Differences in Ca\(^{2+}\)-management between the ventricle of two species of Neotropical teleosts: the jeju, *Hoplerythrinus unitaeniatus* (Spix & Agassiz, 1829), and the acara, *Geophagus brasiliensis* (Quoy & Gaimard, 1824)

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This study analyzed the physiological role of the cardiac sarcoplasmic reticulum (SR) of two neotropical teleosts, the jeju, *Hoplerythrinus unitaeniatus* (Erythrinidae), and the acara, *Geophagus brasiliensis* (Cichlidae). While the *in vivo* heart frequency \((f_H - \text{bpm})\) of acara \((79.6 \pm 6.6)\) was higher than that of the jeju \((50.3 \pm 2.7)\), the opposite was observed for the ventricular inotropism \((F_c - \text{mN/mm}^2)\) at 12 bpm \((\text{acara} = 28.66 \pm 1.86 \text{ vs. jeju} = 36.09 \pm 1.67)\). A 5 min diastolic pause resulted in a strong potentiation of \(F_c\) \((\cong 90\%)\) of strips from jeju, which was completely abolished by ryanodine. Ryanodine also resulted in a \(\cong 20\%\) decrease in the \(F_c\) developed by strips from jeju at both subphysiological \((12 \text{ bpm})\) and physiological \((\text{in vivo})\) frequencies. However, this effect of ryanodine reducing the \(F_c\) from jeju was completely compensated by adrenaline increments \((10^{-9} \text{ and } 10^{-6} \text{ M})\). In contrast, strips from acara were unresponsive to ryanodine, irrespective of the stimulation frequency, and increases in adrenaline concentration \((10^{-9} \text{ and } 10^{-6} \text{ M})\) further increased \(F_c\). These results reinforce the hypothesis of the functionality of the SR as a common trait in neotropical ostariophysian (as jeju), while in acanthopterygians (as acara) it seems to be functional mainly in ‘athletic’ species.

O presente estudo analisou o papel fisiológico desempenhado pelo retículo sarcoplasmático (RS) de duas espécies de teleósteos neotropicais, o jeju, *Hoplerythrinus unitaeniatus* (Erythrinidae), e o acará, *Geophagus brasiliensis* (Cichlidae). Enquanto a frequência cardíaca registrada *in vivo* \((f_H - \text{bpm})\) para o acará \((79.6 \pm 6.6)\) foi superior àquela observada para o jeju \((50.3 \pm 2.7)\), resposta inversa foi verificada para o inotropismo ventricular \((F_c - \text{mN/mm}^2)\) na frequência de estimulação de 12 bpm \((\text{acará} = 28.66 \pm 1.86 \text{ vs. jeju} = 36.09 \pm 1.67)\). Uma pausa diastólica de 5 min resultou em uma expressiva potenciação da \(F_c\) \((\cong 90\%)\) das tiras de jeju, a qual foi completamente abolida pela rianodina. A rianodina também resultou em um decréscimo de \(\cong 20\%\) na \(F_c\) desenvolvida pelas tiras de jeju tanto a frequências sub-fisiológicas \((12 \text{ bpm})\) quanto fisiológicas \((\text{in vivo})\). No entanto, o decréscimo da \(F_c\) promovido pela rianodina foi completamente compensado pela adição de adrenalina \((10^{-9} \text{ e } 10^{-6} \text{ M})\). Em contraste, as tiras de acará foram irrelevantes à rianodina, independentemente da frequência de estimulação utilizada, fazendo com que a adição de adrenalina \((10^{-9} \text{ e } 10^{-6} \text{ M})\) resultasse em incrementos ainda maiores da \(F_c\). Esses resultados reforçam a hipótese de que a funcionalidade do RS seja uma característica comum aos ostariofíseos neotropicais (como o jeju), enquanto nos acantopterígios (como o acará) esta organela parece ser funcional principalmente em espécies ativas.

