The mechanisms of seasonal adaptation in *Drosophila melanogaster*

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ABSTRACT

Environmental heterogeneity is ubiquitous across space and time and can be a form of balancing selection that maintains genetic variation. Deciphering the mechanisms and traits associated with adaptation to environmental heterogeneity is an important task in evolutionary biology. Adaptive evolution and phenotypic plasticity are two important adaptive mechanisms. Gene expression traits provide a great opportunity to study how populations cope with environmental heterogeneity since they allow us to infer physiological functions and assess the adaptive mechanisms. In my dissertation, I address the adaptive mechanisms under seasonal adaptation in the model organism *Drosophila melanogaster* from three perspectives, including genetics, gene expression and ecologically important traits. In Chapter 1, I utilized publicly available datasets to compare the adaptive signals at expression quantitative trait loci (eQTLs) between space and time. I find that the adaptive signals between space and time differ at eQTLs. While adaptation to space across latitudinal clines show strong signals at eQTLs, there is weak seasonal adaptive signal. In addition, seasonal adaptation at eQTLs show idiosyncratic patterns across different populations. These results suggest that adaptation at eQTLs across seasons is likely distinct from that across latitudinal clines. In Chapter 2, I investigate the plasticity in gene expression across 10 seasonal time points using flies reared in an experimental orchard. By modeling gene expression variation across seasons and across associated temperature ranges, I find that seasonal gene expression plasticity is prevalent and that the plastic genes are functionally enriched. Interestingly, the direction of plastic gene expression changes across seasons shows maladaptive signal. In addition, eQTLs associated with plastic genes are depleted for seasonal SNPs, suggesting that plasticity and genetic evolution have limited overlap at the eQTLs. In Chapter 3, I investigate the seasonal plasticity of three fitness traits (body size, developmental time,

fecundity) and assessed whether temperature is associated with their plasticity. I find that seasonal developmental temperatures can elicit phenotypic plasticity in wild seasonal environments. Moreover, I show evidence that seasonal phenotypic plasticity in developmental time and body size are likely adaptive. In general, my work challenges the previous assumption that seasonal adaptation parallels clinal adaptation by showing distinct adaptive signals between space and time at eQTLs. In addition, I show that plasticity in gene expression is prevalent across seasons and that plasticity and genetic evolution likely have limited overlap at eQTLs. However, seasonal plasticity in gene expression shows maladaptive signal. Finally, I show that seasonal developmental temperature in the wild can elicit plastic response in fitness traits and such plasticity could contribute to seasonal population size dynamics. Taken together, my dissertation can advance our understanding of how populations cope with temporal environmental heterogeneity across seasons from the genetics, gene expression, and fitness-related phenotypic levels.

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Introduction

Temporal environmental heterogeneity across seasons imposes strong selection pressures on all living organisms (Dobzhansky and Ayala 1973). Such seasonal selection is viewed as a form of balancing selection that mirrors spatial selection pressures across latitudinal clines and can maintain phenotypic and genetic variation (Rhomberg and Singh 1988; Bergland et al. 2014; Behrman et al. 2015). Populations of organisms can adopt different mechanisms to cope with seasonal environmental shifts. Adaptive evolution and phenotypic plasticity are two well characterized mechanisms (Meyers and Bull 2002). Theory predicts that populations adopt these mechanisms based on the predictability and the scale of environmental changes (Botero et al. 2015). For example, adaptive evolution caused by cross generational genetic change is ideal for organisms with short life spans. For long-lived organisms, phenotypic plasticity can allow the production of various environmentally induced phenotypes within a single generation. Transitioning from one mechanism to another likely lead to a populational collapse (Botero et al. 2015). Therefore, phenotypic plasticity and genetic evolution should have limited overlap.

Gene expression traits allow us to infer physiological functions (Gracey 2007) and thus provides a good perspective to study the adaptive mechanisms. Expression traits have been shown to be important for both inter- and intra- species adaptive evolution (e.g., King and Wilson 1975; Fraser et al. 2010; Juneja et al. 2016; Mack et al. 2018). In addition, the identification of expression quantitative trait loci (eQTLs) and their spatial and temporal allele frequency distributions allows us to assess the evolutionary trajectories of expression variation across space and time (Fraser 2013). However, little is known about whether spatial and temporal gene expression adaptation signals at eQTLs are reflective of each other.

