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Cardiac function in an endothermic fish: cellular mechanisms for overcoming acute thermal challenges during diving

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Understanding the physiology of vertebrate thermal tolerance is critical for predicting how animals respond to climate change. Pacific bluefin tuna experience a wide range of ambient sea temperatures and occupy the largest geographical niche of all tunas. Their capacity to endure thermal challenge is due in part to enhanced expression and activity of key proteins involved in cardiac excitation–contraction coupling, which improve cardiomyocyte function and whole animal performance during temperature change. To define the cellular mechanisms that enable bluefin tuna hearts to function during acute temperature change, we investigated the performance of freshly isolated ventricular myocytes using confocal microscopy and electrophysiology. We demonstrate that acute cooling and warming (between 8 and 28°C) modulates the excitability of the cardiomyocyte by altering the action potential (AP) duration and the amplitude and kinetics of the cellular Ca²⁺ transient. We then explored the interactions between temperature, adrenergic stimulation and contraction frequency, and show that when these stressors are combined in a physiologically relevant way, they alter AP characteristics to stabilize excitation–contraction coupling across an acute 20°C temperature range. This allows the tuna heart to maintain consistent contraction and relaxation cycles during acute thermal challenges. We hypothesize that this cardiac capacity plays a key role in the bluefin tunas' niche expansion across a broad thermal and geographical range.

1. Introduction

The Pacific bluefin tuna (*Thunnus orientalis*) is an apex predator with the largest oceanic home range of all tunas [1–3]. These fish encounter large ambient temperature changes on their travels from the western to eastern Pacific oceans, with archival tags revealing a mean sea surface temperature of 17.36°C ± 0.05, and a range in ambient temperatures from 3.4 to 27°C for juveniles ($n = 78\,000$ observations [3,4]). Pacific bluefin tuna also encounter rapid vertical thermal gradients (10–15°C) when they forage after prey and dive to depths greater than 500 m [5]. Behavioural records from electronic tags indicate bounce diving in juvenile and adult tuna [2,6,7], whereby animals dive repeatedly to depth to forage and then rapidly resurface (figure 1). Bluefin tunas are the most endothermic lineage of the *Thunnus* genus; their countercurrent heat exchangers conserve metabolic heat in the brain, eyes, slow-oxidative red muscles [8,9] and viscera [10], which contributes to their wide oceanic range. Among *Thunnus* only the bluefin lineage ventures into sub-polar and polar seas, and electronic tags in Atlantic bluefins have shown remarkable prolonged occupation of cool surface waters (8–11°C), indicative of a high degree of thermal tolerance [2,11].

Endothermy also poses a physiological challenge for bluefin tuna because their heart receives coronary blood straight from the gills at ambient temperature and is upstream of the countercurrent heat exchangers [9]. Bluefin tunas therefore possess the unique arrangement of a heart operating at fluctuating ambient water temperature delivering blood to warm body tissues. When bluefins are forced to

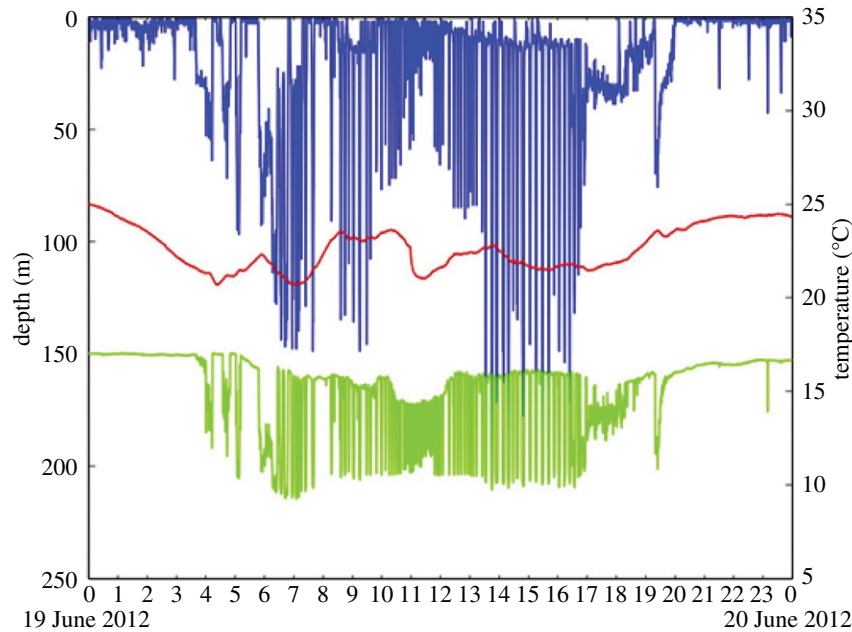


Figure 1. Archival tag record of a juvenile Pacific bluefin tuna off the coast of California, 19–20 June 2012. Archival tag time series shows depth from pressure (blue), internal body temperature from position of archival tag in the peritoneal cavity (red) and ambient water temperature from an external sensor (green). Juvenile bluefin tuna demonstrate rapid bouts of diving associated with foraging behaviours that occur prior to a visceral heat increment of feeding event (peritoneal warming). This bounce diving, where time at depth is short, and interspersed regularly with time at the surface, may be due to cold-induced cardiac bradycardia and subsequent oxygen delivery limitations.

swim outside their thermal neutral zone [12], metabolic rate (O_2 consumption) is elevated, which further increases the circulatory demand placed on the heart. Indeed, bounce diving in juvenile and adult tunas [2,6,7] (figure 1), may be a profound behavioural response related to rewarming and acceleration of cardiac function. This is because cooling at the tuna gill causes a rapid and profound drop in heart rate (bradycardia) [9,13]. *In vivo* measurements with heart rate tags in captive free-swimming Pacific bluefin tuna demonstrate this cold-induced bradycardia; heart rate drops from approximately 60 bpm to approximately 30 bpm when tank water is decreased by 10°C [13]. *In situ* and *in vivo* cardiac studies with Pacific bluefin, yellowfin (*Thunnus albacares*) and albacore (*Thunnus alalunga*) tuna show this cold-induced bradycardia dramatically reduces cardiac output [13–15]. The cardio-depressive effect of acute cooling raises an interesting question: how does the heart maintain function as the fish dives from the relatively warm waters of the sea surface into cooler waters in search of prey?

