

## CARDIORESPIRATORY RESPONSES TO O<sub>2</sub> IN WATER-BREATHING

### FISH

Francisco Tadeu Rantin  
Department of Physiological Sciences  
Federal University of São Carlos  
Via Washington Luiz, km 235 – P.O. Box 676  
13565-905 – São Carlos, SP – Brazil  
Phone: ++55 16 260 8314; Fax: ++55 16 260 8327  
e-mail: frantin@power.ufscar.br

Luiz H. Florindo<sup>1</sup>, Ana L. Kalinin<sup>1</sup>, Stephen G. Reid<sup>2</sup> and William K. Milsom<sup>3</sup>  
1 – Department of Physiological Sciences, UFSCar, São Carlos, SP, Brazil,  
2 – Division of Life Sciences, University of Toronto at Scarborough, Canada.  
3 – Department of Zoology, UBC, Vancouver, Canada.

### Introduction

The respiratory and cardiovascular responses of fish to environmental hypoxia have been studied in a considerable number of species. Hyperventilation (via an increase in both ventilation frequency and amplitude), bradycardia and adjustments in arterial blood pressure are reflex responses to hypoxia that originate from changes in sensory input. Different reflexogenic areas have been proposed to function as O<sub>2</sub>-sensing loci (for a review see Burlerson et al., 1992). Due to the lack of direct histological and physiological evidences for the presence specific O<sub>2</sub> chemoreceptors, several reflexogenic areas have been tested with a variety of indirect techniques (Milsom, 1996). The brain (Jones, 1983), the pseudobranch (Laurent and Rouzeau, 1972), the arterial system (Randall, 1982), the afferent gill vasculature (Smatresk et al., 1986), the first gill arch (Milsom and Brill, 1986; Burlerson and Milsom, 1993) and all the gill arches (Sundin et al., 1999; 2000) have been proposed as loci where the sensory impulses originate. There is also evidence that, in the gill arches, a population of externally oriented (sensing the inspired water) O<sub>2</sub> receptors elicit both ventilatory and cardiovascular reflexes (Randall and Smith, 1967; Saunders and Sutterlin, 1971; Smith and Jones, 1978; Smatresk et al., 1986; Burlerson and Smatresk, 1990b; McKenzie et al., 1991; Burlerson and Milsom, 1993), and another population of internally oriented (arterial sensing) receptors specifically elicit the ventilatory responses (Randall and Smith, 1967; Smith and Jones,

1978; Smatresk et al., 1986; Burluson and Smatresk, 1990b; McKenzie et al., 1991; Burluson and Milsom, 1993).

In teleosts, the reflex hypoxic bradycardia generally arises from stimulation of externally oriented receptors located exclusively within the gills. The distribution and orientation of these receptors, however, is not uniform amongst species. For example, in trout and Atlantic cod these receptors are externally-oriented and are located on the first gill arch (Daxboeck and Holeton, 1978; Smith and Jones, 1978; Fritsche and Nilsson, 1993). In traíra the receptors are on the first arch but are internally-oriented and sense O<sub>2</sub> levels in the arterial blood (Sundin et al., 1999). In catfish they appear to be located on the first three gill arches (Burluson and Smatresk, 1990a) while in tambaqui they appear to be internally- and externally-oriented, and distributed throughout all gill arches (Sundin et al., 2000). In dogfish (an elasmobranch), the receptors are not confined in the gills, but are also present in the oro-branchial cavity and are innervated by the cranial nerves V, VII, IX and X (Butler et al., 1977).

