RESEARCH ARTICLE

Rapid cold hardening improves recovery of ion homeostasis and chill coma recovery time in the migratory locust, Locusta migratoria

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SUMMARY

Chill tolerance of insects is defined as the ability to tolerate low temperature under circumstances not involving freezing of intracellular or extracellular fluids. For many insects chill tolerance is crucial for their ability to persist in cold environments and mounting evidence indicates that chill tolerance is associated with the ability to maintain ion and water homeostasis, thereby ensuring muscular function and preventing chill injury at low temperature. The present study describes the relationship between muscle and haemolymph ion homeostasis and time to regain posture following cold shock (CS, 2 h at -4°C) in the chill-susceptible locust Locusta migratoria. This relationship was examined in animals with and without a prior rapid cold-hardening treatment (RCH, 2 h at 0°C) to investigate the physiological underpinnings of RCH. CS elicited a doubling of haemolymph [K+] and this disturbance was greater in locusts pre-exposed to RCH. Recovery of ion homeostasis was, however, markedly faster in RCH-treated animals, which correlated well with whole-organism performance as hardened individuals regained posture faster than non-hardened individuals following CS. The present study indicates that loss and recovery of muscular function are associated with the resting membrane potential of excitable membranes as attested by the changes in the equilibrium potential for K+ (Eh) following CS. Both hardened and non-hardened animals regained movement once K+ homeostasis had recovered to a fixed level (Eh=−41 mV). RCH is therefore not associated with altered sensitivity to ion disturbance but instead is correlated to a faster recovery of haemolymph [K+].

Key words: Nernst potential, Orthoptera, acute cold shock survival, LT50, reproductive behaviour, threshold potential.

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INTRODUCTION

Thermal tolerance is one of the most important determinants of ectothermic species distribution, including insects (Addo-Bediako et al., 2000; Bale, 2002; Chown and Terblanche, 2006; Angilletta, 2009; Sunday et al., 2010; Hoffmann et al., 2012; Kellermann et al., 2012a; Kellermann et al., 2012b). This is particularly evident when exploring the relationship between cold tolerance and minimal environmental temperature, such that strong correlations exist between cold tolerance and environmental distribution (Kimura, 2004; Chen et al., 2011; Kellermann et al., 2012a). Without the capability to tolerate low temperatures, insect species are likely to suffer cold injury, which may result in increased mortality and/or reduced reproduction (Chen et al., 1987; Wang and Kang, 2003; Koštál et al., 2004; Shreve et al., 2004; Koštál et al., 2006; Overgaard et al., 2007; MacMillan and Sinclair, 2011a). Cold tolerance in insects has classically been divided into freeze tolerance and freeze avoidance on the basis of the insects’ ability to survive ice formation in their extracellular fluid (Zachariaissen, 1985; Lee and Denlinger, 1991; Ramlov, 2000; Sinclair et al., 2003). In reality, however, there are many insect species that do not tolerate low temperatures per se and in these chill-susceptible species there is a loss of function at temperatures considerably above that causing freezing (Bale, 1996; Sinclair, 1999; Nedved, 2000; Koštál et al., 2004; MacMillan and Sinclair, 2011b). This loss of function is manifested in an arrest of movement and at this temperature, termed the critical thermal minimum, the insect will enter a chill coma. Chill coma is fully reversible as long as the cold exposure is moderate in intensity and duration. However, if the cold exposure inducing chill coma is prolonged or severe the insects may accumulate chill injuries that ultimately result in death (Gibert et al., 2001; David et al., 2003; Koštál et al., 2004; Anderson et al., 2005; Koštál et al., 2006; MacMillan and Sinclair, 2011a). Chill sensitivity has been found in a wide range of insect orders including Coleoptera, Diptera, Hemiptera, Orthoptera and Hymenoptera (Lee et al., 1987a; Goller and Esch, 1990; Gibert et al., 2001; Koštál et al., 2004; Powell and Bale, 2004; Jing et al., 2005; Koštál et al., 2006; MacMillan and Sinclair, 2011a) and the chill coma temperature has been shown to be a powerful determinant of intraspecific variation in geographical distribution (Gibert et al., 2001; Kimura, 2004; Macdonald et al., 2004; Anderson et al., 2005; MacMillan and Sinclair, 2011b; Kellermann et al., 2012a). In spite of this, very little is known about the physiology of chill coma.