Adaptive expression plasticity is also an important mechanism for organisms to cope with seasonality. For example, expression plasticity associated with seasonal migration (Johnston et al. 2016), reproductive status (Nakane and Yoshimura 2014), hibernation (Schwartz et al. 2013; Vermillion et al. 2015) and immune response (Dopico et al. 2015) are important seasonal traits in various species. In contrast to being adaptive, plastic responses can also be maladaptive. Maladaptive plasticity can be characterized when the direction of plastic expression is the opposite as ("counter-gradient") evolved changes (e.g., Huang et al. 2022). Examples of adaptive and maladaptive plasticity widely exist in a broad range of species (Ghalambor et al. 2015; Huang and Agrawal 2016; Leonard and Lancaster 2020; Josephs et al. 2021; Huang et al. 2022). However, we lack an understanding of whether seasonal genome-wide expression plasticity shows adaptive or maladaptive signals. Furthermore, we have limited knowledge in whether expression plasticity and genetic evolution have limited overlap at the eQTLs level.

Drosophila melanogaster is an ideal system to study the mechanisms under seasonal adaptation. *D. melanogaster* is broadly distributed across both space and time (Kapun et al. 2021). Phenotypic and genetic latitudinal clines as well as evolutionary changes across seasons are well-documented. For example, lab reared descendants of flies collected in the spring or northern locales are more starvation tolerant and show a wider breadth of thermal tolerance than those who are the descendants of flies collected in fall or southern locales (Schmidt et al. 2005, 2008; Schmidt and Paaby 2008; Behrman et al. 2015). Genetic differences across seasons sometimes parallels those of clinal differences (Bergland et al. 2014; Machado et al. 2021; Rodrigues et al.

2021). Additionally, phenotypic plasticity in response to temperature or other environmental factors are also well characterized in this species (e.g., Zhou et al. 2012; Chen et al. 2015). However, some questions in studying adaptation to seasonality in this species remain to be investigated. First, we lack an understanding of the adaptive signals at eQTLs across seasons and whether it is reflective of clinal adaptation. Second, seasonal plastic gene expression data is needed in order to understand whether expression plasticity show adaptive signals across seasons. Third, we lack the understanding of the plastic response of fitness traits across seasons in wild environments.

With my dissertation, I aim to address the mechanisms under seasonal adaptation in fly populations from multiple levels, including genetics, gene expression and fitness traits. I first ask whether there is strong seasonal adaptive signal at eQTLs and whether it mirrors that of clinal adaptation. Next, to assess the mechanism of plasticity and infer physiological functions, I measured plastic seasonal gene expression variations in a genetically controlled fly population. I created pools of F_1 genotypes from inbred lines and reared them in an experimental orchard in Charlottesville, VA (Morven Farms, VA: 37.96°N, -78.47°W) and also in lab conditions. I performed RNA sequencing experiments on orchard reared flies and modeled seasonal plastic gene expression. Additionally, I measured body size, developmental time, and fecundity on both orchard and lab reared flies to assess phenotypic plasticity across seasons. These computational analysis and experimental results address the sets of questions in my three dissertation chapters.

In my first chapter, I perform a series of computational analyses using publicly available eQTLs (Everett et al. 2020), allele frequency (Machado et al. 2021), and gene expression profiles (Zhou

et al. 2012; Juneja et al. 2016) to compare clinal and seasonal adaptation at eQTLs. I show that eQTLs are enriched for clinal, but not seasonal, SNPs across the genome and inside certain chromosomal inversions. Such a result suggests that eQTLs are more likely to change allele frequencies clinally but not seasonally, and that eQTLs inside the inversions may be the target of clinal selection pressures. In addition, I find that eQTLs change allele frequencies concordantly across a latitudinal cline and in response to starvation and chill-coma. I also observe idiosyncratic patterns in eQTLs allele frequency change amongst individual seasonal populations. Taken together, these results suggest that seasonal adaptative signals at eQTLs is weak and at least partially distinct from that of clinal adaptation.

In my second chapter, I examine seasonal gene expression plasticity using orchard reared flies across seasonal time points and across associated temperature ranges. I find that ~75% of the genome is plastic across seasonal time points or temperature ranges. The plastic genes are functionally enriched for metabolic, biosynthesis, and muscle processes. Interestingly, some functionally grouped genes are likely maladaptive. Maladaptive plasticity suggests that the plastic expressions of genes grouped by certain ecologically relevant functions, such as heat survival and desiccation resistance, could be deleterious. However, such maladaptive plasticity in gene expression could also be trait specific. I further show that eQTLs associated with plastic genes are depleted for seasonal SNPs, suggesting that plasticity and genetic evolution have limited overlap at the eQTLs level. This seasonal gene expression data provides a valuable resource for testing adaptive *vs.* maladaptive plasticity and can advance our understanding of the putative physiological basis for seasonal adaptation.

