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RESEARCH ARTICLE

Temperature effects on Ca^{2+} cycling in scombrid cardiomyocytes: a phylogenetic comparison

Gina L. J. Galli[1,2,*,†], Michael S. Lipnick[1,†], Holly A. Shiels[2] and Barbara A. Block[1]

[1]Hopkins Marine Station of Stanford University, 120 Oceanview Boulevard, Pacific Grove, CA 93950, USA and [2]Faculty of Life Sciences, University of Manchester, Core Technology Facility, 46 Grafton Street, Manchester M13 9NT, UK

*Author for correspondence at present address: School of Medicine, The University of Manchester, Core Technology Facility, 46 Grafton Street, Manchester M13 9NT, UK (ginaljgalli@hotmail.com)

†These authors contributed equally to this work

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SUMMARY

Specialisations in excitation–contraction coupling may have played an important role in the evolution of endothermy and high cardiac performance in scombrid fishes. We examined aspects of Ca^{2+} handling in cardiomyocytes from Pacific bonito (*Sarda chiliensis*), Pacific mackerel (*Scomber japonicus*), yellowfin tuna (*Thunnus albacares*) and Pacific bluefin tuna (*Thunnus orientalis*). The whole-cell voltage-clamp technique was used to measure the temperature sensitivity of the L-type Ca^{2+} channel current (I_{Ca}), density, and steady-state and maximal sarcoplasmic reticulum (SR) Ca^{2+} content ($ssSR_{load}$ and $maxSR_{load}$). Current–voltage relations, peak I_{Ca} density and charge density of I_{Ca} were greatest in mackerel and yellowfin at all temperatures tested. I_{Ca} density and kinetics were temperature sensitive in all species studied, and the magnitude of this response was not related to the thermal preference of the species. SR_{load} was greater in atrial than in ventricular myocytes in the Pacific bluefin tuna, and in species that are more cold tolerant (bluefin tuna and mackerel). I_{Ca} and SR_{load} were particularly small in bonito, suggesting the Na^+/Ca^{2+} exchanger plays a more pivotal role in Ca^{2+} entry into cardiomyocytes of this species. Our comparative approach reveals that the SR of cold-tolerant scombrid fishes has a greater capacity for Ca^{2+} storage. This specialisation may contribute to the temperature tolerance and thermal niche expansion of the bluefin tuna and mackerel.

Key words: L-type Ca^{2+} channel, cardiomyocyte, sarcoplasmic reticulum, mackerel, temperature, tuna.

INTRODUCTION

One of the most important events in vertebrate evolution is the transition from ectothermy to endothermy. This transition is difficult to study because endothermy is a derived characteristic in birds and mammals. The family Scombridae includes highly active regionally endothermic (tunas) and ectothermic (mackerel and bonito) fishes that occupy diverse habitats, including cold, temperate and tropical waters (Gunn and Block, 2001). In addition, within the family Scombridae, the tribe Thunnini contains guilds of closely related tunas with recent common ancestry such as the yellowfin (tropical) and bluefin (cold temperate) tunas (Chow and Kishino, 2010). Thus, scombrid fishes provide a model lineage to investigate specialisations that evolved in response to endothermy, and those that developed in response to thermal niche expansion.

Among tunas, the bluefin tunas have evolved the highest degree of endothermy, possessing internalised slow twitch oxidative (red) muscle and countercurrent heat exchangers that conserve metabolic heat produced in the locomotor musculature, viscera, brain and eyes (Carey and Lawson, 1973; Carey et al., 1984; Linthecum and Carey, 1971). Electronic tagging studies indicate that bluefin tunas occupy a wide thermal niche. Atlantic bluefin tuna range from the frigid waters off Canada in swimming sea surface temperatures (SST) as low as 0.02°C, to the warmer spawning grounds in the Gulf of Mexico (SST as high as 30°C) (Teo et al., 2007; Walli et al., 2009; Lawson et al., 2010). Owing to a high degree of endothermy, the bluefin tuna maintains an elevated body temperature with a mean visceral temperature of 21–23°C and a maximum of over 33°C (Block et al., 2001; Block et al., 2005; Walli et al., 2009). Electronic tagging of juvenile Pacific bluefin in the eastern Pacific demonstrates occupation of waters that average 17.8°C (Kitagawa et al., 2004; Boustany et al., 2010). Maintenance of a thermal excess of 1–10°C above ambient is routine in these juvenile bluefin despite the cool California Current waters (Marcinek et al., 2001a; Kitagawa et al., 2007).

Yellowfin tunas also have internalised slow-twitch muscles but fewer countercurrent heat exchangers (Graham and Dickson, 2004). These fish have a more tropical distribution (Weng et al., 2009; Shaefer et al., 2007). Juvenile yellowfin tunas tracked simultaneously with similar sized Pacific bluefin tunas in the California Current tend to occupy warmer waters to the south (mean SST of 22°C) and are limited in their northern geographic range (Shaefer et al., 2007). The capacity to maintain a thermal excess is not present in the ectothermic sister taxa of the *Thunnus*, such as *Scomber* (mackerel) and *Sarda* (bonito), because they lack countercurrent heat exchangers. Thus, comparative analyses of these fishes with tuna provide an excellent opportunity to examine the evolutionary sequence of events leading to endothermy and concomitant changes in performance and niche utilisation in extant teleosts.

Despite the ability to conserve metabolic heat, tunas face unique physiological challenges; their heart is close to the gills and separate from the supply of the vascular countercurrent heat exchangers (Carey et al., 1984; Brill et al., 1994). The heart also receives cold coronary blood directly from the gills (Brill, 1987; Brill et al., 2001).

Thus, as with mackerel and bonito, the hearts of even the largest tunas operate at ambient water temperatures. Therefore, the tuna heart has an extra physiological burden compared with the mackerel and the bonito, as it must be able to function over a broad thermal range while delivering blood to muscle, viscera and cranial tissues that are often warmer than ambient water temperature and have high metabolic demands.

