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# Reversing sodium differentials between the hemolymph and hindgut speeds chill coma recovery but reduces survival in the fall field cricket, *Gryllus pennsylvanicus*

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## ABSTRACT

Chill-susceptible insects enter the reversible state of chill coma at their critical thermal minimum ($CT_{min}$). During chill coma, movement of $Na^+$ and water from the hemolymph to the gut lumen disrupt ion and water balance. Recovery from cold exposure requires re-establishment of this balance, and failure to do so results in chilling injury or death. We hypothesized that the passive leak of $Na^+$ and consequently water during cold exposure is driven by the [$Na^+$] differential between the gut and hemolymph. To determine the extent to which this [$Na^+$] differential affects cold tolerance, we used artificial diets to load the guts of fall field crickets (*Gryllus pennsylvanicus*) with various concentrations of $Na^+$. Manipulating [$Na^+$] differentials had no effect on the $CT_{min}$, agreeing with recent studies demonstrating that chill coma onset precedes loss of ion balance in the cold). A high [$Na^+$] diet reversed the direction of the [$Na^+$] differential between the gut and hemolymph. Crickets fed a high [$Na^+$] diet recovered from 12 h of chill coma nearly twice as fast as those fed low [$Na^+$] diets. However, the high [$Na^+$] diet was detrimental to survival after prolonged cold exposure (three days at 0 °C). Therefore, while a reduced [$Na^+$] differential helps crickets recover from short-term cold exposure, an increased gut $Na^+$ load itself appears to carry longer-term costs and promotes irreversible chilling injury.

## INTRODUCTION

Most insects are chill-susceptible and die after prolonged exposure to low temperatures well above the point at which they freeze (Overgaard and MacMillan, 2017). At their critical thermal minimum ($CT_{min}$), insects enter chill coma; a reversible state of paralysis caused by cold-induced depolarization (MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). During chill coma, insects gradually lose ion and water homeostasis as $Na^+$ and water move from the hemolymph into the gut (Andersen et al., 2015a; Findsen et al., 2014; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b). As water is lost to the gut, hemolymph volume decreases and hemolymph $K^+$ concentration increases as a result. Muscles bathed in this $K^+$-rich hemolymph further depolarize, which stimulates apoptotic pathways *via* excessive cellular $Ca^{2+}$ influx, leading to cellular (and ultimately whole animal) death (Andersen et al., 2015a; Bayley et al., 2018; Findsen et al., 2014; Koštál et al., 2006; Overgaard and MacMillan, 2017). Upon rewarming, insects re-establish ion and water balance (at a rate dependent on the time spent in chill coma), and the capacity to repair chilling injury dictates survival (MacMillan et al., 2015a; MacMillan et al., 2015c; MacMillan et al., 2012; Overgaard and MacMillan, 2017). An inability to recover ion and water homeostasis results in chilling injury or death, therefore cold tolerance depends in part on prevention of the initial loss of homeostasis and/or efficient redistribution of $Na^+$ and water back to the hemolymph upon rewarming (Findsen et al., 2013; MacMillan et al., 2012). Migration of $Na^+$ and water during chill coma is likely driven by the strength of $Na^+$ differentials (i.e. the difference in $[Na^+]$ between the hemolymph and gut), however we have a limited understanding of how these $Na^+$ differentials determine overall ion and water balance during cold exposure, and ultimately how they affect survival at low temperatures.

The Malpighian tubules and hindgut work in synchrony to maintain ion and water balance in insects (Maddrell and O'Donnell, 1992; O'Donnell et al., 1996). In the Malpighian tubules, the proton pump (V-ATPase) and $H^+$ cation exchangers (amongst other enzymes) transport inorganic cations from the hemolymph to the tubule lumen, driving passive or facilitated transport of water and anions from the hemolymph (Maddrell and O'Donnell, 1992). In the hindgut, ions and water are selectively reabsorbed from the lumen to the hemolymph through paracellular channels of the gut epithelium. The activity of $Na^+/K^+$-ATPase in hindgut epithelial cells produces high $[Na^+]$ in

the paracellular space, which helps drive water through these channels from the gut lumen to the hemolymph (Maddrell and O'Donnell, 1992; O'Donnell and Maddrell, 1983). The movement of $Na^+$ through paracellular channels results in higher hemolymph [$Na^+$] relative to the gut, while movement of water through the channels concentrates feces in the gut lumen and maintains lower osmolality of the hemolymph relative to the hindgut (Dissanayake and Zachariassen, 1980; Zachariassen et al., 2004). At low temperatures, enzyme-mediated active transport is reduced (Gerber and Overgaard, 2018; MacMillan et al., 2015d) and passive gradients between the hemolymph and gut favour net diffusion (i.e. 'leak') of $Na^+$ and water back towards the gut lumen. $Na^+$ and water leak during cold exposure results in the mass disruption of ion and water homeostasis that is characteristic of insect chill coma (Koštál et al., 2006; MacMillan and Sinclair, 2011b). This current model is supported by correlative evidence that more cold-tolerant insects maintain lower basal hemolymph [$Na^+$] (i.e. a lower [$Na^+$] differential between the hemocoel and gut) compared to less cold-tolerant counterparts, which reduces leak of $Na^+$ and water to the gut during cold exposure in cold-tolerant insects (Coello Alvarado et al., 2015; Andersen et al., 2017a; Des Marteaux and Sinclair, 2016; MacMillan et al., 2015b; MacMillan et al., 2015d).

Manipulating ion contents of the insect gut *in vivo* through feeding can modify cold tolerance phenotypes at the whole organism level. For example, feeding *Locusta migratoria* a high $K^+$ wheat diet for 8 d slows chill coma recovery (Andersen et al., 2013), feeding *Drosophila melanogaster* NaCl-enriched diets for 24 h facilitates a faster chill-coma recovery time with no effects on survival, but feeding *D. melanogaster* NaCl-enriched diets for only 1.5 h slows chill coma recovery (Yerushalmi et al., 2016). Experimentally manipulating concentration gradients across tissues *ex vivo* can also affect homeostasis in the cold: at 0 °C, exposure of everted *L. migratoria* gut sacs to fluids with manipulated cation gradients (increased [$Na^+$] and decreased [$K^+$]) did not prevent water absorption from the gut, but exposing gut sacs to high osmolality fluids prevented water reabsorption (Gerber and Overgaard 2018). Clearly, the specific effects of ion differential manipulation *in vivo* or *ex vivo* yields various results, thus the relative contribution of $Na^+$ differentials in determining cold-induced loss of ion homeostasis on both the organismal and tissue level, remains unclear.

The fall field cricket (*Gryllus pennsylvanicus*) is a chill-susceptible insect that enters chill coma at approximately +2.2 °C, and loses water and ion balance within the first 12 h of cold exposure, during which $Na^+$ and water move from the hemolymph to the hindgut (Macmillan and Sinclair, 2011b; Coello Alvarado et al., 2015; Des Marteaux et al., 2017; Des Marteaux and Sinclair, 2016). *Gryllus pennsylvanicus* recover water and ion balance when rewarmed, which involves the active redistribution of $Na^+$ from the gut back into the hemolymph (MacMillan et al., 2012). Cold-acclimated *G. pennsylvanicus* can defend ion and water balance better during chill coma than their warm-acclimated conspecifics, which could be (in part) driven by a lower basal hemolymph [$Na^+$] and thus a reduced $Na^+$ differential between the hemolymph and gut (Coello Alvarado et al., 2015). Cold-acclimated crickets also have increased expression of $Na^+/K^+$ATP-ase and several other hindgut active transporters compared to warm-acclimated crickets, which could allow for faster recovery of $Na^+$ and water homeostasis following a cold exposure (Des Marteaux et al., 2017). Overall, $Na^+$ differentials likely play an important role in determining the extent of damage accrued during, and recovery after chill coma in *G. pennsylvanicus*.

