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RESEARCH ARTICLE

Parallel ionoregulatory adjustments underlie phenotypic plasticity and evolution of *Drosophila* cold tolerance

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ABSTRACT

Low temperature tolerance is the main predictor of variation in the global distribution and performance of insects, yet the molecular mechanisms underlying cold tolerance variation are poorly known, and it is unclear whether the mechanisms that improve cold tolerance within the lifetime of an individual insect are similar to those that underlie evolved differences among species. The accumulation of cold-induced injuries by hemimetabolous insects is associated with loss of Na^+ and K^+ homeostasis. Here we show that this model holds true for *Drosophila*; cold exposure increases haemolymph [K^+] in *D. melanogaster*, and cold-acclimated flies maintain low haemolymph [Na^+] and [K^+], both at rest and during a cold exposure. This pattern holds across 24 species of the *Drosophila* phylogeny, where improvements in cold tolerance have been consistently paired with reductions in haemolymph [Na^+] and [K^+]. Cold-acclimated *D. melanogaster* have low activity of Na^+/K^+-ATPase, which may contribute to the maintenance of low haemolymph [Na^+] and underlie improvements in cold tolerance. Modifications to ion balance are associated with both phenotypic plasticity within *D. melanogaster* and evolutionary differences in cold tolerance across the *Drosophila* phylogeny, which suggests that adaptation and acclimation of cold tolerance in insects may occur through similar mechanisms. Cold-tolerant flies maintain haemolymph osmolality despite low haemolymph [Na^+] and [K^+], possibly through modest accumulations of organic osmolytes. We propose that this could have served as an evolutionary route by which chill-susceptible insects developed more extreme cold tolerance strategies.

KEY WORDS: Critical thermal minimum, Osmotic homeostasis, Sodium pump, Water balance

INTRODUCTION

Low temperature tolerance is especially important for determining geographic range limits of insects (Battisti et al., 2005; Chen et al., 2011; Kellermann et al., 2012). Thus understanding the mechanisms that set thermal limits allows prediction of changes to their distribution and abundance in a changing environment (Pörtner and Farrell, 2008; Hofmann and Todgham, 2010; Andersen et al., 2015; Overgaard et al., 2014). Insect lower thermal limits manifest as entry into chill coma at the critical thermal minimum (CT_{min}), and the subsequent accumulation of chilling injuries that lead to sublethal effects on performance and fitness or death (MacMillan and Sinclair, 2011a; MacMillan et al., 2014). Thermal tolerance can be modulated through phenotypic plasticity or through genetic adaptation, but the former has been suggested to be constrained by the latter; this means that species that have evolved to tolerate more extreme temperatures may be less able to acclimate to changes in temperature (Nyamukondiwa et al., 2011; Stillman, 2003) (but see Calosi et al., 2008; Overgaard et al., 2011). Identifying the mechanisms underlying thermal tolerance would elucidate the reasons for this possible trade-off, and allow for *a priori* estimation of low temperature tolerance, and high-throughput prediction of insect susceptibility to climate change (Williams et al., 2014).

The physiological causes of chill coma and chilling injury have not been thoroughly explored in holometabolous insects, which comprise the majority of terrestrial animal species (Wiegmann et al., 2009), including many threatened by climate change (Chen et al., 2011). The cosmopolitan genus *Drosophila* encompasses 60 million years of evolutionary history (Clark et al., 2007) and has both a broad geographic range and well-described variation in cold tolerance (Kellermann et al., 2012). Variation in cold tolerance among species persists after rearing *Drosophila* species under common conditions, even for many years, which implies an underlying genetic component to cold tolerance that is resilient to laboratory selection and suffers little inbreeding depression (Gilchrist et al., 1997; Ayrinhac et al., 2004; Kellermann et al., 2012; Bechsgaard et al., 2013). Similarly, most laboratory populations of *Drosophila*, including *D. melanogaster*, retain phenotypic plasticity of thermal tolerance traits, including those related to cold (Nyamukondiwa et al., 2011; Overgaard et al., 2011). The CT_{min} is simple to measure in large numbers of flies, and is closely correlated to both the poleward distribution limits of *Drosophila* species, and the sensitivity of flies to chilling injury (Andersen et al., 2015). *Drosophila* are thus an ideal genus in which to explore the mechanisms of thermal tolerance variation in the Holometabola, both within- and among-species.

In hemimetabolous insects, chilling injury is associated with a disruption of ion and water balance (Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b; Findsen et al., 2013). The concentration of Na^+ is high in the extracellular fluid relative to the diet of phytophagous insects, and at low temperatures haemolymph Na^+ leaks across the gut wall. Because water distribution is heavily dependent on Na^+, water follows Na^+ down its concentration gradient, thereby reducing haemolymph volume and consequently increasing extracellular [K^+] (MacMillan and Sinclair, 2011b). Although much Na^+ leaves the haemolymph, the concurrent loss of haemolymph water can result in little change in extracellular [Na^+]. In crickets and locusts, this imbalance is thought to lead to chilling injury, and re-establishment of ion homeostasis is required for recovery of neuromuscular function (MacMillan et al., 2012; Findsen et al., 2014; MacMillan et al., 2014). This net leak of Na^+

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in the cold is thought to occur because the activity of temperature-sensitive enzymatic ion pumps is insufficient to balance passive leak of ions, the rate of which is dependent on the magnitude of the Na^+ gradient (Zachariassen et al., 2004; MacMillan and Sinclair, 2011a).

Failure of Na^+/K^+-ATPase has been specifically hypothesized to be associated with chill coma and chilling injury, because of its primary role in maintaining Na^+ and K^+ homeostasis and electrogenic role in determining the membrane potential (e.g. Hosler et al., 2000; MacMillan and Sinclair, 2011a). At the cellular level, Na^+/K^+-ATPase in the cell membrane consumes ATP to pump $3Na^+$ out of the cell cytoplasm and $2K^+$ in during each reaction cycle, maintaining high intracellular [K^+] and low intracellular [Na^+] (Emery et al., 1998), and also resulting in high extracellular [Na^+]. In insects, Na^+ balance is primarily maintained by the renal system, composed of the Malpighian tubules and hindgut. Expression of mRNA for the α-subunit of Na^+/K^+-ATPase is enriched 7.9- and 5.3-fold (relative to whole body) in the Malpighian tubules and hindgut, respectively, of *D. melanogaster* (Chintapalli et al., 2007). Although fluid secretion at the Malpighian tubules is primarily energized by the proton-motive V-ATPase coupled to a H^+/K^+ exchanger, Na^+/K^+-ATPase in the basolateral membrane of Malpighian tubule principal cells is important in modulating rates of Na^+ and water secretion (Linton and O'Donnell, 1999; Beyenbach et al., 2010). Indeed, inhibition of Na^+/K^+-ATPase in *D. melanogaster* Malpighian tubules elevates [Na^+] of the secreted fluid by as much as 73%, and increases the fluid secretion rate by ~15% (Linton and O'Donnell, 1999). High Na^+/K^+-ATPase activity in the basal membrane of the hindgut is responsible for transporting Na^+ back into the haemolymph of the mosquito *Aedes aegypti* (Diptera: Culicidae) (Patrick et al., 2006), and net Na^+ is transported into the haemolymph in the larval hindgut of *D. melanogaster* (Naikkhwah and O'Donnell, 2012). Thus Na^+/K^+-ATPase in the *Drosophila* renal system appears to maintain high [Na^+] in the haemolymph and limit Na^+ loss to the gut lumen and faeces.

Based on the role of Na^+/K^+-ATPase in ion balance in *Drosophila* and the patterns of ion and water balance disruption in other cold-exposed insects, cold tolerance could potentially be improved by reducing Na^+/K^+-ATPase activity at high or 'normal' temperatures, which would decrease retention of Na^+ under resting conditions, and consequently reduce resting haemolymph [Na^+]. This decrease in haemolymph [Na^+] would reduce the transmembrane and transepithelial Na^+ gradients that drive Na^+ leak and water balance disruption at low temperatures, and thereby improve cold tolerance. This hypothesis is supported by the repeated observation that cold acclimation of insects results in less-polarized muscle Na^+ equilibrium potentials (Koštál et al., 2004; Koštál et al., 2006; Coello Alvarado, 2012).

Although low Na^+/K^+-ATPase activity at relatively high temperatures might reduce resting Na^+ gradients and improve cold tolerance, a failure of this pump in the cold will probably cause net ion leak across cell membranes and epithelia, which would similarly lead to increased haemolymph [K^+] and chilling injury. Reductions in enzyme thermal sensitivity are often associated with adaptation to low temperatures (Somero, 2004; Dong and Somero, 2009; Galarza-Muñoz et al., 2011; Garrett and Rosenthal, 2012). For example, polar and temperate octopus species have Na^+/K^+-ATPase α-subunits that differ in thermal sensitivity; at temperatures greater than 25°C the maximal rate of Na^+/K^+-ATPase does not differ between polar and temperate octopuses, but that of polar octopuses is 4-fold higher at 10°C (Galarza-Muñoz et al., 2011). Thus, cold tolerance may also be improved in *Drosophila* through similar reductions in the thermal sensitivity of Na^+/K^+-ATPase.

In this study, we therefore hypothesized that reduced Na^+/K^+-ATPase activity and decreased haemolymph [Na^+] at rest underlie improvements in insect cold tolerance, both within species (phenotypic plasticity) and among species (genetic adaptation). To test this hypothesis, we used thermal acclimation to induce variation in cold tolerance via phenotypic plasticity in *D. melanogaster*, and 24 species of *Drosophila* whose cold tolerance naturally varies when reared under common conditions, with the CT_{min} used as an index of cold tolerance. We predicted that cold-acclimated *D. melanogaster* and cold-tolerant species would have low haemolymph [Na^+], thereby reducing transmembrane and transepithelial Na^+ gradients at rest, which would consequently protect against Na^+ leak in the cold. We also predicted that cold-tolerant flies would maintain low haemolymph [K^+], which would reduce the impact of any cold-induced loss of Na^+ and water balance on cell survival. Lastly, we hypothesized that cold-tolerant *Drosophila* better maintain their ion gradients when exposed to low temperatures and predicted that cold-adapted and cold-acclimated flies would display reduced thermal sensitivity of Na^+/K^+-ATPase.

