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The role of branchial and orobranchial O_2 chemoreceptors in the control of aquatic surface respiration in the neotropical fish tambaqui (*Colossoma macropomum*): progressive responses to prolonged hypoxia

Luiz H. Florindo[1], Cléo A. C. Leite[2], Ana L. Kalinin[2], Stephen G. Reid[3], William K. Milsom[4] and F. Tadeu Rantin[2,*]

[1]*Department of Zoology and Botany, São Paulo State University–UNESP, São José do Rio Preto, SP, Brazil,* [2]*Department of Physiological Sciences, Federal University of São Carlos, 13565-905 São Carlos, SP, Brazil,* [3]*Division of Life Sciences, University of Toronto at Scarborough, Scarborough, OT, Canada and* [4]*Department of Zoology, University of British Columbia, Vancouver, BC, Canada*

*Author for correspondence (e-mail: ftrantin@power.ufscar.br)

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Summary

The present study examined the role of branchial and orobranchial O_2 chemoreceptors in the cardiorespiratory responses, aquatic surface respiration (ASR), and the development of inferior lip swelling in tambaqui during prolonged (6 h) exposure to hypoxia. Intact fish (control) and three groups of denervated fish (bilateral denervation of cranial nerves IX+X (to the gills), of cranial nerves V+VII (to the orobranchial cavity) or of cranial nerves V alone), were exposed to severe hypoxia ($P_{W_{O_2}}$=10 mmHg) for 360 min. Respiratory frequency (f_R) and heart rate (f_H) were recorded simultaneously with ASR. Intact (control) fish increased f_R, ventilation amplitude (V_{AMP}) and developed hypoxic bradycardia in the first 60 min of hypoxia. The bradycardia, however, abated progressively and had returned to normoxic levels by the last hour of exposure to hypoxia. The changes in respiratory frequency and the hypoxic bradycardia were eliminated by denervation of cranial nerves IX and X but were not affected by denervation of cranial nerves V or V+VII. The V_{AMP} was not abolished by the various denervation protocols. The f_H in fish with denervation of cranial nerves V or V+VII, however, did not recover to control values as in intact fish. After 360 min of exposure to hypoxia only the intact and IX+X denervated fish performed ASR. Denervation of cranial nerve V abolished the ASR behavior. However, all (control and denervated (IX+X, V and V+VII) fish developed inferior lip swelling. These results indicate that ASR is triggered by O_2 chemoreceptors innervated by cranial nerve V but that other mechanisms, such as a direct effect of hypoxia on the lip tissue, trigger lip swelling.

Key words: cardiorespiratory reflex, hypoxia, O_2 chemoresponse, respiratory frequency, heart frequency, ASR, inferior lip swelling, *Colossoma macropomum*.

Introduction

The neotropical fish tambaqui, *Colossoma macropomum*, is a hypoxia tolerant species that 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 (Val and Almeida-Val, 1995). 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).

Several studies have examined the cardio-respiratory responses of tambaqui to hypoxia. The O_2 receptors eliciting the reflex bradycardia and increase in breathing frequency during hypoxia were distributed on all gill arches and sensed changes in both arterial blood and inspired water (Sundin et al., 2000). By contrast, the O_2 receptors that triggered the elevation in systemic vascular resistance and breathing amplitude during hypoxia were extra-branchial. In this study (Sundin et al., 2000), fish were exposed to hypoxia without access to the surface where they could perform ASR, and for only a short period of time (10–30 min), a period too short to induce lip swelling. The cardiorespiratory reflex responses of tambaqui during long-term (6 h) exposure to hypoxia

(P_{O_2}=10 mmHg) were subsequently evaluated (Rantin et al., 2002) and the role of the various O_2 receptors involved in the cardiorespiratory reflex responses in eliciting ASR and the development of lower lip swelling examined. Their data suggest that extrabranchial O_2 receptors participated in the initiation of ASR and the swelling of the inferior lip. The main objective of the present study was to determine whether orobranchial O_2 chemoreceptors innervated by cranial nerves V and VII, could be the extrabranchial receptors involved in these responses.

