



## Research

**Cite this article:** Shiels HA, Galli GLJ, Block BA. 2015 Cardiac function in an endothermic fish: cellular mechanisms for overcoming acute thermal challenges during diving. *Proc. R. Soc. B* **282**: 20141989.  
<http://dx.doi.org/10.1098/rspb.2014.1989>

Received: 11 August 2014

Accepted: 3 December 2014

**Subject Areas:**

physiology, environmental science, behaviour

**Keywords:**

*Thunnus orientalis*, calcium transient, action potential, L-type Ca current, heart rate, adrenaline

**Author for correspondence:**

H. A. Shiels

e-mail: [holly.shiels@manchester.ac.uk](mailto:holly.shiels@manchester.ac.uk)

Electronic supplementary material is available at <http://dx.doi.org/10.1098/rspb.2014.1989> or via <http://rspb.royalsocietypublishing.org>.

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

H. A. Shiels<sup>1</sup>, G. L. J. Galli<sup>2</sup> and B. A. Block<sup>3</sup>

<sup>1</sup>Faculty of Life Sciences, and <sup>2</sup>Faculty of Medical and Human Sciences, The University of Manchester, Core Technology Facility, Grafton Street, Manchester M13 9PL, UK

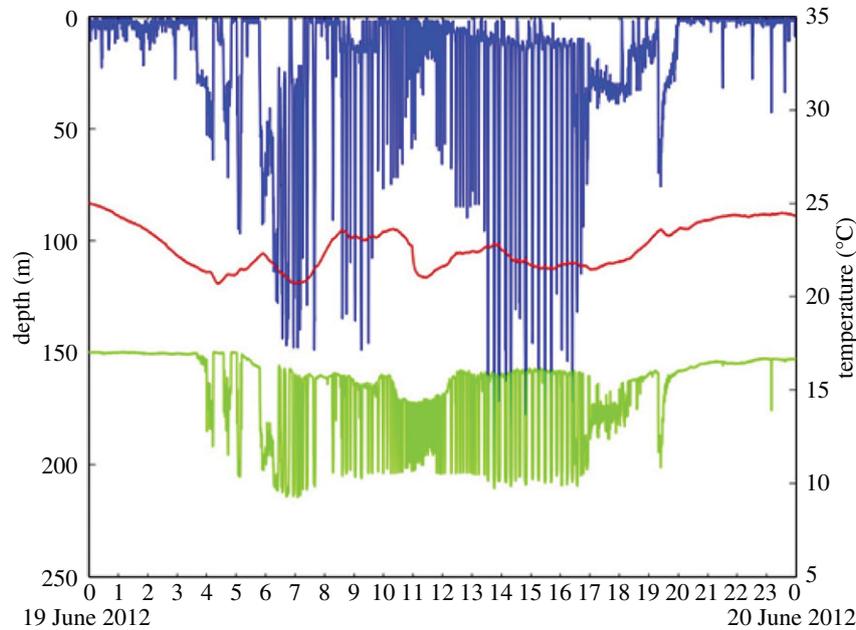
<sup>3</sup>Department of Biology, Tuna Research and Conservation Center, Stanford University, 120 Oceanview Boulevard, Pacific Grove, CA 93950, USA

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 Ca<sup>2+</sup> 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  $10^\circ\text{C}$  [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,  $\text{min}^{-1}$ ). 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  $\text{Ca}^{2+}$  ( $\Delta[\text{Ca}^{2+}]_i$ ) via the process of excitation–contraction coupling. Excitation–contraction coupling links membrane depolarization by the action potential (AP) with the  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  and develop force during excitation–contraction coupling are all known to be acutely temperature-sensitive (e.g. the L-type  $\text{Ca}^{2+}$  current ( $I_{\text{Ca}}$ ) [24,25], the ryanodine receptor [26–29], the SR  $\text{Ca}^{2+}$  ATPase (SERCA) [30–32], the  $\text{Na}^+$ – $\text{Ca}^{2+}$  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  $I_{\text{Ca}}$  [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  $\beta$ -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  $I_{\text{Ca}}$  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  $I_{\text{Ca}}$  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°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  $8.6 \pm 0.8$  kg, mean fork length  $76.1 \pm 2.3$  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<sup>3</sup> 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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  channel current ( $I_{\text{Ca}}$ ).

### (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 MgCl<sub>2</sub>, 3.2 CaCl<sub>2</sub>, 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<sub>2</sub> 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  $\mu\text{M}$  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 *in vivo* temperature dependence [44]: 829 nM at 28°C, 1594 at 14°C and 1922 nM at 8°C. Kinetic analyses of  $\text{Ca}^{2+}$  transients are 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  $\Delta[\text{Ca}^{2+}]_i$ . 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<sub>2</sub>, 5 Na<sub>2</sub>-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°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  $\pm$  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  $\text{Ca}^{2+}$  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  $\text{APD}_{50}$ ) 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  $\text{Ca}^{2+}$  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.

