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["Data Supplement"](#)

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# Warm fish with cold hearts: thermal plasticity of excitation–contraction coupling in bluefin tuna

H. A. Shiels<sup>1,2,\*</sup>, A. Di Maio<sup>2,†</sup>, S. Thompson<sup>2</sup> and B. A. Block<sup>2</sup>

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

<sup>2</sup>Tuna Research and Conservation Center, Hopkins Marine Station of Stanford University, 120 Oceanview Boulevard, Pacific Grove, CA 93950, USA

Bluefin tuna have a unique physiology. Elevated metabolic rates coupled with heat exchangers enable bluefin tunas to conserve heat in their locomotory muscle, viscera, eyes and brain, yet their hearts operate at ambient water temperature. This arrangement of a warm fish with a cold heart is unique among vertebrates and can result in a reduction in cardiac function in the cold despite the elevated metabolic demands of endothermic tissues. In this study, we used laser scanning confocal microscopy and electron microscopy to investigate how acute and chronic temperature change affects tuna cardiac function. We examined the temporal and spatial properties of the intracellular  $\text{Ca}^{2+}$  transient ( $\Delta[\text{Ca}^{2+}]_i$ ) in Pacific bluefin tuna (*Thunnus orientalis*) ventricular myocytes at the acclimation temperatures of 14°C and 24°C and at a common test temperature of 19°C. Acute (less than 5 min) warming and cooling accelerated and slowed the kinetics of  $\Delta[\text{Ca}^{2+}]_i$ , indicating that temperature change limits cardiac myocyte performance. Importantly, we show that thermal acclimation offered partial compensation for these direct effects of temperature. Prolonged cold exposure (more than four weeks) increased the amplitude and kinetics of  $\Delta[\text{Ca}^{2+}]_i$  by increasing intracellular  $\text{Ca}^{2+}$  cycling through the sarcoplasmic reticulum (SR). These functional findings are supported by electron microscopy, which revealed a greater volume fraction of ventricular SR in cold-acclimated tuna myocytes. The results indicate that SR function is crucial to the performance of the bluefin tuna heart in the cold. We suggest that SR  $\text{Ca}^{2+}$  cycling is the malleable unit of cellular  $\text{Ca}^{2+}$  flux, offering a mechanism for thermal plasticity in fish hearts. These findings have implications beyond endothermic fish and may help to delineate the key steps required to protect vertebrate cardiac function in the cold.

**Keywords:** calcium transient; thermal acclimation; cardiomyocyte; sarcoplasmic reticulum; mitochondria

## 1. INTRODUCTION

Pacific bluefin tuna, *Thunnus orientalis*, range across the entire north Pacific Ocean as juveniles. Electronic tagging studies show juvenile Pacific bluefin sojourn into the cool productive waters of the California Current late in year one and occupy this region for several years [1]. While in the eastern Pacific, the bluefin move annually along the North American coast between Baja and California [2], where they experience sea surface temperatures between 11°C and 24°C [1]. Juvenile bluefin tuna can dive to depths of 500 m in search of prey, encountering colder temperatures (5–6°C) and experiencing acute temperature changes of more than 10°C [2]. These data indicate that Pacific bluefin tuna are capable of expansive migrations into northern latitudes and have a thermal tolerance that ranges from 5°C to 24°C.

A unique endothermic physiology contributes to the expansive thermal niche of bluefin tunas. Bluefins have

elevated metabolic rates, defend a thermal neutral zone [3] and conserve metabolic heat with vascular counter-current heat exchangers located in viscera, brain, eyes and muscles (e.g. [4]). The tuna heart is not supplied with a circulatory counter-current heat exchanger and receives coronary blood from the gills at ambient water temperature [5]. Thus, bluefin tunas have the unique physiological arrangement of a heart at ambient water temperature supplying blood to warm endothermic tissues. The thermal sensitivity of the tuna heart may therefore limit the niche occupied by tunas [6]. Indeed, cooling causes bradycardia and an associated reduction in cardiac output in bluefin and yellowfin (*T. albacares*) tunas [7,8]. However, the Pacific bluefin tuna heart generates greater contractile force and maintains cardiac rhythm at colder temperatures than its tropical sister taxa [7,9]. Understanding cardiac thermal sensitivity in the tunas may help to delineate the key steps that protect cardiac function in the cold. We hypothesize that cold tolerance in bluefin tunas is directly related to increased use of the sarcoplasmic reticulum (SR) during excitation–contraction (e–c) coupling in the cardiac myocytes that make up the heart.

Cardiac e–c coupling underlies myocyte contraction and relaxation by controlling the cycling of cellular

\* Author for correspondence ([holly.shiels@manchester.ac.uk](mailto:holly.shiels@manchester.ac.uk)).

† Present address: Department of Molecular, Cell and Developmental Biology, Life Science Building (Room 1203), University of California Santa Barbara, CA 93101, USA.

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$Ca^{2+}$ . The general schema for e–c coupling in fishes differs from that of mammals primarily with regard to the SR (see [10] for a review of e–c coupling in the fish heart). Most fishes do not use the internal  $Ca^{2+}$  stores of the SR for contraction and relaxation, rather,  $Ca^{2+}$  is cycled back and forth across the myocyte (sarcolemmal) membrane with each beat [10]. However, isolated tissue studies have revealed that elevated cardiac performance in fishes is associated with increased use of SR  $Ca^{2+}$  cycling [9,11–16]. Biochemical studies with isolated SR vesicles clearly show that bluefin tuna have more SR  $Ca^{2+}$  ATPase (SERCA) than their warmer sister taxa [17,18] and structural studies demonstrate that an extensive SR is present in bluefin tuna hearts [19]. However, to date, only one study [20] has directly investigated the physiological role of SR  $Ca^{2+}$  during e–c coupling in fish heart, and this study did not examine the effect of temperature.

