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Ion and water balance in Gryllus crickets during the first twelve hours of cold exposure.

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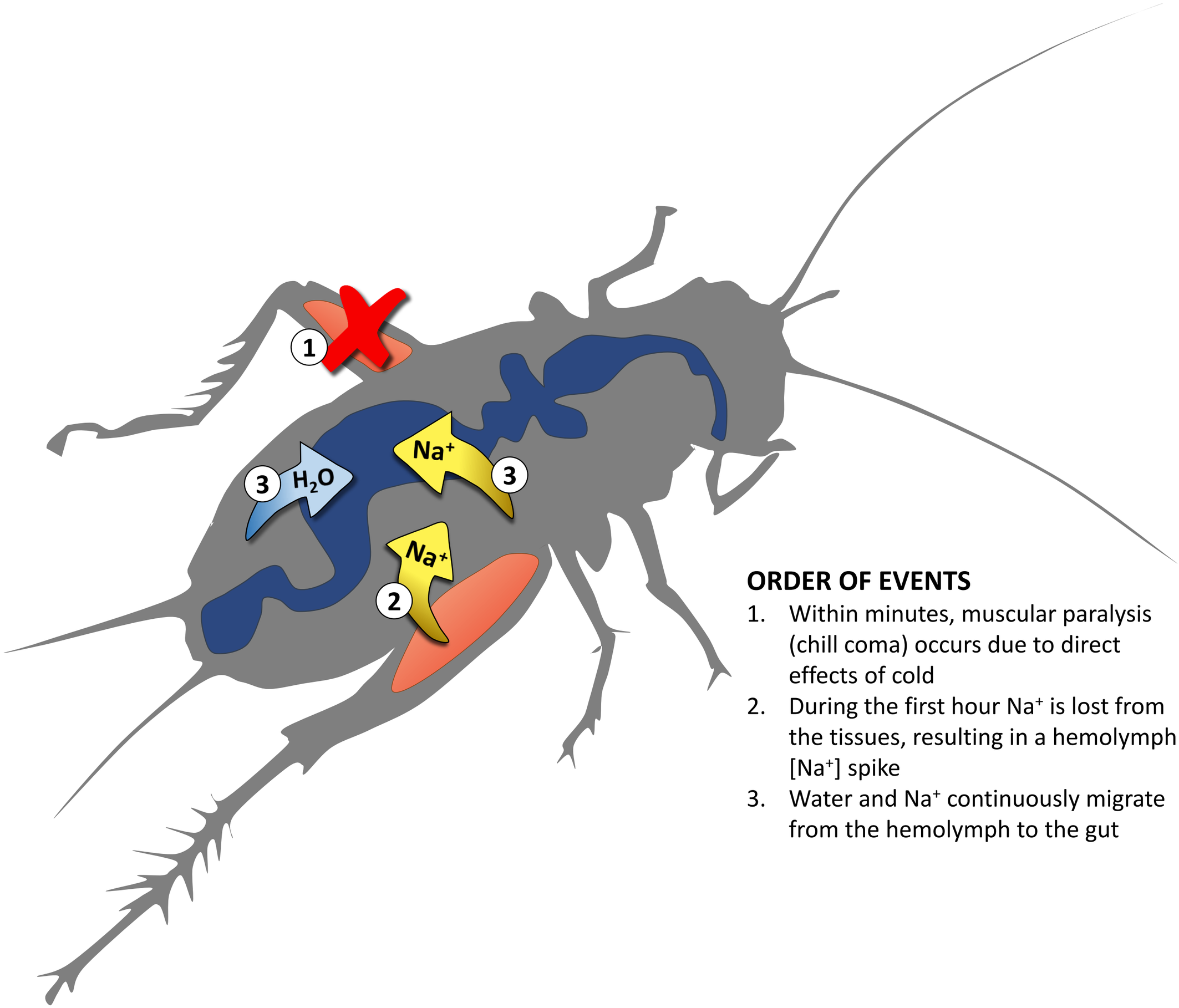
Graphical Abstract (for review)



ORDER OF EVENTS

1. Within minutes, muscular paralysis (chill coma) occurs due to direct effects of cold
2. During the first hour Na^+ is lost from the tissues, resulting in a hemolymph $[Na^+]$ spike
3. Water and Na^+ continuously migrate from the hemolymph to the gut

HIGHLIGHTS

- Insects lose water and ion balance rapidly during chilling
- Patterns of hemolymph [Na^+] in early coma differ from those in late chill coma
- A rise in hemolymph Na^+ in the first hour of chilling may result from tissue leak
- Hemolymph [K^+] increased during chilling but did not account for paralysis
- Chill-tolerant crickets did not defend homeostasis better during 12 h of chilling

Ion and water balance in *Gryllus* crickets during the first twelve hours of cold exposure

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ABSTRACT

Insects lose ion and water balance during chilling, but the mechanisms underlying this phenomenon are based on patterns of ion and water balance observed in later stages of cold exposure (12 or more hours). Here we quantified the distribution of ions and water in the hemolymph, muscle, and gut in adult *Gryllus* field crickets during the first 12 h of cold exposure to test mechanistic hypotheses about why homeostasis is lost in the cold, and how chill-tolerant insects might maintain homeostasis to lower temperatures. Unlike in later chill coma, hemolymph [Na^+] and Na^+ content in the first few hours of chilling actually increased. Patterns of Na^+ balance suggest that Na^+ migrates from the tissues to the gut lumen via the hemolymph. Imbalance of [K^+] progressed gradually over 12 h and could not explain chill coma onset (a finding consistent with recent studies), nor did it predict survival or injury following 48 h of chilling. *Gryllus veletis* avoided shifts in muscle and hemolymph ion content better than *G. pennsylvanicus* (which is less chill-tolerant), however neither species defended water, [Na^+], or [K^+] balance during the first 12 h of chilling. *Gryllus veletis* better maintained balance of Na^+ content and may therefore have greater tissue resistance to ion leak during cold exposure (which could partially explain faster chill coma recovery for that species).

Key Words: insect, chill tolerance, homeostasis, *Gryllus*, ion balance

1. INTRODUCTION

Because insects are ectotherms, many of their physiological processes are directly influenced by ambient temperature. The mechanisms that underlie thermal physiology will therefore determine how climate change impacts insect performance and, consequently, ecosystem function (Sinclair et al., 2003; Chown and Terblanche, 2006; Somero, 2010; Williams et al., 2015). Insect performance is bounded at low temperatures by the critical thermal minimum (CT_{min}), below which insects enter a reversible paralysis called chill coma. Insects lose ion and water homeostasis when in chill coma and regain homeostasis during recovery (Koštál et al., 2004; MacMillan et al., 2012). The ability to survive and maintain homeostasis in the cold is variable and plastic; cold-acclimated or -adapted insect populations sustain water and ion balance at lower temperatures than their warm-acclimated or -adapted counterparts (Gibert and Huey, 2001; Ayrinhac et al., 2004; Koštál et al., 2004; Koštál et al., 2006; Andersen et al., 2014; Coello Alvarado et al., 2015; MacMillan et al., 2015a).

In several insects (including crickets, locusts, and cockroaches), hemolymph Na^+ and water migrate out of the hemolymph during chilling, while hemolymph [K^+] increases (Koštál et al., 2006; MacMillan and Sinclair, 2011; Andersen et al., 2013; Findsen et al., 2014; Coello Alvarado et al., 2015). The migration of Na^+ is likely a result of active ion transport failure and, as water balance is often tightly linked to Na^+ gradients, so too is hemolymph water lost. The decreased hemolymph volume is thought to increase hemolymph [K^+] (MacMillan and Sinclair, 2011). In *Gryllus pennsylvanicus* Burmeister, the largest decrease in hemolymph [Na^+] and increase in hemolymph [K^+] occurs within the first 12 h of cold exposure (MacMillan and Sinclair, 2011). Chill coma onset occurs rapidly (within minutes of cold exposure) and appears to be mechanistically unrelated to processes underlying loss of water and ion homeostasis (Findsen et al., 2014; MacMillan et al., 2014b; Andersen et al., 2015). In particular, previous authors have not observed a loss of homeostasis associated with chill coma paralysis within the first few minutes of cold exposure (Findsen et al., 2014; MacMillan et al., 2014b; Andersen et al., 2015). However, loss of homeostasis during chilling is readily apparent at longer timescales (hours to days) in the context of studies of chill coma recovery time (CCRT) and chilling injury (e.g. Koštál et al., 2006; MacMillan and Sinclair, 2011; Findsen et al., 2013). Thus we do not know

how quickly Na^+ or K^+ balance is lost during cold exposure, or whether the patterns of homeostasis in the initial cold exposure reflect those observed at longer timescales. Similarly, little is known about how ion and water imbalance during chilling relates to or predicts survival and chilling injury (MacMillan et al., 2014b).