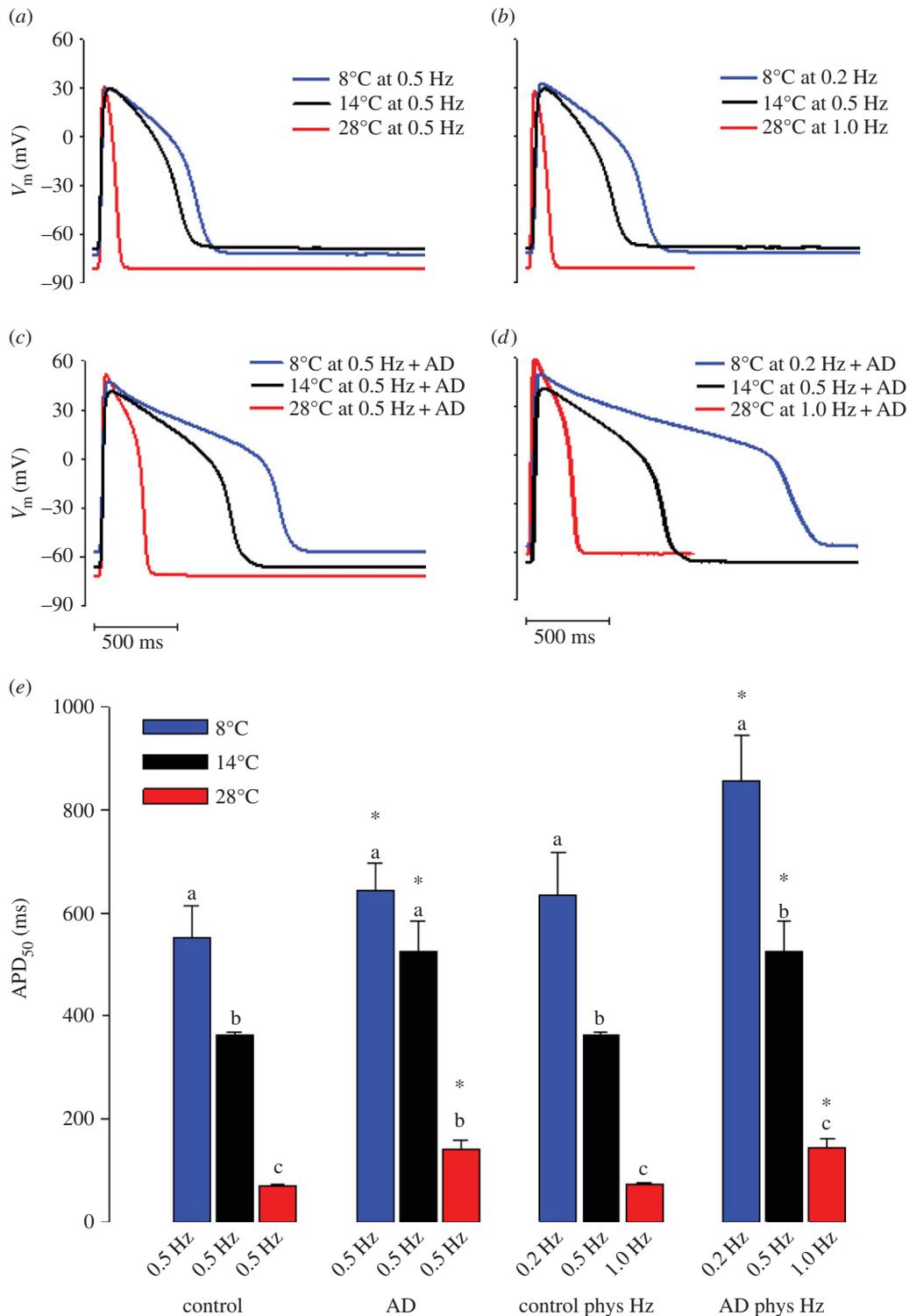
#### (c) Role of $I_{\text{Ca}}$ in acute sensitivity of excitation–contraction coupling in tuna ventricular myocytes

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  $I_{\text{Ca}}$  because it is the predominant  $\text{Ca}^{2+}$  influx pathway in the fish myocyte. There was an increase in the peak amplitude of  $I_{\text{Ca}}$  with acute warming and a decrease in peak amplitude with cooling across a range of membrane potentials (figure 4). The  $I_{\text{Ca}}$  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 ( $\tau$ ) shown in figure 5*a,b*. These temperature-dependent changes in inactivation kinetics resulted in fairly constant  $\text{Ca}^{2+}$  influx into the myocyte on  $I_{\text{Ca}}$  across temperatures between 8 and 24°C (figure 5*c*). This reinforces the importance of the stimulus waveform in generating  $\text{Ca}^{2+}$  influx (and see [27,47]). When the stimulus is 500 ms long and held at 0 mV, as in the  $I_{\text{Ca}}$  experiments,  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  dynamics, including those of  $I_{\text{Ca}}$  (figure 2) [22].