In my third chapter, I assess the plasticity in three fitness traits, including body size, developmental time, and fecundity across 10 seasonal time points in both orchard and lab reared flies. I show that seasonal developmental conditions induce plasticity in these traits by comparing phenotypic values between orchard and lab reared flies. By building mixed effect models, I further show that temperature is an important environmental factor associated with the plastic response of those traits. The seasonal plasticity of these traits could affect the seasonal population size dynamics in the wild and induce seasonal-specific line mean correlation patterns.

In general, my dissertation contributes to advance our understanding of the mechanisms under seasonal adaptation. By showing that seasonal adaptation at eQTLs is idiosyncratic amongst populations and is distinct from that of clinal adaptation, we challenge the previous assumptions that seasonal adaptation should mirror that of spatial adaptation (Rhomberg and Singh 1988; Rodrigues et al. 2021). Our findings also suggest that seasonal adaptive signal at eQTLs is weak and lead to the possibility that plasticity may be an important mechanism for seasonal adaptation. Indeed, we show prevalent gene expression plasticity across seasonal time points and temperature ranges for orchard reared flies. However, genes grouped by ecologically important traits, such as heat survival and desiccation tolerance, show maladaptive signals. Such maladaptive plasticity has been widely observed in many previous studies (e.g., Ghalambor et al. 2015; Huang et al. 2022). Our seasonal plastic gene expression data can be used to strictly test for adaptive *vs.* maladaptive plasticity if paired with future work on measuring expression evolution across the seasons. We also provide evidence that plasticity and genetic evolution compose different sets of genes. Finally, we show seasonal developmental temperature in the experimental orchard can elicit plastic response in fitness traits and that such plasticity could

affect population size dynamics. In addition, we show that phenotypic line mean correlations are seasonal environmentally specific. My dissertation work can further advance our understanding of how populations adapt to seasonality from the genetics, gene expression, and fitness-related phenotypic levels.

CHAPTER 1

(*Evolution, Published*)

Distinct signals of clinal and seasonal allele frequency change at eQTLs in *Drosophila melanogaster*

Short title: Adaptation at eQTLs in *D. melanogaster*

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Abstract

Populations of short-lived organisms can respond to spatial and temporal environmental heterogeneity through local adaptation. Local adaptation can be reflected on both phenotypic and genetic levels, and it has been documented in many organisms. Although complex fitness-related phenotypes have been shown to vary across latitudinal clines and seasons in similar ways in *Drosophila melanogaster* populations, the comparative signals of local adaptation across space and time remains poorly understood. Here, we examined patterns of allele frequency change across a latitudinal cline and between seasons at previously reported expression quantitative trait loci (eQTLs). We divided eQTLs into groups by utilizing differential expression profiles of fly populations collected across latitudinal clines or exposed to different environmental conditions. In general, we find that eQTLs are enriched for clinally varying polymorphisms, and that these eQTLs change in frequency in concordant ways across the cline and in response to starvation and chill-coma. The enrichment of eQTLs among seasonally varying polymorphisms is more subtle, and the direction of allele frequency change at eQTLs appears to be somewhat idiosyncratic. Taken together, we suggest that clinal adaptation at eQTLs is at least partially distinct from seasonal adaptation.

Introduction

Identifying the evolutionary forces that maintain genetic variation in natural populations remains one of the key questions in population genetics (Gillespie 1998; Charlesworth and Charlesworth 2017). One strong diversifying force is environmental heterogeneity (Dobzhansky 1955; McDonald and Ayala 1974; Gillespie 1998), that can result in the selective maintenance of genetic variation within and between populations (Levene 1953; Haldane and Jayakar 1963; Gillespie and Turelli 1989; Turelli and Barton 2004; Charlesworth 2006). Environmental change across the range of many widely distributed species is often associated with latitudinal gradients related to phenology (Viegas et al. 2012; Fjellheim et al. 2014; Kong et al. 2019) and spatial adaptation to temperate environments (Bradshaw et al. 2004). For organisms with short generation times, temporal variation in selection pressures can drive adaptive tracking (Botero et al. 2015). Adaptive tracking has been shown to occur in response to seasonal variation in selection pressures (Dobzhansky and Ayala 1973; Mueller et al. 1985; Rodríguez-Trelles et al. 1996; Ananina et al. 2004; Bergland et al. 2014; Wittmann et al. 2017), and in principle these adaptive fluctuations across seasons should mirror spatial variation because of common selective pressures imposed by seasonality (Singh and Rhomberg 1987).

