

**THE ROLE OF OROBRANCHIAL O₂ CHEMORECEPTORS ON THE
CONTROL OF AQUATIC SURFACE RESPIRATION IN TAMBAQUI,**

Colossoma macropomum.

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Abstract

The present study examined the role of orobranchial O₂ chemoreceptors on cardiorespiratory responses, aquatic surface respiration (ASR), and development of inferior lip swelling in the tambaqui, *Colossoma macropomum*, during long-term exposure to hypoxia. Intact fish (control) and two groups of denervated fish (bilateral denervation of the branches of cranial nerves V or V+VII to the orobranchial cavity) were exposed to severe hypoxia (PwO₂ = 10 mmHg) for 360 min. Respiratory frequency (f_R), ventilation amplitude (V_{AMP}) and heart rate (f_H) were recorded simultaneously with the occurrence of ASR. To verify the effect of hypoxia on the development of the inferior lip swelling, the dimensions of the lips were measured before and after the experiments. Control fish increased f_R , V_{AMP} and total ventilation (\dot{V}_{TOT}), and demonstrated a significant bradycardia during the first 60 min of exposure to hypoxia. Heart rate returned to normoxic levels during the last hour hypoxia. The increases in f_R , V_{AMP} and \dot{V}_{TOT} were not abolished by denervation in both groups of fish (V or V+VII). The data suggest that the population of O₂ receptors located in the oro-branchial cavity is not required for the increase in f_R and V_{AMP} during hypoxia. The hypoxic bradycardia was not abolished by denervation, but denervated tambaqui did not exhibit the recovery in f_H like observed in the intact fish. This suggests

that the orobranchial O₂ chemoreceptors innervated by cranial nerves V and VII may mediate the recovery in heart rate during long-term exposure to hypoxia. After 360 min of exposure to severe hypoxia only the control fish performed ASR. However, both control and denervated fish developed inferior lip swelling. These results suggest that the ASR is regulated by O₂ chemoreceptors located within the orobranchial cavity innervated by the cranial nerves V and VII. Other mechanisms, such as a direct effect of hypoxia on the lip tissue, are also likely to trigger lip swelling.

Introduction

The neotropical fish tambaqui, *Colossoma macropomum*, is a hypoxia tolerant species which employs a variety of behavioral, morphological, physiological and biochemical mechanisms to adapt to widely fluctuating oxygen concentrations in 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 O₂ receptors eliciting the reflex bradycardia and increase in breathing frequency during hypoxia are situated on all gill arches and sense changes in both arterial blood and inspired water. On the other hand, the O₂ 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 O₂ chemoreception and cardiorespiratory reflex responses in fish. Rantin et al. (2002) evaluated the cardiorespiratory reflex responses of tambaqui during long-term (6 h) exposure to hypoxia (PO₂ = 10 mmHg) and compared their data with those (short-term exposure) previously obtained by Sundin et al. (2000). Rantin et al. (2002) also investigated if the O₂ receptors involved in the cardiorespiratory reflex responses were the same as those which trigger ASR and elicit the development of the lower lip swelling. These authors found that: 1. Complete gill denervation failed to abolish the increase in V_{AMP} during hypoxia, suggesting that this reflex response is mediated by extra-branchial O₂ receptors; 2. Hypoxic bradycardia was completely abolished by bilateral denervation of

cranial nerves IX and X and f_H remained constant during the 360 min of experimentation; 3. ASR and inferior lip swelling were not abolished by total gill denervation, as previously observed by Sundin et al. (2000); 4. 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. These data and the previous studies of Sundin et al (2000) and Milsom et al. (2002) suggest the participation of extrabranchial O_2 receptors, possibly located in the orobranchial cavity, on the increase in V_{AMP} in response to hypoxia, the initiation of the ASR and development of the inferior lips swellings. Thus, the main objective of the current study was to determine the exclusive role of orobranchial O_2 chemoreceptors, innervated by the cranial nerves V and VII, on the control of these phenomena.

