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# Thermal dependence of cardiac SR $Ca^{2+}$-ATPase from fish and mammals

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ABSTRACT

The thermal sensitivity of metabolic performance in vertebrates requires a better understanding of the temperature sensitivity of cardiac function. The cardiac sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2) is vital for excitation–contraction (E–C) coupling and intracellular $Ca^{2+}$ homeostasis in heart cells. To better understand the thermal dependency of cardiac output in vertebrates, we present comparative analyses of the thermal kinetics properties of SERCA2 from ectothermic and endothermic vertebrates. We directly compare SR ventricular microsomal preparations using similar experimental conditions from sarcoplasmic reticulum isolated from cardiac tissues of mammals and fish. The experiments were designed to delineate the thermal sensitivity of SERCA2 and its role in thermal sensitivity $Ca^{2+}$ uptake and E–C coupling. $Ca^{2+}$ transport in the microsomal SR fractions from rabbit and bigeye tuna (*Thunnus obesus*) ventricles were temperature dependent. In contrast, ventricular SR preparations from coho salmon (*Onchorhychus kisutch*) were less temperature dependent and cold tolerant, displaying $Ca^{2+}$ uptake as low as 5 °C. As a consequence, the $Q_{10}$ values in coho salmon were low over a range of different temperature intervals. Maximal $Ca^{2+}$ transport activity for each species occurred in a different temperature range, indicating species-specific thermal preferences for SERCA2 activity. The mammalian enzyme displayed maximal $Ca^{2+}$ uptake activity at 35 °C, whereas the fish (tuna and salmon) had maximal activity at 30 °C. At 35 °C, the rate of $Ca^{2+}$ uptake catalyzed by the bigeye tuna SERCA2 decreased, but not the rate of ATP hydrolysis. In contrast, the salmon SERCA2 enzyme lost its activity at 35 °C, and ATP hydrolysis was also impaired. We hypothesize that SERCA2 catalysis is optimized for species-specific temperatures experienced in natural habitats and that cardiac aerobic scope is limited when excitation–contraction coupling is impaired at low or high temperatures due to loss of SERCA2 enzymatic function.



## 1. Introduction

The cardiac sarco/endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA2) isoform plays a major role during excitation–contraction (E–C) coupling, transporting $Ca^{2+}$ from the cytosol into the lumen of the SR at the expense of ATP. The SERCA2 activity promotes relaxation of the heart and is critical for $Ca^{2+}$ loading of the SR. Over the past decade, the importance of $Ca^{2+}$ induced calcium release (CICR) in E–C coupling of cardiac cells has been vigorously examined at the molecular and protein level in numerous vertebrates (Bers, 2002; Vornanen et al., 2002). In mammals, the SR $Ca^{2+}$ release occurs from internal SR stores primarily via the ryanodine receptor (RyR2). Intracellular $Ca^{2+}$ is precisely regulated and the major pathway for removal occurs with an ATP-dependent pump (SERCA2). In rat ventricles, the SERCA2 pump is responsible for removal of ~92% of the cytoplasmic $Ca^{2+}$ while in rabbit the SERCA2 removes ~70% of the $Ca^{2+}$ ions (Bassani et al., 1994; Bers, 2002). The activity of SERCA2 is higher in rat ventricle, a mammal with a significantly higher heart rate, than in rabbit ventricle. This is because of a higher expression levels of SERCA2 pump molecules in rat SR, and $Ca^{2+}$ removal through $Na^+/Ca^{2+}$ exchange is lower, resulting in a balance of ~92% for SERCA2, ~7% for $Na^+/Ca^{2+}$ exchange and ~1% removal by the sarcolemmal $Ca^{2+}$-ATPase and mitochondrial $Ca^{2+}$ uniporter (Bassani et al., 1994; Bers, 2002).

The mechanism of cardiac E–C coupling in fish is more heterogeneous, with significant species-specific differences. In most teleost fishes, the activation of myocyte contraction is initiated by the influx of extracellular $Ca^{2+}$ through sarcolemmal (SL) L-type $Ca^{2+}$ channels and/or $Na^+/Ca^{2+}$ exchange. There is a negligible contribution from the internal SR $Ca^{2+}$ stores (Tibbits et al., 1992; Tiitu and Vornanen, 2001). Heart rates for many fish are relatively low (10–50 beats per minute), and E–C cardiac contraction occurs via extracellular entry

*Abbreviations:* PMSF, Phenylmethanesulfonyl fluoride; EGTA, Ethylene glycol-bis-(2-aminoethyl)-*N,N,N′, N′*-tetraacetic acid

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of $Ca^{2+}$ which is sufficient to keep pace with intracellular $Ca^{2+}$ demands. Physiological and biochemical findings in endothermic species (tunas and sharks), as well as cold-tolerant active fish species (burbot and trout) indicate a significant role for the internal SR $Ca^{2+}$ pool in cardiac E–C coupling (Aho and Vornanen, 1998; Shiels et al., 1999; Tiitu and Vornanen, 2002; Landeira-Fernandez et al., 2004; Castilho et al., 2007; Weng et al., 2005; Shiels et al., 2011; Da Silva et al., 2011). In these active and cold-tolerant fishes, results indicate that in atrial and ventricular myocytes, SR $Ca^{2+}$ cycling is a major contributor to the cytoplasmic $Ca^{2+}$ transient and the major proteins involved in SR $Ca^{2+}$ transport (SERCA2) and $Ca^{2+}$ induced $Ca^{2+}$ release (RyR2) are present. Electron microscopy studies have demonstrated the presence of SR in the heart cells of bluefin tuna (Di Maio and Block, 2008; Shiels et al., 2011), albacore (Breisch et al., 1983), mackerels (Midttun, 1980), burbot (Tiitu and Vornanen, 2002) and trout (Santer, 1985). Fish cardiac myocytes lack T-tubules but ultrastructure images reveal that numerous peripheral couplings, with densities that are associated with RyR2, are present. Biochemical corroboration of significant SR function comes from fish microsomal SR $Ca^{2+}$ uptake measurements in homogenates, which have been found to be comparable to mammals when examined at room temperature (Hove-Madsen et al., 1998).