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

### 4. Discussion

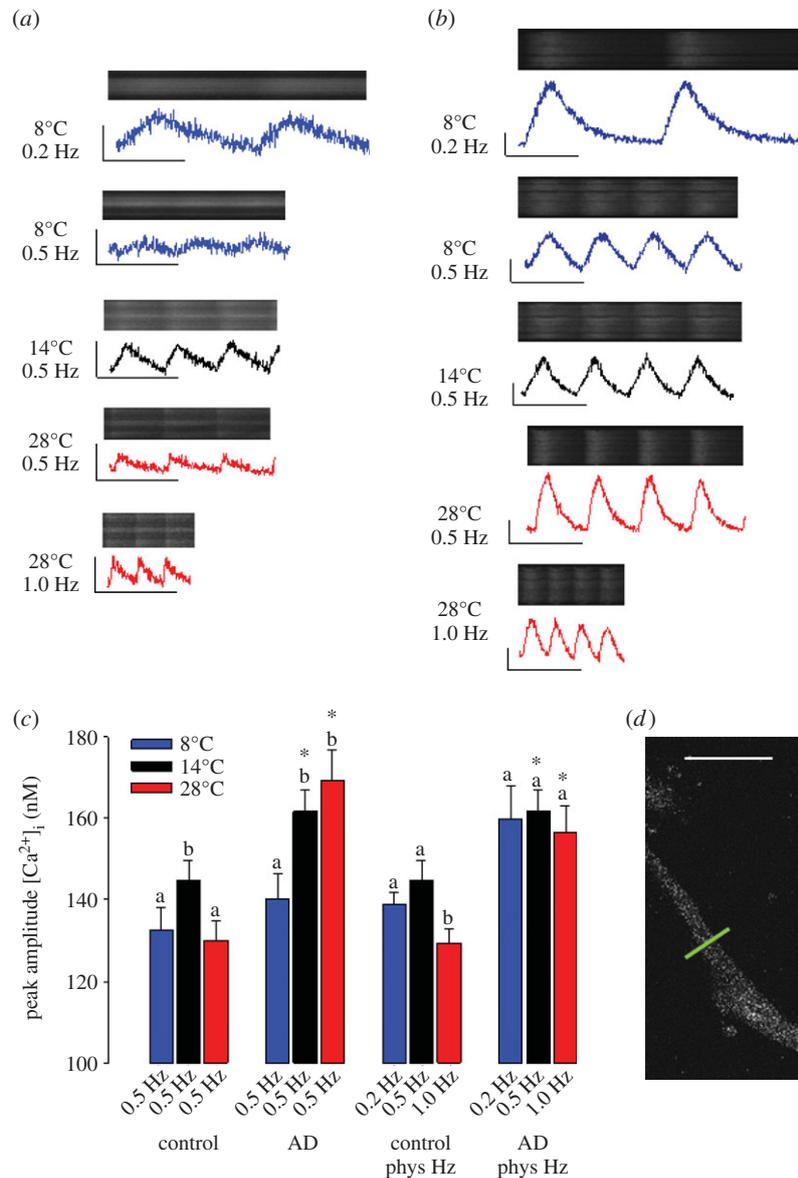
Vertebrate heart function [25,48], including that of tunas [14,32,41], is acutely temperature dependent. Thus, diving into cooler waters could be a challenge for cardiac function, especially given the unique arrangement of bluefin tunas having a warm core and a cardiac system at ambient water temperature. 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  $\text{Ca}^{2+}$  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  $I_{\text{Ca}}$ . 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  $\text{Ca}^{2+}$  influx on the  $I_{\text{Ca}}$  window current offering partial compensation of contractility in the cold. Under all conditions, AD improves myocyte contractility by increasing  $\text{Ca}^{2+}$  influx into the cell. The research demonstrates how cellular  $\text{Ca}^{2+}$  cycling could be preserved via electrical excitability and adrenergic stimulation when a tuna moves across a thermal



