1 **TITLE:** Evolution of age-specific decline in stress phenotypes is driven by both antagonistic
2 pleiotropy and mutation accumulation

3 **RUNNING TITLE**: Age-related change in stress tolerance

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9

Abstract

Efforts to more fully understand and test evolutionary theories of aging have produced distinct predictions for mutation accumulation (MA) and antagonistic pleiotropy (AP) mechanisms. We build on these predictions through the use of association mapping and investigation of the change in additive effects of polymorphisms across age and among traits for multiple stress response phenotypes. We found that cold stress survival with acclimation, cold stress survival without acclimation, and starvation resistance declined with age and that changes in the genetic architecture of each phenotype were consistent with MA predictions. We used a novel test for MA and AP by calculating the additive effect of polymorphisms across ages and found support for both MA and AP mechanisms in the age-related decline in stress tolerance. These patterns suggest both MA and AP contribute to age-related change in stress response and highlight the utility of association mapping to identify genetic shifts across age.

Introduction

The intensity of natural selection changes over an organism's lifespan, having greatest effect early in life, as individuals reach reproductive maturity, and smaller effect as organisms age (Charlesworth, 2001; Charlesworth and Hughes, 1996; Fisher, 1930; Haldane, 1941; Hamilton, 1966; Medawar, 1952; Williams, 1957). Decreased effectiveness of natural selection at old age results in the accumulation of deleterious polymorphisms in populations and leads to decline in age-specific fitness, characteristic of senescence (Hamilton, 1966; Medawar, 1952; Williams, 1957). Senescence is expected to negatively impact phenotypes related to fitness and is thought to have evolved through two non-mutually exclusive genetic mechanisms (Bowler and Terblanche, 2008; Charlesworth, 1994; Ricklefs and Finch, 1995). Under mutation accumulation (MA; Medawar, 1952), decreased effectiveness of natural selection over lifespan allows the retention of deleterious polymorphisms that are only expressed later in life (Ricklefs and Finch, 1995). Under antagonistic pleiotropy (AP; Williams, 1957), genes that are expressed over a wide window of an individual's lifespan have positive effects on fitness at young age and negative effects on fitness at old age (Charlesworth, 2001; Maklakov et al., 2015; Ricklefs and Finch, 1995; Williams, 1957). Both mechanisms rely on the relaxation of natural selection later in an organism's life but have unique predictions for how age-dependent genetic control of phenotypes changes.

Age-related declines and the influence of the MA and AP mechanisms have been well documented for life-history phenotypes such as mortality and fecundity (Bowler and Terblanche, 2008; Charlesworth and Hughes, 1996; Durham et al., 2014; Engström et al., 1989; Hughes et al., 2002; Pletcher et al., 1998; Promislow et al., 1996; Rose, 1984; Rose et al., 1992; Snoke and Promislow, 2003; Tatar et al., 1996), but far less is known about how stress response phenotypes

change with age (Bowler and Terblanche, 2008). Stress response over an organism's lifespan is a critical component of fitness, and is an important modulator of lifespan (Colinet et al., 2015). Variation in stress response can influence the persistence and evolution of populations over short time scales, especially in variable environments (Bergland et al., 2014). In species that experience seasonal change in thermal regime, changes in the demographic structure of populations can also drastically influence the ability of individuals to tolerate stressful temperatures. For example, in *Drosophila melanogaster* and *D. simulans*, populations are primarily composed of young individuals in the spring when temperatures are increasing on average and primarily of older individuals in the fall when temperatures are decreasing on average (Behrman et al., 2015). Thus, measures of thermal tolerance at one point in the season therefore do not reflect the influence of seasonal variation in age on thermal tolerance. Such shifts in the age structure of populations coupled with age related changes in the genetic control of fitness phenotypes have the potential to dramatically influence short- and long-term responses to environmental variation.

The MA and AP aging mechanisms make predictions about age-specific changes in multiple quantitative genetic parameters. Under MA, genetic variance is expected to increase with age because of the expression of age-restricted polymorphisms (Charlesworth, 2001; Charlesworth and Hughes, 1996; Hughes et al., 2002; Leips et al., 2006). These late acting polymorphisms are retained in the population because the individuals that possess them have successfully reproduced, allowing such alleles to evade natural selection (Charlesworth, 2001; Haldane, 1941; Maklakov et al., 2015). Additionally, because the genetic control of the phenotype across ages is independent, the genetic correlation of the phenotype between young and old individuals is expected to be non-negative (Charlesworth, 2001; Maklakov et al., 2015;

Reynolds et al., 2007). In contrast, under the AP hypothesis, age-related change in phenotypes is the result of a genetic trade-off, where polymorphisms that are beneficial early in life are detrimental late in life (Leips et al., 2006; Maklakov et al., 2015). These polymorphisms are retained in the population because of their beneficial effects on fitness at young age (Charlesworth and Hughes, 1996; Leips et al., 2006; Maklakov et al., 2015). AP does not make clear predictions for changes in variance components with age; however, because the same polymorphisms are expected to influence the phenotype with opposite effects across age, the genetic correlation across ages is expected to be negative (Charlesworth and Hughes, 1996; Hughes et al., 2002; Leips et al., 2006).

Despite these clear predictions, the influence of MA and AP and the theory behind these mechanisms does not fully explain age-related change in phenotypes. One reason for this is that the signature of AP may be lost because of small effects or fixation of loci involved in AP leading to ascertainment bias toward MA (Maklakov et al., 2015; Moorad and Promislow, 2009). Further, calculations of genetic variance and correlations are indirect metrics of age-related change in the genetic control of phenotypes (Charlesworth, 2001). In contrast, the use of association mapping allows us to extend these predictions to more explicitly evaluate subtle patterns predicted by the MA and AP theories of aging. Durham et al. (2014) previously used association mapping to identify and compare polymorphisms that are associated with variation in phenotypes measured at multiple ages. This use of association mapping can be extended in two important ways. First, sets of associated polymorphisms can be compared between two different phenotypes across age as well as within phenotypes across age. Second, even if sets of significantly associated polymorphisms are non-overlapping, association mapping allows the

91 evaluation of age-related shifts in the additive effects of associated polymorphisms, thus
92 facilitating the detection of weak antagonistic effects across age or phenotypes.

93 As an example, consider a hypothetical phenotype measured in young and old individuals
94 that is associated with non-overlapping sets of polymorphisms at each age. Two different
95 polymorphisms are associated with the hypothetical phenotype at young age and have positive
96 additive effects on the young phenotype. The polymorphism that is consistent with MA will shift
97 from a significant positive additive effect at young age to an effect that is near zero or of the
98 same sign at old age. In contrast, the polymorphism that is consistent with AP will shift from a
99 positive additive effect at young age to a negative additive effect at old age. Thus, even though
100 association mapping may not detect the antagonistic polymorphism with small effect in old
101 individuals, calculation of additive effects of polymorphisms across age can be used to detect
102 signals of AP (Fig. 1; Maklakov et al., 2015).

103 In the current study, we used a combination of association mapping and quantitative
104 genetic analysis to dissect the variation in age-related changes in four environmental stress
105 response phenotypes and tested the influence of MA and AP. To do this, we measured age-
106 related survival after cold-stress with acclimation and without acclimation, thermal phenotypic
107 plasticity, and starvation resistance in a genetically diverse *D. melanogaster* mapping population
108 from the *Drosophila* Genetic Reference Panel (DGRP; Huang et al., 2014; Mackay et al., 2012).
109 Starvation resistance and cold tolerance are quantitative genetic phenotypes, with heritable
110 variation existing for each (Bowler and Terblanche, 2008; Gerken et al., 2015; Hoffmann et al.,
111 2005; Imasheva et al., 1998; Mackay et al., 2012; Morgan and Mackay, 2006; Overgaard et al.,
112 2010; Schwasinger-Schmidt et al., 2012). However, much of the previous work examining age-
113 related change in cold tolerance has been done in a single genotype (Czajka and Lee, 1990), and

114 little research is available to inform how starvation resistance will change with age (but see
115 (Colinet et al., 2015). We predicted that starvation resistance and cold tolerance (measured as
116 acclimation and non-acclimation survivorship) would decline with age.

117 Genetic variation has also been documented for various forms of thermal phenotypic
118 plasticity (Fallis et al., 2014; Gerken et al., 2015). Short-term acclimation through rapid cold-
119 hardening (RCH score; Lee et al., 1987) is one form of plasticity that occurs when organisms are
120 exposed to a mild thermal stress before experiencing more stressful conditions (Coulson and
121 Bale, 1990; Czajka and Lee, 1990; Gerken et al., 2015; Lee et al., 1987; Powell and Bale, 2005).
122 In flies and other ectothermic species, this pre-treatment usually results in increased cold
123 survivorship and provides a simple model of the physiological response of ectotherms as they
124 respond to episodic fluctuations in temperature (Bozinovic et al., 2011; Coulson and Bale, 1990;
125 Huey et al., 2012; Ju et al., 2011; Niehaus et al., 2012). In most cases, a strong, beneficial
126 acclimation response is detected in young adult individuals (Gerken et al., 2015; Ju et al., 2011;
127 Powell and Bale, 2005; Rajamohan and Sinclair, 2009), but age-related changes in this form of
128 phenotypic plasticity have not been examined. We expected flies to lose the ability to survive
129 acclimation and non-acclimation cold stress at a similar rate, resulting in acclimation scores (the
130 difference in survival with and without cold acclimation) that would not change with age.
131
132 ***Methods***
133 **Fly stocks**
134 The *Drosophila* Genetic Reference Panel (DGRP) was established as a set of natural
135 isogeneic lines founded from a single population in Raleigh, NC (Table S1; Mackay et al., 2012).
136 Stocks were obtained from Bloomington Stock Center and maintained at 25°C on a 12-hour

light-dark cycle on cornmeal-molasses agar sprinkled with active yeast. Parents of experimental flies were sorted over light CO_2 anesthesia and placed into vials containing five individuals of each sex to establish the first experimental block. Females were allowed to mate and lay eggs for three days, after which the parents were transferred to a new set of vials. Egg laying continued in the new vials for three days to establish the second experimental block, and then parents were discarded. Experimental flies were collected on the third day of eclosion and sorted by sex to a density of 10 same-sex individuals per vial. Our experimental design measured responses in "young" and "old" cohorts of flies. Young flies were aged for 1 week (7 days) at 25°C, while old flies were aged for four weeks (28 days) at 25°C. The "old" cohort timing was selected because this was an advanced age time point, but prior to a significant decline in average survivorship among lines (Ivanov et al., 2015). Previous research has also demonstrated reduced fecundity at this age (Leips et al., 2006; Tatar et al., 1996). Experimental flies in the "old" cohort were tipped every third day to new media until flies were tested at 28 days.

