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

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Summary

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

Introduction

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

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

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

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

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

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

Materials and Methods

Fly Stocks

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

Cold Mortality Curves

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

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

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

Water Content

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

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

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

Cold Survival Under Altered Humidity

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

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

Unlike 6°C exposures, at -4°C the mortality curves are very steep and the transition from 0% to 100% mortality occurs rapidly. Because of this, we generated full mortality curves for each genotype at -4˚C for each humidity treatment. Each mortality curve was generated from five time points with data from 60 flies per time point (N=300 flies per mortality curve). All three humidity treatments for each genotype were run in parallel to control for laboratory conditions. Percent survival was scored in each vial after a three-day recovery and LT_{50} was estimated as described above. Adding desiccant at -4°C will decrease the amount of moisture in the air surrounding flies. Increasing the humidity via a moist paper towel presents more of a challenge due to the formation of ice. It is not possible to eliminate the vapor pressure deficit during supercooling because excess water in the air will form ice crystals on available surfaces. However, when the vapor pressure of water in the air is less than the vapor pressure of ice, there will be a net movement of water from the solid phase to the vapor phase. Under these conditions, the presence of ice will increase the amount of water in the air surrounding the fly and decreases the vapor pressure deficit between the insect and the air, relative to the control condition.

Statistical Analyses

Statistical analyses were performed with the R statistical package version 2.10.0 (R Development Core Team, 2008). Logistic regression models were fit using the glm function and post-hoc tests were performed using the TukeyHSD function. LT_{50} (median survival time) and 95% confidence intervals were calculated using the dose.p function from the MASS package (Venables and Ripley, 2002). Post-hoc tests for logistic regression models were performed using the glht function from the multcomp package (Hothorn et al., 2008).

Results

Cold Survival Across Temperatures and Genotypes

Both genotype and temperature had strong effects on survival (Table 1; Figs. 1,2). There were large differences in survival time across the range of temperatures measured; flies at -4°C survived on the order of minutes, with flies living for hours when exposed to 0-4°C and for days at 6°C. If flies experience all low temperatures as a single type of stress or injury, then genotypes with higher survival at one temperature should have higher survival at all temperatures. However, mortality curves generated for each genotype at each of six low temperatures (Fig. 1) revealed large genotypic effects on survival that changed in rank order across temperatures that differed by as little as 2°C (Fig. 2), indicative of temperature-dependent genetic effects (i.e. genotype-by-temperature interactions)(Table 1). The most cold-tolerant genotype at one temperature was not the most cold-tolerant genotype across all temperatures. For example, Canton-S had a significantly higher survival time than Berlin-K at -4°C (P<0.001), 2°C (P<0.001), and 4°C (P=0.004), while Berlin-K had a significantly higher survival time than Canton-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 tests). This suggests that the injury experienced by individual flies that differed in genotype was not the same across these low temperatures, and that different physiological and genetic mechanisms likely mediate cold tolerance across temperature differences as small as 2°C.

Water Content Following Cold Exposure

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

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

Cold Survival at High and Low Humidity

If flies are dying from desiccation during cold exposures, then altering the humidity during cold exposure should alter the rate of water loss and affect how long flies survive the cold. Given the patterns of water content described above, we predicted that altered humidity would affect survival at 6°C, where flies appear to be desiccated when dying, but not at -4°C, where flies appeared to be hydrated when dying. Consistent with these predictions, all genotypes had higher survival when humidity was increased and lower survival when humidity was decreased at 6°C (Fig. 4A). This pattern was reflected in the water contents of flies following the 6°C LT_{50} exposure; water contents were increased relative to controls after cold exposure at higher humidity and decreased relative to controls after cold exposure at lower humidity (Fig. 4B). This indicates that mortality is caused by desiccation at 6°C. However, desiccation does not contribute to mortality at -4°C. At -4°C, increasing humidity had little to no effect on survival, and when decreased humidity had a significant effect, it increased cold survival (Fig. 5). These results confirm that desiccation contributes to mortality at 6°C but not at -4°C. The temperature-dependent genetic effects that we have observed reflect physiological differences in the cause of death at low temperatures, and desiccation is one such cause.

