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**

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

14

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 LT_{50} (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

Introduction

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

Although termites (Isoptera) reach their highest diversity in the tropics, some species appear adapted to colder climates (Eggleton 2000). For example, at least two species of dampwood 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 CTmin of *R. flavipes* collected in the southern

74 United States (Alabama) – with CTmin 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 CTmin 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°C·min^{-1}, and allowed them to equilibrate at 15 °C for 10 min

111 before continuing cooling at 0.25 °C·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 °C·min^{-1}) from room

124 temperature, before being held at 5 °C for 10 min. We then cooled them further at 0.25 °C·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/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 °C·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 °C·min^{-1} to the final exposure temperature, where

148   they were held for one hour before being re-warmed quickly (c. 7°C·min^{-1}) to +5 °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 °C at 0.25 °C·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 °C for 7 d (cold acclimation) or exposed to 4 °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

168	**Results**
169	*Reticulitermes flavipes* workers entered chill coma at approximately +6 °C (Table 1), with the
170	single highest CTmin 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 CTmin (+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 CTmin determinations (Table 1).
184	
185	The LT_{50} 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

Discussion

196 We measured critical thermal mimina 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 to 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   CTmin 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 LT_{50},

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 CTmin 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   CTmin 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 LT_{50} that is decoupled from SCP and CTmin. 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 CTmin, however, closer inspection indicated that this difference was

245   explained by the relationship between body size and CTmin. This implies that larger individuals

246   are more cold tolerant, and that there is some ontogenetic difference in CTmin 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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Acknowledgements

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Tables

366

367 **Table 1:** Mean ± 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 ± 0.35	3.90 ±0.0657[c]	-4.3 ±0.17	3.74 ±0.083
NW11.1	6.1 ± 0.36	3.09 ±0.112[a]	-4.0 ±0.17	3.15 ± 0.113
NW11.2	6.4 ± 0.31	2.91 ±0.143[a]	-4.1±0.15	3.10 ±0.094
Pelee Island				
PI01	8.1 ±0.42	1.69 ± 0.137[b]	-4.4 ±0.15	3.49 ± 0.149
PI02	6.1 ± 0.40	3.08 ± 0.096[a]	-4.6 ± 0.24	3.13 ± 0.132
PI03	5.7 ±0.37	3.12 ± 0.101[a]	-4.3 ±0.47	3.74 ± 0.083

373

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

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

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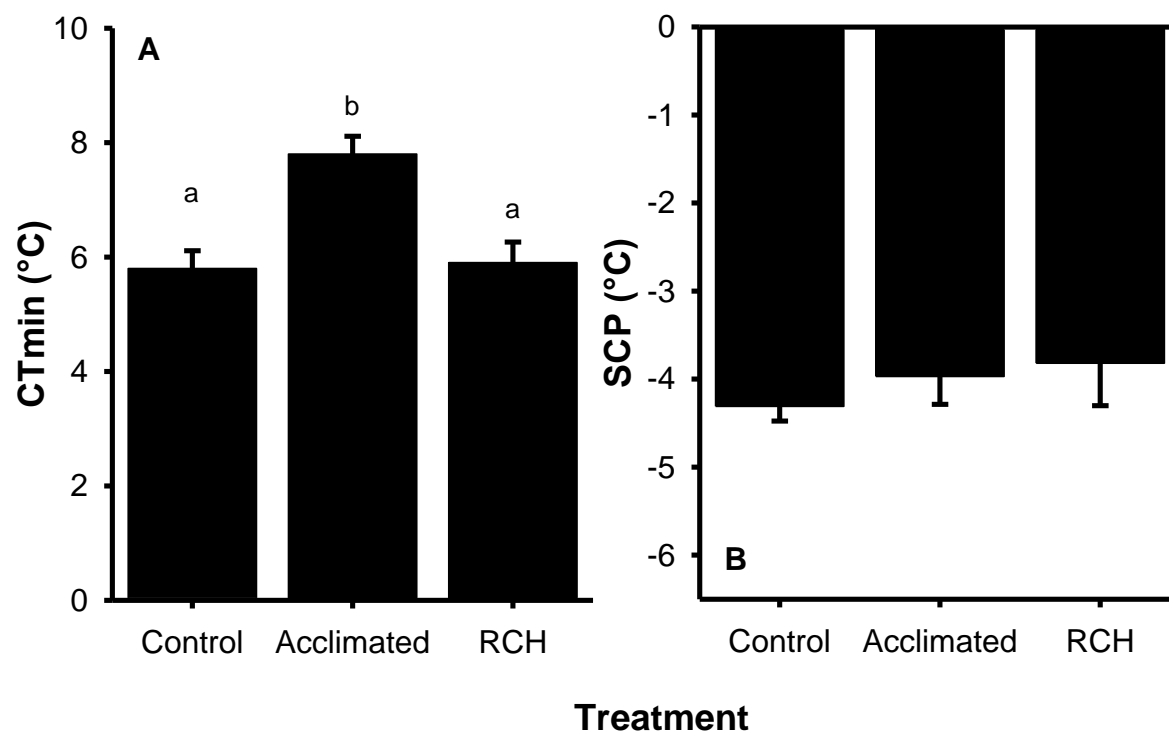
385 (Tukey's *post hoc* test, $p<0.05$); treatment did not significantly alter SCP (see text for statistics).

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387

388   **Figure 1**

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