Here we show that cold exposure below the CT_{min} causes elevated haemolymph [K^+] in *D. melanogaster*, as in other insects, and that within- and among-species improvements in cold tolerance are accompanied by low haemolymph cation concentrations. Despite low concentrations of cations, osmotic homeostasis is maintained, with the changes in extracellular ion balance at low temperatures probably compensated by accumulating other compatible osmolytes. This change in cation homeostasis appears to be associated with reduced Na^+/K^+-ATPase activity at rest (particularly with cold acclimation), but we observed no differences in the thermal sensitivity of the enzyme.

RESULTS

Critical thermal minima

We measured the CT_{min} of adult males and females of 24 *Drosophila* species (supplementary material Table S1), reared under common laboratory conditions and fed a similar diet; and of adult male *D. melanogaster* acclimated for 5 days to 'warm' (i.e. a typical laboratory culture temperature; 21.5°C) and 'cold' (6°C) temperatures. We used phylogenetically independent contrasts (PICs; which account for phylogeny in statistical analyses), to test whether among-species relationships between physiological traits and cold tolerance can be explained by the phylogenetic relationships of our sample species (Garland et al., 1992).

The CT_{min} varied by more than 11°C among species (Fig. 1; supplementary material Fig. S1; Table S2) and had significant phylogenetic signal (K=0.77, P=0.002), meaning closely related species share similar critical thermal minima (Fig. 1). We showed previously that acclimation to 6°C reduced the CT_{min} of male *D. melanogaster* in our laboratory population from 3.4±0.2 to 0.9±0.1°C (Ransberry et al., 2011).

Haemolymph ion concentrations and osmolality

We measured ion concentrations in haemolymph extracted from warm- and cold-acclimated *D. melanogaster*, both at their acclimation temperatures and following a 6 h exposure to 0°C. Acclimation temperature and cold exposure interacted to significantly affect haemolymph [K^+] ($F_{1,71}$=4.6, P=0.036, Fig. 2A). Haemolymph [K^+] did not differ significantly between warm- and cold-acclimated *D. melanogaster* at their respective acclimation temperatures (Tukey's HSD, P=0.291; Fig. 2A), and a 6 h exposure to 0°C significantly increased haemolymph [K^+], but only in warm-acclimated flies (Fig. 2A; Tukey's HSD; P=0.004). By contrast,

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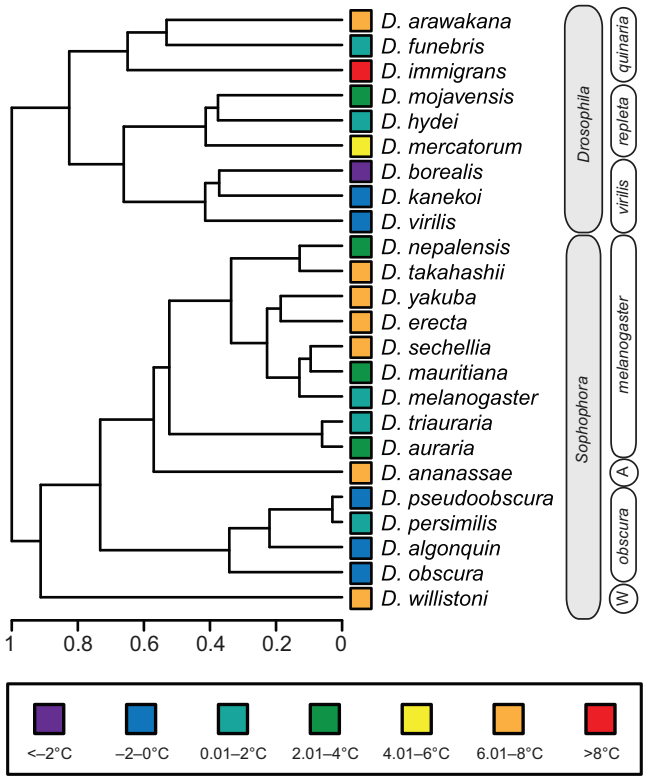

Fig. 1. The critical thermal minimum (CT_{min}) varies widely across the *Drosophila* phylogeny and has strong phylogenetic signal. Phylogeny of members of the genus *Drosophila* used in this study, and among-species variation in the CT_{min}. Branch lengths represent relative time since divergence from a common ancestor. Coloured boxes represent the CT_{min} (temperature at which 80% of flies are knocked down). Vertical labels denote sub-genera (grey) and groups (white) according to current FlyBase designations. A, Ananassae group; W, Willistoni group. See supplementary material Fig. S1 and Table S2 for frequency distributions, sample sizes and summary statistics of CT_{min} values for each species.

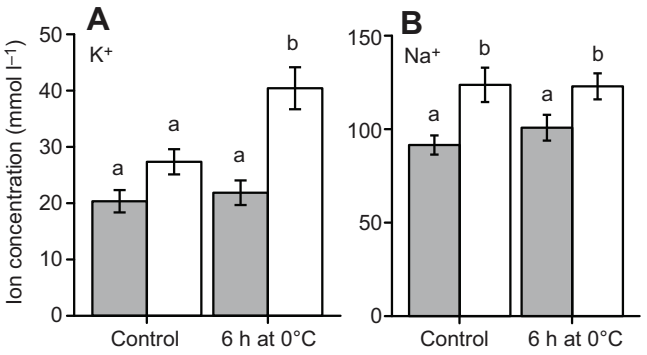

Fig. 2. Cold-acclimated *D. melanogaster* have low haemolymph [Na^+] and defend against the effects of cold exposure on haemolymph [K^+]. Haemolymph K^+ (A) and Na^+ (B) concentrations of cold-acclimated (grey bars) and warm-acclimated (white bars) male *Drosophila melanogaster* at their acclimation temperature (control) and following 6 h at 0°C (mean±s.e.m.). Bars within a panel that share a letter do not significantly differ. N=14–16 (Na^+) and N=15–21 (K^+) per acclimation group per treatment.

cold-acclimated *D. melanogaster* maintained low haemolymph [K^+] during exposure to 0°C (Tukey's HSD; P=0.441; Fig. 2). Haemolymph [Na^+] of cold-acclimated *D. melanogaster* was ~25% lower than that of warm-acclimated flies at their respective acclimation temperature ($F_{1,56}$=14.1, P<0.001), and exposure to 0°C for 6 h had no effect on haemolymph [Na^+] in either acclimation group ($F_{1,56}$=0.4, P=0.544; Fig. 2B).

Haemolymph [K^+] at rest ranged from 16.8±1.4 mmol l^{-1} (*D. auraria*) to 37.6±2.8 mmol l^{-1} (*D. willistoni*; Fig. 3A) among the *Drosophila* species and had significant phylogenetic signal (K=0.70, P=0.014), as some cold-tolerant species groups (such as the obscura and virilis groups) had a consistently low haemolymph [K^+] (supplementary material Table S2). There was a significant positive relationship between the CT_{min} and extracellular [K^+] among species (r=0.72, d.f.=20, P<0.001; Fig. 3A). Resting haemolymph [Na^+] of *Drosophila* species (at 21.5°C) ranged from 47.6±4.7 mmol l^{-1} (*D. immigrans*) to 154.1±6.7 mmol l^{-1} (*D. sechellia*; supplementary material Table S2), and did not have significant phylogenetic signal (K=0.43, P=0.125; Fig. 3B). *Drosophila immigrans* had remarkably low [Na^+], and when this species was included in the correlation of [Na^+] against the CT_{min} the relationship was non-significant (r=0.20, d.f.=20, P=0.371). However, if *D. immigrans* is excluded, there was a significant positive relationship between the CT_{min} and haemolymph [Na^+] (r=0.48, d.f.=19, P=0.029). Thus cold-tolerant species had low concentrations of both Na^+ and K^+ in their haemolymph, and these relationships between ion concentrations and the CT_{min} both remained significant following PIC regression ([K^+]: $F_{1,20}$=12.3, P=0.002; [Na^+] not including *D. immigrans*: $F_{1,19}$=8.5, P=0.008).

We measured the osmolality of haemolymph collected under control conditions to determine whether the observed reductions in ion concentrations in cold-tolerant flies drive reductions in extracellular osmolality. Despite the variation observed in haemolymph ion concentrations, haemolymph osmolality did not significantly differ between warm- (420±23 mOsm) and cold-acclimated (396±23 mOsm) *D. melanogaster* (t_{11}=0.67, P=0.526), and there was no relationship between haemolymph osmolality and CT_{min} among *Drosophila* species before (r=0.16, d.f.=20, P=0.485; Fig. 3C), or after PIC regression ($F_{1,20}$=1.5, P=0.240; supplementary material Fig. S2).

Na^+/K^+-ATPase activity and thermal sensitivity

To examine whether variation in haemolymph ion homeostasis is associated with modulation of Na^+/K^+-ATPase, we measured maximal activity of this ion transporter during a temperature ramp. This approach yielded a complete data set of enzyme V_{max} between 3 and 22°C from each biological replicate prepared from whole-fly homogenates. Na^+/K^+-ATPase activity (V_{max}), the temperature–activity inflection point (IP), and thermal sensitivity (Ts) of activity were extracted from logistic models fitted to Na^+/K^+-ATPase V_{max} (Fig. 4; see Materials and methods for further details).

When measured at a common temperature (21.5°C) Na^+/K^+-ATPase activity of cold-acclimated *D. melanogaster* was 55% lower than that of warm-acclimated flies (t_8=4.4, P<0.001, Fig. 5A). Similarly at 6°C (the cold acclimation temperature) Na^+/K^+-ATPase activity was 54% lower in the cold-acclimated flies. Comparing the groups at their respective acclimation temperature reveals that cold-acclimated *D. melanogaster* had 93% lower Na^+/K^+-ATPase V_{max} than warm-acclimated flies. Thus cold acclimation suppresses Na^+/K^+-ATPase activity beyond that caused by the passive effects of temperature (Fig. 5A).