Previous studies have shown that the transition to chill coma involves a disruption of normal neuro-muscular performance. By examining different species of cockroaches, Anderson and Muchmor showed that the nervous activity is maintained at temperatures below that of chill coma onset (Anderson and Muchmor, 1968). Other studies have demonstrated that the onset of chill coma is associated with loss of muscular excitability in bees and flies (Goller and Esch, 1990; Hosler et al., 2000), suggesting that chill coma is primarily associated with loss of muscular function. Irrespective of whether the temperature-induced loss/recovery of function is related to nervous or muscular function, it seems clear that these processes are strongly correlated with the
simultaneous loss/recovery of extracellular ion homeostasis (Kelty et al., 1996; Zachariassen et al., 2004; Koštál et al., 2006; MacMillan et al., 2012). Similar correlations have been found for the development of chill injury following prolonged cold exposure during chill coma. Thus, failure to maintain extracellular ion homeostasis is strongly associated with the development of chill injury and death in a range of insects (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011a). A recent study on the fall field cricket, *Gryllus pennsylvanicus*, indicated that loss of ion homeostasis might be caused by a failure of osmoregulation between the gut and haemolymph (MacMillan and Sinclair, 2011a). It has also been found that acute cold injury could result in membrane phase changes (Quinn, 1985) and/or damage of crucial proteins (Lee and Chapman, 1987), which could result in severe leakage of ions and cellular lysis, contributing to an overall loss of ion homeostasis. However, it remains to be determined whether the mechanisms behind chill coma and chill injury are related and it is also not well resolved which of the processes take place when entering and recovering from chill coma.

Cold tolerance of insects can be improved by prior exposure to moderate low temperatures. Acclimation effects can be induced during development or long-term exposure (Colinet and Hoffmann, 2012) and improved cold performance may even be significantly enhanced by short-term exposure to sub-lethal cold temperatures or by gradual cooling prior to cold shock (Kelty and Lee, 1999; Kelty and Lee, 2001; Sinclair and Roberts, 2005; Denlinger and Lee, 2010). This latter protective effect of acclimation is termed rapid cold hardening (RCH), and has been demonstrated in many insect orders (Sinclair et al., 2003; Denlinger and Lee, 2010). RCH is among the fastest low-temperature responses (Lee et al., 1987b) and although the mechanisms of RCH are not yet fully understood, it has been found to improve potentially important fitness traits such as survival, activity and reproduction during cold exposure (Chen et al., 1987; Czajka and Lee, 1990; Kelty and Lee, 1999; Rinehart et al., 2000; Wang and Kang, 2003; Shreve et al., 2004; Overgaard et al., 2007). The RCH response might enable insects to enhance cold tolerance in response to predictable (e.g. diurnal) or unexpected decreases in environmental temperature (Chen et al., 1987; Lee and Chapman, 1987; Kelty and Lee, 1999; Koveos, 2001; Kelty, 2007; Overgaard and Sørensen, 2008; Denlinger and Lee, 2010). Using the chill-susceptible locust *Locusta migratoria*, the present study examined the relationship between recovery of ion homeostasis and recovery from chill coma following cold shock (CS, 2 h at −4°C). Moreover, this study investigated how this relationship is affected by prior RCH treatment (2 h at 0°C). Chill coma recovery time, survival and reproductive behaviour were assessed to investigate the effect of RCH on the cold phenotype. Furthermore, recovery from chill coma was correlated with measurements of muscle and extracellular ion concentrations to evaluate how cold shock and hardening affected ion gradients and membrane resting potential. The specific hypotheses tested were as follows: (1) recovery from chill coma is directly related to the re-establishment of ion homeostasis; and (2) RCH enhances chill tolerance through physiological mechanisms, resulting in improved recovery of ion homeostasis.

**MATERIALS AND METHODS**

**Insect rearing**

Fifth instar locusts, *Locusta migratoria* (Linnaeus 1758), were acquired from a commercial supplier (Peter Andersen Aps, Fredericia, Denmark) and kept in cages (0.45 m²) until they reached the adult stage. The cages contained metal grids and egg trays for climbing, moulting and hiding. Locusts were subjected to a 16:8 h light/dark cycle and during day the cages were heated by a sun-simulating lamp, allowing behavioural thermoregulation and resulting in a temperature gradient from 25 to 45°C. During night the temperature was set to 22°C. Locusts were fed daily with wheat sprouts and had a constant supply of wheat bran and water. Locusts were sexed 1–2 days after moulting to the adult stage and separated in males and females until the onset of experimentation. Locusts were used for experiments when they were between 1 and 3 weeks old. Cages were cleaned once a week and debris and uneaten wheat were removed daily.

