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Cold tolerance of the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae), in Ontario.

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1 Physiological Ecology

2 **Cold tolerance of the Eastern Subterranean Termite, *Reticulitermes flavipes* (Isoptera:**

3 **Rhinotermitidae), in Ontario**

4

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13 **Running Head:** Clarke et al.: Cold tolerance of termites in Ontario

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20 Abstract

21 We characterized the cold tolerance of natural populations of Eastern subterranean termite
22 [*Reticulitermes flavipes* (Kollar) (Isoptera, Rhinotermitidae)] in southwestern Ontario, Canada.
23 We measured cold tolerance in workers from six colonies of termites established from Pelee
24 Island in Lake Erie, and Point Pelee National Park. The mean critical thermal minimum
25 (CTmin), at which termites entered chill coma, ranged from +8.1 to +5.7 °C. Mean supercooling
26 points (SCP, the temperature at which individuals freeze) ranged from -4 to -4.6 °C, and did not
27 differ significantly between colonies, nor was SCP dependent on body size. Individuals survived
28 brief exposure to low temperatures, as long as they did not freeze, but internal ice formation was
29 always lethal, suggesting a freeze avoiding strategy. The LT₅₀ (temperature at which 50% of
30 individuals were killed by a 1 h exposure) was -5.1 °C, but all individuals could survive -2 °C for
31 at least 72 h. Low temperature acclimation (12 °C, 7 d) or hardening (4 °C, 2 h) had no impact
32 on the SCP, but acclimation did slightly increase the CTmin – making the termites less cold
33 tolerant. We conclude that *R. flavipes* is not particularly cold tolerant, and likely relies on
34 burrowing deep into the soil to avoid exposure to temperature to extremes.

35

36 **Key Words:** Thermal biology, social insect, chill coma, supercooling point, freeze avoiding,
37 behavioral avoidance

38

Introduction

39 As ectotherms, the body temperatures of insects reflect the temperature of the environment
40 (Harrison et al. 2012), and low temperatures are considered an important determinant of the
41 poleward range limit of many species (Chown and Nicolson 2004). At low temperatures, most
42 insects enter a reversible state of paralysis (chill coma), and the threshold for induction of chill
43 coma is known as the critical thermal minimum (CTmin; MacMillan and Sinclair 2011). When
44 cooled further, insects will freeze at the supercooling point (SCP). While some ('freeze
45 tolerant') species can survive internal ice formation, others cannot. An alternative strategy to
46 freeze tolerance is to depress the SCP to maintain body fluids in a liquid, supercooled state
47 ('freeze avoidant' insects; Chown and Nicolson 2004). However, the majority of insects are not
48 especially cold-hardy and are killed by low temperatures before ice formation occurs (Lee 2010).
49 CTmin and SCP can vary among populations (e.g., Klok and Chown 2003), across seasons (e.g.,
50 Crosthwaite et al. 2011; Vesala and Hoikkala 2011), and with acclimation pre-treatments (e.g.,
51 Rajamohan and Sinclair 2009; Ransberry et al. 2011). Understanding insect thermal limits holds
52 potential for the bottom-up prediction of range expansions with climate change (Gaston et al.
53 2009). However, populations of insects at higher latitudes tend to be more cold tolerant than
54 their lower-latitude counterparts, so among-population variation must be taken into account
55 when predicting range expansion (Sinclair et al. 2012).

56

57 Although termites (Isoptera) reach their highest diversity in the tropics, some species appear
58 adapted to colder climates (Eggleton 2000). For example, at least two species of dampwood
59 termite (Termopsidae) are adapted to the cool temperate zones of Australia – they express

60 cryoprotectant sugars and proteins in response to low temperature (Lacey et al. 2010). One
61 taxonomic family that has the potential for cold adaptation is the subterranean termites
62 (Rhinotermitidae), which have a broad distribution within temperate zones (Pearce and Waite
63 1994). Of these, *Reticulitermes flavipes* is the most northerly distributed and introduced
64 populations have been recorded at latitudes well north of its native range in North America
65 (Urquhart 1954; Esenther and Gray 1968). In Canada, *R. flavipes* is an established urban pest
66 throughout southern Ontario (Kirby 1965). As well as urban populations (Scaduto et al. in
67 press), *R. flavipes* occurs in at least one natural refuge at the southernmost tip of Ontario (Kirby
68 1965; Raffoul et al. 2011).