Cardiac output is the volume of blood (stroke volume, ml) pumped per minute (heart rate, min^{-1}). If stroke volume is experimentally maintained across an acute decrease in temperature, the cold-induced bradycardia drives the collapse of cardiac output in tuna [14,16]. However, at slow cardiac frequencies, stroke volume increases due to increased filling time, which can partially offset the effects of cold. Thus, the interaction between temperature and pacemaker firing rate is an important factor to consider when examining the effect of acute temperature change on heart function. Adrenergic stimulation is also a vital modulator of heart function, altering cardiac inotropy (force), chronotropy (rate) and lusitropy (myocardial relaxation), often in a temperature-dependent manner [17,18]. Adrenaline (AD) has been shown to protect cardiac function under cold-stress and pH-stress in salmonids [19,20], and may play a role in the temperature sensitivity of tuna cardiac performance. However, this has not been tested

experimentally, and the interactions between acute temperature change, cardiac frequency and AD have not been investigated alone or in combination at the cellular level in cardiomyocytes from any species of fish.

Cardiac myocyte contraction and relaxation is controlled by the cycling of cellular Ca^{2+} ($\Delta[\text{Ca}^{2+}]_i$) via the process of excitation–contraction coupling. Excitation–contraction coupling links membrane depolarization by the action potential (AP) with the Ca^{2+} signal that causes contraction of the myofilaments. Acute temperature change in fish and mammals has direct (i.e. Q_{10}) effects on the ion channels and pumps that underlie the cardiac AP. Thus, acute temperature change can disrupt electrical excitation and coordination of the heartbeat [21–23]. The cellular proteins that cycle Ca^{2+} and develop force during excitation–contraction coupling are all known to be acutely temperature-sensitive (e.g. the L-type Ca^{2+} current (I_{Ca}) [24,25], the ryanodine receptor [26–29], the SR Ca^{2+} ATPase (SERCA) [30–32], the Na^+ – Ca^{2+} exchanger [33,34] and the myofilaments [35,36]). However, changes in the shape of the rainbow trout AP have been shown to offset the acute effect of temperature on I_{Ca} [22], suggesting compensatory interplay between thermal sensitivity of the different components of excitation–contraction coupling in fish. The activity of key ion channels and ion pumps involved in excitation–contraction coupling are also modified by AD [17,37] through the β -adrenergic receptor cascade and associated downstream signalling and phosphorylation events. Indeed, AD acting through cAMP-PKA-dependent phosphorylation of proteins has been shown to alter AP duration (APD) in fish heart [38] and to increase $\Delta[\text{Ca}^{2+}]_i$ through augmentation of both I_{Ca} and SERCA function [39]. Thus, acute temperature change and adrenergic stimulation affect the rate and strength of myocyte contraction and relaxation, which culminates in changes in cardiac output.

To define the cellular mechanisms that enable Pacific bluefin tuna hearts to maintain function when encountering

acute temperature change during foraging dives or at frontal edges in surface waters, we investigated the performance of freshly isolated ventricular myocytes. We used the rich electronic tagging data archive from juvenile Pacific bluefin tuna, who often exhibit bounce diving behaviour (figure 1) [5,40], to inform our experimental temperature and frequency range. We then combined confocal microscopy and electrophysiology to explore the interactions between temperature, adrenergic stimulation and contraction frequency on the temporal and spatial properties of $\Delta[\text{Ca}^{2+}]_i$, and the excitation profiles of AP and I_{Ca} in isolated myocytes. We show that acute thermal modulation of contraction frequency, coupled with adrenergic stimulation, modulates AP characteristics to maintain relatively constant Ca^{2+} cycling during acute temperature change. Thus, we demonstrate that compensatory changes in the shape of the AP balance individual effects on ion flux and normalize cellular Ca^{2+} dynamics during a thermal challenge. These cellular cardiac adaptations allow the Pacific bluefin tuna heart to effectively drive contraction and relaxation across a 20°C range, and may contribute to the maintenance of organismal performance over the large thermal niche of the Pacific bluefin tuna.

2. Material and methods

(a) Fish origin and care

Pacific bluefin tuna, *T. orientalis* (mean mass 8.6 ± 0.8 kg, mean fork length 76.1 ± 2.3 cm, $n = 5$, males and females), were captured off the coast of southern California or in Mexican waters off northern Baja as previously described [41]. Fish were held at the Tuna Research and Conservation Centre in Pacific Grove, CA, USA, in 109 m³ circular tanks acclimated to 14°C. Fish were held for at least eight weeks at constant temperature under a 12 L:12 D photoperiod prior to experimentation, and husbandry was provided as previously described [42].

(b) Myocyte isolation

Bluefin tuna ventricular myocytes were isolated as described in detail previously [43] and briefly summarized in the electronic supplementary material.

(c) Experimental protocols

The experiments were designed to simulate the acute changes in temperature, and the corresponding changes in *in vivo* heart rate, that juvenile Pacific bluefin experience during a dive. Based on archival tag data [5,40] and *in vivo* heart rate data from free-swimming tuna [10,13], we set our acute temperature change profile from 8 to 28°C across cardiac frequencies of 0.2–1.0 Hz (12–60 bpm). Myocytes were perfused initially at the bluefin tuna's acclimation temperature of 14°C and stimulated to contract at 0.5 Hz with a field stimulator for Ca^{2+} experiments, or the patch pipette for electrophysiology experiments. Myocyte temperature was controlled with an in-line temperature controller (SC-20, Warner Instruments). Myocytes were then exposed to a series of temperature and frequency changes to simulate those experienced during a dive; APs and Ca^{2+} transients were recorded throughout. First, temperature was increased to 28°C while frequency was held at 0.5 Hz. It took approximately 3 min for the temperature to change and for the recordings to be stable at the new temperature. After recording Ca^{2+} transients and APs at 0.5 Hz, contraction frequency was increased to 1.0 Hz, which is physiologically relevant at 28°C [10,13]. Temperature was then decreased to 8°C and frequency was dropped to 0.2 Hz. After

recordings were made, frequency was increased to 0.5 Hz. Lastly, temperature was returned to 14°C and recovery was assessed. Experiments were performed in either the absence or presence of a high but physiological dose of AD (500 nM [20,38]). Confocal Ca^{2+} imaging and electrophysiological measurements were conducted on separate myocytes, but each underwent the same protocol. Our protocol allowed the direct effect of temperature on AP and Ca^{2+} cycling dynamics to be assessed, as well as the interactive effects of temperature, frequency and adrenergic stimulation. A separate series of experiments were conducted to determine the thermal- and adrenergic-sensitivity of the L-type Ca^{2+} channel current (I_{Ca}).