Insects vary in their ability to maintain ion and water balance in the cold (Koštál et al., 2004; Koštál et al., 2007; MacMillan et al., 2014a; Coello Alvarado et al., 2015; MacMillan et al., 2015a). Our understanding about the mechanisms underlying this variation is incomplete (Gibert and Huey, 2001; Ransberry et al., 2011), but recent studies have revealed a potential role for Na^+ balance. Cold-acclimated *Drosophila melanogaster* Meigen maintain low hemolymph [Na^+] (and consequently low [K^+]) in both warm and cold conditions, and may also exhibit lower Na^+-transport capacity (MacMillan et al., 2014a; MacMillan et al., 2015a). *Gryllus veletis* (Alexander and Bigelow) nymphs maintain Na^+ balance at 0°C, while *G. pennsylvanicus* adults (which are less chill tolerant) lose Na^+ balance at 0°C unless they have undergone prior cold acclimation (Coello Alvarado et al., 2015).

Understanding why insects lose water and ion homeostasis during chilling requires an understanding of the short-term movements of water and ions during cold exposure. Here we explore the patterns of water and ion balance during the first 12 h of cold exposure with the aim of testing and generating mechanistic hypotheses for why homeostasis is lost in the cold, and why chill-tolerant insects are better at maintaining homeostasis at low temperatures. We used two species of field cricket: *Gryllus pennsylvanicus* (which was used to develop the initial model of loss of ion and water homeostasis in the cold), and *G. veletis*, the nymphs of which are more chill-tolerant and maintain ion and water balance at lower temperatures (Coello Alvarado et al., 2015).

2. MATERIALS AND METHODS

Gryllus pennsylvanicus and *G. veletis* colonies originated from individuals collected from the University of Toronto at Mississauga campus, Ontario (2004) and the University of

Lethbridge, Alberta (2010), respectively. We reared crickets under constant summer-like conditions (25°C, 14 L:10 D photoperiod, 70% RH) at the University of Western Ontario Biotron Research Center, as described previously (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). Crickets were housed in transparent plastic containers with stacked cardboard egg cartons for shelter and provided with tap water and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills, Elmira, ON, Canada). We collected eggs in containers of moist vermiculite and sterile sand; *Gryllus veletis* eggs hatched after two weeks, and we placed *G. pennsylvanicus* eggs at 4°C to accommodate an obligate three month diapause (Rakshpal, 1962) before returning them to 25°C to hatch. For all experiments we used adult virgin female *G. pennsylvanicus* and *G. veletis* (approximately 1 and 5 weeks post final molt, respectively). The difference in age reflected a longer development time for *G. veletis*. For one week prior to experiments, crickets were held individually in 177 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh fabric lids and containing egg carton shelters, rabbit food, and water. This isolation prevented cannibalism and any associated changes in gut contents.

2.1 Measurements of chill tolerance

We assessed low temperature performance of *G. pennsylvanicus* and *G. veletis* adult females by measuring the CT_{min}, CCRT, and survival following cold exposure. Measurement of the CT_{min} (N = 20 per species) was as described by (MacMillan and Sinclair, 2011). Briefly, we cooled crickets from room temperature at 0.25°C min^{-1} until the CT_{min} was reached. We defined the CT_{min} as the temperature at which physical stimulus with a metal probe elicited no muscular response. We defined CCRT as the time required for the righting response (a coordinated movement) after 48 h of cold exposure. To measure CCRT and survival of cold exposure, we placed crickets (N = 24 per species) in 15 mL Falcon tubes immersed in an ice-water slurry at 0°C (a temperature that induced chill coma in both *G. pennsylvanicus* and *G. veletis* in preliminary experiments). This time period should not induce substantial mortality; *G. veletis* survive at least five days at 0°C, while *G. pennsylvanicus* suffer approximately 20% mortality after 108 h at 0°C (Coello Alvarado et al., 2015). After 48 h, we moved the crickets to room temperature, placed them on their dorsum in a 6-well plate, and video recorded their recovery for up to 9 h (Hazell et al., 2008). We extracted twitch and righting response times from the video.

Crickets that did not exhibit signs of recovery within 9 h were not included in CCRT analyses. All crickets were then returned to 25°C in individual cups and provided with food, water, and shelter. After 24 h at 25°C, we assessed survival and injury (the latter defined as uncoordinated locomotion or inability to jump when stimulated with a probe) (MacMillan and Sinclair, 2011).

2.2 Cold exposure and dissection

We held crickets at 25°C (control, 0 h) or exposed them to 0°C for a duration of 0.5, 1, 3, 6, or 12 h (N = 14-19 individuals per species per treatment). Size-matching of crickets ensured that mean wet mass did not differ among treatments within each species ($F_{5,83}$ = 0.30, P > 0.9 and $F_{5,89}$ = 0.32, P > 0.9 for *G. pennsylvanicus* and *G. veletis*, respectively). We placed cold-exposed crickets individually into loosely-capped 50 mL plastic tubes suspended in a bath of 50% methanol in water, pre-cooled to 0°C (Lauda Proline RP 3530, Würzburg, Germany). We added a thermocouple in contact with one of the crickets to monitor its body temperature during cold exposure.

Immediately after removal from 0°C we dissected crickets on a Petri dish surrounded by ice within a large Styrofoam box. We punctured the pronotum with an insect pin and collected hemolymph (5-30 μl) with a micropipette, then opened the body cavity by a mid-dorsal incision and collected as much hemolymph from the body as possible by applying gentle pressure to the abdomen. We approximated hemolymph volume gravimetrically by weighing extracted hemolymph and assuming a density equal to water. This method of hemolymph extraction and approximation correlates linearly with inulin dilution estimates for hemolymph volume in *G. pennsylvanicus* (MacMillan et al., 2012). We pinned open the body cavity and removed the gut (from anterior foregut to rectum) into a pre-weighed microcentrifuge tube. We then severed the hind legs and used forceps to extract femur muscles into pre-weighed 0.2 mL microcentrifuge tubes.

To identify potential reservoirs of Na^+ (as we observed increased hemolymph Na^+ content during chilling), we measured Na^+ in the fat body, head, Malpighian tubules, and ovaries from an additional six control *G. pennsylvanicus* females. We calculated tissue water contents calculated

from the difference between the tissue fresh (wet) mass and mass after drying at 70°C for 24 h (muscle, Malpighian tubules, and fat body) or 48 h (gut, head, and ovaries).

2.3 Ion quantification

We assessed ion homeostasis over 12 h of cold exposure by quantifying the concentration and content of Na^+ and K^+ in the hemolymph and tissues. Ion contents indicate bulk movement of Na^+ or K^+ between body compartments (which in turn affects bulk movement of water), while ion concentrations are important for neuromuscular function and as directional predictors of ion leak. We quantified ions as described by MacMillan and Sinclair (2011). Briefly, we digested hemolymph and dried tissues in nitric acid (70%) at room temperature for 24 h (hemolymph, muscle, fat body, and Malpighian tubules), 48 h (gut), or 72 h (head, ovaries). We quantified $[Na^+]$ and $[K^+]$ in the dissolved, diluted hemolymph and tissue samples using an atomic absorption spectrometer (iCE 3300, Thermo Scientific, Waltham, MA, USA). From the measured absorbance, we calculated sample ion concentrations by comparison with standard curves generated from Na^+ and K^+ reference solutions. The water contents of each tissue (assumed to be intracellular water) or hemolymph (assumed to represent extracellular water) allowed us to calculate the ion concentration in the tissue or hemolymph. To determine sample ion content, we corrected ion concentrations for the volume or mass of hemolymph or tissue in the sample.

2.4 Data analysis

We expected that *G. veletis* would exhibit a lower CT_{min} and CCRT, and greater survival following cold exposure than *G. pennsylvanicus* (Coello Alvarado et al., 2015), therefore we made interspecies comparisons of the CT_{min}, CCRT, and survival following cold exposure using one-sided Welch's t-tests. We compared initial and endpoint ion and water measurements as well as trajectories of ion and water balance during cold exposure among species, but we did not make point-by-point comparisons. To compare control ion or water measurements among species, we used two-sided Student's t-tests (if variance was equal) or Welch's t-tests (if variance was unequal). We quantified the relationship between cold exposure time and water or ion balance using generalized least squares models and linear regression. We compared discrete cold exposure time points via one-way ANOVA and Tukey's HSD. We log-transformed cold

exposure times prior to analysis in cases when this transformation improved normality, and used exponential weighting for generalized nonlinear least squares models if variance was unequal across cold exposure times (Gałecki and Burzykowski, 2013). Tissue water and ion contents were positively correlated with tissue dry mass ($P < 0.05$, see Table S1) with the exception of muscle water ($P > 0.1$), therefore we corrected ion contents for tissue dry mass before quantifying the effect of cold exposure on water or ion content (*i.e.* cold exposure effects were modeled with the residuals of water or ion content regressed against tissue dry mass) (MacMillan and Sinclair, 2011). Similarly, because hemolymph volume was positively related to cricket wet mass ($F_{1,85} = 61.89$, $P < 0.001$ and $F_{1,93} = 31.05$, $P < 0.001$ for *G. pennsylvanicus* and *G. veletis*, respectively), we corrected hemolymph volume for cricket wet mass prior to quantifying the effect of cold exposure on hemolymph volume.

