

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

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20 Abstract

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

40

41 Introduction

42 Low temperature tolerance is especially important for determining geographic range limits of
43 insects (Battisti et al., 2005; Chen et al., 2011; Kellermann et al., 2012). Thus, understanding the
44 mechanisms that set thermal limits allows prediction of changes to their distribution and
45 abundance in a changing environment (Andersen et al., in press; Hofmann and Todgham, 2010;
46 Overgaard et al., 2014; Pörtner and Farrell, 2008). Insect lower thermal limits manifest as entry
47 into chill coma at the critical thermal minimum (CT_{min}), and the subsequent accumulation of

48 chilling injuries that lead to sub-lethal effects on performance and fitness or death (MacMillan
49 and Sinclair, 2011a; MacMillan et al., 2014). Thermal tolerance can be modulated through
50 phenotypic plasticity or through genetic adaptation, but the former has been suggested to be
51 constrained by the latter; this means that species that have evolved to tolerate more extreme
52 temperatures may be less able to acclimate to changes in temperature [(Nyamukondiwa et al.,
53 2011; Stillman, 2003), but see: (Calosi et al., 2008; Overgaard et al., 2011)]. Identifying the
54 mechanisms underlying thermal tolerance would elucidate the reasons for this possible trade-off,
55 and allow for *a priori* estimation of low temperature tolerance, and high-throughput prediction of
56 insect susceptibility to climate change (Williams et al., in press).

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

73 In hemimetabolous insects, chilling injury is associated with a disruption of ion and water
74 balance (Findsen et al., 2013; Košťál et al., 2004; Košťál et al., 2006; MacMillan and Sinclair,
75 2011b). The concentration of Na^+ is high in the extracellular fluid relative to the diet of
76 phytophagous insects, and at low temperatures hemolymph Na^+ leaks across the gut wall.
77 Because water distribution is heavily dependent on Na^+ , water follows Na^+ down its

78 concentration gradient, thereby reducing hemolymph volume and consequently increasing
79 extracellular $[K^+]$ (MacMillan and Sinclair, 2011b). Although much Na^+ leaves the hemolymph,
80 the concurrent loss of hemolymph water can result in little change in extracellular $[Na^+]$. In
81 crickets and locusts, this imbalance is thought to lead to chilling injury, and re-establishment of
82 ion homeostasis is required for recovery of neuromuscular function (Findsen et al., 2014;
83 MacMillan et al., 2012; MacMillan et al., 2014). This net leak of Na^+ in the cold is thought to
84 occur because the activity of temperature-sensitive enzymatic ion pumps is insufficient to
85 balance passive leak of ions, the rate of which is dependent on the magnitude of the Na^+ gradient
86 (MacMillan and Sinclair, 2011a; Zachariassen et al., 2004).

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

108 Based on the role of Na^+/K^+ -ATPase in ion balance in *Drosophila* and the patterns of ion and
109 water balance disruption in other cold-exposed insects, cold tolerance could potentially be
110 improved by reducing Na^+/K^+ -ATPase activity at high or “normal” temperatures, which would
111 decrease retention of Na^+ under resting conditions, and consequently reduce resting hemolymph
112 $[\text{Na}^+]$. This decrease in hemolymph $[\text{Na}^+]$ would reduce the transmembrane and transepithelial
113 Na^+ gradients that drive Na^+ leak and water balance disruption at low temperatures, and thereby
114 improve cold tolerance. This hypothesis is supported by the repeated observation that cold-
115 acclimation of insects results in less-polarized muscle Na^+ equilibrium potentials (Coello
116 Alvarado, 2012; Košťál et al., 2004; Košťál et al., 2006).

117 Although low Na^+/K^+ -ATPase activity at relatively high temperatures might reduce resting Na^+
118 gradients and improve cold tolerance, a failure of this pump in the cold will likely cause net ion
119 leak across cell membranes and epithelia, which would similarly lead to increased hemolymph
120 $[\text{K}^+]$ and chilling injury. Reductions in enzyme thermal sensitivity are often associated with
121 adaptation to low temperatures (Dong and Somero, 2009; Galarza-Muñoz et al., 2011; Garrett
122 and Rosenthal, 2012; Somero, 2004). For example, polar and temperate octopus species have
123 Na^+/K^+ -ATPase α -subunits that differ in thermal sensitivity; at temperatures greater than 25°C the
124 maximal rate of Na^+/K^+ -ATPase does not differ between polar and temperate octopuses, but that
125 of polar octopuses is 4-fold higher at 10°C (Galarza-Muñoz et al., 2011). Thus, cold tolerance
126 may also be improved in *Drosophila* through similar reductions in the thermal sensitivity of
127 Na^+/K^+ -ATPase.

