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## Research

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## Physiology

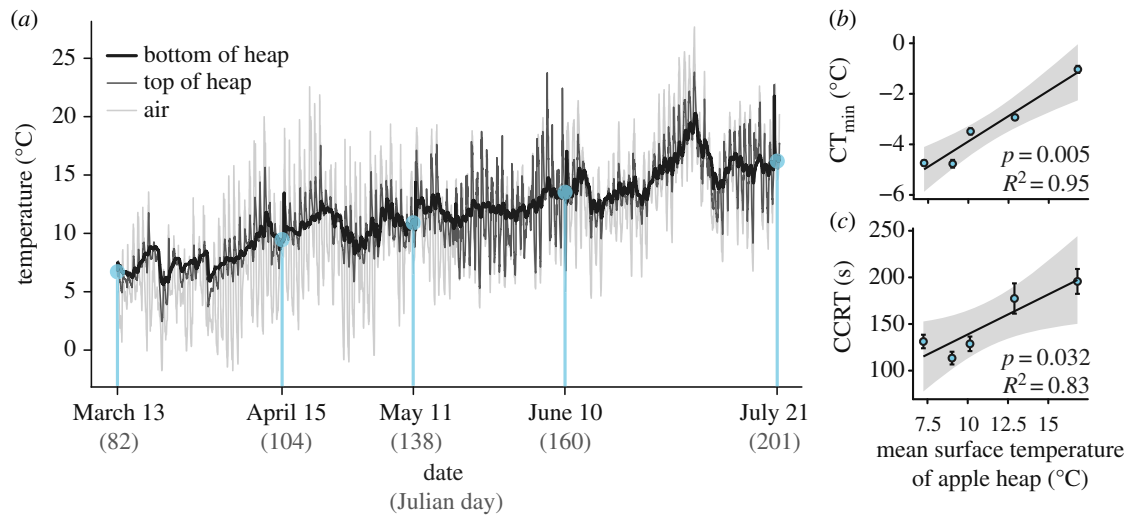
Preservation of potassium balance is strongly associated with insect cold tolerance in the field: a seasonal study of *Drosophila subobscura*Heath A. MacMillan<sup>1,†</sup>, Mads F. Schou<sup>2</sup>, Torsten N. Kristensen<sup>3</sup>  
and Johannes Overgaard<sup>1</sup><sup>1</sup>Department of Bioscience—Zoophysiology, and <sup>2</sup>Department of Bioscience—Genetics, Ecology and Evolution, Aarhus University, 8000 Aarhus, Denmark<sup>3</sup>Department of Chemistry and Bioscience, Aalborg University, 9100 Aalborg, Denmark

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There is interest in pinpointing genes and physiological mechanisms explaining intra- and interspecific variations in cold tolerance, because thermal tolerance phenotypes strongly impact the distribution and abundance of wild animals. Laboratory studies have highlighted that the capacity to preserve water and ion homeostasis is linked to low temperature survival in insects. It remains unknown, however, whether adaptive seasonal acclimatization in free-ranging insects is governed by the same physiological mechanisms. Here, we test whether cold tolerance in field-caught *Drosophila subobscura* is high in early spring and lower during summer and whether this transition is associated with seasonal changes in the capacity of flies to preserve water and ion balance during cold stress. Indeed, flies caught during summer were less cold tolerant, and exposure of these flies to sub-zero temperatures caused a loss of haemolymph water and increased the concentration of  $K^+$  in the haemolymph (as in laboratory-reared insects). This pattern of ion and water balance disruption was not observed in more cold-tolerant flies caught in early spring. Thus, we here provide a field verification of hypotheses based on laboratory studies and conclude that the ability to maintain ion homeostasis is important for the ability of free-ranging insects to cope with chilling.

## 1. Introduction

Thermal tolerance is tremendously important in determining the fundamental niche and distribution of animals [1], and most insects display temporal patterns in cold tolerance that closely match seasonal demands (e.g. [2,3]). Several recent studies have demonstrated that the ability of insects to maintain water and ion homeostasis is crucial for survival (e.g. [4,5]). During cold stress, many insects (including *Drosophila*) enter a chill coma (neuromuscular paralysis) and experience a gradual loss of ionic- and osmoregulatory balance; a net leak of sodium down concentration gradients drives water away from the haemolymph, which concentrates haemolymph potassium and disrupts resting membrane potential [5]. Experiments performed under controlled laboratory conditions have shown that this cascade of events delays recovery from chill coma, and causes chilling injury and death [5,6]. While variation in cold tolerance measures can be directly associated with variation in the capacity to maintain ion homeostasis in the laboratory, it remains unclear whether this homeostatic capacity is associated with the cold tolerance of free-living insects.