We calculated muscle Na^+ and K^+ equilibrium potentials at 23°C (control crickets) and at 0°C (cold-exposed crickets) as described by MacMillan and Sinclair (2011) using the Nernst equation (Nernst, 1888):

$$E = \left(\frac{RT}{zF}\right) \ln\left(\frac{[o]}{[i]}\right) \qquad (1),$$

where R is the universal gas constant, T is the absolute temperature, z is the ionic charge (*e.g.* z for Na^+ or K^+ = 1), F is Faraday's constant, $[o]$ is the ion concentration outside of the muscle (*i.e.* the hemolymph), and $[i]$ is the ion concentration inside the muscle, i.e. our estimate from the tissue.

Descriptive values reported in the text, tables, and figures are given as mean ± s.e.m. Detailed statistics for regression models are included in supplementary material (Table S2). All statistical analyses were performed in R (v3.1.2, R Development Core Team, 2014).

3. RESULTS

Gryllus veletis was more chill tolerant than *G. pennsylvanicus.* The CT_{min} of *G. veletis* (0.7 ± 0.2°C) was lower than that of *G. pennsylvanicus* (2.2 ± 0.13°C) ($t_{36.2} = 7.38$, $P < 0.001$). Following exposure to 0°C for 48 h, *G. veletis* recovered 20-times faster than *G. pennsylvanicus* on average ($t_{8.02} = 4.75$, $P < 0.001$). Sixteen of the 25 *G. pennsylvanicus* never regained righting ability within 9 hours of measuring CCRT (Fig. 1A), and of those half never recovered. Twenty-

four hours after this cold exposure, 84% of *Gryllus pennsylvanicus* crickets were dead or injured, while only 20% of *G. veletis* crickets were injured and none were dead (Fig. 1B).

3.1 Water balance

Under control conditions, hemolymph volume relative to gut water content was lower in *G. veletis* than in *G. pennsylvanicus* (t_{31} = 2.49, P = 0.019). The gut of *G. veletis* accounted for a slightly greater proportion of body fresh mass (11.5 ± 0.9%) compared to *G. pennsylvanicus* (8.2 ± 0.5%) (t_{32} = 3.10, P = 0.004). The volume of hemolymph relative to cricket fresh mass did not differ between species (t_{32} = 1.59, P > 0.1).

Gut water content increased over 12 h of cold exposure for both *G. pennsylvanicus* and *G. veletis* (P = 0.032 and P = 0.004, respectively) (supplementary material, Fig. S1A). Hemolymph volume decreased by 25% in *G. veletis* during 12 h of cold exposure (P = 0.001), whereas the hemolymph of *G. pennsylvanicus* first increased in volume before decreasing slightly, and this decrease was non-significant overall (P = 0.091); supplementary material, Fig. S1B. The water contents of the hemolymph relative to the gut decreased linearly by 23% for *G. pennsylvanicus* and 38% for *G. veletis* (P = 0.009 and P = 0.023, respectively) (Fig. 2A). Muscle water content was unchanged over 12 h of cold exposure for *G. pennsylvanicus* and *G. veletis* (P > 0.3 and P > 0.2).

3.2 Ion balance

The Na^+ gradient between the hemolymph and the gut did not differ between species under control conditions (t_{33} = 0.927, P = 0.361), however both species exhibited linear decreases in the hemolymph-to-gut [Na^+] ratio during 12 h of cold exposure (P < 0.001 and P = 0.002 for *G. pennsylvanicus* and *G. veletis*, respectively) (Fig. 2B). Gut Na^+ content increased by approximately 21% during cold exposure for *G. veletis*, while *G. pennsylvanicus* showed only a similar trend with an approximate increase of 29% (P = 0.032 and P = 0.073, respectively) (Fig. 3). Gut K^+ content did not change over cold exposure time in *G. pennsylvanicus* or *G. veletis* (P > 0.8) despite a decrease in gut [K^+] (P = 0.036 and P = 0.005, respectively).

In the hemolymph of *G. pennsylvanicus*, $[Na^+]$ initially increased (from 110 mM to 130 mM within 0.5 h of cold exposure) before returning to control values by 6 h ($F_{5, 78} = 4.34$, $P < 0.002$) (Fig. 4A). A rise and fall of hemolymph $[Na^+]$ also occurred in cold-exposed *G. veletis* but with a much smaller overall change (from 106 mM to 119 mM) ($F_{5,88} = 2.35$, $P = 0.048$), such that differences among time points were not identified using Tukey's HSD. General patterns of hemolymph $[Na^+]$ during cold exposure in *G. pennsylvanicus* were mirrored by the hemolymph Na^+ content ($F_{5,77} = 2.42$, $P = 0.043$), however a similar trend observed for Na^+ content in the hemolymph of *G. veletis* was non-significant ($F_{5,88} = 2.25$, $P = 0.056$) (Fig. 4C).

We observed an influx of Na^+ to the hemolymph in the first hour of exposure to 0°C, so we quantified $[Na^+]$ and Na^+ content in the ovaries, fat body, head, and Malpighian tubules of *G. pennsylvanicus* under control conditions to identify potential reservoirs of Na^+. The $[Na^+]$ in both the fat body and ovaries exceeded that of the hemolymph, while $[Na^+]$ in the head and Malpighian tubules were lower than the hemolymph (Table 1). The ovaries—which accounted for 32 ± 1.7 % of the adult female body mass—held the largest reservoir of total Na^+. For both species, cold exposure caused linear increases in both hemolymph $[K^+]$ ($P < 0.001$) and K^+ content ($P = 0.037$ and $P < 0.001$ for *G. veletis* and *G. pennsylvanicus*, respectively) (Fig. 5A,C).

Gryllus pennsylvanicus had higher muscle $[K^+]$ compared to *G. veletis* under control conditions ($t_{23.3} = 2.36$, $P = 0.027$). We observed a slight increase in muscle $[K^+]$ for *G. veletis* ($P = 0.049$) over 12 h, however cold exposure had no effect on muscle $[K^+]$ in *G. pennsylvanicus* ($P > 0.4$). Muscle K^+ content was not affected by cold exposure in *G. pennsylvanicus* ($P > 0.3$) or *G. veletis* ($P = 0.080$) (Fig. 5B,D). Muscle $[Na^+]$ in *G. pennsylvanicus* was lower than in *G. veletis* under control conditions ($t_{30.5} = 2.04$, $P = 0.025$). During 12 h of cold exposure, muscle $[Na^+]$ decreased for both *G. pennsylvanicus* and *G. veletis* ($P < 0.001$) and this decrease reflected a loss of muscle Na^+ content ($P < 0.002$ and $P = 0.007$, respectively) (Fig. 4B,D). *Gryllus veletis* appeared to lose muscle Na^+ more slowly than *G. pennsylvanicus*.

Control *G. pennsylvanicus* exhibited higher muscle Na^+ equilibrium potential (by c. 5.5 mV; $t_{33} = 1.92$, $P = 0.032$) and lower muscle K^+ equilibrium potential (by c. 11.5 mV; $t_{23} = 2.38$, $P =$

0.013) compared to *G. veletis* (Fig. 6). We did not observe significant changes in muscle Na^+ potential during 12 h of cold exposure for *G. pennsylvanicus* or *G. veletis* ($F_{5,80}$ = 1.20, P > 0.3 and $F_{5,85}$ = 0.79, P > 0.5, respectively). Muscle K^+ equilibrium potential depolarized from -75.4 mV (*G. pennsylvanicus*) and -63.9 mV (*G. veletis*) to approximately -40 mV in both species after 12 h at 0 °C.

4. DISCUSSION

The mechanisms underlying loss of ion and water balance at low temperatures and the means by which chill-tolerant insects avoid this loss are not fully understood. By observing the ion and water balance in crickets during the first 12 h of cold exposure we have shown that shifts in hemolymph Na^+ balance observed at later stages (days) of cold exposure do not reflect changes in these early stages. We also found that loss of Na^+ balance during chill coma may be driven by a loss of Na^+ from the tissues. While neither species could defend water, $[Na^+]$, or $[K^+]$ balance during cold exposure, shifts in ion contents across the hemolymph and muscle were slower and/or less extensive in the more chill-tolerant cricket (*G. veletis*) compared to the less chill-tolerant cricket (*G. pennsylvanicus*). Our findings support the hypothesis that chill tolerance (as assessed by the CT_{min}, CCRT, and survival of cold exposure) may be associated with a greater resistance of the tissues to ion leak in the cold (MacMillan et al., 2015a).

