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1 **Loss of ion homeostasis is not the cause of chill coma or impaired dispersal in false codling**
2 **moth *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae)**

3

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17 **ABSTRACT**

18 Dispersal is a central requirement of a successful sterile insect release programme, but field-
19 released false codling moth (FCM) typically suffer from poor dispersal ability, especially at
20 low ambient temperatures. Here we test the hypothesis that poor activity and dispersal in FCM
21 is caused by delayed or perturbed recovery of ion and/or water homeostasis after chilling for
22 handling and transport prior to field release. Hemolymph and flight muscle were collected from
23 two treatment groups at three time points that targeted thermal conditions above and below the
24 chill coma induction threshold of $\sim 6^{\circ}\text{C}$: 1) control moths kept at 25°C , 2) moths exposed to
25 3°C or 9°C for 4 h, and 3) moths allowed to recover at 25°C for 24 h after exposure to either
26 3°C or 9°C . We measured concentrations of Na^+ , K^+ and Mg^{2+} in the hemolymph and muscle
27 collected at each time point. Exposure to a chill-coma inducing temperature had little effect
28 overall on ion balance in the hemolymph and flight muscle of false codling moth, but
29 hemolymph $[\text{Na}^+]$ decreased from 10.4 ± 0.4 mM to 6.9 ± 0.7 mM as moths were chilled to 3°C
30 and then increased to 10.4 ± 0.9 mM after the 24 h recovery period. In the 9°C cooling treatment,
31 $[\text{K}^+]$ increased from 8.2 ± 0.5 mM during chilling to 14.1 ± 1.9 mM after the 24 h recovery period.
32 No change were seen in equilibrium potentials in either of the ions measured. Thus, we did not
33 find evidence that water and ion homeostasis are lost by the moths in chill coma and conclude
34 that reduced dispersal in field-released moths is not direct a consequence of the costs of re-
35 establishment of homeostasis.

36 **KEYWORDS:** chill coma recovery time; chilling injury; equilibrium potential; ion balance

37

38 INTRODUCTION

39 The false codling moth (FCM), *Thaumatotibia leucotreta* (Meyrick 1913)
40 (Lepidoptera, Tortricidae), is a polyphagous pest of fruit crops including citrus, stonefruit and
41 pomegranates (Prinsloo & Uys 2015). False codling moth is native to sub-Saharan Africa and
42 is also present on Madagascar, St. Helena and the Indian Ocean islands of Mauritius and La
43 Réunion (EPPO 2014). Due to its wide host range and previous interceptions in the USA
44 (Gilligan *et al.* 2011) and Italy (Mazza *et al.* 2014), false codling moth is a phytosanitary
45 concern for exports from South Africa. Large-scale use of insecticides has resulted in
46 insecticide resistance (Hofmeyr & Pringle 1998) and, together with import restrictions on
47 pesticide residue levels, alternative control methods including orchard sanitation, mating
48 disruption, granulosis virus cover sprays and the Sterile Insect Technique (SIT) have been
49 employed to regulate FCM (Moore & Kirkman 2008; Hofmeyr *et al.* 2015). An SIT program
50 was introduced in the Western Cape Province, South Africa in 2007, resulting in effective
51 control (Hofmeyr *et al.* 2015) and has since been extended to the Northern and Eastern Cape
52 Provinces (Boersma *et al.* 2018). However, the cost of producing and releasing moths means
53 that there is considerable value in improving the quality of moths released in the program
54 (Boersma & Carpenter 2016). In particular, false codling moths are rapidly cooled to 6-10 °C
55 upon collection at the XSIT (Pty) Ltd mass-rearing SIT facility in Citrusdal, South Africa
56 (Hofmeyr & Pretorius 2010), and kept at these low temperatures for up to 48 hours during the
57 rest of the production chain (holding, packaging, irradiation, transport), and are only re-warmed
58 prior to release (Hofmeyr *et al.* 2015). Boersma & Carpenter (2016) showed that this protocol
59 reduces the dispersal distance and numbers recaptured of moths released in citrus orchards,
60 which is in line with other studies reporting decreased performance after longer exposures to
61 low temperatures (Nepgen *et al.* 2015).