Temperature dependency of cardiac SERCA2 activity has been investigated in a few prior studies. Measurements of ventricular SERCA2 activity performed in crude homogenates revealed that cold-acclimated trout (4 °C) display a 2–3 time higher SERCA2 activity than cold-acclimated carp and almost the same activity as homogenates from rat ventricles, when the activity was measured at 25 °C. (Aho and Vornanen, 1998). Contraction kinetics measurements of cold-acclimated trout atrium (CA, 4 °C) indicate a capacity to compensate for the effect of the low temperature by increasing the activity of the SERCA2. Furthermore, it was shown that the force of atrial contraction in the CA trout is sensitive to ryanodine, a pharmacological blocker of the SR $Ca^{2+}$ release channel, at physiological body temperatures (4 °C) and at a physiological pacing rate (0.6 Hz). These findings indicate that the $Ca^{2+}$ stores of SR contribute to cardiac E–C coupling in the trout, a cold active fish, and that the SR of fish hearts is also able to retain its $Ca^{2+}$ load at low body temperatures (Aho and Vornanen, 1999).

Physiological experiments performed in tuna atrial strips (skipjack) with ryanodine, revealed a decrease of ~30% in active force. This data shows that atrial skipjack contraction is also partially dependent upon intracellular $Ca^{2+}$ release from the SR (Keen et al., 1992). Similarly, $Ca^{2+}$ release from the SR in yellowfin tuna isometric muscle preparations also revealed SR activity (Shiels et al., 1999). Ventricular muscle contraction showed a biphasic response to an acute decrease in temperature (from 26 °C to 7 °C) in the bigeye tuna and mahi-mahi; twitch force and kinetic parameters initially increased and then declined (Galli et al., 2009). The magnitude of this response was larger in the bigeye tuna than in the mahi-mahi. Also, cardiac contractile experiments performed at 26 °C, with the ventricular muscle from the bigeye tuna and mahi-mahi, showed that inhibition of SR $Ca^{2+}$ release (with ryanodine) and reuptake (with thapsigargin) decreased twitch force and kinetic parameters only in the bigeye, having no effect on mahi-mahi (Galli et al., 2009).

Recently we reported the microsomal vesicles derived from the SR ventricles from the cold tolerant rainbow trout (*Oncorhynchus mykiss*) retain a SERCA2 enzyme capable of sustaining a high activity at temperatures from 5 °C to 30 °C. This activity was related to the physiology capacity of the trout's heart to operate at low temperatures and thus maintains the swimming capability of this fish in a wide thermal range of temperatures (Da Silva et al., 2011).

In this paper we present comparative analyses of SERCA2 uptake in microsomal vesicles, within the same experimental system to investigate cardiac SR performance in mammals, endothermic and ectothermic fish. Thermal dependence of the ventricular SERCA2 activity from bigeye and skipjack tunas, mahi-mahi, and the coho salmon were measured and compared with the mammalian SERCA2 from rabbit ventricle. Different sensitivities to temperature were observed in these diverse species and we hypothesize that this potentially underlies the thermal sensitivity of E–C coupling and temperature dependence of cardiac performance of the whole animal.

## 2. Material and methods

### 2.1. Animals

Three Skipjack tuna, *Katsuwonus pelamis*, and three mahi-mahi, *Coryphaena hippurus*, with masses between 8 kg and 12 kg and three bigeye tuna, *Thunnus obesus*, with masses between 40 kg and 60 kg were caught on hook and line in the California Current during the same trip in July, 2001. Six coho salmon, *Oncorhynchus kisutch* were captured from the RV Montague and RV Solstice using hook and line in Prince William Sound, Alaska. Temperatures in the late summer waters within the Coho salmon area sampled ranged from 9 °C to 14 °C from 50 m to the surface.. Circle hooks (L2045 20/0 circle hook, Eagle Claw, Denver, CO, USA) were used to avoid gut hooking. Hooks were baited with sardine. In all fish, the heart was removed from the fish after pithing within minutes of being captured. The ventricle was separated, cut into small pieces and freeze-clamped with copper tongs while still beating, then cooled in liquid nitrogen. On land, frozen hearts were stored at −80 °C. Frozen rabbit, *Oryctolagus cuniculus* ventricles were obtained from a commercial source and had been flash-frozen in liquid nitrogen (Pel-Freeze Biologicals, Rogers, AR, USA).