Temperature affects both the rate and strength of contraction and relaxation of cardiac myocytes. Temperature has direct (i.e.  $Q_{10}$ ) effects on the ion channels and pumps that underlie the cardiac action potential [21,22] and that cycle  $Ca^{2+}$  during e–c coupling (e.g.  $I_{Ca}$ : [22,23,24]; ryanodine receptor: [25–27]; SERCA: [17,18,26];  $Na^+$ – $Ca^{2+}$  exchanger: [28]). In an attempt to compensate for the direct effects of temperature on e–c coupling pathways, some animals adjust their phenotype during chronic temperature exposure. For example, in freshwater fishes, cold acclimation (CA) can induce ventricular hypertrophy [29], modify  $K^+$  channel conductance and reduce action potential duration, which enables higher heart rates [21,30]. Importantly, SR function can also increase in the cold. Isolated tissue studies show that CA increases the ryanodine-sensitivity of cardiac isometric force production in rainbow trout, *Oncorhynchus mykiss* [12,14,31]. Biochemical studies indicate that CA increases the activity of the SERCA pump in rainbow trout [11] and morphometric analysis has indicated increased volume of SR following CA in perch (*Perca fluviatilis* [32]).

In this study, we use laser-scanning confocal microscopy to investigate the effect of acute and chronic temperature change on the temporal and spatial properties of the intracellular  $Ca^{2+}$  transient ( $\Delta[Ca^{2+}]_i$ ) in Pacific bluefin tuna ventricular myocytes. We show, to our knowledge, the first physiological evidence for  $Ca^{2+}$ -induced  $Ca^{2+}$ -release [33] during e–c coupling in the tuna heart underscoring the importance of the SR for tuna cardiac performance. We show that acute (less than 5 min) cooling limits cellular  $Ca^{2+}$  cycling but that chronic cold (greater than 4 weeks) invokes a remodelling of cellular  $Ca^{2+}$  flux that offers partial compensation. We provide, to our knowledge, the first functional evidence that enhanced SR  $Ca^{2+}$  cycling underlies the cardiac remodelling associated with cold-tolerance in an endothermic fish. We support these functional findings with electron microscopy and stereology results indicating cold-induced increases in SR volume.

## 2. MATERIAL AND METHODS

### (a) Fish origin and care

Pacific bluefin tuna, *T. orientalis* (mean mass:  $11.1 \pm 0.1$  kg, mean fork length:  $84.5 \pm 2.4$  cm,  $n = 10$ , males and females) were captured from the wild and held as previously described

[22]. Fish were acclimated for at least four weeks and up to eight weeks in two identical 109 m<sup>3</sup> circular tanks; one tank at 24°C (warm acclimated, WA) and one at 14°C (CA) under a 12 L : 12 D photoperiod.

### (b) Myocyte isolation

Myocytes from bluefin tuna were isolated as described previously [22,34] and explained briefly in the electronic supplementary material.

### (c) Confocal imaging

For confocal imaging of  $\Delta[Ca^{2+}]_i$ , ventricular myocytes were loaded with 4  $\mu$ M Fluo-4-AM for 20 min at room temperature. Myocytes were washed by dilution, allowed 30 min for de-esterification and then transferred to a temperature regulated recording chamber on the stage of an Olympus Fluoview confocal microscope. The extracellular solution perfusing the myocytes was precisely controlled with an in-line temperature controller. Myocytes were perfused at their respective acclimation temperatures (14°C and 24°C), and at a common test temperature of 19°C. Temperature changes were accomplished within 2 min. To investigate the role of SR, cells were bathed with solutions containing both the SR  $Ca^{2+}$  release channel inhibitor ryanodine (5  $\mu$ M) and the SR  $Ca^{2+}$  ATPase inhibitor thapsigargin (2  $\mu$ M). Myocytes were stimulated to contract via platinum plate electrodes (50–100 V, 5–15 ms pulses).  $Ca^{2+}$  was imaged by exciting Fluo-4 at 488 nm and detecting emitted fluorescence at wavelengths greater than 505 nm. All line scan images are presented as the original raw fluorescence ( $F$ ) signal. Background fluorescence ( $F_0$ ) was measured in each cell in a region that did not have localized or transient fluorescent elevation.  $F/F_0$  was converted to  $\Delta[Ca^{2+}]_i$  in accordance with Cheng *et al.* [35] and Loughrey *et al.* [36] and corrected for temperature (see [37]). See supplementary material for full details of solutions, confocal set-up and fluorescence calibrations.

### (d) Electron microscopy and stereology

Detailed methodology for fixation and stereology of bluefin tuna heart has been described previously [20]. A brief description is provided in the electronic supplementary material.

## 3. RESULTS

### (a) Effect of acute temperature change on cellular $Ca^{2+}$ cycling

Acute warming accelerated the temporal properties of  $\Delta[Ca^{2+}]_i$  and acute cooling slowed the temporal properties of  $\Delta[Ca^{2+}]_i$  as illustrated in figure 1. The kinetic responses are emphasized in figure 1a (acute warming of CA myocytes from 14°C to 19°C) and figure 1b (acute cooling of WA myocytes from 24°C to 19°C), which shows the time courses for  $\Delta[Ca^{2+}]_i$  at each temperature with the amplitudes normalized. Rapid warming and cooling by 5°C did not affect the amplitude of  $\Delta[Ca^{2+}]_i$  in either WA or CA myocytes (figure 1c).

