

1 Mortality from Desiccation Contributes to a Genotype-by-Temperature Interaction for
2 Cold Survival in *Drosophila melanogaster*

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4 Robert Kobey¹ and Kristi L. Montooth¹

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6 ¹ Department of Biology, Indiana University, Bloomington IN 47405

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8 * author for correspondence:

9 Robert Kobey

10 1001 E 3rd St

11 Bloomington IN 47405

12 rkobey@indiana.edu

13 Office: 812-856-2589

14 Fax: 812-855-6082

15

16 **Summary**

17

18 Survival at cold temperatures is a complex trait, primarily due to the fact that the
19 physiological cause of injury may differ across degrees of cold exposure experienced
20 within the lifetime of an ectothermic individual. In order to better understand how chill-
21 sensitive insects experience and adapt to low temperatures, we investigated the
22 physiological basis for cold survival across a range of temperature exposures from -4°C
23 to 6°C in five genetic lines of the fruit fly, *Drosophila melanogaster*. Genetic effects on
24 cold survival were temperature dependent and resulted in a significant genotype-by-
25 temperature interaction for survival across cold temperature exposures that differ by as
26 little as 2°C. We investigated desiccation as a potential mechanism of injury across these
27 temperature exposures. Flies were dehydrated following exposures near 6°C, while flies
28 were not dehydrated following exposures near -4°C. Furthermore, decreasing humidity
29 during cold exposure decreased survival, and increasing humidity during cold exposure
30 increased survival at 6°C, but not at -4°C. These results support the conclusion that in *D.*
31 *melanogaster* there are multiple physiological mechanisms of cold-induced mortality
32 across relatively small differences in temperature and that desiccation contributes to
33 mortality for exposures near 6°C but not for subzero temperatures. Because *D.*
34 *melanogaster* has recently expanded its range from tropical to temperate latitudes, the
35 complex physiologies underlying cold tolerance are likely to be important traits in the
36 recent evolutionary history of this fruit fly.

37

38 **Introduction**

39

40 Cold tolerance is likely to be an important trait in the recent evolutionary history
41 of the fruit fly, *Drosophila melanogaster* (Schmidt et al., 2005a). While the species
42 originated in tropical Africa, *D. melanogaster* has colonized temperate regions across the
43 world over the last 15,000 years (David and Capy, 1988). Like many insects, *D.*
44 *melanogaster* freezes at temperatures well below the freezing point of water (Czajka and
45 Lee, 1990). Nevertheless, *D. melanogaster* dies within hours even at temperatures that do
46 not freeze tissues (Chen and Walker, 1994; Czajka and Lee, 1990; Novitski and Rush,
47 1949). Despite being susceptible to cold injury, *D. melanogaster* has been extremely
48 successful in colonizing colder environments, and its range now extends as far north as
49 Finland and as far south as Tasmania (Keller, 2007).

50 Temperate populations of *D. melanogaster* tend to be more cold tolerant and more
51 desiccation resistant than tropical and subtropical populations (Bubliy et al., 2002;
52 Davidson, 1990; Karan et al., 1998; Parsons, 1980; Schmidt et al., 2005b). This could
53 indicate that the two traits share a common genetic basis or that temperate environments
54 select for tolerance to both colder and more desiccating environments. However, cold
55 tolerance and desiccation resistance do not always covary with latitude (Dalage et al.,
56 1989; Hoffmann et al., 2001), suggesting that either the genetic underpinnings of or the
57 selection pressures acting on these traits are separable. Selection for either cold tolerance
58 or desiccation resistance in the laboratory can lead to the evolution of cross-tolerances to
59 both stresses (Bubliy and Loeschcke, 2005), indicating that the two tolerances may share
60 a common physiological or genetic basis or that selection acted to increase a general
61 stress response. However, cold exposure and desiccation induce different gene expression
62 profiles (Sinclair et al., 2007a) and the two traits can evolve independently (MacMillan et
63 al., 2009; Sinclair et al., 2007b), suggesting that the genetic correlation between these
64 traits is not absolute.

65 A major challenge to understanding how organisms adapt to cold is the degree to
66 which distinct physiological mechanisms of injury contribute to differences in cold
67 tolerance across a range of cold temperatures that will be experienced in the lifetime of
68 an individual. Cold environments present a number of physiological challenges for chill-

69 sensitive ectotherms, like *Drosophila*, that are able to avoid ice crystal formation at
70 temperatures below 0°C via supercooling of their body water but nonetheless have
71 decreased performance and survival at temperatures well above their supercooling point.
72 This is because injuries caused by acute exposures to subzero temperatures where chill-
73 susceptible species can survive only for short amounts of time may be distinct from the
74 injuries that accumulate as organisms survive longer at chronic exposure to temperatures
75 above 0°C (reviewed by Lee, 2010). Acute exposure to subzero temperatures may
76 damage cellular membranes. As temperature decreases, cellular membranes become more
77 ordered and rigid (Hazel and Williams, 1990), disrupting the function of membrane-
78 bound proteins (Cossins et al., 1981; Hazel, 1972) and resulting in leakage across the
79 membrane when phase transitions occur (Drobnis et al., 1993). The injuries that
80 accumulate during prolonged exposures to temperatures above zero may also be caused
81 by disruptions of the cell membranes that can lead to a loss of ion homeostasis with
82 consequences for the control of the neuromuscular system (Kostal et al., 2007; Lee, 2010;
83 MacMillan & Sinclair, 2011). At cooler temperatures, rates of reaction also slow and
84 enzymes are less effective catalysts (Hochachka and Somero, 2002).