### 2.2. Sarcoplasmic reticulum isolation

Microsomes derived from the SR of ventricles were prepared as previously described (Landeira-Fernandez et al., 2004). Briefly, 5 g of frozen heart tissue was homogenized in 10 vol. of buffer containing 20 mM Mops–Tris pH 7.0, 100 mM KCl, 100 mM NaCl, 1 mM $MgCl_2$ and a cocktail of protease inhibitors (1 mM PMSF, 1 mM pepstatin A, 1 mM iodoacetamide, 1 μM leupeptin, 1 mM benzamidine and 0.1 μM aprotinin) using a Teflon pestle in a 50–100 ml homogenizer chamber. The homogenate was centrifuged at 10,000*g* twice for 20 min in a Sorvall SS34 rotor at 4 °C. The supernatant was filtered through two layers of cheesecloth and then centrifuged at 37,000*g* for 30 min in a Beckman Ti50.2 at 4 °C. The pellet was resuspended in a high ionic strength medium containing 20 mM Mops–Tris pH 7.0 and 0.6 M KCl using a Teflon pestle and then centrifuged at 100,000*g* for 30 min at 4 °C. The pelleted SR microsomes were resuspended in a small volume of cold buffer containing 50 mM Mops–Tris pH 7.0, 50 mM KCl and 0.32 M sucrose, and stored in liquid nitrogen until use.

### 2.3. $Ca^{2+}$ uptake

Unless otherwise specified, 0.6 mg/ml cardiac SR vesicles was added to a cuvette containing 1.5 ml of 50 mM Mops–Tris pH 7.0, 100 mM KCl, 1 mM $MgCl_2$, 10 mM sodium azide, 10 mM potassium oxalate, 5 mM creatine phosphate and 10 μg/ml creatine phosphokinase (as an ATP-regenerating system) and 1.5 μM of the $Ca^{2+}$-sensitive fluorescent dye Fura-2. The cuvette was placed in a Shimadzu RF5301 recording spectrofluorophotometer coupling to a water bath, so that vesicles and buffer could equilibrate to the desire temperature for 2 min (5–35 °C). The $Ca^{2+}$ uptake reaction was initiated by the addition of 1.5 mM MgATP. After the

steady-state was reached, 10 μM of $Ca^{2+}$ was added and the reaction was allowed to reach steady-state again. The reaction was terminated with the addition of 3 μL Triton X-100 or 1.5 μM of the $Ca^{2+}$ ionophore $A_{23187}$, which collapses the $Ca^{2+}$ gradient. Initial rates of $Ca^{2+}$ uptake were calculated from the time for uptake of half the added $Ca^{2+}$ (Landeira-Fernandez et al., 2004).

### 2.4. ATPase activity

The ATP hydrolysis of SR microsomal vesicle preparation was measured using the colorimetric method described by Fiske and Subbarow (1925). $Mg^{2+}$-dependent activity was measured in a medium with no added $Ca^{2+}$ and in the presence of 2 mM EGTA. $Ca^{2+}$-ATPase activity was determined by subtracting $Mg^{2+}$-dependent activity from the activity measured in the presence of both $Mg^{2+}$ and 10 μM $Ca^{2+}$. The reaction medium was composed of 50 mM Mops–Tris pH 7.0, 100 mM KCl, 4 mM $MgCl_2$, 10 mM sodium azide, 3 mM ATP, and 10 μM $Ca^{2+}$ or 2 mM EGTA. In a water bath at the desired temperature (5–35 °C), the reaction was started by the addition of 0.1 mg/ml SR vesicles. At set times, aliquots from the reaction medium were transferred to ice-cold 20% trichloroacetic acid and the rate of ATP hydrolysis per minute was calculated.

### 2.5. Questions and hypothesis of this work

The question we examine is what role does the SERCA2 have in determining the thermal tolerances of the heart. Do endothermic animals have a distinct SERCA2 thermal profile for calcium uptake than ectothermic vertebrates? We hypothesize that SERCA2 function is tuned to distinct species thermal preferences. Thus, cold tolerant vertebrates have a left-shifted function curve, providing increased capacity in the cold, whereas endothermic animals are right shifted-functioning across a warmer thermal range.

In order to address this hypothesis, different animals were used as follows: Rabbit (*O. cuniculus*) was chosen as a model of a mammal which has a high SERCA2 activity and its role during cardiac E–C coupling is well established. The tunas: bigeye (*T. obesus*) and Skipjack (*K. pelamis*) were chosen as a model of endothermic fish with tropical thermal niche (Brill et al., 1999; Holland et al., 1992; Schaefer et al., 2009). The ectothermic mahi-mahi (*C. hippurus*) works as a model of a non-endothermic fish that lives in similar environments to the tropical tunas. The cold active ectothermic coho salmon (*O. kisutch*) is a model of an ectothermic fish that lives in cold waters.

The new data presented in this paper were then compared with previously published results.

Because of the large phylogenetic differences and the different nature of the SR vesicles, we avoid direct comparisons between salmon and tunas; however, the relative activity of the different microsomal preparations, as a percent of maximal activity, is shown in Fig. 5.

### 2.6. Statistics and calculations

All experiments were performed with at least three different tissue preparations and the results were expressed as means ± s.e.m. The number of experiments (n) is shown in parentheses. Statistical significance of differences was assessed by Student's *t*-test or one-way ANOVA using the SigmaPlot/SigmaStat integrated software packages (www.systat.com). Differences were considered significant if $p < 0.05$. When error bars do not appear, it means that they are smaller than the symbols.

## 3. Results

### 3.1. Temperature-sensitivity of SERCA2 function

In order to study the dependence of $Ca^{2+}$ cycling activity of the mammalian SERCA2 on temperature, the rates of $Ca^{2+}$ uptake and ATP hydrolysis in SR microsomes from rabbit ventricles were measured at temperatures from 5 °C to 35 °C (Fig. 1). As expected, vesicles derived from rabbit ventricles were able to catalyze a high rate of $Ca^{2+}$ uptake and ATP hydrolysis (Fig. 1) and had not reached a plateau at 35 °C (Fig. 1). The activity of the mammalian enzyme was very temperature dependent. The rate of $Ca^{2+}$ uptake increased about 40 fold from 5 °C to 35 °C. The rate decreased precipitously as assay temperature decreased and was almost zero at 5 °C (Fig. 1).