### (b) Effect of thermal acclimation on cellular $Ca^{2+}$ cycling

When tested at their respective acclimation temperature, CA myocytes exhibited larger  $\Delta[Ca^{2+}]_i$  (figure 1c) and slower rise times (figure 1e) than WA myocytes. However, decay kinetics and the rate of rise were unchanged

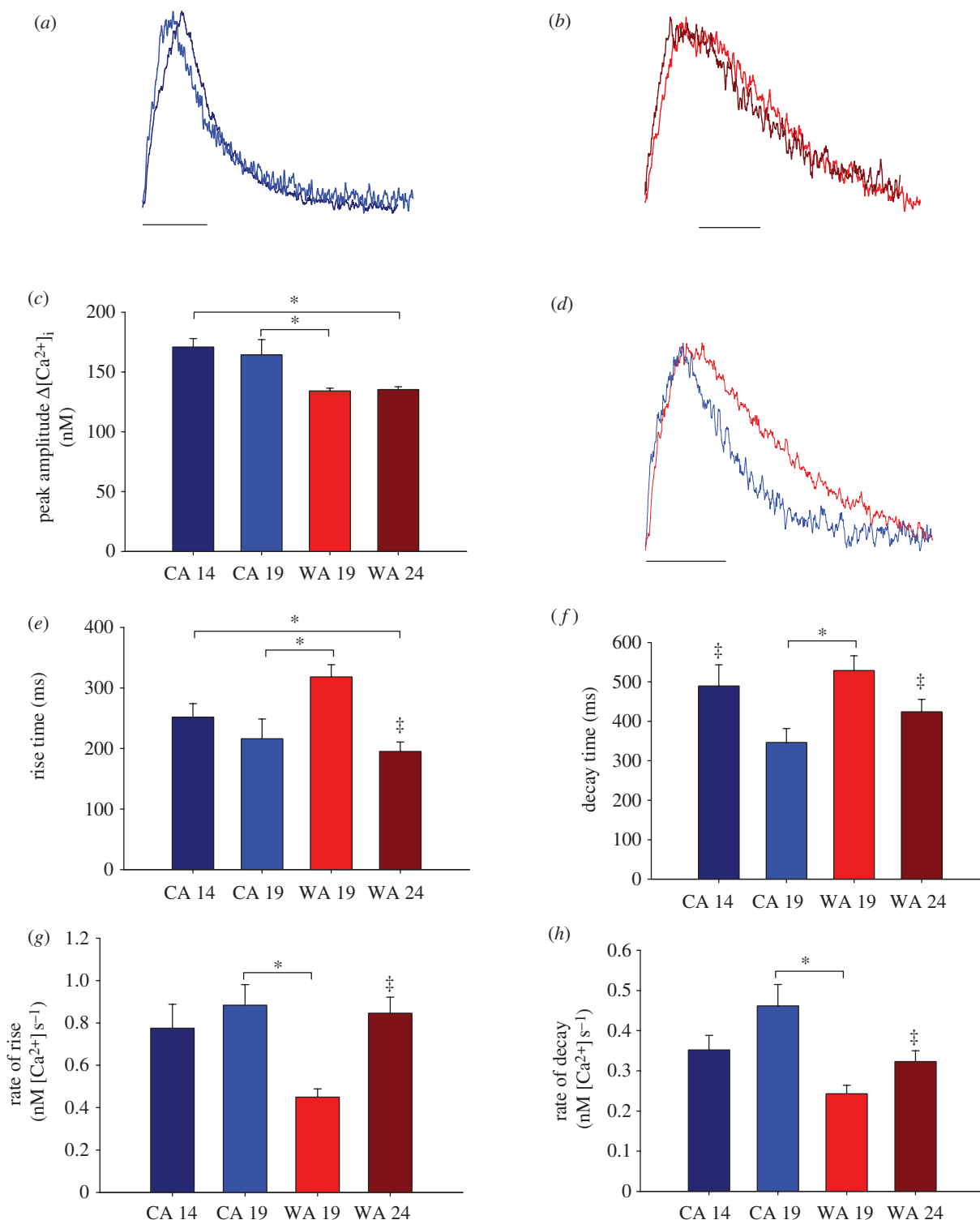


Figure 1. Temporal properties of  $\Delta[Ca^{2+}]_i$  measured transversely across the cell from CA myocytes at their acclimation temperature of  $14^\circ C$  (CA 14, black line) and at the common temperature of  $19^\circ C$  (CA 19, blue line), and from WA myocytes at their acclimation temperature of  $24^\circ C$  (WA 24, brown line) and at the common temperature of  $19^\circ C$  (WA 19, red line). (a) The effects of acute warming on CA myocyte  $\Delta[Ca^{2+}]_i$  kinetics when the time course amplitude of a CA myocyte at  $14^\circ C$  and  $19^\circ C$  are normalized. (b) The effect of acute cooling on WA myocyte  $\Delta[Ca^{2+}]_i$  kinetics when the time course amplitude of a WA myocyte at  $14^\circ C$  and  $19^\circ C$  are normalized. (c) The effects of acute and chronic temperature change on the peak amplitude of  $\Delta[Ca^{2+}]_i$ . (d) The time course of a WA (red line) and CA (blue line) myocyte with the amplitudes normalized at  $19^\circ C$  to emphasize the effect of thermal acclimation on  $\Delta[Ca^{2+}]_i$  kinetics. (e,g) The mean kinetics of the rise and (f,h) the mean kinetics of the fall of  $\Delta[Ca^{2+}]_i$  in response to acute and chronic temperature change. Asterisks (\*) indicate significant effect of acclimation; double-dagger (‡) indicates significant effect of acute temperature change (Student's *t*-test or Mann-Whitney rank sum test,  $p < 0.05$ ;  $n = 6$ –16 cells from four to five animals at each acclimation temperature). Scale bars, (a,b,d) 500 ms.

between WA myocytes tested at  $24^\circ C$  and CA myocytes tested at  $14^\circ C$ , indicating remodelling of cellular  $Ca^{2+}$  flux pathways in response to thermal acclimation.