Here we tested the hypothesis that the extent of ion and water imbalance (and its consequences) during chill coma in *G. pennsylvanicus* is driven by the hemolymph-hindgut [$Na^+$] differential. We fed crickets with diets varying in $Na^+$ concentration, which manipulated gut content such that [$Na^+$] differentials were either not affected (i.e. represent the normal differential where [$Na^+$] is higher in the hemolymph compared to the hindgut), moderately reversed, or greatly reversed the [$Na^+$] (concept illustrated in Figure 1). We measured the effects of each diet on several aspects of low temperature performance and recovery from cold exposure. We predicted that reversing the [$Na^+$] differential (i.e. feeding crickets on medium and high [$Na^+$] diets) between the hemolymph and gut should reduce $Na^+$ leak towards the gut, consequently reduce the amount of active transport required to re-establish [$Na^+$] differentials between the hemolymph and hindgut during recovery from cold exposure, resulting in faster recovery from chill coma and enhanced survival after a long term cold exposure. To control for possible effects of overall high total gut osmolality on cold tolerance, we also fed crickets with diets varying in osmolality but with constant low [$Na^+$].

## MATERIALS & METHODS

### Cricket rearing and maintenance

Our population of *G. pennsylvanicus* was derived from individuals originally collected from the University of Toronto Mississauga campus in 2004 (MacMillan and Sinclair, 2011b). We reared crickets in 60 L bins containing egg cartons for shelter, with water and rabbit food pellets (Little Friends Rabbit Food, Martin Mills Inc., Elmira, ON, Canada) *ad libitum*, under constant summer conditions (25 °C, 14L: 10D photoperiod, 70% RH) according to MacMillan and Sinclair (2011b). Adults had access to containers of a 4:1 mixture of vermiculite and sand for two weeks to lay eggs, and the containers were then transferred to 4 °C for a minimum of three months for the eggs to undergo an obligate diapause and chilling. We then transferred the eggs back to constant summer conditions for hatching and development. Prior to all feeding experiments, we isolated adult females (2-4 months post-hatch), placed them in individual plastic dishes covered with mesh, and fasted them for 24 h.

### Artificial diet preparation and feeding

We developed an artificial diet to load the cricket gut with controlled levels of $Na^+$ and osmolytes. First, we created a base diet composed of ingredients similar to those found in a rearing diet of rabbit food pellets (Supplementary material; Tables S1 and S2). This base diet consisted of 1% agar with casein, cellulose, cholesterol, glucose, xylitol, sodium acetate ($CH_2COONa$), potassium phosphate ($KH_2PO_4$), calcium phosphate ($Ca(H_2PO_4)_2$), and magnesium chloride ($MgCl_2$). For experiments where gut and hemolymph ion concentrations were measured, we added cobalt-EDTA to the diet as an internal standard to verify that crickets had consumed it (Supplementary material; Figure S1).

We altered sodium acetate, xylitol, and water content in the base diet to produce six different diets with differing [$Na^+$] and total osmolyte concentrations (Table 1). Xylitol is not naturally produced or metabolized by insects (Jackson and Nicolson, 2002), thus it was removed from or added to the diet as an osmotic filler, presumably without affecting cricket energy metabolism. Low [$Na^+$], medium [$Na^+$], and high [$Na^+$] diets had 8, 120, and 540 mM $Na^+$, respectively, but similar (1027 ± 133 mOsm) total osmolyte concentrations (Table 1). High

158 osmolality (HO), medium osmolality (MO), and low osmolality (LO) diets had constant [$Na^+$] (7
159 ± 0.2 mM $Na^+$) but differing total osmolyte concentrations (Table 1). The highest osmolality diet
160 had an osmolyte concentration similar to that of the [$Na^+$] diets (Table 1).
161
162 Adult female crickets that were fasted for 24 h ate all six diets, and the food remained in
163 their gut for at least four h (Table 1). Based on this information, we provided individual crickets
164 with a 1.5 mL portion of artificial diet for seven h under constant summer conditions before
165 experiments in which we determined survival after cold exposure, chill coma recovery time, $CT_{min}$,
166 and hemolymph and gut ion and water balance. In addition, we weighed the crickets before and
167 after feeding to verify that they had consumed the artificial diets.
168
169 **Cold tolerance measurements**
170 We measured the $CT_{min}$ according to MacMillan and Sinclair (2011b). We placed six
171 crickets at a time individually into closed 200 mL glass beakers which were surrounded by a
172 Plexiglas enclosure through which an ethylene glycol:water mix (1:1 v:v) was circulated from a
173 programmable refrigerated bath (Model 1157P, VWR International Mississauga, ON, Canada).
174 We recorded the temperature inside each beaker with type-T thermocouples connected to a
175 computer *via* a Picotech TC-08 thermocouple interface and Picolog software (Pico Technology,
176 Cambridge, UK), and cooled the beakers from 21 °C at -0.25 °C.$min^{-1}$. We continuously monitored
177 the body temperature of each cricket with a thermocouple held against the abdomen, and recorded
178 the $CT_{min}$ as the temperature at which no movement could be elicited by poking their abdomen
179 with a blunt probe.
180
181 To measure chill coma recovery time, we placed groups of crickets in 45 mL plastic tubes
182 which were loosely covered, and cooled them from 21 °C to 0 °C at -0.25 °C.min-1 in a
183 programmable refrigerated bath containing a methanol: water mix (50:50 v:v) (Lauda Proline RP
184 3530, Würzburg, Germany) (MacMillan et al., 2012). We held crickets at 0 °C for 12 h, then
185 transferred them back to room temperature on their dorsum in Petri dishes, and recorded the time
186 for crickets to recover by righting themselves.
187

To assess chilling survival, we placed crickets in 45 mL loosely-covered plastic tubes upright in an ice slurry (at 0 °C) for three days. We then transferred crickets to individual plastic dishes with access to rabbit food and water *ad libitum* and allowed them to recover in rearing conditions for 24 h. We assessed survival 48 h after removal from the cold and categorized crickets as either fit (able to walk and jump), injured (alive, but lacking coordination and ability to jump), or dead, after MacMillan and Sinclair (2011b).

**Ion concentration analyses**

After crickets were fed on the artificial diets for seven hours, we either kept crickets at 25 °C (0 h), exposed them to 0 °C for 12 h, or exposed them to 0 °C for 72 h, and then measured ion concentrations in the hemolymph and hindgut. We dissected the 0 h crickets at room temperature (c. 21 °C) and dissected 12 and 72 h cold-exposed crickets on ice. We collected hemolymph in a 200 μL tube containing 100 μL of concentrated nitric acid. We isolated the hindgut from the rest of the digestive tract using microscissors and placed it in a pre-weighed 200 μL tube. We then dried the hindguts in an oven for 48 h at 60 °C before adding 200 μL of concentrated nitric acid to dissolve the tissue (Des Marteaux and Sinclair, 2016). We determined water content in the hindgut gravimetrically, by subtracting dry mass from wet mass.

