Artificial Selection on Chill-Coma Recovery Time in *Drosophila melanogaster*:

Direct and correlated responses to selection

Alison R. Gerken^{1#}, Trudy F.C. Mackay², and Theodore J. Morgan^{1*}

¹Division of Biology and The Ecological Genomics Institute, Kansas State University, Manhattan, KS, 66506 USA.

²Department of Biological Sciences, Program in Genetics and W. M. Keck Center for Behavioral Biology, North Carolina State University Raleigh, NC 27695. USA.

[#]Present Address: College of Medicine, Department of Molecular Genetics and Microbiology, University of Florida, Gainesville, FL, USA

*Corresponding author: Theodore J. Morgan; e-mail: tjmorgan@ksu.edu

Abstract

Artificial selection can be used to create populations with extreme phenotypic responses to environmental stressors. When artificial selection is applied to a single component of a stress response, this selection may result in correlated responses in other stress responses, a phenomenon called cross-tolerance, which is ultimately controlled by the genetic correlations among traits. We selected for extreme responses to cold tolerance by selecting for chill-coma recovery time from a single temperate population of *Drosophila melanogaster*. Chill-coma recovery time is a common metric of low, but non-lethal, cold temperature tolerance. Replicated divergent artificial selection was applied to a genetically variable base population for 31 generations, resulting in two cold resistant, two cold susceptible, and two unselected control lines. To quantify the relationship between selection on chill-coma recovery and other metrics of thermal performance, we also measured survivorship after acute cold exposure, survivorship after chronic cold exposure, survivorship after cold exposure following a pre-treatment period (rapid cold hardening), starvation tolerance, and heat tolerance. We find that chill-coma recovery time is heritable within this population and that there is an asymmetric response to increased and decreased chill-coma recovery time. Surprisingly, we found no cross-tolerances between selection on chill-coma recovery time and the other environmental stress response traits. These results suggest that although artificial selection has dramatically altered chill-coma recovery time, the correlated response to selection on other stress response phenotypes has been negligible. The lack of a correlated response suggests that chill-coma recovery time in these selection lines is likely genetically independent from measures of cold survivorship tested here.

Keywords: Cross-tolerance; chill-coma recovery; *Drosophila melanogaster*; artificial selection

1 INTRODUCTION

Daily and seasonal variation in climate, including temperature, is a stressful environmental factor to which organisms must respond (Harshman et al. 1999, Koehn and Bayne 1989, Pidwirny 2006, Sibly and Calow 1989). For ectotherms, extreme temperatures directly influence most biochemical and physiological functions of the organism (Overgaard and Sorensen 2008), inducing critical physiological processes required to maintain fitness (Brakefield 2003, Hoffmann and Parsons 1991). Low temperatures are particularly damaging as these temperatures will damage cell membranes and decrease overall cellular and physiological function and survivorship (Hazel 1995, Overgaard et al. 2007, Rajamohan and Sinclair 2008).

Understanding how organisms improve tolerance to cold stress has been a primary focus of physiological research both on short-term and long-term time scales (Gerken et al. 2015, Lee et al. 1987, Nyamukondiwa et al 2011, Powell and Bale 2006). General processes that may increase survivorship after cold stresses include within-generation *acclimation* responses caused via thermal pre-exposures to stress (Gerken et al. 2015, Lee et al. 1987, Rajamohan and Sinclair 2008) or the across-generation genetic *adaptation* via shifts in allele frequency caused by the differential survivorship of natural genotypes after a stress exposure. These long-term and short-term cold tolerance mechanisms may be closely related physiologically or genetically, and selection pressures on a single component of cold tolerance may also change (via genetic correlations) other fitness or stress survivorship traits (Gibert et al. 2001, Hoffmann and Parsons 1989, Leips and Mackay 2000). Using artificial selection, it is possible to change the allele frequencies in genomic regions associated with a particular trait as well as to measure the magnitude and direction of genetic correlations between the trait under selection and other

environmental stress response traits (Harshman et al. 1999, Kostal et al. 2012, Mackay et al. 2012, MacMillan et al. 2009, Overgaard and Sorensen 2008, Service 1987).