**Cold exposure**

Twenty-four hours before experiments were performed, male and female locusts were transferred separately to small plastic boxes that were placed in a temperature-regulated room held at 30°C. The animals did not have access to food during this period. At the day of the experiment, locusts were placed in 50 ml plastic containers, with a piece of sponge rubber at the top to prevent them escaping. Locusts were either treated with RCH (2 h at 0°C) or not treated prior to CS (2 h at −4°C). RCH treatment was obtained by placing the plastic tube containing the locusts into an ice bath and the CS was conducted by inserting the plastic tube into a refrigerated glycol bath (Lauda RE 320, Lauda, Lauda-Königshofen, Germany) held at a constant temperature of −4°C. It was assumed that no freezing takes place as long as the temperature is −4°C or higher, even though the literature on supercooling point in adult *L. migratoria* reveals values from −3.5°C to −8°C (Feng et al., 2008; Cloudsley-Thompson, 1978; Cloudsley-Thompson, 1973). This assumption was confirmed by individual inspection of all animals at the −4°C treatment, where no mortality was observed (which would be expected if freezing took place).

Subsequent to the cold exposure, three different experiments were independently performed (Fig. 1): chill coma recovery time, mating success and intracellular and extracellular ion concentration, as described below.

**Chill coma recovery time**

After CS, individuals were removed from the plastic container and placed on a piece of paper, where they were allowed to recover at

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**Fig. 1.** Experimental protocol of locusts treated with rapid cold hardening (RCH, 2 h at 0°C) or untreated prior to cold shock (CS, 2 h at −4°C). Subsequent to these cold exposures, three different experiments were performed independently during the recovery phase. (1) Measurements of recovery time: individuals were allowed to recover at 25°C, and recovery time was registered as the time to regain an upright position. (2) Measurements of mating: the time and proportional occurrence of successful matings for males following CS were measured at 30°C. (3) Measurements of ion status: [K⁺] and [Na⁺] were measured in the haemolymph (extracellular) and muscle tissue (intracellular) at 5, 10, 15, 20, 30 and 60 min after removal from CS treatment.
25°C without any mechanical stimuli. Recovery time was registered as the time it took the locusts to regain neuromuscular function and attain an upright position. This assay of cold tolerance was measured in locusts with and without RCH treatment prior to CS treatment (N=102 CS, N=100 RCH+CS).

Mating success
After CS, comatose individual male locusts were moved to a cage (20×30×20 cm) containing an untreated virgin female. We measured the time it took for the male to resume movement and successfully mate with the female. The time and proportional occurrence of successful matings were measured at 30°C during a 2 h observation period. These experiments were performed for untreated controls (maintained at 30°C), and locusts with and without RCH treatment prior to CS treatment (N=20 Control, N=21 CS, N=19 RCH+CS).

Intracellular and extracellular ion concentration
[K+] and [Na+] were measured in the haemolymph (extracellular) and muscle tissue (intracellular) in untreated control animals and at 5, 10, 15, 20, 30 and 60 min after removal from CS treatment in individuals with and without prior RCH treatment (N=13–19 locusts per treatment/time point).

Haemolymph was sampled by detaching the hindlegs from the animal, and a capillary tube (25 μl) was placed on top of the puncture to allow the haemolymph to flow into the tube. If haemolymph extraction was difficult, a gentle squeeze was applied to the body to obtain enough haemolymph. The haemolymph droplet (1–20 μl) was then transferred by a pipette to a 0.5 ml Eppendorf tube, which was placed in a microcentrifuge (DW-41-230, Radiometer A/S, Brønshøj, Denmark) and spun for 15 s to separate haemolymph from debris. A 1–5 μl sample of haemolymph was then transferred by pipette to a 2 ml buffer solution containing 100 p.p.m. lithium salt (Sherwood Scientific Ltd, Cambridge, UK) and the ion concentrations in the resulting solution were subsequently determined by flame photometry (see below).

Immediately after haemolymph sampling, muscle tissue was dissected from the detached hindlegs, blotted dry of haemolymph and transferred to a pre-weighed 2 ml Eppendorf tube. The Effendorf tube with muscle was closed and re-weighed to obtain muscle wet mass. The muscle sample was then dried for 24 h at 60°C after which dry mass was obtained and muscle water content calculated. The dried muscle was dissolved in 200 μl of milli-Q water and the muscle tissue was homogenized using a tissue lyser (Tissuelyser LT, Qiagen, Hilden, Germany) (50 Hz for 20 min). The sample was centrifuged (13,000 g for 20 min) and 10–20 μl of the supernatant was transferred to three replicates of 2 ml, 100 p.p.m. lithium salt buffer solution. Three samples were made for the muscle to get an average measure of [Na+] because of the low intracellular [Na+]. Muscle [Na+] and [K+] were obtained from the mean of the three samples.

After the preparation described above the [Na+] and [K+] were measured from the haemolymph and muscle samples using a flame photometer (Model 420 Flame Photometer, Sherwood Scientific Ltd, Cambridge, UK) and concentrations were calculated relative to standard samples of known concentration.

