

# Impact of waterborne and trophic mercury exposures on cardiac function of two ecologically distinct Neotropical freshwater fish *Brycon amazonicus* and *Hoplias malabaricus*

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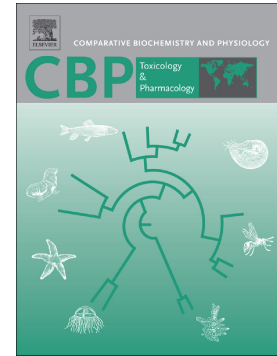
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**Impact of waterborne and trophic mercury exposures on cardiac function of two ecologically distinct Neotropical freshwater fish *Brycon amazonicus* and *Hoplias malabaricus***

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**Running title:** Toxic effects of mercury on cardiac function of fish

**Highlights:**

Mercury exposure by different routes decreased the myocardium performance in vivo and ex vivo.

Mercury exposures harmed cardiac calcium management and contraction/relaxation kinetics.

Inorganic mercury, *via* water or diet, impaired electrical conduction across the heart.

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**Conflict of interest:** The authors declare that they have no conflict of interest.

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## Abstract

Metal pollutants have been considered one of the main factors underlying the depletion of biodiversity in natural populations unbalancing aquatic environments. The aim of this study was to evaluate the effects of exposure to inorganic Hg on myocardial contractility and the electrocardiogram (ECG) of two ecologically distinct Neotropical fish species, namely: matrinxã (*Brycon amazonicus*) and trahira (*Hoplias malabaricus*). Matrinxãs were exposed to a sublethal concentration of 0.1 mg L<sup>-1</sup> of Hg in water for 96h. Trahiras were exposed to dietary Hg doses (0.45 mg of Hg, each 4 days, for 30 days) using juvenile *B. amazonicus* as the prey vehicle. Hg exposures decreased myocardial isometric twitch force development, harmed contraction/relaxation dynamics and cardiac pumping capacity (CPC), and reduced the relative contribution of the calcium stored in the sarcoplasmic reticulum (SR) to excitation contraction (E-C) coupling in both fish species. Analysis of the ECG revealed that Hg impaired electrical conduction across the heart, inducing first degree atrioventricular block and lengthening the plateau phase of action potential duration. In trahira trophic doses of Hg induced a marked bradycardia, increasing the duration of the ventricular action potential and delaying atrial and ventricular depolarization. These findings indicate that both acute and long-term Hg exposure, by different routes is cardiotoxic to matrinxã and trahira. Hg potently impaired intracellular calcium kinetics in the cardiomyocytes, myocardium contractility, and electrical conduction across the heart, all of which can be implicated in decreased cardiac output and putative heart failure.

**Key-words:** fish, mercury, ECG, twitch force, force frequency relationship, excitation-contraction coupling, sarcoplasmic reticulum.

## 1. Introduction

Pollution of the aquatic ecosystems and its toxic effects on aquatic species has been of long-term interest and concern (Taylor, 1996). Mercury (Hg) is one of the most hazardous environmental pollutants occurring naturally and subject to increasing contamination from anthropogenic sources. In the aquatic environments, Hg is found as a metallic or elemental form, inorganic compounds or organic compounds (Black et al., 2007) and each form has a different toxicological profile and biochemical effects. Although organic mercury is the most toxic form, the inorganic mercury is the most common form of mercury released in the aquatic environments by industries (Oliveira Ribeiro et al., 1996). Inorganic Hg compounds have been widely used in the chlor-alkali, chemical and pharmaceutical industries. Particularly, mercuric chloride (HgCl<sub>2</sub>, sublimate corrosive) is widely used as fungicide for wood preservation, as a biocide in the paper industry, in fluorescent lamps, dry battery depolarizer, tanning agent for leather, catalyst in the manufacture of chemicals such as vinyl chloride and disinfectants, dental amalgam fillings among others (Broussard et al., 2002;

Wang et al., 2004; Rustagi and Singh, 2010). High concentrations of Hg (up to 0.05 mg L<sup>-1</sup>) have been found in water samples at the sites with extensive industrial activities (Hypolito et al., 2004; Alinnor, 2005; Bollen et al., 2008). The environmental impact of mercury can be aggravated by ecological disaster such the recent collapse of a mining dam in the Brazilian state of Minas Gerais (near Mariana County in November, 05<sup>th</sup> 2015). The dam burst at an iron ore mine released metal-rich tailing wastes (arsenic, cadmium, copper, chromium, nickel and mercury) in concentrations that endanger human and ecosystem health (IGAM, 2015; Fernandes et al., 2016).

Exposure to inorganic mercury may have harmful effects on fish inducing histopathological damage to liver, kidney, and gills (Oliveira Ribeiro et al., 2000, 2002), oxidative stress (Berntssen et al., 2003; Elia et al., 2003; Monteiro et al., 2010), reduced swimming capability (Vieira et al., 2009), developmental damage and immunotoxicity (Zhang et al., 2016). In mammals, inorganic Hg also produces profound cardiotoxicity (Massaroni et al., 1992; Oliveira et al., 1994; Furieri et al., 2011b) and induces endothelial dysfunction by reducing NO bioavailability (Massaroni et al., 1995; Furieri et al., 2011a). Therefore, mercury, even in the inorganic form, could be considered an important risk factor in cardiovascular disease (Fernandes Azevedo et al., 2012). However, knowledge of the toxicological impact of inorganic mercury on the cardiac function in tropical freshwater fish, under acute and long-term exposure by different routes is limited. In fish, as in all other vertebrates, the cardiovascular system is critical to performance and survival because it ensures the effective distribution/transport of oxygen and nutrients to the tissues (Gamperl and Driedzic, 2009).

Matrinxã, *Brycon amazonicus* (Characiformes, Characidae), is an omnivorous and rheophilic species that inhabits clear and well oxygenated waters. It was originally native to the Amazon basin, but has spread over most Brazilian hydrographic basins (Margarido and Galetti, 1996). Trahira, *Hoplias malabaricus* (Characiformes, Erythrinidae), is a carnivorous freshwater fish found in a large diversity of South American aquatic environments, including lakes, reservoirs, and streams (Bialetzki et al., 2002). Both species constitute a potential resource for local consumption and sport fishing (Craig, 2015; Dias et al., 2012) and are interesting animals to evaluate toxic effects of pollutants in Brazilian ecosystems due to their food chain positions.

Considering the Hg contamination of aquatic ecosystems and scarcity of cardiac physiological studies with native species, we investigated the effects of inorganic mercury on *in vivo* cardiac function and *ex vivo* myocardial contractility after acute waterborne exposure or longer-term exposure by trophic route. This study provided direct evidence of how mercury impacts the heart plus the mechanisms underlying toxic effects of Hg on cardiac muscle. Understanding how Hg impacts the heart of native fish species may provide an important tool for monitoring and predicting the ecotoxicological effects of pollutants in the aquatic environment.

## 2. Materials and Methods

### 2.1 Animal care

Matrinxã, *Brycon amazonicus* (Teleostei, Characiformes, Characidae), and trahira, *Hoplias malabaricus* (Teleostei, Characiformes, Erythrinidae), were obtained from fish culture farms and acclimated for 30 days prior to experimentation in 500 L holding tanks equipped with a continuous supply of well-aerated and dechlorinated water at a constant temperature ( $25 \pm 2$  °C) under natural photoperiod (~12h light:dark cycle). During this period, matrinxãs were fed *ad libitum* with commercial fish pellets (40% of protein) and trahiras were fed at a ratio of 2 % of biomass day<sup>-1</sup> with small alive matrinxãs (~10 g). The water quality parameters were maintained at controlled levels: dissolved oxygen (~ 7.3 mg L<sup>-1</sup>), pH (~7.2), conductivity (~130  $\mu$ S cm<sup>-1</sup>), alkalinity (38-43 mg L<sup>-1</sup> as CaCO<sub>3</sub>), and total hardness (40-50 mg L<sup>-1</sup> as CaCO<sub>3</sub>).

