

*Cardio-Respiratory  
Responses of Fish  
to  
Hypoxia,  
Hypercarbia  
and Temperature*

SYMPOSIUM PROCEEDINGS

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***International Congress on the Biology of Fish***

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## PREFACE

The number and diversity of fish species is greater than in any other vertebrate class. Different species occupy a large variety of habitats that differ widely with respect to temperature, dissolved O<sub>2</sub> and CO<sub>2</sub>, pH and other abiotic variables. Furthermore, these variables can fluctuate both seasonal and/or acutely. Since these variations can be unpredictable, fish must be able to sense the environmental changes, process these information and trigger the appropriate compensatory responses to maintain their cardiovascular and respiratory homeostasis. Although the cardiorespiratory responses of fish to temperature, hypoxia and hypercarbia have been well studied in the last three decades, most of information arose from temperate species. There is a great variability in the descriptions of the mechanisms involved in cardio-respiratory responses to environmental changes. It is not clear to what extent this variability is correlated to habitat and mode of life in different species and, thus, to what extent it might represent selective adaptation. Thus, there is a growing concern about the compensatory mechanisms employed by tropical and neotropical fish, in which extent these mechanisms are common features for this vertebrate class, and if the variability of responses emerges from the phylogenetic background.

This symposium, entitled Cardio-Respiratory Responses of Fish to Hypoxia, Hypercarbia and Temperature, intend not only to reexamine the current information and concepts, but also put together the most recent data, including information on tropical and neotropical species, focusing the mechanisms involved on the cardiovascular and respiratory control in fish and the consequences on the adaptation of these animals to their distinct environments.

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## CONGRESS ACKNOWLEDGEMENTS

This volume is part of the Proceedings of the 6<sup>th</sup> International Congress on the Biology of Fish, held in Manaus, Brazil in August, 2004. Ten years have passed since the first meeting in this series was held in Vancouver, BC, Canada. Subsequent meetings were in San Francisco, California; Baltimore, Maryland; Aberdeen, Scotland; and again in Vancouver, Canada. From those meetings, colleagues from over 30 countries have contributed more than 2,500 papers to the Proceedings of over 80 Congress Symposia, all available for free viewing on the internet.

We would like to extend our sincere thanks to the many people who helped us organize the facilities and program for this 6<sup>th</sup> Congress.

The local arrangements team worked very hard to make this Congress a success. The leaders of those efforts were Vera Almeida Val, Adriana Chippari-Gomes, Nivia Pires Lopes and Maria de Nazare Paula Silva (Local Arrangements); Marcelo Perlingeiro (Executive Secretary) and Maria Angelica Laredo (Fund Raising). The enormous contribution of time and effort that was required has led to an unforgettable experience for the participants, thanks to the imagination, determination and dedication of this team.

Many sponsors helped ensure the success of the meeting through both monetary and in-kind contributions, including: Fundação Djalma Batista, Honda, Merse, Cometais, Turkys Aquarium, Banco da Amazônia, Banco do Brasil, FUCAPI, SEBRAE/AM, IDAM/SEPROR, FAPEAM, SECT-AM, SUFRAMA, PETROBRÁS, CAPES, FINEP, CNPq, the Physiology Section of the American Fisheries Society, UFAM - Federal University of Amazonas, Fisheries and Oceans Canada and INPA - National Institute for Research in the Amazon.

Travel arrangements were ably handled by Atlantic Corporate Travel (special thanks to Maria Espinosa) and Orcal Planet, and the venue for the meeting was the spectacular Tropical Hotel Conference Center in Manaus.

The Student Travel Award Committee of the Physiology Section of the American Fisheries Society, led by Michael Redding, evaluated 65 applications from 15 countries and awarded 40 Travel Grants, after an ambitious and trying fund-raising effort. Special thanks must go the US Department of Agriculture, the US Geological Survey, US National Science Foundation and the World

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The editorial team compiled the short abstracts into an abstract book and formatted and compiled the papers for the Symposium Proceedings. Thanks to Karin Howard, Christie MacKinlay, Anne Martin, Callan MacKinlay and Marcelo Perlingeiro.

In particular, we would like to extend a sincere ‘thank you’ to the organizers of the individual scientific Symposia and their many contributors who took the time to prepare a written submission for these proceedings. Their efforts are very much appreciated. We hope that their participation will result in new insights, new collaborations and new lines of research, leading to new papers to be presented at the 2006 Congress in St. John's, Newfoundland.

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**EFFECTS OF TEMPERATURE AND CALCIUM AVAILABILITY  
ON VENTRICULAR MYOCARDIUM  
FROM NEOTROPICAL TELEOST FISH**

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**Abstract**

Although teleost myocardium contains all the cellular components for EC coupling found in mammalian, the role played by each in force generation are still undetermined. Several studies have demonstrated a correlation between the environmental temperature, level of activity, stimulation frequencies and the sarcoplasmic reticulum (SR) participation in E-C coupling. To address some of these issues, experiments *in vivo* and *in vitro* were performed with 4 teleost species (*Piaractus mesopotamis*, *Prochilodus lineatus*, *Hoplias malabaricus*, and *Synbranchus marmoratus*) at 15, 25 and 35 °C. Our results indicate that these species depend significantly on the intracellular Ca<sup>2+</sup> stores, irrespective of temperature or stimulation frequency. We suggest that the SR participation in E-C coupling is related to the species phylogeny and that a functional SR is a common characteristic of the order Ostariophysi.

**Introduction**

In ectothermic animals, including fish, the cardiac function changes in synchrony with body temperature. Vertical movements in the water column across thermoclines, or diurnal temperature changes in shallow streams may cause significant and rapid changes of body temperature, which will have immediate effects on the heart function. Since these changes are not necessarily

predictable and may be short-lived, the fish heart must have some intrinsic resistance against acute temperature changes (Vornanen et al., 2002). Moreover, anatomical and ultra-structural distinctions between hearts of different species underlie important physiological differences, particularly to the regulation of  $\text{Ca}^{2+}$  delivery to contractile apparatus (Tibbits et al., 1992).

Different fish species inhabit a variety of environments. Depending on their strategy of temperature adaptation, that determine life styles and activity levels, they also display a large variation in cardiac function. The present study examined the effects of acute temperature changes on cardiac function in four neotropical teleost species with different habitats and activity levels: *Piaractus mesopotamicus*, *Prochilodus lineatus*, *Hoplias malabaricus* and *Synbranchus marmoratus*.

Known in Brazil as muçum, the facultative air-breather *S. marmoratus*, burrow in the mud during the dry season they and use their modified buccopharyngeal chamber as an air-breathing organ. Traíra, *H. malabaricus*, a sedentary species highly tolerant to hypoxia, occurs in diverse habitats of Central and South America, from free flowing clear water streams, well up into the valleys, to slow turbid waters, and ponds. Curimatá, *P. lineatus*, and pacu, *P. mesopotamicus*, are active migratory species living in South American rivers and support important fisheries in many parts of the continent.

## Methods

The *in vivo* heart rate ( $f_H$  - bpm) was obtained by electrocardiography (for details see Rantin et al., 1993). After implantation of ECG electrodes, fish were left for a recovery period of 12 h. The  $f_H$  was measured at 25 °C and temperature was stepwise increased ( $10\text{ °C}\cdot\text{h}^{-1}$ ) to 35 °C and, subsequently, gradually reduced to 25 °C.

The *in vitro* preparations were made according to Anelli et al. (2004). Four protocols were developed: 1) Muscle preparations were subjected to a temperature transition from 25 to 35 °C at a physiological extracellular  $\text{Ca}^{2+}$  concentration (1.25 mM). Preparations were maintained at 35 °C for 30 min. Subsequently, the temperature was decreased at the same rate back to 25 °C and kept constant for 30 min. 2) Preparations were allowed to stabilize at 25 or 35°C and the extracellular  $\text{Ca}^{2+}$  was increased (from 1.25 to 11.25 mM) to assess the effect of inotropes on E-C coupling and the potential contribution of transsarcolemmal  $\text{Ca}^{2+}$  transporting mechanisms. 3) Preparations were subjected

to increases in imposed contraction frequency from 0.2 Hz until the frequency in which at least 80% of the strips were still able to contract regularly. In order to analyze the potential contribution of the SR at more physiological frequencies, 10  $\mu\text{M}$  of ryanodine was added to the bath 40 min before alterations in frequency. These protocols were performed at 25 and 35°C. 4) The force developed by ventricle strips upon the first stimulation following a rest period was determined at 25 and 35°C. After a steady-state condition at 0,2 Hz was achieved, stimulation ceased for a period of 5 min. Force development of the first contraction following the resting period was compared to the last contraction in a steady-state train. These experiments were conducted with and without 10  $\mu\text{M}$  ryanodine in the medium. When applied, ryanodine was added to the medium at least 40 min before the diastolic pause.

## Results and Discussion

Figure 1 shows the effects of increases in temperature from 25 to 35 °C and the subsequent recovery to 25 °C on the  $f_H$  (bpm). The  $f_H$  increased significantly at 30 and 35 °C in all the four species analyzed. After the return to 25 °C, the  $f_H$  decreased to values similar to those recorded previously at the same temperature.

The positive chronotropism presented by the four species in response to an acute increase in temperature is similar to the data described for other teleost species. The  $f_H$  of teleost fish is determined by the intrinsic rate of pacemaker cells in the sinus venosus and its extrinsic control by the autonomic nervous system or humoral factors (Laurent et al., 1983).

The resting heart rates in fish are typically between 10 and 60 bpm, not exceeding 120 bpm (Farrell, 1991), like those observed in the present study. However, according to Rantin et al. (1998), resting heart rates follow a  $Q_{10}$  of about 2 during acute temperature transitions in temperate and tropical teleost fish, respectively. Conversely, *P. mesopotamicus* and *H. malabaricus* presented  $Q_{10}$  values of 3.5 and 3.0, respectively, during the transition from 25 to 35 °C, suggesting that increases in  $f_H$  are the main adjustment triggered to maintain the heart performance during increases in temperature (see below).

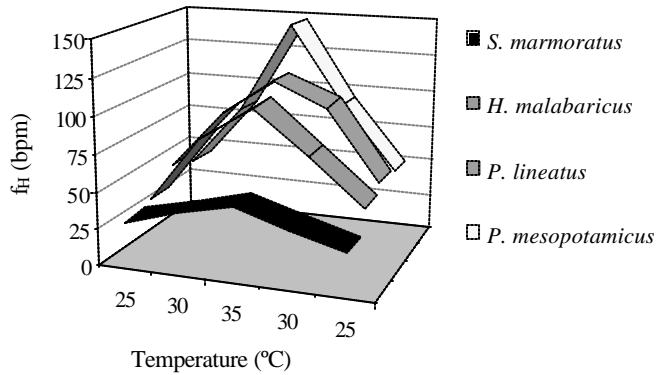


Figure 1: Effects of temperature transition from 25 to 35 °C and subsequent return to 25 °C on *in vivo* heart rate ( $f_H$  - bpm). Mean values (n = 10).

#### *Effects of acute temperature changes on twitch force*

The transition from 25 to 35 °C did not result in significant alterations in twitch force ( $F_c$ ) for *P. mesopotamicus*, *H. malabaricus* and *S. marmoratus* (Figure 2). However, *P. lineatus* presented significant decreases of  $F_c$ , reaching minimum values at 35 °C. During the recovery to 25 °C, the  $F_c$  of the four species increased gradually, achieving the initial values.

Elevating temperature influences myocardial twitch force development by several mechanisms, including increases in  $Ca^{2+}$  sensitivity of the contractile proteins and in the time at which the crossbridges develop maximal force (Harrison & Bers 1990). Moreover, results with unloaded rat cardiac muscle suggest that the cycle length of the actin-myosin interaction shortens, and the frequency of the interaction per myosin head increases with temperature (de Tompe & Keurs 1990). The  $F_c$  changes inversely with acute temperature changes in *Oncorhynchus mykiss* (Shiels & Farrell, 1997), *Lota lota* (Tiitu & Vornanen, 2001), *Carassius carassius* (Vornanen, 1989), *Scomber scombrus* (Shiels et al., 1999), and *Scomber japonicus* (Shiels & Farrell, 2000). The same tendency was recorded to *Bathygobius soporator* (Rantin et al 1998) and *Oreochromis niloticus* (Costa et al., 2000), and to *P. lineatus* in the present study. On the other hand, the maintenance of an unchanged inotropism during acute increases in temperature in *P. mesopotamicus*, *H. malabaricus* and *S. marmoratus*, together with the significant increase in the *in vivo*  $f_H$  reinforces the importance of the chronotropic adjustments in these species.

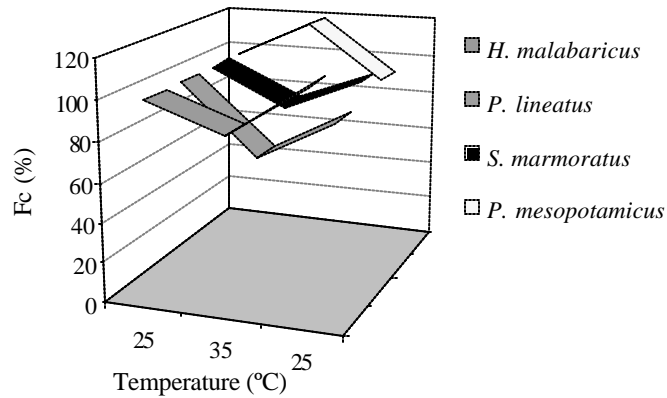


Figure 2. The effect of temperature transition from 25 to 35 °C and subsequent return to 25 °C on twitch force (Fc - % of the initial values). Mean values (n = 10).

#### *Extracellular calcium*

Calcium entry through the sarcolemma is primarily dependent on L-type calcium channels, and the importance of this route of calcium entry was determined by altering extracellular  $\text{Ca}^{2+}$  concentration ( $[\text{Ca}^{2+}]_e$ ). The effects of increments in  $[\text{Ca}^{2+}]_e$  on the peak force of ventricular myocardium were studied at 25 and 35 °C (Figure 3).

At 25 °C, increased  $[\text{Ca}^{2+}]_e$  caused significant increases in Fc of *P. lineatus*, *P. mesopotamicus* and *H. malabaricus* while the Fc of *S. marmoratus* did not change significantly. However, increased concentrations of  $\text{Ca}^{2+}$  caused significant increases in twitch force in all the four species at 35 °C.

