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

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ABSTRACT

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Non-sterile adult moths were obtained from the XSIT (Pty) Ltd mass-rearing SIT facility in Citrusdal, South Africa (Hofmeyr *et al*. 2015) weekly over four weeks and placed in an incubator (BOD-150, MRC Lab Instruments, Holon, Israel) at 25°C in a 150mm petri dish (N=400) without food or water. After 24 h, moths were taken from the incubator, briefly narcotised using CO_2, sexed and placed individually in 2 mL microcentrifuge tubes, which were weighed and transferred to a plastic bag in the bath of a refrigerated circulator (CC410wl, Huber, Berching, Germany) and allowed to equilibrate at 25°C for 30 minutes. We ran two different controlled temperature programs (Figure 1): one program cooling moths to 9°C (A) and another cooling them to 3 °C (B) for four hours, which are above and below the FCM chill coma onset temperature (repeatedly estimated as ~ 6 °C) for this laboratory mass-bred culture (Terblanche *et al*. 2017), respectively. We sampled moths at three time points: at the end of the 30 minute equilibration period ('t1'), which serves as the control; after 4 h at either 3°C or 9 °C ('t2'); and after 24 h recovery at 25°C ('t3'). The 4 h exposure was chosen to be representative of the minimum duration a moth would be chilled prior to release in the field.

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

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

Samples were dissolved in concentrated HNO_3 (100 µL for hemolymph, 500 µL for muscle) for 24 hours before being diluted in deionized water to bring them within measurement range. Sodium (Na^+), Potassium (K^+) and Magnesium (Mg^{2+}) were measured in each diluted sample using an atomic absorption spectrometer (iCE 3000, Thermo Scientific, Waltham, MA, USA; wavelength 180–900 nm), and compared to known standards, as previously described (MacMillan & Sinclair 2011b), to determine the ion concentration.

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

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

RESULTS

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

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

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

DISCUSSION

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

are largely unknown, although hypotheses include changes in ion pumping rates and/or epithelial permeability (MacMillan & Sinclair 2011a; Overgaard & MacMillan 2017; Andersen *et al*. 2018). Here we show that although there is a slight change (decrease) in the Na^+, K^+ and Mg^{2+} concentration in the hemolymph during cold exposure, there is no difference between moths exposed to 3°C or 9°C, and no apparent differences in recovery of ion homeostasis post-cold exposure.

Cold-induced disruption in ion balance has been demonstrated in many insects including *Drosophila melanogaster* (MacMillan *et al*. 2015b), *Pyrrharctia isabella* (Boardman *et al*. 2011), *Gryllus pennsylvanicus* (MacMillan *et al*. 2012), *Manduca sexta*, *Bombyx mori* and *Heliconius cydno* (Andersen *et al*. 2017b). However, false codling moth showed little difference in the concentration of ions between the two treatment groups (3°C or 9°C) in this study and this seems to indicate that although ion homeostasis changes in the cold, that this change is not associated with whether or not the insect was in chill coma. This is consistent with other adult Lepidoptera, where the onset of chill coma had little effect on the transmembrane distribution of Na^+, K^+ and Cl^- (Andersen *et al*. 2017b), and with studies that show that the onset of chill coma has additional causes to loss of ion homeostasis (MacMillan *et al*. 2015c). However, we also see changes in mean [Mg^{2+}] in FCM hemolymph, which was not reported for the other species. The excitability of muscle cells relies on the constant movement of ions across cell membranes to maintain homeostasis. As a result of this lack of ion disruption in false codling moth in chill coma, moths released should recover rapidly from cold exposure, with the primary changes associated with temperature-dependent molecular function (e.g. of contractile apparatus and neuromuscular junctions; MacMillan *et al*. 2015c) rather than the slower and more energetically-costly re-establishment of ion and water homeostasis. Thus, the mechanism of reduced flight performance following chilling or upon release into cooler environments still remains unclear.

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

We did not find evidence that water and ion homeostasis are lost by the moths that enter chill coma to a greater extent than control (reference) group moths, which suggests that loss of ion homeostasis does not drive poor performance in moths previously in chill coma. We cannot rule out some additional differential energetic cost of maintaining ion balance for the moths at 3 °C, but over this time period an ongoing maintenance cost would likely be minor at these temperatures. Thus, we conclude that that ion or water imbalance caused by chill coma inducing temperatures is not the reason that chilled false codling moths disperse poorly upon 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	$\chi 2$	*d.f*	p
Hemolymph					
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	
Muscle					
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	

