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Concurrent effects of cold and hyperkalaemia cause insect chilling injury

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Chilling injury and death are the ultimate consequence of low temperature exposure for chill susceptible insects, and low temperature tolerance is considered one of the most important factors determining insect distribution patterns. The physiological mechanisms that cause chilling injury are unknown, but chronic cold exposure that causes injury is consistently associated with elevated extracellular [K^+], and cold tolerant insects possess a greater capacity to maintain ion balance at low temperatures. Here, we use the muscle tissue of the migratory locust (*Locusta migratoria*) to examine whether chill injury occurs during cold exposure or following return to benign temperature and we specifically examine if elevated extracellular [K^+], low temperature, or a combination thereof causes cell death. We find that *in vivo* chill injury occurs during the cold exposure (when extracellular [K^+] is high) and that there is limited capacity for repair immediately following the cold stress. Further, we demonstrate that that high extracellular [K^+] causes cell death *in situ*, but only when experienced at low temperatures. These findings strongly suggest that that the ability to maintain ion (particularly K^+) balance is critical to insect low temperature survival, and highlight novel routes of study in the mechanisms regulating cell death in insects in the cold.

1. Introduction

Cold tolerance is among the most important factors defining the fundamental niche and distribution of insect species [1–4]. Some species have evolved extraordinary adaptations that allow them to avoid or safely permit freezing of their body fluids, and thereby inhabit some of the harshest environments on the globe [5,6]. Many insects, however, succumb to the effects of chilling at temperatures well above the freezing point of their body fluids. For such chill sensitive insects, including the majority of temperate, subtropical and tropical insect species, chilling causes a loss of homeostasis that leads to paralysis, injury and ultimately death [7–9]. In these species, decreasing temperature is associated with a progressive decrease in activity until the animal eventually enters a state of neuromuscular paralysis (chill coma) related to a progressive depolarization of excitable tissues [10–19]. Chill coma is not a secondary result of injury, as comatose insects fully recover if they are quickly returned to permissive temperatures [10,12,15,20]. However, injury and mortality develop if the cold exposure is prolonged or intense [8,21–24] and chilling injury must, therefore, be associated with physiological phenomena that occur after coma onset. Interestingly, inter- and intraspecific studies have found that the temperature of chill coma onset and sensitivity to chilling injury correlate tightly [25], so although chill coma onset and chill injury are caused by distinct mechanisms, they may yet have some underlying mechanistic relationship [8,15,20,26].

The direct cause of chilling injury in insects is still poorly understood, but several mechanisms have been suggested. Three of the most widely suggested mechanisms are: (i) cold-induced membrane phase changes, (ii) protein denaturation, and finally (iii) a loss of transmembrane ion balance caused by reduced activity of ion-motive pumps. While these three mechanisms could potentially occur in parallel, it is possible to speculate about their relative contribution by considering the processes that initiate each form of injury. First, temperature affects the phase and fluidity of the liquid-crystalline membrane and cooling could, therefore, cause injury through phase changes that

compromise the physical integrity of the membrane [27–29]. Second, cold can induce protein denaturation [30] and numerous studies have found increased expression of heat shock proteins (*hsp*'s) in relation to chill injury [31–34]. Interestingly, the vast majority of these studies find that *hsp* are expressed in the hours following the return to benign temperatures [31,34–36]. While these observations could indicate that protein denaturation is accrued during the reheating it is also possible that increased *hsp* expression is secondary to other sources of injury, but only evident during reheating when temperature allows for transcription and/or translation. A third suggestion regarding chill injury is linked to the consistent observation that chill injury correlates with loss of ion balance, and in particular, increased extracellular [K^+], which, together with temperature, is very important in determining the resting cell membrane potential [8,15,21,22,37–39]. In many insects, low temperature reduces active ion transport, which causes a marked membrane depolarization (because of the lost electrogenic effect of these pumps) [8,15,22]. The reduction in active transport also gradually causes a loss of ion and water balance; Na^+ and water leave the extracellular space while some K^+ leaks towards the haemolymph, causing a progressive increase of extracellular [K^+] and further cell depolarization (e.g. [21,40]). The combined depolarizing effects of low temperature and high extracellular [K^+] are suggested to initiate a debilitating cascade of events [8]. In this scenario, a critical depolarization opens voltage-sensitive Ca^{2+} channels, after which the ensuing Ca^{2+} influx causes an uncontrolled activation of Ca^{2+}-dependent proteases and lipases that ultimately destroy the cell integrity, possibly through activation of apoptotic signalling [8,24,41,42].

