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Abstract

Cold-acclimated insects maintain ion and water balance in the cold, potentially by reducing permeability or increasing diffusion distance across ionoregulatory epithelia such as the rectum. We explored whether cold acclimation induces structural modifications that minimize water and ion diffusion across the rectum and maintain rectal cell integrity. We investigated rectal structure and cytoskeletal stability in chill-susceptible adult *Gryllus pennsylvanicus* crickets acclimated for one week to either warm (25°C) or cold (12°C) conditions. After acclimation, we used light and transmission electron microscopy to examine rectal macromorphology and rectal pad paracellular ultrastructure. We also used fluorescence microscopy and a filamentous-actin (F-actin) specific phalloidin stain to compare the polymerization state of the actin cytoskeleton for each of the acclimation groups before and after a cold shock (1 h at -4°C). Cold acclimation did not alter rectal pad cell density, or the thickness of the rectal pads, muscle, or cuticle. The tortuosity and width of the rectal pad paracellular channels also did not differ between warm- and cold-acclimated crickets. Rectal pad cells had clear basal and apical regions with differing densities of F-actin. Cold shock reduced the density of F-actin in warm-acclimated crickets, whereas cold-acclimated crickets appeared to have unchanged (basal) or enhanced (apical) F-actin density after cold shock. This suggests that while cold acclimation does not modify rectal permeability through structural modifications to increase diffusion distance for water and ions, cold-acclimated crickets have a modified cytoskeleton that resists the depolymerising effects of cold shock.

Key words: Insect, phenotypic plasticity, actin, gut, cold shock, cytoskeleton

Abbreviations:

CT_{min}	Critical thermal minimum
hsp60	Gene encoding heat shock protein 60
NKA	Na^+-K^+ ATPase
RH	Relative humidity

1. Introduction

Chill-susceptible insects lose ion and water homeostasis at low temperatures and may accumulate chilling injuries if the cold exposure is deep or prolonged (Coello Alvarado et al., 2015; Findsen et al., 2014; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b). The loss of homeostasis is thought to result from the failure of active transport to combat Na^+ leak down concentration gradients (MacMillan and Sinclair, 2011a), leading to bulk migration of Na^+ and water from the hemolymph to the gut (Coello Alvarado et al., 2015; MacMillan and Sinclair, 2011b). To recover from cold exposure, insects must re-establish ion and water balance and repair chilling injuries (Findsen et al., 2013; Findsen et al., 2014; MacMillan et al., 2012). Currently, our understanding of the mechanisms underlying loss of homeostasis in the cold, chilling injury, and how these two process are related, is incomplete (MacMillan et al., 2015c; Overgaard and MacMillan, 2017; Rojas and Leopold, 1996; Štětina et al., 2015; Teets and Denlinger, 2013; Yu et al., 2001). Even less is understood about the mechanisms by which cold-adapted or -acclimated insect populations sustain water and ion balance at lower temperatures and avoid chilling injury (Andersen et al., 2017; Ayrinhac et al., 2004; Gibert and Huey, 2001; Koštál et al., 2006; MacMillan et al., 2015b).

Water and ion homeostasis is maintained by the Malpighian tubules (which excrete primary urine) and the rectum (where water and ions from the gut lumen are selectively reabsorbed, Fig. 1). In orthopterans, reabsorption occurs across specialized epithelia of the rectal pads. The lateral borders of rectal pad cells form meandering channels that are intimately-associated with mitochondria, collectively termed the scalariform complex (Noirot-Timothée and Noirot, 1980; Noirot and Noirot-Timothée, 1976; Wall and Oschman, 1970). Sodium-Potassium ATPase (NKA) in the lateral cell membrane generates a high $[Na^+]$ in the paracellular channels, driving water para- and transcellularly from the lumen across the rectal pads (Phillips et al., 1987; Wall et al., 1970). This absorbate enters the hemolymph via one-way valves in the muscle underlying the rectal pads (Oschman and Wall, 1969). Unlike in the Malpighian tubules, where the lumen is isosmotic to the hemolymph (Ramsay, 1954), the rectal pads establish steep osmotic and ionic gradients between the gut lumen and hemolymph (Dow, 1981; MacMillan and Sinclair, 2011b).

