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RESEARCH ARTICLE

Reduced L-type Ca^{2+} current and compromised excitability induce loss of skeletal muscle function during acute cooling in locust

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ABSTRACT

Low temperature causes most insects to enter a state of neuromuscular paralysis, termed chill coma. The susceptibility of insect species to chill coma is tightly correlated to their distribution limits and for this reason it is important to understand the cellular processes that underlie chill coma. It is known that muscle function is markedly depressed at low temperature and this suggests that chill coma is partly caused by impairment in the muscle per se. To find the cellular mechanism(s) underlying muscle dysfunction at low temperature, we examined the effect of low temperature (5°C) on several events in excitation–contraction coupling in the migratory locust (*Locusta migratoria*). Intracellular membrane potential recordings during single nerve stimulations showed that 70% of fibers at 20°C produced an action potential (AP), while only 55% of fibers were able to fire an AP at 5°C. Reduced excitability at low temperature was caused by an ~80% drop in L-type Ca^{2+} current and a depolarizing shift in its activation of around 20 mV, which means that a larger endplate potential would be needed to activate the muscle AP at low temperature. In accordance, we showed that intracellular Ca^{2+} transients were largely absent at low temperature following nerve stimulation. In contrast, maximum contractile force was unaffected by low temperature in chemically skinned muscle bundles, which demonstrates that the function of the contractile filaments is preserved at low temperature. These findings demonstrate that reduced L-type Ca^{2+} current is likely to be the most important factor contributing to loss of muscle function at low temperature in locust.

KEY WORDS: Chill coma, Neuromuscular dysfunction, Excitation–contraction coupling, Contractile force

INTRODUCTION

Insects are ectotherms and therefore are sensitive to their surrounding temperature and enter a state of paralysis – chill coma – when exposed to sufficiently cold environments (MacMillan and Sinclair, 2011). While strong correlations between cold tolerance and insect distribution demonstrate the ecological significance of cold tolerance (Kimura, 2004; Chen et al., 2011; Kellermann et al., 2012; Andersen et al., 2015a), the cellular mechanisms that induce the state of paralysis at low temperature are still not fully resolved. Nevertheless, several studies suggest that chill coma is caused by neuromuscular failure. This neuromuscular dysfunction has been suggested to arise from a failure to maintain membrane potential, neuronal function and contractile function at low temperature (Goller and Esch, 1990; Hosler et al., 2000; Koštál et al., 2004, 2006; MacMillan and Sinclair, 2011; MacMillan et al., 2012, 2014; Andersen et al., 2013; Findsen et al., 2013, 2014). The role of skeletal muscle in chill coma was recently indicated by a study showing that contractile force produced by isolated muscles from locusts is markedly reduced at temperatures that cause chill coma *in vivo* (Findsen et al., 2014). Additionally, recent experiments on locusts and previous studies on *Drosophila*, honey bees and cockroaches have shown that cooling causes depolarization of muscle fibers as a direct consequence of low temperature (Wareham et al., 1974; Hosler et al., 2000; MacMillan et al., 2014; Andersen et al., 2015b) and that this depolarization is associated with reduced action potential (AP) amplitude and excitability (Goller and Esch, 1990; Hosler et al., 2000). Together, these findings point to impairment in the excitation–contraction (E–C) coupling of skeletal muscle as a key element in chill coma.

E–C coupling refers to the series of cellular events that link electrical excitation of a muscle fiber to its contraction. In insect skeletal muscle, E–C coupling starts when an AP reaches the pre-synaptic terminal and triggers release of glutamate into the synaptic cleft (Kerkut et al., 1965; Usherwood and Machili, 1966). Glutamate binds to receptors in the post-synaptic muscle fiber membrane, resulting in a net inward movement of cations (primarily Na^{+}), creating an endplate potential (EPP) in the muscle fiber (Washio, 1972; Salkoff and Wyman, 1983; Collet and Belzunces, 2007). If the EPP is sufficiently large, an AP is formed, driven by current through L-type voltage-gated Ca^{2+} channels (Frolov and Singh, 2013). The influx of Ca^{2+} through L-type Ca^{2+} channels triggers release of Ca^{2+} from the sarcoplasmic reticulum through a Ca^{2+}-induced Ca^{2+}-release mechanism that can be inhibited by ryanodine (Huddart and Oates, 1970; Takekura and Franzini-Armstrong, 2002; Collet, 2009). Finally, the released Ca^{2+} binds to regulatory proteins on the contractile filaments, facilitating contraction of the fiber.

Several steps in E–C coupling have been shown to be temperature sensitive in insect muscle. For example, in muscles of larval *Drosophila*, the L-type Ca^{2+} current that is responsible for the AP upstroke and Ca^{2+} release from internal stores has slower kinetics and markedly reduced amplitude at low temperature (Frolov and Singh, 2013). Additionally, the isometric force production of the contractile proteins is reduced in several insect species at low temperature (Orentlicher et al., 1977; Godt and Lindley, 1982; Iwamoto, 2009). On the basis of these sporadic findings, it is, however, still difficult to determine how E–C coupling fails at low temperature in insect muscle because none of the studies have systematically explored all steps in E–C coupling in one particular insect species. Also, the effect of low temperature on the L-type Ca^{2+} current has previously only been shown in larvae of a holometabolous insect (*Drosophila*) (Frolov and Singh, 2013) and

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the putative impairment has, for example, not yet been explored in adult animals, nor has it been examined in a hemimetabolous insect such as the locust.

To get a better understanding of the cellular mechanisms responsible for arrest of muscle function at the onset of chill coma, the present study examined four aspects of E–C coupling in the extensor tibialis of the chill-susceptible migratory locust (*Locusta migratoria*) at 20 and 5°C. The aspects investigated include: (i) the ability of muscle fibers to generate an AP and an analysis of the effect of temperature on AP waveform and excitability of the muscle fiber, (ii) L-type Ca^{2+} currents, (iii) intracellular Ca^{2+} transients during muscle stimulation and (iv) maximum force of the contractile apparatus. The findings demonstrate that reduced L-type Ca^{2+} current is the major cause of muscle dysfunction at low temperature and a primary cause of muscle paralysis at the onset of chill coma.