Empirical work on *Drosophila melanogaster* has shown parallel differentiations in fitness-related traits across a latitudinal cline and between seasons. Lab reared descendants of flies collected in the spring are more starvation tolerant and show a wider breadth of thermal tolerance, similar to lab reared descendants of flies collected in northern locales (Schmidt et al. 2005, 2008; Schmidt and Paaby 2008; Behrman et al. 2015). Genetic and genomic work has shown that allele

frequency shifts between seasons sometimes show parallel clinal variation (Bergland et al. 2014; Cogni et al. 2014; Paaby et al. 2014; Machado et al. 2021). For instance, inversion frequency of *In(3R)Payne* shows a strong latitudinal cline in North America and stable oscillations between seasons at an orchard in Pennsylvania (Kapun et al. 2016). Candidate adaptive polymorphisms affecting diapause in the gene *couch-potato* show parallel shifts in frequency across space and time: the pro-diapause allele has higher frequency in the spring and in the north, compared to the fall or the south (Cogni et al. 2014).

Although there is growing evidence of parallelism across latitudinal and seasonal gradients in flies, only a small fraction of clinally and seasonally varying SNPs overlap (~3.7%, Rodrigues *et al*. 2021). Such low proportion of overlap could arise from several factors. First, the demographic history of flies collected across a latitudinal cline and between seasons differ (Bergland et al. 2014, 2016): clinally varying polymorphisms may be a consequence of secondary contact and seasonally varying polymorphisms might be affected by severe overwintering bottlenecks. Second, selective forces that vary across latitudinal clines might not exactly mirror those across seasons. Finally, the causal loci of adaptation across latitudinal clines might be different from adaptation across seasons.

To understand the comparative signals between clinal and seasonal adaptation, we studied the spatial and temporal distribution of alleles associated with genetic variation in gene expression. Gene expression variation has been demonstrated to be important for adaptive evolution in many organisms (King and Wilson 1975; Gompel et al. 2005; Fraser et al. 2010; Richards et al. 2012; Fraser 2013; Mack et al. 2018). As a consequence, loci associated with expression (eQTLs)

could show parallel adaptive signals across space and time, and can be used to test hypotheses about local adaptation (Fraser et al. 2011). Knowledge of eQTL identity provides information about the functional significance of non-coding polymorphisms and can therefore be used to provide insight into the function of polymorphisms that vary across space and time. More generally, we can ask whether eQTLs are likely to contribute to rapid spatial and temporal adaptation, and test whether the patterns of allele frequency change are similar at eQTLs between space and time. In addition, knowledge of eQTLs allow us to test hypotheses about the direction of allele frequency change through space and time using information about adaptive differentiation in gene expression (Juneja et al. 2016) and expression plasticity (Zhou et al. 2012).

Materials and Methods

Population allele frequencies and statistics. An overview of data and analysis is explained in Supplemental Figure S1. We used allele frequency estimates at ~1.7M SNPs from 45 samples (Supplemental Table 1) as reported by Machado *et al* (2021). This dataset includes populations sampled along the east coast of North America ("clinal"), and 20 paired spring-fall samples from geographically distributed localities across two continents ("Core 20"). Two paired spring-fall samples (BA_12 and VI_12) from the Core20 were mislabeled (Nunez et al. 2021), we corrected their labeling in our analysis. Machado *et al* (2021) modeled allele frequency change at each SNP across space and time using generalized linear models. The multi-population seasonal model used "spring" and "fall" labels as independent variables and the multi-population clinal model used latitude (hereafter "cross-population"). We used the output of those models to define "seasonal" and "clinal" polymorphisms based on *p*-value and regression coefficients. In general,

we used *p*-values for enrichment tests and the regression coefficients representing the direction of allele frequency change across space and time for directionality tests. We also examined the allele frequency change between spring and fall for each of the Core20 population pairs independently, as well as between Florida and Maine samples to characterize differences between the endpoints of our clinal analysis as described in "Directionality analysis of eQTL frequency" section.