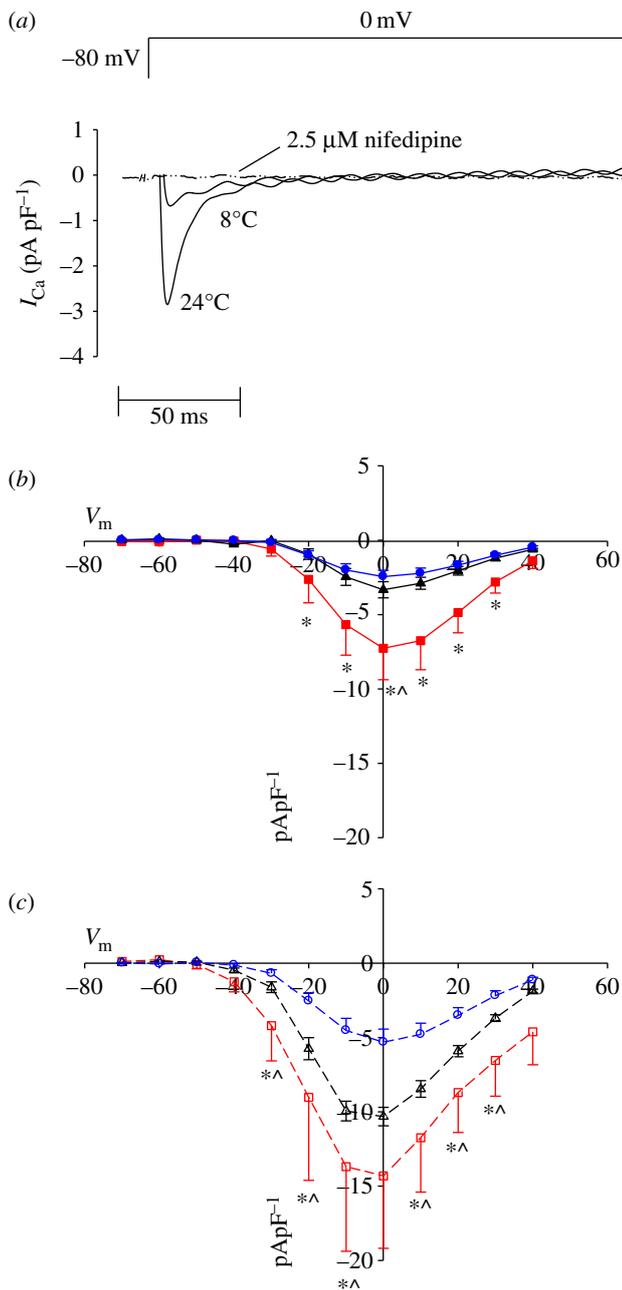
**Figure 3.** Spatial and temporal cellular  $Ca^{2+}$  flux in ventricular myocytes from Pacific bluefin tuna. (a) Representative raw line scan images (top) and corresponding time courses (bottom) showing the effect of acute temperature change and stimulation frequency on temporal and spatial characteristics 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  $\Delta[Ca^{2+}]_i$ . (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  $\mu$ m. 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  $\Delta[Ca^{2+}]_i$  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  $\text{Ca}^{2+}$  channel current ( $I_{\text{Ca}}$ ) in bluefin tuna ventricular myocytes. (a) Representative recording of  $I_{\text{Ca}}$  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  $I_{\text{Ca}}$  recordings were made in the presence of  $0.5 \mu\text{M}$  TTX to inhibit  $\text{Na}^+$ -channels. The  $\text{Ca}^{2+}$ -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 (b)  $I_{\text{Ca}}$  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  $\text{K}^+$  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  $\text{K}^+$  efflux at rest and hyperpolarizes RMP. The combined effect of these two  $\text{K}^+$  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  $\text{K}^+$  fluxes as discussed above, as well as changes in  $I_{\text{Ca}}$  [49].