MATERIALS AND METHODS

Insect rearing

Laboratory-reared locusts, *Locusta migratoria* (Linnaeus 1758) were kept in cages (0.45 m^3) with a 12 h:12 h light:dark cycle. During the day, the cages were heated with a sun-simulating lamp, allowing behavioral thermoregulation within a temperature gradient ranging from 25 to 45°C. During the night, the temperature was set at 22°C. Locusts were fed daily with fresh wheat sprouts and had a constant supply of wheat bran and water. Cages were cleaned once a week and debris and uneaten wheat were removed daily. Experimental animals were between 1 and 4 weeks old at the time of the experiments, measured from the day of imaginal ecdysis. An equal number of males and females was used.

Measurement of contractile force

Muscle preparation for skinned fiber experiments

Locusts were decapitated, the hindlegs were detached, and the top of the femur, the coxa and the trochanter were removed with surgical scissors. The hindlegs were placed in an experimental glass chamber containing a locust Ringer solution (in mmol l^{-1}: 140 NaCl, 10 KCl, 2 $MgCl_2$, 1 NaH_2PO_4, 3 $CaCl_2$, 5 glucose, 20 Hepes buffer; pH 7.15) (Findsen et al., 2014), before further dissection. A lateral cut was made on both sides of the femur and a vertical cut was made approximately 1/3 of the way down the leg. The retractor unguis muscle and associated apodeme were removed so only extensor tibia muscle fibers were left. This procedure of preparation effectively isolates a small piece of the extensor tibia containing approximately 3–4 bundles of fibers that extends to the exoskeleton on each side of the apodeme. The muscle fibers used in these preparations were mainly large and fast, and correspond to the muscles in region 3c of Hoyle's (1978) classification (see his fig. 2). A short stainless-steel hook was gently pushed through one side of the exoskeleton while taking care to avoid damaging the fibers. The hook was fastened to two metal pins embedded perpendicularly in a vertically oriented Plexiglas holder that also contained the platinum electrodes for field stimulation. Another stainless steel hook was pushed through the exoskeleton on the other side of the preparation, and this hook was connected to a force transducer (Grass FT03, range 0.5–100 g, Grass Technologies, Warwick, RI, USA), which could be adjusted vertically to stretch the muscle fibers. The entire muscle preparation was completed within 10 min. After mounting on the holder, the preparation was submerged in a tall water-jacketed glass chamber with the locust Ringer solution. Experimental temperature was controlled using a programmable thermostat (Lauda RE 620, Lauda-Königshofen, Germany) supplying a continuous water flow through the wall of the water-jacketed glass chambers. Preparations rested for 30 min in the locust Ringer solution at 20°C before the start of experiments.

Electrical field stimulation prior to skinning

After mounting, the muscle preparations were adjusted to optimal length such that isometric twitch force was maximal. Contractions were evoked through field stimulation using constant voltage pulses applied via two platinum wire electrodes passing current across the central part of the preparation. Muscle bundles were activated to contract using pulses of 2 ms duration and supra-maximal voltage (24–30 V cm^{-1}). Tetanic contractions were elicited using 1 s trains with a stimulation frequency of 40 Hz. Data were sampled at 1 kHz using an AD converter and software from Cambridge Electronic Design (Power1401, Signal 4.0, Cambridge, UK). After five tetanic contractions, the muscles were exposed to the skinning solution (see below) for 30 min at 20°C.

Skinned fiber preparation

Muscle fiber bundles were chemically skinned using a relaxing solution containing 90 µg ml^{-1} saponin and the following solution (in mmol l^{-1}): 40 EGTA, 20 Hepes, 5.94 $CaCl_2$, 6.49 $MgCl_2$, 127 KOH, 73 KCl, 5 Na_2ATP (pH 7.10; free Ca^{2+}, 50 nmol l^{-1}; free Mg^{2+}, 1 mmol l^{-1}). The saponin degrades the membrane, thereby generating direct access for the bath solution to the contractile filaments.

Measurement of contractile force in skinned preparations

Following skinning, the muscle was exposed to the relaxing solution without saponin. Preparations were then randomly assigned to experiments aiming to test maximum force of the contractile filaments at either 20 or 5°C, ensuring an even number of males and females in each treatment; 5°C is slightly higher than the temperature causing chill coma in locusts (~0°C; Findsen et al., 2014). However, this experimental temperature was the lowest we could achieve logistically in our experimental setup and previous experiments have shown that muscle force production is already declining markedly at 10°C (Findsen et al., 2014). The change from 20 to 5°C was accomplished by ramping the bath at a rate of 0.5°C min^{-1}; hence, 5°C was reached after 30 min, after which measurements were taken. In some experiments, the preparation was first kept at 5°C and then reheated to 20°C before contractile force was measured. This was done to ensure that a difference in force at the two temperatures was not caused by damage induced by low temperature. Following 30 min at either 20 or 5°C, the relaxing solution was changed to a Ca^{2+} solution containing either 1 or 100 µmol l^{-1} of free Ca^{2+}. The 100 µmol l^{-1} free Ca^{2+} (high-Ca^{2+}) solution contained (in mmol l^{-1}): 40 EGTA, 20 Hepes, 40.19 $CaCl_2$, 5.28 $MgCl_2$, 200 KOH, 5 Na_2ATP (pH 7.10; free Ca^{2+}, 100 µmol l^{-1}, free Mg^{2+}, 1 mmol l^{-1}). The 1 µmol l^{-1} free Ca^{2+} (medium-Ca^{2+}) solution was obtained by mixing the high-Ca^{2+} solution and relaxing solution in a 5:1 ratio. The muscle bundles were allowed to reach maximum contractile force, after which the solution was changed back to the relaxing solution, which is almost completely free of Ca^{2+} ([Ca^{2+}]=50 nmol l^{-1}), to allow the filaments to relax. In all experiments, maximum contractile force was compared with the tetanic force at 20°C assessed prior to skinning.

Electrophysiological experiments

Muscle preparation and experimental chamber

In these experiments, the preparations were dissected out as described above to expose only the extensor tibia muscle fibers.

