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1 Effects of cold acclimation on rectal macromorphology, ultrastructure,
2 and cytoskeletal stability in *Gryllus pennsylvanicus* crickets

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17 Abstract

18

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

37

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

39

40 Abbreviations:

41	CT _{min}	Critical thermal minimum
42	<i>hsp60</i>	Gene encoding heat shock protein 60
43	NKA	Na ⁺ -K ⁺ ATPase
44	RH	Relative humidity

45 1. Introduction

46 Chill-susceptible insects lose ion and water homeostasis at low temperatures and may accumulate
47 chilling injuries if the cold exposure is deep or prolonged (Coello Alvarado et al., 2015; Findsen
48 et al., 2014; Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair, 2011b). The loss of
49 homeostasis is thought to result from the failure of active transport to combat Na^+ leak down
50 concentration gradients (MacMillan and Sinclair, 2011a), leading to bulk migration of Na^+ and
51 water from the hemolymph to the gut (Coello Alvarado et al., 2015; MacMillan and Sinclair,
52 2011b). To recover from cold exposure, insects must re-establish ion and water balance and repair
53 chilling injuries (Findsen et al., 2013; Findsen et al., 2014; MacMillan et al., 2012). Currently, our
54 understanding of the mechanisms underlying loss of homeostasis in the cold, chilling injury, and
55 how these two process are related, is incomplete (MacMillan et al., 2015c; Overgaard and
56 MacMillan, 2017; Rojas and Leopold, 1996; Štětina et al., 2015; Teets and Denlinger, 2013; Yu
57 et al., 2001). Even less is understood about the mechanisms by which cold-adapted or -acclimated
58 insect populations sustain water and ion balance at lower temperatures and avoid chilling injury
59 (Andersen et al., 2017; Ayrinhac et al., 2004; Gibert and Huey, 2001; Košťál et al., 2006;
60 MacMillan et al., 2015b).

61
62 Water and ion homeostasis is maintained by the Malpighian tubules (which excrete primary urine)
63 and the rectum (where water and ions from the gut lumen are selectively reabsorbed, Fig. 1). In
64 orthopterans, reabsorption occurs across specialized epithelia of the rectal pads. The lateral borders
65 of rectal pad cells form meandering channels that are intimately-associated with mitochondria,
66 collectively termed the scalariform complex (Noirot-Timotheé and Noirot, 1980; Noirot and
67 Noirot-Timotheé, 1976; Wall and Oschman, 1970). Sodium-Potassium ATPase (NKA) in the
68 lateral cell membrane generates a high $[\text{Na}^+]$ in the paracellular channels, driving water para- and
69 transcellularly from the lumen across the rectal pads (Phillips et al., 1987; Wall et al., 1970). This
70 absorbate enters the hemolymph via one-way valves in the muscle underlying the rectal pads
71 (Oschman and Wall, 1969). Unlike in the Malpighian tubules, where the lumen is isosmotic to the
72 hemolymph (Ramsay, 1954), the rectal pads establish steep osmotic and ionic gradients between
73 the gut lumen and hemolymph (Dow, 1981; MacMillan and Sinclair, 2011b).

74

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

93
94 In addition to inducing a loss of water and ion homeostasis, cold exposure causes multimeric
95 cytoskeletal components (e.g. actin and tubulin) to depolymerize in plants, mammals, and insects
96 (Belous, 1992; Cottam et al., 2006; Job et al., 1982; Kim et al., 2006; Örvar et al., 2000; Pokorna
97 et al., 2004; Russotti et al., 1997), and this is likely to impair gut transport function (Cantiello,
98 1995a; Khurana, 2000; Tilly et al., 1996). Cytoskeletal failure may also cause a loss of cell junction
99 integrity and exacerbate paracellular leak of water and ions (Behrens et al., 1993; Belous, 1992;
100 Gonzalez-Mariscal et al., 1984; Turner et al., 1997), while loss of epithelial rigidity could lead to
101 cell swelling or collapse as water traverses the rectum (Berridge, 1972). Actin is particularly
102 important for the regulation of ion transport (e.g. for localizing enzymes to the membrane and
103 maintaining membrane fluidity (Cantiello, 1995a; Hilgemann, 1997; Khurana, 2000), and
104 membrane (transcellular) permeability (O'Donnell and Maddrell, 1983). Depolymerization of actin
105 filaments activates Na^+ channels in amphibian renal cell lines (Cantiello, 1995b) and, in rats,

