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RESEARCH ARTICLE

Cold-induced depolarization of insect muscle: differing roles of extracellular K^+ during acute and chronic chilling

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ABSTRACT

Insects enter chill coma, a reversible state of paralysis, at temperatures below their critical thermal minimum (CT_{min}), and the time required for an insect to recover after a cold exposure is termed chill coma recovery time (CCRT). The CT_{min} and CCRT are both important metrics of insect cold tolerance that are used interchangeably, although chill coma recovery is not necessarily permitted by a direct reversal of the mechanism causing chill coma onset. Nevertheless, onset and recovery of coma have been attributed to loss of neuromuscular function due to depolarization of muscle fibre membrane potential (V_m). Here we test the hypothesis that muscle depolarization at chill coma onset and repolarization during chill coma recovery are caused by changes in extracellular [K^+] and/or other effects of low temperature. Using *Locusta migratoria*, we measured *in vivo* muscle resting potentials of the extensor tibialis during cooling, following prolonged exposure to −2°C and during chill coma recovery, and related changes in V_m to transmembrane [K^+] balance and temperature. Although V_m was rapidly depolarized by cooling, hemolymph [K^+] did not rise until locusts had spent considerable time in the cold. Nonetheless, a rise in hemolymph [K^+] during prolonged cold exposure further depressed muscle resting potential and slowed recovery from chill coma upon rewarming. Muscle resting potentials had a bimodal distribution, and with elevation of extracellular [K^+] (but not temperature) muscle resting potentials become unimodal. Thus, a disruption of extracellular [K^+] does depolarize muscle resting potential and slow CCRT following prolonged cold exposure. However, onset of chill coma at the CT_{min} relates to an as-yet-unknown effect of temperature on neuromuscular function.

KEY WORDS: Ion balance, Thermal limits, Neuromuscular system, Potassium

INTRODUCTION

In order to make accurate predictions of the sensitivity of species to climate change, we must understand the physiological mechanisms that set critical thermal limits to animal performance and fitness (Hofmann and Todgham, 2010). The majority of insect species are chill susceptible, meaning physiological effects of low temperature that are unrelated to freezing set the lower thermal limits to their survival and fitness (Bale, 1993; Baust and Rojas, 1985). Insects enter a state of complete neuromuscular paralysis, termed chill coma when exposed to temperatures below their critical thermal minimum (CT_{min}) (Mellanby, 1939; Bale, 1996; Sinclair, 1999; Nedvěd, 2000; MacMillan and Sinclair, 2011a; Hazell and Bale, 2011). If the cold exposure is relatively mild or brief, chill coma is reversible, and the time required for an insect to recover its ability to stand following cold stress is termed chill coma recovery time (CCRT) (David et al., 1998). If the cold exposure is more severe, however, chill-susceptible insects progressively accumulate cold-induced injuries (chilling injury) that impair their ability to complete development, stand, walk or engage in mating behaviour (Findsen et al., 2013; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; Rojas and Leopold, 1996).

What measure of cold tolerance is most ecologically relevant and useful for predicting insect distribution is not entirely clear (but see Andersen et al., 2014), but CT_{min}, CCRT and the incidence of injury or death following a cold stress are all commonly used measures (e.g. Ayrinhac et al., 2004; Gaston and Chown, 1999; Gibert et al., 2001; Overgaard et al., 2014) and are generally regarded to be physiologically linked. In particular, CCRT is considered to require a reversal of the physiological state that induces chill coma (David et al., 1998; MacMillan and Sinclair, 2011a), and these two traits are generally considered equivalent measures of insect cold hardiness. Curiously, however, the CT_{min} and CCRT do not always correlate. Such a lack of correlation has been observed for insects that vary in cold tolerance, whether it be cold tolerance variation induced in a single species (*Drosophila melanogaster*) through thermal acclimation (Overgaard et al., 2011; Ransberry et al., 2011) or variation in cold tolerance among *Drosophila* species raised under common conditions (Andersen et al., 2014). These observations suggest that the mechanisms setting the CT_{min} and CCRT are at least partly independent (Findsen et al., 2014; MacMillan et al., 2012).

A striking and consistent effect of low temperature exposure is an increase in hemolymph [K^+] (and a corresponding depolarization of the muscle equilibrium potential for K^+; E_K), which can result from loss of hemolymph [Na^+] and water to the gut lumen (MacMillan and Sinclair, 2011b; MacMillan et al., 2012) and/or leakage of intracellular [K^+] down its concentration gradient from the tissues to the extracellular space (Andersen et al., 2013; Koštál et al., 2004). Chronic cold exposure has been shown to cause ions to leak toward equilibrium in representatives of Blattodea, Orthoptera and Hemiptera (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2012), and even brief cold exposures (e.g. 2 h at −4°C) can induce a marked disturbance of ion homeostasis in the migratory locust [*Locusta migratoria* (Linnaeus 1758)] (Andersen et al., 2013; Findsen et al., 2013).

Flight muscle fibre membrane potential (V_m) is strongly depolarized by exposure to low temperatures in both honeybees and *Drosophila* (Hosler et al., 2000), and V_m is particularly dependent on extracellular [K^+] (Hoyle, 1953; Wood, 1963), so an increase in hemolymph [K^+] could cause muscle depolarization during cooling. This hypothesis is, in part, supported by the observation that chill coma recovery is coincident with the

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reestablishment of normal hemolymph [K^+] in both crickets and locusts (Andersen et al., 2013; Findsen et al., 2013; MacMillan et al., 2012). However, studies of locusts (Findsen et al., 2014), crickets (MacMillan and Sinclair, 2011b) and cockroaches (Koštál et al., 2006) have all shown that insects may enter chill coma before any significant ionic disturbance manifests. This suggests that neuromuscular dysfunction at the CT_{min} is attributed to effects other than a disruption of ion balance, which occurs after the coma has already been induced (Findsen et al., 2014). In support of this alternative hypothesis, *in vitro* tetanic force production of locust muscle is strongly impaired by low temperature alone, even when extracellular [K^+] is maintained at 'normal' values, which indicates that factors other than high [K^+] limit muscle performance in the cold (Findsen et al., 2014).

On the basis of these earlier observations, the present study tests whether depolarization of muscle resting potential during cooling is caused by elevated hemolymph [K^+] or an alternate effect of temperature, and whether recovery from chill coma is dependent on the restoration of muscle V_m, achieved through restoration of low hemolymph [K^+]. We measure *in vivo* V_m in the extensor tibialis muscle of migratory locusts during cooling, prolonged exposure to −2°C and recovery from chill coma, and compare the extent of muscle V_m depolarization with intracellular and extracellular [Na^+] and [K^+]. To validate the *in vivo* findings, we also investigate the effects of temperature and [K^+] on muscle V_m under controlled *in vitro* conditions.

RESULTS

CCRT and survival after exposure to –2°C

The temperature of chill coma (CT_{min}) was not measured here, but all animals were in coma at 0°C when cooled at a rate of 0.15°C min^{-1}, which is also consistent with previous observations from the same colony [CT_{min} approximately 0.5°C (Findsen et al., 2014)]. Chill coma recovery upon the return to room temperature was evaluated using two criteria: (1) time to first extension of legs (indicating resumption of extensor tibialis function) and (2) time to resume a standing position (CCRT). Locusts recovering from an acute cooling to either 0 or −2°C extended their legs within 10 min of being placed at room temperature (Fig. 1B). Prolonged exposure to −2°C significantly increased the time required for leg extension ($F_{7,71}$=16.0, P<0.001; Fig. 1B), which took 19±1 min after 24 h in the cold. Similarly, CCRT was significantly slower with increasing cold exposure duration ($F_{7,54}$=20.2, P<0.001), and was highly variable: an increasing proportion of locusts were unable or unwilling to stand within 90 min of observation following more than 6 h exposure to −2°C (Fig. 1C). Finally, we assessed the incidence of chilling injury 24 h following removal from the cold using a five-point scoring system (see Materials and methods). Locusts had significantly lower survival scores (indicating more extensive chilling injury) as cold exposure duration increased (Kruskal–Wallis; χ^2=59.0, P<0.001; Fig. 1D).