(d) Action potential recordings

APs were recorded from bluefin tuna ventricular myocytes as described previously [43] and summarized in the electronic supplementary material. Briefly, myocytes were superfused with solution containing (mmol) 150 NaCl, 5.4 KCl, 1.5 MgCl₂, 3.2 CaCl₂, 10 glucose, 10 HEPES and pH adjusted to 7.6 via NaOH. The pipette solution contained (mmol) 140 KCl, 5 MgATP, 0.025 EGTA, 1 MgCl₂ and 10 HEPES. The pH was adjusted to 7.2 with KOH. All experiments were conducted in the whole-cell current-clamp mode of the amplifier.

(e) Confocal imaging

Confocal imaging of $\Delta[\text{Ca}^{2+}]_i$ in ventricular myocytes has been described in detail previously [43] and is summarized in the electronic supplementary material. Briefly, myocytes were loaded with 4 μM Fluo-4-AM [41], perfused with the same solution used for the electrophysiology and imaged (excitation at 488 nm, detection more than 505 nm) with an Olympus Fluoview confocal microscope. Repetitive line scans (1000 lines of 512 pixels) were taken every 2–4 ms across the width of the cell. All line scan images are presented as original raw fluorescence (F). Background fluorescence (F_0) was measured in each cell in a region that did not have localized or transient fluorescent elevation. The Kd of Fluo-4 was adjusted for *in vivo* temperature dependence [44]: 829 nM at 28°C, 1594 at 14°C and 1922 nM at 8°C. Kinetic analyses of Ca^{2+} transients are described in detail in [41] and in the electronic supplementary material.

(f) L-type Ca^{2+} channel recordings

I_{Ca} is the major Ca^{2+} influx pathway during the AP plateau in fish myocytes and the major contributor to $\Delta[\text{Ca}^{2+}]_i$. Consequently, we determined the thermal and adrenergic sensitivity of I_{Ca} in a separate series of experiments using standard whole-cell voltage-clamp methodology (see the electronic supplementary material). The pipette solution consisted of (mM) 130 CsCl, 5 MgATP, 15 TEA chloride, 1 MgCl₂, 5 Na₂-phosphocreatine, 10 HEPES and 0.025 EGTA with pH adjusted to 7.2 with CsOH. These studies were conducted with and without isoprenaline (1 μM), a synthetic analogue of AD (see the electronic supplementary material), over an acute temperature range of 8, 19 and 24°C. Analyses of I_{Ca} kinetics and Ca^{2+} entry were calculated as described previously [43,45] and detailed in the electronic supplementary material.

(g) Statistics

Imaging and electrophysiology data are presented as raw data and as means \pm s.e.m. with n equalling the number of cells from five animals. RM ANOVAs (2-factor and 1-factor) were used as indicated in the text or in the figure legends. Significance was accepted at $p < 0.05$. Temperature coefficients (Q_{10} values) for acute temperature changes were calculated for AP upstroke velocity and the integral of APD, according to the equation $Q_{10} = (R_2/R_1)^{10/(T_2-T_1)}$, where

R is the rate and T is the temperature. All other calculations are explained in the electronic supplementary material.

3. Results

(a) Effects of acute temperature change and contraction frequency on action potential duration and $\Delta[\text{Ca}^{2+}]_i$

To study the impacts of acute cooling and warming on cardiac APs and Ca^{2+} dynamics of tuna hearts, ventricular cells were isolated from a captive population of Pacific bluefin (acclimated to 14°C) and subjected to electrophysiological and confocal image analysis. Key results are provided in the figures; more detailed analyses for each variable under each condition are provided in the three electronic supplementary material tables. Acute warming (from 14 to 28°C) shortened the duration of the Pacific bluefin tuna ventricular AP (at 50% repolarization APD_{50}) by approximately 200% ($Q_{10} \sim 3.2$), while acute cooling (from 14 to 8°C) prolonged it by approximately 50% ($Q_{10} \sim 2.3$) (figure 2*a,e*; electronic supplementary material, table S1). These APD responses were exacerbated when contraction frequency was slowed (from 0.5 to 0.2 Hz) during cooling to simulate bradycardia, or accelerated (from 0.5 to 1.0 Hz) during warming to simulate a tachycardia (figure 2*b,e*; electronic supplementary material, table S2). Warming induced a hyperpolarization of the resting membrane potential (RMP) and an increase in the upstroke velocity of the AP ($Q_{10} \sim 1.9$; see electronic supplementary material, tables S1 and S2). This suggests warm temperatures reduce electrical excitability; however, once the activation threshold for an AP is reached, the AP progresses rapidly.

The amplitude of $\Delta[\text{Ca}^{2+}]_i$ was reduced by both cooling and warming from the acclimation temperature of 14°C (figure 3*b,c*). Cooling slowed the rise and fall kinetics of $\Delta[\text{Ca}^{2+}]_i$, whereas warming accelerated these parameters, as indicated by the raw time course traces and mean data (figure 3; electronic supplementary material, tables S1 and S2). Simulating bradycardia at cold temperatures slowed the decay of $\Delta[\text{Ca}^{2+}]_i$, while simulating tachycardia at warm temperatures accelerated the decay of $\Delta[\text{Ca}^{2+}]_i$ (electronic supplementary material, table S2). Taken together, the data indicate acute thermal challenges to 8 or 28°C reduced Ca^{2+} cycling in the ventricular cells, and therefore decreased their contractility in comparison with controls at 14°C.