The V_{max} of Na^+/K^+-ATPase at 21.5°C ranged among species from 14.4±1.7 U g^{-1} (μmol ATP min^{-1} g^{-1}; *D. funebris*) to 75.4±13.3 U g^{-1} (*D. yakuba*; supplementary material Table S2). Although there was a trend for more cold-tolerant species to have low Na^+/K^+-ATPase V_{max}, there was no significant relationship between Na^+/K^+-ATPase

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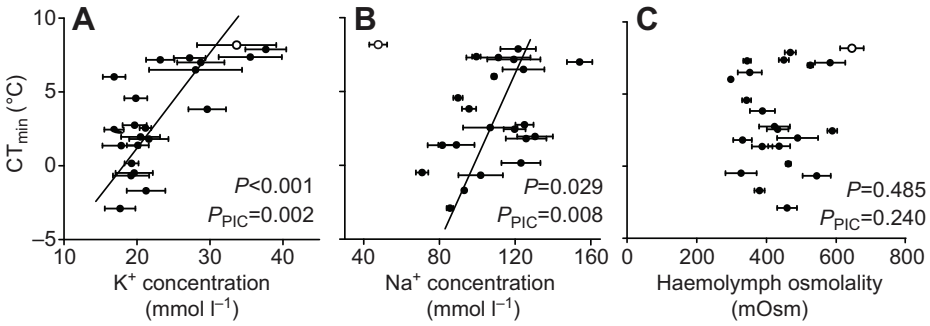

Fig. 3. Cold-adapted *Drosophila* species have low haemolymph [K^+] and [Na^+]. Haemolymph concentrations of K^+ (A) and Na^+ (B) and haemolymph osmolality (C) of species of the genus *Drosophila* are shown in relation to the CT_{min}. Lines denote a significant linear relationship between ion concentration and the CT_{min} among species. *Drosophila immigrans* (open circle) had remarkably low haemolymph [Na^+] and was omitted from the regression for Na^+ (see text for details), but was included in the regressions for [K^+] and osmolality. *P*, *P*-value from correlation of raw data; P_{PIC}, *P*-value from a regression using phylogenetically independent contrasts forced through the origin (supplementary material Fig. S2). *N*=4–8 (K^+) and *N*=3–5 (Na^+). *y*-axis (CT_{min}) error bars (s.e.m.) are obscured by the symbols.

activity and CT_{min} among species (r=0.30, d.f.=21, P=0.162; Fig. 6A). Excluding *D. immigrans*, the relationship between Na^+/K^+-ATPase activity and the CT_{min} approached statistical significance (r=0.42, d.f.=20, P=0.053; Fig. 6A), but we note that here *D. immigrans* did not substantially deviate from the other species. Na^+/K^+-ATPase activity had strong phylogenetic signal (K=1.20, P=0.001), but accounting for phylogeny did not resolve any relationship between Na^+/K^+-ATPase activity at 21.5°C and CT_{min} among *Drosophila* species ($F_{1,21}$=0.2, P=0.670; supplementary material Fig. S2). We did, however, find a positive linear relationship between haemolymph Na^+ concentration and $\log_{10}$-transformed Na^+/K^+-ATPase activity among the *Drosophila* species (r=0.47, d.f.=19, P=0.030; supplementary material Fig. S3).

Contrary to our hypothesis that variation in cold tolerance is driven by changes in the thermal sensitivity of Na^+/K^+-ATPase, neither the inflection point (t_9=1.8, P=0.101) nor thermal sensitivity (t_9=1.2, P=0.261) of Na^+/K^+-ATPase activity differed significantly between warm- and cold-acclimated *D. melanogaster* (Fig. 5B). Similarly, the CT_{min} correlated with neither the thermal sensitivity (r=182, d.f.=21, P=0.403; Fig. 6C), nor the inflection point (r=0.19, d.f.=21, P=0.385; Fig. 6B) of Na^+/K^+-ATPase activity among

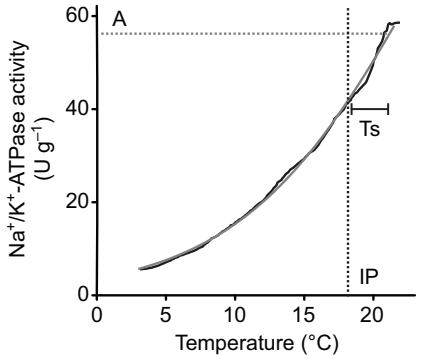

Fig. 4. A logistic equation fitted to temperature effects on maximal activity of Na^+/K^+-ATPase. Example of a logistic model (continuous grey line) fitted to measured Na^+/K^+-ATPase activity of warm-acclimated *Drosophila melanogaster* (continuous black line). The temperature at which the curve stops accelerating and begins decelerating (the inflection point, IP; dotted black line) as well as the thermal sensitivity (Ts) of Na^+/K^+-ATPase were extracted from the model equation (see Materials and methods). Sodium pump activity across the range of temperatures (including at the rearing temperature, 21.5°C; A: dotted grey line) were extracted from model predictions and used to compare activity between acclimation groups (*D. melanogaster*) and among *Drosophila* species.

Drosophila species. There was also no relationship between the PICs of CT_{min} and the inflection point ($F_{1,21}$=1.3, P=0.262) or thermal sensitivity ($F_{1,21}$=3.3, P=0.083; Fig. 6C), and neither the Na^+/K^+-ATPase inflection point (K=0.17, P=0.841) nor thermal sensitivity (K=0.20, P=0.727) had significant phylogenetic signal. Thus we detected no alterations in the shape or position of the *in vitro* activity–temperature curve for Na^+/K^+-ATPase, within or among *Drosophila* species, but instead observed wholesale reductions in pump activity (particularly in cold-acclimated *D. melanogaster*).

Na^+/K^+-ATPase transcript and protein abundance in *D. melanogaster*

Plasticity of Na^+/K^+-ATPase activity can result from transcriptional, translational or post-translational changes (Bertorello et al., 1991; McDonough and Farley, 1993). We examined whether the observed reduction in Na^+/K^+-ATPase activity with cold acclimation in *D. melanogaster* was related to a reduction in mRNA transcript or protein abundance of the Na^+/K^+-ATPase subunits, using high-throughput mRNA sequencing (RNA-seq) and western blotting, respectively. Transcript abundance of the primary Na^+/K^+-ATPase α-subunit gene (*Atpα*) did not differ between acclimation groups (t_9=1.0, P=0.723; supplementary material Fig. S4). *Drosophila melanogaster* has three genes that code for the Na^+/K^+-ATPase β-subunit (*nrv1*, *nrv2* and *nrv3*). Cold-acclimated flies had significantly higher expression of *nrv2* mRNA than warm-acclimated flies (t_9=3.7, P=0.003), but the relative abundance of either *nrv1* (t_9=0.7, P=0.897) or *nrv3* (t_9=1.9, P=0.257; supplementary material Fig. S4) did not differ between warm- and cold-acclimated flies. Similarly, cold acclimation did not change the protein abundance of either the α-subunit ($F_{2,11}$=0.9, P=0.379) or β-subunit ($F_{2,6}$=0.2, P=0.835; supplementary material Fig. S4) of Na^+/K^+-ATPase.

DISCUSSION

Drosophila lose ion balance in the cold

When exposed to 0°C for 6 h, warm-acclimated *D. melanogaster* lost the ability to maintain ion and water homeostasis, leading to an increase in haemolymph [K^+]. This increase in haemolymph [K^+] also occurred during cold exposure in chill-susceptible cockroaches (Koštál et al., 2006), crickets (MacMillan and Sinclair, 2011b), locusts (Findsen et al., 2013) and firebugs (Koštál et al., 2004), which suggests that cold exposure leads to a loss of [K^+] balance in both holometabolous and hemimetabolous chill-susceptible insects.

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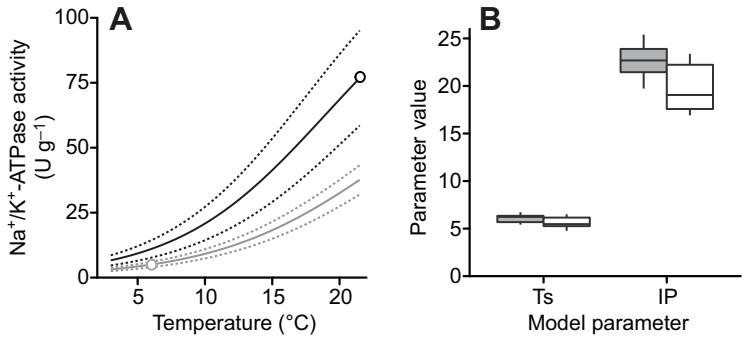

Fig. 5. Cold-acclimated *Drosophila melanogaster* have low Na^+/K^+-ATPase activity. (A) Sodium pump activity of warm-acclimated (21.5°C, black) and cold-acclimated (6°C, grey) *D. melanogaster*. Continuous and dotted lines represent the mean and 95% confidence interval, respectively. Open circles denote mean activity at the acclimation temperature. Cold-acclimated *D. melanogaster* had significantly lower Na^+/K^+-ATPase activity at 21.5°C than warm-acclimated flies. (B) Boxplots of Na^+/K^+-ATPase inflection point (IP) and thermal sensitivity (Ts) values of warm-acclimated (white) and cold-acclimated (grey) *D. melanogaster*. Vertical lines denote the range, and horizontal lines denote the median and quartiles. There was no significant effect of acclimation temperature on IP or Ts. *N*=6 biological replicates per acclimation group (see text for details).

Muscle and nerve resting potentials depend mostly on extracellular $[K^+]$ (Hoyle, 1953; Armstrong et al., 2012). Therefore high haemolymph $[K^+]$ is likely to depolarize cell membranes, prevent neuromuscular signal transmission (Hosler et al., 2000), slow chill-coma recovery (MacMillan et al., 2012; MacMillan et al., 2014) and lead to chilling injury (Koštál et al., 2006; MacMillan and Sinclair, 2011b).

In contrast to the warm-acclimated flies, *D. melanogaster* acclimated to 6°C maintained low extracellular $[K^+]$ after 6 h at 0°C. Phenotypic plasticity in cold tolerance has been consistently associated with an enhanced ability to maintain ion and water balance during cold exposure; cold-acclimated crickets, tropical cockroaches and adult firebugs also maintain haemolymph $[K^+]$ at low levels during cold exposure (Koštál et al., 2004; Koštál et al., 2006; Coello Alvarado, 2012). Thus, like other insects, acclimation to low temperatures appears to improve the ability of *Drosophila* to maintain low haemolymph $[K^+]$ during cold exposure. This ability is probably driven by maintenance of Na^+ and water balance in the haemolymph.