MacMillan and Sinclair (2011) report that hemolymph $[Na^+]$ of *G. pennsylvanicus* adults drops substantially by 12 h of cold exposure and decreases gradually thereafter over 120 h (MacMillan and Sinclair, 2011). However within the first 12 h of cold exposure, we instead observed a rapid increase in hemolymph $[Na^+]$, peaking at 1 h of exposure to 0°C and then returning to control values by 6 h such that there was no net change in $[Na^+]$ by 12 h. Some of this discrepancy could be explained by differences in hemolymph $[Na^+]$ of control crickets (a mean of 110 mM $[Na^+]$ was measured in the present study compared to an approximate 185 mM measured by MacMillan and Sinclair (2011)). Typical orthopteran hemolymph $[Na^+]$ is closer to 91 mM (Piek and Njio, 1979). Food and rearing conditions were identical between the present study and a previous study by MacMillan and Sinclair (2011), however we isolated crickets for one week prior to experiments to prevent cannibalism and any consequent effects on gut ion

content. We also controlled for potential inconsistencies in mating status by ensuring that all females were virgin; gravid females used in the previous study likely exhibit some differences in ovary and/or fat body mass, and this could affect total available tissue Na^+. Finally, CO_2 used for cricket anesthesia in the previous study could affect hemolymph Na^+ balance (Stewart, 1978; Nilson et al., 2006; Matthews and White, 2011). A higher hemolymph [Na^+], as measured by MacMillan and Sinclair (2011) would present a steeper gradient of Na^+ between the hemolymph and gut, favoring greater migration of Na^+ towards the gut (and perhaps this accounted for the rapid drop in hemolymph [Na^+] in the first 12 h).

In the present study, the peak of hemolymph [Na^+] in the first hour of cold exposure reflected a peak in hemolymph Na^+ content and also coincided with increases in gut Na^+ content (at least statistically for *G. veletis*). However by 12 h in the cold we had observed no net change in hemolymph Na^+ content in either species. A net increase in gut Na^+ content without a net decrease in hemolymph Na^+ content was also observed by Coello Alvarado et al. (2015), and suggests that Na^+ may have entered the hemolymph from surrounding tissues before migrating to the gut where it remained. This hypothesis is supported by the loss of muscle Na^+ content observed during cold exposure, which agrees with previous observations for *G. pennsylvanicus* at 12 h in chill coma (MacMillan and Sinclair, 2011). Tissues other than the muscle could also lose Na^+ during cold exposure; the ovaries are a large potential reservoir for Na^+ (and have a higher [Na^+] than the hemolymph). However we did not quantify changes in ovarian Na^+ balance during cold exposure. Quantifying changes in Na^+ balance of non-muscle tissues (*e.g.* fat body, gonads, ganglia) during chill coma would confirm whether a loss of homeostasis in the tissues manifests as imbalance in hemolymph Na^+ content. Male crickets lack ovaries, so it is unclear whether they will exhibit a similar increase in hemolymph Na^+ content during early chill coma, or if the testes act as a potential source of this Na^+.

Cold exposure caused a gradual redistribution of water between the hemolymph and gut, as observed during longer-term cold exposure (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). However, gut water content in *G. pennsylvanicus* increased despite no measurable decrease in hemolymph volume. This phenomenon was also observed in *G. veletis* nymphs over longer cold exposures, and it is possible that dehydration of tissues accounted for the gain of gut

water (Coello Alvarado et al., 2015). Cold-acclimated *Pyrrhocoris apterus* L. bugs lose water from the fat body during chill coma (Koštál et al., 2004), and while we did not observe changes in muscle water content in crickets during chill coma, water could have been lost from the fat body or other tissues and followed Na^+ to the gut.

Cold exposure caused hemolymph [K^+] to increase steadily over 12 h in for both species, reflecting trends observed at longer durations of chilling (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). Increased hemolymph [K^+] in the cold is thought to result from loss of hemolymph volume, rather than changes in hemolymph K^+ content (MacMillan and Sinclair, 2011). Our observations of the initial stages of cold exposure support a gradual loss of hemolymph volume concurrent with a gradual increase in hemolymph [K^+], and without changes in gut K^+ content (similar trends were observed in crickets after a 120 h cold exposure) (MacMillan and Sinclair, 2011; Coello Alvarado et al., 2015). However, we also observed an increase in hemolymph K^+ content during cold exposure. This K^+ was unlikely to be sourced from the muscle; unlike our observation of decreased muscle Na^+ content, muscle K^+ content did not change during cold exposure (similar to the findings of MacMillan and Sinclair (2011)). Potassium could enter the hemolymph from other tissues; *P. apterus* bugs lose K^+ from the fat body when exposed to -5°C (Koštál et al., 2004). Alternatively, the gut contents could act as a source of K^+ as the gut lumen [K^+] is roughly 17-fold higher than the hemolymph and presents a steep gradient for K^+ favoring migration to the hemolymph. Leak of K^+ across the gut may be enhanced during cold exposure due to changes in the permeability of gut epithelium (Motais and Isaia, 1972; Dokladny et al., 2006; Ionenko et al., 2010). Although we did not observe a change in gut K^+ content during early chill coma, small amounts of K^+ lost from the gut could have large impacts on hemolymph K^+ content, accounting for the apparent discrepancy in [K^+] shifts we observed in the hemolymph and gut.

Increased hemolymph [K^+] during cold exposure (which disrupts muscle K^+ equilibrium potential) was initially proposed by MacMillan and Sinclair (2011) to explain chill coma paralysis via loss of muscle resting potential. However recent studies of *Locusta migratoria* L. have shown that chill coma paralysis precedes hemolymph [K^+] imbalance and that low temperatures play a direct role in neuromuscular silencing (Koštál et al., 2006; Findsen et al.,

2014; MacMillan et al., 2014b; Andersen et al., 2015). It is therefore now generally accepted that chill coma onset and loss of homeostasis during cold exposure are mechanistically unrelated. As predicted, we observed that loss of muscle E_K^+ due to hemolymph $[K^+]$ imbalance could not account for a total loss of muscle resting potential. The hypothesized muscle potential threshold for chill coma is between -37 and -45 mV in *D. melanogaster* and *Apis mellifera* L. (Hosler et al., 2000), which is supported by Andersen *et al*. (2015) in locusts. Although chill coma onset is rapid, muscle potential based on $[K^+]$ balance in crickets did not reach -45 mV prior to 7 h in the cold.

4.1 Do more chill-tolerant crickets maintain homeostasis better in the cold?

Gryllus veletis had better low temperature performance (faster CCRT, less injury, and increased survival) than *G. pennsylvanicus*, agreeing with Coello Alvarado *et al*. (2015) who compared chill tolerance of *G. pennsylvanicus* adults with *G. veletis* nymphs. However unlike *G. veletis* nymphs, *G. veletis* adults were not much better than *G. pennsylvanicus* at maintaining water balance and, in most cases, $[Na^+]$ and $[K^+]$ balance during 12 h of cold exposure were similar between the two species. It is not known whether sex or a 6-week age gap in *G. pennsylvanicus* adults accounted for differences in homeostasis observed by Coello Alvarado *et al*. (2015) and the present study. *Gryllus veletis* did, however, exhibit better maintenance of hemolymph Na^+ and K^+ content and to some degree muscle Na^+ content.

Under control conditions and during cold exposure, *G. veletis* contained less water in the hemolymph relative to the gut compared to *G. pennsylvanicus*. This difference was not due to a higher relative gut water content in *G. veletis*. Nevertheless, *G. veletis* did not avoid a loss of water balance over 12 h of cold exposure; the rate of water redistribution from hemolymph to gut was roughly parallel for the two species. This suggests that regulation of ion homeostasis may be more important than water balance for surviving cold exposure.

Hemolymph $[Na^+]$ was similar for both crickets under control conditions but changed less in *G. veletis* during 12 h of cold exposure due to lesser influx of Na^+ to the hemolymph. Coello Alvarado et al. (2015) also observed that *G. veletis* nymphs, and to some degree cold-acclimated *G. pennsylvanicus* adults, avoid this Na^+ influx up to 120 h in the cold. Chill-tolerant insect

tissues may therefore be more resistant to Na^+ leak in the cold; in support of this hypothesis, *G. veletis* lost muscle Na^+ content somewhat more slowly than *G. pennsylvanicus*. This prevention of ion leak could be achieved via enhanced paracellular junctions or otherwise modified epithelial ultrastructure. Additionally (but not necessarily alternatively), *G. veletis* could combat Na^+ leak via enhanced Na^+ pump activity in the cold (Galarza-Muñoz et al., 2011). However, chill tolerance in *Drosophila* is correlated with a decrease in whole-body Na^+-K^+ ATPase activity (MacMillan et al., 2014a). As Na^+-K^+ ATPase maintains higher hemolymph [Na^+] relative to the gut, lower Na^+-K^+ ATPase activity suggests that chill-tolerant insects may reduce Na^+ gradients across the gut. Cold tolerance in *D. melanogaster* is correlated with a reduction in the [Na^+] gradient across the gut, and it is thought that this reduced gradient minimizes the driving force for bulk movement of Na^+ and water from the hemolymph to the gut during cold exposure (MacMillan et al., 2014a; MacMillan et al., 2015a). This hypothesis was not well-supported by our observations, as the mean hemolymph-to-gut [Na^+] ratio in *G. veletis* was not significantly lower than for *G. pennsylvanicus* under control conditions (nor did it appear lower throughout cold exposure). Neither species exhibited a net loss of hemolymph Na^+ content by 12 h of cold exposure, yet both species suffered a loss of hemolymph volume and a rise in hemolymph [K^+].

