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Adaptation to low temperature exposure increases metabolic rates independently of growth rates

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Abstract

Metabolic cold adaptation is a pattern where ectotherms from cold, high-latitude or -altitudes habitats have higher metabolic rates than ectotherms from warmer habitats. When found, metabolic cold adaptation is often attributed to countergradient selection, wherein short, cool growing seasons select for a compensatory increase in growth rates and development times of ectotherms. Yet, ectotherms in high-latitude and –altitude environments face many challenges in addition to thermal and time constraints on lifecycles. In addition to short, cool growing seasons, high-latitude and –altitude environments are characterized by regular exposure to extreme low temperatures, which cause ectotherms to enter a transient state of immobility termed chill coma. The ability to resume activity quickly after chill coma increases with latitude and altitude in patterns consistent with local adaptation to cold conditions. We show that artificial selection for fast and slow chill coma recovery among lines of the fly *Drosophila melanogaster* also affects rates of respiratory metabolism. Cold-hardy fly lines with fast recovery from chill coma had higher respiratory metabolic rates than control lines, with cold-susceptible slow-recovering lines having the lowest metabolic rates. Fast chill coma recovery was also associated with higher respiratory metabolism in a set of lines derived from a natural population. Although their metabolic rates were higher than control lines, fast-recovering cold-hardy lines did not have faster growth rates or development times than control lines. This suggests that raised metabolic rates in high-latitude and altitude species may be driven by adaptation to extreme low temperatures, illustrating the importance of moving “Beyond the Mean”.

Introduction

Metabolic cold adaptation describes a macrophysiological pattern whereby respiratory estimates of metabolic rates of ectotherms from cold environments, typically from high latitudes or altitudes, tend to be elevated relative to those from warm environments (Addo-Bediako and others 2002). Metabolic cold adaptation has been an important and hotly debated topic in ecological and evolutionary physiology for nearly a century (Bullock 1955; Clarke 1993; Fox 1936). Although there are numerous examples of clinal variation in metabolic rate both within and across species that are consistent with metabolic cold adaptation, particularly in terrestrial arthropods and fish (Addo-Bediako and others 2002; Ayres and Scriber 1994; Block and Young 1978; Chappell ; Terblanche and others 2009; Torres and Somero ; White and others 2012; Wohlschlag 1960), other studies find no association between habitat temperature and metabolic rates (Clarke 1993; Clarke and Johnston 1999; Lardies and others 2004; Steffensen 2002). Our purpose in this manuscript is not to assess whether metabolic cold adaptation is a general macrophysiological rule. Although it is not always found, the pattern is general enough that it merits consideration. Rather, our goal is to discuss some of the ultimate, selective mechanisms that may drive metabolic cold adaptation in the context of habitats with high thermal variation, such as those at high latitudes and altitudes (Sunday and others 2011).

When a pattern consistent with metabolic cold adaptation is found, it is most often ascribed to another macrophysiological rule, countergradient variation (Conover and others 2009). Countergradient variation describes a pattern of local adaptation in organismal life histories wherein geographic variation in genotypes counteracts environmental influences, reducing phenotypic variation along an environmental gradient (Conover and Schultz 1995; Levins 1968). In the context of metabolic cold adaptation, this implies that body size and

generation time are preserved along latitudinal or altitudinal thermal gradients, through elevation of rates of growth and development to counter the slowing effects of cool temperatures and short seasons. Countergradient variation is well-enough supported by both intraspecific and interspecific comparisons in terrestrial arthropods, fish, and amphibians to be considered a general macrophysiological rule (Gaston and others 2009; Gotthard and others 2000; Laugen and others 2003; Schultz and others 1996).

Both within and between species, fast growth rates are often correlated with higher rates of respiratory metabolism, presumably due to greater rates of both anabolic and catabolic intermediary metabolism needed to support fast growth (Arendt 1997; Glazier 2015; Metcalfe and Monaghan 2001; Stoks and others 2006). Because of this correlation between metabolic rates and growth rates, latitudinal/altitudinal local adaptation studies that show a pattern consistent with metabolic cold adaptation tend to attribute that pattern to countergradient selection on growth rates and development time (Addo-Bediako and others 2002; Ayres and Scriber 1994; Conover and Schultz 1995; White and others 2012). However, local adaptation to high latitude and altitude sites with high seasonal and thermal variation could entail selection on the ability to be hardy to many stresses beyond time limitation for growth and reproduction. Organisms must frequently balance multiple abiotic and biotic challenges (Sokolova and Pörtner 2007; Todgham and Stillman 2013), thus we expect that local adaptation at high-latitudes and -altitudes will be the product of multifarious selection acting to mitigate both short, cool growing seasons and stressful thermal extremes; necessitating that our conceptual framework for metabolic cold adaptation “moves beyond the mean” to consider the ecological, evolutionary, and mechanistic consequences of thermal variability.

