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
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 Ca²⁺ (Δ[Ca²⁺]) via the process of excitation–contraction coupling. Excitation–contraction coupling links membrane depolarization by the action potential (AP) with the Ca²⁺ signal that causes contraction of the myofilaments. Acute temperature change in fish and mammals has direct (i.e. Q₁₀) 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 Ca²⁺ and develop force during excitation–contraction coupling are all known to be acutely temperature-sensitive (e.g. the L-type Ca²⁺ current (I₅ᵥ) [24,25], the ryanodine receptor [26–29], the SR Ca²⁺ ATPase (SERCA) [30–32], the Na⁺–Ca²⁺ 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 I₅ᵥ [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 Δ[Ca²⁺]i through augmentation of both I₅ᵥ 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 bouncing diving behavior (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 \( \Delta [\text{Ca}^{2+}] \), and the excitation profiles of AP and \( I_{\text{Ca}} \) in isolated myocytes. We show that acute thermal modulation of contraction frequency, coupled with adrenergic stimulation, modulates AP characteristics to maintain relatively constant \( \text{Ca}^{2+} \) 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 \( \text{Ca}^{2+} \) dynamics during a thermal challenge. These cellular cardiac adaptations allow the Pacific bluefin tuna heart to effectively drive contraction and relaxation across a 20\(^\circ\)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, \( T. \text{orientalis} \) (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 m\(^3\) circular tanks acclimated to 14\(^\circ\)C. Fish were held for at least eight weeks at constant temperature under a 12L:12D 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 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 \( \text{in vivo} \) heart rate, that juvenile Pacific bluefin experience during a dive. Based on archival tag data [5,40] and \( \text{in vivo} \) heart rate data from free-swimming tuna [10,13], we set our acute temperature change profile from 8 to 28\(^\circ\)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\(^\circ\)C and stimulated to contract at 0.5 Hz with a field stimulator for \( \text{Ca}^{2+} \) transients were determined for AP upstroke velocity and the integral of \( F \) transients were conducted with and without isoprenaline (1 \( \mu \text{M} \)), a synthetic analogue of AD (see the electronic supplementary material), over an acute temperature range of 8, 19 and 24\(^\circ\)C. Analyses of \( \Delta [\text{Ca}^{2+}] \) transients were conducted in detail in [41] and in the electronic supplementary material.

(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 MgCl\(_2\), 3.2 CaCl\(_2\), 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 MgCl\(_2\) 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 \( \Delta [\text{Ca}^{2+}] \), in ventricular myocytes has been described in detail previously [43] and summarized in the electronic supplementary material. Briefly, myocytes were loaded with 4 \( \mu \text{M} \) 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 \( \text{in vivo} \) temperature dependence [44]: 829 nM at 28\(^\circ\)C, 1594 at 14\(^\circ\)C and 1922 nM at 8 \( ^\circ\)C. Kinetic analyses of \( \Delta [\text{Ca}^{2+}] \) transients were described in detail in [41] and in the electronic supplementary material.

(f) L-type \( \text{Ca}^{2+} \) channel recordings
\( I_{\text{Ca}} \) is the major \( \text{Ca}^{2+} \) influx pathway during the AP plateau in fish myocytes and the major contributor to \( \Delta [\text{Ca}^{2+}] \). Consequently, we determined the thermal and adrenergic sensitivity of \( I_{\text{Ca}} \) 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 MgCl\(_2\), 5 Na\(_2\)-phosphocreatine, 10 HEPES and 0.025 EGTA with pH adjusted to 7.2 with CsOH. These studies were conducted with and without isoprenaline (1 \( \mu \text{M} \)), a synthetic analogue of AD (see the electronic supplementary material), over an acute temperature range of 8, 19 and 24\(^\circ\)C. Analyses of \( I_{\text{Ca}} \) kinetics and \( \text{Ca}^{2+} \) 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 ± 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 \( Q_{10} = (R_2/R_1)^{(T_2-T_1)/10} \), where
3. Results