128 In this study, we therefore hypothesized that reduced Na^+/K^+ -ATPase activity and decreased
129 hemolymph $[\text{Na}^+]$ at rest underlie improvements in insect cold tolerance, both within species
130 (phenotypic plasticity) and among species (genetic adaptation). To test this hypothesis, we used
131 thermal acclimation to induce variation in cold tolerance *via* phenotypic plasticity in *Drosophila*
132 *melanogaster*, and 24 species of *Drosophila* whose cold tolerance naturally varies when reared
133 under common conditions, with the CT_{min} used as an index of cold tolerance. We predicted that
134 cold-acclimated *D. melanogaster* and cold-tolerant species would have low hemolymph $[\text{Na}^+]$,
135 thereby reducing transmembrane and transepithelial Na^+ gradients at rest, which would
136 consequently protect against Na^+ leak in the cold. We also predicted that cold-tolerant flies
137 would maintain low hemolymph $[\text{K}^+]$, which would reduce the impact of any cold-induced loss

138 of Na^+ and water balance on cell survival. Lastly, we hypothesized that cold-tolerant *Drosophila*
139 better maintain their ion gradients when exposed to low temperatures and predicted that cold-
140 adapted and cold-acclimated flies would display reduced thermal sensitivity of Na^+/K^+ -ATPase.

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

149

150 **Results**

151 *Critical thermal minima*

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

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

164 *Hemolymph ion concentrations and osmolality*

165 We measured ion concentrations in hemolymph extracted from warm- and cold-acclimated
166 *D. melanogaster*, both at their acclimation temperatures and following a 6 h exposure to 0°C.
167 Acclimation temperature and cold exposure interacted to significantly affect hemolymph [K⁺]
168 ($F_{1,71}=4.6$, $P=0.036$, Fig. 2A). Hemolymph [K⁺] did not differ significantly between warm- and
169 cold-acclimated *D. melanogaster* at their respective acclimation temperatures (Tukey's HSD,
170 $P=0.291$; Fig. 2A), and a 6 h exposure to 0°C significantly increased hemolymph [K⁺], but only
171 in warm-acclimated flies (Fig 2A; Tukey's HSD; $P=0.004$). By contrast, cold-acclimated
172 *D. melanogaster* maintained low hemolymph [K⁺] during exposure to 0°C (Tukey's HSD;
173 $P=0.441$; Fig. 2). Hemolymph [Na⁺] of cold-acclimated *D. melanogaster* was ca. 25% lower than
174 that of warm-acclimated flies at their respective acclimation temperature ($F_{1,56}=14.1$, $P<0.001$),
175 and exposure to 0°C for 6 h had no effect on hemolymph [Na⁺] in either acclimation group
176 ($F_{1,56}=0.4$, $P=0.544$; Fig. 2B).

177 Hemolymph [K⁺] at rest ranged from 16.8 ± 1.4 mM (*D. auraria*) to 37.6 ± 2.8 mM
178 (*D. willistoni*; Fig. 3A) among the *Drosophila* species and had significant phylogenetic signal
179 ($K=0.70$, $P=0.014$), as some cold-tolerant species groups (such as the Obscura and Virilis
180 groups) had a consistently low hemolymph [K⁺] (Table S2). There was a significant positive
181 relationship between the CT_{min} and extracellular [K⁺] among species ($r=0.72$, $df=20$, $P<0.001$;
182 Fig. 3A). Resting hemolymph [Na⁺] of *Drosophila* species (at 21.5°C) ranged from 47.6 ± 4.7
183 mM (*D. immigrans*) to 154.1 ± 6.7 mM (*D. sechellia*; Table S2), and did not have significant
184 phylogenetic signal ($K=0.43$, $P=0.125$; Fig. 3B). *D. immigrans* had remarkably low [Na⁺], and
185 when this species was included in the correlation of [Na⁺] against the CT_{min} the relationship was
186 non-significant ($r=0.20$, $df=20$, $P=0.371$). However, if *D. immigrans* is excluded, there was a
187 significant positive relationship between the CT_{min} and hemolymph [Na⁺] ($r=0.48$, $df=19$,
188 $P=0.029$). Thus, cold tolerant species had low concentrations of both Na⁺ and K⁺ in their
189 hemolymph, and these relationships between ion concentrations and the CT_{min} both remained
190 significant following PIC regression ([K⁺]: $F_{1,20}=12.3$, $P=0.002$; [Na⁺] not including
191 *D. immigrans*: $F_{1,19}=8.5$, $P=0.008$).