Increased hemolymph [K^+] during cold exposure may lead to chilling injury via signalling disruption and cell death (Rojas and Leopold, 1996; Koštál et al., 2006; MacMillan et al., 2015b), however the accumulation of chilling injuries in adult *Gryllus* crickets was not predicted by the ability to defend hemolymph [K^+] in the first 12 h of cold exposure. It is therefore unclear whether ion imbalance in the first 12 h of chill coma has any great effect on the development of chilling injuries. *Gryllus veletis* did exhibit lesser increases in hemolymph K^+ content compared to *G. pennsylvanicus*, so perhaps the gut epithelium of *G. veletis* is more resistant to changes in ion permeability at low temperatures. This hypothesis could be tested by manipulating the [K^+] gradient between the hemolymph and gut prior to cold exposure by artificial diets, as was attempted in a previous study with *L. migratoria* (Andersen et al., 2013). Preventing leak of K^+ into the hemolymph could also explain faster CCRT in *G. veletis*, as recovery requires reestablishment of water balance in addition to the reversal of any bulk movement of ions that occurred during cold exposure (MacMillan et al., 2012).

Under control conditions, *G. veletis* exhibited a lower muscle Na^+ potential and higher muscle K^+ equilibrium potential compared to *G. pennsylvanicus*. Without direct measurements of muscle resting potential it is unclear whether these differences in Na^+ and K^+ potentials help *G. veletis* delay muscle depolarization in early chill coma or play some role in a more rapid CCRT compared to *G. pennsylvanicus* (MacMillan et al., 2014b; Coello Alvarado et al., 2015). Nevertheless, both species entered chill coma well before muscle K^+ equilibrium potentials had reached the theoretical threshold for chill coma at 7 h of cold exposure.

4.2 Conclusions

After characterizing patterns of ion and water balance in the first 12 h of cold exposure, we can propose some refinements to the current model of homeostasis in the cold. During cold exposure, Na^+ appears to be lost from tissues and enters the hemolymph before ultimately migrating to the gut along with water (the water could originate from the tissues and/or from the hemolymph itself). Loss of hemolymph volume in addition to potential leak of K^+ from the gut to the hemolymph leads to an increase in hemolymph $[K^+]$. This K^+ imbalance does not cause chill coma paralysis, but may negatively affect CCRT.

Chill tolerance based on avoidance of chilling injury was not associated with the ability to defend the balance of water and ion concentrations, however chill-tolerant crickets (*G. veletis*) better defended the balance of Na^+ and K^+ contents compared to less chill-tolerant crickets (*G. pennsylvanicus*). We therefore hypothesize that in addition to the gut epithelium, other tissues (e.g. muscle or ovaries) of chill-tolerant insects have lower permeability to ions in the cold, such that Na^+ does not leak from tissues to the hemolymph and K^+ does not leak across the gut epithelium to the hemolymph. Thus, an important future direction is to quantify the effects of cold on tissue permeability and transport function, with special consideration of ultrastructure and ion pump activities (*e.g.* Na^+-K^+-ATPase and proton pump) in the hindgut and Malpighian tubules, as these tissues are responsible for the bulk of ion and water transport.

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Figure legends

Fig. 1. **Recovery time (A) and cumulative injury and mortality (B) of *G. veletis* and *G. pennsylvanicus* after 24 h of recovery following 48 h in chill coma at 0°C.** A. *N* = 9 and 24 crickets for *G. pennsylvanicus* and *G. veletis*, respectively. B. *N* = 25 crickets per species.

Fig. 2. **Ratio of hemolymph-to-gut water volume (A) and [Na^+] (B) in *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h.** Dashed lines indicate a significant linear relationship between water volume or [Na^+] ratio and cold exposure time. *N* = 11 to 18 crickets per species per time point; see Table S2 for statistics.

Fig. 3. **Content of gut Na^+ (A) and K^+ (B) in *G. pennsylvanicus* and *G. veletis* exposed to 0°C for up to 12 h.** Ion contents are represented as the residuals of a regression of μmoles Na^+ or K^+ against gut dry mass and are expressed as mean mM ± s.e.m. The dashed line indicates a significant relationship between gut ion content and cold exposure time in *G. veletis*. *N* = 13 to 18 per species per time point; see Table S2 for statistics.

Fig. 4. **Balance of Na^+ in the hemolymph (A, C) and muscle (B, D) of *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h.** [Na^+] (A, B) is expressed in mM, while Na^+ content is expressed as total μmoles (C, D). Effects of cold on muscle Na^+ (B, D) were modeled using the residuals of a regression of total μmoles Na^+ against muscle dry mass. Dashed lines indicate significant relationships between muscle Na^+ and 0°C exposure time. Solid lines are used to illustrate trends in hemolymph Na^+ during cold exposure. Different letters indicate differences in mean hemolymph Na^+ of *G. pennsylvanicus* according to Tukey's HSD. Tukey's HSD failed to detect differences among mean for *G. veletis*. Asterisks denote significant differences in Na^+ between species at time = 0 h according to a t-test. *N* = 11 to 18 crickets per species per time point; see Table S2 for statistics.

Fig. 5. **Balance of K^+ in the hemolymph (A, C) and muscle (B, D) of *G. pennsylvanicus* and *G. veletis* crickets exposed to 0°C for up to 12 h.** Potassium concentration (A, B) is expressed in mM, while K^+ content is expressed as total μmoles (C, D). Effects of cold on muscle K^+ (B,

D) were modeled and as the residuals of a regression of total μmoles K^+ against muscle dry mass. Dashed lines indicate significant linear relationships between muscle or hemolymph K^+ and cold exposure time. Asterisks denote significant differences in K^+ between species at time = 0 h according to a t-test (see Table S2 for statistics). *N* = 13 to 18 crickets per species per time point.

Fig. 6. **Na^+ (A) and K^+ (B) potentials (mV) across the muscle cell membrane in *G. pennsylvanicus* and *G. veletis* exposed to 0°C for up to 12 h.** Solid lines are used to illustrate trends in muscle Na^+ potential, but muscle Na^+ potentials did not differ between cold exposure times for either species according to ANOVA. Dashed lines indicate significant relationships between muscle K^+ potential and cold exposure time. Asterisks denote significantly different potentials between *G. pennsylvanicus* and *G. veletis* at exposure time = 0 according to a t-test. *N* = 12 to 18 per species per time point.

Table 1. Content and concentration of Na^+ in the fat body, ovaries, head, Malpighian tubules, and hemolymph of adult *G. pennsylvanicus* crickets in control conditions. *N* = 17 (hemolymph) or 6 (all other tissues).

Tissue	[Na^+] (mM)	Total Na^+ content (µmoles)
Malpighian tubules	65 ± 4.2	0.3 ± 0.03
Head	70 ± 3.8	2.2 ± 0.14
Hemolymph	110 ± 6.6	5.5 ± 0.57
Fat body	123 ± 5.3	0.5 ± 0.05
Ovaries	135 ± 6.0	11.5 ± 0.86

Figure 1

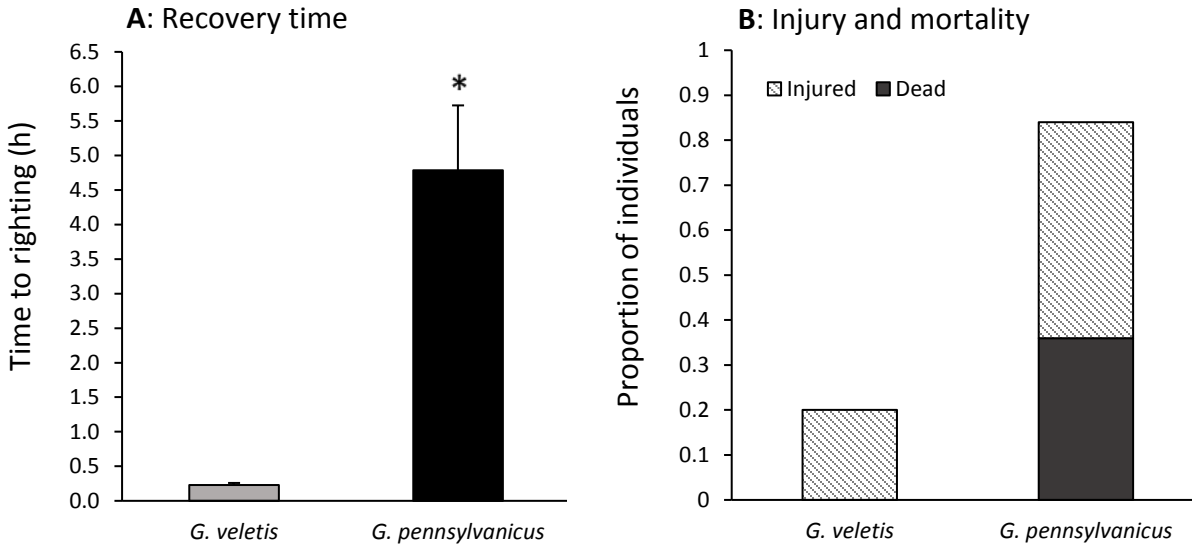

A: Recovery time
Time to righting (h)
6.5
6.0
5.5
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
G. veletis
G. pennsylvanicus
B: Injury and mortality
Proportion of individuals
1
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Injured
Dead
G. veletis
G. pennsylvanicus