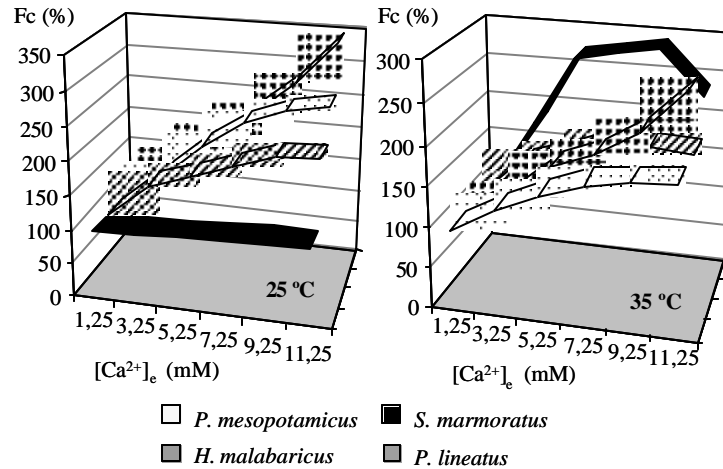


Figure 3. The effect of extracellular calcium ( $[Ca^{2+}]_e$  - mM) on twitch force (Fc - % of the initial values) at 25 and 35 °C. Mean values (n = 10)

Contraction of cardiomyocytes is initiated by the influx of extracellular  $Ca^{2+}$  through the sarcolemma, which stimulates the myofibrils directly and/or opens  $Ca^{2+}$ -release channels in the sarcoplasmic reticulum (Vornanen et al., 2002). The relative importance of these functions varies among taxa. Increases in myocardium Fc during increments in the  $[Ca^{2+}]_e$  were reported for *O. mykiss* (Coyne et al., 2000), *Anguilla rostrata* (Bailey et al., 2000), *B. soporator* (Rantin et al., 1998), *O. niloticus* (Costa et al., 2000), among other species.

Therefore, the elevation in Fc by the increments in the levels of extracellular calcium reveal a great dependence of the extracellular calcium to heart performance, in spite of the intracellular stocks. This dependence of extracellular  $Ca^{2+}$  seems to occur irrespective of temperature as recorded for *O. niloticus* (Costa et al., 2000) at 25 and 40°C. However, in *S. marmoratus* increases in Fc were recorded only at 35°C, demonstrating that contractile force in this fish myocardium is only partially dependent on calcium entry through the sarcolemma.

*Post-rest force development*

Figure 4 shows the effect of a prolonged diastolic pause on post-rest contraction force at different experimental temperatures with and without previous addition of ryanodine to the bath medium.

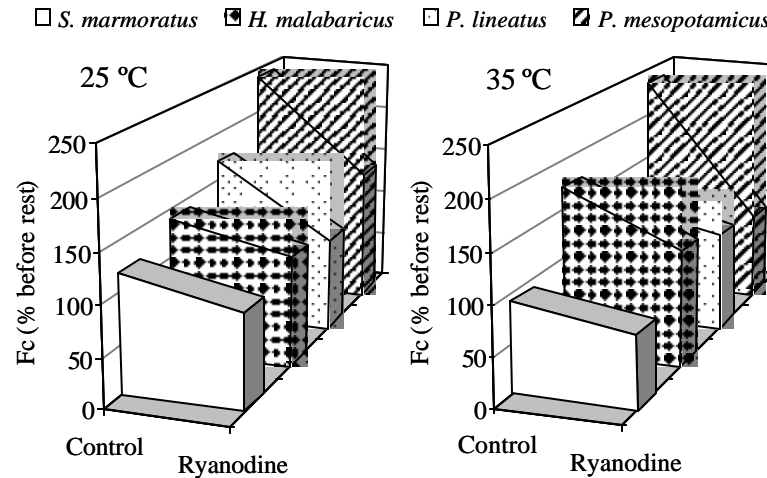


Figure 4. Effect of ryanodine on post-rest force development (Fc - % of values obtained before the rest period) at 25 and 35 °C. Tension is normalized to the last steady state twitch prior to rest (0.2 Hz). Mean values (n = 10).

The post rest potentiation recorded for all the control preparations at 25°C was inhibited by ryanodine. However, at 35°C, force remained unchanged in *S. marmoratus* control preparations, and a significant post rest decay was recorded in the presence of ryanodine.

The post-rest potentiation of twitch force has been related to a greater accumulation of  $Ca^{2+}$  within the SR. In the same direction, the increase of the first twitch force after a resting period is observed in many elasmobranch hearts, consistent with a relatively more developed SR than in teleost fish. Moreover, ryanodine has no effect on the development of post-rest potentiation in most teleost species (Driedzic and Gesser, 1988). An exception to the teleost pattern is the heart of rainbow trouts, in which the SR is anatomically much more developed and the ventricle strips show a post-rest potentiation of force that is strongly reduced by ryanodine, especially at high temperatures (Hove-Madsen and Gesser, 1989; Møller-Nielsen and Gesser, 1992). In spite of this, ryanodine

has no effect on force development when physiological temperatures are considered (Keen et al., 1994; Gesser, 1996; Shiels et al., 1998).

This result contrasts with those obtained in the present study, since ryanodine significantly reduced the post-rest force in all experimental temperatures, considering that these temperatures are normally observed in the species habitats. This implies that, differently from most fish already studied, the ventricle of these species present SRs that are functional at temperatures that are physiologically relevant at *in vivo* conditions.

Considering that *H. malabaricus* and *S. marmoratus* are sedentary species and the SR functionality of the four species, irrespective of temperature, we suggest that the phylogenetic background could explain the presence of a functional SR, since these species belong to the same superorder Ostariophysi. Likewise, phylogenetic aspects appear to underlie the absence of a  $\text{Ca}^{2+}$  releasing SR. Thus, some species that are insensitive to ryanodine belong to distinct taxonomic groups. Nile tilapia, tide pool goby, flounder and sea raven pertain to the superorder Acanthopterygii, while cod and trout are members of the superorder Protoacanthopterygii. Exceptions within the Acanthopterygii are the tunas *Katsuwonus pelamis* (Keen et al. 1992) and *Thunnus albacares* (Shiels et al., 1999) and the Pacific mackerel, *S. japonicus* (Shiels & Farrell, 2000), in which a functional SR is probably related to high levels of aerobic activity.

Another interesting point to be considered is the post-rest decay of twitch force that was observed for *S. marmoratus* at 35°C. A post-rest  $\text{Ca}^{2+}$  depletion has also been consistently observed in rabbit ventricle (Ferraz et al., 2001) and suggests an increased  $\text{Ca}^{2+}$  efflux from the cell during the resting period, probably due to a temperature-dependent increase of  $\text{Na}^+/\text{Ca}^{2+}$  exchange (NCX) activity, while SR  $\text{Ca}^{2+}$  accumulation remains almost unaltered.

#### *Force-frequency relationship*

Figure 5 shows the force-frequency relationship for preparations at 25 and 35 °C.

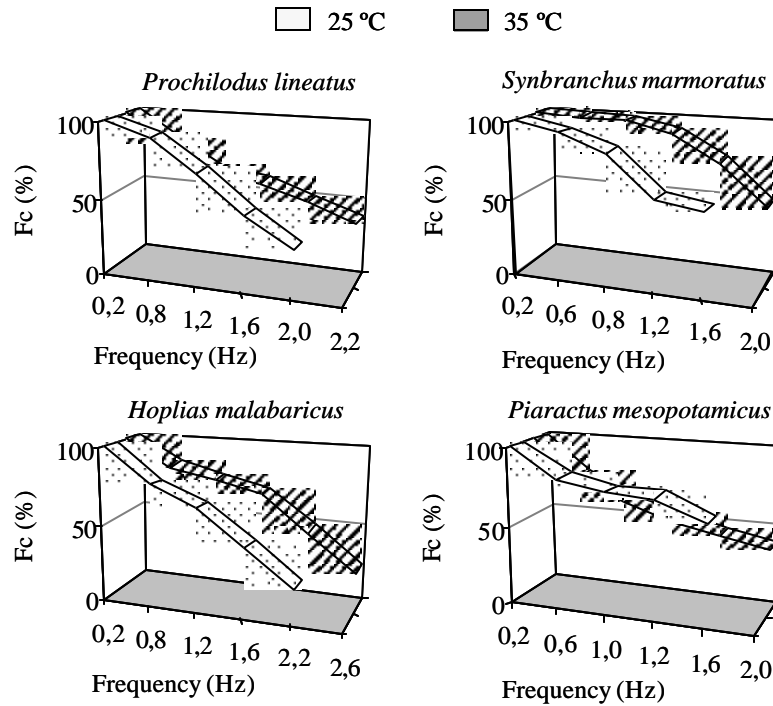


Figure 5. Effect of increases in stimulation frequency on twitch force (F<sub>c</sub> - % of initial values) of ventricle strips from *Prochilodus lineatus*, *Synbranchus marmoratus*, *Hoplias malabaricus* and *Piaractus mesopotamicus* at 25 and 35 °C. Force is normalised to the value for the lowest contraction frequency (0.2 Hz) for each species. Mean values (n = 10).

When normalized values are analyzed, a negative force-frequency relationship is depicted for the 4 species studied. However, when absolute values of force contraction are taken into consideration (Figure 6), decreases in force development of *S. marmoratus* and *H. malabaricus* control preparations were recorded only at 1.6 Hz (25 °C) and in frequencies higher than 1.2 and 1.6 Hz, respectively, at 35 °C. The ryanodine treatment shifted the response curves of these species downwards in both temperatures.

Most teleost fish present a negative force-frequency relationship (Driedzic and Gesser, 1985, 1988; Hove-Madsen and Gesser, 1989; Vornanen, 1989; Shiels

and Farrell, 1997; Rantin et al., 1998; Costa et al., 2000). These animals generally present a SR that does not contribute significantly to tension development when *in vivo* stimulation frequencies and temperatures are considered. However, there are a few species, such as tunas (Keen et al., 1992; Shiels et al., 1999), Atlantic cod (Driedzic and Gesser, 1985) and mackerels (Driedzic and Gesser, 1988; Shiels and Farrell, 2000), that do not show this negative force-frequency response. At least in tunas, this positive force-frequency relationship, associated with a pronounced effect of ryanodine in force development at atrial (Shiels et al., 1999) and ventricle strips (Tibbits, 1996), have suggested that their hearts have a higher reliance on SR  $\text{Ca}^{2+}$  for contraction over its normal temperature and stimulation frequencies range.

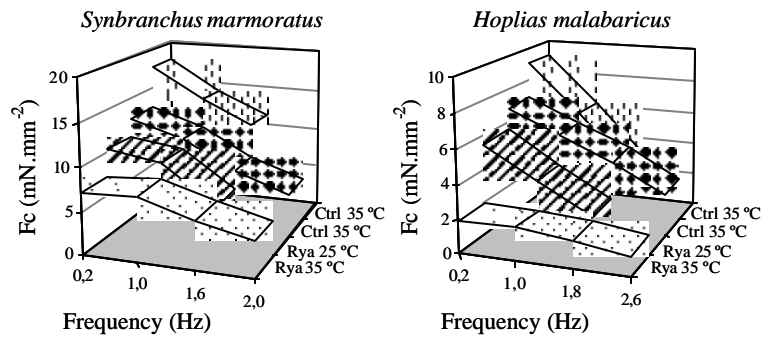


Figure 6. Effect of increases in stimulation frequency on twitch force ( $\text{Fc}$  -  $\text{mN}\cdot\text{mm}^{-2}$ ) of ventricle strips from *Hoplias malabaricus* and *Synbranchus marmoratus* at 25 and 35 °C in control preparations and after addition of 10 mM ryanodine. Mean values ( $n = 10$ ).

A negative force-frequency relationship was also observed in the present study for all the species when normalized values were analyzed. However, the absolute values of the  $\text{Fc}$  developed by *S. marmoratus* and *H. malabaricus* ventricle strips decreased only at frequencies higher than those observed *in vivo*. These findings suggest that these species ventricles have very efficient  $\text{Ca}^{2+}$ -transporting mechanisms, which would allow an adequate delivery of  $\text{Ca}^{2+}$  to myofilaments even after the rise in temperature or frequency-induced curtailment of the time to peak force at physiological conditions. Since ryanodine shifted the force-frequency relationship of both species downwards at 25 and 35°C, it may be proposed that the SR plays a functional role in the  $\text{Ca}^{2+}$  management at frequencies and temperatures observed *in vivo*. Moreover, this

organelle also seems to be important *in vivo* to generate force at acclimation temperature, because ryanodine reduced tension development at the stimulation frequencies situated inside the physiological range. Corroborating these findings, Hochachka and Hulbert (1978) have described an extensive elaboration of the interfibrillar SR of *S. marmoratus*, a striking feature that stands out the heart ultrastructure of this species from that of other fishes. Unfortunately, data on *H. malabaricus* heart ultrastructure are not available.

In conclusion, the studied species seem to present large stores of intracellular activator  $\text{Ca}^{2+}$  that could be a phylogenetic characteristic of the superorder Ostariophysi. These species also depend on extracellular sources of  $\text{Ca}^{2+}$  whose, according to Keen et al. (1992), provide more flexibility to modulate the contraction force.

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**DO THE HIGH HEART FREQUENCIES OBSERVED IN TROPICAL FISH NECESSARILY IMPLY THE PARTICIPATION OF THE SARCOPLASMIC RETICULUM ON CALCIUM MANAGEMENT?**

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**Abstract**

This study analyzed the effect of ryanodine (SR function blocker) on the ventricular isometric force ( $F_c$  -  $\text{mN/mm}^2$ ) and the *in vivo* heart frequency ( $f_H$  - bpm) of two tropical teleosts, the jeju, *Hoplerythrinus unitaeniatus*, and the acara, *Geophagus brasilienses*. While the  $f_H$  of acara ( $79,6 \pm 6,6$ ) was higher than that of jeju ( $50,3 \pm 2,7$ ), the opposite was observed for the  $F_c$  at 12 bpm (acara =  $28,66 \pm 1,86$  versus jeju =  $36,09 \pm 1,67$ ). After a diastolic pause of 5 min, strips from jeju showed a strong potentiation of  $F_c$  (~90%) that was completely abolished by ryanodine. Moreover, a ~20% decrease in  $F_c$  was observed after treatment with ryanodine either in steady-state contractions (12 bpm) or at physiological frequencies. Strips from acara were irresponsive to ryanodine, irrespective of the experimental condition. Thus, in tropical teleosts the higher metabolic demand and, as a consequence, the heart rate, does not seem to be the main factor determining the functionality of the SR, since jeju is much more sedentary than acara. These results reinforce the hypothesis of the functionality of the SR as a common trait in tropical Ostariophysian (as jeju), the opposite being valid to the tropical Acanthopterygian (as acara).

