discussion

### Heart rate and ventilation in intact fish

In this investigation, the  $f_R$  and  $f_H$  of intact fishes in normoxia (Table 1) were slightly elevated compared to



**Fig. 6** Changes in  $f_R$ ,  $V_{AMP}$ ,  $V_{TOT}$  and breathing pattern in *P. mesopotamicus* following external injections of NaCN (1.0 ml of 1.0 mg/ml NaCN). Filled diamond intact group ( $n = 10$ ); filled square IX group ( $n = 10$ ); filled triangle G1 group ( $n = 10$ ); filled circle G4 group ( $n = 8$ ). Open symbols indicate values that are significantly different from initial (normoxic) values for that group and an asterisk indicates significantly differences from the intact group at any given time point following the NaCN injection. Values are means  $\pm$  one standard error. The lines at the top of the graph indicate the times during which animals were breathing episodically (dotted line) or continuously (solid line) for each group

values reported by other investigators for the same species ( $f_H \sim 56$ ; Saint-Paul and Bernardino 1988;  $f_R \sim 53$  and  $f_H \sim 54$ ; Rantin et al. 1998). However, the maximum values of  $f_R$  and the minimum values of  $f_H$  obtained in hypoxia, as well as the threshold  $O_2$  tension at which changes began to occur, were similar to those obtained by others (Saint-Paul and Bernardino 1988; Rantin et al. 1998). The higher resting levels reported in normoxia in the present study could be due to seasonal metabolic differences inherent to this species (Carneiro 1990) or to differences in the levels of stress resulting from the different methodologies employed in the various studies (use of cannulae versus ECG electrodes).

The pacu responded vigorously to hypoxia increasing gill ventilation by about 700% around the critical oxygen tension ( $P_{cO_2}$ ) for this species (35 mmHg Rantin et al.

1998) due to significant increases in  $f_R$  (234%) and  $V_{AMP}$  (241%). When  $P_{wO_2}$  was lowered below an  $O_2$  tension of 20 mmHg, however, total ventilation began to decline. Heart rate began to fall at  $O_2$  tensions below  $P_{wO_2} = 30$  mmHg and fell to  $\sim 30\%$  of starting values at the lowest  $O_2$  tensions. This pattern of response was similar to that previously described by Rantin et al. (1998).

#### Effects of gill denervation on cardio-respiratory variables

There was a trend for  $f_H$  to increase with increasing degrees of gill denervation in resting animals although the increase was only significant in the G4 group. This increase in  $f_H$  was not observed in the tambaqui but did occur in traíra when both species were submitted to a similar protocol (Sundin et al. 1999, 2000). The denervations performed in the present study removed not only the afferent nerve fibres arising from the gills, but also the efferent nerve fibres going to the gills. This makes it difficult to interpret these results. The increase in  $f_H$  could be related to some alteration in the resting vascular tone of the gill vessels or to an alteration of the afferent information from the gills acting on the modulation of the heart activity. When given an injection of atropine, normoxic traíra with either the first gill arch (G1) or all four gill arches (G4) denervated increased  $f_H$ . However, in the G4 group this increase was lower than that observed in the G1 group (Sundin et al. 1999). This suggested that cardiac cholinergic modulation was altered by denervation, although some degree of cardiac control remained even after complete gill denervation. Therefore, it is most likely that the increase in  $f_H$  in the present study was related to both a decrease of branchial vascular resistance and to the removal of sensory information from the gills.

It is interesting to notice that the basal increase in  $f_H$  due to progressive gill denervation is more evident in the pre-injected groups. The  $f_H$  tends to increase reaching similar values to those of G4 group after the beginning of the experimental procedures. After the injections, it is supposed that the basal levels of sympathetic and parasympathetic stimulation to the heart were readjusted. Nevertheless, any argument about the source of this change would be too speculative without appropriate pharmacological study.

Sensory information from the gills also seems to be involved in establishing the resting levels of  $f_R$ . While gill denervation in traíra (Sundin et al. 1999) and tambaqui (Sundin et al. 2000) did not alter resting respiratory frequency, in pacu it decreased resting respiratory frequency indicating that the resting respiratory rate of this animal is influenced by afferent branchial feedback.