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, $PwO_2 \geq 130$ 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 cannula) 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⁻¹) was recorded by connecting the buccal PE catheter to a Narco P-1000B pressure transducer. The output from the pressure transducer was sent 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⁻¹) 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 V (trigeminal) and VII (facial):

For denervation of cranial nerves, fish were anesthetized and moved to the surgical table where they were artificially ventilated as mentioned above. The denervation followed the same protocol described by Milsom et al. (2002). Under stereoscopic microscope (Opto SM 2001, Opto Electronics, São Carlos, SP, Brazil), the palatine branches of cranial nerve VII, as well as all mandibular branches of cranial nerve V innervating the orobranchial cavity were sectioned. This removed sensory information arising from the mouth and buccal cavity. Two small branches of cranial nerve VII were left intact which were sufficient to produce opercular movements that could be monitored as an indication of the frequency and amplitude of ventilation. The opercular branches of VII innervating the floor of the mouth were accessed where they course over the inner surface of the operculum, the palatine branches of VII were accessed through a midline incision in the roof of the mouth. The mandibular branches of V innervating the roof of the mouth were accessed bilaterally by rotating the eyes and cutting the nerves, where they coursed over the back of the orbit, through a small incision in the top of the conjunctiva. In all cases, cranial nerves IX and X to the gills were intact. 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 ASR and respiratory and heart frequencies, an experimental setup similar to that described by Rantin et al. (1998) was employed. The system consisted of two chambers: an upper compartment, where the fish was kept during the experiment, and a lower part, serving to gas the water with N₂. 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 movements were 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 N₂ released from the water and kept a constant atmospheric gas concentration on the water surface, so as to maintain the PO₂ of the surface layer about 5 to 10 Torr higher than in the rest of the tank. The experimental temperature was kept constant ($25 \pm 1^\circ\text{C}$) by means of a thermostat (TRM 10.40, Terroni Equipments Ltd., São Carlos, SP, Brazil) controlling a heater coil placed inside the lower chamber. Movements and behavior were continuously monitored by means of a closed TV circuit (Sharp VL-L 310B video camera and Samsung CN-3355Z monitor) and recorded on videotape (Semp X470 VCR) to verify the occurrence of aquatic surface respiration.

Experimental protocol

The experiments were conducted in two distinct phases: The first with intact fish, and the second with denervated ones (groups V and V+VII). In both phases tambaqui were exposed for a period of 360 min to severe hypoxia ($P_{\text{wO}_2} = 10$ mmHg). The f_{R} and f_{H} were recorded in the last 10 min of each 30 min interval. The f_{R} (breaths.min⁻¹) and f_{H} (beats.min⁻¹) were expressed in absolute values. Since the ventilation amplitude (V_{AMP}) was measured in arbitrary units, V_{AMP} as well as total ventilation ($\dot{V}_{\text{TOT}} = V_{\text{AMP}} \cdot f_{\text{R}}$) were expressed as percentage of the initial values.

To verify the effects of hypoxia on the development of the lip swelling, the dimensions of inferior lips (length and width) of intact and denervated fish were measured before and after exposure to hypoxia.

Statistics

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

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 intact (control) fish f_R increased significantly during the first 60 min. These values remained constant until the end of the experiment. Denervation of cranial nerves V (figure 1A) and V+VII (figure 1B) did not change the increase in f_R in response to hypoxia. Control and denervated fish displayed essentially an identical f_R response to long-term exposure to hypoxia.

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 during the initial 60 min, and these values remained constant until the end of the experiment. However, group V demonstrated a significantly higher V_{AMP} in comparison to the control group during the last 120 min of experimentation, while the V+VII group demonstrated a significantly higher V_{AMP} during the entire experiment. The same tendency was observed for \dot{V}_{TOT} .

Heart rate

The effects of hypoxia on the f_H of intact and denervated fish are shown in figure 4.