85 Desiccation has long been associated with cold injury in insects. For exposures
86 that cause freezing, the increase in electrolyte concentration as ice crystals form may
87 mimic dehydration (Salt, 1961). For cold exposures that do not freeze tissues, desiccation
88 may contribute to cold injury at subzero temperatures during supercooling because the
89 vapor pressure of water is lower for the hemolymph than the air (Holmstrup et al., 2010;
90 Lundheim and Zachariassen, 1993; Zachariassen et al., 2008). Supercooled insects lose
91 water until the depression of the melting point from colligative properties matches the
92 ambient temperature, and many insects avoid freezing by rapidly losing water during
93 supercooling (Holmstrup, 2010). Desiccation may also contribute to mortality at
94 temperatures above the freezing point of water when individuals experience chill coma, a
95 reversible, quiescent state in which insects are unable to move (Mellanby, 1939). Without
96 the ability to feed or drink, insects in chill coma will eventually die from desiccation or
97 starvation if the exposure is not harsh enough to cause fatal injury through some other
98 physiological mechanism. For these reasons, desiccation may contribute to mortality
99 across a range of cold temperatures.

100 Here we report that even across relatively small thermal differences there are
101 significant differences in how five wild-type genotypes of *D. melanogaster* survive cold.
102 These genotype-by-temperature interaction effects suggest that different genetic and
103 physiological mechanisms underlie cold injury at different temperatures between -4°C
104 and 6°C. We find evidence that only particular genotype-temperature combinations result
105 in death from desiccation during cold exposure, indicating that other types of
106 physiological injury are contributing to death across cold temperatures in a genotype-
107 dependent manner. While desiccation contributes to mortality at 6°C, it is not the cause
108 of mortality for subzero exposures. We interpret these results in the context of the
109 colonization of temperate latitudes by *D. melanogaster* and discuss extensions to other
110 chill-susceptible insects.

111

112 **Materials and Methods**

113

114 *Fly Stocks*

115 All experiments included five wild-type, laboratory strains of *D. melanogaster*
116 obtained from the Bloomington Drosophila Stock Center (Bloomington, IN, USA).
117 Canton-S, Hikone-A-S, Oregon-R-C, Berlin-K, and RAL-208 were sampled from
118 different geographic locations and have been in the lab for varying numbers of years with
119 differing levels of inbreeding. We have kept these strains at large population sizes to
120 minimize further inbreeding. Flies were cultured in vials on standard cornmeal-agar
121 media with supplemental yeast. Adult male flies aged 4 to 6 days after eclosion were used
122 in all experiments. Flies were reared at 22°C on a 12-hour light/dark cycle for
123 experiments and for two generations prior to experiments. All experiments were initiated
124 approximately 6 hours into the light cycle to minimize variation caused by circadian
125 rhythm. Because long exposures to carbon dioxide anesthesia are known to affect cold
126 tolerance (Nilson et al., 2006), we minimized the use of carbon dioxide anesthesia for
127 sorting flies to less than 15 minutes.

128

129 *Cold Mortality Curves*

130 Survival was scored and mortality curves were fit for each genotype at each

131 temperature ranging from -4°C to 6°C at 2°C intervals. Pools of ten adult male flies were
132 sorted into 10 mm diameter glass test tubes. The test tubes were placed at low
133 temperatures in a cold bath that could regulate temperature with a precision of $\pm 0.1^{\circ}\text{C}$.
134 Because all flies were in chill coma after cold exposure, flies were transferred to inverted
135 food vials to recover, and we then scored the proportion of flies alive after a three-day
136 recovery at 22°C . To generate mortality curves at each temperature, survival was scored
137 for each genotype for at least five time points with at least five replicate test tubes
138 containing ten flies per time point. Flies sampled at each time point were independent
139 samples of ten males. Time points were chosen using information from the literature and
140 from our own preliminary data such that we would sample vials with complete,
141 intermediate and no survival. Figure S1 shows the complete survival data and time points
142 from which the curves in Figure 1 were fit.

143 Flies from at least three different sibling cohorts and two non-overlapping
144 generations were used to generate mortality curves for each genotype in order to ensure
145 that the estimates were robust to micro-environmental effects. For each cohort, all
146 genotypes were exposed to a single temperature in parallel on the same days to avoid
147 confounding differences between genotypes with day-to-day variation in laboratory
148 conditions. Each mortality curve was estimated from approximately 400 flies per
149 genotype. Curves were fit to the data using logistic regression in the R statistical package
150 version 2.10.0 (R Development Core Team, 2008). From these curves we estimated the
151 duration of exposure predicted to cause 50% mortality, the LT_{50} , with 95% confidence
152 intervals.