**Key words:** Excitation-contraction coupling, Ryanodine, Sarcoplasmic reticulum, Ventricle strips.

**Introduction**

The significance of the sarcoplasmic reticulum (SR) to the contraction-relaxation cycle of the 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, Ca\(^{2+}\) entering the cell during the action potential enhances the release of further Ca\(^{2+}\) from the SR to support the contractile process (Fabiato, 1983). In contrast, in frogs and teleost fish, Ca\(^{2+}\) required for contractility is derived under most circumstances primarily via transport across the sarcolemma, while Ca\(^{2+}\) stored inside the SR plays a secondary (if any) role (Tibbits et al., 1991).
Although present, the role of the SR in the beat-to-beat regulation of contractions 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). Ryanodine is a plant alkaloid that binds specifically and irreversibly to SR Ca²⁺-release channels, thereby reducing the functional importance of SR for the excitation-contraction (E-C) coupling (Nayler et al., 1970; Rousseau et al., 1987; Coronado et al., 1994; Bers, 2001). 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 and ventricle of tunas, ryanodine exerts a clear negative inotropic effect (Keen et al., 1992; Tibbits, 1996; Shiels et al., 1999, 2002), even at physiological frequencies.

These findings suggest that more active fish have a higher Ca²⁺-handling capacity in the cardiac SR than 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 (Olle, 2003; Anelli Jr. et al., 2004; Rivaroli et al., 2006) 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 fish ryanodine had a strong inhibitory effect on postrest force as well as at physiological frequencies (Costa et al., 2004; Rivaroli et al., 2006).

The results indicate a phylogenetical trail 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 neotropical teleosts which are phylogenetically distant and that present different levels of activity: the relatively sedentary jeju, Hoplerythrinus unitaeniatus (Superorder Ostariophysi), and the more active acara, Geophagus brasiliensis (Superorder Acanthopterygii). Moreover, another question to be answered was: to what extent an adrenergic stimulation can compensate the absence of a functional SR or its blockade by ryanodine?

Adrenaline is found in the blood reaching the heart of resting fish at a concentration between 10 and 100 nM (Butler et al., 1986), but its plasmatic levels rise in stressful conditions (e.g., exercise and temperature) to 10 µM (Farrell, 1984). Therefore, further experiments were made to determine whether a tonic adrenergic stimulation (10⁻⁹ M) and an adrenaline concentration observed at fish plasma during stress conditions (10⁻⁶ M) could maximize trans-sarcotlemmal Ca²⁺-influx and then partially or completely compensate the blockade of the SR function by ryanodine in species in which this organelle is functional.
Protocol 1

In this protocol, after the stabilization period (30 min, see above), 10 µM of ryanodine (Sigma) (SR function blocker) was added to the muscle bath, and the twitch force (mN/mm²) developed before (control) and 40 min after adding ryanodine were compared. This experimental protocol was performed to determine if the SR had any physiological role at sub-physiological frequencies (12 bpm).

Protocol 2

The purpose of protocol 2 was to determine whether the ventricle of each species presented a sarcoplasmic reticulum or not that could potentially accumulate calcium during the prolonged rest (see Thomas et al., 1986), as indicated by a postrest potentiation of twitch force (represented as a percentage (%) of the last contraction registered before pause). After a stabilization period at 12 bpm (30 min; see above), ventricle strips from both species were subjected to a prolonged and non-physiological diastolic pause of 5 min, and the first postrest contraction with and without pre-treatment with 10 µM of ryanodine were compared to each other and also to the force developed before rest.

Protocol 3

To determine the relative contribution of the SR in force development over a wide range of frequencies, including in vivo range (as determined by ECG recordings in the in vivo experiments, see above), the force-frequency relationship was determined with and without pre-treatment with 10 µM of ryanodine. Following the 30 min stabilization period, pacing frequency was increased in 12 bpm increments from 12 bpm until the frequency in which the muscle failed to show regular contractions.

Protocol 4

Protocol 4 was similar to protocol 3, but 40 min after adding ryanodine, 10⁻⁶ M of adrenaline (Merck) was added to the bath. After a 7 min stabilization period, ventricle strips were subjected to a force-frequency trial, as described above, and then back to 12 bpm. When the twitch was stabilized again at 12 bpm, adrenaline concentration was then increased to 10⁻⁶ M, and after 7 min, another force-frequency trial was carried out.

Data Presentation and analysis

The inotropic responsiveness of the ventricle strips to each experimental condition was measured by means of the twitch force (Fc - mN/mm² or % initial values), while the in vivo chronotropism is presented as bpm (QRS intervals . min⁻¹).

