Feeding impairs chill coma recovery in the migratory locust (Locusta migratoria)

Jonas Lembcke Andersen *, Anders Findsen, Johannes Overgaard

Zoophysiology, Department of Bioscience, Aarhus University, C.F. Møllers Alle 3, Building 1131, DK-8000 Aarhus C, Denmark

1. Introduction

The physiological adaptations that allow ectothermic animals to thrive under a particular set of thermal conditions are crucial to their distribution and consequently measures of ectothermic thermal tolerance often correlate tightly with latitudinal distribution and/or with the environmental temperature extremes within their distribution range (Angilletta, 2009; Sunday et al., 2010; Hoffmann et al., 2012). Particularly for insects, cold tolerance varies strongly with different species' latitudinal distribution and/or with the minimal environmental temperature experienced (Addo-Bediaoko et al., 2000; Calosi et al., 2010; Kellermann et al., 2012).

From a physiological perspective insect cold tolerance is often divided into freeze tolerant species that are able to tolerate freezing in the extracellular fluid, and freeze-avoiding species that do not tolerate freezing but display specific adaptations that promote their ability to supercool (Zachariassen, 1985; Bale, 1987; Lee, 2010). Some species are, however, unable to cope with low temperatures per se, and such species are often referred to as chill susceptible (Bale, 1996; Nedved, 2000; MacMillan and Sinclair, 2011a). Chill susceptible species lose their ability to coordinate muscular activity at low temperatures (above the temperature that causes freezing) and consequently enter a reversible coma-like state known as chill coma. Both the onset of, and recovery from, chill coma are popular phenotypic traits used to characterise cold tolerance (Gibert et al., 2001; Koštál et al., 2004; MacMillan and Sinclair, 2011a; Findsen et al., 2013). It has been suggested that the neuromuscular failure that characterises chill coma is ultimately caused by a mismatch between passive and active ion transport (Zachariassen et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011a; MacMillan et al., 2012). Thus, onset of chill coma could be associated with a failure to maintain ion homeostasis (particularly K+) across the cell membrane of an excitable cell, depolarising it and thereby causing loss of function in the neuromuscular system (Hoyle, 1954; Goller and Esch, 1990; Hosler et al., 2000; Koštál et al., 2006; Rodgers et al., 2007; Armstrong et al., 2012). Recovery from chill coma has been suggested to involve a reversal of these processes, such that chill coma recovery depends on the animals’ ability to regain water and ion homeostasis, and thereby normal membrane potential and excitability of the neuromuscular cells (MacMillan and Sinclair, 2011a; MacMillan et al., 2012; Findsen et al., 2013). A recent study demonstrated that in crickets (Gryllus pennsylvanicus), cooling caused a large shift of water, Na+ and Ca2+ from the haemolymph to the intestinal lumen while K+ ions remained in the reduced haemolymph causing a
large increase in [K+] (MacMillan and Sinclair, 2011b). Another study found that recovery of extracellular [K+] was tightly coupled to recovery of haemolymph volume in crickets (MacMillan et al., 2012). Cold induced disruption of extracellular ion balance has also been shown in locusts (Locusta migratoria) and rapid cold hardening has been shown to improve recovery rate of ionic mismatch after cold shock (CS) (Findsen et al., 2013). In addition to cold exposure it has previously been shown that large dietary K+ loads can also skew the balance of intra and extracellular ionic distribution in L. migratoria (Hoyle, 1954). Thus a high dietary K+ load resulted in increased extracellular [K+] and this was suggested to cause the sluggish behaviour and reduced muscle force development in vitro observed for postprandial locusts (Hoyle, 1954).

Cold exposure and high dietary K+ therefore represent two independent environmental challenges that are known to compromise ion balance and neuromuscular performance of locusts. The aim of this study was to examine the effects of CS on ion- and water-balance in fed and fasted locusts, thereby combing these two natural and independent stressors (cold exposure and feeding on high [K+] diets). By tracking water shifts and K+ in the animals, we aim to uncover how the ionic mismatch arises and how the animal recovers ion homeostasis and muscular performance following cold exposure. We propose the following three hypotheses: (1) CS will cause a mismatch in ion homeostasis due to a decreased amount of water in the haemolymph, and fed animals will be more negatively affected by CS due to the added stress of increased haemolymph K+. (2) Increased K+ in the system of feeding animals will upset the ionic balance of the animal, leading to a change in chill coma recovery time compared to fasting animals. (3) Recovery from chill coma will be dependent on recovering haemolymph [K’] ([K’]ex), via the recovery of water back into the haemolymph.

