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RESEARCH ARTICLE

Cold acclimation improves chill tolerance in the migratory locust through preservation of ion balance and membrane potential

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ABSTRACT

Most insects have the ability to alter their cold tolerance in response to temporal temperature fluctuations, and recent studies have shown that insect cold tolerance is closely tied to the ability to maintain transmembrane ion gradients that are important for the maintenance of cell membrane potential (V_m). Several studies have therefore suggested a link between preservation of V_m and cellular survival after cold stress, but none has measured V_m in this context. We tested this hypothesis by acclimating locusts (*Locusta migratoria*) to high (31°C) and low temperature (11°C) for 4 days before exposing them to cold stress (0°C) for up to 48 h and subsequently measuring ion balance, cell survival, muscle V_m, and whole animal performance. Cold stress caused gradual muscle cell death, which coincided with a loss of ion balance and depolarization of muscle V_m. The loss of ion balance and cell polarization were, however, dampened markedly in cold-acclimated locusts such that the development of chill injury was reduced. To further examine the association between cellular injury and V_m we exposed *in vitro* muscle preparations to cold buffers with low, intermediate, or high [K^+]. These experiments revealed that cellular injury during cold exposure occurs when V_m becomes severely depolarized. Interestingly, we found that cellular sensitivity to hypothermic hyperkalaemia was lower in cold-acclimated locusts that were better able to defend V_m whilst exposed to high extracellular [K^+]. Together these results demonstrate a mechanism of cold acclimation in locusts that improves survival after cold stress: increased cold tolerance is accomplished by preservation of V_m through maintenance of ion homeostasis and decreased K^+ sensitivity.

KEY WORDS: Cold resistance, Cold stress, Ion homeostasis, Membrane polarization, Chilling injury, *Locusta migratoria*

INTRODUCTION

When cooled sufficiently, most insects lose the ability for coordinated movement and enter a state of complete neuromuscular paralysis, termed chill coma, at a temperature referred to as their critical thermal minimum (CT_{min}) (Mellanby, 1939; Hazell and Bale, 2011; MacMillan and Sinclair, 2011a). During this cold-induced coma, chill-sensitive insects will accumulate cold injury in a manner that is generally dependent on the intensity and duration of the cold stress (Nedvěd et al., 1998; Koštál et al., 2004; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Sensitivity to such cold stress varies enormously amongst insect species adapted to different thermal environments, and accordingly insect cold tolerance is often found to correlate closely with species distribution (Addo-Bediako et al., 2000; Bale, 2002; Kimura, 2004; Sunday et al., 2011; Kellermann et al., 2012; Andersen et al., 2015b). Cold tolerance is also a very plastic trait such that appropriate cold acclimation treatments will markedly improve the cold tolerance of virtually all insect species (Mellanby, 1954; Chen et al., 1987; Gibert and Huey, 2001; Koštál et al., 2004, 2006; Colinet and Hoffmann, 2012; MacMillan et al., 2015b).

The cold tolerance of insects that are not freeze tolerant nor truly freeze avoiding is termed chill tolerance (Zachariassen, 1985; Bale, 1996; Overgaard and MacMillan, 2016). Chill tolerance has been shown to be closely associated with the ability to maintain ion and water balance during cold stress, such that cell membrane potential (V_m) is preserved (Koštál et al., 2004; Zachariassen et al., 2004; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2016). When a chill-susceptible insect is gradually cooled, reduced ion pump activity causes the V_m of excitable tissues to depolarize, and this depolarization often coincides with the onset of chill coma (Pichon and Treherne, 1974; Wareham et al., 1974; Hosler et al., 2000; Andersen et al., 2015a; Overgaard and MacMillan, 2016). Following chill coma onset, hemolymph ion balance is progressively lost, which further depolarizes V_m (MacMillan et al., 2014; Overgaard and MacMillan, 2016). Thus the development of chill injury in insects is almost always associated with increased hemolymph [K^+], which has a marked depolarizing effect on cells, including muscle cells (Hoyle, 1953; Wood, 1963). This notion goes well with the hypothesis put forth by Hochachka (1986) and Boutilier (2001), who proposed that cellular depolarization, potentially caused by disruption of ion homeostasis, could initiate apoptosis or necrotic signalling pathways through a disruption of Ca^{2+} balance: in this model, severe prolonged cellular depolarization would cause the opening of voltage-sensitive Ca^{2+} channels leading to a disastrous increase in intracellular [Ca^{2+}]. The increased intracellular [Ca^{2+}] subsequently causes an activation of Ca^{2+}-dependent lipases and proteases, resulting in cell death (McConkey and Orrenius, 1996; Demaurex and Distelhorst, 2003). In a recent study, MacMillan et al. (2015c) investigated the separate and combined effects of hyperkalemia and low temperature (which both depolarize V_m) on chilling injury in locust muscle and showed that cell death only occurred when the *in vitro* muscle preparations were exposed to both factors simultaneously. Hyperkalemia and low temperature depolarized V_m additively, and only induced injury when applied together. This result suggests that only considerable depolarization of V_m has the potential to initiate the apoptotic/necrotic cascade.

As mentioned above, the majority of chill-susceptible insects have an innate ability to augment their chill tolerance through seasonal or developmental acclimation (Mellanby, 1954; Koštál et al., 2004; Coello Alvarado et al., 2015; MacMillan et al., 2015b), or through rapid cold hardening (the improvement of chill tolerance

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List of symbols and abbreviations

CCRT	chill coma recovery time
CT_{min}	critical thermal minimum
E_X	Nernst potential of ion *X*
V_d	diffusion potential
V_e	electrogenic effect
V_m	resting membrane potential
[*X*]	concentration of ion *X*

after a brief and mild pre-exposure to cold before a more substantial cold challenge; Denlinger and Lee, 2010; Findsen et al., 2013). Several acclimation studies have now observed that cold-acclimated insects either partially or completely retain K^+ balance during cold stress, and thereby avoid injury (Koštál et al., 2004, 2006; Coello Alvarado et al., 2015; MacMillan et al., 2015a,b). A corollary of these observations is that cold-acclimated insects better preserve V_m through improved maintenance of K^+ balance (Koštál et al., 2004; Overgaard and MacMillan, 2017), which in turn prevents cell death and thus ensures organismal survival. This hypothesis, however, remains to be examined directly.

Here, we investigate whether prior cold acclimation affects organismal and cellular survival after cold stress in the migratory locust *Locusta migratoria*. We predicted that cold-acclimated locusts would show improved chill tolerance, and that this improvement would be manifested in both organismal performance and cellular viability. Furthermore, we hypothesize that the benefits of cold acclimation manifest from an improved ability to maintain ion balance and V_m during cold stress.

MATERIALS AND METHODS

Animal husbandry

Pre-adult (fifth instar) locusts (*Locusta migratoria*, Linneaus 1758) were purchased from a commercial supplier (Peter Andersen Aps, Fredericia, Denmark) and held in well-ventilated 0.45 m^2 cages at 20°C with a 12 h:12 h light:dark cycle. Cages contained egg trays for hiding and basking and a 150 W heat lamp allowed for behavioural thermoregulation between approximately 25 and 45°C during light hours. Locusts had *ad libitum* access to wheat bran and water, and fresh wheat sprouts were supplied daily. All experimental animals were kept under these conditions for 2–6 weeks, during which they moulted to the adult stage such that locusts used in this study were adults between 1 and 4 weeks after imaginal ecdysis.