Cold-acclimated insects defend water and ion balance to lower temperatures than warm-acclimated insects. This improved low temperature performance likely arises from enhanced active transport function in the cold and/or reduction in epithelial permeability to minimize water and ion leak (Coello Alvarado et al., 2015; Koštál et al., 2004; MacMillan and Sinclair, 2011a, b). Some of these active transport modifications have been explored recently: seasonally-acquired cold tolerance has been related to whole-body NKA activity in the flies *Drosophila melanogaster* and *Eurosta solidaginis* (Koštál et al., 2007; MacMillan et al., 2015d; MacMillan et al., 2012; McMullen and Storey, 2008), and variation in cold tolerance among *Drosophila* spp. has been correlated with selectivity of cation excretion by the Malpighian tubules (MacMillan et al., 2015a). The insect hindgut is structurally plastic (Hopkins, 1967), however alteration of epithelial permeability via structural modifications in the context of cold acclimation has been relatively unexplored. The insect gut is damaged by cold exposure (Izumi et al., 2005; Philip et al., 2008; Sinclair and Chown, 2005; Yi and Lee, 2003; Yi et al., 2007), and this is likely to exacerbate epithelial leak across the rectum. We hypothesize that cold acclimation reduces epithelial permeability (i.e. reduces water and ion leak) by modifying rectal tissue structure. For example, thickening of the rectal pads and associated tissues (e.g. muscle or inner cuticle) would increase water and ion diffusion distance, while narrowing and/or lengthening of the scalariform complex channels would reduce paracellular permeability across the rectum.

In addition to inducing a loss of water and ion homeostasis, cold exposure causes multimeric cytoskeletal components (e.g. actin and tubulin) to depolymerize in plants, mammals, and insects (Belous, 1992; Cottam et al., 2006; Job et al., 1982; Kim et al., 2006; Örvar et al., 2000; Pokorna et al., 2004; Russotti et al., 1997), and this is likely to impair gut transport function (Cantiello, 1995a; Khurana, 2000; Tilly et al., 1996). Cytoskeletal failure may also cause a loss of cell junction integrity and exacerbate paracellular leak of water and ions (Behrens et al., 1993; Belous, 1992; Gonzalez-Mariscal et al., 1984; Turner et al., 1997), while loss of epithelial rigidity could lead to cell swelling or collapse as water traverses the rectum (Berridge, 1972). Actin is particularly important for the regulation of ion transport (e.g. for localizing enzymes to the membrane and maintaining membrane fluidity (Cantiello, 1995a; Hilgemann, 1997; Khurana, 2000), and membrane (transcellular) permeability (O'Donnell and Maddrell, 1983). Depolymerization of actin filaments activates Na^+ channels in amphibian renal cell lines (Cantiello, 1995b) and, in rats,

unpolymerized actin stimulates renal NKA activity by increasing affinity for Na^+ (Cantiello, 1995a). Because NKA activity is crucial for establishing osmotic gradients within the scalariform complex, loss of actin integrity in the cold could directly hinder water and Na^+ reabsorption. Actin depolymerization is also associated with membrane damage in cold-exposed *Delia antiqua* onion maggots (Kayukawa and Ishikawa, 2009), and loss of cytoskeletal integrity could impede repair of chilling injuries (e.g. epithelial wound closure requires localization of actin to the cell membrane; Fernandez-Gonzalez et al., 2009; Fernandez-Gonzalez and Zallen, 2013; Rodriguez et al., 2003).