When challenged by temperatures below their critical thermal minimum for movement, many ectotherms lose neuromuscular coordination and enter into a state termed chill coma (David and others 2003; MacMillan and Sinclair 2011). If the duration of low temperature is sufficiently brief (usually less than 12 hours), organisms will typically recover fully coordinated movement shortly after returning to warmer temperatures and ultimately continue critical processes like growth and reproduction (MacMillan and Sinclair 2011). The time taken to recover neuromuscular coordination and resume activity after rewarming is termed the chill coma recovery time. Among ectotherms, chill coma and recovery from chill coma have been best studied in terrestrial insects. Chill coma recovery time is ecologically relevant because the time spent recovering from chill coma represents lost opportunities for foraging, dispersal, mating and reproduction; and increased vulnerability to predation, parasitism, and environmental stress (due to loss of ability to behaviorally avoid inhospitable conditions) (David and others 2003; MacMillan and Sinclair 2011). Reinforcing this view, chill coma recovery time shows clear patterns of local adaptation in many terrestrial arthropods wherein populations or species from high-latitudes and -altitudes have faster chill coma recovery time than those from more thermally stable environments (David and others 2003; Sinclair and others 2012). The rationale for this pattern is that insects living in high-latitude and -altitude sites experience greater thermal variability and may frequently enter into chill coma when temperatures fall below critical limits for activity either overnight or as cold fronts sweep in, and then recover from chill coma as temperatures warm again during daytime or as cold fronts dissipate.

A series of quantitative trait locus (QTL), experimental evolution, and genome-wide association studies (GWAS) have shown that chill coma recovery is a strongly heritable quantitative trait underlain by many genomic regions, each with a relatively small effect

(MacKay and others 2012; Morgan and MacKay 2006; Norry and others 2007; Williams and others 2014). From a physiological perspective, insects lose ionic and osmotic homeostasis during chill coma and whole-organism recovery of coordinated movement is correlated with the restoration of ionic and osmotic gradients (Andersen and others 2013; Findsen and others 2013; MacMillan and others 2015; MacMillan and Sinclair 2011; MacMillan and others 2012). Reestablishing ionic and osmotic gradients, and eventually restoration of coordinated movement, is energetically intensive requiring both the availability of ATP and the ability of downstream intermediary metabolic processes to use that ATP to recover homeostasis. Several studies have shown that cold exposure disrupts intermediary metabolism (Koštál and others 2011; Lalouette and others 2007; Michaud and Denlinger 2007) and that low-temperature acclimation can reduce the magnitude of cold-induced perturbations to intermediary metabolism (Colinet and others 2012; Overgaard and others 2007). We have also recently shown that cold adaptation via artificial selection for fast chill coma recovery time in the fly *Drosophila melanogaster* increased the robustness of biochemical networks of intermediary metabolites, permitting enhanced maintenance of metabolic processes during cold exposure and recovery (Williams and others 2014). Given that cold disrupts intermediary metabolism, and that plastic and evolutionary responses to cold enhance the ability to maintain metabolic processes in the cold, could selection for cold-hardiness in highly thermally variable habitats drive the evolution of respiratory metabolic rate in a pattern that is consistent with metabolic cold adaptation, independent of selection on growth rates and development time?

