While much is known about the reflex cardiorespiratory responses of teleost fishes to hypoxia and their ventilatory responses to hypercarbia, there are few reports concerning cardiovascular responses to hypercarbia and surprisingly few studies, on only a handful of species, designed to determine the locations and innervation of the receptors eliciting these reflex responses.

Most teleosts respond to hypoxia with a substantial decrease in heart rate. In teleosts from temperate waters, this bradycardia appears to be triggered by activation of externally oriented branchial receptors (Randall and Smith, 1967; Saunders and Sutterlin, 1971; Smith and Jones, 1978; Smatresk et al., 1986; Burleson and Smatresk, 1990b; McKenzie et al., 1991; Burleson and Milsom, 1993), while in a hypoxia-tolerant neotropical fish, the traira (Hoplias malabaricus), the hypoxic bradycardia is primarily elicited reflexively by activation of internally oriented branchial receptors (Sundin et al., 1999). This may suggest that hypoxia-tolerant species respond primarily to arterial hypoxaemia, while less tolerant fish respond more immediately to aquatic hypoxia.

The distribution of these receptors among the different gill arches also does not seem to be uniform amongst species. For example, in the Atlantic cod (Gadus morhua) (Fritsche and Nilsson, 1989), rainbow trout (Oncorhynchus mykiss) (Smith and Jones, 1978; Daxboeck and Holeton, 1978), coho salmon (O. kisutch) (Smith and Davie, 1984) and traira (Sundin et al., 1999), these receptors are located on the first gill arch, but in the catfish (Ictalurus punctatus) they appear to be located on all of the first three gill arches (Burleson and Smatresk, 1990a). Furthermore, in an elasmobranch, the dogfish (Scyliorhinus canicula), they are not confined to the gills but are also located throughout the orobranchial cavity, where they are innervated by cranial nerves V (trigeminal) and VII (facial) (Butler and Taylor, 1971; Butler et al., 1977). The reasons for these differences are also unclear.

The ventilatory response to hypoxia in teleosts consists of
an increase in both breathing frequency and amplitude. In several studies on various species, denervation of cranial nerves IX and X to the gills and the pseudobranch have failed to eliminate ventilatory responses to hypoxia (Saunders and Sutterlin, 1971; Sundin et al., 1999). However, it has been reported that complete denervation of the branchial branches of cranial nerves IX and X eliminates all the ventilatory response in the catfish (Burleson and Smatresk, 1990a) and the gar (Lepisosteus osseus) (Smatresk, 1989). Thus, the results from fish species studied to date suggest that ventilatory O₂ chemoreceptors do not share common locations and distribution among species, just as the O₂-sensitive chemoreceptors eliciting cardiac reflexes do not.

During exposure to environmental hypercarbia, arterial hypercapnia or intra-arterial injections of acid, fish increase their ventilation rate and/or amplitude (Randall and Jones, 1973; Janssen and Randall, 1975; Randall et al., 1976; Thomas and Le Ruz, 1982; Smith and Jones, 1982; Thomas et al., 1983; Reid et al., 1999). This increase in breathing has been attributed to both indirect and direct effects of the changes in pH/CO₂ (Smith and Jones, 1982; Randall and Taylor, 1991; Perry et al., 1992), but much of the evidence suggests that hypercarbia and/or hypercapnia can cause ventilatory increases directly, independent of changes in arterial oxygen content or plasma catecholamine levels (Butler and Taylor, 1971; Heisler et al., 1988; Perry and Kinkead, 1989; Wood et al., 1990; Burleson et al., 1992). There have been few studies designed to determine the locations and distribution of the receptors involved in the hypercarbic ventilatory response. One such study in a neotropical fish, the traira (Hoplias malabaricus), showed that the hypercarbia-induced increases in both breathing frequency and amplitude arose from receptors with a similar distribution to those that elicited the hypoxic ventilatory responses (Reid et al., 1999). The traira also exhibited a bradycardia and mild hypotension during exposure to environmental hypercarbia (Reid et al., 1999) and, in this case, the distribution of receptors involved in producing the hypoxic and the hypercarbic bradycardia were different; those producing the hypoxic bradycardia were located exclusively in the first gill arch, while those producing the hypercarbic bradycardia were found in other gill arches as well. These data suggested that receptors uniquely sensitive to changes in CO₂/pH may exist in fish and that their distribution may be distinct from that of receptors involved in the hypoxic ventilatory response.

These previous results suggest that studies of the distribution of cardiorespiratory 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. The present study focuses on the cardiorespiratory responses to hypoxia and hypercarbia of another hypoxia-tolerant neotropical fish, the tambaqui (Colossoma macropomum). The tambaqui, however, is an aquatic surface breather that, under conditions of environmental hypoxia, will come to the surface and siphon the well-oxygenated surface layer (see 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 (see Val and Almeida-Val, 1995). While the mechanisms that induce aquatic surface breathing and lip swelling are unknown, this mechanism provides these fish with an alternative strategy to ventilation in hypoxic waters. Thus, the goals of this study were to document respiratory, cardiovascular, behavioural and morphological responses to hypoxia and hypercarbia in Colossoma macropomum and to identify the location (internal and external) and the distribution (branchial and extrabranchial) of O₂-sensitive chemoreceptors involved in these responses. As such, this study was designed to contribute to our knowledge of cardiorespiratory control in fish in general and, by examining differences in receptor distribution promoting reflex responses to hypoxia versus hypercarbia, to help resolve questions concerning the presence or absence of receptors uniquely sensitive to changes in CO₂/pH involved in respiratory control.

