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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 hemolymph [K^+] in *D. melanogaster*, and cold-acclimated flies maintain low hemolymph [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 hemolymph [Na^+] and [K^+]. Cold-acclimated *D. melanogaster* have low activity of Na^+/K^+-ATPase, which may contribute to the maintenance of low hemolymph [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 hemolymph osmolality despite low hemolymph [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.

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 (Andersen et al., in press; Hofmann and Todgham, 2010; Overgaard et al., 2014; Pörtner and Farrell, 2008). 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 sub-lethal 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., in press).

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 (Ayrinhac et al., 2004; Bechsgaard et al., 2013; Gilchrist et al., 1997; Kellermann et al., 2012). 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., in press). *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 (Findsen et al., 2013; Koštál et al., 2004; Koštál et al., 2006; MacMillan and Sinclair, 2011b). The concentration of Na^+ is high in the extracellular fluid relative to the diet of phytophagous insects, and at low temperatures hemolymph Na^+ leaks across the gut wall. Because water distribution is heavily dependent on Na^+, water follows Na^+ down its

concentration gradient, thereby reducing hemolymph volume and consequently increasing extracellular [K^+] (MacMillan and Sinclair, 2011b). Although much Na^+ leaves the hemolymph, the concurrent loss of hemolymph 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 (Findsen et al., 2014; MacMillan et al., 2012; MacMillan et al., 2014). This net leak of Na^+ 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 (MacMillan and Sinclair, 2011a; Zachariassen et al., 2004).

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 (Beyenbach et al., 2010; Linton and O'Donnell, 1999). 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 c. 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 hemolymph of the mosquito (*Aedes aegypti*, Diptera: Culicidae; Patrick et al., 2006), and net Na^+ is transported into the hemolymph 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 hemolymph and limit Na^+ loss to the gut lumen and feces.

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 hemolymph $[Na^+]$. This decrease in hemolymph $[Na^+]$ 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 (Coello Alvarado, 2012; Koštál et al., 2004; Koštál et al., 2006).

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 likely cause net ion leak across cell membranes and epithelia, which would similarly lead to increased hemolymph $[K^+]$ and chilling injury. Reductions in enzyme thermal sensitivity are often associated with adaptation to low temperatures (Dong and Somero, 2009; Galarza-Muñoz et al., 2011; Garrett and Rosenthal, 2012; Somero, 2004). For example, polar and temperate octopus species have Na^+/K^+-ATPase α-subunits that differ in thermal sensitivity; at temperatures greater that 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 hemolymph $[Na^+]$ 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 *Drosophila 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 hemolymph $[Na^+]$, 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 hemolymph $[K^+]$, 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 hemolymph $[K^+]$ in *D. melanogaster*, as in other insects, and that within- and among-species improvements in cold-tolerance are accompanied by low hemolymph 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 (Table S1), reared under common laboratory conditions and fed a similar diet; and of adult male *D. melanogaster* acclimated for five 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, 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°C to 0.9 ± 0.1°C (Ransberry et al., 2011).

Hemolymph ion concentrations and osmolality

We measured ion concentrations in hemolymph 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 hemolymph [K^+] ($F_{1,71}$=4.6, *P*=0.036, Fig. 2*A*). Hemolymph [K^+] did not differ significantly between warm- and cold-acclimated *D. melanogaster* at their respective acclimation temperatures (Tukey's HSD, *P*=0.291; Fig. 2*A*), and a 6 h exposure to 0°C significantly increased hemolymph [K^+], but only in warm-acclimated flies (Fig 2*A*; Tukey's HSD*; P*=0.004). By contrast, cold-acclimated *D. melanogaster* maintained low hemolymph [K^+] during exposure to 0°C (Tukey's HSD; *P*=0.441; Fig. 2). Hemolymph [Na^+] of cold-acclimated *D. melanogaster* was ca. 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 hemolymph [Na^+] in either acclimation group ($F_{1,56}$=0.4, *P*=0.544; Fig. 2*B*).