192 We measured the osmolality of hemolymph collected under control conditions to determine
193 whether the observed reductions in ion concentrations in cold-tolerant flies drive reductions in
194 extracellular osmolality. Despite the variation observed in hemolymph ion concentrations,

195 hemolymph osmolality did not significantly differ between warm- (420 ± 23 mOsm) and cold-
196 acclimated (396 ± 23 mOsm) *D. melanogaster* ($t_{11}=0.67$, $P=0.526$), and there was no relationship
197 between hemolymph osmolality and CT_{\min} among *Drosophila* species before ($r=0.16$, $df=20$,
198 $P=0.485$; Fig. 3C), or after PIC regression ($F_{1,20}=1.5$, $P=0.240$; Fig. S2).

199 *Na⁺/K⁺-ATPase activity and thermal sensitivity*

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

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

214 The V_{\max} of Na^+/K^+ -ATPase at 21.5°C ranged among species from 14.4 ± 1.7 U g⁻¹ (μmol ATP
215 min⁻¹ g⁻¹; *D. funebris*) to 75.4 ± 13.3 U g⁻¹ (*D. yakuba*; Table S2). Although there was a trend for
216 more cold-tolerant species to have low Na^+/K^+ -ATPase V_{\max} , there was no significant
217 relationship between Na^+/K^+ -ATPase activity and CT_{\min} among species ($r=0.30$, $df=21$, $P=0.162$;
218 Fig. 6A). Excluding *D. immigrans*, the relationship between Na^+/K^+ -ATPase activity and the
219 CT_{\min} approached statistical significance ($r=0.42$, $df=20$, $P=0.053$; Fig. 6A), but we note that
220 here, *D. immigrans* did not substantially deviate from the other species. Na^+/K^+ -ATPase activity
221 had strong phylogenetic signal ($K=1.20$, $P=0.001$), but accounting for phylogeny did not resolve
222 any relationship between Na^+/K^+ -ATPase activity at 21.5°C and CT_{\min} among *Drosophila*
223 species ($F_{1,21}=0.2$, $P=0.670$; Fig. S2). We did, however find a positive linear relationship

224 between hemolymph Na⁺ concentration and log₁₀-transformed Na⁺/K⁺-ATPase activity among
225 the *Drosophila* species ($r=0.47$, $df=19$, $P=0.030$, Fig. S3).

226 Contrary to our hypothesis that variation in cold tolerance is driven by changes in the thermal
227 sensitivity of Na⁺/K⁺-ATPase, neither the inflection point ($t_9=1.8$, $P=0.101$) nor thermal
228 sensitivity ($t_9=1.2$, $P=0.261$) of Na⁺/K⁺-ATPase activity differed significantly between warm-
229 and cold-acclimated *D. melanogaster* (Fig. 5B). Similarly, the CT_{min} correlated with neither the
230 thermal sensitivity ($r=0.182$, $df=21$, $P=0.403$; Fig. 6C), nor the inflection point ($r=0.19$, $df=21$,
231 $P=0.385$; Fig. 6B) of Na⁺/K⁺-ATPase activity among *Drosophila* species. There was also no
232 relationship between the PICs of CT_{min} and the inflection point ($F_{1,21}=1.3$, $P=0.262$) or thermal
233 sensitivity ($F_{1,21}=3.3$, $P=0.083$; Fig. 6C), and neither the Na⁺/K⁺-ATPase inflection point
234 ($K=0.17$, $P=0.841$) nor thermal sensitivity ($K=0.20$, $P=0.727$) had significant phylogenetic
235 signal. Thus, we detected no alterations in the shape or position of the *in vitro* activity-
236 temperature curve for Na⁺/K⁺-ATPase, within or among *Drosophila* species, but instead
237 observed wholesale reductions in pump activity (particularly in cold-acclimated
238 *D. melanogaster*).

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

240 Plasticity of Na⁺/K⁺-ATPase activity can result from transcriptional, translational, or post-
241 translational changes (Bertorello et al., 1991; McDonough and Farley, 1993). We examined
242 whether the observed reduction in Na⁺/K⁺-ATPase activity with cold acclimation in
243 *D. melanogaster* was related to a reduction in mRNA transcript or protein abundance of the
244 Na⁺/K⁺-ATPase subunits, using high-throughput mRNA sequencing (RNA-seq) and western
245 blotting, respectively. Transcript abundance of the primary Na⁺/K⁺-ATPase α -subunit gene
246 (*Atp α*) did not differ between acclimation groups ($t_9=1.0$, $P_{adj}=0.723$; Fig. S4). *Drosophila*
247 *melanogaster* has three genes that code for the Na⁺/K⁺-ATPase β -subunit (*nrv1*, *nrv2* and *nrv3*).
248 Cold-acclimated flies had significantly higher expression of *nrv2* mRNA than warm-acclimated
249 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*
250 ($t_9=1.9$, $P_{adj}=0.257$; Fig. S4) did not differ between warm- and cold- acclimated flies. Similarly,
251 cold acclimation did not change the protein abundance of either the α - ($F_{2,11}=0.9$, $P=0.379$) or β -
252 subunit (β : $F_{2,6}=0.2$, $P=0.835$; Fig. S4) of Na⁺/K⁺-ATPase.