**Introduction**

The significance of SR in the contraction-relaxation cycle of cardiac muscle varies greatly among different vertebrate classes, among different species within the same phylogenetic group and during the ontogenetic development of an individual (Aho & Vornanen, 1998). In the mammalian heart,  $\text{Ca}^{2+}$  entering during the action potential enhances the release of further  $\text{Ca}^{2+}$  from the

sarcoplasmic reticulum (SR) to support the contractile process (Fabiato, 1983). In contrast, in frogs and teleost fish,  $\text{Ca}^{2+}$  required for contractility is derived under most circumstances primarily via transport across the sarcolemma (Tibbits et al. 1991).

Although present, the role of SR in the beat to beat regulation of contraction of the teleost heart requires further definition, as ryanodine, which impedes SR function, has a negative impact only at rates below the physiological range of frequencies in most species (Driedzic & Gesser 1994). Species-specific differences are also evident in the inhibition of contraction by ryanodine. In crucian carp heart ryanodine has no effect on ventricular contraction (Vornanen, 1996). In rainbow trout ventricle, ryanodine slightly reduces the force of contraction, especially at high experimental temperatures and at low contraction frequencies (Keen et al. 1994; Shiels & Farrell, 1997), and in the atrium of tuna heart, ryanodine exerts a clear negative inotropic effect (Keen et al. 1992) even at physiological frequencies.

These findings suggest that the more active fish have a higher  $\text{Ca}^{2+}$ -handling capacity in the cardiac SR than the less active species. However, studies on the effect of ryanodine on the ventricular muscle of tropical fish failed to demonstrate a direct relationship among the level of activity and the functionality of the SR, since ventricle strips from some active species (Rivaroli, 2002; Olle, 2003; Anelli Jr. et al., 2004) or fish adapted to acute transitions to high temperatures (Rantin et al., 1998; Costa et al., 2000), did not show a postrest potentiation of twitch force that could be abolished by ryanodine. Indeed, in highly sedentary tropical animals (Rivaroli, 2002; Costa et al., 2004), ryanodine had a strong inhibitory effect on postrest force as well as at physiological frequencies.

The results point to a phylogenetical trait determining the functionality of the SR in tropical fish, rather than its presence being exclusively determined by a high metabolic demand as a result of increased temperatures or activity. Therefore, in the present study we were interested in comparing the effect of ryanodine on the cardiac inotropism of two tropical teleosts phylogenetically distant and that present different levels of activity: the sedentary jeju, *Hoplerythrinus unitaeniatus* (Superorder Ostariophysi), and the more active acara, *Geophagus brasiliensis* (Superorder Acanthopterygii).

## Material and Methods

Adult specimens of jeju, *H. unitaeniatus*, were collected in the Paraná River Basin near Bataguáçu, Mato Grosso do Sul State, while acara, *G. brasiliensis*, were collected from local ponds within the campus area of the Federal University of São Carlos in Southeast Brazil. In the laboratory fish were maintained in 1000 L holding tanks supplied with aerated water at  $25 \pm 1^\circ\text{C}$  (acclimation temperature) for a minimum of four weeks prior to experimentation.

Fish were stunned by a blow to the head, the spine was cut and the heart was carefully removed. Ventricle strips ( $\phi \cong 1$  mm) were excised from the heart and placed into a bathing medium containing (in mM) 100 NaCl, 10 KCl, 1.2 MgSO<sub>4</sub>, 1.5 NaH<sub>2</sub>PO<sub>4</sub>, 27 NaHCO<sub>3</sub>, 2.5 CaCl<sub>2</sub> and 10 glucose and bubbled throughout the experiment with a gas mixture of 98% O<sub>2</sub> and 2% CO<sub>2</sub> (pH 7.5 at 25 °C). Preparations were connected to an isometric force transducer and to a Grass stimulator delivering electrical square pulses with a voltage 50% above the threshold. Twitch tension was allowed to stabilize for about 30 min at 0.2 Hz before each protocol. The temperature of the saline was regulated to  $25 \pm 0.5$  °C with the aid of a recirculating water bath.

In order to detail the capacity for the storage of Ca<sup>2+</sup> in the SR under a wide range of frequencies, the postrest force (after a diastolic pause of 5 min), the force developed in steady-state (12 bpm), and the force-frequency relationship were determined with and without pre-treatment with 10 μM of ryanodine. Moreover, *in vivo* heart rate ( $f_H = \text{QRS complexes/min}$ ) was measured by standard electrocardiography (lead D<sub>I</sub>) in order to estimate the potential contribution of the SR at more physiological frequencies.

## Results

After stabilization at 12 bpm at 25 °C the twitch force developed by ventricle strips of jeju and acara at steady-state (12 bpm) were  $36.09 \pm 1.67$  mN/mm<sup>2</sup> (mean  $\pm$  SE; n = 12) and  $28.66 \pm 1.86$  mN/mm<sup>2</sup> (mean  $\pm$  SE; n = 12), respectively. However, 30 min after the treatment with 10 μM of ryanodine, the twitch force of ventricle strips from jeju decreased ~20%, reaching force values similar to those observed for acara (figure 2). Moreover, the steady-state force developed by ventricle strips from acara after treatment with ryanodine remained unchanged in relation to the control.

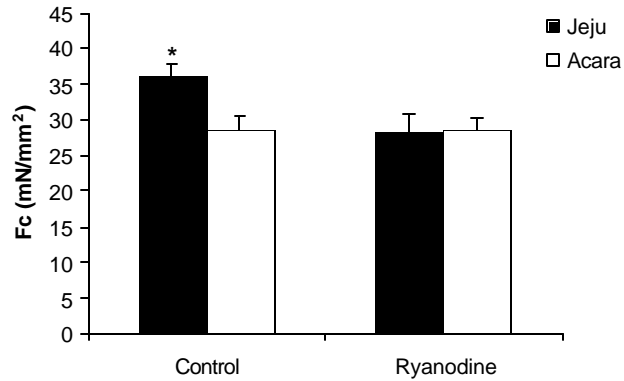


Figure 1 – Twitch force ( $F_c$  -mN/mm<sup>2</sup>; mean + S.E.) developed by ventricle strips from jeju (n = 12) and acara (n = 12) before (control) and after (ryanodine) 30 min of exposure to 10  $\mu$ M of ryanodine during steady-state stimulation (12 bpm). The asterisk above the vertical bar denotes a significant ( $p < 0.05$ ) difference in relation to the other force values.

The relative contribution of the  $Ca^{2+}$  stored in the SR to force generation after a diastolic pause of 5 min is presented in figure 2, where it is compared to the results obtained to other species. The pause resulted in an increase of ~90% in the force developed by ventricle strips from jeju in relation to the steady-state contraction, which was completely abolished by ryanodine (~40% of reduction in relation to the control). In contrast, in ventricle strips from acara there was no changes in twitch force of either control preparations or after treatment with ryanodine.

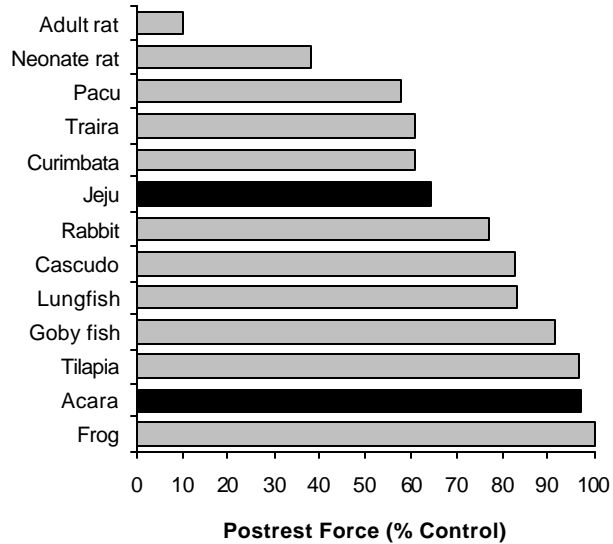


Figure 2 - Effect of 10  $\mu\text{M}$  of ryanodine on the force developed by ventricle strips from jeju ( $n = 12$ ) and acara ( $n = 12$ ) after a 5 min pause ( $F_c$  - % control; mean values). The values obtained for jeju and acara are compared to those described to other species (Rantin et al., 1998: goby fish; Costa et al., 2000: tilapia; Bers, 2001: frog, rabbit, adult and neonate rat; Rivaroli, 2002: cascudo and curimbata; Olle, 2003: traيرا; Anelli Jr. et al., 2004: pacu; Costa et al., 2004: lungfish) at 25  $^{\circ}\text{C}$  (fish) or at room temperature (frog and mammals).

In the force-frequency experiments, the ventricle strips from both animals showed a negative relationship, in spite of the deeper curve presented by jeju (figure 3). However, since the initial absolute values of force were higher for jeju than for acara (figure 1), the force measured at *in vivo* frequency range to each species was higher for jeju ( $\sim 20\text{mN}/\text{mm}^2$  at 50,3 bpm) than for acara ( $\sim 17\text{mN}/\text{mm}^2$  at 79,6 bpm). Additionally, the treatment of ryanodine did not shift up- or downwards the force-frequency curves of either species, but for jeju this curve was shifted to the left in response to ryanodine (from 96 to 72 bpm). At 79,6 bpm ( $f_H$  *in vivo*), the twitch force developed by the ventricle strips from jeju was decreased in  $\sim 4\text{mN}$  after treatment with ryanodine, but the absolute

values of force measured for control and ryanodine strips from acara did not differ from each other in all the range of frequencies tested.

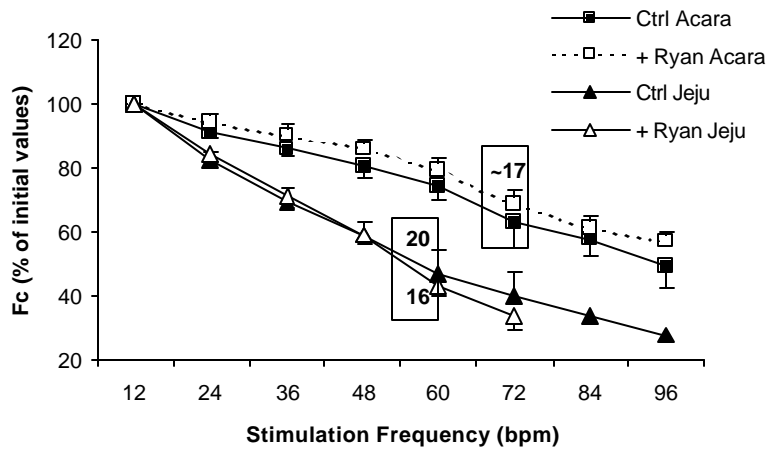


Figure 3 - Effect of increases in frequency on the force (Fc - % of initial values  $\pm$  S.E., n = 10) developed by control preparations (black symbols) and after treatment with 10  $\mu$ M of ryanodine (open symbols) by ventricle strips from jeju (triangles) and acara (squares). The values above the curves denote the approximate absolute force (mN/mm<sup>2</sup>) registered at the frequencies measured *in vivo* for jeju ( $f_H = 50,3 \pm 2,7$  bpm) and acara ( $f_H = 79,6 \pm 6,6$  bpm). In spite of the similar curves obtained for jeju to both treatments, the absolute force was reduced by ~4 mN after treatment with ryanodine at the physiological frequency range.

### Discussion

In temperate teleosts it has been demonstrated that ryanodine channels remain in an “open state” over a longer period of time as a result of the lower testing temperatures (Hove-Madsen et al., 2001). This fact minimizes the role of the SR as a calcium source to the activation of myofibrils (Tiitu & Vornanen, 2001). However, the myocytes of cold-adapted fish developed adaptative mechanisms that led to a relative temperature-insensitivity of sarcolemmal Ca<sup>2+</sup>-transporting systems (particularly L-channels and NCX), maximizing the proportional contribution of transsarcolemmal Ca<sup>2+</sup> fluxes to the

relaxation/contraction cycle (Tibbits et al., 1992; Xue et al., 1999; Kim et al., 2000; Shiels et al., 2000; Elias et al., 2001). This allows  $\text{Ca}^{2+}$  ions to be delivered to myosin in a tax and magnitude compatible with the low heart rates observed in cold-adapted fish (Farrell & Jones, 1992; Driedzic & Gesser, 1994; Lillywhite et al., 1999), assuring their survival in temperatures considered cardioplegic to endotherms, even without the direct participation of the SR on  $\text{Ca}^{2+}$  management.

In contrast, in very active teleosts, as well as in tropical fish, which present considerably higher heart rates, a more direct participation of the SR in the E-C coupling in order to reduce the diffusional distances is predictable. A greater anatomic development of the SR, as well as a potential role of this organelle in the E-C coupling, has been described to rainbow trout, *Oncorhynchus mykiss*, especially at high temperatures and sub-physiological frequencies (Santer, 1974; Hove-Madsen & Gesser, 1989; Hove-Madsen, 1992; Møller-Nielsen & Gesser, 1992; Shiels & Farrell, 1997; Aho & Vornanen, 1998; Hove-Madsen et al., 1998; Lillywhite et al., 1999). Indeed, some recent studies (Harwood et al., 2000; Hove-Madsen et al., 2001) have also demonstrated a direct participation of the SR of trout in force development at more physiological conditions. An even higher contribution of the SR  $\text{Ca}^{2+}$  stores at physiological frequencies was described to the “athletic” scombrids (tunas and mackerels) (Shiels et al., 2002). Corroborating these findings, Rivaroli (2002) and Anelli Jr. et al. (2004) demonstrated the direct contribution of the SR to the ventricular inotropism at physiological frequencies and temperatures in two very active neotropical teleosts, the curimbata, *Prochilodus lineatus*, and the pacu, *Piaractus mesopotamicus*, respectively.