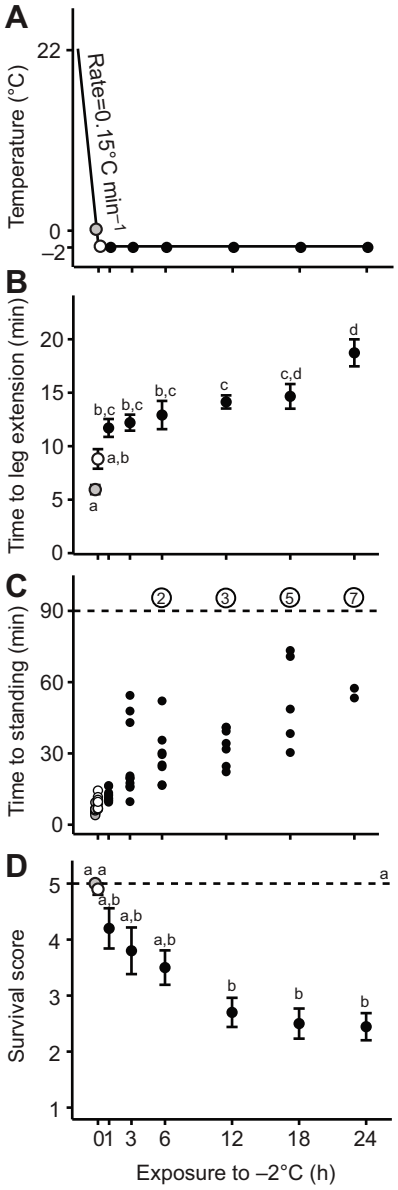

Fig. 1. Cold exposure slows recovery from chill coma and causes chilling injury. Cold exposure regime (A), time to leg extension (B), chill coma recovery time (C) and survival score (D) of *Locusta migratoria*. Locusts were exposed to a temperature ramp from 22 to −2°C at 0.15°C min^{-1} and were removed upon reaching 0°C (grey) or −2°C (open), or following 1–24 h at −2°C (black). Each point in C represents the recovery time of an individual locust, and all other values are means ± s.e.m. Horizontal dashed lines represent the maximum time (90 min) that locusts were observed for chill coma recovery (C) and the mean survival of untreated (control) locusts (D). Circled numbers are the number of locusts that did not stand within 90 min of observation. Values that share a letter within a panel do not significantly differ.

Muscle resting potential during cold exposure and recovery

We measured resting potentials in muscle fibres in response to acute changes in temperature (see Materials and methods for details). The resting potentials in fibres of the extensor tibialis of *L. migratoria* were highly variable (e.g. ranging from −29 to −77 mV at 30°C), and had a bimodal distribution (Fig. 2A inset). To examine the effects of temperature on each group, we separated the muscle fibres by type based on the local minimum value of probability density functions (see Materials and methods). At 30°C, one group of fibres [P1; adopting the naming convention of Jurkat-Rott et al. (Jurkat-Rott et al., 2009)] had a mean resting potential of 62±1 mV, and the other (P2) a mean of 37±4 mV.

Acute chilling significantly depolarized muscle resting potential ($F_{1,303}$=–323.6, P<0.001). Temperature had slightly different effects on the two groups of muscle fibres (interaction: $F_{1,303}$=33.9, P<0.001); a reduction in temperature from 30°C to 0°C caused ca. 26 mV of depolarization in P1 cells and ca. 21 mV of depolarization in P2 cells (Fig. 2A). As seen in supplementary material Fig. S1, the distribution of muscle resting potentials remained bimodal

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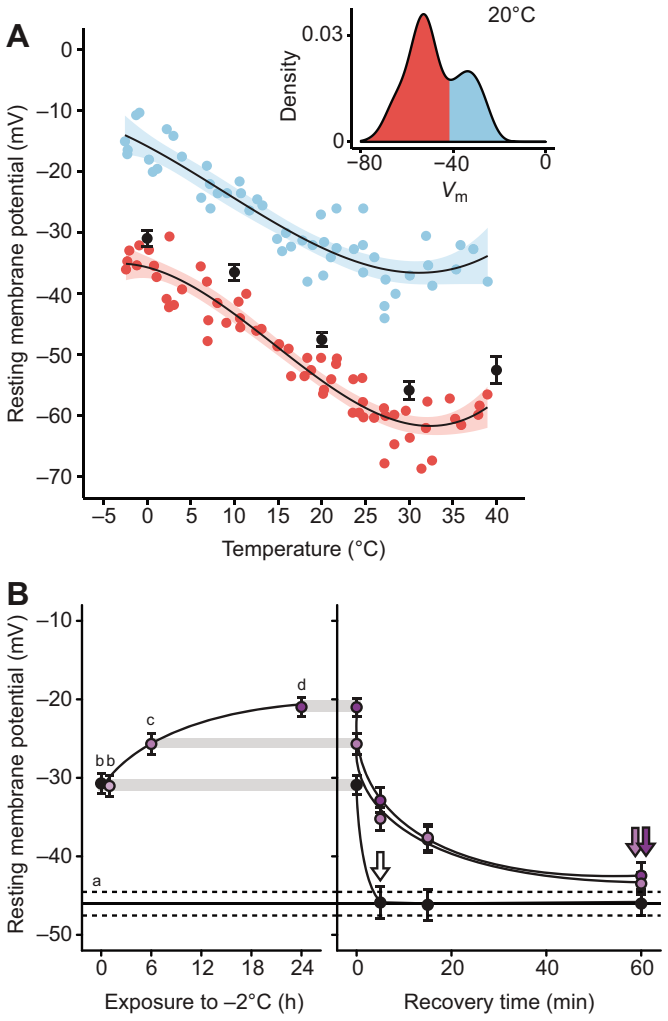

Fig. 2. Muscle resting potential of *L. migratoria* during cold exposure and recovery. Resting potentials (V_m) of fibres of the extensor tibialis muscle were measured *in vivo* during acute and chronic cold exposure (A) or during prolonged exposure and recovery from −2°C (B). (A) Mean resting potentials of two populations of muscle fibres, P1 (red) and P2 (blue), of individual locusts. Fibre populations were separated based on the local minimum of density distributions of resting potential (inset). Solid black lines are third-order polynomial lines of best fit (shaded regions indicate s.e.m.). Closed black circles represent the mean (±s.e.m.) resting potential of all fibres measured, binned by 10°C increments. (B) Membrane potentials during 0, 1, 6 or 24 h exposure to (left) and recovery from (right) −2°C (means ± s.e.m.). Solid and dashed horizontal lines represent the mean (±s.e.m.) muscle resting potentials of locusts kept at room temperature. Values that share a letter (left) do not significantly differ (Kruskal–Wallis rank sum test). Grey shaded regions connect the same data represented in both panels. Arrows (right) indicate the time point of recovery at which muscle resting potential no longer significantly differs from locusts that did not receive a cold exposure (Kruskal–Wallis). Solid black lines are for illustrative purposes and denote a significant change in V_m during cold exposure or recovery based on generalised linear models.

regardless of temperature, and the ratio of the two groups also remained fairly constant (ca. 71% P1 and 29% P2 fibres).

To examine the effects of chronic cold exposure and rewarming on muscle resting potential, we also measured muscle potentials in locusts that experienced up to 24 h at −2°C and during recovery at 22°C after 6 h at −2°C. The initial depolarization of V_m to −34±1 mV with acute cooling was exacerbated upon prolonged cold exposure ($F_{1,24}$=21.8, P=0.001), and reached mean resting potentials of −26±1 and −21±1 mV after 6 or 24 h at −2°C, respectively (Fig. 2B). If locusts were removed from the cold immediately upon reaching −2°C, muscle potentials significantly repolarized ($F_{1,26}$=11.2, P=0.002) within 5 min to match the mean resting potential observed in control locusts at room temperature (Fig. 2B). If left at −2°C for 6 or 24 h, the locust muscle fibres significantly repolarized (6 h: $F_{1,36}$=28.8, P<0.001; 24 h: $F_{1,36}$=28.7, P<0.001) upon return to room temperature, but here the initial repolarization was incomplete after 5 min and was followed by a slower repolarization to near control levels after 60 min of recovery (Fig. 2B).

Although still bimodal upon reaching −2°C, the distribution of V_m became unimodal in the muscle of locusts exposed to 6 or 24 h at −2°C (those exposed to chronic chilling), and remained unimodal after 5 min of recovery at room temperature (despite complete rewarming of the leg; supplementary material Fig. S2). The distributions of V_m in recovering locusts became bimodal again after 15 or 60 min of recovery (supplementary material Fig. S2).

