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Abstract

Cold-acclimated insects defend ion and water transport function during cold exposure. We hypothesized that this is achieved via enhanced active transport. The Malpighian tubules and rectum are likely targets for such transport modifications, and recent transcriptomic studies indicate shifts in Na^+-K^+ ATPase (NKA) and V-ATPase expression in these tissues following cold acclimation. Here we quantify the effect of cold acclimation (one week at 12°C) on active transport in the ionoregulatory organs of adult *Gryllus pennsylvanicus* field crickets. We compared primary urine production of warm- and cold-acclimated crickets in excised Malpighian tubules via Ramsay assay at a range of temperatures between 4 and 25°C. We then compared NKA and V-ATPase activities in Malpighian tubule and rectal homogenates from warm- and cold-acclimated crickets via NADH-linked photometric assays. Malpighian tubules of cold-acclimated crickets excreted fluid at lower rates at all temperatures compared to warm-acclimated crickets. This reduction in Malpighian tubule excretion rates may be attributed to increased NKA activity that we observed for cold-acclimated crickets, but V-ATPase activity was unchanged. Cold acclimation had no effect on rectal NKA activity at either 21°C or 6°C, and did not modify rectal V-ATPase activity. Our results suggest that an overall reduction, rather than enhancement of active transport in the Malpighian tubules allows crickets to maintain hemolymph water balance during cold exposure, and increased Malpighian tubule NKA activity may help to defend and/or re-establish ion homeostasis.

Key words: Insect, *Gryllus*, Malpighian tubules, rectum, sodium pump, proton pump, ion homeostasis, phenotypic plasticity

1. Introduction

Chill-susceptible insects lose ion and water homeostasis at temperatures below their critical thermal minimum (the CT_{min}). This loss of homeostasis progresses over hours to days and appears to be driven by gradual migration of Na^+ down a concentration gradient from the hemolymph to the gut lumen (Coello Alvarado et al., 2015; MacMillan and Sinclair, 2011b; Overgaard and MacMillan, 2017). Water follows the migration of Na^+, leading to decreased hemolymph volume and consequent increase in the concentration of hemolymph K^+ (in addition to Mg^{2+} and Ca^{2+}) (Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; Koštál et al., 2006; MacMillan et al., 2015a; MacMillan and Sinclair, 2011b). This ionic imbalance increases the time required for insects to recover from chill coma (Findsen et al., 2013; Koštál et al., 2007; MacMillan et al., 2014; MacMillan et al., 2012), and likely contributes to the accumulation of chronic chilling injuries (Findsen et al., 2014; Koštál et al., 2006; Lee, 2010; MacMillan et al., 2015b). Defense of water and ion homeostasis during cold exposure is improved with prior mild chilling or cold acclimation (Coello Alvarado et al., 2015; Koštál et al., 2006; MacMillan et al., 2015a), but the mechanisms underlying this plasticity are not well understood.

Insects maintain water and ion balance via the Malpighian tubules (which excrete primary urine) and hindgut (across which selective reabsorption of water and ions occurs; O'Donnell and Simpson, 2008; Phillips et al., 1988). Although the primary urine is isosmotic to the hemolymph, excretion by the Malpighian tubules is dependent on ionic gradients established at the apical cell membrane by active and facilitated cation transporter (Beyenbach, 2003). Transporters include the Na^+-K^+-$2Cl^-$ cotransporter (NKCC, which imports Na^+, K^+, and Cl^- across the basolateral cell membrane), carbonic anhydrase (CA, which provides cytosolic protons), and V-ATPase (which pumps protons to the lumen for future exchange with intracellular cations; Chintapalli et al., 2013; Coast, 2012; Halberg et al., 2015). Highly convoluted, mitochondria-dense paracellular channels in the rectal pads form the scalariform complex, in which membrane-bound Na^+-K^+ ATPase (NKA) establishes a high extracellular $[Na^+]$. This Na^+ concentration gradient within the rectal epithelium drives migration of water from the highly-concentrated rectal lumen to the relatively less-concentrated hemolymph (i.e. against an osmotic gradient overall).

