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## RESEARCH ARTICLE

# The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*

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**SUMMARY**

**To predict the effects of changing climates on insect distribution and abundance, a clear understanding of the mechanisms that underlie critical thermal limits is required. In insects, the loss of muscle function and onset of cold-induced injury has previously been correlated with a loss of muscle resting potential. To determine the cause of this loss of function, we measured the effects of cold exposure on ion and water homeostasis in muscle tissue, hemolymph and the alimentary canal of the fall field cricket, *Gryllus pennsylvanicus*, during an exposure to 0°C that caused chilling injury and death. Low temperature exposure had little effect on muscle osmotic balance but it dissipated muscle ion equilibrium potentials through interactions between the hemolymph and gut. Hemolymph volume declined by 84% during cold exposure whereas gut water content rose in a comparable manner. This rise in water content was driven by a failure to maintain osmotic equilibrium across the gut wall, which resulted in considerable migration of $Na^+$, $Ca^{2+}$ and $Mg^{2+}$ into the alimentary canal during cold exposure. This loss of homeostasis is likely to be a primary mechanism driving the cold-induced loss of muscle excitability and progression of chilling injury in chill-susceptible insect species.**

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Key words: chilling injury, chill coma, osmoregulation, ion homeostasis, water balance, equilibrium potential, alimentary canal.

## INTRODUCTION

The effects of a changing climate on the distribution and abundance of ectotherms will depend on the evolutionary plasticity of physiological mechanisms setting critical thermal limits to activity and fitness (Pörtner et al., 2006; Angilletta, 2009). Although the physiological mechanisms setting such limits are increasingly well known in marine animals, critical thermal limits of terrestrial animals, most notably insects, are very poorly understood. At the critical thermal minimum ($CT_{min}$), an insect enters chill coma, where movement ceases entirely (Semper, 1883; Mellanby, 1939). Prolonged exposure to temperatures that induce chill coma result in a time- and temperature-dependent accumulation of damage (chilling injury), which ultimately results in death. Although some temperate and polar insect species survive exposure to extreme cold, most insects are killed at low temperatures by processes unrelated to freezing (Lee, 2010). Such chill-susceptible species enter chill coma at relatively mild temperatures and die from the accumulation of cellular and whole-animal chilling injury during cold exposure.

When in chill coma, insect nerves are electrically silent and there is a lack of muscle excitability, the latter resulting from a progressive decline in muscle equilibrium potential during cooling (Staszak and Mutchmor, 1973; Goller and Esch, 1990; Hosler et al., 2000). Following prolonged cold exposure, two forms of chilling injury have been described: direct (cold shock) and indirect. Direct chilling injury occurs over a period of minutes to hours and is thought to result from cell membrane phase transitions from the liquid crystalline to the gel phase during rapid cooling (Quinn, 1985; Drobnis et al., 1993). Indirect chilling injury occurs over a period of days to weeks, and proposed mechanisms include changes in membrane phase, mismatching of metabolic pathways, oxidative stress and loss of ion homeostasis (Rojas and Leopold, 1996; Yocum, 2001; Kostál et al., 2004; Kostál et al., 2006). Although the physiological nature of chilling injury is largely unknown, the injury itself can be identified in the animal by a loss of coordination (e.g. impaired gait or inability to right itself when flipped onto its back) or by failure to complete development (Rojas and Leopold, 1996; Kostál et al., 2006; Kostál and Tollarová-Borovanská, 2009).

In insects, ion homeostasis at the organismal level is regulated by the Malpighian tubules and the gut, and varies considerably among species, particularly depending on diet (O'Donnell, 2008). Typically, the Malpighian tubules produce primary urine that is roughly isosmotic with the hemolymph by coupling the action of a V-ATPase and $H^+$-cation exchangers, ultimately driving $K^+$, $Cl^-$ (and $Na^+$, in blood-feeding insects) transcellularly from the hemolymph into the lumen. This process maintains a gradient favoring movement of water (through aquaporins) and waste products (through dedicated transporters) into the lumen of the tubules (Ramsay, 1954; Pannabecker, 1995; O'Donnell, 2009; Spring et al., 2009). Although some resorption of water and ions occurs along the length of the Malpighian tubule, most water and ion resorption in terrestrial insects (specifically locusts) occurs in the hindgut, where $Na^+$-$K^+$-ATPase and an electrogenic $Cl^-$ pump maintain steep local osmotic gradients driving the movement of water and $Na^+$ back into the hemolymph (Ramsay, 1971; Hanrahan and Phillips, 1982).

At the cellular level, ion homeostasis is regulated through ion-motive pumps coupled to channels and/or secondary transporters that strictly maintain differences in the intracellular and extracellular concentrations of physiologically active inorganic ions, particularly

$Na^+$, $K^+$, $Mg^{2+}$, $Ca^{2+}$ and $Cl^-$. ATP-dependent ion pumps (e.g. $Na^+$-$K^+$-ATPase) generate and maintain equilibrium potentials that are utilized by secondary active transporters, such as the $Na^+$-$K^+$-$2Cl^-$ transporter, to move ions against their electrochemical gradients. The bulk movement of ions down their electrochemical gradient is facilitated by ion-specific channels that may or may not be gated (Heimlich et al., 2004; Lang et al., 2007), whereas water movement is facilitated by the presence of aquaporins (Spring et al., 2009).

During exposure to environmental stress, maintenance of ion homeostasis is of great importance; intracellular and extracellular ion abundance largely determine osmotic pressure, and are thus crucial for regulating cellular water balance. When cooled, the activity of ion-motive ATPases would be expected to decline with a $Q_{10}$ between 2 and 3. Conversely, the diffusion of ions down their concentration gradients would be relatively unaffected by temperature (Zachariassen et al., 2004). This would lead to a time- and temperature-dependent loss of ion and water homeostasis at both the cellular and tissue level during cold exposure, provided pathways for such movement (ion channels, aquaporins or paracellular pathways) remain open.

Many important cell functions, including signal transmission in nerve and muscle tissue, rely on coordinated changes in $Na^+$, $K^+$ and $Ca^{2+}$ equilibrium potentials. In addition, alterations to the intracellular abundance of ionic species and their control over intracellular water content are known to play important roles in signaling cascades leading to cellular apoptosis (Mattson and Chan, 2003; Heimlich et al., 2004; Lang et al., 2007). In insects, apoptosis is a known correlate of whole-animal cold-induced injury and rapid plasticity in sensitivity to chilling injury is associated with reduced levels of apoptosis following cold exposure in *Drosophila melanogaster* (Yi et al., 2007).