Tunas that have been studied (bluefin, yellowfin and skipjack) have evolved a robust cardiovascular system. Relative to body size, yellowfin and bluefin tuna hearts are significantly larger than other teleosts, have a thicker ventricular wall and an increased percentage of compact myocardium (Farrell et al., 1992). Furthermore, the tuna heart has large myoglobin reserves (Giovane et al., 1980), a higher oxygen affinity (bluefin tunas) (Marcinek et al., 2001b), high anaerobic enzyme activities (Dickson, 1995; Driedzic and Gesser, 1994) and a highly developed coronary circulation (Farrell et al., 1992), which appears to be reduced in the bonito and almost absent in the mackerel (Davie, 1991). Previous studies have also characterised yellowfin and skipjack tunas as possessing significantly higher intrinsic maximal heart rates (up to 200 beats min^{-1}), which are almost double those found in most teleosts (Brill et al., 2005; Farrell et al., 1992; Keen et al., 1995; Roberts and Graham, 1979). However, recent *in situ* heart measurements in bluefin, yellowfin and albacore tunas (Blank et al., 2004), and *in vivo* measurements in bluefin tunas (Clark et al., 2008), indicate heart rates are primarily 40–130 beats min^{-1}, which is not exceptional among teleosts (Farrell, 1991).

Variation in scombrid cardiovascular performance may be explained in part by specialisations in excitation–contraction (E–C) coupling. It was previously thought that most teleosts cycle Ca^{2+} across the sarcolemma primarily through the L-type Ca^{2+} channel or the Na^{+}/Ca^{2+} exchanger (NCX) (Keen et al., 1992; Shiels and Farrell, 1997; Shiels et al., 2006; Vornanen et al., 2002) with the sarcoplasmic reticulum (SR) contributing negligible amounts of Ca^{2+} for contraction. However, more recent evidence suggests that the role of the L-type Ca^{2+} channel and the SR in teleost E–C coupling depends on a number of factors, including temperature, frequency of contraction, phylogeny and cardiovascular capacity (Hove-Madsen, 1992; Moller-Nielsen and Gesser, 1992; Keen et al., 1994; Shiels and Farrell, 1997; Shiels et al., 1998; Aho and Vornanen, 1999; Rivaroli et al., 2006; Galli et al., 2008). With regards to scombrids, recent physiological and electron microscopy work has demonstrated that the bluefin ventricle and atrium, the bigeye tuna ventricle and the yellowfin tuna atrium have evolved a more advanced 'mammalian-like' form of E–C coupling, with a significant proportion (40–50%) of activator Ca^{2+} used for contraction originating in the SR (DiMaio and Block, 2008; Galli et al., 2008; Shiels et al., 1999; Shiels et al., 2011). Furthermore, biochemical studies indicate bluefin tuna have increased SR Ca^{2+} ATPase (SERCA-2) activity and expression over a range of temperatures when compared with other scombrids (Landeira-Fernandez et al., 2004; Castilho et al., 2007). Lastly, Ca^{2+} entry through the L-type Ca^{2+} channel is greater and inactivation kinetics are faster in atrial myocytes of bluefin tuna than of Pacific mackerel (Shiels et al., 2004). Collectively, these results suggest enhanced Ca^{2+} cycling through E–C coupling pathways may have evolved within the scombrid lineage and may be crucial in the evolution of increased cardiac performance, endothermy and niche expansion. Importantly, these Ca^{2+} pumps and ion channels are all known to be sensitive to acute temperature change (Shiels et al., 2000; Shiels et al., 2002a; Shiels et al., 2002b; Landeira-Fernandez et al., 2004; Castilho et al., 2007; Shiels et al., 2011).

To gain a greater understanding of the evolution of endothermy and cardiovascular performance within scombrids, it is crucial to obtain information on myocyte E–C coupling in a phylogenetic selection of scombrid species. The present study examined the effects of physiologically relevant acute temperature change on aspects of two major E–C coupling pathways [L-type Ca^{2+} channel current (I_{Ca}) density and SR Ca^{2+} content] in ventricular myocytes from four members of the family Scombridae: Pacific bonito (*Sarda chiliensis*), Pacific mackerel (*Scomber japonicus*), yellowfin tuna (*Thunnus albacares*) and Pacific bluefin tuna (*Thunnus orientalis*). Atrial myocytes were also examined in Pacific bluefin tuna. These species encompass both ectothermic (mackerel and bonito) and endothermic (yellowfin and bluefin tuna) species, as well as cold-tolerant (mackerel and bluefin tuna), temperate (bonito) and tropical (yellowfin) species. By using a comparative approach, we aimed to identify specialisations in E–C coupling pathways that could have permitted increased cardiac performance, endothermy and/or increased thermal tolerance.

MATERIALS AND METHODS

Animals

Bluefin tuna (*Thunnus orientali*s, Temminck and Schlegel 1844, *n*=6, fish mass=11.1±0.5 kg), yellowfin tuna (*Thunnus albacares*, Bonnaterre 1788, *n*=4, fish mass=4.33±0.3 kg) and Pacific bonito (*Sarda chiliensis*, Girard 1858, *n*=4, fish mass=1.84±0.4 kg) were caught at sea, by purse or seine or by hook and line, off the coast of San Diego, CA, USA. Fish were held aboard the Shogun fishing vessel in large wells flooded by seawater, or in captive farm pens (bluefin tuna) at Mariculture Del Norte, Ensenada, Mexico. All fish were transported by truck during a 9–11 h transport to the Tuna Research and Conservation Centre (TRCC) in pacific Grove, CA, USA. Pacific mackerel (*Scomber japonicus*, Houttuyn 1782, *n*=10, fish mass=0.52±0.1 kg) were caught in Monterey Bay, CA, USA and held in captivity at Monterey Bay Aquarium and the TRCC. Fish were kept for no less than 3 months prior to experimentation in 109 m^3 tanks at 20±1°C and fed a diet of krill, squid, sardines and enriched gelatine, as previously described (Farwell, 2001). All experimental procedures were conducted in accordance with Stanford University institutional animal use protocols.