In spite of the very low oxygen concentrations to which pacu were exposed ( $P_{\text{wO}_2} = 10$  mmHg), all fish survived the experiments. Even though we did not measure blood gas variables in these fish, it is reasonable to suppose that denervated fish, especially those with all gill arches denervated (G4), had impaired gas exchange. We suspect that these Pacu may have used other strategies to survive such low  $O_2$  tensions when their cardio-respiratory reflexes were depressed by denervation, such as metabolic depression and anaerobic metabolism. Rantin et al. (1998) reported that the  $\dot{V}O_2$  of *P. mesopotamicus* exposed to progressive hypoxia decreased from  $\sim 75$  ml  $O_2$   $kg^{-1}$   $h^{-1}$  in normoxia, to values lower than 15 ml  $O_2$   $kg^{-1}$   $h^{-1}$  at a  $P_{\text{wO}_2}$  of 10 mmHg. This is well below the critical oxygen tension of this species (35 mmHg Rantin et al. 1998). Furthermore, the same authors pointed out that this species can employ anaerobic metabolism during progressive hypoxia, as illustrated by a prominent metabolic rate hysteresis during recovery from hypoxia.

#### Effects of gill denervation on cardiac responses to $O_2$ -chemoreceptor stimulation

Gradual, progressive hypoxia did not induce a significant bradycardia in pacu until the  $P_{\text{wO}_2}$  reached values lower than the critical oxygen tension described for this species (Rantin et al. 1998). Partial or total denervation of the first gill arch (IX and G1 groups) led to an initial increase in  $f_H$  at a  $P_{\text{wO}_2}$  of 50 mmHg and delayed the onset of the bradycardia. In the atlantic cod (*Gadus morhua* Fritsche and Nilsson 1989), trout (*Salmo gairdenri* Smith and Jones 1978), coho salmon (*Oncorhynchus kisutch* Smith and Davie 1984) and traíra (*Hoplias malabaricus* Sundin et al. 1999), denervation of the first gill arch completely abolished the hypoxic bradycardia. This has not been the case in all fish species, however.  $O_2$ -sensitive receptors involved in producing a reflex bradycardia have been found on the first three pairs of gill arches in the catfish (*Ictalurus punctatus* Burslem and Smatresk 1990a), all gill arches in tambaqui (*Colossoma mesopotamicus* Sundin et al. 2000) and on all gill arches and also in the orobranchial cavity in the elasmobranch dogfish (*Scyliorhinus canicula* Butler et al. 1977). It required complete denervation of all gill arches (G4 group) to eliminate the hypoxic bradycardia (despite the alteration in lower hypoxic level) and the cardiac responses to internal and external NaCN in the pacu just as was seen in their close relative, the tambaqui (Sundin et al. 2000).

Despite the fact that the hypoxic bradycardia was eliminated in the G4 group,  $f_H$  decreased in all groups in severe hypoxia. When exposed to severe hypoxia (below the critical  $O_2$  tension— $P_{\text{cO}_2}$ ) as the final  $O_2$  level in

our study, *P. mesopotamicus* presented ECG alterations indicating myocardial ischemia (Rantin et al. 1995). This suggests that the bradycardia that occurred at these low  $O_2$  tensions may have been due to a local effect of hypoxia on the cardiac muscle. A similar bradycardia persisted in tambaqui at this same low  $O_2$  tension even after atropine was administered, suggesting it was not of autonomic origin (Sundin et al. 1999, 2000). In support of this, responses to internal and external injections of NaCN still occurred in the IX and G1 groups, indicating that  $O_2$  chemoreceptors were present on all gill arches, but were absent in the G4 group, indicating that they were confined to the gill arches. Our data, therefore, suggest that in pacu the  $O_2$ -sensitive chemoreceptors involved in producing the reflex bradycardia are located exclusively in the gills and are located on all gill arches.