On the other hand, other studies failed to demonstrate such a direct relationship among level of activity and/or temperature *versus* functionality of the SR. Ventricle strips from Nile tilapia, *Oreochromis niloticus* (Costa et al., 2000), a species well-adapted to temperatures high temperatures such as 35 °C, or from fish that face acute transitions from 25 to more than 40 °C in few hours, such as the tide pool goby *Bathygobius soporator* (Rantin et al., 1998), did not show a postrest potentiation of twitch force that could be inhibited by ryanodine, dismissing the SR participation, even at high temperatures and low frequencies. Interestingly, both species are, as well as acara, Acanthopterygians, in which the E-C coupling has been shown to depend exclusively upon the extracellular  $\text{Ca}^{2+}$  sources at physiological (figure 3), and sub-physiological (figures 1 and 2) frequencies. Moreover, is relevant to emphasize that the cichlids acara and Nile

tilapia have common monophyletic origin (Kullander, 1998), presenting similar modes of life, levels of activity, and habitats.

Additionally, in some highly sedentary tropical fish, as traíra, *Hoplias malabaricus* (Olle, 2003), and cascudo, *Hypostomus regani* (Rivaroli, 2002), ryanodine exerted an inhibitory effect on inotropism at physiological temperatures. Traíra and jeju are erythrinid fish, considered one of the most archaic among the characiforms (Géry, 1977), but jeju is more active than traíra, what could partially account to the greater participation of the SR of jeju. (figure 2).

Due to the fact that a functional SR was found in both sedentary (traíra and cascudo) and active (curimbata and pacu) Ostariophysian fishes, it may be concluded that a functional SR is an ancestral trait of this group of tropical teleosts and not related to activity level. In contrast, in the tropical Acanthopterygians, such as acara, Nile tilapia, and tide pool goby, the opposite seems to have happened. Nevertheless, a larger variety of tropical species from the two superorders remain to be studied to prove the previous statement.

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**O<sub>2</sub> CHEMORECEPTORS INVOLVED IN THE CONTROL OF AIR-  
BREATHING AND AQUATIC SURFACE RESPIRATION IN  
NEOTROPICAL FISH**

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**Abstract**

Our purpose is to review the role of O<sub>2</sub> chemoreceptors involved on the control of respiratory function of air-breathing in jeju, *Hoplerythrinus unitaeniatus*, and aquatic surface respiration in tambaqui, *Colossoma macropomum*, in response to hypoxia. In jeju, denervation of the cranial nerve IX and the pretrematic branch of cranial nerve X serving the first gill arch did not abolish air-breathing in jeju. This response, however, was completely eliminated after branchial denervation. The control of air-breathing in jeju involves O<sub>2</sub> chemoreceptors distributed on all the gill arches. In tambaqui, complete denervation of the gills did not affect ASR, which was abolished after sectioning the cranial nerve V.

**Introduction**

The jeju (*Hoplerythrinus unitaeniatus*, Teleostei, Erythrinidae) is an air-breather and an active fresh water predator distributed in South America. The species is

normally found in streams and shallow waters in tropical and sub-tropical areas where it frequently survives periods of environmental hypoxia. The air-breathing organ (ABO) of jeju is the swim bladder, which is subdivided into an anterior and a posterior section. The posterior section is further subdivided into an anterior respiratory portion, richly vascularized, and a non-respiratory posterior sac. As a facultative air-breather, jeju relies primarily on its gills for gas exchange as long as the water is normoxic or moderately hypoxic, but uses its ABO as a facultative option during severe environmental hypoxia (Carter and Beadle, 1931; Kramer, 1978; Stevens and Holeton, 1978; Withers, 1992). Since jeju can extract O<sub>2</sub> from water (gills) and, alternatively, from air (swim bladder), the species is considered a bimodal respirator (Graham, 1997). The partitioning of O<sub>2</sub> uptake from the air and from the water in jeju was studied by Stevens and Holeton (1978), and its air-breathing pattern was well established by Kramer (1978). However, some cardio-respiratory aspects involved in the transition from water- to air-breathing still remain to be clarified. Lopes (2003) found that the threshold for the air-breathing in jeju coincided with significant increases in the respiratory frequency ( $f_R$ ) and gill ventilation ( $\dot{V}_G$ ), and significant decreases in the O<sub>2</sub> extraction from the ventilatory current (EO<sub>2</sub>) and heart rate ( $f_H$ ). The significant changes in these cardio-respiratory variables also coincided with the critical O<sub>2</sub> tension (PcO<sub>2</sub>) for this species. This author also reported that, in jeju, the O<sub>2</sub> chemoreceptors involved on the control of  $f_R$  and  $f_H$  are distributed to all gill arches and are both internally (monitoring the PaO<sub>2</sub>) and externally (monitoring the PiO<sub>2</sub>) oriented.

The tambaqui (*Colossoma macropomum*, Teleostei, Serrasalminidae) 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 dermal swelling of lower lip. This 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 begins 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). In this species, the O<sub>2</sub> receptors eliciting the reflex bradycardia and increase in breathing frequency during hypoxia are situated on all gill arches and sense O<sub>2</sub> changes in both arterial blood and inspired water (Sundin et al., 2000). On the other hand, the Q 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), a protocol that has been frequently employed in studies on O<sub>2</sub> 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 (PO<sub>2</sub> = 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 O<sub>2</sub> receptors involved in the cardiorespiratory reflex responses and found that they were the same as those which trigger ASR and elicit the development of the lower lip swelling. These authors found that ASR and inferior lip swelling were not abolished by total gill denervation, as previously observed by Sundin et al. (2000). They also observed that both intact and denervated fish developed inferior lip swellings during hypoxia and, at the end of 360 min of exposure to severe hypoxia, there was no difference in lip dimensions between the two groups. These data and the previous studies of Sundin et al (2000) and Milsom et al. (2002) suggest the participation of extrabranchial O<sub>2</sub> receptors, possibly located in the orobranchial cavity, on the increase in V<sub>AMP</sub> 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 location and role the O<sub>2</sub> chemoreceptors involved on the control of air-breathing in jeju and ASR in tambaqui. Furthermore, we compare in which extent the mechanisms controlling air-breathing and ASR in both species are related.

## **Material and Methods**

### *Experimental animals:*

Adult specimens of jeju, weighing  $250 \pm 50$  g, were collected in the Paraná River Basin near Bataguçu, Mato Grosso do Sul State. In the laboratory fish were maintained in 1000 L holding tanks supplied with aerated water (normoxic conditions, PwO<sub>2</sub>  $\geq$  130 mmHg) at  $25 \pm 1^\circ\text{C}$  (acclimation temperature). Fish were fed weekly with live food (smaller live fish of various species), but food was withheld for 2-3 days before trials.

Tambaqui weighing  $657 \pm 39$  g were obtained from the Center of Aquaculture of São Paulo State University (CAUNESP), Jaboticabal, SP. In the laboratory, fish were kept at the same conditions mentioned above. The fish were fed *ad libitum* with commercial food pellets but were fasted for two days prior to experimentation.

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

In order to denervate the cranial nerves IX and X, fish were anesthetized and moved to a surgical table where they were artificially ventilated. The denervation protocol was the same as described by Sundin et al. (2000). Under a stereoscopic microscope (Opto SM 2001, Opto Electronics, São Carlos, SP, Brazil), the operculum was reflected forward, and a small incision (2 cm) was made in the epithelium at the dorsal end of the 1<sup>st</sup> and 2<sup>nd</sup> gill arches where they meet the roof of the opercular cavity. The incision allowed the access to the cranial nerve IX and the pretrematic branches of the nerve X. Cranial nerve IX alone was sectioned (group IX – n = 10). In another group of fish, both cranial nerve IX and the pretrematic branch of cranial nerve X to the first gill arch were sectioned, completely denervating the first gill arch (group G1 – n = 10). In a third group, the cranial nerve IX and all branches of cranial nerve X going to the gills were sectioned, completely denervating all four gill arches (group G4 – n = 10). The same procedure was then performed on the other side so that all denervations were bilateral. The cardiac and visceral branches of the vagus were preserved in all the cases. The healing process in jeju 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.

*Air-breathing frequency and duration - Jeju:*

Air-breathing frequency ( $f_{AR}$ ) and air-breathing duration ( $T_{AR}$ ) were recorded using an experimental setup specially developed for this purpose. The upper part of the experimental chamber consisted of an “inverted funnel”, the neck of which housed an electric bulb positioned in front of a photoelectric cell. To breathe air fish were forced to pass throughout the neck, interrupting the light circuit between the bulb and the photocell. This interruption was detected by a decoder/amplifier in which a square wave was generated and recorded by a data acquisition system (DI 154 Dataq Instruments). Different hypoxic levels were achieved by bubbling the lower part of the experimental chamber with controlled amounts of N<sub>2</sub>. The water PO<sub>2</sub> was continuously monitored by the electrode of a FAC-204A O<sub>2</sub> analyzer connected to a micro-processed system controlling a solenoid valve.

#### *Experimental protocol*

Before experimentation fish were kept overnight period in the experimental chamber. After recording eventual air-breathing episodes during normóxia (140 mmHg), intact fish and fish of all denervated groups were subjected to the following hypoxic tensions: 94, 64, 45, 35, 26 and 17 mmHg for 30 min each tension.

#### *Denervation of cranial nerves V, VII, IX and X - Tambaqui:*

For denervation of cranial nerves V (trigeminal) and VII (facial), fish were anesthetized and moved to the surgical table where they were artificially ventilated. 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 kept intact. The healing process in tambaqui was rapid, and the incisions were covered with “scar tissue” within 24 h. The denervations resulted in two experimental groups: group V (cranial nerve V sectioned) and group V + VII (cranial nerves V + VII sectioned). 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. Denervation of cranial nerves IX and X followed the same procedure as described above for jeju. The complete denervation of cranial nerves IX and X to the gills resulted in the experimental group G4.

#### *ASR frequency and duration - Tambaqui:*

To examine the effects of hypoxia on ASR and development of lip swelling, 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  $N_2$ . 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  $N_2$  released from the water and kept a constant atmospheric gas concentration on the water surface, so as to maintain the  $PO_2$  of the surface layer about 10 mmHg higher than in the rest of the tank. 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 three distinct phases: The first with intact fish, the second with group V, and the third with group V+VII. In all phases tambaqui were exposed for a period of 360 min to severe hypoxia ( $PwO_2 = 10$  mmHg). 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 and Discussion**

Figure 1 shows the air-breathing frequency ( $f_{AR}$ ) of jeju (groups intact, IX, G1 and G4) exposed to graded hypoxia. Bilateral denervation of cranial nerves XI and (group IX) and denervation of the 1<sup>st</sup> gill arch (cranial nerve IX and the preterminal branch of cranial nerve X to the first gill arch - group G1) did not abolish the response. However, after complete gill denervation (group G4) the response was entirely abolished. Although fish of group IX presented significantly lower  $f_{AR}$  in relation to the intact ones, no statistical differences were observed within the curves of intact fish and G1. The air-breathing duration (time spent in air-breathing -  $T_{AR}$ ) also followed the same tendency as observed for  $f_{AR}$  (figure 2).

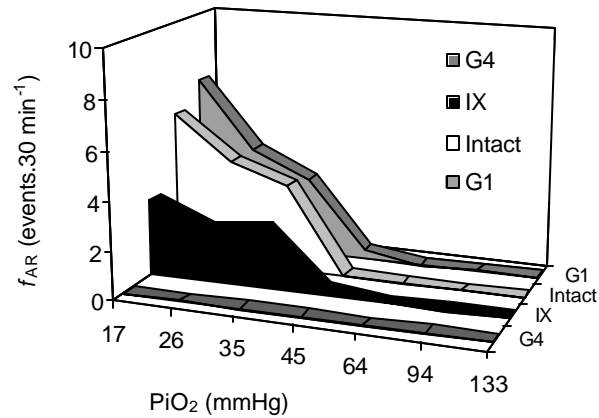


Figure 1. Air-breathing frequency ( $f_{AR}$ ) of jeju, *Hoplerythrinus unitaeniatus* (n = 10) in response to gradual reduction in the  $PiO_2$ .

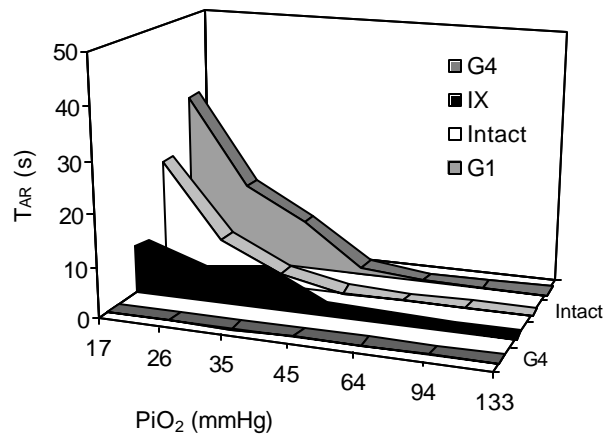


Figure 2. Air-breathing duration ( $T_{AR}$ ) of jeju, *Hoplerythrinus unitaeniatus* (n = 10), in response to gradual reduction in the  $PiO_2$ .

These results indicate that the air-breathing behavior performed by jeju in response to graded hypoxia is elicited by  $O_2$  chemoreceptors, probably distributed by the 2<sup>nd</sup>, 3<sup>rd</sup> and 4<sup>th</sup> gill arches and. These receptors, as previously described by Lopes (2003), are internally and externally oriented and monitor both arterial  $PO_2$  and the  $PiO_2$ , respectively.

Smatresk (1991) obtained similar results with the air-breather *Lepisosteus oculatus*. In this species, the partial denervation of the gills only attenuated the air-breathing frequency, which was totally abolished after complete bilateral section of cranial nerves IX and X. The complete denervation of the gills and pseudobranches of *Amia calva* abolished its accessory air-breathing in response to hypoxia, evidencing that, in this species, the air-breathing behavior is associated with O<sub>2</sub> chemoreceptors innervated by the cranial nerves VII, IX and X (McKenzie et al., 1991).