253 **Discussion**

254 *Drosophila* lose ion balance in the cold

255 When exposed to 0°C for 6 h, warm-acclimated *D. melanogaster* lost the ability to maintain ion
256 and water homeostasis, leading to an increase in hemolymph [K⁺]. This increase in hemolymph
257 [K⁺] also occurred during cold exposure in chill-susceptible cockroaches (Košťál et al., 2006),
258 crickets (MacMillan and Sinclair, 2011b), locusts (Findsen et al., 2013), and firebugs (Košťál et
259 al., 2004), which suggests that cold exposure leads to a loss of [K⁺] balance in both
260 holometabolous and hemimetabolous chill-susceptible insects. Muscle and nerve resting
261 potentials depend mostly on extracellular [K⁺] (Armstrong et al., 2012; Hoyle, 1953). Therefore,
262 high hemolymph [K⁺] is likely to depolarize cell membranes, prevent neuromuscular signal
263 transmission (Hosler et al., 2000), slow chill-coma recovery (MacMillan et al., 2012; MacMillan
264 et al., 2014) and lead to chilling injury (Košťál et al., 2006; MacMillan and Sinclair, 2011b).

265 In contrast to the warm-acclimated flies, *D. melanogaster* acclimated to 6°C maintained low
266 extracellular [K⁺] after 6 h at 0°C. Phenotypic plasticity in cold tolerance has been consistently
267 associated with an enhanced ability to maintain ion and water balance during cold exposure;
268 cold-acclimated crickets, tropical cockroaches, and adult firebugs also maintain hemolymph [K⁺]
269 at low levels during cold exposure (Coello Alvarado, 2012; Košťál et al., 2004; Košťál et al.,
270 2006). Thus, like other insects, acclimation to low temperatures appears to improve the ability of
271 *Drosophila* to maintain low hemolymph [K⁺] during cold exposure. This ability is likely driven
272 by maintenance of Na⁺ and water balance in the hemolymph.

273 *Cold-tolerant flies maintain low hemolymph cation concentrations*

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

280 Decreased hemolymph $[\text{Na}^+]$ could improve cold tolerance by reducing the influence of Na^+
281 gradients on water balance, but only if other osmolytes fill the ‘osmotic gap’ left by Na^+ and
282 thereby maintain hemolymph osmotic pressure (Pierce et al., 1999; Wyatt, 1961). Adult
283 Lepidoptera maintain low hemolymph $[\text{Na}^+]$, in some cases low enough to reverse the muscle
284 Na^+ gradient (Fitzgerald et al., 1996). To maintain hemolymph osmotic balance in the absence of
285 Na^+ , Lepidoptera maintain high hemolymph concentrations of carbohydrates (Wyatt, 1961;
286 Wyatt and Kalf, 1957), and, as a group, maintain muscle excitability to lower temperatures than
287 members of Diptera and Hymenoptera, which have more “conventional” (high) extracellular
288 $[\text{Na}^+]$ (Goller and Esch, 1990; Natchin and Parnova, 1987). Although we observed low cation
289 concentrations in more cold tolerant flies, we observed no differences in hemolymph osmolality
290 between warm- and cold-acclimated *D. melanogaster* nor among *Drosophila* species. Thus,
291 reductions in hemolymph $[\text{Na}^+]$ in *Drosophila* must be paired with accumulations of other
292 extracellular osmolytes.

293 Small accumulations of extracellular osmolytes in chill susceptible insects may protect
294 organismal water balance at low temperatures. A variety of organic solutes act as cryoprotectants
295 at very high concentrations, and are central to the overwintering success of freeze-tolerant and
296 freeze-avoidant insects (Lee, 1991; Storey, 1997). Relatively modest amounts of such
297 compounds, at concentrations unlikely to yield cryoprotection, have been noted to accumulate
298 following cold-exposure in chill-susceptible insects, including *D. melanogaster* (Lee et al., 1987;
299 Overgaard et al., 2007). For example, brief cold exposures that improve subsequent cold
300 tolerance in flesh flies (rapid cold-hardening) increase hemolymph glycerol concentrations from
301 28 to 81 mM, a change that is too small to significantly change the freezing point (Lee et al.,
302 1987). Many cryoprotectants are also osmoprotectants (Sinclair et al., 2013; Teets et al., 2013;
303 Yancey, 2005). Thus, we propose that these relatively small accumulations of organic solutes
304 that have been previously observed serve to maintain or increase hemolymph osmotic pressure in
305 the place of highly permeable osmolytes (particularly Na^+), and thereby permit a decoupling of
306 Na^+ and water balance that promotes low temperature survival. We hypothesize that such a
307 change in osmotic balance may have facilitated the evolution of more extreme cold tolerance
308 phenotypes (such as freeze avoidance and freeze tolerance) associated with accumulations of low
309 molecular weight cryoprotectants.