Hemolymph [K^+] at rest ranged from 16.8 ± 1.4 mM (*D. auraria*) to 37.6 ± 2.8 mM (*D. willistoni*; Fig. 3*A*) 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 hemolymph [K^+] (Table S2). There was a significant positive relationship between the CT_{min} and extracellular [K^+] among species (*r*=0.72, df=20, P<0.001; Fig. 3*A*). Resting hemolymph [Na^+] of *Drosophila* species (at 21.5°C) ranged from 47.6 ± 4.7 mM (*D. immigrans*) to 154.1 ± 6.7 mM (*D. sechellia*; Table S2), and did not have significant phylogenetic signal (K=0.43, *P*=0.125; Fig. 3*B*). *D. 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, df=20, *P*=0.371). However, if *D. immigrans* is excluded, there was a significant positive relationship between the CT_{min} and hemolymph [Na^+] (*r*=0.48, df=19, *P*=0.029). Thus, cold tolerant species had low concentrations of both Na^+ and K^+ in their hemolymph, 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 hemolymph 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 hemolymph ion concentrations,

hemolymph 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 hemolymph osmolality and CT_{min} among *Drosophila* species before (*r*=0.16, df=20, *P*=0.485; Fig. 3*C*), or after PIC regression ($F_{1,20}$=1.5, *P*=0.240; Fig. S2).

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

To examine whether variation in hemolymph 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. 5*A*). 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. 5*A*).

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*; 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 activity and CT_{min} among species (*r*=0.30, df=21, *P*=0.162; Fig. 6*A*). Excluding *D. immigrans*, the relationship between Na^+/K^+-ATPase activity and the CT_{min} approached statistical significance (r=0.42, df=20, *P*=0.053; Fig. 6*A*), 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; Fig. S2). We did, however find a positive linear relationship

between hemolymph Na^+ concentration and log_{10}-transformed Na^+/K^+-ATPase activity among the *Drosophila* species (r=0.47, df=19, *P*=0.030, 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. 5*B*). Similarly, the CT_{min} correlated with neither the thermal sensitivity (r=182, df=21, *P*=0.403; Fig. 6*C*), nor the inflection point (r=0.19, df=21, *P*=0.385; Fig. 6*B*) of Na^+/K^+-ATPase activity among *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. 6*C*), 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_{adj}=0.723; 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_{adj}=0.003), but the relative abundance of either *nrv1* (t_9=0.7, P_{adj}=0.897) or *nrv3* (t_9=1.9, P_{adj}=0.257; Fig. S4) did not differ between warm- and cold- acclimated flies. Similarly, cold acclimation did not change the protein abundance of either the α- ($F_{2,11}$=0.9, *P*=0.379) or β-subunit (β: $F_{2,6}$=0.2, *P*=0.835; 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 hemolymph [K^+]. This increase in hemolymph [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. Muscle and nerve resting potentials depend mostly on extracellular [K^+] (Armstrong et al., 2012; Hoyle, 1953). Therefore, high hemolymph [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 hemolymph [K^+] at low levels during cold exposure (Coello Alvarado, 2012; Koštál et al., 2004; Koštál et al., 2006). Thus, like other insects, acclimation to low temperatures appears to improve the ability of *Drosophila* to maintain low hemolymph [K^+] during cold exposure. This ability is likely driven by maintenance of Na^+ and water balance in the hemolymph.

Cold-tolerant flies maintain low hemolymph cation concentrations

We observed that cold-tolerant *Drosophila* species maintain lower concentrations of both Na^+ and K^+ in their hemolymph, and that modifications to cold tolerance were generally accompanied by inverse modifications in hemolymph 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 hemolymph [K^+] at rest.

Decreased hemolymph [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 hemolymph osmotic pressure (Pierce et al., 1999; Wyatt, 1961). Adult Lepidoptera maintain low hemolymph [Na^+], in some cases low enough to reverse the muscle Na^+ gradient (Fitzgerald et al., 1996). To maintain hemolymph osmotic balance in the absence of Na^+, Lepidoptera maintain high hemolymph concentrations of carbohydrates (Wyatt, 1961; Wyatt and Kalf, 1957), and, as a group, maintain muscle excitability to lower temperatures than members of Diptera and Hymenoptera, which have more "conventional" (high) extracellular [Na^+] (Goller and Esch, 1990; Natochin and Parnova, 1987). Although we observed low cation concentrations in more cold tolerant flies, we observed no differences in hemolymph osmolality between warm- and cold-acclimated *D. melanogaster* nor among *Drosophila* species. Thus, reductions in hemolymph [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 hemolymph glycerol concentrations from 28 to 81 mM, a change that is too small to significantly change the freezing point (Lee et al., 1987). Many cryoprotectants are also osmoprotectants (Sinclair et al., 2013; Teets et al., 2013; Yancey, 2005). Thus, we propose that these relatively small accumulations of organic solutes that have been previously observed serve to maintain or increase hemolymph 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 hemolymph $[Na^+]$ 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) hemolymph $[Na^+]$ 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 substantially impact 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.