If the actin cytoskeleton is damaged by cold, then cold acclimation should protect insects from chilling injury and loss of transport function by stabilizing actin at low temperatures (Gerken et al., 2015; Kayukawa and Ishikawa, 2009; Khurana, 2000; Yi et al., 2007). Acquired cold tolerance appears to involve modification of genes associated with the actin cytoskeleton (Gerken et al., 2015; MacMillan et al., 2016; Teets et al., 2012; Torson et al., 2015), which corroborates this hypothesis. Both cryoprotective dehydration and rehydration in the freeze-avoidant Antarctic midge also cause shifts in actin gene expression (Lopez-Martinez et al., 2009). In the dipterans *Culex pipiens* and *D. antiqua*, acquired cold tolerance is associated with defense of F-actin polymerization during cold exposure (Kayukawa and Ishikawa, 2009; Kim et al., 2006). In *C. pipiens*, improved actin filament stability appears to be driven by upregulation of *actin* genes (Kim et al., 2006). Cold acclimation of *G. pennsylvanicus* modifies the expression of multiple actin-associated genes in the hindgut (Des Marteaux et al., 2017), but how these modifications affect cold tolerance or transport function is unknown.

The fall field cricket, *Gryllus pennsylvanicus* (Burmeister) (Orthoptera: Gryllidae) is a chill-susceptible species that has emerged as a model for understanding cold-induced loss of ion and water balance (Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; MacMillan and Sinclair, 2011b). When exposed to 0°C, *G. pennsylvanicus* exhibits chilling injury in as little as 12 h and mortality at 3-5 d (MacMillan and Sinclair, 2011b), however cold tolerance in this species is plastic; prior cold-acclimation lowers the critical thermal minimum, chill coma recovery time, incidence of injury and mortality following cold shock, and improves defense of ion and water homeostasis in the cold (Coello Alvarado et al., 2015). Cold acclimation in this species also causes

differential expression of multiple cytoskeletal genes in the hindgut, many of which are actin-associated (e.g. actin-stabilizing and actin-to-membrane anchoring proteins, Des Marteaux et al., 2017).

Here we test two hypotheses: 1) that cold acclimation alters the structure of the rectum and/or rectal pad scalariform complex, and 2) that cold acclimation protects cytoskeletal integrity at low temperatures. Using warm- and cold-acclimated *G. pennsylvanicus* we measured the macromorphological characteristics of the rectum (via brightfield microscopy), ultrastructure of the scalariform complex (via transmission electron microscopy), and rectal pad actin polymerization before and after cold shock (via fluorescence confocal microscopy).

2. Materials and Methods

Gryllus pennsylvanicus were reared as described by Des Marteaux and Sinclair (2016). Briefly, we reared crickets under constant summer-like conditions (25°C, 14 light:10 dark photoperiod, 70% RH) 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). We collected eggs in containers of moist vermiculite and sterile sand and placed them at 4°C to accommodate an obligate three-month diapause (Rakshpal, 1962) before returning them to 25°C to hatch. We used adult female crickets at approximately three months post-hatch for all experiments.

2.1 Cold acclimation and cold shock

During acclimation, 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, 14 light:10 dark photoperiod) for one week, while cold-acclimated crickets were housed in a Sanyo MIR 154 incubator (Sanyo Scientific, Bensenville, Illinois) at 12°C, 10 light:14 dark photoperiod for one week. We measured the CT_{min} (n = 10 crickets per acclimation) and chill coma recovery time (n = 9 and 8 for warm- and cold-acclimated crickets, respectively) as described previously (Des Marteaux and Sinclair, 2016). To assess survival of chronic cold exposure, we placed warm-

and cold-acclimated crickets (n = 12 and 10, respectively) in 15 mL Falcon tubes immersed in an ice-water slurry at 0ºC for 72 h. Crickets were then returned to 25°C in transparent cups containing food, water, and shelter, and we assessed mortality and injury (uncoordinated locomotion or the inability to jump when prodded) 24 h later.

Eight crickets (four warm-acclimated and four cold-acclimated) were cold-shocked for cytoskeletal stability measurements (described below). For cold shock, crickets were exposed to -4°C for 1 h in loosely-capped 50 mL plastic tubes suspended in a pre-cooled bath of 50:50 methanol:water (Lauda Proline RP 3530, Würzburg, Germany). To assess whether cold shock affected survival, 40 crickets (20 warm-acclimated, 20 cold-acclimated) were cold-shocked, then returned to cups with food and water at 25°C. Survival was assessed 48 h later.