Materials and methods

Experimental animals

Tambaqui *Colossoma macropomum* Cuvier (body mass 713±40 g, mean ± s.e.m.) 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 aerated water [normoxic conditions, $P_{W_{O_2}} \geqslant 130$ mmHg (17.3 kPa)] at a constant temperature (25°C). The fish were fed *ad libitum* with commercial food pellets but were fasted for 2 days prior to experimentation.

Animal preparation

Fish were anesthetized in a benzocaine solution (100 mg l^{-1}; pre-dissolved in 3 ml of 70% ethanol). After anesthesia, fish were transferred to a surgical table and their gills were artificially ventilated with an aerated, weaker benzocaine solution (50 mg ml^{-1}). Using a Dremel® rotary tool, a hole was drilled through the snout between the nostrils, and a flared cannula (PE-100) was fed from inside the mouth out though the hole and secured with a cuff. The fish were then fitted with ECG electrodes according to the method described (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 to recover in normoxic water ($P_{W_{O_2}} \geqslant 130$ mmHg, 17.29 kPa) at their acclimation temperature (25°C).

Ventilation

Ventilation rate (f_R, breaths min^{-1}) was recorded by connecting the buccal catheter to a Narco P-1000B pressure transducer and a universal coupler (Narco 7189) of a Narco Narcotrace 40 physiograph (Narco Bio-Systems, Houston, TX, USA). Ventilation amplitude (V_{AMP}) was measured in arbitrary units and expressed as percent change from initial values (Sundin et al., 1999; Sundin et al., 2000).

Heart rate

Electrocardiography was used to record heart rate (f_H, beats min^{-1}) by counting the number of QRS complexes min^{-1}. The ECG electrodes were connected to a universal coupler and a third electrode (reference) was immersed in the water of the experimental setup. This preparation produced ECG recordings equivalent to those obtained from bipolar lead I of a standard human electrocardiograph.

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

Fish were anesthetized and placed on a surgical table where they were artificially ventilated as described above. The denervation followed the protocol described (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 1st and 2nd gill arches where they meet the roof of the opercular cavity. The incision allowed access to cranial nerve IX and the branchial branches of cranial nerve X. The branchial nerves of all gill arches were carefully dissected free of connective tissue and cut with fine iris scissors. The cardiac and visceral branches of the vagus were preserved in all cases.

Denervation of cranial nerves V (trigeminal) and VII (facial)

This denervation followed the protocol described (Milsom et al., 2002). Under the stereoscopic microscope, the opercular and 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 innervating the opercular muscles 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 using a video camera attached to the microscope and connected to an ATI Pro interface of a Pentium IBM PC, and 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 (Rantin and Kalinin, 1996; Rantin et al., 1998) was used. 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 N_2.

The water was continuously recirculated 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 air flow above the water surface. This 'air tunnel' removed the excess of N_2 released from the water and kept a constant atmospheric gas concentration on the water surface, so that the P_{O_2} of the surface layer was about 10 mmHg higher than in the rest of the tank. The experimental temperature was kept constant (25±1°C) by a thermostat (TRM 10.40, Terroni Equipments Ltd., São Carlos, SP, Brazil) controlling a heating coil placed inside the lower chamber.

Movements and behavior were continuously monitored by means of a closed circuit TV (Sharp VL-L 310B video camera and Sansung CN-3355Z monitor) and recorded on videotape (Semp X470 VCR) to verify the occurrence of ASR.