Cross-tolerance occurs when selection to a given environmental stressor results in a correlated response to tolerance in another environmental stressor (Harshman and Hoffmann 2000, MacMillan et al. 2009). Cross-tolerance has been demonstrated in a variety of organisms, and is thought to be especially prevalent in insects (Bayley et al. 2001, Ring and Danks 1994, Vermeulen and Loeschcke 2007), although these cross tolerances are inconsistent across all insects (Anderson et al. 2005, Bubliy and Loeschcke 2005, Kristensen et al. 2007, Norry et al. 2007, MacMillan et al. 2009, Phelan et al. 2003) which may be partly attributed to differences in selection methodology (i.e. cold shock treatment (Kristensen et al. 2007, MacMillan et al. 2009) versus chill coma treatment (Anderson et al. 2005). Selection may contribute to the directionality of correlation and cross-tolerance to other traits (MacMillan et al. 2009). For example, correlations have been observed when selection drives an increased heat tolerance which is positively correlated to an increase in cold tolerance, but selection to tolerance to cold does not necessarily carry a correlated response of tolerance to heat (Bubliy and Loeschcke 2005). Furthermore, studies have examined correlations between stress metrics such as cold tolerance and starvation resistance (Bubliy and Loeschcke 2005, Le Bourg 2013, Le Bourg and Massou 2015, Sinclair et al. 2007, Scharf et al 2016), cold selection and heat tolerance capacity (Anderson et al. 2005, Kristensen et al. 2007), diet manipulations and metabolic mechanisms (Clark et al. 2015, Harshman et al. 1999, Sisodia et al. 2015), response to hypoxia and cold tolerance (Benasayah-Meszaros et al. 2015, Boardman et al 2011, Boardman et al. 2015), and

cold survivorship, desiccation resistance, and body size (Benoit et al. 2009, Gantz and Lee 2015, Jumbo-Lucioni et al. 2010, Terhazaz et al. 2015).

The goal in quantifying stress-related trait correlations is to identify genetic correlations among traits and to determine the capacity of selection on one trait to constrain or promote phenotypic evolution of another (Brakefield 2003, Bublly and Loeschcke 2005, MacMillan et al. 2009). As many organisms have shown a wide range of cross-tolerances, several hypotheses have emerged concerning the overlap of genomic regions influencing various stress responses. A lack of correlations between responses to various environmental stresses may suggest that there is no linkage between tolerances to such stresses—all stress responses are genetically independent of one another (Harshman and Hoffmann 2000, Sinclair et al. 2007), or that the experiment is too small to detect a weak correlation caused by the low heritability of each traits. Cross-tolerances may also gradually weaken over time as fitness trade-offs occur or spurious associations (i.e., transient LD) are broken down (Phelan et al. 2003). However, strong cross-tolerances are likely to occur when the underlying quantitative trait loci controlling each environmental stress trait are shared (Bublly and Loeschcke 2005, Norry et al. 2007).

In this study, we specifically examine the cross-tolerances between chill-coma recovery time and five other environmental stress response traits. Chill-coma recovery is a physiological response to non-lethal cold temperatures (David and Capy 1988) that is a genetically variable and adaptive phenotype exhibiting significant clinal variation on multiple continents (Gibert et al. 2001, Kristensen et al. 2007, Rako and Hoffmann 2006) as well as strong habitat associations among species (Gibert et al. 2001). At the end of 31 generations of replicated artificial selection, we measured five environmental stress responses traits on each of the selection and control lines.

These stress responses included acute cold exposure (-6° C for one hour), chronic cold exposure (0° C for 16 hours), a rapid cold hardening pre-treatment followed by cold exposure (two hours at 4° C followed by one hour at -6° C), starvation tolerance, and survival after heat stress (38° C for one hour). Anderson et al. (2005) demonstrated that selection to chill-coma recovery time was mostly independent of stress response to other traits but Bublly and Loeschcke (2005) found correlations between selected traits and starvation and cold shock and heat shock survival. Indeed, artificial, direct selection can lead to a correlated response among traits (Hoffmann and Parsons 1993, Partridge and Fowler 1993). We predict that selection for increased resistance to cold stress via selection on chill-coma recovery time will result in an increased ability to survive multiple environmental stressors while lines selected for a susceptibility to cold tolerance will have decreased survivorship to other environmental stressors.

2 METHODS

2.1 Experimental Flies and Chill-Coma Recovery Time Assay

Mated *D. melanogaster* females were collected from a single population at the Raleigh, NC Farmer's Market using fruit bait and we established a population of 60 isofemale lines in 2004. This sample of isofemale lines was used to establish a genetically variable base population, by crossing the lines in a round robin design in separate culture vials, with four males and four females per vial. Three days after the crosses were initiated, a single inseminated female from each cross was placed in each of two culture bottles to initiate two replicate base populations. The progeny from these culture bottles were designated generation 0. To begin the first generation upon which selection was applied, approximately 25 virgin males and females per

replicate base population were scored for cold tolerance and were used to initiate the three selection regimes (resistant, susceptible, and control) in each base population. Thus resistant line 1, susceptible line 1, and control line 1 were derived from base population 1, while resistant line 2, susceptible line 2, and control line 2 were derived from base population 2.