Highly cold-tolerant insects, such as freeze-avoiding pine beetles (*Rhagium inquisitor*, Coleoptera: Cerambycidae), are able to maintain ion gradients during prolonged exposure to –10°C, whereas freeze-tolerant wood flies (*Xylophagus cinctus*, Diptera: Xylophagidae) permit the redistribution of ions during ice formation in the hemocoel but manage to maintain osmotic neutrality between intracellular and extracellular compartments, thus avoiding a net movement of water (Dissanayake and Zachariassen, 1980; Kristiansen and Zachariassen, 2001; Zachariassen et al., 2004). Chill-susceptible species, however, appear unable to maintain such control. During progressive cooling, flight muscle resting potential decreases exponentially in both *D. melanogaster* (Diptera: Drosophilidae) and *Apis mellifera* (Hymenoptera: Apidae) (Hosler et al., 2000). In chill-susceptible tropical cockroaches (*Nauphoeta cinerea*, Dictyoptera: Blaberidae), muscle equilibrium potentials decline in a manner that correlates with the incidence of chilling injury; however, nearly all changes in ion concentration that drive the loss of potential appear to occur in the hemolymph and not the muscle tissue (Kostál et al., 2006). Such patterns imply that interactions between the hemolymph and tissues other than muscle may be driving the loss of muscle equilibrium potentials during cooling. Studies that have addressed the loss of ion balance during cooling have exclusively measured ion concentrations or their effects on muscle potential, and thus have overlooked the potential for interactive effects of tissue or hemolymph water content and ion abundance (Goller and Esch, 1990; Hosler et al., 2000; Kostál et al., 2004; Kostál et al., 2006; Kostál et al., 2007).

The purpose of the present study was to determine the interdependent roles of the alimentary canal, hemolymph and muscle tissue in the disruption of ion and water balance during chilling in a chill-susceptible insect, the fall field cricket (*Gryllus pennsylvanicus* Burmeister 1838, Orthoptera: Gryllidae). We hypothesized that diffusion of ions and water down their concentration gradients between the hemolymph and gut drive a redistribution of water and the subsequent loss of muscle equilibrium potentials and progression of chilling injury during cold exposure.

## MATERIALS AND METHODS

### Animal rearing

A population of *G. pennsylvanicus* derived from individuals collected in 2004 from the University of Toronto Mississauga campus (43.3°N, 79.4°W) was laboratory reared under constant summer conditions (25°C, 14 h:10 h light:dark, 70% relative humidity) following Judge (Judge, 2010). Briefly, crickets were fed commercial rabbit feed (Little Friends Rabbit Food, Martin Mills Inc., Elmira, ON, Canada) and water *ad libitum*. Adults were given access to 500 ml containers filled with a 4:1 mixture of fine vermiculite and sand for 1-week periods to lay eggs. Containers containing eggs were then transferred to a 4°C cold room for 3 months to accommodate an obligate diapause (Rakshpal, 1962) before being returned to 25°C conditions to complete development. Gravid adult females, approximately 3 weeks post final molt, were used for all experiments.

### Determination of $CT_{min}$ and survival of chronic cold exposure

Critical thermal minima ($CT_{min}$) were determined using a method modified from Klok and Chown (Klok and Chown, 1997). Twelve crickets were placed individually into closed 200 ml glass beakers jacketed in a Plexiglas® enclosure through which an ethylene glycol:water mix (1:1 v:v) was circulated from a programmable refrigerated bath (Model 1157P, VWR International, Mississauga, ON, Canada). The temperature inside each well was monitored with type-T thermocouples connected to a computer *via* a Picotech TC-08 thermocouple interface and PicoLog software (Pico Technology, Cambridge, UK). The chambers were cooled linearly from 25°C at 0.25°C $min^{-1}$, allowing for measurement of temperature within each well within 0.1°C. Crickets were monitored continuously and the $CT_{min}$ was the temperature at which no movement could be elicited from a cricket after harassment from a blunt plastic probe inserted through a small hole in the lid of the beaker.

To assess survival, crickets were placed into individual 14 ml plastic tubes, which were loosely covered (to allow air flow), cooled from 25 to 0°C at 0.25°C $min^{-1}$ and held at 0°C (a temperature where all individuals would be in chill coma) for up to 5 days (120 h). Every 12 h, a group of 10 crickets was removed and placed at 25°C in plastic Petri dishes with access to food and water. Survival and chilling injury were scored 6 and 24 h after removal from cold conditions. Crickets were coaxed to run across a flat bench surface and perform a jump. Crickets that moved in a coordinated manner and performed a jump were scored as fit. Crickets that lacked coordination (encompassing a broad range of severity) or could not jump were scored as chill injured. Crickets that showed no signs of movement were scored as dead (Kostál et al., 2006).

### Tissue sampling

To quantify ion and water content of muscle, hemolymph and gut, crickets were cooled from 25 to 0°C at 0.25°C $min^{-1}$, and held at 0°C as above. Different sets of crickets were used to quantify ion and water contents of: (1) muscle and hemolymph and (2) gut.

Control crickets were immobilized with $CO_2$ and dissected at 22°C, whereas cold-exposed crickets remained in chill coma and were dissected in a 4°C cold room (~3 min per dissection). A small incision was made at the base of each hind leg and hemolymph was

collected using a pipette. The thorax and abdomen were then opened and any additional hemolymph was collected in the same manner. The total volume of hemolymph collected was used as an approximation of hemolymph volume. Quantification of hemolymph volume using radiolabeled inulin dilution (Loughton and Tobe, 1969) was not used because it requires the heart to actively mix the hemolymph (Samaranayaka, 1977), which does not occur when the insect is in chill coma. Muscle tissue was collected from a single hind femur using tweezers and was blotted lightly on a KimWipe to remove any hemolymph remaining on the tissue. All tissue samples were placed into pre-weighed 200 µl PCR tubes.

The foregut (crop and proventriculus), midgut and hindgut were isolated by removing the legs and wings and making a dorsal longitudinal incision from the tip of the abdomen to the base of the head. Junctions between the proventriculus, midgut and hindgut were ligated and dissected out, starting with the hindgut and proceeding forward. This process ensured no mixing of gut contents occurred between sections during the dissection process, and that no gut contents were lost during isolation. Tracheae, Malpighian tubules and fat body surrounding the gut were gently removed and discarded. Gut sections were dabbed lightly on a KimWipe to remove residual hemolymph, the ligatures were removed, and the gut sections placed into pre-weighed 200 µl PCR tubes.