Acclimation and experimental protocol

The present study investigates the physiological consequence of thermal acclimation in locusts with a particular emphasis on how cold tolerance at the organismal and cellular level is related to ion homeostasis and preservation of cellular potential. This was done by comparing how cold and warm acclimation affected (1) organismal cold tolerance, (2) cellular cold tolerance, (3) intracellular and extracellular ion balance, (4) polarization of membrane potential after different durations of chill coma, and (5) cellular sensitivity to hyperkalemia at low temperature.

Pilot experiments revealed considerable difference in cold tolerance between locusts acclimated for 4 days at 11 and 31°C, respectively, and all experiments were therefore focused on the contrast between these two experimental groups. For the acclimation treatment, locusts were transferred from the holding facilities to smaller cages with filter paper and egg trays in the bottom. These cages were then left for 4 days with a 12 h:12 h light:dark cycle at either constant 11°C or constant 31°C during which the animals had *ad libitum* access to wheat bran and water. A 1:1 male:female sex ratio was used for all experiments, unless otherwise stated.

Chill coma recovery time and survival after 24 h recovery

On the day of the experiment, individual locusts from the two acclimation groups were placed in 50 ml polypropylene tubes sealed with foam stoppers and suspended in a water–ice slurry at 0°C in the dark. Animals were then left undisturbed for 0.5, 4, 24 or 48 h before they were returned to room temperature (22°C) and scored for their ability to stand as an estimate of chill coma recovery time (CCRT) (N=8 per treatment). Locusts were encouraged to move/stand by gently blowing air onto them every other minute, and the time for standing was recorded. Observation time was limited to 60 min, and the number of locusts not standing within this time period was noted and these were not included in mean CCRT values. Locusts were returned to cages with *ad libitum* access to food and water, and left for 24 h at 22°C to estimate survival. Survival was scored on a scale of 0 to 5 (0: no movement observed, i.e. dead; 1: able to move but unable to stand; 2: standing, but incapable of walking; 3: able to walk, but in an uncoordinated manner; 4: coordinated movements resulting in normal walking behaviour and sporadic, uncontrolled jumping; 5: normal behaviour including jumps that transition into flight).

Cellular viability after cold exposure

Tissue damage was estimated during control conditions and after 24 and 48 h of cold exposure in locusts from both acclimation groups (N=9–12 per treatment). Tissue damage was estimated in muscle tissue as described in MacMillan et al. (2015c). The head and extremities were removed and a longitudinal incision was made on the ventral side of the thorax to expose the mesothoracic posterior tergocoxal muscle (M90; Snodgrass, 1929). The gut and intestines were carefully removed and the preparation was submerged in approximately 50 ml of a standard locust buffer (in mmol l^{-1}: 140 NaCl, 8 KCl, 3 $CaCl_2$, 2 $MgCl_2$, 1 NaH_2PO_4, 90 sucrose, 5 glucose, 5 trehalose, 1 proline, 10 Hepes, pH 7.2). Fat and tracheal tissue then were carefully removed to fully expose the muscle (approximately 5 min). The muscle was then dissected out and placed on a glass microscope slide with 50 µl of locust buffer after which staining of live and dead cells was performed using a LIVE/DEAD Sperm Viability Kit (Thermo Fisher Scientific, Waltham, MA, USA). Here the locust buffer also contained 38 µmol l^{-1} of SYBR 14 (staining live cells). After 10 min incubation time, 50 µl of locust buffer containing 60 µmol l^{-1} propridium iodide (staining dead cells) was added. After another 10 min of incubation (20 min in total), a glass coverslip was gently placed on top of the muscle sample and the glass slide was immediately placed under a Zeiss Axio Imager.A2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). To visualize SYBR 14 bound to DNA of the live cells (excitation λ_{max}: 475 nm, emission λ_{max}: 516 nm), we used a filter for fluorescein isothiocyanate (excitation λ: 450–490 nm, emission λ: 515–565 nm) and for propridium iodide bound to DNA and RNA of dead cells (excitation λ_{max}: 535 nm, emission λ_{max}: 617 nm) we used a filter for Rhodamine (excitation λ: 534–568 nm, emission λ: 575–640 nm), making live cells appear green and dead cells appear red on images.

Image analysis was performed in Fiji (ImageJ; Schindelin et al., 2012). A 750×750 pixel subsample image from the middle of the muscle was used to avoid the cut ends of the muscle fibre. Individual

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 487-496 doi:10.1242/jeb.150813

red and green images were converted to greyscale, and the automatic threshold function was used to isolate live and dead cell nuclei from the background. The number of live and dead cells was counted using the 'Analyse Particles' function. Manual counting was performed on a random subset of images to validate the automated count. In cases where the automated threshold did not work (i.e. very little damage), a manual threshold was set to minimize background noise.

Intracellular and extracellular concentrations of Na^+ and K^+

Hemolymph and muscle concentrations of Na^+ and K^+ were measured in control locusts of each acclimation group and after 0.5, 4, 24 and 48 h of exposure to 0°C (N=7–8 per treatment). After the cold treatment, hemolymph was sampled from the cervical membrane using capillary tubes, and 5 µl was transferred to microcentrifuge tubes containing 2 ml 100 ppm Li^+ buffer and vortexed. In cases where it was not possible to obtain enough from the cervical membrane, the sample was topped off with hemolymph from the leg, which was collected by gently pressing the femur. Immediately after hemolymph sampling, muscle tissue from the extensor tibialis was collected from the femur of both hindlegs via extrusion, quickly blotted dry of hemolymph on Kimwipes, and transferred to a pre-weighed microcentrifuge tube. Muscle wet mass was estimated by weighing the tubes containing muscle tissue again and samples were then dried in an oven at 60°C for 24 h, after which dry mass and muscle water content were determined by reweighing the tubes containing dried muscle. Dried muscle samples were resuspended in 200 µl Milli-Q water and homogenized using steel beads and a tissue lyser (Tissuelyser LT, Qiagen, Hilden, Germany) at 50 Hz for 20 min. Samples were then spun down at 10,000 ***g*** for 10 min, and 5 and 20 µl of the supernatant from each sample was transferred to separate microcentrifuge tubes containing 2 ml of a 100 ppm Li^+ buffer solution. Two different volumes of supernatant were used to keep sample concentrations of Na^+ and K^+ within the sensitivity range of the flame photometer (Model 420, Sherwood Scientific Ltd, Cambridge, UK) used for measurements of both intracellular and extracellular Na^+ and K^+. All measurements were calibrated against standards of known concentrations. Muscle ion concentrations were calculated using the muscle water content. Muscle ion concentrations were corrected on the assumption of a 4% residual hemolymph volume in muscle tissue (MacMillan et al. 2014):

$$\text{Corrected}[X]_\text{i} = \frac{[X]_\text{i} - (0.04 \cdot [X]_\text{o})}{0.96}, \quad (1)$$

where $[X]_\text{i}$ is the measured muscle concentration of ion X and $[X]_\text{o}$ is the measured hemolymph concentration of ion X. Using hemolymph and muscle concentrations of Na^+ and K^+, their respective equilibrium potential (E_Na and E_K) were calculated using the Nernst equation:

$$E_X = \frac{RT}{zF} \cdot \ln\left(\frac{[X]_\text{o}}{[X]_\text{i}}\right), \quad (2)$$

where R is the gas constant, T is the temperature in Kelvin (295 K for controls and 273 K for cold-exposed locusts), z is the ionic charge of ion X, and F is Faraday's constant.