Here we tested whether fast recovery from chill coma was associated with higher respiratory metabolic rates across two complementary sets of lines of the fly *D. melanogaster*, both derived from a mid-latitude site in Raleigh, North Carolina USA that experiences

substantial seasonal thermal variation. The first set was a series of isogenic lines that represent naturally segregating variation in chill coma recovery time (*Drosophila melanogaster* Genetic Reference Panel; MacKay and others 2012). The second was a set of replicated experimental evolution lines artificially selected for fast or slow chill coma recovery time, as well as unselected control lines. We tested whether chill-coma recovery time was associated with a shift in the relationship between temperature and metabolic rate by testing lines across a series of temperatures from very low (0 ºC) to relatively high (25 ºC). Across both sets of lines, fast chill coma recovery was associated with higher respiratory metabolism at temperatures above 15 ºC. Using the artificial evolution lines, we further tested whether lines selected for fast chill coma recovery time that had higher respiratory metabolic rates, also had higher growth rates and shorter development times. Although lines selected for fast chill coma recovery had higher respiratory metabolism than either control or slow-recovering lines, cold-hardy fast-recovering lines did not have higher growth rates or development times than control lines. Because chill coma recovery time is a cold-hardiness trait expected to be a target of selection in habitats with high thermal variation, we propose that selection on cold-hardiness could drive patterns of respiratory metabolism that are consistent with metabolic cold adaptation independent of countergradient variation in life history traits.

Methods

Fly stocks

We used two complementary genetic resources, both originating from a natural population in Raleigh, North Carolina, USA. The first was the *Drosophila melanogaster* genetic reference panel (MacKay and others 2012); a fully genotyped panel of inbred lines representing

genetic variation segregating within the population at the time of founding the lines. We chose 6 lines from either tail of the distribution of chill coma recovery times, representing naturally occurring combinations of alleles associated with extreme cold-hardiness and -susceptibility. The second genetic resource was a set of replicate experimental evolution lines derived from the same base population, and selected in the laboratory for fast versus slow recovery from chill coma (Williams and others 2014). The selection process generated substantial and genetically fixed differences in chill coma recovery times, with hardy lines recovering after an average of 6.1 and 5.8 min (replicate lines 1 and 2), compared to 12.4 and 23.7min for susceptible flies (Williams and others 2014). All flies were reared on standard cornmeal-agar-molasses medium under controlled density as previously described (Williams and others 2014).

Respirometry

For respirometry experiments, >1-day-old female flies were sorted into groups of 20 under light CO_2 anaesthesia and then left to recover for 2-4 days. All experiments were performed on 5-8 day old mated females, reared at 25 °C. We used a Sable Systems International (SSI) respirometry system (Las Vegas, NV, USA) with an Oxzilla II oxygen (O_2) analyzer (SSI) and a LiCor 7000 carbon dioxide (CO_2) analyzer (Lincoln, NE, USA). We collected data using a UI2 interface (SSI) at a frequency of 1 Hz. Incurrent air was scrubbed of water vapor and CO_2 using a drierite-ascarite-drierite column, excurrent gas was scrubbed of water vapor before entering the CO_2 analyzer using a magnesium perchlorate column, and CO_2 and water vapor were scrubbed before the O_2 analyzer using an ascarite-magnesium perchlorate column. We corrected data to a reading taken through an empty baseline chamber at the beginning and end of each recording to correct for instrument drift.

Thermal performance curves for metabolic rate

We used stop-flow respirometry to measure $\dot{V}CO_2$ and $\dot{V}O_2$ as estimates of metabolic rate in groups of 10 female flies at 0, 15, 20 and 25 °C, with each group of flies measured only once at one temperature (Supplementary Material). Respiratory exchange ratios were calculated as the ratio of $\dot{V}CO_2$:$\dot{V}O_2$. Differences in activity levels among lines could confound metabolic rate estimates, so we estimated minimum costs of transport for each line in separate experiments (Supplementary Material), and subtracted these costs from metabolic rate estimates to ensure that activity was not driving the patterns we saw. We did not measure activity at 0 °C because all flies are in chill coma (inactive) within a few minutes of exposure to 0 °C. After each measurement, groups of flies were frozen and weighed to 0.01 µg using a microbalance.

All statistical analyses were performed in R 3.2.2 (R Core Team 2015). Preliminary data exploration was performed as recommended by Zuur *et al.* (2010). $\dot{V}O_2$ was $\log_{10}$-transformed to improve normality. We fit general linear mixed models describing $\dot{V}O_2$ as a function of temperature, natural logarithm of temperature, and temperature as a 2^{nd} or 3^{rd} order polynomial, and compared the fit using AIC (where $\Delta AIC > 2$ is justification for preferring a more complex model). After the most parsimonius form of temperature was determined, we added random effects of syringe, run, and replicate population nested within cold hardiness, plus nested permutations of these random effects. We ascertained which combination of random effects was most parsimonius using AIC as above. We then fit additional fixed effects of cold hardinessand the interaction between selection and temperature (assessing whether the thermal sensitivity of metabolic rate differed among lines), with mass as a covariate. We simplified the model (including pooling factor levels) using AIC as above to determine the minimal adequate model (Crawley 2007).