In most species, the ventilatory response to hypoxia consists of an increase in both ventilation frequency and amplitude. In early studies, the denervation of cranial nerves IX and X to the gills and pseudobranch failed to completely abolish the ventilatory responses to hypoxia, suggesting the occurrence of central O<sub>2</sub> chemoreceptors that regulate ventilation (Satchell, 1961; Saunders and Sutterlin, 1971). However, attempts to correlate ventilatory responses to hypoxia with O<sub>2</sub> chemoreception by the CNS have failed (Rovainen, 1977; Hedrick et al., 1991; Milsom et al., 2002). On the other hand, complete denervation of cranial nerves IX and X to the gills abolished the hypoxic ventilatory response in two species that lack a pseudobranch, the gar (Smatresk, 1989) and catfish (Burluson and Smatresk, 1990a). The failure of branchial denervation to abolish ventilatory reflexes in other species leads to several questions (Sundin et al., 1999). Are there species differences in the presence/absence and distribution of extra-branchial chemoreceptors, or do the different experimental results arise from failure to completely denervate all branchial nerves? Furthermore, other important questions still remain. What populations of receptors are present and what exactly is their stimulus modality? How sensitive are the receptors to low levels of oxygen and how do they transduce low levels of PO<sub>2</sub> into a signal to the brain? It is clear that more studies on a variety of species are needed to delineate a general picture of the location and relative roles of O<sub>2</sub>-chemosensitive areas in cardiorespiratory reflexes in fish. These studies will need to elucidate the distribution of internal and external receptors among branchial and extra-branchial sites as well as

identify the corresponding cardiorespiratory reflexes elicited by each receptor population (Sundin et al., 1999).

Given this background, our aim was to extend our studies (Sundin et al., 2000; Milsom et al., 2002) on chemoreceptor control of cardio-respiratory reflexes in a hypoxic tolerant neotropical species, the tambaqui *Colossoma macropomum*. This species employs a variety of behavioral, morphological, physiological and biochemical mechanisms to adapt to the widely fluctuating oxygen concentrations of its habitat (Rantin and Kalinin, 1996). To alleviate the effects of hypoxia, this species performs aquatic surface respiration (ASR) facilitated by the development of lower lip dermal swelling. The lower lip is not involved in gas exchange but serves as a mechanical structure that enhances skimming of the well-aerated surface water across the gills (Saint-Paul, 1988). Tambaqui immediately begin ASR even in moderate hypoxia (50 – 70 mmHg), and the frequency of ASR increases as the environment becomes more hypoxic. However, the complete development of the swollen lip takes 3 h or more (Rantin and Kalinin, 1996). A previous study on this species (Sundin et al., 2000) demonstrated that the O<sub>2</sub> receptors which elicit the reflex bradycardia and increase in breathing frequency are situated on all gill arches and sense changes in both arterial blood and inspired water. On the other hand, the O<sub>2</sub> receptors that trigger the elevation in systemic vascular resistance and breathing amplitude during hypoxia are extra-branchial. In the aforementioned experiments, fish were exposed to hypoxia for a short period of time (10 to 30 min). Such a protocol has been used in the majority of studies on O<sub>2</sub> chemoreception and cardiorespiratory reflex responses in fish. However, in the present study, the main goal was to evaluate the cardiorespiratory reflex responses of tambaqui during a long-term (6 h) exposure to hypoxia (PO<sub>2</sub> = 10 mmHg) and compare these responses with those (short-term exposure) previously obtained by Sundin et al. (2000). Furthermore, we also investigated if the O<sub>2</sub> receptors involved in the cardiorespiratory reflex responses are the same as those which trigger ASR and elicit the development of the lower lip swelling.

## **Material and Methods**

### *Experimental animals:*

Tambaqui (Wt = 657 ± 39 g) were obtained from the Center of Aquaculture of São Paulo State University (CAUNESP), Jaboticabal, SP, Brazil. In the laboratory, fish were kept in 1000 L holding tanks supplied with a continuous flow of dechlorinated and well-aerated water (normoxic conditions, PwO<sub>2</sub> ≥ 130

mmHg) at a constant temperature (25°C). The fish were fed *ad libitum* with commercial food pellets but were fasted for two days prior to experimentation.