106 unpolymerized actin stimulates renal NKA activity by increasing affinity for Na⁺ (Cantiello,
107 1995a). Because NKA activity is crucial for establishing osmotic gradients within the scalariform
108 complex, loss of actin integrity in the cold could directly hinder water and Na⁺ reabsorption. Actin
109 depolymerization is also associated with membrane damage in cold-exposed *Delia antiqua* onion
110 maggots (Kayukawa and Ishikawa, 2009), and loss of cytoskeletal integrity could impede repair
111 of chilling injuries (e.g. epithelial wound closure requires localization of actin to the cell
112 membrane; Fernandez-Gonzalez et al., 2009; Fernandez-Gonzalez and Zallen, 2013; Rodriguez et
113 al., 2003).

114
115 If the actin cytoskeleton is damaged by cold, then cold acclimation should protect insects from
116 chilling injury and loss of transport function by stabilizing actin at low temperatures (Gerken et
117 al., 2015; Kayukawa and Ishikawa, 2009; Khurana, 2000; Yi et al., 2007). Acquired cold tolerance
118 appears to involve modification of genes associated with the actin cytoskeleton (Gerken et al.,
119 2015; MacMillan et al., 2016; Teets et al., 2012; Torson et al., 2015), which corroborates this
120 hypothesis. Both cryoprotective dehydration and rehydration in the freeze-avoidant Antarctic
121 midge also cause shifts in actin gene expression (Lopez-Martinez et al., 2009). In the dipterans
122 *Culex pipiens* and *D. antiqua*, acquired cold tolerance is associated with defense of F-actin
123 polymerization during cold exposure (Kayukawa and Ishikawa, 2009; Kim et al., 2006). In *C.*
124 *pipiens*, improved actin filament stability appears to be driven by upregulation of *actin* genes (Kim
125 et al., 2006). Cold acclimation of *G. pennsylvanicus* modifies the expression of multiple actin-
126 associated genes in the hindgut (Des Marteaux et al., 2017), but how these modifications affect
127 cold tolerance or transport function is unknown.

128
129 The fall field cricket, *Gryllus pennsylvanicus* (Burmeister) (Orthoptera: Gryllidae) is a chill-
130 susceptible species that has emerged as a model for understanding cold-induced loss of ion and
131 water balance (Coello Alvarado et al., 2015; Des Marteaux and Sinclair, 2016; MacMillan and
132 Sinclair, 2011b). When exposed to 0°C, *G. pennsylvanicus* exhibits chilling injury in as little as 12
133 h and mortality at 3-5 d (MacMillan and Sinclair, 2011b), however cold tolerance in this species
134 is plastic; prior cold-acclimation lowers the critical thermal minimum, chill coma recovery time,
135 incidence of injury and mortality following cold shock, and improves defense of ion and water
136 homeostasis in the cold (Coello Alvarado et al., 2015). Cold acclimation in this species also causes

137 differential expression of multiple cytoskeletal genes in the hindgut, many of which are actin-
138 associated (e.g. actin-stabilizing and actin-to-membrane anchoring proteins, Des Marteaux et al.,
139 2017).

140
141 Here we test two hypotheses: 1) that cold acclimation alters the structure of the rectum and/or
142 rectal pad scalariform complex, and 2) that cold acclimation protects cytoskeletal integrity at low
143 temperatures. Using warm- and cold-acclimated *G. pennsylvanicus* we measured the
144 macromorphological characteristics of the rectum (via brightfield microscopy), ultrastructure of
145 the scalariform complex (via transmission electron microscopy), and rectal pad actin
146 polymerization before and after cold shock (via fluorescence confocal microscopy).

147

148 2. Materials and Methods

149 *Gryllus pennsylvanicus* were reared as described by Des Marteaux and Sinclair (2016). Briefly,
150 we reared crickets under constant summer-like conditions (25°C, 14 light:10 dark photoperiod,
151 70% RH) in transparent 60 L plastic containers with stacked cardboard egg cartons for shelter, tap
152 water, and *ad libitum* commercial rabbit food (Little Friends Original Rabbit Food, Martin Mills,
153 Elmira, ON, Canada). We collected eggs in containers of moist vermiculite and sterile sand and
154 placed them at 4°C to accommodate an obligate three-month diapause (Rakshpal, 1962) before
155 returning them to 25°C to hatch. We used adult female crickets at approximately three months
156 post-hatch for all experiments.