During cold exposure, active transport of ions across ionoregulatory epithelia is thought to be exceeded by passive leak of ions down their concentration gradients. Cold-acclimated insects are therefore expected to defend water and ion homeostasis by reducing epithelial permeability (to minimize water and ion leak) and/or by enhancing active ion transport at lower temperatures (MacMillan and Sinclair, 2011a). The latter hypothesis is supported by shifts in the transcription of genes encoding the ion pumps that drive epithelial transport in cold-acclimated *Drosophila melanogaster* (MacMillan et al., 2015c; MacMillan et al., 2016) and fall field crickets [*Gryllus pennsylvanicus* (Burmeister), Orthoptera: Gryllidae] (Des Marteaux et al., 2017). Although cold acclimation increased hindgut NKA mRNA in *G. pennsylvanicus*, V-ATPase mRNA in the Malpighian tubules was instead downregulated with cold acclimation. These transcriptional changes suggest that cold acclimation reduces active transport across the Malpighian tubules while enhancing active transport across the rectum.

We hypothesized that cold acclimation: 1) reduces excretion rates by decreasing Malpighian tubule V-ATPase activity, and 2) increases NKA activity in the rectum (which we expect would enhance reabsorption of Na^+ and water). To test these hypotheses, we compared Malpighian tubule excretion rates (a proxy for active transport) of warm- and cold-acclimated insects, and related recent findings of acclimation-attributed transcriptional changes in NKA and V-ATPase (Des Marteaux et al., 2017) to functional changes in tissue transport via enzyme activity assays in homogenized Malpighian tubules and recta. For this work we used warm- and cold-acclimated *G. pennsylvanicus*; an emerging model system for the study of cold tolerance plasticity and its relation to water and ion homeostasis (Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; MacMillan and Sinclair, 2011b; MacMillan et al., 2012).

2. Materials and methods

2.1 Insect rearing and acclimation

Crickets were reared as described by Des Marteaux and Sinclair (2016). Briefly, crickets were housed in transparent 60 L plastic containers with stacked cardboard egg cartons for shelter, tap water, and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills, Elmira, ON, Canada) and developed under constant summer-like conditions (25°C, 14L:10D photoperiod, 70% RH). Crickets laid eggs in containers of moist vermiculite and sterile sand which were placed at 4°C to accommodate an obligate three-month diapause

(Rakshpal, 1962) before being returned to 25°C to hatch. We used adult female crickets at approximately three months post-hatch for all experiments.

Crickets were isolated in 180 mL transparent cups (Polar Plastics, Summit Food Distributors, London, ON, Canada) with mesh fabric lids, containing egg carton shelters, rabbit food, and water. Warm-acclimated crickets remained in summer-like conditions (25°C, 14L:10D) for the week, while cold-acclimated crickets were placed in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, Illinois) at 12°C, 10L:14D for one week. This acclimation regime lowers the CT_{min} (by 1.7°C), speeds chill coma recovery time 3.5-fold, and reduces the incidence of both mortality and chilling injury following chronic cold exposure (Des Marteaux et al., submitted).

2.2 Dissections

Crickets were pinned through the pronotum and the body cavity was opened by mid-dorsal incision. The Malpighian tubules were removed as a bundle by detaching the ureter from the gut with forceps. The rectum was severed from the rest of the gut with microscissors. Both tissues were immediately placed in droplets of simple Ringer's solution specific to *G. pennsylvanicus* hemolymph: (in mM) 110 Na+, 8.5 K+, 6 Mg2+, 7 Ca2+, 144.5 Cl-, pH 7.6 (derived from Des Marteaux and Sinclair, 2016). Any adhering fat body or tracheae were removed from organs. For Ramsay assays, individual Malpighian tubules were detached from the bundle by severing with forceps as close as possible to the ampulla (where multiple tubules coalesce towards the ureter; Wall et al., 1975).

For enzyme activity assays, entire Malpighian tubule bundles were blotted on tissue paper, flash frozen in liquid nitrogen, and stored at -80°C until use. Recta were cut open with microscissors to empty the lumen of fecal material, blotted on a tissue, and stored on ice for enzyme activity assays performed on the same day. Each replicate for Malpighian tubule enzyme activity assays was comprised of entire Malpighian tubule bundles pooled from five crickets. For enzyme activity assays in the recta, each replicate was comprised of pooled organs from 5-10 crickets (21°C assays) or 8-11 crickets (6°C assay).