**Figure 1.** Seasonal changes in field temperature and chilling tolerance of *D. subobscura* living in close association with a heap of discarded apples. (a) Air temperature (light grey) and the temperature at the bottom (black) and top (i.e. surface; dark grey) of the heap of apples were recorded during the transition from spring to summer. Adult *D. subobscura* were collected approximately every four weeks (circles with vertical lines). (b)  $CT_{\min}$  and (c) CCRT measured in flies collected during the experimental period (mean  $\pm$  s.e.) regressed against mean temperature at the surface of the heap in the 24 h preceding collection. Shaded areas represent 95% confidence intervals of regressions. (Online version in colour.)

Field-caught insects vary seasonally in their cold tolerance (e.g. [3,7]) but will, in contrast to laboratory-reared insects, experience a range of complex environmental cues that influence their physiology (e.g. mean temperature, day length, precipitation, resource availability and thermal variability). Furthermore, individuals collected from the wild at different time points will potentially also differ genetically and vary in factors such as age, reproductive status, hydration and dietary/feeding status. In this study, we collected *Drosophila subobscura* living in a heap of discarded and rotting apples over a period of four months and quantified seasonal changes in cold tolerance and the capacity for maintenance of ion and water homeostasis during cold stress. In doing so, we provide the first field test of whether iono- and osmoregulatory capacity varies seasonally, and whether such variance provides a mechanistic explanation for variation in the cold tolerance of a free-ranging insect.

## 2. Material and methods

Adult *D. subobscura* were collected between 9.00 and 11.00 on five dates throughout the spring and summer of 2015 (figure 1a) at Karensminde orchard in Jutland, Denmark (55°56'42.46" N, 10°12'4.31" E) from a heap of discarded apples as previously described [3]. At this location, *D. subobscura* vary in age and mating status in the spring, but are not in a reproductive diapause [3]. Air temperature, as well as the temperatures of the surface and interior of the apple heap, were recorded using data loggers (iButton, Maxim Integrated). Flies were aspirated from the heap surface into plastic vials containing 7 ml of *Drosophila* medium (oatmeal, sugar, yeast and agar) and placed in a mobile temperature-controlled cabinet pre-set to the air temperature at the time of collection ( $\pm 0.5^\circ\text{C}$ ; Sable Systems International). Flies were transported to Aarhus University, where females were identified without anaesthesia, kept in the temperature cabinet and used for experiments within 1 h of collection. Flies were difficult to find on July 21st, so sample sizes were smaller and haemolymph volume was not measured on this date (see below).

The critical thermal minimum ( $CT_{\min}$ ) and chill coma recovery time (CCRT) were measured on each collection date in  $n = 18\text{--}20$  flies (in all cases except July 21st, where  $n = 7$ ). The  $CT_{\min}$  was