In vivo membrane potential measurement

Resting membrane potential (V_m) was measured in the extensor tibialis muscle fibres of locusts using Clark borosilicate glass microelectrodes (1B100F-4; World Precision Instruments, Sarasota, FL, USA) pulled using a Flaming-Brown P-97 electrode puller (Sutter Instruments, Novato, CA, USA) to have a resistance of 5–10 MΩ. Using a chlorinated silver wire placed in the hemolymph as our reference electrode, membrane potentials were measured using an Electro 705 electrometer (World Precision Instruments) connected to a Micro1401-3 data acquisition system and recorded using Spike2 (version 8) (both from Cambridge Electronic Design, Cambridge, UK).

In order to measure V_m after prolonged cold exposure, locusts were fixed to a glass microscope slide in a putty made of beeswax, resin and paraffin oil (17:2:1 by volume) and were placed inside 50 ml polypropylene tubes. The tubes containing individual locusts on glass slides were then suspended in an ice–water slurry to maintain them at 0°C. V_m was measured in both acclimation groups in control conditions (22°C) and after 1, 4, 24 and 48 h (compared with other measurements, the time point 0.5 h was replaced with 1 h for V_m measurements due to logistics of setting up animals and equipment simultaneously). For measurement, locusts were placed on a pre-cooled plate to maintain a body temperature of 0°C. Extensor tibialis fibres were accessed by gently cutting open the cuticle as described by MacMillan et al. (2014). The exposed muscle fibres were penetrated using the glass electrode, and when a sudden drop in potential was recorded this was interpreted as having entered a cell, and the drop in potential was noted as the membrane potential of the given cell if this could be repeated for a minimum of three times. Thus the V_m of the cell is recorded as the mean of at least three representative repeats. For each individual, varying amounts of cells were measured depending on time and success rate (N=8 per treatment giving rise to V_m measurements from a total of 361 cells).

In vitro tissue damage

The effect of extracellular K^+ on cellular viability was investigated using the same assay as described in the 'Cellular viability after cold exposure' section. However, for the *in vitro* experiments the mesothoracic posterior tergocoxal muscle was dissected out and placed at 0°C for 24 h before the live/dead staining. Thus to examine the effect of $[K^+]$ on cold viability the muscle was carefully dissected out and incubated in 100 µl of standard locust buffer with either 10, 20 or 30 mmol l^{-1} K^+ (when K^+ was elevated, Na^+ was reduced to maintain osmolality). The glass slide was placed under a plastic lid to minimize evaporation during the 24 h period after which cell survival was estimated (N=6–8 per treatment).

In vitro resting membrane potential

To investigate if increasing extracellular $[K^+]$ affected the maintenance of V_m in the two acclimation groups we performed a series of *in vitro* examinations of resting membrane potential at 0°C and with varying extracellular K^+ concentration. V_m measurements were performed as described above; however, for these measurements we used the mesothoracic posterior tergocoxal muscle instead of extensor tibialis muscle fibres as this was also the muscle used for measurement of tissue damage. Here the mesothoracic posterior tergocoxal muscle was exposed and the open thorax secured in a 30 ml glass dish with elastomer in the bottom. The preparation was submerged in locust buffer with either 10, 20 or 30 mmol l^{-1} K^+ and the glass dish was placed on a pre-cooled aluminium plate to reach 0°C (cooling took 15–20 min). Approximately 30 min after muscle dissection, V_m was measured as described above, but here the reference electrode was placed in the buffer. Membrane potential was measured in several cells in the muscle bundle (3:2 male:female, N=8 per treatment resulting in 268 V_m measurements from 48 locusts).

Journal of Experimental Biology (2017) 220, 487-496 doi:10.1242/jeb.150813

Data analysis

All data analysis was performed in R version 3.3.1 (http://r-project.org). The effects of acclimation temperature and cold exposure were tested for in all experiments using two-way ANOVA. In case data was non-normal distributed (estimated from box-plots and Shapiro–Wilk tests), a log-transformation was performed to achieve normality. In experiments where sex was noted, this was included as a factor and removed from the model if no effect was found, and where sex was not recorded (CCRT, survival score and ion balance data) an equal amount of males and females were used. All values presented are means±s.e.m. and the level of significance was assumed to be 0.05 in all analyses.

RESULTS

Chill coma recovery time and survival after cold exposure

After brief periods of cold exposure (0.5 h), the locusts recovered from chill coma to a standing position (CCRT) within 5–10 min, while longer exposure times increased CCRT substantially (main effect of cold exposure time: $F_{2,38}$=30.4, P<0.001; Fig. 1A). Cold-acclimated locusts always recovered considerably faster than their warm-acclimated conspecifics (effect of acclimation group: $F_{1,38}$=9.7, P=0.004; Fig. 1A). Furthermore, after 48 h of cold exposure, none of the warm acclimated locusts could recover from coma within an hour, and from the cold acclimation group the average recovery time was 50.4 min for the five out of eight locusts that recovered within the first 60 min (this point has been removed in the statistical analysis due to missing values for comparison). A significant interaction of acclimation treatment and cold exposure duration on CCRT (interaction: $F_{2,38}$=4.7, P=0.015) further indicates that cold acclimation not only improved recovery times, but did so with increasing effect as the cold exposure duration increased.

A very similar result was observed when chill tolerance was estimated from the survival score obtained following 24 h of recovery at room temperature (Fig. 1B). As expected, the duration of cold exposure had large and significant effects on the performance of recovered animals (effect of cold exposure: $F_{3,56}$=14.1, P<0.001). Brief periods of cold exposure (≤4 h) had marginal effects on the performance of recovered animals (most behaved normally and could all walk the following day) while survival score decreased markedly following 24 and 48 h of cold exposure after which several animals died or were unable to stand. Similar to the CCRT measurements, cold-acclimated locusts showed significantly higher survival scores after recovery following cold exposure (effect of acclimation: $F_{1,56}$=9.5, P=0.003) and did so in a similar way after all exposure durations (interaction effect: $F_{3,56}$=0.7, P=0.530).

In vivo cell survival

In vivo cell viability was estimated at representative time points during the 48 h of cold exposure (Fig. 1C). Estimates of cellular survival in untreated animals (effect of dissection) remained high in both acclimation groups (~90%) while increasing cold exposure duration decreased cellular survival significantly (effect of cold exposure time: $F_{2,55}$=14.4, P<0.001). Cold-acclimated animals tended to suffer less tissue damage during the cold stress, but the effect of acclimation temperature did not reach the criteria of statistical significance (effect of acclimation: $F_{1,55}$=3.5, P=0.067).