We prepared samples for atomic absorption spectrometry (AAS) as previously described by MacMillan and Sinclair (2011b). Briefly, hemolymph and dried hindgut samples were digested in concentrated nitric acid at room temperature (21 °C) for 24 and 48 h, respectively, then vortexed and diluted in double distilled $H_2O$ to bring the samples within a measurable range of the AAS (iCE 3300, Thermo Scientific, Waltham MA, USA). To calculate ion concentrations in the samples, we compared their absorption values to standard curves for $Na^+$, $K^+$ and Cobalt ($Co^{2+}$: to measure Cobalt-EDTA internal standards) generated using standards of known concentration diluted in nitric acid (for detailed calculations see supplementary material Table S4).

## Data analysis

We conducted all statistical analyses using R software v3.5.2 (R Development Core Team, 2014). $Na^+$ differentials were taken as the difference in $[Na^+]$ between the hemolymph and hindgut. We compared $Na^+$ differentials, $CT_{min}$, and chill coma recovery time among diet treatments using a one-way ANOVA followed by a Tukey's HSD *post hoc* comparison. We used a Fisher's exact test to compare differences in the proportion of crickets that were injured, recovered, or dead after cold exposure among diet treatment groups. We used two-way ANOVAs with Tukey's HSD *post hoc* comparisons to compare $Na^+$ differentials, hemolymph $[Na^+]$, hindgut $[Na^+]$, hindgut $Na^+$ content, hemolymph $[K^+]$, and hindgut water content among diet and temperature treatment groups. To meet the assumptions of ANOVA, we log-transformed any data that was non-normally distributed or was non-homogenous prior to analyses.

# RESULTS

## Verification of modified gut content in crickets fed $Na^+$ and osmolality diets

Loading the gut with low $[Na^+]$ diets resulted in a $[Na^+]$ differential that represented the normal physiological conditions, such that the hemolymph $[Na^+]$ was higher in the hemolymph, compared to the gut. Loading the gut with medium $[Na^+]$ diets increased gut $[Na^+]$ such that it was slightly higher than hemolymph $[Na^+]$, but not significantly higher than loading the gut with low $[Na^+]$ diets ($F_{2,21} = 7.9$, $p = 0.25$; Fig. 2). Loading the gut with high $[Na^+]$ diets increased gut $[Na^+]$ such that it was significantly higher than hemolymph $[Na^+]$, thus the $[Na^+]$ differential between the hemolymph and hindgut was significantly reversed prior to cold exposure in the high $[Na^+]$ diet. ($F_{2,21} = 7.9$, $p < 0.001$; Fig. 2). Loading cricket guts with diets of varying osmolyte concentrations did not affect $[Na^+]$ differentials between the hemolymph and hindgut ($F_{2,21} = 1.4$, $p = 0.26$; Fig. 2).

## Effect of dietary manipulations on cold tolerance of crickets fed $Na^+$ and osmolality diets

The $CT_{min}$ did not differ among crickets fed on various $[Na^+]$ or osmolyte diets ($Na^+$ diets: $F_{2,\ 21} = 0.14$, $p = 0.87$; osmolality diets: $F_{2,\ 21} = 0.69$, $p = 0.52$; Fig. 3A). However, crickets fed on high $[Na^+]$ diets recovered from chill coma more rapidly than those fed on lower $[Na^+]$ diets ($F_{2,41}$

= 4.4, $p$ = 0.019; Fig. 3B). Conversely, crickets fed on the low [$Na^+$] diet showed greater survival after a three-day exposure to 0 °C compared with those fed on medium or high [$Na^+$] diets (Fisher's exact test, $p$ = 0.004; Fig. 4A, Figure S2). Changing the total osmolyte concentration of the gut did not affect chill coma recovery time ($F_{2,42}$ = 0.29, $p$ = 0.75; Fig. 3A) or survival after three days of exposure to 0 °C (Fisher's exact test, $p$ = 0.76; Fig. 4B). The diets themselves appeared to have no immediate adverse effect, as all crickets fed on low, medium, and high [$Na^+$]/osmolality diets at 25 °C for seven hours survived at least three days (Supplementary material; Table S3).

**Dietary $Na^+$ effects on ion and water balance after cold exposure**

Prior to cold exposure, hemolymph [$Na^+$] was approximately 140 ± 13 mM for all crickets regardless of diet $Na^+$ content. By 12 h of exposure to 0 °C, [$Na^+$] differentials between the hemolymph and hindgut of all crickets (i.e. across all [$Na^+$] diets) nearly equilibrated; a small difference in [$Na^+$] remained between the hemolymph and hindgut ($F_{4,54}$ = 10.2, $p$ = <0.001; Fig. 5A). This pattern persisted at 72 of cold exposure. At 12 h of cold exposure, hemolymph [$Na^+$] had decreased significantly (to roughly 76 ± 15 mM; $F_{2,56}$ = 92, $p$ < 0.001; Fig. 5C), and this decrease did not differ significantly among the different [$Na^+$] diets ($F_{4,56}$ = 1.3, $p$ = 0.27 Fig. 5C). By 72 h of cold exposure, hemolymph [$Na^+$] had nearly returned to pre cold-exposure values across all [$Na^+$] diets (($F_{2,56}$ = 92, $p$ < 0.001; Fig. 5C), and hemolymph [$Na^+$] increased significantly more after 72 h of cold exposure in crickets fed on medium and high [$Na^+$] diets compared to those fed on low [$Na^+$] diets (Tukey's HSD, $p$ < 0.001). Gut [$Na^+$] had decreased in all crickets at 12 h of cold exposure, and remained similarly low at 72 h of cold exposure ($F_{2,60}$ = 40.6, $p$ < 0.001; Fig. 5E). Hindgut $Na^+$ initially decreased in all crickets after 12 h of cold exposure ($F_{2,60}$ = 6.2, $p$ < 0.001; Fig. 6A), but crickets fed on medium and high [$Na^+$] diets still had higher hindgut $Na^+$ content compared to those fed on low [$Na^+$] diets (Tukey's HSD, $p$ < 0.001). At 72 h of cold exposure, hindgut $Na^+$ content increased crickets fed on all diets, but more so in crickets fed on medium and high [$Na^+$] diets ($F_{2,60}$ = 6.2, $p$ < 0.001; Fig. 6A).

Hemolymph [$K^+$] increased during cold exposure ($F_{2,54}$ = 115, $p$ < 0.001; Fig. 7A), and this increase was most pronounced between 12 and 72 h. Such changes in hemolymph [$K^+$] were consistent across the different [$Na^+$] diets ($F_{2,54}$ = 1.6, $p$ = 0.22). Gut water volume increased during cold exposure in a similar pattern to hemolymph [$K^+$]; the increase was most pronounced

between 12 and 72 h of cold exposure, however this increase in gut water content was not significant ($F_{2,60} = 0.11$, $p = 0.60$; Fig. 8A). There was slightly more gut water at 72 h of cold exposure in crickets fed on high [$Na^+$], and this increase in gut water was almost significant ($F_{2,60} = 0.60$, $p = 0.06$; Fig. 8A).

