

RESEARCH ARTICLE

Why do insects enter and recover from chill coma? Low temperature and high extracellular potassium compromise muscle function in *Locusta migratoria*

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ABSTRACT

When exposed to low temperatures, many insect species enter a reversible comatose state (chill coma), which is driven by a failure of neuromuscular function. Chill coma and chill coma recovery have been associated with a loss and recovery of ion homeostasis (particularly extracellular $[K^+]_o$) and accordingly onset of chill coma has been hypothesized to result from depolarization of membrane potential caused by loss of ion homeostasis. Here, we examined whether onset of chill coma is associated with a disturbance in ion balance by examining the correlation between disruption of ion homeostasis and onset of chill coma in locusts exposed to cold at varying rates of cooling. Chill coma onset temperature changed maximally 1°C under different cooling rates and marked disturbances of ion homeostasis were not observed at any of the cooling rates. In a second set of experiments, we used isolated tibial muscle to determine how temperature and $[K^+]_o$, independently and together, affect tetanic force production. Tetanic force decreased by 80% when temperature was reduced from 23°C to 0.5°C, while an increase in $[K^+]_o$ from 10 mmol l⁻¹ to 30 mmol l⁻¹ at 23°C caused a 40% reduction in force. Combining these two stressors almost abolished force production. Thus, low temperature alone may be responsible for chill coma entry, rather than a disruption of extracellular K⁺ homeostasis. As $[K^+]_o$ also has a large effect on tetanic force production, it is hypothesized that recovery of $[K^+]_o$ following chill coma could be important for the time to recovery of normal neuromuscular function.

KEY WORDS: Cold tolerance, Critical thermal minimum, Grasshoppers, Isometric force, Membrane potential, Neuromuscular junction

INTRODUCTION

When exposed to a critical low temperature, many insect species will enter a coma-like state (Bale, 1996; Sinclair, 1999; Nedved, 2000; Košťál et al., 2004; MacMillan and Sinclair, 2011b). This comatose state is termed chill coma and is manifested in a complete arrest of movement (Mellanby, 1939). Chill coma can be fully reversible depending on the intensity and duration of the low temperature exposure. However, if the cold exposure is prolonged or severe, the insects may accumulate direct chilling injuries, for

example in the form of membrane phase transitions, leading to irreparable cellular injury (Quinn, 1985). Moreover, prolonged cold exposure may lead to indirect chilling injuries, often manifested in dissipation of transmembrane ion gradients and ion distribution (Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair, 2011a). Although the mechanisms behind chill coma and chill injury may share physiological similarities, it is also possible that the linkage between chill coma entry and chill injury development is of correlative nature.

The cold tolerance of chill-sensitive insects can be measured in several ways. These include the temperature of chill coma entry (the critical thermal minimum, CT_{min}) (Kelty and Lee, 2001; Terblanche et al., 2007; Overgaard et al., 2011a), chill coma recovery time, which measures the time it takes the insects to recover from a comatose state (Gibert et al., 2001; Anderson et al., 2005; Košťál et al., 2006; MacMillan et al., 2012; Findsen et al., 2013), and measures of chill mortality caused by indirect chilling injuries (Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair, 2011a). These traits may be related in regards to their underlying mechanisms (Košťál et al., 2004; MacMillan and Sinclair, 2011b) and several of these measures are known to describe ecologically relevant variation linked to distribution, adaptation and/or acclimation. Thus, variation in chill tolerance measurements is found at both the intraspecific (Macdonald et al., 2004; Anderson et al., 2005; Košťál et al., 2006; Overgaard et al., 2011a; Colinet and Hoffmann, 2012) and interspecific level (Goller and Esch, 1990; Gibert et al., 2001; Kimura, 2004; Overgaard et al., 2011b; Kellermann et al., 2012).

Despite the popularity and power of the aforementioned phenotypic traits, very little is still known about the physiological mechanisms underlying entrance into and recovery from chill coma. The principal hypothesis regarding chill coma pertains to the observation that muscle membrane potential is severely depolarized with low temperature, and this causes a loss of muscle excitability, which ultimately leads to a chill coma. Thus, Hosler et al. (Hosler et al., 2000) examined chill coma in *Apis mellifera* and *Drosophila melanogaster* and found that the muscle resting membrane potential (V_m) depolarized during cooling. Even though the two insect species entered chill coma at different temperatures (*A. mellifera*: 10°C, *D. melanogaster*: 5°C), the resting V_m measured at the onset of chill coma was depolarized to a similar level in the two species (*A. mellifera* approximately -40 mV and *D. melanogaster* approximately -45 mV). Moreover, entry into chill coma in both species was accompanied by a loss of muscle excitability and severely reduced muscle action potentials, and similar observations have been made in a number of Lepidopteran and Hymenopteran species (Esch, 1988; Goller and Esch, 1990). Together, these findings point to a critical value of V_m where muscle excitability is lost such that the insect enters chill coma.

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Received 21 October 2013; Accepted 23 December 2013

List of symbols and abbreviations

CT_{\min}	critical thermal minimum (chill coma temperature)
E_K	equilibrium potential for K^+
E_{Na}	equilibrium potential for Na^+
TTX	tetrodotoxin
V_m	membrane potential
$[X]_i$	concentration of ion X in the intracellular fluid (muscle)
$[X]_o$	concentration of ion X in the extracellular fluid (haemolymph)

In addition to the studies examining chill coma entry, other studies have focused on the recovery from chill coma under the assumption that the processes occurring during recovery describe a reversal of the processes that take place during the onset of chill coma (MacMillan and Sinclair, 2011b). Prolonged exposure to cold has been shown to cause a large disturbance in ion homeostasis in many insect species (Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair, 2011a; MacMillan et al., 2012), and even short periods below the CT_{\min} are associated with a marked disturbance of ion homeostasis (Andersen et al., 2013; Finsen et al., 2013). Thus, when locusts are exposed to temperatures below the chill coma threshold for 2 h, the extracellular $[K^+]$ ($[K^+]_o$) increases up to 3-fold compared with that of untreated controls (Andersen et al., 2013; Finsen et al., 2013). The disturbance in $[K^+]_o$ seen after short and prolonged cold exposure in insects probably occurs because cooling affects active (temperature-sensitive electrogenic pumps) and passive (ion channels) ion transport processes differently (MacMillan and Sinclair, 2011b). This may compromise water and ion regulation and cause dissipation of ion gradients between intracellular and extracellular compartments (Košťál et al., 2004; MacMillan and Sinclair, 2011b; Andersen et al., 2013). The observed increases in $[K^+]_o$ following cooling are likely to have a large effect on muscle V_m as resting V_m in many insects, including locusts, is largely determined by the equilibrium potential of K^+ (E_K) (Hoyle, 1953; Wood, 1963). In fact, reducing temperature can be envisaged to cause membrane depolarization through several cellular mechanisms. Firstly, as mentioned above, low temperature may cause membrane depolarization through run down of transmembrane ion gradients, reflecting increased ion leak from tissues compared with active re-uptake mechanisms. Secondly, a reduction in temperature should, according to simple calculations of V_m , within the constraints of the Goldman regime, lead to a depolarization even for maintained membrane permeabilities and ion gradients. Thirdly, reduced active ion transport through electrogenic transport mechanisms (e.g. Na^+/K^+ -ATPase) will reduce the electrogenic contribution of these pumps to V_m . The exact mechanistic relationship between membrane depolarization, muscle action potential amplitude and muscle function still remains to be described and other physiological processes could also be involved. Thus, loss of muscle excitability could also be connected to failure in nervous signal propagation or in neuromuscular transmission. Some (but not all) chill coma events in locusts and fruit flies are associated with a halt in neuronal activity possibly caused by high $[K^+]_o$ surrounding the nervous system (Rodgers et al., 2010; Armstrong et al., 2012). In addition to membrane depolarization leading to disturbed ion homeostasis, low temperature per se can be envisaged to affect multiple membrane proteins involved in determining muscle fibre excitability (Frolov and Singh, 2013).

Here, we conducted two sets of experiments to evaluate the effect of low temperature per se and the effect of disrupted ion homeostasis for entry into chill coma. In the first set of experiments, we exposed the chill-susceptible locust *Locusta migratoria* (Linnaeus 1758) to low temperature using different cooling rates, as

this is known to markedly affect the CT_{\min} of other insect species (Kelty and Lee, 1999; Overgaard et al., 2006; Terblanche et al., 2007; Overgaard et al., 2011b). We measured the CT_{\min} as well as the intracellular and extracellular ion composition with the hypotheses that (1) the onset of chill coma (CT_{\min}) is associated with a disturbance of ion balance and (2) differences in CT_{\min} between different cooling rates can be explained by different degrees of ionic disturbance. In a second experiment, we studied the isolated and combined effects of low temperature and high potassium on muscular function in *in vitro* muscle preparations. The specific hypotheses tested were as follows: (1) low temperature and/or increased $[K^+]_o$ will decrease tetanic force production; (2) the effect can be partitioned to the muscle and is independent of nervous function; and (3) high potassium and low temperature will have an additive or synergistic effect as would be assumed if both are related to a depolarization of V_m .