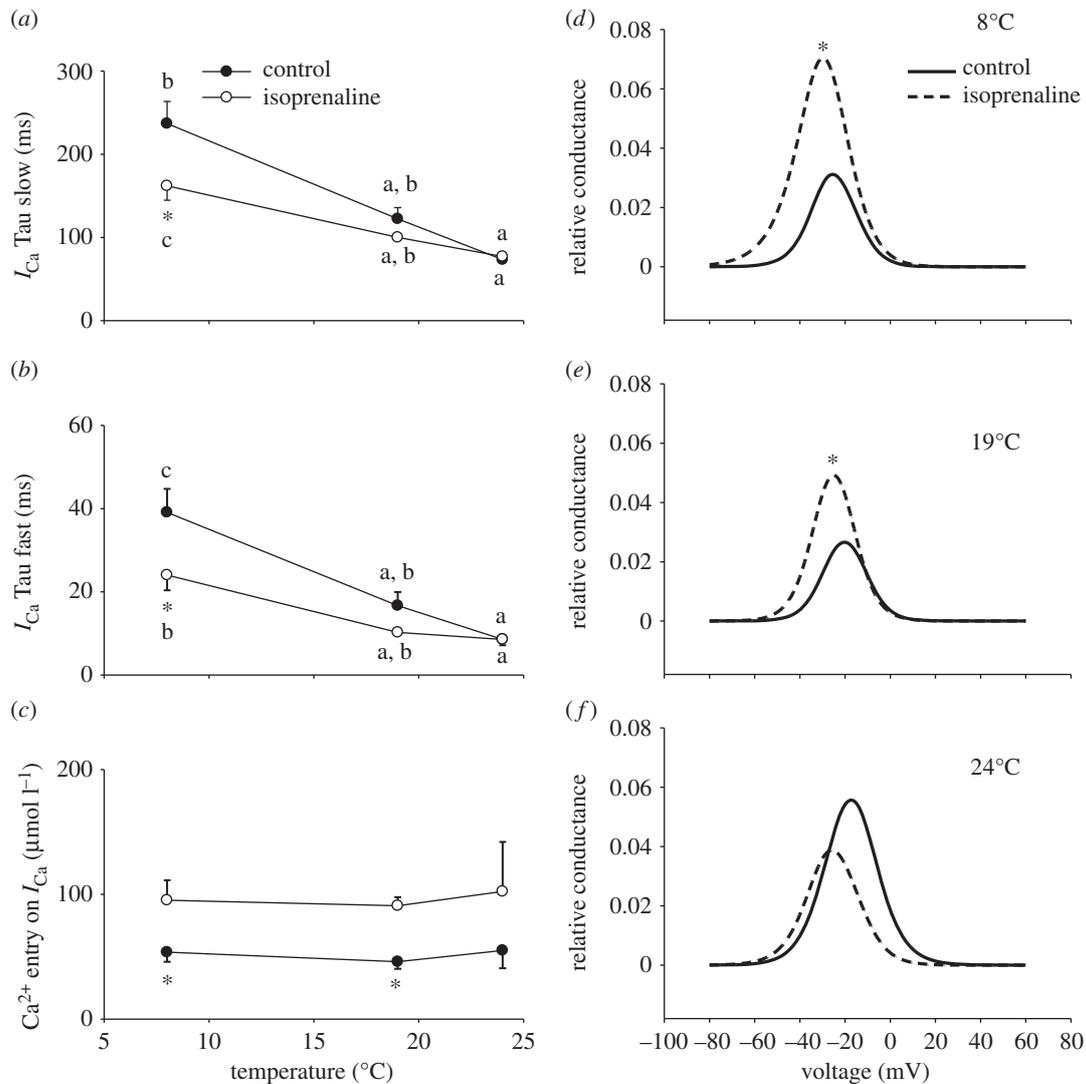
### (b) Acute temperature change and $\text{Ca}^{2+}$ dynamics

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  $\text{Ca}^{2+}$  influx on  $I_{\text{Ca}}$  (figure 5). The reduction in  $\Delta[\text{Ca}^{2+}]_i$  may be due to a reduction in  $\text{Ca}^{2+}$  release from the intracellular stores of the SR. Release of SR  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  stores for excitation–contraction coupling [41], so the reduction in  $\Delta[\text{Ca}^{2+}]_i$  may be due to a reduction in  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  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  $\text{Ca}^{2+}$  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 $\text{Ca}^{2+}$ 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  $\text{Ca}^{2+}$  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  $I_{\text{Ca}}$  [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 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  $\text{Ca}^{2+}$  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 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.

**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 Rodrigue, Jake Noguiera and Dr Tim Clark for their help collecting and maintaining the bluefin. We thank Prof. Stuart Thompson for experimental advice.

**Funding statement.** This study was supported by a grant from the National Oceanic and Atmospheric Administration grants to BA.B., and the Monterey Bay Aquarium Foundation.