**Dietary osmolality effects on ion and water balance after cold exposure**

There were no significant changes in the [$Na^+$] differential between the hemolymph and hindgut after either 12 or 72 h of cold exposure ($F_{4,54} = 2.5$, $p = 0.09$; Fig. 5B) The type of osmolality diet had no significant effect on hemolymph [$Na^+$] at a given time point ($F_{2,56} = 2.3$, $p = 0.11$; Fig 5D). Hemolymph [$Na^+$] had decreased at 12 h of cold exposure (most notably in medium and low osmolality diets; $F_{2,56} = 9.1$, $p < 0.001$; Fig 5D), but hemolymph [$Na^+$] approximately returned to pre-cold exposure values at 72 h (Tukey's HSD, $p < 0.001$). Gut [$Na^+$] decreased markedly and significantly by 12 h cold exposure for all osmolality diets and returned to pre-cold levels by 72 h ($F_{4,60} = 4.4$, $p = 0.016$; Fig. 5F)

Hindgut $Na^+$ content initially decreased in crickets fed on all osmolality diets at 12 h of cold exposure but returned to control levels at 72 h of cold exposure ($F_{4,59} = 5.1$, $p < 0.001$; Fig. 6B). There were no differences in hindgut $Na^+$ content among crickets fed on different osmolality diets ($F_{2,59} = 0.72$, $p = 0.16$; Fig. 6B). Hemolymph [$K^+$] had increased significantly by 12 h of cold exposure for all osmolality diets (most notably in the low osmolality diet), and remained elevated at 72 h of cold exposure ($F_{4,50} = 3.1$; $p = 0.024$; Fig. 7B).

At a given cold exposure time, hemolymph [$K^+$] did not differ significantly among crickets fed on low, medium or high osmolality diets ($F_{2,50} = 1.8$, $p = 0.17$; Fig. 7B). Hindgut water volume did not change among crickets fed on either low, medium or high osmolality diets ($F_{2,60} = 0.83$, $p = 0.44$; Fig. 8B), or after any length of cold exposure ($F_{2,60} = 2.25$, $p = 0.11$; Fig. 8B).

## DISCUSSION

To test the hypothesis that the extent of water and ion balance lost during chill coma (and its consequences for cold tolerance) is driven by the $[Na^+]$ differential between the hemolymph and gut, we manipulated $[Na^+]$ differentials using artificial diets with varying $[Na^+]$. Reversing this $[Na^+]$ differential with various $Na^+$ diets did not completely prevent leak of $Na^+$ from the hemolymph into the hindgut during cold exposure as we predicted. The reversed $[Na^+]$ differential had no effect on the $CT_{min}$, but decreased chill coma recovery time after a short (12 h) cold exposure. This suggests that reversal of the $[Na^+]$ differential allowed crickets to re-establish $Na^+$ homeostasis during recovery from short term cold exposure faster, and thus reduce chill coma recovery time, which aligns with our initial predictions. Conversely, the reversed $[Na^+]$ differential led to a reduction in survival following a longer (72 h) cold exposure. Therefore, although a high gut $Na^+$ load may allow crickets to recover from chill coma faster after a short-term cold exposure, this dietary stress likely exacerbates chilling injuries accumulated during prolonged cold exposure.

To control for the inadvertent effects of high osmolality in our $[Na^+]$ diets, we also assessed homeostasis and cold tolerance in crickets fed on diets with varying osmolalities. We found that varying osmolality in the cricket gut affected neither the $CT_{min}$, chill coma recovery after a short (12h) cold exposure, nor survival after a long (72h) cold exposure. There were no significant effects of dietary osmolality on gut $[Na^+]$, hemolymph $[Na^+]$, hemolymph $[K^+]$ or $Na^+$ content in crickets after either 12 or 72 h of cold exposure, thus we can rule out any changes in ion and water distribution observed in crickets fed on varying $[Na^+]$ as being driven by manipulated osmotic differentials. Gut $Na^+$ content in crickets fed on either low, medium or high osmolality diets did not surpass that of crickets fed on low $[Na^+]$ diets (Figure 6A). This further supports our use of diets with varying osmolality as controls, because all three osmolality diets had the same $[Na^+]$ as our low $[Na^+]$ diet.

**Reversing the [$Na^+$] differential has various effects on cold tolerance and homeostasis during cold exposure**

Reversing the [$Na^+$] differential did not affect the $CT_{min}$, and this agrees with the standing hypothesis that the passive movement of ions and water down gradients is not mechanistically linked to chill coma onset (Overgaard and MacMillan, 2017). For example, recent studies demonstrate that chill coma onset is driven by processes unrelated to $K^+$ and $Na^+$-driven muscle depolarization (Andersen et al., 2015a), and chill coma paralysis in *G. pennsylvanicus* precedes changes in hemolymph [$K^+$] (Des Marteaux and Sinclair, 2016). Similarly, some *Drosophila* species enter chill coma before the theoretical chill coma muscle potential threshold (~-40 mV) is reached (Andersen et al., 2015b). Potentially, chill-induced spreading depolarization in the central nervous system (rather than hyperkalemia) sets the $CT_{min}$ (at least for *Drosophila,* Andersen and Overgaard, 2019; and *L. migratoria,* Robertson et al., 2017).

We predicted that reversing the hemolymph-hindgut [$Na^+$] differential would prevent leak of $Na^+$ to the hindgut during short-term cold exposure, thereby decreasing chill coma recovery time (as less time and energy would be required to restore a more minor disruption in homeostasis). When [$Na^+$] differentials were manipulated by artificial diets, hindgut $Na^+$ content decreased at 12 h of cold exposure, but increased at 72 h of cold exposure. We hypothesize that when the [$Na^+$] differential was reversed (i.e. when hindgut [$Na^+$] exceeded hemolymph [$Na^+$]), $Na^+$ instead migrated from the hindgut into the hemolymph during the first 12 h of cold exposure. This is perhaps not surprising, given that $Na^+$ is expected to leak down a concentration gradient during chill coma (Macmillan and Sinclair, 2011b). Although we did not observe a corresponding increase in hemolymph [$Na^+$] at 12 h, it is possible that a bulk influx of $Na^+$ also drew water concomitantly from the surrounding tissues (Des Marteaux and Sinclair, 2016); however we must limit this speculation in the absence of hemolymph volume measurements. We also speculate that overall gut contents might have leaked from the gut to the hemolymph during the first 12 h of chill coma; because crickets ate a substantial amount of diet, their guts were full and thus a high hydrostatic pressure could have opposed the electrochemical gradients driving water and ions into the gut from the hemolymph (MacMillan and Sinclair, 2011b). Alternatively, crickets may be expelling excess hindgut $Na^+$ some time between 0-12 h either through defecation or regurgitation.

Despite faster chill coma recovery times in crickets fed on medium and high [$Na^+$] diets, we did not observe a lower gut $Na^+$ content, higher hemolymph [$Na^+$] or any differences in gut water content or hemolymph [$K^+$] compared to crickets fed on low [$Na^+$] diets. We speculate that this faster chill coma recovery could be driven by patterns of ion and water balance during the first 12 h of chill coma. Because we observed an equilibrium of $Na^+$ differentials after 12 h of cold exposure, it is difficult to link faster chill coma recovery with changes in ion balance. Thus, future experiments should measure $Na^+$ flux between the hemolymph, hindgut, and surrounding tissues at several time points during this first 12 h of cold exposure to determine how a high [$Na^+$] diet affects the movement of $Na^+$ in early chill coma, and the distribution of $Na^+$ among tissues in the absence of active transport should be traced more effectively.

