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Effects of exercise training on excitation-contraction coupling, calcium dynamics and protein expression in the heart of the Neotropical fish *Brycon amazonicus*

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ABSTRACT

Matrinxã (*Brycon amazonicus*) is a great swimming performance teleost fish from the Amazon basin. However, the possible cardiac adaptations of this ability are still unknown. Therefore, the aim of the present work was to investigate the effects of prolonged exercise (EX group - 60 days under 0.4 BL·s^{-1}) on ventricular contractility by (i) *in-vitro* analysis of contractility comparing the relative roles of sodium/calcium exchanger (NCX) and sarcoplasmic reticulum (SR) in the excitation-contraction (E-C) coupling and (ii) molecular analysis of NCX, sarcoplasmic reticulum Ca^{2+} ATPase (SERCA2) and phospholamban (PLB) expression and quantification. The exercise training significantly improved twitch tension, cardiac pumping capacity and the contraction rate when compared to controls (CT). Inhibition of the NCX function, replacing Na^+ by Li^+ in the physiological solutions, diminished cardiac contractility in the EX group, reduced all analyzed parameters under both high and low stimulation frequencies. The SR blockage, using 10 μM ryanodine, caused ~50% tension reduction in CT at most analyzed frequencies while in EX, reductions (34–54%) were only found at higher frequencies. SR inhibition also decreased contraction and relaxation rates in both groups. Additionally, higher post-rest contraction values were recorded for EX, indicating an increase in SR Ca^{2+} loading. Higher NCX and PLB expression rates and lower SERCA2 rates were found in EX. Our data indicate that matrinxã presents a modulation in E-C coupling after exercise-training, enhancing the SR function under higher frequencies. This was the first study to functionally analyze the effects of swimming-induced exercise on fish cardiac E-C coupling.

1. Introduction

Some fishes are usually described as “active” due to their remarkable swimming capacity to overcome high stream speeds and/or maintain sustained swimming for extended periods (Bushnell and Jones, 1994; Ballantyne and Robinson, 2011). In laboratory conditions, the approach to expose fish to different levels and periods of swimming activity is defined as exercise-training and consists of submitting fish to a controlled water flow (see Kieffer, 2010).

Several cardiovascular adjustments in fishes induced to extended exercise-training include increases in stroke volume (Farrell et al., 1991), hematocrit (Gallaugher et al., 2001), tissue perfusion (Sanger and Potscher, 2000) and relative ventricular size (Davison, 1997). Additionally, Castro et al. (2013), studied Atlantic salmon (*Salmo salar*) after 70 days under 1.31 body lengths·s^{-1} (BL·s^{-1}) swimming exercise and found increases in cardiac gene expression rates of Ca^{2+} handling proteins. However, no functional data on the exercise-induced effects in fish cardiac contractility is currently available.

Cardiac excitation–contraction coupling (E-C) is the process from electrical excitation of the myocyte to the contraction of the heart, and depends on a dynamic Ca^{2+} influx ($[Ca^{2+}]_i$) and efflux through cardiomyocytes (Bers, 2002). This process initiates with the activation of the sarcolemmal voltage-gated slow Ca^{2+} channels or L-type channels (LTCCs) and consequent Ca^{2+} influx (Bers, 2008). In adult mammals, a large fraction of Ca^{2+} is stored inside the sarcoplasmic reticulum (SR), which is released to the cytoplasm *via* Ca^{2+} activation of the SR ryanodine receptors (RyR), a process known as Ca^{2+}-induced Ca^{2+} release (CICR) (Lanner et al., 2010; Eisner et al., 2012). During relaxation, the Ca^{2+} fraction released from SR is transported back by the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), which is regulated by phospholamban (PLN) and the small amount of remaining Ca^{2+} is extruded to the extracellular medium by the Na^+-Ca^{2+} exchanger (NCX) and sarcolemmal Ca^{2+}-ATPase (Bers, 2000).