### Sample preparation and analysis

Sample tubes containing tissues were weighed to obtain wet mass, dried in an oven at 70°C for 48 h and reweighed to determine dry mass. Nitric acid (200 µl) was added to each sample tube and samples were kept at 22°C for 24 h with occasional mixing until the tissue was completely dissolved. Samples were diluted with double-distilled $H_2O$ to bring them within the measurable range of the atomic absorption spectrometer (iCE 3300, Thermo Scientific, Waltham MA, USA) and absorption values were compared with standard curves of $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ generated from diluted standards containing nitric acid.

### Data analysis

Muscle cell membrane equilibrium potentials for each ion were calculated for each cricket sampled using the Nernst equation:

$$E = \left(\frac{RT}{zF}\right) \ln \frac{[\text{Extracellular}]}{[\text{Intracellular}]}, \quad (1)$$

where $R$ is the universal gas constant, $T$ is the absolute temperature, $z$ is the ionic charge, $F$ is Faraday's constant, [Extracellular] is the ion concentration in the hemolymph and [Intracellular] is the ion concentration in the muscle tissue.

Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, NC, USA). Ion (µmol) and water (µl) content data from control (no cold exposure) crickets and those exposed to 0°C for the shortest duration (6 or 12 h) were compared using *t*-tests, and table-wide *P*-values were adjusted with false discovery rate (FDR) correction using the PROC MULTTEST function. Use of FDR in this manner controls for increased likelihood of type I error when making multiple comparisons (Benjamini and Hochberg, 1995). This approach was taken because clear differences were observed in the direction and rate of change of ion and water content between the first 6 or 12 h of cold exposure and prolonged cold exposure. If there was a significant difference between control crickets and those that experienced the shortest duration of cold exposure, implying a rapid cold-induced change, control crickets were excluded from regression analysis. Ion and water content data of both raw and ln-transformed data were then analyzed using the PROC GLM function and the model with the highest $R^2$ value was used within each data set. Tissue dry mass was used as a covariate to account for sample mass as appropriate. As the effect of dry mass on ion and water content was always significant ($P<0.001$), only the effects of exposure time are reported in such cases. Degrees of freedom, values of statistics and precise *P*-values are presented in the supplementary material (supplementary material Tables S1–S3), with just the level of significance reported in the text. All values reported for descriptive purposes are sample means ± s.e.m.

## RESULTS

### $CT_{min}$ and survival at 0°C

When cooled at 0.25°C min$^{-1}$, female crickets entered chill coma at 2.3±0.1°C. All crickets exposed to 0°C were in chill coma when removed to room temperature for survival analysis. When assessed following 6 h of recovery at 25°C, chilling injury was noted after as little as 12 h at 0°C, and 60% of crickets were injured following 60 h (3 days) of exposure (Fig. 1A). Following 120 h (5 days) at 0°C and 24 h of recovery time, all of the crickets were dead (Fig. 1B). Individuals from several time points scored as injured 6 h after warming were dead 24 h following warming, whereas one individual (of 10 crickets cold-exposed for 48 h) scored after 6 h as injured was later scored as fit (Fig. 1A,B).

### Hemolymph and muscle ion concentration

Hemolymph [$Na^+$] declined from 189.4±19.7 to 127.0±5.4 mmol l$^{-1}$ during the first 12 h at 0°C ($P<0.05$), then continued to drop linearly ($P<0.001$) but at a slower rate during continued cold exposure (Fig. 2A). By contrast, hemolymph [$K^+$] increased significantly from

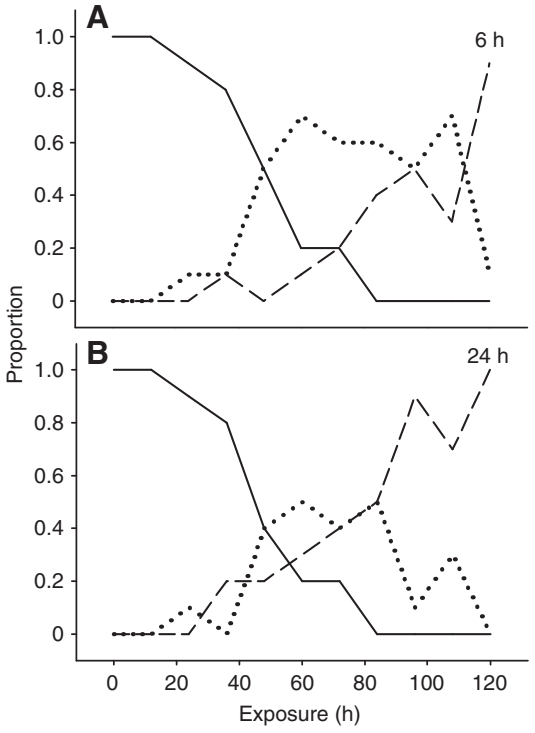


Fig. 1. Survival of *Gryllus pennsylvanicus* 6 h (A) and 24 h (B) after removal from exposure to 0°C for up to 120 h. Survival data are presented as a proportion of fit (solid line), injured (dotted line) and dead (dashed line) individuals. Fit crickets were defined as able to move in a coordinated manner and perform a jump. Injured crickets were defined by a lack of coordination or inability to jump. Dead crickets did not move at all. $N$=10 crickets per 12 h period.