Results are presented as mean ± S.E. (n = 12). In all experiments, levels of significance with respect to the initial values obtained in the same experimental protocol were assessed with One-way Analysis of Variance (ANOVA) followed by Bartlett’s test for homogeneity of variances and the Tukey-Kramer multiple comparisons test (p < 0.05). Additionally, the Mann-Whitney test was used to compare the results obtained at different protocols (p < 0.05).

The resting heart rate measured in vivo by electrocardiography at acclimation temperature (25°C) was 50.3 ± 2.7 bpm for jeju (mean ± S.E.; n = 12) and 79.6 ± 6.6 bpm for acara (mean ± S.E.; n = 12). Additionally, after stabilization at 12 bpm at acclimation temperature (25°C), the twitch force developed by ventricle strips of jeju and acara at steady-state (12 bpm) were 36.09 ± 1.67 mN/mm² (mean ± S.E.; n = 12) and 28.66 ± 1.86 mN/mm² (mean ± S.E.; n = 12), respectively. However, 40 min after treatment with 10 µM of ryanodine, the twitch force of ventricle strips from jeju decreased approximately 20%, reaching force values similar to those observed for acara (Table 1). In contrast, the steady-state force (12 bpm) developed by ventricle strips from acara after treatment with ryanodine remained unchanged (p > 0.05) in relation to the control.

The relative contribution of the Ca²⁺ stored in the SR to force generation after a diastolic pause of 5 min with and without (control) pre-treatment with 10 µM of ryanodine is presented in Fig. 1. The non-physiological diastolic pause resulted in an increase of 90% (p < 0.05) in the force developed by ventricle strips from jeju in relation to the steady-state contractions. This postrest potentiation of the twitch force developed by ventricle strips from jeju was completely abolished by ryanodine. In contrast, the postrest contraction force of control and ryanodine-treated ventricle strips from acara remained unchanged (p > 0.05) in relation to the steady-state contractions.

In the force-frequency experiments, ventricle strips from both species showed a negative force-frequency relationship (Fig. 2), with a steeper curve presented by jeju in all protocols. However, for control preparations, initial absolute values of the force developed by strips from jeju were higher than those observed in acara (Table 1, Fig. 2a), while the force measured when the in vivo frequency was reached became similar for jeju (20.40 ± 0.94 mN/mm² at 50.3 bpm) and acara (17.13 ± 1.11 mN/mm² at 79.6 bpm). Furthermore, treatment with ryanodine did not shift up- or downwards the force-frequency curve of acara. In contrast, this curve was shifted downwards (initial from 36.09 ± 1.67 to 28.34 ± 2.44 mN/mm²) and also to the left (maximal regular contraction is decreased from 96 to 72 bpm) for jeju ventricle strips in response to ryanodine. At 50.3 bpm (in vivo fᵉᵣ), the twitch force developed by the ventricle strips from jeju was decreased by 4 mN (to 15.91 ± 0.37 mN/mm²) after being treated with ryanodine.

When applied at steady-state contractions (12 bpm) after pre-treatment with 10µM of ryanodine, a tonic dose of adrenaline (10⁻⁶ M) failed to change inotropic responsiveness of the strips from both species (Table 1). However, when an adrenaline concentration usually observed in the plasma in response to stressful conditions (10⁻⁴ M) was added to the bath, strips from acara and jeju showed, respectively, a 50% and 225% increase in Fc under steady-state stimulation frequency.