RESULTS **CT_{\min}**

In the first set of experiments, the effect of different cooling rates on CT_{\min} was determined. While the different cooling rates resulted in significantly different CT_{\min} (ANOVA, $F_{3,96}=17.05$, $P<0.01$), these differences were small in absolute terms, with the maximal variation between the average CT_{\min} values being $\sim 1^\circ\text{C}$ (Fig. 1A). There was no directional response related to cooling rate as locusts cooled at the slowest ($0.02^\circ\text{C min}^{-1}$) and the highest ($0.18^\circ\text{C min}^{-1}$) rate had similar CT_{\min} ($-0.35\pm 0.12^\circ\text{C}$ versus $-0.37\pm 0.13^\circ\text{C}$; ANOVA, $F_{3,96}=17.05$, Tukey's *post hoc* test, NS) while cooling rates of 0.04 and $0.1^\circ\text{C min}^{-1}$ resulted in CT_{\min} that were significantly different from those of all other treatments (ANOVA, $F_{3,96}=17.05$, Tukey's *post hoc* test, $P<0.05$) (Fig. 1A).

Ion concentrations and Nernst equilibrium potentials at onset of chill coma

To test whether onset of chill coma was associated with a disruption of K^+ homeostasis, we measured K^+ balance once the locust had reached -1°C . We found a significant interaction between cooling rate and temperature on $[K^+]_o$ (two-way ANOVA, $F_{6,103}=5.86$, $P<0.0001$; supplementary material Fig. S1A). This interaction was caused because $[K^+]_o$ reached higher values at -1°C for the locusts exposed to the slowest cooling rate. Although significant, the increase in $[K^+]_o$ for the slowest cooling rate ($0.02^\circ\text{C min}^{-1}$) was modest, rising from $8.3\pm 0.5\text{ mmol l}^{-1}$ at 20°C to $13.3\pm 0.9\text{ mmol l}^{-1}$ at -1°C . We found no difference in intracellular K^+ concentration ($[K^+]_i$) between any treatments (supplementary material Fig. S1B). Despite the small increase observed in $[K^+]_o$ for the locusts cooled at $0.02^\circ\text{C min}^{-1}$ there was no significant difference in E_K at -1°C between locusts exposed to different cooling rates (one-way ANOVA, $F_{3,31}=2.26$, NS) (Fig. 1B). However, as expected, E_K did alter with temperature in all treatment groups, changing from $-69.2\pm 1.2\text{ mV}$ at 20°C to $-60.3\pm 2.1\text{ mV}$ at -1°C (two-way ANOVA, $F_{2,93}=16.92$, Tukey's *post hoc* test, $P<0.05$; supplementary material Fig. S3A). No correlation was found between E_K and CT_{\min} as the regression did not deviate from zero (linear regression, $F_{1,2}=2.023$, NS) (Fig. 1C).

Extracellular Na^+ ($[Na^+]_o$) was not affected by temperature (two-way ANOVA, $F_{2,105}=0.90$, NS) but the treatment group (cooling rate) had a significant effect as the $[Na^+]_o$ in locusts cooled at $0.02^\circ\text{C min}^{-1}$ was consistently higher than that in locusts cooled at $0.1^\circ\text{C min}^{-1}$ (two-way ANOVA, $F_{3,105}=3.48$, Tukey's *post hoc* test, $P<0.05$; supplementary material Fig. S2A). All other cooling rates were not different from each other. Again we found no change in

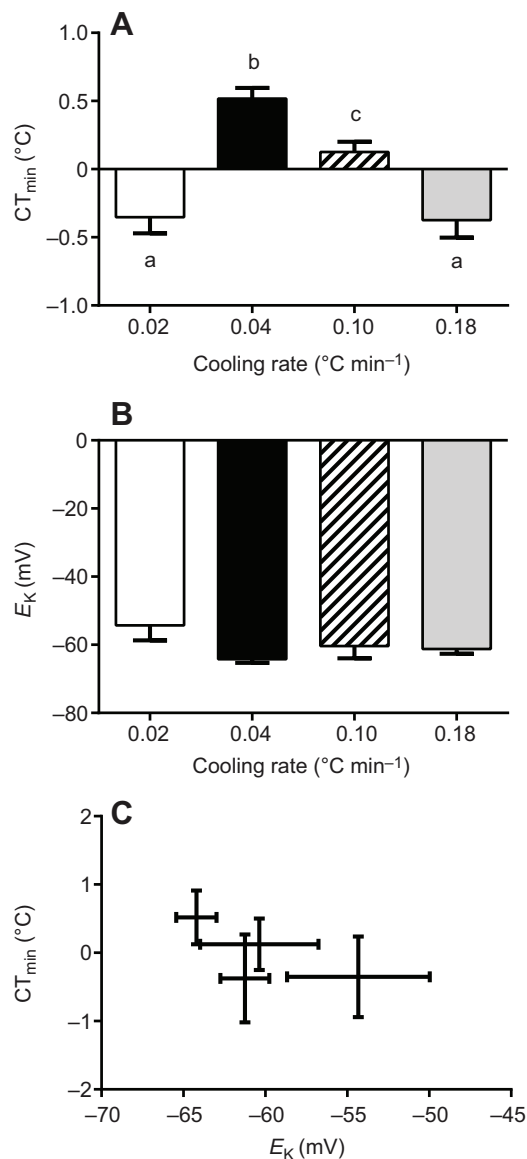


Fig. 1. CT_{min} and E_K measurements of *Locusta migratoria* at four different cooling rates (0.02, 0.04, 0.1 and 0.18°C min⁻¹). (A) Chill coma temperature (CT_{min}) was assessed as the temperature at which the animal was paralysed by cold and was non-responsive. Bars represent means \pm s.e.m. of 25 locusts for each cooling rate treatment. Different letters indicate groups that differ significantly. (B) Equilibrium potential of K⁺ (E_K) at -1°C for four different cooling rates (0.02, 0.04, 0.1 and 0.18°C min⁻¹). (C) CT_{min} plotted against E_K values at -1°C. Values are means \pm s.e.m. Additional information can be found in supplementary material Figs S1–S3.

intracellular Na⁺ concentration ($[Na^+]_i$) between any treatment groups or temperatures (supplementary material Fig. S2B). E_{Na} was significantly reduced with temperature (two-way ANOVA, $F_{2,100}=3.60$, $P<0.03$; supplementary material Fig. S3B).

Control series of force measurements

A force–frequency relationship was initially established using stimulation frequencies between 2 and 150 Hz on muscle preparations at 23 and 0.5°C (Fig. 2A). As shown by representative traces at the two temperatures in Fig. 2B, maximum force was obtained at around 120 Hz at both 23 and 0.5°C but at all frequencies the force was markedly reduced at the lowest

temperature. When the observations of force at the different frequencies were related to the maximum force at the two temperatures, and this relative force was plotted against frequency, the data were well described by sigmoidal functions (Fig. 2C). Such fits provided estimates of the frequency that resulted in half the maximum force (f_{50}). This analysis showed that lowering the temperature to 0.5°C tended to cause a leftwards shift of the fits with f_{50} being 19.5 \pm 2.3 Hz at 23°C versus 14.6 \pm 6.3 Hz at 0.5°C (unpaired t -test, $t_8=0.835$, $P=0.43$). On this basis it was concluded that determination of force using 60 Hz trains was suitable to produce tetanic contractions at both temperatures, being 88 \pm 2% and 93 \pm 6% of the maximum force at 23 and 0.5°C, respectively (Fig. 2C).

During 260 min of incubation, where the muscle preparations were stimulated at 60 Hz every 10 min, tetrodotoxin (TTX)-treated groups had a drop in force of 51 \pm 7%, after which the force stabilized at around 160 min (Fig. 2D). The force production in the untreated group was also relatively stable with time, although we observed an average drop in force of 16.5 \pm 6.8% from the start to the end of the experiment (5 h in total) (Fig. 2D).

Isolated effects of temperature and increased $[K^+]_o$ on muscle force production

Comparing groups with and without TTX allowed us to investigate whether effects of high K⁺ and low temperature were mediated through a failure to stimulate the motor nerve or to dysfunction of factors distal to the motor nerve including neuromuscular transmission, excitation–contraction (E–C) coupling in the muscle and altered force-generating capacity. As force stabilized at around 160 min in the TTX-treated groups (Fig. 2D), it was set to 100% at this time point such that the effects of subsequently lowering temperature or increasing extracellular K⁺ could be normalized to the force measured at this time point. Decreasing temperature from 23 to 0.5°C had a dramatic effect on force production, which was reduced by 80% of the pre-treatment value (Fig. 3A). The relative reduction in force was similar in the TTX-treated and non-treated muscles (t -test, $t_9=0.403$, $P=0.70$) (Fig. 3B). Increasing extracellular K⁺ from 10 to 30 mmol l⁻¹ decreased force by around 40% and again there was no difference in the response between TTX-treated and non-treated preparations (t -test, $t_{10}=1.233$, $P=0.25$) (Fig. 3C,D).