Ion balance during cold exposure

There were only small changes in muscle ion concentrations while [Na^+] and [K^+] in the hemolymph changed substantially during cold exposure (Fig. 2A,B,D,E): control values for cold- and warm-acclimated locusts were similar in hemolymph and muscle [Na^+] (hemolymph: 76.0±3.2 and 78.9±1.7 mmol l^{-1}, muscle: 24.2±2.2 and 20.3±2.1 mmol l^{-1}, for 11 and 31°C, respectively), whereas [K^+] was slightly different between the groups (hemolymph: 16.4±2.0 and 11.7±0.7 mmol l^{-1}, muscle: 144.1±4.8 and 134±3.8 mmol l^{-1}, for 11 and 31°C, respectively). Despite a small

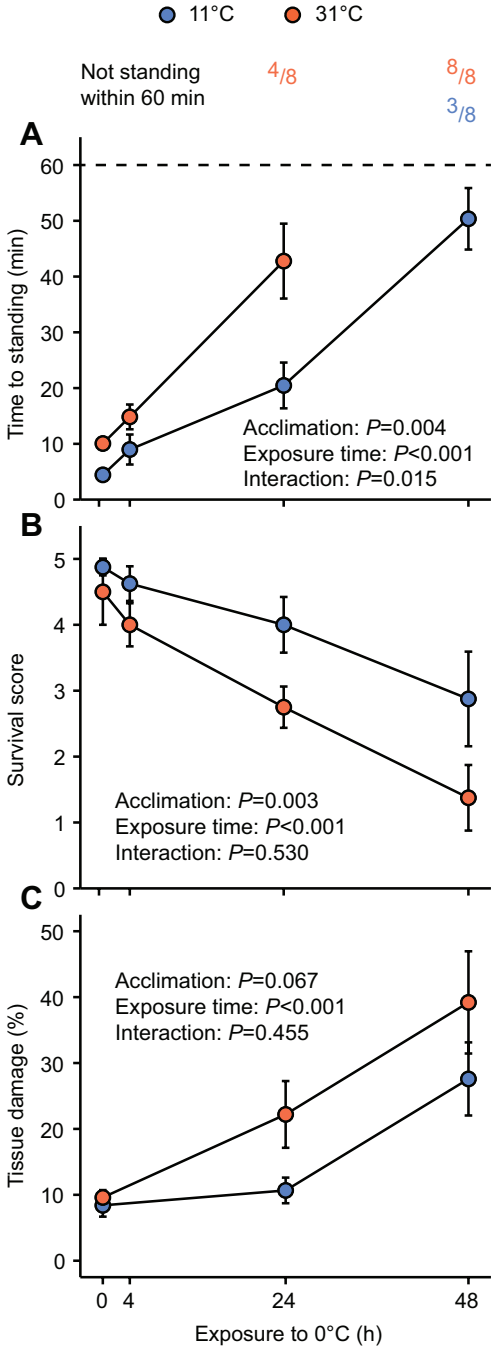

Fig. 1. Organismal and cellular chill tolerance of *Locusta migratoria* acclimated to 11 and 31°C. (A) Chill coma recovery time (measured as time to standing) and (B) survival scores of cold- (blue) and warm- (orange) acclimated *L. migratoria* after exposure to 0°C for different durations and 24 h recovery at 22°C. The numbers above panel A indicate the number of locusts that were not standing within 60 min of observation. (C) Damage accumulated in *L. migratoria* muscle cells in the mesothoracic posterior tergocoxal muscle following 0, 24 or 48 h of 0°C exposure. The horizontal dashed line in A indicates the maximum time that locusts were observed for the ability to stand (CCRT). Values are means±standard error, and error bars not visible are obscured by the symbols.

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 487-496 doi:10.1242/jeb.150813