Nernst potential calculation
The Nernst potential (E,) was calculated on the basis of [K+] and [Na+] in the extracellular (haemolymph) and intracellular (muscle) fluid using the following equation:

\[ E = \frac{RT}{zF} \ln \left( \frac{X_o}{X_i} \right), \]

where \( R \) is the gas constant (8.314 JK\(^{-1}\)mol\(^{-1}\)), \( T \) is the absolute temperature in Kelvin (298 K as all animals were assumed be at room temperature at the time of sampling), \( z \) is the charge on each ion and \( F \) is the Faraday constant (96,487 C per mole), \( [X_o] \) and \( [X_i] \) are the concentration of ion \( X \) in the extracellular fluid (haemolymph) and intracellular (muscle) fluid, respectively. This calculation assumes that [K+] and [Na+] in the muscle fluid resembles the concentrations in the extracellular fluid, although this is only an approximation because a small fraction of extracellular fluid remains in the interstitial fluid of the muscle (Wood, 1963).

Acute CS survival
As a separate experiment, acute CS survival was measured by exposing locusts to \(-4, -5, -6, -7 \) or \(-8^\circ C \) for 2 h (N=50 per temperature point). All temperatures were tested on both non-hardened and RCH-treated (2 h at 0°C) individuals. Survival was measured 44 h after removal from CS, and locusts that were standing or responded to mechanical stimuli were defined as alive; others were scored as dead.

Statistics
All statistical analysis was performed using Graphpad Prism 6.0, except that for the acute CS survival data, which was performed in RStudio, R Version 2.15.1 (R Development Core Team, 2012). Recovery times were not normally distributed, so we compared among treatments using a Mann–Whitney U-test. Time differences in male mating success between controls, non-RCH and RCH individuals were compared using a one-way ANOVA followed by Tukey’s post hoc multiple comparison test. Pairwise comparisons of mating proportion between treatment groups (Control, CS, RCH+CS) were tested using Chi-square tests and Bonferroni corrections were used to correct for multiple testing.

An ANCOVA analysis were performed to compare the slopes of the best-fit linear regressions to test for differences in the temporal development of ion concentrations and Nernst potentials between non-hardened and hardened locusts. Best-fit linear regressions were only performed on the data points between 5 and 20 min, as this is the period where the recovery of movement takes place.

A one-way ANOVA followed by Tukey’s post hoc multiple comparison test was used to compare ion concentrations and Nernst potentials between non-hardened, hardened and untreated locusts at 5 and 60 min, respectively.

The effect of CS temperature on acute CS survival was fitted to a generalized linear model (GLM) assuming a binomial distribution and using a logit-link function (logistic regression) in R. Associated LT\(_{50}\) (the temperature at which 50% of the animals were dead) for the two treatments groups was calculated with standard error using the dose.p application for R. All values are presented as means ± s.e.m., and where data are not normally distributed the median is also given. \( P<0.05 \) was considered statistically significant.

RESULTS

Effect of CS and RCH on chill coma recovery time, reproductive behaviour and survival
RCH significantly reduced locust chill coma recovery time following a CS (2 h at \(-4^\circ C \)) (Fig. 2A), such that locusts receiving RCH treatment prior to CS recovered more than 2 min faster than animals exposed directly to CS (13.3±0.3 min, median 12.80 min, versus 15.9±0.5 min, median 14.15 min, Mann–Whitney U-test, \( U=3631, n_1=102, n_2=100, P<0.001 \)). Exposure to CS significantly increased the time before successful mating, as cold-exposed
animals were ~50% slower to successfully mate compared with untreated control animals (control 38.6±9.1 min, CS 62.1±8.4 min, RCH+CS 72.3±6.5 min). This difference was, however, only significant for the RCH+CS males compared with the control animals (ANOVA, $F_{2,30}=4.06$, Tukey’s post hoc test, $P<0.05$). Although there was no difference in time to mating between RCH+CS and CS treatments, the RCH treatment resulted in a larger proportion of males being able to successfully mate compared with the non-hardened group (Fig. 2B). Thus, 53% of the RCH-treated males successfully mated while only 29% of the males from the CS group succeeded in mating following CS. This difference was, however, not significant as we only found a significant difference in mating proportion between control and CS treatment groups (Chi-square, $z=3.639$, $P<0.001$).

Finally, RCH significantly decreased the LT$_{50}$ for CS survival (i.e. increased cold tolerance) (GLM, $z=5.18$, $P<0.001$), with RCH-treated individuals having a LT$_{50}$ of $-6.6±0.1°C$ whereas non-hardened individuals had a LT$_{50}$ of $-5.9±0.1°C$ (Fig. 2C).