157 2.1 Cold acclimation and cold shock

158 During acclimation, crickets were isolated in 180 mL transparent cups (Polar Plastics, Summit
159 Food Distributors, London, ON, Canada) with mesh fabric lids, containing egg carton shelters,
160 rabbit food, and water. Warm-acclimated crickets remained in summer-like conditions (25°C, 14
161 light:10 dark photoperiod) for one week, while cold-acclimated crickets were housed in a Sanyo
162 MIR 154 incubator (Sanyo Scientific, Bensenville, Illinois) at 12°C, 10 light:14 dark photoperiod
163 for one week. We measured the CT_{min} ($n = 10$ crickets per acclimation) and chill coma recovery
164 time ($n = 9$ and 8 for warm- and cold-acclimated crickets, respectively) as described previously
165 (Des Marteaux and Sinclair, 2016). To assess survival of chronic cold exposure, we placed warm-

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

170

171 Eight crickets (four warm-acclimated and four cold-acclimated) were cold-shocked for
172 cytoskeletal stability measurements (described below). For cold shock, crickets were exposed to -
173 4°C for 1 h in loosely-capped 50 mL plastic tubes suspended in a pre-cooled bath of 50:50
174 methanol:water (Lauda Proline RP 3530, Würzburg, Germany). To assess whether cold shock
175 affected survival, 40 crickets (20 warm-acclimated, 20 cold-acclimated) were cold-shocked, then
176 returned to cups with food and water at 25°C . Survival was assessed 48 h later.

177

178 *2.2 Rectal macromorphology and ultrastructure*

179 Warm-acclimated crickets (mean \pm s.e.m. mass: 491 ± 17 mg) and cold-acclimated crickets (506
180 ± 36 mg) were size-matched for measurements ($t_{4,3} = 0.36$, $P = 0.73$). Warm- and cold-acclimated
181 crickets were secured to Sylgard-lined Petri dish by a pin through the pronotum and dissected. We
182 opened the body cavity by a mid-dorsal incision and pinned the body open to remove the rectum
183 with microscissors. The severed rectum was placed in a droplet of Ringer's solution (110 Na^+ , 8.5
184 K^+ , 6 Mg^{2+} , 7 Ca^{2+} , 144.5 Cl^- , pH 7.6, concentrations in mM, derived from measurements of *G.*
185 *pennsylvanicus*; Des Marteaux and Sinclair, 2016) and the fecal material flushed out with a
186 Ringer's-filled 5 mL syringe.

187

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

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

202

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

221 *2.3 Cytoskeletal stability*

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

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

234

235 We imaged rectal pad F-actin with Zeiss LSM 5 Duo Vario confocal microscope and ZEN Pro
236 software (Carl Zeiss Microscopy GmbH, Jena, Germany). F-actin fluorescence was converted to
237 grayscale for analysis using ImageJ. Fluorescence data file names were encrypted and the
238 measurements were made by an author (JRS) who was blind to the treatment of each cricket.
239 Because F-actin density was consistently higher in the basal region of the rectal pads (generally
240 between the nuclei and basal lamina) compared to the apical region (between the nuclei and apical
241 border), apical and basal F-actin intensities were measured separately. Approximately five rectal
242 pads (but ranging from one to six) were measured for each cricket. For each rectal pad measured,
243 four to five 80 μ m² regions in each of the apical and basal areas were haphazardly selected to
244 quantify grey pixel intensity. The grey pixel density of the background (regions where no tissue
245 was present) were measured in two 80 μ m² regions per section and subtracted this value from rectal
246 pad measurements. Grey pixel intensity was averaged first for each section, then averaged for the
247 whole individual prior to analysis. Grey pixel intensity measurements were natural log-
248 transformed prior to analysis to meet the assumption of normality, and these measurements were
249 compared for warm- and cold-acclimated crickets using a three-way ANOVA in R. The
250 standardized effect size for acclimation was calculated as the difference in grey pixel intensity
251 between warm and cold acclimated rectal pads divided by their pooled standard deviation. The
252 standardized effect size for cold shock was calculated as the difference in grey pixel intensity of
253 cold shocked and non-cold shocked rectal pads divided by their pooled standard deviation.