The loss and recovery of extracellular ion balance

We measured concentrations of Na^+ and K^+ in the hemolymph and muscle tissue of locusts ramped to and held at −2°C for up to 24 h, and also following recovery at room temperature (for 5, 15 or 60 min) in locusts exposed to 6 h at −2°C. Cold exposure significantly elevated hemolymph [K^+] from 8.1±0.3 mmol l^{-1} under control conditions to 31.0±1.4 mmol l^{-1} after 24 h at −2°C ($F_{3,45}$=22.0, P<0.001; Fig. 3A). The same cold exposure also reduced hemolymph [Na^+] from 77.2±0.8 to 62.3±3.3 mmol l^{-1} ($F_{3,45}$=4.7, P=0.005). Intracellular [K^+] concentration was not altered by cold exposure ($F_{3,44}$=0.8, P=0.482), and remained close to 105 mmol l^{-1}, but cold exposure caused a small but significant reduction in intracellular [Na^+] from 26.4±2.4 to 18.6±1.1 mmol l^{-1} ($F_{3,44}$=3.1, P=0.037; Fig. 3B).

When locusts were removed to room temperature following 6 h at −2°C, extracellular [K^+] recovered significantly from 18.3±1.2 to 13.6±0.9 mmol l^{-1} ($F_{4,53}$=23.9, P<0.001), but had not reached control [K^+] concentrations (8.1±0.3 mmol l^{-1}) after 60 min of recovery (Fig. 3A). Intracellular [Na^+] did not change significantly during recovery from 6 h at −2°C ($F_{4,54}$=2.4, P=0.058), but intracellular [K^+] increased significantly during chill coma recovery from 106.0±1.8 to 123.1±4.0 mmol l^{-1} ($F_{4,55}$=5.1, P=0.001; Fig. 3B). Locust muscles also accumulated ~10% of their wet mass in additional water during the 60 min recovery period ($F_{4,55}$=7.0, P<0.001; supplementary material Fig. S3).

The net effects of the observed changes in ion concentrations were used to estimate muscle equilibrium potentials for K^+ (E_K) and Na^+ (E_{Na}). E_K was progressively depolarized during chilling from −65±2 to −55±1 mV when temperature was reduced from 20 to −2°C, and further to −32±4 mV after 24 h at −2°C ($F_{3,45}$=39.3, P<0.001). In contrast, E_{Na} did not significantly change from ca. +28 mV ($F_{3,45}$=0.6, P=0.625; Fig. 3C), because of parallel reductions in extracellular and intracellular [Na^+]. During recovery from 6 h at −2°C, E_{Na} remained relatively stable, although there was a significant difference in E_{Na} between the 5 and 60 min recovery time points ($F_{4,52}$=2.9, P=0.030; Fig. 3C). Muscle E_K was restored from −42±2 to −56±2 mV during the 60 min recovery period that followed the 6 h at −2°C ($F_{4,55}$=30.6, P<0.001). Although E_K was repolarized during recovery, the decrease in hemolymph [K^+] (Fig. 3A) and increase in muscle intracellular [K^+] we observed (Fig. 3B) were not sufficient to completely re-

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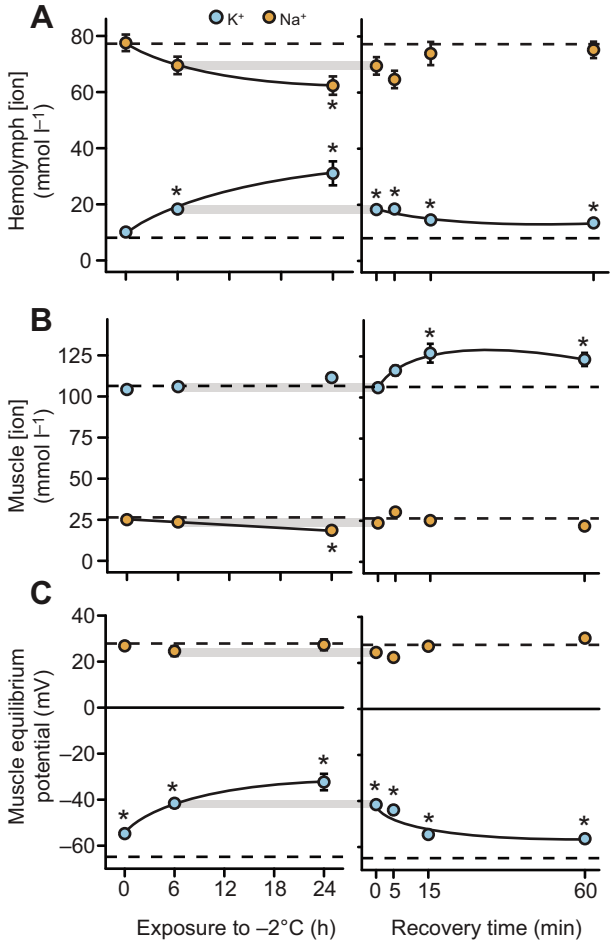

Fig. 3. The loss and recovery of ion balance during cold exposure and recovery. Hemolymph (A) and muscle (B) concentrations and calculated equilibrium potentials (C) of Na^+ (orange) and K^+ (blue) in *L. migratoria* in relation to duration of exposure to −2°C (left) and recovery time at room temperature following 6 h at −2°C (right). Dashed lines represent the mean value from locusts that did not receive cold exposure, and asterisks denote values that significantly differ from this line (based on Tukey's HSD). Shaded regions connect the same data represented in both panels. Solid black lines connecting points illustrate a significant effect of exposure duration or recovery time on ion concentration or equilibrium potential. Data presented are means ± s.e.m. Error bars that are not visible are obscured by the symbols.

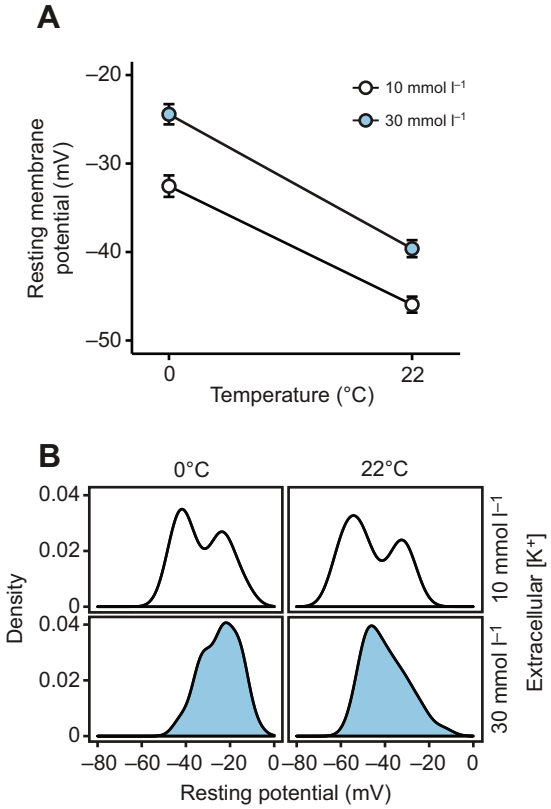

Fig. 4. The effects of hyperkalemia and low temperature on muscle resting potential *in vitro*. Mean (±s.e.m.) values (A) and probability density distributions (B) of resting membrane potentials in the extensor tibialis muscle of *L. migratoria* measured at 22 and 0°C in the presence of either 10 mmol l^{-1} (open) or 30 mmol l^{-1} K^+ (blue). Both low temperature and high $[K^+]$ significantly depolarized muscle V_m, with no interaction (see Results for details of analysis).

establish E_K to resting levels after 60 min of recovery at room temperature (Fig. 3C).