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

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% ($Q_{10} \approx 3.2$), while acute cooling (from 14 to 8°C) prolonged it by approximately 50% ($Q_{10} \approx 2.3$) (figure 2b,c; 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 tachycardia (figure 2b,c; 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 ($Q_{10} \approx 1.9$; 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 $\Delta [Ca^{2+}]_i$ was reduced by both cooling and warming from the acclimation temperature of 14°C (figure 3b,c). Cooling slowed the rise and fall kinetics of $\Delta [Ca^{2+}]_i$, 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 $\Delta [Ca^{2+}]_i$, while simulating tachycardia at warm temperatures accelerated the decay of $\Delta [Ca^{2+}]_i$ (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 $\Delta [Ca^{2+}]_i$ 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 2c–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 2d, and AD Phys Hz in figure 2e), APD was further prolonged by approximately 55%. This increase in APD reduced the effect of acute temperature changes on $\Delta [Ca^{2+}]_i$, and resulted in a similar $\Delta [Ca^{2+}]_i$ amplitude at 8, 14 and 28°C (AD Phys Hz in figure 3d). Because $\Delta [Ca^{2+}]_i$ 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 $\Delta [Ca^{2+}]_i$, 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 4a and the mean values for fast and slow time constants ($\tau$) shown in figure 5a,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 5c). 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 5a,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 5c). One consequence of prolonging APD is that it provides enough time for Ca$^{2+}$ influx into the myocyte on $I_{Ca}$ across temperatures between 8 and 24°C. Because $I_{Ca}$ window current (figure 5d–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 5a,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
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 Ca²⁺ to enter the heart cell during the AP plateau. This reduces [Ca²⁺]_{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 Δ[Ca²⁺], and depresses contractility. However, in vivo, cold temperatures induce bradycardia, which we show here prolongs APD. This can increase Ca²⁺ influx on the I_{Ca} window current offering partial compensation of contractility in the cold. Under all conditions, AD improves myocyte contractility by increasing Ca²⁺ influx into the cell. The research demonstrates how cellular Ca²⁺ cycling could be preserved via electrical excitability and adrenergic stimulation when a tuna moves across a thermal

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 ± 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.
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 \( \Delta [Ca^{2+}] \) 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 \( (Q_{10} \sim 3.1) \). 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 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 \( \Delta([Ca^{2+}] \), (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 \mu m.

All data are means ± 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, \( p < 0.05. \)
inhibit Na\(^+\)I to 14 (2.5 \(\text{mM}\)) on the L-type Ca\(^2+\) channel. The effect of acute temperature change and adrenergic stimulation on the L-type Ca\(^2+\) channel current \(I_{\text{Ca}}\) in bluefin tuna ventricular myocytes is shown in Figure 4. 

Figure 4. The effect of acute temperature change and adrenergic stimulation on the L-type Ca\(^2+\) channel current \(I_{\text{Ca}}\) in bluefin tuna ventricular myocytes. (a) Representative recording of \(I_{\text{Ca}}\) from a myocyte (Cm, 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 \(I_{\text{Ca}}\) 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 \(I_{\text{Ca}}\). The effect of acute temperature change on the \(I_{\text{Ca}}\) current–voltage relationship in the absence (filled symbols) and presence (open symbols) of 1 \(\mu\text{M}\) isoprenaline. Blue circles, 8°C; black triangles, 19°C; red squares, 24°C. Means ± s.e.m. are from \(n = 4–8\) myocytes from three fish. Asterisks indicate 24°C is significantly different from 8°C; circumflexes (\(^\wedge\)) 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 \(I_{\text{K1}}\), 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 \(I_{\text{K1}}\) 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 \(I_{\text{Ca}}\) [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 ∆[Ca\(^{2+}\)], is reduced during cooling from 14 to 8°C (figure 4), which cannot be explained by Ca\(^{2+}\) influx on \(I_{\text{Ca}}\) (figure 5). The reduction in ∆[Ca\(^{2+}\)] 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 \(I_{\text{Ca}}\) [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 ∆[Ca\(^{2+}\)] may be due to a reduction in Ca\(^{2+}\)-induced Ca\(^{2+}\) release. In rainbow trout atrial myocytes, warming increased the amplitude of \(I_{\text{Ca}}\), but the very short APD resulted in an overall reduction in Ca\(^{2+}\) influx, which reduced ∆[Ca\(^{2+}\)], [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\(^{2+}\) influx 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 \(I_{\text{Ca}}\) [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 scrombrids. 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 ∆[Ca\(^{2+}\)] across a 20°C acute temperature change. We suggest increased Ca\(^{2+}\) influx on the \(I_{\text{Ca}}\) 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 gorbuscha [38]) and turtle (Trachemys scripta elegans [54]). The mechanism underlying the adrenergic prolongation of the
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$^{2+}$] 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.

**Acknowledgements.** We thank the staff of the Tuna Research and Conservation Center and the Monterey Bay Aquarium and the Captains and crew of the F/V Shogun. We thank Mr Charles Farwell, Alex Norton,
Louis Rodriguez, Jake Noguiera and Dr Tim Clark for their help collecting and maintaining the bluefin. We thank Prof. Stuart Thompson for experimental advice.

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