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The preparations were placed in an experimental glass chamber containing locust Ringer solution and with a Sylgard® (Dow Corning, Midland, MI, USA) silicone bottom, enabling the preparation to be fastened to the bottom by small needles pushed through the exoskeleton into the Sylgard®. The experimental chamber was installed in a Nikon upright microscope (Eclipse FN1, DFA, Glostrup, Denmark) and the muscle fibers and electrodes were visualized on a TV screen using a Nikon DS-Vil camera (Eclipse FN1, DFA). A custom-built nerve electrode connected to a constant current isolated stimulator (model DS3, Digitimer Ltd, Herts, UK) was placed inside the femur close to the trochanter and the nerve could be stimulated with local field stimulation. Experiments were carried out at 20 and 5°C. Experimental temperature was controlled using a programmable thermostat (Lauda RE 320, Lauda, Lauda-Königshofen, Germany) supplying a continuous flow of cooling liquid through the wall of the water-jacketed glass chamber. The low temperature was reached within 30 min by ramping the bath (approximately 0.2°C min^{-1}).

Electrode setup for measurements of muscle fiber potentials

The membrane potential responses to nerve stimulation were measured in individual muscle fibers with intracellular electrodes. The electrodes were filled with 2 mol l^{-1} potassium citrate and had a resistance of 10–20 MΩ. Recordings were performed using a voltage-clamp amplifier (TEC-O5X, NPI, Tamm, Germany). Experimental protocols for nerve stimulation and sampling of data (30 kHz) were controlled using Signal 5 and a Power 1401 interface (Cambridge Electronic Design Ltd).

Ca^{2+} currents measured using a three-electrode voltage clamp

We used a previously described, three-electrode voltage-clamp method to measure Ca^{2+} currents (Adrian et al., 1970; Ashcroft and Stanfield, 1982). Briefly, two electrodes were used to record the membrane potential at two distances from the apodemal end of the fiber (l and $2l$). The membrane potential recordings at these two positions were referred to as V_{m1} and V_{m2}, respectively. The third electrode, inserted at $2l$+l', was used to pass current. V_{m1} was used as feedback for the voltage clamp using the TEC-05X two-electrode clamp, while V_{m2}, the voltage-recording electrode closest to the current electrode, was not controlled by the clamp. V_{m2} was connected to a SEC-05X amplifier (NPI) (see Adrian et al., 1970). All electrodes were filled with 2 mol l^{-1} potassium citrate, which resulted in resistances of 5–20 MΩ. Currents were elicited by 500 ms voltage steps from a holding potential of −80 mV to potentials between −80 and +10 mV in 10 mV steps with an inter-pulse interval of 1.5 s. The solution used during the voltage-clamp experiments contained (in mmol l^{-1}): 10 Hepes, 4 CsCl, 5 glucose, 9 NaCl, 1 4-aminopyridine, 2 $CaCl_2$, 123 TEA-Cl_2 (titrated with CsOH, pH 7.10). Blebbistatin was added to the solution (final concentration 100 µmol l^{-1}) to reduce contraction. The membrane currents (I_m, in A cm_2^{-}) were calculated from:

$$I_m = \frac{a(V_{m2} - V_{m1})}{3R_i l^2}, \quad (1)$$

where a is the fiber radius and R_i is the sarcoplasmic resistivity (Ω cm). In all experiments, the inter-electrode distance l was set at 600 µm and l' was 50 µm. R_i was assumed to be 180 Ω cm in the voltage-clamp solution at 20°C and 300 Ω cm at 5°C (Adrian et al., 1970; Ashcroft, 1980). Fiber radius was calculated using cable theory from the electrical response to a 10 mV hyperpolarization (Adrian et al., 1970). To do this, we first obtained intracellular resistance per unit length of fiber (r_i, Ω cm^{-1}) by considering the fiber as a finite cable. The current injected from the current electrode (I_0) will then be described by:

$$I_0 = \frac{V_{m1}\cosh((2l + l')/\lambda)(\tanh(k/\lambda) + \tanh((2l + l')/\lambda))}{r_i\,\lambda\,\cosh(l/\lambda)}, \quad (2)$$

where k is the distance between the current electrode and the end of the fiber with no inserted voltage recording electrodes and the length constant λ is given by:

$$\lambda = l\sqrt{\frac{3V_{m1}}{2(V_{m1} - V_{m2})}}. \quad (3)$$

By first calculating λ, it was then possible to calculate fiber radius from:

$$a = \sqrt{R_i/\pi r_i}. \quad (4)$$

Linear leak was subtracted using the current recordings when applying hyperpolarizing steps from −80 to −90 mV. For voltage steps larger than 10 mV, this leak current was scaled as appropriate.

Measurement of Ca^{2+} transients

Muscle preparations for measuring Ca^{2+} transients were obtained as for electrophysiological experiments. Experiments were carried out at 20 and 5°C. In these experiments, two electrodes were used – one to measure the membrane potential response to nerve stimulation, as described above, and the other to ionophoretically inject Ca^{2+}-sensitive dye (Fluo-4, K-salt) into the fiber. The two electrodes were connected to a TEC-05X two-electrode voltage-clamp amplifier (NPI) and placed inside the same muscle fiber. To ensure that both electrodes were placed in the same fiber, a small current was delivered from the electrode containing the fluorescent dye; if the electrodes were placed in the same fiber, a deflection would be observed in the recordings by the other electrode. Before injecting the Ca^{2+}-sensitive dye, background fluorescence of the fiber was measured by a photomultiplier (TILL Photonics, Gräfelfing, Germany), connected to the microscope. Ca^{2+}-sensitive dye was then injected from the electrode into the fiber for 90 s by applying a small sinusoidal current. Following loading of the muscle fiber, it was stimulated by nerve stimulation and the membrane potential response and Ca^{2+} transient were sampled with the photomultiplier (30 kHz) using Signal 5 and a Power 1401 interface (Cambridge Electronic Design Ltd). The dye was excited at 488 nm using a polychrome V monochromator system (TILL Photonics) and the dye fluorescence was collected between 510 and 580 nmol l^{-1} using the photomultiplier. Fluorescence values were expressed as $\Delta F/(F-F_0)$, F being the resting fluorescence, F_0 the background fluorescence and ΔF the change in fluorescence from the baseline following stimulation.

Statistics

All statistical analysis was performed in Graphpad Prism 6.0. Data are presented as means±s.e.m. and $P<0.05$ was considered statistically significant. Statistical differences between two groups were obtained using Student's two-tailed t-test and for comparisons between more than two groups a one-way ANOVA followed by Tukey's *post hoc* multiple comparison test was used.