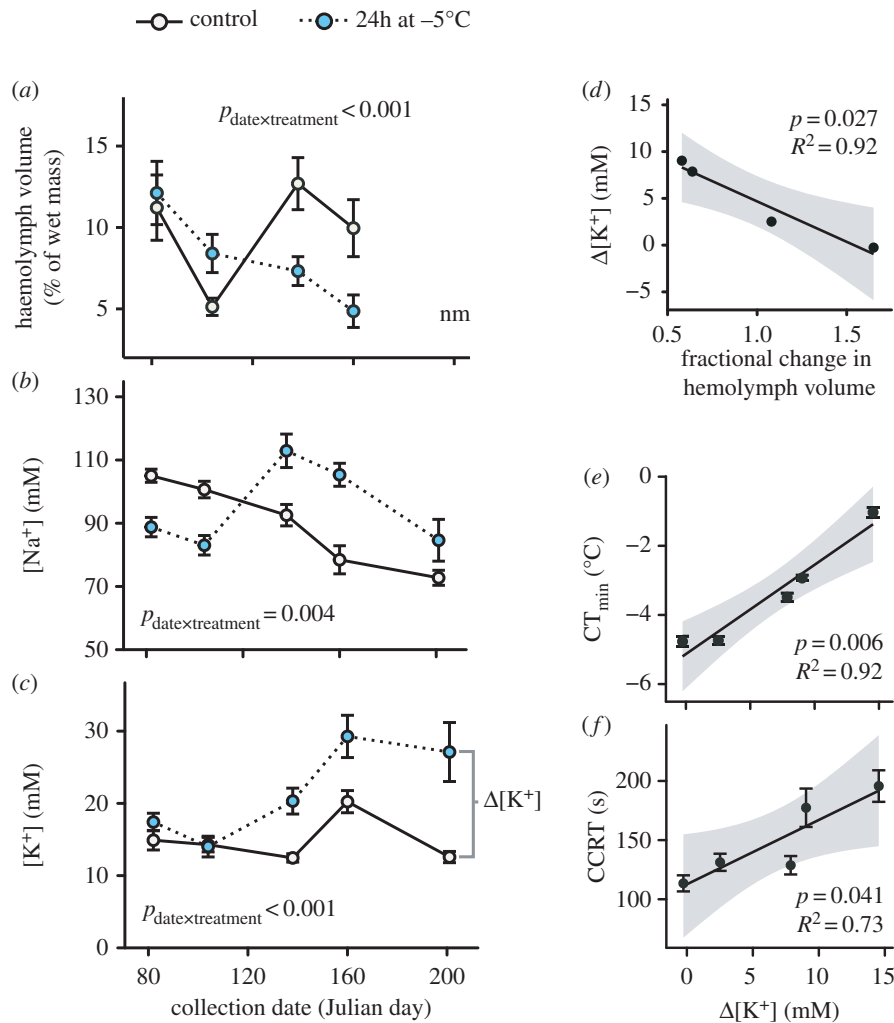
measured by placing flies individually in 5 ml glass vials that were then submerged in a 1:1 mixture of ethylene glycol and water circulated by a temperature-controlled bath pre-set to  $15^\circ\text{C}$ . Temperature was then decreased at  $0.1^\circ\text{C min}^{-1}$  and the flies were continually observed until they lost all capacity for movement (and thus had entered chill coma ( $CT_{\min}$ )). CCRT was measured on a separate set of flies. Flies were loaded into vials (as above) and submerged in an ethylene glycol: water (1:1) mixture pre-cooled to  $-5^\circ\text{C}$ . After 24 h at  $-5^\circ\text{C}$ , the flies were moved to a laboratory bench at  $23^\circ\text{C}$  and the time for flies to stand on all six legs was recorded as the CCRT. To ensure that CCRT directly measured the recovery of neuromuscular function, we tapped the vials on the laboratory bench every 30 s. Note that the onset of coma ( $CT_{\min}$ ) is not directly caused by loss of ion balance [8], but is tightly correlated to (and generally less variable than) other cold tolerance measures that are mechanistically coupled to ion balance (including CCRT and survival indices).

For each round of sampling, haemolymph volume was measured in control flies taken directly from the field, and later from flies that spent 24 h at  $-5^\circ\text{C}$  (as above;  $n = 17\text{--}20$  per ion, treatment and collection date). Haemolymph volume was measured using a blotting method [6,9] where fly mass is measured before and after haemolymph removal. Concentrations of  $\text{Na}^+$  and  $\text{K}^+$  were measured in the haemolymph of flies taken directly from their collection temperature and following 24 h at  $-5^\circ\text{C}$  ( $n = 11\text{--}15$  per ion  $\times$  treatment  $\times$  collection date combination; except July 21st, where  $n = 6\text{--}7$ ). Cold-exposed flies were pre-loaded into 200- $\mu\text{l}$  pipette tips for rapid haemolymph sampling by antennal ablation. Haemolymph was immediately deposited under paraffin oil for measurement of  $\text{Na}^+$  or  $\text{K}^+$  activity using ion-selective electrodes as previously described [6].

Data analysis was completed in R (v. 3.2) [10] using linear models (lm) and generalized linear models (glm), with body mass included as a covariate in the case of haemolymph volume.

## 3. Results and discussion

The apple heap provided a thermally buffered microhabitat for *D. subobscura* that also allowed for some behavioural thermoregulation. Thus, average temperatures at the base of the heap (indicative of larval developmental temperature) increased gradually from  $6.7^\circ\text{C}$  to  $16.5^\circ\text{C}$  during the four-month period.



**Figure 2.** Water and ion balance in *D. subobscura* before (i.e. control) and immediately after 24 h at  $-5^{\circ}\text{C}$  on five dates during the spring–summer transition. (a) Haemolymph volume, (b) haemolymph  $[\text{Na}^+]$  and (c) haemolymph  $[\text{K}^+]$  measured in flies before and after cold exposure. The net change in haemolymph  $[\text{K}^+]$  with cold exposure ( $\Delta[\text{K}^+]$ ) was significantly associated with the net change in haemolymph volume (d), as well as both indices of chilling tolerance: (e) the  $\text{CT}_{\text{min}}$  and (f) CCRT. Shaded areas represent 95% confidence intervals of regressions. All values are means ( $\pm$  s.e.). Haemolymph volume was not measured (nm) on July 21st. (Online version in colour.)

Throughout this period, the surface temperature of the heap (where the adult flies were collected) had clear diurnal temperature cycles allowing for some behavioural thermoregulation of the mobile adult flies (figure 1a). The changing season had a marked effect on  $\text{CT}_{\text{min}}$ , which increased with the progression of spring, such that the  $\text{CT}_{\text{min}}$  was significantly and positively related to the temperature at the surface of the apple heap in the 24 h before fly collection (figure 1b). Our other measure of cold tolerance (CCRT) followed a very similar trend (figure 1c).