Conclusion

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 likely maintain osmotic balance by accumulating other organic and inorganic osmolytes in their hemolymph 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 tradeoffs 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 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 21.5 ± 0.5°C and at 50 ± 5% relative humidity with a 13:11 h (L:D) light 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 five 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 at 6 ± 0.5°C conditions (cold-acclimated) for five 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 aluminum baffles to which the flies cling (similar to the design of Huey et al., 1992). The temperature of the column was controlled by circulating a 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 v5.20.1 (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 (Fig. S1).

Hemolymph collection

Adult *Drosophila* were positioned for hemolymph 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 hemolymph 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 hemolymph was less than 2 min.

Hemolymph ion concentration and osmolality

Droplets of hemolymph 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. Hemolymph 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^+), 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 mold before hemolymph ion concentrations and osmolality were measured, and so were not included in this analysis. Hemolymph 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 Co., Novato, USA). Pulled micropipettes were silanized at 300°C with N,N-dimethyltrimethylsilylamine vapour for 1 h and backfilled with 100 mM 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 backfilled with 0.5 M 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).

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

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

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

Hemolymph osmolality was measured using a Clifton Nanolitre 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 anesthesia, snap frozen in liquid nitrogen vapor, and stored at -80°C. Na^+/K^+-ATPase activity was measured in 6 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 mM imidazole, 0.2% w/v Na^+-deoxycholate, 10 mM β-mercaptoethanol, 2 mM EDTA, pH 7.5) with a Tissue-Tearor (Biospec Products, Bartlesville, OK, USA) using four 10 s bursts each followed by 20 s rests 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 approximately 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 two 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 mM ATP solution. Final conditions for the assay were: 70 mM imidazole (pH 7.5), 140 mM NaCl, 30 mM KCl, 7 mM $MgCl_2$, 4 mM phosphoenolpyruvic acid, 300 µM NADH, 5 mM 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 v3.0 (Agilent Technologies). Four replicate cuvettes of each *Drosophila* sample were run, two of which contained 1 mM 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, St. Neots, United Kingdom), 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 (Fig. S5*A*). Activity of Na^+/K^+-ATPase was determined as the difference between the rates of cuvettes containing 1 mM ouabain and cuvettes that did not contain ouabain (Fig. S5*B*). For each replicate, rates of change in absorbance (OD min^{-1}) were smoothed using a 15-point sliding window in LoggerPro (v3.8.4, Vernier Software Inc., Beaverton, OR, USA).

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

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

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 *Drosophila melanogaster* acclimated to 21.5°C (Fig. S5*C*). 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 mol ATP consumed min^{-1} 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 (Fig. S5*C*).

The abundance of Na^+/K^+-ATPase proteins and transcripts were determined using Western blots and high-throughput mRNA sequencing, respectively (see methods supplement for details).

Data analysis

All data analyses were completed in R v.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. Hemolymph 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 hemolymph 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 hemolymph $[Na^+]$ 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.

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-neighbor distance. Node ages were standardized using a semi-parametric method for use in statistical analyses.

Phylogenetically-independent contrasts (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; 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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Author Contributions

HAM, BJS, JFS and AD conceived and designed the research. HAM, LVF, and AN performed the experiments. HAM and BJS interpreted and analyzed the data, HAM and BJS drafted the manuscript, and all authors revised the manuscript.

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Figure Captions

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 subgenera (grey) and groups (open) according to current FlyBase designations. A: Ananassae group, W: Willistoni group. See 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 hemolymph [Na^+] and defend against the effects of cold exposure on hemolymph [K^+]. Hemolymph K^+ (A) and Na^+ (B) concentrations of cold-acclimated (grey bars) and warm-acclimated (open bars) male *Drosophila melanogaster* at their acclimation temperature (Control) and following 6 h at 0°C (mean ± sem). 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.