### 2.2 Experimental Design

For waterborne Hg exposure, specimens of *B. amazonicus* (body mass =  $139.3 \pm 8.6$  g, mean  $\pm$  SE) were divided into two experimental groups: control (Ctrl; n = 24) and HgCl<sub>2</sub>-exposed (wHg; n = 24) at the sublethal concentration (~20% of LC<sub>50</sub>-96 h, as previously established by Monteiro et al., 2010) of 0.1 mg L<sup>-1</sup> of Hg (HgCl<sub>2</sub> > 99.5 % purity, Sigma-Aldrich) for 96h in static holding tanks (see details in Monteiro et al., 2010). Hg concentrations were evaluated by Cold Vapor Atomic Fluorescence Spectrometry (CV-AFS) according to the USEPA 1631 detection technique (USEPA 2002). This concentration was chosen based on the concentration range often detected in water bodies near industrialized areas (from 0.01 to 0.23 mg L<sup>-1</sup>) as reported by Alinnor (2005), Bollen et al. (2008), Hypolito et al. (2004).

For trophic Hg exposure, specimens of *H. malabaricus* (body mass =  $230.2 \pm 23.5$  g, mean  $\pm$  SE) were divided into two treatment groups: control (Ct, n = 24) and Hg exposed group, *via* contaminated food (tHg, n = 24). The food supply consisted of four live young specimens of *B. amazonicus* (5-80 g) at each 96h (from clean water or following exposure to 0.1 mg of HgCl<sub>2</sub> during 96h), corresponding to 2% of the total body weight (bw) per day, over 30 consecutive days. This food ratio was based on the studies of Rios et al. (2005) and the experimental design is fully described in Monteiro et al. (2013a). The total Hg concentration in these young matrinxãs was analyzed by a Cold Vapor Atomic Absorption Spectrometry (CV-AAS) following the method recommended by the United States Pharmacopeia (USP 2000). Considering the average mass of the prey, each *H. malabaricus* received about 0.45 mg of Hg every 96h. This tested dose of total Hg was estimated from fish collected in impacted areas (Francesconi and Lenanton, 1992; Alho and Vieira, 1997; Hylander et al., 2000). The trophic contamination carried out in the present study refers exclusively to Hg inorganic forms, since no methyl mercury levels exceeding 1  $\mu$ g kg<sup>-1</sup> were detected in the matrinxãs exposed to HgCl<sub>2</sub> and provided as prey for the trahiras. Methyl mercury

was analyzed by gas chromatography mass spectrometry according to the method of AOAC (2006).

In the present study, both water and food Hg concentrations caused a metal bioaccumulation in different tissues of matrinxã and trahira, as described in Monteiro et al. (2010; 2013a). In brief, the concentration of Hg in the tissues after waterborne exposure and trophic exposure occurred as follow: gills ( $\sim 17.8 \text{ mg kg}^{-1}$ ) > liver ( $\sim 10.5 \text{ mg kg}^{-1}$ ) >> heart ( $\sim 0.7 \text{ mg kg}^{-1}$ )  $\approx$  white muscle ( $\sim 0.6 \text{ mg kg}^{-1}$ ) for *B. amazonicus*; and gills ( $\sim 1.5 \text{ mg kg}^{-1}$ ) > liver ( $\sim 1.1 \text{ mg kg}^{-1}$ ) >> white muscle ( $\sim 0.2 \text{ mg kg}^{-1}$ )  $\approx$  heart ( $\sim 0.1 \text{ mg kg}^{-1}$ ) for *H. malabaricus*.

### 2.3 Ex vivo Experiments

In order to analyze of the cardiac excitation-contraction coupling in cardiac myocytes, fish (n = 12 per experimental group) were euthanized by concussion followed by spinal cord transection and the hearts were carefully excised and immediately transferred to a Ringer solution containing (mM): 125.0 NaCl, 2.5 KCl, 0.94 MgSO<sub>4</sub>, 1.0 NaH<sub>2</sub>PO<sub>4</sub>, 30.0 NaHCO<sub>3</sub>, 1.5 CaCl<sub>2</sub>, 10 glucose at pH 7.4. Strips of ventricular muscle with a maximal thickness of 3 mm (mean mass  $2.90 \pm 0.27 \text{ mg}$ ) were excised then tied at each end to two metal rings and immersed in a bath containing the Ringer solution at 25°C, continuously bubbled with a gas mixture of 2% CO<sub>2</sub> and 98% O<sub>2</sub>. One ring was attached to a LETICA isometric force transducer (Letica Corporation, USA), through a stainless steel wire and the other was tied around a platinum electrode connected to an AVS 100D stimulator (Solução Integrada Ltda., Brazil) which delivered square electrical pulses of 8 ms and a voltage 50% above that eliciting maximal twitch force. Preparations were stretched to obtain a twitch tension at the maximum of the length-twitch tension relation.

After the stabilization period (40 min at 0.2 Hz or 12 bpm), twitch force were recorded by dedicated data-acquisition software (Soft & Solutions, Solução Integrada Ltda., São Paulo, Brazil). The length and wet mass of each strip were measured and isometric force (F<sub>c</sub>) relative to cross-sectional area (mN mm<sup>-2</sup>) was calculated assuming a muscle density of 1.06-mg·mm<sup>-3</sup> (Layland et al., 1995). Time-dependent parameters such as the rates of contraction and relaxation, represented by the maximum rate of tension rise (+dF/dt – mN mm<sup>-2</sup> s<sup>-1</sup>) and maximum rate of tension decline (-dF/dt – mN mm<sup>-2</sup> s<sup>-1</sup>), were also determined.

#### 2.3.1 Experimental protocols: force-frequency relationship and post-rest behavior

The effect of stimulation frequency on the contractility of fish heart was studied by increasing pacing rate from 0.2 Hz (12 bpm) until the frequency at which at least 80% of the strips were still able to contract regularly. The cardiac pumping capacity (CPC) at each stimulation frequency was calculated as the product of F<sub>c</sub> and heart rate as previously described by Matikainen and Vornanen (1992). Thereafter, post-rest potentiation was measured at 0.2 Hz. Electrical stimulation was switched off during pause intervals of 10, 30, 60, and 300 s and isometric twitch tension was

analyzed at the first beat upon re-stimulation. The relative potentiation was evaluated as the ratio of post-rest contraction and last contraction in a steady-state train. Post-rest contractile behavior reflects the amount of  $\text{Ca}^{2+}$  stored within and released from the SR in the first contraction after rest (Pieske et al., 1996).

To evaluate the role of SR  $\text{Ca}^{2+}$  stores in excitation-contraction coupling, the experiments described above were repeated after addition of the alkaloid ryanodine ( $10 \mu\text{M}$ ) to the bath 30 min before each protocol to inhibit SR function (Mill et al., 1998). Ryanodine promotes continuous leakage of the stored  $\text{Ca}^{2+}$  in SR and disrupts SR  $\text{Ca}^{2+}$  release by the  $\text{Ca}^{2+}$  pool that enters the cardiomyocyte through sarcolemmal  $\text{Ca}^{2+}$  channels (Bassani et al., 1999).