Age-related stress responses

Cold stress responses

We measured three cold stress responses on 101 DGRP lines at young and old age (Table S1). We measured acclimation survival using a rapid cold hardening treatment that consisted of a two-hour exposure to 4°C immediately prior to cold shock at -6°C for one hour (Gerken et al., 2015; Lee et al., 1987). Following cold shock, the flies were transferred to fresh media and allowed to recover at 25°C for 24 hours (Fig. S1A). We also measured non-acclimation survival by transferring flies directly (without acclimation) to -6°C for one hour (Fig. S1B). As with the acclimation treatment, the non-acclimated flies were transferred to fresh media following cold

stress and allowed to recover for 24 hours at 25°C. After 24 hours, the proportion of flies that had survived each treatment was recorded by counting the number of individuals in each vial that were capable of coordinated movement (flying or walking). Acclimation score (or rapid cold hardening capacity) was calculated by subtracting non-acclimation survivorship from acclimation survivorship (Gerken et al., 2015). A total of four replicates per sex, line, age, and cold stress treatment were measured in two experimental blocks with 10 individuals per vial replicate.

Starvation resistance

We measured starvation resistance in 164 DGRP lines, including the 101 lines used in the cold stress response experiments, at young and old age (Table S1). Young and old flies were maintained on standard media until they were one or four weeks of age, respectively. At one or four weeks of age, flies were transferred to starvation media (1.5% agarose) and maintained at 25°C. Vials were monitored every four hours, and average time of death per vial was recorded as the response. A total of three replicates per sex, line, and age were measured with 10 individuals per vial replicate.

Cold-stress responses in physiologically aged individuals

We conducted an additional experiment to test for variability in the rate of senescence among DGRP lines. For this experiment, 10 lines were randomly selected from the 101 included in the cold stress experiment (Table S1), and experimental flies were obtained as described previously. Physiologically-aged flies were set up at a density of 20 individuals per vial and maintained at 25°C until the number of flies per vial and line reached approximately 50%

183 (Td50). At this point, acclimation survivorship, non-acclimation survivorship, and acclimation
184 score were measured for the physiologically aged flies and compared to that of the
185 chronologically aged (four-week-old) flies.
186
187 **Data analysis**
188 *Genetic variation*
189 Genetic variation among all lines was analyzed via mixed-model ANOVA for the cold
190 stress responses (acclimation survivorship, non-acclimation survivorship, and acclimation score)
191 and starvation resistance. The model for each analysis included the main effects of age and sex,
192 as well as interactions, with block (for cold stress responses only) and line as random effects.
193 Specific effects of sex by age interactions were tested with Tukey's HSD post hoc test, with an
194 experiment-wide $\alpha = 0.05$.
195 To assess the effect of variation in rate of aging on cold stress tolerance (i.e.
196 physiological vs chronological cold stress phenotypes), linear regression was used to compare
197 the mean cold stress responses of four-week-old flies with cold stress response of flies at their
198 line specific Td50. However, because none of the physiologically-aged flies survived non-
199 acclimated cold stress, we only compared acclimation survivorship in this analysis.
200
201 *Genome-wide association analysis*
202 We used association mapping to identify regions of the genome that were significantly
203 associated with variation in acclimation survivorship, non-acclimation survivorship, acclimation
204 score, and starvation resistance. Association mapping was performed on each age and phenotype
205 separately, and significance was assigned at $-\log_{10}(5)$ (Durham et al., 2014; Gerken et al., 2015;

206 Mackay et al., 2012). Shifts in genetic architecture across age and phenotype were assessed by
207 comparing the significant polymorphisms associated with each age-specific phenotype. We
208 performed gene ontology (GO) enrichment analysis using FlyMine (Lyne et al., 2007) to
209 determine whether specific classes of genes or pathways were overrepresented in the loci
210 associated with each phenotype and age.
211
212 *Quantitative genetic analyses*
213 Heritability, variance components, and genetic correlations were estimated using the
214 program H2boot, which applies bootstrap resampling to quantitative genetic data (Phillips,
215 1998). Acclimation survivorship, non-acclimation survivorship, acclimation score, and starvation
216 resistance for one- and four-week-old flies were treated as eight phenotypes. Data were analyzed
217 using a one-way ANOVA, resampling lines 10,000 times with replacement. Because DGRP lines
218 are inbred homozygous lines, reported heritability estimates are broad sense, and were estimated
219 as:
220
221 $$H^2 = \sigma_L^2/(\sigma_L^2 + \sigma_E^2),$$
222
223 where σ_L^2 is the among line homozygous genetic variance component, and σ_E^2 is the
224 environmental variance component. The coefficient of homozygous genetic variance was used to
225 assess the effect of age on changes in homozygous genetic variance, and was estimated as:
226
227 $$CV_G = 100(\sqrt{\sigma_L^2})/\overline{z}_\iota,$$
228

where $\overline{z_i}$ is the phenotype mean. Genetic correlations across ages were estimated as:

$$R_G = \sigma^2_{L1,4}/\sqrt{(\sigma^2_{L1}\sigma^2_{L4})},$$

where $\sigma^2_{L1,4}$ is the covariance component of the phenotype across the two ages tested, σ^2_{L1} is the among line homozygous genetic variance component of the phenotype for one-week-old flies, and σ^2_{L4} is the among line homozygous genetic variance component of the phenotype for four-week-old flies. Variance component and genetic correlation estimates were reported as the average of the 10,000 bootstraps, and the variation in the estimate was used to generate standard errors for each term.

The additive effect of an allele (α) for associated polymorphisms was calculated as one-half the difference in phenotypic mean of lines grouped according to homozygous genotype, corrected for *Wolbachia* infection and TE insertions (Falconer and Mackay, 1996; Huang et al., 2014):

$$\alpha = (\alpha_1 - \alpha_2)/2.$$

Allele class was designated as major if allele frequency exceeded 50% in the experimental population. Standardized allele effects were calculated as the additive effect divided by the standard deviation of the phenotype:

$$a = \alpha/\sigma_P.$$

252 In the DGRP, evidence of MA and AP was examined four ways. First, if MA contributes
253 to population-level decline in a phenotype, late acting alleles will inflate CV_G in four-week-old
254 flies. Second, if MA is responsible for age-specific decline in phenotypes, unique regions of the
255 genome should be associated with the phenotype at young and old age. Under AP, regions of the
256 genome that are associated with the phenotype in young and old individuals should overlap.
257 Third, under MA, we expect the genetic correlation between ages for each phenotype to be non-
258 negative, due to the expectation that the additive effects at different ages are independent
259 (Hughes et al., 2002; Leips et al., 2006), while under AP, we expect the genetic correlation
260 between ages for each phenotype to be negative because regions of the genome associated with
261 the phenotype in young and old individuals overlap (Charlesworth and Hughes, 1996; Maklakov
262 et al., 2015). Finally, under MA, the additive effects of polymorphisms that are associated with a
263 phenotype in young individuals are expected to have additive effects on the phenotype in old
264 individuals that are smaller but of the same sign (Fig. 1), while under AP, polymorphisms
265 associated with a phenotype in young individuals are expected to have additive effects on the
266 phenotype in old individuals that are of the opposite sign (Fig. 1).
267 In addition to testing these predictions of MA and AP within each phenotype across age,
268 we also tested the role of MA and AP in age-related change between phenotypes. As above for
269 each phenotype, we assessed the level of overlap of polymorphisms and genetic correlations
270 between phenotypes and calculated the additive effects of polymorphisms associated with each
271 phenotype on every other phenotype and age in our study. For example, the additive effects of
272 polymorphisms associated with acclimation survival at one week were calculated for both the
273 one and four-week non-acclimation survival and starvation resistance responses.
274

Tests for selection

We used the QTL sign test (QTLST) to the test the direction of the additive effects of associated polymorphisms identified through association mapping. The QTLST was developed by (Orr, 1998) to determine whether the signs of QTL effects were indicative of directional selection acting on a phenotype. The probability for rejecting the null hypothesis that selection does not influence the phenotype was calculated as in Orr (1998):

$$P = \sum_{i=n_{+obs}}^{n} Pr\{n_+ = i | 2\sum G_1 \geq R\},$$

In this study, we treated n as the number of associated polymorphisms detected for each phenotype, n_{+obs} as the number of these polymorphisms that had a positive additive effect, and G as a vector of all additive effects. Because association mapping does not involve generation of a mapping population from distinct lines, R was simply the standard deviation of the phenotype of the population. An exponential distribution of the polymorphism effects was assumed in our adaptation of this model as in applications of QTLST to QTL data. Because QTLST is sensitive to high variance among additive effects, we also performed the QTLST-EE, which assumes that each of the additive effects are equal (Anderson and Slatkin, 2003; Rice and Townsend, 2012). Analyses were done in R using code adapted from Muir et al. (2014) (Hope, 2013; Muir et al., 2014; R Core Team, 2015; Wickham, 2009).