2. Materials and methods

2.1. Animal maintenance

Locusts (L. migratoria), originally purchased from Peter Andersen A/S, Denmark, were reared in-house and kept in containers of 0.45 m³ with hiding places and nets for climbing. The animals were exposed to a light:dark cycle of 16:8 and heat was supplied by a 150 W spotlight bulb allowing for behavioural thermoregulation between 25 and 45 °C. Dark cycle temperature was controlled to 22 °C. Locusts had ad libitum access to water and were fed daily with 8 day-old wheat sprouts, grown in commercial growth medium (Vermiculite). During rearing (but not during experiments) the food was supplemented with apple and wheat bran. Males and females were kept in separate containers thereby ensuring that all experimental animals were virgins.

2.2. Experimental protocols

Prior to experiments, locusts of both sexes were fasted for a minimum of 24 h, with access to water, before being randomly placed in individual cages (30 × 20 × 20 cm) with or without access to a high potassium diet (wheat sprouts). Animals used in the different experimental groups were of similar body size prior to feeding (Kruskall–Wallis one-way ANOVA on Ranks; P = 0.082). After a 70 min period for feeding or fasting the locusts were transferred to 50 ml plastic containers and acutely exposed to a 2 h CS treatment in a glycol cooling bath pre-set at −4 °C (Lauda RE 320, Lauda-Königshofen, Germany). After CS, the locusts were quickly returned to room temperature to either access cold tolerance (Chill coma recovery time) or ion- and water-balance after 5 or 15 min, respectively. During handling or sampling we noted no cases of freezing in the locusts due to the CS treatment.

2.3. Chill coma recovery time

Immediately after CS treatment, the fed and fasted locusts were placed on their sides, in clean individual cages at room temperature (≈24 °C), and the time of recovery (chill coma recovery time) was recorded. Since chill coma recovery time relies both on the physiological ability of the animal to regain a standing position, as well as the animals “motivation” to move, a small puff of air was applied to the animal each minute. For this particular metric we also included a treatment group that received a diet with an intermediary K+ content (peeled apple). However, for logistical reasons all further examinations were focused on the two experimental groups that received either fresh wheat or no food.

2.4. Haemolymph and tissue sampling during chill coma recovery

Haemolymph samples were obtained to investigate the effects of diet, cold exposure, and recovery on haemolymph volume, [Na+] and [K+]. Locusts were weighed for fasting fresh mass followed by feeding and CS. Fed mass was recorded immediately after CS, and the locusts were then placed at ≈24 °C for either 5 or 15 min of recovery before measurements of haemolymph volume and haemolymph ions. Immediately before sampling, the “recovery score” (responsiveness) of the locust was noted as either coma (0), responsive to prodding (1) or standing (2). At the time of sampling a small noose was tightened around the postcoppocut region such that the grasshopper was decapitated and the oesophagus was sealed. The extractable amount of haemolymph was collected within 2 min from an opening in the neck membrane, between the head and thorax, using capillary tubes (10 or 25 μl). The thorax was then opened allowing remaining haemolymph to be collected with pre-weighed pieces of filter paper. Haemolymph from the capillary tubes was transferred to a 1.5 ml Eppendorf and spun for 10 s at 2000×g to remove debris (Sprout™, Heathrow Scientific™, Illinois, USA) and 2.5 or 5.0 μl of the haemolymph was transferred to 2.0 ml of 100 ppm lithium salt buffer solution (Sherwood Scientific Ltd, Cambridge, UK) for later analysis of [K+] and [Na+] (see Section 2.5). Total haemolymph volume was estimated from the product of the mass gain in the filter paper, mass of the spun down haemolymph and the volume of haemolymph used for analysis (assuming that 1 μl haemolymph = 1 mg). After sampling of haemolymph the hind legs were detached and the leg muscle was isolated and blotted dry before muscle wet mass was recorded. Similarly, the intestinal tract was isolated and transferred to a pre-weighed Eppendorf tube to determine wet mass. Muscle and intestine samples were subsequently placed in a drying oven and left for ≈48 h at 55 °C before dry mass was obtained. The total handling time for the entire sampling procedure was less than 4 min, and all haemolymph were sampled within a maximum of 3 min.