62 The field performance of ectothermic insects is influenced not only by the immediate
63 environmental temperature, but also by their thermal history (Terblanche 2014). Stotter &
64 Terblanche (2009) showed that exposing false codling moth to sub-freezing temperatures (-
65 6°C-0°C) for long time periods (2-10h) is lethal and that exposure to 0°C for 2h resulted in
66 only 80% survival. The recapture rate of mass-reared false codling moth decreases if they are
67 chilled prior to release (Boersma & Carpenter 2016), but acclimation does improve cold
68 tolerance (Boersma *et al.* 2018) and dispersal under cool conditions (Boersma *et al.* in prep.).
69 Assuming that the acclimation response is costly to the overall fitness of moths (Boersma *et al.*
70 2018), we expect that there are trade-offs associated with mitigating the negative impacts of
71 low temperature exposure and/or improving release under cool conditions. However, presently
72 we do not understand the physiological mechanisms underlying low temperature performance
73 of FCM (see also Boardman *et al.* 2017), which means that we lack clear physiological markers
74 for the thresholds and costs associated with non-lethal cold exposure.

75 Below their critical thermal minimum (CT_{min} ; 6-10 °C in FCM depending on ramping
76 rate; Terblanche *et al.* 2017), insects typically enter a reversible state of muscle paralysis (chill
77 coma). The onset of chill coma is driven by temperature-dependent cellular and neuronal
78 mechanisms (Overgaard & MacMillan 2017; Andersen *et al.* 2018), but ion balance is disrupted
79 while the insect is in chill coma, and a resulting increase in extracellular $[K^+]$ depolarises the
80 muscle (Overgaard & MacMillan 2017). This disruption of ion homeostasis can lead to chilling
81 injury (Košťál *et al.* 2006; Coello Alvarado *et al.* 2015) associated with cellular depolarisation
82 induced by the opening of Ca^{2+} channels (Bayley *et al.* 2018), and loss of ion balance may be
83 metabolically expensive to reverse (MacMillan *et al.* 2012). This can, at least in part, cause the
84 costly effects of cold on the overall performance of insects following recovery from chill coma
85 (MacMillan & Sinclair 2011a).

86 Cold acclimation appears to reduce the onset or impact of this loss of ion homeostasis
87 in various ways (MacMillan *et al.* 2016; Des Marteaux *et al.* 2018a,b; Yerushalmi *et al.* 2018),
88 resulting in improved resistance to chilling injury. However, this general model of ion
89 homeostasis and its loss in the cold has been derived largely from work on model Diptera
90 (Kristiansen & Zachariassen 2001; MacMillan *et al.* 2015a,b) and Orthoptera (Košťál *et al.*
91 2006; MacMillan & Sinclair 2011b; MacMillan *et al.* 2012; Andersen *et al.* 2017a), and there
92 is only limited work on ion balance in Lepidoptera (McCann & Wira 1967; Wareham *et al.*
93 1975; Layne & Peffer 2006 Boardman *et al.* 2011; Andersen *et al.* 2017b).