Moreover, the force-frequency curves tended to be
Table 1. Twitch force values (Fc - mN/mm², mean values ± S.E.; n = 12) developed by ventricle strips from acara and jeju at different stimulation frequencies (12, *in vivo*, and 96 bpm) in response to different treatments. Ctrl: without previous addition of adrenaline or ryanodine; + Ryan: pre-treated with 10 µM of ryanodine; T-Adr + Ryan: treated with 10-9 M of adrenaline and 10 µM of ryanodine; A-Adr + Ryan: treated with 10-6 M of adrenaline and 10 µM of ryanodine. I: irregular recording (i.e., at least 20% of preparations were unable to contract regularly at the respective frequency). #: different from Ctrl; a: different from + Ryan; b: different from T-Adr+Ryan; c: different from A-Adr+Ryan at the same stimulation frequency (Mann-Whitney non-parametric test). p < 0.05.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acara</th>
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<th>Jeju</th>
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<td></td>
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<td>12</td>
<td>79.6</td>
<td>96</td>
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<td>17.13 ± 1.11</td>
<td>14.06 ± 1.87</td>
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</tr>
<tr>
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</tr>
<tr>
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<td>33.30 ± 1.33</td>
<td>27.35 ± 2.54</td>
<td>33.35 ± 2.65</td>
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<tr>
<td>A-Adr + Ryan</td>
<td>44.27 ± 4.93</td>
<td>33.57 ± 3.74</td>
<td>26.40 ± 2.07</td>
<td>92.16 ± 7.91</td>
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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 minimizes the role of the SR as a calcium source to the activation of myofibrils (Tittu & Vornanen, 2001). However, the myocytes of cold-adapted fish developed adaptive mechanisms that lead to a relative temperature-insensitivity of sarcolemmal Ca²⁺-transporting systems (particularly L-channels and NCX), maximizing the proportional contribution of transsarcolemmal Ca²⁺ 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 Ca²⁺ to be delivered to myosin at a rate 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 in Ca²⁺ management.

In contrast, in very active temperate 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 diffusion 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 for rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792), especially at high temperatures and sub-physiological frequencies (Santer, 1974; Hove-Madsen & Gesser, 1989; Hove-Madsen, 1992; Möller-Nielsen & Gesser, 1992; Shiels & Farrell, 1997; Åho & Vornanen, 1998; Hove-Madsen et al., 1998; Lillywhite et al., 1999). Indeed, more 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 in more physiological conditions. An even higher contribution of the SR Ca²⁺ stores at physiological frequencies was described for the ‘athletic’ scombrids (tunas and mackerels) (Shiels et al., 2002). Corroborating these findings, Rivaroli et al. (2006) and Anelli Jr. et al. (2004) demonstrated the direct contribution of the SR to the ventricular inotropism at physiological frequencies and

Fig. 1. Effect of 10 µM of ryanodine on the force developed by ventricle strips from jeju (n = 12) and acara (n = 12) after a 5 min pause (Fc - % of the force developed at 12 bpm; mean values ± S.E.). The asterisk above the vertical bar denotes a difference in relation to the steady-state force (before rest) (ANOVA followed by Tukey-Kramer parametric test), while the asterisk above the horizontal line indicates the effect of ryanodine on the twitch force developed after the prolonged diastolic pause (Mann-Whitney non-parametric test). p < 0.05.
temperatures in two very active neotropical teleosts, the curimbata, *Prochilodus lineatus* (Valenciennes, 1837), and the pacu, *Piaractus mesopotamicus* (Holmberg, 1887), respectively.

On the other hand, other studies failed to demonstrate such a direct relationship among the level of activity and/or temperature vs functionality of the SR. Ventricle strips from Nile tilapia, *Oreochromis niloticus* (Linnaeus, 1758), a species well-adapted to high temperatures such as 35ºC, or from fish that face acute transitions from 25 to more than 40ºC in a few hours, such as the tide-pool goby *Bathygobius soporator* (Valenciennes, 1837), 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 (Rantin et al., 1998; Costa et al., 2000). Interestingly, both species (as well as acara) are tropical acanthopterygians, in which the E-C coupling has been shown to depend exclusively upon the extracellular Ca²⁺ sources at physiological (Fig. 2), and subphysiological (Fig. 1) frequencies. Moreover, it is worth mentioning that cichlids such as acara (South American) and Nile tilapia (African) have a common monophyletic origin (Kullander, 1998), presenting similar modes of life, levels of activity, and habitats.

Moreover, in some highly sedentary tropical fish, such as traira, *Hoplias malabaricus* (Bloch, 1794), studied by Olle (2003), and cascudo, *Hypostomus regani* (Ihering, 1905), studied by Rivaroli (2002), ryanodine exerted an inhibitory effect on inotropism at physiological temperatures (Fig. 3). Traira and jeju are erythrinid fish and show a similar reliance on SR Ca²⁺ stores.