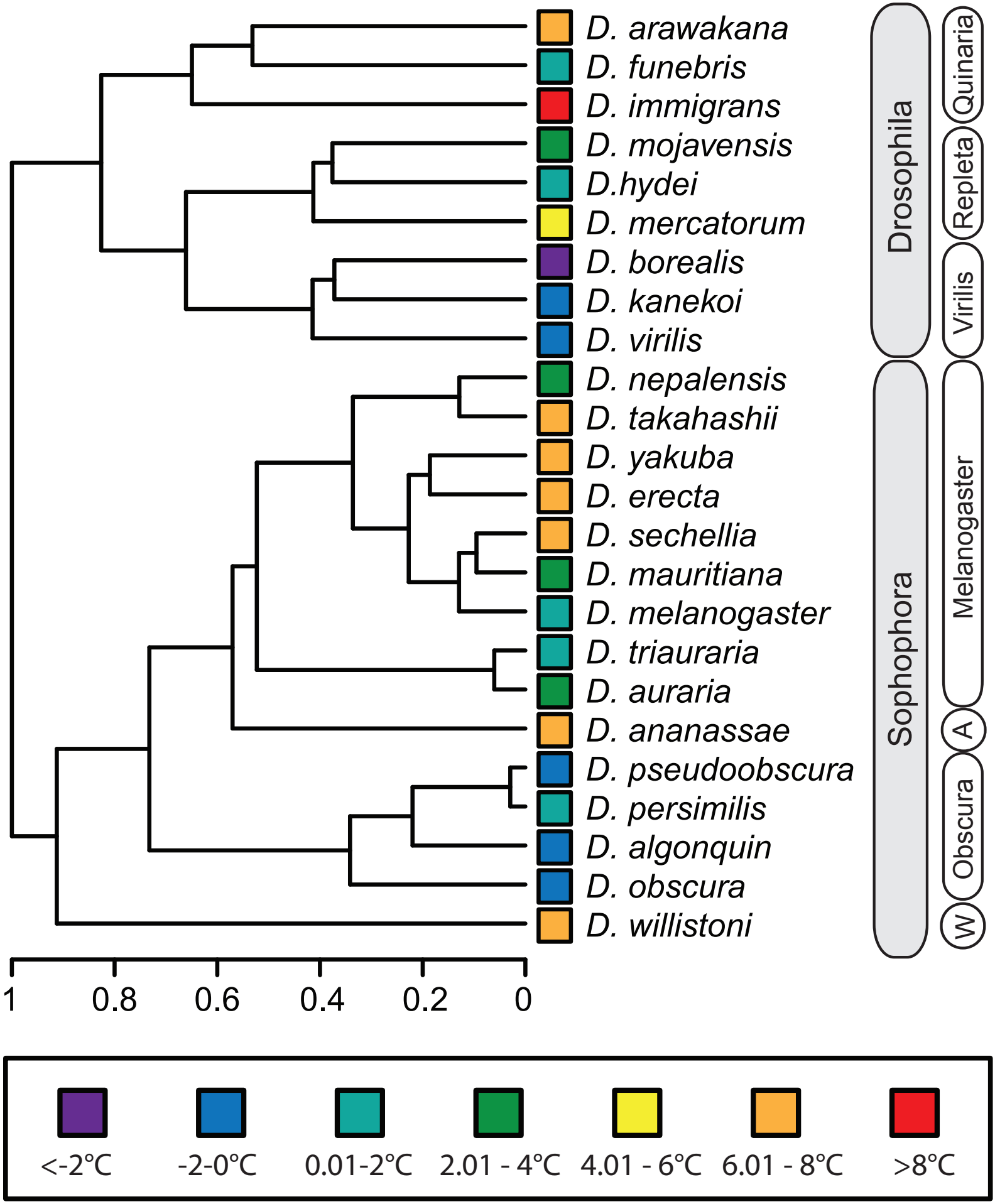
Fig. 3. Cold adapted *Drosophila* species have low hemolymph [K^+] and [Na^+]. Hemolymph concentrations of K^+ (A) and Na^+ (B) and hemolymph osmolality (C) of species of the genus *Drosophila* are shown in relation to the CT_{min}. Lines denote a significant linear relationship between ion concentration and the CT_{min} among species. *Drosophila immigrans* (open circle) had remarkably low hemolymph [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 (Fig. S2). *n*=4-8 (K^+) and *n*=3-5 (Na^+). Y-axis (CT_{min}) error bars (sem) are obscured by the symbols.