Materials and methods

Experimental animals

For these experiments, juvenile tambaqui Colossoma macropomum (Cuvier, 1818) (659±32 g; mean ± S.E.M., N=28) were obtained from CEPTA (Tropical Fish Research Centre)/IBAMA in Pirassununga SP, Brazil, and transported to the Federal University of São Carlos. These fish are third- or fourth-generation descendants of native tambaqui taken from the Amazon in 1993. Animals were maintained outdoors in fibreglass aquaria supplied with aerated and dechlorinated City of São Carlos tapwater. Temperature was maintained at 25 °C, and the animals were exposed to a natural photoperiod. Fish were fed ad libitum every second day, and experiments were performed between September and October.

Animal preparation

Animals were anaesthetized in an aqueous solution of benzocaine (100 mg l⁻¹) predissolved in 2 ml of 70% ethanol. During surgery, the gills were ventilated with a second solution of benzocaine (50 mg l⁻¹) gassed with air. Impedance electrodes were sutured to each operculum to monitor the breath-by-breath displacement of the operculum and measure ventilation rate (fV) and an index of ventilation amplitude (VAMP). Using a dremel tool, a hole was drilled through the snout between the nostrils, and a flared cannula (PE 160) was fed from inside the mouth out through the hole and was secured with a cuff. This allowed administration of NaCN and HCl solutions into the buccal cavity to stimulate putative O₂ and CO₂/pH chemoreceptors (external) on the gills monitoring the respiratory water. A second cannula (PE 50) was inserted into the afferent branchial artery of the third gill arch and advanced towards the ventral aorta. This cannula was used to measure ventral aortic blood pressure (PVA) and heart rate (fHt) as well
as to inject solutions of saline, NaCN and HCl to stimulate putative O₂ and CO₂/pH chemoreceptors (internal) that monitor the blood.

The operculum was reflected forward, and a small incision (approximately 1 cm) was made in the epithelium at the dorsal end of the first and second gill arches where they meet the roof of the opercular cavity. This permitted access to cranial nerve IX (glossopharyngeal) and the pretrematic branch of cranial nerve X (vagus) innervating the first gill arch (G1 group, N=10). Tambaqui do not have a pseudobranch. For complete branchial denervation, the incision was enlarged approximately 1 cm in the caudal direction. The branchial nerves to all gill arches were carefully dissected free of connective tissue and cut with fine iris scissors (G4 group, N=7). In all cases, the cardiac and visceral branches of the vagus (X) were left intact. In the control group (N=11), the nerves were left intact, but in three of these animals the nerves were exposed (sham operation) but not sectioned. There was no sham effect. The healing process in this species was rapid, and the incision was covered with ‘scar tissue’ within 24 h. All denervations were confirmed post mortem by autopsy.

After surgery, the animals were manually ventilated with aerated water, and as soon as they showed signs of coming out of the anaesthesia they were placed into individual cylindrical tubes housed within larger experimental tanks (approximately 80 l). Mesh covered the ends of the cylindrical tube. This facilitated rapid equilibration of the water within the tube with the water in the holding tank. A large slit on top of the tubes permitted the impedance leads/cannulae to exit the tank. The tank was covered to maintain a dark and quiet environment for the fish. Animals were allowed to recover for a minimum of 24 h prior to experimentation.

**Experimental protocols**

Following the 24 h recovery period, the opercular impedance leads were connected to an impedance converter to measure \( f_V \) (breaths min\(^{-1}\)) and \( V_{AMP} \) (arbitrary units). The afferent branchial artery cannula was connected to a pressure transducer to measure \( P_{VA} \) (kPa) and \( f_H \) (beats min\(^{-1}\)). The partial pressure of oxygen in the water (\( P_{WO2} \)) was monitored continuously with an oxygen electrode (FAC 001 O₂ electrode and FAC 204A oxygen analyser) supplied, via siphon, with a steady flow of water from the experimental chamber. The electrode was calibrated with solutions of sodium bisulphate in borax (\( P_{O2}=0 \) kPa) and air-equilibrated water (\( P_{O2}=18.6 \) kPa; 25 °C). Water pH was continuously measured with a pH electrode calibrated with standard solutions. Prior to initiating the experimental protocol, the fish were left undisturbed for approximately 30 min to allow \( f_V, V_{AMP}, f_H \) and \( P_{VA} \) to stabilize at steady levels.