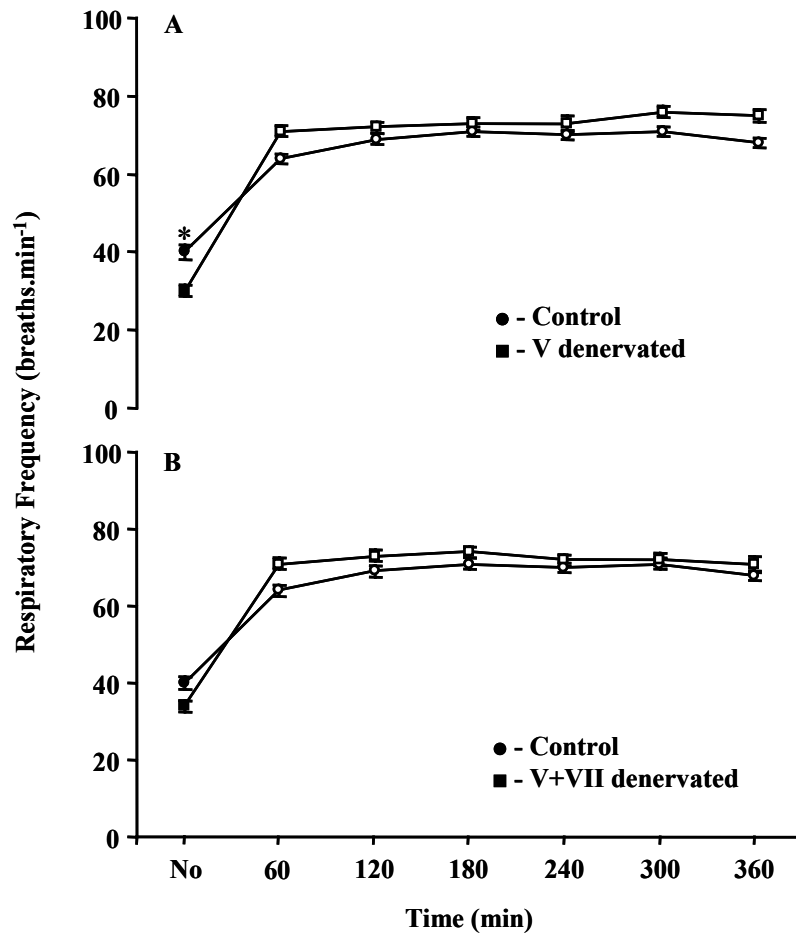


Figure 1. The effects of hypoxia ($PwO_2 = 10$ mmHg) on the f_R of intact (control) and denervated (**A** - cranial nerve V; **B** - cranial nerves V+VII) tambaqui, *Colossoma macropomum*. Open symbols represent a statistical difference from normoxia. The data are shown as the mean \pm 1 SEM.