The SR preparations isolated from bigeye tuna ventricles also retain a membrane-bound protein that was able to transport $Ca^{2+}$ at the expense of ATP hydrolysis (Fig. 2). Transport was totally abolished in the presence of 1 μM thapsigargin (a specific inhibitor of SERCA2 pump that has no effect on plasma membrane $Ca^{2+}$-ATPase); (data not shown). The rate of $Ca^{2+}$ uptake and ATP hydrolysis catalyzed by bigeye tuna ventricular SERCA2 displayed an activity several times

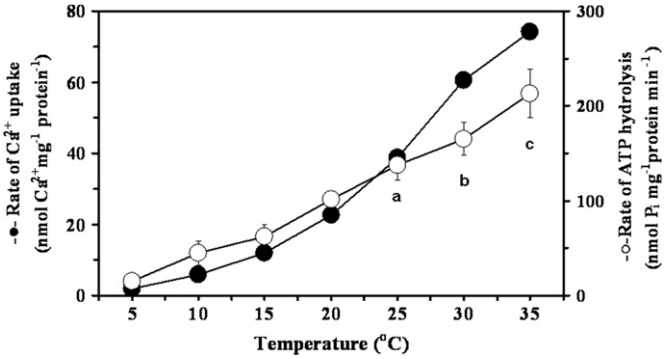


**Fig. 1. Temperature-dependence of SERCA2 activity from *Oryctolagus cuniculus*.** The experimental conditions are described in Section 2. Closed circles represent the rate of $Ca^{2+}$ uptake and open circles represent the rate of ATP hydrolysis. The values represent the average ± s.e.m. of six experiments performed with three preparations from three individual animals. 'a' means that is not statistically different from 'b' and 'b' is not statistically different from 'c', but 'a' and 'c' are statistically different ($p < 0.05$). All the other values are statistically different ($p < 0.05$).

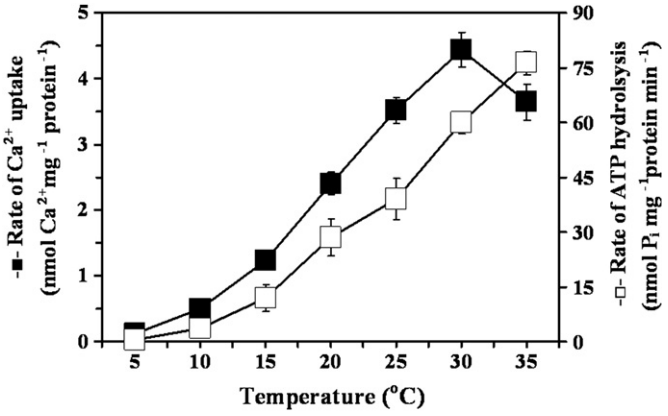


**Fig. 2. Temperature-dependence of SERCA2 activity from *Thunnus obesus* ventricles.** The experimental conditions are described in Section 2. Closed squares represent the rate of $Ca^{2+}$ uptake and open squares the rate of ATP hydrolysis. The values represent the average ± s.e.m. of at least three experiments performed with preparations from three individual fish. All the values are statistically different ($p < 0.05$).

lower than the rabbit isoform (Fig. 2) which is in agreement with results found with other tuna ventricular preparations (Landeira-Fernandez et al., 2004). Similar to the mammalian isoforms, the SERCA2 from bigeye tuna ventricles also displayed enzyme activity with marked temperature dependence, increasing by a factor greater than 20 fold from 5 °C to 30 °C (Fig. 2). In contrast to rabbit SR, the maximal rate of $Ca^{2+}$ uptake catalyzed by the SERCA2 from bigeye ventricles was found at 30 °C (Fig. 2). Unlike the $Ca^{2+}$ uptake activity, the rate of ATP hydrolysis catalyzed by the bigeye tuna microsomes continued to rise at 35 °C (Fig. 2). At these high temperatures the SERCA2 pump from bigeye tuna ventricular microsomes appears to be partially uncoupled. Due to the low enzyme activity displayed by the mahi-mahi and skipjack tuna ventricular SR vesicles, we were not able to measure the enzyme activity of SERCA2 from these fish at different temperatures. We present only the data found at 25 °C as a comparison with other vertebrates.

In order to investigate the involvement of the SERCA pump in the cardiac E–C coupling of a cold-tolerant and active teleost fish, we examined enzyme function in salmon. The temperature dependence of the cardiac SERCA2 pump from coho salmon was measured from 5 °C to 30 °C. Vesicles derived from the SR of coho salmon ventricles were able to transport $Ca^{2+}$ at the expense of ATP (Fig. 3) and were inhibited 100% by thapsigargin (data not shown). The coho salmon SERCA2 pump displayed a unique temperature vs. activity profile that was unlike that from tunas and rabbit. The maximal rate of $Ca^{2+}$ uptake catalyzed by the salmon SERCA2 pump was attained at 25 °C (Fig. 3). As the temperature of the medium decreased, the pump rate decreased fivefold but it was still able to transport a significant amount of $Ca^{2+}$ even at 5 °C. (Fig. 3). Similar to $Ca^{2+}$ uptake the rate of ATP hydrolysis catalyzed by the salmon SERCA2 pump also displayed a maximum activity at 25 °C, showing a coupling between ATP hydrolysis and $Ca^{2+}$ uptake. At 35 °C the rate of ATP hydrolysis dropped precipitously (Fig. 3).