94 There have been several studies of the effects of cold on ion balance in Lepidopteran
95 larvae (Layne & Peffer 2006; Boardman *et al.* 2011, Andersen *et al.* 2017b), but the unusual
96 ion balance strategies of Lepidopteran larvae (Sutcliffe 1963) mean that these may not be
97 extrapolated to adults. Andersen *et al.* (2017b) investigated ion balance in the adults of
98 *Manduca sexta* and *Heliconius cydno* when exposed to cold temperatures over short and longer
99 periods (0-48 hours). They showed that an acute cold exposure inducing chill coma had very
100 little effect on the transmembrane distribution of K⁺ and Na⁺ in the species of adult moths
101 investigated. Moreover, exposure over longer time periods increased hemolymph [K⁺]. They
102 conclude that their data supports that the maintenance of ion balance is important for cold
103 tolerance and recovery from chill coma in these Lepidoptera. However, no measurements have
104 been made on false codling moth adults, and the extent to which ion and/or water homeostasis
105 in the cold can be used as a marker (or target) for improving the use of cold in rearing and
106 release systems remains unexplored. Furthermore the physiological mechanism(s) determining
107 performance costs of cold acclimation in moths destined for release under cool conditions
108 remains unclear.

109 Here we assess whether a loss of ion and/or water homeostasis plays a role in chill coma
110 onset and recovery, and hence, low temperature activity thresholds in false codling moth. If

111 FCM ion balance is similar to that of other insects in the cold, then we may be able to use the
112 chill coma models developed on these other taxa to understand the effects of low temperature
113 on ion balance to expedite improvement of the use and mitigation of cold in the FCM SIT
114 program. We thus hypothesise that adult FCM lose ion and water balance in the cold, that this
115 loss of ion balance is associated with exposure to chill coma inducing temperatures, and that it
116 is reversed upon recovery in warm conditions.

117 **MATERIALS AND METHODS**

118 Non-sterile adult moths were obtained from the XSIT (Pty) Ltd mass-rearing SIT
119 facility in Citrusdal, South Africa (Hofmeyr *et al.* 2015) weekly over four weeks and placed in
120 an incubator (BOD-150, MRC Lab Instruments, Holon, Israel) at 25°C in a 150mm petri dish
121 (N=400) without food or water. After 24 h, moths were taken from the incubator, briefly
122 narcotised using CO₂, sexed and placed individually in 2 mL microcentrifuge tubes, which
123 were weighed and transferred to a plastic bag in the bath of a refrigerated circulator (CC410wl,
124 Huber, Berching, Germany) and allowed to equilibrate at 25°C for 30 minutes. We ran two
125 different controlled temperature programs (Figure 1): one program cooling moths to 9°C (A)
126 and another cooling them to 3 °C (B) for four hours, which are above and below the FCM chill
127 coma onset temperature (repeatedly estimated as ~ 6 °C) for this laboratory mass-bred culture
128 (Terblanche *et al.* 2017), respectively. We sampled moths at three time points: at the end of the
129 30 minute equilibration period ('t1'), which serves as the control; after 4 h at either 3°C or 9
130 °C ('t2'); and after 24 h recovery at 25°C ('t3'). The 4 h exposure was chosen to be
131 representative of the minimum duration a moth would be chilled prior to release in the field.

132 We removed and discarded a single leg from each moth and placed the remaining moth
133 body head-first into a pre-weighed 0.6 mL microcentrifuge tube, and spun them at 5900 ×g for
134 5 min at either 3°C or 9 °C (depending on the treatment) to expel the hemolymph. We measured

135 hemolymph volume with a calibrated microcapillary tube (1-5 μ L, Sigma Aldrich) and pooled
136 samples from different moths to give samples of 5 μ L (usually 5-20 moths). We weighed the
137 microcentrifuge tubes with hemolymph, dried them for 24 hours at 60°C and reweighed them
138 to determine dry mass to determine the volume of hemolymph collected. After collecting
139 hemolymph, we dissected flight muscle on ice and pooled muscle for the same individuals as
140 for the hemolymph collection, and we dried the samples and weighed them as for hemolymph.
141 We shipped the dried samples from the laboratory in South Africa to The University of Western
142 Ontario, Canada for ion content determination.