2.5. Measurements of [Na+] and [K+]

Dried intestine and muscle samples were suspended in 400 and 200 μl of milli-Q water, respectively, and homogenised using a tissue-lyser set at 50 oscillations/s for 20 min (Tissuelyser LT, Qiagen, Hilden, Germany). Debris were centrifuged down at 20,000 × g for 15 min (Sigma 3–18 K, Sigma Laborzentrifugen GmbH, 37520 Osterode am Harz), and the supernatant was transferred to a new Eppendorf tube. Supernatant of homogenised intestine was diluted by transferring either 2.5 or 5.0 μl into 2 ml of lithium salt buffer, and for the muscle lysate 10 or 20 μl was transferred. A similar procedure was used to assess the ionic contents of the different diets (K+ content of apple = 95.3 ± 9.1 μmol gwwt⁻¹ and K+ content of 'wheat = 249.4 ± 24.6 μmol g-wwt⁻¹). The [Na+] and [K+] measurements were performed on a flame photometer (Model 420 Flame Photometer, Sherwood Scientific Ltd, Cambridge, UK). All
concentrations (tissues, haemolymph and diets) were calibrated against standard curves based on samples of known ion concentration. Intracellular muscle concentrations and total intestinal concentrations (a mix of intestinal- tissue and content) were calculated using the original water content of muscle and intestinal samples obtained from the wet and dry mass measurements.

For muscle tissue the equilibrium potentials, $E_K$ and $E_{Na}$, where calculated from the relationship between muscle and haemolymph concentration using:

$$E_X = \frac{RT}{zF} \ln \left( \frac{[X]_{\text{ex}}}{[X]_{\text{in}}} \right)$$

where $X$ is the ion in question (Na$^+$ or K$^+$), $R$ is the gas constant ($8.314 \text{ J K}^{-1} \text{ mol}^{-1}$), $T$ is the absolute temperature (297 K as all animals were assumed to be at room temperature at the time of sampling), $z$ is the ionic charge, $F$ is the Faraday constant ($96,487 \text{ coulombs mol}^{-1}$), $[X]_{\text{ex}}$ is the haemolymph concentration and $[X]_{\text{in}}$ is the muscle concentration. This calculation is based on the assumption of similar ionic composition of whole muscle tissue and the true intracellular compartments, using the assumption that the volume of the interstitial fluid is minimal as found by Wood (1963).

2.6. Statistics

All statistics were performed with SigmaPlot 11.0 (Systat Software, Inc. IL, USA). Effects of time (cold-treatment) and diet (feeding state) were analysed using a two-way ANOVA with a post hoc Bonferroni test employed to identify differences in treatment groups, using subsequent t-statistics. Prior to analysis outliers were removed using Grubbs’ tests (see Ref. GraphPad, 2013). Similarity in body size was analysed using a Kruskal–Wallis One-way ANOVA on Ranks. Similarity in ingested amount of food, as well as differences in recovery score, were analysed using a nonparametric one-way ANOVA and the differences in $K^+$ content of apple and wheat were analysed using a two-tailed t-test. All presented values are means ± SEM and level of significance is set to $\alpha = 0.05$.

3. Results

3.1. Chill coma recovery time

Recovery time varied significantly between locusts that were either fasted or fed on apple or wheat, ($t_{(2, 83)} = 3.195, P = 0.046$, Fig. 1A). This difference was not related to the amount of food consumed which was similar between the apple and the wheat group, respectively 74.7 ± 15.1 and 84.4 ± 11.8 mg food g$^{-1}$ (bm$^{-1}$) ($t_{(16)} = 0.504, P = 0.621$). The effect of dietary K$^+$ was also observed in recovery score from the locusts used for haemolymph and tissue sampling. Locusts fed on a wheat meal prior to CS had a significantly lower average recovery score (closer to chill coma) after both 5 and 15 min of recovery ($F_{(2, 83)} = 5.975, P = 0.004$, Fig. 1B).

3.2. Ion concentrations and equilibrium potentials

Cold shock elicited a significant change in $[K^+]_{\text{ex}}$ across cold-treatment (Two-way ANOVA, $F_{(2, 83)} = 42.08, P < 0.001$, Fig. 2A) and the changes in $[K^+]_{\text{in}}$ were similar in fed and fasted locusts (Two-way ANOVA, $F_{(2, 83)} = 0.082, P = 0.775$). Five min after the CS, $[K^+]_{\text{in}}$ was increased to twice the value of the untreated control ($t = 8.21, P < 0.001$) but the recovery of $[K^+]_{\text{in}}$ was quick such that there was little difference between control values and those measured after 15 min of recovery ($t = 2.38, P = 0.058$). Despite a tendency for slower recovery of $[K^+]_{\text{in}}$ in the wheat fed locusts, there was no significant interaction between feeding and cold-treatment (Two-way ANOVA, $F_{(2, 83)} = 1.33, P = 0.269$), that is, there was no significant difference in the recovery rate. The disturbance of muscle $[K^+]$ was smaller in relative terms (Fig. 2B) but did change significantly across cold-treatment (Two-way ANOVA, $F_{(2, 80)} = 26.38, P < 0.001$). Responses to cold-treatment differed between feeding states as seen from a significant interaction between cold-treatment and feeding state (Two-way ANOVA, $F_{(2, 80)} = 3.35, P = 0.040$). Cold shock elicited a 10% decrease in muscle $[K^+]_{\text{in}}$ when measured 5 min after CS ($t = 7.25, P < 0.001$). But after 15 min of recovery the fasted locusts had returned to control values ($t = 2.32, P = 0.345$), whereas $[K^+]_{\text{in}}$ in the fed locusts was still significantly different from the control ($t = 4.79, P = 0.001$). Thus, fed locusts displayed a slower recovery of muscle $[K^+]$ than fasted locusts.