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

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

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

335 Reductions in the effects of temperature on enzyme activity can allow for comparatively higher
336 rates of catalysis at low temperatures (Galarza-Muñoz et al., 2011). However, we found no
337 evidence to support the hypothesis that cold-acclimated and cold-adapted *Drosophila* better
338 maintain ion balance at low temperatures through reductions in the thermal sensitivity of

339 Na^+/K^+ -ATPase. Although the estimates of Na^+/K^+ -ATPase V_{\max} we obtained *in vitro* are
340 reliable indicators of relative differences in Na^+/K^+ -ATPase activity, they may not approximate
341 rates of ion transport *in vivo*, where local substrate and co-factor concentrations and the
342 immediate membrane environment could substantially impact ion transport rates and thermal
343 sensitivity. Adaptation and acclimation to low temperatures in *Drosophila* have been associated
344 with decreased saturation of phospholipid fatty acids, which would increase membrane fluidity
345 and maintain ion pump function at low temperatures (Ohtsu et al., 1993; Ohtsu et al., 1998;
346 Overgaard et al., 2005; Overgaard et al., 2008; but see MacMillan et al., 2009). Comparative
347 analyses of Na^+/K^+ -ATPase activity functioning within the membrane bilayer are needed to
348 elucidate the role of these influences on ion balance in the cold. Because the physiological
349 mechanisms underlying insect ionoregulation are diverse (O'Donnell, 2008), the reductions in
350 Na^+/K^+ -ATPase activity observed here may be one of several mechanisms of ionoregulation that
351 contribute to cold tolerance. The roles, for example, of other ion-motive pumps (i.e. H^+ -ATPase
352 and Ca^{2+} -ATPase) and exchangers, ion channels and aquaporins, paracellular routes of ion and
353 water transport, and the hormones that regulate them remain to be explored in the context of
354 insect cold acclimation and adaptation.

355 *Conclusion*

356 Cold-acclimated *D. melanogaster* and cold-tolerant *Drosophila* species have reduced reliance on
357 Na^+ as an extracellular cation and more cold-tolerant species also maintain lower extracellular
358 $[\text{K}^+]$. Cold-tolerant flies likely maintain osmotic balance by accumulating other organic and
359 inorganic osmolytes in their hemolymph to replace Na^+ as the primary determinant of water
360 balance. These changes to ionic and osmotic homeostasis would limit Na^+ and water migration,
361 maintain K^+ balance in the cold, and thereby improve cold tolerance. Thus, similar
362 ionoregulatory mechanisms appear to underlie both phenotypic plasticity and cold tolerance
363 evolution, which could lead to tradeoffs between basal and plastic responses to cold, and suggest
364 an evolutionary route for the cryoprotectant-mediated cold tolerance strategies of freeze
365 tolerance and freeze avoidance.

366 **Materials and methods**

367 *Animal origins and husbandry*

368 We examined a total of 24 species from the genus *Drosophila*. Complete information on stock
369 origins is presented in Table S1. All species were maintained on the same banana, barley malt
370 and yeast-based medium (Nyamukondiwa et al., 2011) except that the diet of four cactophilic
371 species (*D. mojavensis*, *D. obscura*, *D. persimilis* and *D. pseudoobscura*) was supplemented with
372 2.1 g L⁻¹ *Opuntia ficus-indicta* powder (OroVerde Export, Morelos, Mexico). With the exception
373 of cold-acclimated *D. melanogaster* (described below), all flies were maintained at a constant
374 21.5 ± 0.5°C and at 50 ± 5% relative humidity with a 13:11 h (L:D) light cycle. Newly-eclosed
375 adult *Drosophila* were transferred, without anesthesia, to fresh 35 mL vials containing food
376 medium. Following transfer, adults were returned to 21.5°C for five days before use in
377 experiments. For the *D. melanogaster* acclimation experiments, virgin males were collected
378 under light CO₂ anesthesia (<10 min) on the day of their emergence, divided randomly into two
379 groups which were placed at either 21.5°C (warm-acclimated), or at 6 ± 0.5°C conditions (cold-
380 acclimated) for five days to acclimate.