*Animal preparation:*

Fish were anesthetized in a benzocaine solution (100 mg/L) previously dissolved in 3 mL of ethanol (70%). After anesthesia, fish were transferred to a surgical table and their gills were artificially ventilated with a weaker benzocaine solution (50 mg/mL) gassed with air. Using a Dremel® rotary tool, a hole was drilled through the snout between the nostrils, and a flared cannula (PE-100; buccal canulae) was fed from inside the mouth out through the hole and was secured with a cuff. The fish were then fitted with ECG electrodes according to the method described by Glass et al. (1991). One electrode (+) was inserted and sutured in a ventral position between the gills and the heart, and a second (-) in a ventrocaudal position close to the pelvic fins. After surgery, the fish were placed in the experimental chamber for at least 24 h of recovery in normoxic water ( $PwO_2 \geq 130$  mmHg; 25 °C).

*Ventilation rate:*

Ventilation rate ( $f_R$  - breaths·min<sup>-1</sup>) was recorded by connecting the buccal PE catheter to a Narco P-1000B pressure transducer, coupled to a universal coupler (Narco 7189) of a Narco Narcotrace 40 (Narco Bio-Systems, Houston, TX, USA) physiograph.

*Heart rate:*

Electrocardiography was used to record heart rate ( $f_H$  - beats·min<sup>-1</sup>) by counting the number of QRS complexes per minute. The ECG electrodes were connected to another universal coupler of the same physiograph. A third electrode (reference) was immersed in the water of the experimental setup. This preparation allowed ECG recordings equivalent to bipolar lead I of standard electrocardiography.

*Denervation of cranial nerves IX (glossopharyngeal) and X (vagus):*

In order to denervate the cranial nerves, fish were anesthetized and moved to the surgical table where they were artificially ventilated. The denervation protocol was the same as described by Sundin et al. (2000). Under a stereoscopic microscope (Opto SM 2001, Opto Electronics, São Carlos, SP, Brazil), the operculum was reflected forward, and a small incision (2 cm) was made in the epithelium at the dorsal end of the 1<sup>st</sup> and 2<sup>nd</sup> gill arches where they meet the roof of the opercular cavity. The incision allowed the access to the cranial nerve IX and the pretrematic branches of the nerve X. The branchial nerves of all gill

arches were carefully dissected free of connective tissue and cut with fine iris scissors (Group G4). The cardiac and visceral branches of the vagus were preserved in all the cases. The healing process in tambaqui was rapid, and the incisions were covered with “scar tissue” within 24 h. All denervations were documented with a video camera attached to the microscope and connected to an ATI Pro interface of a Pentium IBM PC. Denervations were confirmed *post mortem* by autopsy. After surgery, fish were ventilated with aerated water, and as soon as they showed signs of arousal from anesthesia, they were transferred to the experimental system where they recovered for 24 h in normoxic water prior to experimentation.

*Experimental system:*

To simultaneously examine the effects of hypoxia on cardiorespiratory variables and ASR, an experimental setup similar to that described by Rantin et al. (1996; 1998) was used. The system consisted of two chambers, an upper compartment, where the fish was kept during the experiment, and a lower compartment, in which the water was gassed with N<sub>2</sub> in order to lower the PO<sub>2</sub>. The water was continuously re-circulated from the lower to the upper compartment. The shape of the upper chamber allowed fish to remain on the bottom or move up to the surface to perform ASR whereas lateral movement was restricted. This compartment was also equipped with two ventilators to maintain a unidirectional flow of air above the water surface. This "air tunnel" removed the excess of N<sub>2</sub> released from the water and kept a constant atmospheric gas concentration on the water surface, so that the PO<sub>2</sub> of the surface layer remained 5 to 10 mmHg higher than the bulk air in the rest of the tank. The experimental temperature was kept constant (25 ± 1°C) with a thermostat-controlled heater coil (TRM 10.40, Terroni Equipments Ltd., São Carlos, SP, Brazil) placed inside the lower chamber. Movements and behavior were continuously monitored via of a closed circuit TV (Sharp VL-L 310B video camera and Sansung CN-3355Z monitor) and recorded on videotape to verify the occurrence of aquatic surface respiration.