**Effects of CS and RCH on ion homeostasis**

In order to investigate whether recovery from chill coma is related to the re-establishment of haemolymph ion homeostasis, we measured the concentration of Na$^+$ and K$^+$ in haemolymph and muscle during the recovery phase following a CS (5, 10, 15, 20, 30 and 60 min after CS). These measurements were performed on both hardened and non-hardened animals to examine the effect of RCH on the recovery of ion homeostasis. CS elicited more than a doubling of haemolymph [K$^+$] ([K$^+$]$_0$), which increased from a control value of 14.7±0.7 mmol l$^{-1}$ to 32.8±1.1 mmol l$^{-1}$ for RCH+CS and 28.1±1.3 mmol l$^{-1}$ for CS 5 min after cold exposure (Fig. 3A). This initial increase in [K$^+$]$_0$ was significantly larger for animals that had received a 2 h RCH pre-treatment prior to the CS (ANOVA, $F_{2,45}=88.20$, Tukey’s post hoc test, $P<0.05$, Fig. 3A). Recovery of ion homeostasis was, however, markedly faster in RCH-treated animals, as the slope of the best-fit line was significantly different from that of the CS treatment (RCH+CS $-0.86±0.13$ mmol l$^{-1}$ min$^{-1}$ versus CS $-0.28±0.13$ mmol l$^{-1}$ min$^{-1}$, ANCOVA, $F_{1,121}=10.47$, $P<0.01$). Neither of the treatments reached control values within 60 min of recovery, but 60 min after CS there was no difference between hardened and non-hardened individuals. The source of the increased extracellular K$^+$ was unlikely to be the muscle (intracellular) K$^+$ stores as [K$^+$]$_i$ was stable within the first 20 min after cold exposure (Fig. 3B, slopes not significantly different from zero) and not significantly different from control (RCH+CS 122.3±2.6 mmol l$^{-1}$, CS 126.0±2.5 mmol l$^{-1}$ and control 129.9±3.4 mmol l$^{-1}$). However, for the CS-treated locusts the [K$^+$]$_i$ decreased later such that 30 min after CS the [K$^+$]$_i$ was 5–10 mmol l$^{-1}$ lower and the levels remained lowered until the end of the experiment, 60 min after the CS treatment (ANOVA, $F_{2,41}=6.98$, Tukey’s post hoc test, $P<0.05$, Fig. 3B).

CS elicited a decrease in haemolymph [Na$^+$] ([Na$^+$]$_0$) from control values of 96.4±2.2 mmol l$^{-1}$ to 66.0±2.3 mmol l$^{-1}$ for RCH+CS and 74.8±2.6 mmol l$^{-1}$ for CS (Fig. 3C), 5 min after cold exposure. Thus, hardened individuals had a significantly higher initial disturbance of [Na$^+$]$_0$ than CS animals (ANOVA, $F_{2,40}=42.36$, Tukey’s post hoc test, $P<0.05$, Fig. 3C). Although the hardened locusts initially recovered some of the [Na$^+$]$_0$, both treatment groups continued to have a significant reduction in [Na$^+$]$_0$, 60 min after the CS (Fig. 3C, RCH+CS 76.8±2.4 mmol l$^{-1}$ versus CS 74.0±3.2 mmol l$^{-1}$). The disturbance in [Na$^+$]$_0$ could not be attributed to muscle [Na$^+$] ([Na$^+$]$_m$), as this was largely unaffected by the CS treatments. However, RCH+CS animals had a slightly reduced [Na$^+$]$_0$, 60 min after the CS treatment (ANOVA, $F_{2,41}=5.73$, Tukey’s post hoc test, $P<0.05$, Fig. 3D).

**Nernst potential**

Because of the large increase in [K$^+$]$_0$, the Nernst equilibrium potential for K$^+$ ($E_k$), depolarized dramatically following CS (Fig. 4A). The initial depolarization in $E_k$ was greater for RCH+CS than for CS individuals (control $-56.7±1.1$ mV, RCH+CS $-34.0±0.8$ mV, CS $-38.9±1.3$ mV, ANOVA, $F_{2,45}=131.3$, Tukey’s post hoc test, $P<0.05$). However, RCH-treated animals recovered faster as the slope of the best-fit line was significantly different between hardened and non-hardened animals (slope $-1.01±0.15$ versus $-0.30±0.16$ mV min$^{-1}$, ANCOVA, $F_{1,121}=10.64$, $P<0.01$). At 60 min after the CS, the hardened and non-hardened individuals had reached the same $E_k$, but both treatment groups had still not fully recovered $E_k$ to control values (control $-56.7±1.1$ mV, RCH+CS $-47.4±1.5$ mV, CS $-47.4±1.6$ mV, ANOVA, $F_{2,41}=15.89$, Tukey’s post hoc test, $P<0.05$). In Fig. 4A, we estimated the $E_k$ at recovery using the average time to recover for the two treatment
groups (see Fig. 2). From this analysis we found that recovery in the two treatment groups occurred when the animals had reached the same $E_K$ (RCH+CS $-$14.1 mV, CS $-$14.1 mV).