Results

Phenotypic responses

All phenotypes measured in this study were variable across ages, lines, and sexes (Fig. 2; Table S2). Two-way interactions among these effects were also significant, except for age by sex in non-acclimation survivorship (Table S2). The three-way interaction between age, line, and sex explained a significant amount of variation for acclimation survivorship and starvation resistance as well (Table S2). On average, acclimation and non-acclimation survivorship and starvation resistance decreased with age as expected (Fig. 2A, D, J; Table S2). However, age-related decline was stronger in non-acclimation survivorship compared to acclimation survivorship, resulting in an average acclimation score that increased significantly with age (Fig. 2G; Table S2). When the sex-specific cold tolerances were analyzed, male flies maintained their capacity to survive the acclimation treatment across age (Fig. 2B; adj. $P = 0.64$). However, this maintenance across age was not observed in the non-acclimation treatment (Fig. 1E; adj. $P < 0.001$). In females, flies tended to lose the survival capacity at an equal rate for both acclimation and non-acclimation treatments (Fig. 2B, E). As a result of these sex-specific age-related responses, female acclimation score did not change with age (adj. $P = 0.46$; Fig. 2H), but male acclimation score increased (adj. $P < 0.001$). Thus, the population-level increase in acclimation score was likely driven by retention of cold tolerance in the acclimated male flies. Post hoc comparisons of sex-specific starvation resistance revealed that the age-related average decrease in starvation resistance was primarily driven by a significant decrease in resistance in females (adj. $p < 0.001$; Fig. 2K).

Genotype-specific responses for each phenotype were highly variable (Fig. 2, right column; Table S3); an age-related increase in stress resistance was observed for some lines, while responses in other lines remained constant or decreased (Fig. 2, right column; Table S2). Negative acclimation scores were obtained for several lines screened at one week of age,

suggesting that the acclimation treatment had a detrimental effect on survivorship; however, the vast majority of lines responded positively to this treatment (Fig. 2I). When screened at four weeks, the negative acclimation effect largely disappeared as only two lines had acclimation scores below 0. This change in the pattern of cold tolerance with age may have important implications for the role of plasticity in maintaining stress response with age.

Variation in senescence

To assess the relationship between chronological and physiological age, we measured acclimation survival, non-acclimation survival, and acclimation score on ten randomly selected lines from the DGRP (Table S1). Non-acclimation survival of physiologically-aged flies was 0 in all lines, so only acclimation survivorship was compared between the physiologically-aged (Td50) flies and the chronologically-aged (four-week-old) flies. The average acclimation survivorship of the ten lines selected for the physiological aging experiment was comparable to that of the 101 lines. The average proportion survived following acclimation for one-week old flies from the ten lines was 74.0 ± 8.1 S.E. compared to 63.4 ± 1.1 S.E. in 101 lines, while the average proportion survived following acclimation in four-week old flies was 52.2 ± 9.9 S.E. compared to 55.2 ± 1.2 S.E. in 101 lines. Regression analysis indicated that acclimation survivorship measured in four-week old flies was a good predictor of acclimation survivorship in flies that reached a similar physiological age ($R = 0.83$; $P < 0.003$; Fig. 3; Table S4). While longevity does vary among DGRP lines, variation in lifespan did not significantly alter the rank order of acclimation survival among the lines. This suggests that variation in longevity among the DGRP lines does not influence the age-related change in detected in phenotypes between young and old flies.

343

344 **Genetic architecture**

345 In one-week-old flies, association mapping identified 24 polymorphisms and 23
346 genes associated with acclimation survival, 22 polymorphisms and 14 genes associated with non-
347 acclimation survival, 45 polymorphisms and 23 genes associated with acclimation score, and 20
348 polymorphisms and 9 genes associated with starvation resistance (Table 1). In four-week-old
349 flies, association mapping identified 31 polymorphisms and 28 genes associated with acclimation
350 survival, 69 polymorphisms and 48 genes associated with non-acclimation survival, 26
351 polymorphisms and 6 genes associated with acclimation score, and 27 polymorphisms and 22
352 genes associated with starvation resistance (Table 1). Surprisingly, no polymorphisms or genes
353 were shared within phenotype across age or between phenotypes (Fig. S2; Table S5).

354 Several polymorphisms were associated with genes that have been previously associated
355 with cold-, starvation-, or age-related phenotypes, and were distributed across the phenotypes
356 measured in this study (Table S5 and references therein). Out of all genes identified in our study
357 (Table 1, Table S5), 54 have been previously associated with cold acclimation or with a cold-
358 sensitive phenotype in *Drosophila*, 18 have been previously associated with starvation response
359 or stress, and 59 have been previously associated with aging or lifespan. For example, *Cht2*,
360 involved in chitin binding, has been previously associated with cold acclimation response
361 (MacMillan et al., 2016) and was associated with four-week starvation resistance in this study.
362 *Meltrin*, associated with one-week starvation resistance in our study, has been previously
363 associated with cold acclimation response and age-specific fitness (Durham et al., 2014;
364 MacMillan et al., 2016). *CG10916*, associated with four-week non-acclimation survival in our
365 study, has previously been associated with determination of adult lifespan as well as cold

366 acclimation (MacMillan et al., 2016; Paik et al., 2012; Vermeulen et al., 2013). Several genes
367 (28) were also associated with oxidative stress resistance, which has been associated with aging
368 and senescence (Schwarze et al., 1998). For example, *decay*, *rg*, and *Pde1c* have been previously
369 associated with oxidative stress and were associated with four-week acclimation survival or one-
370 week non-acclimation survival in our study (Table S5). Additional details describing the function
371 of each gene and associated references are listed in Table S5. Despite the previous reporting of
372 genes that are associated with aging or stress phenotypes, no gene ontology (GO) categories
373 were overrepresented following enrichment analysis,.
374
375 **Evolutionary theories of aging and the decline in stress response**
376 *Shifting genetic architecture within phenotypes across age*
377 Each phenotype was associated with a unique set of polymorphisms across age and
378 among phenotypes (Table 1, Fig. S2, Table S5). Thus, the lack of overlap of associated
379 polymorphisms across age within phenotypes suggests the genetic architecture shifted and that
380 genetic control of the phenotypes was age-specific. MA not only predicts that associated
381 polymorphisms at each age are unique, but also that associated polymorphisms have positive
382 additive effects in young individuals that remain positive or approach 0 in older individuals.
383 Conversely, AP predicts associated polymorphisms with positive additive effects in young
384 individuals will have negative additive effects in old individuals. We calculated the additive
385 effects of all significantly associated polymorphisms for the stress response phenotypes that
386 declined with age (acclimation survivorship, non-acclimation survivorship, and starvation
387 resistance; Fig. 4) for the one- and four-week response. When additive effects of the associated
388 polymorphisms in one-week-old flies were calculated for the same phenotype in four-week-old

389 flies, the additive effects were either closer to 0 or of the same sign (i.e. not antagonistic; Fig. 4;
390 Table S6). The reverse comparison resulted in the same pattern; for example, additive effects of
391 four-week acclimation survival polymorphisms on one-week acclimation survival were smaller
392 and closer to 0 than the additive effects of four-week acclimation survival polymorphisms on
393 four-week acclimation survival (Fig. 4; Table S6). This pattern of unique associated
394 polymorphisms and additive effects that decrease with age was observed for each of the
395 phenotypes that declined with age (starvation resistance, acclimation survival, and non-
396 acclimation survival) and is consistent with MA.

397 MA also predicts that, for phenotypes that decline with age, the coefficient of genetic
398 variance (CV_G) will increase with age and that the genetic correlation between the phenotype in
399 young individuals will be non-negative (either not different from 0 or positively correlated) with
400 the phenotype in old individuals. While AP does not have predictions specific to the coefficient
401 of genetic variance (Houle et al., 1994; Partridge and Barton, 1993), AP does predict a negative
402 genetic correlation between the phenotype in young individuals and in old individuals. Variance
403 component analysis provided further support for MA for each phenotype. We observed increase
404 in CV_G for each phenotype, although the increase was slight for starvation resistance ($CV_{G,\ young}$
405 = 13.51 versus $CV_{G,\ old}$ 14; Table 1). Also consistent with MA predictions, genetic correlations
406 for each phenotype across age were either significantly positive (acclimation survival: $R_G = 0.70$
407 ± 0.3 S.E.; starvation resistance: $R_G = 0.69 \pm 0.3$ S.E.), or not statistically different from 0 (non-
408 acclimation survival: $R_G = 0.43 \pm 0.3$ S.E.; Table S8). The lack of negative genetic correlations
409 for each phenotype between young and old individuals combined with the increase in the
410 coefficient of genetic variance with age provides additional evidence that supports the MA
411 mechanism.

412

413 *Shifting genetic architecture between phenotypes with age*

414 Comparisons of associated polymorphisms for each phenotype measured in this study
415 demonstrated unique genetic control for each phenotype across age. However, the lack of
416 overlap in associated polymorphisms does not necessarily mean that the polymorphisms detected
417 for each phenotype do not influence variation in other phenotypes at other ages, but rather that
418 the additive effect was too small to be detected by association mapping. Polymorphisms
419 associated with one phenotype (e.g. one-week acclimation survival) may have small but
420 important additive effects on other phenotypes (e.g. four-week starvation resistance), and if this
421 is the case, the interpretation is similar to the comparison of additive effects within phenotypes
422 across age reported above (e.g. Fig. 1). If the additive effect is close to 0 and of the same sign,
423 this supports the MA mechanism; if the additive effect is different from 0 and of the opposite
424 sign, this supports the AP mechanism (Fig. 1).