The  $O_2$  chemoreceptors involved in producing the hypoxic bradycardia appear to sense the change in the external water coursing over the gills in many teleosts (tench *Hemibarbus americanus* Saunders and Sutterlin 1971; trout *Onchorhynchus* sp., Daxboeck and Holeton 1978; Smith and Jones 1978; cod *Gadus morhua*, Fritsche and Nilsson 1989; adriatic sturgeon *Acipenser naccarii*, McKenzie et al. 1995) as well as in elasmobranchs (dogfish *Scyliorhinus canicula*, Barret and Taylor 1984). In some teleosts, however, the receptors are internally oriented, sensing changes in  $O_2$  tension in the blood passing through the gills (traíra *Hoplias malabaricus*, Sundin et al. 1999). Some other species have chemoreceptors internally and externally oriented, sensing both water and blood (tambaqui *Colossoma macropomum*, Sundin et al. 2000). In the present study, both internal and external injections of NaCN resulted in bradycardia also denoting the existence of chemoreceptor populations sensitive to  $O_2$  changes in both water and blood. Furthermore, while progressive gill denervation led to a progressive reduction in the degree of bradycardia, suggesting the presence of  $O_2$  chemoreceptors sensitive to external stimuli on all gill arches, the magnitude of the bradycardia resulting from internal stimuli was not reduced by the denervation of either IX or the first gill arch (G1). This could indicate that the first gill arch does not possess internally oriented  $O_2$  chemoreceptors that participated in the hypoxic bradycardia, or that the receptors on the other arches are capable of producing a full response.

It is interesting to note that there was a tachycardia present in the denervated groups at moderate levels of hypoxia that was not present in the intact group. This suggests there is a receptor population innervated by the IX cranial nerve acting to reduce heart rate at these higher  $O_2$  tensions that normally masks this effect. This tachycardia persisted even in the completely gill denervated group (G4). It would suggest that it arose from either the

pseudobranch or from receptors outside the gills; or that the increased activity of the respiratory mechanoreceptors resulting from the elevated ventilation was enough to drive up the heart rate at this level of  $P_{wO_2}$ . This response has not been reported in other species.

Effects of gill denervation on respiratory responses to  $O_2$ -chemoreceptor stimulation.

The magnitude of respiratory response to hypoxia decreased progressively as the degree of gill denervation increased suggesting there are  $O_2$  chemoreceptors associated with the ventilatory response present on all gill arches in pacu. Nevertheless, complete gill denervation did not abolish all of the increases in  $f_R$  or  $V_{AMP}$ , nor the associated switch from episodic to continuous breathing.

Many other studies on  $O_2$  chemoreception have failed to eliminate the ventilatory response to hypoxia by gill denervation (Hughes and Shelton 1962; Saunders and Sutterlin 1971; Davis 1971; Bamford 1974; Jones 1983). For some, as in the present study, total gill denervation did not remove afferent input arising from the pseudobranch (see Smatresk 1989 for review). In trout the pseudobranch is responsive to decreases in  $O_2$  (Laurent and Rouzeau 1972) and is innervated by the VII cranial nerve (the facial nerve) as well as the IX cranial nerve (Nilsson 1984). In other studies where the pseudobranch was denervated, the respiratory response to hypoxia was completely abolished (the bowfin *Amia cavia*, McKenzie et al. 1991; the channel catfish *Ictalurus punctatus*, and the gar *Lepisosteus osseus*, Smatresk 1989). Both traíra (Sundin et al. 1999) and tambaqui (Sundin et al. 2000; Milsom et al. 2002), however, which do not possess pseudobranchs, showed increases in  $V_{AMP}$  in response to hypoxia following complete gill denervation, arising from receptors that were externally oriented and sensed changes in  $O_2$  in the water. In the tambaqui, these receptors were shown to be innervated by cranial nerves V and VII (Milsom et al. 2002). Thus, it is possible that the remaining response in pacu originated from receptors in the pseudobranch or at extrabranchial sites.