Cold-tolerant flies maintain low haemolymph cation concentrations

We observed that cold-tolerant *Drosophila* species maintain lower concentrations of both Na^+ and K^+ in their haemolymph, and that modifications to cold tolerance were generally accompanied by inverse modifications in haemolymph ion concentrations across the *Drosophila* phylogeny (because these relationships remained significant following PIC analysis). Similarly, cold-acclimated *D. melanogaster* constitutively maintain low extracellular $[Na^+]$, but acclimation has no effect on haemolymph $[K^+]$ at rest.

Decreased haemolymph $[Na^+]$ could improve cold tolerance by reducing the influence of Na^+ gradients on water balance, but only if other osmolytes fill the 'osmotic gap' left by Na^+ and thereby maintain haemolymph osmotic pressure (Wyatt, 1961; Pierce et al., 1999). Adult Lepidoptera maintain low haemolymph $[Na^+]$, in some cases low enough to reverse the muscle Na^+ gradient (Fitzgerald et al., 1996). To maintain haemolymph osmotic balance in the absence of Na^+, Lepidoptera maintain high haemolymph concentrations of carbohydrates (Wyatt and Kalf, 1957; Wyatt, 1961), and, as a group, maintain muscle excitability to lower temperatures than members of Diptera and Hymenoptera, which have more 'conventional' (high) extracellular $[Na^+]$ (Natochin and Parnova, 1987; Goller and Esch, 1990). Although we observed low cation concentrations in more cold-tolerant flies, we observed no differences in haemolymph osmolality between warm- and cold-acclimated *D. melanogaster*, nor among *Drosophila* species. Thus, reductions in haemolymph $[Na^+]$ in *Drosophila* must be paired with accumulations of other extracellular osmolytes.

Small accumulations of extracellular osmolytes in chill-susceptible insects may protect organismal water balance at low temperatures. A variety of organic solutes act as cryoprotectants at very high concentrations, and are central to the overwintering success of freeze-tolerant and freeze-avoidant insects (Lee, 1991; Storey, 1997). Relatively modest amounts of such compounds, at concentrations unlikely to yield cryoprotection, have been noted to accumulate following cold-exposure in chill-susceptible insects, including *D. melanogaster* (Lee et al., 1987; Overgaard et al., 2007). For example, brief cold exposures that improve subsequent cold tolerance in flesh flies (rapid cold-hardening) increase haemolymph glycerol concentrations from 28 to 81 mmol l^{-1}, a change that is too small to significantly change the freezing point (Lee et al., 1987). Many cryoprotectants are also osmoprotectants (Yancey, 2005; Sinclair et al., 2013; Teets et al., 2013). Thus we propose that these relatively small accumulations of organic solutes that have been

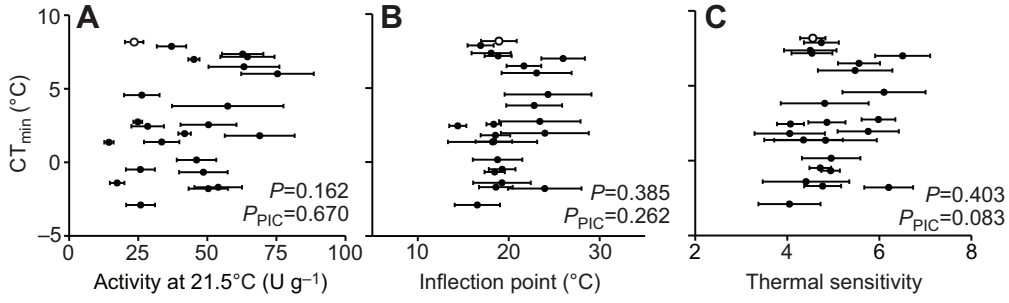

Fig. 6. Cold-tolerant *Drosophila* species tend to have low Na^+/K^+-ATPase activity. Mean (±s.e.m.) Na^+/K^+-ATPase activity at 21.5°C (A), inflection point (B) and thermal sensitivity (C) of species of the genus *Drosophila* in relation to the CT_{min}. *Drosophila immigrans* (open circle) had low Na^+/K^+-ATPase activity but was retained in the analysis of activity at 21.5°C. *P*, *P*-value from correlation of raw data; P_{PIC}, *P*-value from a regression of phylogenetically independent contrasts forced through the origin (supplementary material Fig. S2). *N*=3–6 biological replicates per species. CT_{min} error bars (s.e.m.) are obscured by the symbols.

previously observed serve to maintain or increase haemolymph osmotic pressure in the place of highly permeable osmolytes (particularly Na^+), and thereby permit a decoupling of Na^+ and water balance that promotes low temperature survival. We hypothesize that such a change in osmotic balance may have facilitated the evolution of more extreme cold tolerance phenotypes (such as freeze avoidance and freeze tolerance) associated with accumulations of low molecular weight cryoprotectants.

Cold-tolerant flies tend to have reduced Na^+/K^+-ATPase activity

Cold-acclimated *D. melanogaster* had significantly reduced Na^+/K^+-ATPase activity, and the low haemolymph [Na^+] observed in cold-adapted *Drosophila* species was positively correlated with maximal activity of Na^+/K^+-ATPase *in vitro*. The ubiquitous Na^+/K^+-ATPase plays important roles in both cellular and whole-organism ion balance, but importantly functions in the *Drosophila* renal system to maintain high extracellular [Na^+] (see Introduction). Our observations are thus consistent with our expectation that Na^+/K^+-ATPase activity influences (but does not wholly determine) haemolymph [Na^+] and may play a role in determining thermal tolerance through modification of resting [Na^+] gradients. Given the complexity of insect renal physiology and the fact that our estimates of Na^+/K^+-ATPase activity were necessarily based on whole-organism homogenates, the observation that Na^+/K^+-ATPase activity does not directly correlate with the CT_{min} among species is perhaps not surprising, and suggests that selection for cold tolerance does not act directly on resting Na^+/K^+-ATPase activity.

Phenotypic plasticity of Na^+/K^+-ATPase activity in *D. melanogaster* does not appear to be achieved through changes in mRNA or protein abundance, as we observed no differences in the abundance of either the α- or β-subunit proteins and no changes in the expression of subunit transcripts except for a modest increase in one β-subunit isoform (*nrv2*). Alternatively, modulation of Na^+/K^+-ATPase activity may involve differential expression of the 11 tissue-specific *Atpα* alternative transcripts that are known (but could not be distinguished accurately in our RNA-seq experiment because of the close similarity in sequence among isoforms) (Marygold et al., 2013), or post-translational modification. For example, suppression of Na^+/K^+-ATPase activity though reversible phosphorylation is associated with winter diapause in the goldenrod gall fly (McMullen and Storey, 2008). We suggest that post-translational modification or alternative isozyme expression reduce Na^+/K^+-ATPase activity and improve organismal cold tolerance through reduced Na^+ gradients.

Reductions in the effects of temperature on enzyme activity can allow for comparatively higher rates of catalysis at low temperatures (Galarza-Muñoz et al., 2011). However, we found no evidence to support the hypothesis that cold-acclimated and cold-adapted *Drosophila* better maintain ion balance at low temperatures through reductions in the thermal sensitivity of Na^+/K^+-ATPase. Although the estimates of Na^+/K^+-ATPase V_{max} we obtained *in vitro* are reliable indicators of relative differences in Na^+/K^+-ATPase activity, they may not approximate rates of ion transport *in vivo*, where local substrate and co-factor concentrations and the immediate membrane environment could have a substantial impact on ion transport rates and thermal sensitivity. Adaptation and acclimation to low temperatures in *Drosophila* have been associated with decreased saturation of phospholipid fatty acids, which would increase membrane fluidity and maintain ion pump function at low temperatures (Ohtsu et al., 1993; Ohtsu et al., 1998; Overgaard et al., 2005; Overgaard et al., 2008) (but see MacMillan et al., 2009). Comparative analyses of Na^+/K^+-ATPase activity functioning within the membrane bilayer are needed to elucidate the role of these influences on ion balance in the cold. Because the physiological mechanisms underlying insect ionoregulation are diverse (O'Donnell, 2008), the reductions in Na^+/K^+-ATPase activity observed here may be one of several mechanisms of ionoregulation that contribute to cold tolerance. The roles, for example, of other ion-motive pumps (i.e. H^+-ATPase and Ca^{2+}-ATPase) and exchangers, ion channels and aquaporins, paracellular routes of ion and water transport, and the hormones that regulate them remain to be explored in the context of insect cold acclimation and adaptation.

Conclusions

Cold-acclimated *D. melanogaster* and cold-tolerant *Drosophila* species have reduced reliance on Na^+ as an extracellular cation and more cold-tolerant species also maintain lower extracellular [K^+]. Cold-tolerant flies probably maintain osmotic balance by accumulating other organic and inorganic osmolytes in their haemolymph to replace Na^+ as the primary determinant of water balance. These changes to ionic and osmotic homeostasis would limit Na^+ and water migration, maintain K^+ balance in the cold, and thereby improve cold tolerance. Thus similar ionoregulatory mechanisms appear to underlie both phenotypic plasticity and cold tolerance evolution, which could lead to trade-offs between basal and plastic responses to cold, and suggest an evolutionary route for the cryoprotectant-mediated cold tolerance strategies of freeze tolerance and freeze avoidance.

MATERIALS AND METHODS

Animal origins and husbandry

We examined a total of 24 species from the genus *Drosophila*. Complete information on stock origins is presented in supplementary material Table S1. All species were maintained on the same banana, barley malt and yeast-based medium (Nyamukondiwa et al., 2011), except that the diet of four cactophilic species (*D. mojavensis*, *D. obscura*, *D. persimilis* and *D. pseudoobscura*) was supplemented with 2.1 g l^{-1} *Opuntia ficus-indicta* powder (OroVerde Export, Morelos, Mexico). With the exception of cold-acclimated *D. melanogaster* (described below), all flies were maintained at a constant temperature of 21.5±0.5°C, and at 50±5% relative humidity with a 13 h:11 h light:dark cycle. Newly eclosed adult *Drosophila* were transferred, without anesthesia, to fresh 35 ml vials containing food medium. Following transfer, adults were returned to 21.5°C for 5 days before use in experiments. For the *D. melanogaster* acclimation experiments, virgin males were collected under light CO_2 anesthesia (<10 min) on the day of their emergence, divided randomly into two groups which were placed at either 21.5°C (warm-acclimated) or 6±0.5°C conditions (cold-acclimated) for 5 days to acclimate.