Cardiomyocyte preparation

Ventricular myocytes from all species were isolated by adaptation of protocols previously developed for bluefin tuna (Shiels et al., 2004; Galli et al., 2009). Because atrial tissue is known to be more SR dependent than ventricular tissue in rainbow trout (Aho and Vornanen, 1999), we also isolated atrial myocytes from bluefin tuna to test whether the same holds true for this species and to serve as a measure of the sensitivity of our analyses of SR Ca^{2+} content. Fish were killed by pithing and decapitation (for mackerel) and severing of the spinal cord (for tuna), and the heart was removed intact [mean mass (in g): bluefin tuna, 37.6±1.4; mackerel, 1.14±0.2; bonito=3.99±0.5; yellowfin tuna, 17.1±4.9]. The bulbus arteriosus was cannulated and the heart was retrogradely perfused with an oxygenated, Ca^{2+}-free isolation solution to promote cardiomyocyte relaxation and clear the heart of blood. For the tuna and bonito, the coronary artery was also cannulated and perfused, whereas that of the mackerel was not, because of size constraints. Isolation solution was perfused from a height of 60 cm for ~10 min for mackerel, ~15 min for bonito, and ~20 min for tuna. Proteolytic enzymes were then added to the isolation solution and run through the cannulated bulbus arteriosus for ~20 min in mackerel and ~30 min in tuna and bonito. After enzymatic digestion, the ventricle and atrium were

dissected and placed into separate Petri dishes containing fresh isolation solution. Tissues were gently triturated with a Pasteur pipette to free cardiomyocytes, and cells were stored at 20°C for the duration of the experiment (up to 8 h).

Solutions

All solutions used were adapted from those previously developed for Pacific bluefin tuna (Shiels et al., 2004). The isolation solution consisted of (in mmol l^{-1}) 100 NaCl, 10 KCl, 1.2 KH_2PO_4, 4 $MgSO_4$, 50 taurine, 20 glucose and 10 Hepes, with pH adjusted to 6.9 with KOH at 20°C. The enzymatic digestion solution comprised collagenase (type IA), trypsin (type IX) and fatty-acid-free BSA added to the isolation solution. The extracellular solution used for current recordings contained (in mmol l^{-1}) 150 NaCl, 5.4 CsCl, 1.2 $MgSO_4$, 0.4 NaH_2PO_4, 3.2 $CaCl_2$, 10 glucose and 10 Hepes, adjusted to pH 7.7 with CsOH. Tetrodotoxin (TTX; 1 μmol l^{-1}) was added to the extracellular solution just before perfusion of cells in the bath in order to block fast Na^+ channels. Caffeine (10 mmol l^{-1}) was similarly added just before use to promote release of SR Ca^{2+} stores. The pipette solution consisted of (in mmol l^{-1}) 130 CsCl, 5 MgATP, 15 tetraethylammonium chloride, 1 $MgCl_2$, 5 sodium phosphocreatine, 10 Hepes and 0.025 EGTA with pH adjusted to 7.2 with CsOH. CsCl and tetraethylammonium chloride were incorporated into the solutions in order to block K^+ currents. Thapsigargin (2 μmol l^{-1}) and ryanodine (10 μmol l^{-1}) were added to the extracellular solution to block caffeine transient currents for control purposes. Nifedipine (2.5 μmol l^{-1}) was similarly added to block I_{Ca}. Except for TTX (purchased from Tocris Bioscience, Ellisville, MO, USA), all chemicals were purchased from Sigma-Aldrich (Dorset, UK).

Experimental procedure

Procedures for obtaining and analysing I_{Ca} and SR Ca^{2+} characteristics were adapted from established methods for fish preparations (Shiels et al., 2004; Shiels et al., 2006; Vornanen, 1997; Vornanen, 1998). A sample of cardiomyocytes, suspended in isolation solution, were added to the recording chamber and allowed to settle and attach to the bottom for ~5 min. The cell chamber was superperfused at 23°C with extracellular solution containing TTX at a rate of ~2 ml min^{-1}. Voltage clamp experiments were conducted in the whole-cell configuration using a PC 505B amplifier (Warner Instruments, Hamden, CT, USA) with a 202B headstage (Warner Instruments). Micropipettes were made from borosilicate glass (Garner Glass, Claremont, CA, USA) using a P-87 pipette puller (Sutter Instrument Company, Novato, CA, USA) and had resistances of 2.35±0.03 MΩ when filled with pipette solution. Junction potentials were zeroed prior to seal formation, and pipette capacitance was compensated for once a gigaohm seal was achieved. Access to the cell was obtained by rupturing the membrane patch with a brief voltage pulse. Capacitive transients were compensated for by adjusting the series resistance and cell capacitance compensation circuits. Mean series resistance was 9.7±0.5 MΩ. Currents were low-pass filtered at a frequency of 2 kHz and analysed offline using Clampfit 9.2 (Axon Instruments, Sunnyvale, CA, USA).

Measurement of I_{Ca}

I_{Ca} was activated using the protocol illustrated in Fig. 1. Current–voltage relationships were elicited by depolarizing the cell from –80 mV to +60 mV using 500 ms square pulses in 10 mV increments. The amplitude of the L-type Ca^{2+} channel was calculated as the difference between the maximal inward current deflection and the current at the end of the depolarizing pulse. I_{Ca} was normalised for cell size by dividing I_{Ca} by cell capacitance to give I_{Ca} density in pA pF^{-1}. Current–voltage curves were obtained at 15, 19 and 23°C by adjusting superfusate temperature through a temperature-controlled rapid solution changer (SC-20 in-line heater/cooler, CL-100 bipolar temperature controller; Warner Instruments). When possible, the effects of all three temperatures were measured in each cell. However, because of problems in maintaining a high quality seal (in the gigaohm range), some cardiomyocytes were only tested at one or two of the experimental temperatures. The order of temperature exposure was reversed (perfused first at 15°C and then warmed to 19 and 23°C) in some experiments, and this did not affect the results. Inactivation kinetics of I_{Ca} and the time constants of fast (τ_f) and slow inactivation (τ_s) were obtained by fitting a second-order (mackerel and tuna) or first-order (bonito, τ) exponential curve to the decaying portion of the I_{Ca} using the Chebyshev procedure (Clampfit software, Axon Instruments). Charge density of I_{Ca} (Q_{Ca}) was calculated as the integral of the peak I_{Ca} for each current–voltage curve and is given in pC pF^{-1}. This value was used to calculate the change in total cellular Ca^{2+} resulting from Ca^{2+} influx through L-type Ca^{2+} channels ($\Delta[Ca^{2+}]$) using cell volume (Vornanen, 1997). Cell volume was calculated from the measured cell capacitance and the surface:volume ratio of the cells. Cardiomyocytes were considered to be flat elliptical cylinders with an axis ratio of 1:2 for the elliptical cross section (Vornanen, 1997; Vornanen, 1998). $\Delta[Ca^{2+}]$ was expressed as a function of non-mitochondrial volume (Vornanen, 1997; Vornanen, 1998).