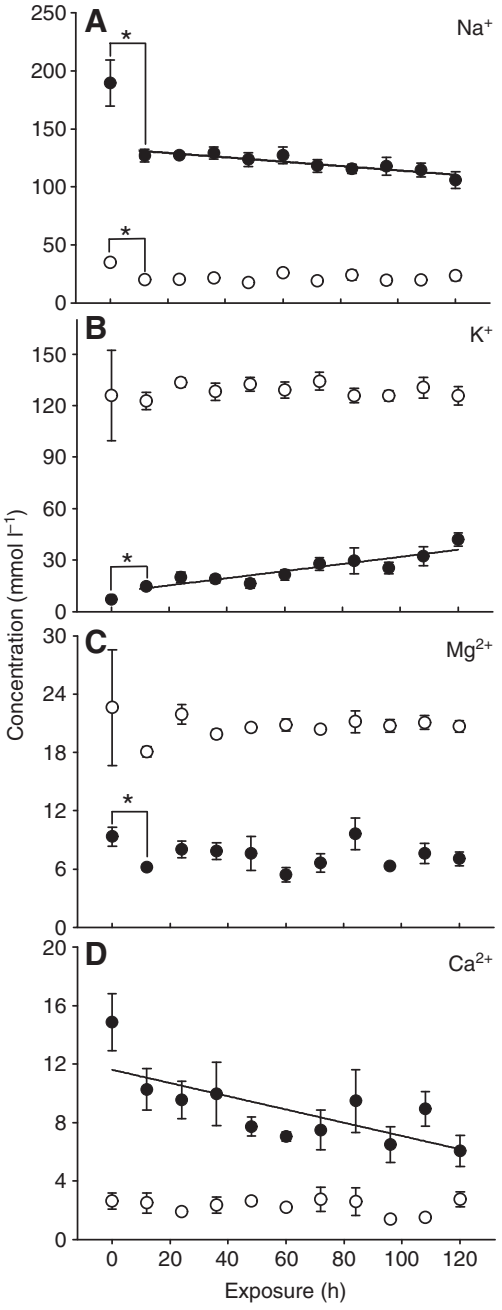


Fig. 2. Concentration (mM; mean ± s.e.m.) of $Na^+$ (A), $K^+$ (B), $Mg^{2+}$ (C) and $Ca^{2+}$ in hemolymph (closed circles) and femur muscle tissue (open circles) of *G. pennsylvanicus* during exposure to 0°C for up to 120 h. Asterisks denote significant changes in ion concentration within the first 12 h (see Results), and lines denote significant linear relationships between time at 0°C and ion concentration. *N*=6 crickets per 12 h period. Error bars that are not visible are obstructed by the symbols.

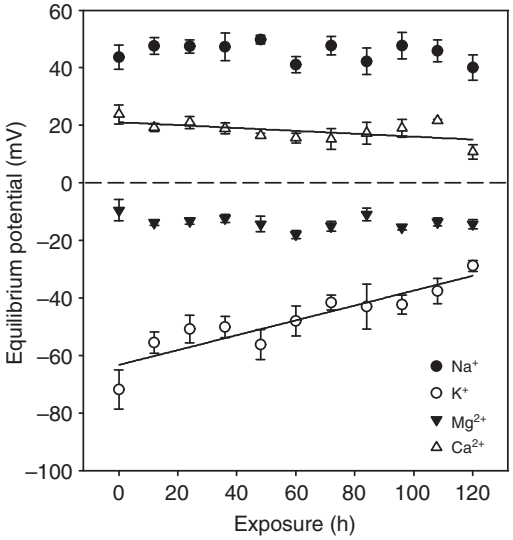


Fig. 3. Calculated equilibrium potentials (mV; mean ± s.e.m.) of $Na^+$, $K^+$, $Mg^{2+}$ and $Ca^{2+}$ of muscle cell membranes of *G. pennsylvanicus* during prolonged exposure to 0°C. No significant changes in equilibrium potentials in the first 12 h were found. Lines denote significant linear relationships between cold exposure duration and equilibrium potentials. *N*=6 crickets per 12 h period. Error bars that are not visible are obstructed by the symbols.

7.0±0.9 to 14.7±1.7 mmol $l^{-1}$ during the first 12 h at 0°C ($P$<0.01) and continued to increase to 41.9±3.9 mmol $l^{-1}$ following 120 h at 0°C ($P$<0.001; Fig. 2B). Hemolymph [$Mg^{2+}$] significantly declined during the first 12 h ($P$<0.05); however, there was no further effect of prolonged cold exposure and mean [$Mg^{2+}$] returned to control levels at several time points throughout cold exposure ($P$>0.05; Fig. 2C). Hemolymph [$Ca^{2+}$] did not significantly decline during the first 12 h of cold exposure ($P$>0.05), but it declined significantly from 14.9±1.9 to 6.1±1.1 mmol $l^{-1}$ during prolonged cold exposure ($P$<0.001; Fig. 2D). In the muscle tissue, [$Na^+$] declined significantly from 35.0±2.8 to 20.3±1.9 mmol $l^{-1}$ during the first 12 h of cold exposure ($P$<0.01), although there was no continued effect of exposure duration ($P$>0.05; Fig. 2A). Cold exposure did not significantly alter [$K^+$], [$Mg^{2+}$] or [$Ca^{2+}$] during the first 12 h of cold exposure, nor did it affect them for the duration of cold exposure ($P$>0.05; Fig. 2B–D).

No significant changes in $Na^+$, $K^+$, $Mg^{2+}$ or $Ca^{2+}$ muscle equilibrium potentials ($E_{Na}$, $E_K$, $E_{Mg}$ and $E_{Ca}$, respectively) were observed during the first 12 h of cold exposure ($P$>0.05). However, the absolute $E_K$ significantly dropped from –71.7±3.3 to –29.4±2.7 mV following exposure to 0°C for 120 h ($P$<0.001; Fig. 3). Similarly, $E_{Ca}$ significantly declined from 23.4±1.6 mV under control conditions to 11.4±2.32 mV following 120 h at 0°C ($P$<0.05). $E_{Na}$ and $E_{Mg}$ did not significantly change during cold exposure ($P$>0.05; Fig. 3).

### Tissue water content

Muscle water content did not significantly change during the first 12 h of cold exposure or over the duration of cold exposure ($P$>0.05; Fig. 4A). Hemolymph volume significantly decreased in an exponential manner from an initial mean volume of 40.8±2.6 µl to 6.4±2.1 µl in crickets exposed to 72 h or longer at 0°C ($P$<0.001; Fig. 4B). Over the same time period, total gut water content increased from an initial mean volume of 66.2±6.7 µl to 95.7±13.5 µl in crickets exposed to 0°C for 72 h or longer ($P$<0.05; Fig. 4B). The decrease in hemolymph volume and increase in total gut volume during the first 6 h of cold exposure were not statistically significant ($P$>0.05). Following 120 h at 0°C, crickets lost 16.10±1.73 mg of body mass, a significant change ($P$<0.001).