eQTL identity. Our study used eQTLs identified by Everett *et al* (2020) that are also polymorphic among the clinally and seasonally sampled populations. Everett *et al* (2020) identified eQTLs using RNASeq data on pre-genotyped inbred DGRP lines against SNPs with > 0.05 allele frequency and < 25% missing phenotypes for both sexes (3-5 day mated, whole body). We grouped eQTLs into female-specific, male-specific, and non-sex biased based on their association with each of ~4,000 genes and novel transcribed regions (NTRs), hereafter referred to as "genes". Of the 104,592 autosomal eQTLs (SNPs) originally identified (Everett et al. 2020), 72,389 were identified as polymorphic among the clinal and seasonal dataset. The high proportion of SNPs shared between the DGRP lines and the wild populations that we study reflects the recent shared evolutionary history of the DGRP and the other North American populations that we use to study spatial and temporal patterns of allele frequency change.

Matched controls. For enrichment and directionality analyses, we compared eQTLs to sets of matched control SNPs (hereafter "controls") that were not identified as eQTLs themselves. For each eQTL, we identified 1000 control SNPs matched for chromosomal arm, heterozygosity (binned by 0.05), and inversion status classified as "breakpoint" (±0.5Mb around known

inversion breakpoints), “inside” the inversion region and excluding breakpoint regions, “outside” the inverted region and excluding break regions, of 6 cosmopolitan inversions (Corbett-Detig and Hartl 2012; Bergland et al. 2014; Machado et al. 2021). Heterozygosity, ranging from 0 to 0.5 with an increment of 0.05, for each SNP was estimated from the DGRP. These sets of controls are used throughout, unless otherwise noted.

Genome-wide enrichment analysis. We tested if eQTLs are enriched for clinal or seasonal SNPs relative to controls based on their ranked clinal or seasonal *p*-value quantiles. For the test set of eQTLs or each of the 1000 sets of control SNPs, we used the counts of SNPs above and below a range of *p*-value quantiles (0.001 to 0.5) to calculate 1000 odds-ratios. We calculated the odds ratio (*OR*) as AD/BC, where A and C are the counts of eQTLs (A) or controls (C) below or equal to a certain ranked *p*-value quantile, and B and D are the counts of eQTLs (B) or controls (D) above a certain ranked *p*-value quantile. We $\log_2$ transformed odds-ratio and calculated confidence interval as 1.96 x standard deviation of the mean (1000 sets). In addition, to break linkage amongst the eQTLs, we randomly sampled one eQTL per 10kb for 100 times and re-performed the enrichment analysis.

Inversion analysis. To test if eQTLs located inside (~ 19.5k), near the breakpoints (~ 4.3k) or outside (~ 59.6k) of cosmopolitan inversions *In(2L)t*, *In(2R)NS*, *In(3L)P*, *In(3R)K*, *In(3R)P* and *In(3R)Mo* are enriched for clinal or seasonal SNPs, we partitioned each chromosomal arm into “break-point”, “inside”, “outside” for each inversion separately. We performed the enrichment analysis using top 5% clinal or seasonal *p*-value quantile for eQTLs, and their matched controls.

Gene-specific enrichment analysis. To determine whether the genome-wide enrichment signals observed are driven by specific genes, we partitioned the eQTLs (both *cis*- and *trans*-) by genes. For each gene, we calculated the proportion of its eQTLs that are in the top 5% of clinal or seasonal SNPs. We calculated the gene specific enrichment as an odds-ratio (described above) relative to matched controls. We only included genes with at least one eQTL in the top 5% of clinal (1,093 genes) or seasonal (1,158 genes) *p*-value quantiles for this analysis.

Directionality analysis of eQTL frequency. To test whether allele frequency change at eQTLs across space and time matches known patterns of differential expression, we performed a directionality analysis by calculating concordance scores. The concordance score is the fraction of eQTLs or controls that change allele frequency across space or time in the predicted manner. We defined 3 outcomes from the analysis: 1. Concordant: when concordance score is significantly higher than the null expectation of 50%; 2. Discordant: when concordance score is significantly lower than the null, indicating the opposite directions as expected; 3. Neutral: when the concordance score is not significantly different from the null.