Measurement of SR Ca^{2+} content

By rapidly changing between ports on the rapid solution changer, caffeine (10 mmol l^{-1}) was briefly (~1 s) administered to induce SR Ca^{2+} release. Ca^{2+} released by the SR is extruded from the cell *via* the NCX, resulting in an inward Na^+ current (I_{NCX}). This Na^+ current is proportional (3:1) to the Ca^{2+} released by the SR, and thus the time integral of the I_{NCX} can be used to calculate the Ca^{2+} released (in pC) by the SR (Varro et al., 1993). This value was adjusted for cell size by dividing it by the cell capacitance (pC pF^{-1}). Integrating the I_{NCX} and cell volume gave total Ca^{2+} released by the SR (in μmol l^{-1}). Cell volumes were calculated by extrapolating surface area measurements from cell capacitance (in pF) and using previously developed calculations for surface:volume ratios and specific membrane capacitance (Vornanen, 1997). Preliminary studies in each species and at each temperature showed that at least 25 pulses from –80 mV to 0 mV (500 ms) were necessary to achieve a steady-state SR Ca^{2+} load ($ssSR_{load}$). Therefore, 25 pulses were used in all subsequent experiments when measuring $ssSR_{load}$. Maximal SR Ca^{2+} load ($maxSR_{load}$) was calculated as the caffeine-induced I_{NCX} after a square wave pulse from –80 to +50 mV for 6–9 s. $ssSR_{load}$ and $maxSR_{load}$ Ca^{2+} loads were measured in ventricular myocytes from all species at each test temperature (15, 19 and 23°C, as described in the previous section). Additionally, as SR dependence is known to be higher in atrial than in ventricular muscle, we measured $ssSR_{load}$ and $maxSR_{load}$ in atrial myocytes from one of the scombrid species, the bluefin tuna. This allowed assessment of the sensitivity of our SR_{load} measurements.

Statistics

With the exception of original traces and voltage protocols, data are given as means ± s.e.m. *N*-values are the number of cells and are given in each figure legend. A one-way ANOVA with the Student–Newman–Keuls test was used to evaluate the effects of

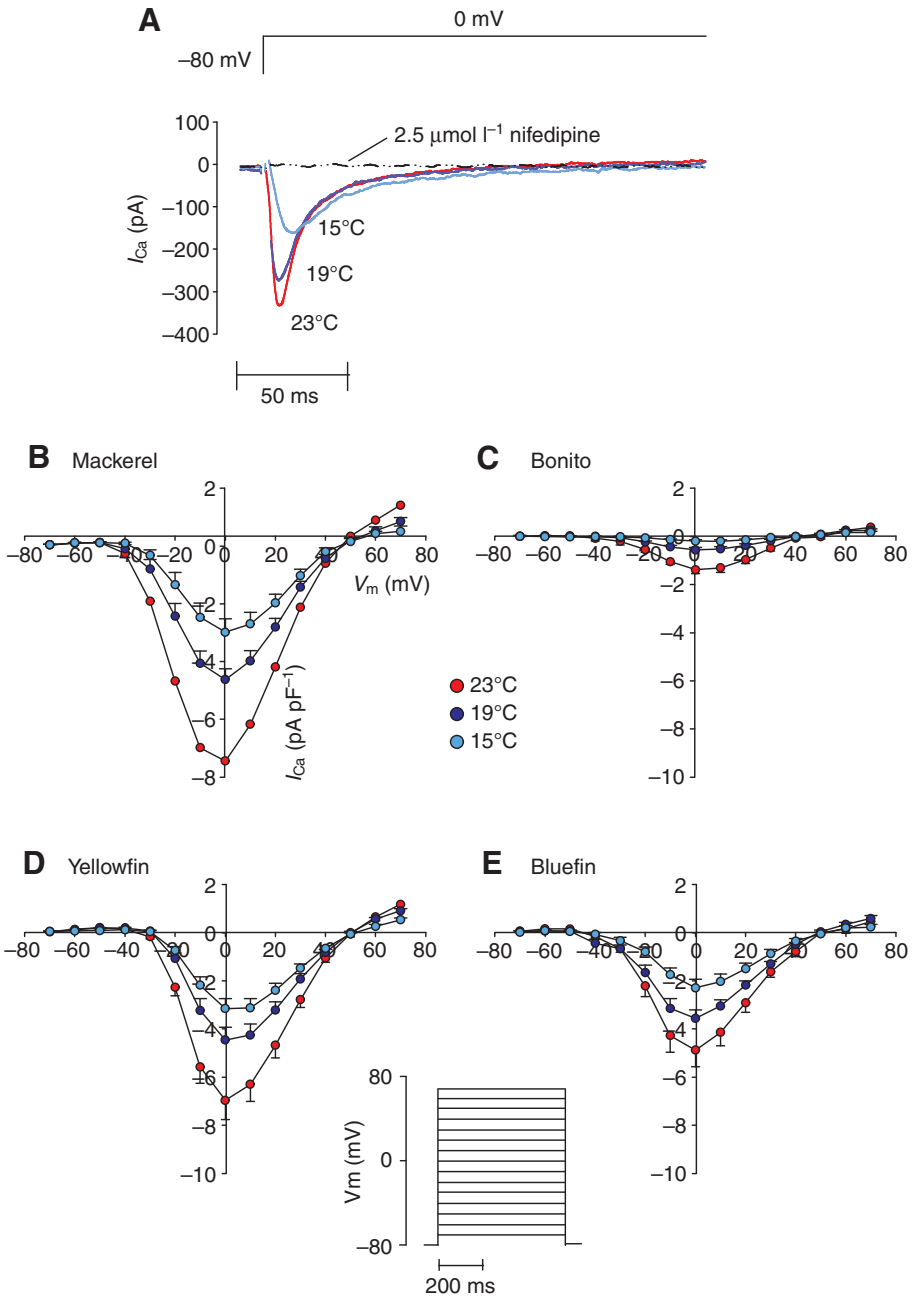

Fig. 1. Effect of acute temperature changes on L-type Ca^{2+} channel current–voltage relationships in ventricular myocytes from the Pacific mackerel, bonito, yellowfin tuna and Pacific bluefin tuna. (A) Representative trace and voltage protocol for examining the temperature sensitivity of the L-type Ca^{2+} channel current (I_{Ca}) in a ventricular myocyte from the mackerel (cell capacitance was 34 pF). I_{Ca} was completely abolished with 2.5 µmol l^{-1} nifedipine (dotted line). (B–E) The current–voltage relationship for I_{Ca} for each scombrid. The voltage protocol is given in the inset between D and E. Red circles, 23°C; dark blue circles, 19°C, light blue circles, 15°C. Statistical analysis is discussed in the text. Values are means ± s.e.m. The number of cells examined at 23, 19 and 15°C, respectively were: bluefin tuna 13, 10, 9; mackerel 15, 16, 10; bonito 16, 17, 15; yellowfin tuna 20, 18, 17. V_m, membrane potential.