Measurement of CT_{min}

The CT_{min} of each *Drosophila* species was quantified as previously described (Ransberry et al., 2011). Adult flies were transferred into a custom-built, temperature-controlled 150×25 cm glass column containing aluminium baffles to which the flies cling [similar to the design of Huey et al. (Huey et al., 1992)]. The temperature of the column was controlled by circulating an ethylene glycol:water (1:1) mixture through the column jacket from a refrigerated circulating bath (model 1157P, VWR International, Radnor, PA, USA). The temperature inside the column was independently monitored by four type-T thermocouples (two at both the top and bottom of the column) and a TC-08 interface connected to a computer running Picolog version 5.20.1 (Pico Technology, St Neots, UK). Adult flies were released into the column where they clung to the baffles, and the temperature was held at 21°C for 15 min before being reduced at 0.1°C min^{-1}. At their CT_{min}, flies lost the ability to cling to the baffles and fell into a collecting tube containing soapy water that was changed every 1°C (10 min). Flies collected from the column were frozen, and later sorted according to sex and counted. For the interspecific comparisons a mean of 206 (range: 78–695) flies were

used to determine the CT_{min} of each sex in each *Drosophila* species (supplementary material Fig. S1).

Haemolymph collection

Adult *Drosophila* were positioned for haemolymph sampling using a custom-made apparatus previously described (MacMillan and Hughson, 2014). Briefly, adult flies were moved directly from their rearing vial and positioned head-first in a 10 µl pipette tip through a system of rubber tubing by air flow. The end of the pipette tip was then removed to expose the antennae, an antenna was amputated at its first segment, and a clear droplet of haemolymph was secreted. The pipette tip, with the fly and droplet attached, were immediately removed from the rest of the device and the droplet was placed under hydrated paraffin oil for measurement of Na^+ and K^+ concentrations by the ion-selective microelectrode technique or osmolality by nanoliter osmometry (see below). The time from removal of a fly from its acclimation temperature or from 0°C to measurement of ion concentration or osmolality of the haemolymph was less than 2 min.

Haemolymph ion concentration and osmolality

Droplets of haemolymph from adult flies were used to measure extracellular ion concentrations and osmolality. Different flies were used to measure each trait and a single droplet was taken from a single fly. Haemolymph ion concentrations and osmolality were measured in 4–8 (K^+), 3–5 (Na^+) and 3–8 (osmolality) droplets from flies of each species and osmolality was measured in 5–7 droplets from *D. melanogaster* males from each acclimation group. Ion concentrations were measured in 14–16 (Na^+) and 15–21 (K^+) droplets per treatment (control and 6 h at 0°C) from flies in each acclimation group of *D. melanogaster*. The *D. kanekoi* and *D. algonquin* lines were lost to mould before haemolymph ion concentrations and osmolality were measured, and so were not included in this analysis. Haemolymph Na^+ and K^+ concentrations were measured in all *Drosophila* at rearing temperature (21.5±1°C). For the cold exposure, *D. melanogaster* were transferred to microcentrifuge tubes and submerged in an ice-water slurry (0°C) for 6 h. Flies that received a cold exposure were sampled immediately following removal from the cold to 21.5°C.

Ion concentration was measured using an ion-selective microelectrode (ISME) technique using pulled glass microelectrodes front-filled with ionophore cocktails (Jonusaite et al., 2011). Ion-selective microelectrodes were constructed by pulling borosilicate glass capillaries [TW-150-4, World Precision Instruments (WPI), Sarasota, FL, USA] to a tip diameter of ~5 µm using a P-97 Flaming Brown micropipette puller (Sutter Instruments, Novato, CA, USA). Pulled micropipettes were silanized at 300°C with *N*,*N*-dimethyltrimethylsilylamine vapour for 1 h and backfilled with 100 mmol l^{-1} KCl or NaCl. Microelectrodes were then front-filled with ionophore cocktails for either K^+ (K^+ ionophore I, cocktail B, Sigma-Aldrich, St Louis, MO, USA) or Na^+ (Na^+ ionophore X; Messerli et al., 2008) and dipped in a solution of polyvinylchloride (Sigma-Aldrich) in tetrahydrofuran (Sigma-Aldrich). A borosilicate glass (IB200F-4, WPI) reference electrode back-filled with 0.5 mol l^{-1} KCl was used to complete the circuit. Voltage was recorded using a ML 165 pH amplifier and PowerLab 4/30 data acquisition system connected to a computer running LabChart 6 software (AD Instruments, Colorado Springs, CO, USA).

Haemolymph ISME voltages were converted to ion concentration by reference to calibration solutions of known concentration using Eqn 1:

$$[h] = [c] \times 10^{\Delta V/S}, \quad (1)$$

where $[h]$ is the active ion concentration in the haemolymph, $[c]$ is the concentration in one of the calibration solutions, ΔV is the voltage difference between the calibration solution and haemolymph, and S is the slope of the voltage response to a tenfold concentration difference in calibration solutions.

Haemolymph osmolality was measured using a Clifton Nanoliter Osmometer (Clifton Technical Physics, Hartford, NY, USA). Small droplets obtained as described above (~20 nl) were suspended in wells filled with type B immersion oil under a Nikon SMZ 1500 microscope equipped with a Nikon Digital Sight DS-Fil camera connected to software NIS-Elements D2.30 SP4 Laboratory Imaging software (Nikon Corporation, Tokyo, Japan) and rapidly cooled until frozen. The droplets were then warmed slowly until the temperature at which one last crystal remained visible, before the crystal was warmed again to determine the melting point (i.e. the temperature at which the last crystal disappeared). The melting point was used to determine osmolality, as one mole of solute will decrease melting point by 1.86°C.

Na^+/K^+-ATPase activity and transcript and protein abundance

To quantify maximal Na^+/K^+-ATPase activity, whole flies (~80 mg of pooled adults; 20–80 flies, depending on species) were transferred to 1.7 ml microcentrifuge tubes without anaesthesia, snap frozen in liquid nitrogen vapour, and stored at −80°C. Na^+/K^+-ATPase activity was measured in six biological replicates of each temperature acclimation group of *D. melanogaster*, and 3–6 biological replicates of each species were used for the interspecific analysis. *Drosophila arawakana* was excluded from this analysis because of insufficient sample size (*N*=1). Frozen *Drosophila* were weighed to obtain pooled fresh mass in pre-weighed 2 ml microcentrifuge tubes before being homogenized on ice in 1 ml of homogenization buffer (25 mmol l^{-1} imidazole, 0.2% w/v Na^+-deoxycholate, 10 mmol l^{-1} β-mercaptoethanol, 2 mmol l^{-1} EDTA, pH 7.5) with a Tissue-Tearor (Biospec Products, Bartlesville, OK, USA) using four 10 s bursts each followed by 20 s rest on ice. Homogenized samples were sonicated (Virsonic 100; VirTis, Gardiner, NY, USA) following the same timing of bursts and rests, and centrifuged at 7000 ***g*** for 5 min at 4°C. Size-exclusion filtration columns, which permit the passage of proteins larger than ~50 kDa, were prepared by plugging the tip of a 3 ml plastic syringe barrel with glass wool and adding 3 ml of Sephadex G-50 (GE Healthcare, Waukesha, WI, USA). Columns were stored at 4°C for a maximum of 2 weeks before use, and were conditioned by eight passes of 300 µl of homogenization buffer and 1 min centrifugations (500 ***g***). A conditioned column was placed into a clean 5 ml plastic test tube, a 300 µl aliquot of supernatant derived from the homogenate was added to the column, and the column within the tube was centrifuged at 500 ***g*** for 1 min to draw the sample through the column and into the tube.

Filtered fly homogenates were diluted 7-fold in homogenization buffer immediately before a 20 µl aliquot was added to a 1 ml cuvette containing 880 µl of assay buffer initially at 23°C. The reaction was initiated by the addition of 100 µl of a 50 mmol l^{-1} ATP solution. Final conditions for the assay were: 70 mmol l^{-1} imidazole (pH 7.5), 140 mmol l^{-1} NaCl, 30 mmol l^{-1} KCl, 7 mmol l^{-1} $MgCl_2$, 4 mmol l^{-1} phosphoenolpyruvic acid, 300 µmol l^{-1} NADH, 5 mmol l^{-1} ATP, 50 U ml^{-1} pyruvate kinase (EC: 2.7.1.40) and 50 U ml^{-1} lactate dehydrogenase (EC: 1.1.1.27).

Maximal activity of Na^+/K^+-ATPase was measured across a range of temperatures using a thermally dynamic, spectrophotometric assay. The absorbance of NADH at 340 nm was recorded using a Cary 100 Bio spectrophotometer with a Cary Peltier-effect temperature controller (Agilent Technologies, Santa Clara, CA, USA) connected to a computer running WinUV Thermal Application version 3.0 (Agilent Technologies). Four replicate cuvettes of each *Drosophila* sample were run, two of which contained 1 mmol l^{-1} ouabain (a specific inhibitor of Na^+/K^+-ATPase). Temperature inside a dummy cuvette was monitored by a ceramic temperature probe (Agilent Technologies) interfaced with the spectrophotometer, which controlled the rate of temperature change. A type-T thermocouple was also suspended in the dummy cuvette and connected to a TC-08 interface (Pico Technology), which measured the temperature inside the cuvette every second for the duration of each sample run. The rate of temperature change inside the cuvette was consistent throughout the temperature ramp (supplementary material Fig. S5A). Activity of Na^+/K^+-ATPase was determined as the difference between the rates of cuvettes containing 1 mmol l^{-1} ouabain and cuvettes that did not contain ouabain (supplementary material Fig. S5B). For each replicate, rates of change in absorbance (OD min^{-1}) were smoothed using a 15-point sliding window in LoggerPro (version 3.8.4, Vernier Software Inc., Beaverton, OR, USA).