Water content of the foregut and midgut tended to increase following 6 h of cold exposure, although these increases were

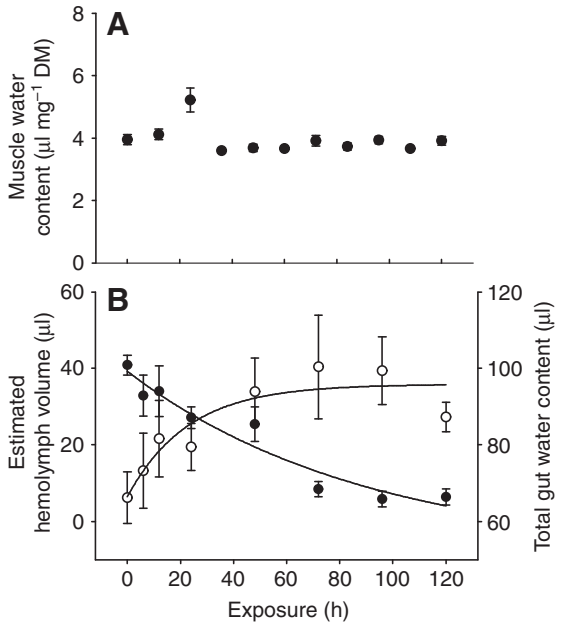


Fig. 4. Mean ± s.e.m. (A) muscle water content (expressed as µl $mg^{-1}$ of muscle dry mass [DM]) and (B) hemolymph volume (closed circles) and total gut water content (open circles) (expressed in µl) of *G. pennsylvanicus* during exposure to 0°C for up to 120 h. Cold exposure did not significantly affect muscle water content. Hemolymph water content and total gut water content fell and rose, respectively, in an exponential manner that was statistically significant (see text for statistics). Total water accumulation in the gut accounts for *ca.* 86% of lost hemolymph volume. *N*=6 and 8 crickets per sampling period for muscle water content and hemolymph and gut analysis, respectively. Error bars that are not visible are obstructed by the symbols.

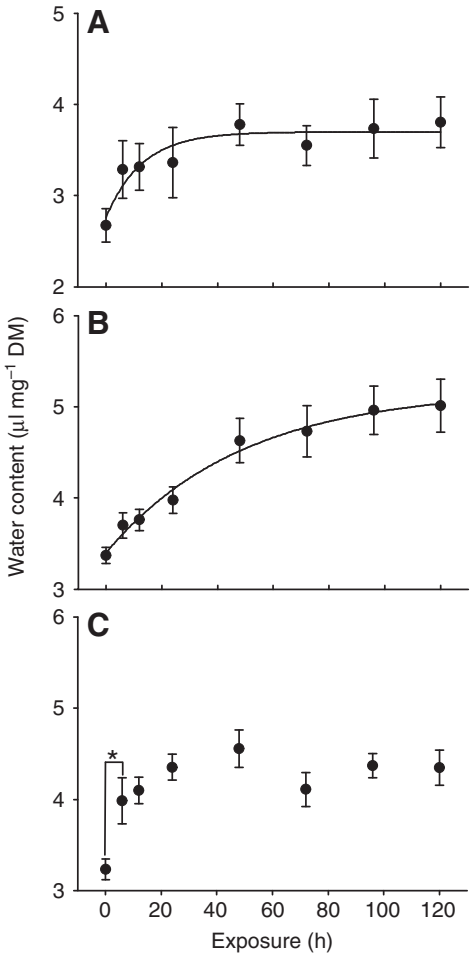


Fig. 5. Mean ± s.e.m. (A) foregut, (B) midgut and (C) hindgut water content (expressed as µl $mg^{-1}$ tissue DM) of *G. pennsylvanicus* exposed to 0°C for up to 120 h. Exposure to 0°C caused a significant accumulation of water in all regions of the gut. Asterisk denotes a significant effect of initial cold exposure and lines of best fit denote a significant relationship between water content and time at 0°C (see text for statistics). *N*=8 crickets per sampling period.

marginally non-significant ($P$=0.070 and $P$=0.051, respectively). Total cold exposure duration, however, caused a significant exponential rise in both foregut ($P$<0.05) and midgut ($P$<0.001) water content (Fig. 5A,B). Foregut water content rose from an initial relative volume of 2.67±0.18 to 3.80±0.28 µl $mg^{-1}$ following 120 h at 0°C, whereas midgut water content increased from 3.37±0.09 to 5.01±0.29 µl $mg^{-1}$ within the same time period. Cold exposure significantly increased hindgut volume from 3.23±0.12 to 3.98±0.25 µl $mg^{-1}$ within the first 6 h of exposure ($P$<0.01; Fig. 5C). Following this initial increase, hindgut water content remained high with increased cold exposure duration ($P$>0.05; Fig. 5C).

### Hemolymph and gut ion content

Total hemolymph $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ declined exponentially with increased cold-exposure duration ($P$<0.001; Fig. 6A,C,D). In contrast, total hemolymph $K^+$ did not change significantly with increasing time spent at 0°C ($P$>0.05; Fig. 6B). Changes in hemolymph $Na^+$ ($P$=0.068), $K^+$ ($P$=0.085), $Mg^{2+}$ ($P$=0.068) and $Ca^{2+}$ ($P$=0.085) over the first 12 h were all marginally non-significant, with $K^+$ tending to increase in the hemolymph whereas all other ions tended to decrease (Fig. 6). The total decrease in hemolymph ion content was approximately 87, 81 and 90% for $Na^+$, $Mg^{2+}$ and $Ca^{2+}$, respectively.

Cold exposure significantly increased foregut $Na^+$ ($P$<0.01), but this effect was not significant within the first 6 h ($P$>0.05; Fig. 7A). Exposure to 0°C caused a significant rise in midgut $Na^+$ during the first 6 h ($P$<0.01) that continued in an exponential manner with prolonged cold exposure ($P$<0.05, Fig. 7B). In the hindgut, $Na^+$ increased significantly from 0.23±0.02 to 0.45±0.03 µmol $mg^{-1}$ during the first 6 h of cold exposure ($P$=0.001); however, this increase was followed by an exponential decrease in hindgut $Na^+$ between 6 and 120 h at 0°C, during which $Na^+$ levels in the hindgut fell to 0.29±0.03 µmol $mg^{-1}$ ($P$<0.001; Fig. 7C).

Foregut $K^+$ did not significantly change during the first 6 h of cold exposure or with increased cold-exposure duration ($P$>0.05; Fig. 7D). In the midgut, $K^+$ significantly declined during the first 6 h and then increased during prolonged cold exposure in a linear manner ($P$<0.05; Fig. 7E). Hindgut $K^+$ did not significantly change during the first 6 h or over the entire duration of cold exposure ($P$>0.05; Fig. 7F).

Cold exposure did not significantly affect foregut or hindgut $Mg^{2+}$, either during the first 6 h or over the entire duration of cold exposure ($P$>0.05; Fig. 7G,I). Midgut $Mg^{2+}$ increased significantly in an exponential manner over the duration of cold exposure ($P$<0.001), although this increase was not significant within the first 6 h ($P$>0.05; Fig. 7H).