#### 2.4 *In vivo Experiments*

Fish ( $n = 12$  per experimental group) used for electrocardiogram (ECG) recordings were anaesthetized by immersion in a solution of benzocaine ( $0.5 \text{ g L}^{-1}$ ) and then placed on a surgical tray. Using sterile techniques, two ECG electrodes (for details see Monteiro et al., 2013) were inserted under skin in the ventral midline approximately 2 cm apart, one just anterior to the heart and the second caudal to the heart. The procedure took about 5 minutes. Recovery from anesthesia was achieved by gill/skin irrigation with anesthetic-free water. Each fish was then placed in the experimental tank for 12h enclosed in an open flow-through respirometer supplied with normoxic water ( $140 \text{ mmHg}$ ) at  $25 \text{ }^\circ\text{C}$  and allowed to fully recover from the operation. The electrodes were connected to a bioamplifier and a ground electrode was immersed in the experimental tank. ECG was recorded at 1 KHz, amplified and filtered using the bioamplifier and PowerLab data acquisition supplied by ADInstruments, Brazil. Besides heart rate ( $f_H$ ), ECG recordings were used to determine: a) duration of the P wave, QRS complex, and T wave (atrial depolarization, ventricular depolarization and ventricular repolarization duration, respectively); b) RR intervals (time measurement between the R wave of one heart beat and the R wave of the preceding heart beat); c) PR interval (time for impulse propagation from atrium to ventricle); d) QT interval (duration of ventricular action potential); and e) ST segment (representing phase two or the plateau of the action potential).

#### 2.5 *Statistical analysis*

Results are presented as means  $\pm$  S.E.M. Data from control and Hg exposed groups were tested for statistically significant differences using the Student t-test. The Kolmogorov and Smirnov method was applied to evaluate normality of the samples and the F-test was applied for homogeneity of variances. Analysis of variance one-way Anova followed by Dunnett's post hoc tests was used to check the existence of significant variations between the values obtained at different stimulation frequencies for the same experimental group. Differences between means at a 5% ( $P < 0.05$ ) level were considered significant.



### 3. Results

The effects of increasing stimulation frequency on contractile parameters of both experimental groups are shown in Fig. 1. Mercury exposure reduced the maximum sustained frequency of ventricular strips of both species, from 120 bpm to 96 bpm. Matrinxã and trahira ventricle strips showed a negative force frequency relationship, revealed by the significant and progressive reduction of  $F_c$  values from 24 and 48 bpm (Figs. 1A and 1D), respectively, and reaching minimum values at the highest sustained frequencies. For matrinxã, the changes in the  $+dF/dt$  with the increased stimulation frequency were similar to that observed in the contractile force under control conditions or after exposure to Hg. The  $+dF/dt$  values decreased significantly above 60 e 72 bpm in Ctrl and wHg groups, respectively, when compared to initial values (Fig. 1B), while  $-dF/dt$  was maintained constant in the range of tested frequencies (Fig. 1C). However, increases in the stimulation frequency had no effect on  $+dF/dt$  and  $-dF/dt$  of trahira ventricle strips (Figs. 1E and 1F). Both Hg exposure routines significantly decreased the force development and the contraction and relaxation rates at all frequencies studied. Nevertheless, the decreases in contraction force and rates of contraction and relaxation of matrinxã preparations, caused by wHg exposure (~ 48%, 48%, and 51%, respectively), were more prominent than those caused by tHg exposure in trahira (~ 20%, 35%, and 32%, respectively).

Fig. 1

Cardiac pumping capacity (CPC) is shown in Fig. 2. For matrinxã (Fig. 2A), CPC increased significantly above 24 bpm (0.4 Hz) in relation to the initial values of 12 bpm in both the Ctrl and wHg groups. For trahira (Fig. 2B), the optimum frequency for the pumping capacity in the C group ranged between 48 and 120 bpm (0.8 to 2.0 Hz) while it ranged between 48 and 96 bpm in the tHg group. Statistical analysis revealed that the CPC values of the Hg groups (waterborne and trophic exposure) were lower than the control groups at all stimulation frequencies analyzed in both species (50% and 32% respectively).

Fig. 2

The relative participation of sarcoplasmic reticulum (SR) in total force-associated  $Ca^{2+}$  flux as a function of the stimulation frequency is presented in Fig. 3. This variable was assessed as the effect of ryanodine on isometric tension development in isolated ventricular strips for each species and treatment. A leftward and downward shift in the force frequency relationship was detected after treatment with ryanodine in both control and Hg groups. The proportion of the SR  $Ca^{2+}$  contribution

to excitation-contraction coupling in the myocardium of control groups of matrinxã and trahira was approximately 81% and 69% respectively (Figs. 3A and 3D). Following Hg exposure the SR contribution to tension generation revealed by treatment with ryanodine, fell to around 51% in matrinxã and 53% in trahira (Figs. 3B and 3E). Thus, mercury exposures *per se* reduced the relative SR contribution to a rise in intracellular  $Ca^{2+}$  in matrinxã (~ 30%) and trahira (~ 16%) at all stimulation frequencies analyzed (Figs. 3C and 3F).

Fig. 3

Fig. 4 illustrates the post-rest contraction behavior of cardiac strips from matrinxã (Fig. 4A) and trahira (Fig. 4B) when stimulated at 0.2 Hz at varied diastolic periods after interruption of repetitive stimulation, with or without ryanodine. Both control and Hg groups showed a significant post-rest potentiation which was completely abolished by ryanodine. The amplitude of the first contraction after the prolonged diastolic interval (10 - 300 sec) was higher than the preceding contraction in all experimental groups (Ctrl, wHg, Ct, and tHg). Maximum post-rest potentiation occurred after 300 sec, with increments of 31% and 42% for matrinxã and trahira, respectively, under control conditions, and 17% and 12% after waterborne or trophic Hg exposure, respectively. In the control groups the post-rest potentiation values were significantly higher in matrinxã ventricle strips after rest periods of 30 - 300 sec, while in trahira this potentiation was higher during steady stimulation (0.2 Hz).only following a rest period of 300 sec.

Fig. 4

Changes in the ECG recordings of matrinxã (*Brycon amazonicus*) and trahira (*Hoplias malabaricus*) after Hg exposures are shown in Table 1 and Figures 5 and 6.

For waterborne Hg exposure, the T wave duration decreased from 167 to 103 ms (- 38%) while PR interval and ST segment were elongated from 81 to 91 ms (12%) and from 168 to 212 ms (26%), respectively. These alterations are evidenced in Fig. 5.

Table 1

Fig. 5

On the other hand, the ECG pattern of *H. malabaricus* was profoundly influenced by Hg trophic exposure. Heart rate was significantly slowed down to 39.8  $beats\ min^{-1}$  (- 27%) and T wave duration was shortened by 50% while the duration of P wave and QRS complex was found to be significantly higher (from 93 to 106 ms and from 69 to 93 ms, respectively). Concomitantly, RR, PR and QT intervals, and ST segment were also significantly prolonged by 38, 13, 20, and 34%,

respectively. In addition, QRS complexes were frequently directed downward, opposite to the polarity of the P and T waves as shown in Fig. 6.

Fig. 6

#### 4. Discussion

This study on two South American freshwater teleost fish (matrinxã, *B. amazonicus*, and trahira, *H. malabaricus*) is the first to demonstrate that inorganic mercury, whether accumulated from the external environment or from the diet (waterborne or trophic exposure), impairs *in vivo* heart function and *ex vivo* myocardial contractility, falling E-C coupling efficiency and contraction/relaxation kinetics.