Figure 3 presents the time spent by tambaqui in ASR in response to acute hypoxia (PiO<sub>2</sub> = 10 mmHg). Complete denervation of cranial nerves IX and X failed to abolish the ASR behavior, which was completely abolished by bilateral section of cranial nerve V (group V). The V+VII denervated group also did not perform ASR. Denervation of cranial nerves V and V+VII, however, 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.

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 O<sub>2</sub> 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 O<sub>2</sub> chemoreceptors located within the orobranchial cavity innervated by cranial nerve V, as suggested by Sundin et al (2000) and Milsom et al. (2002).

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. This suggests that the formation of inferior lip swelling in tambaqui is either controlled by O<sub>2</sub> receptors located outside the gills and orobranchial cavity or results from a direct effect of hypoxia/hypoxemia on the lip tissue itself.

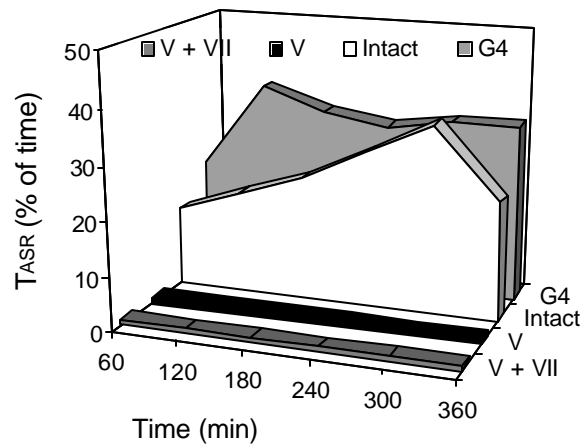


Figure 3. Time spent in ASR ( $T_{ASR}$ ) by intact (control,  $n = 10$ ) and denervated (group G4,  $n = 10$ ; group V,  $n = 8$ ; group V+VII,  $n = 8$ ) tambaqui, *Colossoma macropomum*, during exposure to severe hypoxia ( $PwO_2 = 10$  mmHg) for 360 min.

Our data document that the control of ASR in response to hypoxia is related to  $O_2$  chemoreceptors that are distinct from those that elicit air-breathing. Both mechanisms allow the fish to achieve more oxygen, when confronted with ambient hypoxia, but the receptors seem to have evolved independently.

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**CARDIORESPIRATORY AND TISSUE ADENOSINE RESPONSES TO  
HYPOXIA AND REOXYGENATION IN THE SHORTHORN SCULPIN,  
MYOXOCEPHALUS SCORPIOUS**

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**EXTENDED ABSTRACT ONLY – DO NOT CITE**

Adenosine is a product of ATP breakdown that exerts protective effects on tissues during hypoxia. Accumulation of adenosine under hypoxia is well documented in mammals but cardiac adenosine content has not been measured in fish, despite the importance attributed to its actions (Nilsson and Holmgren, 1992). Exogenous adenosine is widely used in investigations of cardiovascular physiology in fish but its effects are variable and concentration-dependent. For these reasons, it is important to characterize adenosine levels in fish.

The purpose of this study was to characterize adenosine levels in a teleost fish under hypoxia and reoxygenation. Investigations were carried out on the shorthorn sculpin, *Myoxocephalus scorpius*, a North-temperate species that exhibits considerable hypoxia resistance. Adenosine content was measured by HPLC in heart and brain tissue from animals exposed to acute hypoxia and to graded hypoxia and reoxygenation at 8°C. Plasma lactate and ions were measured to gain insight into the relationship between known hypoxia-inducible parameters and tissue adenosine levels. Cardiorespiratory parameters were also

recorded under graded hypoxia and reoxygenation using a ventral aortic flow probe and impedance electrodes.

#### *Cardiorespiratory response to hypoxia / reoxygenation*

Cardiorespiratory data for sculpin exposed to hypoxia and reoxygenation are presented in Figure 1. Ventilation rate ( $f_v$ ) increased slightly at 5.6 mg·l<sup>-1</sup> dissolved oxygen (DO<sub>2</sub>) saturation but subsequently declined and remained significantly lower than normoxic levels throughout hypoxia and reoxygenation. Hypoxia resulted in a significant decrease in heart rate ( $f_H$ ), by more than 60% after 6 h at 2.0 mg·l<sup>-1</sup> DO<sub>2</sub>. Bradycardia was accompanied by a 65% decline in cardiac output (Q), to a minimum of 8.8 ml·min<sup>-1</sup> following 5 h at 2.0 mg·l<sup>-1</sup> DO<sub>2</sub>. Stroke volume (SV) was high and was maintained under hypoxia, indicating that no attempt was made to defend blood flow. Both  $f_H$  and Q recovered to initial normoxic levels following reoxygenation.

#### *Metabolic response to hypoxia / reoxygenation*

Tissue adenosine levels and plasma parameters are presented in Figure 2. Acute hypoxia elevated plasma Ca<sup>2+</sup> and K<sup>+</sup> but had no effect on lactate. During graded hypoxia, plasma lactate and Ca<sup>2+</sup> were elevated over initial normoxic concentrations after 4 h at 2.0 mg·l<sup>-1</sup> DO<sub>2</sub>, while K<sup>+</sup> levels were unaffected. Cardiac adenosine content was similar to values for mammalian heart and brain levels were comparable to those in shark. Adenosine content was unaffected by acute hypoxia. Adenosine was maintained in heart under graded hypoxia and showed no clear trend of accumulation. Brain adenosine fluctuated throughout hypoxia and was significantly higher after 4 h of exposure to 2.0 mg·l<sup>-1</sup> DO<sub>2</sub> than after either 2 or 6 h of exposure, but was not different from initial normoxic levels.

Following 6 h at 2.0 mg·l<sup>-1</sup> DO<sub>2</sub> some animals were subjected to reoxygenation. Reoxygenation for 0.5 h resulted in a significant decrease in cardiac adenosine content compared to levels observed at 6 h at 2.0 mg·l<sup>-1</sup> DO<sub>2</sub> that was no longer evident after 1 h reoxygenation. Brain adenosine content was unaffected. Plasma K<sup>+</sup> increased at reoxygenation and lactate remained elevated.

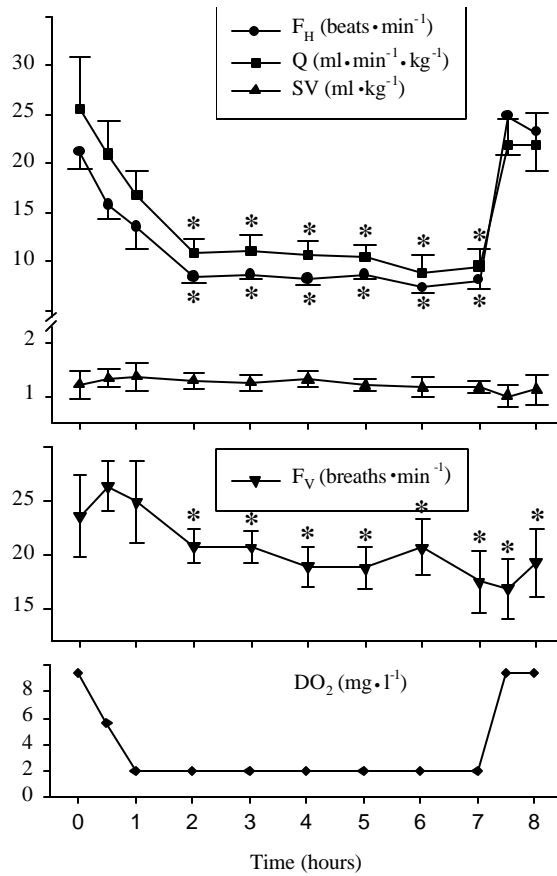


Figure 1. Cardiorespiratory parameters for *M. scorpius* subjected to hypoxia and reoxygenation. \* indicates significant difference from initial normoxic values ( $P < 0.05$ ).

## Discussion

This study provides the first information regarding cardiac adenosine levels in fish. Sculpin maintained cardiac adenosine during extended hypoxia. In contrast, adenosine can increase up to 55-fold during ischemia in mammalian heart (Mullane and Bullough, 1995). Elevated lactate concentrations indicate that hypoxia was sufficient to impair aerobic metabolism, suggesting that the lack of

adenosine accumulation cannot be ascribed to adequate oxygen supply. The lack of cardiac adenosine accumulation is probably due to the observed depression of cardiac activity. A drop in cardiac activity should reduce tissue oxygen requirements and defend high-energy phosphate levels. Given that plasma lactate levels were elevated throughout hypoxia it seems reasonable that anaerobic metabolism was robust enough to defend ATP levels.

Brain adenosine levels did increase significantly after 4 h at 20% DO<sub>2</sub> and were higher than cardiac levels as a whole, reflecting an inability of the brain to maintain ATP levels. In the freshwater turtle, *Trachemys scripta*, extracellular adenosine levels fluctuate by several fold during long term anoxia (Lutz and Kabler, 1997). It is possible that sculpin exhibit similar fluctuations but that the sampling methods employed here do not provide the resolution to elucidate this phenomenon.

#### **Acknowledgements**

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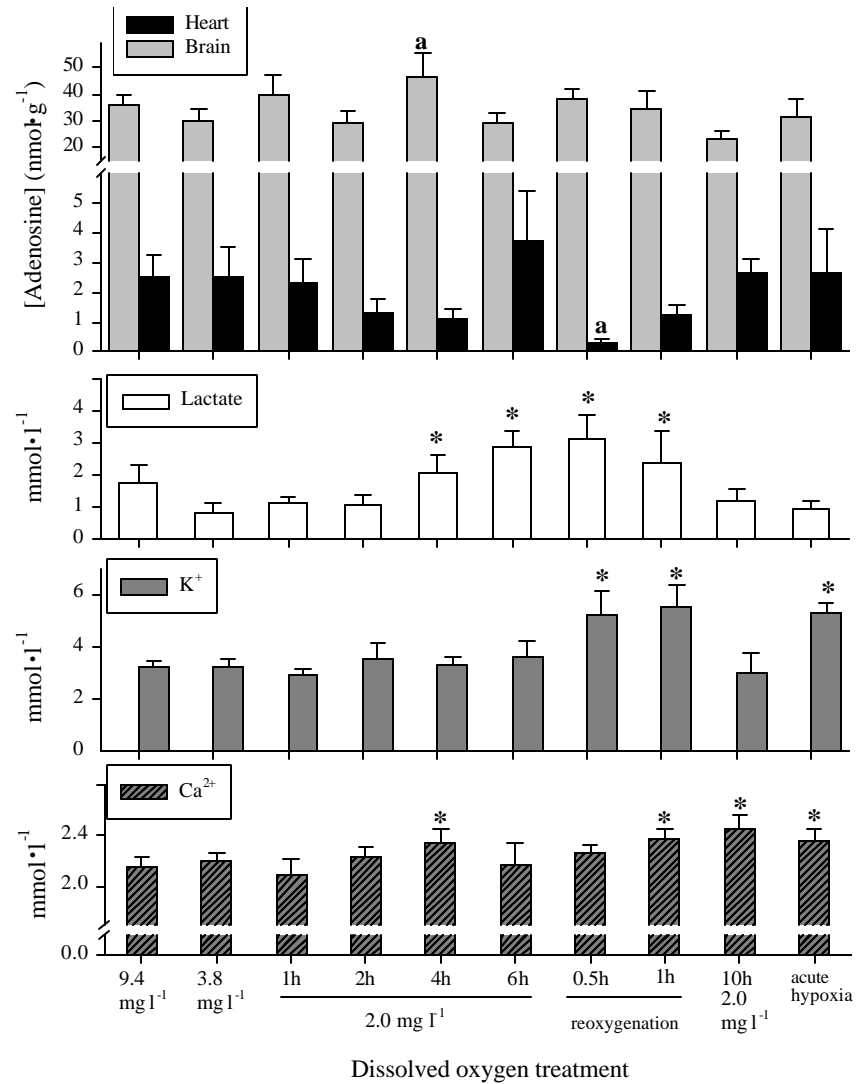


Figure 2. Tissue adenosine and plasma lactate, K<sup>+</sup>, and Ca<sup>2+</sup> levels in *M. scorpius* subjected to acute hypoxia and to graded hypoxia and reoxygenation (N=8 for all treatments). <sup>a</sup> indicates significant difference from measured value

immediately previous ( $P < 0.05$ ). \* indicates significant difference from values measured under initial normoxia ( $P < 0.05$ ).....

**THE INFLUENCE OF CHANGES IN ACTIVITY LEVEL  
AND TEMPERATURE  
ON CARDIO-RESPIRATORY CONTROL,  
A STUDY OF THE SHORT-HORNED SCULPIN  
(*MYOXOCEPHALUS SCORPIUS*) AND  
THE BLACK COD (*PARANOTO THENIA ANGUSTATA*)**

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**EXTENDED ABSTRACT ONLY: DO NOT CITE**

It has previously been shown, through pharmacological blockade and nerve transection, that the degree of cardiac vagal or adrenergic tone on the fish heart varies greatly between species and with activity levels, temperature or oxygen level within species (Taylor, 1992). Consequently, a systematic study of variations in tonic control of the heart in fishes, at a range of temperatures and activity levels, may serve to clarify the mechanisms of beat-to-beat control of the fish heart.

In an initial study (Campbell et al, 2004) we explored the use of Power Spectral Analysis in examining the neural influences on the teleost heart. The short-horned sculpin (*Myoxocephalus scorpius*) was chosen as an example of a labriform swimmer of limited aerobic scope associated with sit-and-wait predation. We also explored the neuranatomy of the vagal sensory and motor columns and the role of glutamate as a neurotransmitter in the modulation of vagal control of ventilation, heart rate and blood pressure in the sculpin (Sundin et al, 2004). In order to explore the influence of ecological factors such as temperature on heart rate, ventilation and oxygen uptake, this study was extended to the sub-Antarctic black cod (*Paranotothenia angustata*).