Experimental protocol

The experiments were conducted in two phases: the first with the fish intact, and the second with the fish denervated (groups IX+X, V and V+VII). Thus, for each fish, the protocol consisted of an initial surgery and recovery overnight. On the second day they were exposed to severe hypoxia (Pw_{O_2}=10 mmHg) for 360 min, following which they were returned to normoxic water and allowed to recover. Recovery took approximately 1 h but fish were allowed to rest overnight. On the third day, fish were denervated and kept undisturbed for a post-surgical recovery period of 24 h. The denervated fish were then subjected to the same level of hypoxia for another 360 min. Separate groups of 10 fish each were used for each denervated group. For each of the V and V+VII groups, two animals underwent all surgical procedures but the nerves were not transected (shams). The responses of these animals in the second run were no different from those of the intact animals in the first run and so the first run for each animal was used as the control for the second trial post-denervation. The f_R and f_H values were recorded during the last 10 min of each 30 min interval.

To verify the effects of hypoxia on the swelling of the inferior lip, the dimensions of the inferior lip (length and width) were measured using graduated calipers before and after exposure to hypoxia. An initial measurement in intact fish was made in the anesthetized animals during the implantation of the buccal cannula and EKG electrodes, and in denervated fish during the denervation procedures. Measurements in both groups were again made by re-anesthetizing the fish briefly at the end of the hypoxia protocol, before returning the fish to aerated water. Before and after the hypoxic exposure, the images of the inferior lips were digitized by means of a video camera coupled to the microscope (Opto SM 2001, Opto Electronics) and connected to an ATI Pro interface of a Pentium IBM PC. The digitized images were used to determine the area of the inferior lips by means of a computer program (Jandel Sigma Plot, Image Measurement Software, Version 3.0, Jandel Corporation).

Statistics

To compare changes in each variable over time at each O_2 tension, a one-way repeated-measures analysis of variance (ANOVA) was performed followed by a Tukey–Kramer multiple comparison test. Differences were considered significant if $P<0.05$. A paired t test was employed to compare the areas of the inferior lips of intact and denervated fish during normoxia and hypoxia. Values are presented as means ± s.e.m. The commercial package GraphPad InStat v. 3.0 (GraphPad Software Inc.) was used to carry out statistical analyses.

Results

Ventilation rate (f_R) and ventilation amplitude (V_{AMP})

Fig. 1 shows the changes in f_R of intact (control) and denervated fish as a function of the length of time of exposure to severe hypoxia (Pw_{O_2}=10 mmHg). In intact fish, f_R increased significantly (60%; $P<0.001$) in the first 60 min of experimentation, after which it remained nearly constant for the last 300 min (Fig. 1A). Bilateral denervation of cranial nerves IX and X produced a small increase in f_R and abolished totally the increases in f_R during hypoxia.

Fig. 1 also illustrates the effects of severe hypoxia on f_R during the 360 min of exposure in fish with denervation of cranial nerve V (Fig. 1B) and cranial nerves V+VII (Fig. 1C). Again, in these fish while the gills were intact, f_R increased significantly during the first 60 min and these values remained constant until the end of the experiment. Denervation of cranial nerves V and V+VII did not alter this response. Control and denervated fish displayed essentially identical increases in f_R to long-term exposure to hypoxia.

Fig. 2 depicts the effect of severe hypoxia on the V_{AMP} of intact and IX and X denervated (Fig. 2A), V denervated (Fig. 2B) and V and VII denervated (Fig. 2C) fish during 360 min. In the intact fish V_{AMP} increased significantly during the first 60 min of exposure to severe hypoxia and remained elevated until the end of the experimental time (360 min). The V_{AMP} was not abolished by denervation of cranial nerves IX+X, V and V+VII.

Heart rate

Fig. 3 illustrates the f_H of intact (control) and denervated fish in normoxia (time 0) and during exposure to severe hypoxia (Pw_{O_2}=10 mmHg) for 360 min. The intact fish demonstrated a significant bradycardia in the first hour of exposure to hypoxia. This bradycardia decreased gradually, returning to values not significantly different from those seen in normoxia by the last 60 min of experimentation. After bilateral denervation of cranial nerves IX and X the bradycardia was completely abolished and the values of f_H recorded for denervated fish were always higher than those observed in the intact fish (Fig. 3A).