Remodelling of ventricular myocytes with thermal acclimation is best revealed by comparing the temporal properties of cellular  $Ca^{2+}$  cycling in WA and CA

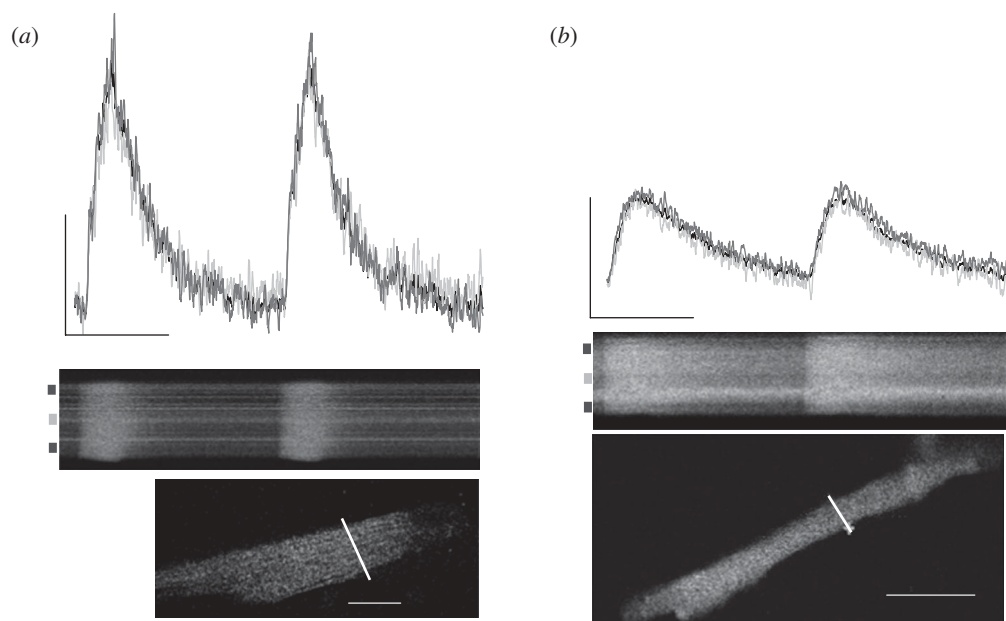


Figure 2. Spatial and temporal cellular  $\text{Ca}^{2+}$  flux in ventricular myocytes from thermally acclimated Pacific bluefin tuna. Representative time courses (top) and corresponding raw line scan images (below) show temporal and spatial characteristics of  $\text{Ca}^{2+}$  flux in a (a) CA and (b) WA myocyte. Markings on left side of line scan images show regions (cell centre, light grey lines; cell periphery, dark grey lines) for spatial characterization (see electronic supplementary methods for details). The time course of the  $\text{Ca}^{2+}$  transient across the whole width of the cell is given by the black time course line, which is partially obscured by the overlapping time courses at the cell centre and periphery. Images below the line scans show the corresponding myocyte loaded with Fluo-4 AM. The white line indicates position of scan. The white scale bar is  $20\ \mu\text{m}$  in both images. Time course scale is  $100\ \text{nM} [\text{Ca}^{2+}]$  by  $1\ \text{s}$ . Line scans are  $2500\ \text{lines} \times 512\ \text{pixels}$ .

myocytes at a common test temperature. Figure 1 shows mean data for  $\Delta[\text{Ca}^{2+}]_i$  peak amplitude (figure 1c) and kinetics (figure 1e–h) across the width of CA and WA myocytes at the common temperature of  $19^\circ\text{C}$ . CA increased the amplitude of  $\Delta[\text{Ca}^{2+}]_i$  and accelerated the kinetics of contraction and relaxation when compared with WA. These kinetic differences are illustrated in figure 1d, where  $\Delta[\text{Ca}^{2+}]_i$  amplitudes are normalized. The increased rate of  $\text{Ca}^{2+}$  cycling after CA is consistent with an upregulation of SR function in the cold.

Remodelling of cellular  $\text{Ca}^{2+}$  flux in response to thermal acclimation could involve spatial changes in the time course of the transient across the width of the myocyte. This was investigated by monitoring  $\text{Ca}^{2+}$  flux in the cell centre and cell periphery as well as across the whole width of the cell in WA and CA myocytes at  $19^\circ\text{C}$ . Figure 2 shows the time course and corresponding raw line scan image of cellular  $\text{Ca}^{2+}$  flux across the width of a CA (figure 2a) and WA (figure 2b) myocyte at  $19^\circ\text{C}$ . There was a uniform rise in the  $\text{Ca}^{2+}$  wavefront across the width of the cell in both acclimation groups as indicated by the line scan images and the unchanged time course of the transients at cell periphery when compared with cell centre. This suggests that  $\text{Ca}^{2+}$  is rapidly propagated from the cell periphery to the cell centre in both acclimation groups at  $19^\circ\text{C}$  indicating  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$ -release during e–c coupling. Thermal acclimation does not alter spatial characteristics of the  $\text{Ca}^{2+}$  transient when SR function is intact.

#### (c) The role of SR in CA myocytes

The increased rate of  $\text{Ca}^{2+}$  cycling after CA is illustrated in figures 1 and 2, and is consistent with an upregulation

of SR function in the cold [11,38]. To investigate this possibility, the SR was inhibited in CA myocytes at  $14^\circ\text{C}$ , which caused a slowing of the rise and fall of  $\Delta[\text{Ca}^{2+}]_i$  and a decline in amplitude. Effects on kinetics are emphasized in figure 3c, where the amplitude of the transients before and after SR inhibition has been normalized. These temporal differences are supported by spatial differences in the rise of the  $\text{Ca}^{2+}$  wavefront after SR inhibition. The  $\text{Ca}^{2+}$  wavefront rose faster ( $p < 0.05$ , Mann–Whitney rank sum test;  $n = 11$  cells) in the cell periphery ( $381 \pm 33\ \text{ms}$ ) than the cell centre ( $501 \pm 50\ \text{ms}$ ) after SR inhibition. This is evidenced visually by the more v-shaped rise in the  $\text{Ca}^{2+}$  wavefront in the raw line scan image in figure 3b when compared with the uniform rise in the  $\text{Ca}^{2+}$  wavefront across the width of the cell in figure 3a.