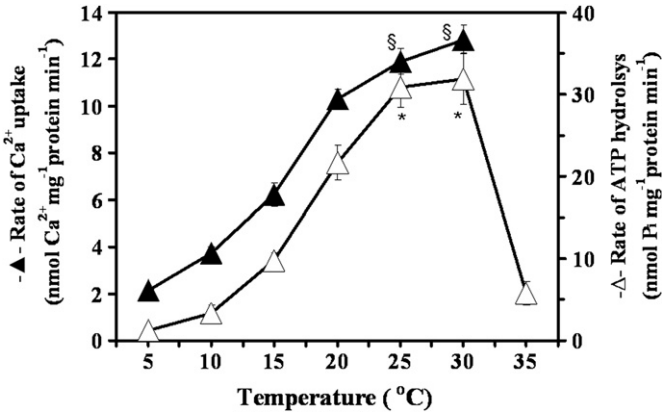


**Fig. 3. Temperature-dependence of SERCA2 activity from *Oncorhynchus kisutch* ventricles.** The experimental conditions are described in Section 2. Closed triangles represent the rate of $Ca^{2+}$ uptake and Open triangles the rate of ATP hydrolysis. The values represent the average ± s.e.m. of three experiments performed with preparations from three individual fish. * means that values are not statistically different ($p < 0.05$). § means that values are not statistically different ($p < 0.05$). All the other values are statistically different ($p < 0.05$).

### 3.2. Comparative aspects of SERCA2 function

To compare SR $Ca^{2+}$ cycling capacity across species the rate of $Ca^{2+}$ uptake catalyzed by the SERCA2 vesicles derived from the ventricles of different fish was examined using the same experimental conditions (25 °C) (Fig. 4). There was a gradient of activity among the *Thunnus* species from cold-tolerant to tropical species; with Pacific bluefin (*Thunnus orientalis*) having the highest activity followed by albacore (*Thunnus alalunga*), bigeye (*Thunnus obesus*) and yellowfin (*Thunnus albacares*) tunas. The rate of $Ca^{2+}$ transport catalyzed by SERCA2 from bigeye ventricles was slightly, but significantly higher than for yellowfin tuna (Fig. 4).

For comparison with the *Thunnus* species we also isolated SR vesicles from the ventricles from *Katsuwonnus pelamus*, the skipjack tuna, and the ectothermic mahi-mahi, *Coryphaena hippurus*, which occupies a warm temperate and tropical habitat similar to yellowfin and skipjack tunas. Both species showed inhibition of ATP-dependent uptake with thapsigargin (data not shown). The $Ca^{2+}$ uptake activity found in the SR vesicles from mahi-mahi ventricles was similar to that from the tropical yellowfin tuna but quite different from the tropical skipjack tuna, which together with the ectothermic mackerel (*Scomber japonicus*) displayed the lowest measurable SERCA2 activity (Fig. 4).

The $Ca^{2+}$ uptake activity catalyzed by different SERCA2 isoforms showed distinct temperature dependency among mammals and fishes measured in our laboratory using assays conducted under similar conditions. The rate of $Ca^{2+}$ uptake catalyzed for each preparation was normalized as 100% at its maximal temperature activity (Fig. 5). The mammalian SERCA2 isoforms from rat and rabbit had a similar temperature-dependent profile with

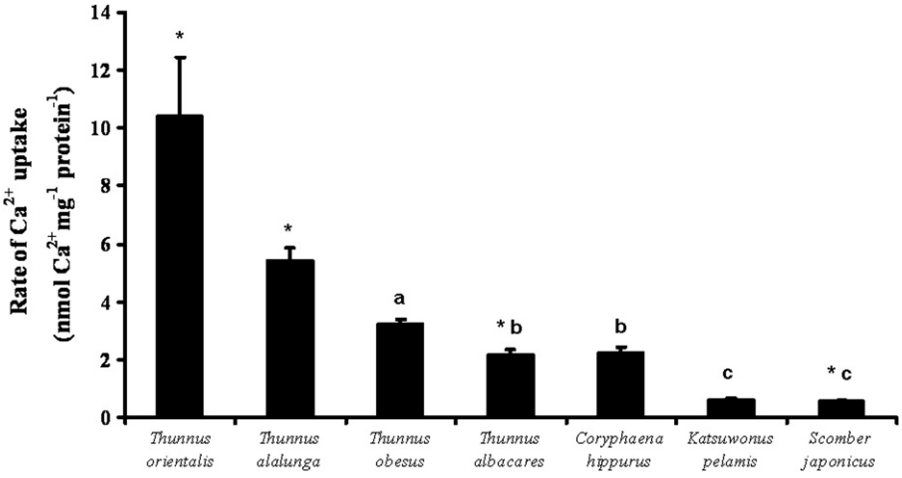


**Fig. 4. Rate of $Ca^{2+}$ uptake catalyzed by SERCA2 ventricles from different fish.** The experimental conditions are described in Section 2. The temperature of the medium was 25 °C. *These data were taken from Landeira-Fernandez et al. (2004). The values represent the average ± s.e.m. of at least three experiments performed with preparations from three individual fish. Different letters above columns indicate statistical differences ($p < 0.05$).