First, the animals were subjected to a series of internal (into the branchial artery cannula) and external (into the snout cannula) injections of NaCN and HCl to stimulate putative O₂ and CO₂/pH chemoreceptors. Injections of the vehicle alone (0.9 % NaCl for internal and water for external injections) served as controls. The injections were administered in the following order: (1) internal saline, (2) internal NaCN (0.5 ml of 2 mg ml\(^{-1}\) NaCN in saline), (3) internal HCl (0.125 mmol l\(^{-1}\) in 0.3 ml of saline), (4) external water, (5) external NaCN (1 ml of 2 mg ml\(^{-1}\)NaCN in water) and (6) external HCl (0.125 mmol l\(^{-1}\) in 0.5 ml of water). In each case, the cannula was flushed with 0.5 ml of saline (for internal injections) or 1.0 ml of water (for external injections) to ensure complete drug delivery. After each injection, cardiorespiratory variables were recorded for 3 min. If pre-injection levels of \( f_V, V_{AMP}, P_{VA} \) and \( f_H \) were not restored within that period, subsequent injections were delayed until these variables returned to previous levels or stabilized at new levels.

Next, the animals were subjected to abrupt, progressive environmental hypoxia by shutting off the airflow and gassing the tank with nitrogen. The \( P_{WO2} \) was lowered from an air-saturated level of 18.6 kPa (140 mmHg; 25 °C) to 1.3 kPa (10 mmHg) over approximately 10 min. At this point, the nitrogen flow was halted, airflow was restored and the water \( P_{O2} \) gradually returned to normoxic levels. Finally, the animals were subjected to abrupt, progressive environmental hypercarbia by gassing the tank with 5 % CO₂. Initial hypercarbic experiments demonstrated that equilibrating the water with 0.1 %, 0.25 %, 0.5 % or 0.75 % CO₂ had no effect on ventilation or heart rate/blood pressure, whilst 1.25 % and 2.5 % CO₂ elicited very modest changes. Consequently, the animals were exposed to 5.0 % CO₂ such that the water pH fell from approximately 7.0 to 5.0 over 10 min. This reliably stimulated ventilation in all control animals. The animals were then returned to normocapnic conditions, and cardiorespiratory variables were monitored until the water pH returned to at least 6.5. These changes in pH and \( P_{O2} \) (both magnitude and rate of change) are well within physiological ranges for these fish. For example, measurements made at different times during the annual flood cycle in the Amazon and Orinoco rivers, in which these fish are typically found, show pH ranging from 3.8 to 8.0 with dissolved O₂ concentration in the range 5.1-0 mg l\(^{-1}\) through the water column. During the dry periods, levels of CO₂ are lower, and pH and O₂ levels are higher, in these waters (Val and Almeida-Val, 1995). Since these experiments were designed to study the effects of progressive branchial denervation on cardiorespiratory responses, rapid progressive changes in pH and \( P_{O2} \) were used to produce strong responses whose modulation by denervation would be most apparent. Removal of a modest response by denervation would be much less convincing.

Because of a persistent decrease in \( f_H \) during hypoxia and hypercarbia in the G4 fish (see Results), these animals were subsequently treated with atropine (1 mg kg\(^{-1}\), Sigma), and the experiments were repeated to test for direct effects of hypoxia, hypercarbia and NaCN on the heart. This procedure should block receptor-mediated reflex responses at the heart and reveal whether the bradycardia demonstrated by the G4 fish was due to a direct effect of each treatment on the heart.

**Buccal and opercular pressure versus respiratory impedance**

Our primary measure of breathing was the change in...
electrical impedance measured across the orobranchial cavity. To confirm the accuracy of our impedance measurements, we alternately measured buccal and opercular pressures in three fish, using appropriate cannulae, and simultaneously measured respiratory impedance. To stimulate breathing further under these conditions, we injected NaCN (1 ml of 500 μg ml⁻¹ NaCN) into the mouth through the buccal cannula.

*Lip swelling and aquatic surface respiration*

Intact and G4 fish (the same fish that had been used in the protocols described above) were further exposed to environmental hypoxia (P₂O₅=2.0 kPa) for approximately 3.5 h (time taken to reduce P₂O₅ was approximately 0.5 h). Before these experiments, however, all instrumentation except the P₂VA cannula was removed under anaesthesia, and the animals were transferred to a large glass aquarium where they were allowed to recover for 1–2 days. Three sides of the aquarium were covered to minimize disturbance, but one side was left open to allow visual observation of the fish. Hypoxia was induced by gassing the water with nitrogen, and a fan directed a constant stream of air across the surface of the water to prevent this gas from accumulating above the surface. The effects of denervation on the degree of inferior lip swelling and the incidence of aquatic surface respiration were monitored visually. Tambaqui normally develop inferior lips fully within approximately 2–3 h of exposure to hypoxia (Braum and Junk, 1982; Val and Almeida-Val, 1995).