temperature on maximal and steady-state SR Ca^{2+} content, I_{Ca} density and I_{Ca} inactivation kinetics. Data were considered significantly different if $P<0.05$.

RESULTS

Cardiomyocyte size

The size of scombrid cardiomyocytes was compared by measuring the cell capacitance. Bluefin and yellowfin ventricular cardiomyocytes had a significantly smaller cell capacitance than the bonito and the mackerel (Table 1).

Characterisation of I_{Ca} in scombrid cardiomyocytes

In all species a voltage step from –80 mV to 0 mV in the presence of TTX gave rise to a slow activating and inactivating I_{Ca} that varied in amplitude and kinetics according to temperature (Fig. 1A). To confirm this current originated from the L-type Ca^{2+} channel we applied nifedipine (2.5 µmol l^{-1}), a specific L-type Ca^{2+} channel inhibitor, that completely abolished I_{Ca} (Fig. 1A dotted line).

I_{Ca}–voltage relationships and kinetics

The temperature dependence of the I_{Ca}–voltage relationships for each scombrid species tested are shown in Fig. 1B–E. Activation of I_{Ca} began at –40 mV, peaked at ~0 mV and reversed at approximately –50 mV. Peak I_{Ca} amplitude was similar between mackerel and yellowfin tuna at each temperature tested (Fig. 1B,D). I_{Ca} inactivation kinetics for these species were also similar (Table 1), and as a result, Q_{Ca} (the charge carried by I_{Ca}) was not significantly different between the two species over the range of temperatures tested (Table 1). Peak I_{Ca} density and Q_{Ca} in bonito myocytes was significantly smaller than in bluefin tuna, yellowfin tuna and mackerel myocytes at all temperatures tested (Fig. 1B–D and

Table 1. L-type Ca^{2+}channel charge density and inactivation kinetics from scombrid ventricular myocytes

	Cell capacitance (pF)	Temp (°C)	*N*	Q_{Ca} (pC pF^{-1})	$\Delta[Ca^{2+}]$ (μmol l^{-1})	τ_s (ms)	τ_f (ms)	τ (ms)
Bluefin tuna	41.8±1.3[b,d]	23	13	0.2±0.02	47.8±5.0	113.1±9.0[b]	14.0±1.9	–
		19	10	0.14±0.01*[,a,b]	33.8±1.8*[,a,b]	112.6±13.7	13.6±1.4[b]	–
		15	9	0.13±0.01*[,a,b]	31.7±1.5*[,a,b]	128.9±27.1	38.5±18.4*	–
Yellowfin tuna	38.7±2.1[c,e]	23	19	0.2±0.01	47.0±3.1	118.1±13.1[c]	12.8±1.3	–
		19	18	0.18±0.01	43.0±2.6	110±11.2	16.1±1.3	–
		15	17	0.19±0.01	44.1±2.6	124.4±11.0	21.5±1.4*	–
								–
Mackerel	56.6±2.3	23	15	0.23±0.02	53.3±6.9	63.6±5.3	14.2±1.1	–
		19	16	0.21 ±0.02	51.1±3.9	90.8±5.5*	20.0±1.6*	–
		15	10	0.2±0.02	47.0±4.43	130.7±20.2*	28.6±5.6*	–
Bonito	57.4±3.2	23	13	0.07±0.01	16.4±2.12	–	–	39.6±2.9
		19	15	0.04±0.01*[,d,e,f]	9.0±0.9*[,d,e,f]	–	–	78.4±9.3*
		15	10	0.03±0.01*[,d,e,f]	5.2±1.9*[,d,e,f]	–	–	139.3±11.5*

Values are means ± s.e.m. *N* is the number of cells.

$\Delta[Ca^{2+}]$, change in the total cellular $[Ca^{2+}]$ expressed as a function of non-mitochondrial cell volume, as calculated by Vornanen et al. (Vornanen et al., 1998); Q_{Ca}, charge density of L-type Ca^{2+} channel current; τ_f and τ_s, time constant of fast and slow inactivation, respectively. In bonito ventricular myocytes, inactivation was best fit with a single exponential (τ).

*A significant difference between 23 and 19 or 15°C. Letters indicate a significant difference between the following groups: [a]bluefin and yellowfin tuna; [b]bluefin tuna and mackerel; [c]yellowfin tuna and mackerel; [d]bluefin tuna and bonito; [e]yellowfin tuna and bonito; [f]mackerel and bonito; $P<0.05$, Student's *t*-test.

Table 1). The decay of bonito I_{Ca} was better fit by a single exponential equation. Bluefin tuna peak I_{Ca} was larger than that of bonito, but significantly smaller than the mackerel and yellowfin tuna peaks (Fig. 1E). Furthermore, bluefin tuna I_{Ca} inactivation kinetics were generally slower than those of mackerel and yellowfin tuna, leading to a smaller Q_{Ca} at 19 and 15°C (Fig. 1B–D, Table 1).