The effects of hypoxia on the f_H of intact fish and fish following denervation of cranial nerves V, or V+VII, are also shown in Fig. 2B,C, respectively. Intact fish exhibited a significant bradycardia during the first 60 min of exposure to

10 mmHg O_2, which decreased gradually, with f_H returning to normoxic values during the final 60 min of exposure. Bilateral denervation of branches of cranial nerves V or V+VII did not abolish the hypoxic bradycardia. However, in both denervated groups the f_H did not return to normoxic levels at the end of the experiment as was observed in the control fish.

ASR and inferior lips swellings

The effects of severe hypoxia on ASR and swelling of the inferior lips are illustrated in Figs 4 and 5, respectively. Both intact fish and fish following denervation of cranial nerves IX and X, performed ASR and developed inferior lip swelling during the exposure to severe hypoxia. ASR frequency (events h^{-1}) increased significantly in intact and IX and X denervated fish during the first hour and reached peak values after 180 min of exposure to severe hypoxia (17 and 11, respectively) (P<0.05). While the peak levels of ASR in terms of events h^{-1} were not sustained, the amount of time the fish spent at the water surface was, ranging from 40% to 60% over the last 3 h in both groups (Fig. 4B).

ASR was completely abolished by bilateral section of cranial nerve V (Fig. 4) and cranial nervesV+VII (not shown).

Denervation of cranial nerves V, V+VII or IX+X did not

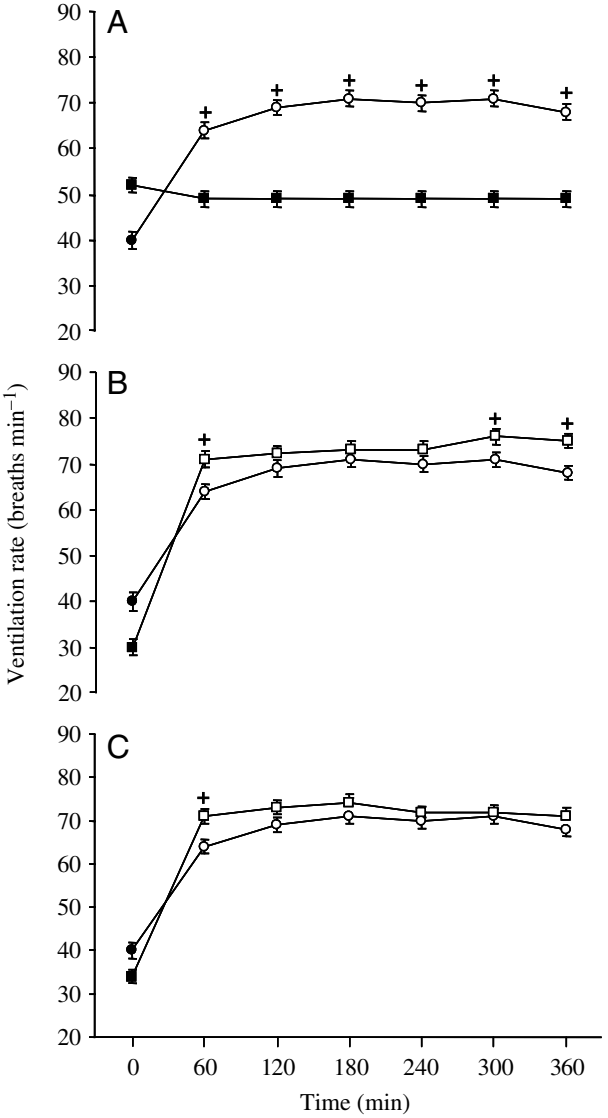

Fig. 1. Ventilation rate (f_R) of tambaqui, *Colossoma macropomum*, exposed to normoxia ($P_{W_{O_2}}$=140 mmHg at time zero) and then hypoxia ($P_{W_{O_2}}$=10 mmHg) for 360 min. Intact (circles; N=10) and denervated fish (squares): (A) cranial nerves IX+X denervated (N=10), (B) cranial nerve V denervated (N=10) and (C) cranial nerves V+VII denervated (N=10). Values are mean ± s.e.m. Open symbols indicate values that are significantly (P<0.05) different from time zero. +Intact and denervated fish are significantly different.