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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691 Table 1. Logistic regression analysis of survival across temperatures

	d.f.	Deviance	Residual d.f.	Residual deviance	*P*
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

Figure Legends

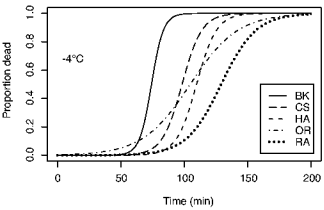
Figure 1. Fitted mortality curves for five genotypes at six exposure temperatures from -4°C to 6°C. Genotypes are Berlin-K (BK), Canton-S (CS), Hikone-A-S (HK), Oregon-R-C (OR), and Raleigh-208 (RA). Curves were fit using logistic regression with survival data from ~400 individual male flies of the same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

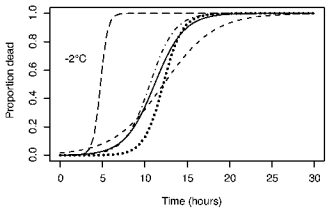
Figure 2. Genotype-by-temperature effects on cold survival. A. The LT_{50} (median survival time ± 95% CI) at each of six exposure temperatures from -4°C to 6°C was estimated from mortality curves for each of five genotypes: Berlin-K (BK), Canton-S (CS), Hikone-A-S (HK), Oregon-R-C (OR), and Raleigh-208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

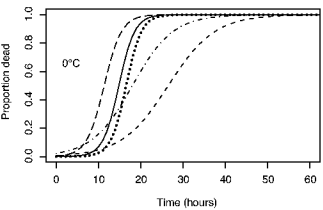
Figure 3. Water content of individual adults following LT_{50} exposures at each of six temperatures compared to desiccated and to hydrated control flies. Proportion of water (mean ± SE) was measured by subtracting dry weight from wet weight and dividing by wet weight. Bars labeled A were not significantly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

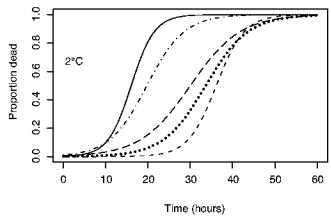
Figure 4. Effects of altered humidity on survival and water content at 6°C. A. The proportion (mean ± SE) of flies surviving a 6°C LT_{50} in either dry, control or hydrated conditions. B. The water content (mean ± SE) of flies surviving a 6°C LT_{50} in either dry, control or hydrated conditions. Tukey's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

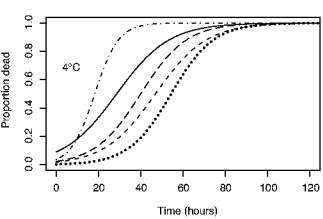
Figure 5. Effects of altered humidity on survival and water content at -4°C. The LT_{50} (median survival time ± 95% CI) was estimated for all five genotypes at -4°C with decreased (dry), control and increased (wet) humidities. Tukey's post-hoc tests, * $P<0.05$, ** $P<0.01$, *** $P<0.001$

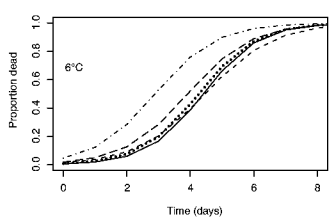

-4°C
BK
CS
HA
OR
RA
Proportion dead
Time (min)

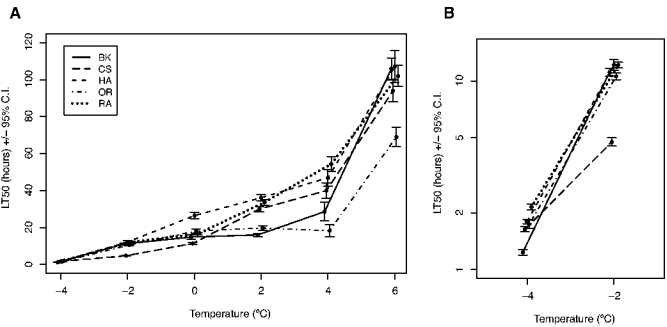

-2°C
Proportion dead
Time (hours)