153

154 *Water Content*

155 To determine whether flies that die from cold exposure have water contents
156 similar to or distinct from flies that die from desiccation, we measured the water content
157 of 15 individual males of each genotype that were exposed to each cold temperature for a
158 duration corresponding to the LT_{50} value for the genotype. Approximately 50% of flies
159 should survive an LT_{50} exposure, but the majority of flies should either be near death or
160 recently dead. Thus, measuring the water content following an LT_{50} exposure
161 approximates water content at or near the time of death. Immediately following cold

162 exposure, flies were stored at -80°C for less than 24 hours and then weighed to determine
163 wet mass. Flies were allowed to thaw for 2-3 minutes before weighing. We then dried the
164 flies at 55°C overnight and weighed dry mass. Storing flies at -80°C for 24 hours has
165 very little effect on the mass of a fly and is consistent across genotypes, decreasing mass
166 by less than 3.5% (Fig. S2). Masses were measured to the nearest 0.001 mg using a
167 Sartorius ME5 microbalance (Sartorius, Goettingen, Germany). We quantified the water
168 content of flies after LT_{50} exposure as the difference between their wet and dry masses
169 (Gibbs et al., 1997). Water contents were converted to a proportion by dividing by the
170 wet mass.

171 In order to compare the water contents of flies after an LT_{50} cold exposure to
172 those of flies that died from desiccation, we measured the water content of flies that died
173 from desiccation. We placed individual male flies from each genotype in vials with
174 Drierite brand desiccant (Xenia, Ohio, USA) at 22°C and monitored them every hour
175 until death. At death, flies were stored at -80°C and water contents were measured as
176 described above. Fifteen flies were measured for each genotype. Water contents were
177 also measured for fed, hydrated control males of the same age, but with no LT_{50} or
178 desiccant exposure.

179

180 *Cold Survival Under Altered Humidity*

181 We measured survival at 6°C and -4°C with altered humidity to determine if low
182 water contents following LT_{50} exposures were the cause of mortality. For the high
183 humidity treatment, pools of ten 4-6 day old adult males were placed in test tubes
184 containing a moist paper towel with a rayon ball separating the flies from the paper towel.
185 Pools of flies for the low humidity treatment were placed in test tubes containing Drierite
186 brand desiccant (Xenia, Ohio, USA) with a rayon ball separating the flies from the
187 desiccant. The sides of the test tubes were wiped down to remove any residues left behind
188 by the desiccant. Control flies were placed in test tubes with a rayon ball separating the
189 flies from the bottom of the test tube. For each genotype, all three humidity treatments
190 were run in parallel so as not to confound treatment effects with any day-to-day variation
191 in laboratory conditions. For 6°C exposures, 80 flies per treatment were exposed to low
192 temperature in a cold bath for a duration corresponding to the calculated LT_{50} for each

193 genotype. Water content was measured as described immediately following the cold
194 exposures in fifteen flies for each genotype and treatment.

195 Unlike 6°C exposures, at -4°C the mortality curves are very steep and the
196 transition from 0% to 100% mortality occurs rapidly. Because of this, we generated full
197 mortality curves for each genotype at -4°C for each humidity treatment. Each mortality
198 curve was generated from five time points with data from 60 flies per time point (N=300
199 flies per mortality curve). All three humidity treatments for each genotype were run in
200 parallel to control for laboratory conditions. Percent survival was scored in each vial after
201 a three-day recovery and LT₅₀ was estimated as described above. Adding desiccant at -
202 4°C will decrease the amount of moisture in the air surrounding flies. Increasing the
203 humidity via a moist paper towel presents more of a challenge due to the formation of
204 ice. It is not possible to eliminate the vapor pressure deficit during supercooling because
205 excess water in the air will form ice crystals on available surfaces. However, when the
206 vapor pressure of water in the air is less than the vapor pressure of ice, there will be a net
207 movement of water from the solid phase to the vapor phase. Under these conditions, the
208 presence of ice will increase the amount of water in the air surrounding the fly and
209 decreases the vapor pressure deficit between the insect and the air, relative to the control
210 condition.

211

212 *Statistical Analyses*

213 Statistical analyses were performed with the R statistical package version 2.10.0
214 (R Development Core Team, 2008). Logistic regression models were fit using the glm
215 function and post-hoc tests were performed using the TukeyHSD function. LT₅₀ (median
216 survival time) and 95% confidence intervals were calculated using the dose.p function
217 from the MASS package (Venables and Ripley, 2002). Post-hoc tests for logistic
218 regression models were performed using the glht function from the multcomp package
219 (Hothorn et al., 2008).