425 To test for the presence of pleiotropic effects of associated polymorphisms on other
426 phenotypes measured in this study, we calculated the average standardized additive effect of
427 associated polymorphisms for each phenotype on every other phenotype and age (Fig. 4; Table
428 S6). For example, the additive effects of the set of associated polymorphisms with acclimation
429 survival in one-week-old flies were calculated for one- and four-week non-acclimation survival
430 and one- and four-week starvation resistance (Fig. 4A). Confidence intervals were used to
431 determine if the calculated average additive effects were different from 0 (Table S6).

432 Approximately half of the calculated average effects were not different from 0 (55.6%),
433 and 27.8% of the comparisons resulted in average effects with a sign opposite that of the average
434 effect of the polymorphisms in the phenotype with which they were significantly associated

435 (suggesting an antagonistic relationship; Table S6). The antagonistic effects of polymorphisms
436 between phenotypes were often, but not always, reciprocal (Fig. 4). For example, polymorphisms
437 associated with one-week acclimation survival had an average antagonistic additive effect on
438 starvation resistance at both ages (Fig. 4A), while polymorphisms associated with four-week
439 starvation resistance had an average antagonistic additive effect on only one-week acclimation
440 survival (Fig. 4F). In an aging context, evidence from additive effect comparisons support both
441 AP and MA across age, depending on the phenotypes being compared; however, MA was more
442 common based on apparent independence of additive effects (average additive effects were not
443 different from 0).

444 Phenotypes that appeared to be antagonistically pleiotropic such that polymorphisms
445 increased the phenotype in young flies but decreased the phenotype in old flies include
446 acclimation survival and starvation resistance (Fig. 4A, B, F) and non-acclimation survival and
447 starvation resistance (Fig. 4C, D, F). Polymorphisms associated with one-week starvation
448 resistance did not have an antagonistic effect on average on any other phenotype, although
449 several individual polymorphisms did have antagonistic effects on other phenotypes (Fig. 4E;
450 Table S6). The antagonistic relationship between one-week acclimation survival and four-week
451 starvation resistance was further supported by a significant negative genetic correlation between
452 traits across ages (R_G = -0.47 ± 0.2 S.E.; Table S8). However, all other combinations of
453 phenotypes involved effects and genetic correlations that were not different from 0 (Fig. 4; Table
454 S6, S8), and were thus more consistent with the predictions of MA.

455

456 **Evidence of selection and phenotypic trade-offs**

With the exception of acclimation score, the additive effects of the majority of polymorphisms significantly associated with the phenotypes measured in our study were of the same sign (i.e. most additive effects were positive or negative; Fig. 4 and 5, Table S5). To determine if more additive effects of positive sign were associated with the phenotype than expected by chance, we used the QTLST to test for evidence of selection. More positive additive effects than expected by chance were observed for one-week acclimation survival, non-acclimation survival, and acclimation score suggesting selection increased these phenotypes in the founding population of the DGRP (Fig. 4 and Fig. 5A, C, E). The QTLST was also significant for four-week acclimation survival, non-acclimation survival, and both one- and four-week starvation resistance, suggesting selection has acted to decrease these phenotypes (Fig. 4 and Fig. 5B, D, G, H). However, because the effectiveness of natural selection is expected to decline with age, this significant result is likely the result of a correlated response resulting from selection on young phenotypes. The signs of the additive effects of polymorphisms associated with acclimation score in four-week-old flies were more mixed than any other phenotype, leading to a non-significant QTLST for this phenotype (Fig. 4 and Fig. 5F).

Discussion

Genetic variation in age-specific decline in stress tolerance

As expected, the average phenotypic responses for most phenotypes declined with age, with the exception of plasticity measured as acclimation score (Fig. 2). For each phenotype, we observed significant genetic variation across ages, with some genotypes exhibiting increased stress resistance with age. We investigated variation in longevity among DGRP lines by comparing cold tolerance responses measured at four weeks to those in physiologically aged flies

480 at the point when the population reached Td50. We know that lifespan for virgin female flies
481 varies from approximately 20 days to approximately 80 days in the DGRP (Ivanov et al., 2015).
482 This variation could result in an inequivilence of line comparisons at four weeks. Based on
483 (Ivanov et al., 2015) longevity estimates, some lines may be only half way through their life span
484 while others may be closer to the end of their life span at four weeks of age. Furthermore, genetic
485 shifts known to be associated with senescence would be further advanced in lines that age more
486 quickly (Pletcher et al., 2002). The significant positive correlation in acclimation survival
487 between chronologically aged (four-week) flies and physiologically aged (Td50) flies suggests
488 that, despite variation in the rate of senescence among lines, the four-week point is representative
489 of how an "old" fly responds to cold stress. Additionally, this subset of the DGRP lines reached
490 Td50 well after four weeks of age (Table S4), indicating that the four-week aging period does
491 not lead to many lines entering a late-life plateau in mortality and that our lines were likely in the
492 aging phase (Charlesworth, 2001; Curtsinger, 2016)

493 The only phenotype in our study that behaved unexpectedly and did not decline with age
494 was plasticity measured as acclimation score. Acclimation score was significantly higher in four-
495 week-old flies (Fig. 2G), where we expected this phenotype to remain constant across age. The
496 age-related response in acclimation score was driven by the male response. Four-week-old male
497 flies had a stronger age-related decline in non-acclimation survival compared to acclimation
498 survival. Therefore, the observed increase in acclimation score for our population has at least two
499 interpretations. First, plasticity at the population level may increase with age, potentially as a
500 compensatory mechanism to overcome the overall loss of basal cold tolerance (Fig. 2F).
501 Throughout the season, natural populations of *D. melanogaster* are expected to be composed of
502 increasingly old individuals such that by the time temperatures begin to cool at the beginning of

503 the fall season, a greater proportion of populations is composed of older individuals (Behrman et
504 al., 2015). If older individuals are less cold tolerant, they may still be able to tolerate cold
505 temperature exposures through increased capacity for adaptive plasticity through acclimation.
506 Second, acclimation pretreatment appears to have had a less detrimental effect on survival in
507 four-week-old flies compared to one-week-old flies (Fig. 2I). In one-week-old flies, acclimation
508 improved survival in the majority of lines; however, several lines (14%) had negative
509 acclimation scores, indicating that exposure to 4°C prior to the -6°C exposure was more
510 damaging than the -6°C exposure alone (Fig. 2I; Gerken et al., 2015). Only 2% of lines tested
511 had negative acclimation scores at four weeks suggesting that acclimation may be more likely to
512 either improve or not affect survival following cold stress at four weeks. Shifts in the effect of
513 acclimation on survival were observed when genotypes were reared under different
514 developmental conditions or experienced altered thermal regimes (Gerken et al., 2015; Kelty and
515 Lee, 2001); our results suggest that the age of the individual has an important influence on the
516 effect of acclimation on survival as well.
517
518 **MA describes age-related change within individual phenotypes**
519 When each phenotype was considered separately across age, we found support for MA,
520 satisfying predictions based on analysis of quantitative genetic parameters (Charlesworth, 2001;
521 Charlesworth and Hughes, 1996; Hughes et al., 2002; Leips et al., 2006; Reynolds et al., 2007).
522 First, the coefficient of genetic variance increased with age for each phenotype (Table 1). The
523 increase in CV_G in starvation resistance was less drastic than other phenotypes, but when sexes
524 were analyzed separately, the increase was more dramatic (Table S7). An increase in CV_G
525 indicates that a greater proportion of the phenotypic variance can be explained genetically at four

526 weeks of age (Charlesworth, 2001; Charlesworth and Hughes, 1996; Houle et al., 1994) and this
527 increase is consistent with the hypothesis that the age-related decline in the phenotype is the
528 result of the accumulation of deleterious age-specific polymorphisms that influence variation in
529 the phenotype (Charlesworth, 2001; Charlesworth and Hughes, 1996; Engström et al., 1989).

530 Second, MA predicts that a phenotype is controlled by unique sets of genes across age
531 (Charlesworth, 2001, 1994; Charlesworth and Hughes, 1996; Maklakov et al., 2015; Medawar,
532 1952; Partridge and Barton, 1993; Rose, 1991). We detected unique sets of polymorphisms that
533 were associated with each phenotype across age. This is consistent with age-specific association
534 patterns presented by (Durham et al., 2014) who determined age-related change in fecundity was
535 also influenced by MA. The genetic independence of phenotypes across age was further
536 supported by the non-significant or significantly positive genetic correlations for each phenotype
537 across age (Table S8). While positive genetic correlations suggest that the genetic control of the
538 phenotype across age is not independent (inconsistent with MA), Charlesworth (2001) and
539 Maklakov et al. (2015) suggest that positive pleiotropy can lead to significantly positive genetic
540 correlations across age under MA.