Myofilaments are activated during the E-C coupling in response to an increased cytosolic  $\text{Ca}^{2+}$  concentration which can occur in response to mobilization of these ions from the sarcoplasmic reticulum (SR) as well as by influx through the sarcolemma (SL), which can occur *via* L-type  $\text{Ca}^{2+}$  channels and/or  $\text{Na}^+/\text{Ca}^{2+}$  exchanger (NCX) acting in the reverse mode. On the other hand, cardiac muscles relax when intracellular  $\text{Ca}^{2+}$  content is reduced back to its diastolic resting levels by  $\text{Ca}^{2+}$  transportation out of the cell *via* the sarcolemmal  $\text{Ca}^{2+}$ -ATPase and, in addition, mainly by the NCX and/or its accumulation inside the SR, which occurs by pumping activity *via* SERCA - sarcoplasmic-endoplasmic  $\text{Ca}^{2+}$ -ATPase (Kalinin et al., 2009). Regardless of stimulation frequency (including the *in vivo* frequency range, Table 1) the ventricular strips of Hg-exposed animals presented a reduced contraction force compared with controls. This reduction in performance may result from a decrease in  $\text{Ca}^{2+}$  transients, including reduced calcium-induced calcium release (CIRC) from the SR. In both species studied, Hg exposures shifted the force frequency relationship to the left and downward, reflecting a lower amplitude  $\text{Ca}^{2+}$  transient. The negative staircase effect, characteristic of the teleosts force frequency relationship, may be explained by the frequency dependent decline in  $\text{Ca}^{2+}$  transient due to a decreased transsarcolemmal  $\text{Ca}^{2+}$  influx (*via* L-type  $\text{Ca}^{2+}$  channel or NCX reverse mode) or a reduced SR  $\text{Ca}^{2+}$  release associated with a shortest time for mechanical restitution at high frequencies (Shiels et al., 2002). The decrease in cardiac contractility and in maximum pacing frequency represents an important and deleterious effect of Hg on matrinxã and trahira heart function.  $\text{Hg}^{2+}$  oxidizes sulfhydryl groups of  $\text{Na}^+/\text{K}^+$ -ATPase under *in vitro* and *in vivo* conditions (Kade, 2012). The inhibition of the sarcolemmal  $\text{Na}^+$ ,  $\text{K}^+$ -ATPase results in a digitalis-like effect, increasing the intracellular  $\text{Na}^+$  levels and thus, reducing the NCX activity (Oliveira et al., 1994).

Under Hg exposures, the negative force-frequency relationship becomes significantly steeper at physiological frequencies, denoting a mercury negative lusitropic effect. Accordingly, ventricular strips from both species exhibited slower rates of contraction and relaxation, indicating that the

beat-to-beat intracellular  $\text{Ca}^{2+}$  cycling dynamics was impaired by Hg. Moreover, Hg exposures attenuated the cardiac pump capacity (CPC = product of heart rate and force) curves. The calculated CPC can be used as an index of power output for isolated heart preparations since it integrates the effects of changes in force and changes in stimulation frequency (Shiels et al., 1999). Thus, Hg exposures, *via* water or food-chain, decreased the optimum frequency for power output in both species and this is probably related to the slower rates of tension development and relaxation of the ventricular strips. Previous studies have demonstrated that inorganic Hg functionally disrupts E-C coupling in the mammalian heart by inhibition of the sarcolemmal  $\text{Na}^+/\text{K}^+$ -ATPase, SERCA, and myosin ATPase (Hechtenberg and Beyersmann, 1991; Vassallo et al., 1999; Moreira et al., 2003). These actions might also be the way the metal exerts its effects on the fish heart, acting at several sites on the myocytes.

The survival of vertebrates depends on uninterrupted heart function and efficient adjustments of cardiac output under different physiological or adverse conditions (Driedzic and Gesser, 1994). According to Vornanen et al. (2002), the fish cardiac myocytes E-C coupling is a greatly variable and flexible process that enables fish to have an appropriate cardiac scope to take advantage of a diverse range of environments. The efficiency of the heart is critically dependent on the myocardial contractility which, in turn, depends on the intracellular  $\text{Ca}^{2+}$  homeostasis. After Hg exposure, *matrinxã* and *trahira* hearts were unable to maintain a normal cardiac output due to Hg-induced myocardial contractility dysfunction. Since strategies for modulating cardiac output to meet cardiovascular demands are crucial, the impact of Hg on cardiovascular performance of both fish species can compromise their survival.

The post-rest potentiation of force occurs due to the accumulation of calcium ions in the SR terminal structures during the rest period. The SR  $\text{Ca}^{2+}$  content tends to increase with increased duration of pauses, resulting in an increased  $\text{Ca}^{2+}$  release in the first contraction after rest (Kondratyeva et al., 2012). Thus, changes in post-rest contractile force result primarily from changes in the amplitude of the intracellular  $\text{Ca}^{2+}$  transients, reflecting the SR capacity of calcium storage (Mill et al., 1992; Pieske et al., 1996). In *matrinxã* and *trahira*, SR  $\text{Ca}^{2+}$ -stores have a predominant role on cardiac contractility since ryanodine effectively abolished post-rest potentiation and reduced contraction force. The relative contribution of the SR  $\text{Ca}^{2+}$  to force generation varies remarkably among species (Bers, 2002), mostly in fish (Vornanen et al., 2002; Haverinen and Vornanen, 2009; Korajoki and Vornanen, 2013). In mammalian cardiac muscle, SR plays a key role as source of activator  $\text{Ca}^{2+}$  meanwhile in fish cardiac myocytes, the extracellular  $\text{Ca}^{2+}$  cycling is generally more important than intracellular  $\text{Ca}^{2+}$  stores (Driedzic and Gesser, 1994; Vornanen et al., 2002). In this study, although force-frequency relationship was negative, the substantial role of SR  $\text{Ca}^{2+}$ -release in force generation (70-80%) is similar to humans and rabbits, suggesting that *matrinxã* and *trahira* can be alternative and attractive models to study myocardial contractility.

The importance of the SR as a source of  $\text{Ca}^{2+}$  to force development was attenuated after Hg exposures (*via* water or food) probably due to a reduced  $\text{Ca}^{2+}$  reuptake caused by a SERCA2a dysfunction. Consequently, a decreased amount of activator  $\text{Ca}^{2+}$  will be available for the next contraction. These results highlight the Hg-induced negative inotropic effect. In accordance with these possibilities, inorganic mercury is a strong inhibitor of SERCA activity in rabbit cardiac muscle (Hechtenberg and Beyersmann, 1991). In isolated rat ventricular tissue, the exposure to mercury reduced the SERCA/phospholamban ratio, decreasing the SR  $\text{Ca}^{2+}$  uptake and thus, contributing to a calcium overload and contractility dysfunction (Furieri et al., 2011b).