Figure 2

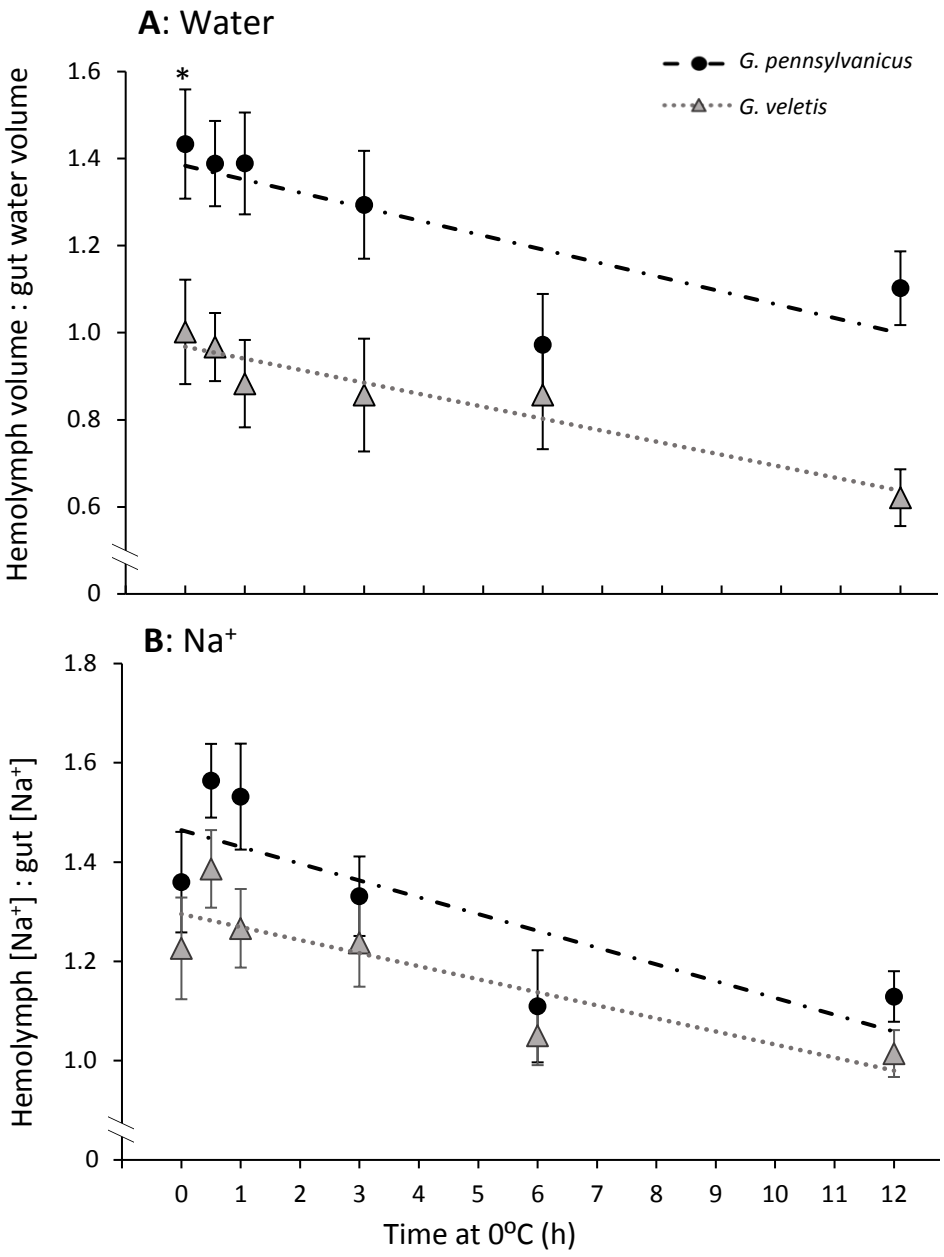

A: Water
G. pennsylvanicus
G. veletis
*
Hemolymph volume : gut water volume
1.6
1.4
1.2
1.0
0.8
0.6
0
B: Na+
Hemolymph [Na+] : gut [Na+]
1.8
1.6
1.4
1.2
1.0
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time at 0°C (h)

Figure 3

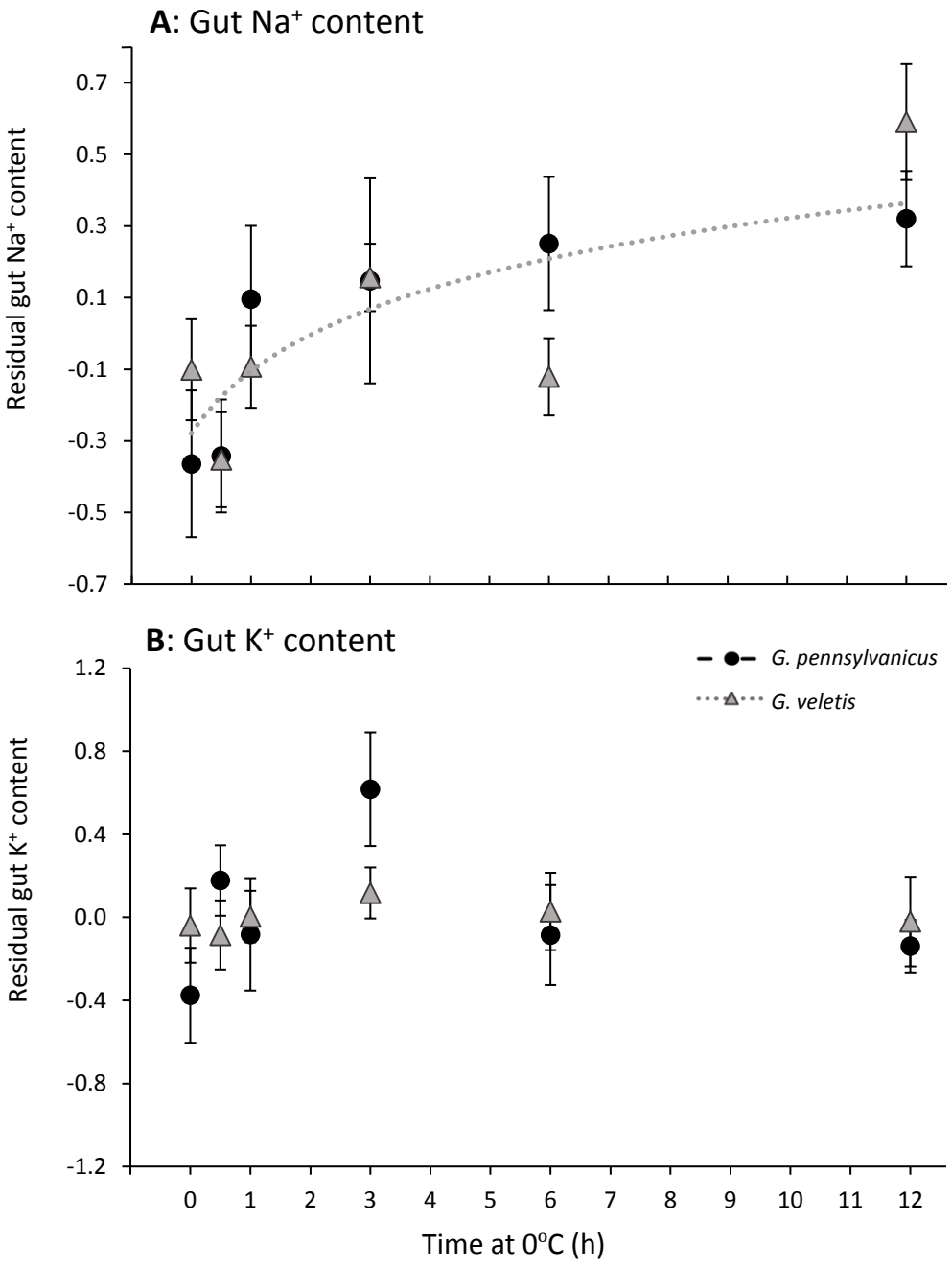

A: Gut Na+ content
Residual gut Na+ content
0.7
0.5
0.3
0.1
-0.1
-0.3
-0.5
-0.7
B: Gut K+ content
Residual gut K+ content
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
G. pennsylvanicus
G. veletis
0
1
2
3
4
5
6
7
8
9
10
11
12
Time at 0°C (h)