## References

- Marcinek DJ, Blackwell SB, Dewar H, Freund EV, Farwell C, Dau D, Seitz AC, Block BA. 2001 Depth and muscle temperature of Pacific bluefin tuna examined with acoustic and pop-up satellite archival tags. *Mar. Biol.* **138**, 869–885. (doi:10.1007/s002270000492)
- Lawson GL, Castleton MR, Block BA. 2010 Movements and diving behavior of Atlantic bluefin tuna *Thunnus thynnus* in relation to water column structure in the northwestern Atlantic. *Mar. Ecol. Prog. Ser.* **400**, 245–265. (doi:10.3354/Meps08394)
- Kitagawa T, Boustany AM, Farwell CJ, Williams TD, Castleton MR, Block BA. 2007 Horizontal and vertical movements of juvenile bluefin tuna (*Thunnus orientalis*) in relation to seasons and oceanographic conditions in the eastern Pacific Ocean. *Fish Oceanogr.* **16**, 409–421. (doi:10.1111/j.1365-2419.2007.00441.x)
- Block BA *et al.* 2011 Tracking apex marine predator movements in a dynamic ocean. *Nature* **475**, 86–90. (doi:10.1038/nature10082)
- Boustany AM, Matteson R, Castleton M, Farwell C, Block BA. 2010 Movements of Pacific bluefin tuna (*Thunnus orientalis*) in the Eastern North Pacific revealed with archival tags. *Prog. Oceanogr.* **86**, 94–104. (doi:10.1016/j.pcean.2010.04.015)
- Schaefer KF, Block B. 2007 Movements, behavior, and habitat utilization of yellowfin tuna (*Thunnus albacares*) in the northeastern Pacific Ocean, ascertained through archival tag data. *Mar. Biol.* **152**, 503–525. (doi:10.1007/s00227-007-0689-x)
- Graham JB, Dickson KA. 2004 Tuna comparative physiology. *J. Exp. Biol.* **207**, 4015–4024. (doi:10.1242/jeb.01267)
- Linthicum DS, Carey FG. 1972 Regulation of brain and eye temperatures by the bluefin tuna. *Comp. Biochem. Physiol. A* **43**, 425–433. (doi:10.1016/0300-9629(72)90201-0)
- Brill RW, Bushnell PG, Block BA, Stevens ED. 2001 The cardiovascular system of tunas. In *Tunas: physiology, ecology and evolution* (eds WS Hoar, DJ Randall, AP Farrell), pp. 79–120. San Diego, CA: Academic Press.
- Clark TD, Taylor BD, Seymour RS, Ellis D, Buchanan J, Fitzgibbon QP, Frappell PB. 2008 Moving with the beat: heart rate and visceral temperature of free-swimming and feeding bluefin tuna. *Proc. R. Soc. B* **275**, 2841–2850. (doi:10.1098/rspb.2008.0743)
- Walli A, Teo SLH, Boustany A, Farwell CJ, Williams T, Dewar H, Prince E, Block BA. 2009 Seasonal movements, aggregations and diving behavior of Atlantic bluefin tuna (*Thunnus thynnus*) revealed with archival tags. *PLoS ONE* **4**, e0006151. (doi:10.1371/journal.pone.0006151)
- Blank JM, Morrisette JM, Farwell CJ, Price M, Schallert RJ, Block BA. 2007 Temperature effects on metabolic rate of juvenile Pacific bluefin tuna *Thunnus orientalis*. *J. Exp. Biol.* **210**, 4254–4261. (doi:10.1242/jeb.005835)
- Clark TD, Farwell CJ, Rodriguez LE, Brandt WT, Block BA. 2013 Heart rate responses to temperature in free-swimming Pacific bluefin tuna (*Thunnus orientalis*). *J. Exp. Biol.* **216**, 3208–3214. (doi:10.1242/jeb.086546)
- Blank JM, Morrisette JM, Landeira-Fernandez AM, Blackwell SB, Williams TD, Block BA. 2004 In situ cardiac performance of Pacific bluefin tuna hearts in response to acute temperature change. *J. Exp. Biol.* **207**, 881–890. (doi:10.1242/jeb.00820)
- Korsmeyer KE, Lai NC, Shadwick RE, Graham JB. 1997 Heart rate and stroke volume contribution to cardiac output in swimming yellowfin tuna: response to exercise and temperature. *J. Exp. Biol.* **200**, 1975–1986.
- Galli Gina LJ, Shiels Holly A, Brill Richard W. 2009 Temperature sensitivity of cardiac function in pelagic fishes with different vertical mobilities: yellowfin tuna (*Thunnus albacares*), bigeye tuna (*Thunnus obesus*), mahimahi (*Coryphaena hippurus*), and swordfish (*Xiphias gladius*). *Physiol. Biochem. Zool.* **82**, 280–290. (doi:10.1086/597484)