In this study, we used a combination of *in vivo* and *in situ* experiments to investigate the primary cause of chill injury in an insect, the locust. Using a live/dead assay on muscle tissue, we first examined the progressive development of injury in intact animals during chronic exposure to cold and following recovery. Because chronic cold exposure is inevitably associated with increased extracellular [K^+] in locusts, we followed these experiments using an *in situ* approach, in which we could isolate the effects of chronic cold and hyperkalaemia. Using the combined *in vivo* and *in situ* results, we discuss if injury is likely to be associated with membrane phase changes (injury will develop with duration of cold exposure and be independent of K^+), protein denaturation (injury will primarily develop during the recovery from cold and be independent of K^+) or membrane depolarization (injury will develop primarily when cold and hyperkalaemia are combined, through their additive effects on cell resting potential).

2. Material and methods

(a) Animal husbandry

Female locusts (*Locusta migratoria*, Linnaeus 1758) used in this study were purchased as newly emerged adults from a commercial supplier (Peter Andersen Aps, Fredericia, Denmark), and placed into 0.45 m^3 cages in a room at 22°C on a 12 L : 12 D cycle. Adult locusts had ad libitum access to fresh wheat sprouts, wheat bran and water and were held with cardboard egg cartons to facilitate hiding. A 150 W heat lamp that was on during the day created a thermal gradient from 25 to 45°C to allow for behavioural thermoregulation. Locusts were maintained under these conditions for 7–14 days before being used in experiments.

(b) Chill coma recovery time and chilling injury

Chill coma recovery time (CCRT) and the degree of chilling injury were measured as previously described [15]. Briefly, locusts were individually placed in 50 ml polypropylene centrifuge tubes that were suspended in a mixture of ethylene glycol and water (1 : 1 v/v) in a bath at 20°C. After a 15 min hold at 20°C the temperature (according to thermocouples measuring air temperature in three random tubes) was reduced to −2°C at −0.15°C min^{-1} and then maintained at −2°C for up to 72 h. Ten locusts were removed to record CCRT and survival immediately upon reaching −2°C, and after 24, 48 and 72 h at −2°C. The locusts were positioned on their side on a table lined with paper and were observed for up to 90 min at 22°C. We recorded time to recovery of muscle function (identified as a controlled extension of either hind leg) and time required for a locust to voluntarily stand on its feet (typically called CCRT). Following recovery, each locust was placed back into its tube, and given wheat bran and water, upon which the locusts were maintained at 22°C for 24 h. We assessed chilling injury the following day by removing a locust from its tube and coaxing it to walk and jump on a filter paper surface. Injury was recorded on a 5-point scale: 5, able to stand, walk and jump in a coordinated manner; 4, able to stand, walk and/or jump, but some lack of coordination; 3, able to stand, but unable or unwilling to walk or jump; 2, moving, but unable to stand; 1, no movement observed (i.e. dead) [15]. Control locusts were given no cold exposure and were all given a score of 5 owing to the absence of neuromuscular injury. To examine whether the degree of injury changed with time after the cold exposure, we also assessed chilling injury in groups of 10 locusts that were only given 3 h of recovery at 22°C following exposures to −2°C for 24 and 48 h. Locusts given 72 h at −2°C were all considered dead as they were never observed to move. Accordingly, we did not measure ion balance in locusts given 72 h at −2°C, although we did measure the degree of muscle damage immediately following the cold stress.

(c) Haemolymph [K^+]

We measured concentrations of K^+ in the haemolymph of locusts under control conditions, upon reaching −2°C, following 24 h and 48 h at −2°C, and following 3 h and 24 h recovery at 22°C from a 24 h or 48 h cold exposure ($n = 7–10$ locusts per treatment). Haemolymph samples (greater than 5 µl) were collected using capillary tubes (approx. 10–25 µl) from an opening in the neck membrane, between the head and the thorax. A 5 µl aliquot of the haemolymph was then transferred by pipette to a microcentrifuge tube containing 2 ml of 100 ppm lithium buffer. The concentration of K^+ in diluted haemolymph samples was measured using a Sherwood flame photometer (Model 420, Sherwood Scientific Ltd, Cambridge, UK) from reference to prepared standards of known concentration as previously described [43].