*Data analysis*

Cardiovascular and respiratory variables were analyzed for a 1 min control period immediately prior to the initiation of hypoxia, for the final 30 s of the hypoxic exposure (water P₂O₅=1.3 kPa) and after 30 min of recovery as well as immediately prior to the initiation of hypercarbia, for the final minute of the hypercarbic exposure (water pH=5.0) and after 30 min of recovery from hypercarbia. During the injection experiments, data were analyzed for a 30 s control period immediately prior to an injection of water, saline, NaCN or HCl and at 10 s intervals for the first minute post-injection. During the second and third minute post-injection, data were analyzed for a 30 s period each minute. Maximum responses are reported.

fh, P₂VA and fV are reported as absolute values. Since VAMP was measured in arbitrary units, VAMP and total ventilation (VₖT=VAMP×fV) are reported as a percentage change from the control value. In the second series of experiments, either the animals performed aquatic surface respiration or they did not. No quantification, in terms of frequency or time spent at the surface, was made. The extent of lip formation was estimated visually.

*Statistical analyses*

The data are reported as the mean ±1 standard error of the mean (S.E.M.). Differences in resting values for each variable between the intact animals and the denervated groups, before and after treatment with atropine, were tested by unpaired and paired Student’s t-tests, where appropriate. Data were compared using one-way repeated-measures analysis of variance (ANOVA) to test for the significance of changes in response to each stimulus. If significant differences (P≤0.05) were found, a Dunnett’s multiple-comparison test was used as a post-hoc test. To evaluate the effect of selective denervations on the responses to the different treatments, a two-way repeated-measures ANOVA was used.
Results

Ventilatory mechanics: buccal and opercular pressure versus respiratory impedance

Tambaqui skimmed water at the surface of open tanks under hypoxic conditions. At this time, the inferior lip became swollen and was used as a funnel to direct the surface film of water into the mouth. The mouth remained agape the entire time the fish was breathing the surface water. On close examination, we observed that there was no water reflux through the mouth during buccal contraction despite the fact the mouth was agape. This was because these fish possess loose epithelial flaps attached to the upper and lower jaw that can act as a ‘pocket valve’ to prevent reflux when the pressure in the buccal cavity exceeds ambient pressure. Fig. 1A,B shows the opening to the mouth from the front with the mouth open wide and the mouth flap open (Fig. 1A) and closed (Fig. 1B). These flaps are drawn open, presumably by negative pressure, during buccal expansion and are forced to close during buccal contraction. Fig. 1C depicts a fish skimming water at the surface of the tank, while Fig. 1D shows the swollen lower lip from a dorsal view.

Our primary measure of breathing was the change in electrical impedance measured across the orobranchial cavity. In trying to determine the source of apparent recording artefacts arising from injections into the mouth through the buccal cannula, we discovered that the buccal and opercular rhythms could vary independently. Thus, to confirm the accuracy of our impedance measurements, we alternately measured buccal and opercular pressures in three fish, using appropriate cannulae, and simultaneously measured respiratory impedance to examine the effects of the buccal/opercular asynchrony on our impedance measurements.

Fig. 2 illustrates simultaneous pressure and impedance recordings obtained in one fish. Note that the buccal pressure fluctuations (Fig. 2A, initial portion of upper trace) are synchronous with the changes in respiratory impedance (a coupling ratio of 1:1), but that opercular pressure fluctuations tend to be asynchronous (coupling ratios from 1:1 to 1:1.6, equivalent to approximately 2:3). When breathing was stimulated by the injection of NaCN into the respiratory water flow, the frequency and amplitude of the impedance fluctuations (as well as the buccal pressure fluctuations, which are not shown) increased, while the rate and magnitude of the opercular pressure fluctuations were unchanged (Fig. 2B). Similar results were obtained in all three animals studied.

Aquatic surface breathing and lip swelling

During hypoxia, tambaqui with access to the water surface initiated aquatic surface respiration and, to aid skimming of the surface water, their lower lips swelled to form a funnel (Fig. 1C,D). From Table 1 it is clear that, even when the nerves to all gill arches were sectioned, the tambaqui still performed aquatic surface respiration.

Table 1. Effect of total gill denervation on the incidence of aquatic surface respiration and the degree of lower lip swelling in tambaqui

<table>
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<tr>
<th>Individual</th>
<th>Lip swelling</th>
<th>ASR</th>
<th>Individual</th>
<th>Lip swelling</th>
<th>ASR</th>
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ASR, aquatic surface respiration.
+++ , Fully developed; ++, half developed; +, poorly developed; *fish performs ASR.
aquatic surface respiration and began to develop swelling of the lower lip in response to hypoxia. Subjectively, the degree of swelling of the lower lip, however, was attenuated.

**Effects of progressive denervation on resting levels of cardiorespiratory variables**

There was a trend for resting $f_H$ and $f_V$ to increase with progressive levels of denervation of the branchial arches (G1 and G4); however, progressive denervation had no statistical effect on resting $P_{VA}$, $f_H$ or $f_V$ (Table 2).