Based on the present data we can also suggest that there may be internally oriented  $O_2$  chemoreceptors innervated by the IX cranial nerve that inhibit ventilation during hypoxic exposure in pacu. Although the IX group did not show an enhanced respiratory response to progressive hypoxia, there was a significant increase in  $V_{AMP}$  and  $V_{TOT}$  relative to intact fish in response to the internal NaCN injections. This was also associated with a delay in the return of  $V_{AMP}$  to pre-injection values. Similar results have been reported by Sundin et al. (1999) in traíra, with the exception that in that study the chemoreceptors appeared to

be externally oriented and affected only the respiratory frequency.

Comparison of tambaqui and pacu.

We hypothesized that receptor distribution and locations in pacu would be similar to those of tambaqui, a closely related species that inhabits a similar environment. Both species are derived from the same ancestral line within the family Characidae, a line that also includes the piranhas. They are large fruit and seed eaters with strong jaws that procure most of their food from flooded forests during the rainy season. During the low water season they retreat to turbid water rivers or get trapped in marginal and/or “várzea” lakes and ponds in the Pantanal and Amazon basin (Goulding 1980). They are extremely tolerant of hypoxic conditions as long as they have access to the water surface where they undertake aquatic surface respiration (Carter and Beadle 1931; Kramer et al. 1978; Saint-Paul and Bernardinho 1988). The distribution and locations of the receptors involved in the cardiac responses (i.e., the hypoxic bradycardia) were identical in the two species (Sundin et al. 2000; present study). The distribution and locations of receptors within the gills involved in respiratory responses were also identical in the two species (Sundin et al. 2000; Milsom et al. 2002; present study). We observed an inhibitory respiratory input arising from receptors in the first gill arch innervated by the IXth cranial nerve in this study not reported in tambaqui, but then the IXth cranial nerve was not denervated in isolation in that species (Sundin et al. 2000). Pacu have a covered pseudobranch, not present in its relative tambaqui. Both species have receptors located outside the gills that are involved in the respiratory response. In tambaqui these are all externally oriented, located in the orobranchial cavity and innervated by cranial nerves V and VII (Milsom et al. 2002). In pacu they are both internally and externally oriented but their locations remain unknown, possibly in the pseudobranch and at extrabranchial sites. If the internal responses arose from the pseudobranch and the external responses arose from the orobranchial cavity, then the differences between the two species could reduce to nothing more than the presence or absence of the pseudobranch.

The complex distribution of  $O_2$  chemoreceptor populations involved in respiratory and cardiac responses to hypoxia and/or hypoxemia in fish remain difficult to explain. We began this study on the premise that studies of the distribution of cardio-respiratory chemoreceptor populations for a variety of species, adapted to different habitats, would help us develop testable hypothesis concerning the evolution, phylogeny and adaptive

significance of these receptors and their distribution. The overall similarities between tambaqui and pacu, and the relatively small differences, suggest that it may prove rewarding to continue to try to map locations and distribution as a function of phylogeny, habitat and ecological niche. All are bound to play a role in the process. Unfortunately, short-term plasticity due to history of exposure (acute vs. chronic) etc., may also lead to changes in responsiveness continuing to make this a difficult task. While such intra-specific differences are not easy to understand, our data, along with those from previous studies on tambaqui (Sundin et al. 2000, Milsom et al. 2002), do not support the hypothesis that hypoxia tolerant species are more sensitive to systemic hypoxemia while hypoxia-intolerant species are more sensitive to environmental hypoxia.