Our results are also corroborated by those of Oliveira and Vassalo (1992) and Oliveira et al. (1994) which reported a progressive reduction of the post-rest potentiation with increased concentrations of  $\text{HgCl}_2$  in isolated rat cardiac muscle, showing that SR function was depressed by mercury in a dose-dependent manner. According to Pieske et al. (1996), a reduced SR  $\text{Ca}^{2+}$  storage ability, alterations in SR  $\text{Ca}^{2+}$  release channels and/or excessive  $\text{Ca}^{2+}$  leak *via* ryanodine receptors (RyR2s) may contribute to abnormal  $\text{Ca}^{2+}$  handling and diminished post-rest potentiation. This uncontrolled CIRC has been related to a spectrum of cardiac disease states ranging from triggered and reentrant arrhythmias to heart failure as result from  $\text{Ca}^{2+}$  overload and/or sensitization of RyR2s by enhanced luminal  $\text{Ca}^{2+}$  leading to spontaneous  $\text{Ca}^{2+}$  releases (Györke and Carnes, 2008). Moreover, the *ex vivo* effects of Hg on myocardium contractility were further supported by *in vivo* experiments with these two different Hg-exposure routes (Monteiro et al., 2013b). We demonstrated that inorganic Hg impaired cardiorespiratory function in these species, promoting tachypnea in *matrinxã* and, in *trahira*, bradypnea associated with a decreased metabolic rate ( $\text{O}_2$  uptake), and severe bradycardia. Another aspect that should be mentioned is that these different doses and routes of inorganic Hg-exposure caused oxidative stress damages and mercury bioaccumulation in the cardiac tissues of both species under same experimental conditions (for details, see Monteiro et al., 2010, 2013a).

Inorganic Hg strongly influenced the contractile performance of cardiac muscle and impaired the electrical conduction activity in both fish species. Hg exposures induced: shortening of the T wave duration that represents the repolarization of the ventricles; prolongation of the PR interval, conventionally known as first-degree atrioventricular block; and lengthening of the ST segment that reflects the duration of ventricular action potential. The electrocardiographic findings indicate a cation imbalance leading to an altered excitability and, consequently, a limited heart pumping capacity. Massaroni et al. (1992; 1995) described similar electrical responses to inorganic mercury in rats and suggested that these changes may be related to the depression of  $\text{Na}^+, \text{K}^+$ -ATPase and of SR activity produced by mercury. In addition, the *H. malabaricus* ECG recordings also showed a negative chronotropic response after longer-term and trophic Hg exposure with reduced RR intervals, delayed atrial and ventricular depolarization (increased P and QRS duration), lengthened duration of ventricular electrical systole, including depolarization and repolarization (prolongation of

the QT interval), and a QRS complex with P and T waves apparently reversed in polarity. Altered QRS duration and morphology may indicate myocardial fibrosis and bundle branch block (Farraj et al., 2011), while QT interval prolongation is associated with delayed repolarization of cardiac myocyte action potential, thereby predisposing individuals to heart failure (Yu et al., 2010). The inversion of the QRS complex after Hg exposure may represent a real change in the direction of ventricular depolarization, though is difficult to explain in functional terms and likely to result in profound cardiac dysfunction. The recorded changes in the ECG waveforms after mercury exposure raise several questions that invite further investigation.

The *in vivo* cardiac activity was harmed by exposure to mercury in different ways depending on the route and/or time of exposure. Variations exist across species in terms of susceptibility. Further, in *Hoplias malabaricus*, a longer-term exposure could also contribute to the pronounced effects of inorganic Hg on cardiac rhythm and electrical conduction. Indeed, metal toxicity depends upon the absorbed dose, the route of exposure and duration of exposure, i.e. acute or chronic as well as the age, gender, genetics, and nutritional status of exposed individuals (Tchounwou et al., 2012; Jaishankar et al., 2014).

In summary, our results provide clear evidence that different routes of exposure to inorganic Hg disturb electrical and mechanical performance of the heart of two fish species with different life styles and ecological demands. Hg disrupted E-C coupling and potentially impaired the contraction force and maximal cardiac pumping capacity by interfering with SR Ca<sup>2+</sup> stores and reducing the SR importance to E-C coupling. So, mercury produces negative inotropic effects in fish heart resulting in weakened contractility and, consequently, decreased cardiac output. Moreover, Hg harmed heart electrophysiological mechanisms leading to atrioventricular conduction block and depolarization/repolarization events abnormally delayed. The Hg-induced dysregulation on myocardial Ca<sup>2+</sup> handling and electrical conduction have been implicated in heart failure and hemodynamic abnormalities, which can affect energy-demanding activities in fish such as swimming, foraging or reproduction.

**Ethics in Animal Experimentation.** All of the experiments were performed in compliance with Brazilian laws. This study was performed under the approval of the Committee of Ethics in Animal Experimentation (CEUA - Approval #04/2007) and Committee of Environmental Ethics (CEA - Approval #003/2006) of the Federal University of São Carlos and in accordance with the national guidelines for the care and use of laboratory animals.

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## Legends

**Figure 1.** Ventricular strips from: matrinxã (A, B, and C) and trahira (D, E, and F). Effects of increases in stimulation frequency on isometric twitch force ( $F_c$ ; A and D), rates of contraction ( $+dF/dt$ ; B and E) and relaxation ( $-dF/dt$ ; C and F) developed by ventricular strips of control (Hg-free water = Ctrl or Hg-free diet = Ct,  $n = 12$ ) and mercury exposed (*via* water = wHg or trophic exposure = tHg,  $n = 12$ ). Mean values  $\pm$  SEM. Open symbols denote a significant difference in relation to the values obtained at 0.2 Hz ( $P < 0.05$ ), while asterisks indicate significant differences between experimental groups at the same frequency ( $P < 0.05$ ).

**Figure 2.** Cardiac pumping capacity (CPC) developed by ventricular strips of control (Hg-free water = Ctrl or Hg-free diet = Ct,  $n = 12$ ) and mercury exposed (*via* water = wHg or trophic exposure = tHg,  $n = 12$ ) specimens of matrinxã (A) and trahira (B). Mean values  $\pm$  SEM. Open symbols denote a significant difference in relation to the values obtained at 0.2 Hz ( $P < 0.05$ ), while asterisks indicate significant differences between experimental groups at the same frequency ( $P < 0.05$ ).

**Figure 3.** Role of sarcoplasmic reticulum (SR) in force generation of ventricular strips from specimens of matrinxã (A, B and C) and trahira (D, E and F) at different stimulation frequencies: A and D are controls (Hg-free water = Ctrl or Hg-free diet = Ct,  $n = 12$ ) B and E are mercury exposed (*via* water = wHg or trophic exposure = tHg,  $n = 12$ ) in absence or presence of 10  $\mu$ M ryanodine (RYAN).  $F_c$  as a proportion of control are shown in C and F. Mean values  $\pm$  SEM. Open symbols denote a significant difference in relation to the values obtained at 0.2 Hz ( $P < 0.05$ ), while asterisks indicate significant differences between experimental groups at the same frequency ( $P < 0.05$ ).

**Figure 4.** Post-rest force contraction of ventricular strips of matrinxã (A) and trahira (B) from control (Hg-free water = Ctrl or Hg-free diet = Ct,  $n = 12$ ) or mercury exposed groups (*via* water = wHg or trophic exposure = tHg,  $n = 12$ ) with or without ryanodine (RYAN, 10 $\mu$ M) after rest periods of 10, 30, 60, and 300 seconds. Amplitude was expressed as a percentage of the previous steady-state contraction (0.2 Hz or 12 bpm), which is indicated by the dotted line. Mean values  $\pm$  SEM. \* indicates significant difference ( $P < 0.05$ ) in relation to steady-state force, # indicates significant difference ( $P < 0.05$ ) between Ctrl and wHg or Ct and tHg groups.

**Figure 5.** Electrocardiogram tracings of matrinxã, *Brycon amazonicus*, from (A) control (Hg-free water = Ctrl) or (B) mercury exposed group (*via* water = wHg) showing reduced T wave duration and prolonged PR intervals and ST segments.