**Responses to abrupt hypoxia, hypercarbia and NaCN and HCl injections in intact animals**

In Fig. 3A, B, original recordings of $f_V$ and $V_{AMP}$ and of $f_H$ and $P_{VA}$ in a sham-operated fish (intact gill innervation) during abrupt, progressive hypoxia are shown. It is evident from this figure that, when the water $P_O_2$ falls (past approximately 16 kPa in this figure), $f_H$ begins to decrease whilst both $f_V$ and $V_{AMP}$ increase. Upon the restoration of airflow, $f_H$ increased rapidly and rose to substantially greater levels than observed prior to the initiation of hypoxia. Ventilation ($f_V$ and $V_{AMP}$) was slower to respond to the restoration of $O_2$ levels in the water.

Fig. 4A shows original recordings depicting the changes in ventilation ($f_V$ and $V_{AMP}$) and in $f_H$ and $P_{VA}$ during the abrupt transition from normocarbia (air) to severe hypercarbia (water pH 5.0) and back to normocarbia in one control fish. It is evident from the extracted data (Fig. 4B) that, when the water was gassed with 5.0% CO₂, causing the water pH to fall from 7.0 to 5.0, $f_H$ clearly decreased whilst $P_{VA}$, $f_V$ and $V_{AMP}$ increased. These changes (bradycardia and hyperventilation) were typical responses of tambaqui to environmental hypercarbia prior to any gill denervation.
Both internal and external NaCN injections also produced reflex bradycardia and increased ventilation (Figs 5, 7, 8, 9). Acid injections, both internal and external, were notably without effect (data not shown). It is possible that the dose of acid used was not sufficient to produce significant pH changes at the receptor sites. Alternatively, it is possible that the receptors mediating the hypercarbic responses are primarily stimulated by CO₂ rather than extracellular H⁺ per se. Fish did not tolerate stronger doses of acid well, however, preventing us from pursuing this further. The lack of response to the acid injections also makes it impossible to determine whether the receptors mediating the hypercarbic response were internally or externally oriented.

**Cardiovascular reflexes**

Direct stimulation of oxygen-sensitive receptors (hypoxia, internal and external injections of NaCN) produced a rapid decrease in \( f_\text{H} \) that remained after the first gill arch had been denervated (Fig. 5). After all the gill arches had been denervated (G4 fish), hypoxia and internally (but not externally) injected NaCN still produced a significant, small,
but more slowly developing, bradycardia. By pre-treating the fish with atropine, we tested whether this response was a cholinergic reflex response or a nonspecific direct effect on the heart. Despite atropine treatment, this small, slowly developing bradycardia still remained (Fig. 5).

Hypercarbia (exposure to 5.0% CO₂) with the gill innervation intact also caused heart rate to decrease. This response was abolished in the G1 denervated group; there was a trend for heart rate to decrease during hypercarbia in this group, but this decrease was not statistically significant. This was not affected by total gill denervation (G4) nor by pre-treatment with atropine (1 mg kg⁻¹) to block cholinergic reflex responses on the heart (Fig. 5).

Despite the reflex hypoxic bradycardia, there were no significant changes in \( P_{VA} \) in any of the fish groups in response to hypoxia (Fig. 6). When NaCN was injected into the blood, all groups of animals maintained or slightly increased (although not significantly) \( P_{VA} \) (Fig. 6). Changes in \( P_{VA} \) following external NaCN injection reflected the changes in \( f_H \).

Thus, as \( f_H \) markedly fell from 47 to 20 beats min⁻¹ in control and G1 fish (Fig. 5), \( P_{VA} \) also fell (Fig. 6). Just like the bradycardia, this pressure decrease was abolished in G4 animals.

The effects of hypercarbia on \( P_{VA} \) are also illustrated in Fig. 6. In the control group, despite the bradycardia, there was a progressive increase in blood pressure. While this response appeared unchanged following denervation of the first gill arch (G1), this increase was no longer statistically significant. Blood pressure again increased significantly in the G4 fish during hypercarbia (pH 5.0), despite the tendency for heart rate to decrease. Identical changes occurred in this group following pre-treatment with atropine, although these changes were again not significant because of high interindividual variability.

**Respiratory reflexes**

Hypoxia and internal NaCN produced a rapid increase in \( f_V \) in both the control and the G1 fish (Fig. 7) that was abolished by complete branchial denervation. External NaCN also
Branchial chemoreceptors in tambaqui produced a rapid increase in $f_V$ in the control and G1 fish that was not eliminated by complete branchial denervation; external NaCN still increased $f_V$, albeit more slowly. In the control and G1 fish, ventilation rate also increased during exposure to hypercarbia. Ventilation rate in the G4 fish did not increase significantly during hypercarbia.

Hypoxia and internal NaCN significantly elevated $V_{AMP}$ in all experimental groups (control, G1 and G4), and there were no differences in responses to the treatments detected between the groups (Fig. 8). External NaCN injection also increased $V_{AMP}$ in the intact animals and to a lesser extent in the G1 and G4 fish.