Self-starting logistic models were fitted to Na^+/K^+-ATPase V_{max} estimates (U g^{-1}) across the range of measurement temperatures (using the SSlogis() function in R):

$$R = \frac{\text{Max}}{1 + e^{(\text{IP}-T)/\text{Ts}}}, \quad (2)$$

where T is temperature and R is the empirically determined rate of ATP turnover per minute at that temperature, Max is the model-derived logistic

asymptote, IP is the model-derived inflection point of the curve, and Ts is model-derived sensitivity of Rate to temperature (see Fig. 4). The IP parameter is a measure of the position of the temperature–activity curve on the temperature axis, and can be used to detect cold- or warm-shifts in the enzyme activity–temperature relationship. The Ts parameter is a measure of enzyme thermal sensitivity.

Logistic functions provided a good fit to the data (Fig. 4). As Max in this equation represents the enzyme reaction rate at $T=\infty$, and enzymes denature at high temperatures, this parameter is not biologically relevant and was not used in further analyses. Estimates of Ts and IP were obtained for each sample from the fitted models, and rates across the range of temperatures (3–21.5°C) were extracted from models using the predict() function in R (which allowed us to standardize all rates to common temperature intervals).

To confirm that the thermally ramped assays described above did not yield different results from a more traditional method, the activity of Na^+/K^+-ATPase was also measured at five static temperatures (6, 11, 13.3, 17.6 and 21.5°C) in $N=4$ samples of male *D. melanogaster* acclimated to 21.5°C (supplementary material Fig. S5C). The methods of sample preparation and the final assay conditions for the static-temperature Na^+/K^+-ATPase assays were the same as those used for the thermally dynamic assay. Once the temperature inside the dummy cuvette was stable at the set temperature (determined from the thermocouple trace), the reaction was initiated by the addition of ATP as in the thermally dynamic assay. Rates of Na^+/K^+-ATPase activity in OD min^{-1} over a 10 min period of recording were converted to moles of ATP consumed per minute using the Beer–Lambert law. Static and dynamic methods of the Na^+/K^+-ATPase assay yielded similar estimates of Na^+/K^+-ATPase activity in male *D. melanogaster* acclimated to 21.5°C (supplementary material Fig. S5C).

Na^+/K^+-ATPase protein abundance

The abundance of the Na^+/K^+-ATPase α- and β-subunit proteins was quantified by western blot in seven (α) and four to five (β) biological replicates of each acclimation group of *D. melanogaster*. Twenty adult male flies were freshly homogenized using a glass Dounce homogenizer in 1 ml of buffer containing detergent and protease inhibitors (160 mmol l^{-1} Tris-HCl, 0.2 % w/v Na^+-deoxycholate, 1 mmol l^{-1} phenylmethanesulfonylfluoride, 5 µg ml^{-1} aprotinin and leupeptin, pH 6.8). Homogenized samples were centrifuged at 7000 ***g*** and 4°C for 5 min. A 200 µl aliquot was reserved for protein quantification, and a second 200 µl aliquot was added to an equal volume of a 2× concentrated loading buffer to yield final loading conditions [4 mol l^{-1} urea, 4% w/v sodium dodecyl sulfate (SDS), 4% v/v β-mercaptoethanol, 4% v/v glycerol, 0.005% w/v bromophenol blue)]. Samples were loaded (15 µg soluble protein) on SDS-polyacrylamide gels (10% w/v resolving gel, 5% w/v stacking gel) in a SE600 electrophoresis unit (Hoefer Inc., Holliston, MA, USA). Loaded samples were separated by electrophoresis (120 V for ~2.5 h) in running buffer (25 mmol l^{-1} Tris-base, 192 mmol l^{-1} glycine, 0.3% w/v SDS, pH 8.6) and resolved proteins were wet transferred at 4°C to polyvinylidene fluoride (PVDF) membranes (Biotrace, Pall Corporation, Port Washington, NY, USA) at 15 V overnight (Newington et al., 2011). The following morning membranes were soaked in blocking buffer (20 mmol l^{-1} Tris-base, 150 mmol l^{-1} NaCl, 0.05% v/v Tween 20, 3% w/v BSA, 1.5% w/v dry blotting milk, pH 7.5) for 1 h at 22°C. Blocked membranes were washed (3×5 min) in wash buffer (20 mmol l^{-1} Tris-base, 150 mmol l^{-1} NaCl, 0.05% v/v Tween 20, pH 7.5). Membranes were incubated in primary antibody buffer (20 mmol l^{-1} Tris-base, 150 mmol l^{-1} NaCl, 0.05% v/v Tween 20, 3% w/v BSA, 0.05% w/v sodium azide, pH 7.5) for 1 h at 22°C. The primary antibody buffer contained mouse antibodies targeting either the α- [a5; 1:500; antigen species: chicken; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA, USA] or β-subunit (nrv5F7; 1:50; antigen species: *Drosophila*; DSHB) of Na^+/K^+-ATPase (Lebovitz et al., 1989; Sun and Salvaterra, 1995). Probed membranes were then washed (3×5 min, as above), incubated in blocking buffer containing goat anti-mouse IgG horseradish peroxidase conjugate secondary antibody (1:10,000; Bio-Rad Laboratories Inc., Hercules, CA, USA), and washed again. Following 1 min incubation in SuperSignal West Pico chemiluminescent substrate (Thermo Fisher Scientific, Rockford, IL, USA), images of immunoblots were produced using a ChemiDoc digital imaging system (Bio-Rad). After probing, membranes were stained for total protein as a loading control following described methods (Welinder and Ekblad, 2011). Membranes were washed (2×5 min), stained with 0.1% Coomassie R-350 in methanol:water (1:1) for 1 min, and destained with acetic acid:ethanol:water (1:5:4) for 20 min. Following destaining, the membranes were air-dried and scanned at 600 d.p.i. on a flatbed scanner. Densitometric quantification of immunoblots and total protein images were completed by measuring band and total lane intensity in ImageJ.

Na^+/K^+-ATPase mRNA abundance

The abundance of transcripts coding for Na^+/K^+-ATPase subunits was quantified using high-throughput mRNA sequencing (RNA-seq). Frozen warm- and cold-acclimated male *D. melanogaster* (five biological replicates per acclimation temperature, each containing 25 flies) were homogenized by pestle and mortar over liquid nitrogen in 1 ml of TRIzol reagent. Homogenized samples were centrifuged at 12,000 ***g*** for 5 min at 4°C and the top 200 µl (lipids) discarded. Total RNA extraction from samples was completed using a RNeasy lipid tissue mini kit (Qiagen, Hilden, Germany). Chloroform (200 µl) was added to the tubes, samples were shaken for 15 s, and left at 22°C for 3 min. Samples were centrifuged at 12,000 ***g*** for 15 min at 4°C, the upper (aqueous) phase transferred to a new tube with 1 volume of 70% ethanol, and vortexed (30 s). A 700 µl aliquot of the sample was centrifuged (8000 ***g*** for 15 s at 22°C) through an RNeasy Mini spin column (Qiagen) in a 2 ml collection tube. The effluent was discarded and the sample (bound to the column) was washed with 700 µl of RW1 buffer (Qiagen), and 2×500 µl of RPE buffer (Qiagen) using the same centrifuge settings. The column was placed into a new collection tube and RNase-free water (50 µl) was centrifuged through the column (8000 ***g*** for 1 min at 22°C) twice to release the RNA from the column. Total mRNA was purified from RNA, and cDNA libraries were prepared using a TruSeq Stranded mRNA preparation kit (Illumina, San Diego, CA, USA) following the TruSeq RNA Sample Prep version 2 protocol (Illumina). Briefly, mRNA was purified by poly-A selection using magnetic beads bound to poly-T oligo-nucleotides and chemically fragmented. Fragmented mRNA was reverse-transcribed into single-stranded cDNA by reverse transcriptase and the RNA on the opposite strand was replaced with DNA to yield double-stranded (ds) cDNA. Overhangs at the 3′- and 5′-ends of ds cDNA were cleaved and repaired by exonuclease and DNA polymerase, respectively.

Data analysis

All data analyses were completed in R version 3.1 (R Development Core Team, 2013). The temperature at which 80% of flies had fallen from the temperature-controlled column (CT_{min}) was determined using accelerated failure time models (Therneau and Grambsch, 2000) and the aggregate() function. Haemolymph ion concentrations and osmolality were compared among treatments and acclimation groups of *D. melanogaster* by ANOVA with group and treatment as factors, followed by Tukey's HSD. The relationships between haemolymph ion concentration or osmolality and the CT_{min} among *Drosophila* species were determined by Pearson's product-moment correlation, with line equations for plotting provided by ranged major axis model II regressions using the lmodel2 package (Legendre, 2013). Reaction rates (V_{max}) at 21.5°C, Ts and IP of Na^+/K^+-ATPase, were compared between warm- and cold-acclimated *D. melanogaster* using *t*-tests. Among *Drosophila* species, the relationships between these variables and the CT_{min} were tested by correlation, with RMA model II linear regression used for plotting purposes, as was the relationship between haemolymph [Na^+] and Na^+/K^+-ATPase activity. The abundance of each Na^+/K^+-ATPase subunit was compared between warm- and cold-acclimated *D. melanogaster* using an ANCOVA, with total protein abundance included as a covariate.

Transcriptome data manipulation and statistical analyses were completed in Galaxy (Goecks et al., 2010) following the analysis workflow of Trapnell et al. (Trapnell et al., 2012). Illumina adapter sequences were clipped from reads using FastQ Clipper (part of the FastX Toolkit), and clipped reads were aligned to the genome of *D. melanogaster* (Ensembl build 5.25) using TopHat (version 2.0.8; Trapnell et al., 2009), with alignment limited to known splice junctions. Cufflinks was used to assemble and count transcript reads for each sample and Cuffmerge was used to merge assembled

transcripts for all biological replicates into a single reference transcriptome (version 2.0.2) (Trapnell et al., 2010). Na^+/K^+-ATPase subunit transcript expression was compared between warm- and cold-acclimated flies using Cuffdiff (Trapnell et al., 2013).