381 *Measurement of CT_{min}*

382 The CT_{min} of each *Drosophila* species was quantified as previously described (Ransberry et al.,
383 2011). Adult flies were transferred into a custom-built, temperature-controlled 150 × 25 cm glass
384 column containing aluminum baffles to which the flies cling (similar to the design of Huey et al.,
385 1992). The temperature of the column was controlled by circulating a ethylene glycol:water (1:1)
386 mixture through the column jacket from a refrigerated circulating bath (model 1157P, VWR
387 International, Radnor, PA, USA). The temperature inside the column was independently
388 monitored by four type-T thermocouples (two at both the top and bottom of the column) and a
389 TC-08 interface connected to a computer running Picolog v5.20.1 (Pico Technology, St. Neots,
390 UK). Adult flies were released into the column where they clung to the baffles, and the
391 temperature was held at 21°C for 15 min before being reduced at 0.1°C min⁻¹. At their CT_{min},
392 flies lost the ability to cling to the baffles and fell into a collecting tube containing soapy water
393 that was changed every 1°C (10 min). Flies collected from the column were frozen, and later

394 sorted according to sex and counted. For the interspecific comparisons a mean of 206 (range: 78
395 - 695) flies were used to determine the CT_{min} of each sex in each *Drosophila* species (Fig. S1).

396 *Hemolymph collection*

397 Adult *Drosophila* were positioned for hemolymph sampling using a custom made apparatus
398 previously described (MacMillan and Hughson, 2014). Briefly, adult flies were moved directly
399 from their rearing vial and positioned head-first in a 10 μ L pipette tip through a system of rubber
400 tubing by air flow. The end of the pipette tip was then removed to expose the antennae, an
401 antenna was amputated at its first segment, and a clear droplet of hemolymph was secreted. The
402 pipette tip, with the fly and droplet attached, were immediately removed from the rest of the
403 device and the droplet was placed under hydrated paraffin oil for measurement of Na^+ and K^+
404 concentrations by the ion selective microelectrode technique or osmolality by nanoliter
405 osmometry (see below). The time from removal of a fly from its acclimation temperature or from
406 $0^{\circ}C$ to measurement of ion concentration or osmolality of the hemolymph was less than 2 min.

407 *Hemolymph ion concentration and osmolality*

408 Droplets of hemolymph from adult flies were used to measure extracellular ion concentrations
409 and osmolality. Different flies were used to measure each trait and a single droplet was taken
410 from a single fly. Hemolymph ion concentrations and osmolality were measured in 4-8 (K^+), 3-5
411 (Na^+) and 3-8 (osmolality) droplets from flies of each species and osmolality was measured in 5-
412 7 droplets from *D. melanogaster* males from each acclimation group. Ion concentrations were
413 measured in 14-16 (Na^+), 15-21 (K^+) droplets per treatment (control and 6 h at $0^{\circ}C$) from flies in
414 each acclimation group of *D. melanogaster*. The *D. kanekoi* and *D. algonquin* lines were lost to
415 mold before hemolymph ion concentrations and osmolality were measured, and so were not
416 included in this analysis. Hemolymph Na^+ and K^+ concentrations were measured in all
417 *Drosophila* at rearing temperature ($21.5 \pm 1^{\circ}C$). For the cold exposure, *D. melanogaster* were
418 transferred to microcentrifuge tubes and submerged in an ice-water slurry ($0^{\circ}C$) for 6 h. Flies
419 that received a cold exposure were sampled immediately following removal from the cold to
420 $21.5^{\circ}C$.