69

70 Because of their ability to excavate extensive tunnels, subterranean termites are expected to
71 avoid low temperatures by digging into the soil, where temperatures are buffered. Experimental
72 colonies do dig deeper or avoid cold soil (Strack and Myles 1997; Hu and Song 2007). Hu &
73 Appel (2004) report seasonal variation in the CT_{min} of *R. flavipes* collected in the southern
74 United States (Alabama) – with CT_{min} decreasing from c. +4.2-4.9 °C in summer to c. +1.5 °C
75 in winter. Moreover, these authors report that *R. flavipes* can survive brief exposure (a few
76 seconds) to temperatures below -5 °C, but are killed after longer or colder exposures (Hu and
77 Appel 2004). Davis and Kamble (1994) reported that SCPs increased from -7.4 to -5.5 °C after
78 cold acclimation. Combined with an LLT of c. -5.5 to -6.5 °C when briefly exposed to cold,
79 Davis and Kamble (1994) concluded that *R. flavipes* is freeze tolerant, although they did not
80 present direct evidence of survival of internal ice formation.

81

82 In this study we characterize the low temperature performance of field-collected populations of
83 *R. flavipes* from Ontario. Specifically, we estimate the CT_{min} and SCP for this economically-
84 important species that is a well-known invasive pest in North America. From this information,
85 we infer the most likely cold tolerance strategy that this species uses to persist at the northern
86 edges of its range. We also determine the effect of prior cold exposure on the potential to
87 acclimate to, and recover from, acute cold exposure. From our analyses we speculate on the
88 potential for climate-limited range expansion of this species within North America.

89

90

Materials and Methods

91 **Animal collection and care**

92 We collected termites from free-living populations in Point Pelee National Park (41.9°N,
93 82.5°W; n=3 colonies, NW10, NW11.1, NW11.2) and Pelee Island (41.5°N, 82.4°W; n= 3
94 colonies, PI01, PI02, and PI03) Ontario, as described in Raffoul et al. (2011). From these
95 collections we established contained laboratory colonies of *R. flavipes* in 6 L plastic bins with
96 soil as substrate and cardboard and plywood for food. We misted colonies regularly to maintain
97 humidity, and maintained colonies under ambient light at room temperature (c. 18-24 °C). We
98 used large workers (2-4mg) of mixed sex in all experiments, and held termites in separate dishes
99 containing moistened sand for 24 hrs prior to our physiological assays to standardise feeding
100 state.

101 **Cold tolerance**

102 We determined the CT_{\min} of $n=18$ termites from each colony using a method adapted from Klok
103 and Chown (2003). Specifically, we removed individual termites from their natal colony,
104 weighed them (UMX-5, Mettler, Columbus, OH), and placed each termite into a 200 ml glass
105 beaker that was jacketed in an acrylic enclosure cooled by a 50:50 mix of ethylene glycol:water
106 circulated from a programmable refrigerated bath (VWR Signature, PolyScience, IL). We
107 monitored the temperature inside each chamber with type-T thermocouples (36 AWG, Omega,
108 Laval, QC, Canada) connected to a computer via a Picotech TC-08 thermocouple interface and
109 PicoLog software (PicoLog 5.21.1; Pico Technology, Cambridge, UK). We cooled groups of six
110 termites from 25 to 15 °C at $0.5^{\circ}\text{C}\cdot\text{min}^{-1}$, and allowed them to equilibrate at 15 °C for 10 min
111 before continuing cooling at $0.25^{\circ}\text{C}\cdot\text{min}^{-1}$. Beyond this temperature, we began to probe for
112 activity by gently flipping individual termites with a fine wire every 10 s. We recorded the CT_{\min}
113 as the temperature where the termite could no longer right itself from the dorsal position (after
114 Klok and Chown 2003). We compared the observed CT_{\min} among the six populations using an
115 ANCOVA, with termite mass (mg) as a covariate. We performed all statistical analyses using the
116 R statistical package (R Core Team 2012).