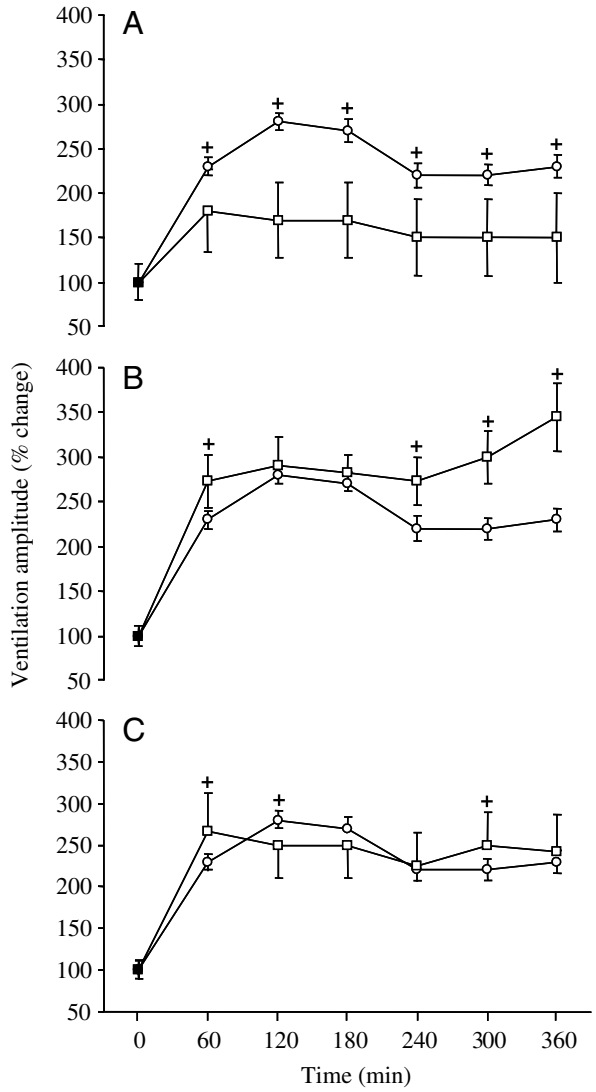

Fig. 2. Ventilation amplitude (V_{AMP}) of tambaqui, *Colossoma macropomum*, exposed to normoxia ($P_{W_{O_2}}$=140 mmHg at time zero) and then hypoxia ($P_{W_{O_2}}$=10 mmHg) for 360 min. Intact (circles; N=10) and denervated fish (squares): (A) cranial nerves IX+X denervated (N=10), (B) cranial nerve V denervated (N=10) and (C) cranial nerves V+VII denervated (N=10). Values are mean ± s.e.m. Open symbols indicate values that are significantly (P<0.05) different from time zero. +Intact and denervated fish are significantly different.

affect the development of inferior lip swelling in response to severe hypoxia. All groups of tambaqui developed the same, significant (P<0.05) degree of inferior lip swelling (Fig. 5).

Discussion

Effects of denervation on the cardio-respiratory variables of normoxic tambaqui

Sundin et al. reported that complete denervation of the four gill arches (cranial nerves IX and X) did not alter the heart rate or breathing frequency of normoxic tambaqui (Sundin et al., 2000). They observed discrete increases in both f_R and f_H, but without statistical significance. In the present study, however, these increases in both f_R and f_H were significant. This may reflect the much longer post-surgical recovery period used in the present study (24 h). In traíra, *Hoplias malabaricus*, an increase in f_H and a non-significant decrease in blood pressure were also recorded after complete bilateral gill denervation (Sundin et al., 1999). Considering that this denervation abolishes both afferent (sensory) and efferent (motor) innervation of the gills, it is not clear if the changes in f_H originated from removal of sensory feedback or removal of motor tonus to systemic arteries (i.e. a reflex increase in heart rate due to a fall in blood pressure), i.e. whether these change represent cause or effect.