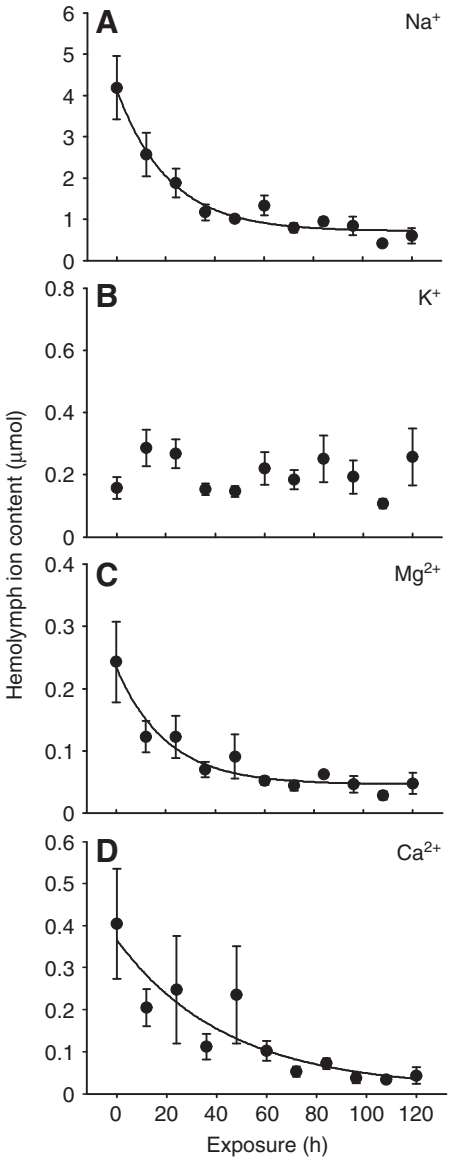


Fig. 6. Mean ± s.e.m. total (A) $Na^+$, (B) $K^+$, (C) $Mg^{2+}$ and (D) $Ca^{2+}$ content of the hemolymph of *G. pennsylvanicus* during exposure to 0°C for up to 120 h. $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ significantly declined during cold exposure, whereas there was no significant effect of cold exposure on total hemolymph $K^+$ (see text for statistics). *N*=6 crickets per sampling period. Error bars that are not visible are obstructed by the symbols.

In a similar manner to $Mg^{2+}$, $Ca^{2+}$ content did not significantly change in the foregut or hindgut during the first 6 h or over the entire duration of cold exposure ($P$>0.05; Fig. 7J,L). Midgut $Ca^{2+}$, however, increased significantly in an exponential manner over the duration of cold exposure ($P$<0.001), although this increase was not significant within the first 6 h ($P$>0.05; Fig. 7K).

### Hemolymph color

Of the 40 crickets exposed to 48 h or longer of cold exposure, six (15%) had black hemolymph at the time of dissection. Those individuals that had black hemolymph had significantly higher hemolymph volume (8.79±2.29 μl) than those that had pale yellow hemolymph (2.78±0.56 μl, $t_{36}$=3.90, $P$<0.001). Individuals with black hemolymph also had significantly lower midgut water content (13.76±1.82 μl) than individuals with yellow (normal) hemolymph (33.70±1.88 μl, $t_{36}$=5.12, $P$<0.001).

## DISCUSSION

Exposure of *G. pennsylvanicus* to 0°C caused a redistribution of water between the hemolymph and the gut. The volume of extractable hemolymph declined by approximately 84% of its initial volume in crickets that were injured and killed by exposure to 0°C. This loss of water from the hemolymph occurred in an exponential manner that strongly correlated with an increase in water in the gut. A mean of 34 μl was lost from the hemolymph, whereas approximately 24 μl of water was gained in the gut during cold exposure. The remaining *ca.* 10 μl of water from the hemolymph was likely lost from the body through desiccation during cold exposure, which would account for the majority of the 16 mg decrease in body mass over 120 h at 0°C that we observed.

### Osmotic balance of the hemolymph and muscle

In the hemolymph, $[Na^+]$ and $[Ca^{2+}]$ both declined whereas $[K^+]$ increased and $[Mg^{2+}]$ was unchanged with time spent at 0°C. Within the first 12 h of cold exposure, hemolymph $[Na^+]$ declined by 62 mmol $l^{-1}$. However, this rapid drop in hemolymph $[Na^+]$ had little effect on $E_{Na}$ of the muscle, as muscle $[Na^+]$ declined by a similar proportion at the same time. Surprisingly, despite all of the interactions occurring between the hemolymph and gut during cold exposure, muscle water and ion content otherwise appeared to be unchanged during cold exposure, as no other effects of cold exposure were found on the muscle tissue. This effect of cold exposure on muscle $[Na^+]$ may be a protective response of temperate insects, and may act to prevent muscle $E_{Na}$ from declining while large amounts of $Na^+$ were being lost from the hemolymph. Overall, cold exposure did not significantly affect muscle $E_{Na}$ in *G. pennsylvanicus*; this is in contrast to tropical cockroaches, in which $E_{Na}$ also declines with duration of cold exposure (Kostál et al., 2006).

Hemolymph $Na^+$, $Mg^{2+}$ and $Ca^{2+}$ all decreased in an exponential manner with increased cold exposure duration. Ultimately, the magnitude of loss of $Na^+$ and $Ca^{2+}$ from the hemolymph was larger than the loss of volume, such that hemolymph concentrations of both ions decreased. Although some changes in hemolymph ion concentration reflect the correct direction of ion movement into or out of the hemolymph, alterations in hemolymph volume can strongly affect interpretations of ion drift. For example, although $Mg^{2+}$ content decreased overall, the concomitant loss of volume resulted in hemolymph $[Mg^{2+}]$ remaining stable during cold exposure. Similarly, although $[K^+]$ increased with time spent at 0°C, no significant change in total hemolymph $K^+$ occurred. Thus, the increase in hemolymph $[K^+]$ and absolute decrease in $E_K$ at the muscle were driven by the decrease in hemolymph volume, and not by an increase in the amount of extracellular $K^+$.