To further examine the depressive effects of low temperature and increased K⁺ on force production, we investigated the response to intermediate values of $[K^+]_o$ (20 mmol l⁻¹ K⁺) and temperature (10°C) (Fig. 4) of an additional set of TTX-treated preparations. When exposed to 10°C, force was reduced to a level intermediate to the force at 23 and 0.5°C (23°C: 100 \pm 0%; 10°C: 61 \pm 8%; 0.5°C: 19 \pm 5%; ANOVA, $F_{2,13}=63.79$, Tukey's *post hoc* test, $P<0.05$) (Fig. 4A). Similarly, 20 mmol l⁻¹ K⁺ reduced force to a level that was intermediate to those at 10 and 30 mmol l⁻¹ K⁺, respectively (10 mmol l⁻¹: 100 \pm 0%; 20 mmol l⁻¹: 82 \pm 2%; 30 mmol l⁻¹: 56 \pm 2%; ANOVA, $F_{2,15}=31.53$, Tukey's *post hoc* test, $P<0.05$) (Fig. 4B).

Combined effect of temperature and increased $[K^+]_o$ on muscle force production

In a continuation of the experiments examining the isolated effects of temperature or increased extracellular K⁺ on force generation, we next tested the combined effect of temperature and $[K^+]_o$ on the force produced by TTX-treated preparations. The combined treatment of 10°C and 20 mmol l⁻¹ K⁺ elicited a larger decrease in force than either of the treatments in isolation (10°C: 61 \pm 8%; 10°C and 20 mmol l⁻¹ K⁺: 27 \pm 7%; 20 mmol l⁻¹ K⁺: 82 \pm 2%; ANOVA, $F_{2,13}=19.33$, Tukey's *post hoc* test, $P<0.05$) and a similar result was seen when combining 0.5°C and 30 mmol l⁻¹ K⁺ (0.5°C: 19 \pm 5%;

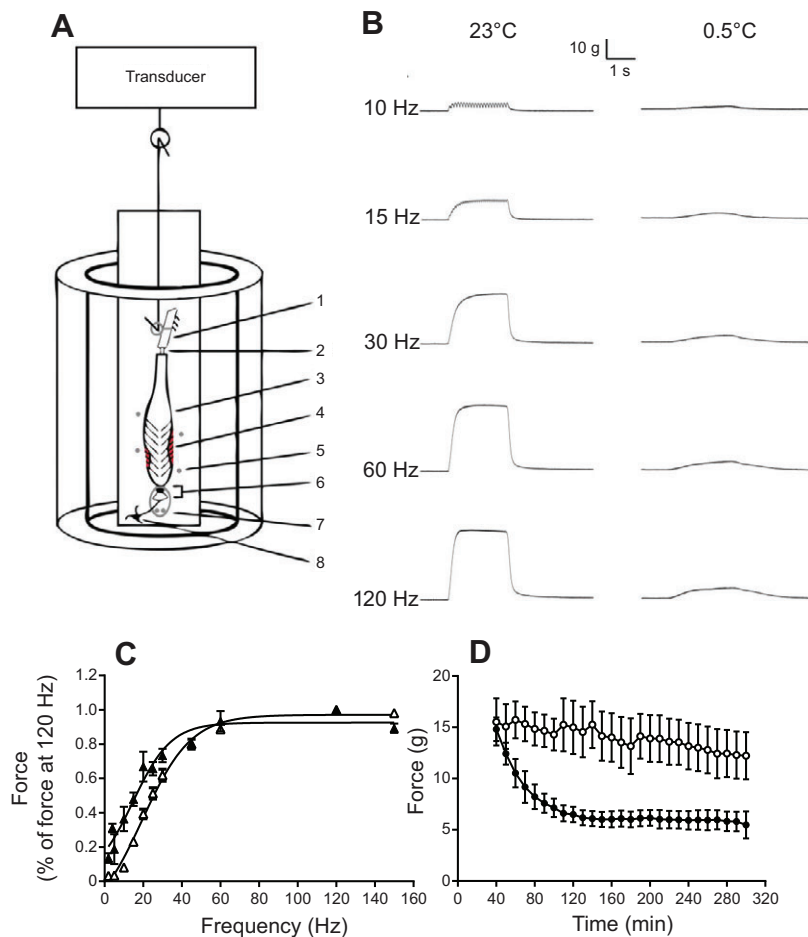


Fig. 2. Schematic diagram of the muscle preparation from the hindleg of *L. migratoria* and control series of tetanic force production. (A) The muscle preparation consists of the tibia (1), femur (3), coxa and trochanter (6) and nerve ending from the methathoracic ganglion (8). The coxal end is fastened with suture to two metal pins (7) embedded perpendicularly in a vertically oriented Plexiglas plate that also contained the wire electrodes (5) for field stimulation. The tibial end was fastened to a stainless steel hook connected to a force transducer. To expose the muscle tissue to the electrical field stimulation, two small incisions were made in the exoskeleton (4). To avoid the build-up of force in the bending springs positioned in the joint section and to secure a straight pull in the transducer, the cuticle in this area was removed and the tendons were exposed (2). The whole preparation was submerged in a water-jacketed glass chamber containing standard locust saline.

(B) Representative traces of force from one preparation excited with pulses at frequencies between 10 and 120 Hz at two experimental temperatures. (C) Force–frequency relationship obtained using stimulation frequencies between 2 and 150 Hz at 23°C (open triangles) and 0.5°C (filled triangles). Continuous lines represent Boltzmann curves fitted to data. Symbols represent means \pm s.e.m. of four to six preparations. (D) Tetanic force in response to 60 Hz stimulation for 2 s given every 10 min to preparations with (filled circles) and without tetrodotoxin (TTX, open circles). In the preparations with TTX, the compound was added to a concentration of $1 \mu\text{mol l}^{-1}$ at 40 min and maintained at this concentration throughout the experiment. Symbols represent means \pm s.e.m. of four muscles in each treatment.

0.5°C and $30 \text{ mmol l}^{-1} \text{ K}^+$: $5 \pm 3\%$; $30 \text{ mmol l}^{-1} \text{ K}^+$: $56 \pm 2\%$; ANOVA, $F_{2,13}=14.32$, Tukey's *post hoc* test, $P < 0.05$) (Fig. 4). Thus, the combined effects of 10°C and $20 \text{ mmol l}^{-1} \text{ K}^+$ resulted in a $73 \pm 7\%$ reduction in force production, which is a larger reduction in

force than would be expected by simple additive action of the two parameters (i.e. evaluated from multiplying the effect of K^+ with that of reduced temperature: $61\% \times 82\% = 50\%$). This apparent synergistic effect was also seen when combining 0.5°C and $30 \text{ mmol l}^{-1} \text{ K}^+$,