143 Samples were dissolved in concentrated HNO₃ (100 μ L for hemolymph, 500 μ L for
144 muscle) for 24 hours before being diluted in deionized water to bring them within measurement
145 range. Sodium (Na⁺), Potassium (K⁺) and Magnesium (Mg²⁺) were measured in each diluted
146 sample using an atomic absorption spectrometer (iCE 3000, Thermo Scientific, Waltham, MA,
147 USA; wavelength 180–900 nm), and compared to known standards, as previously described
148 (MacMillan & Sinclair 2011b), to determine the ion concentration.

149 To determine the ion content for each tissue (μ mol/mg tissue), we multiplied the ion
150 concentration by the water content of that sample. We also calculated the muscle equilibrium
151 potential for each ion using the Nernst equation, as previously described (MacMillan & Sinclair
152 2011b). All data were analysed using Statistica v13 (StatSoft, Inc., Tulsa, USA). We examined
153 the effects of a temperature exposure below and above the chill coma onset threshold for FCM
154 at different time points. Ion concentration (mM) and content (μ mol) in the hemolymph as well
155 as ion concentration (mM) and ion content (μ mol/mg) in the muscle were compared at 3°C and
156 9°C at different time points (t1, t2, t3) using a generalized linear model (GLZ) where
157 parametric assumptions were violated (normality and homogeneity of variance) and a factorial
158 ANOVA where these assumptions were not violated. A Kruskal-Wallis test was used to test
159 for normality and homogeneity of variances were confirmed by plotting raw residuals over the

160 predicted values. Muscle equilibrium potential (mV) were compared using a Kruskal-Wallis
161 ANOVA by ranks for Na^+ and Mg^{2+} and a factorial ANOVA for K^+ . Significance levels were
162 set at 0.05 and if significant p-values were found we made use of Tukey's HSD post-hoc test
163 and 95% confidence intervals to identify homogenous groups.

164 **RESULTS**

165 Concentrations of Na^+ , K^+ , and Mg^{2+} all decreased slightly during cold exposure (t2),
166 and increased after rewarming (t3; Table 1; Figure 2; Table S1). However, we observed this
167 pattern in moths exposed to both 3 °C (*i.e.* in chill coma), and 9 °C, suggesting that the effect
168 was not related to chill coma (Table 1; Table S1; Figure 2). Muscle [Na^+], [K^+] and [Mg^{2+}] did
169 not significantly change between time points in either cooling regime (Table 1; Table S1;
170 Figure 2).

171 Ion concentrations can remain stable in the face of bulk redistribution because of
172 management of water volume (MacMillan & Sinclair 2011b), but there was also no significant
173 change in ion content of muscle or hemolymph when moths were cooled to 3°C or 9°C (Table
174 S2; Figure S1). Consequent to this stability of ion balance, there was no disturbance in the
175 muscle equilibrium potential for [Na^+] ($H_1=0.299$, $p=0.585$), [K^+] ($F_{1,43}=0.0003$, $p=0.986$) and
176 [Mg^{2+}] ($H_1=1.798$, $p=0.180$) during or after cooling to below (3°C) or above (9°C) their chill
177 coma induction temperatures (Table S1; Figure 3).

178 Finally, neither hemolymph volume nor water content of the muscle changed with low
179 temperature exposure or over time (Figure S2).

180 **DISCUSSION**

181 Recovery from chill coma has been used as a measure of how well insects respond to a
182 cold exposure and is often used as a metric of performance (David *et al.* 2003; Sinclair *et al.*
183 2012; Andersen *et al.* 2015). However, the mechanisms that govern recovery from chill coma

184 are largely unknown, although hypotheses include changes in ion pumping rates and/or
185 epithelial permeability (MacMillan & Sinclair 2011a; Overgaard & MacMillan 2017; Andersen
186 *et al.* 2018). Here we show that although there is a slight change (decrease) in the Na⁺, K⁺ and
187 Mg²⁺ concentration in the hemolymph during cold exposure, there is no difference between
188 moths exposed to 3°C or 9°C, and no apparent differences in recovery of ion homeostasis post-
189 cold exposure.