2.3 Active transport across the Malpighian tubules (Ramsay assay)

The rate of primary urine excretion (a proxy for active transport function) was quantified by Ramsay assay (Ramsay, 1954), using methodology modified from Rheault and O'Donnell

(2004). Assays were carried out using a custom acrylic enclosure. The top surface of the enclosure contained four, flat-bottomed wells (3.5 cm diameter, 2.5 cm depth) lined with Sylgard 184 (Paisley Products of Canada Inc., Scarborough, ON) and filled with paraffin oil. Well temperature was monitored with type-T thermocouples connected to Picotech TC-08 interface and processed by PicoLog software (Pico Technology, Cambridge, UK). The enclosure was connected to a refrigerated circulator (Model 1157P, VWR International, Mississauga, ON, Canada) filled with a 1:1 mixture of ethylene glycol and water.

Four blocks (5 x 2.5 mm) of Sylgard 184 were affixed to the bottom of each well in the enclosure, and a shallow incision was made by razorblade medially on the top edge of each block. A 10 μL droplet of Ringer's (with 4 mM glucose and 15 mM HEPES added, buffered to pH 7.6) was added 3 mm from each block and one Malpighian tubule was placed individually into each droplet. The proximal end of each tubule was pulled from the droplet through the paraffin oil and 'cleated' into the incision on the edge of a block. The region of tubule between the droplet and block was gently punctured using a dissecting pin to produce an initial bead of primary urine. This first bead was discarded after 15 min. Each tubule was then allowed to excrete through this puncture for 2 h and the diameter of each bead and the length of tubule within the droplet were measured using a microscope with an ocular micrometer. The sum of the bead diameters (assumed to be spherical) was used to calculate volume ($\pi d^3/6$) excreted per hour, and corrected to the length of tubule within the droplet. Malpighian tubule excretion rate was measured at 24, 16, 12, 8, and 4°C (n = 4, 5, 5, 6, and 2 crickets per treatment, respectively). The excretion rate for each cricket was the mean of the excretion rates measured from six individual Malpighian tubules.

2.4 NKA and V-ATPase activity assays

We measured NKA and V-ATPase activity in homogenates of recta and Malpighian tubules from warm- and cold-acclimated crickets using an NADH-linked activity assay as described by Jonusaite et al. (2011) (n = 5-10 per enzyme/organ/acclimation combination). Pooled tissues were diluted in 400 μL in SEID buffer (in mM: 150 sucrose, 10 EDTA, 50 imidazole, and 2.5 Na^+-deoxycholate, pH 7.3) and homogenized on ice for 10 s with a 7 mm attachment on a Polytron PT 10-35 homogenizer (Kinetica, USA). Homogenates were centrifuged at 10000 × g for 10 min at 4°C and the supernatant was collected. Supernatants were diluted 5-fold further with SEID for use in activity assays. A reaction buffer was comprised (in mM) of 47 NaCl, 2.6 $MgCl_2$, 10.5 KCl, 50 imidazole, 0.27 NADH, 2.6 ATP, and 2.1 phosphoenolpyruvate, with 3