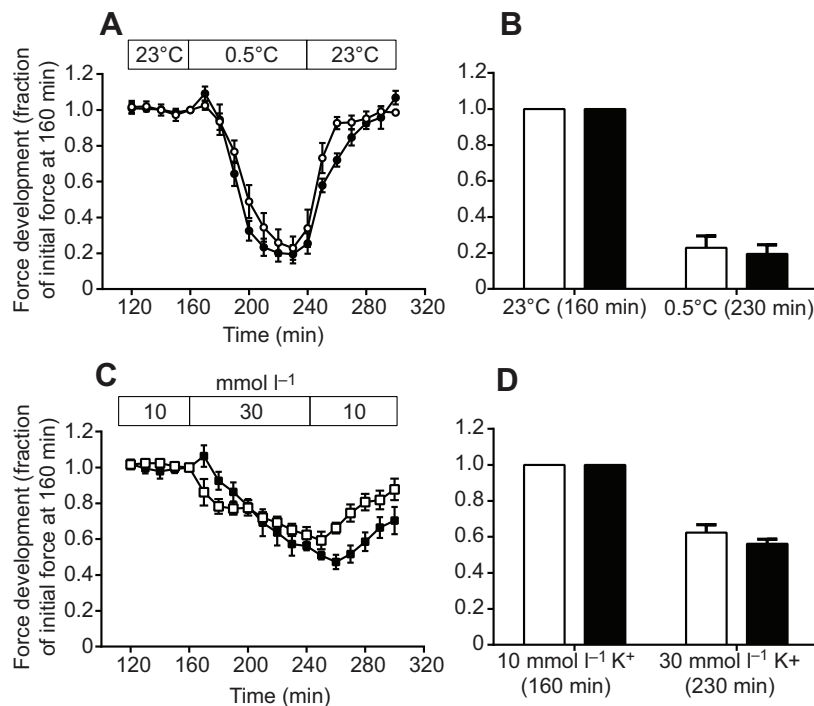


Fig. 3. Tetanic force production at different temperatures and $[\text{K}^+]$ with and without TTX. (A) Tetanic force production was elicited every 10 min in preparations with (filled circles) and without TTX (open circles). Force production was normalized to that obtained at 160 min and temperature was lowered gradually from 23°C to 0.5°C from 160 min. The experimental temperature (0.5°C) was reached within 50 min and the preparation was allowed to stabilize for the following 30 min before being returned quickly to 23°C at 240 min. Symbols represent means \pm s.e.m. of five to six preparations in each treatment. (B) Tetanic force production at 0.5°C normalized to force at 23°C for TTX-treated (filled bars) and non-treated (open bars) preparations. Bars represent means \pm s.e.m. of five to six preparations in each treatment. (C) Tetanic force production was elicited every 10 min in preparations with (filled squares) and without TTX (open squares). Force production was normalized to that obtained at 160 min and $[\text{K}^+]_o$ was increased from 10 to 30 mmol l^{-1} from the 160th minute. The experimental K^+ concentration (30 mmol l^{-1}) was maintained at this level for 80 min before returning to 10 mmol l^{-1} at the 240th minute. Symbols represent means \pm s.e.m. of six preparations in each treatment. (D) Tetanic force production at $30 \text{ mmol l}^{-1} \text{ K}^+$ normalized to force at $10 \text{ mmol l}^{-1} \text{ K}^+$ for TTX-treated (filled bars) and non-treated (open bars) preparations. Bars represent means \pm s.e.m. of six preparations in each treatment.

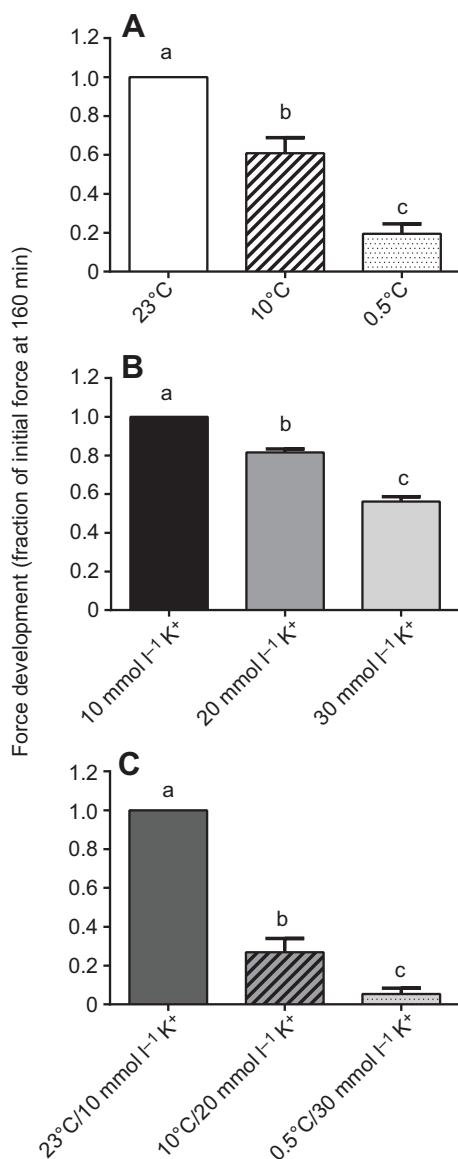


Fig. 4. Tetanic force production at combinations of different temperatures and $[K^+]_o$ with TTX present. (A) Tetanic force production measured at 23, 10 and 0.5°C. Force production at the different temperatures is normalized to force at 23°C. (B) Tetanic force production measured at 10, 20 and 30 mmol l⁻¹ K⁺. Force production at the different potassium concentrations is normalized to force at 10 mmol l⁻¹. (C) Tetanic force production measured at combinations of lowered temperature and increased $[K^+]_o$ (23°C/10 mmol l⁻¹ K⁺, 10°C/20 mmol l⁻¹ K⁺ and 0.5°C/30 mmol l⁻¹ K⁺). Force production at the different combined treatments is normalized to force at 23°C/10 mmol l⁻¹ K⁺. All treatments were performed on TTX-treated preparations. Bars represent means \pm s.e.m. of five to seven preparations in each treatment. Different letters indicate groups that differ significantly.

which resulted in a force production of $5 \pm 3\%$ (the calculated additive value would have been: $19\% \times 56\% = 11\%$) (Fig. 4C).

DISCUSSION

In the present study we conducted two different sets of experiments to investigate whether temperature and/or disruption of ion homeostasis are the principal causes of chill coma entry in the chill-susceptible species *L. migratoria*. Entry into chill coma (CT_{min}) changed moderately with different cooling rates. However, the

differences between cooling rates were not associated with a marked disturbance of ion homeostasis. Furthermore, we investigated chill coma at the tissue level by examining the isolated and combined effects of low temperature and high $[K^+]_o$ on muscular function. It has previously been suggested that low temperature per se as well as perturbation of ion homeostasis associated with chronic low temperature can both induce loss of neuromuscular function and entrance into chill coma in insects (MacMillan and Sinclair, 2011b). In support of this notion, we demonstrated that both low temperature and high $[K^+]_o$ can have large negative effects on muscle tetanic force production of the hindleg muscle of *L. migratoria*. Thus, lowering the temperature from 23°C to 0.5°C resulted in an 80% drop in force while an increase in $[K^+]_o$ from 10 to 30 mmol l⁻¹ resulted in a 40% reduction in force. Furthermore, we found that the combination of these stressors resulted in a synergistic depression that almost eliminated force production, with only 5% of initial force production remaining.

CT_{min} and ion disturbance

CT_{min} has been used as a measure of cold tolerance in many insect species (Goller and Esch, 1990; Hosler et al., 2000; Gibert and Huey, 2001; Terblanche et al., 2008; Kellermann et al., 2012), and several studies have shown that CT_{min} can be altered by using different cooling rates (Kelty and Lee, 1999; Terblanche et al., 2007). For example, CT_{min} is lowered in *D. melanogaster* when slow cooling rates, that allow for rapid cold hardening, are used (Kelty and Lee, 1999), whereas slow cooling of tsetse flies resulted in a poorer tolerance (higher CT_{min}) probably due to accumulation of cold damage (Terblanche et al., 2007). Although it has been suggested that loss of ion homeostasis may be the cause of chill coma (MacMillan and Sinclair, 2011a; MacMillan and Sinclair, 2011b; MacMillan et al., 2012), few studies have directly examined the physiological causes of the onset of chill coma. In the present study, we found little evidence to suggest that perturbation of ion homeostasis is the cause of chill coma. Thus, at the temperature at which the locusts enter chill coma, both intracellular and extracellular ion balance are almost unchanged relative to the control situation. Moreover, the small degree of perturbation that we did observe does not correlate with CT_{min} . These findings point to a mechanism responsible for the entrance into chill coma (CT_{min}) for locusts that is not related to altered ion balance of muscle tissue. Nevertheless, given the very different effect of cooling rates seen in different insects (*Drosophila*, tsetse flies and locusts) it is still possible that different causes exist for different species.

Isometric force production

Tetanic force was used in this study as a measure of muscle performance in the locust. As attested by the force–frequency relationship, tetanic force is reached at both high and low temperature when using a stimulation frequency of 60 Hz (Fig. 2B,C). Because the temporal development of tension is temperature dependent we used a long stimulation train (2 s) to ensure that tetanic force was also reached at low temperatures (Fig. 2B).

Several studies have examined muscle performance in ectotherms at different temperatures. Jumping and running performance has for example been extensively studied in frogs and lizards (Bennett, 1984; Hirano and Rome, 1984; Marsh and Bennett, 1985; Navas et al., 1999; John-Alder et al., 1989), while flight muscle performance has been the main focus with regard to thermal effects on muscle function in locusts (Weis-Fogh, 1956; Neville and Weis-Fogh, 1963). In these studies, it has been shown that the power output is

highly temperature sensitive as a result of the temperature sensitivity of rate-limiting processes that affect the temporal development of tension and maximal velocity of shortening (Bennett, 1984). In contrast, maximal tetanic force contraction is rarely used as a proxy of muscle performance in ectothermic animals because tetanic force is generally temperature independent within a broad range of temperatures (Bennett, 1984; John-Alder et al., 1989; Barclay and Robertson, 2000). For ectothermic animals, the R_{10} values of tetanic force production are often close to 1 (Bennett, 1984) (R_{10} expresses the temperature effect in a similar manner to Q_{10} , but concerns quantities such as force production instead of rates of reactions), such that an animal should be capable of applying an equal force regardless of its body temperature (Bennett, 1984). Thermal independence of tetanic force production has also been the general observation in insects, where orthopteran flight and cicada tymbal muscles have R_{10} values of 1.1 (Heinrich, 1981). Similarly, Barclay and Robertson (Barclay and Robertson, 2000) observed in locusts that tibial force was almost unchanged over a temperature interval of 25°C (from 20 to 45°C), but that force dropped sharply at higher temperatures as a consequence of heat stress. Thus, the development of maximal tension may be slower at low temperature, but across a broad range of temperatures the maximal tetanic force is often found to be relatively constant. Such thermal independence of maximal force production is important in many static situations such as standing and maintaining normal posture.