(b) Effects of adrenaline on action potential duration and $\Delta[\text{Ca}^{2+}]_i$ in tuna ventricular myocytes

Adrenergic stimulation is known to protect the fish heart during environmental perturbation [20]. In Pacific bluefin tuna ventricular cells, AD depolarized RMP, particularly in the cold, suggesting AD increases excitability and offsets the depressive effect of cold on ion flux (figure 2; electronic supplementary material, tables S1 and S2). AD also prolonged APD at all temperatures (figure 2*c–e*). The percentage increase in APD with adrenergic stimulation was greater at warmer (approx. 100% at 28°C and approx. 45% at 14°C) than colder (approx. 15% at 8°C) temperatures. When adrenergic stimulation was coupled with bradycardia at 8°C (figure 2*d*, and AD Phys Hz in figure 2*e*), APD was further prolonged by approximately 55%. This increase in

APD reduced the effect of acute temperature changes on $\Delta[\text{Ca}^{2+}]_i$ and resulted in a similar $\Delta[\text{Ca}^{2+}]_i$ amplitude at 8, 14 and 28°C (AD Phys Hz in figure 3*d*). Because $\Delta[\text{Ca}^{2+}]_i$ amplitude is a good index of force production [46], these data suggest cardiac contractility can be maintained across a wide and acute thermal gradient in the bluefin tuna ventricle through frequency- and AD-dependent modulation of excitation–contraction coupling.

(c) Role of I_{Ca} in acute sensitivity of excitation–contraction coupling in tuna ventricular myocytes

Having shown acute temperature change and adrenergic stimulation alter APD and the amplitude of $\Delta[\text{Ca}^{2+}]_i$, we next investigated the role of I_{Ca} because it is the predominant Ca^{2+} influx pathway in the fish myocyte. There was an increase in the peak amplitude of I_{Ca} with acute warming and a decrease in peak amplitude with cooling across a range of membrane potentials (figure 4). The I_{Ca} current inactivated more slowly with cooling and more rapidly with warming as indicated by raw current traces in figure 4*a* and the mean values for fast and slow time constants (τ) shown in figure 5*a,b*. These temperature-dependent changes in inactivation kinetics resulted in fairly constant Ca^{2+} influx into the myocyte on I_{Ca} across temperatures between 8 and 24°C (figure 5*c*). This reinforces the importance of the stimulus waveform in generating Ca^{2+} influx (and see [27,47]). When the stimulus is 500 ms long and held at 0 mV, as in the I_{Ca} experiments, Ca^{2+} influx on this current is fairly constant. However, we know from the first series of experiments that acute temperature change alters the duration of the AP and the voltage at which the plateau occurs (figure 2), and this affects Ca^{2+} dynamics, including those of I_{Ca} (figure 2) [22].

At all temperatures, adrenergic stimulation increased the amplitude of I_{Ca} (figure 4). It also accelerated the time constants of inactivation at the cooler temperatures (figure 5*a,b*), which reduced time for Ca^{2+} influx. This effect is, however, overwhelmed by the increased current amplitude as evidenced by the overall increase Ca^{2+} entry on I_{Ca} at all temperatures after AD (figure 5*c*). One consequence of prolonging APD is that it provides enough time for I_{Ca} to recover from inactivation and reopen during the extended AP plateau. This is called the I_{Ca} window current. Figure 5*d–f* shows that bluefin tuna have a sizeable I_{Ca} window current, which, when investigated with a 500 ms square depolarizing pulse, is larger at warmer temperatures and after adrenergic stimulation (figure 5*a,b*). Of course, *in vivo* the shortening of APD by acute warming (figure 2) would limit the contribution of this Ca^{2+} -window current influx path, whereas the prolongation of APD with cooling would increase it.

4. Discussion

Vertebrate heart function [25,48], including that of tunas [14,32,41], is acutely temperature dependent. Thus, diving into cooler waters could be a challenge for cardiac function, especially given the unique arrangement of bluefin tunas having a warm core and a cardiac system at ambient water temperature. In this study, we used laser-scanning confocal microscopy and electrophysiology to investigate mechanisms that could protect cardiomyocyte function during acute temperature change. Our aim was to simulate with isolated heart cells what may be