The changes in intra and extracellular $[K^+]$ resulted in a similar $E_K$ for fed and fasted locusts, under control conditions ($E_K$ for the fasted and the wheat fed group was $-62.08 ± 1.45$ and $-64.07 ± 2.44$ mV, respectively; $t = 0.75, P = 1$; Fig. 2C). In both treatment groups, $E_K$ changed with recovery time (Two-way ANOVA, $F_{(2, 83)} = 62.65, P < 0.001$) while there was no overall effect of feeding-state alone (Two-way ANOVA, $F_{(1, 83)} = 0.37, P = 0.548$). There was, however, a significant interaction between cold-treatment and feeding state (Two-way ANOVA, $F_{(2, 83)} = 4.07, P = 0.021$) due primarily to a notably faster recovery of $E_K$ in the fasted locusts compared to the fed locusts. Thus, the two treatment groups were characterised by the same initial disturbance leading to a markedly depolarised $E_K$ (18 mV increase) after 5 min of recovery (fasted; $t = 7.27, P < 0.001$, fed; $t = 7.92, P < 0.001$). However,

![Figure 1](https://example.com/figure1.png)

**Figure 1.** A: Chill coma recovery time following cold shock (2 h at $-4$ °C) - Locust were fasted or fed two diets with different K$^+$ content, apple ($95.32 ± 9.11$ μmol g$^{-1}$ bm$^{-1}$) and wheat ($249.35 ± 24.55$ μmol g$^{-1}$ bm$^{-1}$); B: Average recovery score (0 = coma, 1 = responsive to prodding, 2 = standing) of fasted and wheat-fed locusts after 5 or 15 min of recovery from cold shock (2 h at $-4$ °C). Dissimilar letters identify treatments that are significantly different and all values are mean ± SEM.
recovery towards control values was significantly faster in fasting locusts that attained values that were no longer significantly different from control values 15 min after CS (\(t = 1.52, P = 1\)). Though significantly decreased from values at 5 min of recovery (\(t = 3.50, P = 0.012\)), fed locusts only recovered \(\approx 7\) mV towards control values after 15 min (\(t = 4.82, P < 0.001\)).

The mean \(E_k\) corresponding to the two feeding states’ chill coma recovery time (Fig. 1A) is depicted with dotted lines in Fig. 2C. As mentioned above there was a significant difference in chill coma recovery time between fasted and fed animals, but less than 2 mV difference in \(E_k\) at the time of recovery as inferred from the linear regression (\(E_k = -52.5\) and \(-50.9\) mV, respectively).

The changes in [\(\text{Na}^+\)] showed a reversed pattern compared to [\(\text{K}^+\)]. Haemolymph [\(\text{Na}^+\)] ([\(\text{Na}^+]_{\text{Ha}}\)] changed significantly with cold-treatment (Two-way ANOVA, \(F_{(2, 84)} = 15.36, P < 0.001\), Fig. 2D) but the recovery differed between the feeding states as attested by the significant interaction between feeding state and cold-treatment (Two-way ANOVA, \(F_{(2, 84)} = 3.87, P = 0.025\)). Haemolymph [\(\text{Na}^+\)] was similar for the two feeding states under control conditions (\(t = 0.13, P = 1\)), and decreased by 25% immediately following CS. Haemolymph [\(\text{Na}^+\)] recovered faster in the fasted locust and was similar to control values after 15 min of recovery (\(t = 0.77, P = 1\)). In contrast, fed locusts did not recover [\(\text{Na}^+]_{\text{Ha}}\] within this period (\(t = 3.48, P = 0.012\)) such that [\(\text{Na}^+]_{\text{Ha}}\] is significantly different between feeding states after 15 min of recovery (\(t = 3.04, P = 0.048\)).