2.2 Rectal macromorphology and ultrastructure

Warm-acclimated crickets (mean ± s.e.m. mass: 491 ± 17 mg) and cold-acclimated crickets (506 ± 36 mg) were size-matched for measurements ($t_{4.3}$ = 0.36, P = 0.73). Warm- and cold-acclimated crickets were secured to Sylgard-lined Petri dish by a pin through the pronotum and dissected. We opened the body cavity by a mid-dorsal incision and pinned the body open to remove the rectum with microscissors. The severed rectum was placed in a droplet of Ringer's solution (110 Na^+, 8.5 K^+, 6 Mg^{2+}, 7 Ca^{2+}, 144.5 Cl^-, pH 7.6, concentrations in mM, derived from measurements of *G. pennsylvanicus*; Des Marteaux and Sinclair, 2016) and the fecal material flushed out with a Ringer's-filled 5 mL syringe.

We used brightfield microscopy to visualize rectal macrostructure in cross-section. Recta were fixed in 10% formalin and embedded, mounted, cross-sectioned, and treated with Movat's stain (Movat, 1955) at the Robarts Research Institute (Molecular Pathology Facility, London, ON, Canada). We captured images of the cross-sections with an AxioImager Z1 Microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), and used Image-Pro Premier software (Media Cybernetics Inc, Rockville, MD) to measure macrostructural features. We counted the number of nuclei (as a proxy for epithelial cell density) and measured the length of all six rectal pads for each cricket (Fig. 3C). Nuclei count per rectal pad was averaged across the six rectal pads for each cricket. For each rectal pad, we made five width measurements (at regular intervals). For the entire cross-

section we made sequential clockwise measurements of outer circular muscle width (at least 75 measurements per section) and cuticle width (at least 30 measurements per section) at regular intervals. We used the grand mean (± s.e.m.) for each metric for each cricket to compare warm- and cold-acclimated individuals (n = 4 per treatment) with Welch's t-tests in R (v3.2.2, R Development Core Team, 2015).

We used transmission electron microscopy (TEM) to visualize the ultrastructure of the mid-cell scalariform complexes in rectal pad epithelia. Recta were fixed (2.5% glutaraldehyde, 0.1 M cacodylate, pH 7.4) and then stained with 1% osmium tetroxide (pH 7.4), with three washes in 0.1 M cacodylate buffer (pH 7.4). Recta were then serially dehydrated in acetone and embedded in Epon-Araldite resin (Electron Microscopy Sciences, Fort Washington, PA, USA), which was polymerized at 60°C for 48 hr. We cut 0.5 μm sections and stained with 2% uranyl acetate (20 min) followed by Reynold's lead citrate (1 min) (Graham and Orenstein, 2007). We imaged sections with a Philips CM10 Transmission Microscope (Philips Electron Optics, Eindhoven, The Netherlands) and AMT Advantage digital imaging system with Hamamatsu Orca 2 MPx HRL Camera (Advanced Microscopy Techniques, Woburn, MA). TEM data file names were encrypted and the measurements were made by an author (JRS) who was blind to the treatment of each cricket. We measured scalariform complex tortuosity (n = 3 cold-acclimated and 4 warm-acclimated crickets, 1-9 different scalariform complexes measured per cricket) and channel widths (n = 4 crickets per treatment, 2-8 channels measured per cricket) using ImageJ software (Schindelin et al., 2015). Tortuosity was quantified as the length of scalariform complex channel relative to the length of a straight trajectory between each end of the channel. We compared measurements from warm- and cold-acclimated crickets using Welch's t-tests in R. All reported values in the text are means ± s.e.m.

2.3 Cytoskeletal stability

To determine whether cold acclimation protects cytoskeletal stability at low temperatures, we quantified polymerized actin density in recta of warm- and cold-acclimated crickets with and without cold shock (described above, n = 4 crickets per treatment combination). Recta were dissected from each cricket and flushed with insect Ringer's (as above). We fixed recta in 4% paraformaldehyde overnight at 4°C and then embedded, cross-sectioned, and mounted the tissues on slides. Tissues were deparaffinized in xylene and progressively rehydrated to 70% ethanol

before rinsing in water and PBS. To stain for filamentous actin (F-actin), we first permeabilized rectal sections for 5 min using 0.1% Triton X-100 in PBS and then applied Background Sniper (Biocare Medical, Concord, CA) for 5 min to reduce background florescence. Sections were stained in PBS with 2.5 % Alexa-Fluor 488 Phalloidin for F-actin and then DAPI (0.6 μM) for nuclei. We used PermaFluor mountant (Thermo Scientific, Mississauga, ON) to reduce fading and stored slides at 4°C until imaging.