The independent and combined effects of K^+ and low temperature on V_m

To examine the individual and possible interactive effects of low temperature and high extracellular $[K^+]$ on muscle resting potential, we measured V_m in the extensor tibialis muscle *in vitro* using a simulated locust hemolymph saline with low and high $[K^+]$ (10 or 30 mmol l^{-1} K^+). Both low temperature ($F_{3,23}$=3.2, P=0.004) and high extracellular $[K^+]$ ($F_{3,23}$=3.4, P=0.003) independently depolarized muscle fibres, with no interaction ($F_{3,23}$=0.5, P=0.590; Fig. 4A). Cooling muscles from 22 to 0°C depolarized muscle fibres by ca. 14 mV, and elevating $[K^+]$ from 10 to 30 mmol l^{-1} caused ca. 8 mV of depolarization. Increasing $[K^+]$ also caused the bimodal distribution of resting membrane potential to become unimodal (Fig. 4B), as was observed *in vivo* in locusts that had elevated hemolymph $[K^+]$ following 6 or 24 h at −2°C (supplementary material Fig. S2).

DISCUSSION

Chilling depolarizes insect muscle fibres

Most insects lose their ability for coordinated motion when cooled. There are several possible explanations for this loss of neuromuscular function at low temperature (see further discussion below), but the currently favoured hypothesis suggests that a temperature-induced depolarization of V_m drives a loss of muscle excitability, leading to chill coma (Esch, 1988; Findsen et al., 2014; Goller and Esch, 1990; Hosler et al., 2000; MacMillan and Sinclair, 2011a). Similar to previous studies on temperature and muscle resting potential, we found that chilling caused a severe depolarization of muscle resting potential (V_m) in the extensor tibialis of locusts (Fig. 2A). This observation is in keeping with Hosler et al. (Hosler et al., 2000), who noted a similar effect of temperature on V_m in the flight muscles of both honeybees (*Apis mellifera*) and fruit flies (*Drosophila melanogaster*). These two species differed in chill coma onset temperature (10 and 5°C, respectively) but had a similar V_m at their respective chill coma temperatures (−40 and −45 mV), which led the authors to suggest that this resting potential may represent a critical V_m at which muscle excitability is lost (Hosler et al., 2000). In the present study, the V_m of locust extensor tibialis fibres were variable, however, and many fibres had a more positive V_m than this

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suggested threshold, even at high temperatures (Fig. 2A). The breadth of the V_m distribution (which was consistent regardless of temperature; supplementary material Fig. S1) would thus ensure that if a threshold resting potential for muscle failure exists, different fibres would cross the threshold at different temperatures, leading to a gradual reduction in the proportion of fibres that are excitable during cooling. The difference in the distribution of muscle potentials observed in this study and by Hosler et al. (Hosler et al., 2000) could be attributed to interspecies variation or differences in muscle fibre types, but it could also be an artefact of the manner in which V_m was sampled. In the present study we used two criteria for our measurements of V_m: a sharp change in potential upon entry into a fibre and that electrode resistance was not altered after entry into a cell. By contrast, Hosler et al. (Hosler et al., 2000) only accepted fibres with a resting potential between −60 and −70 mV at 24°C. Such fibres would represent the most hyperpolarized population of cells found in the extensor tibialis of locusts (P1), which indeed depolarized beyond ca. −40 mV at the temperature that induces chill coma (ca. 0°C; supplementary material Fig. S4).

Chill coma is not caused by a disruption of K^+ balance

The present study extends the observation that insect muscle cells are depolarized during chilling to Orthoptera, but also shows that this initial depolarization is largely independent of extracellular $[K^+]$. Hemolymph $[K^+]$ of locusts that had just reached −2°C did not differ from that of control locusts, and locusts removed from the cold immediately recovered muscle potential when their body temperature was rewarmed to room temperature (Fig. 2B). This follows previous observations that hemolymph $[K^+]$ is not immediately altered during cooling, despite insects crossing their CT_{min} and entering chill coma (Findsen et al., 2014; Koštál et al., 2006; MacMillan and Sinclair, 2011b). This supports the notion that the depolarization of muscle V_m, suspected to be a possible cause of chill coma onset, is not necessarily caused by high extracellular $[K^+]$ (Findsen et al., 2014).

It has been well demonstrated that the muscle cell resting potential of insects is highly sensitive to extracellular $[K^+]$, owing to the high permeability of the muscle cell membrane to K^+ (Fig. 4) (Hoyle, 1953; Wood, 1963), but a number of other factors contribute to V_m in muscle cells. Resting potential can be considered a simple sum of currents creating a diffusion potential (V_d) as expressed by the Goldmann equation plus an electrogenic contribution (V_e) caused by the uneven pumping of charged ions by ion-motive pumps (Casteels et al., 1973; Djamgoz, 1987). The contributions of temperature, ion concentrations and the permeability of the membrane to each ion determine V_d, as illustrated by the (simplified) Goldmann equation:

$$V_d = \frac{RT}{F}\ln\left(\frac{P_{Na}[Na^+]_o + P_K[K^+]_o + P_{Cl}[Cl^-]_i}{P_{Na}[Na^+]_i + P_K[K^+]_i + P_{Cl}[Cl^-]_o}\right), \quad (1)$$

where R is the universal gas constant, T is temperature, F is Faraday's constant, P_{ion} is permeability of the membrane to the ion, $[ion]_o$ is the concentration of the ion in the extracellular compartment and $[ion]_i$ is the intracellular concentration. From this equation, it is evident that changes in ion concentration or permeability can affect V_d and also that lowering temperature directly affects V_d. However, cooling from 30 to 0°C (303 to 273K) can only explain ca. 4–5 mV of the ca. 25 mV of V_m depolarization observed during cooling (Fig. 2A). Cooling locusts to −2°C did not immediately alter $[Na^+]_o$ or $[Na^+]_i$ of the muscle (Fig. 3B), and Cl^- is generally regarded to be passively distributed in most insect muscles and therefore contribute little to V_m (Djamgoz, 1987; Fitzgerald et al., 1996; Rheuben, 1972). If the remaining 20 mV of depolarization is driven by a change in V_d, it may thus result from a cold-induced increase in P_{Na}, or decrease in P_K, which would drive membrane potential toward the muscle equilibrium potential for Na^+ (E_{Na}=+28 mV; Fig. 3C) (Wareham et al., 1974).

The V_e results from the current induced by an imbalance of charge moved across the cell membrane by primary ion pumps, such as Na^+/K^+-ATPase, Ca^{2+}-ATPase or V-ATPase (Emery et al., 1998; Rheuben, 1972). V_e is given by the equation:

$$V_e = i_p R_m , \quad (2)$$

where i_p is the net current transported and R_m is membrane resistance. Rates of catalysis by ion pumps are slowed by temperature with a Q_{10} of between 2 and 3 (Galarza-Muñoz et al., 2011), which would reduce i_p and thus depolarize V_m (Findsen et al., 2014; Hosler et al., 2000; MacMillan and Sinclair, 2011a; Zachariassen et al., 2004). Slowing of Na^+/K^+-ATPase is often cited as a possible cause of neuromuscular failure at extreme temperatures (Hosler et al., 2000; MacMillan and Sinclair, 2011a; Rodgers et al., 2010; Sinclair et al., 2004), but to our knowledge the contribution of Na^+/K^+-ATPase to the V_m of insect muscle has not been studied in relation to temperature. Cooling has been shown to increase the effective R_m of muscle fibres of both a moth (Rheuben, 1972) and the desert locust (Kornhuber and Walther, 1987). Assuming this is also the case for muscles of the migratory locust, the proportional decrease in the active ion transport current (i_p) would have to be greater than the proportional increase in membrane resistance in order for chilling to depolarize V_e. Otherwise, a similar V_e could be produced by the smaller current (Rheuben, 1972). Thus, we suggest that depolarization of muscle V_m during cooling is caused by either an increase in P_{Na}:P_K, a slowing of electrogenic pumps that is greater in magnitude than a concurrent increase in membrane resistance, or some combination thereof. Such changes in membrane resistance, ion pump activity and ion permeability may manifest directly from temperature effects on tertiary and quaternary structure of ion pumps and channels, or indirectly through temperature effects on the conformational order of the lipid bilayer (Else and Wu, 1999; Hazel, 1995; Overgaard et al., 2008).