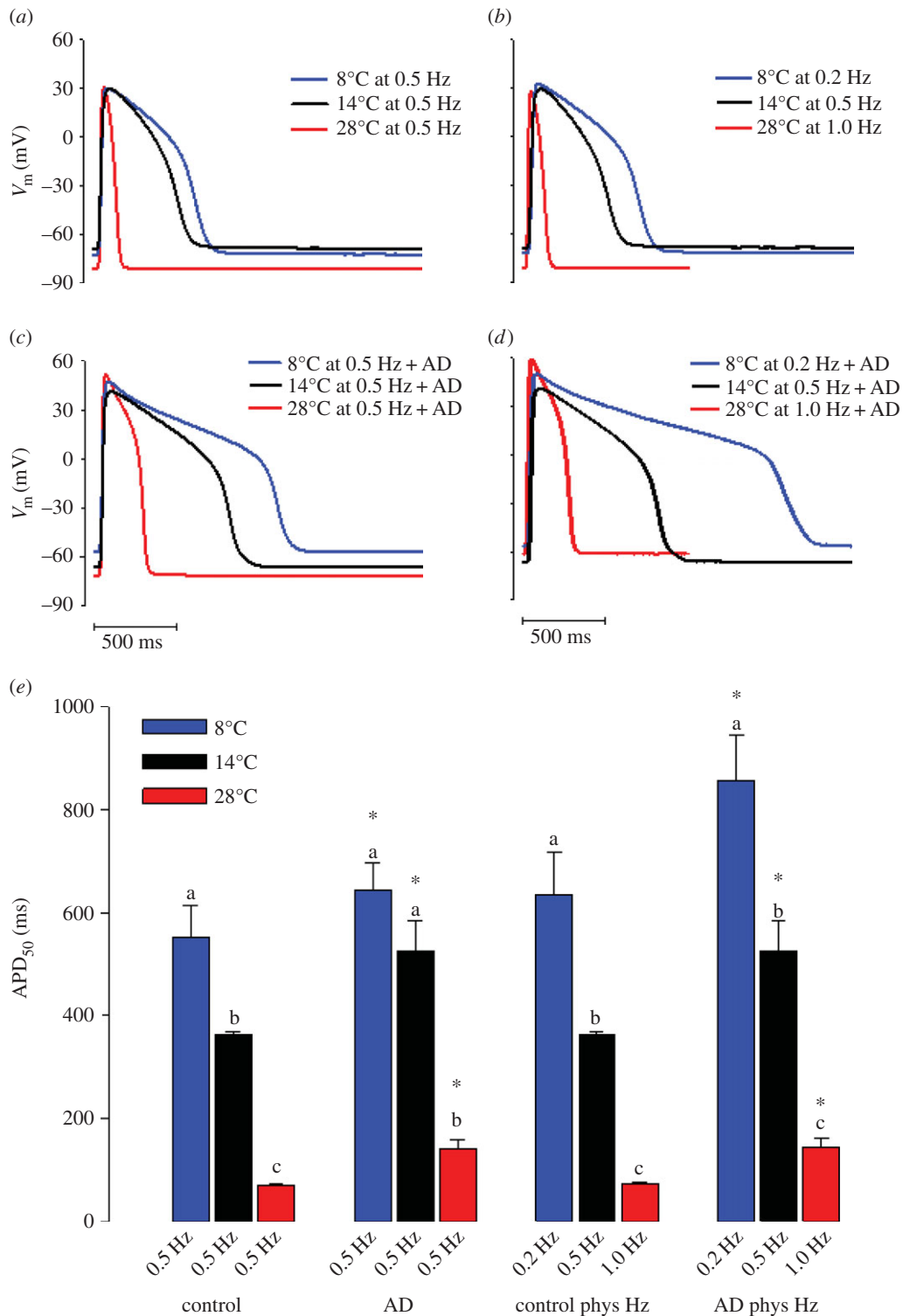


Figure 2. The effect of acute temperature change on the ventricular AP from the Pacific bluefin tuna. Responses (a) under control conditions at a constant frequency of 0.5 Hz, (b) under control conditions at a physiologically relevant contraction frequency for each temperature, (c) in the presence of 500 nM AD at a constant stimulation frequency of 0.5 Hz and (d) with 500 nM AD and at a physiologically relevant contraction frequency for each temperature; (e) mean data \pm s.e.m. for each condition. All means are $n = 10$ ventricular myocytes from five fish. Dissimilar letters indicate effects of temperature, asterisks indicate effects of adrenergic stimulation. Two-way RM ANOVA, $p < 0.05$.

occurring in the whole animal during a dive or thermal front experience. Our results provide new information about thermal performance of the endothermic bluefin tuna cardiomyocyte and can be summarized as follows. First, acute warming (from 14 to 28°C) shortens APD and reduces the time available for Ca^{2+} to enter the heart cell during the AP plateau. This reduces $\Delta[\text{Ca}^{2+}]_i$ and reduces contractility, but may be compensated for *in vivo* by the faster heart rates that accompany warming [13,14]. Acute cold (8°C) prolongs APD and reduces the

amplitude of I_{Ca} . This also reduces $\Delta[\text{Ca}^{2+}]_i$ and depresses contractility. However, *in vivo*, cold temperatures induce bradycardia, which we show here prolongs APD. This can increase Ca^{2+} influx on the I_{Ca} window current offering partial compensation of contractility in the cold. Under all conditions, AD improves myocyte contractility by increasing Ca^{2+} influx into the cell. The research demonstrates how cellular Ca^{2+} cycling could be preserved via electrical excitability and adrenergic stimulation when a tuna moves across a thermal