Effect of temperature on I_{Ca}–voltage relationships and kinetics

In this and subsequent sections, Q_{10} values have been calculated across the range of 23 to 15°C. In all species tested, acute reductions in temperature from 23 to 19 to 15°C led to a significant decrease in peak I_{Ca} density (Fig. 1). Although the temperature sensitivity of I_{Ca} in mackerel and yellowfin tuna was relatively high (Q_{10}=3.1 and 2.7, respectively), a cold-induced slowing of I_{Ca} kinetics (τ_s and τ_f) allowed Q_{Ca} to remain unaffected by acute temperature reductions (Q_{10}=1.1 and 1.2, respectively; Table 1). In bluefin tuna, the cold-induced slowing of I_{Ca} kinetics was not significant, and as a result, reductions in temperature led to both a significant decrease in peak I_{Ca} (Q_{10}=2.6) and Q_{Ca} (Q_{10}=1.8; Table 1). In bonito, although τ was significantly slower at cold temperatures, the dramatic effect of cold temperature on peak I_{Ca} (Q_{10}=11.0) caused Q_{Ca} to decline significantly (Q_{10}=4.23; Table 1).

Steady state and maximal SR_{load}

Atrial cardiomyocytes are known to more SR dependent than ventricular cardiomyocytes, relying more strongly on SR Ca^{2+} contributions. We assessed $ssSR_{load}$ and $maxSR_{load}$ in both ventricular and atrial cardiomyocytes from the bluefin tuna. Application of caffeine caused the cells to contract strongly and induced a large inward current (I_{NCX}), as shown in Fig. 2A (ventricular myocyte) and B (atrial myocyte). This current was completely abolished by pre-incubation with SR inhibitors (10 μmol l^{-1} ryanodine and 2 μmol l^{-1} thapsigargin) for 5 min (not shown). The time integral of this current, as shown in Fig. 2C (ventricular myocyte) and D (atrial myocyte), was used to assess SR Ca^{2+} content at the time of caffeine application at each temperature.

Under stead-state conditions, bluefin tuna atrial myocytes had significantly larger SR_{load} values compared with ventricular myocytes (Fig. 2E,G), consistent with increased SR content in atrial cells. This result was also apparent under maximal conditions, but only significant at 23°C (Fig. 2F,H).

Having verified our technique, we went on to measure SR_{load} in the other scombrid cardiomyocytes. $ssSR_{load}$ and $maxSR_{load}$ was greater in mackerel and bluefin ventricular myocytes compared with yellowfin tuna and bonito (Figs 2 and 3). This difference was found to be statistically significant at all temperatures tested, except at 19°C, where bluefin $ssSR_{load}$ was not significantly different from that of bonito ($P>0.05$). No significant differences in $ssSR_{load}$ or $maxSR_{load}$ were observed between mackerel and bluefin tuna, or between yellowfin tuna and bonito, at any temperature tested (Figs 2 and 3).

Effects of temperature on SR_{load}

The $ssSR_{load}$ in ventricular and atrial cardiomyocytes from the bluefin tuna decreased significantly when the temperature was reduced from 21 to 15°C (Q_{10}=2.1 and 2.2, respectively), and was more temperature sensitive within the upper temperature range (19–21°C; Fig. 2E,G). Ventricular $maxSR_{load}$ was significantly decreased between 21 and 15°C (Q_{10}=1.8), whereas atrial $maxSR_{load}$ was only temperature sensitive at the lower temperature range (15–19°C; Fig. 2F,H). For the other scombrids, $ssSR_{load}$ in ventricular myocytes was only sensitive to temperature in bonito (Q_{10}=1.3), whereas $maxSR_{load}$ decreased significantly with a temperature reduction from 21 to 15°C in the bonito and yellowfin (Q_{10}=1.7 and 1.8, respectively) and was more temperature sensitive in the upper thermal range (Fig. 3).

DISCUSSION

The major findings from our study are, (1) the magnitude of ventricular I_{Ca} density does not correlate with endothermy, cardiovascular capacity or thermal niche expansion, (2) SR Ca^{2+} content is greater in the more cold-tolerant species (bluefin tuna and mackerel), and (3) bonito utilises a different E–C coupling strategy from mackerel and tunas.

Scombrid I_{Ca}–voltage relationships, kinetics

Species-specific variability in the relative contribution of I_{Ca} to force development has previously been demonstrated in scombrids and