*Experimental protocol*

Two groups of experimental animals were studied. The first with the gill innervation intact, and the second with the gills denervated. In both groups, tambaqui were exposed for a period of 360 min to severe hypoxia (PwO<sub>2</sub> = 10 mmHg). The  $f_R$  and  $f_H$  were recorded in the last 10 min of each 30 min interval. The  $f_R$  (breaths.min<sup>-1</sup>) and  $f_H$  (beats.min<sup>-1</sup>) were expressed in absolute values. Since the ventilation amplitude ( $V_{AMP}$ ) was measured in arbitrary units, the  $V_{AMP}$  as well as the total ventilation ( $\dot{V}_{TOT} = V_{AMP} \cdot f_R$ ) were expressed as percentage

of the initial values. To verify the effects of hypoxia on the development of lip swelling, the dimensions of the inferior lips (length and width) were measured before and after exposition to hypoxia.

#### Statistics

The data are presented as as mean  $\pm$  SEM. The data were analyzed using a repeated measures analysis of variance (ANOVA) followed by a Tukey-Kramer multiple comparison test.  $P < 0.05$  was taken to be the level of significance.

### Results

#### *Respiratory frequency ( $f_R$ ), Ventilation amplitude ( $V_{AMP}$ ) and Total Ventilation ( $\dot{V}_{TOT}$ )*

Figure 1 illustrates the effects of severe hypoxia on  $f_R$  during exposure to 360 min of hypoxia ( $PwO_2 = 10$  mmHg). In the intact fish (control)  $f_R$  increased significantly (approximately 60 %) during the first 60 min. These values remained constant until the end of the experiment. Bilateral denervation of cranial nerves IX and X completely abolished the increase in  $f_R$ . Under normoxic conditions, the  $f_R$  of denervated fish was higher than the control group.

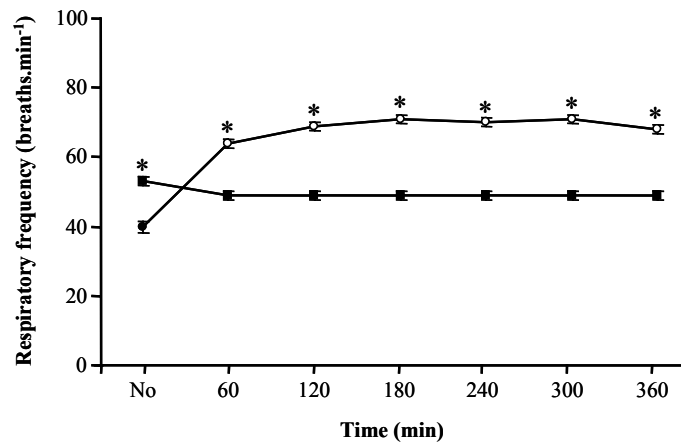


Figure 1. The effects of hypoxia ( $PwO_2 = 10$  mmHg) on the  $f_R$  of tambaqui. ● - control fish ( $n = 8$ ); ■ - denervated fish ( $n = 8$ ). Open symbols - statistical differences from normoxia (No); \* - statistical differences between the control and denervated fish (mean  $\pm$  1 SEM).

The effects of long-term exposure to hypoxia on  $V_{AMP}$  and  $\dot{V}_{TOT}$  are illustrated in figures 2 and 3, respectively. During hypoxia both  $V_{AMP}$  and  $\dot{V}_{TOT}$  increased significantly in the intact fish, reaching maximum values after 120 min of exposure to hypoxia and remained constant for the last 240 min of experimentation. Total denervation attenuated, but did not abolish the increases in  $V_{AMP}$  and  $\dot{V}_{TOT}$  during hypoxia. Both parameters increased significantly in the first 60 min, after which they remained constant for the last 300 min of exposure to hypoxia. During hypoxia, the  $\dot{V}_{TOT}$  of intact group was always significantly higher than that in the denervated group.

#### *Heart rate*

The effects of hypoxia on the  $f_H$  of intact and denervated fish are shown in figure 4. Intact fish exhibited a significant bradycardia in the 60 first min of exposure to 10 mmHg O<sub>2</sub>. At this point  $f_H$  increased gradually, and during the last 120 min of the experiment it was similar to the normoxic values.

Bilateral denervation of cranial nerves IX and X abolished the reflex bradycardia. However, denervated fish always had a higher  $f_H$  than the control group.