Due to the fact that a functional SR was found in both sedentary (traira and cascudo) and active (curimbata and pacu) ostariophysian fish, it may be suggested that a
functional SR is an ancestral trait of this group of tropical teleosts and not related only to the level of activity. In contrast, in tropical acanthopterygians, such as acara, Nile tilapia, and tide pool goby, the opposite seems to happen. Nevertheless, a larger variety of tropical species from the two superorders remain to be studied to prove the previous statement. The relative contribution of the Ca\(^{2+}\) stored in the SR of acara and jeju heart strips to force generation after a diastolic pause of 5 min is presented in Fig. 3, where it is compared to the results obtained to other species.

In spite of the species-specific variability in SR Ca\(^{2+}\)-dependency discussed previously, the strong positive inotropism caused by adding 10\(^{-6}\) M of adrenaline to the muscle bath (Fig. 3c) fully compensated the SR blockade by ryanodine for the jeju ventricle strips and increased the twitch force even further which was observed for control preparations of both species. Moreover, when treated with a tonic dose of adrenaline (10\(^{-9}\) M), ventricle strips from jeju and acara were able to recover the twitch force observed before treatment with ryanodine when in vivo frequencies were reached (Table 1; Fig. 2b). This responsiveness to adrenaline demonstrates that both species, regardless of relying or not on intracellular Ca\(^{2+}\)-stores to regulate the cardiac inotropism on a beat-to-beat basis, have developed mechanisms that assure the maintenance of inotropism. Additionally, as adrenaline was only applied in the presence of ryanodine, it cannot be excluded that it may have had a larger effect in the absence of ryanodine in ventricle strips from jeju.

In our experiments we studied the effects of adrenaline, even though adrenaline and noradrenaline are found in similar concentrations in the blood and heart tissue/nerve endings at rest (Abrahamsson & Nilsson, 1978; Pennec & LeBras, 1984). The selection of adrenaline as the sole adrenergic agonist was based on the fact that adrenaline is generally 10 times more effective than noradrenaline in stimulating adrenoceptors (Ask et al., 1981; Farrell et al., 1996).

The observed inotropic effects of adrenaline are complex. A redistribution of cellular Ca\(^{2+}\) is likely to be involved (Grossmann & Furchgott, 1964; Niedergerke & Page, 1977), as well as a stimulation of glycolysis (Williamson, 1964). The cardiac effects of adrenaline are mediated through a \(\beta\)-adrenoceptor signaling pathway that, in fish, involves \(\beta\_2\)-adrenoceptors (Ask et al., 1981; Temma et al., 1986; Gamperl et al., 1994).

There are several mechanisms of adrenergic modulation for heart performance and some of them are relevant to the regulation of inotropism. The stimulation of \(\beta\)-adrenergic receptors causes the phosphorylation of the SL L-type Ca\(^{2+}\) channels (Shiels et al., 1998) and also stimulates Na\(^{+}/K\^-\) exchanger (Hove-Madsen & Gesser, 1989), increasing transarcolemmal Ca\(^{2+}\) fluxes. Furthermore, Boller & Pott (1989) demonstrated that Ca\(^{2+}\) transportation through SR is also increased by catecholamines in mammalian hearts. However, given that in our experiments SR function was blocked by ryanodine previously to adrenaline addition, the effects of adrenaline could be attributed only to increases in Ca\(^{2+}\) transportation through sarcolemma.

In contrast to what is described to birds and mammals, in which adrenaline is unable to compensate the negative effect of SR blockade by solely increasing sarcolemmal Ca\(^{2+}\)-transportation, a \(\beta\)-adrenergic stimulation in response to stressful conditions in ventricle strips from jeju can potentially enhance the inotropic responsiveness of the heart when increases on cardiac performance are required. Considering the previous observations, it can be suggested that the ventricular myocytes of acara depend exclusively on extracellular Ca\(^{2+}\), while intracellular Ca\(^{2+}\)-stores seem to be relatively important to force development in the ventricle from jeju. In the former, adrenaline simply improves transarcolemmal Ca\(^{2+}\) fluxes, while in the latter it could potentially act on sarcolemmal and SR fluxes.

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