We predicted that reversing the [$Na^+$] differential between the hemolymph and hindgut would reduce bulk movement of water towards the gut during cold exposure, but this is not what we observed. Irrespective of the [$Na^+$] in the hindgut, or the length of time exposed to 0 °C, hindgut water volume did not increase appreciably. There was a slight increase in water volume between 12 and 72 h of cold exposure, but it was not statistically significant. We speculate that this small volume of water influx to the gut between 12 and 72 h, irrespective of diet, could be driven by cold-induced changes to gut permeability during chill coma. Water likely leaks into the gut through paracellular channels, and the integrity of junctions in these paracellular channels decreases with cold exposure (MacMillan et al., 2017). Thus, in this experiment, cold-induced epithelial barrier disruption could be more important than the hemolymph-gut [$Na^+$] differential in determining the flow of bulk water in *G. pennsylvanicus*, despite our initial predictions.

We predicted that reversing hemolymph-hindgut [$Na^+$] differentials would reduce the extent of hyperkalemia in the hemolymph during cold exposure, which is not what we observed. This suggests that faster chill coma recovery time in crickets fed on medium and high-[$Na^+$] diets was not driven by the extent of cold-induced hyperkalemia *per se,* but rather by the extent of hypernatria in the hindgut. Faster chill coma recovery time in chill susceptible insects, including *G. veletis* crickets (Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016), is often associated with lower basal levels of hemolymph [$Na^+$]. When compared to *G. pennsylvanicus*, *G. veletis* have lower basal hemolymph [$Na^+$] and consequently a reduced [$Na^+$] differential between

the hemolymph and hindgut. (Des Marteaux and Sinclair, 2016). At 0 °C, *G. veletis* maintain $Na^+$ and water balance better than *G. pennsylvanicus*, but both species suffer increased hemolymph [$K^+$] to the same extent during cold exposure (Des Marteaux and Sinclair, 2016), which is concordant with our observations in crickets fed on varying [$Na^+$] diets.

**Reversing [$Na^+$] differentials impairs survival after prolonged cold exposure**

Manipulating the hemolymph-hindgut [$Na^+$] differential reduced survival after a prolonged (72 h) cold exposure, which contradicts our prediction that crickets fed on a high [$Na^+$] diet would exhibit less leak of $Na^+$ towards the gut and therefore have increased survival after cold exposure. This is surprising given that crickets fed on high [$Na^+$] diets recovered more quickly from chill coma, and fast chill coma recovery time is often correlated with increased survival at low temperatures (Andersen et al., 2015b; Andersen et al., 2017a). We speculate that poor survival following prolonged cold exposure reflects accumulated $Na^+$ stress (discussed below).

Although hindgut water content at 72 h of cold exposure was statistically unaffected by the diets, hindgut [$Na^+$] and $Na^+$ content were higher in all crickets at 72 h relative to 12 h. Furthermore, the greatest increase in both [$Na^+$] and $Na^+$ content was observed for crickets fed on high [$Na^+$] diets. We hypothesize that such a high $Na^+$ content was difficult to regulate during recovery from cold exposure, and thus contributed to higher mortality. Because the hindgut epithelium (rectal pads) drives reabsorption of $Na^+$ at permissive temperatures, it is likely that the cold-attributed reduction in the activity of epithelial $Na^+/K^+$ ATPase prevented adequate regulation of excess gut $Na^+$. Dietary salt stress causes gene expression changes in the insect renal system similar to some of those seen with cold stress (Des Marteaux et al., 2017; MacMillan et al., 2016; Stergiopoulos et al., 2009), so it is possible that the combination of the two stresses had an antagonistic effect, leading to reduced survival. Furthermore, although we focused on the hindgut as an important site for maintaining ion and water balance in the cold, the Malpighian tubules also play an important tole in insect cold and salt tolerance (Andersen et al., 2017b; Yerushalmi et al., 2018). Dietary salt stress can stimulate rates of urine secretion through the release of diuretic factors (such as capa neuropeptides) which are also released during recovery from cold exposure (MacMillan et al., 2018; Terhzaz et al., 2015). We speculate that both cold and salt stress exacerbated tissue injury, ultimately limiting survival after a prolonged cold exposure.

## CONCLUSIONS AND FUTURE DIRECTIONS

It has been well established that the maintenance of ion homeostasis is key for survival of chill-susceptible insects at low temperatures (Overgaard and MacMillan, 2017). Here we show that modifying [$Na^+$] differentials between the hemolymph and the hindgut can partially improve insect performance following short-term cold exposure by facilitating a faster recovery from chill coma. However, irreversible chilling injury during longer-term cold exposure appears to be exacerbated by dietary $Na^+$ stress. We provide further evidence that the ion differential between the hemolymph and hindgut plays a role in determining insect cold tolerance, but that the $Na^+$ differential across the gut does not appear to drive movement of water, or consequently affect the development of hemolymph hyperkalemia during chill coma.

Because insects maintain homeostasis, experimentally manipulating $Na^+$ differentials between the hemolymph and hindgut was challenging, and did not yield the results we might expect for several reasons. First, we measured end point ion concentrations and not ion flux, thus it was difficult to conclude how our manipulations affected the movement of ions during cold exposure. Second, we only focused on how manipulating differentials affected ion and water balance at the hindgut, however it is possible that our manipulations changed the function of other components of the renal system (e.g. the Malpighian tubules or other gut segments). Thus, future studies should aim to measure ion fluxes directly, and across multiple tissues.

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561 **TABLES**

562 **Table 1**. Mean consumption of each experimental diet by individual *G. pennsylvanicus* crickets

563 over 7 h, and total $Na^+$ and osmolyte concentrations of each diet.

| | **Diets with varying [$Na^+$]** | | | **Diets with varying osmolality** | | |
|---|---|---|---|---|---|---|
| **Diet** | **Mean diet consumption (mg ± SD)** | **[$Na^+$] (mM)** | **[total] (mOsm $kg^{-1}$)** | **Mean diet consumption (mg ± SD)** | **[$Na^+$] (mM)** | **[total] (mOsm $kg^{-1}$)** |
| Low | 59 ± 29 | 8.13 | 794.37 | 29±16 | 7.80 | 200.25 |
| Medium | 59 ± 28 | 119.88 | 971.86 | 42±25 | 7.50 | 374.136 |
| High | 16 ± 5 | 540.45 | 1315.07 | 53±24 | 7.31 | 954.95 |