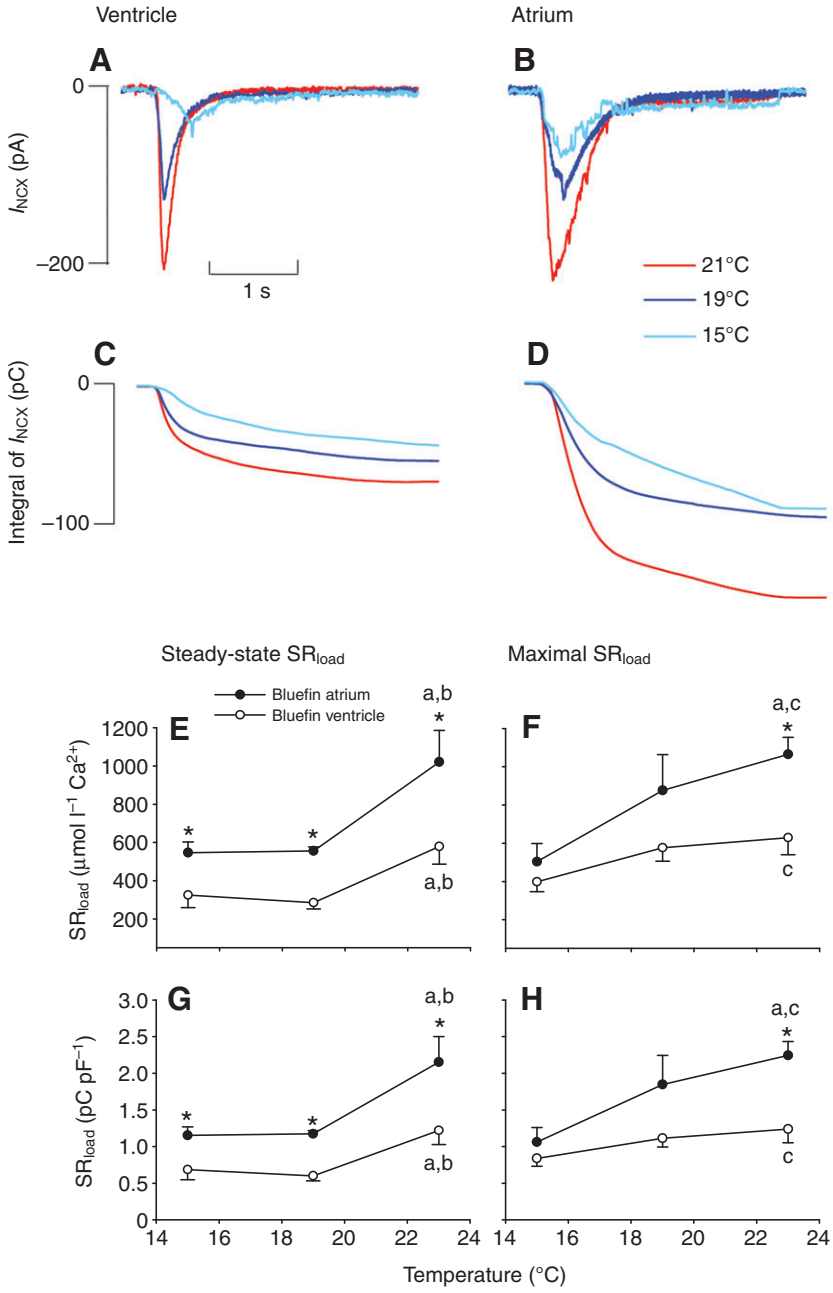

Fig. 2. Effects of acute temperature change on steady-state and maximal sarcoplasmic reticulum Ca^{2+} content (SR_{load}) in atrial and ventricular myocytes from the Pacific bluefin tuna. (A–D) Representative steady-state Na^+/Ca^{2+} exchange current (I_{NCX}) recordings in pA (A,B), and corresponding charge transfer (integral of I_{NCX}) in pC (C,D). (E–H) Mean data (± s.e.m.) for ventricular and atrial steady-state and maximal SR_{load} and corresponding charge transfer. *Difference between the ventricular and atrial myocytes. Significant effects of temperature on SR_{load} within a species are shown by different letters, [a]23 *vs* 15, [b]23 *vs* 19, [c]19 *vs* 15°C. $P<0.05$, one-way analysis of variance, Student–Newman–Keuls *post hoc* test. The number of cells (N) at 21, 19 and 15°C, respectively, for steady-state SR_{load} were: ventricle N=12, 10 and 8, atrium N=11, 7 and 6; for maximal SR_{load}, ventricle N=10, 11, 9 and atrium N=6, 7, 6.

supports the hypothesis that differences in cardiac performance may be related to differences in I_{Ca} density (Shiels et al., 2004). However, in the present study I_{Ca} and Q_{Ca} density were greatest in mackerel and yellowfin at all temperatures tested. This result suggests the magnitude of I_{Ca} density does not correlate with the evolution of cardiovascular capacity, endothermy or thermal niche expansion.

Interestingly, bonito peak I_{Ca} and Q_{Ca} was dramatically lower than the other scombrids at all temperatures tested. In fact, peak I_{Ca} density in bonito ventricular myocytes tested at 23°C was three times and six times lower than that recorded in ventricular myocytes of trout (tested at 21°C and acclimated to 17°C) and carp (tested at 21°C and acclimated to 23°C) (Vornanen, 1997; Vornanen, 1998). Similar results on L-type Ca^{2+} channel characteristics found in bonito have been reported in burbot ventricular myocytes (Shiels et al., 2006). Although direct comparisons are difficult because of different experimental conditions, low I_{Ca} and Q_{Ca} density in burbot was related to a smaller density of sarcolemmal L-type Ca^{2+} channels (Tiitu and Vornanen, 2003), whereas I_{NCX} density was robust and thought to be the primary pathway for sarcolemmal Ca^{2+} influx (Shiels et al., 2006). We propose that the NCX might be the primary pathway for sarcolemmal Ca^{2+} influx in the bonito, and might provide the majority of activator Ca^{2+} to the myofilaments (see SR discussion below).

Scombrid I_{Ca} temperature sensitivity

All scombrid fishes showed significant reductions in I_{Ca} with decreasing temperature. In the mackerel and yellowfin, the slowing of inactivation kinetics of I_{Ca} offset the negative effects of temperature, resulting in a temperature-insensitive Ca^{2+} flux (Q_{10}=1.2 for mackerel, Q_{10}=1.1 for yellowfin). This effect has been observed in most other teleosts examined to date (Shiels et al., 2000),

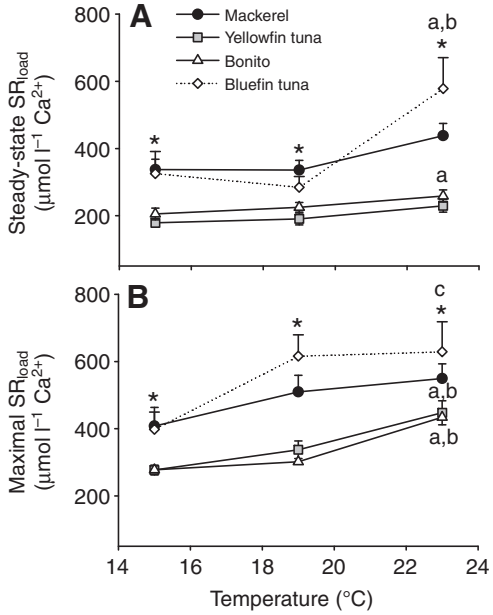

Fig. 3. Effects of acute temperature change on steady-state and maximal sarcoplasmic reticulum Ca^{2+} content (SR_{load}) in mackerel, yellowfin tuna, bonito and Pacific bluefin tuna ventricular myocytes. Here we have converted charge transfer (in pC) to the total change in intracellular Ca^{2+} (in $\mu mol\,l^{-1}$) as explained in the Materials and methods. Data are means ± s.e.m. * A significant difference between mackerel and bluefin *vs* yellowfin and bonito. No significant differences were observed between yellowfin and bonito or bluefin and mackerel. Significant effects of temperature on SR_{load} within a species are shown by different letters; [a]23 *vs* 15, [b]23 *vs* 19, [c]19 *vs* 15°C. $P<0.05$, one-way analysis of variance, Student–Newman–Keuls *post hoc* test. The number of cells (*N*) at 21, 19 and 15°C, respectively, steady-state SR_{load} were: mackerel N=11, 13, 8; yellowfin tuna N=10, 13 and 10; bonito N=10, 8 and 9; bluefin tuna N=12, 10 and 8; for maximal SR_{load}; mackerel N=12, 12 and 9; yellowfin tuna N=10, 12 and 11; bonito N=8, 9, 10, bluefin tuna N=10, 11 and 9.

and may be a common protective mechanism against the depressive effects of the cold. In contrast, the Ca^{2+} carried by I_{Ca} in the bluefin tuna and mackerel appears to be temperature sensitive with distinctly different affects (Q_{10}=1.7 and 4.2, respectively). It is possible that temperature-induced alterations in heart rate and action potential shape and/or duration in these species may offset the negative effects of cold temperature on I_{Ca} (see Limitations of the study and future directions).