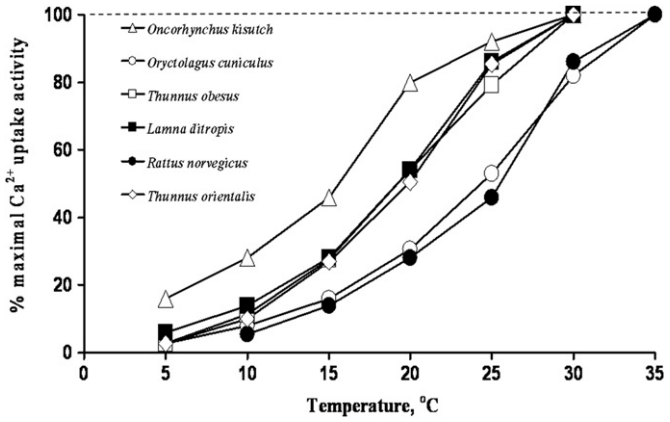


**Fig. 5. Temperature-dependence profile catalyzed by ventricle SERCA2 activity.** The experimental conditions are described in Section 2. Symbols represent: (△) *Oncorhynchus kisutch*; (■) *Lamna ditropis*; (◇) *Thunnus orientalis*; (□) *Thunnus obesus*; (●) *Rattus norvegicus* and (○) *Oryctolagus cuniculus*. The data from *Thunnus orientalis* were taken from Landeira-Fernandez et al. (2004) and data for *Rattus norvegicus* and *Lamna ditropis* were from Weng et al. (2005). The other values are from Figs. 1–3. The dotted line represents the maximal 100% activity, which was calculated using the temperature of 30 °C for the fish species and 35 °C for the mammals. The 100% values were: 12.8 nmol $Ca^{2+}$ $mg^{-1}$ protein $min^{-1}$ for *Oncorhynchus kisutch*; 63.3 nmol $Ca^{2+}$ $mg^{-1}$ protein $min^{-1}$ for *Lamna ditropis*; 12.2 nmol $mg^{-1}$ protein $min^{-1}$ for *Thunnus orientalis*; 4.43 nmol $Ca^{2+}$ $mg^{-1}$ protein $min^{-1}$ for *Thunnus obesus*; 127.8 nmol $Ca^{2+}$ $mg^{-1}$ protein $min^{-1}$ for *Rattus norvegicus* and 74.2 nmol $Ca^{2+}$ $mg^{-1}$ protein $min^{-1}$ for *Oryctolagus cuniculus*.

**Table 1**
Values of $Q_{10}$ for $Ca^{2+}$ uptake found with different ventricular SR preparations.

| Animal | Temperature interval | | | |
|---|---|---|---|---|
| | **10–20** °C | **15–25** °C | **20–30** °C | **25–35** °C |
| [a]**Rabbit** | # 3.9 ± 0.25 | § 3.3 ± 0.28 | ***A*** 2.8 ± 0.21 | 2.0 ± 0.17 |
| [a]**Bigeye** | # 4.3 ± 0.23 | § 2.9 ± 0.31 | ***B*** 1.9 ± 0.16 | – |
| [b]**Salmon shark** | # 4.0 ± 1.31 | § 3.3 ± 0.34 | ***C*** 2.6 ± 0.99 | – |
| [c]**Bluefin** | # 4.9 ± 0.34 | § 3.2 ± 0.40 | ***D*** 2.0 ± 0.17 | – |
| [a,*]**Salmon** | 2.7 ± 0.18 | 1.9 ± 0.12 | 1.3 ± 0.13 | – |

# and § mean that the values are not statistically different from each other. ***A*** means that the value is not statistically different from ***C*** but it is statistically different from ***B*** and ***D*** ($p < 0.05$). ***B, C*** and ***D*** are not statistically different from each other.

[a] Data from Figs. 1–3 of this work.
[b] Data from Weng et al. (2005).
[c] Data from Landeira-Fernandez et al. (2004).
* means that the values found with salmon are statistically different from all other animals in all temperature range ($p < 0.05$).

maximal activity at 35 °C. In these mammalian cardiac preparations, at temperatures lower than 35 °C, the rate of $Ca^{2+}$ uptake decreased significantly and at 20 °C only 30% of the mammals' SERCA2 activity remained (Fig. 5). In contrast, the SERCA2 isolated from the ventricular tissues of different fish species had their maximal activity at 30 °C. Among fish, the endothermic fish, i.e., Pacific bluefin tuna, bigeye tuna and salmon shark (*Lamna ditropis*), showed properties of enzyme function and temperature sensitivities that were similar to the mammalian isoforms, as they were also very temperature-dependent. However at 20 °C, SERCA2 activity remaining in the endothermic fishes was about 50% of its maximal activity. The SERCA2 isolated from salmon ventricle microsomes was less temperature-dependent and at 20 °C, the enzyme activity of coho salmon was approaching maximal, being 80% of its maximal activity (Fig. 5).

In Table 1, the $Q_{10}$ values of the different SR vesicle preparations are shown. In all cases, as the temperature of the medium increased, the $Q_{10}$ values decreased. In the mammal (rabbit) and bigeye tuna, at temperatures below 25 °C the $Q_{10}$ values were high and varied between 3.0 and 4.0 (Table 1). Similar high values were described for other species, such as yellowfin, albacore and bluefin tuna ventricles (Landeira-Fernandez et al., 2004) and atrium (Castilho et al., 2007); rat and salmon shark ventricles and atrium SERCA2 tissues (Weng et al., 2005). Importantly, in contrast to mammals and the tunas, the SERCA2 from coho salmon displayed significantly lower $Q_{10}$ values over a range of different temperature intervals (Table 1). The $Q_{10}$ values for coho salmon varied from 1.3 to 2.7 over temperature intervals of 20–30 °C and 10 °C–20 °C, respectively (Table 1). Although the experiments were done under different experimental conditions, these values are more similar to those found with the ventricle SERCA2 from trout in which the $Q_{10}$ values were 1.19 ± 0.08 (20–30 °C temperature interval) and 1.25 ± 0.06 (10–20 °C temperature interval) (Da Silva et al., 2011).