(d) Muscle damage

Given that a loss of coordination or the capacity for movement is the most evident and oft-cited indication of chilling injury in insects, we use muscle tissue as a model. We quantified muscle damage in locusts at room temperature (control), those that had just reached −2°C, locusts that experienced 24, 48 or 72 h at −2°C, and locusts that experienced 24 or 48 h at −2°C and were given 3 or 24 h to recover at 22°C before dissection ($n = 6$ locusts per experimental group). In all cases, dissections were carried out at 22°C within 2 min. The head was removed and a ventral longitudinal incision was made through the meso-and metathoracic ganglia, after which the legs and wings were removed. The ventral incision was extended down the abdomen

and the thorax was carefully pinned open in a 75 ml dish with an elastomer filled base (Sylgard 184, Dow Corning Corp., Midland, MI, USA). The gut and ovaries were removed and discarded before 50 ml of locust buffer (final concentrations: 140 mM NaCl, 8 mM KCl, 3 mM $CaCl_2$, 2 mM $MgCl_2$, 1 mM NaH_2PO_4, 90 mM sucrose, 5 mM glucose, 5 mM trehalose, 1 mM proline, 10 mM HEPES, pH 7.2) was added to the preparation.

Forceps were used to carefully tear away the fat body and air sacs obscuring the thoracic musculature, and the mesothoracic posterior tergocoxal muscle (muscle M90; [44]) was isolated. This bifunctional muscle (being a remoter of the mesothoracic leg during walking and elevator of the forewing during flight) was chosen as it is easily identified and removed with minimal damage caused to the muscle body. We cut the distal tendon, immediately before cutting through the muscle body as close as possible to the dorsal body wall using microscissors. Forceps were then used to extract the muscle and remove any large trachea still attached to the muscle body. The extracted muscle was placed on a clean glass slide in a 50 µl droplet of fresh buffer. The proximal end of the muscle (which was mechanically damaged by the scissors) served as a staining control for dead tissue, and was confirmed in every preparation before imaging.

To quantify damage, we used the LIVE/DEAD Sperm Viability Kit (Thermo Fischer Scientific, Waltham, MA, USA), with modification to the method described by Yi & Lee [45]. The buffer surrounding the muscle was removed by micropipette, and the muscle was incubated in 50 µl of 38 µM SYBR 14 in locust buffer (10 min), after which 50 µl of 60 µM propridium iodide in buffer was added (for a further 10 min). A coverslip was gently placed over the sample, and the centre of the muscle body was imaged using a Zeiss Axio Imager.A2 fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany). We used a filter developed for fluorescein isothiocyanate (excitation: 450–490 nm, emission: 515–565 nm) to visualize SYBR 14 bound to DNA of live cells (excitation λ_{max}: 475 nm, emission λ_{max}: 516 nm) and filter for Rhodamine (excitation $\lambda = 534$–568 nm, emission $\lambda = 575$–640 nm) to visualize propridium iodide bound to DNA and RNA of dead cells (excitation λ_{max}: 535 nm, emission λ_{max}: 617 nm). Thus, live cells in the images appear green, and dead cells appear red.

Image analysis was completed in Fiji, a distribution of ImageJ [46]. A 750 × 750 pixel subsample from the centre of each image was used for analysis to avoid the cut end of the muscle and focus on several fibres in focus in the centre of the muscle body. For each image, the green (SYBR 14) or red channel (propridium iodide) was isolated and converted to greyscale. Image thresholds were determined using the automatic threshold applet in Fiji with default settings (iterative intermeans). In some cases, particularly red images that contain very little damaged tissue (i.e. controls), this automated approach failed, and the threshold was manually set in a manner that minimized background noise while maximizing any apparent signal. We noted that damage to the muscle could appear as a few large red features, or many smaller nuclei stained red, and we thus analysed the data in the form of both total fluorescing area and number of fluorescing particles. Choice of method had no impact on any of our conclusions, so we present an average proportion of damage, and include full results of both analysis methods in the electronic supplementary material, figure S1. Composite images for presentation in the figures were created using the Merge Channels function, which combined the red and green channels of the two images. For presentation in the figures, the saturation and brightness of the red and green channels were manually adjusted for visual clarity, and raw versions of all images are included in the electronic supplementary material.