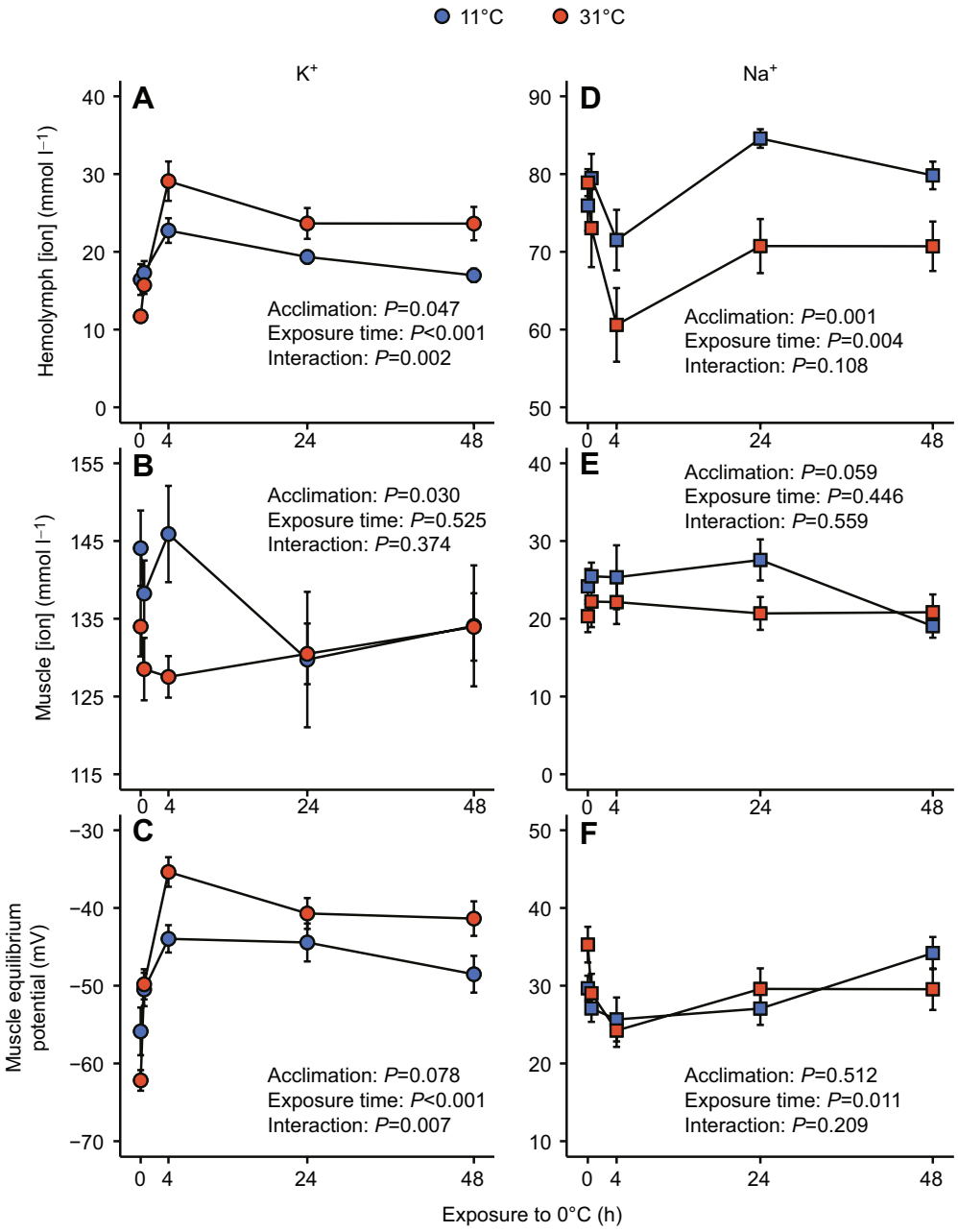

Fig. 2. Ion balance and muscle equilibrium potential of cold-exposed *Locusta migratoria* acclimated to 11 and 31°C. Concentrations of K^+ (circles) and Na^+ (squares) in the hemolymph (A,D) and muscle (B,E) of locusts exposed to 0°C for up to 48 h. Locusts were acclimated to 11°C (blue) and 31°C (orange) prior to the cold exposure. (C) and (F) Depict the muscle equilibrium potentials (Nernst potential) of K^+ and Na^+, respectively. Values are means±standard error, and error bars not visible are obscured by the symbols.

trend for an increase in hemolymph [K^+] for particularly the warm-acclimated locusts, neither hemolymph [Na^+] nor [K^+] changed significantly after a brief cold exposure (0.5 h) when all animals had just entered chill coma. By contrast, extracellular ion concentrations changed considerably during the following hours (effects of cold exposure on hemolymph [ion]: Na^+: $F_{4,68}$=4.2, P=0.004; K^+: $F_{4,68}$=16.6, P<0.001). After 4 h of cold exposure hemolymph [Na^+] had decreased to ~72 mmol l^{-1} ($\Delta Na^+_{11°C}$=−4 mmol l^{-1}, −5.3%) in the cold-acclimated locusts and to ~61 mmol l^{-1} ($\Delta Na^+_{31°C}$=−18 mmol l^{-1}, −22.7%) in their warm-acclimated conspecifics, whilst K^+ increased to ~23 mmol l^{-1} ($\Delta K^+_{11°C}$=+7 mmol l^{-1}, +43.8%) in the cold-acclimated locusts and to 29 mmol l^{-1} ($\Delta K^+_{31°C}$=+17 mmol l^{-1}, +106.3%) in the warm-acclimated locusts. After the initial drastic change in hemolymph ion composition, ion balance recovered partially in warm-acclimated locusts and considerably in the cold-acclimated conspecifics during the prolonged exposure (24 and 48 h).

Muscle [Na^+] and [K^+] (Fig. 2B,E) remained constant within acclimation groups during the initial and prolonged exposure (effects of cold exposure on muscle [ion]: [Na^+]: $F_{4,68}$=0.9, P=0.446; [K^+]: $F_{4,68}$=0.8, P=0.525) with overall average values of 24.3±1.4 mmol l^{-1} (cold) and 21.2±0.4 mmol l^{-1} (warm) for Na^+ and 138.4±3.0 mmol l^{-1} (cold) and 130.9±1.3 mmol l^{-1} (warm) for K^+ during the entirety of the exposure period. Moreover, muscle [K^+] differed between cold- and warm-acclimated locusts during prolonged cold exposure (effect of acclimation on muscle [K^+]: $F_{1,68}$=4.899, P=0.030), and despite the very similar values for muscle Na^+, we observed a tendency that muscle Na^+ was higher in cold-acclimated locusts (effect of acclimation on muscle [Na^+]: $F_{1,68}$=3.7, P=0.059).

Equilibrium potentials for K^+ and Na^+ were calculated from hemolymph and muscle concentrations, respectively (Fig. 2C,F). The equilibrium potential for Na^+ (E_{Na}) depolarized similarly in both acclimation groups (approximately 10 mV) during cold

Journal of Experimental Biology (2017) 220, 487-496 doi:10.1242/jeb.150813

exposure (effect of exposure time: $F_{4,67}$=3.552, P=0.011) followed by a slight gradual recovery during prolonged cold exposure. The equilibrium potential for K^+ for cold- and warm-acclimated locusts had values of −55.9±3.1 and −62.2±1.3 mV, respectively, in the untreated controls, and following cold exposure E_K quickly depolarized to −50.5±2.1 and −49.8±1.9 mV (0.5 h). This was followed by a further depolarization to −44.0±2.4 and −35.4 ±1.9 mV after 4 h (effect of exposure time: $F_{4,67}$=26.1, P<0.001). We found a small yet non-significant effect of acclimation on E_K during cold exposure (effect of acclimation: $F_{1,68}$=3.2, P=0.078), but a strong interaction between exposure time and acclimation temperature (interaction: $F_{4,68}$=3.8, P=0.007), suggesting that E_K was slightly less polarized in cold-acclimated locusts at room temperature but depolarized less than their warm-acclimated conspecifics during cold stress. After the initial depolarization at 4 h, the E_K of both groups repolarized slightly and even more so in the cold-acclimated locusts, but E_K never recovered completely in either group.

Maintenance of membrane potential during prolonged cold exposure

As expected, cold exposure depolarized V_m in extensor tibialis muscle fibres (effect of cold exposure: $F_{4,351}$=61.8, P<0.001; Fig. 3): during the first hour, V_m had depolarized from *ca* −42 mV to −28 mV in cold-acclimated locusts and from *ca* −41 mV to *ca* −30 mV in the warm-acclimated conspecifics; at ≥4 h it had depolarized further to approximately −25 mV and −20 mV in cold- and warm-acclimated locusts, respectively. Thus the cold-acclimated locusts better maintained muscle polarization during cold exposure (effect of acclimation: $F_{1,351}$=10.8, P=0.001). The different temporal development of V_m in the two acclimation groups was also indicated by the significant interaction term (interaction: $F_{4,351}$=4.2, P=0.003), suggesting that muscle cells of warm-acclimated locusts tend to depolarize more after the initial (1 h) depolarization compared with their cold-acclimated conspecifics, which depolarize more during the acute exposure (0 to 1 h).