Metabolic rate of individual flies during cold exposure and recovery

We used open-flow respirometry to measure VCO_2 as an estimate of metabolic rates in individual *D. melanogaster* continuously during cold exposure and recovery using open-flow respirometry (Supplementary material, Fig. S1). It is not possible to measure VO_2 in individual flies due to the relatively low sensitivity of oxygen compared to carbon dioxide analyzers. From these data, we estimated metabolic rates at the following time points: 1) Before cold exposure (25 °C); 2) During cold exposure (0 °C); 3) Rewarming, the time taken for the temperature to completely equilibrate to 25 °C (~20 min); 4) Early Recovery, the first 1.5h after temperature had equilibrated; and 5) Late Recovery, the final 1.5h of 4h recovery. These time points are indicated on Fig. S1 (1-5 along top axis). For each time period, metabolic rates for each individual were averaged and analyzed using mixed general linear models as described above (*Thermal performance curves for metabolic rate*), except that size (thorax length; Supplementary Material) was measured using a dissecting microscope with eye piece reticule and used as a covariate instead of mass. To characterize variability in metabolic rates over time, we calculated rolling standard deviations on 1500 second windows for each individual (zoo package; Zeileis and Grothendieck 2005). The size of the window is shown in the black box in Fig. S1. These rolling standard deviations were averaged over the early and late recovery time points as previously described, and analyzed using the same linear model framework.

Growth rates and development time

Eggs were collected from population cages of each line using grape juice agar plates with a thin coat of liquid yeast. Plates were left for two hours in the cages and then immediately submerged in 70% ethanol to stop egg development and stored in a 4°C chamber. Groups of 100 eggs were dried and weighed (Cahn C-35 microbalance (±1 μg), Orion Research, Inc., Boston, MA) to obtain the average egg dry mass for each line, which was used as a starting mass to calculate

growth rates. Groups of 30 eggs from each line of flies (collected from the growth medium) were each placed into 5 replicate vials for each temperature treatment, and were checked for pupation or adult emergence at 5pm each day. Date of pupation or adult emergence was recorded and used to calculate pupal and adult development time. Adult flies were collected the day they emerged and frozen at -20°C. After all flies had emerged, they were dried individually at 60°C for 48 hours and a random subset of the population of six females per replicate was weighed. The use of the random subset was required due to the large population size of the experiment. Growth rates were obtained for each line by calculating the difference between adult and egg dry mass divided by the total development time. Growth rate data were analyzed using general linear mixed models as described above (*Thermal performance curves for metabolic rate*), with starting mass of eggs as a covariate.

Results

Thermal performance curves for metabolic rate

Cold-hardy and cold-susceptible flies did not differ in mass when reared at 25 °C (normal rearing conditions for all flies used in respirometry experiments) ($p>0.1$, Fig. 1A-B). In flies from all lines, $\dot{V}O_2$ increased with temperature ($F_{2,45}=371.7$, $p < 0.0001$), with the relationship best described by a 2nd degree polynomial (Fig. 1C-D). In the experimental evolution lines, $\dot{V}O_2$ was more thermally sensitive in cold-hardy flies, with higher $\dot{V}O_2$ at warm temperatures but lower $\dot{V}O_2$ at 0 °C in cold-hardy compared to -susceptible flies, and control flies were intermediate (hardiness × temperature: $F_{4,98}=10.6$, $p < 0.0001$, Fig. 1C). At 0 °C, metabolic rates were lowest in cold-hardy flies and highest in -susceptible, with controls intermediate (Fig. 1E). The mass-scaling of metabolic rate varied as a function of temperature: respiration rates increased with mass at warm temperatures, but showed progressively shallower scaling exponents as