To test the independent and combined effects of extracellular $[K^+]$ and temperature on muscle viability, we use semi-intact locust preparations. Locusts (with the head, extremities gut and ovaries removed) were pinned open in a 75 ml dish at room temperature as described above and 50 ml of a buffer was added. The buffer either had low $[K^+]$ (8 mM, identical to that used above) or high $[K^+]$ (30 mM KCl, with NaCl reduced to 118 mM to maintain osmolality) and was pre-warmed or cooled to 22 or 0°C, respectively. Although our *in situ* experiment was not 'fully crossed' *per se*, we argue that 3 h at 22°C is comparable in 'biological time' with 24 h at 0°C (assuming a Q_{10} of 2.5). More importantly, however, the conditions were chosen for their biological relevance; the two buffers used simulate the extracellular conditions of a control locust (low $[K^+]$) and a locust that had been at −2°C for 48 h (high $[K^+]$) based on the results of the prior ion measurements. The semi-intact preparations were then held at either 22°C for 3 h (which simulated control conditions [8 mM] or conditions immediately following rewarming [30 mM]) or 24 h at 0°C, after which the tergocoxal muscle was removed and treated as above to quantify cellular damage. Although this design does not allow us to examine whether an exposure to 30 mM $[K^+]$ for 24 h causes damage, this situation does not occur during or following cold exposure, so is not biologically relevant.

(e) Data analysis

All data analysis was completed in R v. 3.0.3 [47]. The effects of cold exposure duration on time to leg movement and CCRT, and the effects of cold exposure and recovery time on haemolymph $[K^+]$ were analysed by one-way analysis of variance followed by Tukey's HSD. Locust survival scores were compared using a Kruskal–Wallis rank-sum test followed by multiple comparison using the kruskalmc() function in the pgirmess package [48]. Analysis of covariance was used to analyse the number of red fluorescing features or red fluorescing area, with treatment included as a factor and total green features or area included as covariates. *Post hoc* analysis of differences among groups for illustrative purposes was done with one-way analysis of variance on per cent damage among treatment groups, followed by Tukey's HSD. Muscle damage in semi-intact preparations was compared in the same manner, but with temperature and $[K^+]$ concentration (and their interaction) included as factors. *Post hoc* analysis was done by pairwise *t*-tests on the per cent damaged area, with *p*-values corrected for false discovery [49].

3. Results and discussion

(a) Chronic exposure to −2°C causes chilling injury, slows chill coma recovery and elevates extracellular $[K^+]$

Cold-induced injury and death have repeatedly been associated with increased extracellular potassium concentrations in a variety of distantly related chill susceptible insects [8,21,22,43,50]. Chill tolerant congeners of these species, or members of the same species that have been cold acclimated, by contrast, maintain 'normal' extracellular $[K^+]$, and suffer little injury during a comparable cold exposure [22,23,26,51]. Taken together, this body of literature suggests that elevated extracellular $[K^+]$ may be a primary cause of chilling injury and death, but most previous evidence is of a correlative nature.

The findings in this study are all in keeping with the prior literature on ion balance and chilling injury. Locusts that were exposed to −2°C were in chill coma when removed from the cold, and the time required to recover the ability to move the hind legs ($F_{2,27} = 34.5$, $p < 0.001$) and voluntarily stand ($F_{2,19} = 49.75$, $p < 0.001$) both increased with increasing