Cooling had the largest effect on muscle $E_K$ and $E_{Ca}$, which declined by 43 and 12 mV, respectively. The absolute decline observed in $E_K$ would be sufficient to dissipate muscle membrane potential, as membrane potential closely follows $E_K$ in non-lepidopteran insects (Hoyle, 1953; Wood, 1957; Leech, 1986). In addition, the re-polarizing current of action potentials at the insect muscle membrane is generated by an outward potassium current, but unlike vertebrates, the rising phase is generated not by the movement of $Na^+$ but by an inward $Ca^{2+}$ current that is highly dependent on hemolymph $[Ca^{2+}]$ (Hoyle, 1953; Patlak, 1976; Ashcroft, 1981; Collet and Belzunces, 2007). Thus the rise in $[K^+]$ and fall in $[Ca^{2+}]$ in the hemolymph can together explain the drop in resting potential and loss of excitability of insect muscle tissue

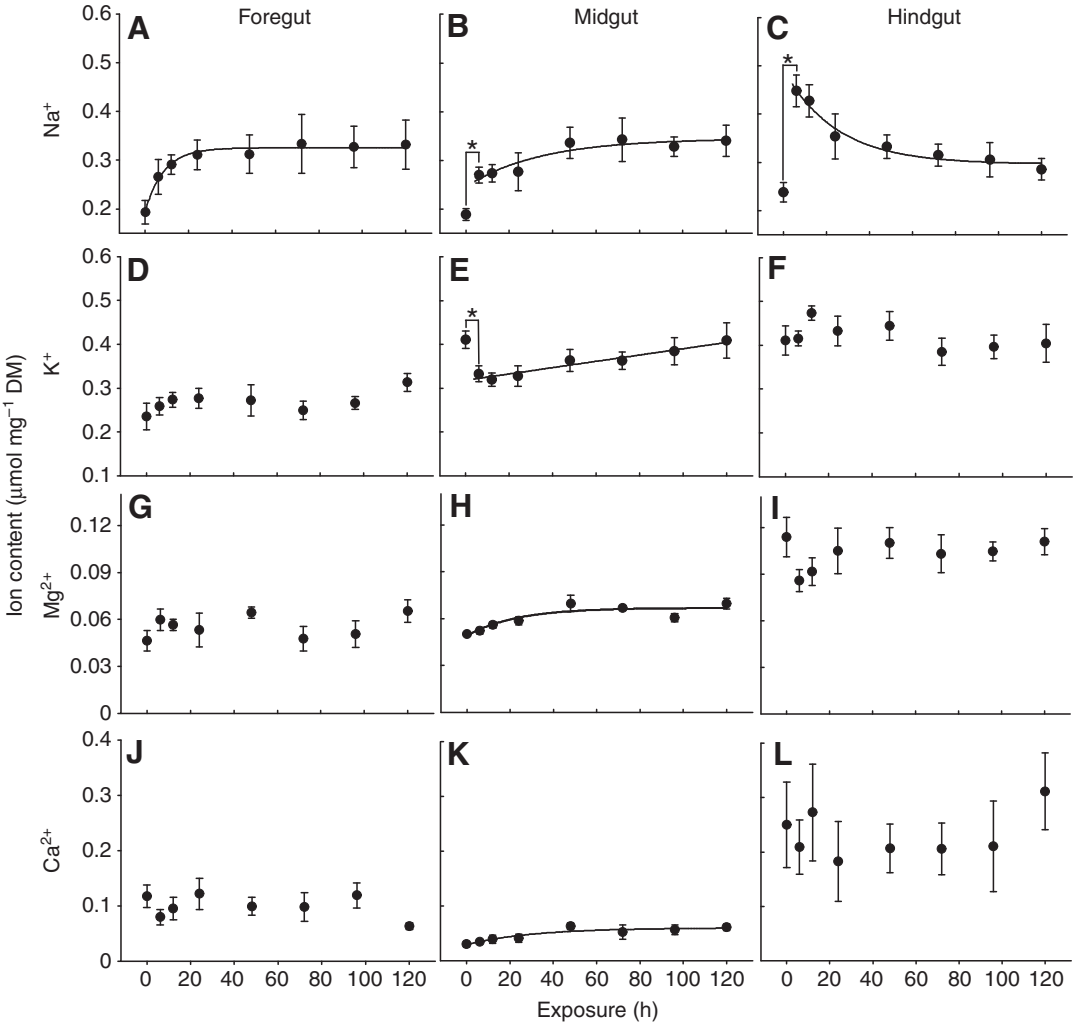


Fig. 7. Mean ± s.e.m. (A–C) $Na^+$, (D–F) $K^+$, (G–I) $Mg^{2+}$ and (J–L) $Ca^{2+}$ of the foregut (left) midgut (centre) and hindgut (right) of *G. pennsylvanicus* during exposure to 0°C for 120 h. Significant changes in ion content (expressed as μmol $mg^{-1}$ DM) within the first 6 h of cold exposure are denoted with an asterisk. Lines of best fit indicate significant effects of cold-exposure duration on ion content (see text for statistics). Error bars that are not visible are obstructed by the symbols.

that likely plays a role in both chill-coma onset and the progression of chilling injury (Esch, 1988; Hosler et al., 2000; Kostál et al., 2006).

In the insect nervous system, the brain, ventral ganglia and large isolated nerves are all protected from direct exchange of solutes with the hemolymph by the blood–brain barrier, which maintains ion concentrations in the neuronal microenvironment that are very different from those of the hemolymph (Treherne and Schofield, 1979). Because of the blood–brain barrier, whole tissue estimates of ion abundance in the nervous system, such as we present for other tissues, are not sufficient to measure osmotic phenomena of the nervous system, and direct measurements with microelectrodes are required. The nervous system may be important in chill coma and chilling injury; for example, in semi-intact preparations of locusts (*Locusta migratoria*; Orthoptera: Acrididae), gradual chilling is associated with a rise in extracellular $[K^+]$ in the nervous system that sometimes coincides with physical arrest (Rodgers et al., 2010). The initial disruption of neuronal and gut osmotic balance during chilling may occur by similar effects of temperature on enzymes responsible for the maintenance of ion balance. In addition, ion balance within the nervous system is ultimately achieved through interaction with the hemolymph, thus both rapid and long-term changes in hemolymph $[Na^+]$, $[K^+]$ and $[Ca^{2+}]$ during cold exposure noted here may potentiate local effects within the nervous system.

### Osmotic balance of the alimentary canal

Both water and cations primarily moved from the hemolymph into the gut during cold exposure. The rate of accumulation of water in each of the gut segments closely followed the accumulation of $Na^+$ in the same segment, although the different patterns in the gut sections suggest that little mixing of gut contents between sections occurs during cold exposure. Phytophagous insects, including locusts and mantids (and, therefore, probably crickets), tend to have lower levels of $Na^+$ in the crop than in the hemolymph, owing to the low $Na^+$ content of plants (Dow, 1981; Hatle et al., 2002). In the foregut, no significant changes in the amount of $K^+$, $Mg^{2+}$ or $Ca^{2+}$ were found. This is consistent with the role of the foregut as a solute-impermeable storage area. Foregut $Na^+$, however, was low under control conditions and increased exponentially, reaching a plateau following roughly 24 h of cold exposure, implying that $Na^+$ migrated into the foregut during chilling and that foregut $Na^+$ is actively regulated under control conditions.