In some fish species, the longer and thinner cardiomyocyte architecture reduces the diffusional distance to the extracellular medium, making the sarcolemmal Ca^{2+} influx *via* LTCCs and/or reverse mode

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NCX sufficient to promote the contraction without significant participation of SR (Gillis and Tibbits, 2002; Vornanen, 1997, 1999). Nevertheless, a great diversity in this process can be found among fish species (Vornanen et al., 2002; Shiels and Sitsapesan, 2015) and essential SR contributions for Ca^{2+} cycling were described during atrial and ventricular contractions in tunas (Keen et al., 1992; Shiels et al., 1999; Shiels and Farrell, 2000; Galli et al., 2011) and for atrial contraction in salmonids (Gesser, 1996; Hove-Madsen et al., 1998).

According to Shiels and Galli (2014), a greater SR participation in the cardiac E-C coupling of highly active species appears to be a general principle that can be applied not only to fish, but also to all vertebrates (Shiels and Galli, 2014). However, sedentary fish species as the Neotropical trahira, *Hoplias malabaricus* (Rivaroli et al., 2006) and marble swamp eel, *Synbranchus marmoratus* (Rocha et al., 2007) also depend on SR Ca^{2+} stores for cardiac contraction, suggesting a relationship between the phylogeny of these species and the SR functionality. Additionally, only one study has analyzed the ventricular E-C coupling of a very active Neotropical fish (*Prochilodus lineatus*), showing evidence that the SR is important for tension development (Rivaroli et al., 2006).

Thus, in their natural environments, tropical fish are frequently exposed to the stream flow, swimming upstream or maintaining their position against the current. This imposition demands constant effort, possibly triggering modulations on cardiac E-C coupling that may not be found in sedentary species. Additionally, most studies on exercise physiology in fish have been conducted on salmonids while tropical fishes have largely been ignored (Kieffer, 2010).

Matrinxã, *Brycon amazonicus* (Spix & Agassiz, 1829), is a Neotropical teleost fish native of lotic (mean water speed 1–2 $m{\cdot}s^{-1}$) and warm (ranging between 24 and 32 °C) rivers in the Amazon basin (Almeida-Val et al., 2005; Gensac et al., 2016). This species presents rheophilic habits, performs seasonal upstream migrations (Nakauth et al., 2016) and is able to achieve high swimming speeds (~6 $BL{\cdot}s^{-1}$) even under hypoxic conditions (Ferreira et al., 2010, 2013). Indeed, prolonged exercise-training regimes (2–3 $BL{\cdot}s^{-1}$ for 30–72 days) were able to improve the body growth rate and mass gain of juvenile matrinxãs (Hackbarth and Moraes, 2006; Ferreira et al., 2013).

The present study characterizes for the first time the ventricular tension generation and contractile dynamics in exercised fish. We performed (i) *in-vitro* analysis of contractility, accessing the roles of NCX and SR, and (ii) protein expression analysis of NCX, SERCA2 and PLB in matrinxã under sedentary conditions and after continuous exercise-training.

2. Materials and methods

2.1. Animals

Specimens of *B. amazonicus* (n = 32) were obtained from the Santa Candida Fish Farm (Santa Cruz da Conceição SP, Brazil) and were kept in 500 L holding tanks supplied with a circulation of dechlorinated and continuously aerated water (Pw O_2 > 120 mmHg) for at least 60 days of stabilization before submission to the different protocols. During this period, fish were fed *ad libitum* with commercial fish pelleted feed (30% protein).