Mean data are provided in figure 4 and show how SR inhibition reduces the peak amplitude of  $\Delta[\text{Ca}^{2+}]_i$ , and slows the kinetics of contraction and relaxation in CA myocytes at  $14^\circ\text{C}$ . There were no significant effects of SR inhibition on the amplitude or kinetics of  $\Delta[\text{Ca}^{2+}]_i$  after acute warming (figure 4), indicating that acute warming negates the need for increased  $\text{Ca}^{2+}$  cycling through the SR and suggests that sarcolemmal  $\text{Ca}^{2+}$  is sufficient for activation of these myocytes. Indeed, acute warming from  $14^\circ\text{C}$  to  $19^\circ\text{C}$  (under control conditions with SR function intact) tended to accelerate the kinetics of  $\Delta[\text{Ca}^{2+}]_i$  as indicated by the normalized recordings in figure 1a.

#### (d) The role of SR in WA myocytes

Inhibiting the SR in WA myocytes at  $24^\circ\text{C}$  slowed the kinetics of  $\Delta[\text{Ca}^{2+}]_i$  rise as shown in figure 3e–h, but did not

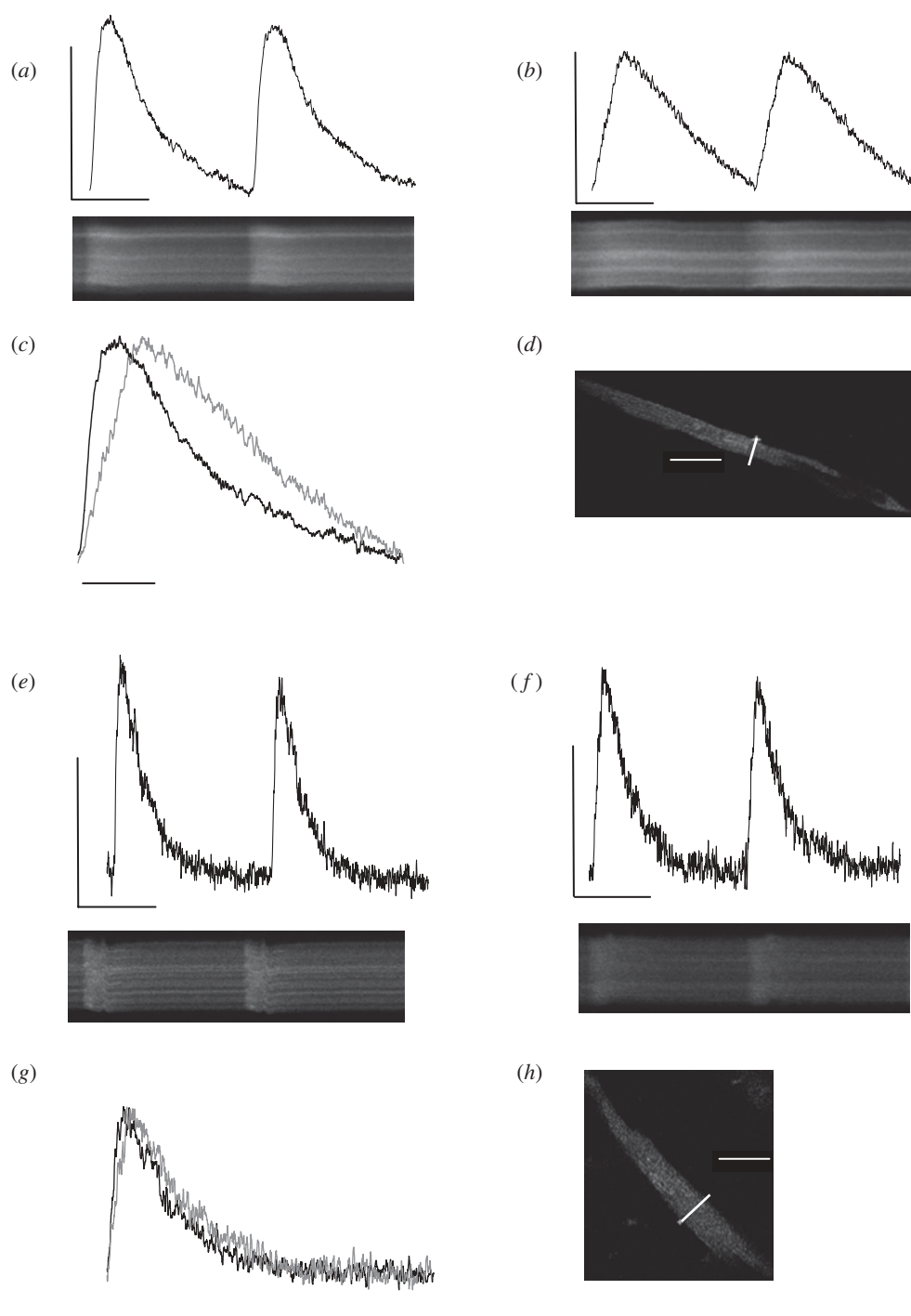


Figure 3. The effect of SR inhibition on cellular  $Ca^{2+}$  flux in bluefin tuna ventricular myocytes. (a–d) Data from a CA myocyte tested at its acclimation temperature of  $14^{\circ}C$ . Figure shows the transient time course (top) and the corresponding raw line scan image (bottom) under control conditions (a) and after SR inhibition (b) for the myocyte loaded with Fluo-4 AM shown in (d). The effects of SR inhibition on  $Ca^{2+}$  cycling kinetics at  $14^{\circ}C$  are emphasized when the amplitudes are normalized (c). (e–h) Data from a WA myocyte tested at its acclimation temperature of  $24^{\circ}C$ . (e) Transient time course (top) and the corresponding raw line scan image (bottom) under control conditions and after SR inhibition (f) for the myocyte loaded with Fluo-4 AM shown in (h). (g) The effect of SR inhibition on kinetics as the amplitudes are normalized. The white line in (d, h) indicates position of scan; the white scale bar is  $50\ \mu m$ . Time course scale in is  $100\ nM\ [Ca^{2+}]$  by 1 s. Line scans are  $2500\ lines \times 512\ pixels$ . Scale bars, (c, g) 500 ms.

affect the amplitude or decay. Mean data are given in the electronic supplementary material. These results lend further support to the idea that warm temperatures reduce the need for  $Ca^{2+}$  cycling through the SR in bluefin tuna ventricular myocytes when contraction frequency is constant at 0.5 Hz.