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

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

$$436 \quad [h] = [c] \times 10^{\frac{\Delta V}{S}} \quad (1)$$

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

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

450 *Na⁺/K⁺-ATPase activity and transcript and protein abundance*

451 To quantify maximal Na⁺/K⁺-ATPase activity, whole flies (~80 mg of pooled adults; 20-80 flies,
452 depending on species) were transferred to 1.7 mL microcentrifuge tubes without anesthesia, snap
453 frozen in liquid nitrogen vapor, and stored at -80°C. Na⁺/K⁺-ATPase activity was measured in 6
454 biological replicates of each temperature acclimation group of *D. melanogaster* and 3-6
455 biological replicates of each species were used for the interspecific analysis. *Drosophila*
456 *arawakana* was excluded from this analysis because of insufficient sample size ($n=1$). Frozen
457 *Drosophila* were weighed to obtain pooled fresh mass in pre-weighed 2 mL microcentrifuge
458 tubes before being homogenized on ice in 1 mL of homogenization buffer (25 mM imidazole,
459 0.2% w/v Na⁺-deoxycholate, 10 mM β-mercaptoethanol, 2 mM EDTA, pH 7.5) with a Tissue-
460 Tearor (Biospec Products, Bartlesville, OK, USA) using four 10 s bursts each followed by 20 s
461 rests on ice. Homogenized samples were sonicated (Virsonic 100; VirTis, Gardiner, NY, USA)
462 following the same timing of bursts and rests and centrifuged at 7000 × *g* for 5 min at 4°C. Size-
463 exclusion filtration columns, which permit the passage of proteins larger than approximately 50
464 kDa, were prepared by plugging the tip of a 3 mL plastic syringe barrel with glass wool and
465 adding 3 mL of Sephadex G-50 (GE Healthcare, Waukesha, WI, USA). Columns were stored at
466 4°C for a maximum of two weeks before use, and were conditioned by eight passes of 300 μL of
467 homogenization buffer and 1 min centrifugations (500 × *g*). A conditioned column was placed
468 into a clean 5 mL plastic test tube, a 300-μL aliquot of supernatant derived from the homogenate
469 was added to the column, and the column within the tube was centrifuged at 500 × *g* for 1 min to
470 draw the sample through the column and into the tube.

471 Filtered fly homogenates were diluted 7-fold in homogenization buffer immediately before a
472 20 μL aliquot was added to a 1 mL cuvette containing 880 μL of assay buffer initially at 23°C.
473 The reaction was initiated by the addition of 100 μL of a 50 mM ATP solution. Final conditions
474 for the assay were: 70 mM imidazole (pH 7.5), 140 mM NaCl, 30 mM KCl, 7 mM MgCl₂, 4 mM
475 phosphoenolpyruvic acid, 300 μM NADH, 5 mM ATP 50 U mL⁻¹ pyruvate kinase (EC:
476 2.7.1.40), and 50 U mL⁻¹ lactate dehydrogenase (EC: 1.1.1.27).

477 Maximal activity of Na⁺/K⁺-ATPase was measured across a range of temperatures using a
478 thermally-dynamic, spectrophotometric assay. The absorbance of NADH at 340 nm was

479 recorded using a Cary 100 Bio spectrophotometer with a Cary Peltier-effect Temperature
480 Controller (Agilent Technologies, Santa Clara, CA, USA) connected to a computer running
481 WinUV Thermal Application v3.0 (Agilent Technologies). Four replicate cuvettes of each
482 *Drosophila* sample were run, two of which contained 1 mM ouabain (a specific inhibitor of
483 Na⁺/K⁺-ATPase). Temperature inside a dummy cuvette was monitored by a ceramic temperature
484 probe (Agilent Technologies) interfaced with the spectrophotometer, which controlled the rate of
485 temperature change. A type-T thermocouple was also suspended in the dummy cuvette and
486 connected to a TC-08 interface (Pico Technology, St. Neots, United Kingdom), which measured
487 the temperature inside the cuvette every second for the duration of each sample run. The rate of
488 temperature change inside the cuvette was consistent throughout the temperature ramp (Fig.
489 S5A). Activity of Na⁺/K⁺-ATPase was determined as the difference between the rates of cuvettes
490 containing 1 mM ouabain and cuvettes that did not contain ouabain (Fig. S5B). For each
491 replicate, rates of change in absorbance (OD min⁻¹) were smoothed using a 15-point sliding
492 window in LoggerPro (v3.8.4, Vernier Software Inc., Beaverton, OR, USA).

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

$$495 \quad R = \frac{Max}{1 + e^{(IP - T)/Ts}} \quad (2)$$

496 where T is temperature and R is the empirically-determined rate of ATP turnover per minute at
497 that temperature, Max is the model-derived logistic asymptote, IP is the model-derived inflection
498 point of the curve, and Ts is model-derived sensitivity of $Rate$ to temperature (see Fig. 4). The IP
499 parameter is a measure of the position of the temperature-activity curve on the temperature axis,
500 and can be used to detect cold- or warm-shifts in the enzyme activity-temperature relationship.
501 The Ts parameter is a measure of enzyme thermal sensitivity.

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

508 To confirm that the thermally-ramped assays described above did not yield different results from
509 a more traditional method, the activity of Na⁺/K⁺-ATPase was also measured at five static
510 temperatures (6, 11, 13.3, 17.6, and 21.5°C) in n=4 samples of male *Drosophila melanogaster*
511 acclimated to 21.5°C (Fig. S5C). The methods of sample preparation and the final assay
512 conditions for the static-temperature Na⁺/K⁺-ATPase assays were the same as those used for the
513 thermally-dynamic assay. Once the temperature inside the dummy cuvette was stable at the set
514 temperature (determined from the thermocouple trace), the reaction was initiated by the addition
515 of ATP as in the thermally-dynamic assay. Rates of Na⁺/K⁺-ATPase activity in OD min⁻¹ over a
516 10 min period of recording were converted to mol ATP consumed min⁻¹ using the Beer-Lambert
517 law. Static and dynamic methods of the Na⁺/K⁺-ATPase assay yielded similar estimates of
518 Na⁺/K⁺-ATPase activity in male *D. melanogaster* acclimated to 21.5°C (Fig. S5C).