Gill ventilatory responses to hypoxia

The ventilatory response to severe hypoxia that was observed in the present study included a significant and sustained increase in breathing frequency. As in other studies (Sundin et al., 2000; Milsom et al., 2002), in the present study we found that total gill denervation in tambaqui abolished the increase in f_R in response to short-term exposure to hypoxia.

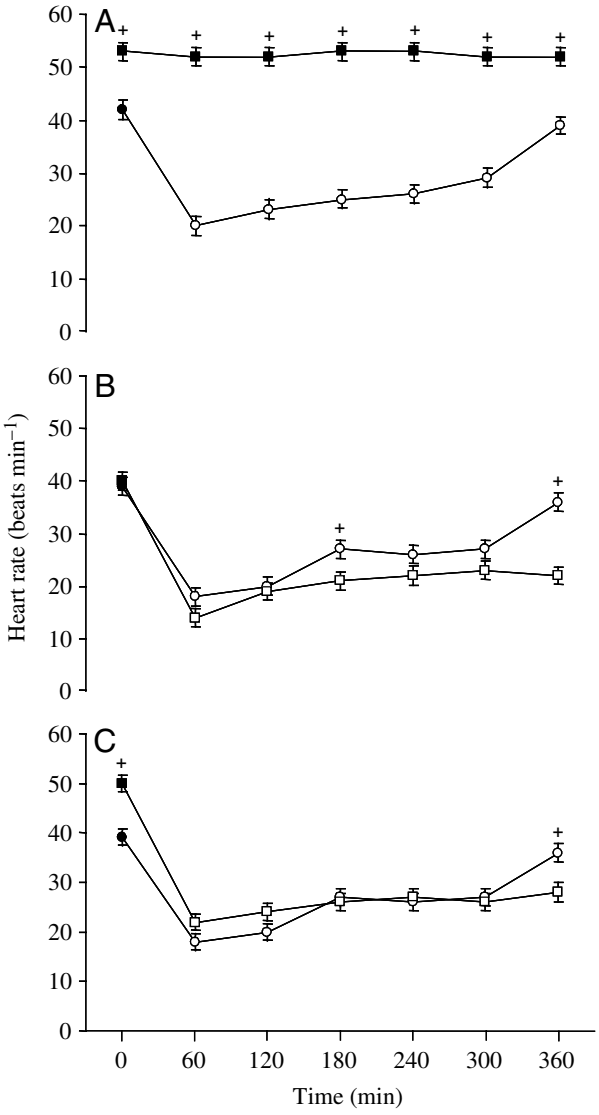

Fig. 3. Heart rate (f_H) of tambaqui, *Colossoma macropomum*, exposed to normoxia ($P_{W_{O_2}}$=140 mmHg at time zero) and then hypoxia ($P_{W_{O_2}}$=10 mmHg) for 360 min. Intact (circles; N=10) and denervated fish (squares): (A) cranial nerves IX+X denervated (N=10), (B) cranial nerve V denervated (N=10) and (C) cranial nerves V+VII denervated (N=10). Values are mean ± s.e.m. Open symbols indicate values that are significantly (P<0.05) different from time zero. $^+$Intact and denervated fish are significantly different.