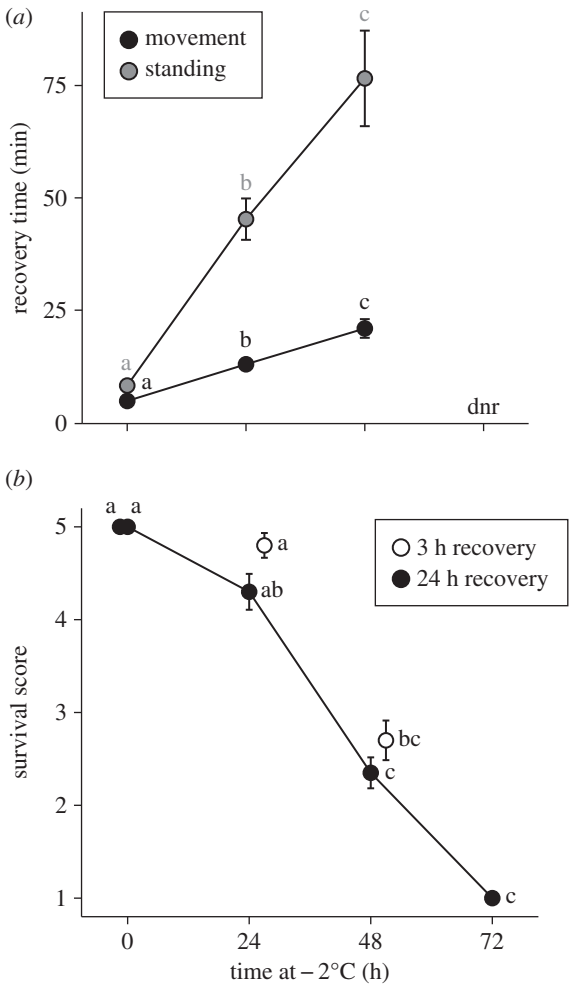

Figure 1. Prolonged exposure to −2°C slows recovery from chill coma and causes chilling injury in locusts. Recovery from chill coma and incidence of chilling injury in female locusts following exposure to −2°C for varying lengths of time. (*a*) Chill coma recovery time (mean ± s.e.m.), measured as the ability to extend the hind legs (black) or voluntarily stand (grey). Locusts that experienced 72 h at −2°C did not recover (dnr) any ability to move within 90 min of observation. (*b*) Survival scores of locusts given 3 h (white) and 24 h to recover at 22°C before assessment of injury (5, healthy; 1, dead; see Material and methods for details of scoring). Means that share a letter do not significantly differ (see Material and methods for details).

duration of cold exposure. After 48 h at −2°C, however, only 2 of the 10 animals were able to stand within 90 min of observation (although all were observed to move), and none of the animals that were at −2°C for 72 h showed any signs of movement (figure 1*a*). Duration of time at −2°C significantly impacted the degree of whole animal injury ($\chi^2_6 = 70.6$, $p < 0.001$) (figure 1*b*). Survival scores of the animals deteriorated progressively until 72 h at −2°C where all of the locusts were dead, and this result was independent if the survival score was assessed 3 h or 24 h after removal from the cold (figure 1*b*). The progressive development of injury was closely associated with a gradual increase in extracellular [K^+] ($F_{7,68} = 9.1$, $p < 0.001$; figure 2*a*) and recovery at 22°C was associated with a rapid (< 3 h) restoration of K^+ balance, as has been previously observed in this species [40,43]. Also as previously noted, mean haemolymph [K^+] did not fully return to control levels within 24 h of recovery [15]. Taken together, chilling injury is closely associated with the magnitude of the cold-induced hyperkalaemia, and a rapid recovery of [K^+] balance upon rewarming does not appear to impact the degree of chilling injury observed at the whole animal level.

(b) Chronic cold exposure causes muscle damage and repair occurs slowly following rewarming

To examine whether the observed development of chilling injury was reflected at the cellular level, we used the mesothoracic posterior tergocoxal muscle as a model tissue to quantify muscle damage following cold exposure and recovery (figure 2*b–k*). Control locusts had minimal damage to their muscles (less than 4% dead tissue), and prolonged exposure to −2°C caused muscle injury. Cold exposure treatment strongly impacted the proportion of propridium ioidide staining (red, dead) to SYBR 14 staining (green, alive; feature counts: $F_{8,44} = 4.5$, $p < 0.001$; area: $F_{8,44} = 4.7$, $p < 0.001$). The degree of injury to the muscle increased with increasing cold exposure duration, up to a maximum of approximately 51% damage in locusts that spent 72 h in the cold (figure 2*b*; electronic supplementary material; figure S1). Recovery time had a comparatively small effect on the degree of muscle damage, as locusts recovered for 24 h at 22°C after 24 or 48 h at −2°C recovered only some of the damage induced by cold exposure (figure 2*b*). Thus, damage to locust muscles appears to be accrued during the cold exposure, rather than following removal to warmer temperatures, and within the short time frame used here (1 day) there is only limited capacity for cellular repair and/or clearance of dead cells. The extent of tissue damage and slow course of recovery observed following chronic cold exposure could explain the repeated observation that cold exposed insects appear unable to completely recover low haemolymph [K^+] in the hours following a cold stress (figure 2*a*; [12,15,43]). Most probably, continuous ion leak from damaged cells keeps the insects from completely restoring their original K^+ balance.