Ventilation amplitude did not increase significantly in any group during hypercarbic exposure (Fig. 8). There was a tendency for ventilation amplitude to increase in some individuals in the control and G1 groups, but there was tremendous variability in this response. Some fish (7/11 in the control group and 6/10 in the G1 group), such as the one depicted in Fig. 4, showed large increases in breathing amplitude, while others (4/11 in the control group and 4/10 in the G1 group) showed no change or a decrease in amplitude.

Because of the amplitude responses, $V_{TOT}$ also increased in all the groups during hypoxia, with the increase in the control fish being substantially greater than in the G1 and the G4 fish (Fig. 9). Internal and external NaCN injections caused rapid increases in $V_{TOT}$ in both the control and G1 groups, but only external NaCN treatment produced an increase in $V_{TOT}$ in the G4 group. During hypercarbia, the control group exhibited a doubling of $V_{TOT}$, which was attenuated in the G1 group and absent in the G4 group.

**Discussion**

**Ventilatory mechanics: buccal and opercular pressure versus respiratory impedance**

While observing tambaqui breathing during hypoxia in an open tank, we noticed that these fish possessed a flap-like valve in the mouth (Fig. 1A,B). This flap appears to be composed of...
thin epithelial sheets, which extend from the margins of the upper and lower jaw and act like a pocket valve. These flaps collapse against the roof and floor of the mouth during the negative pressure expansion phase of the buccal cycle (Fig. 1A), but fill with water and close, sealing the entrance to the mouth, during the positive-pressure compression phase (Fig. 1B). As such, they prevent reflux of water back through the open mouth, allowing the fish to ventilate the gills efficiently while still maintaining the mouth gape. It also undoubtedly serves to prevent disturbance of the surface layer. Since the top 1–5 mm of the water column can be the sole source of oxygen during periods of extreme hypoxia in natural habitats, this valve mechanism may serve to prevent disturbances of the surface layer that would make aquatic surface respiration pointless.

We also inadvertently discovered that the buccal rhythm did not always coincide with the rate of opercular expansion and compression. In examining this further, we discovered that buccal pressure cycles were synchronous with the changes in respiratory impedance (a coupling ratio of 1:1) and that both responded well to respiratory stimuli. Opercular pressure fluctuations, however, could, from time to time, be asynchronous with the changes in respiratory impedance (coupling ratios 1:1–2:3) and did not respond to respiratory stimuli. This suggests the possibility that there may be separate central rhythm generators for buccal and opercular rhythms (which are often entrained) and that only the buccal rhythm generator responds to aquatic respiratory stimuli (unfortunately no internal injections of NaCN were given in this experimental series). This intriguing suggestion requires further research. This is quite different from the dissociation seen between buccal and opercular movements in air-breathing fish, where opercular movements stop while buccal movements continue during air breaths. In the tambaqui, the opercular rhythm does not simply stop but continues at a distinctly different rate from that of the respiratory impedance (buccal rhythm).

**Responses to hypoxia: lip swelling and aquatic surface breathing**

To ascertain whether the aquatic surface respiration and lip swelling were induced by receptors located in the gills, free-swimming tambaqui were exposed to hypoxia prior to and after complete branchial denervation. Our results clearly show that fish still displayed aquatic surface respiration following complete branchial denervation which, subjectively, was indistinguishable from the behaviour exhibited by intact fish. Denervated fish did show a clear reduction in the degree of swelling of the lower lip compared with intact animals, however, indicating that lip swelling
Branchial chemoreceptors in tambaqui resulted from the stimulation of both branchial and extrabranchial receptors.

Cardiovascular responses: heart rate

Acute and rapidly induced hypoxia or hypercarbia produced a marked bradycardia in the present study. During hypoxic exposure, progressive denervation reduced the magnitude of the bradycardia, but did not eliminate it completely. When all branchial nerves were sectioned in the G4 group of tambaqui, the bradycardia that was still present was not eliminated by pre-treatment with atropine, indicating that the sustained bradycardia was not a receptor-mediated vagal reflex response. Branchial motor nerves control the positioning of the gill curtain in the respiratory water flow and the distribution of blood flow through the gill filaments (Nilsson, 1984), and a large reduction in arterial $P_{O_2}$ following complete bilateral denervation of cranial nerves IX and X has been reported in the sea raven (Hemitripterus americanus) (Saunders and Sutterlin, 1971). Therefore, it is likely that the hypoxaemia developed in the G4 fish during hypoxic exposure could have been particularly severe and that this could directly have affected the heart.

A hypoxic bradycardia is a typical response in fish, but in contrast to most teleosts, in which this reflex arises from receptors located on the first gill arch (Atlantic cod, Fritsche and Nilsson, 1989; rainbow trout, Smith and Jones, 1978; Daxboeck and Holeton, 1978; Smith and Davie, 1984; traira, Sundin et al., 1999), the component of this response that appeared to be reflex in tambaqui was not abolished by denervation of the IXth and Xth cranial nerves to only the first gill arch. Other examples where $O_2$-sensitive receptors eliciting cardiac reflexes are situated outside the first gill arch are the channel catfish, in which these receptors are found on the first three gill arches (Burleson and Smatresk, 1990a), and an elasmobranch, the dogfish shark (Scyliorhinus canicula), in which they are found on all the gill arches as well as within the orobranchial cavity (Butler et al., 1977). It is not yet clear what these differences in distribution reflect since, at present, they cannot be correlated with degree of hypoxia tolerance, habitat preference or phylogenetic position.