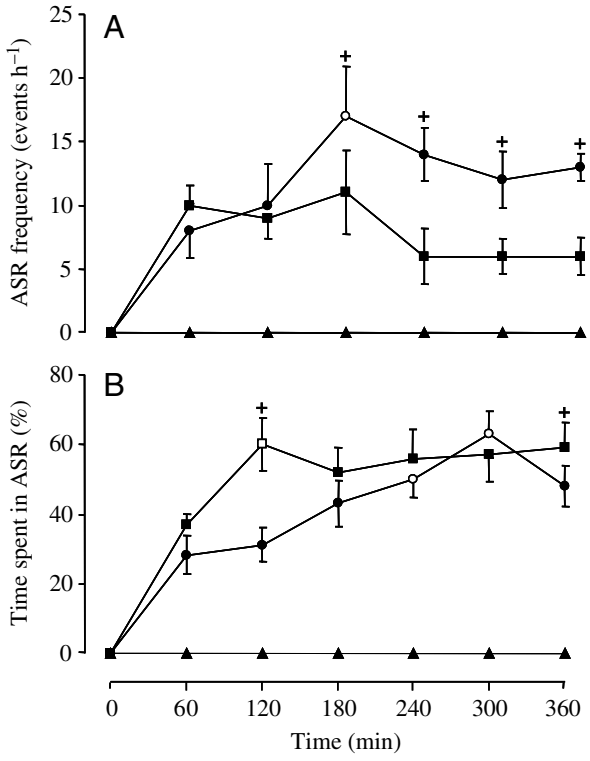

Fig. 4. (A) ASR frequency and (B) time spent in ASR (time spent at the surface) of tambaqui, *Colossoma macropomum*, exposed to normoxia ($P_{W_{O_2}}$=140 mmHg at time zero) and then severe hypoxia ($P_{W_{O_2}}$=10 mmHg) for 360 min. Circles, intact fish (N=8); squares, fish with cranial nerves IX+X denervated (N=8); triangles, fish with cranial nerve V denervated (N=8). Values are means ± s.e.m. Open symbols indicate values that are significantly (P<0.05) different from time zero. $^+$Intact and denervated fish are significantly different.

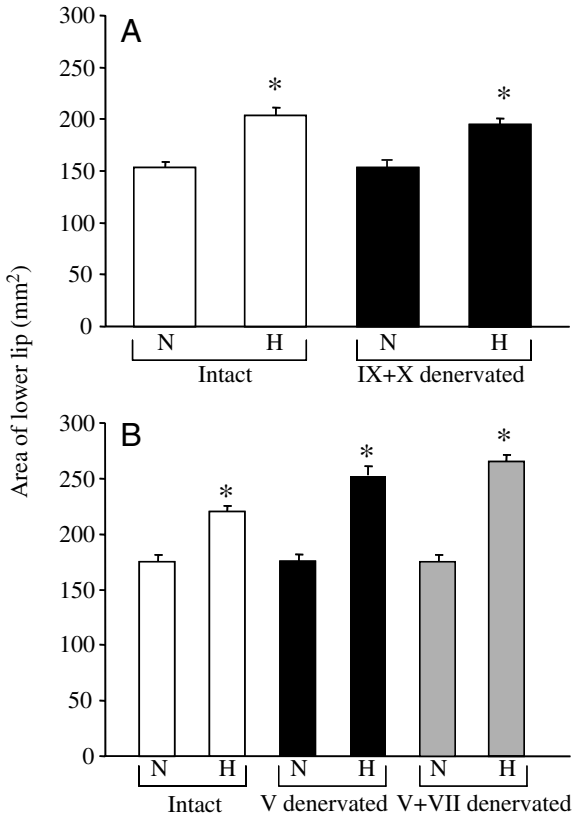

Fig. 5. Area of lower lip of tambaqui, *Colossoma macropomum*, exposed to normoxia and then 360 min of severe hypoxia ($P_{W_{O_2}}$=10 mmHg). (A) Open bars, intact fish (N=8); filled bars, fish with cranial nerves IX+X denervated (N=8). (B) Open bars, intact fish (N=8); filled bars, fish with cranial nerve V denervated (N=8); grey bars, fish with cranial nerves V+VII denervated (N=8). N, normoxia; H, hypoxia; Values are means ± s.e.m. *Values are significantly (P<0.05) different from the normoxic values.