184 $U.mL^{-1}$ lactate dehydrogenase (E.C. 1.1.1.27) and 3.75 $U.mL^{-1}$ pyruvate kinase (E.C. 2.7.1.40),
185 pH 7.5.
186
187 Duplicate wells on a 96-well plate each received 10 μL of dilute supernatant and 200 μL of
188 either assay buffer, assay buffer with 5 mM ouabain (to inhibit NKA), or assay buffer with 10
189 mM bafilomycin A1 (to inhibit V-ATPase). NADH absorbance (at 340 nm) of the reaction at
190 21°C was then measured each minute for 30 min in a Multiskan Spectrum spectrophotometer
191 and SkanIt Software (v2.2) (Thermo Scientific, Wilmington, DE, USA), simultaneously for all
192 samples. Total protein concentrations of dilute sample supernatants were quantified by
193 Bradford assay against albumin standards (Kruger, 1994). Enzyme activities were calculated as
194 the difference in rates between reactions with and without enzyme inhibitors, corrected for total
195 protein abundance.
196
197 ## 2.5 Hindgut NKA activity at low temperature
198 To determine whether cold acclimation alters rectal NKA activity during cold exposure we
199 quantified NKA activity in homogenized recta from warm- and cold-acclimated crickets at 6°C
200 using assays modified from MacMillan et al. (2015c). Briefly, recta were diluted in 14 volumes
201 of homogenization buffer (25 mM imidazole, 10 mM β-mercaptoethanol, 0.2% w/v Na^+-
202 deoxycholate, pH 7.5), homogenized with a Polytron PT 10-35, and sonicated with a Virsonic
203 100 (VirTis, Gardiner, NY, USA). Tissues were homogenized and sonicated each in four, 10 s
204 bursts followed by 20 s on ice. Homogenates were then centrifuged at 7000 × *g* for 5 min at
205 4°C and the supernatant was collected. Aliquots (300 μL) of supernatant were filtered through
206 a size-exclusion column (a 3 mL syringe barrel plugged with glass wool, containing 3 mL of
207 Sephadex G50, and equilibrated with homogenization buffer) by centrifuging at 500 × *g* for 1
208 min. The total protein concentrations of filtered supernatants were quantified by Bradford assay
209 against albumin standards.
210
211 We added 10 μL of filtered sample to each of four ultra-micro cuvettes; one pair of cuvettes
212 then received 350 μL of reaction buffer (30 mM KCl, 156 mM NaCl, 7.8 mM $MgCl_2$, 74 mM
213 imidazole, pH 7.5), while a second pair of cuvettes received 350 μL of reaction buffer also
214 containing 1.0 mM ouabain. Phosphoenolpyruvate, NADH, lactate dehydrogenase, and
215 pyruvate kinase were then added (final reaction concentrations of 4 mM, 300 mM, 20 $U.mL^{-1}$,
216 and 20 $U.mL^{-1}$, respectively). Reactions were initiated by adding 40 μL of 50 mM ATP in
217 reaction buffer.

NADH absorbance of each reaction was recorded five times per minute for 20 min at 21°C or 6°C (n = 6 biological replicates per acclimation) in a Cary 100 Bio spectrophotometer (Varian, Palo Alto, CA, USA) using WinUV Thermal Application software (v3.0, Agilent Technologies). Temperature was maintained with a Cary Temperature Controller (Varian, Palo Alto, CA, USA). To monitor temperature, a type-T thermocouple connected to a TC-08 interface was placed in a blank microvolume cuvette containing water. Enzyme activities were calculated as the difference in rates between reactions with and without ouabain, corrected for total protein abundance.

2.6 Data analyses

We compared the Malpighian tubule excretion rates (Ramsay assays) of warm- and cold-acclimated crickets by two-way ANOVA. Enzyme activities of warm- and cold-acclimated crickets were compared by t-tests (or Welch's t-tests when variance differed between acclimation treatments). Values reported in the text are means ± s.e.m. All statistical analyses were performed in R (v3.3.3, R Development Core Team, 2017).

3. Results

3.1 Active transport across the Malpighian tubules

The rate of fluid excretion by the Malpighian tubules decreased with temperature ($F_{1,40}$ = 102, P < 0.001). The Q_{10}s of secretion rate for warm- and cold-acclimated tubules were 2.2 and 1.9, respectively (calculated between 15.4°C and 24.8°C). Rates of fluid excretion by cold-acclimated crickets were approximately 35% slower compared to warm-acclimated crickets based on a linear model ($F_{1,40}$ = 20.5, P < 0.001; Fig. 1). We observed no significant interaction between temperature and acclimation ($F_{1,40}$ = 0.046, P > 0.8).

3.2 Enzyme activities in the Malpighian tubules

The Malpighian tubules of cold-acclimated crickets had higher NKA activity relative to warm-acclimated crickets at 21°C (t_{15} = 2.19, P = 0.045; Fig. 2a). We did not observe a decrease in Malpighian tubule V-ATPase activity with cold acclimation (t_9 = 1.21, P = 0.26). Total protein abundance did not differ between warm- and cold-acclimated Malpighian tubules (t_{24} = 0.47, P = 0.64).

3.3 Enzyme activities in the rectum

NKA activity in homogenized recta was unaffected by cold acclimation at 21°C (t_{15} = 0.78, P = 0.45; Fig. 2b). Rectal NKA activity at 6°C was low (0.0094 ± 0.0026 μmol/mg.min and 0.0070 ± 0.0020 μmol/mg.min for warm- and cold-acclimated crickets, respectively) and did not differ between acclimations ($t_{9.4}$ = 0.74, P = 0.48). Similarly, V-ATPase activity was equivalent in the recta of warm- and cold-acclimated crickets (t_{17} = 1.45, P = 0.16; Fig. 2b).