Table I: Dimensions of the inferior lips of intact and denervated tambaqui during normoxia and after exposure to severe hypoxia (10 mmHg) for 360 min. Significant differences (\*) were observed between values for normoxia and hypoxia in both groups. The data are reported as the mean  $\pm$  SEM.

	<b>Intact</b>		<b>Denervated</b>	
	Length (mm)	Width (mm)	Length (mm)	Width (mm)
<b>Normoxia</b>	7.4 $\pm$ 0.4	27.7 $\pm$ 0.7	7.3 $\pm$ 0.4	27.9 $\pm$ 0.5
<b>Hypoxia</b>	8.1 $\pm$ 0.2*	29.9 $\pm$ 0.7*	7.8 $\pm$ 0.4*	29.9 $\pm$ 0.6*

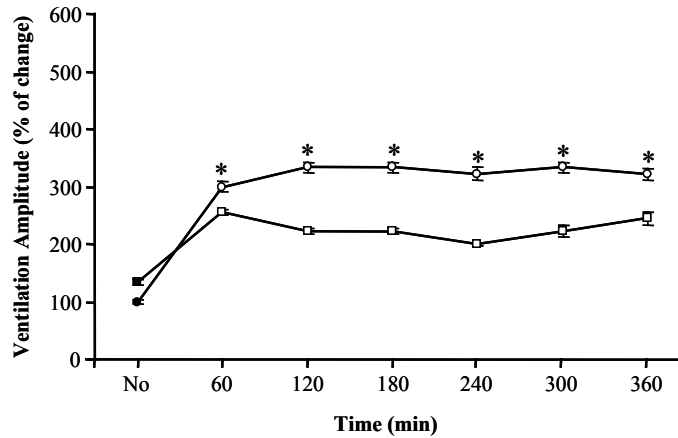


Figure 2. The effects of hypoxia ( $PwO_2 = 10$  mmHg) on the  $V_{AMP}$  of tambaqui. ● - control fish ( $n = 8$ ); ■ - denervated fish ( $n = 8$ ). Open symbols - statistical differences from normoxia (No); \* - statistical differences between the control and denervated fish (mean  $\pm 1$  SEM).

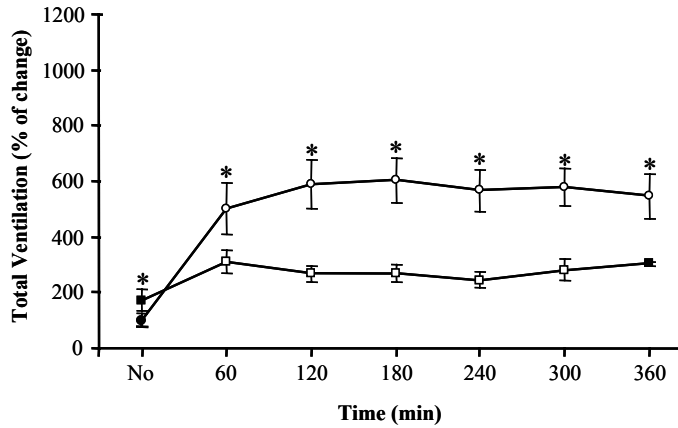


Figure 3. The effects of hypoxia ( $PwO_2 = 10$  mmHg) on the  $V_{TOT}$  of tambaqui. ● - control fish ( $n = 8$ ); ■ - denervated fish ( $n = 8$ ). Open symbols - statistical differences from normoxia (No); \* - statistical differences between the control and denervated fish (mean  $\pm 1$  SEM).