$Na^+$ was the only ion in the hindgut that changed in abundance with cooling, increasing rapidly during the first 6 h followed by a relatively slower exponential decline. The hindgut contains feces with a low water content, which is driven by local cycling of ions ($Na^+$, $K^+$, and $Cl^-$) that drive absorption of water from the hindgut against the prevailing osmotic gradient (Phillips, 1964; Goh and Phillips, 1978; Phillips et al., 1982). Thus, the rectal lumen possesses both high water permeability and a large osmotic gradient that together explain the rapid movement of both water and $Na^+$ into the hindgut lumen during cold exposure. Cold-tolerant adult firebugs (*Pyrrhocoris apterus*, Hemiptera: Pyrrhocoridae) exposed to –5°C for up to 60 days caused an accumulation of not only $Na^+$ and water but also $K^+$ in the hindgut (Kostál et al., 2004). Thus, accumulation of ions and water in the hindgut also occurs in cold-hardy insects; however, by allowing $K^+$ to accumulate in the gut system along with $Na^+$, cold-hardy insects may protect hemolymph [$K^+$] and avoid the effects of $K^+$ accumulation on survival at relatively mild low temperatures.

The midgut was most sensitive to low-temperature exposure, as the concentration of all four ions significantly changed with time spent at 0°C. $Ca^{2+}$, $Mg^{2+}$ and $Na^+$ levels all increased exponentially in the midgut with exposure to 0°C. The influx of both $Na^+$ and $Ca^{2+}$ into the midgut appears to coincide with their loss from the hemolymph. However, although a total of 0.37±0.11 μmol of $Ca^{2+}$ was lost from the hemolymph after 120 h at 0°C, only 0.17±0.10 μmol of this is accounted for by influx into the midgut, implying that some $Ca^{2+}$ may have migrated elsewhere in the body. Although $Mg^{2+}$ moved into the midgut during cold exposure, its concentration in the hemolymph was unaffected, suggesting that $Mg^{2+}$ was driven into the midgut during cold exposure by the potential produced by the reduction of hemolymph volume, and likely remained in equilibrium between the hemolymph and midgut. Midgut $K^+$ content significantly decreased during the first 6 h and then increased linearly for the remainder of the cold exposure. In larval Lepidoptera, a V-ATPase and $K^+$-$2H^+$-antiport combination is utilized to actively transport $K^+$ from the hemolymph to the midgut (Chamberlin, 1990; Wieczorek et al., 1991; Klein et al., 1996). Although, to our knowledge, such a mechanism has not been described in Orthoptera, if $K^+$ is actively transported out of the midgut of *G. pennsylvanicus*, it could explain the initial drop in $K^+$ in the midgut during exposure to 0°C. As hemolymph volume decreased with continued cold exposure, however, hemolymph [$K^+$] likely surpassed the midgut [$K^+$], driving an efflux of $K^+$ from the hemolymph to the midgut. The combined increases in midgut $Na^+$, $Mg^{2+}$ and $Ca^{2+}$, along with the drift of $K^+$ toward initial levels, likely increased the osmotic pressure in the midgut during cold exposure, subsequently driving the influx of water. The midgut is likely to be the most sensitive region of the gut to physical damage from water accumulation; whereas the foregut and hindgut are derived from ectoderm and thus have a cuticular lining to provide structural support, the midgut does not (Dow, 1986).

After 48 h or more at 0°C, several crickets had dark brown or black hemolymph. Darkening of the hemolymph is caused by activity of phenoloxidase, which causes a melanization of the hemolymph that is important in both wound healing and the encapsulation of invading organisms in the hemocoel (Gillespie et al., 1997; Hoffmann et al., 1999). The inactive form of phenoloxidase (prophenoloxidase), which becomes activated during pathogen invasion, has been isolated and characterized in a number of invertebrate species, including an orthopteran (Cherqui et al., 1996), and can be activated at temperatures as low as 0°C (Evans, 1967). In our study, individuals that had dark hemolymph also had significantly reduced midgut volume and significantly increased hemolymph volume compared with crickets with yellow hemolymph. The hemolymph was also thicker and more difficult to extract from the hemocoel in crickets with black hemolymph. Thus, we postulate that increasing volume of the midgut during cold exposure ruptured the midgut in some crickets, spilling its contents into the hemocoel. Melanization in response to foreign material from the gut would then darken the hemolymph. Such physical damage to the gut is probably not the primary cause of chilling injury, as only six out of 40 crickets had darkened hemolymph, despite 100% mortality from 120 h at 0°C.

The observed patterns of water and ion accumulation in the gut imply that gut–hemolymph interactions may play a major role in indirect chilling injury of chill-susceptible insects. In contrast to chill-susceptible species, highly cold-tolerant insects that survive supercooling of their body fluids are known to cease feeding and void or eliminate their gut in preparation for winter (Lee et al., 1996). This strategy acts to remove factors that promote ice formation at low temperatures. However, we suggest that highly cold-tolerant insects may also avoid detrimental alterations in hemolymph ion and water content through removal of the gut or its contents.

## Conclusions

Exposure to 0°C caused migration of the majority of hemolymph $Na^+$, $Mg^{2+}$, $Ca^{2+}$ and water into the alimentary canal of a chill-susceptible insect. These patterns of ion and water movement caused hemolymph [$Na^+$] and [$Ca^{2+}$] to decrease and hemolymph [$K^+$] to increase, driving absolute decreases in $E_K$ and $E_{Ca}$ at the muscle tissue. The observed changes in hemolymph ions and water content are likely to affect tissue viability following removal from the cold and be closely linked with symptoms of indirect chilling injury. Future directions for understanding the mechanisms of chilling injury may include investigating the biochemical mechanisms regulating ion and water movement between compartments during cold exposure and the means by which insects modulate their cold tolerance through changes in gut permeability to ions and water.

## LIST OF SYMBOLS AND ABBREVIATIONS

| | |
|---|---|
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| $CT_{min}$ | critical thermal minimum |
| $E_{Ca}$ | equilibrium potential of sodium |
| $E_K$ | equilibrium potential of potassium |
| $E_{Mg}$ | equilibrium potential of magnesium |
| $E_{Na}$ | equilibrium potential of sodium |
| $Q_{10}$ | temperature coefficient |
| [X] | concentration of X |

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