SR Ca^{2+} content across species and temperatures

The $ssSR_{load}$ in scombrid ventricular myocytes at 23°C ranged between 229 and 578 $\mu mol\,l^{-1}$ Ca^{2+}. These values are similar to recently published data from warm-acclimated (18°C) freshwater teleosts: $ssSR_{load}$ in ventricular myocytes from the rainbow trout, burbot and crucian carp ranged between 332 and 540 $\mu mol\,l^{-1}$ at 18°C (Shiels et al., 2002b; Haverinen and Vornanen, 2009). Although these results suggest scombrid $ssSR_{load}$ is not exceptional among teleosts, the values presented here are three- to ninefold larger than those reported for rabbit (87 $\mu mol\,l^{-1}$ at 22°C) and rat (64 $\mu mol\,l^{-1}$ at 23°C) (Walden et al., 2009) ventricular myocytes. Furthermore, our values for $maxSR_{load}$ suggest scombrid ventricular myocytes can store up to 628 $\mu mol\,l^{-1}$ of Ca^{2+} in the SR, which is tenfold larger than mammalian SR_{load} under steady-state conditions. Thus, bluefin tunas and cold-tolerant active teleosts in general, appear to have an exceptional capacity to store Ca^{2+} in the SR.

The large SR Ca^{2+} storage capacity of fish compared with mammalian cardiomyocytes does not appear to relate to differences in total SR volume (DiMaio and Block, 2008; Shiels et al., 2011; Bowler and Tirri, 1990). Rather, Haverinen and Vornanen suggest functional differences in Ca^{2+} management within the SR results in a higher total Ca^{2+} capacity (Haverinen and Vornanen, 2009). For example, the fish ryanodine receptor appears to have a lower affinity for Ca^{2+} binding than mammals, which would stabilise SR Ca^{2+} content (Vornanen, 2006). Potentially the cooler ambient temperatures experienced by fish cardiomyocytes (compared with mammals) requires higher Ca^{2+} content to initiate and maintain Ca^{2+}-release events (Haverinen and Vornanen, 2009). Support for this contention comes from recent imaging of intracellular Ca^{2+} in bluefin tuna myocytes, which shows both steady-state SR Ca^{2+} utilisation during E–C coupling and increased SR Ca^{2+} utilisation in the cold (Shiels et al., 2011).

Species-specific differences in SR Ca^{2+} content do show significant variations within the scombrid lineage, which provides some evidence for linkage between thermal tolerance and niche expansion. Ventricular steady-state and maximal SR Ca^{2+} loads decrease in the order of bluefin tuna>mackerel>yellowfin tuna>bonito, with no statistical difference between bluefin tuna and mackerel. As far as the bluefin tuna is concerned, our finding of a comparatively large SR Ca^{2+} storage capacity is consistent with previous studies indicating an enhanced role for the SR in E–C coupling. Ultrastructural studies have revealed a significant SR density in bluefin tuna cardiomyocytes (DiMaio and Block, 2008), and protein work has demonstrated enhanced expression of SERCA-2 compared with other scombrids (Landeira-Fernandez et al., 2004; Landeira-Fernandez et al., 2007). Most recently, fluorescent microscopy has confirmed that the bluefin tuna SR releases Ca^{2+} on a beat-to-beat basis, contributing activator Ca^{2+} for contraction (Shiels et al., 2011). Thus, the bluefin tuna SR has an enhanced capacity to store, release and reuptake Ca^{2+} from the cytosol, which contributes to the generation of increased cardiac force and frequencies of contraction in this species.

Also consistent with previous studies on the significance of the SR in scombrids, the yellowfin tuna had significantly smaller SR_{load} values than the bluefin tuna. Isolated ventricular muscle from the yellowfin tuna has been shown to be unresponsive to SR inhibition with ryanodine and thapsigargin (Galli et al., 2008). Furthermore, SERCA-2 protein expression in the yellowfin tuna ventricle is significantly less than in the bluefin tuna (Landeira-Fernandez et al., 2004). Thus, the geographical distribution in warm warmer waters of the yellowfin tuna may not require the specialisations in E–C coupling that are seen in the bluefin tuna heart.

The equally large SR Ca^{2+} storage capacity of the mackerel and bluefin tuna was a somewhat surprising result in our study. Prior work suggested the Pacific mackerel has a lower expression of SERCA-2 than yellowfin, albacore and bluefin tuna (Landeira-Fernandez et al., 2004). However, ryanodine receptors have been identified in the mackerel, using western blot analysis (Castilho et al., 2007), and ryanodine has been shown to inhibit ventricular force of contraction by 20% (Shiels and Farrell, 2000). Although our results suggest SR Ca^{2+} loading was more temperature sensitive in the bluefin tuna and mackerel (higher Q_{10} values), $ssSR_{load}$ and $maxSR_{load}$ were larger at any given temperature than in the more tropical species, the bonito and yellowfin. A large and maintained capacity to store Ca^{2+} in the SR at cold temperatures may allow these species to maintain Ca^{2+} cycling and cardiac function during exploitation of cold waters.

Limitations of the study and future directions

Our study aimed at identifying differences between scombrids in the capacity of their cardiomyocytes to move Ca^{2+} through the L-type Ca^{2+} channel and store Ca^{2+} in the SR. By using square-wave pulses at a single frequency to measure I_{Ca}, we were able to isolate these differences without interspecific variation in action potential shape or frequency. However, temperature independently affects the frequency of contraction and action potential shape and/or duration, which in turn can have profound affects on $I_{Ca,}$ (Shiels et al., 2000) and SR loading (Shiels et al., 2002b). Thus, future experiments should be aimed at assessing I_{Ca} properties with physiologically relevant frequencies of contraction and action potentials as the command waveform.

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