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## References

- Bamford OS (1974) Oxygen reception in the rainbow trout (*Salmo gairdneri*). *Comp Biochem Physiol* 48:69–76
- Barret DJ, Taylor EW (1984) Changes in heart rate during progressive hypoxia in the dogfish, *Scyliorhinus canicula* L.: evidence for a venous oxygen receptor. *Comp Biochem Physiol* 78:697–703
- Burleson ML, Milsom WK (1993) Sensory receptors in the first gill arch of rainbow trout. *Resp Physiol* 93:97–110
- Burleson ML, Smatresk NJ (1990a) Effects of sectioning cranial nerves IX and X on cardiovascular and ventilatory reflex responses to hypoxia and NaCN in channel catfish. *J Exp Biol* 154:407–420
- Burleson ML, Smatresk NJ (1990b) Evidence for two oxygen sensitive chemoreceptor loci in Channel catfish. *Phys Zool* 63:208–221
- Butler PJ, Taylor EW, Short S (1977) The effect of sectioning cranial nerves V, VII, IX and X on the cardiac response of the dogfish *Scyliorhinus canicula* to environmental hypoxia. *J Exp Biol* 69:233–245
- Carneiro DJ (1990) Efeito da Temperatura na exigência de proteína e energia em dietas para alevinos de pacu. *Piaractus mesopotamicus* (Homberg, 1887). Theses, Ph.D. on physiological sciences, UFSCar, SP, Brazil
- Carter GS, Beadle LC (1931) The fauna of the swamps of the Paraguayan Chaca in relation to its environment. II. Respiratory adaptations in the fishes. *J Lin Soc Zool* 37:327–368
- Davis JC (1971) Circulatory and ventilatory responses of rainbow trout (*Salmo gairdneri*) to artificial manipulation of gill surface area. *J Fish Res Board Can* 28:1609–1614
- Daxboeck C, Holeton GF (1978) Oxygen receptors in the rainbow trout, *Salmo gairdneri*. *Can J Zool* 56:1254–1256
- Florindo LH, Reid SG, Kalinin AL, Milsom WK, Rantin FT (2004) Cardiorespiratory reflexes and aquatic surface respiration in the neotropical fish tambaqui (*Colossoma macropomum*): acute responses to hypercarbia. *J Comp Physiol* 174:319–328
- Florindo LH, Leite CAC, Kalinin AL, Reid SG, Milsom WK, Rantin FT (2006) The role of branchial and orobranchial O<sub>2</sub> chemoreceptors in the control of aquatic surface respiration in the neotropical fish tambaqui (*Colossoma macropomum*): progressive responses to prolonged hypoxia. *J Exp Biol* 209:1709–1715
- Fritsche R, Nilsson S (1989) Cardiovascular responses to hypoxia in the Atlantic cod, *Gadus morhua*. *Exp Biol* 48:153–160
- Gilmour KM, Milsom WK, Rantin FT, Reid SG, Perry SF (2005) Cardiorespiratory responses to hypercarbia in tambaqui *Colossoma macropomum*: chemoreceptor orientation and specificity. *J Exp Biol* 208:1095–1107
- Goulding M (1980) The fishes and the forest: Explorations in Amazonian Natural History. University of California Press, Berkeley 280p
- Hughes GM, Shelton G (1962) Respiratory mechanisms and their nervous control in fish. *Adv Comp Physiol Biochem* 1:275–364
- Jones DR (1983) Ontogeny and Phylogeny of the oxygen response. *Proc Physiol Soc NZ* 3:79–81
- Kramer DL, McClure M (1982) Aquatic surface respiration, a widespread adaptation to hypoxia in tropical freshwater fishes. *Env Biol Fish* 7:47–55
- Kramer DL, Lindsey CC, Moodie GEE, Stevens ED (1978) The fish and the aquatic environment of the central Amazon basin, with particular reference to respiratory patterns. *Can J Zool* 56:717–729
- Krylov SS, Anichkov SV (1968) The effect of metabolic inhibitors on carotid chemoreceptors. In: Torrance RW (ed) Arterial chemoreceptors. Blackwell Scientific, Oxford, pp 103–114
- Laurent P, Rouzeau JD (1972) Afferent neural activity from the pseudobranch of teleosts. Effects of PO<sub>2</sub>, pH, osmotic pressure and Na<sup>+</sup> ions. *Resp Physiol* 14:307–331
- McKenzie DJ, Burleson ML, Randall DJ (1991) The effects of branchial denervation and pseudobranch ablation on cardio-ventilatory control in an air-breathing fish. *J Exp Biol* 161:347–365
- McKenzie DJ, Taylor EW, Bronzi P, Bolis CL (1995) Aspects of cardioventilatory control in the Adriatic sturgeon (*Acipenser naccarii*). *Resp Physiol* 100:45–53
- Milsom WK, Reid SG, Rantin FT, Sundin L (2002) Extrabranchial chemoreceptors involved in respiratory reflexes in the neotropical fish *Colossoma macropomum* (the tambaqui). *J Exp Biol* 205:1765–1774
- Nilsson S (1984) Innervation and pharmacology of the gills. In: Hoar WS, Randall DJ (eds) Fish physiology, vol XA. Academic, New York, pp 185–227
- Randall DJ, Smith JC (1967) The regulation of cardiac activity in fish in a hypoxic environment. *Physiol Zool* 40:104–113
- Rantin FT, Kalinin AL (1996) Cardiorespiratory function and aquatic surface respiration in *Colossoma macropomum* exposed to graded and acute hypoxia. In: Val AL, Almeida-Val VMF, Randall DJ (eds) Physiology and biochemistry of the fishes of the Amazon. INPA, Manaus, pp 169–180
- Rantin FT, Kalinin AL, Guerra CDR, Maricondi-Massari M, Verzola RMM (1995) Electrocardiographic characterization of myocardial function in normoxic and hypoxic teleosts. *Braz J Med Biol Res* 28:1277–1289
- Rantin FT, Guerra CDR, Kalinin AL, Glass ML (1998) The influence of aquatic surface respiration (ASR) on cardio-respiratory function of the serrasalmid fish *Piaractus mesopotamicus*. *Comp Biochem Physiol A* 119:991–997
- Reid SG, Sundin L, Florindo LH, Rantin FT, Milsom WK (2003) Effects of afferent input on the breathing pattern continuum in the tambaqui (*Colossoma macropomum*). *Respir Physiol Neurobiol* 136:39–53
- Reid SG, Perry SF, Gilmour KM, Milsom WK, Rantin FT (2005) Reciprocal modulation of O<sub>2</sub> and CO<sub>2</sub> cardiorespiratory chemoreflexes in the tambaqui. *Respir Physiol Neurobiol* 146:175–194