#### (e) Effects of thermal acclimation on morphology

Electron microscopy revealed that the abundance of SR profiles was higher in CA when compared with WA ventricular myocytes (figure 5) as quantified by morphometric analysis using stereological techniques (table 1). CA increased overall SR surface area to volume ratio by

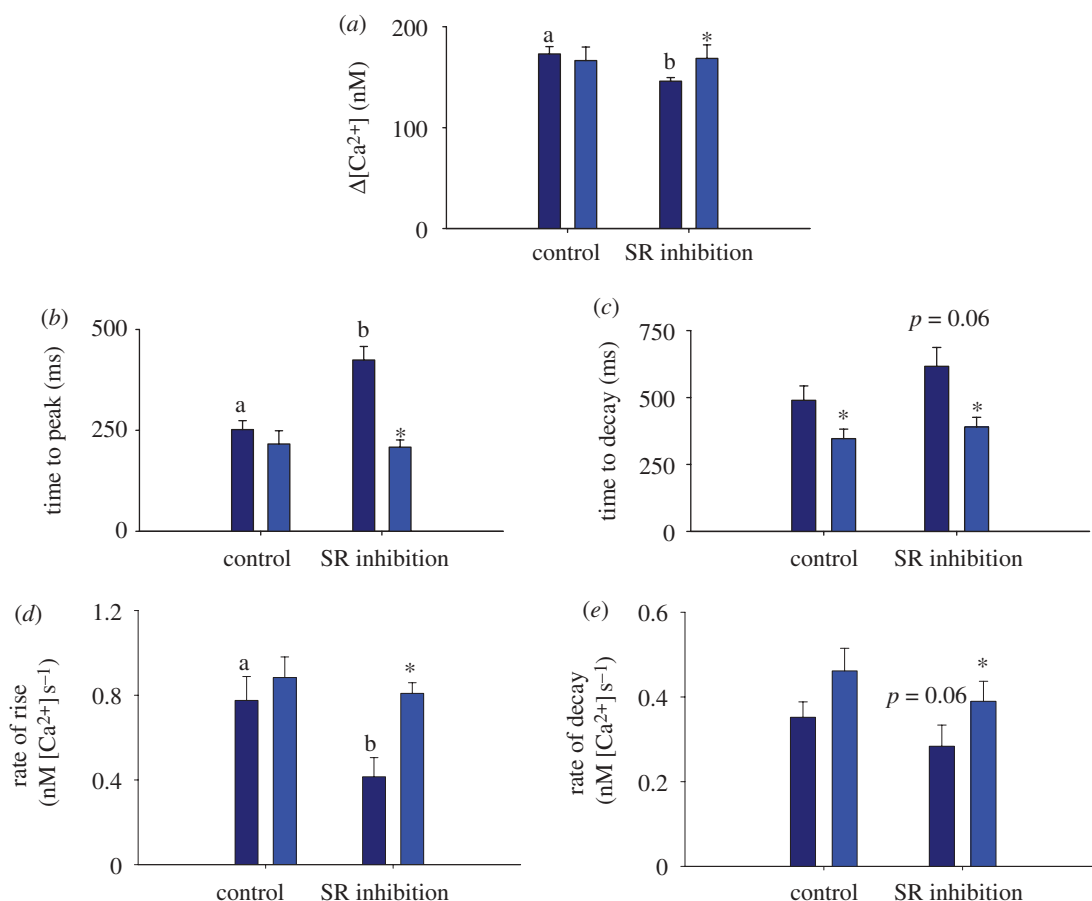


Figure 4. The effect of SR inhibition and acute warming on the temporal properties of  $\Delta[Ca^{2+}]_i$  in CA ventricular myocytes from the Pacific bluefin tuna. (a) At 14°C, SR inhibition decreased the peak amplitude and slowed the kinetics of  $\Delta[Ca^{2+}]_i$  (compare dark blue bars; dissimilar letters indicate significant effect of SR inhibition). (b–e) When CA myocytes were acutely warmed to 19°C, SR inhibition had no effect on  $\Delta[Ca^{2+}]_i$  (compare light blue bars; asterisks indicate significant effect of acute warming);  $p < 0.05$ ; Student's *t*-test or Mann–Whitney rank sum test. Data are means  $\pm$  s.e.m.,  $n = 6$ –16 cells from four to five animals at each acclimation temperature.