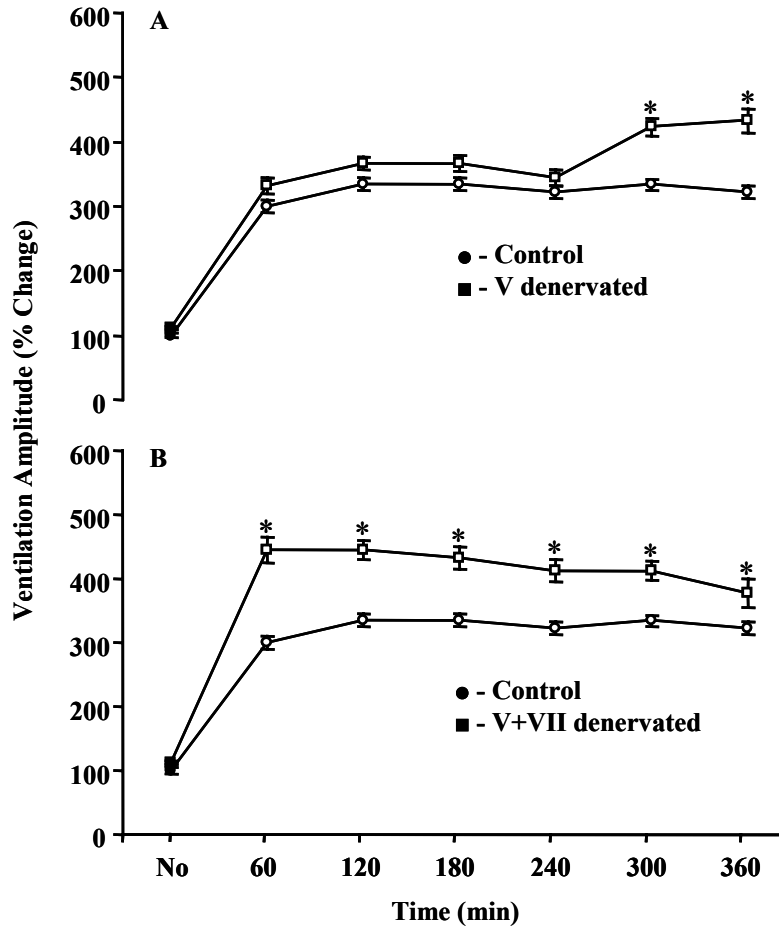


Figure 2. The effects of hypoxia ($P_{wO_2} = 10$ mmHg) on the V_{AMP} of intact (control) and denervated (A - cranial nerve V; B - cranial nerves V+VII) tambaqui, *Colossoma macropomum*. Open symbols represent a statistical difference from normoxia while an asterisk (*) represents a statistical difference between the control and denervated fish. The data are shown as the mean \pm 1 SEM.

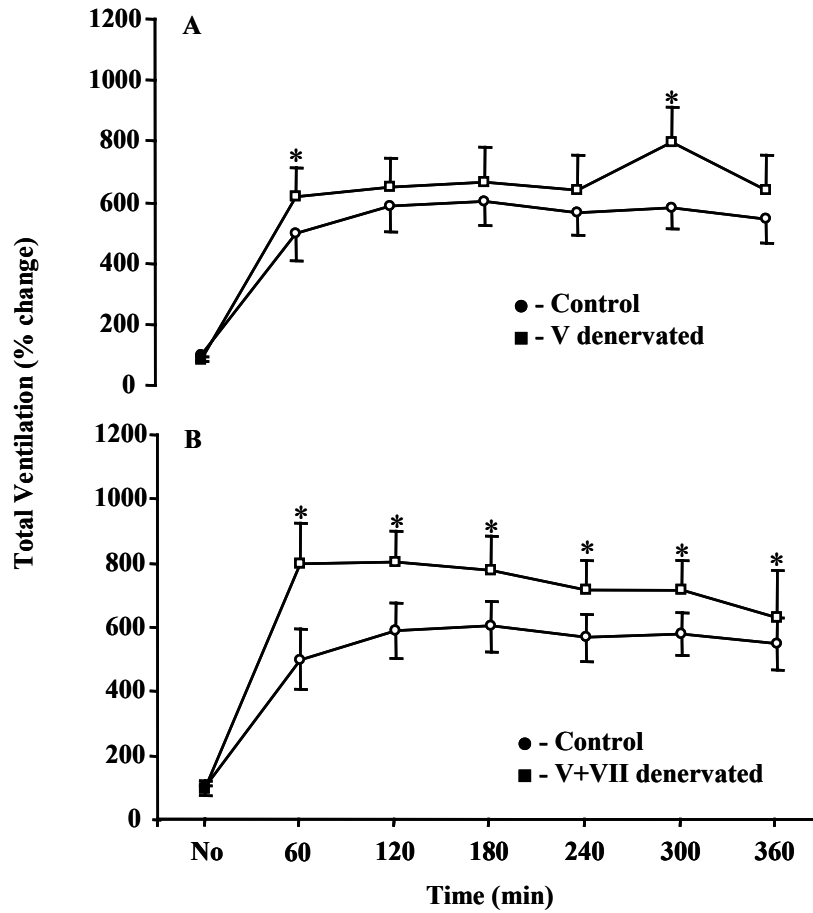


Figure 3. The effects of hypoxia ($PwO_2 = 10$ mmHg) on the \dot{V}_{TOT} of intact (control) and denervated (A - cranial nerve V; B - cranial nerves V+VII) tambaqui, *Colossoma macropomum*. Open symbols represent a statistical difference from normoxia while an asterisk (*) represents a statistical difference between the control and denervated fish. The data are shown as the mean ± 1 SEM.