Effect of hyperkalemia on membrane potential and cellular viability after 24 h at 0°C

The effect of hyperkalemia ([K^+]: 10, 20 and 30 mmol l^{-1}) on cellular viability and membrane potential was investigated *in vitro* after exposing muscle preparations to 24 h of 0°C in a standard locust buffer with varying K^+ concentrations (Fig. 4). Because of the more invasive dissection used for *in vitro* experiments, the cellular level of tissue damage was slightly elevated compared with *in vivo* estimates from control animals (compare Fig. 1C and Fig. 4A). Thus cell mortality *in vivo* was *ca* 10% and *in vitro ca* 20% in controls when the locust saline contained 10 mmol l^{-1} [K^+] (Figs 4A and 1C). Twenty-four hours of cold incubation at increasing extracellular [K^+] (20 or 30 mmol l^{-1}) increased the amount of muscle damage (effect of extracellular [K^+]: $F_{2,35}$=7.8, P=0.002). Interestingly, the effect of hyperkalemia was smaller in cold-acclimated locusts compared with their warm-acclimated conspecifics (effect of acclimation: $F_{1,35}$=6.8, P=0.013), and although non-significant, there was a strong tendency for the

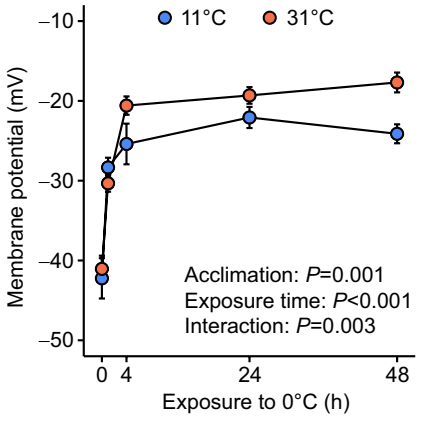

Fig. 3. Muscle membrane potential of cold-exposed *Locusta migratoria* acclimated to 11 and 31°C. *In vivo* membrane potential measured in the femoral muscle cells of locusts at control conditions (22°C, at 0 h) and after 1, 4, 24 and 48 h of cold exposure at 0°C. Orange symbols represent 31°C-acclimated locusts and blue symbols 11°C-acclimated locusts. Values are means±standard error of values of individual cells.

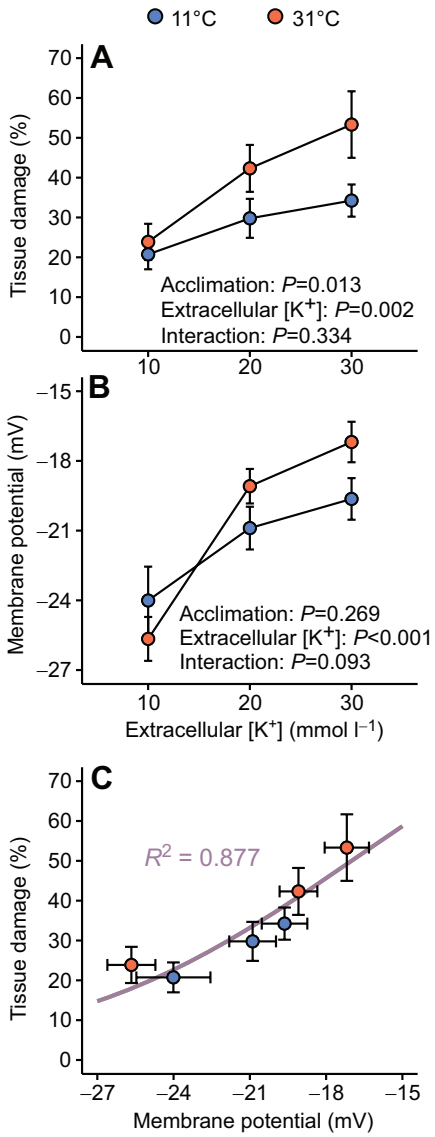

Fig. 4. Estimates of *in vitro* cellular mortality and membrane potential in cold-exposed muscle preparations exposed to different extracellular concentrations of K^+. Muscle damage and muscle membrane potential were measured in the mesothoracic posterior tergocoxal muscle in a locust buffer containing 10, 20 or 30 mmol l^{-1} of K^+. (A) *In vitro* tissue damage after 24 h at 0°C, (B) V_m in muscle cells measured immediately after submersion and cooling to 0°C, and (C) possible correlation between V_m and tissue damage indicated by the fitted sigmoidal function (purple line).

Journal of Experimental Biology

Journal of Experimental Biology (2017) 220, 487-496 doi:10.1242/jeb.150813

muscles of cold-acclimated locusts to be less sensitive to increasing extracellular [K^+] (interaction effect: F=2.5, P=0.088).

To test whether the different sensitivity to [K^+] between cold- and warm-acclimated locusts was related to the membrane potential, V_m was measured in the mesothoracic posterior tergocoxal muscle *in vitro* by acutely exposing the muscle to 0°C whilst incubating in locust buffer with control [K^+] (10 mmol l^{-1}) or hyperkalemia (20 or 30 mmol l^{-1} K^+) and measuring immediately thereafter to imitate the conditions experienced during prolonged cold stress. As seen in Fig. 4B, V_m was −24.0±1.5 mV (11°C acclimated) and −25.7±0.9 mV (31°C acclimated) in control locusts, which is comparable to the 1 h *in vivo* cold treatment condition and muscle cells depolarized with increasing [K^+] (effect of [K^+]: $F_{2,256}$=23.8, P<0.001). While there was no effect of acclimation (effect of acclimation: $F_{1,256}$=1.2, P=0.269) we again observed a (non-significant) tendency for increasing extracellular [K^+] to cause greater depolarization in the muscles of the warm-acclimated locusts relative to their cold-acclimated conspecifics (interaction: $F_{2,262}$=2.5, P=0.093). Here, we also found a significant effect of sex (effect of sex: $F_{1,256}$=7.8, P=0.006) and an interaction between sex and acclimation temperature (interaction: $F_{1,256}$=8.4, P=0.004) such that cold-acclimated females were particularly better at preserving V_m (see Fig. S1). Thus when cell damage is plotted as a function of V_m (Fig. 4C, effect of sex included in Fig. S1) we find that the two acclimation groups fall on a single continuous sigmoidal line correlating polarization of V_m with cellular viability (purple line, Fig. 4C).

DISCUSSION

Cold acclimation improves organismal and cellular survival

Cold acclimating locusts for 4 days at 11°C improves their chill tolerance markedly compared with locusts maintained at 31°C. CCRT increased continuously with more prolonged cold exposure but cold-acclimated locusts always recovered faster than their warm-acclimated conspecifics (Fig. 1A). This is consistent with previous observations after cold acclimation and rapid cold hardening in orthopterans and other insect orders such as *Diptera* and *Hemiptera* (Koštál et al., 2007; MacMillan and Sinclair, 2011a; Findsen et al., 2013; Coello Alvarado et al., 2015; MacMillan et al., 2015b). Survival of the locusts gradually decreased as cold exposure times became longer and again the cold-acclimated locusts always performed better after recovery from cold stress (Fig. 1B). In previous studies, rapid cold hardening has been shown to improve the chill tolerance of locusts such that the brief hardening improved survival after severe cold stress (Wang and Kang, 2003; Findsen et al., 2013). This is also somewhat similar to the fall field cricket *Gryllus pennsylvanicus*, where the proportion of dead crickets after chronic cold stress (>60 h) was lower in animals that had experienced cold acclimation or rapid cold hardening prior to the cold stress (Coello Alvarado et al., 2015). Cold acclimation of the firebug *Pyrrhocoris apterus* (Koštál et al., 2004) and a tropical cockroach *Nauphoeta cinerea* (Koštál et al., 2006) also resulted in increased survival rates after chronic cold stress, whereas both developmental and adult acclimation to cold independently and synergistically aided in improving survival after prolonged cold stress in the fruit fly *Drosophila melanogaster* (Colinet and Hoffmann, 2012; MacMillan et al., 2015b). Thus as expected and reported in numerous previous studies, cold acclimation clearly improves organismal cold tolerance.