## 4. Discussion

### 4.1. Mammalian SERCA2 and cold adaptation

The cycling of intracellular calcium in the heart via SR is critical for optimal metabolic performance of vertebrates. Thermal sensitivity of the cardiomyocytes across vertebrates is directly related to the E–C coupling process. Here we show that $Ca^{2+}$ removal by the SERCA2 is one of the most temperature sensitive processes in the heart. However, our study implies that different classes of vertebrates express a SERCA2 isoform that has thermal properties tuned for the niche they occupy. In this paper, we investigate the role of the SERCA2 in regulating this thermal sensitivity by conducting comparative studies of the SR microsomes from fish and mammals in similar experimental conditions. We directly compare SR ventricular microsomal preparations from mammals, endothermic fish and ectothermic fish, in order to better understand the impact of this thermal sensitivity on intracellular $Ca^{2+}$ removal and E–C coupling. We report that in all vertebrate cardiac SR microsomal preparations, there is a decrease in both $Ca^{2+}$ uptake and ATP hydrolysis as the temperature of the medium declines. This result indicates potential impairment of contractile performance in a cooling heart due to decreased capacity to load the SR with $Ca^{2+}$.

The rate of $Ca^{2+}$ uptake catalyzed by the SR vesicles from rabbit ventricles was similar to that found by Fukomoto et al. (1991), although the rate of ATP hydrolysis in their study was significantly lower than in ours. The differences in ATP activity may be associated with the different experimental conditions used. The rabbit SERCA2 $Ca^{2+}$ uptake response to temperature was similar to previous data found with the rat SERCA2 (Weng et al., 2005). On the other hand, the activity of the rabbit SERCA2 uptake was 20–30% lower than the SERCA2 from rat ventricles measured in similar conditions (Weng et al., 2005). This is consistent with the literature, which shows that in rat ~90% of the $Ca^{2+}$ used during cardiac E–C coupling comes from the SR, while in rabbits this value decreases up to 70%, implying less reliance on SERCA2 pathways consistent with the slower rabbit heart rates, compared with rats (Bers, 2002). Moreover, these results are also in accordance with the literature that shows that the SERCA2 activity from rat has a higher activity than rabbit, using different experimental conditions from ours and consistent with allometric relationships (Su et al., 2003).

In mammalian atrial preparations (human), measurements of SERCA2 activity decreased significantly (88 fold for $Ca^{2+}$ uptake and 35 fold for ATP hydrolysis) when the temperature was reduced from 37 °C to 12 °C (Labow et al., 1993). This research

also demonstrated that storage of the mammalian heart tissue at 4 °C for 4 h was sufficient to reduce the rate of $Ca^{2+}$ uptake by 50%. Low temperatures in mammalian myocyte SR preparations have also been shown to be deleterious, causing prolonged channel opening of the ryanodine receptor (RyR), with resulting arrhythmia and cold-induced contractile dysfunction (Bridge, 1986; Bers, 1991). The results suggest that cold reduces the SR load in mammals which is most likely due to higher probability of RyR channel opening and a reduced capacity for loading via SERCA2. Importantly, cold tolerant mammals exhibit cardiovascular specializations to low body temperature (5–7 °C) inclusive of increased activity of SERCA2 in a similar fashion to endothermic cold tolerant fish (salmon sharks, bluefin tuna). These SR and SERCA2 specializations would help to maintain cardiac function at temperatures which larger mammals would exhibit heart failure.

### 4.2. Endothermic fish

The genus *Thunnus* consists of a rapid radiation of closely related species inhabiting pelagic environs from the tropical to sub-polar seas. Recent physiological laboratory studies indicate that specializations of the cardiac physiology can be linked to the capacity of some species (bluefins) to function in the cold (Blank et al., 2004, 2007, Galli et al., 2009, 2011, Shiels et al., 2011).

The bluefin species range to the highest temperate and sub-polar latitudes, experiencing over time, occupation of the coolest waters for prolonged durations. Tropical tunas such as yellowfin and bigeye tunas occupy lower latitude environs entering cool waters only when foraging at depth (Holland et al., 1992; Brill et al., 1999; Schaefer and Fuller, 2002). The bluefin tunas are unique among all teleost fish for their degree of endothermic physiology, high metabolic rate and increased niche breadth (Blank et al., 2004, 2007; Block et al., 2001, 2005, 2011; Lawson et al., 2010). Despite the fact that tuna are capable of internal warmth, the heart is continuously exposed to ambient water temperatures as the retia mirabilia are down stream of the gills and the heart is in close proximity to the ventral surface. Furthermore, the heart receives cold blood from the coronary circulation. Physiological experiments on the in situ hearts indicate that the Pacific bluefin tuna can maintain beat to beat contraction at temperatures as low as 2 °C (Blank et al., 2004) while the closely related sub-tropical tuna yellowfin exhibit arrhythmias at 8–10 °C. (Blank et al., 2002). Skipjack tunas and mahi-mahi are tropical species that are found predominantly in warmer waters (18–28 °C) (Schaefer and Fuller, 2002, 2007; Schaefer et al., 2009).