4. Discussion

We hypothesized that cold-acclimated insects should defend hemolymph volume by slowing fluid excretion rates of Malpighian tubules, and that this would be driven by a reduction in V-ATPase activity. Cold acclimation may have modified active transport across the Malpighian tubules, manifesting as a reduction in fluid excretion rate at both low and optimal temperatures. However, lower rates of fluid excretion were not related to modified V-ATPase activity, rather these slowed rates corresponded with an increase in NKA activity. Although we expected cold acclimation to increase rectal NKA activity (a means of enhancing water and ion reabsorption), we observed no such change at either 6°C or 21°C.

Cold acclimation reduces fluid excretion rates of the Malpighian tubules

Fluid excretion by the Malpighian tubules is driven by active ion transporters, most of which are temperature-sensitive (Dietz et al., 2001; Galarza-Muñoz et al., 2011; O'Donnell and Simpson, 2008; Somero, 2004). MacMillan and Sinclair (2011a) hypothesized that cold acclimation modifies active ion transport such that ion pumping rates are maintained at lower temperatures compared to warm-acclimated insects; however, we show that the Malpighian tubules of cold-acclimated crickets excrete fluid more slowly across a range of temperatures. In *Eurosta solidaginis* larvae, seasonal acclimatization (between September and December) also corresponds with a reduction in the rate of Malpighian tubule transport (Yi and Lee, 2005). By reducing active transport across the Malpighian tubules, cold-acclimated orthopterans may retain hemolymph volume (i.e. mitigate leak of water) during cold exposure. However, this mechanism may not be conserved among insect lineages; in *D. melanogaster*, the Malpighian tubules of cold-acclimated individuals instead excrete fluid more rapidly than warm-acclimated individuals (Yerushalmi et al., 2017), and knockdown of diuretic capa peptides also slows chill coma recovery (Terhzaz et al., 2015). While we expect that active transport modification is

likely to underlie the changes in fluid excretion, it is also possible that cold acclimation reduces Malpighian tubule fluid excretion by reducing epithelial permeability (e.g. by modifying cell junctions or the expression/localization of aquaporins) (Spring et al., 2009).

Proton pumping drives net cation transport across the Malpighian tubules, and V-ATPase is central to this process (Chintapalli et al., 2013; Klein, 1992). Although V-ATPase mRNA abundance is reduced in the Malpighian tubules of cold-acclimated crickets (Des Marteaux et al., 2017), cold acclimation did not reduce the activity of this enzyme in the present study. Decreased fluid excretion rates may therefore involve modification of other enzymes (e.g. NKA or perhaps CA). Carbonic anhydrase mRNA abundance is reduced in the Malpighian tubules of cold-acclimated crickets (Des Marteaux et al., 2017), suggesting that CA is a candidate for this modification. Carbonic anhydrase in the Malpighian tubules provides protons for transport by V-ATPase and potentially the counterions (H^+ and HCO_3^-) for import of hemolymph Na^+ and Cl^- (Beyenbach and Piermarini, 2011; Chintapalli et al., 2013; Wessing et al., 1997). Although we did not measure CA activity in warm- and cold-acclimated crickets, decreased activity of this enzyme could drive decreased primary urine excretion in cold-acclimated crickets. Because CA is a thermally-insensitive enzyme (Feller and Gerday, 1997), cold exposure alone would not be expected to reduce activity.

Cold acclimation increased Malpighian tubule NKA activity, and this should have multiple effects on water and ion balance in the hemolymph. NKA activity in the Malpighian tubules appears to be antidiuretic; in *Rhodnius*, ouabain (an inhibitor of NKA) stimulates transport of Na^+ and fluid to the Malpighian tubule lumen (Maddrell and Overton, 1988), and the diuretic hormone 5-HT inhibits NKA activity (Grieco and Lopes, 1997). It is proposed that NKA inhibition leads to the accumulation of intracellular Na^+, favoring transport of Na^+ and water to the lumen (Caruso-Neves and Lopes, 2000). Increased NKA activity in the Malpighian tubules of cold-acclimated crickets could therefore account in part for the decreased primary urine production rate. NKA activity in the Malpighian tubules also regulates selectivity of excreted cations. For example, inhibition of NKA by ouabain increases the Na^+:K^+ ratio of the primary urine in *Acheta domesticus* crickets (Coast, 2012). Under optimal temperatures (e.g. 21°C), increased Malpighian tubule NKA in cold-acclimated crickets may thereby hasten the removal of K^+ and re-establish low hemolymph [K^+] during recovery from cold exposure (Beyenbach, 2003). As we would predict, chill-tolerant *Drosophila* spp. Also excrete primary urine with lower Na^+:K^+ ratios compared to chill-susceptible species (MacMillan et al., 2015a). Although