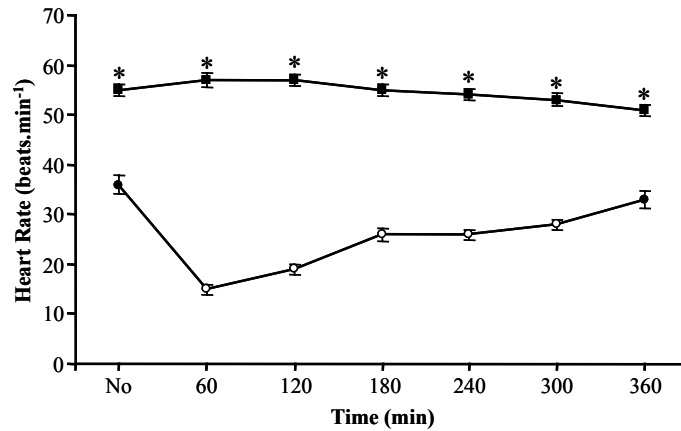


Figure 4. The effects of hypoxia ( $PwO_2 = 10$  mmHg) on the  $f_H$  of tambaqui. ● - control fish ( $n = 8$ ); ■ - denervated fish ( $n = 8$ ). Open symbols - statistical differences from normoxia (No); \* - statistical differences between the control and denervated fish (mean  $\pm 1$  SEM).

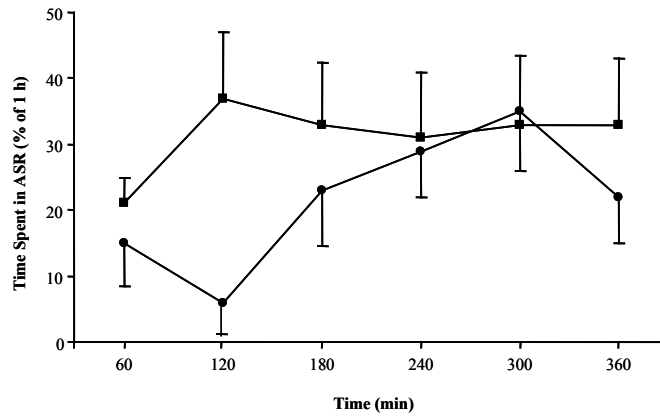


Figure 5. Time spent in ASR by tambaqui exposed to hypoxia ( $PwO_2 = 10$  mmHg) during 360 min. ● - control fish ( $n = 8$ ); ■ - denervated fish ( $n = 8$ ). Data are shown as the mean  $\pm$  SEM.

#### *ASR and inferior lips swellings*

The effects of severe hypoxia on ASR and swelling of the inferior lips are reported in figure 5 and table I, respectively. Both the intact and denervated groups performed ASR during the 6 h of exposure to severe hypoxia. Furthermore, both groups also developed a swelling of the inferior lips.

### **Discussion**

#### *Effects of denervation on the cardiorespiratory variables*

In previous study (Sundin et al., 2000), complete denervation of cranial nerves IX and X to the gills did not significantly affect any cardiorespiratory variable during normoxia. However, in the present study, significant increases in  $f_R$  and  $f_H$  were observed in the denervated fish under normoxic conditions. Given that this protocol of denervation removes all afferent sensory inputs from the gills, it appears as if these reflex responses are controlled by sites other than chemoreceptors on the gills.

#### *Respiratory responses to severe hypoxia*

Rantin and Kalinin (1996) reported that tambaqui increase the  $f_R$  by about 89% in response to acute hypoxia (10 mmHg). A similar response was observed in the present study (figure 1). Sundin et al. (2000) and the present study demonstrated that the  $O_2$  receptors involved in increasing  $f_R$  during hypoxia are restricted to the gills and distributed on all the gill arches. Sundin et al. (2000) also reported that both internal and external injections of NaCN (to pharmacologically stimulate  $O_2$  chemoreceptors) caused a rapid increase in  $f_R$ . This leads to the conclusion that, in this species, the  $O_2$  of both the inspired water and arterial blood are monitored by branchial  $O_2$  receptors that trigger changes in  $f_R$ .

Complete gill denervation failed to abolish the increase in  $V_{AMP}$  during hypoxia (figure 2), suggesting that this reflex response is mediated by extra-branchial  $O_2$  receptors. These results are in agreement with those of Saunders and Sutterlin (1971) for the searaven. Evidence of extra-branchial  $O_2$  receptors eliciting increases in  $V_{AMP}$  in response to hypoxia was previously observed in traíra (Sundin et al., 1999). In this species, however, receptors located within the gills also contribute significantly to such increases (Sundin et al., 1999). Conversely, complete denervation of catfish gills abolished all the ventilatory responses (Burlison & Smatresk, 1990a).