To measure chill-coma recovery time, the trait upon which selection was applied, we utilized the assay presented in Morgan and Mackay (2006). Specifically, 50 male and 50 female flies were transferred without anesthesia into empty vials and placed in chambers containing melting ice ($0^{\circ}\text{C} \pm 0.5$) for 3 hours. After 3 hours, individuals were removed from the cold treatment and returned to room temperature ($23^{\circ}\text{C} \pm 0.5$), and the chill-coma recovery time was measured by recording the amount of time until an individual was able to stand on its legs.

All of the individuals screened in this study were young flies (i.e., 5-7 days old), as previous studies have found that chill-coma recovery time changes as a function of age (David et al. 1998). Experimental individuals were maintained at 25°C and 60% humidity and a 12:12h light/dark cycle until they were used in the experimental assay. We scored chill-coma recovery time every generation, rather than every other generation as we have been unable to detect any evidence of ‘carry-over’ effects, i.e., detrimental influence of the cold treatment on future success (Anderson et al. 2005, Harrison et al. 2011), for cold tolerance (data not shown). However, if ‘carry over’ effects do exist within these or other populations the result of these effects will be the underestimation of the genetic variation in the trait rather than the overestimation.

2.2 Artificial Selection Regime

Beginning at generation one, 50 virgin male and female progeny were collected from each of the six lines every generation. These individuals were assayed for chill-coma recovery time as described above. In the two replicate resistant lines the 20 males and females with the fastest recovery time were used as the breeders for the next generation, while in the replicate susceptible lines the 20 males and females with the slowest recovery time were used as the breeders for the next generation, and in the control lines 20 males and females were randomly chosen with respect to chill-coma recovery time and used as the breeders for the next generation. Individuals that did not recover from chill-coma in the 90-minute observation period were given a score of 90 minutes. Artificial selection was applied in the manner for 31 generations.

2.3 Correlated Responses to Selection

After 31 generations of selection and approximately 20 generations without artificial selection, where flies were reared at 25°C with 12/12 light/dark schedule and ad libitum access to food and mates, five environmental stress response phenotypes were measured to test for the presence of correlated responses to selection. These include chronic cold exposure, acute cold exposure, acclimated cold exposure, starvation resistance, and survival after heat stress. We also measured chill-coma recovery time at this time to ensure that the response to selection was stable after artificial selection was ceased, these contemporaneous chill-coma recovery times are used for assessment of correlated responses to selection. For heat and cold stress assays, flies were placed in empty vials during the low or high temperature treatment to prevent flies becoming stuck in the food. After each temperature treatment flies were placed into vials containing fresh food and

survivorship was measured 24 hours later. Individuals were scored as alive if they retained the ability to stand or right oneself after a physical knockdown and the phenotype was recorded as a proportion of dead per vial (Colinet and Hoffmann 2010, Kelty and Lee 2001, MacMillan et al. 2009, Overgaard et al. 2005).

2.3.1 Heat and Cold Stress Response Traits

Survival after heat stress was measured on all selection lines by exposure to 38° C for one hour. Each line was tested separately for males and females, with 10 individuals per vial (n=3 vials per sex/line). Chronic cold exposure was performed by exposing the six selection lines to 0° C for 16 hours in order to simulate a long-term exposure to freezing temperatures (Gibert et al. 2001, Sinclair et al. 2007). Each line was tested separately for males and females, with 10 individuals per vial (n=3 vials per sex/line). Acute cold exposure was assayed on the selection lines as a percentage of dead flies after an exposure of flies to -6° C for one hour (Kelty and Lee 2001). Flies were sorted into vials with 10 individuals per vial separated by sex (n=3 vials per sex/line).

A rapid-cold hardening response (RCH) is characterized by a change in survivorship following a short non-lethal pre-treatment exposure before a cold shock (Gerken et al. 2015, Lee et al. 1987, Nyamukondiwa et al. 2011, Overgaard and Sorensen 2008). RCH has been shown to be genetically variable in natural populations of *D. melanogaster* (Gerken et al. 2015) and is important in natural populations of insects (Nyamukondiwa et al. 2011, Overgaard and Sorensen 2008, Worland and Convey 2001, Kristensen et al. 2007). To measure RCH, flies were separated into vials with 10 individuals by sex (n=3 vials per sex/line). Prior to cold exposure, flies were transferred into empty vials and placed in a cold room for exposure to 4° C for two hours. After

two hours, flies were placed directly into -6°C for one hour. RCH was calculated as the difference between non-hardened survivorship and acclimated survivorship.