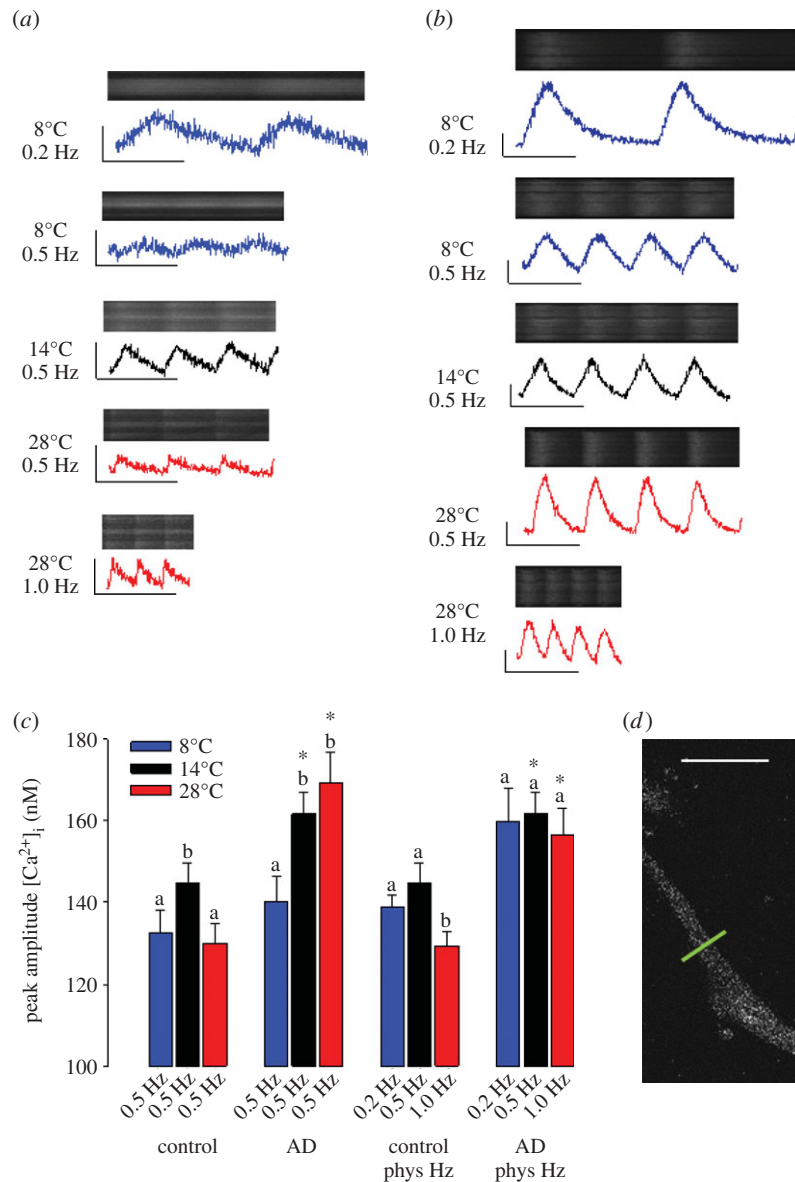


Figure 3. Spatial and temporal cellular Ca^{2+} flux in ventricular myocytes from Pacific bluefin tuna. (a) Representative raw line scan images (top) and corresponding time courses (bottom) showing the effect of acute temperature change and stimulation frequency on temporal and spatial characteristics of Ca^{2+} under control conditions. All recordings are taken from the same cell. (b) Same as (a) but in a different cell and in the presence of 500 nM AD. Time course scale is $1 F/F_0$ by 3 s (see Material and methods for details). (c) Effect of acute temperature change, stimulation frequency and adrenergic stimulation on the mean amplitude of $\Delta[\text{Ca}^{2+}]_i$. (d) Confocal image of a bluefin tuna ventricular myocyte loaded with Fluo-4 AM. Green line indicates position of scan. White scale bar is 20 μm . All data are means \pm s.e.m. for $n = 8-11$ ventricular myocytes from five fish. Dissimilar letters indicate effects of temperature, asterisks indicate effects of adrenergic stimulation. Two-way RM ANOVA, $p < 0.05$.

gradient and its heart rate changes. Whether these responses are unique to tuna or shared by other fish is not clear, and awaits similar investigations in oceanic and freshwater species. However, as $\Delta[\text{Ca}^{2+}]_i$ is directly related to contractility and force production in fish [49], our results suggest cardiac output would decrease without compensation by other mechanisms when a bluefin tuna leaves its 'thermal neutral zone' (i.e. its acclimation temperature) and moves into cooler waters. These results may explain why tunas with high thermal and metabolic demands would move back to the surface to rewarm (i.e. bounce dive; figure 1).

(a) Acute temperature change, frequency change and electrical activity

Consistent with previous studies in mammals and fish [21,23,50,51], we show that acute cooling prolongs APD in

bluefin tuna myocytes, while acute warming shortens it. The temperature dependence of APD shortening ($Q_{10} \sim 3.2$) was greater than that of APD prolongation ($Q_{10} \sim 2.3$), which is similar to what has been described previously for *T. orientalis* over a narrower temperature range [21] and agrees with previous work in a number of freshwater fish species [23]. Interestingly, the bluefin tuna APD (at any given temperature) is less than that of most freshwater species [23] and has been implicated in its thermal acclimatory response [21]. The delayed-rectifier K^+ channel current (I_{Kr}) is the major repolarizing current in ventricular myocytes and strongly influences APD [23,50,52]. In bluefin tuna acclimated to 14°C, I_{Kr} channel current density (and gene expression [51]) is strongly modified by acute temperature change (Q_{10} of approx. 3.1 [21]). Cooling decreases current density, which slows K^+ efflux from the myocyte, inhibiting repolarization and prolonging APD. The inverse occurs with warming, leading to APD shortening and a reduction in