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RESULTS

Effects of low temperature on tetanic force in non-skinned muscle bundles and maximum force in skinned bundles

Tetanic force was first determined at 20°C, then at 5°C and then after reheating to 20°C. Reduction in temperature was associated with a marked decline in force, and reheating caused a partial restoration of force (Fig. 1A). Similar observations were obtained for nine bundles, and on average the reduction in temperature caused around a 90% drop in force that could be recovered to 53% upon reheating (Fig. 1C).

To evaluate whether this loss of force at low temperature reflected altered function of the contractile filaments, experiments were next conducted using muscle bundles that had been chemically skinned by exposure to saponin. Prior to skinning, the tetanic force at 20°C was determined and this force was used for normalization of the maximum force that the bundle could produce after skinning. This skinning procedure removes the surface membranes, providing full access to the intracellular space, which is then effectively controlled by the surrounding solution. After skinning, the preparations were therefore maintained in solutions that mimicked the intracellular environment in muscle fibers, and it was possible to test their capacity to generate force by exposing them to bathing solutions with 100 or 1 µmol l^{-1} free Ca^{2+}. Typical recordings of force in skinned muscle bundles are shown in Fig. 1B. These recordings illustrate the general observations that the skinned muscle bundles retained their capacity to produce force at low temperature. In the traces shown in Fig. 1B, the contractions were induced by transferring the skinned muscle bundle to a solution that contained 100 µmol l^{-1} free Ca^{2+}. In another series of experiments, contractions were induced by exposing the bundles to solutions that contained 1 µmol l^{-1} free Ca^{2+}. Very similar observations were obtained in the two series of experiments, suggesting that 1 µmol l^{-1} free Ca^{2+} is sufficient to saturate the contractile apparatus at both temperatures. The data shown in Fig. 1D confirm that the capacity of the bundles to produce force was not affected by lowering the temperature.

Effects of low temperature on EPPs and APs in response to nerve stimulation

The well-maintained force-generating capacity of the contractile filaments at low temperature suggested that cold-induced loss of muscle function reflected an inability of the muscle fibers to release sufficient Ca^{2+} following nervous activation of the muscle fibers. Pursuing this line of thinking, the effect of low temperature on neuromuscular transmission and AP waveform was next determined. Intracellular electrodes were placed in individual muscle fibers and the motor nerve was then stimulated with a single pulse. The membrane potential response to nerve stimulation varied substantially at both temperatures, but each muscle fiber only produced one kind of potential and was only used once: as illustrated by overlaid recordings at 20 and 5°C, the observations could be categorized into EPPs (Fig. 2A), APs with a single peak and monotonous repolarization (hereafter called ‘simple APs’; Fig. 2B), APs with a plateau (Fig. 2C) or APs with two peaks

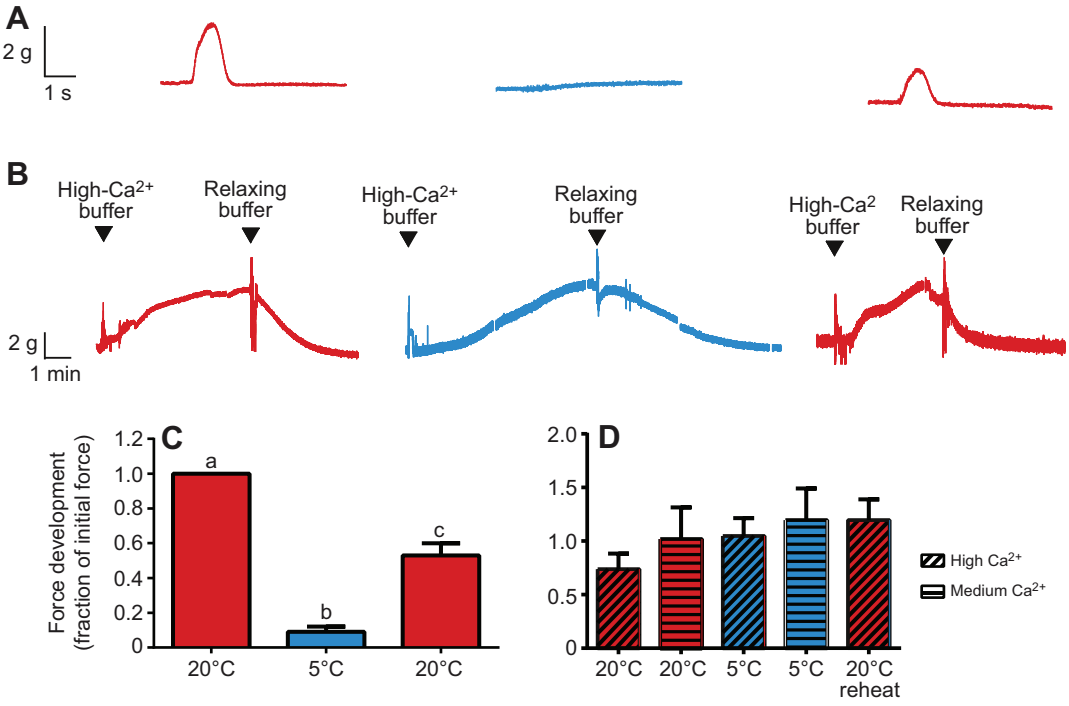

Fig. 1. Effect of low temperature on tetanic force in intact muscle bundles and maximum force in skinned bundles. (A) Typical recordings of tetanic force following field stimulation of an intact (non-skinned) muscle bundle from the extensor tibialis first at 20°C (red), then at 5°C (blue) and following reheating to 20°C (red). (B) Typical recordings of force in a saponin-treated (skinned) muscle bundle from the extensor tibialis exposed to high-Ca^{2+} buffer (100 µmol l^{-1} free Ca^{2+}) at 20°C (red), 5°C (blue) and following reheating to 20°C (red). After reaching maximum force in high-Ca^{2+} buffer, the filaments were transferred to a relaxing buffer that was almost completely free of Ca^{2+} ([Ca^{2+}]=50 nmol l^{-1}) to get the filaments to relax. (C) Normalized force development in field-stimulated muscle bundles as shown in A at normal (20°C) and low (5°C) temperature and then reheated to 20°C. Force was normalized to the force measured following field stimulation just before cooling. Bars represent means±s.e.m. of nine preparations. Different letters indicate that groups differ significantly (one-way ANOVA, Tukey’s *post hoc*). (D) Force development in saponin-treated (skinned) locust muscle at high and low temperature, and at 100 µmol l^{-1} (high) and 1 µmol l^{-1} (medium) free [Ca^{2+}]. Force in skinned preparations was normalized to the tetanic force measured at 20°C prior to saponin treatment. Bars represent means±s.e.m. of 9–12 preparations.