254

255 3. Results

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

261 3.1 Rectal macromorphology and ultrastructure

262 Cold acclimation had no discernible effects on rectal pad length ($t_{4.3} = 1.47$, $P = 0.21$) or width
263 ($t_{4.7} = 0.67$, $P = 0.54$; Figs. 3, 4A), nor did it alter the thickness of the cuticle ($t_{5.9} = 0.11$, $P = 0.92$)
264 or outer circular muscle ($t_{4.7} = 0.01$, $P = 0.99$; Figs. 3,4B). Mean nuclear density of cold-acclimated
265 rectal pad cross sections (55 ± 5 nuclei per pad) did not differ from that of warm-acclimated rectal
266 pads (57 ± 4 nuclei per pad; $t_{3.3} = 0.26$, $P = 0.81$). Paracellular channel structure differs between
267 apical (Fig. 5) and mid-cell regions (Fig. 6) of the rectal pads. Tortuosity of the mid-cell
268 scalariform complexes did not differ between warm-acclimated crickets ($5.3 \pm 0.4 \mu\text{m}/\mu\text{m}$) and
269 cold-acclimated crickets ($5.2 \pm 1.5 \mu\text{m}/\mu\text{m}$) ($t_{2.3} = 0.105$, $P = 0.925$). Similarly, cold acclimation
270 did not alter the mid-cell scalariform channel width (26.4 ± 1.5 nm in warm-acclimated crickets,
271 29.3 ± 1.7 nm in cold-acclimated crickets) ($t_{4.8} = 0.89$, $P = 0.42$; Fig. 6).

272 3.2 Cytoskeletal stability

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

279
280 F-actin density in the basal region following cold shock was higher in cold-acclimated crickets
281 compared to warm-acclimated crickets ($F_{1,14} = 11.4$, $P = 0.004$; Tukey's HSD $P < 0.001$,
282 standardized effect size = 0.71). Although we found a significant interaction between acclimation

283 and cold shock in the apical region ($F_{1,14} = 5.1$, $P = 0.04$), we could not determine the driver of
284 this interaction using Tukey's HSD. All crickets survived cold shock, regardless of acclimation.
285

286 4. Discussion

287
288 Cold acclimation is hypothesized to cause a restructuring of insect ionoregulatory tissues to reduce
289 permeability, thereby preventing loss of water and ion homeostasis during cold exposure (Des
290 Marteaux et al., 2017; MacMillan and Sinclair, 2011a; Overgaard and MacMillan, 2017).
291 Structural modifications that enhance cytoskeletal stability in the cold are also expected to protect
292 ionoregulatory tissues chilling injury and loss of transport function (Kayukawa and Ishikawa,
293 2009; Kim et al., 2006; Teets et al., 2012; Torson et al., 2015). By comparing rectal tissue structure
294 of warm- and cold-acclimated crickets we show that cold acclimation does not modify rectal
295 macromorphology or the structure of the rectal pad scalariform complex. However, cold
296 acclimation does modify the cytoskeleton such that actin polymerization is protected (and even
297 enhanced) following cold shock.

298 *4.1 Rectal macromorphology and scalariform complex ultrastructure are not targets of* 299 *cold acclimation*

300 We hypothesized that cold acclimation could reduce hindgut permeability (thereby minimizing
301 leak during cold exposure) by thickening the rectal pads, inner cuticle, and/or rectal musculature
302 to increase diffusion distance. Cold acclimation could also narrow the scalariform complex
303 channels and/or increase the tortuosity of those channels. The benefit of the former to maintain
304 water and ion homeostasis would be two-fold; narrowed channels could prevent leak of Na^+ and
305 water from the hemolymph, while decreased channel volume would reduce the NKA activity
306 required to achieve a given $[\text{Na}^+]$ within the channels (thereby mitigating effects of reduced
307 enzyme activity in the cold). In the latter scenario, increased channel tortuosity would increase
308 diffusion (leak) distance. However, we observed none of these predicted structural changes,
309 indicating that if cold acclimation modifies tissue permeability this modification is achieved by
310 other means.
311

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

319
320 Cold acclimation may cause other hindgut structural modifications that were not apparent using
321 the methods we employed. Cell junctions (e.g. tight and occluding junctions) are temperature-
322 sensitive (Behrens et al., 1993; Gonzalez-Mariscal et al., 1984; Turner et al., 1997) and their failure
323 may contribute to paracellular water and ion leak during cold exposure. Narrowing or reducing the
324 thermal sensitivity of cell junctions in the apical (or basal) rectal pad regions (Fig. 5) could
325 therefore reduce paracellular permeability (Adam, 2015; Hartsock and Nelson, 2008). Indeed, the
326 expression of multiple genes encoding components of tight and adherens (septate) junctions (e.g.
327 *vinculin*, *partitioning defective protein 3*, *protein shroom*, *α -actinin*, and *casein kinase II*) are
328 altered by cold acclimation in crickets (Des Marteaux et al., 2017). In flies, acquired cold tolerance
329 is also accompanied by shifts in the expression of genes involved in cellular adhesion (MacMillan
330 et al., 2016; Teets et al., 2012).