Fig. 8. The effects on ventilation amplitude (percentage change from starting values) of abrupt hypoxia (A), abrupt hypercarbia (B) and injections of NaCN either internally into the ventral aorta (C) or externally into the respiratory water (D). The data are shown as the mean ± S.E.M. The grey columns depict the final response and the black columns represent the recovery values. Columns marked with an asterisk are significantly different ($P<0.05$) from starting values (100%) in that condition. See Table 2 for values of $N$.

Injections of NaCN into the blood or the respiratory water produced a rapid bradycardia in both the control and G1 fish, suggesting that both internally and externally oriented $O_2$ chemoreceptors are involved in eliciting the reflex decrease in $f_H$ in tambaqui. Similar results have been obtained in the bimodally breathing gar (Lepisosteus osseus) (Smatresk et al., 1986). In contrast, these receptors appear to be exclusively externally oriented in most water-breathing teleosts (for review, see Burleson et al., 1992) but internally oriented in traira (Sundin et al., 1999). These intra-species differences in
interbranchial distribution are even harder to interpret than their intrabranchial distribution and do not support the suggestion that hypoxia-tolerant species are more sensitive to arterial hypoxaemia while less tolerant species are more sensitive to aquatic hypoxia.

In the case of hypercarbia, the fall in heart rate in the G1 and G4 fish was not significant, suggesting that the reflex was elicited by branchial receptors confined to the first gill arch. Thus, the distribution of receptors producing the hypercarbic bradycardia in tambaqui appears to be different from that of the receptors mediating the hypoxic bradycardia and strengthens arguments that changes in CO₂/pH may act as independent cardiac stimuli in fish. A similar conclusion was drawn in the only other study of which we know that documents the distribution of chemoreceptors mediating hypercarbic bradycardia in a teleost (Reid et al., 1999). The receptors involved in eliciting the hypercarbic bradycardia in traira, however, were distributed differently from those in tambaqui. While the receptors mediating the hypercarbic bradycardia in tambaqui are located on the first arch, those mediating the hypoxic bradycardia are located possibly on all gill arches; in traira, the situation is reversed, with the hypercarbic bradycardia being mediated by receptors possibly on all gill arches and the hypoxic bradycardia being initiated by receptors on only the first arch.

Cardiovascular responses: blood pressure

Although hypoxia in teleost fish commonly produces hypertension, as a result of hypoxic bradycardia, some fish species exhibit a constant blood pressure or even hypotension (see Fritsche and Nilsson, 1993; Sundin et al., 1999). In tambaqui following internal and external NaCN treatment and hypoxia, the only significant change in blood pressure was a decrease of approximately 1 kPa in the control and the G1 fish caused by external injection of NaCN. Judging from these results, it appears that tambaqui have good barostatic control and can maintain blood pressure within a narrow range despite large changes in \( f_H \). Reflex corrections to maintain blood pressure could include either increased cardiac stroke volume or systemic vascular resistance or both.

The overall effects of hypercarbia on blood pressure were quite small. In all groups of fish, however, there was a mild hypertension (a 15–20% increase in blood pressure). This was significant in the control and total-gill-denervated groups despite a significant bradycardia in the control fish. This could only arise from a concomitant increase in stroke volume or systemic vascular resistance and, since this still occurred in...
animals after complete gill denervation, it must have been mediated by receptors outside the gills or by direct effects of the changes in pH/PCO₂ on the vasculature. Extrabranchial receptors appear to trigger increases in systemic vascular resistance during hypoxia in other teleosts (see Sundin et al., 1999), and these or similar receptors may be involved here.

**Ventilatory responses: ventilation rate**

Hypoxia is a powerful respiratory stimulant in all fish (for references, see Shelton et al., 1986; Fritsche and Nilsson, 1993) and, as has been reported previously, tambaqui increase resting fV from approximately 35 to 55–66 breaths min⁻¹ in response to acute exposure to low oxygen tensions (Rantin and Kalinin, 1996). The denervation experiments in the present study revealed that the receptors involved in producing this increase are solely branchial and are probably distributed on all the gill arches. Both internal and external injections of NaCN were able to mimic hypoxia and rapidly increased fV by a comparable amount. On the basis of these results, we conclude that the branchial O₂-sensitive chemoreceptors mediating the increase in fV monitor both the blood and the respiratory water. There is a striking similarity between the distribution and location of the receptors triggering the hypoxic bradycardia and the increase in fV in this species (see above), raising the possibility that the same receptor population mediates both reflex responses. This is not the case for most other species since, in most, the receptors responsible for producing the fall in heart rate are found only on the first gill arch (see above).