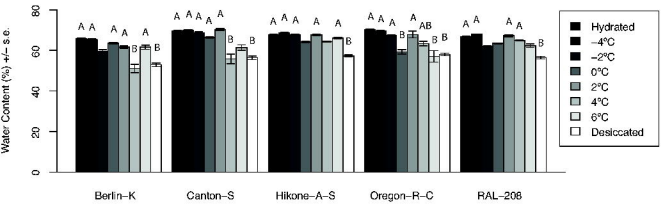

0°C
Proportion dead
Time (hours)

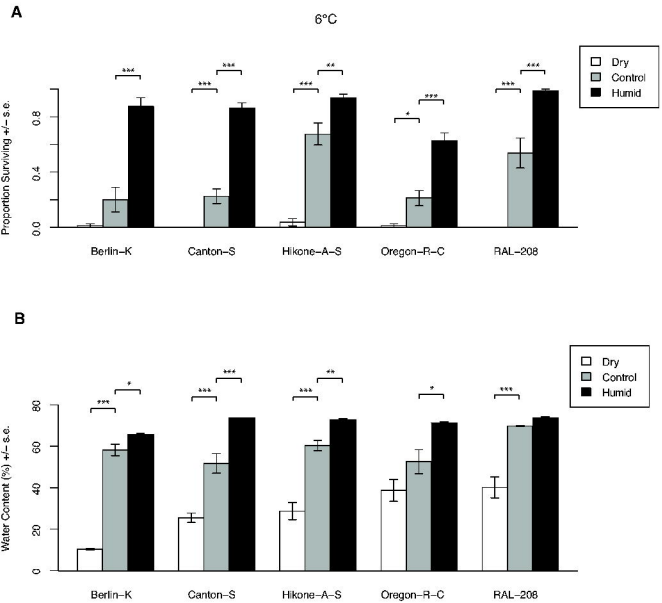

2°C
Proportion dead
Time (hours)


4°C
Proportion dead
Time (hours)


6°C
Proportion dead
Time (days)


A
B
BK
CS
HA
OR
RA
LT50 (hours) +/- 95% C.I.
Temperature (°C)
0
20
40
60
80
100
120
−4
−2
0
2
4
6
1
2
5
10


Water Content (%) +/− s.e.
0
20
40
60
80
A A
A A B A B
A A A A A B B
A A A A A
A A A A AB B B
A A A A B
Berlin−K
Canton−S
Hikone−A−S
Oregon−R−C
RAL−208
Hydrated
−4°C
−2°C
0°C
2°C
4°C
6°C
Desiccated
A: Similar to hydrated (P>0.05)
B: Similar to desiccated (P>0.05)


A
6°C
Dry
Control
Humid
Proportion Surviving +/− s.e.
0.0
0.4
0.8
***
***
***
**
*
***
***
***
Berlin−K
Canton−S
Hikone−A−S
Oregon−R−C
RAL−208
B
Dry
Control
Humid
Water Content (%) +/− s.e.
0
20
40
60
80
***
*
***
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***
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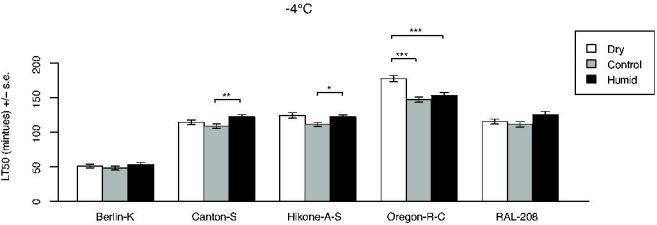
*
***
Berlin−K
Canton−S
Hikone−A−S
Oregon−R−C
RAL−208


-4°C
LT50 (minutes) +/- s.e.
0
50
100
150
200
**
*
***
***
Dry
Control
Humid
Berlin-K
Canton-S
Hikone-A-S
Oregon-R-C
RAL-208