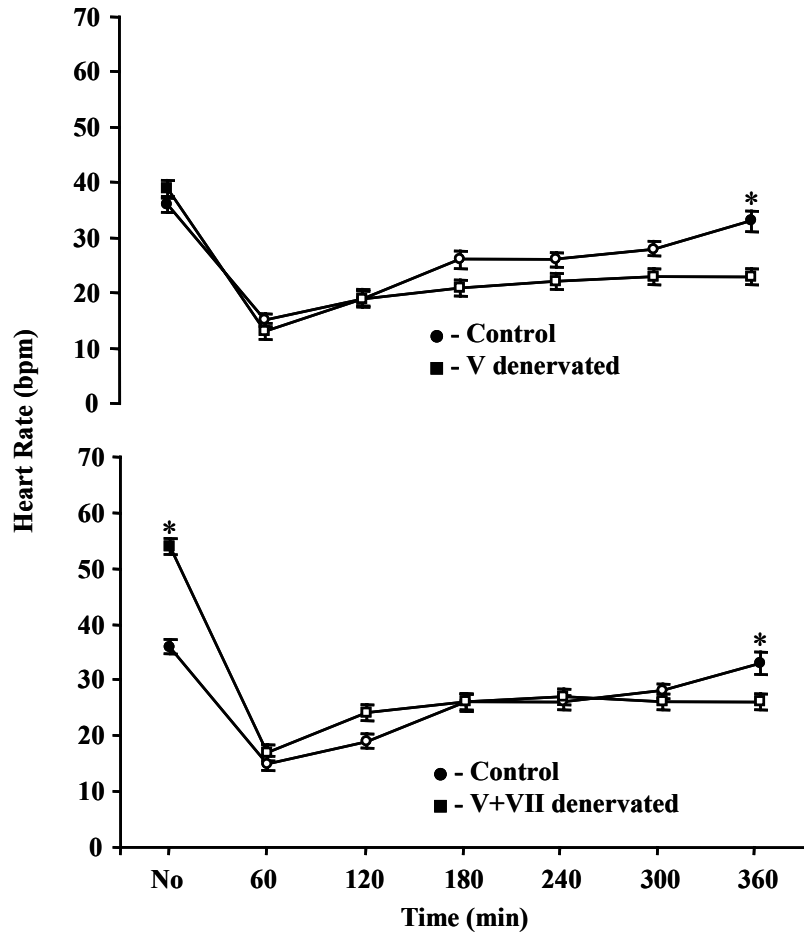


Figure 4. The effects of hypoxia ($PwO_2 = 10$ mmHg) on the f_H of intact (control) and denervated (A - cranial nerve V; B - cranial nerves V+VII) tambaqui, *Colossoma macropomum*. Open symbols represent a statistical difference from normoxia while an asterisk (*) represents a statistical difference between the control and denervated fish. The data are shown as the mean \pm 1 SEM.

Intact fish exhibited a significant bradycardia during the first 60 min of exposure to 10 mmHg O₂. At this point f_H increased gradually, and during the last 60 min of the experiment it was similar to the normoxic values. Bilateral denervation of branches of the cranial nerves V and V+VII did not abolish the hypoxic bradycardia. However, in both denervated groups the f_H did not recover the normoxic value at the end of the experiment as was observed for the control fish.

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. ASR was completely abolished by bilateral section of cranial nerve V (group V). The V+VII denervated group did not perform ASR.

Denervation of cranial nerves V and V+VII did not affect the development of inferior lip swelling in response to severe hypoxia. Intact and denervated groups of tambaqui developed inferior lip swelling in the same proportion (table I).

Table I. Dimensions of the inferior lips of intact and denervated (groups V and V+VII) 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 all groups. The data are reported as the mean \pm SEM.