Ventilation rate also increased significantly during hypercarbia in intact fish. This is consistent with other studies on a wide variety of fish (dogfish Scyliorhinus stellaris, Heisler et al., 1988; skate Raja oscillata, Wood et al., 1990; rainbow trout, Kinkead and Perry, 1991; Gilmour and Perry, 1994; traira, Reid et al., 1999). The levels of CO₂ required to produce these responses in tambaqui, as well as in traira, are substantially greater than in more temperate fishes (4.0–5.3 kPa versus 0.7 kPa), and this may reflect the extremely acidic waters (pH 3.5–5.0) in which these fish often live. The increase in ventilation rate on exposure to hypercarbia was still present in fish following denervation of the first gill arch, but not following total denervation of all gill arches. The receptors mediating the hypercarbic tachypnoea in the other species studied so far, the traira (Reid et al., 1999), as well as the receptors mediating the hypoxic tachypnoea in both the traira and the tambaqui (Sundin et al., 1999), are all confined to the gills. Thus, it is possible that the same receptors are involved in mediating the increase in ventilation rate during both hypercarbia and hypoxia in both species.

**Ventilatory responses: ventilation amplitude**

Even complete branchial denervation failed to attenuate significantly the increase in VAMP induced by hypoxia, clearly showing that activation of extrabranchial receptors alone is sufficient to produce this increase. These results agree with earlier studies in tench (Tinca tinca), sea raven and traira in which ventilatory responses were unaffected by complete gill denervation (Hughes and Shelton, 1962; Saunders and Sutterlin, 1971; Sundin et al., 1999). In contrast, in the channel catfish and the longnose gar, complete branchial denervation abolished all the ventilatory responses (Smatresk, 1989; Burleson and Smatresk, 1990a).

As with the hypoxic stimulus, complete gill arch denervation did not attenuate the increase in VAMP elicited by internal or external NaCN injections. This suggests that there is a contribution to the increase in VAMP from extrabranchial receptors that are both externally and internally oriented. Several potential locations have been suggested as the site for extrabranchial oxygen receptors. They may exist in the orobuccal cavity, innervated by cranial nerves V and VII (Hughes and Shelton, 1962; Butler et al., 1977), or be located within the central nervous system (Satchell, 1961; Saunders and Sutterlin, 1971). Since all attempts to evoke a ventilatory response by central stimulation of the brain in vitro or in vivo have failed (Rovainen, 1977; Kawasaki, 1980; Hedrick et al., 1991), support for the existence of central chemoreceptors in fish is weak. They may also be located in the heart or in the ventral aorta innervated by the cardiac and visceral branches of the vagus nerve since these branches were not sectioned in the present study (Smatresk et al., 1986).

Studies on other fish species have typically found increases in ventilation amplitude in response to hypercarbia (Heisler et al., 1988; Wood et al., 1990; Kinkead and Perry, 1991; Gilmour and Perry, 1994; Reid et al., 1999). In tambaqui, hypercarbia produced a variety of changes in ventilation amplitude in different individuals. The net result was that there were no significant changes in mean ventilation amplitude in response to hypercarbia in any group. In the control and G1 fish, seven out of 11 and six out of 10 animals, respectively, increased ventilation amplitude in each group. In several animals (such as the one depicted in Fig. 4), the increases were substantial. After complete gill denervation, only one out of seven animals showed any notable increase in ventilation amplitude. Given this variability, it is difficult to draw firm conclusions, but the data suggest that increases in ventilation amplitude do occur in some animals and that, for the most part, this appears to be a response to stimulation of branchial receptors.

**Concluding remarks**

The results of the present study do not reveal a simple picture of cardiorespiratory chemoreceptor control in tambaqui. The responses of this species to hypoxia and hypercarbia appear to involve several putative receptor populations, in different locations, that have different central projections producing different reflex motor outputs. The basis for the intraspecies variability in the location and distribution of branchial and extrabranchial chemoreceptors in fish that have been studied remains elusive. More observational data of this sort will be needed, for a variety of species adapted to different habitats, before testable hypothesis concerning the evolution, phylogeny and adaptive significance of differences
in the distribution of cardiorespiratory chemoreceptors can be formulated. Finally, while there is some evidence to suggest that the receptors involved in producing the hypercarbic bradycardia and increase in ventilation amplitude, in those animals that showed an increase in ventilation amplitude, were different from the O₂-sensitive receptors that elicited similar changes in response to hypoxia, the distributions of receptors involved in producing increases in systemic vascular resistance and breathing frequency during both hypercarbia and hypoxia were similar. Viewed subjectively, this evidence is not overwhelming because the differences appear to be more quantitative than qualitative. Furthermore, even if there are differences in distribution, the data do not necessarily imply that the hypercarbic responses arise from receptors that are insensitive to hypoxia, but that only some are also sensitive to hypercarbia and have a different distribution from those that are not. True resolution of this question will require single-fibre recordings from putative branchial receptors.

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References