temperature decreased, being completely mass-independent at 0 °C (mass × temperature: $F_{2,98}$=21.4, p < 0.0001; Fig. S2A). We replicated these patterns using lines of flies originating from the DGRP: hardy flies had higher thermal sensitivity of $\dot{V}O_2$, with higher $\dot{V}O_2$ than susceptible lines at warm temperatures, but both hardy and susceptible lines had similar $\dot{V}O_2$ in the cold (hardiness × temperature: $F_{2,136}$=3.0, p =0.05, Fig. 1D,F). Mass scaling exponents for metabolic rate again decreased as a function of temperature, parallel to the pattern seen in the experimental evolution lines (mass × temperature: $F_{2,136}$=5.5, p = 0.005; Fig. S2B). Respiratory exchange ratios (RERs) were extremely variable at 0 °C, and ranged from 0.3 – 1.3 (Fig. S3A). Across the warmer temperatures (15-25 °C), RERs decreased with increasing temperature (Experimental Evolution lines: $F_{1,19}$=7.9, p=0.01, Fig. S3B; DGRP: $F_{1,35}$=12.1, p=0.001, Fig. S3C), with values centered around 1 at 25 °C but increasing to a mean of 1.1 by 15 °C. For the Experimental Evolution lines, hardy flies had lower RERs than control and susceptible (which were pooled in the best model; $F_{1,82}$=8.9, p=0.004), while by contrast in the DGRP hardy flies had higher RER than susceptible flies ($F_{1,108}$=6.9, p=0.001).

To rule out differential activity as a potential driver of the observed differences in metabolic rate, we estimated the distance walked by each line of flies during a typical respirometry recording, and used these data to calculate the average metabolic costs of transport (Supplementary Material). In the experimental evolution lines, hardy flies had similar costs of transport to control flies, while susceptible flies had reduced costs of transport (mostly due to reduced activity levels, Fig. S4). In the DGRP, hardy and susceptible flies had similar costs of transport at 20-25 °C, but lower costs of transport at 15 °C. Thus, in neither set of lines was increased activity of hardy lines driving the increased metabolic rates of cold hardy flies. To confirm this, we subtracted the estimated costs of transport from measured metabolic rates to estimate metabolic costs that were

independent of activity (dotted lines in Fig. 1C-D), and reanalyzed these activity-corrected data. Analysis of activity-corrected $\dot{V}O_2$, or raw or activity-corrected $\dot{V}CO_2$, gave identical conclusions for all analyses, confirming that the pattern of higher metabolic rates in cold hardy flies was not driven by higher activity levels in these lines (Supplementary material, Fig. S4).

Metabolic rate during cold exposure and recovery

Metabolic rates of all flies decreased abruptly during cold exposure, then gradually recovered back towards pre-cold rates, but in most cases did not fully recover to before cold levels (e.g. Fig. S1). Hardy flies had a distinct trajectory of metabolic rates during cold exposure and recovery compared to susceptible flies: we replicated our finding of higher metabolic rates before cold, dropping to lower rates during cold, and recovering to higher levels than did susceptible flies during recovery (cold hardiness × time: $F_{8,101} = 2.3$, $p = 0.033$; Fig. 2A). Standardizing metabolic rates of each individual to the average "Before cold" metabolic rate for that individual confirmed that hardy flies dropped their metabolic rates to a greater degree during cold exposure, and additionally revealed that although absolute metabolic rates were higher in hardy than susceptible flies during recovery, the metabolic rates of hardy flies remained suppressed relative to their "before cold" rates during recovery, while susceptible flies returned to close to their before cold rates by the late recovery phase (cold hardiness × time: $F_{6,80} = 3.0$, $p = 0.013$; Fig. 2B). Many of the flies showed a distinctly cyclic pattern of respiration that started during either the early or late recovery phases (Fig. S5), and the onset of this pattern was independent of the onset of activity (for example see Fig. S1). There was no significant difference between hardy and susceptible flies in the variance of $\dot{V}CO_2$ during the early or late recovery period ($F_{2,40} = 3.3$, $p = 0.095$), although it is possible that we lacked power to detect an effect were it there.

Growth rates and development times

Growth rates increased with increasing temperature, and were similar among lines at the normal laboratory rearing temperature (25 °C; Fig. 3). On either side of that temperature, chill-susceptible flies had lower growth rates than hardy and control flies, which were statistically indistinguishable (rearing temperature × cold hardiness [hardy and control pooled]: $F_{3,678}$= 8.6, p <0.0001, Fig. 3). The slower growth rates of susceptible flies resulted from longer development times, while development time did not differ between hardy and control lines (Supplementary material, Fig. S6).