190 Cold-induced disruption in ion balance has been demonstrated in many insects
191 including *Drosophila melanogaster* (MacMillan *et al.* 2015b), *Pyrrharctia isabella* (Boardman
192 *et al.* 2011), *Gryllus pennsylvanicus* (MacMillan *et al.* 2012), *Manduca sexta*, *Bombyx mori*
193 and *Heliconius cydno* (Andersen *et al.* 2017b). However, false codling moth showed little
194 difference in the concentration of ions between the two treatment groups (3°C or 9°C) in this
195 study and this seems to indicate that although ion homeostasis changes in the cold, that this
196 change is not associated with whether or not the insect was in chill coma. This is consistent
197 with other adult Lepidoptera, where the onset of chill coma had little effect on the
198 transmembrane distribution of Na⁺, K⁺ and Cl⁻ (Andersen *et al.* 2017b), and with studies that
199 show that the onset of chill coma has additional causes to loss of ion homeostasis (MacMillan
200 *et al.* 2015c). However, we also see changes in mean [Mg²⁺] in FCM hemolymph, which was
201 not reported for the other species. The excitability of muscle cells relies on the constant
202 movement of ions across cell membranes to maintain homeostasis. As a result of this lack of
203 ion disruption in false codling moth in chill coma, moths released should recover rapidly from
204 cold exposure, with the primary changes associated with temperature-dependent molecular
205 function (e.g. of contractile apparatus and neuromuscular junctions; MacMillan *et al.* 2015c)
206 rather than the slower and more energetically-costly re-establishment of ion and water
207 homeostasis. Thus, the mechanism of reduced flight performance following chilling or upon
208 release into cooler environments still remains unclear.

209 In this study we only exposed moths to an acute (four hour) exposure to a chill coma
210 induction temperature. A number of studies investigating chronic exposure to cold
211 temperatures in insects have shown a disruption in ion balance after exposures longer than four
212 hours (Košťál *et al.* 2006; Des Marteaux & Sinclair 2016; Andersen *et al.* 2017a). During the
213 SIT program, FCM can spend at least four hours at low temperatures during handling and
214 shipping, so these chronic cold exposures may further influence the distribution of ions in false
215 codling moth. Furthermore, moths released as part of a SIT program must be competitive
216 immediately upon release and are not allowed prolonged periods of time to recover from a cold
217 exposure.

218 We did not find evidence that water and ion homeostasis are lost by the moths that enter
219 chill coma to a greater extent than control (reference) group moths, which suggests that loss of
220 ion homeostasis does not drive poor performance in moths previously in chill coma. We cannot
221 rule out some additional differential energetic cost of maintaining ion balance for the moths at
222 3 °C, but over this time period an ongoing maintenance cost would likely be minor at these
223 temperatures. Thus, we conclude that that ion or water imbalance caused by chill coma
224 inducing temperatures is not the reason that chilled false codling moths disperse poorly upon
225 subsequent release in the field.

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353 **TABLES**

354 **Table 1** Statistical tests of the effects of low temperature treatment (3°C and 9°C) and time
 355 point (before, during and 24 h recovery after cold exposure) on hemolymph and muscle ion
 356 concentration in false codling moth (*Thaumatotibia leucotreta*).

Ion	Effect	F	χ^2	d.f	p
<i>Hemolymph</i>					
Na ⁺	Treatment	0.051		1	0.822
	Time point	11.42		2	<0.001
	Treatment*Time point	0.658		2	0.523
	Error			45	
K ⁺	Treatment		1.084	1	0.298
	Time point		14.85	2	<0.001
	Treatment*Time point		1.255	2	0.534
Mg ²⁺	Treatment	0.132		1	0.718
	Time point	6.217		2	<0.05
	Treatment*Time point	0.233		2	0.793
	Error			45	
<i>Muscle</i>					
Na ⁺	Treatment		0.759	1	0.384
	Time point		1.914	2	0.384
	Treatment*Time point		0.809	2	0.667
K ⁺	Treatment		2.64	1	0.104
	Time point		1.1	2	0.577
	Treatment*Time point		1.37	2	0.504
Mg ²⁺	Treatment	2.867		1	0.097
	Time point	0.613		2	0.547
	Treatment*Time point	0.959		2	0.391
	Error			42	