331
332 To reduce transcellular permeability, cold acclimation could reduce the abundance or membrane-
333 localization of aquaporins and/or ion channels (Köttgen et al., 2005; Spring et al., 2009). Shifts in
334 the type or abundance of aquaporins appear to be important for acquired freeze tolerance in
335 dipterans (Philip and Lee, 2010; Philip et al., 2008), however aquaporin transcript abundance was
336 not altered with cold acclimation in crickets (Des Marteaux et al., 2017). Aquaporin and ion
337 channel function can also be regulated by post-translational modifications (Seo and Lee, 2004),
338 which we have not investigated. Cold acclimation does reduce the expression of $\text{Na}^+\text{-K}^+\text{-2Cl}^-$
339 cotransporter and organic anion/cation transporters in cricket hindguts (Des Marteaux et al., 2017).
340 Whether transcriptional or post-translational shifts correlate with a reduction in ion channel
341 abundance and hindgut permeability requires verification. Diffusion could also be minimized via
342 modifications to membrane viscosity (Hazel, 1995)

343 4.2 Cold acclimation protects the cytoskeleton from cold shock

344 Both cold acclimation and seasonally-acquired cold tolerance correlate with shifts in cytoskeletal
345 gene expression, and actin stability appears to be particularly important for survival of cold
346 exposure (Gerken et al., 2015; Kim et al., 2006; Teets et al., 2012; Torson et al., 2015). For
347 example, both diapausing and cold-acclimated *D. antiqua* onion maggots defend actin
348 polymerization in the cold (Kayukawa and Ishikawa, 2009). In cold-acclimated *Gryllus* crickets,
349 genes promoting actin stability are upregulated in the hindgut specifically (Des Marteaux et al.,
350 2017). Here we demonstrate that these transcriptional changes likely protect F-actin against
351 depolymerization in the rectal pads during cold shock. Contrary to our predictions, cold shock
352 alone (-4°C for 1 hr) did not significantly depolymerize F-actin in the cricket rectal pads. This cold
353 shock also caused no injury or mortality, and we hypothesize that a more severe cold exposure (i.e.
354 one that causes injury or death) could cause a more substantial cytoskeletal depolymerization.

355

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

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

376 *4.3 Conclusions*

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

384

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394

395

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590

591

592 **Figure captions**

593

594 **Figure 1. A schematic of the rectum of an orthopteran insect (in cross section) (A) with a**
595 **simplified schematic of water and ion transport across the rectal pad epithelium (B, detail).**

596 An apical semi-permeable cuticle filters the absorbate initially. The lateral borders of the epithelial
597 cells in the mid and basal regions are mitochondria-dense and form meandering channels, together
598 comprising the scalariform complex. High $[Na^+]$ within the channels (established by NKA) favors
599 migration of water and ions from the lumen to the hemolymph (otherwise against osmotic and ionic
600 gradients between the hemolymph and gut). The absorbate passes through valves in the muscle
601 surrounding the gut prior to entry into the hemolymph.

602

603 **Figure 2. Improved cold tolerance of adult *G. pennsylvanicus* crickets following one week of**
604 **cold acclimation.** Cold acclimation decreased the CT_{min} (A), chill coma recovery time following
605 12 h at $0^\circ C$ (B), and mortality following three days at $0^\circ C$ (C) (see text for statistics).

606

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

613

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

618

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

626

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

634 complex. This magnification was used to measure channel width. Sections A and C belonged to
635 warm-acclimated crickets, while sections B and D belonged to cold-acclimated crickets.
636

637 **Figure 7. Effect of cold acclimation and cold shock on the density of filamentous actin in the**
638 **rectal pads of adult *G. pennsylvanicus* crickets.** Warm-acclimated (A, B) and cold-acclimated
639 crickets (C, D) were either not cold shocked (control) (A, C) or cold shocked (B, D) at -4°C for 1
640 h. Images represent one of four crickets from each treatment combination. F-actin was stained with
641 phalloidin (green). ap - apical region, ba - basal region, cm - circular muscle, cu - cuticle, lu - gut
642 lumen, nu - nuclei (blue).
643

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

650
651