The Nernst equilibrium for Na\(^+\) ($E_{Na}$) was less affected than the $E_K$ but was decreased following CS (Fig. 4B). No difference between cold-treated animals was seen after 5 min (RCH+CS 29.9±1.6 mV versus CS 30.6±2.0 mV, ANOVA, $F_{2,44}=17.53$, Tukey’s post hoc test, n.s.) and recovery time was the same for RCH+CS and CS animals as no difference was seen in the slope of the best-fit lines. $E_{Na}$ fully recovered for both hardened and non-hardened locusts within 60 min as there was no significant difference between treatment groups and controls (Fig. 4B, RCH+CS 40.5±1.2 mV, CS 38.1±1.9 mV and control 41.5±1.2 mV, ANOVA, $F_{2,40}=1.67$, n.s.).

**DISCUSSION**

Locusts (*L. migratoria*) exposed to temperatures below 0°C enter a state of chill coma. This coma is fully reversible if the cold exposure is mild, but mortality levels increase if the 2h exposure involves temperatures below $-5^\circ\text{C}$. Acute CS survival and recovery following cold exposure is significantly improved by RCH treatment prior to CS exposure (Fig. 2).

The present study shows that the recovery from chill coma in locusts is tightly correlated to the re-establishment of ion homeostasis following cold exposure. Moreover, we demonstrated that RCH improves the ability of the locusts to recover ion homeostasis following cold exposure. Thus, recovery of muscular function following chill coma is retained once a specific level of extracellular ion homeostasis is re-established, and the physiological mechanisms underlying RCH are therefore likely to be related to mechanisms facilitating improved regulation of extracellular ion composition.

**Effects of RCH on important fitness traits**

It is well established from many species of chill-sensitive insects that rapid cold hardening can improve potentially important fitness traits such as survival, activity and reproduction following cold exposure (Chen et al., 1987; Czajka and Lee, 1999; Kelty and Lee, 1999; Rinehart et al., 2000; Shreve et al., 2004; Overgaard et al., 2007; Denlinger and Lee, 2010; MacMillan and Sinclair, 2011b). RCH prior to CS has also been shown to improve the survival of eggs and first instar hoppers of *L. migratoria* (Wang and Kang, 2003; Wang and Kang, 2005) and here we have demonstrated that RCH also improves acute CS survival and shortens recovery time in adults of *L. migratoria* following CS (Fig. 2). The migratory locust has a broad geographical distribution from tropical to temperate zones and is found in Africa, Asia and Australia (Uvarov, 1966). For the Asian populations, it has been reported that winter temperature can be low for extended periods; thus, the RCH effect found in overwintering eggs might help the locusts survive such extremes (Wang et al., 2006). This also applies to first instar hoppers and adults, which might experience low temperatures in early spring or late autumn, where temperature can fluctuate by 20–40°C on a daily basis (Wang and Kang, 2003; Tanaka and Zhu, 2008). Such fluctuations could result in mortality and it is likely that the RCH effect on recovery and acute CS survival, found in this study, enhances the cold tolerance of wild locusts exposed to diurnal or unexpected decreases in environmental temperature (Chen et al., 1987; Lee et al., 1987a; Kelty and Lee, 1999; Koveos, 2001; Kelty, 2007; Overgaard and Sørensen, 2008; Denlinger and Lee, 2010). Importantly, the observed benefit of RCH on recovery and acute CS survival occurred despite the fact that hardened individuals had been exposed to cold for 4h (2h at 0°C followed by 2h at $-4^\circ\text{C}$), whereas the non-hardened individuals had only been exposed to cold for 2h (at $-4^\circ\text{C}$).

Previous studies on other insect species have also shown that RCH can help to preserve complex responses such as mating and reproduction (Shreve et al., 2004; Overgaard et al., 2007). A beneficial effect of RCH on mating behaviour was not observed in this study as the time to successfully mate was not different between hardened and non-hardened individuals. However, we observed a tendency towards a higher proportion of successful mating in RCH-treated males (Fig. 2B), although this was not significant in the limited data set.