We imaged rectal pad F-actin with Zeiss LSM 5 Duo Vario confocal microscope and ZEN Pro software (Carl Zeiss Microscopy GmbH, Jena, Germany). F-actin fluorescence was converted to grayscale for analysis using ImageJ. Fluorescence data file names were encrypted and the measurements were made by an author (JRS) who was blind to the treatment of each cricket. Because F-actin density was consistently higher in the basal region of the rectal pads (generally between the nuclei and basal lamina) compared to the apical region (between the nuclei and apical border), apical and basal F-actin intensities were measured separately. Approximately five rectal pads (but ranging from one to six) were measured for each cricket. For each rectal pad measured, four to five 80 μm^2 regions in each of the apical and basal areas were haphazardly selected to quantify grey pixel intensity. The grey pixel density of the background (regions where no tissue was present) were measured in two 80 μm^2 regions per section and subtracted this value from rectal pad measurements. Grey pixel intensity was averaged first for each section, then averaged for the whole individual prior to analysis. Grey pixel intensity measurements were natural log-transformed prior to analysis to meet the assumption of normality, and these measurements were compared for warm- and cold-acclimated crickets using a three-way ANOVA in R. The standardized effect size for acclimation was calculated as the difference in grey pixel intensity between warm and cold acclimated rectal pads divided by their pooled standard deviation. The standardized effect size for cold shock was calculated as the difference in grey pixel intensity of cold shocked and non-cold shocked rectal pads divided by their pooled standard deviation.

3. Results

Cold acclimation for one week reduced the CT_{min} by approximately 1.7°C ($t_{13.2}$ = 7.9, $P < 0.001$), reduced the chill coma recovery time by 50 min (3.5-fold faster recovery; $t_{8.9}$ = 7.0, $P < 0.001$), and improved survival of chronic cold exposure (only 30% of cold-acclimated crickets were injured by this cold exposure, while 50 and 33% of warm-acclimated crickets were injured and killed, respectively; Fig. 2).

3.1 Rectal macromorphology and ultrastructure

Cold acclimation had no discernible effects on rectal pad length ($t_{4.3}$ = 1.47, P = 0.21) or width ($t_{4.7}$ = 0.67, P = 0.54; Figs. 3, 4A), nor did it alter the thickness of the cuticle ($t_{5.9}$ = 0.11, P = 0.92) or outer circular muscle ($t_{4.7}$ = 0.01, P = 0.99; Figs. 3,4B). Mean nuclear density of cold-acclimated rectal pad cross sections (55 ± 5 nuclei per pad) did not differ from that of warm-acclimated rectal pads (57 ± 4 nuclei per pad; $t_{3.3}$ = 0.26, P = 0.81). Paracellular channel structure differs between apical (Fig. 5) and mid-cell regions (Fig. 6) of the rectal pads. Tortuosity of the mid-cell scalariform complexes did not differ between warm-acclimated crickets (5.3 ± 0.4 μm/μm) and cold-acclimated crickets (5.2 ± 1.5 μm/μm) ($t_{2.3}$ = 0.105, P = 0.925). Similarly, cold acclimation did not alter the mid-cell scalariform channel width (26.4 ± 1.5 nm in warm-acclimated crickets, 29.3 ± 1.7 nm in cold-acclimated crickets) ($t_{4.8}$ = 0.89, P = 0.42; Fig. 6).