2.3.2 Starvation Tolerance

Starvation tolerance was measured in the selection lines by utilizing starvation vials (1% agar medium) with no nutritional value but allowing the flies to obtain ingestible moisture (Bubliy and Loeschcke 2005, Harshman et al. 1999, MacMillan et al. 2009). Flies were separated by sex and line ($n=3$ vials per sex/line). At the beginning of the experiment, flies were transferred to vials containing the starvation medium. Flies were kept at 25°C and were counted every four hours for dead flies until all flies had died (Sinclair et al. 2007). Each individual fly's time of death in hours was recorded as the phenotypic value for starvation.

2.4 Analyses

2.4.1 Quantitative Genetic Analysis of the Selection Response

Estimates of the realized heritabilities for chill-coma recovery time were computed for each of the replicate susceptible and resistant selection lines by regression of the cumulative selection response (as a deviation from the control) on the cumulative selection differential based on the data from generation 0 to 31 (Falconer and Mackay 1996). Within replicate heritability estimates are most appropriate because the resulting resistant and susceptible lines are derived from identical replicate base populations.

At generation 31 or the time when the correlated traits were assayed, we used a nested model analysis of variance (ANOVA) to determine the effect of selection on chill-coma recovery

time, chronic cold exposure, acute cold exposure, acclimated cold exposure, starvation resistance, and survival after heat stress. Each of these phenotypes was analyzed using the following mixed model: $y = \mu + Sel + Line(Sel) + Sex + Sel \times Sex + Line(Sel) \times Sex + \varepsilon$ where y is the phenotype, Sel is the fixed effect of selection regime (susceptible, control, or resistant), $Line(Sel)$ is the random effect of replicate line nested within selection regime (replicate population 1 or 2), Sex is the fixed effect of sex, and ε is the residual variance. The primary terms of interest in the analysis are the Sel , $Line(Sel)$, $Sel \times Sex$, $Line(Sel) \times Sex$ as these reflect general or sex-specific genetic differences as a result of selection (significant differences among the selection regimes) or random genetic drift (significant differences among lines within each selection group). The significance of each of these fixed or random effects was determined using the appropriate denominator mean squares from the mixed model. For example, the significance of selection was determined using the mean square of line nested with selection.

2.4.2 Analysis of Correlated Responses to Selection

For the correlated trait analyses, we used the same model as above on three separate data sets per trait: (1) all three selection regimes; (2) the control vs. the cold resistant selection regime; and (3) the control vs. the cold susceptible selection regime. Each of these analyses was performed to explore the possibility of symmetric and asymmetric correlated responses to selection. In many fitness-related traits, such as cold tolerance, asymmetrical responses are often observed as natural populations have selected against deleterious traits, represented by recessive alleles (Frankham 1990). Observing selection on these populations often results in a greater change in the deleterious (cold susceptible) lines and a smaller change in the naturally favorable (cold

resistant) lines, i.e. an asymmetrical response (Frankham 1990) and can occur at either the same or different loci. Symmetrical selection responses occur in both selection directions relative to a control group when the system is under stabilizing selection (Nagylaki 1989) and is the result of the evolution of different alleles at the same loci. Because sex differences were not significant at generation 31 of selection, we pooled our analyses of males and females. Heritability was calculated for each environmental stress response using the lmer package in R statistical software (version 2.13).

ANOVAs provide a baseline comparison of treatment and selection interaction effects while correlations between stress tolerance assays were conducted using Pearson's product-moment correlations. Correlations were performed to compare chill-coma recovery time to acute cold survivorship, chronic cold survivorship, time to death under starvation, survivorship following heat treatment, and survivorship following RCH and an acute cold stress. We also tested correlations with and without the control lines in order to test selection lines independently. If correlations are significant we can identify a cross-tolerance effect of selection on chill-coma recovery and the other environmental stress. Directionality of correlations indicates if the cross-tolerance is a trade-off (negative) or a true cross-tolerance effect (positive). All analyses were performed using R statistical software (version 2.13).

3 RESULTS

3.1 Direct Selection Response

The result of 31 generations of replicated selection for cold susceptibility and cold resistance is shown in Fig. 1A. There were no significant differences in mean chill-coma recovery time

among the three selection groups at generation 1 ($F_{2,3} = 2.43$ $P = 0.23$). The selection response was highly asymmetrical in the direction of cold susceptibility. The first consistent significant difference between the susceptible and resistant lines was measured at generation 9 ($F_{2,3} = 18.46$ $P = 0.0206$) and at generations 30 and 31 there is a highly significant divergence among the all of the selection groups (Table 1; Gen30: $F_{2,3} = 25.34$, $P = 0.013$; Gen 31: $F_{2,3} = 22.88$, $P = 0.015$). All times to recovery are reported as a mean $\pm$ standard error of the mean. At generation 31 the resistant lines recovered on average after 7 minutes 45 seconds (± 48 seconds), the susceptible lines recovered on average after 60 minutes 8 seconds (± 51 seconds), while the random bred controls recovered on average after 15 minutes 8 seconds (± 50 seconds).