The capacity of the northern bluefin tuna hearts to operate at low temperatures is correlated with the presence of a higher SERCA2 enzyme activity and content in the heart (Fig. 4 and Landeira-Fernandez et al., 2004). Bluefin microsomal vesicles from atrium and ventricle have SERCA2 activity ~5–6 times higher than that of yellowfin tuna (Landeira-Fernandez et al., 2004; Castilho et al., 2007). Recent confocal microscopy experiments examining $Ca^{2+}$ transients in Pacific bluefin tuna after cold exposure also indicate that in response to cold acclimation the bluefin tuna remodel the SR content and increase their capacity for $Ca^{2+}$ cycling possible through greater activity of SERCA2 and ryanodine receptors (Shiels et al., 2011; Galli et al., 2011). In these closely related species, increased SERCA2 function appears to be directly related to increased niche breadth.

The niche expansion linkage to cardiac physiology has also been demonstrated in salmon sharks (*Lamna ditropis*). Salmon sharks are members of the endothermic shark family Lamnidae, and display a high SERCA2 activity, with rates as high as in mammalian (rat) ventricular SR vesicles, when examined at 25 °C (Weng et al., 2005). These sharks occupy waters above 60°N and are found in winter in seas as cold as 2–8 °C for prolonged durations while maintaining an endothermic metabolism.

The results in the present study from fish and sharks suggest that cold tolerance may result in an increase in the activity of the SERCA2 protein in tunas and lamnid sharks. The comparative results demonstrate that the highest levels of $Ca^{2+}$ uptake activity occurs amongst tunas that inhabit the coldest environmental waters.

### 4.3. Coho salmon SERCA2

By directly comparing the fish and mammalian preparations using similar techniques, the experiments reveal that coho salmon have a cardiac SR with $Ca^{2+}$ cycling capacity that is significantly higher at low temperatures. Coho transport is 11.5 nmol$Ca^{2+}$/mg/min and rabbit uptake is 40.0 at 25 °C, but at 10 °C they are almost the same (~4–6 nmol$Ca^{2+}$/mg/min). This cardiac SR uptake capacity may underlie the capacity of the salmon to have high aerobic performance in cooler northern latitude waters. The $Ca^{2+}$ uptake rates for the salmon microsomal preparations are similar to previous measurements for rainbow trout (*Oncorhynchus mykiss*); (Da Silva et al., 2011; Aho and Vornanen, 1998, 1999). The results presented here indicate that coho salmon also function across a wide thermal range with increased performance in the cold. We hypothesize that this is due to increased activity of the SERCA2 enzyme although differences in amino-acids sequences may also play a role and remains to be investigated in fish.

### 4.4. Temperature-sensitivity of SERCA2

The effects of the temperature on enzyme activity can be quantified by calculating the temperature coefficient or $Q_{10}$ of the process. For many physiological processes such as rates of respiration and enzymatic process, $Q_{10}$ values near 2.0 or slightly higher are observed when thermal effects are studied within the species' physiological range of body temperatures (Hochachka and Somero, 2002). At high temperatures at which lethal injury may ensue, $Q_{10}$ values may be less than 1.0, indicating that increases in temperature are damaging the enzyme system and leading to what may be an irreversible loss of function. At relatively low temperatures, $Q_{10}$ values may became much larger than 2.0 indicating there may be a change in the properties of the underlying biochemical systems, such that the energy barriers to the processes are increased (Hochachka and Somero, 2002). In this work, $Q_{10}$ values near 2.0 were found in different temperature ranges (10–20 °C; 15–25 °C and 20–30 °C), depending on the physiological temperature of the animal species studied. For rabbits, an endothermic animal that maintains optimal tissue temperatures close to 37 °C, SERCA2 $Q_{10}$ values near 2.0 were found at the highest temperature interval examined (25 °C to 35 °C), in bigeye tuna heart preparations, this value was in the range of 20–30 °C and in salmon, a $Q_{10}$ value near 2.0 was found in a lower range of temperatures, between 15 °C and 25 °C (Table 1). At temperatures above or below this range the $Q_{10}$ values, were different from 2.0, i.e., at lower temperatures the values were higher than 2.0 and at the optimal temperature the values were lower than 2.0. The in vitro results indicate that the different SERCA2 isoforms from the ectothermic and endothermic animals were specialized for maximal enzymatic activity in different physiological ranges, with fish peaking in activity below the thermal capacity of mammals. These data demonstrate that fish and rabbit SERCA2 isoforms, have different kinetics properties.

## 5. Conclusions

The analyses of SERCA2 function in fish provided here help to define physiological factors that establish cardiac performance in fish which in turn relates directly to the organisms' thermal tolerance limits. This study suggested a correlation between a species' temperature preferences/tolerance and increased in SERCA2 activity. This increases the capacity to load SR with $Ca^{2+}$ in the cold ensuring a significant $Ca^{2+}$ release from SR upon excitation when $Ca^{2+}$ ions are diffusing slowly. Future studies should examine the primary sequences of SERCA2 among closely related fish as well as the properties of the SR $Ca^{2+}$release channel during function of the myocyte at cold temperatures. Remarkably, cardiomyocytes from fish and mammals use similar cryoprotective techniques, increased expression of SERCA2 enzyme activity, to reduce cardiac dysfunction in the cold – suggesting common mechanisms across vertebrate E–C coupling underlie cardiac arrhythmia at low temperatures.

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