541 Positive pleiotropy (Maklakov et al., 2015) expands on MA predictions in that, instead of
542 limiting polymorphisms to very narrow windows of effect, polymorphisms can influence a wider
543 window of ages but with lower additive effects (Maklakov et al., 2015). Thus, the positive
544 genetic correlations across age are the result of the associated polymorphisms having a slightly
545 wider window of age-specific effects that ultimately influence the phenotype at other ages. This
546 pattern was observed for acclimation and non-acclimation survival and starvation resistance
547 across age; for all phenotypes, the four-week associated polymorphisms had negative additive
548 effects on the one-week phenotype that were smaller than the effect of the polymorphisms on the

549 four-week phenotype. This suggests that four-week polymorphisms that led to decline in each
550 phenotype do have small pleiotropic effects (in the same direction) at one week of age. The
551 failure of natural selection to remove polymorphisms that have small negative effects early in life
552 and larger negative effects later in life is one way in which senescence can evolve and is
553 consistent with expectations of positive pleiotropy under MA (Maklakov et al., 2015; Wachter et
554 al., 2014, 2013).

555 Our third piece of evidence to support MA comes from our novel approach of calculating
556 the additive effects of polymorphisms across age. We calculated the additive effects of
557 polymorphisms associated with the one-week phenotype in the four-week phenotype data (and
558 vice versa). Under MA, we expected the additive effect of the one-week polymorphisms to be
559 smaller and in the same direction (i.e. they have the same sign) when the additive effects were
560 calculated for the four-week response data (Maklakov et al., 2015). If the sign of a particular
561 polymorphism had flipped (a polymorphism with a positive effect in one age had a negative
562 effect in the other age), this would have suggested that an antagonistic relationship existed and
563 would have supported AP (Maklakov et al., 2015). For all phenotypes, the calculated additive
564 effects of age-specific polymorphisms across age were either closer to 0 and/or in the same
565 direction, providing definitive support for the role of MA in the age-related decline in the stress
566 responses measured (Fig. 4).

567

568 **MA and AP describe age-related variation between phenotypes**

569 Our novel extension of association mapping through the calculation of additive effects of
570 polymorphisms across phenotypes allowed us to investigate the role MA and AP on age-specific
571 responses between phenotypes as well. Though each phenotype and age was associated with a

572 unique set of polymorphisms (consistent with predictions for MA), we found support for AP
573 between several phenotypes (Fig. 4). We observed a significantly negative phenotypic
574 correlation between one-week acclimation survival and both one- and four-week starvation
575 resistance, corroborating a pattern reported by (Hoffmann et al., 2005) (Table S8). We also
576 observed a significantly negative genetic correlation between one-week acclimation survival and
577 four-week starvation resistance (Table S8), suggesting that AP (between one-week acclimation
578 survival and four-week starvation resistance) influenced age-related change in these phenotypes.
579 When the additive effects of polymorphisms associated with four-week starvation were
580 calculated for both one-week cold tolerance phenotypes (Table S6), all four-week starvation
581 resistance associated polymorphisms, which had negative additive effects on four-week
582 starvation resistance, had positive additive effects on one-week acclimation survival and one-
583 week non-acclimation survival (Fig. 4F). The change in sign of the additive effects of four-week
584 starvation resistance associated polymorphisms on both of the one-week cold tolerance
585 phenotypes is strong evidence to support the role of AP in age-related decline in starvation
586 resistance.

587 Additional examples of AP existed between phenotypes in our data as well and were
588 identified through confidence interval analysis of additive effects (Fig. 4; Table S6). Specifically,
589 one-week acclimation and non-acclimation survival polymorphisms had positive effects on their
590 respective phenotypes across age but negative additive effects on four-week starvation resistance
591 (Fig. 4A – D; Table S6). This pattern is consistent with that discussed above and again suggests
592 that age-related change in starvation resistance is influenced by AP with cold tolerance.
593 Interestingly, four-week acclimation and non-acclimation survival polymorphisms had largely
594 positive additive effects on one-week starvation resistance (Fig. 4B, D; Table S6). This pattern

595 suggests that AP may also be contributing to age-related decline in acclimation and non-
596 acclimation survival. With evidence from our examination of acclimation and non-acclimation
597 survival across age (discussed above), and the apparent role of MA and positive pleiotropy for
598 age-related change within these phenotypes, it is evident that it may not be possible to fully
599 disentangle the roles of MA and AP on the age-related decline in phenotypes. In essence,
600 polymorphisms that increase one phenotype in young individuals and decrease another
601 phenotype in old individuals through AP may also contribute to age-related change within the
602 phenotype through positive pleiotropy under MA. These results demonstrate the need for caution
603 in interpreting the lack of overlap in significant associated polymorphisms as support for MA in
604 isolation of other evidence because AP may still be playing an important role in the age-related
605 change in phenotypes.

606 We also found support for the role of MA between phenotypes across age. Age-related
607 change in acclimation survival and non-acclimation survival appears to be evolving largely
608 independently of the other phenotype under MA as we found either non-significant or
609 significantly positive phenotypic and genetic correlations between all age combinations of these
610 phenotypes (Table S6, S8). The calculated additive effects of polymorphisms between
611 phenotypes and ages uphold this interpretation to a large degree as well (Fig. 4A – D; Table S6).
612 One-week acclimation survival polymorphisms, which had positive effects on one-week
613 acclimation survival, all had positive additive effects when calculated for both one- and four-
614 week non-acclimation survival (Fig. 4A). On average, additive effects of polymorphisms
615 associated with four-week acclimation survival had additive effects that were not different from
616 zero, although some individual polymorphisms did have antagonistic effects on one- and four-
617 week non-acclimation survival. All but two polymorphisms associated with one-week non-

618 acclimation survival had additive effects of the same sign on one- and four-week acclimation
619 survival, and all polymorphisms associated with four-week non-acclimation survival had
620 additive effects of the same sign on one- and four-week acclimation survival. While the small
621 number of individual polymorphisms with antagonistic additive effects across phenotype may
622 impact age-related change in these cold tolerance phenotypes, it likely that this impact is small in
623 comparison to the role of MA and positive pleiotropy.
624
625 **Natural selection shapes phenotypic variation across age**
626 Our data recapitulate previously reported relationships between different measures of
627 cold tolerance and starvation resistance (Table S8; Gerken et al., 2015; Hoffmann et al., 2005;
628 Sinclair and Roberts, 2005); however, we have added another layer to these relationships by
629 considering the influence of age and natural selection on these phenotypes. Specifically, the
630 direction of the additive effects of polymorphisms associated with each phenotype may reflect
631 the role natural selection has played in the evolution of the phenotype. Through applying a basic
632 sign test (QTLST probabilities) to the additive effects of the associated age-specific
633 polymorphisms, it is evident that the signs of the effects are not randomly associated with the
634 frequency of the allele (Fig 5A – E, G, H). If a phenotype is evolving neutrally, polymorphisms
635 that influence the phenotype are equally likely to have positive or negative effects (Anderson and
636 Slatkin, 2003; Orr, 1998; Rice and Townsend, 2012). Signatures of directional selection are
637 detected when this null hypothesis is rejected (Anderson and Slatkin, 2003; Muir et al., 2014;
638 Orr, 1998; Rice and Townsend, 2012; Rieseberg et al., 2002). Because we observed an
639 overabundance of major alleles with positive effects on one-week acclimation and non-
640 acclimation survival, this suggests that natural selection favored polymorphisms that increase

641 cold tolerance phenotypes in the population from which the DGRP was established (Fig. 4A, C
642 and 5A, C; Table S5). This finding is consistent with evidence of selection for cold tolerance in
643 natural populations of *D. melanogaster* (Bergland et al., 2014), as well as previous reports of
644 majority positive additive effects of polymorphisms associated with chill coma recovery
645 (Mackay et al., 2012).

646 Conversely, most of the polymorphisms associated with cold tolerance at four weeks of
647 age were negative (Fig. 4B, D), indicating that the major alleles decreased survival following
648 cold stress. While the QTLST was significant for these late-acting polymorphisms, it is very
649 unlikely that natural selection directly led to this pattern. Instead, polymorphisms associated with
650 acclimation and non-acclimation survival in old individuals likely arose through mutation and
651 were maintained in the population because their negative effect on survival in young individuals
652 was small relative to the four-week additive effects (Charlesworth, 2001; Houle et al., 1994;
653 Maklakov et al., 2015)Fig. 4B, D). Similarly, in both young and old individuals, most of the
654 additive effects of starvation resistance associated polymorphisms were negative (Fig. 4E, F).
655 This pattern suggests that age-related change in starvation resistance is influenced by positive
656 selection on acclimation and non-acclimation survival in young individuals. Alternatively, the
657 effectiveness of natural selection on starvation resistance may be constrained by pleiotropy
658 between one-week acclimation survival and one-week starvation resistance (many one-week
659 acclimation and non-acclimation polymorphisms had negative effects on one-week starvation
660 resistance; Fig. 4A, C). Some positive selection on starvation resistance in young individuals
661 may provide a mechanism for the retention of four-week acclimation and non-acclimation
662 survival associated polymorphisms that have increasingly negative effects with age.
663

664 **Implications for evolutionary theories of aging**

665 Efforts to more fully understand and test evolutionary theories of aging have encouraged
666 expansion and clarification of predictions of both MA and AP mechanisms (Charlesworth, 2001;
667 Houle et al., 1994; Maklakov et al., 2015; Reynolds et al., 2007; Wachter et al., 2014, 2013). Our
668 novel extension of existing methods not only revealed the relative importance of MA and AP for
669 age-related change in stress response, but also verified recent hypotheses that present expansions
670 on the theory of MA. When originally formulated, the MA mechanism predicted that fitness was
671 controlled by polymorphisms that had very narrow windows of effect (Charlesworth and
672 Hughes, 1996; Medawar, 1952; Rose, 1991), but evidence from several studies has indicated that
673 it is more likely that polymorphisms which contribute to late-life decline in fitness and age-
674 related change in phenotypes have wide windows and increasingly large effects across age
675 (Houle et al., 1994; Maklakov et al., 2015). Patterns of additive effects of associated
676 polymorphisms identified in our study align well these elaborations of MA predictions and
677 provide empirical support for positive pleiotropy. In addition, we provide evidence supporting
678 the potential for positive genetic correlations between young and old phenotypes under MA,
679 demonstrating through patterns of additive effects of polymorphisms that, while the size of the
680 effect may change across age, the direction of the effect is often preserved (Maklakov et al.,
681 2015; Reynolds et al., 2007).