we did not measure Malpighian tubule enzyme activities at low temperatures, enhanced NKA activity during cold exposure could prevent or delay imbalance of hemolymph Na^+, water, and K^+ during chill coma (both reducing the CCRT and the energetic costs of re-establishing ionic and osmotic gradients; MacMillan et al., 2012).

Rectal NKA and V-ATPase activities are unchanged by cold acclimation

Because cold exposure results in leak of Na^+ and water towards the gut, an obvious hypothesis is that cold acclimation enhances the activity of rectal pad NKA to mitigate this leak. Increased rectal NKA activity at higher temperatures (i.e. during rewarming) should also speed up re-establishment of Na^+ and water balance thereby reducing chill coma recovery time. An increase in hindgut NKA transcript abundance for cold-acclimated *G. pennsylvanicus* (Des Marteaux et al., 2017) certainly supports this hypothesis. However, we found no evidence of increased rectal NKA activity at 6°C or 21°C. Similarly, although rectal V-ATPase transcript abundance decreases in cold-acclimated *G. pennsylvanicus* (Des Marteaux et al., 2017), V-ATPase activity in rectal homogenates was unchanged by cold acclimation in the present study. The significance of altered NKA and V-ATPase transcript abundance in cold-acclimated *G. pennsylvanicus* therefore remains in question, and further illustrates the point that mRNA abundance does not necessarily reflect increased enzyme abundance (Gygi et al., 1999).

Other enzymes controlling reabsorption across the rectum could be modified by cold acclimation, however many remain to be identified (Chintapalli et al., 2013; O'Donnell and Simpson, 2008). This poses a challenge for predicting how modification of hindgut water or ion transporters may affect transport in the cold. First, active transport across the rectal pads of warm- and cold-acclimated insects should be compared (e.g. via Ussing chamber; Ussing and Zerahn, 1951; Clarke, 2009 or everted-sac technique (Barthe et al., 1998; Lechleitner et al., 1989) to determine whether rectal transport is modified by cold acclimation overall. The specific enzymatic targets of cold acclimation (and their relative contribution to altered transport function) could then be determined by comparing active transport rates across the rectum with and without selective enzyme inhibitors (Bertram et al., 1991; Clarke, 2009; Hanrahan et al., 1984).

An organ-specific role for NKA in cold acclimation?

Acquired cold tolerance is associated with a reduction in whole-body NKA activity in *D. melanogaster* (MacMillan et al., 2015c) and goldenrod gall fly larvae (*Eurosta solidaginis*)

(McMullen and Storey, 2008). However, the functional significance of modified active transport should depend on the specific enzyme and organ in which that modification occurs. In the Malpighian tubules of cold-acclimated *G. pennsylvanicus* we instead observed increased NKA activity, and this should prevent loss of hemolymph volume during cold exposure. It is possible that cold acclimation modifies transport function differently in dipterans than in orthopterans, but we suspect that this contrast is because NKA is ubiquitously expressed and comparisons of whole-body NKA activity are not informative for predicting how cold acclimation affects transport function in specific ionoregulatory organs.

Changes in total protein abundance could not explain increased Malpighian tubule NKA activity, but we did not measure NKA abundance specifically. It is also possible that the abundance of NKA increases proportionally with decreased abundance of other enzymes (e.g. V-ATPase) such that total protein abundance is unaffected. Alternately, cold-acclimated crickets could express NKA isozymes with different activities or thermal sensitivities (Blanco, 2005; Galarza-Muñoz et al., 2011). NKA activity could also be altered by changes in membrane fluidity {Koštál, 1998 #516}, post-transcriptional modifications (e.g. via RNA editing; Colina et al., 2010) or by post-translational modifications (e.g. phosphorylation or dephosphorylation; McMullen and Storey, 2008; Poulsen et al., 2010; Seo and Lee, 2004). Kinase-mediated phosphorylation is already proposed to reduce NKA activity in overwintering goldenrod gall flies (McMullen and Storey, 2008). However, we do not know the extent to which these modifications persist under present assay conditions. Enzyme activity assays for homogenates are also unlikely to capture differences based on modified recruitment of enzymes to the membrane or modified cytoskeletal structure (Khurana, 2000; Lai and Jan, 2006). A first step may be to determine the effect of cold acclimation on the phosphorylation state of target ion transporters (Pavlides et al., 2011).