Using a live/dead assay we tested whether faster recovery and higher survival following a cold stress were related to cellular viability (Fig. 1C). As hypothesized, we found that cold acclimation also improved cellular survival, as muscle cells from cold-acclimated animals had sustained less tissue damage after ≥24 h of cold stress. This observation is consistent with earlier work by Yi and Lee (2003), who found that seasonal cold acclimation and rapid cold hardening improved cellular survival in the freeze-tolerant gall fly larvae *Eurosta solidaginis*. A similar trend has been observed in the flesh fly *Sarcophaga crassipalpis* (Yi and Lee, 2004; Teets et al., 2013). Yi and Lee (2004) further observed that improvement of cellular survival occurred even if the rapid cold hardening was given to isolated tissues *in vitro* (i.e. salivary glands and Malphigian tubules). Hence, various lengths of cold acclimation all show the potential to provide cellular protection to severe cold stress and this protection is at least partially independent of humoral and nervous regulation.

Cold acclimation improves ion homeostasis during cold stress

Numerous studies have shown how chronic cold stress changes hemolymph [K^+] and [Na^+] in locusts (Andersen et al., 2013; Findsen et al., 2013) and other insects (Koštál et al., 2004, 2006; MacMillan and Sinclair, 2011b; MacMillan et al., 2014, 2015b; Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016) and consistent with these earlier observations we found that ion balance of locusts was disrupted during prolonged cold stress (≥4 h). Specifically we found that muscle ion concentrations generally remained constant (Fig. 2B,E) while hemolymph ion concentrations underwent considerable changes in the form of significant decreases in hemolymph [Na^+] and increases in hemolymph [K^+]. Furthermore, we noted that the changes in hemolymph ion balance associated with cold stress were smaller in cold-acclimated locusts (Fig. 2A,D), which is also consistent with previous observations of cold-stressed insects (Koštál et al., 2004, 2006, 2007; Coello Alvarado et al., 2015; MacMillan et al., 2015b).

The causes and consequences of perturbed ion balance in insects is complex and has been discussed extensively in recent papers (MacMillan and Sinclair, 2011b; MacMillan et al., 2015b; Overgaard and MacMillan, 2017). The general picture that has emerged suggests that a leak of hemolymph Na^+ (and water) towards other tissues will cause hemolymph volume to decrease, which then secondarily increases hemolymph [K^+] (Koštál et al., 2004; MacMillan and Sinclair, 2011b; MacMillan et al., 2015b; Overgaard and MacMillan, 2017). The ensuing increase in hemolymph [K^+] then contributes to membrane depolarization, which causes cellular and organismal failure (see discussion below). The present study has not measured aspects of water balance and it is therefore beyond the scope of the present study to speculate on why cold-acclimated locusts maintain ion balance better. However, we note that both hemolymph [Na^+] and [K^+] were preserved better in cold-acclimated locusts, which is consistent with the hypothesis that hemolymph [K^+] is defended because the leak of hemolymph [Na^+] (and water) is reduced in cold-adapted/acclimated animals (Koštál et al., 2004; Coello Alvarado et al., 2015; MacMillan et al., 2015a,b).

The changes in ion concentrations we observed during cold stress gave rise to changes in the equilibrium potentials for Na^+ and K^+ (E_{Na} and E_K, respectively). As previously observed in cold-stressed locusts, we found that E_K depolarized considerably, while E_{Na} remained fairly constant during the prolonged cold stress (Fig. 2C,F) (MacMillan et al., 2014). Cold-acclimated locusts retained a more polarized E_K than their warm-acclimated conspecifics because they were able to better maintain transmembrane K^+ balance, a pattern also observed in crickets (Coello Alvarado et al., 2015), fire bugs

(Koštál et al., 2004), and cockroaches (Koštál et al., 2006). Thus cold acclimation improves the maintenance of E_K, which correlates well with the increased rates of survival and performance after cold stress (Overgaard and MacMillan, 2017). This relationship between E_K and survival is often inferred from the association between E_K and membrane potential (V_m), since the distribution of K^+ is the principal determinant of insect membrane potential (Hoyle, 1953; Wood, 1963). However, to our knowledge no previous study has examined the influence of cold acclimation on preservation of V_m directly.

Chill-tolerant locusts are better at preserving V_m

Membrane potential (V_m) is a complex trait, which represents the sum of the diffusion potential (V_d) and the electrogenic effect (V_e). The diffusion potential is the largest component of V_m and can be approximated by the Goldman–Hodgkin–Katz equation (Goldman, 1943; Hodgkin and Katz, 1949); V_d is dependent on the uneven distribution of ions across cell membrane, and the relative permeabilities of these ions, as well as a thermodynamic component (i.e. temperature is a parameter in the Goldman–Hodgkin–Katz equation that describes the diffusion potential). The electrogenic effect is a potential generated by the activity of ion pumps transporting an uneven number of charges across cell membranes (i.e. the Na^+/K^+ pump) and the potential thus depends on the amount of ion pumps and their activity, which is highly temperature dependent (Rheuben, 1972; Pichon and Treherne, 1974; Djamgoz, 1987; Fitzgerald et al., 1996).

After acute exposure to 0°C (1 h), V_m had depolarized significantly by ∼14 and ∼11 mV in cold- and warm-acclimated locusts, respectively (Fig. 3). At this time point we observed a small (non-significant) disturbance in ion balance and only in the 31°C acclimation group. Acute changes in ion balance can therefore only partly explain the magnitude of membrane depolarization measured and the depolarization of membrane potential must therefore also be caused by the thermodynamic component of V_d, a reduced electrogenic effect and/or by changes to the relative permeability of ions (Wareham et al., 1974; MacMillan et al., 2014; Overgaard and MacMillan, 2017). After 4 h of cold exposure, V_m had depolarized further by ∼3 mV and ∼10 mV in cold- and warm-acclimated locusts and plateaued in both acclimation groups. This 'additional' depolarization was smaller in cold-acclimated locusts compared with their warm-acclimated conspecifics (Fig. 3) and is probably mainly associated with the smaller changes in E_K associated with cold-induced increase in extracellular $[K^+]$ (Hoyle, 1953; Wood, 1963), which was only increased by ∼5 mmol l^{-1} in the cold-acclimated locusts compared with ∼14 mmol l^{-1} in the warm-acclimated locusts (see Fig. 2).

Preservation of membrane potential during cold stress correlates with improved cellular survival and organismal performance

The observations discussed above are entirely consistent with the general picture that has emerged from several previous studies of insect cold tolerance and cold acclimation. Cold acclimation improves organismal performance and cellular viability after cold stress, and this is associated with an improved maintenance of ion balance. While previous studies have suggested that 'improved ion homeostasis' preserves membrane potential, the present study is the first to demonstrate this link through direct measurements of V_m.