**Fig. 3.** (A) Haemolymph K\(^+\) concentration ([K\(^+\)]\(_{o}\)). (B) muscle K\(^+\) concentration ([K\(^+\)]\(_{i}\)). (C) haemolymph Na\(^+\) concentration ([Na\(^+\)]\(_{o}\)) and (D) muscle Na\(^+\) concentration ([Na\(^+\)]\(_{i}\)) following cold exposure with (black circles) and without (grey squares) RCH in locusts. Concentrations were measured at 5, 10, 15, 20, 30 and 60 min after CS treatment. Black and grey lines denote the best-fit linear relationship between ion concentration and recovery time between 5 and 20 min for hardened and non-hardened individuals, respectively. Values from control (not cold exposed) are shown as open circles (mean) and dotted (±s.e.m.) lines. Letters at 5 and 60 min denote statistically significant differences between treatment groups: a, difference between non-hardened and hardened individuals; b, difference between non-hardened and hardened controls; and c, difference between hardened individuals and controls. Values are means ± s.e.m. Error bars that are not visible are obstructed by the symbols.
Disturbance of ion homeostasis coincides with development of chill coma

The onset of chill coma involves a disruption of normal neuromuscular performance due to a loss of extracellular ion homeostasis (Goller and Esch, 1990; Hosler et al., 2000). It is somewhat unclear whether this loss of function is primarily nervous or muscular; however, mounting evidence suggests that the failure of these excitable tissues is strongly correlated with, in particular, the increase in extracellular \([K^+]\) (Anderson and Mutchmor, 1968; Kelty et al., 1996; Koštál et al., 2006; MacMillan and Sinclair, 2011b; Armstrong et al., 2012). This was also found in the present study, as \([K^+]\) increased and \([Na^+]\) decreased in the haemolymph following exposure to chill coma. Importantly, the present study demonstrates that the recovery of normal function after chill coma is associated with a re-establishment of ion homeostasis in the extracellular compartments, particularly with respect to \([K^+]\). A similar finding was recently reported for the fall field cricket, where chill coma recovery was also tightly coupled to the establishment of extracellular \([K^+]\) (MacMillan et al., 2012). In that study, it was suggested that putative changes in recovery of ion homeostasis following recovery were also to be manifested in faster recovery of function (MacMillan et al., 2012). In the present study, we confirm this physiological link between chill coma and extracellular ion homeostasis through the RCH treatment, where the locusts recover extracellular \([K^+]\) more rapidly than non-hardened individuals. This is directly translated to a faster recovery of normal function in this treatment group (Fig. 2).

During chill coma the Nernst equilibrium potential for both \(Na^+\) and \(K^+\) changes as a result of the altered ion balance between haemolymph and muscle. The \(E_K\) is the principal determinant of locust muscle membrane potential (Hoyle, 1953), and a combination of the increased haemolymph \([K^+]\) and unchanged muscle \([K^+]\) resulted in an increase in \(E_K\) to \(-38.9\) mV for non-hardened and \(-34.0\) mV for RCH-treated individuals 5 min after CS ended. The \(E_K\) at which the animals recovered was almost identical for the two treatments (approximately \(-41\) mV, Fig. 4), suggesting that RCH is not associated with an altered threshold potential but instead a faster recovery rate. In a previous study by Koštál and others (Koštál et al., 2006), it was found that chill injury in the tropical cockroach occurred at an \(E_K\) of approximately \(-40\) mV; likewise, Hosler and others (Hosler et al., 2000) found, when examining flies and bees, that these species entered chill coma at a membrane potential \(V_m\) close to \(-40\) mV, irrespective of different chill coma temperatures. It is interesting that separate studies on different species have identified a similar \(E_K/V_m\) where the insect enters or recovers from chill coma. This could point to an evolutionary set point for \(V_m/E_K\).

In accordance with a number of previous studies investigating the relationship between chill injury and ion homeostasis, we found little change in ion status in the muscle tissue (Koštál et al., 2006; MacMillan and Sinclair, 2011a). Thus, the changes observed in the haemolymph cannot be attributed to the movement of ions between muscle tissue and haemolymph. Instead, the observed change in haemolymph \([K^+]\) may be contributed by a relocation of ions and water between the haemolymph and the gut during cold exposure. This mechanism was described in a recent study examining cold tolerance in the fall field cricket (MacMillan and Sinclair, 2011a). In their case, it was found that total \(K^+\) content in the haemolymph did not change during cold exposure but the concentration increased because of a reduction in the total haemolymph volume. This observation indicates that \(K^+\) ions did not move towards the haemolymph; instead, water moved from the haemolymph to the gut. The present study cannot confirm whether this is also the mechanism behind increased \([K^+]_o\) in \(L.\ migratoria\) as the volume of haemolymph was not measured; however, preliminary data suggest that such movement is part of the explanation for the increased \([K^+]_o\) (J.L.A., A.F. and J.O., unpublished results). Movement of water during cold exposure is probably a result of \(Na^+\) diffusing into the intestinal lumen, thereby drawing water with it as suggested by MacMillan and Sinclair (MacMillan and Sinclair, 2011a). However, the precise mechanism behind this water movement is not fully understood and further studies are needed to explore this relationship as it may be crucial for the cold sensitivity of many insects. Although muscle \([K^+]\) was almost unaffected by cold exposure, we found a small decrease after 30 and 60 min of recovery, which could possibly indicate that a minor disruption of muscle \([K^+]\) homeostasis is associated with the resumption of
movement and muscular activity. However, further studies are needed to verify this hypothesis.