(c) Synergistic effects of cold and hyperkalaemia cause cell death

The exact cause of chill injury in insects is poorly resolved but has most often been associated with membrane damage/phase change, protein denaturation or uncontrolled cellular depolarization. These potential causes are difficult to separate *in vivo* as cold is likely to induce multiple perturbations concurrently. We postulated that if injury were associated with membrane phase changes or cold-induced protein denaturation, cell death would develop at low temperature and be independent of extracellular [K^+]. We did not assess protein denaturation or *hsp*s levels in the present study. However, the vast majority of studies investigating the transcription/translation of *hsps* following cold stress in insects find that this takes place after the cold exposure and not during the exposure. While delayed *hsp* expression could suggest that damage occurs during recovery, it also possible that *hsp* expression is simply delayed to the recovery period because the processes of expression are compromised by low temperature. Nevertheless, if injury develops directly as a cause of cold-induced protein denaturation, then we would expect injury to develop during the cold exposure and/or during recovery from cold but still be independent of extracellular [K^+].

Finally, if cell membrane depolarization is the primary cause of chill injury, we predict that injury will develop

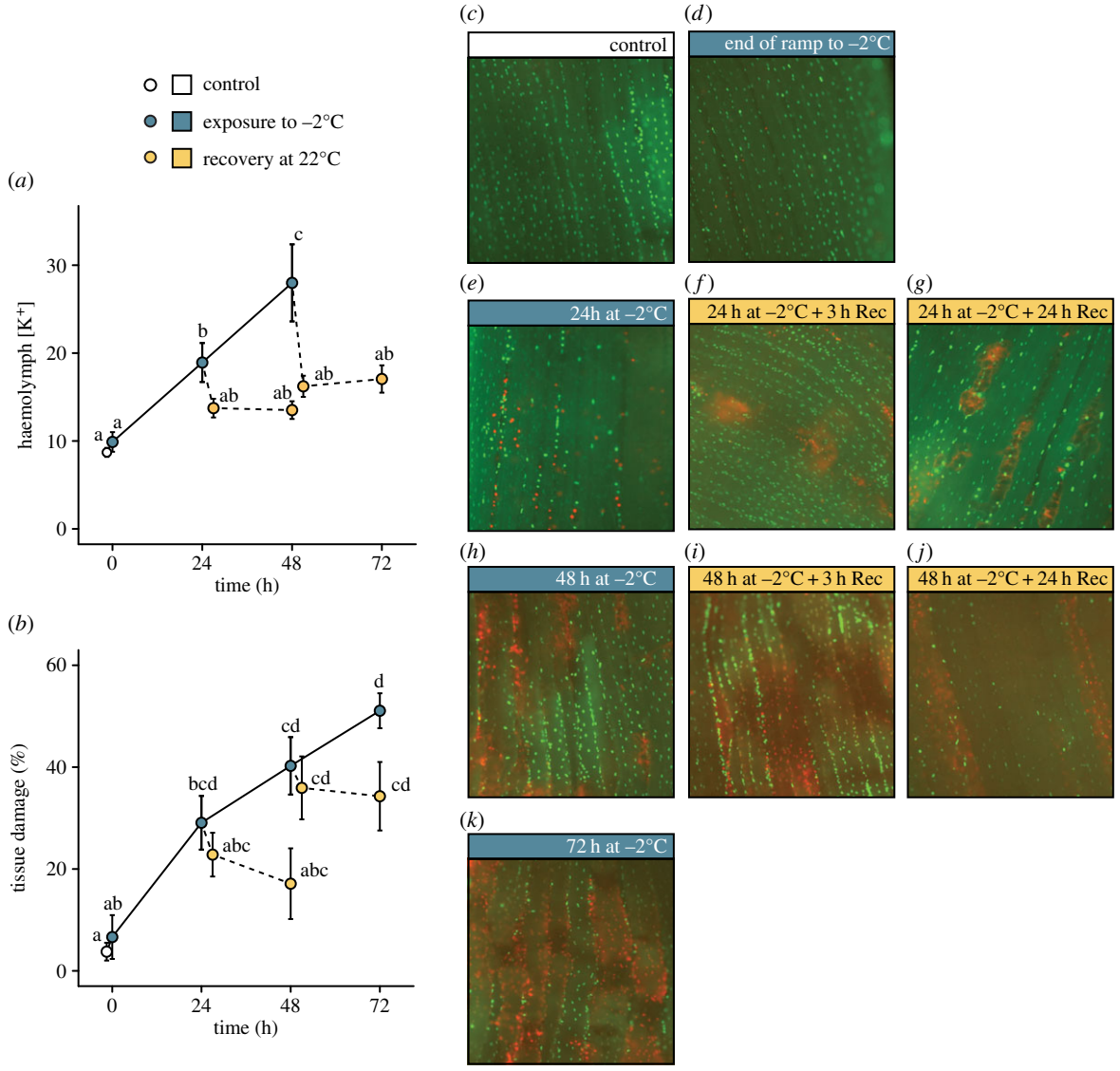

Figure 2. Prolonged cold exposure *in vivo* increases extracellular [K^+] and causes muscle damage in the locust. (*a*) Concentrations of K^+ (mean ± s.e.m.) in the haemolymph of locusts that were sampled from their rearing conditions (control; open circles), sampled after 0 (sampled immediately upon reaching −2°C), 24 or 48 h at −2°C (blue circles) or recovered at room temperature for 3 or 24 h after spending 24 or 48 h at −2°C (orange circles). Error bars that are not visible are obscured by the symbols. (*b*) Per cent damage (mean ± s.e.m.) in the mesothoracic posterior tergocoxal muscle (M90) of female locusts that were sampled from their rearing conditions (control; open circles), sampled after 0 (sampled immediately upon reaching −2°C), 24 or 48 h at −2°C (blue circles), or recovered at room temperature for 3 or 24 h after spending 24 or 48 h at −2°C (orange circles). Proportions displayed are an average of two damage