117

118 We measured the SCP of $n=14-16$ termites from each colony. For this analysis, we weighed
119 each termite, and then suspended them by their abdomens with silicone grease on type-T
120 thermocouples (interfaced to a computer as above) within 1.5 ml microcentrifuge tubes. We then
121 placed each tube into a well in an aluminium block cooled with methanol circulated from a
122 Lauda Proline RP855 refrigerated circulator (Lauda-Brinkmann, Wurtzburg, Germany). Groups
123 of individually suspended termites were cooled rapidly (approximately $0.5^{\circ}\text{C}\cdot\text{min}^{-1}$) from room
124 temperature, before being held at 5 °C for 10 min. We then cooled them further at $0.25^{\circ}\text{C}\cdot\text{min}^{-1}$

125 until all the termites had frozen. We inferred the supercooling point for each termite as the
126 lowest temperature reached before beginning of the exotherm from the release of the latent heat
127 of crystallisation (Lee 1991). We compared the observed SCP among the six populations using
128 an ANOVA in R.

129

130 To determine the cold tolerance strategy, we distinguished whether chilled termites were killed
131 by ice formation or simply by cold exposure. We suspended eight individual termites from a
132 single colony (PI03) from thermocouples, and cooled these termites as in the SCP assay above.
133 Once we observed the exotherms from four of the termites (half the individuals), we removed all
134 eight of the termites to room temperature and recorded survival (movement of legs) after 5 min.
135 If all termites survive it would suggest that this population of *R. flavipes* is freeze tolerant. If
136 termites survive cold exposure, but not ice formation, this would indicate freeze avoidance, and
137 if all termites were killed by the cold exposure regardless of whether or not they had frozen, it
138 would suggest chill susceptibility (Crosthwaite et al. 2011).

139

140 We determined the lower lethal temperature (LLT) and temperature at which 50 % of individuals
141 were killed (LT_{50}) by examining survival of groups of termites exposed to temperatures between
142 +5 and -8 °C. Because of the large sample size required, we performed this survivorship
143 analysis only on termites from a single colony (PI03). We weighed termites ($n=8/\text{temperature}$)
144 and placed each individual onto moistened sand in a 1.5mL microcentrifuge tube. We cooled
145 each group in an aluminum block (as above) at approximately $0.5\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ from room
146 temperature to +5 °C, at which point we held the termites for 10 min. After this equilibration

147 period, we further cooled the termites at $0.25\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ to the final exposure temperature, where
148 they were held for one hour before being re-warmed quickly (c. $7^{\circ}\text{C}\cdot\text{min}^{-1}$) to $+5\text{ }^{\circ}\text{C}$. We then
149 removed the termites to room temperature and assessed survival (normal behaviour and
150 coordinated movement) after 24 h. LT_{50} (the temperature at which 50% of termites were
151 expected to survive) was calculated using a logistic regression in R.

152

153 Because our cold exposures were relatively short, we determined the effects of exposure time on
154 survival of a mild sub-zero temperature. Here, we placed groups of five individuals onto moist
155 sand in microcentrifuge tubes, and cooled each group from 25°C to $-2\text{ }^{\circ}\text{C}$ at $0.25\text{ }^{\circ}\text{C}\cdot\text{min}^{-1}$ and
156 held for 6, 12, 24, or 72 hours before being rapidly re-warmed to room temperature. Survival
157 was assessed, as previously described, after 24 h.

158

159 We determined whether two measures of cold tolerance (CT_{min} and SCP) responded to low
160 temperature acclimation or displayed a rapid response similar to rapid cold-hardening (Lee and
161 Denlinger 2010). Termites ($n=20$ for each treatment) from PI03 were either placed in an
162 incubator at $12\text{ }^{\circ}\text{C}$ for 7 d (cold acclimation) or exposed to $4\text{ }^{\circ}\text{C}$ for 2 h (rapid cold-hardening).
163 The acclimated or hardened termites were then divided into two groups of ten individuals and
164 SCP or CT_{min} determined as above. CT_{min} and SCP of acclimated and RCH-pre-treated termites
165 were compared to values from non-pretreated individuals from the PI03 population using
166 ANOVA in R.

167

Results

168
169 *Reticulitermes flavipes* workers entered chill coma at approximately +6 °C (Table 1), with the
170 single highest CT_{min} of +12.1 °C in a small (0.84 mg) individual from PI01. The single lowest
171 value, by contrast, was +3.1 °C, which was recorded in a larger individual (3.9 mg) from colony
172 NW10. The mean CT_{min} (+8.1 °C) of workers from the PI01 colony was higher than that of the
173 other colonies, but an ANCOVA revealed that this was explained primarily by body mass
174 (workers chosen from this colony were considerably smaller than those from other colonies;
175 $F_{1,101}=27.293$, $P<0.001$, Tukey's *post hoc* test $p<0.05$), and when body mass was taken into
176 account, there was no significant difference among the colonies ($F_{5,101}=1.203$, $p=0.313$; Table 1).