The main purpose of this study was to test whether seasonal changes in cold tolerance were associated with a fly's capacity to preserve ion balance during cold exposure. We hypothesized that the gradual loss of cold tolerance over the spring–summer transition would be accompanied by a loss of homeostatic capacity at low temperature. This was clearly confirmed as exposure to  $-5^{\circ}\text{C}$  for 24 h caused a loss of haemolymph water and increased  $[\text{K}^+]$ , but only in flies caught in the summer months. Haemolymph volume of field-caught flies varied widely depending on the date of collection (figure 2a), possibly owing to differences in water availability in the days and hours preceding collection. Importantly, however, we found a significant interactive effect of collection date and treatment on haemolymph volume (glm:  $F_{4,149} = 3.7$ ,  $p = 0.003$ ). Flies collected in the spring months (March and

April), which had a lower  $\text{CT}_{\text{min}}$ , had unchanged or higher haemolymph volume following the cold stress. By contrast, flies collected in the summer months (June and July) lost haemolymph volume during the cold stress (figure 2a).

Changes in haemolymph volume during cold stress have been associated with a drift of  $\text{Na}^+$  away from the haemolymph compartment, which causes  $\text{K}^+$  to be concentrated in the reduced volume [5,6]. We found significant interactive effects of collection date and treatment on both haemolymph  $[\text{Na}^+]$  (glm:  $F_{4,108} = 16.6$ ,  $p < 0.001$ ; figure 2b) and  $[\text{K}^+]$  (glm:  $F_{4,111} = 5.0$ ,  $p = 0.001$ ; figure 2c). As hypothesized, more cold-tolerant flies collected in March/April maintained low haemolymph  $[\text{K}^+]$  following a cold stress. By contrast, more chill sensitive flies collected in May–July suffered an increase in haemolymph  $[\text{K}^+]$  during cold exposure. In these flies,  $[\text{K}^+]$  almost doubled in relation to the simultaneous (ca 50%) reduction in haemolymph volume. In contrast to our hypothesis, however, we found that both cold- and warm-acclimatized flies lost  $\text{Na}^+$  from the haemolymph during cold exposure. In cold-acclimatized flies,  $[\text{Na}^+]$  decreases with no change in haemolymph volume and in warm-acclimatized flies  $[\text{Na}^+]$  increases by 10–15% while volume was reduced considerably more (again representing a net loss of  $\text{Na}^+$ ). Under controlled laboratory conditions, we previously showed that both inter- and intraspecific variances in

Na<sup>+</sup> regulation is tightly associated with regulation of haemolymph volume [5,6], but in the field-caught flies we find this association to be much less clear. The role of Na<sup>+</sup> in osmotic balance may thus differ under field conditions where flies are likely to experience much larger variation in dietary and hygric conditions. We hypothesize that flies active in the spring may have higher levels of organic osmolytes (higher haemolymph osmolality) such that the contribution of Na<sup>+</sup> to water balance is similar across seasons, and/or have higher levels of intracellular [Na<sup>+</sup>] such that the Na<sup>+</sup> gradient across membranes (and the tendency for Na<sup>+</sup> leak at low temperatures) is less. Regardless of the proximate cause of volume reductions, we found that these reductions were closely associated (figure 2*d*) with an increase in haemolymph [K<sup>+</sup>], which was in turn associated with cold tolerance (figure 2*e,f*) as previously demonstrated for laboratory-reared drosophilids [5,6].

In conclusion, we have demonstrated that the cold tolerance of free-living chill-sensitive insects is related to their ability to regulate ion and water balance in the cold. We cannot yet say why the insects progressively lose this ability during the spring–summer transition, but we speculate that increased demand for food, reproduction and activity may be at odds with the physiological demands or priorities of

iono- and osmoregulatory systems. Thus, it is possible that seasonal acclimatization to higher temperatures, rapid nutrient acquisition and higher reproductive activity come at the cost of a tight and cost-effective ion regulation that is needed in the cold.

**Ethics.** Experiments presented herein were performed on insects and are thus not subject to animal care and use committee approval.

**Data accessibility.** Data accompanying this article have been deposited in the Dryad.org digital depository at the following: <http://dx.doi.org/10.5061/dryad.1qj22>.

**Authors' contributions.** All authors conceived of and designed the study. All authors collected the data. H.A.M. and M.F.S. analysed the data. H.A.M. and J.O. drafted the manuscript, and all authors revised the manuscript and approved of its final form. All authors agree to be held accountable for the content herein and approved the final version of the manuscript.

**Competing interests.** We have no competing interests.

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