**Figure 6.** Electrocardiogram tracings of trahira, *Hoplias malabaricus* from (A) control (Hg-free diet = Ct) or (B) mercury exposed group (trophic exposure = tHg) showing that the QRS complex was frequently directed downward and prolonged besides increases in the P wave duration, PR and QT intervals and ST segments.

ACCEPTED MANUSCRIPT

Figure 1

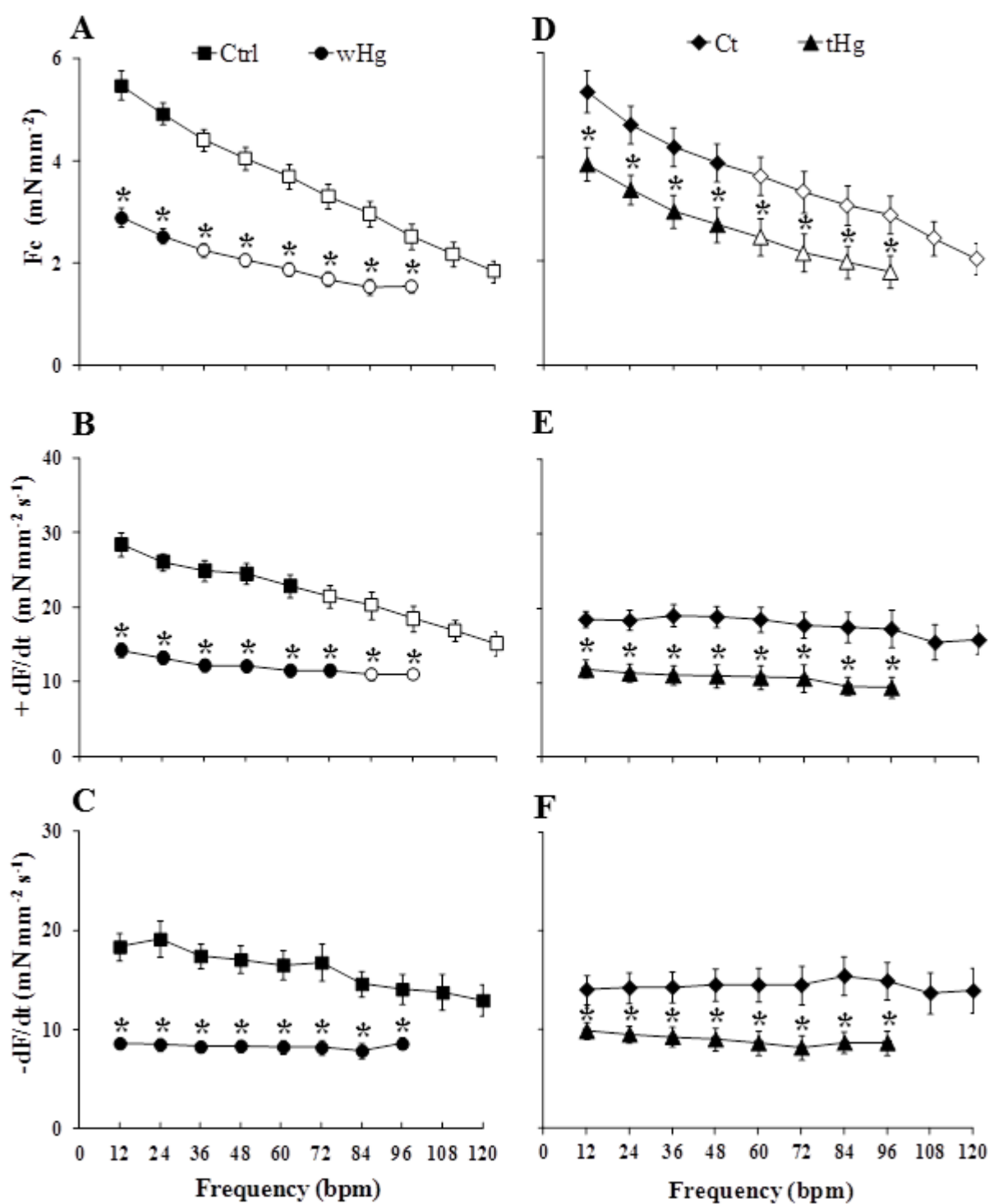


Figure 2

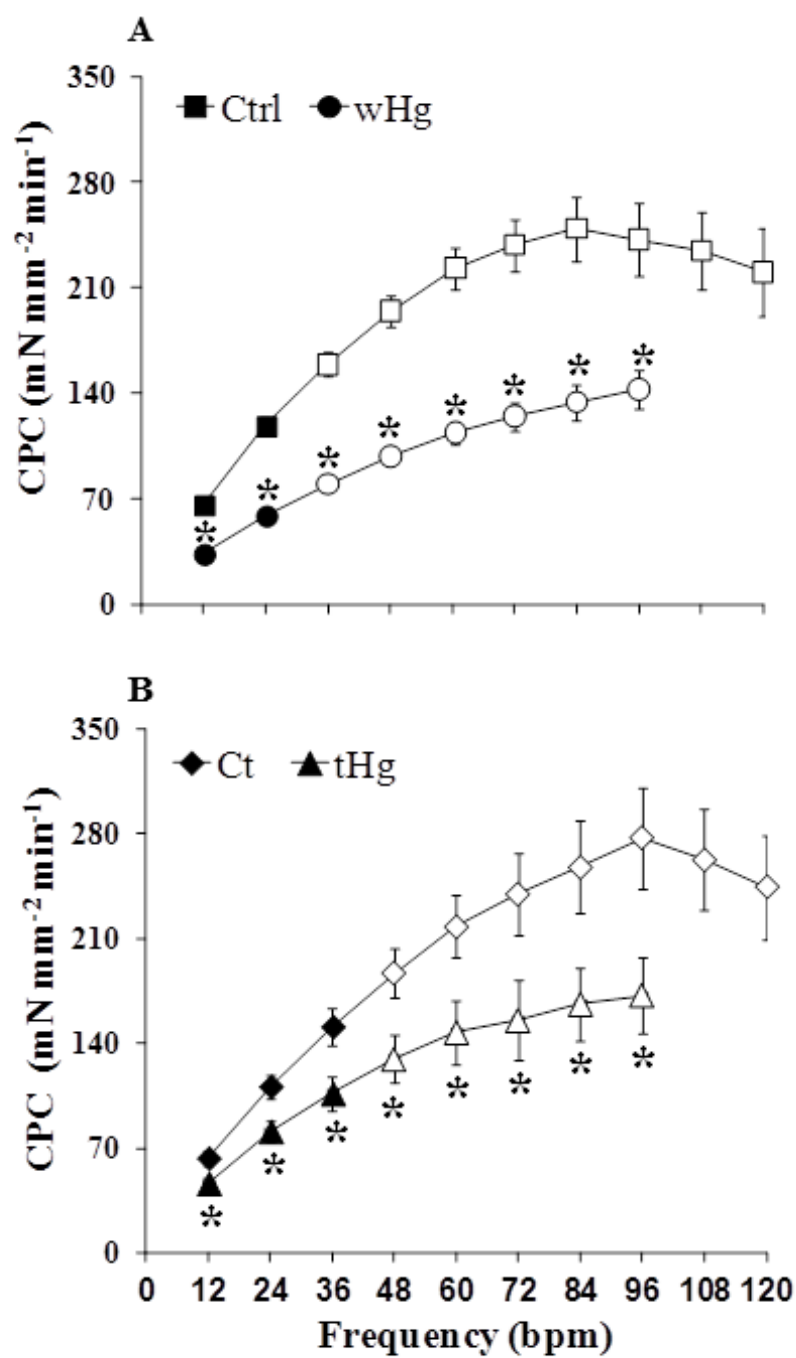




Figure 3

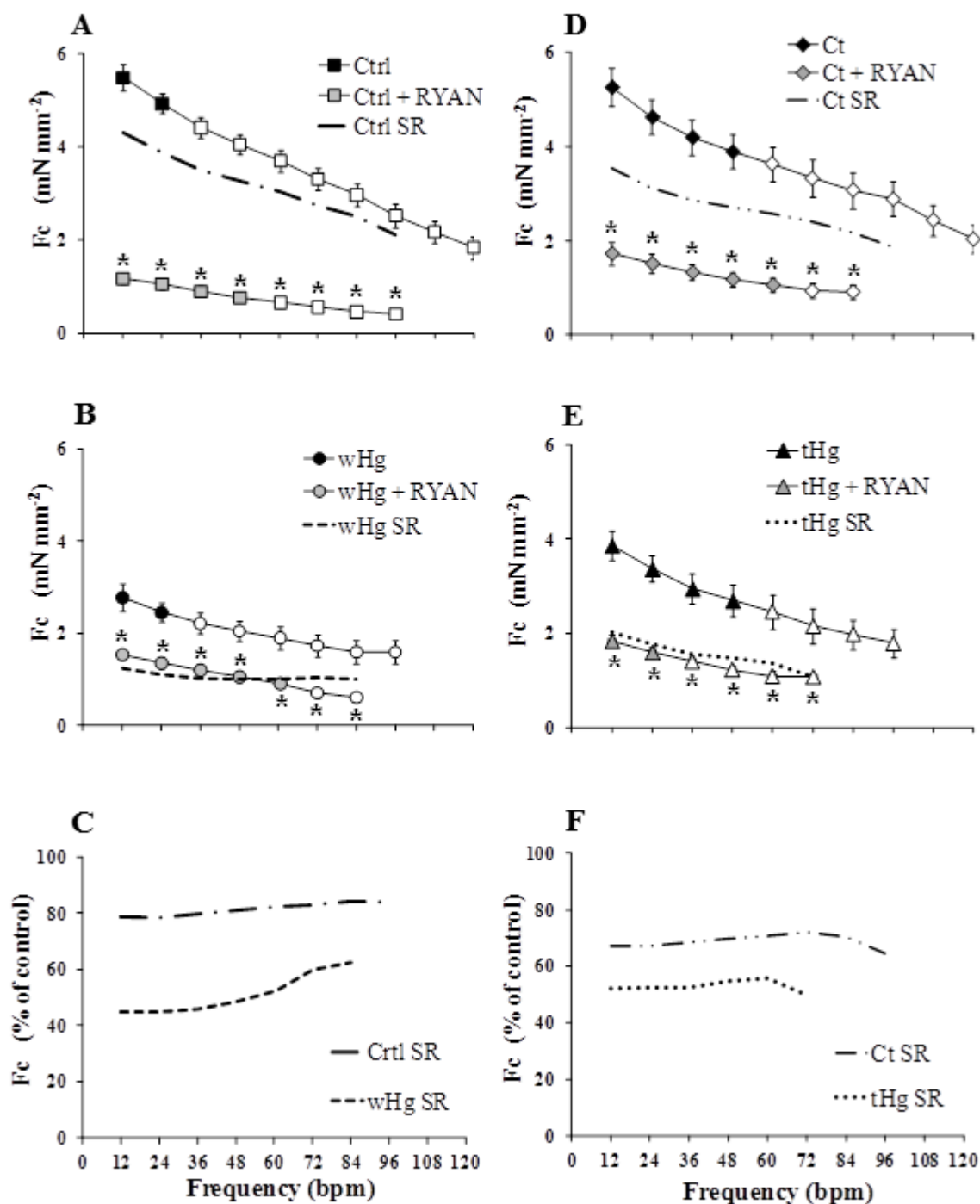


Figure 4

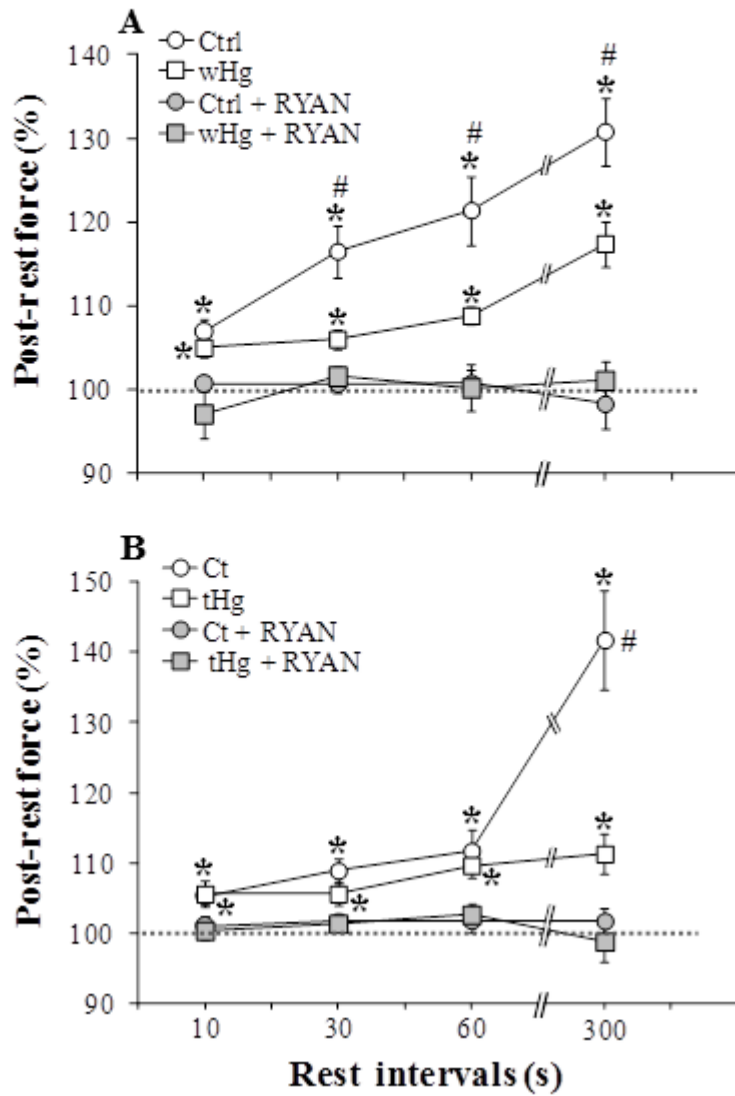
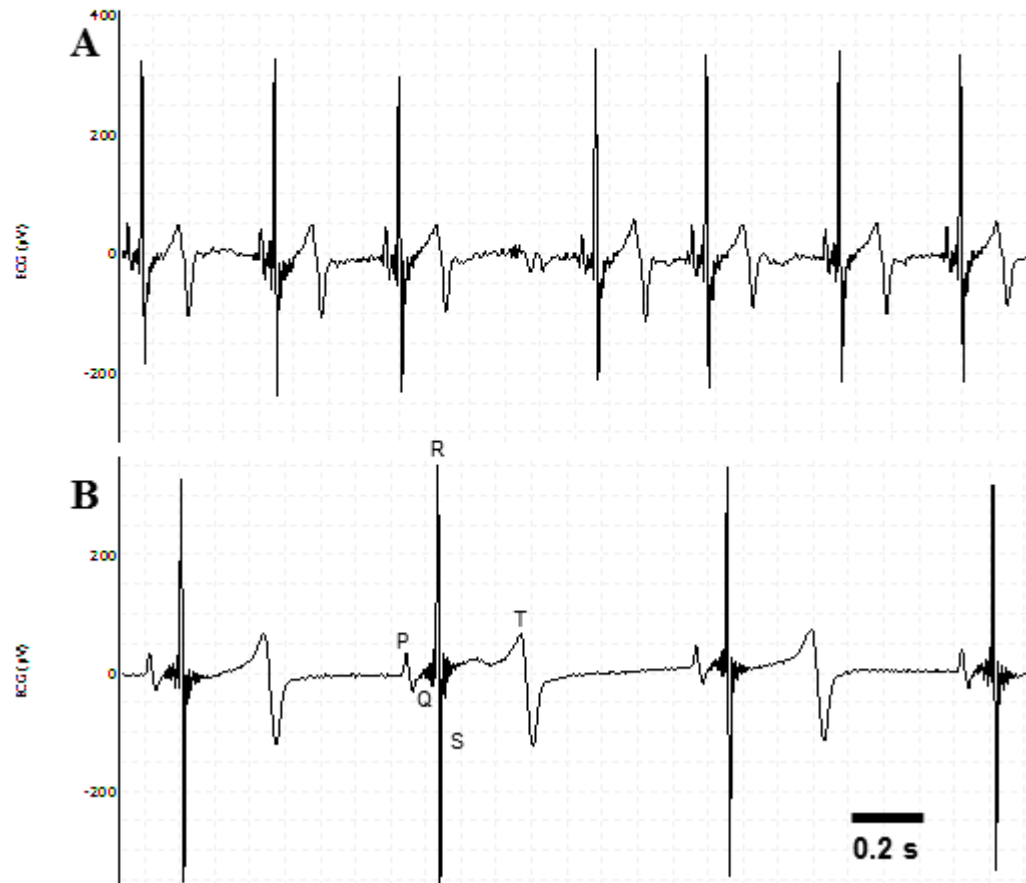