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## FIGURES

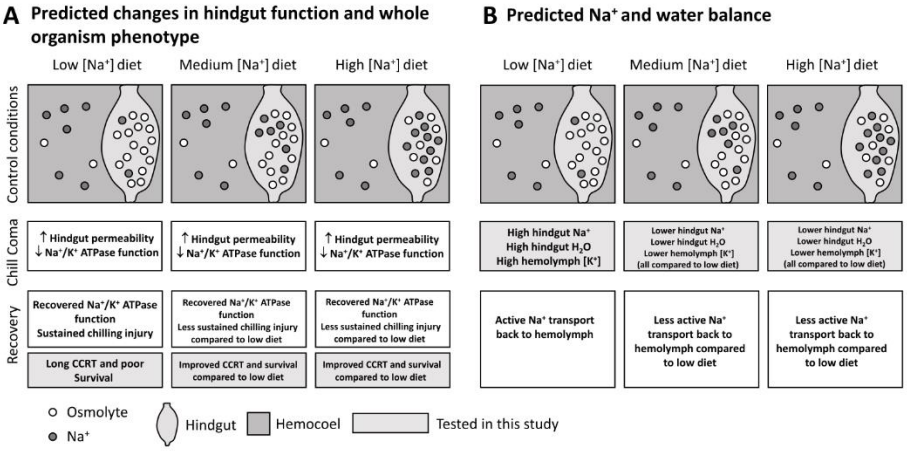
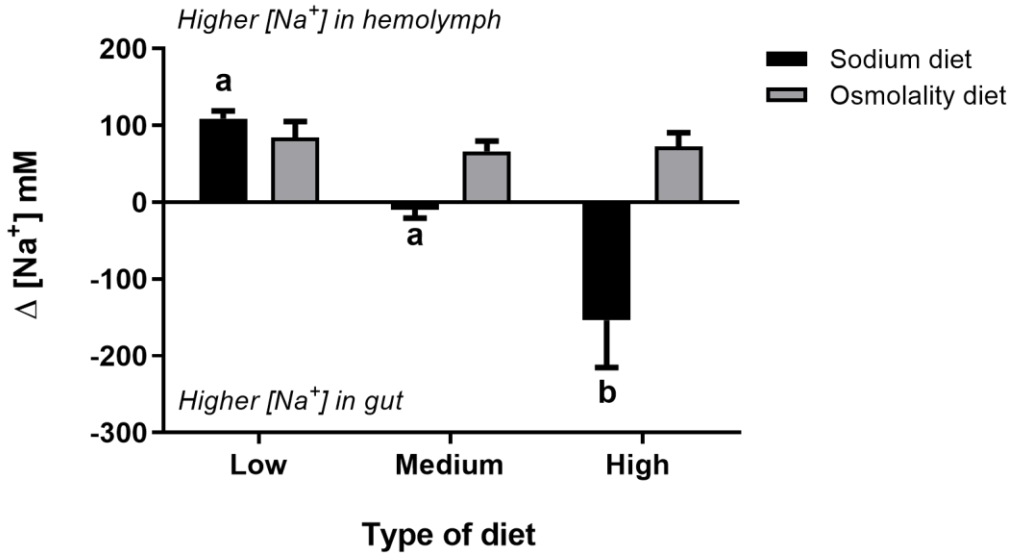



**Figure 1.** A schematic of [$Na^+$] diet manipulations with (A) predicted changes in hindgut function and whole organism phenotype and (B) predicted changes in $Na^+$ and water balance in crickets fed on the different diets. Before crickets enter chill coma (i.e. in control conditions), we assume they maintain $Na^+$ ion and water homeostasis when fed on all diets, and that our diet manipulations persist (see supplementary material for gut retention times of artificial diet). During chill coma, hindgut epithelial permeability increases and $Na^+/K^+$-ATPase activity is reduced. Hindgut permeability and $Na^+/K^+$-ATPase activity are independent of ion differentials between the hemolymph and gut, therefore we predict that they will be unaffected by the different diets. During chill coma, increased hindgut permeability and failure of active transport are thought to drive movement of $Na^+$ (and consequently water) into the gut. We therefore predict that reversing $Na^+$ differentials between the hemolymph and hindgut in crickets fed on medium and high [$Na^+$] diets should reduce/prevent leak of $Na^+$ and water from the hemolymph into the hindgut. We also predict that less active transport will be required to re-establish [$Na^+$] differentials in crickets fed on medium and high [$Na^+$] diets during recovery from chill coma, which will drive faster chill coma recovery time and improve survival following recovery from chill coma.




583

584 **Figure 2.** [$Na^+$] differentials (Δ [$Na^+$]) between the hemolymph and hindgut of *G. pennsylvanicus*
585 crickets fed on diets with varying [$Na^+$] (black) and osmolality (grey) for 7 h, as well as those kept
586 under control conditions (25 °C). The differential is calculated as the difference in [$Na^+$] between
587 the hemolymph and hindgut, thus all values > 0 indicate higher [$Na^+$] in the hemolymph relative
588 to the hindgut, and values < 0 indicate higher [$Na^+$] in the hindgut relative to the hemolymph. Error
589 bars represent ± s.e.m and different letters denote significant differences among diets according to
590 Tukey's HSD ($p < 0.05$). $N$=8 crickets per diet.

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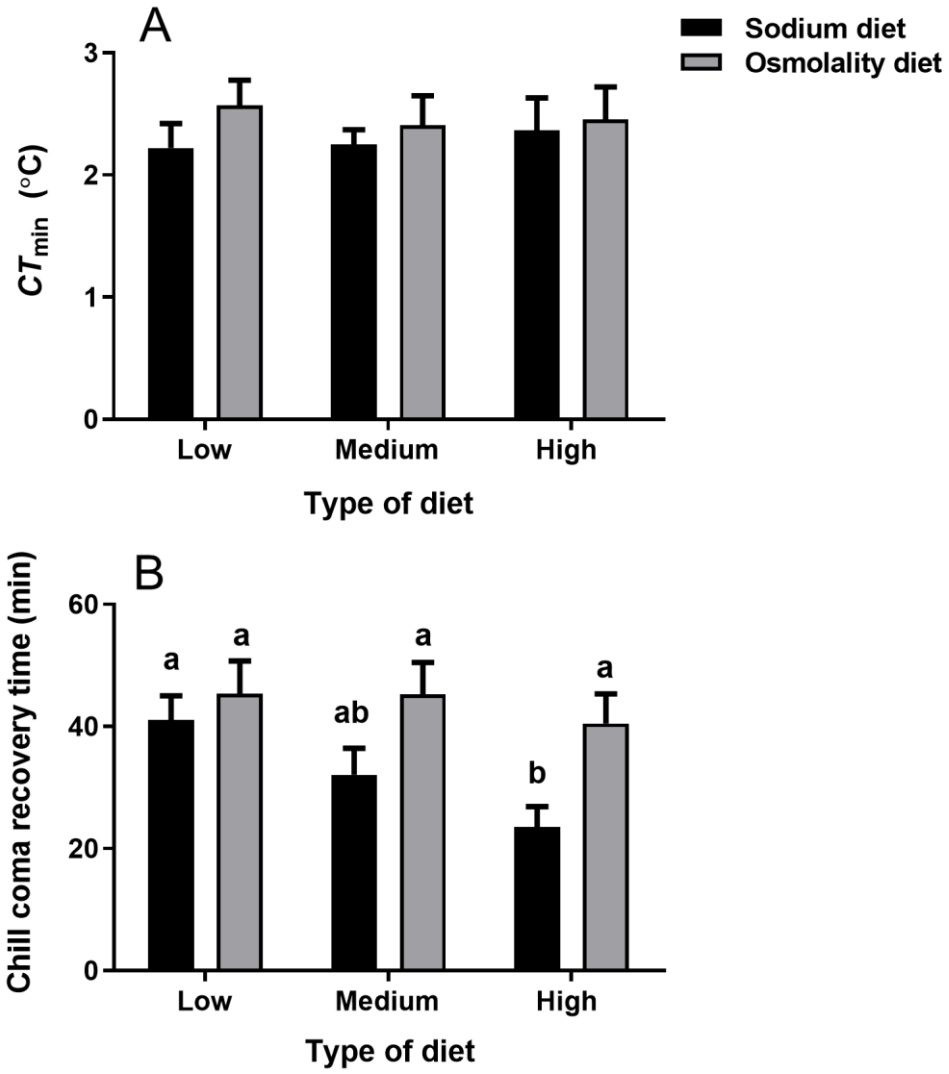
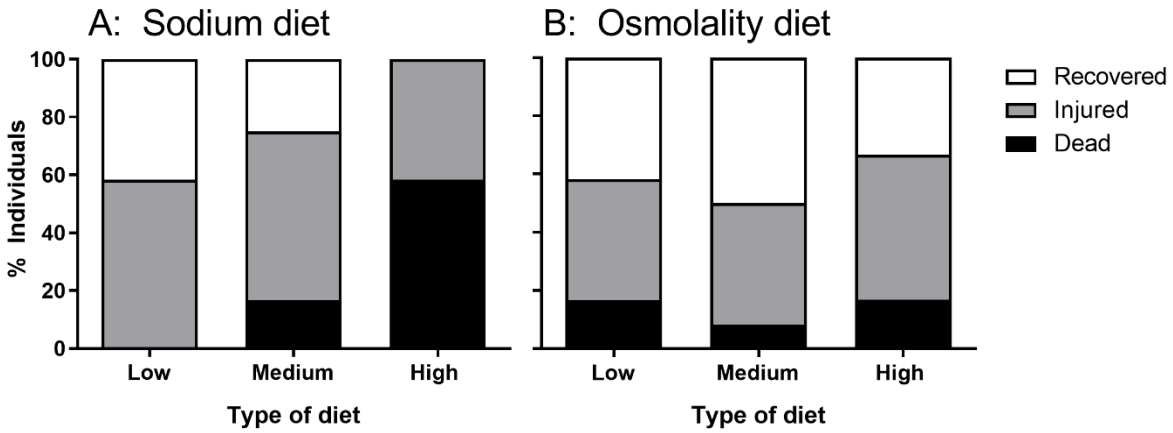