220

221 **Results**

222

223 *Cold Survival Across Temperatures and Genotypes*

224 Both genotype and temperature had strong effects on survival (Table 1; Figs. 1,2).
225 There were large differences in survival time across the range of temperatures measured;
226 flies at -4°C survived on the order of minutes, with flies living for hours when exposed to
227 $0-4^{\circ}\text{C}$ and for days at 6°C . If flies experience all low temperatures as a single type of
228 stress or injury, then genotypes with higher survival at one temperature should have
229 higher survival at all temperatures. However, mortality curves generated for each
230 genotype at each of six low temperatures (Fig. 1) revealed large genotypic effects on
231 survival that changed in rank order across temperatures that differed by as little as 2°C
232 (Fig. 2), indicative of temperature-dependent genetic effects (i.e. genotype-by-
233 temperature interactions)(Table 1). The most cold-tolerant genotype at one temperature
234 was not the most cold-tolerant genotype across all temperatures. For example, Canton-S
235 had a significantly higher survival time than Berlin-K at -4°C ($P<0.001$), 2°C ($P<0.001$),
236 and 4°C ($P=0.004$), while Berlin-K had a significantly higher survival time than Canton-
237 S at -2°C ($P<0.001$), 0°C ($P<0.001$), and 6°C ($P=0.013$) (P -values from Tukey's post-hoc
238 tests). This suggests that the injury experienced by individual flies that differed in
239 genotype was not the same across these low temperatures, and that different
240 physiological and genetic mechanisms likely mediate cold tolerance across temperature
241 differences as small as 2°C .

242

243 *Water Content Following Cold Exposure*

244 We used LT_{50} exposures to provide a snapshot of the physiological state of flies
245 of different genotypes near death from cold exposure. We compared the water contents of
246 flies given an LT_{50} cold exposure with the water contents of flies that died from
247 desiccation to determine whether the hydration state of flies dying from cold was
248 consistent with that of flies that we knew had died from desiccation at room temperature
249 (Fig. 3). If desiccation is the cause of mortality, water content following cold exposure
250 should be similar to desiccated flies and significantly lower than unstressed, hydrated
251 controls. For exposures at temperatures near -4°C , most genotypes had water contents
252 that were significantly higher than desiccated flies and not significantly different from
253 hydrated controls (Fig. 3). All genotypes had water contents that were significantly
254 higher than desiccated flies for exposures below 0°C (Fig. 3). For exposures at

255 temperatures near 6°C, most genotypes had water contents that were significantly lower
256 than hydrated controls (Fig. 3), and some genotypes had water contents that were not
257 significantly different from desiccated flies (Fig. 3). Thus, the overall trend across
258 genotypes was for flies to have less water following exposures to higher cold
259 temperatures, leading us to hypothesize that desiccation contributes to mortality at
260 temperatures near 6°C, but not at subzero exposures.

261

262 *Cold Survival at High and Low Humidity*

263 If flies are dying from desiccation during cold exposures, then altering the
264 humidity during cold exposure should alter the rate of water loss and affect how long
265 flies survive the cold. Given the patterns of water content described above, we predicted
266 that altered humidity would affect survival at 6°C, where flies appear to be desiccated
267 when dying, but not at -4°C, where flies appeared to be hydrated when dying. Consistent
268 with these predictions, all genotypes had higher survival when humidity was increased
269 and lower survival when humidity was decreased at 6°C (Fig. 4A). This pattern was
270 reflected in the water contents of flies following the 6°C LT₅₀ exposure; water contents
271 were increased relative to controls after cold exposure at higher humidity and decreased
272 relative to controls after cold exposure at lower humidity (Fig. 4B). This indicates that
273 mortality is caused by desiccation at 6°C. However, desiccation does not contribute to
274 mortality at -4°C. At -4°C, increasing humidity had little to no effect on survival, and
275 when decreased humidity had a significant effect, it increased cold survival (Fig. 5).
276 These results confirm that desiccation contributes to mortality at 6°C but not at -4°C. The
277 temperature-dependent genetic effects that we have observed reflect physiological
278 differences in the cause of death at low temperatures, and desiccation is one such cause.

279

280 **Discussion**

281

282 It has been previously suggested that cold, but non-freezing, temperature
283 exposures might produce two qualitatively different types of cold injury depending on the
284 exposure temperature (Chen and Walker, 1994). Three pieces of evidence support this
285 conclusion: (1) mortality occurs within minutes or hours at subzero temperatures but

286 takes days to occur at higher temperatures (Chen and Walker, 1994); (2) acclimation
287 treatments that increase survival at subzero temperatures do not increase survival at 0°C
288 (Chen and Denlinger, 1992); and (3) selection for survival at -7°C results in higher
289 survival at -7°C but not 0°C, while selection at 0°C increases survival at both
290 temperatures (Chen and Walker, 1994). While these two types of cold exposure are often
291 interpreted to represent differences in the physiological mechanism of injury (Lee, 2010;
292 Nedved, 2000; Sinclair and Roberts, 2005), these different temperature exposures have
293 also been interpreted as a single physiological mechanism of injury acting at different
294 rates (Morris and Watson, 1984). Our findings support that both genetic effects and the
295 mechanisms of physiological injury that cause death differ across a range of cold
296 temperature exposures, although the interesting possibility remains that similar cellular
297 structures (e.g. the cell membranes) are compromised across this range of cold.