We included two differential expression datasets for this directionality analysis. One dataset identified genes that show parallel differential expression in females between populations derived from high and low latitudes in Australia and North America and reared in a common environment (Juneja et al. 2016), hereafter referred to as "latitudinal DE genes". We used female-specific (n = 1,392) and non-sex-biased (n = 880) eQTLs because Juneja *et al* (2016) measured differential expression only in females. Of the 159 genes identified by Juneja *et al* (2016), we used 39 that overlapped with the eQTL dataset. The second dataset identified genes

differentially expressed in response to heat-shock (57 genes), chill-coma (16), starvation (28), high-temperature (19) and low-temperature (20) amongst an outbred panel derived from the DGRP (Zhou et al. 2012). We used non-sex-biased eQTLs (n = 4,844 in total) for this dataset.

To calculate concordance scores at eQTLs, we combined the sign of allelic effects at eQTLs (i.e., up- or down-regulating) with the observed change in gene expression in the two differential expression datasets. For example, genes with higher expressions in northern populations compared to southern ones, we hypothesized that the eQTLs associated with an increase in gene-expression should be more common in northern than southern populations. The converse would be the case for genes that are more highly expressed in southern populations. Fly populations collected in the spring are thought to be more "winter-adapted" than those collected in fall (Bergland et al. 2014), and thus we hypothesized that spring-fall comparisons would mirror north-south comparisons.

We applied a similar approach to genes expressed in response to several environmental treatments (Zhou et al. 2012). We hypothesized that for genes upregulated following chill-coma, starvation, or low-temperature exposure, the associated upregulating eQTL alleles will be more common in the north and in the spring, relative to the south or the fall. Low temperature stands for constant low temperature treatment (18 ºC) whereas chill coma stands for acute 3-h on ice followed by 1-h recovery treatment (Zhou et al. 2012). Our assumption is that populations in the north or in the spring are more likely to experience both constant low temperature and acute chill shocks since they live in an environment with comparatively low temperature and more likely to encounter chill shock. Conversely, we hypothesized that for genes upregulated following heat-

shock and high-temperature exposure, the upregulating alleles would be less common in the north and in the spring.

We then calculated concordance scores. We examined directionality based on the cross-population clinal and seasonal models. To determine whether the cross-population concordance signals observed are driven by specific genes, we partitioned the eQTLs by genes. For each gene, we calculated concordance score using clinal or seasonal cross-population models.

In addition, we also examined eQTL directionality between pairs of populations. For the spatial comparison, we compared allele frequencies between Florida and Maine. For the seasonal comparisons, we compared allele frequencies between spring and fall within a sampling locality. The locality specific seasonal comparison is meant to assess the consistency of allele frequency change between seasons across populations. We generated expected distributions using 1000 control-sets.

Empirical p-values. For the enrichment and directionality analysis, we calculated empirical *p*-values. Let S be the observed value and S_0 be the expected distribution generated by 1000 sets of controls, N is the total number of tests, and defined

$$p = (1+\sum(S \geq S_0))/(N+1)$$

$$emp.p = 2 \times \min (p, 1-p)$$

(Davison and Hinkley 1997). For $emp.p = 0$ in our tests, we report $emp.p < 0.001$.

Results

Genome-wide Enrichment Test. To understand clinal and seasonal allele frequency change at eQTLs, we examined whether eQTLs are enriched for clinal or seasonal SNPs. We find significant enrichment of clinal SNPs in 44/45 of our tests (adjusted $emp.p \leq 0.003$, Figure 1, Supplemental Table 2) but not seasonal SNPs for female-, male-, and non-sex-biased eQTLs across a range of *p*-value quantiles. To test if this result is affected by linkage-disequilibrium, we randomly sampled one eQTL per 10kb, and again show enrichment of clinal SNPs in female-, male-, and non-sex-biased eQTLs across a range of clinal *p*-value quantiles (Figure S2). The decrease in clinal enrichment among the down-sampled eQTLs (Figure 1 *vs.* Figure S2) suggests that clinal eQTLs are heterogeneously distributed throughout the genome at the most stringent clinal *p*-value quantiles. However, the general trend of clinal enrichment signals is not solely affected by linkage.