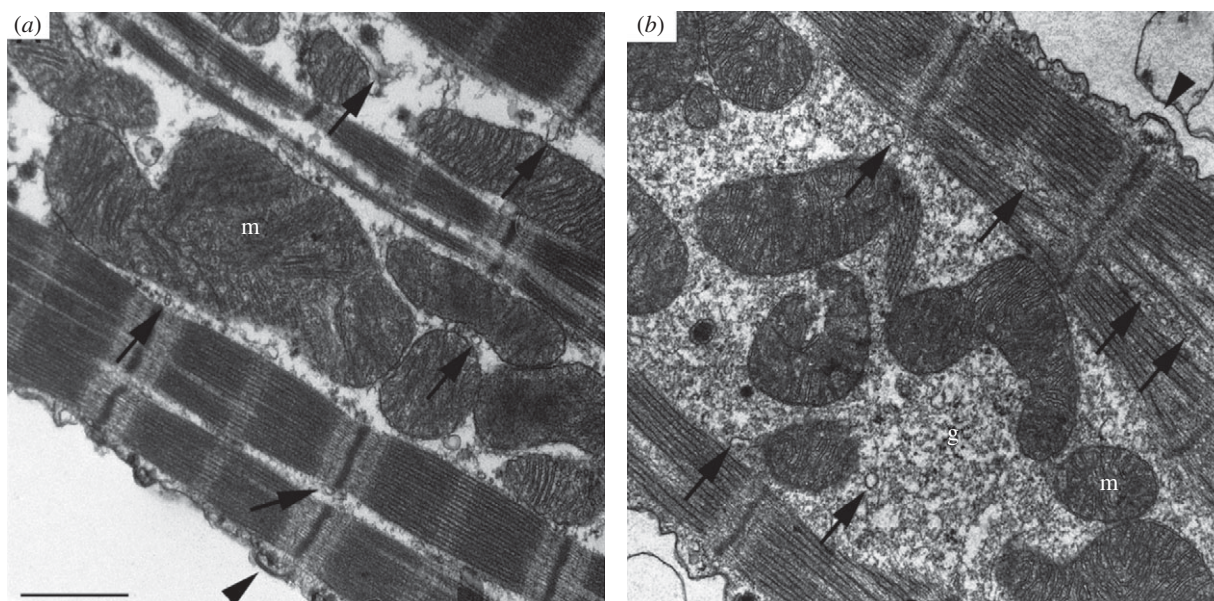


Figure 5. Comparison of longitudinal thin sections of (a) WA and (b) CA Pacific bluefin tuna ventricular myocytes. Two forms of sarcoplasmic reticulum (SR) are evident. Free SR (fSR) is the  $Ca^{2+}$  pumping SR domain and is situated around the myofibrils or in narrow gaps between the myofilaments (arrows). Junctional SR (jSR) is also apparent and appears in the form of wide cisternae in association with the sarcolemma, forming peripheral couplings associated with  $Ca^{2+}$  release (arrowheads). Both fSR and jSR are combined to provide mean SR data in table 1. The jSR cisternae contain dense aggregates of the calcium-binding protein calsequestrin, which is often located at the opposite side of the surface membrane. CA cells also had a greater mitochondrial density and appeared to contain more glycogen droplets than WA cells. Stereological quantifications are provided in table 1 (scale bar, 1  $\mu$ m).

Table 1. Volume and surface measurements of Pacific bluefin tuna ventricular myocytes. Stereological data of SR volume and surface area of cold acclimated (CA) and warm acclimated (WA) myocytes. (All values are expressed as mean  $\pm$  s.d. from  $n = 62$  cells from each animal with  $n = 4$  (CA) and  $n = 5$  (WA) animals. SR, sarcoplasmic reticulum; M, mitochondria; Cyto, cytoplasm. Symbols indicate significant differences between CA and WA with Student's  $t$ -test at  $*p = 3.36 \times 10^{-3}$ ;  $**p = 3.97 \times 10^{-6}$ ;  $***p = 0.02$  and  $****p = 0.005$ .)

	SR			mitochondria	
	volume fraction ( $V_{SR}/V_{tot}$ )	SR-vol/cyto-vol ( $V_{SR}/V_{Cyto}$ )	surface area ( $S_{SR}/V_{tot}$ ) ( $mm^2 mm^{-3}$ )	volume fraction ( $V_M/V_{tot}$ )	m-vol/cyto-vol ( $V_M/V_{Cyto}$ )
CA	$0.037 \pm 0.01^*$	$0.05 \pm 0.01^{**}$	$0.16 \pm 0.06^{***}$	$0.28 \pm 0.14^{****}$	$0.49 \pm 0.3^{****}$
WA	$0.026 \pm 0.01$	$0.036 \pm 0.01$	$0.15 \pm 0.03$	$0.24 \pm 0.09$	$0.31 \pm 0.2$

approximately 7 per cent, and SR volume relative to total volume by approximately 42 per cent, or relative to cytosolic volume by approximately 38 per cent (table 1). CA also increased mitochondrial content (table 1) and appeared to increase glycogen droplets (figure 5).

Remodelling of SR membranes and cellular mitochondrial volume was associated with morphological changes in the myocyte. The mean width measured using confocal microscopy across the single isolated ventricular myocyte (at the centre point of cell length) showed that CA myocytes ( $15.5 \pm 1.45 \mu m$ ,  $n = 31$ ) were wider than ( $p < 0.001$ , Mann-Whitney Rank sum test) WA myocytes ( $8.45 \pm 0.73 \mu m$ ,  $n = 34$ ). We did not observe an effect of CA on the ratio of whole heart (ventricle, atrium and bulbus arteriosus) mass to body mass (CA,  $0.37 \pm 0.02\%$ ; WA,  $0.37 \pm 0.01\%$ ).

#### 4. DISCUSSION

In bluefin tuna, thermal gradients of approximately  $10^\circ C$  are common between ambient sea surface and internal body temperatures, and can increase to approximately  $20^\circ C$  when diving into cool waters [1,2,39]. Thus, the bluefin tuna heart must supply the high oxygen demand of warm tissues while functioning across a wide range of temperatures. In this study, confocal imaging of live contracting ventricular myocytes has been employed to test the hypothesis that cold tolerance in bluefin tuna is directly related to SR use during e-c coupling. We provide structural and functional evidence for robust SR involvement in e-c coupling in the bluefin tuna heart and suggest that this arrangement is important in preserving heart function when diving into cooler waters. We then show that chronic cooling induced remodelling of cellular  $Ca^{2+}$  flux through further enhancement of SR function consistent with thermal compensation. We suggest that this remodelling is important for robust cardiac function of tuna in cold northerly latitudes and that it may be a conserved trait for cardio-protection from the cold across vertebrate species (see below [23,38,40,41]).

##### (a) Bluefin tuna e-c coupling

Bluefin tuna hearts are dependent upon SR  $Ca^{2+}$  cycling during e-c coupling. We found a homogeneous rise in the  $Ca^{2+}$  wavefront across the width of the bluefin tuna myocyte, irrespective of temperature. This indicates that voltage-dependent  $Ca^{2+}$  entry triggers  $Ca^{2+}$  release from peripheral junctional SR that subsequently induces  $Ca^{2+}$  release in more central

regions of the cell through propagated  $Ca^{2+}$ -induced  $Ca^{2+}$ -release (i.e. [33]). Our functional model is supported by morphological evidence for  $Ca^{2+}$ -induced  $Ca^{2+}$ -release in bluefin tuna myocytes [19], recent electrophysiological studies [21,34], and biochemical studies on isolated SR vesicles [17,18].