Other effects of cooling that could cause chill coma

Although we provide further evidence for depolarization of muscle V_m with chilling, it remains unclear whether chill coma onset is indeed caused by this depolarization, because the existing evidence supporting this is all of a correlative nature (Findsen et al., 2014; MacMillan and Sinclair, 2011a). The effects of temperature on other aspects of neuromuscular signal transmission and excitation–contraction coupling have received little attention, but may strongly contribute to the cold-induced decrease in muscle performance (Findsen et al., 2014; MacMillan and Sinclair, 2011a). Spontaneous electrical activity continues in the central nervous system of cockroaches below chill coma onset temperature (Anderson and Mutchmor, 1968). In both *Drosophila* and locusts, however, exposure to low temperatures causes rapid surges of extracellular K^+ that can lead to neuronal silence (Armstrong et al., 2012; Rodgers et al., 2010), and the role of the nervous system in chill-coma recovery has not been studied, so it remains unclear how nervous system function may influence the CT_{min} and CCRT. At the level of the *Drosophila* synapse, Ca^{2+} clearance rates are slowed at high temperatures, and signal transmission is impaired because of high thermal sensitivity of synaptic Ca^{2+}-ATPase (Klose et al., 2009).

Although signal transmission at the synapse has not been studied in insects at low temperatures, it has been suggested that slowing of Ca^{2+}-ATPase and/or reduced membrane fluidity at the synapse could lead to neuromuscular silence (MacMillan and Sinclair, 2011a). In larval *Drosophila* muscles, L-type Ca^{2+} channels are responsible for both the rising phase of action potentials and excitation–contraction coupling, and these channels have slower kinetics and conductance rates for Ca^{2+} in the cold (Frolov and Singh, 2013). Lastly, a convincing body of literature from ectothermic vertebrates suggests that cold impairs myosin ATPase function, and that thermal adaptation of polar and temperate species is associated with improved low-temperature performance of the contractile apparatus (e.g. Johnston and Altringham, 1985; Mutungi and Johnston, 1987). Thus, many aspects of neuromuscular signal transmission and muscle excitation–contraction coupling could be impaired during exposure to low temperatures, and the CT_{min} could be set by the effects of temperature on any one (or combination) of these aspects in addition to depolarization of muscle V_m.

The role of K^+ in chilling injury and chill coma recovery

When we held locusts at −2°C for up to 24 h, we observed a further 10 mV of V_m depolarization (measured *in vivo*; Fig. 2B). We simulated the increase in $[K^+]$ *in vitro* by comparing V_m at 10 and 30 mmol l^{-1} K^+, which caused 8 mV of V_m depolarization at 0°C (Fig. 4A). Thus, in contrast to depolarization observed during acute cooling, the additional depolarization observed during chronic cold exposure can be almost completely attributed to increased $[K^+]$ in the hemolymph that depolarizes the muscle equilibrium potential for E_K. Given that cooling has already substantially depolarized the muscle fibres, this further depolarization may lead to injury in muscle and other tissues through activation of apoptotic signalling cascades (Boutilier, 2001; Hochachka, 1986). It is worth noting, however, that the supposed mechanistic link between high levels of cell death observed in insect tissues following cold exposure (Yi and Lee, 2011; Yi et al., 2007) and the progressive loss of ion and water balance at low temperatures (Findsen et al., 2013; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b) remains an unconfirmed hypothesis.

In contrast to locusts that were immediately removed from the cold upon reaching −2°C, locusts that experienced 6 or 24 h at −2°C only partly recovered V_m in the first 5 min following removal from the cold, and only approached 'normal' muscle V_m after 60 min of recovery at room temperature (Fig. 2B). This means that after resting potential has been disrupted by chronic cold exposure, recovery is separated into two phases (fast and slow). The fast phase of V_m recovery is driven by a rapid reversal of the (undetermined) temperature effect on muscle resting potential (see discussion above). By contrast, the slow phase of V_m recovery is attributable to the gradual recovery of normal ion balance and particularly the distribution of K^+ (Andersen et al., 2013; Findsen et al., 2013; MacMillan et al., 2012). The restoration of E_K is driven by both a reduction in hemolymph $[K^+]$ (Fig. 3A) that depends on restoration of whole-organism ion and water balance, and a simultaneous rise in intracellular $[K^+]$ facilitated by local transport mechanisms at the muscle cell membrane (supplementary material Fig. S3) (Andersen et al., 2013; MacMillan et al., 2012).

The independent effects of temperature and K^+ on muscle resting potential

As discussed above, we observed that muscle fibres of the extensor tibialis could be separated into two groups, characterised by a relatively high or low V_m. Although cooling depolarized the mean V_m, it did not alter the modality of muscle resting potentials, which remained bimodal regardless of temperature (supplementary material Fig. S1). High $[K^+]$ also depolarized fibres in the extensor tibialis, but the mean magnitude of this effect was smaller than the depolarization caused by temperature alone (Fig. 4A). Interestingly, muscle resting potentials become unimodal *in vitro* under conditions of high extracellular $[K^+]$ (Fig. 4B). In addition, *in vivo* distributions of V_m were unimodal after locusts had spent 6 or 24 h at −2°C (which also caused increased extracellular $[K^+]$) and remained unimodal after rewarming (after 5 min of recovery). Membrane potentials only began to appear bimodal again after 15 min of recovery, whereupon $[K^+]$ levels had reduced toward resting levels (Fig. 3C; supplementary material Fig. S2). Thus, the effect of temperature on V_m was similar in all fibres, while the effect of elevated $[K^+]$ seems to either 'transform' one population of fibres to the other or more strongly depolarize one group of fibres. Similar bimodal distributions of muscle V_m have been described in both humans and rats (Jurkat-Rott et al., 2009). In mammals, these two modes represent fibres that switch states depending upon external $[K^+]$, such that a proportion of the fibres are more depolarized than would be expected by the Nernst equilibrium for K^+ (i.e. V_m is more reliant on the distribution of other ions such as Na^+). Our findings suggest that fibres of the locust extensor tibialis muscle can similarly adopt one of two states, the relative proportions of which are sensitive to external $[K^+]$. How alleviation of this secondary effect of extracellular $[K^+]$ may be important for muscle excitability and force generation during chill coma recovery remains to be explored. Temperature and high extracellular $[K^+]$ both reduce tetanic force production in the locust extensor tibialis (Findsen et al., 2014), and it is possible that this reduction in force is driven by the independent depolarizing effects of cooling and a loss of ion balance. Low temperature and high $[K^+]$, however, synergistically reduce muscle force (Findsen et al., 2014), and we found no significant interaction in the effects of these two factors on V_m (Fig. 4A). This discrepancy further suggests that temperature effects on muscle performance are not entirely V_m dependent.

Conclusions

Both low temperature and high $[K^+]$ independently depolarize locust muscle fibres, but these effects are disconnected in time during a cold exposure. Because ion homeostasis is maintained during acute cooling, it will be the 'temperature effect' that is most relevant for when and at what temperature the animal enters chill coma. In contrast, we provide further evidence that the recovery from chill coma is highly dependent on the 'potassium effect', and that a progressive rise in $[K^+]$ is correlated with the development of chilling injury. Thus, the phenomena of chill coma, chilling injury and chill coma recovery appear to be mechanistically independent, which explains why the CT_{min} and CCRT are poorly correlated (Overgaard et al., 2011; Ransberry et al., 2011; Andersen et al., 2014). As such, investigation of the evolution and plasticity of insect cold tolerance may yield different molecular targets of cold tolerance selection depending on the measure of cold tolerance used, and accurate integration of this information will require careful study of the mechanisms underlying each of the traits: CT_{min}, CCRT and chilling injury. Toward this goal, we suggest that the following questions are most critical to address: (1) what is the cause of low temperature depolarization; (2) why and how does $[K^+]$ affect the distribution of muscle V_m; (3) how are other aspects of excitation–contraction coupling affected by low temperature and high extracellular $[K^+]$; and (4) does cell depolarization at low temperatures cause cell death and chilling injury?

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MATERIALS AND METHODS

Animal husbandry

A breeding colony of locusts (*Locusta migratoria*) was established from fifth instar nymphs purchased from a commercial supplier in 2012 (Peter Andersen Aps, Fredericia, Denmark). Locusts were reared in cages (0.45 m^3) containing metal screening and egg trays to facilitate hiding and moulting in a room at 22°C (12 h:12 h light:dark cycle). During the day, a 150 W heat lamp was used to create a thermal gradient from 25 to 45°C, thereby allowing for behavioural thermoregulation. Locusts were fed daily with fresh wheat sprouts and had access to wheat bran and water *ad libitum*. One to two days after imaginal ecdysis, adult locusts were separated by sex and all locusts used for experiments were 7–14 days post-final moult. Throughout all experiments we used locusts of both sexes in a 1:1 ratio, but as we observed no differences related to sex this factor was not included in the final analysis.