177
178 All individuals that froze were killed, whereas those that did not freeze survived brief exposure
179 to temperatures as low as -4.2°C (Table 2), indicating that *R. flavipes* is not freeze tolerant.
180 Mean supercooling points ranged from -4.0 to -4.6 °C, and did not differ among colonies
181 ($F_{5,82}=1.541$, $p=0.186$; Table 1). There was no effect of mass on SCP ($F_{1,81}=0.554$, $p=0.459$),
182 although variation in mass of workers was lower for those used for SCP experiments than those
183 used in CT_{min} determinations (Table 1).

184
185 The LT₅₀ of workers from PI03 was -5.1 ± 0.08 °C, and the lowest temperature survived for 1 h
186 by any individual termite was -5.3 °C. All termites (n=5) recovered from exposure of up to 72 h
187 at -2 °C (data not shown).

188

189 Mass was not a significant predictor of SCP or CTmin in acclimation experiments on workers
190 from PI03, and was excluded from analyses. Acclimation and RCH had no effect on the SCP of
191 *R. flavipes* workers ($F_{2,30}=0.722$, $p=0.494$; Figure 1), but low temperature acclimation did elevate
192 the CTmin of workers by nearly 2 °C ($F_{2,35}=8.33$, $p=0.001$; Figure 1), making them less cold
193 tolerant.

194

195

Discussion

196 We measured critical thermal minima of c. +6 °C, which is lower than the 13 °C value reported
197 by Sponsler and Appel (1991), but higher than the +1.5 - 4 °C observed by Hu and Appel (2004)
198 for subterranean termites collected in Alabama. Hu and Appel (2004) used a cooling rate of 1
199 °C·min⁻¹ in their determinations, which likely led to a lower estimate of CTmin (Terblanche et
200 al. 2007), and may not be as ecologically relevant as the slower rate that we used (0.5 °C·min⁻¹).
201 Dampwood termites (Termopsidae), which more typically inhabit temperate environments,
202 sampled from cold areas of Australia are estimated to have a CTmin below -3 °C. In contrast to
203 subterranean termites that can burrow deep underground, the dampwood termites tend to occupy
204 epigeal microhabitats with minimal thermal buffering (Lacey et al. 2010), which perhaps has
205 selected for lower CTmin. By contrast, *R. flavipes* workers can dig to avoid low temperatures
206 (Strack and Myles 1997; Hu and Song 2007). Soil temperatures recorded at c. 5 cm depth at a
207 location on Pelee Island close to our collection site are below the CTmin estimated from this
208 study, and remain so for the entire winter (H. Udaka & B.J. Sinclair, unpublished observations).
209 Husby (1980) reports that *R. flavipes* nests can be 75 - 95 cm below the surface in southwestern

210 Ontario, and we expect that this depth should yield stable temperatures, that are above the
211 CT_{min} for much of the winter.

212

213 Davis and Kamble (1994) observed mean SCPs below the LLT and a slight increase in SCP
214 during the winter in *R. flavipes* in Nebraska, and suggested that this was consistent with *R.*
215 *flavipes* being freeze tolerant. Davis and Kamble (1994) did not directly observe survival on ice
216 formation, and although a high SCP is observed in most freeze tolerant insects (e.g. Sinclair and
217 Chown 2005), we found that every individual in which ice formation occurred was killed.
218 Individuals that did not freeze survived, and the mean SCP was only slightly above the LT₅₀,
219 suggesting that *R. flavipes* in Ontario is probably freeze-avoiding. While it is possible that the
220 Nebraska population is indeed freeze tolerant, we suggest that further investigations, including
221 the direct observation of individuals that unequivocally survive internal ice formation, are
222 necessary to draw this conclusion. It is more likely that the relatively high SCP and (therefore)
223 low cold tolerance of this species, both in Ontario and Nebraska, reflects a lack of selection for
224 tolerance to very low temperatures because of its ability to behaviorally avoid seasonal cold.