In the present study, we used tetanic force production as a reasonable proxy for the muscular dysfunction characteristic for chill coma, where it is always observed that the insects lose the ability to maintain posture and movement (Mellanby, 1939; MacMillan and Sinclair, 2011b). When examining the temperature interval between 23 and 0.5°C, we observed a larger thermal effect on tetanic force production than previously reported for insects (the calculated R_{10} is ~2). This demonstrates a clear thermal dependency in locust muscle ability to generate force at low temperatures, which contrasts with earlier observations on locusts (Weis-Fogh, 1956; Neville and Weis-Fogh, 1963) but is similar to the marked reduction in force at critically high temperatures (Barclay and Robertson, 2000).

Mechanisms behind loss of muscle function when entering chill coma

Previous studies on chill-sensitive insects have found that onset of chill coma is correlated with a large depolarization of V_m (Hosler et al., 2000). This change in V_m coincides with a reduction in muscle action potentials and it is therefore possible that chill coma is associated with a loss of muscle excitability caused by membrane depolarization (Goller and Esch, 1990; Hosler et al., 2000). We did not measure muscle V_m in this study but it is possible that a marked depolarization could have been taking place at low temperature. This could arise as a consequence of lowered temperature per se, as a result of an altered transmembrane chemical gradient for K^+ and a depressed electrogenic contribution from the temperature-sensitive ion-motive pumps (Hosler et al., 2000). Nevertheless, the exact mechanistic basis of the impaired force production remains to be understood as it is largely unknown whether and how the depolarized V_m causes such a large depression in muscle action potentials and force (Esch, 1988; Goller and Esch, 1990; Hosler et al., 2000). Alternatively, it is possible that low temperature has other effects on E–C coupling. A recent study on fruit flies demonstrated that the kinetics of the L-type Ca^{2+} -channels, responsible for producing the depolarization during a muscle action potential, are very sensitive to temperature (Frolov and Singh, 2013). Altered opening kinetics of the L-type Ca^{2+} -channels could cause a decrease

in force at low temperature but further studies are needed to determine whether this is also relevant for locust muscle at low temperature. Regardless of the exact cause of the low temperature effect, it remains clear from the present study that temperature alone has a considerable negative effect on muscle force.

Previous studies have suggested that a principal cause of chill coma entry and depolarized V_m was related to a disturbance of ion regulation (see discussion below) (MacMillan and Sinclair, 2011a; MacMillan and Sinclair, 2011b; MacMillan et al., 2012). However, the present findings suggest that temperature itself may be the principal cause of entry into chill coma. This assumption is supported by the fact that we did not observe any disruption of ion homeostasis at the onset of chill coma when locusts are gradually cooled, as presented above. Thus, disruption of ion homeostasis is principally established while the locusts are in chill coma and not before they enter chill coma (Andersen et al., 2013; Findsen et al., 2013). Similar observations have been made for a tropical cockroach, where low temperature caused chill coma before disruption of ion homeostasis developed (Košťál et al., 2006). Ongoing studies performing parallel measurements of membrane potential and ion concentration at chill coma onset will hopefully aid in the understanding of the exact mechanisms involved in the onset and recovery of chill coma in locusts.

Mechanisms behind recovery of muscle function following chill coma

In addition to a large effect of temperature on force production, we also observed a negative effect of increased $[K^+]_o$ on force production. The effect of increased K^+ on the muscle tissue is not surprising as high levels of K^+ have been shown to affect muscle excitability, V_m and tension in locusts (Hoyle, 1953; Hoyle, 1954). Thus, dissipation of the transmembrane K^+ gradient causes a marked depolarization of V_m , which may lead to a similar disruption of muscle force production to that produced by cold exposure. Even though we suggest that disruption of ion homeostasis is not the principal cause of entry into chill coma, it is possible that cold-induced dissipation of ion gradients could play a significant role for the recovery from chill coma. Cold-induced disruption of ion homeostasis, especially $[K^+]_o$, has been observed in many chill-susceptible insect species where the degree of disruption is often dependent on the exposure time to low temperature and/or the acclimation status of the animals (Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair, 2011a; MacMillan et al., 2012; Andersen et al., 2013; Findsen et al., 2013). Recent studies of locusts have also shown that chill coma recovery time is correlated with the recovery of 'normal' K^+ balance (Andersen et al., 2013; Findsen et al., 2013) and that the recovery time after cold coma is dependent on the magnitude of the dissipation of ion gradients (Košťál et al., 2004; Košťál et al., 2006; MacMillan et al., 2012). The correlation between chill coma recovery time and recovery of $[K^+]_o$ homeostasis found in these earlier studies combined with the negative effect of increased $[K^+]_o$ on muscle force production presented here suggests that recovery of K^+ balance could be a prerequisite for recovery of muscle function and therefore also for chill coma recovery.

In the present study we found a synergistic effect of low temperature and high K^+ on the impairment of muscle force production. As discussed above, low temperature and high $[K^+]_o$ may affect force production through common mechanisms related to V_m (or possibly through other processes in E–C coupling), but similar results could also be obtained if temperature and ion homeostasis had their main effect on different parts of the E–C

coupling process. Nonetheless, we suggest that during the initial development of chill coma, the direct effect of low temperature is the main cause of impaired muscle function. During prolonged cooling, a dissipation of ion gradients further depresses this impairment such that muscle function is still impaired upon return to a benign temperature until appropriate ion homeostasis is regained. This model is entirely consistent with the observations of previous studies investigating the putative causes of chill coma in insects (Košťál et al., 2004; MacMillan et al., 2012; Andersen et al., 2013; Finsden et al., 2013), but further studies, particularly regarding the mechanistic basis of muscle impairment, are needed to verify this hypothesis.

Do both temperature and high $[K^+]_o$ affect muscle and nervous tissue?

We used direct field stimulation to induce muscular contraction in the present study. This kind of stimulation may electrically excite the muscle cells directly or it may induce muscle contraction indirectly through electrical excitation of associated motor neurons. The tetanic contractions we observe therefore stem from a mixed population of muscle cells that were excited either directly by the field stimulation or indirectly through the nerve. Because some of the cells are excited through the nerves, any change in force production could in principle relate to changes in excitation and propagation of neuronal action potentials. To test this possibility, we performed experiments in the presence and absence of TTX, which is a potent blocker of nervous function but has no effect on locust muscle (Washio, 1972; Orchard et al., 1981; Collet, 2009). Application of TTX reduced overall force production to about half that in untreated muscle, indicating that approximately half of the muscle cells were exclusively dependent on nervous signal transmission (Fig. 2D). Importantly, we consistently observed that treatments with low temperature or hyperkalaemia caused a similar relative drop in muscle force production in preparations with and without TTX (Fig. 3). This indicates that low temperature and high $[K^+]_o$ affect the muscle's ability to generate force directly, as any additional contribution of the nervous signal propagation would have caused a difference in the relative decrease in force between the TTX-treated and non-treated preparations. The observation that the signal transmission is relatively unaffected by the cold treatment is in agreement with previous findings from chill-sensitive insects where Anderson and Mutchmor (Anderson and Mutchmor, 1968) observed that the nervous tissue is still excitable below the chill coma temperature in three species of cockroaches. However, it should be mentioned that in the present experimental system we cannot rule out the possibility that field stimulation was able to directly trigger pre-synaptic release of neurotransmitters independently of excitation of an action potential in the nerves. If this was the case then loss of muscle function in TTX-treated preparations could reflect compromised neuromuscular transmission. It has, for example, been shown in some ectothermic animals (*Rana pipiens* and *Pachygrapsus*) that the neuromuscular junction is more sensitive to cold than the muscle itself (Jensen, 1972; Stephens, 1990). As the effect of K^+ on muscle function is the same in the presence and absence of TTX, we conclude that increased extracellular $[K^+]_o$ has no major effect on the nervous signal propagation under these experimental conditions. This is somewhat surprising as it is known that high $[K^+]_o$ has a dramatic effect on nervous membrane potential (Hoyle, 1953). However, Hoyle (Hoyle, 1953) also pointed out that the connective sheath found surrounding the nervous tissue in locusts is an effective barrier to the diffusion of K^+ ions, and it is possible that the

concentrations used here are not sufficient to affect signal propagation. Previous locust studies have reported that entrance into chill coma is associated with a halt in neuronal activity as a result of high $[K^+]_o$ (~67 mmol l^{-1}) in the extracellular fluid of the metathoracic ganglion (Rodgers et al., 2010). This pattern has also been observed in *Drosophila*, where $[K^+]_o$ in the extracellular environment increased almost tenfold (from 5 mmol l^{-1} to 47 mmol l^{-1}) when the animal entered chill coma (Armstrong et al., 2012). However, high $[K^+]_o$ and depressed neuronal activity are not always observed in relation to locust chill coma (Rodgers et al., 2010), indicating that $[K^+]_o$ is not solely the reason for impaired nerve function and chill coma entry. Additionally, there may be differences in the effect of $[K^+]_o$ in the central and peripheral nervous system.