CCRT and chilling injury

To measure CCRT and the incidence of chilling injury following cold exposure, locusts were placed inside 50 ml polypropylene centrifuge tubes and suspended in a programmable circulating temperature bath set at 20°C. The bath contained a mixture of ethylene glycol and water (1:1 v/v) and temperature was adjusted according to the recordings of eight thermocouples placed in random tubes to record the air temperature experienced by the locusts inside the tubes. Following a 15 min hold at 20°C, the temperature was reduced to −2°C at −0.15°C min^{-1} (ca. 150 min), after which temperature was maintained at −2°C for up to 24 h. At several time points, 10 locusts (five of each sex) were removed from the bath and placed at room temperature (22°C) to observe recovery. Thus locusts were sampled when temperature reached 0 and −2°C, and after being held at −2°C for 1, 3, 6, 12, 18 and 24 h. Locusts removed from the cold were gently taken out of the tube using tweezers and positioned on their side onto a table lined with filter paper. The 10 locusts were observed for 90 min by two observers who recorded time to recovery of muscle function (identified as a controlled extension of either hind leg) and CCRT of each individual (defined as standing). Following recovery, each locust was placed back into its 50 ml tube with wheat bran and water and returned to 22°C. Injury was assessed 24 h following removal from the cold by removing the locusts from their tube and coaxing them to walk and jump on a filter paper surface. The incidence of chilling injury was recorded on a five-point scale as follows: 5: able to stand, walk and jump in a coordinated manner; 4: able to stand, walk and/or jump, but some lack of coordination; 3: able to stand, but unable or unwilling to walk or jump; 2: moving, but unable to stand; 1: no movement observed (i.e. dead). Ten locusts were placed inside 50 ml tubes with food and water and assessed under the same conditions without having experienced any cold to control for recovery conditions. None of these locusts showed any signs of injury.

In vivo measurement of muscle cell resting potential

Muscle cell potentials were recorded using Clark borosilicate glass microelectrodes (GC100TF; Warner Instruments, Hamden, CT, USA) pulled to a tip resistance of 5–10 MΩ using a Flaming-Brown P-97 electrode puller (Sutter Instruments Co., Novato, CA, USA). A chlorinated silver wire reference was used to complete the circuit. The electrodes were connected to an Electro 705 differential electrometer (World Precision Instruments Inc., Sarasota, FL, USA), and a 1401 Micro3 data acquisition system connected to a computer running Spike2 software (v8, Cambridge Electronic Design, Cambridge, UK).

To record muscle cell potential during cooling, locusts were held in place on their ventral surface in a putty made from beeswax, resin and paraffin oil (17:2:1 v/v/v) that was mounted on a plastic plate cooled by a programmable temperature bath. The spiracles along the left side of the abdomen were left free of the putty to permit gas exchange. The left hind leg was embedded in the putty with its dorsal surface of the femur left accessible for dissection and a thermocouple (type K, connected to a computer via a TC-08 interface, Pico Technologies, St Neots, UK) was embedded in the putty in contact with the ventral surface of the femur. Locust sampled at temperatures between 20 and 40°C were positioned individually on the pre-warmed plate and left for 10 min to stabilize body temperature (T_b) before sampling. To sample locusts during a temperature ramp from 20 to −2°C, groups of four locusts were positioned on the plate in putty at 20°C. Following a 15 min hold at 20°C, the temperature was ramped as in the CCRT and chilling injury experiment, and membrane potentials were sampled in random order as their individual target temperatures were reached.

To access fibres of the extensor tibialis muscle, a small (~5 mm) incision was made lateral to the most dorsal cuticular ridge of the femur. Two incisions perpendicular to the first incision were made to create a 'window' into the underlying muscle bundles that was held open by a pin without damaging muscle attachment points or tracheal oxygen supply. The reference electrode was gently hooked under the cuticle in contact with the hemolymph and one muscle fibre was penetrated in each of six visible fibre bundles using the glass microelectrode. A sharp drop in the measured potential without any change in electrode resistance identified the presence of the electrode inside a cell. Six muscle cells from different fibre bundles were sampled from each of 62 locusts at their individual target temperature (ca. 3–5 min per animal; 370 fibres in total). Immediately following measurement, the thermocouple was removed from the ventral surface of the femur and placed inside the opening in the leg to accurately confirm muscle temperature, which was always ±0.5°C of temperature of the putty.

Measurement of muscle cell potential following prolonged exposure to −2°C was conducted as described above, but locusts were individually positioned in putty on a glass microscope slide before being placed inside a 50 ml tube and cooled as described in the CCRT and chilling injury experiment (n=6 locusts per cold exposure). A thermocouple was embedded in the putty immediately adjacent to the leg. Following 1, 6 or 24 h at −2°C, the glass slide with the locust attached was removed from the bath and secured on the plate, which was pre-cooled to maintain locust T_b at −2°C throughout sampling. To measure the recovery of muscle potential during rewarming following 6 and 24 h at −2°C, the same method was used, but the plate was maintained at 22°C and recovery time was measured from the time at which the locust was placed on the pre-warmed plate (n=6 locusts per cold exposure/recovery time). The rate of T_b rise measured in this experiment was somewhat faster than the rate of T_b recovery in locusts removed from the bath and positioned on a laboratory bench at 22°C. Consequently, locusts had rewarmed to >20°C 5 min after removal from the cold (supplementary material Fig. S2).

In vitro muscle cell resting potential measurement

Resting potentials of fibres in the extensor tibialis were also measured *in vitro* in isolated locust femurs to isolate the effects of temperature and high K^+ on the muscle. A single hind femur was removed from locust maintained at rearing conditions. The muscle fibres were exposed as described for the *in vivo* measurements and the dissected leg was quickly positioned in a double-walled glass Petri dish containing standard locust buffer [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)], the temperature of which was controlled by a programmable temperature bath circulating a 1:1 (v/v) mixture of ethylene glycol through the walls and base of the dish. To maintain high oxygen availability, ambient air was gently bubbled through the buffer solution. Following a 5 min equilibration period, six muscle resting potentials were measured from each leg as described above. The buffer solution was then extracted from the dish using a syringe, and replaced with a buffer containing 30 mmol l^{-1} K^+ (to simulate the high hemolymph [K^+] observed following 24 h at −2°C) and 120 mmol l^{-1} Na^+ (to maintain osmotic neutrality). After 5 min of equilibration in the high K^+ saline, six muscle potentials were recorded. These experiments using normal and high levels of K^+ were performed at both 22 and 0°C.

Measurement of intra- and extracellular ion concentrations

Concentrations of Na^+ and K^+ were measured in the hemolymph and muscle tissue of locusts under control conditions, upon reaching −2°C, following 6 and 24 h at −2°C, and following 5, 15 and 60 min of recovery at 22°C from a 6 h exposure to −2°C (n=11–13 locusts per treatment). All cold exposures were completed as described in the CCRT and chilling injury experiment. Hemolymph samples (>5 µl hemolymph) were collected using capillary tubes (10–25 µl) from an opening in the neck membrane, between the head and thorax. A 5 µl aliquot of the hemolymph was then transferred by pipette to a microcentrifuge tube containing 2 ml

of 100 ppm lithium buffer (Sherwood Scientific Ltd, Cambridge, UK). Immediately after hemolymph collection, muscle tissue was dissected from both hindlegs, blotted dry of hemolymph on a kimwipe, and transferred to a pre-weighed 2 ml microcentrifuge tube (total sampling time was ca. 30 s per locust). The tube containing muscle tissue was weighed to obtain muscle wet mass, dried for 24 h at 60°C and reweighed to determine dry mass and muscle water content. The dried muscle was dissolved in 200 µl of milli-Q water, homogenized using a tissue lyser (Tissuelyser LT, Qiagen, Hilden, Germany) at 50 Hz for 20 min, and then centrifuged (10,000 ***g*** for 20 min). From each sample, aliquots of 5 and 20 µl of the supernatant were then transferred to separate 2 ml 100 ppm lithium buffer solutions. The two different sample volumes were used to ensure that both intracellular [Na^+] and [K^+] could be determined within the sensitivity range of the flame photometer. [Na^+] and [K^+] was measured from the hemolymph and muscle samples using a Sherwood flame photometer (Model 420, Sherwood Scientific Ltd, Cambridge, UK) and concentrations were calculated relative to standard samples of known concentration. Intracellular muscle concentrations were calculated using muscle water content and corrected assuming 4% hemolymph by muscle volume using a simple weighted average of intracellular and extracellular concentration (MacMillan et al., 2012):