Figure 4

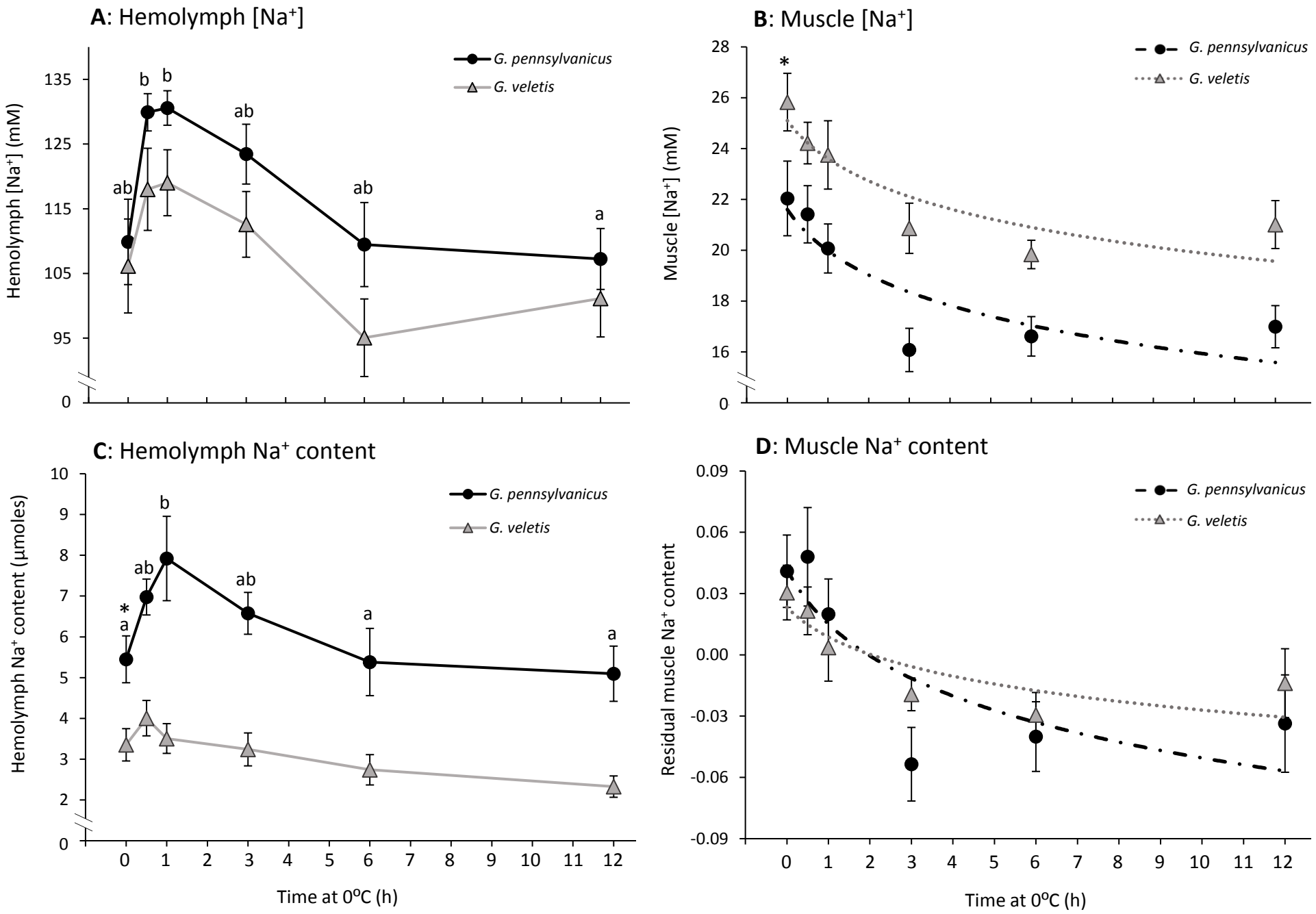

A: Hemolymph [Na+]
Hemolymph [Na+] (mM)
G. pennsylvanicus
G. veletis
B: Muscle [Na+]
Muscle [Na+] (mM)
C: Hemolymph Na+ content
Hemolymph Na+ content (µmoles)
Time at 0°C (h)
D: Muscle Na+ content
Residual muscle Na+ content
Time at 0°C (h)

Figure 5

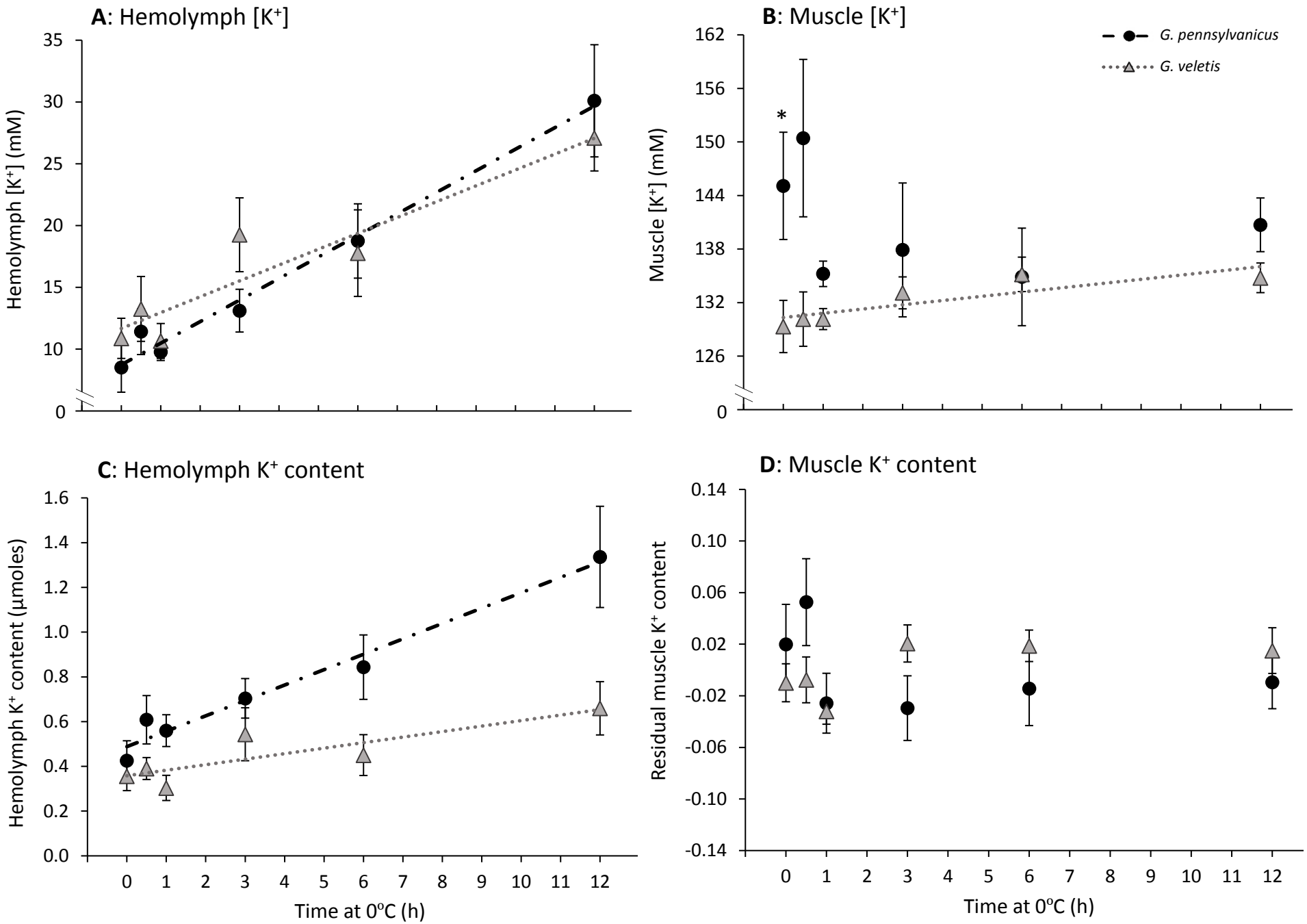

A: Hemolymph [K+]
Hemolymph [K+] (mM)
B: Muscle [K+]
Muscle [K+] (mM)
G. pennsylvanicus
G. veletis
*
C: Hemolymph K+ content
Hemolymph K+ content (µmoles)
Time at 0°C (h)
D: Muscle K+ content
Residual muscle K+ content
Time at 0°C (h)