Phylogenetically independent contrasts

We calculated PICs using a phylogeny constructed by combining two recently published *Drosophila* trees (Fig. 1). A recent comprehensive phylogeny of the family Drosophilidae (van der Linde et al., 2010) contains all but four of the species used in this study, and was used as the base for our tree. Extraneous species were trimmed from the van der Linde et al. tree (van der Linde et al., 2010), and the four additional species (*D. borealis*, *D. kanekoi*, *D. nepalensis* and *D. triauraria*) were added from a second phylogeny (Strachan et al., 2011) with branch lengths standardized to the rest of the tree using the ratio of nearest-neighbour distance. Node ages were standardized using a semi-parametric method for use in statistical analyses.

PICs of species trait means were generated in R using the pic() function in the ape package (Paradis et al., 2004). Tests of relationships between the CT_{min} and physiological traits while controlling for phylogeny were conducted using linear regressions of PICs forced through the origin (Garland et al., 1992) (supplementary material Fig. S2). Species traits were also tested for phylogenetic signal – a measure of the tendency for related species to have similar trait values – by the *K*-statistic (Blomberg et al., 2003).

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Competing interests

The authors declare no competing or financial interests.

Author contributions

H.A.M., B.J.S., J.F.S. and A.D. conceived and designed the research. H.A.M., L.V.F. and A.N. performed the experiments. H.A.M. and B.J.S. interpreted and analysed the data, H.A.M. and B.J.S. drafted the manuscript, and all authors revised the manuscript.

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Supplementary material

Supplementary material available online at
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References

Andersen, J. L., Manenti, T., Sørensen, J. G., MacMillan, H. A., Loeschcke, V. and Overgaard, J. (2015). How to assess *Drosophila* cold tolerance: chill coma temperature and lower lethal temperature are the best predictors of cold distribution limits. *Funct. Ecol.* **29**, 55-65.

Armstrong, G. A. B., Rodríguez, E. C. and Meldrum Robertson, R. (2012). Cold hardening modulates K^+ homeostasis in the brain of *Drosophila melanogaster* during chill coma. *J. Insect Physiol.* **58**, 1511-1516.

Ayrinhac, A., Debat, V., Gibert, P., Kister, A.-G., Legout, H., Moreteau, B., Vergilino, R. and David, J. R. (2004). Cold adaptation in geographical populations of *Drosophila melanogaster*: Phenotypic plasticity is more important than genetic variability. *Funct. Ecol.* **18**, 700-706.

Battisti, A., Stastny, M., Netherer, S., Robinet, C., Schopf, A., Roques, A. and Larsson, S. (2005). Expansion of geographic range in the pine processionary moth caused by increased winter temperatures. *Ecol. Appl.* **15**, 2084-2096.

Bechsgaard, J. S., Hoffmann, A. A., Sgró, C., Loeschcke, V., Bilde, T. and Kristensen, T. N. (2013). A comparison of inbreeding depression in tropical and widespread *Drosophila* species. *PLoS ONE* **8**, e51176.

Bertorello, A. M., Aperia, A., Walaas, S. I., Nairn, A. C. and Greengard, P. (1991). Phosphorylation of the catalytic subunit of Na^+,K^+-ATPase inhibits the activity of the enzyme. *Proc. Natl. Acad. Sci. USA* **88**, 11359-11362.

Beyenbach, K. W., Skaer, H. and Dow, J. A. T. (2010). The developmental, molecular, and transport biology of Malpighian tubules. *Annu. Rev. Entomol.* **55**, 351-374.

Blomberg, S. P., Garland, T., Jr and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* **57**, 717-745.

Calosi, P., Bilton, D. T. and Spicer, J. I. (2008). Thermal tolerance, acclimatory capacity and vulnerability to global climate change. *Biol. Lett.* **4**, 99-102.

Chen, I.-C., Hill, J. K., Ohlemüller, R., Roy, D. B. and Thomas, C. D. (2011). Rapid range shifts of species associated with high levels of climate warming. *Science* **333**, 1024-1026.

Chintapalli, V. R., Wang, J. and Dow, J. A. T. (2007). Using FlyAtlas to identify better *Drosophila melanogaster* models of human disease. *Nat. Genet.* **39**, 715-720.

Clark, A. G., Eisen, M. B., Smith, D. R., Bergman, C. M., Oliver, B., Markow, T. A., Kaufman, T. C., Kellis, M., Gelbart, W.; Drosophila 12 Genomes Consortium (2007). Evolution of genes and genomes on the *Drosophila* phylogeny. *Nature* **450**, 203-218.

Coello Alvarado, L. E. (2012). *Ion Homeostasis and Variation in Low Temperature Performance in the Fall and Spring Field Crickets (Orthoptera: Gryllidae),* Paper 969. University of Western Ontario, Electronic Thesis and Dissertation Repository. Available at: http://ir.lib.uwo.ca/etd/969.

Dong, Y. and Somero, G. N. (2009). Temperature adaptation of cytosolic malate dehydrogenases of limpets (genus Lottia): differences in stability and function due to minor changes in sequence correlate with biogeographic and vertical distributions. *J. Exp. Biol.* **212**, 169-177.

Emery, A. M., Djamgoz, M. B., Ready, P. D. and Billingsley, P. F. (1998). Insect Na^+/K^+-ATPase. *J. Insect Physiol.* **44**, 197-210.

Findsen, A., Andersen, J. L., Calderon, S. and Overgaard, J. (2013). Rapid cold hardening improves recovery of ion homeostasis and chill coma recovery time in the migratory locust, *Locusta migratoria*. *J. Exp. Biol.* **216**, 1630-1637.

Findsen, A., Pedersen, T. H., Petersen, A. G., Nielsen, O. B. and Overgaard, J. (2014). Why do insects enter and recover from chill coma? Low temperature and high extracellular potassium compromise muscle function in *Locusta migratoria*. *J. Exp. Biol.* **217**, 1297-1306.

Fitzgerald, E., Djamgoz, M. and Dunbar, S. (1996). Maintenance of the K^+ activity gradient in insect muscle compared in Diptera and Lepidoptera: contributions of metabolic and exchanger mechanisms. *J. Exp. Biol.* **199**, 1857-1872.

Galarza-Muñoz, G., Soto-Morales, S. I., Holmgren, M. and Rosenthal, J. J. C. (2011). Physiological adaptation of an Antarctic Na^+/K^+-ATPase to the cold. *J. Exp. Biol.* **214**, 2164-2174.

Garland, T., Harvey, P. H. and Ives, A. R. (1992). Procedures for the analysis of comparative data using phylogenetically independent contrasts. *Syst. Biol.* **41**, 18-32.

Garrett, S. and Rosenthal, J. J. C. (2012). RNA editing underlies temperature adaptation in K^+ channels from polar octopuses. *Science* **335**, 848-851.

Gilchrist, G. W., Huey, R. B. and Partridge, L. (1997). Thermal sensitivity of *Drosophila melanogaster*: evolutionary responses of adults and eggs to laboratory natural selection at different temperatures. *Physiol. Zool.* **70**, 403-414.

Goecks, J., Nekrutenko, A., Taylor, J. and Team, T. G. (2010). Galaxy: a comprehensive approach for supporting accessible, reproducible, and transparent computational research in the life sciences. *Genome Biol.* **11**, R86.

Goller, F. and Esch, H. (1990). Comparative study of chill-coma temperatures and muscle potentials in insect flight muscles. *J. Exp. Biol.* **150**, 221-231.

Hofmann, G. E. and Todgham, A. E. (2010). Living in the now: physiological mechanisms to tolerate a rapidly changing environment. *Annu. Rev. Physiol.* **72**, 127-145.

Hosler, J. S., Burns, J. E. and Esch, H. E. (2000). Flight muscle resting potential and species-specific differences in chill-coma. *J. Insect Physiol.* **46**, 621-627.

Hoyle, G. (1953). Potassium ions and insect nerve muscle. *J. Exp. Biol.* **30**, 121-135.

Huey, R., Crill, W., Kingsolver, J. and Weber, K. (1992). A method for rapid measurement of heat or cold resistance of small insects. *Funct. Ecol.* **6**, 489-494.

Jonusaite, S., Kelly, S. P. and Donini, A. (2011). The physiological response of larval *Chironomus riparius* (Meigen) to abrupt brackish water exposure. *J. Comp. Physiol. B* **181**, 343-352.

Kellermann, V., Loeschcke, V., Hoffmann, A. A., Kristensen, T. N., Fløjgaard, C., David, J. R., Svenning, J.-C. and Overgaard, J. (2012). Phylogenetic constraints in key functional traits behind species' climate niches: patterns of desiccation and cold resistance across 95 *Drosophila* species. *Evolution* **66**, 3377-3389.

Koštál, V., Vambera, J. and Bastl, J. (2004). On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in the adults of *Pyrrhocoris apterus*. *J. Exp. Biol.* **207**, 1509-1521.

Koštál, V., Yanagimoto, M. and Bastl, J. (2006). Chilling-injury and disturbance of ion homeostasis in the coxal muscle of the tropical cockroach (*Nauphoeta cinerea*). *Comp. Biochem. Physiol.* **143B**, 171-179.

Lebovitz, R. M., Takeyasu, K. and Fambrough, D. M. (1989). Molecular characterization and expression of the (Na^+ + K^+)-ATPase alpha-subunit in *Drosophila melanogaster*. *EMBO J.* **8**, 193-202.

Lee, R. E. (1991). Principles of insect low temperature tolerance. In *Insects at Low Temperature* (ed. R. E. Lee and D. L. Denlinger), pp. 17-36. New York, NY: Chapman and Hall.

Lee, R. E., Jr, Chen, C.-P. and Denlinger, D. L. (1987). A rapid cold-hardening process in insects. *Science* **238**, 1415-1417.

Legendre, P. (2013). *lmodel2: Model II Regression*. R package version 1.7-2. Available at: http://CRAN.R-project.org/package=lmodel2.

Linton, S. M. and O'Donnell, M. J. (1999). Contributions of K^+:Cl^- cotransport and Na^+/K^+-ATPase to basolateral ion transport in malpighian tubules of *Drosophila melanogaster*. *J. Exp. Biol.* **202**, 1561-1570.