298 It is known that cold survival in *D. melanogaster* differs between genetic strains,
299 for acute versus chronic cold exposure and with different acclimation treatments (Chen
300 and Walker, 1994; Rajamohan and Sinclair, 2008). Here we investigated basal cold
301 survival in five genotypes across six, densely sampled cold temperatures, allowing us to
302 quantify strong genotype-by-temperature interactions on cold survival across a relatively
303 continuous gradient of cold exposure. Furthermore, we show that this genotype-by-
304 environment interaction for cold survival is caused by differences in physiological injury
305 across this gradient. Across genotypes, water content following potentially lethal
306 exposures tended to decrease with increasing temperatures. For most genotypes, flies
307 dying from 6°C exposures had water contents identical to flies that died from desiccation.
308 Combined with the strong effect of humidity on survival at 6°C, but not at -4°C, these
309 data indicate that desiccation contributes to mortality for milder cold exposures but not
310 for subzero exposures in *D. melanogaster*. This pattern supports the conclusion that there
311 are at least two classes of cold injury in *D. melanogaster*, with desiccation contributing to
312 death at milder, cold temperatures. This is consistent with the timescales at which flies
313 are dying across these temperatures. In the presence of desiccant, genotypes had median
314 survival times of approximately 10 to 20 hours at room temperature and some individual
315 flies survived for 1-2 days (data not shown). Flies exposed to temperatures from 0-6°C
316 survived 1-4 days, consistent with the expectation that desiccation survival times should

317 be longer at lower temperature (Dalage et al., 1989) and in the absence of desiccant. The
318 rate of water loss would have to be 10 or 20 times higher for desiccation to cause
319 mortality at -4°C , as flies only survive a few hours under these extreme conditions.

320 These findings may reveal why investigations of cross-tolerances for desiccation
321 and cold tolerances in *D. melanogaster* using different temperature exposures have
322 yielded conflicting results. Selection for survival at -5°C does not increase desiccation
323 resistance (MacMillan et al., 2009), and selection for desiccation resistance does not
324 increase cold survival for exposures near -5°C (Sinclair et al., 2007b). The lack of a
325 correlated response to selection suggests that desiccation does not contribute to mortality
326 for exposures near -5°C , and we find that flies show no sign of dehydration following
327 potentially lethal -4°C exposures. Selection for desiccation resistance increases cold
328 survival at 0.5°C , and selection for cold resistance at 0.5°C increases survival at low
329 humidity (Bubliy and Loeschcke, 2005). In our experiments, three of the five genotypes
330 were partially or fully dehydrated following the 0°C exposure and had water contents
331 significantly lower than hydrated controls. While there are likely to be additional reasons
332 for the different results obtained by artificial selection experiments in the literature
333 (Gibbs, 2002; Harshman and Hoffmann, 2000), we suggest that differences in exposure
334 temperature explain some of these differences. Furthermore, our findings indicate that
335 mechanisms of desiccation resistance, such as increased glycogen stores (Gefen et al.,
336 2006), likely contribute to the evolution of cold tolerance in temperate natural
337 populations that regularly experience cold temperatures above 0°C .

338 Even if the proximate cause of mortality at 0°C is not dehydration, we find that
339 partial dehydration is occurring in most genotypes at temperatures near 0°C . This may
340 magnify other types cold injury and affect other fitness components of flies that are able
341 to survive a single bout of cold exposure. Partial dehydration increases mortality at low
342 temperature in the springtail, *Orchesella cincta*, even when the loss of water is not
343 sufficient to cause death on its own (Nedved et al., 1998). Partial dehydration also has
344 negative effects on life history traits in insects, such as the fecundity of mosquitos
345 (Benoit et al., 2010; Canyon et al., 1999). In *D. melanogaster*, less desiccation-resistant
346 males have lower mating success in arid environments (Gefen and Gibbs, 2009). Yet,

347 female fecundity is not lowered by desiccation (Albers and Bradley, 2006; Sepulveda et
348 al., 2008), suggesting that the physiological consequences of bouts of desiccating cold-
349 exposure may be sex-specific. Thus, the selection pressure experienced by flies at
350 temperatures near 0°C may increase desiccation resistance due to the correlated effects of
351 dehydration on reproductive success, regardless of whether mortality is due to
352 dehydration. Selection in natural populations likely acts on suites of traits that not only
353 affect survival during cold exposure, but also mediate the lasting effects of cold injury on
354 fitness traits once the cold exposure has passed. For example, the female reproductive-
355 diapause phenotype in *D. melanogaster* is at higher frequency in high-latitude
356 populations and is correlated with a suite of stress resistance traits that include higher
357 cold survival and starvation resistance (Schmidt et al., 2005a; Schmidt et al., 2005b).

358 The differences among genotypes in water content after cold exposure,
359 particularly at temperatures just above 0°C, indicate that flies of different genotypes may
360 be dying from different physiological causes at the same temperature. For example, flies
361 of the Hikone-A genotype were hydrated when dying across the entire range of cold
362 exposures, while the water contents of the Berlin-K, Oregon-R and Canton-S genotypes
363 dying at 4°C were similar to desiccated flies. This suggests that Hikone-A individuals die
364 from injury other than desiccation at a temperature where other genotypes are dying from
365 desiccation. This may be because Hikone-A is a more desiccation resistant genotype,
366 allowing for increased survival at temperatures where the capacity to resist desiccation
367 becomes increasingly important for survival. Yet, Hikone-A was not the most cold
368 tolerant genotype at all non-subzero temperatures, highlighting the physiological
369 complexity of surviving cold. Thus, while types of cold injury may differ across a range
370 of low temperatures, in any given population or species, different genotypes experiencing
371 the same thermal environment may also die from distinct or combined physiological
372 stresses.