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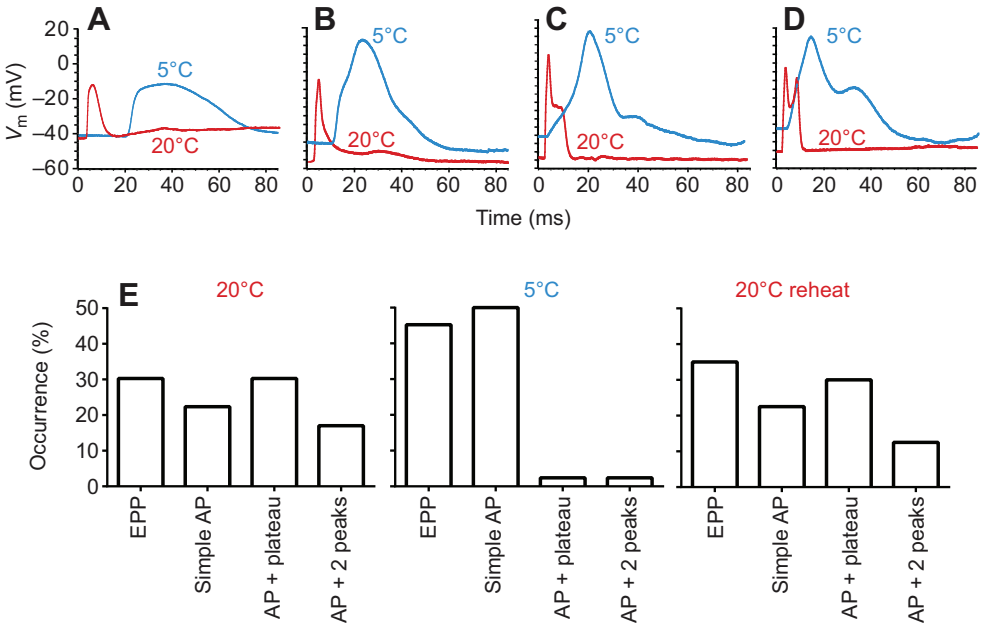

Fig. 2. Effects of low temperature on endplate potentials and action potentials in response to nerve stimulation. Four categories of electrical potentials were identified following nervous stimulation of the muscle preparation. These were endplate potentials (EPPs; A), simple action potentials (APs; B), APs with a plateau (C) and APs with two peaks (D). All categories of electrical potentials were observed at both normal (20°C) and low (5°C) temperature, although the relative occurrence differed (see below). Contractions of the muscle fiber increased notably from EPPs (little or no contraction), to simple APs (medium contraction), to APs with a plateau (medium/large contraction), to APs with two peaks (large/long contraction). V_m, membrane potential. (E) Occurrence of the different electrical potentials at normal (20°C; 19 individuals, 76 fibers) and low (5°C; 9 individuals, 42 fibers) temperature, and following reheating to 20°C (20°C reheat; 8 individuals, 40 fibers).

(Fig. 2D). This categorization of the recordings was evaluated by the observer and it was done to enable quantification of the data. Nevertheless, the four categories of recordings most likely represent a continuum of electrical potentials and not four types per se. It should be noted, however, that APs with two peaks were observed at both normal and low temperature, and were always found in highly polarized cells (V_m below −50 mV). Muscle cells producing APs with two peaks have also been observed in crustaceans (Wiersman, 1967). In addition, the cells producing APs with two peaks also produced a large amount of force (see below).

All four types of signal were markedly prolonged at low temperature and the rates of depolarization and repolarization were clearly reduced. In addition, the occurrence of the different signals changed substantially when the temperature was lowered. Fig. 2E shows the distribution of signals in individual muscle fibers examined at the two experimental temperatures. While APs with a plateau or two peaks were commonly observed at 20°C, very few muscles exhibited them at 5°C (Fig. 2E). Small increases in EPPs and APs were seen when lowering the temperature. Visual inspection of the contraction of fibers was done by live imaging with the microscope camera: from this, it was clear that the contractile response was most pronounced for fibers that produced APs with a plateau or two peaks. Following this notion, the recordings at 20 and 5°C were repeated but with prior injection of a Ca^{2+}-sensitive dye (Fluo-4), enabling the simultaneous recording of the electrical response to nerve stimulation and the consequent rise in cytosolic Ca^{2+} (see below). This type of experiment therefore reveals whether the different types of APs were associated with alterations in Ca^{2+} release.

Relationship between electric potentials and Ca^{2+} transients from the muscle fibers in the extensor tibialis

Recordings from a fiber that generated an EPP in response to nerve stimulation are shown in Fig. 3A. This contrasts with the other fibers in Fig. 3B–D, which all generated APs. The APs could again be divided into simple APs (Fig. 3B), APs with a plateau (Fig. 3C) or APs with two peaks (Fig. 3D). From the fluorescence signals it was clear that the amplitude of the Ca^{2+} transients increased markedly from EPPs through to APs with two peaks (Fig. 3A-D lower panel). On average, Ca^{2+} transients ($\Delta F/F$) for APs with two peaks were seven times larger than Ca^{2+} transients for EPPs (2.2±0.8 versus 0.3±0.1, one-way ANOVA, $F_{3,16}$=6,75, Tukey's *post hoc* test, P<0.05). A significant difference was also observed between APs with two peaks and simple APs (2.2±0.8 vs 0.8±0.2, one-way ANOVA, $F_{3,16}$=6,75, Tukey's *post hoc* test, P<0.05) while the other groups did not differ significantly (Fig. 3E). From these observations it was apparent that failure to excite APs and the lower occurrence of APs with a plateau or two peaks could contribute substantially to the loss of force at low temperature. Ca^{2+} transients were also measured at low temperature following nervous stimulation. However, at 5°C it proved very difficult to load the fibers with dye by the iontophoretic method, and a reasonable loading was only achieved in three fibers. Nevertheless, the data from these fibers were very clear. In none of the fibers did nerve stimulation elicit a detectable rise in cytosolic Ca^{2+}. Fig. 3F shows recordings from one of these fibers in which nerve stimulation did elicit an EPP but there was no detectable rise in cytosolic Ca^{2+}.