- Shiels HA, Vornanen M, Farrell AP. 2003 Acute temperature change modulates the response of  $I_{Ca}$  to adrenergic stimulation in fish cardiomyocytes. *Physiol. Biochem. Zool.* **76**, 816–824. (doi:10.1086/378918)
- Keen JE, Viazon DM, Farrell AP, Tibbits GF. 1993 Thermal acclimation alters both adrenergic sensitivity and adrenoceptor density in cardiac tissue of rainbow trout. *J. Exp. Biol.* **181**, 27–47.
- Farrell AP, MacLeod KR, Chancey B. 1986 Intrinsic mechanical properties of the perfused rainbow trout heart and the effects of catecholamines and extracellular calcium under control and acidotic conditions. *J. Exp. Biol.* **125**, 319–345.
- Farrell AP, Milligan CL. 1986 Myocardial intracellular pH in a perfused rainbow trout heart during extracellular acidosis in the presence and absence of adrenaline. *J. Exp. Biol.* **125**, 347–359.
- Galli GLJ, Lipnick MS, Block BA. 2009 Effect of thermal acclimation on action potentials and sarcolemmal  $K^+$  channels from Pacific bluefin tuna cardiomyocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R502–R509. (doi:10.1152/ajpregu.90810.2008)
- Shiels HA, Vornanen M, Farrell AP. 2000 Temperature-dependence of L-type  $Ca^{2+}$  channel current in atrial myocytes from rainbow trout. *J. Exp. Biol.* **203**, 2771–2780.
- Haverinen J, Vornanen M. 2009 Responses of action potential and  $K^+$  currents to temperature acclimation in fish hearts: phylogeny or thermal preferences? *Physiol. Biochem. Zool.* **82**, 468–482. (doi:10.1086/590223)
- Galli GLJ, Lipnick MS, Shiels HA, Block BA. 2011 Temperature effects on  $Ca^{2+}$  cycling in scombrid cardiomyocytes: a phylogenetic comparison. *J. Exp. Biol.* **214**, 1068–1076. (doi:10.1242/jeb.048231)
- Herve JC, Yamaoka K, Twist VW, Powell T, Ellory JC, Wang LC. 1992 Temperature dependence of electrophysiological properties of guinea pig and ground squirrel myocytes. *Am. J. Physiol.* **263**, 177–184.
- Hove-Madsen L, Llach A, Tort L. 2001 The function of the sarcoplasmic reticulum is not inhibited by low temperatures in trout atrial myocytes. *Am. J. Physiol.* **281**, R1902–R1906.
- Shiels HA, Vornanen M, Farrell AP. 2002 Temperature dependence of cardiac sarcoplasmic reticulum function in rainbow trout myocytes. *J. Exp. Biol.* **205**, 3631–3639.
- Sitsapesan R, Montgomery RAP, Macleod KT, Williams AJ. 1991 Sheep Cardiac sarcoplasmic-reticulum calcium-release channels: modification of conductance and gating by temperature. *J. Physiol.* **434**, 469–488.
- Korajoki H, Vornanen M. 2013 Temperature dependence of sarco(endo)plasmic reticulum  $Ca^{2+}$ -ATPase expression in fish hearts. *J. Comp. Physiol. B* **183**, 467–476. (doi:10.1007/s00360-012-0724-1).
- Korajoki H, Vornanen M. 2012 Expression of SERCA and phospholamban in rainbow trout (*Oncorhynchus mykiss*) heart: comparison of atrial and ventricular tissue and effects of thermal acclimation. *J. Exp. Biol.* **215**, 1162–1169. (doi:10.1242/jeb.065102)
- Aho E, Vornanen M. 1998  $Ca^{2+}$ -ATPase activity and  $Ca^{2+}$  uptake by sarcoplasmic reticulum in fish heart: effects of thermal acclimation. *J. Exp. Biol.* **201**, 525–532.
- Landeira-Fernandez A, Morrisette JM, Blank JM, Block BA. 2004 Temperature dependence of  $Ca^{2+}$ -ATPase (SERCA2) in the ventricles of tuna and mackerel. *Am. J. Physiol.* **286**, R398–R404. (doi:10.1152/ajpregu.00392.2003)
- Shiels HA. 2006 Sarcolemmal ion currents and sarcoplasmic reticulum  $Ca^{2+}$  content in ventricular myocytes from the cold stenothermic fish, the burbot (*Lota lota*). *J. Exp. Biol.* **209**, 3091–3100. (doi:10.1242/jeb.02321)
- Llach A, Tibbits GF, Sedarat F, Tort L, Hove-Madsen L. 2001 Low temperature reduces  $Na^+$ - $Ca^{2+}$  exchange rate but not SR  $Ca^{2+}$  release in trout atrial myocytes. *Biophys. J.* **80**, 585A.