	Intact fish	V denervated	V+VII denervated
	Length (mm)	Length (mm)	Length (mm)
Normoxia	7.4 \pm 0.4	7.5 \pm 0.2	7.5 \pm 0.3
Hypoxia	8.1 \pm 0.2*	8.6 \pm 0.3*	8.1 \pm 0.4*
	Width (mm)	Width (mm)	Width (mm)
Normoxia	27.7 \pm 0.5	30.1 \pm 0.6	30.5 \pm 0.7
Hypoxia	29.9 \pm 0.4*	33.6 \pm 0.8*	34.4 \pm 0.9*

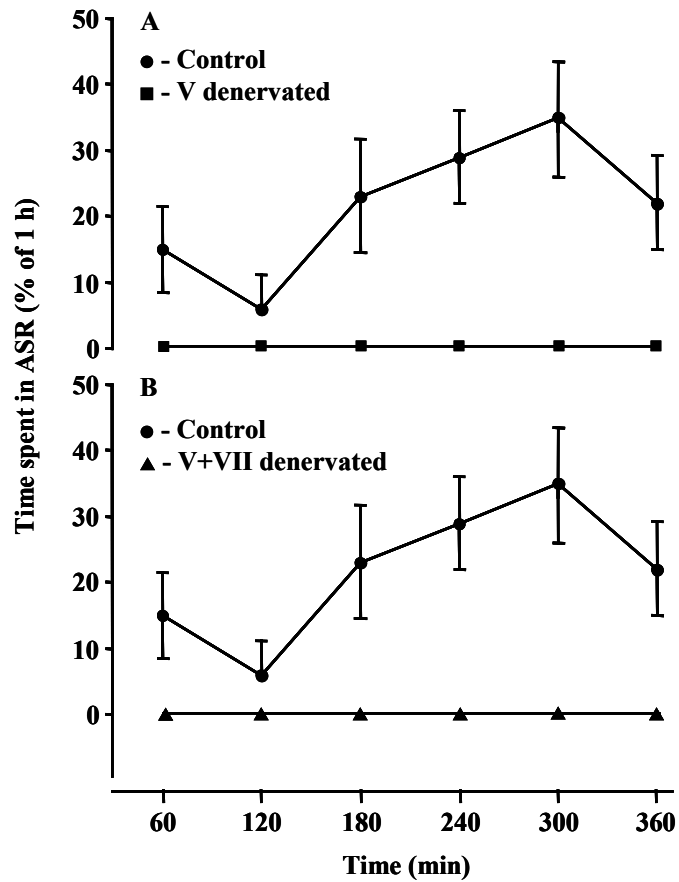


Figure 5. Time spent in ASR by intact (control, n = 8) and denervated (group V, n = 8; group V+VII, n = 8) tambaqui, *Colossoma macropomum*, during exposure to severe hypoxia ($PwO_2 = 10$ mmHg) for 360 min. Data are shown as the mean \pm SEM.

Discussion

Respiratory responses to severe hypoxia

Total gill denervation did not abolish ventilatory responses to hypoxia in tench (Hughes and Shelton, 1962), sea raven (Saunders and Sutterlin, 1971) and traíra (Sundin et al., 1999). On the other hand, changes in ventilatory variables were completely abolished by gill denervation of channel catfish (Smatresk, 1989) and gar (Burlison and Smatresk, 1990). Sundin et al. (2000), Milsom et al. (2002) and Rantin et al. (2002) demonstrated that total gill denervation of tambaqui abolished the increases in f_R in response to short- and long-term exposure to hypoxia. In the studies of Sundin et al. (2000) and Milsom et al. (2002), gill denervation did not eliminate the increase in V_{AMP} during short-term exposure to hypoxia. This finding was corroborated by the data of Rantin et al. (2002) for long-term hypoxic exposure. According to Milsom et al. (2002) the hypoxia-induced increase in V_{AMP} could only be eliminated following denervation of both the gills and the orobranchial cavity. In the present study only the orobranchial nerves (V and V + VII) were sectioned, while the branchial (gill) innervation was preserved. Thus denervation of the oro-branchial cavity (V and VII) alone is not sufficient to prevent the increase in ventilation during hypoxia. It would appear as if the two populations of O_2 chemoreceptors (gill and oro-branchial) function in a redundant manner such that either population alone is sufficient to produce an increase in ventilation. However, while the increase in ventilation mediated by the gill chemoreceptors is due to an increase in both f_R and V_{AMP} (see Sundin et al., 2000; Milsom et al., 2002), the oro-branchial chemoreceptors only trigger increases in ventilation amplitude.