Realized heritabilities (h^2) of chill-coma recovery time were computed from the regressions of the cumulative selection response (R) on the cumulative selection differential (S) for each selection line (Fig. 1B) as well as for the divergence between selection lines within each replicate base population (Falconer and Mackay 1996). Estimates of h^2 ($\pm$ SE of the regression coefficient) were $h^2 = 0.046$ (± 0.019 ; $P = 0.026$) and $h^2 = 0.10$ (± 0.025 ; $P = 0.0003$) for replicate 1 and 2 in the resistant lines, respectively. Estimates of h^2 for replicate 1 and 2 of the susceptible lines, respectively, were $h^2 = 0.12$ (± 0.009 ; $P < 0.0001$) and $h^2 = 0.066$ (± 0.008 ; $P < 0.0001$). Heritabilities estimated from the divergence were $h^2 = 0.11$ (± 0.008 ; $P < 0.0001$) and $h^2 = 0.070$ (± 0.007 ; $P < 0.0001$).

3.2 Correlated Responses in Environmental Stressors

After 20 generations without selection, the resistant and susceptible lines maintained their differential response to chill-coma recovery selection. These populations were then used to test

cross-tolerance to our five environmental traits. The correlated responses to selection were compared to the re-measured chill-coma recovery time at generation 20 ($F_{2,3} = 89.59$, $P=0.0021$; Fig. 1C). At generation 20 (Fig. 1C), control lines had an average chill-coma recovery time of 14 minutes and 25.8 seconds (± 38.4 seconds), the resistant lines had an average recovery time of 6 minutes and 57 seconds (± 37.2 seconds), and the susceptible lines had an average recovery time of 42 minutes and 22.2 seconds (± 37.8 seconds).

There was no significant difference in chronic cold tolerance among selection regimes ($F_{2,3} = 0.76$, $P = 0.54$; Fig. 2A). Chronic cold tolerance had heritability of 0.14 and showed a negative association with chill-coma recovery time but it was not significant (Table 2). The resistant selection regime had an average proportion dead of 0.52 ± 0.25 , the susceptible selection had an average of 0.39 ± 0.24 , and the control populations had an average of 0.51 ± 0.27 . There was also no significant difference among selection regimes for acute cold tolerance ($F_{2,3} = 0.10$, $P = 0.90$; Fig. 2B; Table 2). Acute cold tolerance and chill-coma recovery time was not related in this population and showed a very low correlation (Table 2) which could be attributed to the undetectable heritability of acute cold tolerance in these lines. The resistant selection regime had an average proportion dead of 0.53 ± 0.42 , the susceptible selection had an average of 0.51 ± 0.45 , and the control populations had an average of 0.54 ± 0.39 .

There was also no difference among selection regimes in the rapid cold hardening response ($F_{2,3} = 0.32$, $P = 0.75$; Fig. 2C). Rapid cold hardening had heritability of 0.078 and had little correlation to chill-coma recovery time and the association was not significant (Table 2). The resistant selection regime had an average proportion dead of 0.06 ± 0.10 , the susceptible selection had an average of 0.06 ± 0.12 , and the control populations had an average of $0.09 \pm$

0.14. Survival response to heat stress did not differ among selection regimes ($F_{2,3}= 0.46$, $P = 0.66$; Fig. 2D). Heat stress had a heritability of 0.29 and had no correlation to chill-coma recovery time (Table 2). The resistant selection regime had an average survival after heat stress of 0.88 ± 0.17 , the susceptible selection had an average of 0.77 ± 0.33 , and the control populations had an average of 0.72 ± 0.29 .

Among selection regimes, starvation time to death was nearly significantly different ($F_{2,3}= 6.47$, $P = 0.081$; Fig. 2E) and there were significant differences in responses for all comparisons (resistant to control, $P < 0.0001$; resistant to susceptible, $P < 0.0001$; susceptible to control, $P < 0.0001$). However, an examination of the means of the selection regimes reveals that both the resistant and susceptible lines have survival means less than the control lines and thus there is no correlation between starvation and chill-coma recovery time (Table 2). Starvation had a heritability of 0.068. The control populations had an average of 68.83 ± 20.49 hours, while the resistant selection regime had an average time to death of 63.97 ± 17.57 hours, the susceptible selection had an average of 59.13 ± 16.32 hours. Thus, this significant selection term is likely caused by inbreeding effect of the selection process and not a true correlated response to selection, as both the susceptible and resistant lines are less starvation resistant than the control lines.