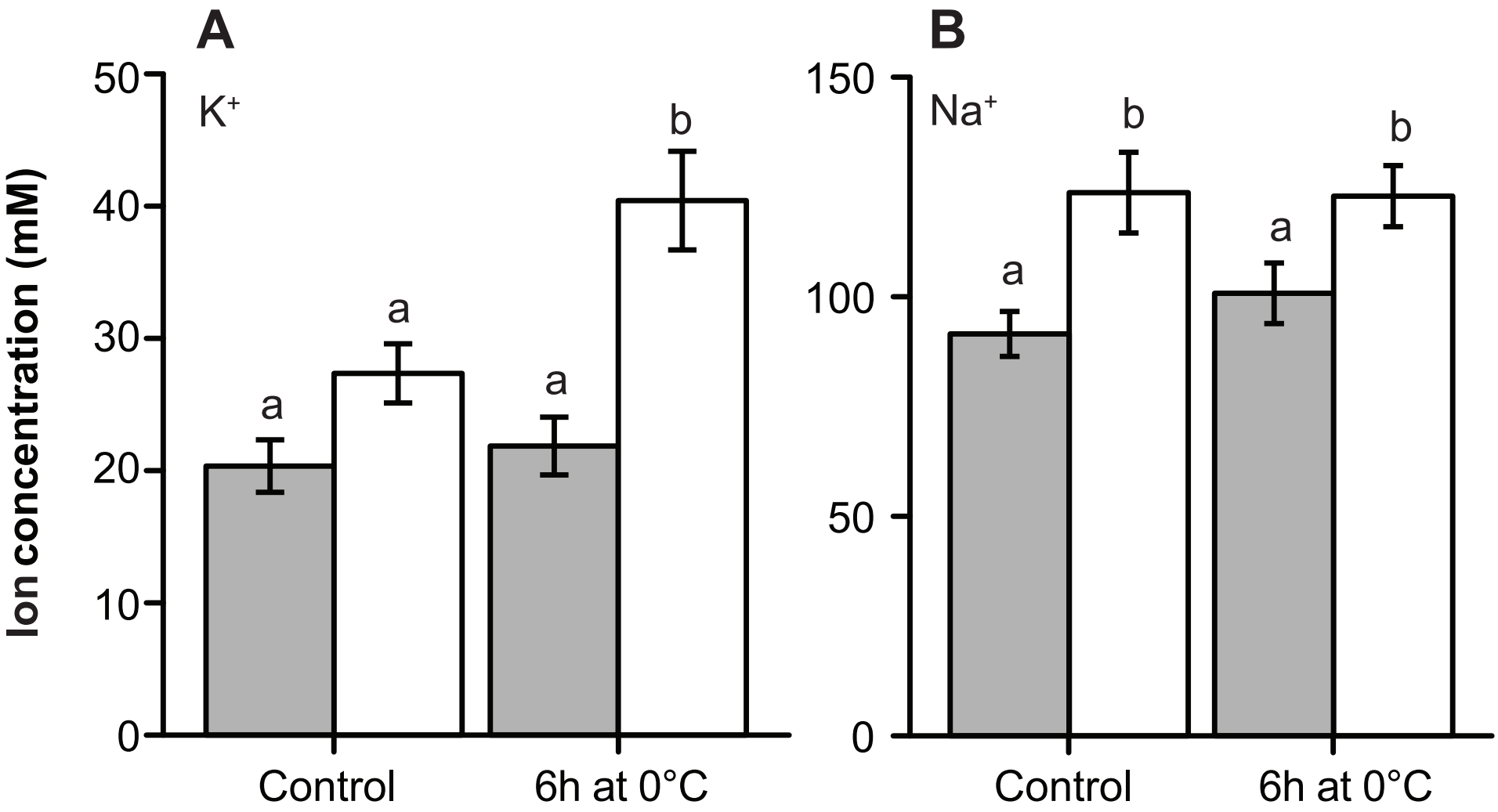
Fig. 4. A logistic equation fitted to temperature effects on maximal activity of Na^+/K^+-ATPase. Example of a logistic model (solid grey line) fitted to measured Na^+/K^+-ATPase activity of warm-acclimated *Drosophila melanogaster* (solid black line). The temperature at which the curve stops accelerating and begins decelerating (the inflection point;