- Saint-Paul U, Bernardinho G (1988) Behavioral and ecomorphological responses of Neotropical Pacu *Piaractus mesopotamicus* (Teleostei, Serrasalminidae) to oxygen-deficient waters. *Exp Biol* 48:19–26
- Saunders RL, Sutterlin AM (1971) Cardiac and respiratory response to hypoxia in the searaven, *Hemipterus americanus*, an investigation of possible control mechanism. *J Fish Res Board Can* 28:491–503
- Shelton G, Jones DR, Milsom WK (1986) Control of breathing in ectothermic vertebrates. In: *Handbook of physiology*, vol 2. pp 857–909
- Smatresk NL (1989) Chemoreflex control of respiration in an air breathing fish. In: Lahiri S, Foster RE II, Davies RO, Pack AI (eds) *Chemoreceptors and chemoreflexes in breathing cellular and molecular aspects*. Oxford University Press, London, pp 29–52
- Smatresk NL, Burleson ML, Azizi SQ (1986) Chemoreflexive responses to hypoxia and NaCN in longnose gar: evidence for two chemoreceptive loci. *Am J Physiol* 251:116–125
- Smith FM, Davie PS (1984) Effects of sectioning cranial nerves IX and X on the cardiac response to hypoxia in the coho salmon, *Oncorhynchus kisutch*. *Can J Zool* 62:766–768
- Smith FM, Jones DR (1978) Localization of receptors causing hypoxic bradycardia in trout (*Salmo gairdenri*). *Can J Zool* 56:1260–1265
- Sundin L, Reid SG, Kalinin AL, Rantin FT, Milsom WK (1999) Cardiovascular and respiratory reflexes: the tropical fish, traira (*Hoplias malabaricus*) O<sub>2</sub> chemoresponses. *Respir Physiol* 116:181–199
- Sundin L, Reid SG, Rantin FT, Milsom WK (2000) Branchial receptors and cardio-respiratory reflexes in a neotropical fish, the tambaqui (*Colossoma macropomum*). *J Exp Biol* 203:1225–1239
- Val AL, Almeida-Val VMF (1995) *Fishes of the Amazon and their environments. Physiological and biochemical features*. Springer, Heidelberg, 224 pp