Effect of low temperature on Ca^{2+} currents

The failure to excite APs and the corresponding decrease in Ca^{2+} transients at low temperature could possibly be explained by reduced function of L-type voltage-gated Ca^{2+} channels at low temperature. To examine this, we measured Ca^{2+} currents at 20 and 5°C using three-electrode voltage clamp. Fig. 4A,B shows representative recordings of Ca^{2+} currents in two fibers from the same bundle at the two temperatures. It can be seen that low temperature was associated with a marked decline in peak Ca^{2+} current. Similar observations were obtained from 12 (20°C) and 16 (5°C) fibers, and on average the peak currents were approximately 5 times larger at 20°C than at 5°C. In addition, the *I–V* relationship in Fig. 4C shows that activation of the channels was right-shifted ~20 mV at 5°C. This means that a larger depolarization was required to activate the channels and trigger an AP at low temperature.

DISCUSSION

In the chill-susceptible locust, *L. migratoria*, there is a close association between the onset of chill coma and arrest of muscle

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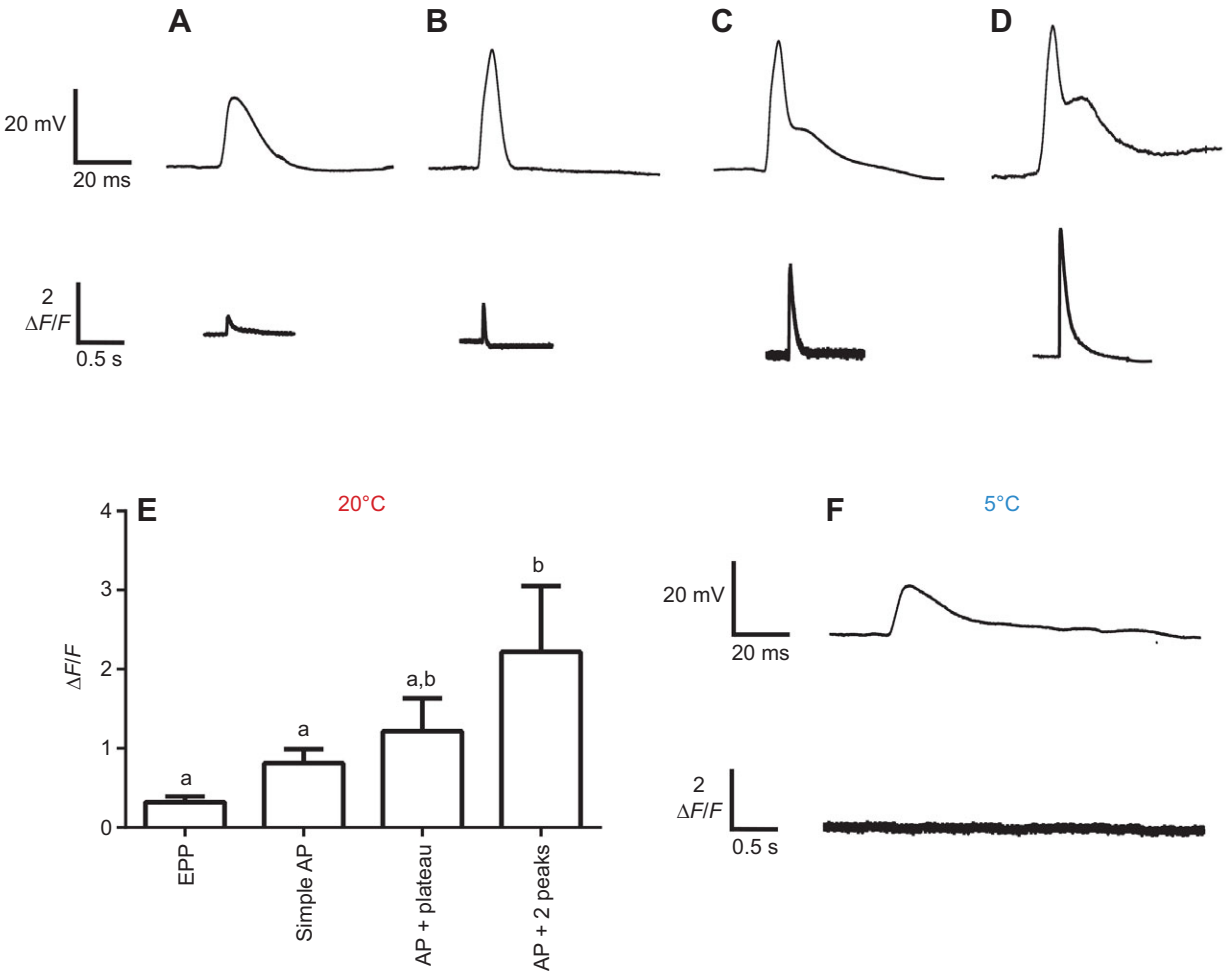

Fig. 3. Relationship between electrical potentials and Ca^{2+} transients from muscle fibers in the extensor tibialis. (A–D) Typical recordings of Ca^{2+} transients from fibers eliciting excitatory EPPs (A), simple APs (B), APs with a plateau (C) and APs with two peaks (D) (upper traces). Ca^{2+} transients are reported from the change in Ca^{2+} fluorescence relative to the background of the unstimulated fiber ($\Delta F/F$, lower traces). All experiments were conducted at 20°C. (E) Average Ca^{2+} transients ($\Delta F/F$) resulting from the different categories of potentials at 20°C; bars represent means±s.e.m. of 3–6 fibers. Different lowercase letters indicate groups that differ significantly (one-way ANOVA, Tukey's *post hoc*). (F) Recording of a Ca^{2+} transient at 5°C (lower trace) from a fiber eliciting EPPs (upper trace).

function (Findsen et al., 2014). To determine the cellular mechanisms responsible for this arrest of muscle function at low temperature, in the present study we conducted four series of experiments in muscle fibers from *L. migratoria* to examine: (i) the ability of muscle fibers to generate an AP and the effect of temperature on AP waveform and excitability of the muscle fiber, (ii) L-type Ca^{2+} currents, (iii) intracellular Ca^{2+} transients during muscle stimulation and (iv) maximum force of the contractile apparatus. The experiments demonstrate that cold-induced depression of tetanic force in the extensor tibialis (90% drop in force) is not caused by a reduced capacity to produce force by the contractile filaments. Instead, the depressed muscle function at low temperature appears to be caused by greatly reduced Ca^{2+} release during E–C coupling. The depressed Ca^{2+} release at low temperature resulted from altered muscle fiber excitability secondary to loss of L-type Ca^{2+} channel function at low temperature. Thus, the Ca^{2+} current was found to be greatly reduced and activated at more depolarized membrane potentials at low temperature. Collectively, our study suggests that depression of the L-type Ca^{2+} current plays a key role in muscle arrest and onset of chill coma in chill-susceptible insects.