There was no effect of feeding state on muscle [\(\text{Na}^+\)] (Two-way ANOVA, \(F_{(1, 79)} = 0.29, P = 0.590\)) while cold-treatment significantly affected muscle [\(\text{Na}^+\)] in both treatment groups (Two-way ANOVA, \(F_{(2, 79)} = 15.18, P < 0.001\)). Thus, muscle [\(\text{Na}^+\)] increases more than 50% following CS, and subsequently muscle [\(\text{Na}^+\)] shows a slight (but non-significant) tendency towards recovery from 5 to 15 min (\(t = 1.01, P = 0.946\)).

The equilibrium potential of [\(\text{Na}^+\)] changed significantly across cold-treatment (Two-way ANOVA, \(F_{(2, 82)} = 51.27, P < 0.001\), Fig. 2F) but there was no significant interaction between feeding state and cold-treatment (Two-way ANOVA, \(F_{(2, 82)} = 2.43, P = 0.094\)). Locusts displayed a significant decrease in \(E_{\text{Na}}\) from controls after CS (\(t = 10.11, P < 0.001\)) and recovered towards control values between 5 and 15 min of recovery (\(t = 3.48, P = 0.002\)).

3.3 Water balance and ion distribution

The increase in [\(\text{K}^+]_{\text{lex}}\) found 5 min after CS seems to be driven entirely by a reduction in haemolymph volume after cold-treatment (Two-way ANOVA, \(F_{(2, 85)} = 20.58, P < 0.001\), Fig. 3A). Thus, the total extracellular [\(\text{K}^+\)] content remains constant after CS (Two-way ANOVA, \(F_{(2, 84)} = 0.52, P = 0.593\), Fig. 3B), and showed no effect of feeding state (Two-way ANOVA, \(F_{(1, 85)} = 0.34, P = 0.563\)) and no interaction between feeding state and cold-treatment (Two-way ANOVA, \(F_{(2, 85)} = 0.75, P = 0.475\)). The increase in [\(\text{K}^+]_{\text{lex}}\) is therefore driven by the significant decrease in haemolymph volume to \(\approx 50\%\) of control values after 5 min of recovery (\(t = 6.40, P < 0.001\) which was similar in both fed and fasted animals, (Fig. 3A). The extracellular [\(\text{Na}^+\)] content (Fig. 3C) follow the trends of the water shift, displaying a significant 50% reduction due to cold-treatment (Two-way ANOVA, \(F_{(2, 85)} = 32.13, P < 0.001\)). There was a tendency for faster recovery of haemolymph [\(\text{Na}^+\)] in fasted animals but we found no interaction between feeding state and cold-treatment (Two-way ANOVA, \(F_{(2, 85)} = 1.01, P = 0.369\)). The recovery of haemolymph volume and total [\(\text{Na}^+\)] content are of proportional size for the fed locusts (Fig. 3A and C) such that [\(\text{Na}^+]_{\text{lex}}\] is unchanged during recovery (\(t = 0.28, P = 1\), Fig. 2D). In contrast, \(\text{Na}^+\) content recovers at a faster rate than haemolymph volume in the fasted locusts such that [\(\text{Na}^+]_{\text{lex}}\) recovers to control conditions within 15 min (\(t = 0.77, P = 1\), Fig. 2D).

Intestinal water volume was the only parameter that displayed significantly different control values between fed and fasted locusts (Two-way ANOVA, \(F_{(1, 81)} = 108.12, P < 0.001\), Fig. 3D), however, there were no changes during cold-treatment.
Two-way ANOVA, $F(2, 81) = 1.47, P = 0.236$) and no interaction between feeding state and cold-treatment (Two-way ANOVA, $F(2, 81) = 0.74, P = 0.479$). A similar pattern was seen for total intestinal $K^+$ in feeding state (Two-way ANOVA for feeding state, $F(1, 85) = 88.09, P < 0.001$; Fig. 3E). There was no significant changes in total intestinal $K^+$ related to the cold-treatments (Two-way ANOVA, $F(2, 85) = 1.04, P = 0.359$), although the two dietary groups differed in response as attested by a significant interaction between cold-treatment and feeding state (Two-way ANOVA, $F(2, 85) = 3.26, P = 0.043$). Feeding state significantly affected total intestinal $Na^+$ content (Two-way ANOVA, $F(1, 85) = 8.68, P = 0.004$; Fig. 3F), as did cold-treatment (Two-way ANOVA, $F(2, 85) = 8.80, P < 0.001$), but no significant interaction between cold-treatment and feeding state was found (Two-way ANOVA, $F(1, 85) = 0.13, P = 0.880$). The pattern of disturbance in total intestinal $Na^+$ displays a 25% increase in intestinal content compared to controls ($t = 4.18, P < 0.001$), but a non-significant decline towards control values from 5 to 15 min of recovery ($t = 1.31, P = 0.580$).