682 Our use of calculated additive effects also allowed us to overcome difficulties in
683 characterizing the role of AP in age-related change in phenotypes. The isolated analysis of the
684 coefficient of genetic variance and genetic correlation is problematic because the signature of AP
685 may be too small to detect due to near fixation of segregating alleles at the antagonistic loci
686 (Moorad and Promislow, 2009; Partridge and Barton, 1993; Schnebel and Grossfield, 1988). For

this reason, association mapping may be particularly biased against the detection of AP loci. However, by comparing the effects of polymorphisms calculated for each age and phenotype pair, we are able to overcome this bias against polymorphisms that have small effects.

Very few studies present convincing evidence of the influence of both MA and AP on age-related change within and among phenotypes (but see Leips et al., 2006), but we have demonstrated that both mechanisms contribute to age-related change in stress response. It is clear from our results that individual polymorphisms that are significantly associated with phenotypes at different ages can contribute to age-related decline within and among phenotypes in patterns that are consistent with both MA and AP. Thus, the evolution of senescence and associated decline in fitness is influenced by a combination of natural selection acting on correlated phenotypes that have non-independent antagonistic genetic architectures, as well as the accumulation of polymorphisms with negative effects that strengthen with age. It is likely that similar patterns will be observed for other phenotypes related to fitness as well, adding to our understanding of how evolution of aging and multivariate evolution are tightly intertwined.

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Conflict of Interest

No competing interests declared.

710

711 **Author Contributions**

712 E.R.E and T.J.M. conceived the experimental framework and prepared the manuscript. E.R.E.

713 performed the research and the statistical analyses.

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Tables and Figures

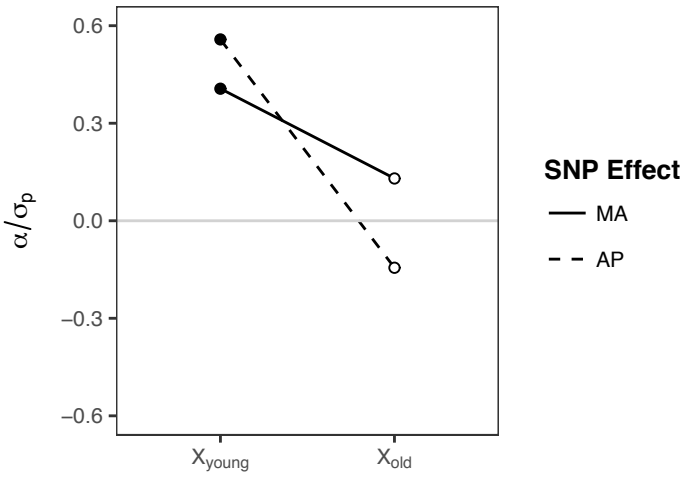
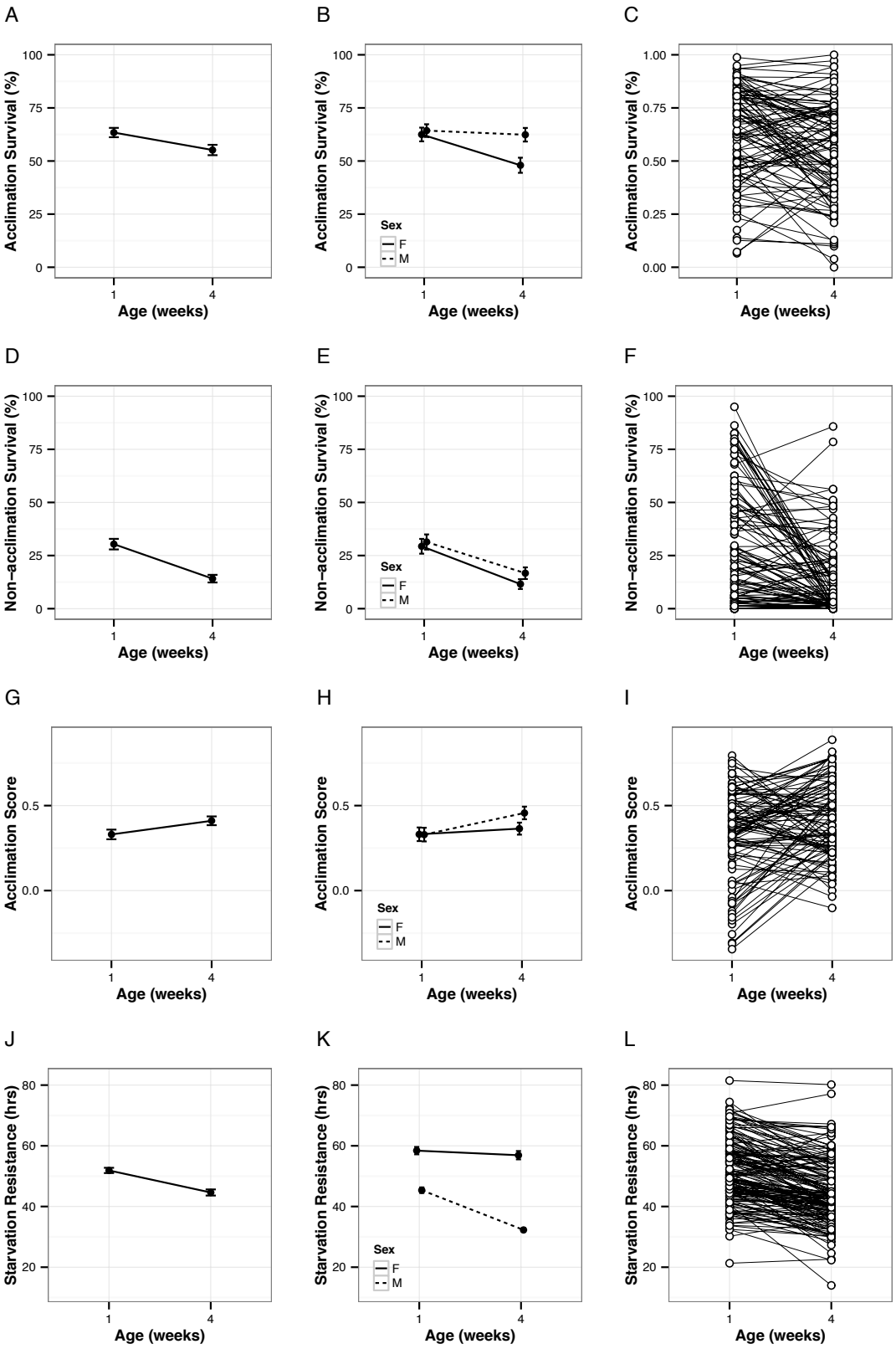


Figure 1. An example of how calculated additive effects (α/σ_p) of polymorphisms identified through association mapping can be used to characterize the role of mutation accumulation (MA) and antagonistic pleiotropy (AP) on the age-related change in hypothetical phenotype *X*. Two polymorphisms that are associated with phenotype *X* in young individuals (X_{young}) but not in old individuals (X_{old}). The additive effects of the associated polymorphisms at young ages are represented by the closed symbols. The open symbols indicate the additive effects of X_{young} polymorphisms on the phenotype at old age (X_{old}). The polymorphism connected with a solid line demonstrates the shifting effect across age consistent with MA, where the polymorphism has a small additive effect of the same sign (positive) on the phenotype at old age (X_{old}) as the effect of the polymorphism on X_{young}. The polymorphism connected with a dashed line illustrates an AP pattern, where the polymorphism has an additive effect that is of the opposite sign (negative) on X_{old} compared to the effect of the polymorphism on X_{young}.



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916 **Figure 2**. Plots of mean phenotypes across age are shown with 95% CI for the total population
917 (left column) and by sex (middle columns); among-line variation observed for each phenotype is
918 shown as line averages (right column). A. Average acclimation survival declined significantly
919 with age ($F_{1,1212} = 49.5$, $P < 0.001$). B. Males retained their ability to survive the acclimation
920 treatment, while acclimation survival significantly declined in females (age by sex: $F_{1,1212} =$
921 28.9, $P < 0.001$). C. Acclimation survival significantly varied among the 101 DGRP lines (age
922 by line: $F_{100,1212} = 3.21$, $P < 0.001$). D. Average non-acclimation survival declined significantly
923 with age ($F_{1,1212} = 215.4$, $P < 0.001$). E. Non-acclimation survival significantly declined in both
924 females and males to a similar degree (age by sex: $F_{1,1212} = 1.98$, $P = 0.16$). F. Non-acclimation
925 survival significantly varied among the 101 DGRP lines (age by line: $F_{100,1212} = 5.88$, $P < 0.001$).
926 G. Average acclimation score increased significantly with age ($F_{1,1212} = 25.13$, $P < 0.001$). H.
927 Acclimation score significantly increased in males, but remained consistent across age for
928 females (age by sex: $F_{1,1212} = 8.68$, $P < 0.01$). I. Acclimation score significantly varied among
929 the 101 DGRP lines (age by line: $F_{100,1212} = 3.18$, $P < 0.001$). J. Average starvation resistance
930 decreased significantly with age ($F_{1,1623} = 893.0$, $P < 0.001$). K. Starvation resistance
931 significantly decreased in both sexes, but to a larger degree in females (age by sex: $F_{1,1623} =$
932 567.0, $P < 0.001$). L. Starvation resistance significantly varied among the 164 DGRP lines (age
933 by line: $F_{163,1623} = 6.0$, $P < 0.001$).
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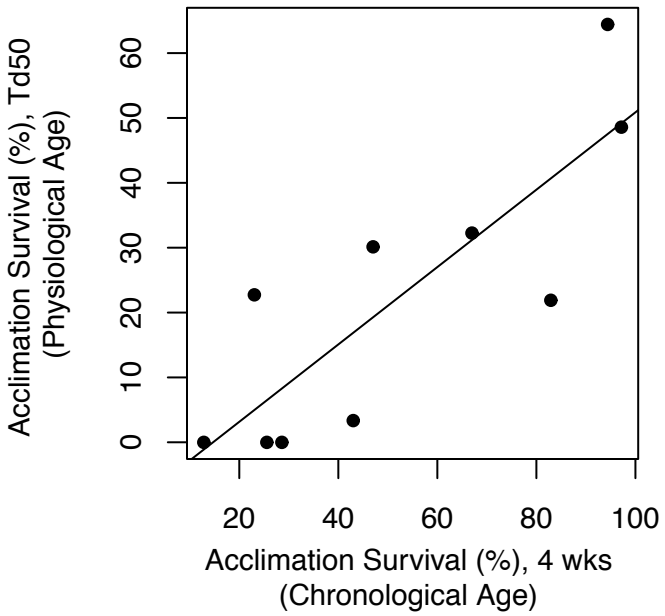