Discussion

Here, we show that lines of *Drosophila melanogaster* flies with faster recovery from chill coma, a common metric of cold hardiness, also have higher respiratory metabolism at warm temperatures permissive for activity (15-25 ºC). We demonstrated this pattern in both a set of experimental evolution lines artificially selected for fast or slow recovery from chill coma (compared to unselected controls), and also a series of lines derived from the *Drosophila melanogaster* Genetic Reference Panel. Both sets of lines were originally derived from a mid-latitude population that represent naturally segregating genetic variation for many traits, including cold hardiness (MacKay and others 2012). Chill coma recovery time shows clear patterns of local adaptation across terrestrial arthropod species, wherein populations from high latitude and high altitude sites show faster recovery from chill coma than populations from less cold and thermally variable habitats (David and others 2003; Sinclair and others 2012). Therefore, selection on the ability to recover activity after cold knockdown at high latitudes and

altitudes may drive higher respiratory metabolism and thus contribute to the pattern of metabolic cold adaptation in terrestrial arthropods.

Interestingly, while cold-hardy, fast-recovering lines had higher respiratory metabolic rates than control and susceptible lines at warm temperatures permissive for fly activity (15-25 ºC), respiratory metabolism dropped to a greater degree upon cold exposure in cold-hardy compared to control or susceptible lines. Artificial selection for cold-hardiness via fast chill coma recovery thus altered the relationship between temperature and metabolic rate, wherein metabolic rates increased more quickly with temperature in hardy lines than control lines, followed by cold-susceptible lines. Similarly, fast-recovering, cold-hardy lines from the *Drosophila* Genetic Reference Panel also had a higher thermal sensitivity of respiratory metabolism than cold-susceptible lines. Although most studies of metabolic cold adaptation in insects only assess metabolism at one or two shared temperatures (Addo-Bediako and others 2002), our observations are consistent with studies that have found intraspecific and interspecific variation in metabolic rate-temperature relationships associated with living in cold habitats (Chappell 1983; Terblanche and others 2009). Our work differs from other studies on metabolic rate-temperature relationships because we include both metabolic rates at relatively high temperatures where flies can be active (15-25 ºC) and a stressful low temperature that is below the threshold for activity (0 ºC). Despite the difficulty of quantifying metabolic rates of small insects at low temperatures, we included this measure at 0 ºC specifically because we think it critical to understand how selection during the cold exposure may act on metabolic rate.

When considering patterns of respiratory metabolism during adaptation to cold stress, selection could alter metabolism at three different points: before cold exposure so that animals have been selected to be better prepared to resist cold stress, during cold exposure, and during

recovery from cold so that animals are better able to tolerate perturbation from cold stress (Williams and others 2014). By following respiratory metabolism across each of these time points, we showed that cold-hardy lines have greater metabolic plasticity than control or susceptible lines. Specifically, cold-hardy lines have higher respiratory metabolism before the cold but they have lower respiratory metabolism at 0 ºC than control or susceptible lines, suggesting greater metabolic plasticity in response to cold stress. During recovery from cold stress, the respiratory metabolic rates of cold-hardy lines increased again faster than control or susceptible lines, but even after 4 h of recovery hardy lines did not completely recover to their pre-cold respiratory metabolic rates whereas control and susceptible lines did return to near their pre-stress metabolic rates. This suggests that metabolic suppression was present during recovery from cold in hardy flies. This is in line with previous findings that plastic responses to cold exposure do not necessarily increase metabolic costs in chill-susceptible insects (Basson and others 2012; but see MacMillan and others 2012)

The potential benefits of increased metabolic plasticity during adaptation to cold stress become clear when considering the mechanisms that may underlie chill coma and recovery responses. During chill coma insects accumulate osmotic, ionic, and metabolic imbalances that increase in severity with duration spent in chill coma (Andersen and others 2013; Findsen and others 2013; MacMillan and others 2015; MacMillan and Sinclair 2011). Metabolic, ionic, and osmotic homeostasis must be reestablished during recovery from chill coma, along with repair of other types of cellular damage that may accrue with time at low temperature. Reestablishing homeostasis and repairing cold-induced damage is energetically expensive from both the perspectives of energetic currency to do work and anabolic substrates to effect repairs (MacMillan and others 2012). Given that respiratory rates reflect rates of intermediary