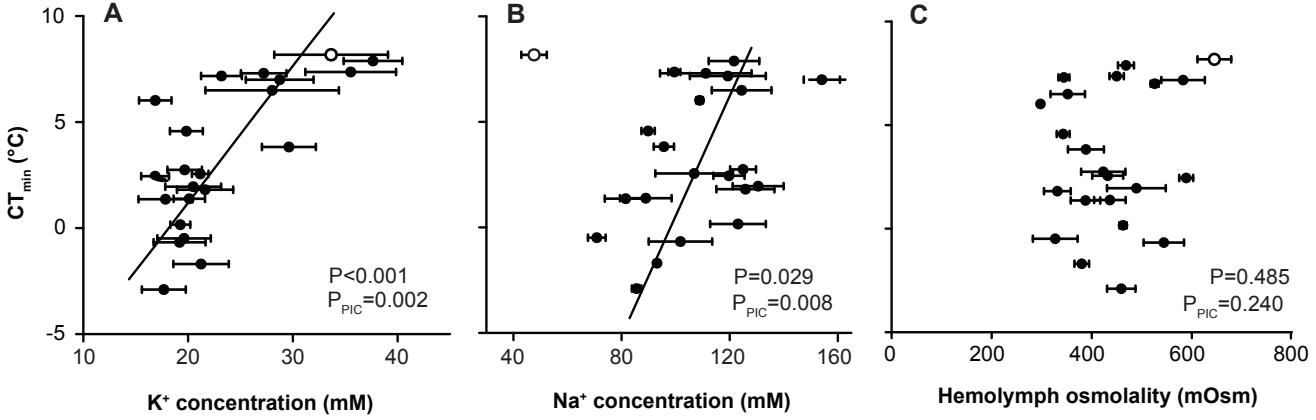
IP; dotted black line) as well as the thermal sensitivity (*Ts*) of Na^+/K^+-ATPase were extracted from the model equation (see materials and methods). Sodium pump activity across the range of temperatures (including at the rearing temperature, 21.5°C; *A*: dotted grey line) were extracted from model predictions and used to compare activity between acclimation groups (*D. melanogaster*) and among *Drosophila* species.

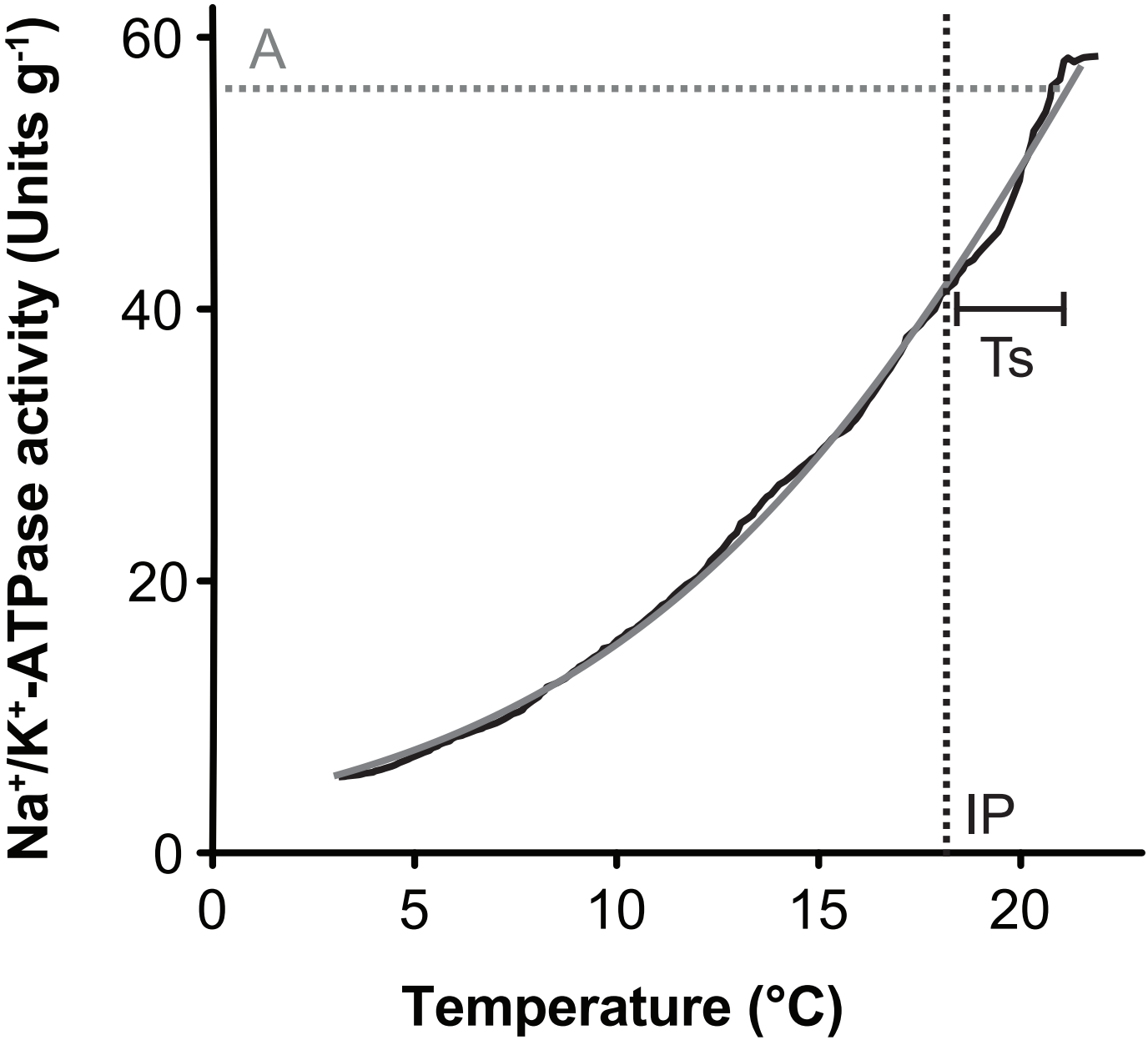
Fig. 5. Cold-acclimated *Drosophila melanogaster* have low Na^+/K^+-ATPase activity. (A) Sodium pump activity of warm-acclimated (21.5°C, black) and cold-acclimated (6°C, grey) *D. melanogaster*. Solid lines and dotted lines represent the mean and 95% CI, respectively. Open circles denote mean activity 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 (open) 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).