In contrast to other studies on cold tolerance in Orthopteran species (MacMillan and Sinclair, 2011a), we found that haemolymph [Na+] decreased and muscle [Na+] was unchanged following CS, resulting in a decreased $E_Na$ (Fig. 4B). This has also been observed in the tropical cockroach (Koštál et al., 2006), and points to an impairment of Na+ regulation during cold exposure. MacMillan and Sinclair found a simultaneous decrease in muscle and haemolymph [Na+] following cold exposure in the fall field cricket and proposed that this simultaneous decrease could have an adaptive value as it preserves $E_Na$ (MacMillan and Sinclair, 2011a). The reasons for the species difference between the migratory locust and fall field cricket are unclear, but may relate to either a difference in cold exposure time and/or the temperate/tropical distributions of these species, but further experiments are needed to verify this.

Possible mechanisms behind impaired ion homeostasis and RCH effect during cold exposure

As mentioned above, a central requirement for chill tolerance is the maintenance of ion homeostasis, which is achieved through osmoregulatory mechanisms. In insects the osmoregulation is maintained via the Malpighian tubule and the gut. Excess K+ is removed from the haemolymph through the secondary active transport based on V-ATPase activity, and water follows osmotically (Edney, 1977; Bradley, 2008). It is possible that temperature-induced reductions in the activity of the V-ATPase could lead to increased haemolymph K+ during cold exposure. In addition, water reabsorption of terrestrial insects, including locusts, is driven by ion-motive pumps in the hindgut. This active transport establishes steep osmotic gradients, driving Na+ and water from the intestinal lumen and back into the haemolymph (Ramsay, 1971; Hanrahan and Phillips, 1982). Hence, the observed increase in K+ associated with cold exposure could be related to reduced active transport in the gut causing reductions in haemolymph volume and leading to a secondary increase in haemolymph [K+]. Irrespective of whether active transport is involved in the reduction of haemolymph volume and increase in haemolymph [K+], it has been clearly demonstrated that the increase in haemolymph [K+] is correlated to chill injury in the fall field cricket (MacMillan and Sinclair, 2011a). It is possible that the protective effect of the RCH treatment on recovery arises from an activation of osmoregulatory mechanisms in the hardened animals, resulting in a faster re-establishment of ion homeostasis. Future studies are needed to confirm this hypothesis and it is also unclear whether putative differences in osmoregulatory performance are related to altered hormonal stimulation of antiidiuretic hormones or other processes. Thus, the differences in recovery of ion homeostasis could be related to some of the classical adaptations associated with insect cold tolerance. A number of studies have, for example, found that RCH in insects is associated with membrane alterations or modest increases in the levels of cryptoprotecants (Michaud and Denlinger, 2007; Denlinger and Lee, 2010; Overgaard et al., 2005; Lee et al., 2006; Overgaard et al., 2006; MacMillan et al., 2009). Membrane composition is of great importance for the functions of the embedded ion motive pumps (Wu et al., 2004) and even modest increases in cryptoprotectant levels are also known to help preserve membrane integrity at low temperature (Cacela and Hinch, 2006). Future studies are, however, needed to investigate these possibilities further. For example, it is known that RCH can still take place in the absence of protein synthesis (Misener et al., 2001).

Concluding remarks

The transition to chill coma involves a disruption of normal neuro-muscular performance, which is probably caused by failure of the osmoregulatory system at low temperature. The present study demonstrates that chill coma recovery is tightly correlated to the re-establishment of haemolymph ion homeostasis and that RCH treatment enhances chill tolerance by reducing the time to recover ion homeostasis. This positive effect of RCH also translates to higher levels of biological organization in the form of improved acute CS survival. The physiological responses underlying chill tolerance are not fully understood, but mounting evidence highlights the importance of the osmoregulatory system of insects for cold tolerance.

LIST OF SYMBOLS AND ABBREVIATIONS

[X]i concentration of ion i in the intracellular fluid (muscle)
[X]e concentration of ion i in the extracellular fluid (haemolymph)
CS cold shock
$E_K$ equilibrium potential for K+
$E_Na$ equilibrium potential for Na+
LT50 temperature at which 50% of the animals are dead
RCH rapid cold hardening
$a_i$ membrane potential

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AUTHOR CONTRIBUTIONS

J.O., S.C. and A.F. conceived and designed the research; A.F. and J.L.A. performed the experiments; A.F. and J.L.A. described and analyzed the data; A.F. and J.O. wrote the paper.

COMPETING INTERESTS

No competing interests declared.

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