4 DISCUSSION

Artificial selection is a powerful tool used to shift the allele frequencies in a population. Such selection typically results in populations with trait values that lie on extreme ends of the phenotypic distribution. In our experiment, we were able to successfully select *Drosophila*

melanogaster with increased and decreased cold tolerance, as measured by chill-coma recovery times from a single temperate population. Chill-coma recovery is an ecologically relevant and adaptive trait in *D. melanogaster* as it enables the fly to cope with prolonged periods at non-lethal cold temperatures (David et al. 1998, Kelty 2007). The selection procedure produced replicate sets of lines that were both susceptible and resistant to chill-coma recovery time and dramatically different between the selection regimes. The response to selection was more dramatic in the direction of susceptibility than resistance, possibly reflecting natural allelic frequencies in relation to deleterious (cold susceptible) traits having largely recessive alleles, and favorable (cold resistant) traits having alleles that are high in frequency in natural populations, thus dampening the effects of artificial selection on such favorable traits (Frankham 1990). During artificial selection, the deleterious effects (i.e. survivorship, competition) are reduced creating an environment where these deleterious phenotypes can persist. This is what may drive the asymmetrical effects between resistant and susceptible selection lines. Our finding of asymmetry is also consistent with asymmetric responses to selection on other traits that are closely associated with fitness (Edwards et al. 2006, Krebs and Loeschke 1997, Mackay et al. 2005). The response was also more variable in the susceptible lines than in the resistant lines, which is also consistent with selection on traits associated with fitness. That is when artificial selection is applied to a fitness trait the possible genetic changes that reduce fitness by selection on deleterious alleles are less than the possible shifts in beneficial alleles that increase fitness.

From the selection response we were also able to estimate the realized heritability for chill-coma recovery by regressing the cumulative selection response (R) on the cumulative selection differential (S) for each replicate pair of selection lines. Because we imposed our

artificial selection regime on replicate base populations derived from a single temperate population we are able to estimate the heritability of chill-coma recovery time without the confounding effects of admixed populations or linkage disequilibrium, which will inflate estimates of heritability. The heritability estimates for chill-coma recovery time were low and consistent with those estimated for most physiological or behavioral traits (Mousseau and Roff 1987, Roff 1997). Our estimates are dramatically lower than those estimated by Hoffmann et al. (2005) and Morgan and Mackay (2006). This is however to be expected because both of these studies estimated heritability for chill-coma recovery time using admixed study populations, thus likely causing an inflation in the level of transient linkage disequilibrium and increasing the estimated heritability. In addition, Morgan and Mackay (2006) used recombinant inbred lines for which allele frequencies were around 0.5, which may also account for differences in heritability estimates.

We did not find a significant cross-tolerance effect of our selection pressures to any of the traits assayed here. However, we find that for starvation, the selected lines, both resistant and susceptible, show a marginally shorter time to death than the control lines. This pattern is similar to the cross-tolerance that Bubliy and Loeschcke (2005) demonstrated and may suggest that starvation is linked to several stress related traits. The slight increase in susceptibility to starvation may suggest that the selection pressures overall are creating poor performance to other stressors and not specifically in the direction of chill-coma recovery time. However, the reduction in performance to stresses does not follow for any of the others assayed here and further analyses of overall life-history characteristics may be useful in demonstrating overall genotypic performance due to selection for chill-coma recovery time. MacMillan et al (2009)

also found cold tolerance to be independent of other stress traits and suggests that any cross-tolerances found may be coincidental. Recent work from Sinclair et al. (2013) suggest that although there may not be shared mechanisms there may be evolutionary potential for cross-talk in stress traits, resulting in similar physiological responses.

A correlated response to selection depends not only on the genetic correlations, but on the narrow sense heritabilities of the focal and the correlated traits. The realized heritability of chill-coma recovery time is low (0.046 to 0.12) most likely due to low allele frequencies contributing to susceptibility of chill coma and epistatic gene action (Chown et al. 2009). Similarly the correlated traits' estimated heritabilities are also low (0.00, 0.14, and 0.078 for acute cold tolerance, chronic cold tolerance, and RCH response; 0.068 for starvation; and 0.29 for heat tolerance) and thus, the correlated response in these traits is constrained to be low even if the underlying genetic correlation is high. Previous work has also shown heritability of cooling tolerance to be low (0.08 ± 0.17 in fish; Charo-Karisa et al. 2005) while chronic cold tolerance can be quite high (0.44) and RCH capacity to be similar to heritabilities found here (0.14; Gerken et al. 2015). Additionally, Gerken et al. (2015) examined a subset of the traits analyzed here and did not detect significant genetic correlations among many cold tolerance traits in a study focused on hundreds of genotypes. Thus, the low heritability levels of individual traits under direct selection may also suggest that these traits are genetically independent from chill-coma recovery. Additionally, the degree of stress examined here may not have been strong enough to detect significant differences among the selection and control lines. Thus, a statistically detectable significant response between selection lines and control lines may have been missed

374 simply due to a lower severity of stress used here than what is needed to show differential stress
375 response between selection regimes.