MacMillan, H. A. and Hughson, B. N. (2014). A high-throughput method of hemolymph extraction from adult *Drosophila* without anesthesia. *J. Insect Physiol.* **63**, 27-31.

MacMillan, H. A. and Sinclair, B. J. (2011a). Mechanisms underlying insect chill-coma. *J. Insect Physiol.* **57**, 12-20.

MacMillan, H. A. and Sinclair, B. J. (2011b). The role of the gut in insect chilling injury: cold-induced disruption of osmoregulation in the fall field cricket, *Gryllus pennsylvanicus*. *J. Exp. Biol.* **214**, 726-734.
MacMillan, H. A., Guglielmo, C. G. and Sinclair, B. J. (2009). Membrane remodeling and glucose in *Drosophila melanogaster*: a test of rapid cold-hardening and chilling tolerance hypotheses. *J. Insect Physiol.* **55**, 243-249.
MacMillan, H. A., Williams, C. M., Staples, J. F. and Sinclair, B. J. (2012). Reestablishment of ion homeostasis during chill-coma recovery in the cricket *Gryllus pennsylvanicus*. *Proc. Natl. Acad. Sci. USA* **109**, 20750-20755.
MacMillan, H. A., Findsen, A., Pedersen, T. H. and Overgaard, J. (2014). Cold-induced depolarization of insect muscle: differing roles of extracellular K^+ during acute and chronic chilling. *J. Exp. Biol.* **217**, 2930-2938.
Marygold, S. J., Leyland, P. C., Seal, R. L., Goodman, J. L., Thurmond, J., Strelets, V. B., Wilson, R. J.; FlyBase consortium (2013). FlyBase: improvements to the bibliography. *Nucleic Acids Res.* **41**, D751-D757.
McDonough, A. A. and Farley, R. A. (1993). Regulation of Na,K-ATPase activity. *Curr. Opin. Nephrol. Hypertens.* **2**, 725-734.
McMullen, D. C. and Storey, K. B. (2008). Suppression of Na^+/K^+ -ATPase activity by reversible phosphorylation over the winter in a freeze-tolerant insect. *J. Insect Physiol.* **54**, 1023-1027.
Messerli, M. A., Kurtz, I. and Smith, P. J. S. (2008). Characterization of optimized Na^+ and Cl^- liquid membranes for use with extracellular, self-referencing microelectrodes. *Anal. Bioanal. Chem.* **390**, 1355-1359.
Naikkhwah, W. and O'Donnell, M. J. (2012). Phenotypic plasticity in response to dietary salt stress: Na^+ and K^+ transport by the gut of *Drosophila melanogaster* larvae. *J. Exp. Biol.* **215**, 461-470.
Natochin, Y. V. and Parnova, R. G. (1987). Osmolarity and electrolyte concentration of hemolymph and the problem of ion and volume regulation of cells in higher insects. *Comp. Biochem. Physiol.* **88A**, 563-570.
Newington, J. T., Pitts, A., Chien, A., Arseneault, R., Schubert, D. and Cumming, R. C. (2011). Amyloid beta resistance in nerve cell lines is mediated by the Warburg effect. *PLoS One* **6**, e19191.
Nyamukondiwa, C., Terblanche, J. S., Marshall, K. E. and Sinclair, B. J. (2011). Basal cold but not heat tolerance constrains plasticity among *Drosophila* species (Diptera: Drosophilidae). *J. Evol. Biol.* **24**, 1927-1938.
O'Donnell, M. (2008). Insect excretory mechanisms. *Adv. In Insect Phys.* **35**, 1-122.
Ohtsu, T., Katagiri, C., Kimura, M. T. and Hori, S. H. (1993). Cold adaptations in *Drosophila*. Qualitative changes of triacylglycerols with relation to overwintering. *J. Biol. Chem.* **268**, 1830-1834.
Ohtsu, T., Kimura, M. T. and Katagiri, C. (1998). How *Drosophila* species acquire cold tolerance – qualitative changes of phospholipids. *Eur. J. Biochem.* **252**, 608-611.
Overgaard, J., Sørensen, J. G., Petersen, S. O., Loeschcke, V. and Holmstrup, M. (2005). Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *J. Insect Physiol.* **51**, 1173-1182.
Overgaard, J., Malmendal, A., Sørensen, J. G., Bundy, J. G., Loeschcke, V., Nielsen, N. C. and Holmstrup, M. (2007). Metabolomic profiling of rapid cold hardening and cold shock in *Drosophila melanogaster*. *J. Insect Physiol.* **53**, 1218-1232.
Overgaard, J., Tomčala, A., Sørensen, J. G., Holmstrup, M., Krogh, P. H., Šimek, P. and Kostál, V. (2008). Effects of acclimation temperature on thermal tolerance and membrane phospholipid composition in the fruit fly *Drosophila melanogaster*. *J. Insect Physiol.* **54**, 619-629.
Overgaard, J., Kristensen, T. N., Mitchell, K. A. and Hoffmann, A. A. (2011). Thermal tolerance in widespread and tropical *Drosophila* species: does phenotypic plasticity increase with latitude? *Am. Nat.* **178 Suppl. 1**, S80-S96.
Overgaard, J., Kearney, M. R. and Hoffmann, A. A. (2014). Sensitivity to thermal extremes in Australian *Drosophila* implies similar impacts of climate change on the distribution of widespread and tropical species. *Glob. Chang. Biol.* **20**, 1738-1750.
Paradis, E., Claude, J. and Strimmer, K. (2004). APE: Analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**, 289-290.
Patrick, M. L., Aimanova, K., Sanders, H. R. and Gill, S. S. (2006). P-type Na^+/K^+-ATPase and V-type H^+-ATPase expression patterns in the osmoregulatory organs of larval and adult mosquito *Aedes aegypti*. *J. Exp. Biol.* **209**, 4638-4651.
Pierce, V. A., Mueller, L. D. and Gibbs, A. G. (1999). Osmoregulation in *Drosophila melanogaster* selected for urea tolerance. *J. Exp. Biol.* **202**, 2349-2358.
Pörtner, H. O. and Farrell, A. P. (2008). Ecology. Physiology and climate change. *Science* **322**, 690-692.
R Development Core Team (2013). *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna, Austria. Available at: http://www.R-project.org/.
Ransberry, V. E., MacMillan, H. A. and Sinclair, B. J. (2011). The relationship between chill-coma onset and recovery at the extremes of the thermal window of *Drosophila melanogaster*. *Physiol. Biochem. Zool.* **84**, 553-559.
Sinclair, B. J., Ferguson, L. V., Salehipour-shirazi, G. and MacMillan, H. A. (2013). Cross-tolerance and cross-talk in the cold: relating low temperatures to desiccation and immune stress in insects. *Integr. Comp. Biol.* **53**, 545-556.
Somero, G. N. (2004). Adaptation of enzymes to temperature: searching for basic 'strategies'. *Comp. Biochem. Physiol.* **139B**, 321-333.
Stillman, J. H. (2003). Acclimation capacity underlies susceptibility to climate change. *Science* **301**, 65.
Storey, K. B. (1997). Organic solutes in freezing tolerance. *Comp. Biochem. Physiol.* **117A**, 319-326.
Strachan, L. A., Tarnowski-Garner, H. E., Marshall, K. E. and Sinclair, B. J. (2011). The evolution of cold tolerance in *Drosophila* larvae. *Physiol. Biochem. Zool.* **84**, 43-53.
Sun, B. and Salvaterra, P. M. (1995). Characterization of nervana, a *Drosophila melanogaster* neuron-specific glycoprotein antigen recognized by anti-horseradish peroxidase antibodies. *J. Neurochem.* **65**, 434-443.
Teets, N. M., Kawarasaki, Y., Lee, R. E., Jr and Denlinger, D. L. (2013). Expression of genes involved in energy mobilization and osmoprotectant synthesis during thermal and dehydration stress in the Antarctic midge, *Belgica antarctica*. *J. Comp. Physiol. B* **183**, 189-201.
Therneau, T. M. and Grambsch, P. M. (2000). *Modeling Survival Data: Extending The Cox Model* (*Statistics for Biology and Health)*. New York, NY: Springer.
Trapnell, C., Pachter, L. and Salzberg, S. L. (2009). TopHat: discovering splice junctions with RNA-Seq. *Bioinformatics* **25**, 1105–1111.
Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., Salzberg, S. L., Wold, B. J. and Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat. Biotechnol.* **28**, 511–515.
Trapnell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., Pimentel, H., Salzberg, S. L., Rinn, J. L. and Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nat. Protoc.* **7**, 562–578.
Trapnell, C., Hendrickson, D. G., Sauvageau, M., Goff, L., Rinn, J. L. and Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat. Biotechnol.* **31**, 46–53.
van der Linde, K., Houle, D., Spicer, G. S. and Steppan, S. J. (2010). A supermatrix-based molecular phylogeny of the family Drosophilidae. *Genet. Res.* **92**, 25-38.
Welinder, C. and Ekblad, L. (2011). Coomassie staining as loading control in Western blot analysis. *J. Proteome Res.* **10**, 1416-1419.
Wiegmann, B. M., Kim, J. and Trautwein, M. D. (2009). Holometabolous insects (Holometabola). In *The Timetree of Life* (ed. S. B. Hedges and S. Kumar), pp. 260-263. Oxford: Oxford University Press.
Williams, C. M., Henry, H. A. L. and Sinclair, B. J. (2014). Cold truths: how winter drives responses of terrestrial organisms to climate change. *Biol. Rev. Camb. Philos. Soc.* [Epub ahead of print] doi: 10.1111/brv.12105.
Wyatt, G. R. (1961). The biochemistry of insect hemolymph. *Annu. Rev. Entomol.* **6**, 75-102.
Wyatt, G. R. and Kalf, G. F. (1957). The chemistry of insect hemolymph. II. Trehalose and other carbohydrates. *J. Gen. Physiol.* **40**, 833-847.
Yancey, P. H. (2005). Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *J. Exp. Biol.* **208**, 2819-2830.
Zachariassen, K. E., Kristiansen, E. and Pedersen, S. A. (2004). Inorganic ions in cold-hardiness. *Cryobiology* **48**, 126-133.