Thermal sensitivity of isometric force production and contractile function

The present study confirmed a large drop (90% reduction) in tetanic force when temperature was lowered from 20°C to 5°C. Upon reheating to 20°C, force was found to recover to 53% of that prior to cooling. A similar depressing effect of low temperature on muscle function was found in a previous study on locust muscles but in that study the force recovered fully upon reheating to 20°C (Findsen et al., 2014). The difference in recovery between these two studies may reflect the use of different preparations. The present study used isolated muscle bundles, whereas whole leg muscles were used in our earlier study (Findsen et al., 2014). It is possible that muscle bundles are more fragile than the entire leg muscle, considering the more extensive dissection procedure required to obtain them. These preparations could be more prone to structural damage during the cooling and reheating protocol.

To evaluate whether the cold-induced reduction in tetanic force was caused by loss of function of the contractile proteins per se, we performed experiments with chemically skinned muscle bundles that could be made to contract when exposed to bath solutions containing 100 µmol l^{-1} free Ca^{2+}. These experiments (Fig. 1D)

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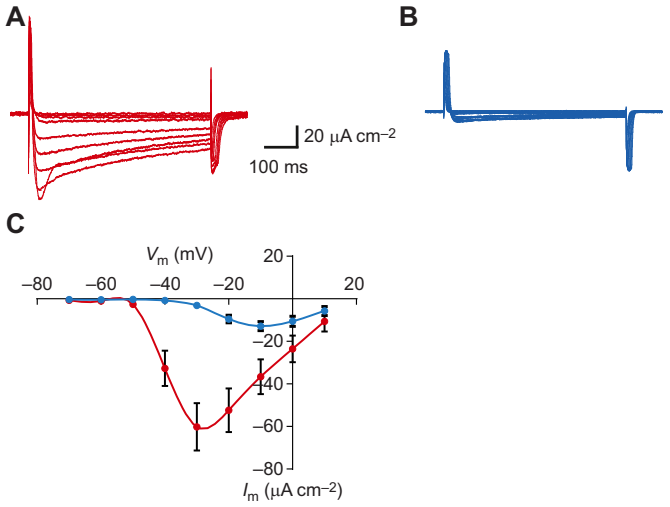

Fig. 4. Effects of low temperature on Ca^{2+} currents in muscle fibers of the extensor tibialis. (A,B) Typical recordings of Ca^{2+} currents at 20°C (A) and 5°C (B) associated with depolarizing voltage steps, from a holding potential of −80 mV. The steps were from −70 mV to +10 mV in 10 mV steps. Step duration was 500 ms and inter-step duration was 1.5 s. (C) Average membrane current (I_m)–voltage (V_m) relationship (±s.e.m.) for the Ca^{2+} current at 20°C (red, 6 individuals, 13 fibers) and 5°C (blue, 5 individuals, 17 fibers).

clearly demonstrated that low temperature had little effect on the maximum force produced by the skinned preparations. Similar measures of maximum force were obtained when the preparations were activated with 1 rather than 100 μmol l^{-1} Ca^{2+} in the bath. On this basis, it appears that there were no gross shifts in Ca^{2+} sensitivity with temperature of the contractile filaments within this large range of Ca^{2+} concentrations. Our observations do, to some extent, contrast earlier findings on locust flight muscle, where binding of Ca^{2+} to troponin was found to be compromised at low temperature, resulting in lower force production (Iwamoto, 2009). We cannot exclude the possibility that low temperature shifts Ca^{2+} sensitivity in the range of concentrations beyond those examined here. However, it should be noted that leg and wing muscles are known to have different thermal sensitivities, suggesting that different steps in E–C coupling induce loss of muscle function in the two types of muscle. Hence, most insects cease to fly at temperatures that are above their chill coma temperature (Mellanby, 1939; Lehmann, 1999; Frazier et al., 2008). In addition, contrasting results for temperature-dependent Ca^{2+} sensitivity of the contractile filaments has been found in other ectothermic animals (Bennett, 1984). In studies on skinned muscle fibers from crayfish and frog (*Rana pipiens*), it has been shown that Ca^{2+} sensitivity changes with temperature (Orentlicher et al., 1977; Godt and Lindley, 1982), whereas in the giant acorn barnacle (*Balanus nubilus*), similar Ca^{2+} sensitivities at 4 and 20°C have been observed (Ashley and Moisescu, 1977). The differences could be associated with different muscle types in the animals. This notion is supported by findings in rats in which the Ca^{2+} sensitivity dropped at low temperature for fast-twitch fibers (5 versus 25°C) but remained unaltered for slow-twitch fibers in the temperature range from 3 to 35°C (Stephenson and Williams, 1981).