35. Gillis TE. 2012 Evolution of the regulatory control of the vertebrate heart: the role of the contractile proteins. In *Ontogeny and phylogeny of the vertebrate heart* (ed. DST Wang), pp. 125–145. Heidelberg, Germany: Springer.
36. Gillis TE, Marshall CR, Xue XH, Borgford TJ, Tibbits GF. 2000 Ca<sup>2+</sup> binding to cardiac troponin C: effects of temperature and pH on mammalian and salmonid isoforms. *Am. J. Physiol.* **279**, R1707–R1715.
37. El-Armouche A, Eschenhagen T. 2009 Beta-adrenergic stimulation and myocardial function in the failing heart. *Heart Fail. Rev.* **14**, 225–241. (doi:10.1007/s10741-008-9132-8)
38. Ballesta S, Hanson LM, Farrell AP. 2012 The effect of adrenaline on the temperature dependency of cardiac action potentials in pink salmon *Oncorhynchus gorbuscha*. *J. Fish Biol.* **80**, 876–885. (doi:10.1111/j.1095-8649.2011.03187.x)
39. Bovo E, Dvornikov AV, Mazurek SR, de Tombe PP, Zima AV. 2013 Mechanisms of Ca handling in zebrafish ventricular myocytes. *Pflugers Arch.* (doi:10.1007/s00424-013-1312-2)
40. Kitagawa T, Kimura S, Nakata H, Yamada H. 2007 Why do young Pacific bluefin tuna repeatedly dive to depths through the thermocline? *Fish. Sci.* **73**, 98–106. (doi:10.1111/j.1444-2906.2007.01307.x)
41. Shiels HA, Di Maio A, Thompson S, Block BA. 2011 Warm fish with cold hearts: thermal plasticity of excitation–contraction coupling in bluefin tuna. *Proc. R. Soc. B* **278**, 18–27. (doi:10.1098/rspb.2010.1274)
42. Farwell CJ, Block BA, Stevens ED. 2001 Tunas in captivity. In *Tunas: physiology, ecology and evolution* (eds WS Hoar, DJ Randall, AP Farrell), p. 391. San Diego, CA: Academic Press.
43. Shiels HA, Blank JM, Farrell AP, Block BA. 2004 Electrophysiological properties of the L-type Ca<sup>2+</sup> current in cardiomyocytes from bluefin tuna and Pacific mackerel. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **286**, R659–R668. (doi:10.1152/ajpregu.00521.2003)
44. Woodruff ML, Sampath AP, Matthews HR, Krasnoperova NV, Lem J, Fain GL. 2002 Measurement of cytoplasmic calcium concentration in the rods of wild-type and transducin knock-out mice. *J. Physiol.* **542**, 843–854. (doi:10.1113/jphysiol.2001.013987)
45. Vornanen M. 1997 Sarcolemmal Ca influx through L-type Ca channels in ventricular myocytes of a teleost fish. *Am. J. Physiol.* **41**, R1432–R1440.
46. Yue DT. 1987 Intracellular [Ca<sup>2+</sup>] related to rate of force development in twitch contraction of heart. *Am. J. Physiol.* **252**, H760–H770.
47. Shiels HA, Vornanen M, Farrell AP. 2002 Effects of temperature on intracellular [Ca<sup>2+</sup>] in trout atrial myocytes. *J. Exp. Biol.* **205**, 3641–3650.
48. Puglisi JL, Yuan WL, Bassani JWM, Bers DM. 1999 Ca<sup>2+</sup> influx through Ca<sup>2+</sup> channels in rabbit ventricular myocytes during action potential clamp: influence of temperature. *Circ. Res.* **85**, E7–E16. (doi:10.1161/01.RES.85.6.e7)
49. Harwood CL, Howarth FC, Altringham JD, White E. 2000 Rate-dependent changes in cell shortening; intracellular Ca<sup>2+</sup> levels and membrane potential in single isolated rainbow trout (*Oncorhynchus mykiss*) ventricular myocytes. *J. Exp. Biol.* **203**, 493–504.
50. Vornanen M, Rykkyne A, Nurmi A. 2002 Temperature-dependent expression of sarcolemmal K<sup>+</sup> currents in rainbow trout atrial and ventricular myocytes. *Am. J. Physiol.* **282**, R1191–R1199. (doi:10.1152/ajpcell.00475.2001)
51. Wang SQ, Cao HM, Zhou ZQ. 1997 Temperature dependence of the myocardial excitability of ground squirrel and rat. *J. Therm. Biol.* **22**, 195–199. (doi:10.1016/S0306-4565(97)00010-7)
52. Hassinen M, Haverinen J, Vornanen M. 2008 Electrophysiological properties and expression of the delayed rectifier potassium (ERG) channels in the heart of thermally acclimated rainbow trout. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **295**, R297–R308. (doi:10.1152/ajpregu.00612.2007)
53. Patrick SM, White E, Brill RW, Shiels HA. 2011 The effect of stimulation frequency on the transmural ventricular monophasic action potential in yellowfin tuna *Thunnus albacares*. *J. Fish Biol.* **78**, 651–658. (doi:10.1111/j.1095-8649.2010.02869.x)
54. Nielsen JS, Gesser H. 2001 Effects of high extracellular [K<sup>+</sup>] and adrenaline on force development, relaxation and membrane potential in cardiac muscle from freshwater turtle and rainbow trout. *J. Exp. Biol.* **204**, 261–268.
55. Boyett MR. 1978 An analysis of the effect of the rate of stimulation and adrenaline on the duration of the cardiac action potential. *Pflugers Arch.* **377**, 155–166. (doi:10.1007/BF00582846)
56. Taggart P, Sutton P, Chalabi Z, Boyett MR, Simon R, Elliott D, Gill JS. 2003 Effect of adrenergic stimulation on action potential duration restitution in humans. *Circulation* **107**, 285–289. (doi:10.1161/01.CIR.0000044941.13346.74)
57. Quadbeck J, Reiter M. 1975 Cardiac action potential and inotropic effect of noradrenaline and calcium. *Naunyn Schmiedebergs Arch. Pharmacol.* **286**, 337–351. (doi:10.1007/BF00506649)
58. Veldkamp MW, Verkerk AO, van Ginneken AC, Baartscheer A, Schumacher C, de Jonge N, de Bakker JM, Opthof T. 2001 Norepinephrine induces action potential prolongation and early after depolarizations in ventricular myocytes isolated from human end-stage failing hearts. *Eur. Heart J.* **22**, 955–963. (doi:10.1053/euhj.2000.2499)