In mammalian myocytes, the homogeneous rise in the  $Ca^{2+}$  wavefront is attributed to the T-tubular system which couples the SR to the sarcolemma throughout the entire cell volume [42]. Bluefin tuna ventricular myocytes lack T-tubules [19] similar to all other non-mammalian ventricular myocytes. We suggest that the rapid propagation of the  $Ca^{2+}$  signal between neighbouring ryanodine receptors in the bluefin heart gives rise to this homogeneous  $Ca^{2+}$  wavefront in the absence of T-tubules (e.g. figure 2). The small physical dimensions (shallow depth  $< 10 \mu m$ , narrow width  $< 20 \mu m$ ) of the myocytes will also play a role in the spatio-temporal characteristics of the bluefin  $Ca^{2+}$  transient. However, in rainbow trout myocytes, which have similar dimensions, the  $Ca^{2+}$  transient wavefront is not homogeneous, presumably owing to less robust coupling between SR  $Ca^{2+}$  release units [20].

##### (b) The role of SR in thermal remodelling

We show here that prolonged cold exposure increases the amplitude and accelerates the kinetics of  $\Delta[Ca^{2+}]_i$ , thereby offering partial compensation for the direct effects of cold. These physiological responses are consistent with a cold-induced increase in SR function. Increased SR function in the cold has been documented in eurythermal rainbow trout [12,14,31], cold stenothermal burbot [28,43], and hibernating [23,38,41] and torpid [40] mammals, suggesting that enhanced SR function may be a conserved trait for cardioprotection in the cold across vertebrates.

Our study shows that CA increases both the rate of rise and the rate of decay of the  $Ca^{2+}$  transient indicating thermal compensation in both SR  $Ca^{2+}$  uptake and SR  $Ca^{2+}$  release mechanisms. An increase in SERCA function could account for both of these responses and previous biochemical studies have indicated that SERCA function is the feature of e-c coupling that distinguishes the Pacific bluefin tuna from other scombrids [18,19]. However, our study does not distinguish the precise mechanism for increased SR function after CA. A number of mechanisms could be involved including: (i) greater amount of SR; (ii) greater activity of SERCA; (iii) greater activity of ryanodine receptors; (iv) changes in SR luminal  $Ca^{2+}$ ; or (v) changes in accessory proteins (i.e. phospholamban, triadin, junctin, FKBP) regulating SR  $Ca^{2+}$  cycling.

Our structural data clearly show increased free and junctional SR after CA in the bluefin ventricle, which is in line with earlier studies on CA perch [32]. This alone could account for the increased rate and amplitude of  $\Delta[Ca^{2+}]_i$  in the bluefin, however SR vesicles from rainbow trout have demonstrated increased maximal  $Ca^{2+}$  uptake velocity after CA [11], suggesting that enzyme activity could also be enhanced. By contrast, no effect of temperature acclimation has yet been observed for the expression or subcellular localization of the ryanodine receptor in trout [44] or carp [45]. This may indicate that the ryanodine receptor plays less of a role than SERCA in the thermal remodelling response in fishes. To date, there have, to our knowledge, been no studies investigating the effect of thermal acclimation on SR accessory proteins in fishes. However, recently, Korajoki & Vornanen [46] showed expression of the SR luminal  $Ca^{2+}$  buffer, calsequestrin, does not vary with acclimation in the rainbow trout heart. Detailed biophysical studies combined with molecular biological approaches are required to discern the precise molecular mechanism for enhanced SR function in the CA bluefin heart.

In the hearts of most ectotherms, action potential shape is significantly altered by thermal acclimation to avoid temperature-induced disturbances in electrical excitability [31]. Interestingly, previous work with Pacific bluefin tuna showed no change in action potential duration with thermal acclimation [22]. Similarly, ventricular myocytes from Siberian hamsters (*Phodopus sungorus*) showing daily bouts of torpor (from 37 to approximately 15°C) exhibit an increased amplitude of  $\Delta[Ca^{2+}]_i$  and an acceleration of rise and fall kinetics, with no change in action potential duration, when compared with non-torpid littermates [40]. This may suggest that animals that experience rapid changes in temperature on a daily cycle (e.g. diving tuna or torpid mammals) do not compensate seasonally via action potential excitability, but rather via SR  $Ca^{2+}$  flux mechanisms.

#### (c) Acute effects of temperature on $\Delta[Ca^{2+}]_i$

Electronic tagging of juvenile Pacific bluefin tuna show that they experience rapid temperature changes of 5–10°C daily as they dive through the stratified water column [1,2]. In isolated ventricular myocytes, a 5°C acute temperature change altered the kinetics of  $\Delta[Ca^{2+}]_i$ , consistent with changes in force development in isolated strips of tuna cardiac muscle [9,16]. *In vivo* and *in situ* studies indicate that bluefin tuna exhibit a robust bradycardia in response to rapid cooling [7]. We hypothesize that it is the combined effect of acute temperature change on the inotropic and chronotropic output of the heart and the challenges of meeting the oxygen demands of the warm body that induce myocyte remodelling during prolonged cold exposure.

#### (d) Effect of thermal acclimation on heart morphology

In addition to the effect of CA on SR content, we observed increased mitochondrial density in bluefin tuna ventricular myocytes after CA. Adjustments in mitochondrial properties and capacities are crucial for acclimation to the cold in some fishes as are increases in cardiac glycogen storage [47]. These data suggest that

remodelling in cellular energetics may accompany the remodelling in cellular  $Ca^{2+}$  flux in the bluefin tuna heart. The increase we observed in myocyte width with CA is consistent with the increase in cell capacitance (a measure of cell surface area) reported previously for CA bluefin tuna myocytes [21].

All procedures used in these experiments were in accordance with Stanford University institutional animal use protocols and adhered to the United Kingdom Home Office Animals Scientific Procedures Act of 1986 for Schedule 1 killing of fish.

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