357

358

359 **FIGURE LEGENDS**

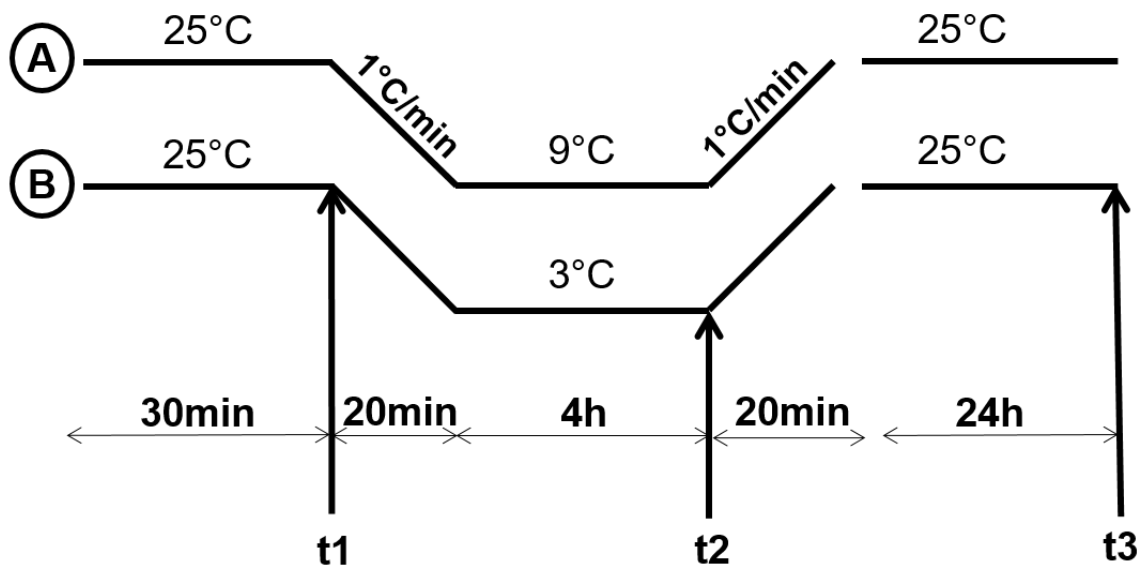
360 **Figure 1** Experimental design: False codling moth (*Thaumatotibia leucotreta*) were exposed to
361 a 9°C (A, above the chill coma induction threshold temperature of 6°C) and 3°C (B, below chill
362 coma induction temperature) for 4 h. Moths were sampled at three time points (t1, t2, t3),
363 before, during and 24 h after recovery from a cold exposure.

364

365 **Figure 2** Mean ion concentration in the hemolymph for (A) Na⁺, (B) K⁺, (C) Mg²⁺ and muscle
366 (D) Na⁺, (E) K⁺, (F) Mg²⁺ of false codling moth (*Thaumatotibia leucotreta*) in two cooling
367 treatments (3°C and 9°C) at three different time points before (t1), during (t2) and 24 h after
368 recovery (t3) from a cold exposure. Letters indicate significant differences in hemolymph ion
369 concentrations. There were no significant differences in ion concentrations in the muscle across
370 treatments and time points. Vertical bars indicate 95% confidence intervals.