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FIGURE LEGENDS

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

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

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

FIGURES

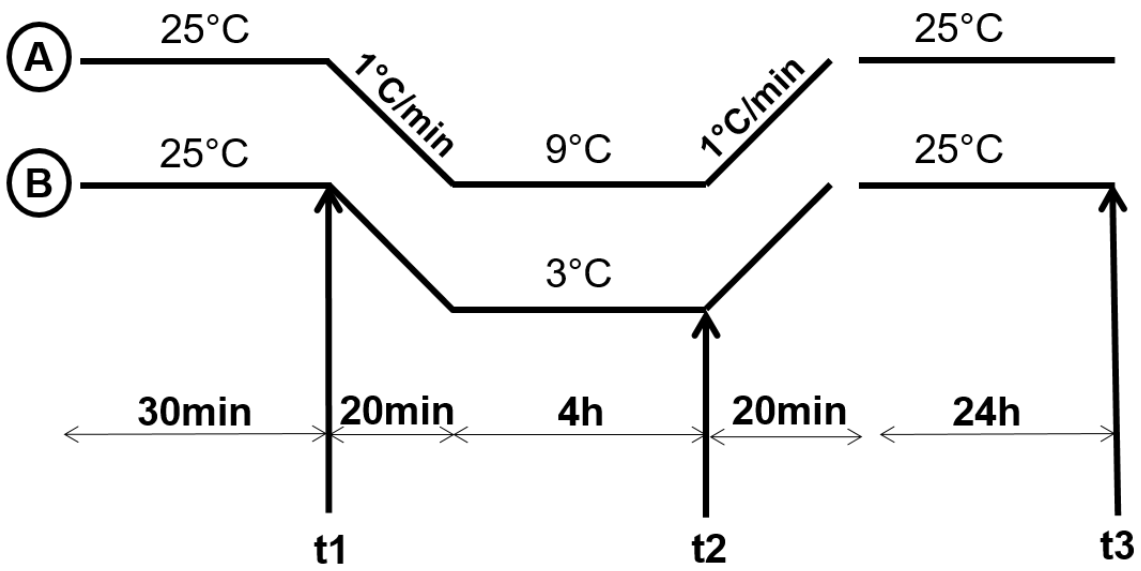
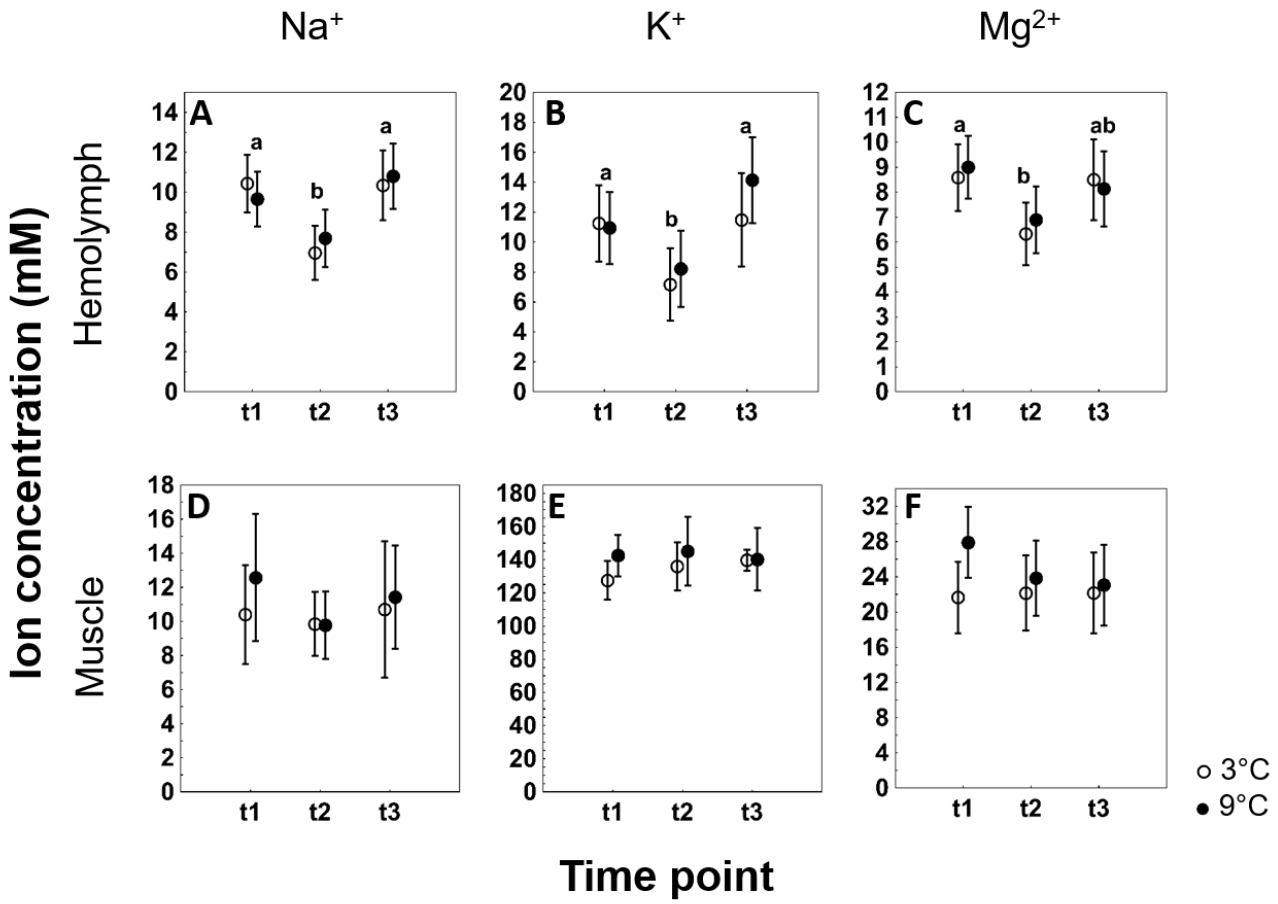
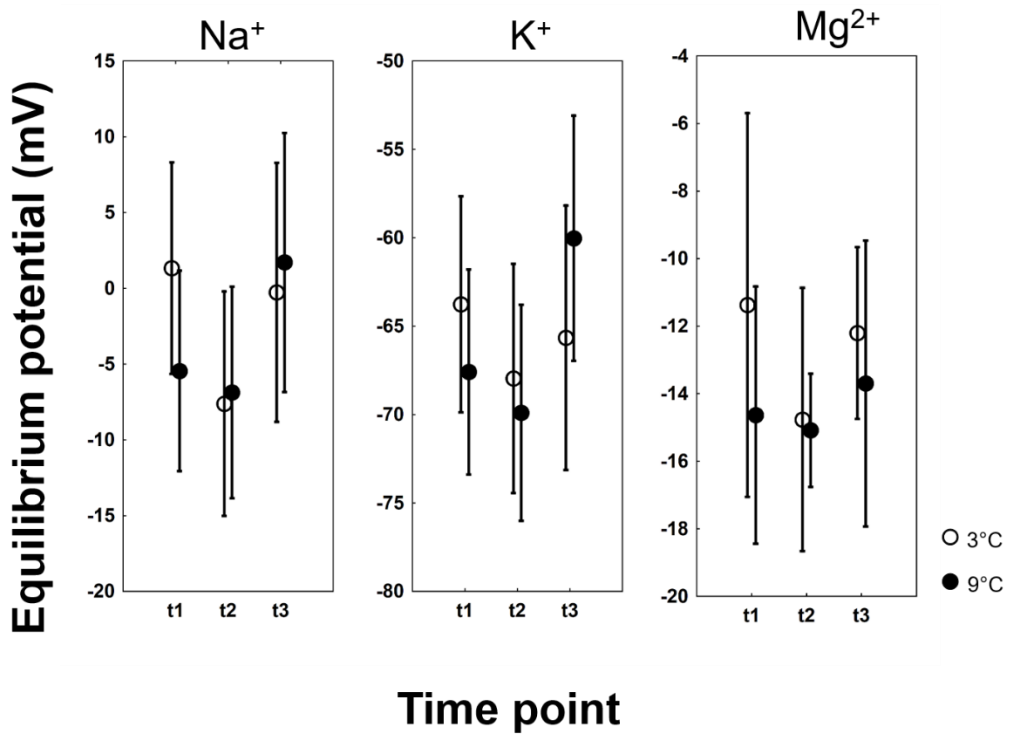


Figure 1. Experimental design: False codling moth (*Thaumatotibia leucotreta*) were exposed to a 9°C (A, above the chill coma induction threshold temperature of 6°C) and 3°C (B, below chill coma induction temperature) for 4 h. Moths were sampled at three time points (t1, t2, t3), before, during and 24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



382

383 **Figure 2.** Mean ion concentration in the hemolymph for (A) Na^+, (B) K^+ , (C) Mg^{2+} and muscle (D) Na^+, (E) K^+, (F) Mg^{2+} of false codling moth
384 (*Thaumatotibia leucotreta*) in two cooling treatments (3ºC and 9ºC) 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 (3ºC and 9ºC) 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.