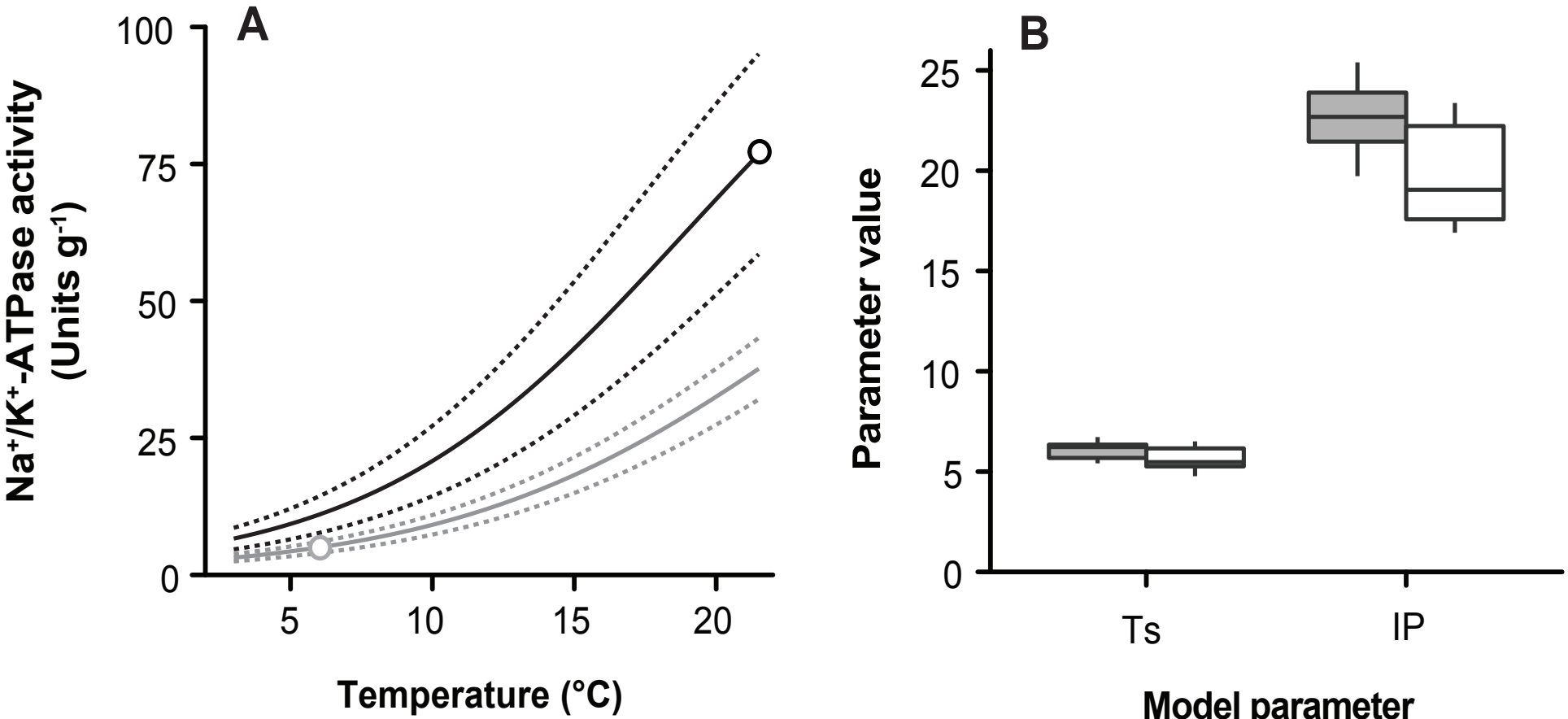
Fig. 6. Cold tolerant *Drosophila* species tend to have low Na^+/K^+-ATPase activity. Mean (± sem) Na^+/K^+-ATPase activity at 21.5°C (A) inflection point (B) and thermal sensitivity (C) of species of the genus *Drosophila* in relation to the CT_{min}. *Drosophila immigrans* (open circle) had low Na^+/K^+-ATPase 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 (Fig. S2). *n*=3-6 biological replicates per species. CT_{min} error bars (sem) are obscured by the symbols.