225

226 Insects can modify their cold tolerance over both the short term (e.g. rapid cold-hardening) and
227 through longer-term acclimation, including seasonal acclimatisation (Lee 2010; Lee and
228 Denlinger 2010). Such plasticity has been observed in freeze-avoiding (e.g., Crosthwaite et al.
229 2011), freeze tolerant (e.g., Sinclair 1997; Lee et al. 2006) and in chill-susceptible insects (e.g.
230 Ransberry et al. 2011). Some seasonal variation in cold tolerance has been reported previously
231 for field-collected *R. flavipes* (Hu and Appel 2004), and acclimation in the laboratory has also

232 been reported to improve cold tolerance (Davis and Kamble 1994; Cabrera and Kamble 2001).
233 Thus, it was surprising that the only response to our acclimation and RCH treatments was a
234 slight increase in CT_{min} in individuals exposed to 12 °C for one week. Although many insects
235 use photoperiod cues to initiate modification of cold tolerance (Tauber et al. 1986) most insects
236 do show some plasticity in response to modified temperatures (e.g., Lee and Denlinger 2010),
237 and it is unlikely that a subterranean insect would respond solely to photoperiod. Thus, it
238 appears that *R. flavipes* populations in Ontario have limited or no ability to decrease their SCP or
239 CT_{min} in response to short- or medium-term cold exposure. However, we did not determine
240 whether the cold acclimations improved survival of low temperature exposure, and it is possible
241 that there is plasticity in LT₅₀ that is decoupled from SCP and CT_{min}. However, since the SCP
242 represents the lethal temperature in freeze avoiding species, we would expect a change in
243 thermal tolerance to be accompanied by a change in SCP. We initially detected a significant
244 among-colony difference in CT_{min}, however, closer inspection indicated that this difference was
245 explained by the relationship between body size and CT_{min}. This implies that larger individuals
246 are more cold tolerant, and that there is some ontogenetic difference in CT_{min} within the
247 population. Such a relationship could determine the instars that are able to overwinter, and
248 influence the overwinter success of colonies.

249

250 Our results point to the importance of understanding field behavior for explaining the current
251 distribution of *R. flavipes* and for predicting the ability of this species to expand its northward
252 distribution. Important questions to address in field studies include: 1) What are the thermal
253 conditions deep in the soil at the overwintering site?; 2) which age/size classes overwinter?; and
254 3) Is there mortality over winter, and is this caused by cold, or by reduced energy supply? In

255 addition to going underground during winter, there may be other behaviors that allow
256 subterranean termites to persist in colder habitats. First, *R. flavipes* in Ontario shows a strong
257 affinity for urban environments where human habitations likely provide a temperature refuge
258 during cooler months (Scaduto et al. in press). It is for this reason that this species is best known
259 in Canada as an urban pest. Second, northern populations appear to forego alate dispersal flights
260 and instead found new colonies via neotenic reproduction (inbreeding by immatures) and colony
261 budding (Grace 1996). This ‘short cut’ to colony reproduction that differs from their more
262 southerly counterparts is presumably a response to colder, shorter seasons that characterize
263 northern latitudes, and that render independent colony founding by alates less successful.

264

265 We conclude that geographically separate *R. flavipes* populations in SW Ontario have consistent
266 SCP and CTmin. Our in-depth study of one colony indicates that *R. flavipes* is freeze avoidant,
267 but the generally poor cold tolerance suggests that this species likely relies on behavioral
268 avoidance of low winter temperatures to survive the winter. Studies of termites in the field will
269 be needed to determine whether there are other aspects of overwintering biology that could
270 restrict the northern distribution of this species, but the moderate acute cold tolerance, buffered
271 soil temperatures, and environmentally-responsive reproductive behavior, suggests that the
272 distribution of *R. flavipes* in SW Ontario – and therefore other parts of mid-western North
273 America – is unlikely to be restricted by acute low temperatures.

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276

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366

Tables

367 **Table 1:** Mean \pm sem critical thermal minimum (CTmin) and supercooling point (SCP) of
 368 *Reticulitermes flavipes* from six different colonies from two locations in southern Ontario. The
 369 table also shows the mean body mass for each experimental group of individuals. Different
 370 superscripts denote significant pairwise differences in mass (Tukey's HSD after ANCOVA). See
 371 text for detailed statistics.