metabolism, in this context higher metabolic rates before and after cold stress may support greater substrate flux through critical pathways of intermediary metabolism at warm temperatures to help individuals better resist cold-induced perturbations to metabolism and to recover metabolic homeostasis and repair damage more quickly after cold perturbation. Reduction of intermediary metabolism, and particularly aerobic catabolism, is a common response to many stressors beyond cold (Guppy and Withers 1999; Storey and Storey 1990). The ability of cold-hardy lines to reduce intermediary metabolism during cold stress to a greater degree than control or susceptible lines may help to reduce the magnitude of metabolic perturbation during cold exposure, thus leaving cold hardy flies better prepared to recover homeostasis. Indeed, allied work on these same experimental evolution lines has found that cold-hardy lines suffer less cold-induced perturbations in their levels of metabolites than susceptible lines (Williams and others 2014), and that this is associated with higher rates of catabolism and anabolism (Williams and others submitted). Unfortunately, the literature on metabolic cold adaptation in terrestrial arthropods is currently lacking in studies that include metabolic responses at all three times (before, during, and after cold stress), although several studies measure metabolic rates either during or after cold stress (Basson and others 2012; Stevens and others 2010). We do not know whether the pattern of increased metabolic plasticity we observed here is a general aspect of metabolic cold adaptation, but we encourage other authors to include time series data of respiratory metabolism across a thermal perturbation when considering metabolic adaptation to cold stress.

Respiratory exchange ratios (RERs) were close to 1 at room temperature, suggesting that flies were primarily relying on carbohydrate metabolism (Lighton 2008). RERs increased with decreasing temperature in both sets of lines, which may indicate an increasing predominance of

anabolic relative to catabolic processes as temperatures cool (Lighton 2008). At low temperatures, RERs were extremely variable. For these experiments we used stop-flow respirometry, which allows gas to build up and then be injected into the system in a bolus, thus low oxygen concentrations are not a possible explanation for this variability at 0 °C. Inspection of $\dot{V}CO_2$ and $\dot{V}O_2$ at 0 °C show similar levels of variation, further supporting that oxygen measurements had sufficient resolution. It is thus possible that the highly variable RERs at 0 °C reflect true biological differences in the response to low temperatures, but this requires further investigation. Differences in RER between hardy and susceptible flies were inconsistent across the Experimental Evolution lines and the DGRP, so we conclude that RER does not seem to be related to cold hardiness in these lines.

One of the caveats for using experimental evolution approaches in the laboratory to study mechanisms of adaptation in the field is that one may inadvertently artificially select for a trait that they did not intend (Gibbs 1999). Is it possible that we inadvertently artificially selected for high levels of activity that are driving the observed patterns of respiratory metabolism rather than cold tolerance? We quantified activity, distance walked, and walking speed in flies from each of the experimental evolution lines and the naturally derived lines and used these data to estimate costs of transport (Supplementary Material). These data rule out increased activity levels as a driver of increased metabolic rates in hardy flies. Among our experimental evolution lines, the cold-hardy lines did not differ from the control lines in costs of transport, although the susceptible lines did have reduced costs of transport due to lower activity level (Fig. S3). Thus, the higher respiratory metabolic rates we observed in hardy vs. control lines at temperatures permissive for movement were not driven by activity. In the DGRP, hardy flies had lower activity at cool temperatures; the opposite to the pattern we saw for metabolic rate. We are

confident that our results represent a specific response of increased respiratory metabolism to selection for hardiness to cold stress that is consistent with patterns of metabolic cold adaptation.

Abundant nutrient availability in laboratory selection studies can also ameliorate life history trade-offs that may prevent the evolution of phenotypes in nature – therefore in the wild, hardy flies with increased metabolic demands may experience decreased ability to invest in other fitness components such as reproduction or somatic maintenance (Zera and Harshman 2001). In this sense, laboratory selection experiments indicate what can happen in response to a selective pressure, but not necessarily what will happen in the wild (Gibbs 1999). We did not assay other measures of fitness such as fecundity or viability – it is entirely possible that these fitness measures may be reduced in cold hardy flies, indicating a functional trade-off between cold hardiness and reproduction. Alternately, the cost may come in the form of increased nutrient requirements, requiring longer foraging periods or increased efficiency of digestion, absorption, or catabolism.