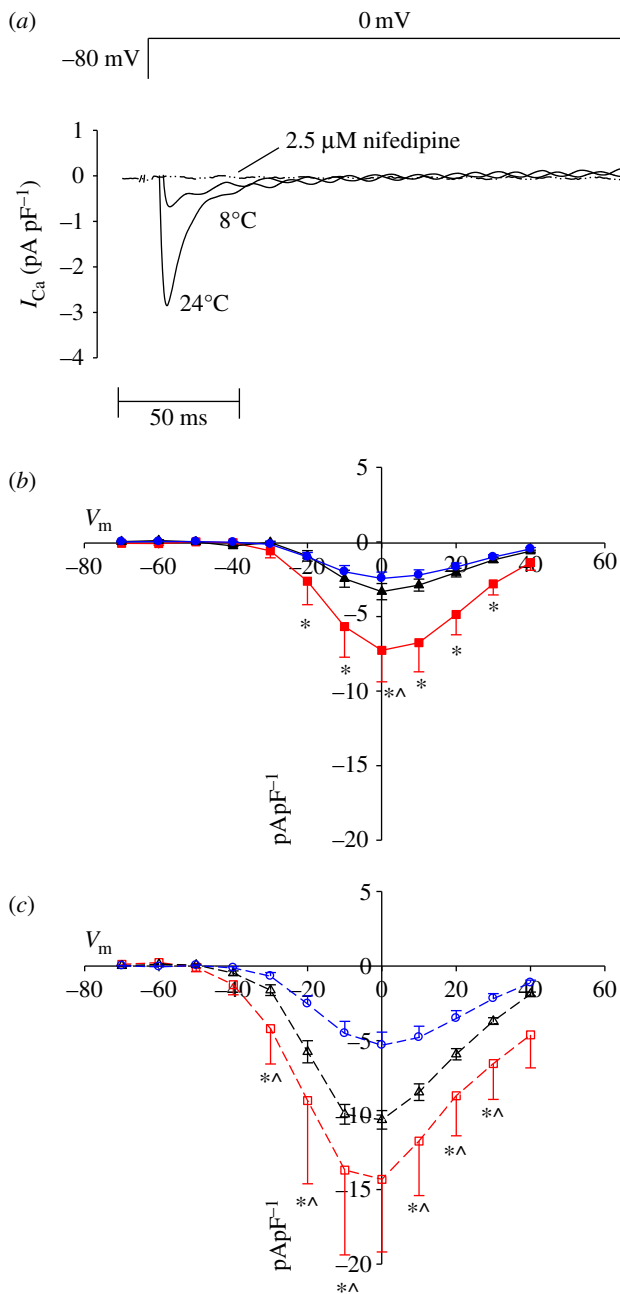


Figure 4. The effect of acute temperature change and adrenergic stimulation on the L-type Ca^{2+} channel current (I_{Ca}) in bluefin tuna ventricular myocytes. (a) Representative recording of I_{Ca} from a myocyte (C_m , 63.3 pF) acclimated to 14°C showing the effect of acute cooling from 24 to 8°C. Membrane voltage was depolarized from a holding potential of -80 to 0 mV for 500 ms as indicated. All I_{Ca} recordings were made in the presence of $0.5 \mu\text{M}$ TTX to inhibit Na^+ channels. The Ca^{2+} -channel inhibitor nifedipine was applied at $2.5 \mu\text{M}$ to confirm isolation of I_{Ca} . The effect of acute temperature change on the (b) I_{Ca} current–voltage relationship in the absence (filled symbols) and (c) presence (open symbols) of $1 \mu\text{M}$ isoprenaline. Blue circles, 8°C; black triangles, 19°C; red squares, 24°C. Means \pm s.e.m. are from $n = 4$ –8 myocytes from three fish. Asterisks indicate 24°C is significantly different from 8°C; circumflexes (^) indicate 19 and 8°C are different (two-way RM ANOVA, $p < 0.05$).

the AP plateau phase. We also show that acute warming leads to hyperpolarization of bluefin tuna ventricular myocyte RMP (electronic supplementary material, tables S1 and S2). This can be explained by the effect of acute temperature change on the inward-rectifier K^+ current (I_{K1}), the current that sets the RMP in the heart cell. Experiments by Galli *et al.* [21] on Pacific bluefin tuna demonstrate that acute warming from 14 to 19°C increases

I_{K1} density in ventricular myocytes, which increases K^+ efflux at rest and hyperpolarizes RMP. The combined effect of these two K^+ currents is that myocytes are more excitable (i.e. RMP is close to threshold for AP firing) and are depolarized for longer during acute cooling. This should offset the direct effects of cold temperatures on excitatory (depolarizing) currents and work to preserve cardiac function during descent into cold waters.

We also show frequency-dependent changes in APD at any given temperature (figure 2; electronic supplementary material, table S2). A reduction in APD with increased frequency has been reported previously for other fish including the yellowfin tuna [53] and the rainbow trout (*Oncorhynchus mykiss* [49]). The change in electrical restitution as heart rate changes is a common feature of vertebrate hearts and probably relates to the frequency dependence of K^+ fluxes as discussed above, as well as changes in I_{Ca} [49].

(b) Acute temperature change and Ca^{2+} dynamics

The prolonged APD at cold temperatures should provide partial compensation for the direct effect of cold on the ion fluxes that make up the AP. However, we show that $\Delta[\text{Ca}^{2+}]_i$ is reduced during cooling from 14 to 8°C (figure 4), which cannot be explained by Ca^{2+} influx on I_{Ca} (figure 5). The reduction in $\Delta[\text{Ca}^{2+}]_i$ may be due to a reduction in Ca^{2+} release from the intracellular stores of the SR. Release of SR Ca^{2+} is triggered by the rate and amplitude of I_{Ca} [41,43], and thus may fail at acutely cooler temperatures (figure 4). Earlier work by the authors has shown that tuna use SR Ca^{2+} stores for excitation–contraction coupling [41], so the reduction in $\Delta[\text{Ca}^{2+}]_i$ may be due to a reduction in Ca^{2+} -induced Ca^{2+} release. In rainbow trout atrial myocytes, warming increased the amplitude of I_{Ca} , but the very short APD resulted in an overall reduction in Ca^{2+} influx, which reduced $\Delta[\text{Ca}^{2+}]_i$ [47]. Whether this response could be altered via adrenergic stimulation at warm temperatures is not clear, but loss of adrenergic efficacy at warm temperatures in trout heart [17] suggests this is unlikely.

(c) Adrenaline, electrical activity and Ca^{2+} dynamics

AD is known to protect the fish heart function under conditions of stress [19,20], thus we hypothesized that it could play a role in alleviating the direct effect of temperature on the ion channels underlying cardiac excitation–contraction coupling. Adrenergic stimulation increased Ca^{2+} flux at all temperatures but was less effective at stimulating contractility in the cold. This finding from tuna is opposite to that observed in rainbow trout atrial myocytes where acute cooling increased the adrenergic sensitivity of I_{Ca} [17]. The mechanism underlying this difference is not known; however, it supports the idea that acute warming is stressful for many salmonid species, whereas acute cooling is stressful for many scombrids. We found that across all temperatures, when combined with physiologically realistic changes in contraction frequency, high but physiological (500 nm [20,38]) adrenergic stimulation stabilized $\Delta[\text{Ca}^{2+}]_i$ across a 20°C acute temperature change. We suggest increased Ca^{2+} influx on the I_{Ca} window current may be involved (see below), but future work should also examine the role of the SR in acute thermal adaptation.