assessment methods (based upon feature counts and proportional area; see Material and methods for details and the electronic supplementary material, figure S1 for independent results of each method). Means that share a letter do not significantly differ (Tukey's HSD). (*c–k*) Example composite images of live (green, stained with SYBR 14) and dead cells (red, stained with propridium iodide) of each treatment group. Colour of image headings denote control (white) cold exposed (blue) and recovering locusts (orange). (Online version in colour.)

primarily when cold and hyperkalaemia are combined as these treatments have additive effects on cell resting potential; cold depolarizes the membrane owing to a decreased electrogenic contribution from ion pumps [15], and increased [K^+] also depolarizes the membrane as membrane potential is particularly sensitive to K^+ distribution [37].

To investigate these options, we independently manipulated extracellular [K^+] *in situ*, using physiologically relevant concentrations mimicking haemolymph conditions under control conditions (8 mM) and after 48 h at −2°C (30 mM). We found a significant interaction between treatment (3 h at 22°C or 24 h at 0°C) and [K^+], regardless of the analysis method used (feature counts: $F_{1,27} = 29.4$, $p < 0.001$; area: $F_{1,27} = 10.8$, $p = 0.003$; see Material and methods for details of analysis). The high [K^+] buffer caused muscle damage, but only when applied during the low temperature exposure (figure 3). Given that low temperature and high extracellular [K^+] depolarize muscle fibres additively in this species [15], this finding suggests that the combined effects of temperature and high [K^+] may damage the muscle fibres through their combined effects on resting cell potential. Importantly, this result also highlights that cold *per se* does not induce injury at relatively mild low temperatures, nor do relevant levels of hyperkalaemia when applied at room temperature for a biologically relevant time period. While, the absence of any notable damage in cells exposed to cold only suggests that cold-induced denaturation or membrane phase changes are unlikely candidates of chill injury at these (mild) cold exposures, it remains possible that these factors play a larger role during more extreme cold exposures.

Although we cannot distinguish between apoptotic and necrotic cell death with our assay, cold stress causes cell death