Because fast growth rates are correlated with higher rates of respiratory metabolism (Arendt 1997; Glazier 2015; Metcalfe and Monaghan 2001; Stoks and others 2006), higher rates of respiratory metabolism in terrestrial arthropods from high latitudes and altitudes are often attributed to countergradient selection for fast growth during short, cool growing seasons (Ayres and Scriber 1994; Gotthard and others 2000; Laugen and others 2003; Schultz and others 1996). We tested whether our selection regime for cold hardiness that increased respiratory metabolism also produced correlated changes in growth rates and development time. Neither growth rates nor development time differed between cold-hardy and control lines, although cold-susceptible lines had lower growth rates that were attributable to longer development times at all temperatures other than 25 ºC (Fig. 3). Countergradient selection on life histories with latitude and altitude is a

common enough pattern in ectotherms to be considered a general macroecological rule (Conover and others 2009; Gaston and others 2009). We agree that selection for fast growth rates via countergradient selection is a potentially important force that could drive patterns of metabolic cold adaptation in terrestrial arthropods (Addo-Bediako and others 2002; White and Kearney 2013). However, our work shows that artificial selection for cold-hardiness via fast recovery from chill coma can also drive the evolution of higher rates of respiratory metabolism independent of either growth rates or activity. Because chill coma recovery time shows latitudinal clines consistent with local adaptation within and between *Drosophila* species and other widely distributed terrestrial arthropods (David and others 2003; Sinclair and others 2012), we propose that natural selection on rapid recovery from chill coma recovery, and perhaps other energetically costly aspects of cold hardiness, could also be an important force shaping patterns of metabolic cold adaptation. Acclimatization in response to low mean temperatures at high-latitudes or -altitudes could augment this elevation of metabolic rate (e.g. Berrigan and Partridge 1997; Terblanche and others 2005).

Conclusions

High-latitude and high-altitude environments are characterized by shorter, cooler growing seasons and greater thermal variability (Sunday and others 2011). Understanding patterns of local adaptation to these environments, and the potential for climate change to impact organisms in these environments will necessitate “moving beyond the mean” to consider how selection imposed by thermal variation may affect organisms. Here we show that selection imposed by exposure to and recovery from an extreme low temperature event can shape respiratory metabolism in a manner consistent with patterns of metabolic cold adaptation, without altering either activity or growth rates. This work suggests an alternative selective pressure that could

lead to higher respiratory metabolism in high-latitude and high-altitude environments and emphasizes the importance of considering the multifarious nature of selection that can be experienced by organisms across the entire lifecycle.

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Data availaibility

Data will be deposited in Dryad upon acceptance.

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Figures

Figure 1 – Size and metabolic rates of female *Drosophila melanogaster* from Experimental Evolution lines (A,C,E) or the Drosophila melanogaster Genetic Reference Panel (DGRP; B,D,F). For all panels, N = 5-7/line/temperature, with 6 (DGRP) or 2 (Experimental Evolution) replicate lines of flies for each level of cold hardiness. A-B) Boxplots showing mass of lines that vary in cold hardiness. C-D) Oxygen consumption of cold-hardy (black triangles), control (dark grey circles) or cold-susceptible (light grey squares) female flies. Dotted lines indicate remaining oxygen consumption when the estimated cost of walking was removed. Values are mean ± SEM. E-F) Magnification of metabolic rates at 0 °C. . Significant effects are from general linear models described in results, main effects are not given where there is a significant higher-order interaction term.

Figure 2 – CO_2 production (as a proxy for metabolic rate) of cold-hardy (white), control (light grey), or cold-susceptible (dark grey) or individual female *Drosophila melanogaster* during cold exposure and recovery. A) CO_2 production rates and B) change in CO_2 production rates compared to the same individual before cold exposure. All time points are relative to a 3h cold exposure at 0 °C; Early = first 2h of recovery period, Late = subsequent 2h of recovery period (see Fig. S1 for time periods). N= 8 for each box (two replicate selection lines pooled for display purposes, but accounted for in models, see text). Significant effects are from general linear models described in results, main effects are not given where there is a significant higher-order interaction term.

Figure 3 – Growth rates of cold-hardy (black), -susceptible (light grey) and control (dark grey) female flies, from an experimental evolution experiment. N ≥ 5 replicate vials of 30 eggs/vial/line, resulting in ≥ 30 surviving adults for each line. Values are mean ±

SEM of two replicate experimental evolution lines for each level of cold hardiness (accounted for in models as a random effect). Significant effects are from general linear models described in results, main effects are not given where there is a significant higher-order interaction term.