3.2 Cytoskeletal stability

The density of F-actin was higher in the cytoplasm basal to nuclei compared to the cytoplasm apical to the nuclei (Fig. 7). These differences may reflect greater density of organelles (e.g. mitochondria) in the scalariform complexes of the basal cell regions (Khurana, 2000). Cold shock alone did not reduce F-actin density (P = 0.098, standardized effect size = -0.53; Fig. 8). Cold acclimation enhanced F-actin following cold shock in the basal cell region ($F_{1,14}$ = 10.7, P = 0.006) but not in the apical region ($F_{1,14}$ = 0.93, P = 0.35).

F-actin density in the basal region following cold shock was higher in cold-acclimated crickets compared to warm-acclimated crickets ($F_{1,14}$ = 11.4, P = 0.004; Tukey's HSD $P < 0.001$, standardized effect size = 0.71). Although we found a significant interaction between acclimation

and cold shock in the apical region ($F_{1,14}$ = 5.1, P = 0.04), we could not determine the driver of this interaction using Tukey's HSD. All crickets survived cold shock, regardless of acclimation.

4. Discussion

Cold acclimation is hypothesized to cause a restructuring of insect ionoregulatory tissues to reduce permeability, thereby preventing loss of water and ion homeostasis during cold exposure (Des Marteaux et al., 2017; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017). Structural modifications that enhance cytoskeletal stability in the cold are also expected to protect ionoregulatory tissues chilling injury and loss of transport function (Kayukawa and Ishikawa, 2009; Kim et al., 2006; Teets et al., 2012; Torson et al., 2015). By comparing rectal tissue structure of warm- and cold-acclimated crickets we show that cold acclimation does not modify rectal macromorphology or the structure of the rectal pad scalariform complex. However, cold acclimation does modify the cytoskeleton such that actin polymerization is protected (and even enhanced) following cold shock.

4.1 Rectal macromorphology and scalariform complex ultrastructure are not targets of cold acclimation

We hypothesized that cold acclimation could reduce hindgut permeability (thereby minimizing leak during cold exposure) by thickening the rectal pads, inner cuticle, and/or rectal musculature to increase diffusion distance. Cold acclimation could also narrow the scalariform complex channels and/or increase the tortuosity of those channels. The benefit of the former to maintain water and ion homeostasis would be two-fold; narrowed channels could prevent leak of Na^+ and water from the hemolymph, while decreased channel volume would reduce the NKA activity required to achieve a given [Na^+] within the channels (thereby mitigating effects of reduced enzyme activity in the cold). In the latter scenario, increased channel tortuosity would increase diffusion (leak) distance. However, we observed none of these predicted structural changes, indicating that if cold acclimation modifies tissue permeability this modification is achieved by other means.

A four-week cold acclimation regime altered tissue structural and cytoskeletal gene expression in *G. pennsylvanicus* hindguts previously (Des Marteaux et al., 2017) and crickets in the present study were acclimated for only one week. However, one week of cold acclimation is as effective at enhancing cold tolerance as a four-week acclimation. Therefore, we assume that any tissue structural modifications involved in prevention of chilling injury or re-establishment of water and ion homeostasis during recovery from cold exposure should be apparent within one week of cold acclimation.

Cold acclimation may cause other hindgut structural modifications that were not apparent using the methods we employed. Cell junctions (e.g. tight and occluding junctions) are temperature-sensitive (Behrens et al., 1993; Gonzalez-Mariscal et al., 1984; Turner et al., 1997) and their failure may contribute to paracellular water and ion leak during cold exposure. Narrowing or reducing the thermal sensitivity of cell junctions in the apical (or basal) rectal pad regions (Fig. 5) could therefore reduce paracellular permeability (Adam, 2015; Hartsock and Nelson, 2008). Indeed, the expression of multiple genes encoding components of tight and adherens (septate) junctions (e.g. *vinculin*, *partitioning defective protein 3*, *protein shroom*, *α-actinin*, and *casein kinase II*) are altered by cold acclimation in crickets (Des Marteaux et al., 2017). In flies, acquired cold tolerance is also accompanied by shifts in the expression of genes involved in cellular adhesion (MacMillan et al., 2016; Teets et al., 2012).