$$\text{corrected } [X]_\text{i} = \frac{[X]_\text{i} - (0.04[X]_\text{o})}{0.96}, \quad (3)$$

where $[X]_\text{o}$ is the concentration of the ion measured in the extracellular fluid and $[X]_\text{i}$ is the concentration of the ion measured in the muscle. Muscle equilibrium potentials for Na^+ and K^+ were then calculated from [K^+] and [Na^+] in the extracellular and muscle intracellular fluid (corrected values) using the Nernst equation:

$$E_\text{X} = \frac{RT}{zF} \ln \frac{[X]_\text{o}}{[X]_\text{i}}, \quad (4)$$

where R is the universal gas constant, T is temperature in Kelvin (−2°C for all samples taken directly from the cold treatment and 22°C for all other samples prior to cold treatment or during recovery), z is the ionic charge and F is the Faraday constant.

Data analysis

All data analysis was completed in R v. 3.0.1 (R Development Core Team, 2013). The effects of duration of cold exposure on time to leg movement, as well as cold exposure duration and recovery time on hemolymph and muscle ion concentrations and muscle equilibrium potentials, were each analysed by one-way ANOVA followed by Tukey's honestly significant difference (HSD) test for pairwise analysis among time points. Locust survival scores were compared among cold exposure durations using a Kruskal–Wallis rank-sum test followed by multiple comparisons using the kruskalmc() function in the pgirmess package (Giraudoux, 2013).

Muscle potentials measured *in vivo* during a temperature ramp had a bimodal distribution (Fig. 2A inset; supplementary material Fig. S1). To examine the effect of temperature on the two apparent groups of muscle fibres, we generated density distributions of muscle resting potentials (binned by 10°C increments; Gaussian error distribution) and used the minimum value between modes as a discriminating resting potential to separate the two datasets (all distributions and discriminating potentials are presented in supplementary material Figs S1 and S2). The effect of temperature on resting potential was analysed by generalised linear model with the animal sampled treated as a random effect. Following prolonged exposure to −2°C, muscle equilibrium potentials became unimodal (supplementary material Fig. S2), so the effects of cold exposure duration and recovery on muscle potentials were calculated without separating the resting potentials by fibre type. The effects of cold exposure duration and recovery time (from each cold exposure duration) were independently analysed using generalised linear models with cold exposure duration or recovery time treated as a fixed factor and the animal sampled treated as a random effect. Resting potentials were also compared among cold exposure durations and recovery time points using Kruskal–Wallis tests (ignoring the effect of the animal sampled) to generate *post hoc* comparisons shown in the figures. The effects of temperature and extracellular [K^+] on *in vitro* muscle potentials were analysed by generalised linear model, with both factors treated as fixed. All values reported in the text are means ± s.e.m.

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Competing interests

The authors declare no competing financial interests.

Author contributions

H.A.M., A.F., T.H.P. and J.O. designed and conceived the research; H.A.M., A.F. and J.O. performed the experiments; H.A.M. and J.O. described and analysed the data; H.A.M. and J.O. drafted the manuscript; and all authors revised the manuscript.

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Supplementary material

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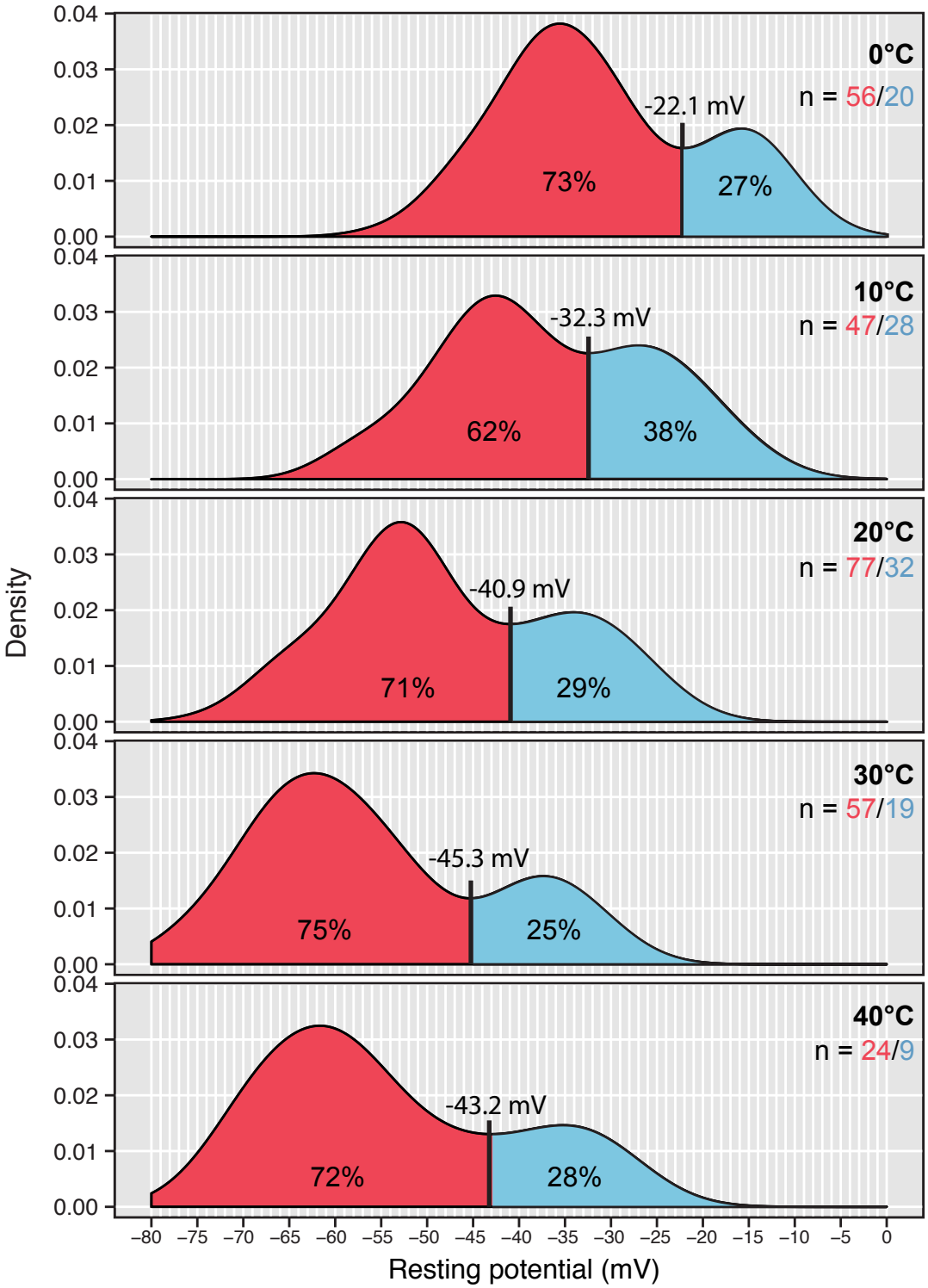

Fig. S1. Probability density distributions (Gaussian error) of muscle resting potentials measured in the extensor tibialis (*in vivo*) of *Locusta migratoria* during acute temperature exposure. Resting potentials were binned by 10°C increments to determine characteristic potentials (vertical lines) that were used to discriminate between P1 (red) and P2 (blue) fibres. Distributions of resting potentials were bimodal regardless of temperature and the proportions of fibres classified as P1 and P2 were relatively stable (% values inside distributions). *n*, number of fibres in each group at a given temperature. Cooling significantly depolarized muscle resting potential (shifted the entire distribution to the right; see Results for details of statistics).