Water content relative to dry muscle mass differed with cold-treatment (Two-way ANOVA, $F(2, 81) = 9.34, P < 0.001$, Fig. 3G) in fed animals, while fasted animals had similar values across cold-treatments. We found a significant interaction between feeding state and cold-treatment (Two-way ANOVA, $F(2, 81) = 3.46, P = 0.036$), with similar values in both the fed and fasted animals after 5 min of recovery ($t = 0.214, P = 1$) and different values after 15 min ($t = 3.04, P = 0.048$). That is; the fed animals recovered muscle water slower than the fasted animals. The initial increase in muscle water content, had no effect on dry muscle potassium content (Fig. 3H) which remained similar across cold-treatments (Two-way ANOVA, $F(2, 85) = 0.54, P = 0.947$) and between feeding states (Two-way ANOVA, $F(1, 85) = 0.64, P = 0.425$). The increase in muscle cell water content was likely driven by the large increase in sodium content found after the cold-treatment (Two-way ANOVA, $F(2, 85) = 20.95, P < 0.001$, Fig. 3I). Although there is a trend towards recovery in the fasting locusts we found no significant interaction between feeding state and cold-treatment (Two-way ANOVA, $F(2, 85) = 1.04, P = 0.359$).
ANOVA, $F_{2, 84} = 2.21, P = 0.116$) and neither an effect of feeding state in itself (Two-way ANOVA, $F_{1, 84} = 0.12, P = 0.735$).

4. Discussion

4.1. Ion homeostasis and chill coma recovery

The onset and recovery of chill coma in insects has repeatedly been correlated to the loss and recovery of ion-homeostasis and neuromuscular excitability (Anderson and Mutchmor, 1968; Hosler et al., 2000; Koštál et al., 2006; Rodgers et al., 2007; MacMillan and Sinclair, 2011a; Armstrong et al., 2012; MacMillan et al., 2012; Findsen et al., 2013). Similarly it has been demonstrated that the progression of chill injury is correlated with the degree of disturbance of particularly extracellular ion composition (Koštál et al., 2004; MacMillan and Sinclair, 2011b). Although chill coma and chill injury may be somewhat different phenomena (MacMillan and Sinclair, 2011a), these findings suggest that preservation of ion homeostasis is important for several metrics of cold tolerance in chill sensitive insects. Accordingly, cold acclimation and rapid cold hardening, which are known to improve cold recovery and survival, have also been shown to significantly improve the ability to maintain and/or recover ion homeostasis during and following cold exposure (Koštál et al., 2004, 2006; Armstrong et al., 2012; Findsen et al., 2013). To obtain further knowledge about the mechanisms underlying recovery from chill coma, we investigated the mechanistic connection between ion-homeostasis and cold tolerance. This was done by exposing the chill sensitive migratory locust (L. migratoria) to cold as well as letting them feed, since food in the gut is also reported to affect $[K^+]_{ex}$ and neuromuscular function (Hoyle, 1954).

Consistent with earlier reports concerning a range of chill sensitive insects, the present study demonstrated that CS induced large increases in $[K^+]_{ex}$, leading to a significant increase in the equilibrium potential for $K^+$ (Fig. 2C) (Koštál et al., 2004, 2006; MacMillan and Sinclair, 2011a; Armstrong et al., 2012; MacMillan et al., 2012; Findsen et al., 2013). Earlier reports have also found that a high potassium diet increased $[K^+]_{ex}$ (Hoyle, 1954). Given $[K^+]_{ex}$ is of large importance for the resting membrane potential (Hoyle, 1953), it was suggested that the sluggish jumping and muscle performance of post-prandial locusts was caused by the increased $[K^+]_{ex}$ and an associated reduction in neuromuscular function (Hoyle, 1954). The present study, however, did not find an increased $[K^+]_{ex}$ after feeding. Instead, we observed that fed locusts had a tendency towards slower recovery of $[K^+]_{ex}$ and $E_{K}$ and a significantly slower $[K^+]_{in}$, $[Na^+]_{in}$, and $K^+$ equilibrium potential recovery after CS (Fig. 2A–D and F). The overall effect of feeding on the cold tolerance is consistent with a recent study on fed first-instar nymphs of Australian plague locusts (Chortoicetes terminifera), showing that food is an added stress during cold, as survival after CS was significantly higher in fasted animals (Woodman, 2010). It is possible that the higher mortality of fed animals found in the study by Woodman (2010) relates to processes other than chill injury, as the presence or absence of gut content is also an important factor for the supercooling ability in insects (Powell, 1976; Samme and Block, 1982; Zachariassen, 1985; Ramløv, 2000; Salin et al., 2000; Bale, 2002; Colinet et al., 2007). However, in the present study we never observed frozen animals and our data therefore confirm the hypothesis that the combination of CS and food lowered the recovery score and increased the chill coma recovery time (Fig. 1A and B) through the interactive effects on ion and water homeostasis by the treatments.