Conclusions

Chill coma temperature has been used in many studies and has been shown to be a good proxy for cold tolerance and distribution. The present study showed that CT_{min} changed slightly with cooling rate, but the differences in CT_{min} were small between the different cooling treatments ($>1^\circ C$) and there was no pattern of slow or fast cooling rates being associated with high or low CT_{min} . Moreover, no relationship was found between ion balance and CT_{min} .

When examining the direct effects of cold and high $[K^+]_o$, we used tetanic force production as a proxy for muscle function as this, together with neural factors, is important for the insect's ability to maintain posture. Surprisingly, we found that low temperature in isolation exerts a large depression on tetanic force production, which suggests that low temperature in itself may be responsible for chill coma entry. During chill coma a large disturbance in ion homeostasis, especially $[K^+]_o$, has previously been observed, and in combination with the depressive effect of increased K^+ on tetanic force production, we hypothesize that recovery of chill coma is likely to be influenced by the recovery of ion homeostasis following cold as this might be essential for the animal to recover normal muscle function.

The reduced tetanic force as a result of low temperature and high $[K^+]_o$ could be related to the increased V_m caused by both these stressors, leading to loss of muscle excitability. However, it remains to be understood whether and how these stressors specifically affect the E–C coupling in locust (insect) muscle. Thus, further studies examining the consequences of low temperature and high K^+ on the electrophysiological processes in muscle E–C coupling are needed to uncover the reasons for the impaired muscle function observed at chill coma.

MATERIALS AND METHODS

Insect rearing

Laboratory-reared locusts, *L. migratoria*, were kept in cages (0.45 m³) with a 12 h:12 h light:dark cycle. During the day, the cages were heated with a sun-simulating lamp allowing behavioural 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. Locusts were sexed within 5 days of reaching adulthood (stage 6) (Uvarov, 1966), and males and females were subsequently separated, thereby ensuring that all experimental animals were virgins. Both male and female locusts were used for experiments and all animals were between 1 and 5 weeks old at the time of the experiments, measured from the day of imaginal ecdysis.

CT_{min}

A custom-built acrylic glass box with 25 separate compartments (7×7×6 cm) was installed inside a cooling incubator (Termaks, model KB8182/KB8400).

A bar was mounted on each side of the box and the bars could be fitted into a hole on each side of the incubator, allowing for full rotation of the box inside the closed incubator. A low-heat-producing LED light was fitted to the back of the incubator to illuminate the inside. This setup allowed observation of the 25 locusts during cooling while the box was turned intermittently to see whether the animals could respond. Twenty-four hours before the experiments began, male and female locusts were transferred separately to small plastic boxes and placed in a temperature-controlled room (22°C). The animals had access to water but no food. To quantify CT_{\min} , 25 locusts were allocated to individual compartments (each with a small piece of water-soaked paper to ensure a water source). The box was placed at 20°C after which cooling was applied at one of four cooling rates (0.02, 0.04, 0.1 and 0.18°C min⁻¹). Chill coma temperature (CT_{\min}) was defined as the temperature when no more activity was observed.

Measurement of intracellular and extracellular concentrations

Na⁺ and K⁺ concentrations were measured at 20, 10 and -1°C to estimate the degree of ionic disturbance in the intracellular and extracellular compartment (muscle tissue and haemolymph) (-1°C was chosen as this is where all animals had entered chill coma). For these measurements, locusts were placed in 50 ml plastic tubes, with a piece of sponge rubber at the top to prevent the locust escaping. The tubes were placed in a refrigerated glycol bath (Lauda RE 320, Lauda, Lauda-Königshofen, Germany) held at a constant 20°C and after 30 min equilibration the bath started cooling at one of the four cooling rates. Haemolymph samples were taken with a capillary tube (25 µl) from behind the head of the animal. If sufficient haemolymph could not be obtained in this manner, additional haemolymph was taken from the hindlegs. Extraction of muscle tissue samples and measurement of ion concentration were performed as described elsewhere (Findsen et al., 2013).

Nernst equilibrium potential (E_x)

The Nernst potentials for K⁺ and Na⁺ were calculated on the basis of [K⁺] and [Na⁺] in the extracellular (haemolymph) and intracellular (muscle) fluid using the following equation:

$$E = \left(\frac{RT}{zF} \right) \ln \left(\frac{X_o}{X_i} \right), \quad (1)$$

where R is the gas constant (8.314 J K⁻¹ mol), T is the absolute temperature in Kelvin, which is 293.15 K (20°C), 283.15 K (10°C) and 272.15 K (-1°C), depending on the sample temperature, z is the charge on each ion and F is the Faraday constant (96,487 C per gram-equivalent charge). $[X]_o$ and $[X]_i$ are the concentration of the ion in the extracellular fluid (haemolymph) and intracellular (muscle) fluid, respectively. This calculation assumes that [K⁺] and [Na⁺] in the muscle fluid resemble the concentrations in the intracellular fluid, although this is only an approximation because a small fraction of extracellular fluid remains in the interstitial fluid of the muscle (Wood, 1963).

Muscle preparation

Locusts were decapitated and a small incision was made in the ventral thoracic wall to expose the metathoracic ganglion. The nerves going from the ganglion to the hindleg were cut and exposed, after which a muscle preparation consisting of a small part of the coxa, trochanter, femur and half of the tibia was isolated, while ensuring that the hindleg motor neurons remained attached to the muscle preparation. The cuticle around the joint section and semi-lunar process was removed so the tibia and femur were loosened from each other to avoid the build-up of force in the bending springs positioned in the joint section and to secure a straight pull in the transducer (Fig. 2A). The tibia and femur were still connected by the two tendons connecting the extensor tibia and flexor tibia muscles with the tibia. A suture was fastened between the trochanter and femur and another suture was secured around the tibia. Two small incisions were made on the dorsal and ventral part of the femur exoskeleton as the exoskeleton insulates muscle tissue from electrical field stimulation (Fig. 2A). The coxal end was fastened with suture to two metal pins embedded perpendicularly in a vertically oriented Plexiglas plate that also contained the wire electrodes for

field stimulation. The tibial end of the muscle was fastened with suture to a stainless steel hook connected to a force transducer (Grass FT03 range 0.5–100 g, Grass Technologies, Warwick, RI, USA) (Fig. 2A). The transducer could be adjusted vertically to stretch the muscle. The entire muscle preparation was prepared within 10 min and submerged in a water-jacketed glass chamber with standard locust Ringer solution containing (in mmol l⁻¹): 140 NaCl, 10 KCl, 2 MgCl₂, 1 NaH₂PO₄, 3 CaCl₂, 5 glucose, 20 Hepes buffer; pH 7.15 (7.05 at 0.5°C) (Hoyle, 1953). In experiments where extracellular potassium was increased, an equivalent amount of Na⁺ was omitted to maintain iso-osmolarity. 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. In all experiments, the preparations were mounted for isometric contractions in the standard locust saline and equilibrated at 23°C for at least 30 min before starting the experiments.

Electrical stimulation and measurement of isometric force

After incubation, the muscle preparations were adjusted to optimal length such that isometric twitch force production 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 muscle. Muscles were activated to contract using pulses of 1 ms duration and supra-maximal voltage (24–30 V cm⁻¹). Tetanic contractions were elicited every 10 min throughout the experiments using 2 s trains with a stimulation frequency of 60 Hz.

Data were sampled at 1 kHz using an AD converter and software from Cambridge Electronic Design (Power1401, Signal 4.0, Cambridge, UK).

Control series of force measurements

Prior to the experimental series investigating the isolated and combined effects of hyperkalaemia and temperature, we performed a series of control experiments to examine and validate the experimental system. These control series were divided into three different experiments. Firstly, a force–frequency relationship was performed at both 23°C and 0.5°C to ensure that maximum force was reached when stimulating at 60 Hz for 2 s. Secondly, the stability of the preparation was examined to investigate whether muscle force production changed over time. This was performed on preparations with and without TTX (which blocks nervous function but has no effect on locust muscle) (Washio, 1972; Orchard et al., 1981) to further test whether a reduction in force with time was associated with reduced muscle function or failure in the excitation of associated motor neurons.