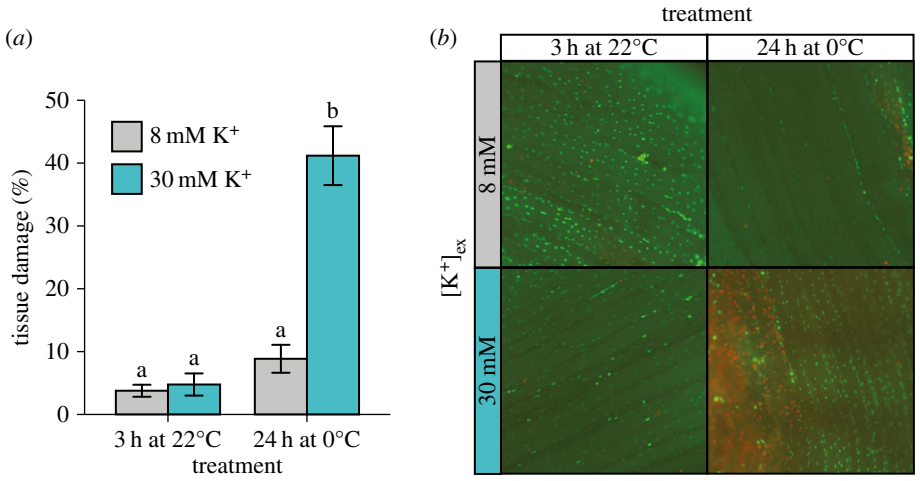

Figure 3. Low temperature and high extracellular [K^+] interact to cause muscle damage *in situ*. (*a*) Per cent damage (mean ± s.e.m.) in mesothoracic posterior tergocoxal muscle (M90) of muscle preparations exposed to 3 h at 22°C or 24 h at 0°C in either a low (8 mM; grey bars) or high (30 mM, teal bars) [K^+] buffer. Bars that share a letter do not significantly differ (false discovery rate corrected pairwise *t*-tests). Proportions displayed are an average of two damage assessment methods (based upon feature counts and proportional area; see Materials and methods for details and the electronic supplementary material, figure S1 for independent results of each method). (*b*) Example composite images of live (green, stained with SYBR 14) and dead cells (red, stained with propridium iodide) of each treatment group. (Online version in colour.)

via apoptosis in the flight muscles of *Drosophila melanogaster* [24], and cell membrane depolarization (via disruption of the [K^+] gradient) is an early event in apoptosis [52]. We suggest that depolarization of cell resting potential probably leads to activation of an inward Ca^{2+} current. Calcium is a central regulator of apoptosis, and excessive increases in intracellular [Ca^{2+}] are expected to trigger the apoptotic signalling cascade [53,54]. Others have also highlighted how hypothermic and hyperkalaemic depolarization impair intracellular Ca^{2+} balance in vertebrates, but there it was emphasized how this loss of calcium regulation caused a debilitating activation of lipases and proteases leading to cell death [41,42]. Regardless of the downstream cascade, we suggest that cold exposure causes the following: (i) cooling causes cellular depolarization through a reduction in electrogenic ion pumping, which may be directly associated with onset of chill coma but not chill injury (see discussion in [13,15]); (ii) a chronic reduction in primary ion transport causes a loss of water and ion balance (see discussion in [15,19]), resulting in a progressive developing hyperkalaemia that further depolarizes the tissue; and (iii) extensive cellular depolarization activates voltage-sensitive Ca^{2+} channels, and the ensuing disruption of intracellular Ca^{2+} balance causes uncontrolled activation of proteases and lipases and/or activation of apoptotic signalling that ultimately cause tissue damage. This model is consistent with observations that: (i) the degree of injury and hyperkalaemia are often well correlated [8,21–23,55], (ii) injury does not develop early in a cold exposure [21,22], and (iii) cold adaptation and cold acclimation responses mitigate hyperkalaemia during cold exposure and are associated with improved survival at low temperature [8,22,23,26,55]. We note that the physiological mechanisms described in this cascade are conserved across terrestrial insects, and that elevated extracellular [K^+] is closely correlated with injury across a wide range of insect groups. We, therefore, suggest that our model of injury applies to the majority of chill susceptible insects. We emphasize, however, that low temperature alone (in the absence of increased extracellular [K^+]) is likely to cause injury at more extreme low temperatures through other mechanisms, such as cell membrane phase changes or cold-induced protein denaturation. Future study on chilling injury should be concentrated on how depolarization of the cell membrane potential may directly or indirectly impact Ca^{2+} regulation and/or activation of the cell death machinery, and the (probably) complex manner that temperature, time and increased haemolymph [K^+] interact to cause injury.

Data accessibility. Supplementary data are available through Dryad http://dx.doi.org/10.5061/dryad.4c0s0.

Authors' contributions. All the authors contributed to the design of the experiment; H.M. completed the experiments and statistical analysis; H.M. and J.O. drafted the manuscript, and all the authors edited the manuscript. All the authors gave final approval for publication.

Competing interests. We have no competing interests.

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