Previous studies have found that the recovery from cold is related to the recovery of $E_{K}$, usually to a value around $-40$ mV (Hosler et al., 2000; MacMillan et al., 2012; Findsen et al., 2013). The slower return towards resting potentials of both $K^+$ and $Na^+$ in the fed animals, compared to the fasted animals (Fig. 2C and F), clearly shows that there is an added stress associated with a full gut. In the present study, $E_{K}$ at chill coma recovery time was between $-50$ and $-55$ mV (Fig. 2C). This value is below the $-40$ mV found in other studies, but the similar $E_{K}$ at recovery for the two treatment groups qualitatively confirms earlier observations of a “critical-$E_{K}$” value. Thus, the findings of the present study are consistent with our earlier observation, comparing rapid cold hardened and unhardened locusts, where it was found that chill coma recovery of the animal is associated with a specific threshold value of $E_{K}$ (Findsen et al., 2013). The difference in “critical-$E_{K}$” between the present and previous study is unclear but could be related to differences in the protocol used to assess chill coma recovery time.

4.2. Water- and ion-balance during chill coma recovery

The Malpighian tubules of insects usually exploit secondary active ion transport to take up water and ions from the haemolymph, which results in an iso-osmotic fluid high in $K^+$ that is secreted to the gut (Ramsay, 1954; Maddrell and O’Donnell, 1992). Much of this transport is dependent on the activity of a $H^+$-pumping $V$-ATPase facilitating the removal of $K^+$ from the haemolymph (Maddrell and O’Donnell, 1992; O’Donnell et al., 1996). The fluid secreted from the Malpighian tubules is transported through the ileum and into the rectum where important ions and molecules are reabsorbed into the haemolymph, thereby producing a hyperosmotic and high $[K^+]_{in}$ content of hindgut (Edney, 1977; Harrison et al., 2012). In the rectum, under normal conditions, $Na^+$–$K^+$-ATPases and electrogenic Cl$^-$-pumps facilitate reabsorption of $Na^+$ and water back to the haemolymph (Ramsay, 1971: Hanraban and Phillips, 1982). Lowering body temperature to $–4^\circ C$ will depress the activity of the ion motive pumps facilitating transport of $K^+$ and $Na^+$ (and the associated water). This depression in active pumping will potentially cause an imbalance of ions and water entering or leaving the intestinal lumen, unless the passive transport processes are reduced proportionally (MacMillan and Sinclair, 2011a). Similarly, in the cells, a reduction in $Na^+$–$K^+$-ATPase activity will lead to a net influx of $Na^+$ and water from the extracellular compartment and a net efflux of $K^+$ from the intracellular compartment (Randall et al., 2001). In a related study on chill tolerance in the fall field cricket (G. pennsylvaniae), MacMillan and Sinclair (2011b) found that the increase in $[K^+]_{ex}$ associated with cold exposure could be explained entirely by a relocation of water from the haemolymph to the intestine. Thus, after CS, the same amount of $K^+$ was dissolved in a reduced haemolymph volume, while $Na^+$ and water shifted into the intestine. The present study partially confirms this observation in L. migratoria, as we found that the doubling of haemolymph $[K^+]_{ex}$ after CS (Fig. 2A) was associated with a 50% reduction in extracted haemolymph volume (Fig. 3A), while total $K^+$ content remained constant (Fig. 3B). However, unlike the study of G. pennsylvaniae (MacMillan and Sinclair, 2011b), we did not find that the water lost from the haemolymph relocated to the intestine since there was only a slight and non-significant increase in intestinal water volume following CS in L. migratoria (Fig. 3D). Moreover, we found that the subsequent recovery of $[K^+]_{ex}$ could not be explained by water movement alone. For example, haemolymph water volume had only recovered by 33% after 15 min of recovery while the $[K^+]_{ex}$ was more than 90% recovered towards control values in the fasted animals (Fig. 3A and Fig. 2A, respectively). This suggests that $[K^+]_{ex}$ is regulated by other means than just haemolymph water volume and that active removal of $K^+$ from the haemolymph may also be an important parameter for recovery of $[K^+]_{ex}$ in L. migratoria. Such removal of excess $K^+$ could for example be facilitated by secondary active transport of $K^+$ in the Malpighian tubules (Maddrell and O’Donnell, 1992; O’Donnell,
et al., 1996; Koštál et al., 2007) or by increased Na\(^{+}\)-K\(^{+}\)-ATPase activity of other tissues.