373 While differential contributions of desiccation to survival contribute to the
374 genotype-by-temperature effects that we observed, our findings also indicate that other
375 physiological and genetic mechanisms are responding to cold injury in populations of
376 flies that regularly experience subzero temperatures. We observed a complex pattern of
377 genotype survival times across temperatures that differ by as little as 2°C and across

378 temperatures from -4°C to 4°C where flies are not entirely dying from desiccation. These
379 observations suggest that there may be more than just two classes of cold injury. In other
380 words, the genetic effects do not fall into two qualitatively different categories that would
381 be indicative of two genetic mechanisms responding to two qualitatively different types
382 of cold injury (i.e. desiccated and non-desiccated). Other physiological mechanisms
383 implicated in cold injury without freezing include loss of membrane fluidity (Lee et al.,
384 2006; Overgaard et al., 2008; Shreve et al., 2007), oxidative stress (Joanisse and Storey,
385 1996; Lalouette et al., 2011; Rojas and Leopold, 1996), loss of ion homeostasis (Kostal et
386 al., 2004; Kostal et al., 2007), protein misfolding (Rinehart et al., 2007), and the
387 induction of cell death pathways (Yi et al., 2007), any of which could potentially
388 contribute to the differential mortality of genotypes across temperatures. At the lowest
389 temperatures, we cannot rule out the possibility that inoculative freezing caused by ice
390 crystal formation on the external cuticle of flies contributes to mortality (Lee, 2010).
391 However, if flies are dying from inoculative freezing, the effects are not immediate; flies
392 sampled after 1 hour at -4°C have nearly 100% survival. Further investigation of these
393 sources of injury across a range of cold temperatures will provide insight into the
394 underlying basis for the physiological and genetic complexity of cold tolerance that we
395 have observed.

396 Some of the proposed cellular mechanisms of cold injury may either result from
397 or be similar to physiological injuries caused by desiccation. Dehydration results in the
398 increased concentration of solutes within cells and the hemolymph. Cold exposure has
399 been associated a loss of ion homeostasis caused by leaky membranes (Drobnis et al.,
400 1993) and the inability to regulate ion homeostasis at the organismal level during cold
401 exposure (Kostal et al., 2004; Kostal et al., 2007; MacMillan and Sinclair, 2011). The
402 loss of ion homeostasis during cold exposure may have effects that are similar to the
403 effect of dehydration on ion balance. Ion imbalance may affect protein folding (Record et
404 al., 1998), and the association of heat shock protein expression with both cold and
405 desiccation may be the result of a shared response to misfolded proteins (Benoit et al.,
406 2010; Burton et al., 1988; Hayward et al., 2004; Kostal et al., 2009; Peterson et al., 1990;
407 Rajamohan and Sinclair, 2008; Sinclair et al., 2007a). Both extreme dehydration and low
408 temperature can have similar effects on membrane fluidity (Crowe et al., 1992).

409 However, *D. melanogaster* does not survive the levels of extreme dehydration that are
410 typically necessary to significantly affect membrane fluidity (Crowe et al., 1992). Thus,
411 we might expect that the adaptations that confer tolerance of cold injury may share a
412 common genetic basis with desiccation tolerance.

413 While simple, sudden low temperature exposures do not mimic the variable and
414 complex conditions experienced by flies in nature, these experiments do inform our
415 understanding of cold tolerance in natural populations. The physiological and genetic
416 complexity that we find underlying cold tolerance across temperatures suggest that there
417 are unlikely to be genotypes that are superior at surviving the full range of cold injuries
418 that may be experienced even across a fairly narrow range of temperatures. Thus, the
419 evolution of cold tolerance is likely to involve tradeoffs that favor generalist strategies
420 that maximize fitness across a range of cold temperatures at latitudes with seasonal
421 cooling. Furthermore, natural populations experience fluctuating thermal environments,
422 and bouts of extreme cold may be interrupted by milder conditions with an opportunity to
423 repair injuries. Our findings from single temperature exposures inform our understanding
424 of the transient injuries that would need to be repaired in a fluctuating environment, and
425 highlight the large role that partial dehydration will likely play in the physiological
426 fitness costs of temperatures that fluctuate around 0°C. Multiple sub-lethal exposures to
427 low temperature are known to decrease fecundity in *D. melanogaster* (Marshall and
428 Sinclair, 2010). While this has been interpreted as a tradeoff in the allocation of energy
429 stores that maximizes fitness, the loss of reproductive fitness may also be due to
430 accumulated injuries of transient dehydration stress during these exposures (Benoit et al.,
431 2010). Even if dehydration is not the primary cause of death, desiccation resistance
432 mechanisms likely contribute to increased fitness in natural populations of *D.*
433 *melanogaster* experiencing transient subzero exposures, in addition to the clear
434 advantages of desiccation resistance that we find for flies at cold temperatures above 0°C.