Thermal sensitivity of muscle excitability and its impact on Ca^{2+} transients

Loss of muscle excitability has been suggested to be one of the main causes for loss of muscle function in insects when entering chill coma (Esch, 1988; Goller and Esch, 1990; Hosler et al., 2000; MacMillan et al., 2014). In the present study, we stimulated the motor nerve using field stimulation and then measured the ensuing electrical response in muscle fibers at both high and low temperature. Some fibers only generated EPPs while other fibers generated APs in response to the EPP. Fibers with different AP waveform morphologies were identified (Fig. 2B–D) and characterized as simple APs, APs with a plateau or APs with two peaks. The depolarization during the AP is known in many insect species (honey bees, *Drosophila*, stick insect, dragonflies, damselflies) to be generated by the activation of L-type voltage-gated Ca^{2+} channels and not voltage-gated Na^{+} channels as in insect nerves or mammalian skeletal muscle. The influx of Ca^{2+} through the L-type Ca^{2+} channels triggers release of Ca^{2+} from the sarcoplasmic reticulum through a Ca^{2+}-induced Ca^{2+}-release mechanism (Huddart and Oates, 1970; Ashcroft and Stanfield, 1982; Takekura and Franzini-Armstrong, 2002; Collet and Belzunces, 2007; Collet, 2009; Frolov and Singh, 2013). The repolarizing current of the AP is primarily driven by two K^{+} currents – an early (I_A) and a delayed (I_K) outward current (Ashcroft and Stanfield, 1982; Salkoff and Wyman, 1983; Gorczynska et al., 1996). The early outward current has been shown to be Ca^{2+} activated, and hence to be more strongly activated with a large influx of Ca^{2+}. The observations of APs with two peaks could therefore reflect that a valley developed and caused the two peaks because a large, albeit transient, activation of I_A occurred. This agrees with the two-peaked APs being associated with the largest Ca^{2+} release events and, presumably, the most potent I_A activation.

Lowering the temperature resulted in a marked prolongation of both EPPs and APs (Fig. 2A–D). More interestingly, fibers that generated APs with a plateau or two peaks were almost absent at low temperature (Fig. 2E). Instead, low temperature resulted in a much higher proportion of fibers generating EPPs and simple APs. This clearly indicates that low temperature depresses the fiber's active membrane responses such that fibers become less excitable. Importantly, the occurrence of membrane responses after nerve stimulation at low temperature shows that the nerve and synapse are still functioning at low temperature. This well-preserved nerve function agrees with previous studies performed on cockroaches and locusts, where it has been shown that propagation of nerve signals can occur below chill coma temperature (Anderson and Mutchmor, 1968; Findsen et al., 2014). While we have shown that nervous and synaptic function is maintained at low temperature, we cannot exclude the possibility that low temperature caused neuronal dysfunction in the central nervous system. Previous studies on locusts and *Drosophila* have reported that entry into chill coma is sometimes associated with neuronal silence of the metathoracic ganglion and brain (Rodgers et al., 2010), and this halt in neuronal activity is in some instances coupled to an increase in $[K^{+}]$ in the extracellular environment of the neurons (Armstrong et al., 2012).

The loss of muscle fiber excitability that was observed at low temperature in this study reflected a clear depression in the L-type Ca^{2+} current. Thus, lowering of temperature resulted in a marked decrease in peak current and a right-shift of the membrane potential that was required to activate the current. Such effects of low temperature on Ca^{2+} current have also been found in other insect species. Frolov and Singh (2013) found that maximum Ca^{2+} current was approximately 4 times lower at 4°C compared with that at 21°C in larval *Drosophila*, and that the potential where peak current was highest was right-shifted from −15 mV at 21°C to 0 mV at 4°C. While the membrane potentials for maximum current activation in larval *Drosophila* are depolarized compared with our findings, the magnitudes of current depression and right-shift of activation at low

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temperature are very similar between the two studies. This suggests that the L-type Ca^{2+} current appears to be equally affected by low temperature in larvae and adult insects and this observation also suggests that the thermal sensitivity of these channels represents a general response as it is found in both dipterans (holometabolous insects) and orthopterans (hemimetabolous insects). In accordance with our observations, the peak Ca^{2+} current in stick insects was observed at around −10 mV (Ashcroft and Stanfield, 1982). The peak current in the stick insect at 4°C was, however, 2.5 times larger than what we found at 5°C, but this could be explained by the 2.5 times larger concentration of extracellular Ca^{2+} used in that study (Ashcroft and Stanfield, 1982).

Reduced L-type Ca^{2+} current can thus explain the reduced excitability at low temperature because it is this current that is responsible for the AP upstroke. It also explains the reduced force production at low temperature because this current triggers Ca^{2+} release via a Ca^{2+}-induced Ca^{2+}-release mechanism. The reduced Ca^{2+} current at low temperature would therefore result in a less efficient release of Ca^{2+} from the sarcoplasmic reticulum. Collectively, there are substantial data to support the conclusion, both from the current and previous studies (Ashcroft and Stanfield, 1982; Frolov and Singh, 2013), that depression of L-type Ca^{2+} current magnitude and a right-shift in its activation occur at temperatures where onset of muscle paralysis and chill coma are known to develop.

Conclusions

In the present study, we have examined the thermal sensitivity of several events in the E–C coupling of locust muscle fibers to determine the cellular mechanisms responsible for arrest of muscle function at temperatures close to the onset of chill coma. The data clearly indicate that cold-induced loss of leg muscle function is linked to the inability of the muscle fibers to release sufficient Ca^{2+} following nervous activation because low temperature caused a marked depression of the L-type Ca^{2+} current, which is responsible for both setting the excitability of the fibers and triggering release of Ca^{2+} from the sarcoplasmic reticulum. It is possible that this loss of L-type Ca^{2+} current is exacerbated, given that during cooling the muscle fibers become depolarized, possibly leading to inactivation of the channels. Depolarization of muscle fibers at low temperature has been observed in several studies of chill-sensitive insects and this depolarization is consistently associated with chill coma in various insects (Hosler et al., 2000; MacMillan et al., 2014). While our study strongly suggests that reduced function of L-type Ca^{2+} channels is central for the onset of chill coma, it cannot be excluded that other temperature-sensitive mechanisms also contribute to the arrest of movement at low temperature.

Chill coma onset has been shown to be a good proxy for cold tolerance in insects (Kimura, 2004; Chen et al., 2011; Kellermann et al., 2012; Andersen et al., 2015a), and the present study suggests that depression of the L-type Ca^{2+} current at low temperature is an important cellular mechanism for the onset of chill coma. Although other temperature-sensitive cellular mechanisms could also be contributing to loss of force at low temperature, it would be of great interest to examine the effect of acclimation on L-type Ca^{2+} currents. In addition, examining cold tolerance in different species could aid in further determining the different cellular mechanisms that are responsible for loss of muscle function and how evolution has shaped their cold tolerance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.F., J.O. and T.H.P. designed and conceived the research; A.F. and T.H.P. performed the experiments; A.F. and T.H.P. described and analyzed the data; A.F., T.H.P. and J.O. wrote the paper.

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