592

593 **Figure 3.** $CT_{min}$ (A) and chill coma recovery time after 12 h exposure to 0°C (B) for *G.*
594 *pennsylvanicus* crickets fed on experimental diets varying in [$Na^+$] (black) or total osmolality
595 (grey). For each treatment-diet combination, the $CT_{min}$ and chill coma recovery time was measured
596 for $N$=8 and $N$=15 crickets, respectively. Bars with different letters on the same panel are
597 significantly different from one another (Tukey's HSD; $p < 0.05$).




598

599 **Figure 4.** Proportion of *G. pennsylvanicus* crickets recovered, injured, and dead after exposure to
600 0°C for three days and 48 h recovery at 24 °C. Crickets had been fed on diets with varying [$Na^+$]
601 (A) and osmolality (B) prior to the cold exposure. *N*=12 crickets per diet.

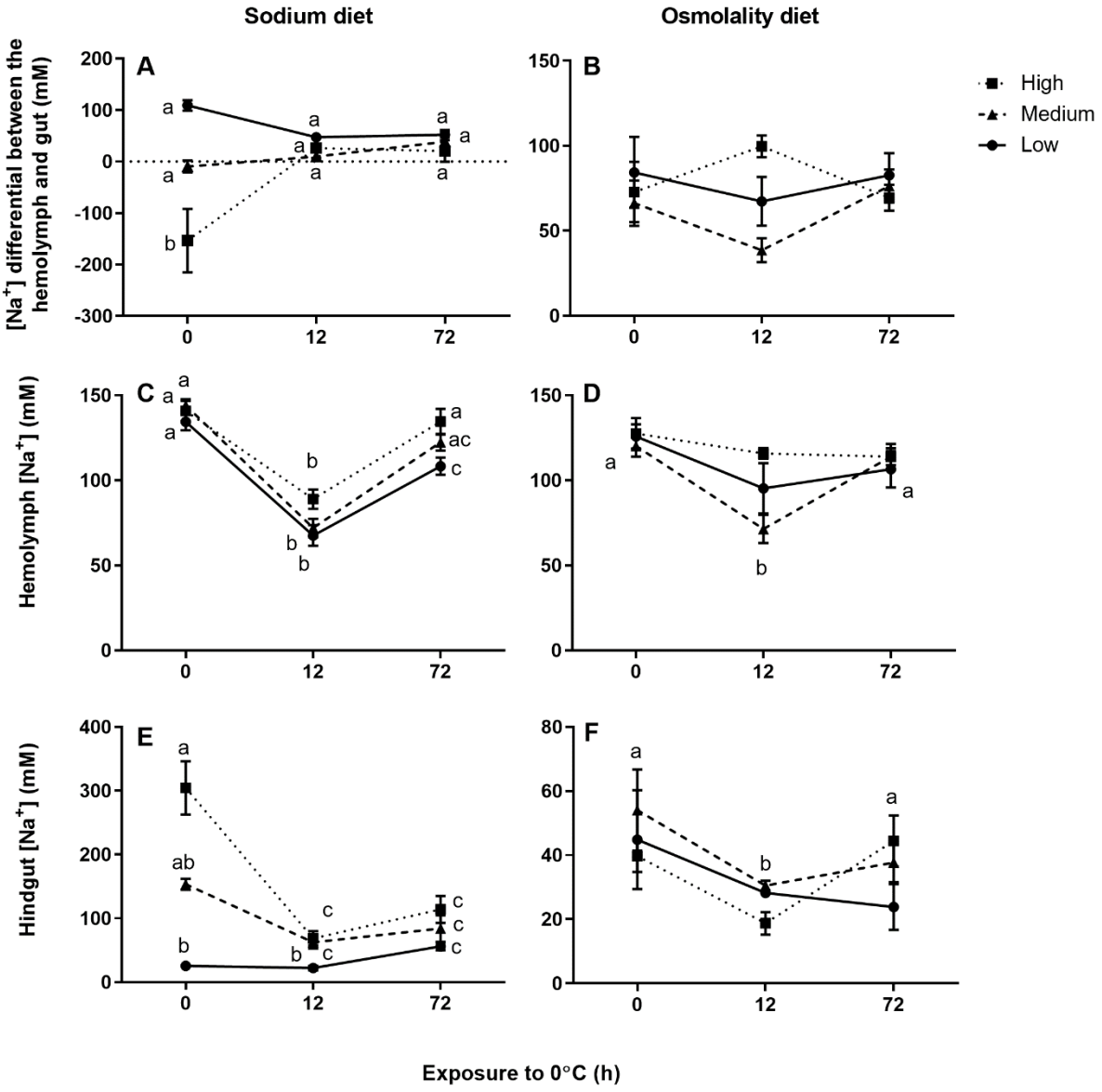
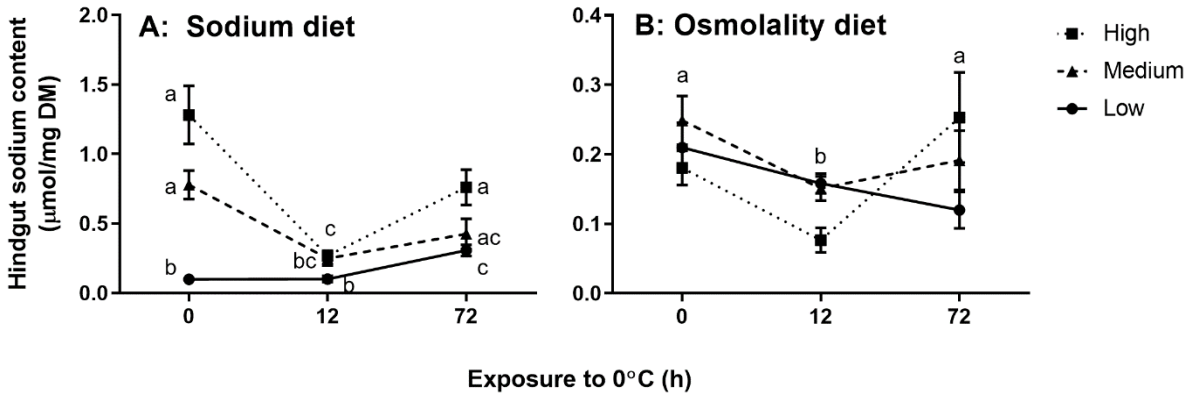