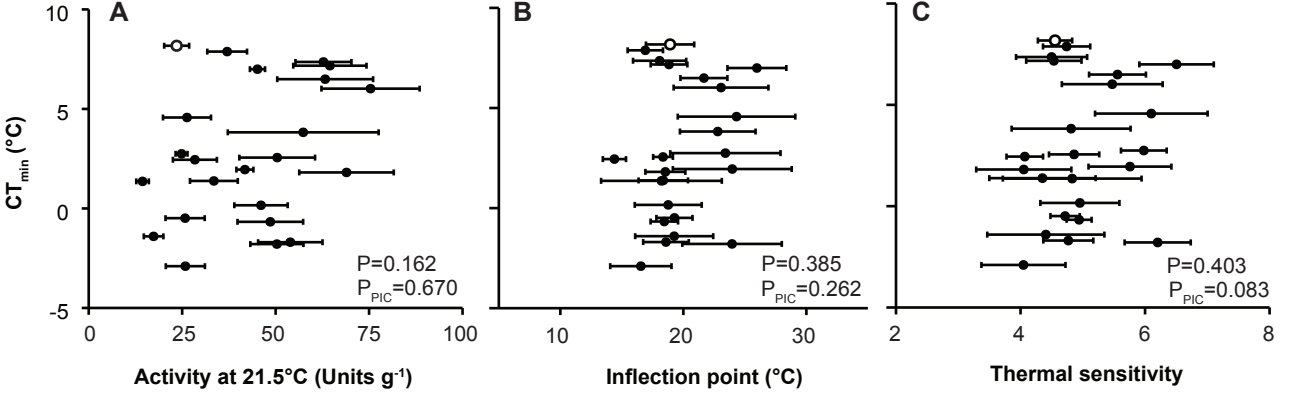

D. arawakana
D. funebris
D. immigrans
D. mojavensis
D.hydei
D. mercatorum
D. borealis
D. kanekoi
D. virilis
D. nepalensis
D. takahashii
D. yakuba
D. erecta
D. sechellia
D. mauritiana
D. melanogaster
D. triauraria
D. auraria
D. ananassae
D. pseudoobscura
D. persimilis
D. algonquin
D. obscura
D. willistoni
1
0.8
0.6
0.4
0.2
0
Drosophila
Sophophora
Quinaria
Repleta
Virilis
Melanogaster
A
Obscura
W
<-2°C
-2-0°C
0.01-2°C
2.01 - 4°C
4.01 - 6°C
6.01 - 8°C
>8°C


A
B
Ion concentration (mM)
K+
Na+
50
40
30
20
10
0
150
100
50
0
a
a
b
a
a
b
Control
6h at 0°C
Control
6h at 0°C


A
B
C
CTmin (°C)
10
5
0
-5
10
20
30
40
P<0.001
PPIC=0.002
K+ concentration (mM)
40
80
120
160
P=0.029
PPIC=0.008
Na+ concentration (mM)
0
200
400
600
800
P=0.485
PPIC=0.240
Hemolymph osmolality (mOsm)


A
60
40
20
0
Ts
IP
0
5
10
15
20
Na+/K+-ATPase activity (Units g-1)
Temperature (°C)


A
100
75
50
25
0
5
10
15
20
Na+/K+-ATPase activity
(Units g-1)
Temperature (°C)
B
25
20
15
10
5
0
Parameter value
Ts
IP
Model parameter


A
B
C
10
5
0
-5
CT_{min} (°C)
P=0.162
P_{PIC}=0.670
P=0.385
P_{PIC}=0.262
P=0.403
P_{PIC}=0.083
0
25
50
75
100
10
20
30
2
4
6
8
Activity at 21.5°C (Units g^{-1})
Inflection point (°C)
Thermal sensitivity