435 Additionally, insects in nature likely acclimate to cooling thermal environments
436 during their lifetime to increase cold tolerance. Interestingly, the beneficial effects of cold
437 acclimation treatments can differ across temperatures (Chen and Denlinger, 1992; Chen
438 and Walker, 1994; Rajamohan and Sinclair, 2008). While our study only measured basal
439 cold survival, our results provide a possible explanation for the differential effects of

440 acclimation on cold survival. If different physiological mechanisms of injury underlie
441 survival across cold exposures, we might expect that not all cold acclimation treatments
442 will have the same effects on survival across temperatures. Consistent with this, cold
443 acclimation treatments do not all have the same effects on gene expression (Goto 2000;
444 Goto, 2001; Qin et al. 2005; Sinclair et al., 2007a) or on physiological traits (Overgaard
445 et al., 2005; Overgaard et al., 2006; Overgaard et al., 2007; Overgaard et al., 2008;
446 Tomcala et al., 2006). For example, exposure to 0°C results in a gene expression profile
447 different from the gene expression profile induced by desiccation (Sinclair et al., 2007a).
448 Acclimation at 0°C increases survival at -5°C (Czajka and Lee, 1990), which would not
449 be expected to cause injury by desiccation based on our results at -4°C. Thus, it is logical
450 for acclimation at 0°C to have a different gene expression profile from desiccation.
451 Although a profile of gene expression does not take into account all of the physiological
452 changes that occur, we would predict that acclimation at 0°C does not increase survival at
453 6°C based on this data. A more thorough investigation of the differential effects of these
454 acclimation treatments on survival will inform our understanding of the physiological
455 mechanisms of injury across temperatures and the plastic responses that protect against
456 injury.

457 These results also have implications for the evolution of cold tolerance strategies
458 in other small ectotherms. There has been a great deal of research on freezing as a critical
459 temperature and cold tolerance strategies for either avoiding freezing or tolerating
460 freezing (Bale, 1993). However, *D. melanogaster* does not freeze until temperatures are
461 near -20°C (Czajka and Lee, 1990). The identification of different physiological causes of
462 death at different temperatures above -20°C indicates that there are other critical
463 temperatures between freezing and chill coma at which the physiological cause of death
464 changes. Elucidating the physiological mechanisms of injury at these temperatures, the
465 effect of acclimation on these critical temperatures, the adaptations to these
466 physiologically different stresses, and any tradeoffs between adaptive strategies is likely
467 to uncover novel strategies for maximizing fitness in cold environments. The frequency
468 and duration with which an environment crosses these threshold temperatures will
469 influence the cold tolerance strategies favored by natural selection.

470

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472

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477

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689
690

691 Table 1. Logistic regression analysis of survival across temperatures

	d.f.	Deviance	Residual d.f.	Residual deviance	<i>P</i>
Null			1039	9003.2	
Time	1	1043.80	1038	7959.4	< 0.001
Temperature	1	15.36	1037	7944.1	< 0.001
Genotype	1	13.92	1036	7930.1	< 0.001
Time x Temperature	1	437.29	1035	7492.9	< 0.001
Time x Genotype	1	0.78	1034	7492.1	0.377
Temperature x Genotype	1	39.66	1033	7452.4	< 0.001
Time x Temperature x Genotype	1	0.71	1032	7451.7	0.401

692

693 **Figure Legends**

694 **Figure 1.** Fitted mortality curves for five genotypes at six exposure temperatures from -
 695 4°C to 6°C. Genotypes are Berlin-K (BK), Canton-S (CS), Hikone-A-S (HK), Oregon-R-
 696 C (OR), and Raleigh-208 (RA). Curves were fit using logistic regression with survival
 697 data from ~400 individual male flies of the same genotype per curve.

698

699 **Figure 2.** Genotype-by-temperature effects on cold survival. A. The LT_{50} (median
 700 survival time \pm 95% CI) at each of six exposure temperatures from -4°C to 6°C was
 701 estimated from mortality curves for each of five genotypes: Berlin-K (BK), Canton-S
 702 (CS), Hikone-A-S (HK), Oregon-R-C (OR), and Raleigh-208 (RA). The genotype-by-
 703 temperature interaction can be seen visually by the crossing reaction norms that connect
 704 genotype LT_{50} estimates across cold temperature exposures. B. A close up reveals
 705 crossing genotype reaction norms even at the coldest temperatures.