Oxygen consumption ( $MO_2$ ) and heart rate ( $f_H$ ), measured as the ECG and expressed as R-R interval, were monitored simultaneously in the short-horned sculpin. Anaesthesia and minor surgery to place e.c.g. recording electrodes caused an initial increase in  $MO_2$ . This was accompanied by a decrease in mean R-R interval and in heart rate variability (HRV), measured as the standard deviation in the R-R interval (SDRR). Mean R-R interval increased to a steady state value 72 h post-surgery, but mean SDRR took 120 h to stabilise. The progressive, post-surgical recovery in  $MO_2$  showed a good correlation with both mean R-R interval and the mean SDRR, although a more significant covariance existed between the decrease in  $MO_2$  and increase in SDRR.

Power spectral analysis (PSA) applied to recordings of instantaneous heart rate showed no spectral peaks immediately after surgery, though high and low frequency peaks were identified 120 h post-surgery. Bilateral cardiac vagotomy abolished the variability in beat-to-beat interval and both peaks, suggesting that much of the regulation of heart rate and HRV in sculpin was under parasympathetic, cholinergic control that was withdrawn as a result of surgical and handling stress. These data suggest that sculpin use  $f_H$  as a way of moderating oxygen consumption, fine-tuned on a beat-to-beat basis by cholinergic control.

The locations of the vagal sensory (Xs) and motor (Xm) areas in the medulla were established by the ortho- and retrograde axonal transport of the neural tract tracer Fast Blue, following its injection into the ganglion nodosum. Unilateral microinjection of glutamate into defined locations in the Xs caused marked changes in  $f_H$ , blood pressure, ventilation frequency or amplitude. Often these responses occurred simultaneously in different combinations, but occasionally, they appeared singly, suggesting specific projections into the Xs, for each cardiorespiratory variable and local determination of the modality of the response. Response patterns related to chemoreceptor reflex activation were predominantly located rostral of obex, whereas patterns related to baroreceptor activation were more caudal, around obex.

The e.c.g, ventilation rate and oxygen consumption were measured simultaneously in the black cod, during 96 h periods of recovery from surgical intervention or feeding. Both caused an elevation of  $MO_2$ , accompanied by shortened R-R and V-V intervals. Over the subsequent 96 h there was a progressive reduction in  $MO_2$  which showed a significant correlation with increases in R-R and V-V interval. Vagotomy caused a similar decrease in R-R

and V-V intervals, but  $MO_2$  did not increase to the same extent. With time post-vagotomy R-R or V-V interval did not show any significant increases and  $MO_2$  did not significantly alter from immediately after vagotomy.

The present study revealed, for these two species, that mean heart rate ( $f_H$ ) varied directly with  $MO_2$ . However, in the sculpin, while mean R-R interval recovered at a faster rate than  $MO_2$ , SDRR recovered at the same rate as  $MO_2$ , following disturbance. Thus, there was a close correlation between HRV and  $MO_2$ , implying that HRV may serve to optimise respiratory gas exchange (Taylor, 1992). We conclude that PSA is a useful method of determining HRV in fish, and that HRV is a more sensitive measure of recovery from disturbance than  $f_H$  alone.

This study highlights the importance of cholinergic innervation of the heart in *M. scorpius*. Stimulation of the vagus caused cardiac inhibition similar to that observed in dogfish (Agnisola et al, 2003), suggesting that cholinergic innervation has a major influence on the cardiac pacemaker. In addition, sectioning of the vagus nerve led to an increase in  $f_H$  and abolition of the HRV in resting fish. A glutamate-induced bradycardia was NMethyl-D-Aspartate (NMDA)-receptor dependent and atropine-sensitive, providing evidence that glutamate is a putative player in the reflex control of the heart.

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**HEMOGLOBIN SICKLING**  
**IN BOREAL FISHES:**  
**ADAPTATION TO THE COLD?**

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**Abstract**

Recent studies of fish red blood cells found that a regular para crystalline array of hemoglobin (Hb) tetamers formed under low oxygen conditions in 2 species of boreal fishes, Arlantic cod (*Gadus morhua*) and toadfish (*Opsanus tau*). This phenomenon is termed hemoglobin gelation and its physiological characteristics and importance to survival of boreal fishes is unknown. Preliminary data was obtained on the frequency and physiological nature of the phenomenon of hemoglobin (Hb) gelation in red blood cells of fishes that inhabit cold-water temperate and Arctic environments. Over 25 species have been examined to date and only cold-water boreal fishes exhibited Hb gelation. The present study tests the hypothesis that hemoglobin gelation within fish red blood cells may be a feature of normal fish respiratory physiology and may be adaptive in extreme cold-water environments. We will present data on the temperature and pH dependence of gelation and oxygen binding characteristics of purified Hb from Atlantic cod. Experiments included ligand binding kinetics using stopped flow spectrophotometry, oxygen equilibrium studies using tomometry, and optical microscopy and SEM characterization of Hb crystalline structures. These data tests if gelation is dependent on the surrounding intracellular matrix. Preliminary findings on purified Hb suggest that gelation is an intrinsic property of the Hb and occurs under conditions which are likely to be found in vivo. The differences in type and severity of Hb gelation may directly affect physiology and survival of boreal fishes, but too few species have been assayed to determine the extent of Hb gelation and no data exists on the physiological conditions that cause gelation in boreal fishes.

## **Introduction**

A regular paracrystalline array of hemoglobin (Hb) tetramers formed under low oxygen conditions in the red blood cells of 2 species of boreal fishes, Atlantic cod (*Gadus morhua*) and toadfish (*Opsanus tau*) (Harosi et al. 1998). This phenomenon, termed hemoglobin gelation, its physiological characteristics and importance to survival of boreal fishes is unknown. Gelation results in Hb crystals that can rupture cells and possibly result in fish mortality. The differences in type and severity of Hb gelation may directly affect physiology and survival of boreal fishes, but too few species have been assayed to determine the extent of Hb gelation and no data exists on the physiological conditions that cause gelation in boreal fishes. This study set out to sample a large number of boreal and Arctic fish species and will provide an accurate assessment of the distribution of gelation within boreal fishes and any correlation with genetic variation among and within species.

## **Methods and Materials**

Methods and materials included observation of Hb gelation in red cells using whole red blood cells mounted on a microscope slide and sealed with a cover slip which resulted in low PO<sub>2</sub> conditions as fish red cells are metabolically active. The formation of the Hb crystals was recorded using a high-resolution video-camera connected to a computer equipped with image analysis software (Optimas 6.1) and images were stored digitally. Portions of all blood samples were preserved for TEM in 4% glutaraldehyde. Hemoglobin components from red cells were purified and separated using standard published procedures and these basic procedures include cell lysis, stripping and ion removal, gel permeation, chromatography and ion exchange chromatography.

## **Results and Discussion**

Results showed that of the 27 species tested only 8 species exhibited strong Hb gelation frequencies, while 2-4 species seemed to have some capacity to gelate (Table 1). In contrast the remaining species, which comprised the majority of fishes tested, exhibited no Hb gelation. The species of fishes that exhibited strong Hb gelation were all in or related to gadiform fishes or cod fishes, one of the most diverse and ecologically successful groups of fishes in the north Atlantic.

Although the phenomenon of gelation of hemoglobin within some teleost fish erythrocytes has been noted in earlier literature (Thomas, 1971), it is only very recently (Harosi et al, 1998) that a systematic characterization of this process utilizing microscopy and physical-chemical methods has been initiated.

Our current work significantly expands our understanding of the scope and characteristics of this gelation phenomenon. Comparison of data from light microscopy and TEM of whole cells with studies of purified hemoglobin components confirms that changes in the erythrocyte morphology and ultrastructure upon gelation can be correlated with formation of higher order aggregates in intracellular hemoglobin. Whole cells gelate *in vitro* when allowed to deoxygenate themselves as a result of their own metabolic activity. The corresponding purified hemoglobins crystallize when deoxygenated gasometrically or with sodium dithionite. Primary sequences of COD hemoglobin  $\alpha$  and  $\beta$  (Tipping and Birley, 2001) reveal an interesting pattern of substitution of conserved amino acids when compared to other known fish hemoglobin structures. Homology modelling of the  $\beta$  chains suggests that three of these substitutions occur in very close proximity to each other and one ( $\beta 56$ ) probably results in a new surface cysteine, possibly capable of forming hemoglobin polymers through intermolecular disulfide formation. Precedence for these types of interactions in hemoglobins has been previously established (Tondo et al., 1974) and is consistent with the report of the presence of paracrystalline arrays of hemoglobin in unsickled red blood cells of cod (Thomas, 1971). Other possible synergistic interactions and aggregation might be stimulated by events linked to hemoglobin function and RBC physiology such as oxygen concentration, pH, temperature.

The physiological and/or pathophysiological significance of fish RBC gelation and awaits confirmation that the phenomenon can take place *in vivo*. Possible selective parameters might include improvement in oxygen binding/delivery characteristics or a lowering of blood viscosity.

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Figure 1. Shows a panel of normal and sickled (gelated) Atlantic cod red cells.

Normal cod RBCs  $\rightarrow$

Gelated cod RBCs  $\rightarrow$

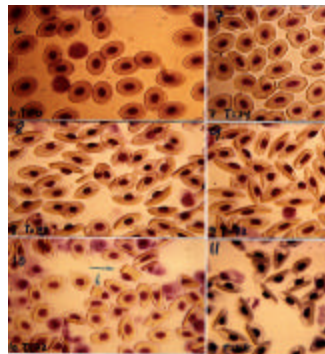


Table 1: Boreal fish species that have been sampled for Hb gelation and the presence of gelation in fresh whole red blood cells in whole mount preparations.

Species name	Positive Hb gelation
1) Cod ( <i>Gadus morhua</i> )	Yes
2) Haddock ( <i>Melanogrammus aeglefinus</i> )	Yes
3) Pollock, saithe ( <i>Pollachius virens</i> )	Yes
4) Silver Hake ( <i>Merluccius bilinearis</i> )	Yes
5) Red Hake ( <i>Urophycis chuss</i> )	Yes
6) Redfish ( <i>Sebastes fasciatus</i> )	Yes
7) Winter Flounder ( <i>Pleuronectes americanus</i> )	Yes?crystals RBC edge
8) Summer Flounder ( <i>Paralichthys dentatus</i> )	Yes?crystals RBC edge
10)Longhorn Sculpin ( <i>Myoxocephalus octodecemspinosus</i> )	No
11) Shorthorn Sculpin ( <i>Myoxocephalus scorpius</i> )	No
12) Sea Raven ( <i>Hemitripterus americanus</i> )	No
13) Ocean pout ( <i>Macrozoarces americanus</i> )	No
14) Mackerel ( <i>Scomber scombrus</i> )	No
15) Herring ( <i>Clupea harengus harengus</i> )	No
16) Norwegian Labrid ( <i>Ctenolabrus rupestris</i> )	No
17) Norwegian Mudfish ( <i>Pomatoschistus microps</i> )	Yes
18) Dogfish ( <i>Squalus acanthias</i> )	No
19) Toadfish ( <i>Opsanus tau</i> )	Yes
20) Tautog ( <i>Tautoga onitis</i> )	Yes (larval only)
21) Killifish ( <i>Fundulus heteroclitus</i> )	No
22) Sheepshead minnow ( <i>Cyprinodon variegatus</i> )	No
23) Blueback herring ( <i>Alosa aestivalis</i> )	No
24) Atlantic silverside ( <i>Menidia menidia</i> )	No
25) Smooth dogfish ( <i>Mustelis canis</i> )	No
26) Little skate ( <i>Raja erinacea</i> )	No
27) Common white sucker ( <i>Catostomus commersoni</i> )	No
28) Arctic cod ( <i>Boreogadus saida</i> )	Yes
29) Spiny eelpout ( <i>Notocanthus sp.</i> )	No
30) Arctic eelpout ( <i>Lycodes reticulatus</i> )	No
31) Arctic Sculpin ( <i>Cottuncullus microps</i> )	No
32) Turbot ( <i>Reinhardtius hippoglossoides</i> )	hippoglossoides)



**CARDIAC FUNCTION IN ATLANTIC WOLFFISH (ANARHICHAS  
LUPUS) EXPOSED TO ACUTE TEMPERATURE  
AND HYPOXIA CHALLENGES**

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**EXTENDED ABSTRACT ONLY - DO NOT CITE**

**Introduction**

An *in vivo* study was performed to examine the effect of increased environmental temperature and hypoxia on the cardiac performance of the Atlantic wolffish (*Anarhichas lupus*). These environmental factors are known to strongly affect the cardiac physiology of pelagic (active) fish species, but few studies have been conducted on benthic (sedentary) marine fishes. Further, although several species of wolffish in the North Atlantic are listed as endangered and *Anarhichas lupus* has been identified as a species with great aquaculture potential, their physiology is virtually unknown.

**Material and Methods**

This study was performed with *Anarhichas lupus* (N=8; 729±177 g) that were reared at the Ocean Sciences Centre (MUN) and acclimated to 8°C for 1 month before experimentation. Cardiovascular parameters (cardiac output, Q; heart rate, F<sub>H</sub>; and stroke volume, S<sub>V</sub>) during temperature and hypoxia challenges, were measured using blood flow probes (2.0 or 2.5 SB, Transonic® Systems; Ithaca, NY) implanted around ventral aorta. Fish were first submitted to the temperature challenge: where water temperature was increased from 6 to 16 °C,

at the rate of 2 °C/hour, and then rapidly decreased (in 1 hour) to 8 °C. After the temperature challenge, fish were transferred to a small insulated tank where water oxygen could be tightly controlled, and allowed to rest for 20h in water of 100% oxygen saturation. The hypoxic challenge was initiated by lowering oxygen saturation to 80% (by gassing with 100% N<sub>2</sub>) over 20 min. and maintaining water oxygen levels at this value for an additional 20 min. Thereafter, the water oxygen content was lowered by 10%, every 20 min, until a water oxygen level of 20% saturation was achieved. This level of hypoxia was maintained for 30 min., and the hypoxia challenge was ended by gradually increasing the water oxygen content back to 100% saturation over a 30 min. period. Cardiovascular parameters were recorded continuously throughout the temperature and hypoxia experiments, and for 1 hour after water oxygen levels were restored to 100% saturation.

All values presented are means ± S.E. One-way repeated measures analyses of variance (ANOVA) were applied to analyse the effects of temperature and oxygen on cardiac function. Dunnet's test was applied to determine when cardiac output and heart rate became significantly lower than at 100% O<sub>2</sub>. The significance level used was  $P < 0.05$ .