5. Conclusions

Cold acclimation reduces fluid excretion rates of Malpighian tubules, suggesting an overall reduction in active transport across the insect Malpighian tubules. Decreased excretion rates were not attributed to a reduction in V-ATPase activity (as predicted by transcriptomic changes), but may in part result from increased Malpighian tubule NKA activity. Rectal NKA activity was unchanged by cold acclimation (also contrary to observations of increased hindgut NKA transcript abundance). Modification of Malpighian tubule transport is therefore an

387 important aspect of acquired cold tolerance; by reducing primary urine production, cold-
388 acclimated crickets should mitigate loss of hemolymph volume at low temperatures. Upon
389 rewarming, enhanced NKA activity should allow cold-acclimated insects to re-establish ion
390 balance more rapidly by preferentially retaining hemolymph Na^+ content and excreting
391 hemolymph K^+.
392
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394
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402
403

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539

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Figure captions

Figure 1. Effect of cold acclimation on fluid excretion rate by the Malpighian tubules in adult *G. pennsylvanicus* crickets. Fluid excretion was measured on isolated tubules using the Ramsay assay (n = 12 to 36 tubules per temperature-acclimation combination). The effects of assay temperature and acclimation on excretion rate were both significant according to two-way ANOVA (see text for statistics). Trend lines represent linear models for each acclimation treatment.

Figure 2. Effect of cold acclimation on the activity of Na^+-K^+ ATPase (NKA) and V-ATPase in homogenized Malpighian tubules (A) and recta (B) of *G. pennsylvanicus* crickets. Activity rates were measured at 21°C via NADH-linked assays, and given as moles of ADP converted per hour (corrected for protein concentration in homogenates). Replication for pooled warm- and cold-acclimated Malpighian tubule homogenates was 10 and 7 (NKA) and 6 and 5 (V-ATPase), respectively. Replication for pooled warm- and cold-acclimated rectal homogenates was 8 and 9 (NKA) and 9 and 10 (V-ATPase), respectively. Significant differences in the activity of a given enzyme between warm- and cold-acclimated tissues is represented by an asterisk.