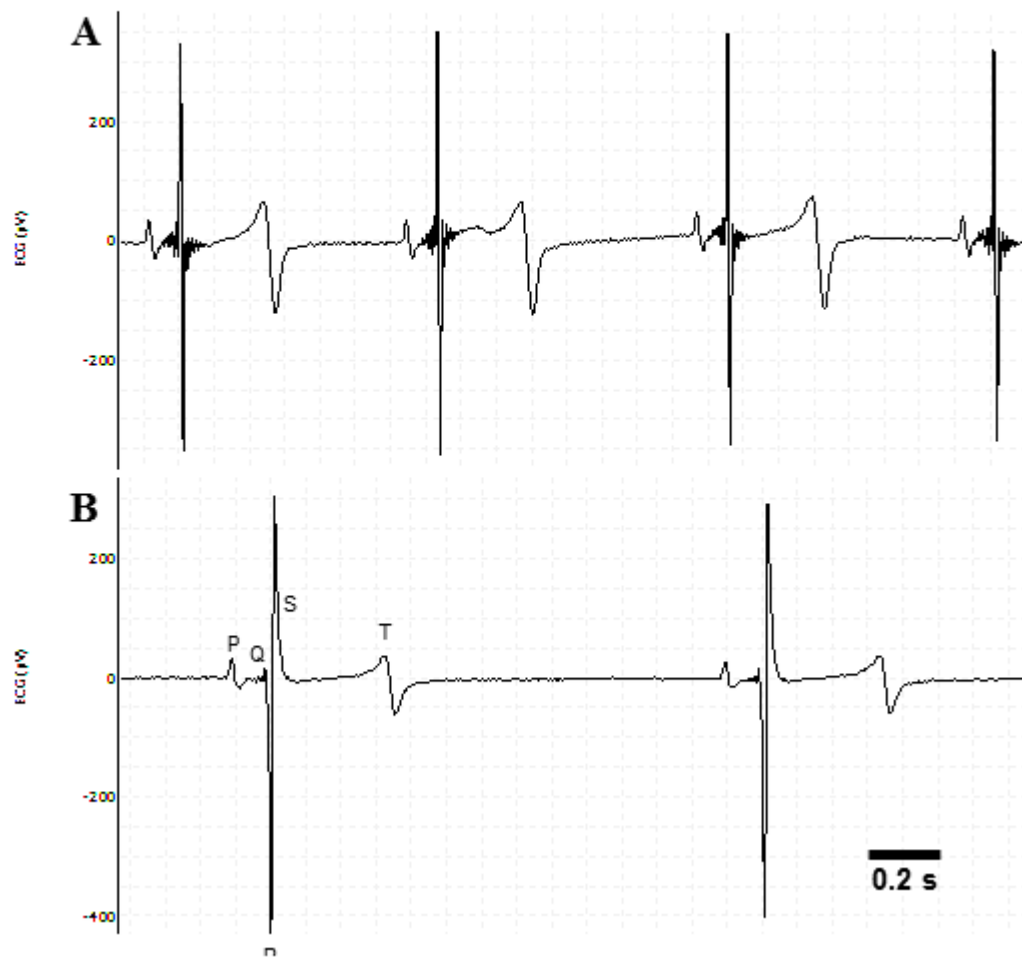


Figure 5



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Figure 6



ACCEPTED

**Table 1.** Effects of Hg exposures on different variables of *in vivo* ECG of matrinxã (*Brycon amazonicus*) and trahira (*Hoplias malabaricus*) Results are means  $\pm$  SEM. # indicates significant differences between experimental groups ( $P < 0.05$ ).

ECG parameters	<i>Brycon amazonicus</i>		<i>Hoplias malabaricus</i>	
	Ctrl	wHg	Ct	tHg
$f_H$ (beats $\text{min}^{-1}$ )	65.0 $\pm$ 3.7	73.4 $\pm$ 5.5	54.6 $\pm$ 5.2	39.8 $\pm$ 3.13 #
RR intervals (ms)	877.7 $\pm$ 46.7	856.0 $\pm$ 43.5	1252.5 $\pm$ 118.2	1732.6 $\pm$ 145.4 #
P duration (ms)	41.6 $\pm$ 2.7	38.0 $\pm$ 1.7	93.0 $\pm$ 2.9	106.3 $\pm$ 3.8 #
QRS duration (ms)	65.6 $\pm$ 4.7	63.5 $\pm$ 2.7	69.1 $\pm$ 8.1	93.2 $\pm$ 4.0 #
T duration (ms)	167.3 $\pm$ 17.9	103.5 $\pm$ 8.7 #	344.1 $\pm$ 33.5	175.4 $\pm$ 24.9 #
PR interval (ms)	81.2 $\pm$ 1.6	91.2 $\pm$ 3.8 #	190.0 $\pm$ 5.7	213.9 $\pm$ 5.9 #
QT interval (ms)	373.9 $\pm$ 9.4	373.5 $\pm$ 14.9	587.7 $\pm$ 28.0	704.1 $\pm$ 38.6 #
ST segment (ms)	168.4 $\pm$ 15.8	211.7 $\pm$ 6.5 #	283.6 $\pm$ 31.6	379.0 $\pm$ 28.8 #