376   Using these lines we have the potential to examine costs and benefits of this artificial
377 selection response on an ecologically important trait (Chown et al. 2009, Lande 1979). However,
378 the low number of selection lines may inhibit us from finding significant correlations to other
379 physiological stress traits, due to the low power to detect genetic correlations in a small sample
380 of natural genetic variation. Furthermore, chill-coma is a physiological response to a non-lethal
381 temperature exposure for a moderate amount of time while the other environmental traits we
382 tested were either at lethal temperatures or extended exposures to adverse conditions. As shown
383 by the lack of cross-tolerance among the environmental stressors tested and chill-coma recovery
384 time, we can further hypothesize that recovery time and survival mechanisms are not linked for
385 this set of environmental stressors. A recent review of various cold tolerance responses and
386 correspondence to ecological variation (Andersen et al. 2015) showed that cold tolerance metrics
387 – such as time to 50% lethality, critical thermal minimums, and chill-coma recovery time – are
388 often highly correlated to one another as well as the ecological characteristics of distribution.
389 Survivorship, therefore, may not be the best metric for testing correlated responses of cold
390 tolerance phenotypes.

391   Although we did not observe any cross-tolerance of chill-coma recovery time to our
392 environmental stress responses, we must note that experiments involving few lines have limited
393 statistical power and thus decrease our ability to detect correlations among such stress responses.
394 However, several artificial selection populations with two replicates have provided substantial
395 scientific value and our lines have the potential to do the same (MacMillan et al. 2009, Manning

1961, Telonis-Scott et al. 2006). Future experiments should work to expand on the numbers of naturally varying lines to increase power of detection of potential cross-tolerance effects. One such resource would be the *Drosophila melanogaster* Genetic Reference Panel (DGRP), a community tool for the analysis of phenotypic variation and the underlying genetics of quantitative traits (Mackay et al. 2012). Not only will we be able to increase our power to detect potential cross-tolerance between phenotypes but we will also have access to the complex genetic basis of traits using association mapping techniques (Mackay et al. 2012). However, cross-tolerance of starvation resistance and chill coma recovery time in these lines is also low, consistent with the results shown here (Mackay et al. 2012). Gerken et al. (2015) also found that there as very little correlation to chill-coma recovery and survivorship traits in the DGRP, but that survivorship and plasticity had high correlations. This suggests that the low correlations between chill-coma recovery and survivorship traits here may not be due to low statistical power but may be a biological property of different cold tolerance traits.

4.1 Conclusions

In conclusion, we find that chill-coma recovery time is highly heritable within this population of temperate insects. We also find an asymmetric response to selection, suggesting that beneficial alleles for decreased chill-coma recovery time (resistance lines) are fixed within this population, but that more deleterious alleles for increased recovery time (susceptibility lines) have a greater response to selection as well as higher variability in selection between our two replicate lines. In addition, we find no cross-tolerance or correlated response to selection to chill-coma recovery time and our stress traits described here. This lack of correlation could be due to

the low power of our system, but the low levels of correlation suggest that these traits are probably independent of one another.

Although our analyses may lack robust statistical power, these lines selected for increased and decreased chill-coma recovery time are important for understanding pathways of convergent evolution in cold tolerance traits (Barrett 2001, Clark and Worland 2008). Understanding the variance between the susceptibility lines may increase our understanding of convergent evolution at the molecular level; metabolomics analyses of the selection lines have provided information on the physiological and metabolic changes brought on by selection to chill-coma recovery time (Williams et al. 2014). Increasing the numbers of naturally varying lines will increase our power to detect cross-tolerances and thus, future research on these environmental stressors will include a large set of independently segregating lines.

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440 6 Vitae

441 Alison Gerken completed her Ph.D. in biology from Kansas State University studying the
442 ecological and evolutionary genomics of cold tolerance. She is currently a post-doctoral research
443 associate at the University of Florida working with statistical genomics. She was a Graduate
444 Assistance in Areas of National Need Fellow and a National Science Foundation GK-12 Fellow.
445 Her research interests include modeling population level responses to stress and the genes
446 associated with population-level variation in stress responses.