#### *Cardiac responses to severe hypoxia*

Severe hypoxia rapidly induces a pronounced bradycardia in tambaqui (Rantin and Kalinin, 1996; Sundin et al., 2000). In the current study, this bradycardia was maintained during the initial 120 min of hypoxia, after which  $f_H$  started to increase and returned to the control values in the last 60 min of experimentation. An explanation for the return of  $f_H$  to normoxic (control) values is not obvious. According to Gesser and Poupa (1983) and Gesser (1985), the negative inotropic and chronotropic effects in isolated heart strips can be improved during acidosis with an increase in extra-cellular  $Ca^{2+}$  and adrenergic stimulation. The humoral release of catecholamines during hypoxia may account for the increase in heart rate (for a review see Randall and Perry, 1992). Burtleson and Milsom (1995) demonstrated that the administration of 100 nmol/kg of noradrenaline caused a significant increase in the  $f_H$  of rainbow trout. These authors also demonstrated that increases in noradrenaline concentration did not cause any further increase in  $f_H$ , but prolonged this response. Thus, it is possible that an elevation of circulating catecholamines may have caused the increase in  $f_H$ , with a subsequent return to control values after about 300 min of experimentation.

Sundin et al. (2000) observed that complete denervation of the gill arches reduced, but did not abolish, the hypoxic bradycardia of tambaqui even after pre-treatment with atropine. This indicated that the bradycardia was maintained by mechanisms other than vagal reflexes to the heart. Similarly, hypoxia caused a bradycardia in traíra even after complete gill denervation (Sundin et al., 1999). It is possible that hypoxemia had a direct effect on the heart of both tambaqui and traíra, leading to the decrease in heart rate. This idea is corroborated by the electrocardiographic findings of Glass et al. (1991) for carp, Rantin et al. (1993) for traíra and trairão, and Rantin et al. (1995) for pacu. In all these species, exposure to severe hypoxia, below their critical  $O_2$  tensions, caused a bradycardia accompanied by electrocardiographic evidences of myocardial ischemia. In the present study, the hypoxic bradycardia was completely abolished by bilateral gill denervation (figure 4). As such, any adverse effects of hypoxemia on the myocardium do not appear to be manifest during long-term (360 min) exposure to hypoxia.

#### *ASR and swelling of the lower lip*

As previously observed by Rantin and Kalinin (1996), severe hypoxia induced both ASR and inferior lip swelling in tambaqui with intact gill innervation. Additionally, we observed that ASR and inferior lip swelling were not abolished by total gill denervation, as previously demonstrated by Sundin et al. (2000). These authors also reported that, compared to control fish, denervated fish

exhibited a considerable reduction in the inferior lip swelling, suggesting that the lip swelling resulted, in part, from the stimulation of both branchial and extra-branchial O<sub>2</sub> receptors. Nevertheless, the present data clearly demonstrates that both intact and denervated fish developed inferior lip swellings during hypoxia and, at the end of 360 min of exposure to severe hypoxia, there was no difference in lip dimensions between the two groups (Table I). As such, while branchial and extrabranchial chemoreceptors may play a role in the initiation of lip swellings, their activation is not essential for this phenomenon to occur. Other mechanisms, such as a direct effect of hypoxia on the lip tissue, are also likely to trigger lip swelling.

In this study the intact fish began to perform ASR after 240 min of exposure to hypoxia, while the denervated fish started to perform ASR after 120 min (figure 5). This suggests that, although the control of ASR is not associated with afferent inputs from the gills, denervation altered this behavioral response. It is possible that the removal of motor tone to the gills caused a partial collapse of the gill curtain which, in turn, impaired O<sub>2</sub> transfer across the gills resulting in a more severe hypoxemia than experienced by the control fish. In turn, this may have led to the accelerated performance of ASR. Given that gill denervation did not prevent ASR, it is clear that ASR is mediated by extra-branchial O<sub>2</sub> receptors, possibly located in the orobranchial cavity.

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