### **Results and Discussion**

Changes in the wolffish's cardiovascular parameters (cardiac output, heart rate and stroke volume) during acute exposure to elevated temperatures and hypoxia are presented in Figures 1 and 2. Cardiac output was unchanged from 6 to 8°C but increased linearly ( $P < 0.05$ ) as temperature was increased from 8 to 16°C ( $Q_{10}$  of 1.52). This increase was due solely to an elevation in heart rate ( $Q_{10}$  of 1.79) because stroke volume showed a slight, but non-significant decrease. Similar results have been reported for other benthic fish species (winter flounder, Cech *et al.*, 1976; and lingcod, Stevens *et al.*, 1972) and the rainbow trout (Brodeur *et al.*, 2001)

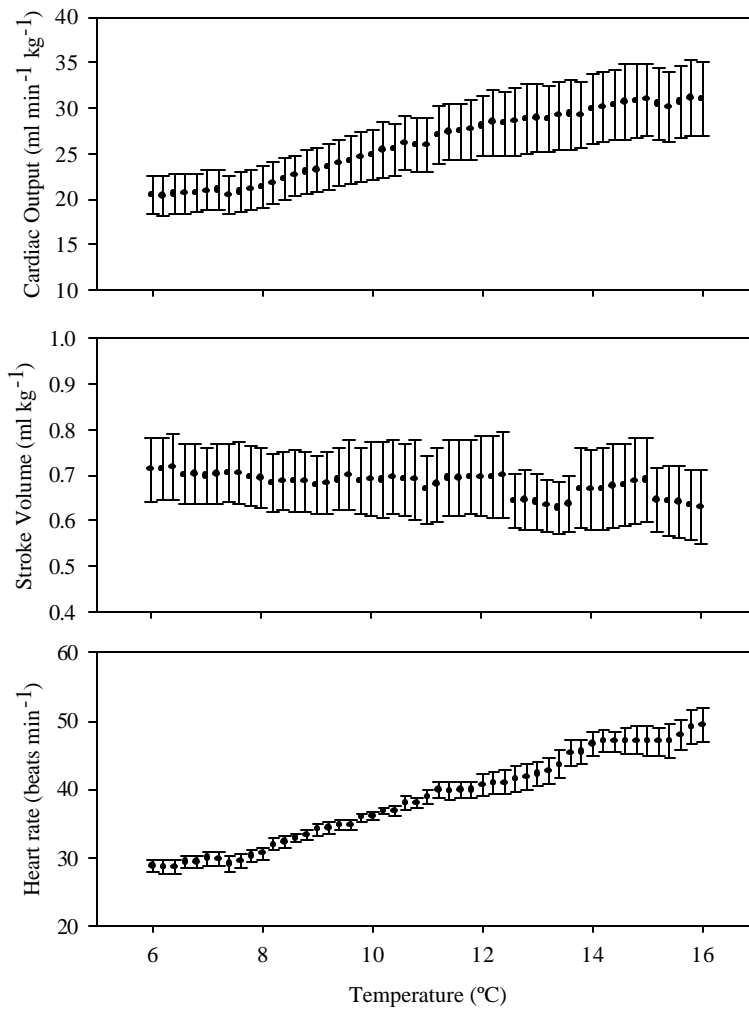
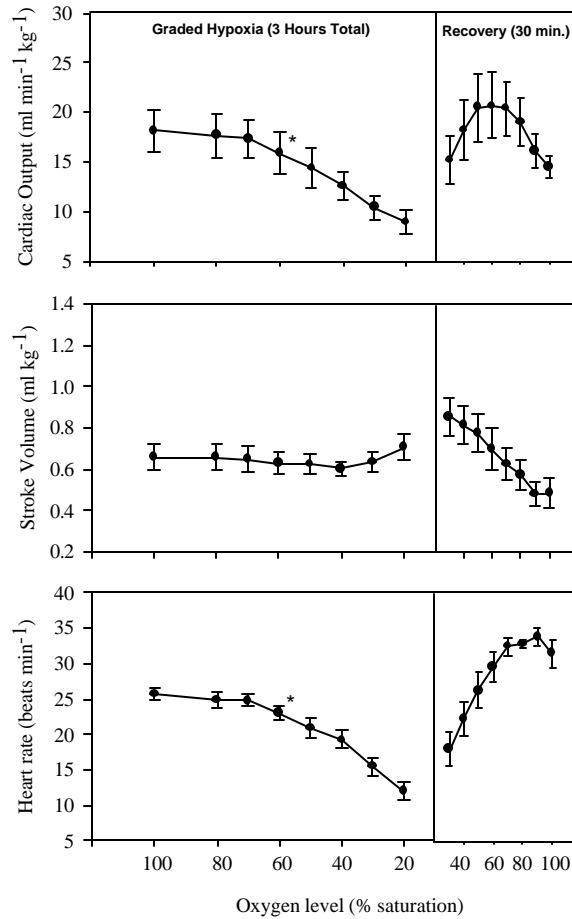


Figure 1 - Cardiac variables in the wolffish (*A. narhichas lupus*) during an acute temperature challenge (6 to 16°C at 2°C h<sup>-1</sup>). Reported values are means ± S.E. (N = 8).



**Figure 2.** Cardiac variables in the wolffish (*A. narhichas lupus*) during graded hypoxia (final O<sub>2</sub> level 20% saturation) and the 30 min. recovery period. Reported values are means ± S.E. (N = 8). \* Indicates the O<sub>2</sub> level when the cardiac variable became significantly lower than at 100% O<sub>2</sub>.

Exposure to severe hypoxia induced a marked bradycardia in the wolffish (Fig. 2). This bradycardia began between 60 and 70% saturation, with F<sub>H</sub> decreasing from 25.8±0.9 beats min<sup>-1</sup> at 100% saturation to 12.0±1.2 beats min<sup>-1</sup> at 20% saturation. This drop in F<sub>H</sub> also resulted in a considerable decrease in cardiac

output, from  $18.1 \pm 2.1$  to  $9.0 \pm 1.2$  ml min<sup>-1</sup> kg<sup>-1</sup>, as stroke volume was not altered by hypoxic exposure. These data suggest that the critical oxygen level for the wolffish is 60-70% saturation, and that this species does not defend cardiac output (by increasing SV) in the face of acute decreases in water oxygen content. The critical oxygen level where bradycardia occurs in the wolffish is in agreement with data on lingcod (Farrell, 1982) and short-horned sculpin (McCormack and Driedzic, this symposium), but considerably lower than for rainbow trout (O<sub>2</sub>crit 80%) (Gamperl *et al.*, 1994). In contrast to most other teleost species (trout, lingcod; cod, Fritsche and Nilsson, 1992, Exp. Biol. 48: 153) SV did not increase in an attempt to maintain Q during bradycardia. This result is surprising, but not unique, as an almost identical response in Q and SV is seen in the short-horned sculpin (McCormack and Driedzic, this symposium) during acute hypoxic exposure. Clearly, the pattern of changes in SV and Q with hypoxia is not related to pelagic vs. benthic lifestyles (the lingcod is a benthic species).

In summary, the Atlantic wolffish modulates HR, but not SV, in response to acute changes in oxygen and temperature, and this results in an 'atypical' Q response (decrease) in the face of decreased water oxygen levels. Whether cardiovascular responses to other challenges (e.g. exercise, stress) are different from those reported for other teleost species is unknown, as is whether the lack of an increase in SV (to defend Q) during hypoxia is characteristic of hypoxia-tolerant teleost species.

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**O<sub>2</sub> CHEMORECEPTORS INVOLVED  
IN THE CONTROL OF  
CARDIORESPIRATORY FUNCTION  
OF PACU, PIARACTUS MESOPOTAMICUS,  
IN RESPONSE TO GRADED HYPOXIA.**

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**Abstract**

Many of reflexogenic areas have been indirectly tested as O<sub>2</sub>-sensitive loci in fish. Among these areas, only the gills, pseudobranch and the orofaringeal cavity were confirmed. However, these sites showed a considerable intra-specific variation on the pattern of distribution, localization, orientation, and on which modality of cardio-respiratory response they are specifically related. We examine the site of O<sub>2</sub> chemoreception involved in the cardio-respiratory reflex responses to hypoxia in the neotropical fish pacu, *Piaractus mesopotamicus*. To analyze in which gill arches the O<sub>2</sub> chemoreceptors were localized, we compared the cardiorespiratory responses (heart rate, arterial blood pressure, respiratory frequency, ventilation amplitude and the respiratory pattern) of intact and gill-denervated fish (cranial nerves IX and X) during graded hypoxia. The O<sub>2</sub> receptors involved in the cardiac responses are located exclusively in the gills and are distributed to all gill arches. The receptors involved in the respiratory responses to hypoxia are also distributed in all gill arches. Furthermore, extrabranchial receptors were also evidenced. The respiratory

pattern was maintained in denervated fish, suggesting that this function is under control of receptors located elsewhere outside of the gills.

## **Introduction**

The ecological thrive of some species depends particularly on their skills in detecting O<sub>2</sub> level changes and quickly use this information to properly adjust the cardio-respiratory activity (Fritsche and Nilsson, 1993).

The O<sub>2</sub> chemoreceptor in water-breathing fish are localized in structures like orobranchial cavity and gills, and these receptors are innervated by the cranial nerves V, VII, IX and X (Randall and Jones, 1973; Daxboeck and Holeton, 1978; Smith and Davies, 1984; Smatresk et al. 1986; Burleson and Smatresk, 1990a; 1990b; Burleson and Milsom, 1993; Sundin et al., 1999; 2000; Milsom et al., 2002). Due to the lack of histological evidences on the exact site of the O<sub>2</sub> chemoreceptor, a variety of reflexogenic areas have been tested as possible O<sub>2</sub>-sensitive loci using many indirect techniques. In spite of the recent increase in the number of studies, the knowledge on fish O<sub>2</sub> chemoreception is still based on the data obtained for few species (for a review, see Milsom, 1996). Among neotropical fish, the O<sub>2</sub> chemoresponses of few species have been studied: the erythrinid fish traíra, *Hoplias malabaricus* (Sundin et al., 1999), and jeju, *Hoplerythrinus unitaeniatus* (Rantin et al., 2004), and the serrasalmid tambaqui, *Colossoma macropomum* (Sundin et al., 2000, Milsom et al., 2002).

The aim of this study was to identify the site (branchial and/or extrabranchial) of O<sub>2</sub>-sensitive receptors related to the cardiorespiratory responses to hypoxia in the neotropical fish pacu, *Piaractus mesopotamicus*.

## **Material and Methods**

Specimens of pacu, *Piaractus mesopotamicus* (Wt = 557 ± 82g) were obtained from fish farms near Sao Carlos, SP, Brazil. In the laboratory fish were maintained in 1000 L holding tanks supplied with aerated water (normoxic conditions, PwO<sub>2</sub> ≥ 130 mmHg) at 25 ± 1°C (acclimation temperature). Fish were daily fed, but food was withheld for 48 h before trials.

Before and during the surgical procedure fish were anaesthetized and artificially ventilated with aerated benzocaine solutions (0,1 g·L<sup>-1</sup> and 0,05 g·L<sup>-1</sup> respectively).

In order to record the cardiorespiratory variables (respiratory frequency - fR, ventilation amplitude - VAMP, arterial blood pressure – Pa, and heart rate - fH), the fish had the roof of the mouth (through the dorsal palate) and the caudal artery cannulated (PE 100 and PE 50, respectively).

To analyze in which gill arches the  $O_2$  chemoreceptors were localized, we compared the cardiorespiratory responses during graded hypoxia of 4 experimental groups: the Intact group, composed by 7 intact and 3 sham operated fish (which results did not differ from those of the intact animals); group IX, with the cranial nerve IX to the gills sectioned; group G1, with the cranial nerve IX and first branch of nerve X to the gills sectioned; group G4, with the cranial nerve IX and all branches of cranial nerve X to the gills sectioned. For details of cannulations and denervation of cranial nerves, see the study of Sundin et al. (2000). After the surgery, the fish were allowed to recover for a minimum of 24 h prior to experimentation. All denervations were confirmed “post mortem”.

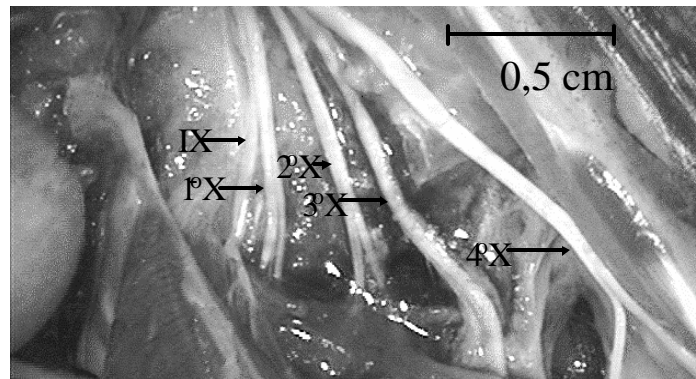


Figure 1. General view of the dorsal area of the left branchial chamber of pacu, *P. mesopotamicus*, showing the branches of the cranial nerves IX e X to the gills.

After the recovery period, the buccal and caudal catheters were connected to pressure transducers of a data acquisition system to obtain the fR and VAMP, Pa and fH, respectively. Before starting the experiments, a period of 30 min was observed to assure that all the cardiorespiratory parameters were on the basal level. After this, the PwO<sub>2</sub> was stepwise reduced from the O<sub>2</sub> saturation level (normoxia - 140 mmHg) to 100, 70, 50, 30 and 20 mmHg. Fish remained for 10 min in each experimental PwO<sub>2</sub>. The water temperature of the experimental chamber was maintained always at 25°C.

The cardiorespiratory parameters were recorded for 1min in normoxia and during the last minute of each hypoxic tension. The fR and fH were expressed in breaths·min<sup>-1</sup> and bpm, respectively. The VAMP and VTOT (VTOT = VAMP:fR) were calculated as a percentage change of the control value.

The data are presented as median ± SE. The Kruskal-Wallis and Wilcoxon tests were employed to detect significant differences between normoxic and hypoxic values within the same experimental group. The difference between the Intact group and the denervated ones were tested using the Mann-Whitney test. The Cochran's test was used to verify the change between the episodic and continuous ventilation pattern. Values were considered statistically different when P < 0.05.