447 Trudy Mackay is a professor in the Department of Biological Sciences at NC State University.
448 Her laboratory focuses on understanding the genetic and environmental factors affecting
449 variation in quantitative traits, using *Drosophila* as a model system. Her laboratory seeks to
450 identify the genetic loci at which segregating and mutational variation occurs, allelic effects and
451 environmental sensitivities, and the causal molecular variants. Her research utilizes mutagenesis
452 and analysis of natural variation, and systems genetics analyses. She is a Fellow of the American
453 Academy of Arts and Sciences and the Royal Society and a member of the US National
454 Academy of Sciences.

455 Ted Morgan is an Associate Professor of Biology at Kansas State University. His laboratory
456 focuses on finding the loci and genetic networks in ecologically-relevant phenotypic variation
457 and the processes that have influenced molecular genetic variation at these functional loci. He
458 uses *Drosophila melanogaster* as a genetic model for understanding how the molecular variation
459 influences ecologically relevant phenotypic variation in nature. In addition, his laboratory
460 combines information from metabolomics and cellular responses to cold tolerance for a whole-
461 organism evolutionary analysis of the response to cold stress.

462

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Figure Captions

Fig. 1. Phenotypic response to selection for chill-coma recovery time. (A) Mean chill-coma recovery time (minutes) of the selection lines (y-axis) versus generation of selection on the x-axis. The light and dark blue lines are the replicate susceptible lines, light and dark grey lines are the replicate control lines, and light and dark red lines are the replicate resistant lines. (B) Regressions of the cumulative selection response ($\bar{R}$) (y-axis) on cumulative selection differential ($\bar{S}$) for divergence between high and low selection lines (x-axis). Open circles and solid line are for replicate 1, while closed circles and dashed line are replicate 2. Heritabilities (h^2) estimated from divergence are highlighted for each replicate in the legend box. (C) Bar graph of chill-coma recovery time in minutes (y-axis) for the selected lines at generation 31 of selection (Gen31) and 20 generations post-selection (Gen51). Error bars represented standard error of the mean. The selection lines have maintained their differences in chill-coma recovery time. Gray are control lines, red are resistant to cold lines, and blue are susceptible lines. Filled bars are 20 generations post selection (Gen51).

Fig. 2. Environmental stress responses. The mean proportion of dead for cold and heat tolerance assays and the mean time to death for starvation is reported. Error bars represent standard errors on the mean. Letters above bars are significantly different means ($P < 0.05$). There are no consistent significant differences among selection regimes for each of the stresses, although for starvation differences between susceptible and resistant to control lines are observed. Selection lines are represented by replicates for resistant 1 and 2 and susceptible 1 and 2. A) Proportion dead following exposure to an acute cold stress of -6°C for 1 hour. B) Proportion dead following exposure to chronic cold stress for 0°C for 16 hours. C) Proportion dead following pretreatment at 4°C for 2 hours followed by exposure for 1 hour at -6°C , known as rapid-cold hardening (RCH). D) Proportion dead following exposure to an acute heat stress of 38°C for 1 hour. E) Time to death during starvation stress.

673 **Table 1. ANOVA of chill-coma recovery time.**

<i>Source</i>	<i>Generation 30</i>				<i>Generation 31</i>			
	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>	<i>df</i>	<i>MS</i>	<i>F</i>	<i>P</i>
<i>Selection</i>	2	122798.0	25.34	0.0132	2	159196.0	22.88	0.0153
<i>Line(Selection)</i>	3	4848.9	62.92	0.0033	3	6961.2	1.66	0.3445
<i>Sex</i>	1	819.6	10.62	0.0468	1	26.8	0.01	0.9414
<i>Selection x Sex</i>	2	45.9	0.60	0.6057	2	9.4	0.00	0.9978
<i>Line(Selection) x Sex</i>	3	77.1	0.43	0.7339	3	4205.4	30.13	<0.0001
<i>Error</i>	615	180.6			596	139.6		

674
675
676 **Table 2. Correlations between chill-coma recovery time in selected lines and various**
677 **environmental stressors.**

<i>Trait</i>	<i>Correlations</i>					
	<i>Selection</i>		<i>With Control Lines</i>		<i>Without Control Lines</i>	
	<i>F_{2,3}</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
<i>Acute Cold</i>	0.10	0.91	0.04	0.94	0.23	0.76
<i>Starvation</i>	6.47	0.082	-0.60	0.21	-0.58	0.42
<i>RCH</i>	0.32	0.75	-0.18	0.72	-0.03	0.93
<i>Chronic Cold</i>	0.76	0.54	-0.60	0.20	-0.57	0.43
<i>Heat</i>	0.46	0.67	-0.13	0.81	-0.25	0.74

Figure 1

Figure 2

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