Some of the Na\(^{+}\) and water lost from the haemolymph after cold, relocated to the muscle (Fig. 3G and I). This relocation can explain approximately half of the 20 μl haemolymph g-bm\(^{-1}\) lost from the extracellular volume. Hence, the excess water in the leg muscles after CS amounted to 2.5 μl of fluid per 8 mg-dwt of leg muscle, assuming a similar water content and water shift in the 43 mg-dwt of flight muscle (Beenakkers, 1965), would represent a total of approximately 9 μl of water moving into the muscle cells of a 1 g animal. Consistent with processes described above, total Na\(^{+}\) in the haemolymph dropped 3.4 μmol after CS (Fig. 3C). This Na\(^{+}\) was found to be relocated to the intestine and muscle (around 0.7 and 3 μmol increase, respectively, assuming a total muscle mass of 51 mg-dwt g-bm\(^{-1}\)). Following 15 min of recovery, Na\(^{+}\) and water content did show signs of recovery towards controls (Fig. 3A, C and G), although E\(\text{Na}_{\text{in}}\) was far from control values at the time the animals regained neuromuscular function (Fig. 2F).

With the available data, we were unable to locate all the missing haemolymph and ions after CS. It is likely that some of it has relocalized to other organ systems which we did not investigate in the present study. For example, it was suggested in a study of the chill sensitive firebug (Pyrrhocoris apterus) that some water may move into tissues like the fat body or gonads during CS (Koštál et al., 2004). Another possibility is that water actually moves into the gut lumen, and is regurgitated or defecated during CS or handling. We did not, however, notice signs of such regurgitation or defecation in the test tubes used for CS, and few animals were noted to lose gut/oesophagus content during handling.

The present study confirms that disruption of water balance causes a dramatic increase in [K\(^{+}\)]\(_{\text{ex}}\) during cold exposure, which leads to a considerable change in resting membrane potential (Hoyle, 1953), impairing neuromuscular function. The water and ion shifts between muscle and haemolymph during CS are likely to be due to an uncompensated decrease in activity of active ion transport (V-ATPase and Na\(^{-}\)–K\(^{+}\)-ATPase) and our data suggest that both recovery of water volume and active removal of K\(^{+}\) are important during recovery of [K\(^{+}\)]\(_{\text{ex}}\). When comparing the cold-treatment and feeding state groups we did not find that the added stress of cold, relocation to the intestine and muscle (around 0.7 and 3 μmol increase, respectively, assuming a total muscle mass of 51 mg-dwt g-bm\(^{-1}\)). Following 15 min of recovery, Na\(^{+}\) and water content did show signs of recovery towards controls (Fig. 3A, C and G), although E\(\text{Na}_{\text{in}}\) was far from control values at the time the animals regained neuromuscular function (Fig. 2F).

5. Conclusions

Cold shock disrupts ion homeostasis, where in particular disturbance of K\(^{+}\) balance is thought to disrupt neuromuscular excitability. We found that locusts fed a high K\(^{+}\) diet had an inferior cold tolerance as measured from the chill coma recovery time. The increased chill coma recovery time was not caused by a more dramatic disturbance of ion-homeostasis, but rather due to a slower recovery of water, Na\(^{+}\) and K\(^{+}\) homeostasis. Consistent with earlier studies (MacMillan and Sinclair, 2011b; MacMillan et al., 2012) we found that water balance could be the cause for initial K\(^{+}\) disturbance during CS, but we also found that recovery of water and [K\(^{+}\)]\(_{\text{ex}}\) was decoupled in L. migratoria, suggesting that both reestablishment of haemolymph volume and active removal of K\(^{+}\) is responsible for the regaining of [K\(^{+}\)]\(_{\text{ex}}\) after CS.

Acknowledgements

We are grateful to Kirsten Krommann for all the help in the laboratory and for the valuable comments of two anonymous referees. The research was funded by a Sapere Aude DFF-Starting grant (JO) from The Danish Council for Independent Research | Natural Sciences.

References


Comparative Biochemistry and Physiology. A. Molecular Integrated Physiology 147, 231–238.