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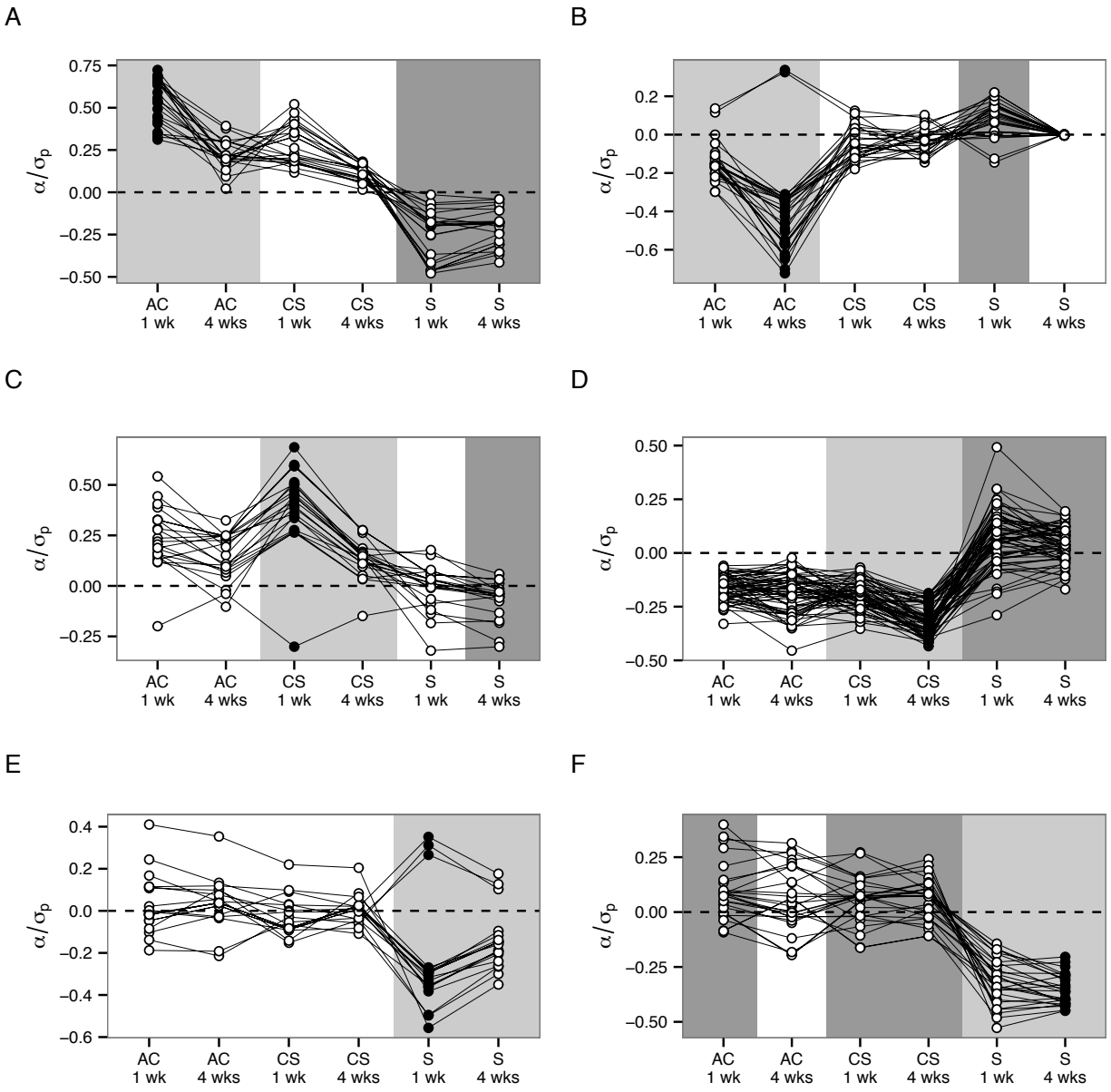
936 **Figure 3**. Stress tolerance of 4-week-old flies was correlated with stress tolerance of flies that
937 were a similar physiological age (Td50). Acclimation and non-acclimation survivorship was
938 measured for 10 DGRP lines that were aged to four weeks (chronological age) and until
939 populations in experimental vials reached 50% of the starting population (physiological age).
940 Physiologically-aged flies did not survive the non-acclimation treatment. Acclimation
941 survivorship in four-week old flies is a good predictor of acclimation survivorship in flies that
942 are approximately the same physiological age ($R = 0.833$, $P < 0.003$). Points shown indicate
943 average acclimation survival for the 10 DGRP lines.



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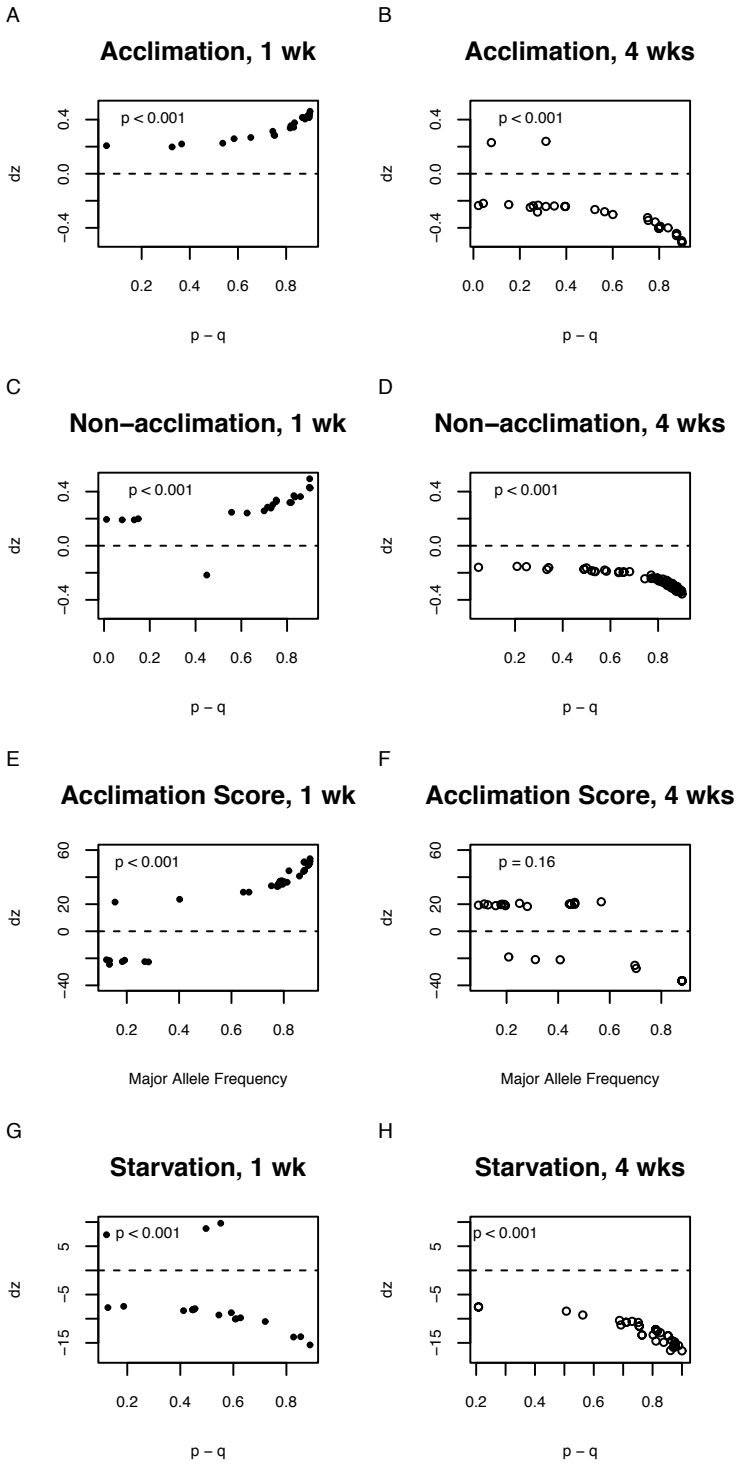
945 **Figure 4**. Standardized additive effects (α/σ_p) of polymorphisms associated with each
946 phenotype across age (A – F). In each plot, black solid points indicate which age and phenotype
947 the polymorphisms were significantly associated with. Open points indicate the calculated
948 additive effects of those polymorphisms on each of the other phenotypes. The light grey shading
949 highlights the 'within phenotype' change in the additive effect of polymorphisms across age.
950 Darker grey shading highlights 'between phenotype' comparisons where antagonistic effects of