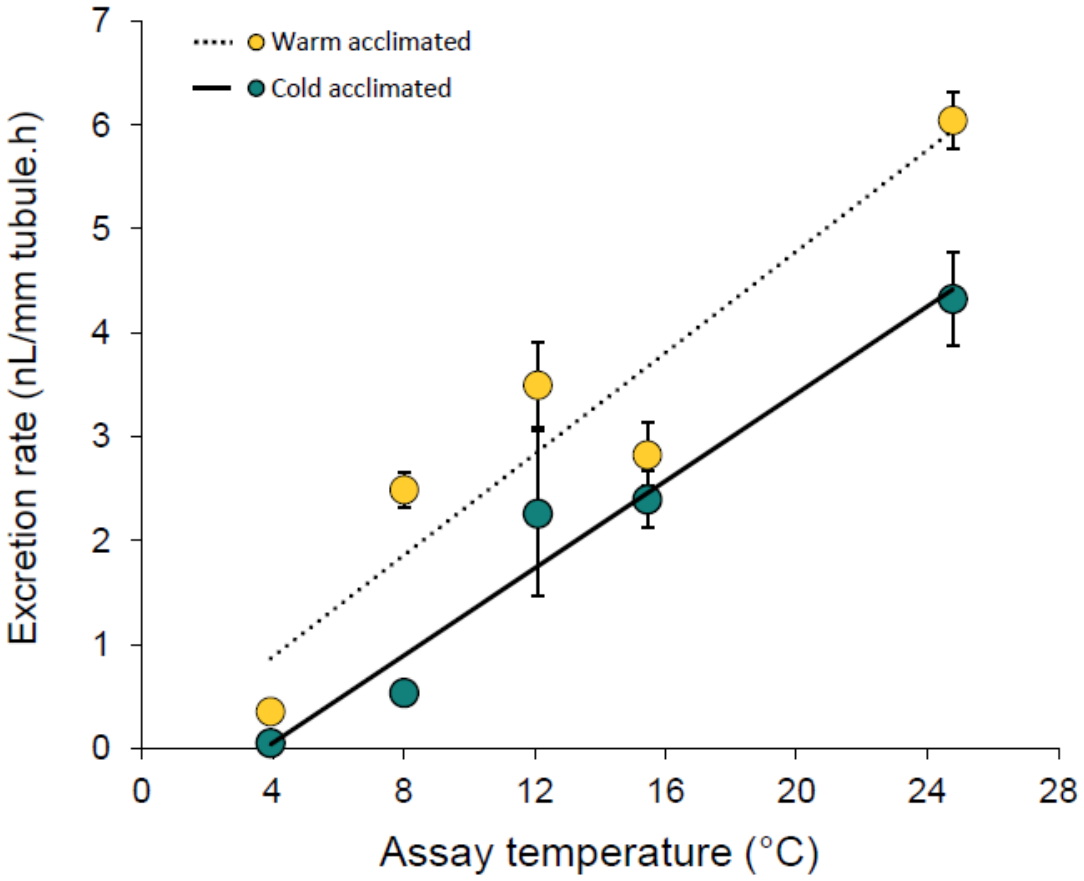

Figure 1

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561

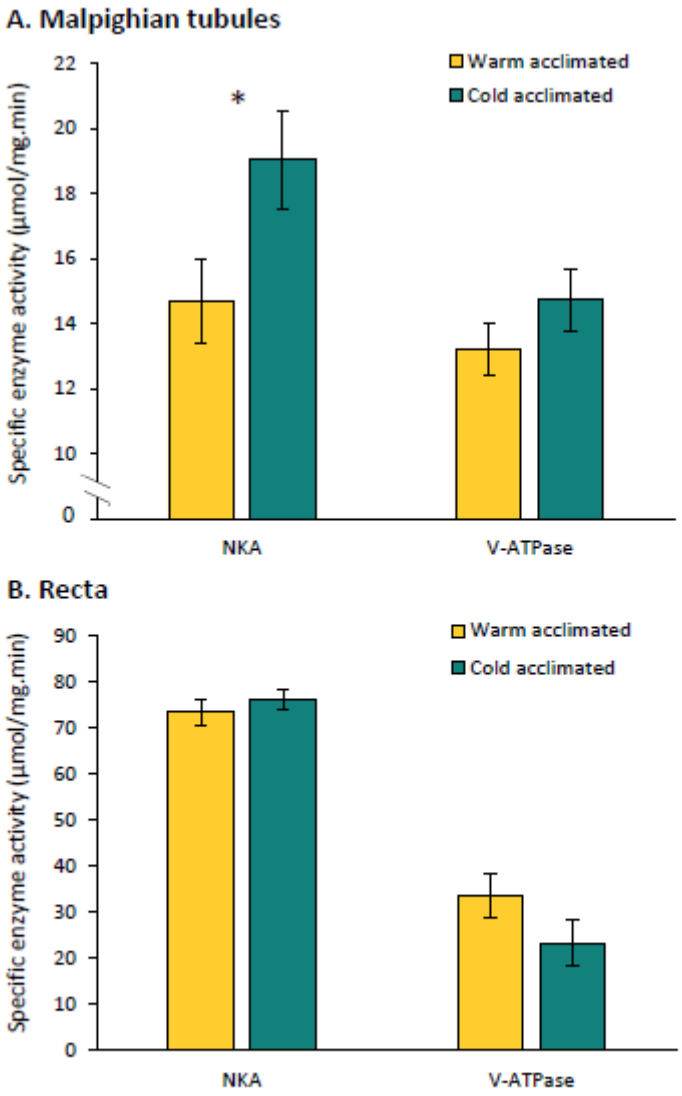
A. Malpighian tubules
Warm acclimated
Cold acclimated
Specific enzyme activity (µmol/mg.min)
22
20
18
16
14
12
10
0
*
NKA
V-ATPase
B. Recta
Warm acclimated
Cold acclimated
Specific enzyme activity (µmol/mg.min)
90
80
70
60
50
40
30
20
10
0
NKA
V-ATPase

Figure 2

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