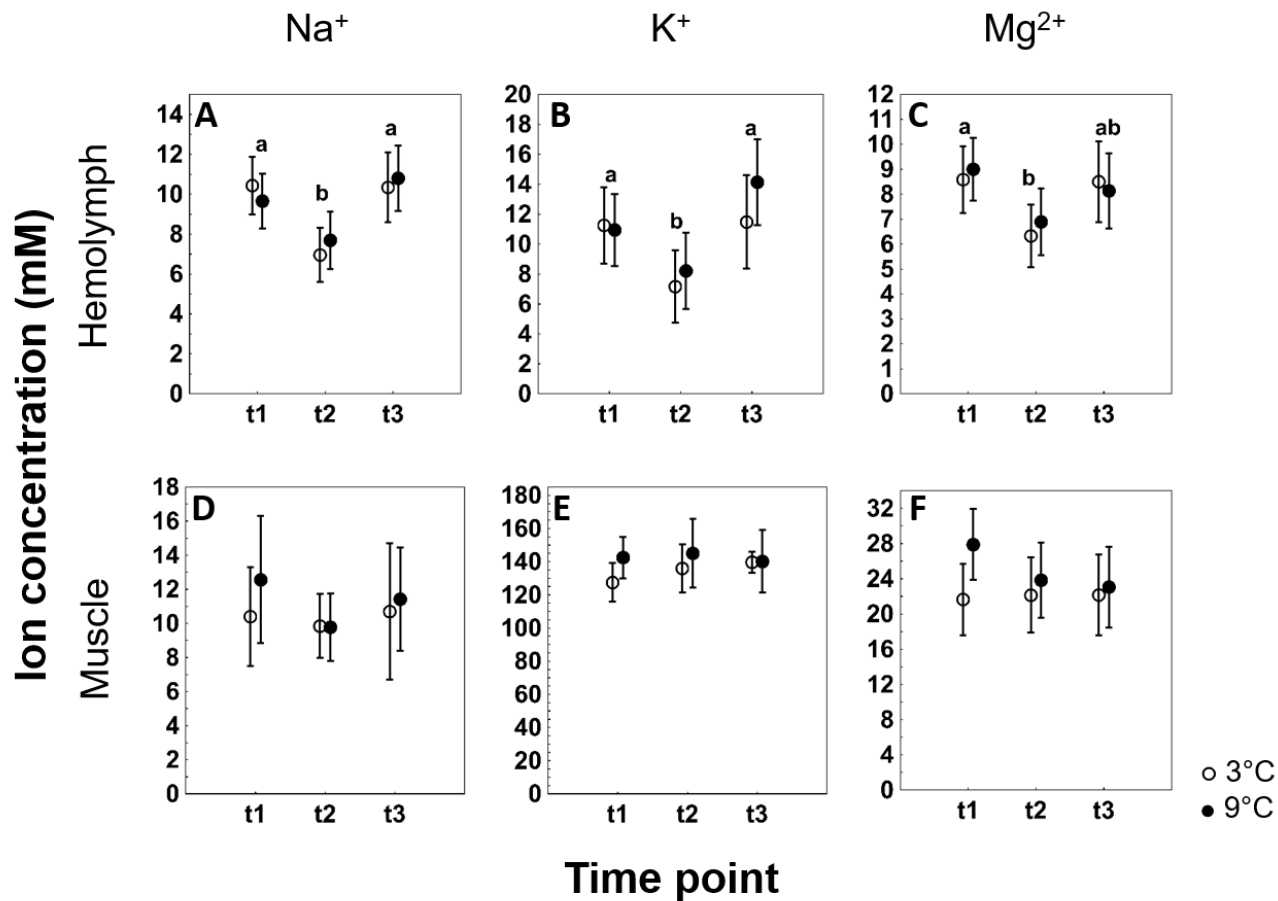
371

372 **Figure 3** Mean equilibrium potential (mV) in the muscle tissue of false codling moth
373 (*Thaumatotibia leucotreta*) in two cooling treatments (3°C and 9°C) at three different time
374 points before (t1), during (t2) and 24 h after recovery (t3) from cold exposure. Vertical bars
375 indicate 95% confidence intervals.