## Results

The bilateral denervation of the gills caused a significant increase in fH only in the group G4. The denervation did not affect fR during normoxia (table I). The Intact group displayed hypoxic bradycardia below the PwO<sub>2</sub> of 50 mmHg. This bradycardia was more evident in O<sub>2</sub> tensions below the critical oxygen tension for this specie (PcO<sub>2</sub> = 35 mmHg; Rantin et al., 1998). Conversely, the groups XI and G1 increased their fH in O<sub>2</sub> tensions between 70 and 50 mmHg. Below these tensions this variable decreased significantly. In these groups the fH was always higher than in the Intact group (figure 2). The group G4 presented hypoxic bradycardia only in the more hypoxic tension of 10 mmHg. The hypoxic bradycardia was recorded for all groups in this O<sub>2</sub> tension.

The hypoxia elicited respiratory changes in all experimental groups. The groups Intact, IX and G1 progressively increased their respiratory parameters fR, VAMP and, consequently, the VTOT, in O<sub>2</sub> tensions below 100 mmHg (figure 3). The denervated fish presented lower values of fR when compared to the Intact group. Even with the four gill arches denervated, the group G4 presented

ventilatory response to hypoxia. Despite of this, the magnitude of changes in ventilatory variables was lower than that recorded for the groups IX and G1. Furthermore, fR, VAMP and VTOT started to increase in lower  $P_{O_2}$  tensions than that of other groups ( $P_{wO_2} = 70$  mmHg for group G4 and 100 mmHg for groups Intact, IX and G1).

All groups showed episodic respiration (normoxic rest pattern), which changed to continuous respiration below the  $O_2$  tension of 100mmHg.

Table I. Cardiorespiratory parameters of *P. mesopotamicus* (Intact and denervated groups: IX, G1 e G4) during normoxia ( $P_{wO_2} = 140$  mmHg). Points are median  $\pm$  standard error. \* - denotes significant differences ( $P < 0,05$ ) in relation to the Intact group.

	Intact (n = 10)	IX (n = 10)	G1 (n = 10)	G4 (n = 8)
fH (bpm)	73 $\pm$ 6	77 $\pm$ 4	79 $\pm$ 3	82 $\pm$ 3 *
fR (breaths·min <sup>-1</sup> )	67 $\pm$ 4	64 $\pm$ 7	58 $\pm$ 4	60 $\pm$ 6

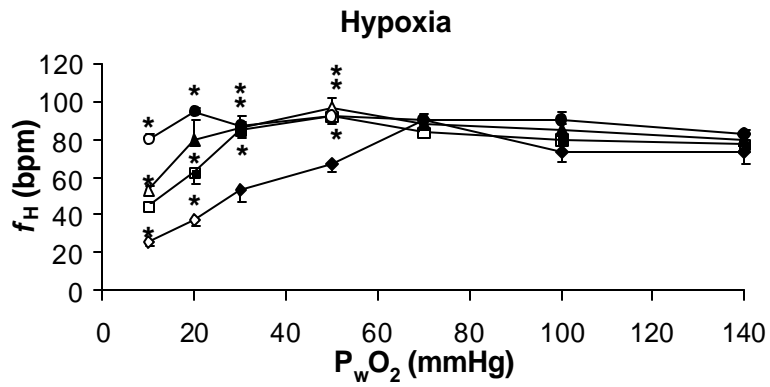


Figure 2. Heart frequency (fH) of *P. mesopotamicus* as a function of the PwO<sub>2</sub>.  
 ◆ - Group Intact (n = 10); ■ - Group IX (n = 10); ▲ - Group G1 (n = 10);  
 ● - Group G4 (n = 8). Points are median ± standard error. The open symbols mean statistical difference in relation to the normoxic values. \* - denotes significant differences (P < 0,05) in relation to the Intact group.

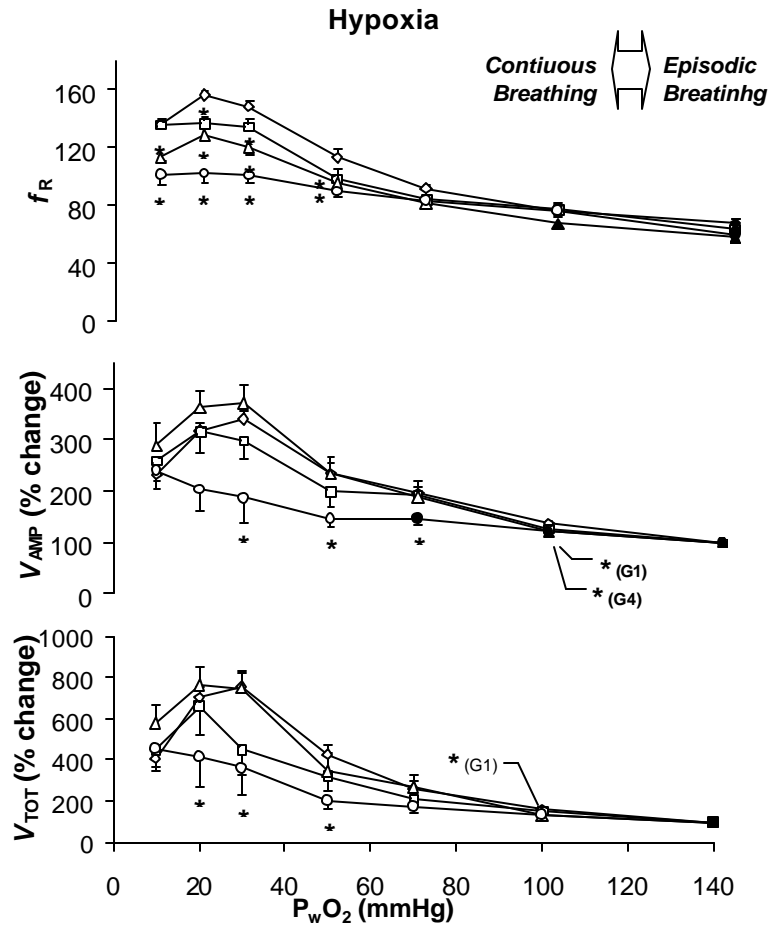


Figure 3. Ventilatory variables ( $f_R$ ,  $V_{AMP}$  e  $V_{TOT}$ ) and respiratory pattern changes of *P. mesopotamicus* in response to graded hypoxia.  $\blacklozenge$  - Group Intact ( $n = 10$ );  $\blacksquare$  - Group IX ( $n = 10$ );  $\blacktriangle$  - Group G1 ( $n = 10$ );  $\bullet$  - Group G4 ( $n = 8$ ). Points are median  $\pm$  standard error. The open symbols mean statistical difference in relation to the normoxic values. \* - denotes significant differences ( $P < 0,05$ ) in relation to the Intact group.

## Discussion

In the current study the normoxic fish presented fR and fH (table I) slightly higher than those obtained by Rantin et al. (1998) for the same specie (fR =  $53 \pm 7$  e fH ~ 54). However, the highest fR and lowest fH values during hypoxia, and the O<sub>2</sub> tension in which they started to change were similar to those reported by these authors. These differences could be attributed to seasonal variations, level of stress, body size, and different experimental protocols.

### *Cardiac reflexes*

Graded hypoxia did not elicit bradycardia in pacu until a PwO<sub>2</sub> similar to the PcO<sub>2</sub> for this species. Both partial (group IX) and total (G1) denervation of the first gill arch retard the response and also were responsible for an increase in fH in the PwO<sub>2</sub> = 50 mmHg. This results differ from those obtained for Atlantic cod, *Gadus morhua* (Fritsche and Nilsson, 1989), trout, *Onchorhynchus mykiss* (Smith and Jones, 1978), and traíra, *Hoplias mlabaricus* (Sundin et al., 1999). In these species the first gill arch denervation abolished the hypoxic bradycardia. However, some species present O<sub>2</sub> chemoreceptors involved on the fH control distributed also in other sites. Catfish has these receptors in the three first gill arches (Burlerson and Smatresk, 1990a). In tambaqui and pacu as well, the hypoxic bradycardia was not abolished by complete denervation of the first gill arch (Sundin et al., 2000). Both species also presented distinct responses between the groups G1 and G4. This means that pacu, like the tambaqui, posses O<sub>2</sub> chemoreceptors involved in the fH control distributed in all gill arches.

Complete gill denervation abolish the hypoxic bradycardia, unless under PwO<sub>2</sub> of 10 mmHg. According to Rantin et al. (1998), the bradycardia observed in pacu exposed this O<sub>2</sub> levels was accompanied by electrocardiographic alterations, suggesting myocardial impairment. Under the same O<sub>2</sub> tension, tambaqui presented a bradycardia that persisted even after atropine administration (Sundin et al., 2000). Sundin et al. (1999) also reported that, at a PwO<sub>2</sub> of 20 mmHg, traíra developed hypoxic bradycardia even after complete gill denervation and atropine administration. Thus, the bradycardia presented by pacu of group G4 could be a consequence of myocardial impairment (for a review, see Rantin et al., 1995) rather than a reflex originated in the O<sub>2</sub> chemoreceptors. Based on the present results, the gills are the unique O<sub>2</sub>-sensitive sites involved on the mediation of the hypoxic bradycardia.

According to Burlleson et al. (1992), most of water-breathing teleosts present cardiac response mediated by O<sub>2</sub> chemoreceptors externally oriented and located in the first gill arch. Some species support this statement, such as *Hemirhamphus americanus* (Saunders and Sutterlin, 1971), *Onchorhynchus mykiss* (Daxboeck and Holeton, 1978; Smith and Jones, 1978), and *Gadus morhua* (Fritsche and Nilsson, 1989). Nevertheless, Sundin et al. (1999) reported that traíra, *Hoplias malabaricus*, has O<sub>2</sub> chemoreceptors internally oriented, but restrict to the first gill arch. The tambaqui, *Colossoma macropomum* has internally and externally oriented O<sub>2</sub> receptor distributed in all the gill arches (Sundin et al., 2000). The similarity of the O<sub>2</sub> chemoreception in both pacu and tambaqui indicates that the distribution and location of the O<sub>2</sub> receptors are not exceptions among species. To date, the unique rule for the O<sub>2</sub> chemoreceptors involved on the cardiac reflex responses to hypoxia is that they are located only in the gills.

#### *Respiratory reflexes*

Pacu changed the magnitude of the respiratory responses with progressive denervation. Groups IX and G1 presented lower capacity to increase fR in response to the hypoxia. This indicates the presence of O<sub>2</sub> receptors in the first gill arch. The difference between the response of G1 and G4 also indicates the presence of O<sub>2</sub> receptors in the other gill arches. The complete gill denervation reduced the general response intensity but did not abolish the responses of the ventilatory variables (fR, VAMP, VTOT) and the alteration in the respiratory pattern from episodic to continuous. This indicates the presence of O<sub>2</sub> chemoreceptors distributed in all the gill arches, and also suggests the existence of extrabranchial O<sub>2</sub> chemoreception in this specie.

As to the extrabranchial sites for the fR response, pacu differs from most of the species already studied. The groups G1 and G4 developed, respectively, 83 and 74,2 % of the Intact maximal response, demonstrating the relevance of the extrabranchial receptors for the mediation of this reflex. Sundin et al. (2000) showed that tambaqui with all gill arches denervated increased fR after buccal injection of NaCN. The similarity of the responses reported for both species supports the hypothesis that the extrabranchial O<sub>2</sub> receptors in pacu could be located in the oropharyngeal cavity. Differently from tambaqui, pacu presents pseudobranches, a structure recognized as an O<sub>2</sub>-sensitive site in other species. Eventually, it is possible that the extrabranchial O<sub>2</sub> receptors are internally and/or externally oriented. This was validated with another experimental series conducted in our laboratory with this species (unpublished data).

In the groups IX and G1, hypoxia did not affect the initial increase in VAMP as observed in the Intact group, except for the over-increasing tendency observed in group IX. In the group G4, the beginning of the VAMP hypoxic response was delayed, but not abolished. This reveals the presence of O<sub>2</sub> chemoreceptors related to control of VAMP in all the gill arches and also in extrabranchial sites. Moreover, the VAMP increasing tendency shown by the group IX could indicate the presence of receptor involved on the inhibition of the VAMP in response to hypoxia. Likewise, Sundin et al. (1999) suggested that traíra presents externally oriented receptors in the first gill arch that could exert an inhibitory effect on the fR and VAMP. Experiments on internal and external stimulation with NaCN confirmed the presence of these receptors in pacu (unpublished account).

Extrabranchial O<sub>2</sub> receptors related to the VAMP were also detected in traíra (Sundin et al., 1999) and tambaqui (Sundin et al., 2000; Milsom et al., 2002). In both species these receptors are externally oriented. Since, like in tambaqui, the O<sub>2</sub> chemoreceptors involved on the control of the VAMP are located in the oropharyngeal cavity, it is possible that, in pacu, the receptors eliciting this response are also localized in the oropharynx and/or in the pseudobranch.

To date, only the pseudobranches and oropharynx were confirmed as O<sub>2</sub>-sensitive extrabranchial sites. Moreover, all the attempts to induce respiratory response by central stimulation did not result in consistent data (Roivainen, 1977; Wilkies et al., 1981; Hendric et al., 1991; Milsom et al., 2002).

The responses of pacu to hypoxia seem to involve many chemoreceptors populations, in different site, have different central projection producing distinct motor effects. And also, the possibility that the same receptor may be involved in more than one of the reflexes studied here cannot be rejected.

It is also possible that the respiratory pattern, changing from episodic to continuous as PwO<sub>2</sub> decreases, results from the interaction between fR and VAMP alterations rather than from reflexes initiated in specific O<sub>2</sub> chemoreceptors.

Finally, based on the available data, it is difficult to determine patterns for the complex distribution of the O<sub>2</sub> chemoreceptor in teleosts. The basis related with the inter-specific variability of O<sub>2</sub> chemoreceptors populations that have been studied remains elusive. More information of this sort is still necessary for a variety of species from various habitats, before compose feasible hypothesis

concerning the influence of the evolution, phylogeny and adaptive differences in the distribution of O<sub>2</sub> chemoreceptors (Sundin et al., 2000).

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