The causal relationship between membrane depolarization and cellular viability has been proposed to stem from an imbalance in Ca^{2+} balance such that constant severe depolarization of V_m causes cellular apoptosis or necrosis through a catastrophic increase in intracellular $[Ca^{2+}]$ (Hochachka, 1986; Boutilier, 2001). Teets et al. (2013) demonstrated how small increases in intracellular $[Ca^{2+}]$ occurred when temperature was gradually lowered and suggested that Ca^{2+} balance could aid cold sensing in insects. The cell membrane depolarization that occurs when insects are gradually cooled could be responsible for this influx of Ca^{2+} into the cytosol, which further corroborates the hypothesis of a certain threshold for membrane depolarization and intracellular $[Ca^{2+}]$ needs to be passed before initiating cell death (Nicotera and Orrenius, 1998; Bortner et al., 2001). Hence lowering temperature chronically causes the membrane potential to depolarize to a degree that may affect the status of voltage-gated Ca^{2+} channels sufficiently to cause a debilitating increase in intracellular $[Ca^{2+}]$ (Hochachka, 1986; Nicotera and Orrenius, 1998; Bortner et al., 2001; Boutilier, 2001). In our study we observed that chill injury continued to increase even after V_m had stabilized (after approximately 4 h); this indicates that it is not only the degree of polarization, but also the duration of the depolarized state that determines the amount of chill injury. Consistent with this idea we found that cold-acclimated locusts maintained polarization of V_m better, and the same animals also showed improved cellular and organismal survival. In a previous study by MacMillan et al. (2015c) we showed that only the combined effect of hyperkalemia and chilling causes injury, which suggested that V_m must be depolarized past a critical threshold level to induce necrotic/apoptotic events. Our results support the hypothesis that by maintaining low hemolymph $[K^+]$ during cold stress, cold-acclimated locusts better preserve V_m, which ensures that cellular viability and organismal performance is maintained during chilling. Warm-acclimated locusts, however, were unable to maintain low hemolymph $[K^+]$, resulting in a greater proportion of cells crossing the hypothetical V_m threshold, and cellular and organismal mortality thus ensued. Moreover, our study shows that the amount of time spent in cold stress is a key factor for the degree of chill injury as cellular mortality continued to increase after V_m had already plateaued (approximately 4 h), such that more time spent in this depolarized state increased mortality. This also demonstrates how the depolarization precedes the cellular mortality, further corroborating our conceptual model.

Cold acclimation lowers cellular K^+ sensitivity in locusts

As discussed above, cold acclimation improves homeostatic control with respect to ion balance, which has direct implications for the improvement of chill tolerance through improved maintenance of cellular polarization and viability. However, it is obviously possible that cold acclimation also improves performance at the cellular level in ways that are independent of ion balance. A number of studies using *in vitro* tissue preparations have, for example, shown how different forms of cold acclimation can improve cellular viability (Yi and Lee, 2003, 2004; Teets et al., 2013). Such improvements occur independently of extracellular ion balance as the *in vitro* experiments are conducted in standardized insect buffer, which also ensured the independence of neuronal and humoral signalling. To examine if cold acclimation also improves cellular cold tolerance in locusts, we measured cellular viability of *in vitro* muscle preparations after 24 h of cold exposure in cold- and warm-acclimated locusts, respectively. These experiments were conducted at 10 (control), 20 or 30 mmol l^{-1} extracellular $[K^+]$ and as expected, increasing extracellular $[K^+]$ increased tissue damage (Fig. 4A). However, we also noted that the cellular sensitivity to hyperkalemia was lower in muscles from cold-acclimated locusts compared with their warm-acclimated conspecifics. To examine whether this 'additional' cellular cold tolerance occurred

independently of membrane potential, we measured V_m directly in similar *in vitro* preparations acutely exposed to the same three levels of hyperkalemia at 0°C. Surprisingly, we found a trend that muscle membrane polarization was affected differently in the two acclimation groups such that hyperkalemia caused a stronger depolarization in warm-acclimated locust muscle (Fig. 4B). However, when these two factors (cellular viability and cellular polarization) are analysed together, we still find a strong association in which viability begins to increase when V_m approaches the same 'critical level' (V_m L_{50}=−17±0.8 mV from the sigmoidal fit; Fig. 4C). As discussed above, this 'critical level' for cellular mortality is unlikely to represent a fixed value, as the probability of cell death is also likely to depend on the amount of time spent in a depolarized state. As all of our *in vitro* studies were conducted over similar durations, our data still suggest that the depolarization of V_m affects muscle cell viability in the same way in the two acclimation groups. Both groups had a similar 'threshold V_m' if analysed separately (V_m L_{50}=−15.5±0.1 and −17.6±0.6 mV for cold- and warm-acclimated locusts, respectively). That said, we note a small tendency for cold-acclimated locusts to acquire less injury at the same depolarized state (Fig. 4C), and interestingly we also found an effect of sex on cellular polarization (see Fig. S1). Further studies are needed to investigate these sources of variation, but here we clearly demonstrate a relationship between *in vitro* cell viability and polarization that reinforces the hypothesized model in which depolarization initiates an apoptotic/necrotic cascade (Hochachka, 1986; Boutilier, 2001; MacMillan et al., 2015c; Overgaard and MacMillan, 2017). These results also raise three important questions: (1) why might the V_m of cold-acclimated cells be less affected by hyperkalemia?; (2) can this relationship be exposed through manipulation of other factors than K^+ balance during hypothermia?; and (3) does this relationship between injury and membrane potential hold for other (i.e. non-excitable) tissues?

As discussed above, cell membrane potential is a complex trait that is dependent on several factors including ion pump activity (the electrogenic effect), transmembrane ion distribution and the relative permeability of the major ions, as well as on thermodynamic effects on V_d (the diffusion potential). In our *in vitro* experiments, we find that cold-acclimated cells are less affected by extracellular [K^+], suggesting that one or several of the other factors determining V_m must also be different. It is beyond the scope of the present study to identify which factors underlie the reduced K^+ sensitivity in cold-acclimated muscles, but factors such as increased Cl^- permeability or increased electrogenic activity of ion pumps could be involved (Wareham et al., 1974; Djamgoz, 1987; Fitzgerald et al., 1996; Overgaard and MacMillan, 2017). Either way, our results indicate that cold acclimation not only reduces the perturbation of K^+ homeostasis but also reduces the cellular sensitivity to K^+.

Conclusions

In the present study, we have shown at several levels of biological integration how cold acclimation improves survival after cold stress. We demonstrate for the first time how increased organismal and cellular performance after cold acclimation is directly linked to preservation of V_m. Our data strongly suggest that cold acclimation serves to preserve V_m through improved ionic regulation, but also through decreased cellular sensitivity to K^+ balance. Together these observations highlight two important questions to guide future research: why is V_m important for maintenance of cellular viability in insects?; and why is cell membrane potential less affected by K^+ balance in a cold-acclimated insect? Clearly, elucidating the physiology of insect cold tolerance will benefit both fundamental and applied science in terms of understanding the core determinants of ectotherm cold tolerance.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

This study was conceived and designed by M.K.A. and J.O. Experiments were carried out by M.K.A and R.F. M.K.A. and H.A.M. analysed the data. M.K.A. and J.O. wrote first draft, and all authors contributed to and approved the final version.

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Supplementary information

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