To reduce transcellular permeability, cold acclimation could reduce the abundance or membrane-localization of aquaporins and/or ion channels (Köttgen et al., 2005; Spring et al., 2009). Shifts in the type or abundance of aquaporins appear to be important for acquired freeze tolerance in dipterans (Philip and Lee, 2010; Philip et al., 2008), however aquaporin transcript abundance was not altered with cold acclimation in crickets (Des Marteaux et al., 2017). Aquaporin and ion channel function can also be regulated by post-translational modifications (Seo and Lee, 2004), which we have not investigated. Cold acclimation does reduce the expression of Na^+-K^+-$2Cl^-$ cotransporter and organic anion/cation transporters in cricket hindguts (Des Marteaux et al., 2017). Whether transcriptional or post-translational shifts correlate with a reduction in ion channel abundance and hindgut permeability requires verification. Diffusion could also be minimized via modifications to membrane viscosity (Hazel, 1995)

4.2 Cold acclimation protects the cytoskeleton from cold shock

Both cold acclimation and seasonally-acquired cold tolerance correlate with shifts in cytoskeletal gene expression, and actin stability appears to be particularly important for survival of cold exposure (Gerken et al., 2015; Kim et al., 2006; Teets et al., 2012; Torson et al., 2015). For example, both diapausing and cold-acclimated *D. antiqua* onion maggots defend actin polymerization in the cold (Kayukawa and Ishikawa, 2009). In cold-acclimated *Gryllus* crickets, genes promoting actin stability are upregulated in the hindgut specifically (Des Marteaux et al., 2017). Here we demonstrate that these transcriptional changes likely protect F-actin against depolymerization in the rectal pads during cold shock. Contrary to our predictions, cold shock alone (-4°C for 1 hr) did not significantly depolymerize F-actin in the cricket rectal pads. This cold shock also caused no injury or mortality, and we hypothesize that a more severe cold exposure (i.e. one that causes injury or death) could cause a more substantial cytoskeletal depolymerization.

By quantifying F-actin polymerization before and after cold shock, we show that cold shock can enhance F-actin polymerization if crickets were first cold acclimated. Increased polymerization and distribution of actin with cold exposure was also observed in the midguts of *C. pipiens* (an effect which is more pronounced in diapausing individuals; Kim et al., 2006). It is unclear how cold exposure directly enhances actin polymerization, but this enhancement (and stabilization) could involve phosphorylation of cytoskeletal components (Colinet et al., 2017). Therefore, cytoskeletal modification appears to be shared among diapause and cold acclimation processes. Which molecules actually promote actin stability is unclear; defense of actin polymerization in the cold correlates with *actin* expression in *C. pipiens*, *hsp60* expression in *D. antiqua*, and a plethora of cytoskeleton-associated genes in *G. pennsylvanicus* (Des Marteaux et al., 2017; Kayukawa and Ishikawa, 2009; Kim et al., 2006). The cytoskeleton includes multiple accessory proteins in addition to actin and tubulin, and transcriptional changes in one of these components may not necessarily alter overall cytoskeletal stability. To identify which proteins enhance cytoskeletal stability in the cold, cold tolerance and actin polymerization could be compared for insects with or without knockdown or mutations of candidate cytoskeletal components. We expect that loss of cytoskeletal structure will contribute to transport failure, chronic chilling injury, and/or failure to clear or repair damaged cellular components (Fernandez-Gonzalez and Zallen, 2013; Findsen et al., 2014; Kayukawa and Ishikawa, 2009; Koštál et al., 2006; Lee, 2010; Monastyrska et al., 2009).

Thus, the next step is to understand the mechanisms that link defense of cytoskeletal structure during chronic cold exposure to the direct or indirect prevention of chilling injuries.

4.3 Conclusions

We aimed to demonstrate the functional significance of modified hindgut tissue and cell structural gene expression following cold acclimation. We hypothesized that structural changes to reduce rectal epithelial permeability should prevent water and ion leak during cold exposure, however these permeability changes do not appear to involve modification of rectal macromorphology or rectal pad scalariform complex ultrastructure. Cold acclimation does protect actin from depolymerization at low temperatures, which supports a role for cytoskeletal modification in preventing cellular chilling injury and maintaining transport function in the insect rectum.