We show AD prolongs APD in tuna ventricle, which is in agreement with recent studies using sharp electrode impalement of ventricle tissue of pink salmon (*Oncorhynchus gorbusha* [38]) and turtle (*Trachemys scripta elegans* [54]). The mechanism underlying the adrenergic prolongation of the

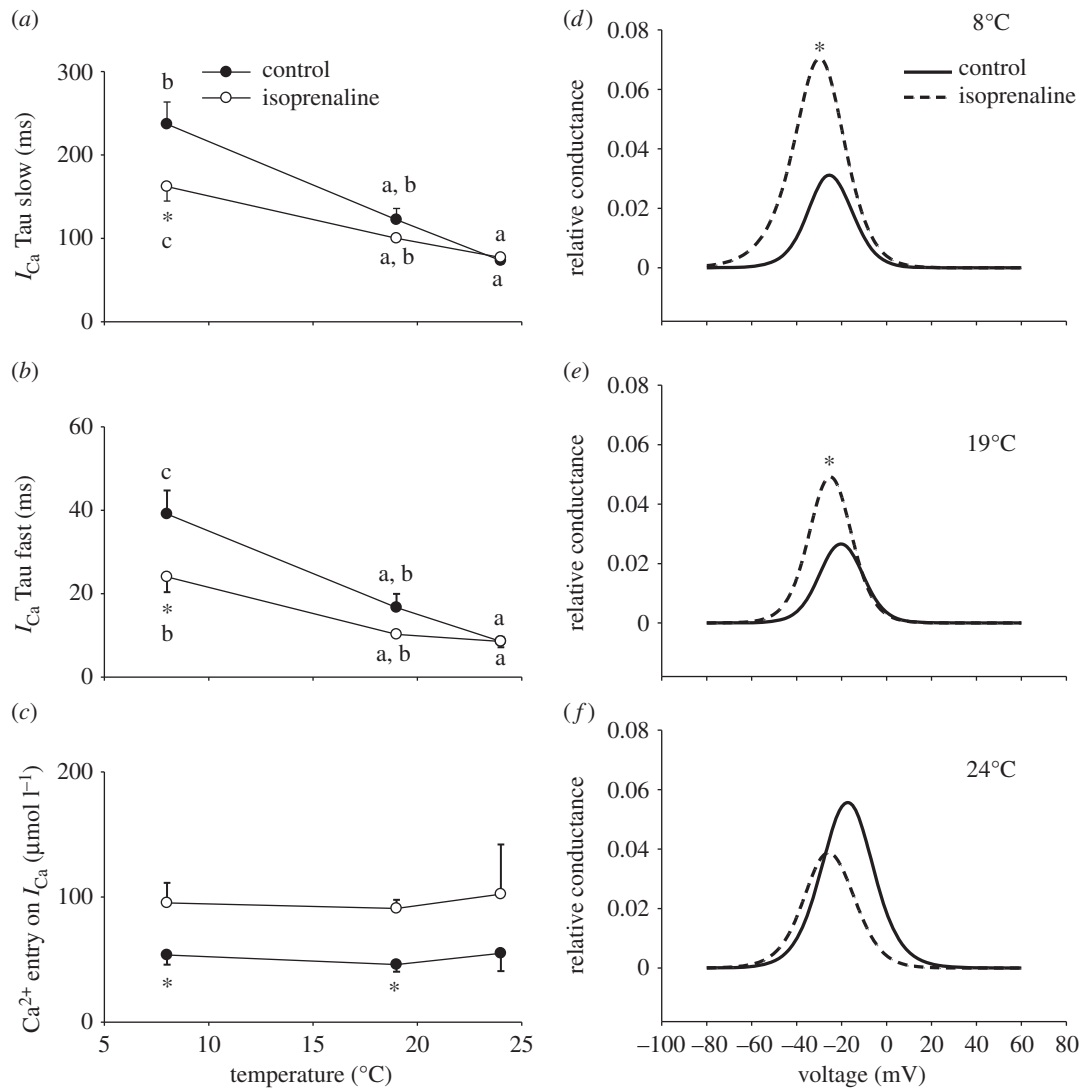


Figure 5. The effect of acute temperature change and adrenergic stimulation ($1 \mu\text{M}$ isoprenaline) on kinetics of I_{Ca} in ventricular myocytes from bluefin tuna. (a) The fast and (b) slow time constant of I_{Ca} inactivation; (c) Ca^{2+} influx on I_{Ca} expressed per litre of myofibrillar volume [45]; (d–f) I_{Ca} window current at each temperature, with and without adrenergic stimulation. The window current is the non-inactivating current that occurs during the AP plateau due to Ca^{2+} channels recovering from inactivation. These curves are a product of the Boltzmann fits to steady-state activation and steady-state inactivation parameters of I_{Ca} (see electronic supplementary material, text, figure S1 and table S3, for details). Means \pm s.e.m. are from $n = 4–8$ myocytes from three fish. Asterisks signify significant effect of adrenergic stimulation and dissimilar letters indicates a significant effect of temperature (two-way ANOVA, $p < 0.05$).

APD in fish (and reptiles) is unknown. In mammals, adrenergic stimulation can either increase or decrease APD [55–57]. The prolongation of APD with AD in mammals is often attributed to an increase in I_{Ca} and the greater influx of Ca^{2+} on the Ca^{2+} -window current [58]. We show AD increases I_{Ca} in bluefin tuna myocytes (figure 4) and increases the amplitude of the I_{Ca} window current (figure 5), especially at cold temperatures when the APD is significantly prolonged. Thus, we suggest that an increase in the window current may underlie the adrenergically induced APD prolongation in the current study. The concomitant increase in $\Delta[Ca^{2+}]_i$ supports this hypothesis, but further work is required to be definitive.

(d) Perspectives

Pacific bluefin tuna, like all members of the bluefin lineage, have expanded their niches vertically and geographically into the coldest waters experienced by members of the *Thunnus* genus. Cardiac function is maintained in cooler waters, and thus niche expansion of this species is realized in comparison with closely related tunas (i.e. yellowfin

tuna) [4]. The penetration into higher latitudes and the capacity to endure acute thermal challenges during dives [2] is clearly a function of the cardiac tolerances to both acute warming and acute cooling. In this study, we demonstrate that acute thermal modulation of contraction frequency, coupled with adrenergic stimulation, alters AP characteristics to maintain relatively constant Ca^{2+} cycling during rapid temperature change. These mechanistic strategies act synergistically to preserve cardiac function across temperatures and when combined with behavioural routines like bounce diving, may contribute to robust organismal performance across a large thermal niche. Comparative work with other scombrids, as well as species of freshwater fishes, is currently lacking. Such studies would help clarify whether these physiological traits are present in all fishes or specifically pronounced in the bluefin tuna lineage.

Ethical statement. Experimental procedures were conducted in accordance with Stanford University institutional animal use protocols.

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