2.2. Swimming-induced exercise

After acclimation to laboratory conditions, animals were anesthetized by immersion in 0.1 $g{\cdot}L^{-1}$ benzocaine solution and were then weighed and measured. Fish were randomly divided into two experimental groups (n = 16 each) and placed in two round 100 L experimental tanks. The central portion of each tank was blocked with a plastic cylinder (25 cm diameter and 80 cm height) and the upper part of it was fixed to an end of a horizontal water pipe, also connected to a water pump (BCR-2010, 1cv, Schneider). The medial portion of the horizontal pipe was coupled to two other diagonally drilled pipes, disposed vertically, 15 cm distant from each other. The water pump was able to aspirate water from the lower portion of the tank and eject it into this pipe system, generating a circular water flow. The water speed was adjusted by limiting the pump output flow. Tanks were maintained with a continuous renewal of water, constant aeration (Pw O_2 > 120 mmHg) and controlled temperature (25.5 ± 0.15 °C). The water temperature was chosen as it was considered adequate for matrinxã farming (Frascá-Scorvo et al., 2001; Guimarães and Storti-Filho, 2003). The number of animals per tank was selected based on the fact that this species presents better feeding behavior when in groups of 15 animals or higher (authors observation). The exercised group (EX, Wb = 266 ± 13 g; Lb = 25.5 ± 0.4 cm) was maintained in a circular water flow of 10.25 ± 0.15 $cm{\cdot}s^{-1}$ speed, ~0.4 body length per second ($BL{\cdot}s^{-1}$). The mean water speed inside the tank was kept around 10.3 ± 0.5 $cm{\cdot}s^{-1}$ closer to the center, 10.5 ± 0.2 $cm{\cdot}s^{-1}$ in the medial portion and 9.93 ± 0.6 $cm{\cdot}s^{-1}$ in the edge of the tank. The control group (CT, Wb = 260 ± 16 g; Lb = 25.1 ± 0.4 cm) was maintained in a free swimming condition under static water. Throughout the protocols, both groups were fed *ad libitum* once a day with commercial pelleted feed for fish (30% protein) until 24 h before euthanasia. In both tanks (CT and EX), algae and excrement were removed with siphon (3 times a week during ~20 min) when the water flow was interrupted in the EX tank. The water flow was also interrupted in EX during the daily feeding period (~10 min) and then restarted. Both groups were kept under these conditions for 60 days.

2.3. Euthanasia and Relative Ventricular Mass (RVM)

After completing the experimental period, fish from both groups were euthanized by cervical fracture and spinal cut and then weighed and measured. The ventricles were carefully removed and weighed (Wv - g) to obtain the ventricular mass (mean ± SEM) which was expressed as a percentage of body mass (relative ventricular mass, RVM - % of Wb) and immediately transferred to an ice-cold physiological solution. Subsequently, ventricles were divided into the two experimental approaches: *in vitro* measurements of tension contraction and protein expression (immediately frozen at − 80 °C).

2.4. In vitro experiments

Ten animals from each experimental protocol (n = 10) were randomly designated for the *in vitro* analysis. Four 1 mm thickness strips (mean length 2.5 ± 0.2 mm and mass 3.0 ± 0.2 mg) were excised from each ventricle and transferred to an oxygenated bathing medium containing (mM): 125 NaCl, 2.5 KCl, 0.94 $MgCl_2$, 1.0 NaH_2PO_4, 30 $NaHCO_3$, 3.0 $CaCl_2$, 10 glucose and pH adjusted to 7.4 with H_2SO_4. The saline was continuously bubbled with a gas mixture of 2% CO_2 and 98% O_2 and the temperature was kept at 25 °C by a temperature-controlled water bath.

The ends of the strips were attached to two metal split rings: one free and another coupled to a 10 cm metal thread. The ends of the metal threads were attached to isometric force transducers (Grass FT.03 Transducer, Grass Technologies, West Warwick, RI, USA) and the rings, at the other end, were attached to platinum electrodes connected to a Grass S88 stimulator which delivered electrical square pulses. The stimulation was standardized at 8 ms and 90 V.

Preparations were stretched to obtain the maximum induced tension and stimulated at 0.2 Hz (12 bpm) for 40 min to stabilization before tension recordings. The stimulation was increased until the frequency of at least 80% of the strips was still able to contract regularly. Data were recorded and analyzed using AcqKnowledge MP150 (Biopac Systems Inc., USA). The length and wet mass of each strip were measured and the isometric twitch tension (Tt) relative to the cross-sectional area ($mN.mm^{-2}$) was calculated assuming a muscle density of 1.06 $mg{\cdot}mm^{-3}$ (Layland et al., 1995). The Tt values of each contraction were divided by their respective values of time-to-peak tension (TPT-

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