706

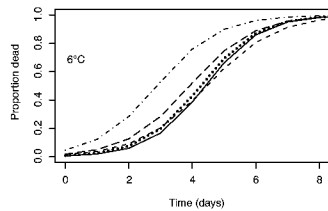
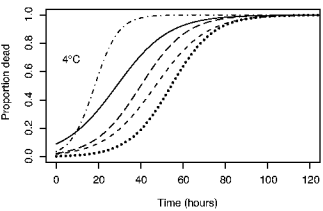
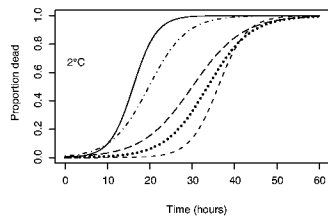
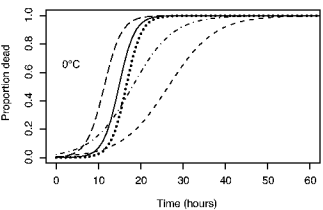
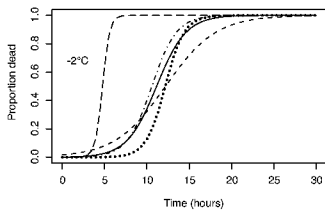
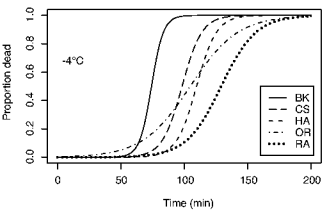
707 **Figure 3.** Water content of individual adults following LT_{50} exposures at each of six
 708 temperatures compared to desiccated and to hydrated control flies. Proportion of water
 709 (mean \pm SE) was measured by subtracting dry weight from wet weight and dividing by
 710 wet weight. Bars labeled A were not significantly different from hydrated flies (Tukey's
 711 post-hoc test, $P>0.05$). Bars labeled B were not significantly different from desiccated
 712 flies (Tukey's post-hoc test, $P>0.05$). Bars labeled AB were not significantly different
 713 from hydrated or desiccated flies (Tukey's post-hoc test, $P>0.05$). Unlabeled bars were
 714 significantly different from both hydrated and desiccated flies (Tukey's post-hoc test,
 715 $P<0.05$)

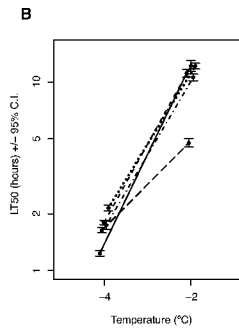
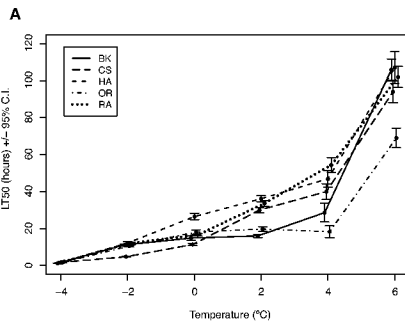
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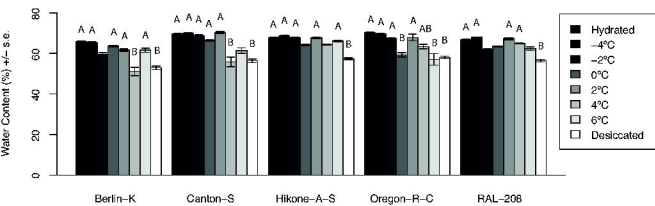
717 **Figure 4.** Effects of altered humidity on survival and water content at 6°C. A. The
 718 proportion (mean \pm SE) of flies surviving a 6°C LT_{50} in either dry, control or hydrated
 719 conditions. B. The water content (mean \pm SE) of flies surviving a 6°C LT_{50} in either dry,
 720 control or hydrated conditions. Tukey's post-hoc tests, * $P<0.05$, ** $P<0.01$, ***
 721 $P<0.001$.

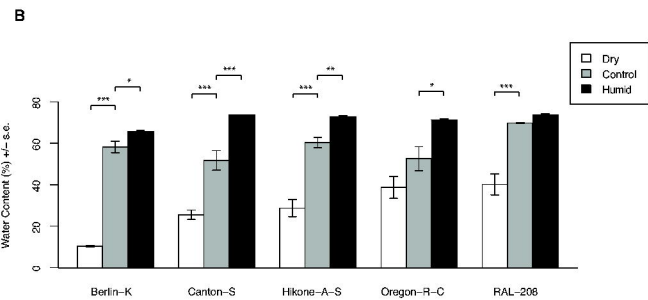
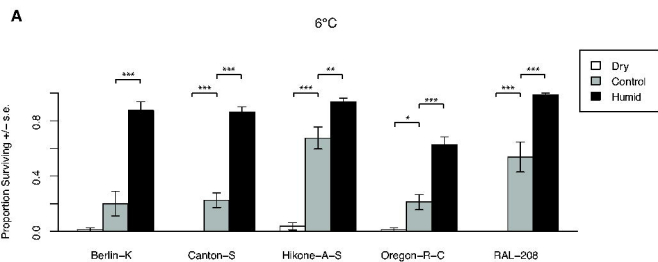
722

723 **Figure 5.** Effects of altered humidity on survival and water content at -4°C. The LT₅₀
724 (median survival time ± 95% CI) was estimated for all five genotypes at -4°C with
725 decreased (dry), control and increased (wet) humidities. Tukey's post-hoc tests, * $P < 0.05$,
726 ** $P < 0.01$, *** $P < 0.001$









LT50 (minutes) \pm s.e.