Previous studies have failed to demonstrate central O_2 chemoreception in water-breathing fish (Kawasaki, 1980; Hedrick et al., 1991; McKenzie et al., 1991). Furthermore, Milsom et al. (2002) were unable to induce changes in respiration in the tambaqui by superfusing the brain with hypoxic, hyperoxic, hypercapnic, acidic or alkaline solutions. Additionally, the data of Milsom et al. (2002) does not support a role for circulating catecholamines as a causal factor in the hypoxic ventilatory response (Randall and Taylor, 1991) since the exogenous application of catecholamines inhibited ventilation. This suggests that if hypoxia becomes severe enough to cause a release of catecholamines from chromaffin tissue into the circulation, the net effect would be to depress ventilation. This is consistent with the ideas advanced by Perry et al. (1991). In the present study no ventilatory depression was observed, possibly due to the fact that tambaqui is a very hypoxia-tolerant species. It is possible that longer-term exposure may ultimately result in a decrease in ventilation.

Heart rate

Sundin et al., (2000) demonstrated that the O₂ chemoreceptors eliciting the bradycardia during short-term exposure to hypoxia are situated on all gill arches and sense changes in both the blood and inspired water. Rantin et al. (2002) observed that, during long-term exposure to severe hypoxia, the f_H of intact tambaqui returns to the normoxic values after 300 min of experiment. In the current study, the denervation of the palatine branches of cranial nerve VII, as well as all mandibular branches of cranial nerve V innervating the orobranchial cavity abolished such a response. This implies that, in tambaqui, there are O₂ chemoreceptors involved in regulating the gradual return of f_H to normocardia, during long-term exposure to hypoxia. Furthermore, the data suggest that these chemoreceptors are located in the orobranchial cavity and are innervated by cranial nerves V and VII. Such a situation would be similar to the dogfish, in which cardiac function is controlled by O₂ receptors innervated by branches of the cranial nerves V, VII, IX and X (Butler et al., 1977).

With some exceptions, such as the sea raven (Saunders and Sutterlin, 1971) and five-bearded rockling (Fritsche, 1990), most teleosts exhibit a reflex bradycardia in response to hypoxia. Dogfish (Butler et al., 1977) and traíra (Sundin et al., 1999) exposed to moderate hypoxia exhibit a reflex bradycardia that gradually returns to normocardia. However, in the majority of the species so far studied, the bradycardia is sustained during the entire hypoxic period. To what extent this is due to vagal (or adrenergic) reflexes as opposed to a direct effect of hypoxemia on the myocardium is not clear. It is currently not possible to determine if O₂ chemoreceptors, located in the orobranchial cavity, are actually involved in the return of the f_H of tambaqui to normocardia during hypoxia, although the data do imply that this is the case. Further studies are required to address this issue as well as any possible regulation by circulating catecholamines.

ASR and development of inferior lip swelling

As previously observed by Rantin and Kalinin (1996) and Rantin et al. (1998), severe hypoxia induces ASR in tambaqui with intact gill and oro-branchial innervation. In this species, ASR was not abolished by bilateral denervation of the branches of cranial nerves IX and X to the gills (Sundin et al. 2000; Rantin et al. 2002), suggesting that branchial O₂ chemoreceptors are not involved in triggering this behavioural response. However, in the current study, denervation of the mandibular branches of cranial nerve V innervating the orobranchial cavity abolished such a response. This finding confirms that ASR is controlled by O₂ chemoreceptors located within the orobranchial cavity innervated by cranial nerve V.

The inferior lip swelling induced in tambaqui by severe hypoxia was not abolished by denervation of either cranial nerves IX and X to the gills (Sundin et al. 2000; Rantin et al., 2002) or by denervation of cranial nerves V and VII to the orobranchial cavity (present study). This suggests that the formation of inferior lip swelling in tambaqui is either controlled by O₂ receptors located outside the gills and orobranchial cavity or results from a direct effect of hypoxia/hypoxemia on the lip tissue itself.

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Acknowledgements

This study was supported by FAPESP (Proc. 98/13112-0 and 00/12382-5). L.H. Florindo received a PhD fellowship from CNPq. The authors thank the CAUNESP, Jaboticabal, SP, for providing the fish.