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

521 *Data analysis*

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

537 *Phylogenetically-independent contrasts*

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

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

552

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561

562 **Author Contributions**

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

566

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761 **Figure Captions**

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

770 **Fig. 2. Cold-acclimated *D. melanogaster* have low hemolymph $[Na^+]$ and defend against the**
771 **effects of cold exposure on hemolymph $[K^+]$.** Hemolymph K^+ (A) and Na^+ (B) concentrations
772 of cold-acclimated (grey bars) and warm-acclimated (open bars) male *Drosophila melanogaster*
773 at their acclimation temperature (Control) and following 6 h at $0^{\circ}C$ (mean \pm sem). Bars within a
774 panel that share a letter do not significantly differ. $n=14-16$ (Na^+) and $n=15-21$ (K^+) per
775 acclimation group per treatment.

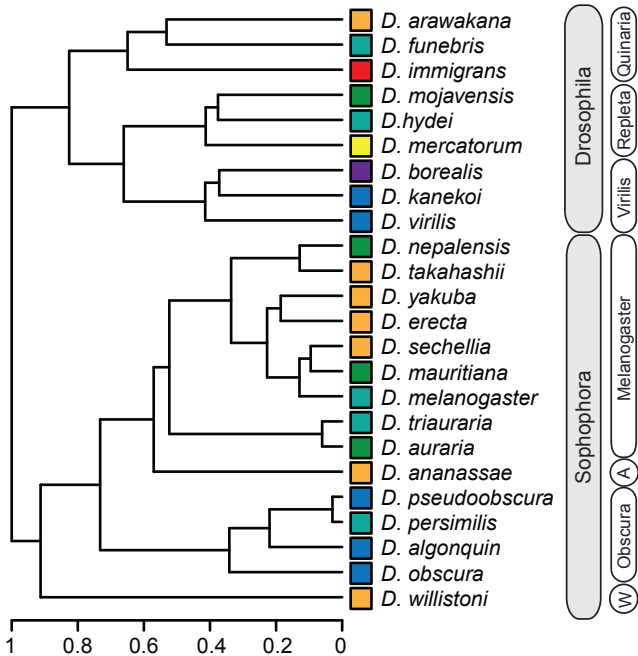
776
777 **Fig. 3. Cold adapted *Drosophila* species have low hemolymph $[K^+]$ and $[Na^+]$.** Hemolymph
778 concentrations of K^+ (A) and Na^+ (B) and hemolymph osmolality (C) of species of the genus
779 *Drosophila* are shown in relation to the CT_{min} . Lines denote a significant linear relationship
780 between ion concentration and the CT_{min} among species. *Drosophila immigrans* (open circle) had
781 remarkably low hemolymph $[Na^+]$ and was omitted from the regression for Na^+ (see text for
782 details), but was included in the regressions for $[K^+]$ and osmolality. P : P -value from correlation
783 of raw data, P_{PIC} : P -value from a regression using phylogenetically-independent contrasts forced
784 through the origin (Fig. S2). $n=4-8$ (K^+) and $n=3-5$ (Na^+). Y-axis (CT_{min}) error bars (sem) are
785 obscured by the symbols.

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

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

795 **Fig. 5. Cold-acclimated *Drosophila melanogaster* have low Na⁺/K⁺-ATPase activity.** (A)
796 Sodium pump activity of warm-acclimated (21.5°C, black) and cold-acclimated (6°C, grey)
797 *D. melanogaster*. Solid lines and dotted lines represent the mean and 95% CI, respectively. Open
798 circles denote mean activity at the acclimation temperature. Cold-acclimated *D. melanogaster*
799 had significantly lower Na⁺/K⁺-ATPase activity at 21.5°C than warm-acclimated flies (B)
800 Boxplots of Na⁺/K⁺-ATPase inflection point (*IP*) and thermal sensitivity (*T_s*) values of warm-
801 acclimated (open) and cold-acclimated (grey) *D. melanogaster*. Vertical lines denote the range
802 and horizontal lines denote the median and quartiles. There was no significant effect of
803 acclimation temperature on *IP* or *T_s*. *n*=6 biological replicates per acclimation group (see text for
804 details).

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



<-2°C



-2-0°C



0.01-2°C



2.01-4°C



4.01-6°C



6.01-8°C



>8°C