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Figure captions

Figure 1. A schematic of the rectum of an orthopteran insect (in cross section) (A) with a simplified schematic of water and ion transport across the rectal pad epithelium (B, detail). An apical semi-permeable cuticle filters the absorbate initially. The lateral borders of the epithelial cells in the mid and basal regions are mitochondria-dense and form meandering channels, together comprising the scalariform complex. High [Na^+] within the channels (established by NKA) favors migration of water and ions from the lumen to the hemolymph (otherwise against osmotic and ionic gradients between the hemolymph and gut). The absorbate passes through valves in the muscle surrounding the gut prior to entry into the hemolymph.

Figure 2. Improved cold tolerance of adult *G. pennsylvanicus* crickets following one week of cold acclimation. Cold acclimation decreased the CT_{min} (A), chill coma recovery time following 12 h at 0°C (B), and mortality following three days at 0°C (C) (see text for statistics).

Figure 3. Representative rectal cross sections from warm-acclimated (A) and cold-acclimated (B) adult *G. pennsylvanicus*. We compared the rectal macromorphology (C) of crickets that were cold-acclimated (12°C, 10 light:14 dark photoperiod for one week) with those that were warm-acclimated (25°C, 14 light:10 dark photoperiod for one week). Sections were stained with Movat's stain: nuclei/elastin (black), ground substance/mucin (blue), cytoplasm/muscle (red).

Figure 4. Effect of cold acclimation on the macromorphology of *G. pennsylvanicus* rectal pads, as measured by brightfield microscopy of stained rectal cross sections. Neither the mean length or width of rectal pads (A) nor the mean thickness of the rectal cuticle and circular muscles (B) differed between warm- and cold-acclimated crickets.

Figure 5. Representative cross sections of *G. pennsylvanicus* rectal pads imaged by TEM. A) a meandering apical paracellular channel (pc_a) separates two rectal pad epithelial cells. lu - lumen. B) the apical and mid-cell intersection is marked by a shift in lateral cell border characteristics; the paracellular channel in close association with mitochondria (m) forms the scalariform complex (sc_m) in the mid-cell region. C) ladder-like structure of the tightly-opposed apical paracellular channel. Sections A and C belonged to warm-acclimated crickets, while section B belonged to a cold-acclimated cricket.

Figure 6. Example cross-sections of *G. pennsylvanicus* rectal pads for ultrastructure measurements. Sections represent the mid-cell region (in the vicinity of the nuclei) and were imaged by TEM at increasing magnification from A to D. A) epithelial cells (ec) are each bordered by meandering scalariform complexes. One nucleus (nu) is visible. B) scalariform complex (sc) between the nuclei of two epithelial cells. This approximate magnification was used for measurements of scalariform tortuosity. C) close association of mitochondria (m) with the paracellular channel (ch) of the scalariform complex. D) the paracellular channel of a scalariform

complex. This magnification was used to measure channel width. Sections A and C belonged to warm-acclimated crickets, while sections B and D belonged to cold-acclimated crickets.

Figure 7. Effect of cold acclimation and cold shock on the density of filamentous actin in the rectal pads of adult *G. pennsylvanicus* crickets. Warm-acclimated (A, B) and cold-acclimated crickets (C, D) were either not cold shocked (control) (A, C) or cold shocked (B, D) at -4°C for 1 h. Images represent one of four crickets from each treatment combination. F-actin was stained with phalloidin (green). ap - apical region, ba - basal region, cm - circular muscle, cu - cuticle, lu - gut lumen, nu - nuclei (blue).

Figure 8. Effect of cold acclimation and acute cold exposure on the polymerization state of actin in *G. pennsylvanicus* cricket rectal pads. Actin polymerization was measured by fluorescence of F-actin. Both warm- and cold-acclimated crickets were either cold shocked (exposed to -4°C for 1 h) or not cold shocked (control). Significant effects of acclimation, cold shock (treatment), or their interaction are indicated above each figure in italics. Error bars represent the s.e.m. Note differing scales for apical (A) and basal (B) regions.