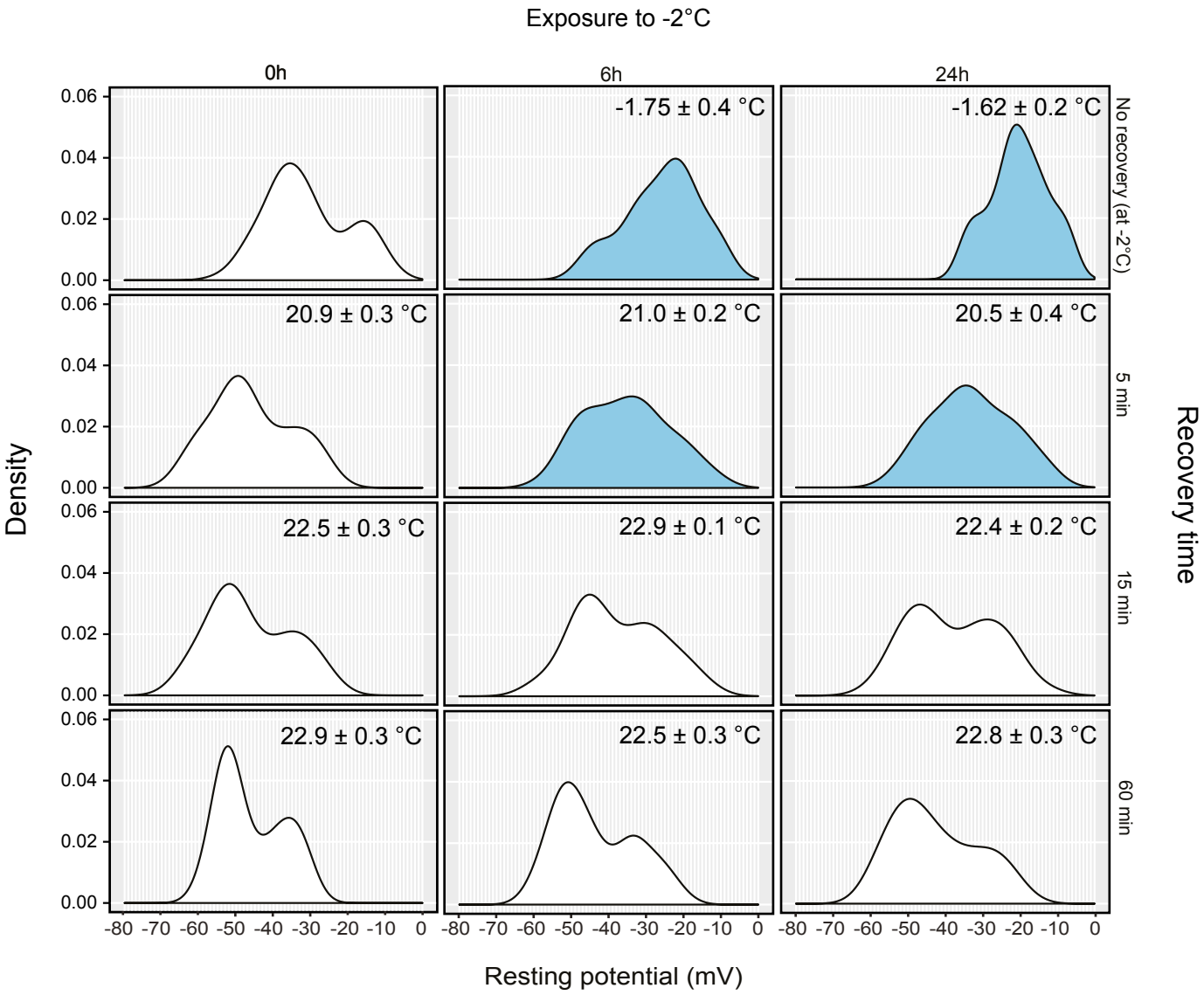

Fig. S2. Probability density distributions (Gaussian error) of muscle resting potentials (V_m) measured *in vivo* in the extensor tibialis of *Locusta migratoria* during prolonged cold exposure and recovery from cold exposure at 22°C. Temperatures in the upper right corner of each panel are the mean (±s.e.m.) temperature measured inside the femur, measured immediately after resting potential measurements were made. Distributions of V_m remained bimodal in locusts that were cooled to –2°C and immediately rewarmed (left). By contrast, locusts that had experienced prolonged cold exposure had unimodal V_m distributions (blue) that remained unimodal even after rewarming. These distributions became bimodal again after 15 or 60 min of recovery at 22°C.

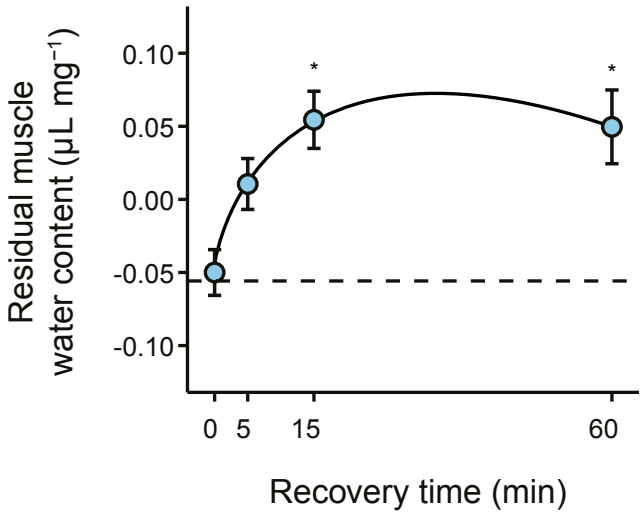

Fig. S3. Residual water content (expressed as per mg mean muscle wet mass) of muscle tissue from the femur of *Locusta migratoria* under control conditions (dashed line = mean) and during recovery from 6 h at –2°C (blue circles; mean ± s.e.m.). Tissue dry mass was a strong predictor of water content (R^2=0.96, P<0.001), so we tested for changes in water content during chill-coma recovery using residuals of the regression of water content against dry mass. Locust muscle did not accumulate water during cold exposure (data not shown) but significantly accumulated water during recovery ($F_{4,55}$=7.0, P<0.001). Stars denote a significant difference in water content relative to control locusts at room temperature (based on Tukey's HSD). Solid black line is for illustrative purposes only.

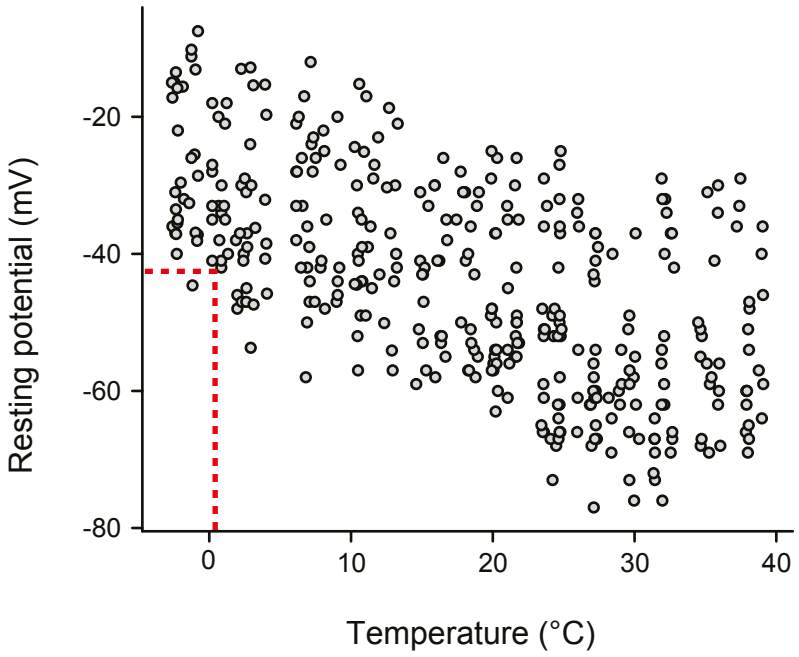

Fig. S4. Individual resting potentials (n=370 fibres in 62 animals) in the extensor tibialis of *Locusta migratoria* measured at –2 to 40°C. Vertical and horizontal dashed red lines denote the chill-coma onset temperature of *L. migratoria* [0.5°C (Findsen et al., 2014)] and the approximate theoretical excitability threshold of insect muscles [–40 to –45 mV (Hosler et al., 2000)]. Only the most polarized of fibres in the extensor tibialis were observed to cross this threshold at the chill-coma onset temperature.