polymorphisms were found based on analysis of 95% confidence interval around the average additive effect (Table S6). Unshaded sections of each plot indicate calculated additive effects that are consistent with MA relative to the additive effects of the polymorphisms on their associated phenotype. In each plot, AC indicates acclimation survival, CS indicates non-acclimation survival, and S indicates starvation resistance. A. Polymorphisms associated with acclimation survival of one-week-old flies all had positive additive effects on the phenotype that became smaller when the additive effects were calculated for four-week-old acclimation survival and non-acclimation survival of one- and four-week-old flies. Polymorphisms had antagonistic effects on starvation resistance at both ages. B. Polymorphisms associated with acclimation survival of four-week-old flies had smaller additive effects on every other phenotype except one-week starvation resistance, where the effects were largely antagonistic. C. Polymorphisms associated with non-acclimation survival of one-week-old flies had smaller additive effects on every other phenotype except four-week starvation resistance, where the effects were largely antagonistic. D. Polymorphisms associated with non-acclimation survival of four-week-old flies had smaller additive effects on every other phenotype except starvation resistance at both ages, where the effects were largely antagonistic. E. Polymorphisms associated with one-week starvation resistance had smaller additive effects on every other phenotype. F. Polymorphisms associated with four-week starvation resistance had antagonistic effects on every other phenotype except acclimation survival in four-week-old flies and starvation resistance in one-week-old flies.



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972 **Figure 5**. Plots of allele frequency (p – q) against the effect of significant polymorphisms
973 reflected as change in phenotype (dz). Inset numbers are QTLST probabilities that directional
974 selection influenced the phenotype.

975 **Table 1**. Quantitative genetic estimates (± S.E.) for all phenotypes as they vary with age and the
976 number of polymorphisms (generalized as SNPs) and genes significantly associated with each
977 phenotype identified by GWAS with a threshold of –log10(5). All heritabilities reported are
978 broad-sense and are greater than 0.

Phenotype	No. Lines	Age (weeks)	Mean	H^2	CV_G	CV_E	No. SNPs	No. Genes
Acclimation	101	1	63.4 (1.1)	0.26 (0.04)	22.68	38.58	24	23
Acclimation	101	4	55.2 (1.2)	0.19 (0.04)	25.60	52.70	31	28
Non-acclimation	101	1	30.4 (1.3)	0.33 (0.04)	58.76	84.52	22	14
Non-acclimation	101	4	14.1(0.9)	0.26 (0.05)	83.86	141.09	69	48
Acclimation Score	101	1	33.03 (1.4)	0.20 (0.03)	50.53	101.67	45	23
Acclimation Score	101	4	41.1 (1.3)	0.14 (0.02)	31.94	79.03	26	6
Starvation	164	1	51.89 (0.4)	0.34 (0.03)	13.51	18.66	20	9
Starvation	164	4	44.62 (0.5)	0.13 (0.02)	14.00	36.45	27	22

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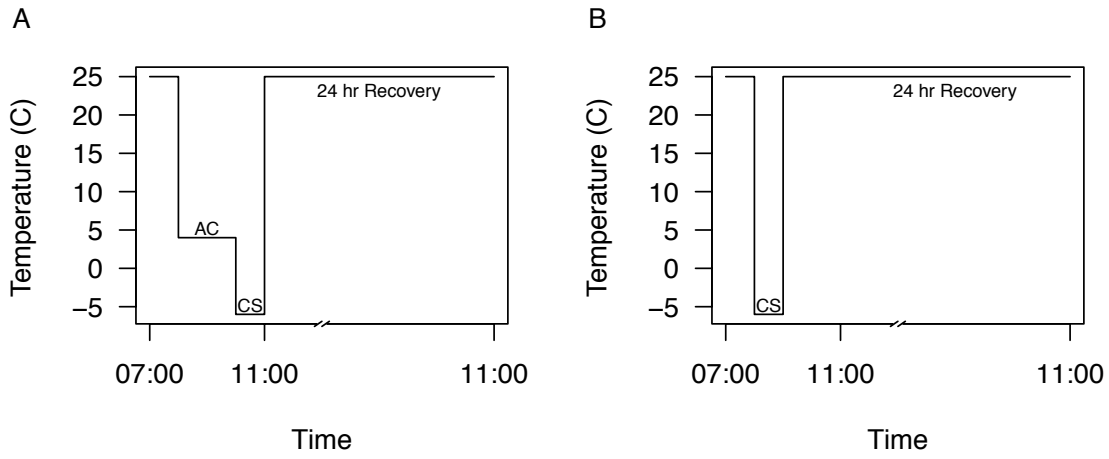
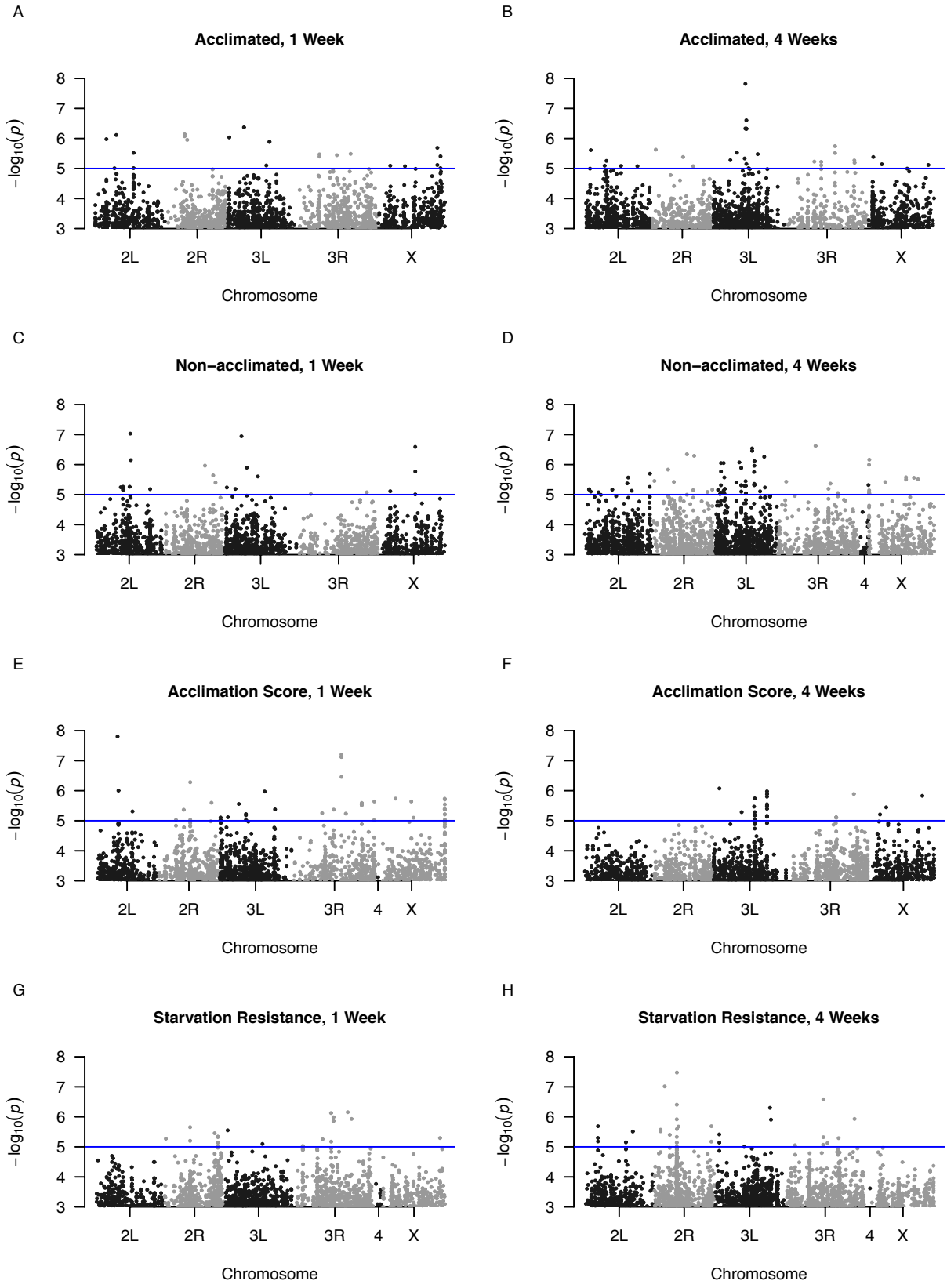


Figure S1. Graphical representation of acclimation (A) and non-acclimation treatment (B). Flies were maintained at 25°C during rearing and recovery, and lights on occurred at 07:00hrs. A. Flies were transferred from 25°C to 4°C for two hours for the acclimation (AC) treatment and then were transferred immediately to -6°C for one hour for the cold shock treatment (CS). Flies were placed on fresh media and allowed to recover for 24 hours at 25°C. B. Flies were transferred to -6°C for one hour for the cold shock treatment (CS) and were allowed to recover at 25°C for 24 hours on fresh media.



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990 **Figure S2**. Manhattan plots of each phenotype. The significance threshold is indicated by the

991 horizontal line at –log(5).