Isolated and combined effect of temperature and extracellular potassium on muscle force production

One experimental series was designed to investigate the isolated effects of low temperature (10 and 0.5°C) and high potassium (20 and 30 mmol l⁻¹ K⁺) on isometric force production. The second experimental series aimed at investigating the combined effects of high potassium and low temperature (30 mmol l⁻¹ K⁺ and 0.5°C or 20 mmol l⁻¹ K⁺ and 10°C) on isometric force production. Throughout all experiments the muscle performed tetanic contractions every 10 min. The general protocol for the two experimental series was as follows. After incubation, the muscle was field stimulated for 40 min until muscle force stabilized. TTX was then added (1 µmol l⁻¹) in half of the preparations to investigate whether the effects of high potassium/low temperature were mediated through a failure to excite action potentials in the motor nerve or a reduced function of the muscle itself. The preparations were then allowed to stabilize for 2 h while being stimulated every 10 min to produce tetanic contractions. After this period the preparation was exposed to a treatment (lowered temperature or increased extracellular potassium or a combination of both). The preparations were kept under these experimental conditions for the next 80 min before they were returned to normal buffer and temperature for 60 min, after which the experiment was terminated.

We chose 0.5°C as the lowest temperature as this temperature is within the range of the CT_{\min} of this locust species (see Results). Additionally, the highest [K⁺] of 30 mmol l⁻¹ used in this study has been observed in locusts after short (2 h) cold exposure (Findsen et al., 2013).

Statistical analysis

All statistical analysis was performed in Graphpad Prism 6.0. Prior to analysis, outliers of CT_{\min} and E_K measurements were removed using Grubbs' tests (GraphPad, 2013). A one-way ANOVA followed by a Tukey's *post hoc* multiple comparison test was used to compare CT_{\min} values at the different cooling rates and a similar analysis was performed with respect to E_K at -1°C . Two-way ANOVA followed by Tukey's *post hoc* multiple comparison test was used to compare the effect of temperature and cooling rate on $[K^+]_o$, $[Na^+]_o$, $[K^+]_i$, $[Na^+]_i$, E_K and E_{Na} . A linear regression analysis was performed on E_K and CT_{\min} to test whether the variation in CT_{\min} could be explained by the variation in E_K .

For the muscle experiments, a Boltzmann sigmoidal curve was fitted to the force–frequency data and f_{50} values for 23 and 0.5°C were compared using an unpaired *t*-test. A similar *t*-test was also used to compare the remaining relative force between TTX-treated and non-treated preparations after the treatments (low temperature, high $[K^+]_o$). Thus, the remaining force of all preparations was reported relative to the force immediately before the experimental treatment (at 160 min). Differences between groups within each treatment (temperature, extracellular potassium and combination of both) were compared using a one-way ANOVA followed by a Tukey's *post hoc* multiple comparison test. A similar test was also used to compare the combined and isolated effect of the treatments.

All data are presented as means \pm s.e.m., and $P < 0.05$ was considered statistically significant.

Acknowledgements

We are grateful for technical assistance from Kirsten Kromand, and a special thanks to Vibeke Uhre and Marianne Stürup-Johansen for guidance and support in the lab. Thanks to Heath Macmillan for valuable comments on the manuscript.

Competing interests

The authors declare no competing financial interests.

Author contributions

A.F., T.H.P., A.G.P., O.B.N. and J.O. designed and conceived the research; A.F. and A.G.P. performed the experiments; A.F., A.G.P. and J.O. described and analysed the data; A.F. and J.O. drafted the manuscript; A.F., T.H.P., O.B.N. and J.O. revised the manuscript.

Funding

This research was funded by a Sapere Aude DFF-Starting grant (to J.O.) from The Danish Council for Independent Research | Natural Sciences and by a grant (to A.F.) from the Faculty of Science and Technology of Aarhus University.

Supplementary material

Supplementary material available online at <http://jeb.biologists.org/lookup/suppl/doi:10.1242/jeb.098442/-DC1>

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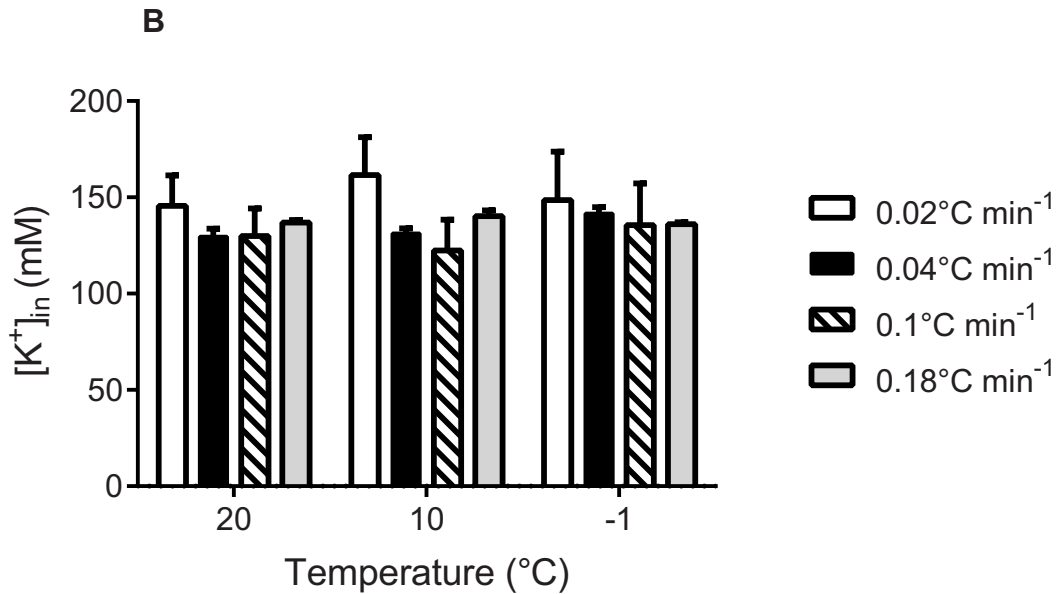
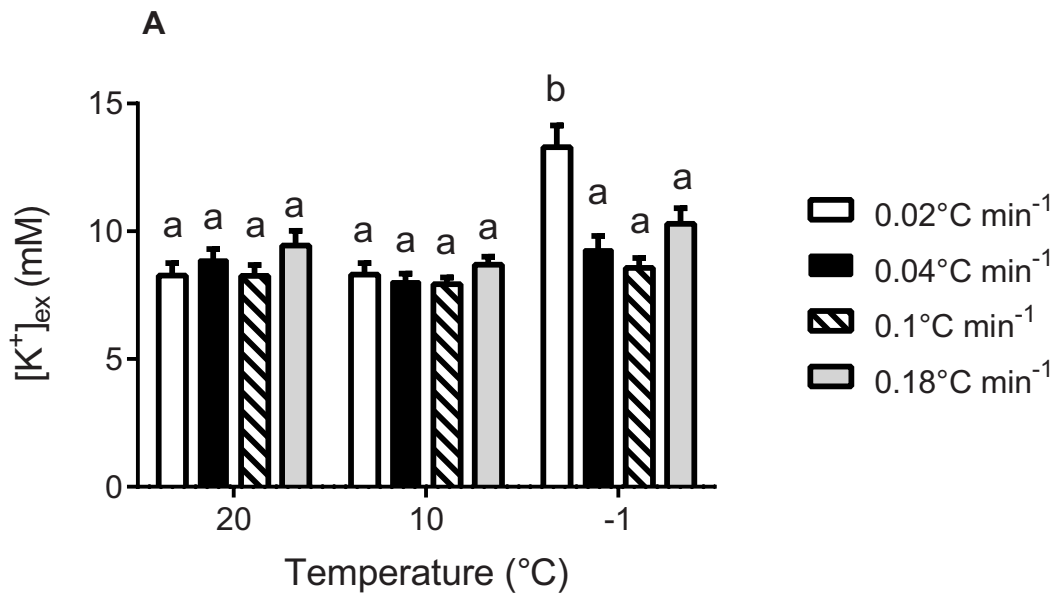


Fig. S1. Extra- and intracellular concentrations of K^+ at different temperatures and cooling rates. (A) Extracellular $[K^+]_{ex}$ measured at three temperatures during different cooling rates. Bars represent means \pm s.e.m. of 8-10 samples in each treatment. A significant interaction was found between cooling rate and temperature using a Two-way ANOVA. The means flanked by different letters within a temperature group are statistically different at $P < 0.05$. (B) Intracellular $[K^+]_{in}$ measured at three temperatures during different cooling rates. Bars represent means \pm s.e.m. of 8-10 samples in each treatment.