372

Colony	CTmin (°C)	Mass (mg)	SCP (°C)	Mass (mg)
Point Pelee				
NW10	6.0 \pm 0.35	3.90 \pm 0.0657 ^c	-4.3 \pm 0.17	3.74 \pm 0.083
NW11.1	6.1 \pm 0.36	3.09 \pm 0.112 ^a	-4.0 \pm 0.17	3.15 \pm 0.113
NW11.2	6.4 \pm 0.31	2.91 \pm 0.143 ^a	-4.1 \pm 0.15	3.10 \pm 0.094
Pelee Island				
PI01	8.1 \pm 0.42	1.69 \pm 0.137 ^b	-4.4 \pm 0.15	3.49 \pm 0.149
PI02	6.1 \pm 0.40	3.08 \pm 0.096 ^a	-4.6 \pm 0.24	3.13 \pm 0.132
PI03	5.7 \pm 0.37	3.12 \pm 0.101 ^a	-4.3 \pm 0.47	3.74 \pm 0.083

373

374 **Table 2:** Survival of individual *Reticulitermes flavipes* workers that did (SCP provided) or did
 375 not experience internal ice formation. All individuals that froze died (indicated by '-'), and all
 376 that did not freeze survived (indicated by '+'). The minimum temperature experienced by
 377 unfrozen individuals was -4.2 °C.

378

Supercooling point (°C)	Survival after rewarming
not reached	+
not reached	+
not reached	+
not reached	+
-1.6	-
-3.1	-
-4.2	-
-4.6	-

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380

381

Figure legends

382

Figure 1: Effect of acclimation (7 days at 12 °C) and rapid cold-hardening (2 h at 4 °C) on

383

critical thermal minimum (A) and supercooling point (B) of *Reticulotermes flavipes* workers.

384

Differing letters indicate statistically significant differences in CTmin among treatments

385

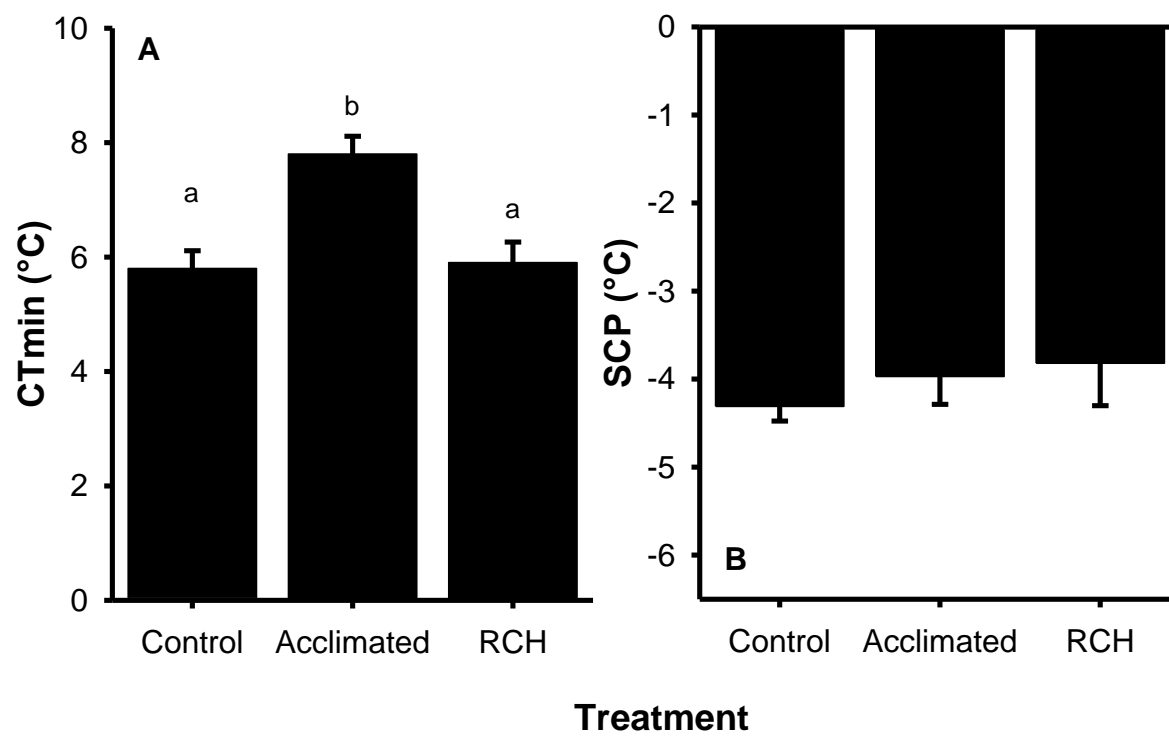
(Tukey's *post hoc* test, $p < 0.05$); treatment did not significantly alter SCP (see text for statistics).

386

387

388 **Figure 1**

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390