Figure 6

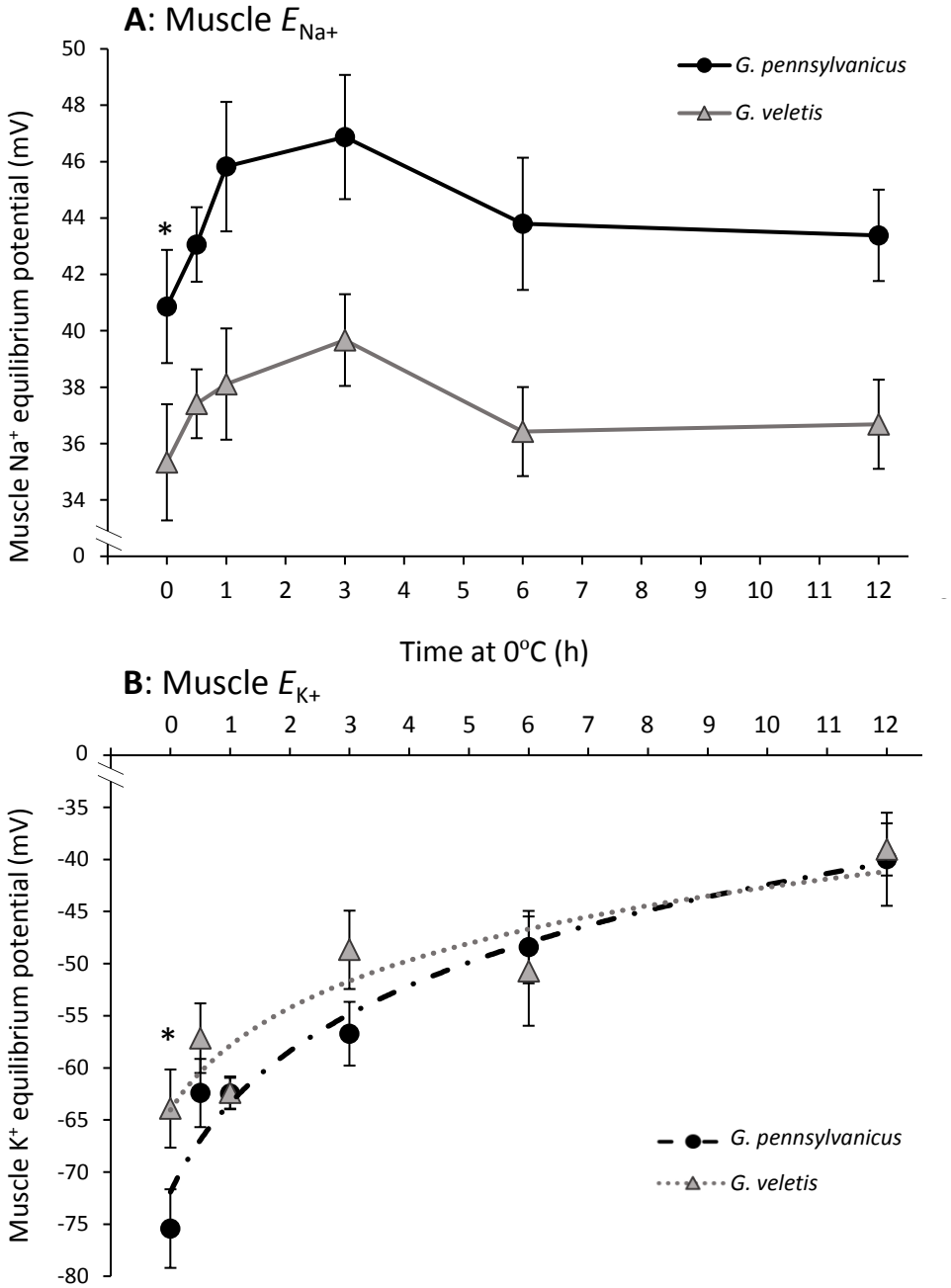
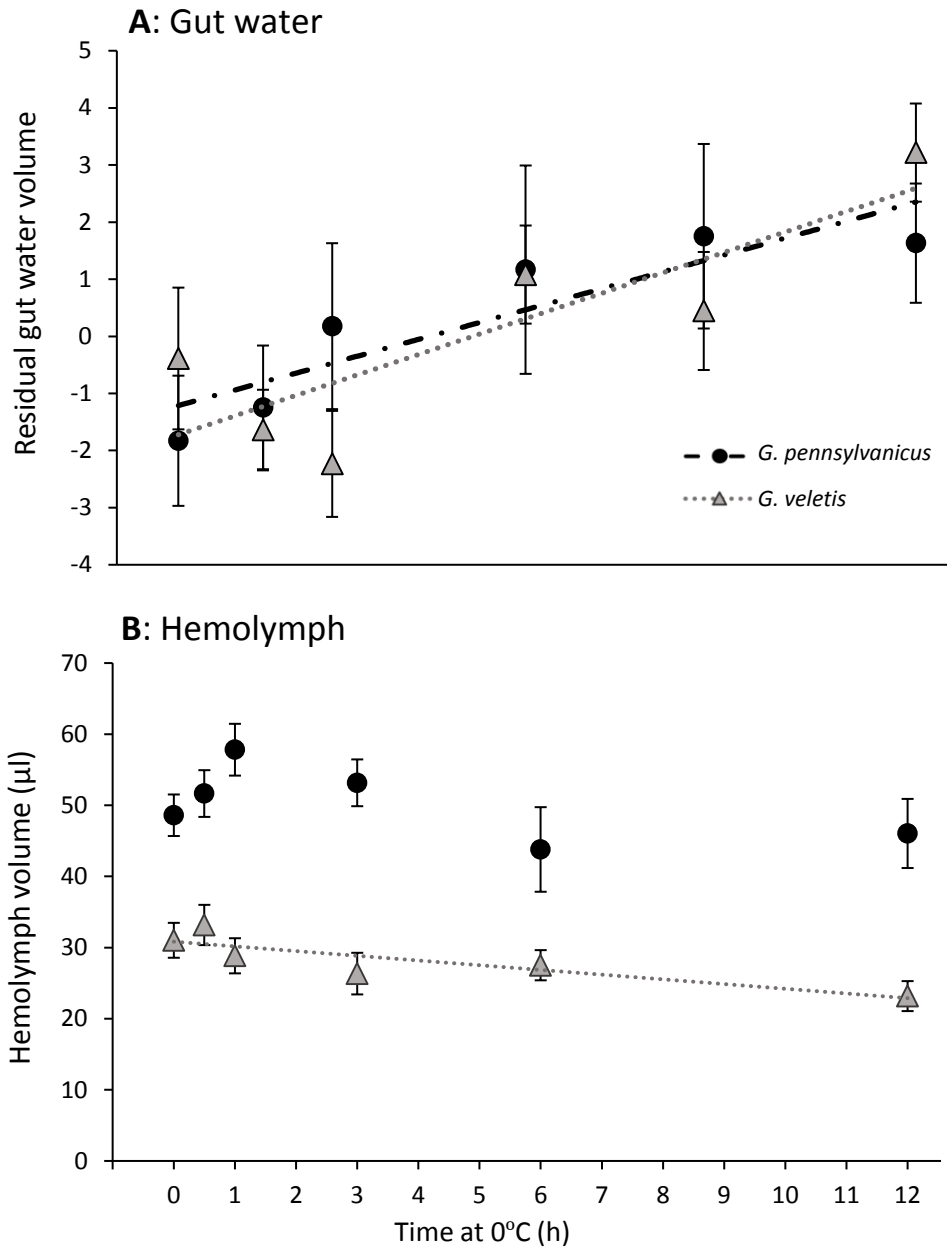

A: Muscle E_{Na+}
Muscle Na^+ equilibrium potential (mV)
50
48
46
44
42
40
38
36
34
0
*
G. pennsylvanicus
G. veletis
0 1 2 3 4 5 6 7 8 9 10 11 12
Time at 0°C (h)
B: Muscle E_{K+}
0 1 2 3 4 5 6 7 8 9 10 11 12
0
-35
-40
-45
-50
-55
-60
-65
-70
-75
-80
Muscle K^+ equilibrium potential (mV)
*
G. pennsylvanicus
G. veletis

Figure S1


A: Gut water
Residual gut water volume
5
4
3
2
1
0
-1
-2
-3
-4
G. pennsylvanicus
G. veletis
B: Hemolymph
Hemolymph volume (µl)
70
60
50
40
30
20
10
0
0 1 2 3 4 5 6 7 8 9 10 11 12
Time at 0°C (h)

Supplementary Information

Click here to download e-component: Ions in early chill coma - Supplemental Information.docx