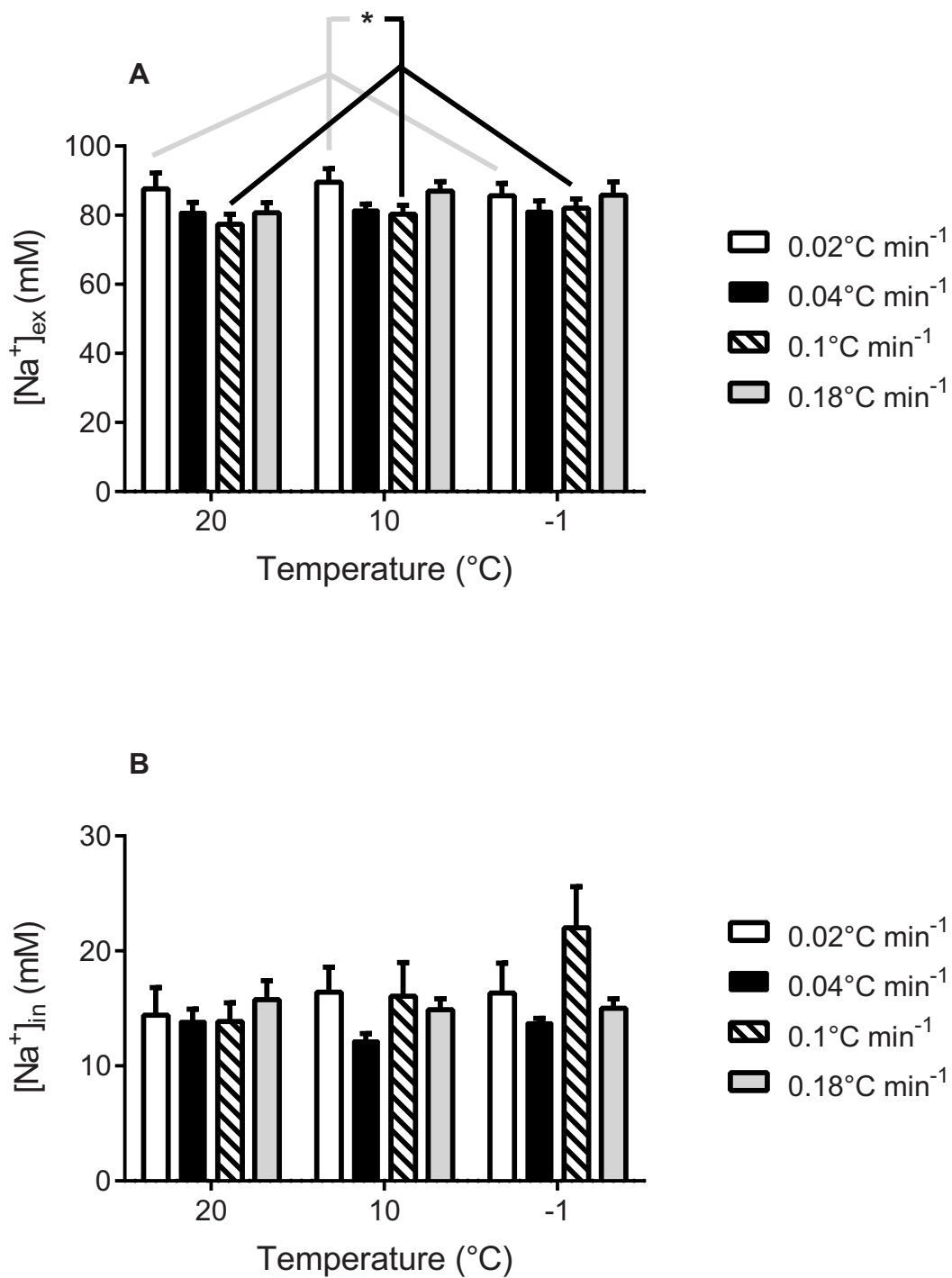


Fig. S2. Extra- and intracellular concentrations of Na⁺ at different temperatures and cooling rates. (A) Extracellular [Na⁺] measured at three temperatures during different cooling rates. Bars represent means ± s.e.m. of 8-10 samples in each treatment. A significant effect of cooling rate was found using a Two-Way ANOVA. Statistical differences ($P < 0.05$) between cooling rates are indicated by *. (B) Intracellular [Na⁺] measured at three temperatures during different cooling rates. Bars represent means ± s.e.m. of 8-10 samples in each treatment.

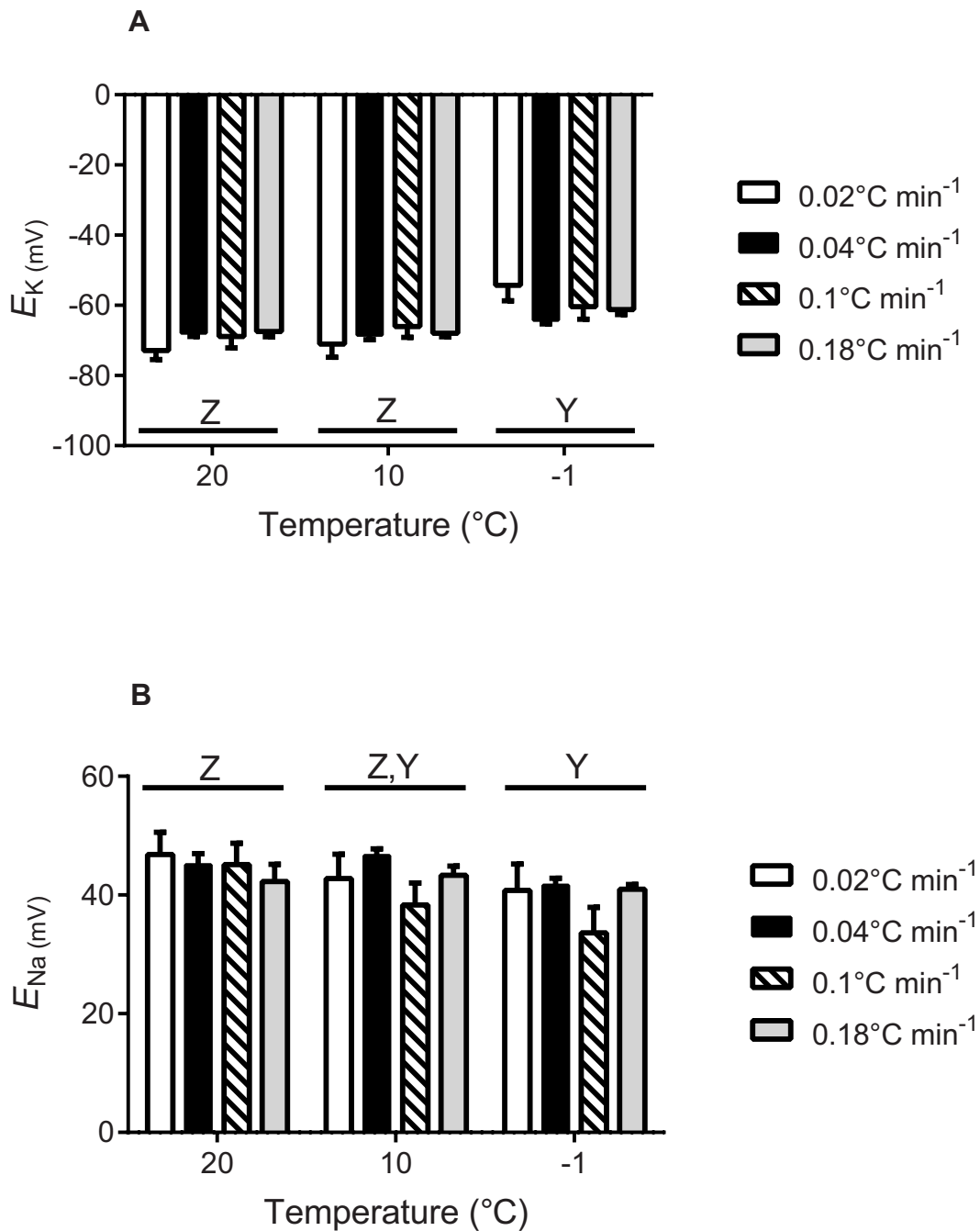


Fig. S3. Nernst potential for K^+ and Na^+ at different temperatures and cooling rates. (A) Nernst potential for K^+ (E_K) calculated from the intra- and extracellular $[\text{K}^+]$ at three temperatures during different cooling rates. Bars represent means \pm s.e.m. of 8-10 samples in each treatment. A significant effect of temperature was found using a Two-way ANOVA. Dissimilar letter indicate groups that differ significantly ($P < 0.05$). (B) Nernst potential for Na^+ (E_{Na}) calculated from the intra- and extracellular $[\text{Na}^+]$ at three temperatures during different cooling rates. Bars represent means \pm s.e.m. of 8-10 samples in each treatment. A significant effect of temperature was found using a Two-way ANOVA. Dissimilar letter indicate groups that differ significantly ($P < 0.05$)