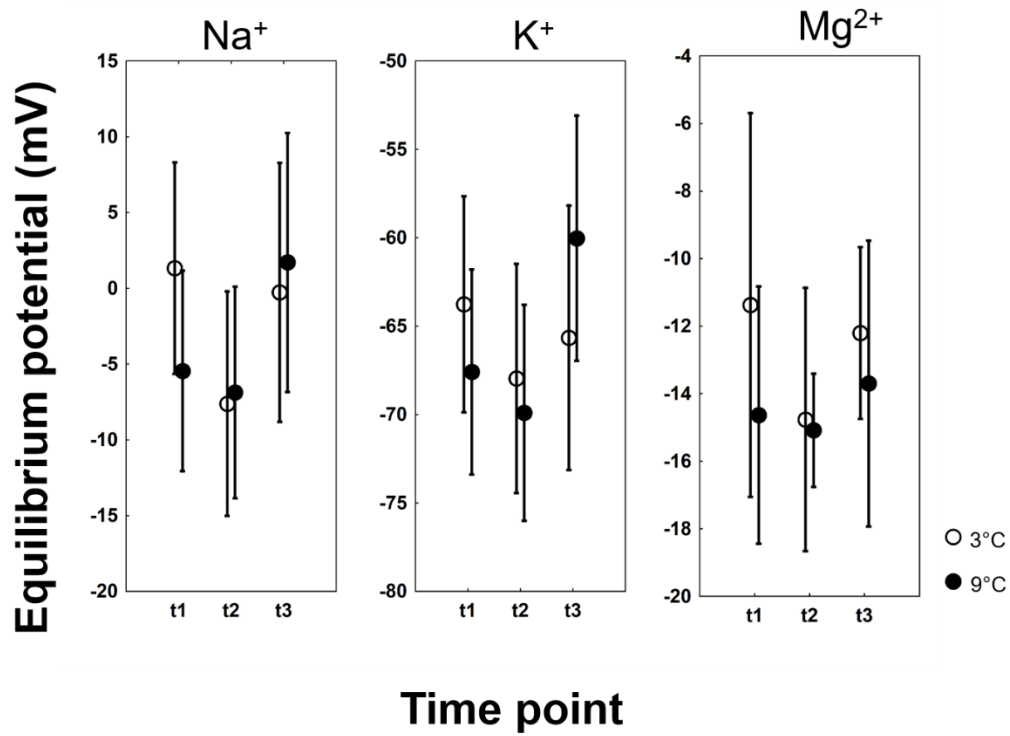
377

378 **Figure 1.** Experimental design: False codling moth (*Thaumatotibia leucotreta*) were exposed
 379 to a 9°C (A, above the chill coma induction threshold temperature of 6°C) and 3°C (B, below
 380 chill coma induction temperature) for 4 h. Moths were sampled at three time points (t1, t2, t3),
 381 before, during and 24 h after recovery from a cold exposure.



382

383 **Figure 2.** Mean ion concentration in the hemolymph for (A) Na⁺, (B) K⁺, (C) Mg²⁺ and muscle (D) Na⁺, (E) K⁺, (F) Mg²⁺ of false codling moth
 384 (*Thaumatotibia leucotreta*) in two cooling treatments (3°C and 9°C) at three different time points before (t1), during (t2) and 24 h after recovery (t3)
 385 from a cold exposure. Letters indicate significant differences in hemolymph ion concentrations. There were no significant differences in ion
 386 concentrations in the muscle across treatments and time points. Vertical bars indicate 95% confidence intervals.



387

388 **Figure 3.** Mean equilibrium potential (mV) in the muscle tissue of false codling moth
 389 (*Thaumatotibia leucotreta*) in two cooling treatments (3°C and 9°C) at three different time
 390 points before (t1), during (t2) and 24 h after recovery (t3) from cold exposure. Vertical bars
 391 indicate 95% confidence intervals.