We found that it also abolished the long-term increase in f_R. We did not quantify changes in respiratory amplitude in this study, however. According to Milsom et al., the hypoxia-induced increase in ventilation amplitude (V_{AMP}) could only be eliminated following denervation of both the gills and the orobranchial cavity (Milsom et al., 2002). This may explain the V_{AMP} variation observed in all denervation protocols, since in the present study gills and orobranchial cavity were not denervated simultaneously, as was done in the earlier study (Milsom et al., 2002). Total gill denervation did not abolish ventilatory responses to hypoxia in tench (Hughes and Shelton, 1962), sea raven (Saunders and Sutterlin, 1971) or traíra (Sundin et al., 1999). By contrast, the hypoxic ventilatory response was completely abolished by gill denervation in channel catfish (Burleson and Smatresk, 1990) and gar (Smatresk, 1989). Clearly there are large species differences in the role of the branchial chemoreceptors in this response.

Milsom et al. also suggested that circulating catecholamines did not contribute to the hypoxic ventilatory response (Randall and Taylor, 1991) in tambaqui since the exogenous application of catecholamines inhibited ventilation (Milsom et al., 2002). 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. (Perry et al., 1992). Since no ventilatory depression was observed in the present study, this suggests that the tambaqui were not undergoing severe stress even at the levels of hypoxia we applied (10 mm Hg), possibly reflecting the extreme hypoxia-tolerance of this species (Perry et al., 2004).

Heart rate responses to hypoxia

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. Also, in the majority of species so far studied, the bradycardia is sustained during the entire hypoxic period. In species such as the dogfish (Butler et al., 1977) and traíra (Sundin et al., 1999), however, the heart rate gradually returns to normoxic levels despite sustained hypoxia. This was also the case with the tambaqui in the present study; during long-term exposure to severe hypoxia, the f_H of intact tambaqui returned to normoxic values over 300 min of exposure. The O_2 chemoreceptors eliciting the bradycardia during short-term exposure to hypoxia are situated on all gill arches (Sundin et al., 2000) and they sense changes in both the blood and inspired water. Hypoxic bradycardia was completely abolished by denervation of the branchial branches of cranial nerves IX and X (Sundin et al., 2000). Our data also confirm this. Interestingly, however, while denervation of the opercular and palatine branches of cranial nerve VII, as well as all mandibular branches of cranial nerve V, innervating the orobranchial cavity, did not affect the initial bradycardia, it did abolish the slow return of heart rate to starting values. Since heart rates were not different between intact and denervated fish at 300 min, and the heart rate did not statistically return to starting values in the intact fish at 360 min in these trials either, it is difficult to read too much into this. That said, heart rates were significantly lower in the denervated fish at 360 min compared to intact fish and the reasons for this are not clear, but could possibly be the result of a more severe hypoxaemia in the denervated fish compared to controls. Without blood gas data we cannot confirm this.

ASR and development of inferior lip swelling in response to severe hypoxia

Severe hypoxia rapidly induced ASR and slowly induced the development of inferior lip swelling in intact tambaqui. These results are in agreement with previous studies of others (Saint-Paul, 1988; Val and Almeida-Val, 1995; Rantin and Kalinin, 1996; Rantin et al., 1998). Furthermore, fish with cranial nerves IX and X denervated also performed ASR and developed inferior lip swelling, which is in agreement with the data of Sundin et al. (Sundin et al., 2000). However, denervation of the mandibular branches of cranial nerve V innervating the orobranchial cavity completely abolished ASR.

And, while one previous study (Sundin et al., 2000) also found that the development of inferior lip swelling in branchial denervated fish was considerably lower than in intact fish, in the present study it was observed that the development of inferior lip swelling was practically the same in the intact and denervated fish. The inferior lip swelling induced in tambaqui by severe hypoxia was also not abolished by denervation of cranial nerves V and VII to the orobranchial cavity in the present study. This suggests that the formation of inferior lip swelling in tambaqui either (1) can be elicited by any one group of the O_2 chemoreceptors at multiple sites (we did not denervate all of V, VII, IX and X simultaneously), (2) is controlled by O_2 receptors located outside the gills and orobranchial cavity, or (3) results from a direct effect of hypoxia/hypoxemia on the lip tissue itself. Given the time course of the response and the data collected to date, we favour the latter possibility.

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