**Figure 5.** Mean ± s.e.m difference in [$Na^+$] between the hemolymph and hindgut (A, B), hemolymph [$Na^+$] (C, D), and hindgut [$Na^+$] (E, F) for *G. pennsylvanicus* crickets fed on diets with varying [$Na^+$] or total osmolality and exposed to 0 °C for 0, 12 or 72 h. Circles represent crickets fed on low [$Na^+$]/osmolality diets, triangles represent crickets fed on medium [$Na^+$]/osmolality diets, and squares represent crickets fed on high [$Na^+$]/osmolality diets. Points with different letters on the same panel are significantly different according to Tukey's HSD ($p < 0.05$). *N*=5-8 crickets per diet per temperature treatment.




610

611 **Figure 6.** Mean ± s.e.m hindgut $Na^+$ content of *G. pennsylvanicus* crickets fed on diets with
612 varying [$Na^+$] or total osmolality and exposed to 0 °C for 0, 12 or 72 hr. Circles represent crickets
613 fed on low [$Na^+$]/osmolality diets, triangles represents crickets fed on medium [$Na^+$]/osmolality
614 diets, and squares represents crickets fed on high [$Na^+$]/osmolality diets. Points with different
615 letters on the same panel are significantly different according to Tukey's HSD ($P < 0.05$). $N$=5-8
616 crickets per diet per temperature treatment.

617

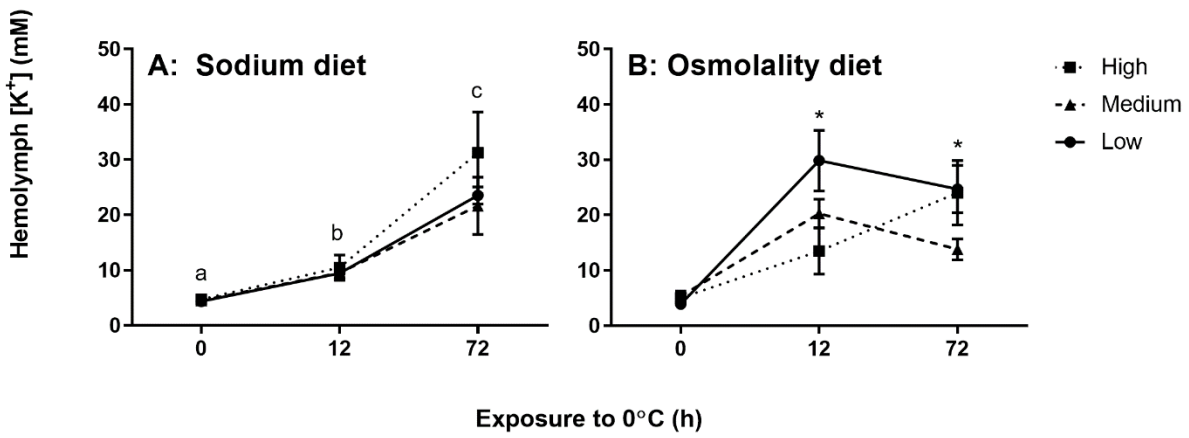
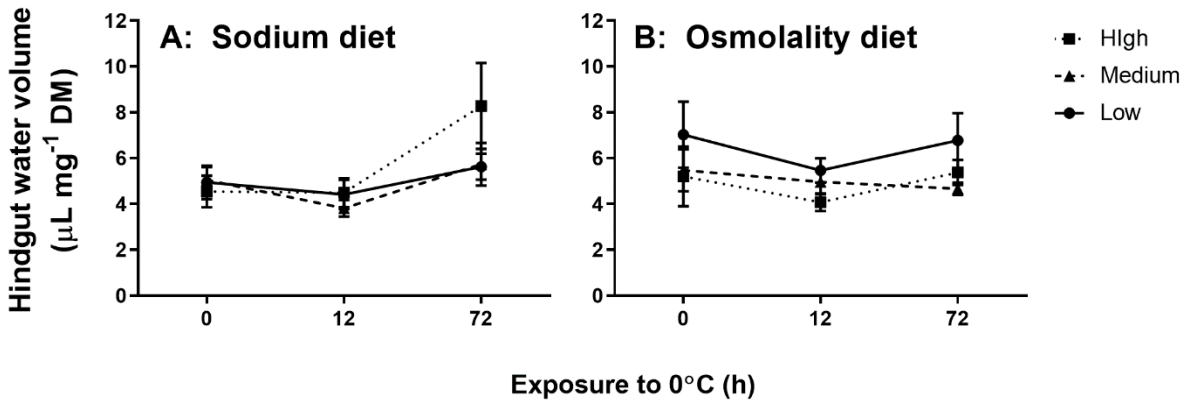



**Figure 7.** Mean ± s.e.m hemolymph [$K^+$] of *G. pennsylvanicus* crickets fed on diets with varying [$Na^+$] and osmolality and exposed to 0 °C for 0, 12 or 72 h. Circles represent crickets fed on low [$Na^+$]/osmolality diets, triangles represent crickets fed on medium [$Na^+$]/osmolality diets, and squares represent crickets fed on high [$Na^+$]/osmolality diets. Different letters on panel A denote significant differences among time points according to Tukey's HSD ($p$<0.05), and asterisks on panel B denote significant differences among time points according to Tukey's HSD ($p$<0.05). $N$=5-8 crickets per diet per temperature treatment.




627

628 **Figure 8.** Mean ± s.e.m hindgut water volume expressed as µl $mg^{-1}$ dry hindgut mass of *G.*
629 *pennsylvanicus* crickets fed on diets with varying [$Na^+$] and osmolality and exposed to 0 °C for 0,
630 12 or 72 h. Circles represent crickets fed on low [$Na^+$]/osmolality diets, triangles represent crickets
631 fed on medium [$Na^+$]/osmolality diets, and squares represent crickets fed on high [$Na^+$]/osmolality
632 diets. Asterisks denote significant differences among time points according to Tukey's HSD
633 ($p$<0.05). $N$=5-8 crickets per diet per temperature treatment.

634