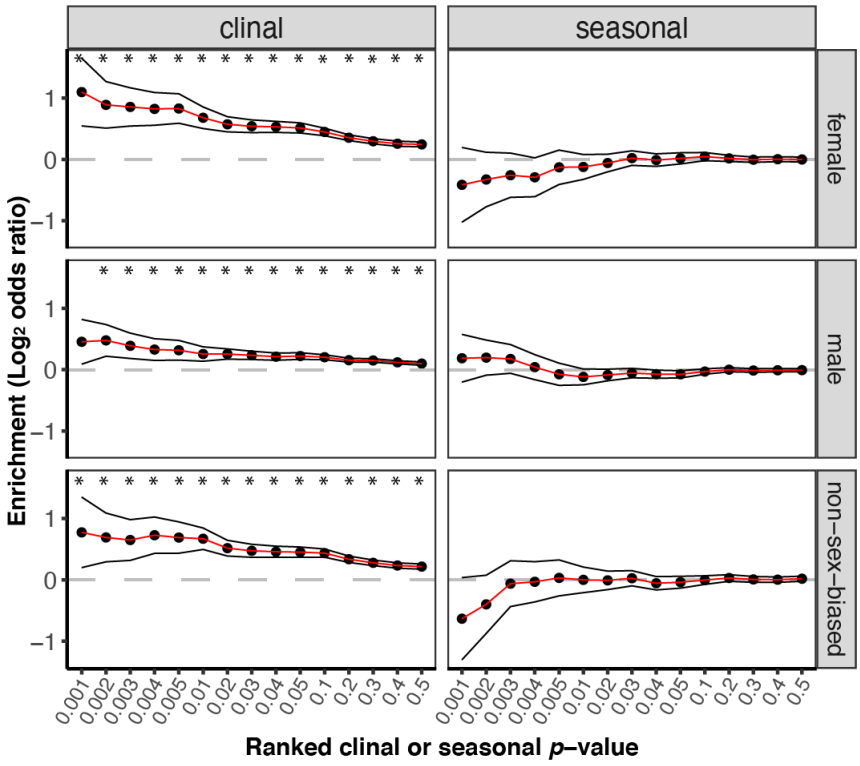

Figure 1. Enrichment of clinal or seasonal SNPs in female-, male-, and non-sex-biased eQTLs genome-wide. The x-axis is ranked clinal (left) or seasonal (right) *p* value thresholds. The y-axis is enrichment, calculated as the $\log_2$(odds ratio) of eQTLs having ranked clinal or seasonal *p* values below or equal to certain thresholds compared to controls based on matching parameters. Black dots represent average $\log_2$(odds ratio) over 1000 bootstraps. Black lines are confidence intervals, represented by 1.96 standard deviations of the mean over 1000 bootstraps. Asterisks indicate significant enrichment after Bonferroni correction for 15 tests (empirical $p \leq 0.003$).

Next, we tested whether inversion status of the eQTLs (inside, outside or near breakpoints of 6 cosmopolitan inversions) affect clinal or seasonal enrichment signals. We show chromosome-wide enrichment signals of clinal SNPs in eQTLs on chromosomal arms 2L (non-sex-biased: *emp.p* < 0.001), 2R (all: *emp.p* < 0.001), 3L (all: *emp.p* < 0.001), and 3R (female-, non-sex-biased: *emp.p* < 0.001), and enrichment of seasonal SNPs in eQTLs on chromosomal arm 3R (female-: *emp.p* < 0.001). eQTLs near inversion breakpoints for *In(3R)P* (female-, non-sex-

biased: *emp.p* < 0.001), *In(3R)Mo* (non-sex-biased: *emp.p* < 0.001), *In(3R)K* (female-: *emp.p* < 0.001), or within inverted regions for *In(3L)P* (all: *emp.p* < 0.001), *In(2L)t* (female-: *emp.p* < 0.001), *In(3R)P* (non-sex-biased: *emp.p* < 0.001), *In(3R)Mo* (female-: *emp.p* < 0.001), *In(3R)K* (female-: *emp.p* < 0.001) are enriched for clinal SNPs. We do not observe enrichment signals for seasonal SNPs in eQTLs near inversion breakpoints or within inverted regions (Figure 2A).

To address whether the enrichment signals are driven by a limited number of genes, or by many genes, we performed gene-specific enrichment analysis. We find that 23.79% and 10.10% of genes included in the analysis are significantly enriched (*emp.p*≤0.05) for clinal and seasonal eQTLs, respectively (Figure 2B). In addition, there is a strong excess of genes that are significantly enriched for clinal or seasonal eQTLs, compared to genes which are depleted for clinal or seasonal eQTLs, respectively (proportions > 0.5, $p < 0.05$, Figure 2B). Our results suggest that the genome-wide enrichment signals are not driven by a small number of genes (Supplemental Table 3).

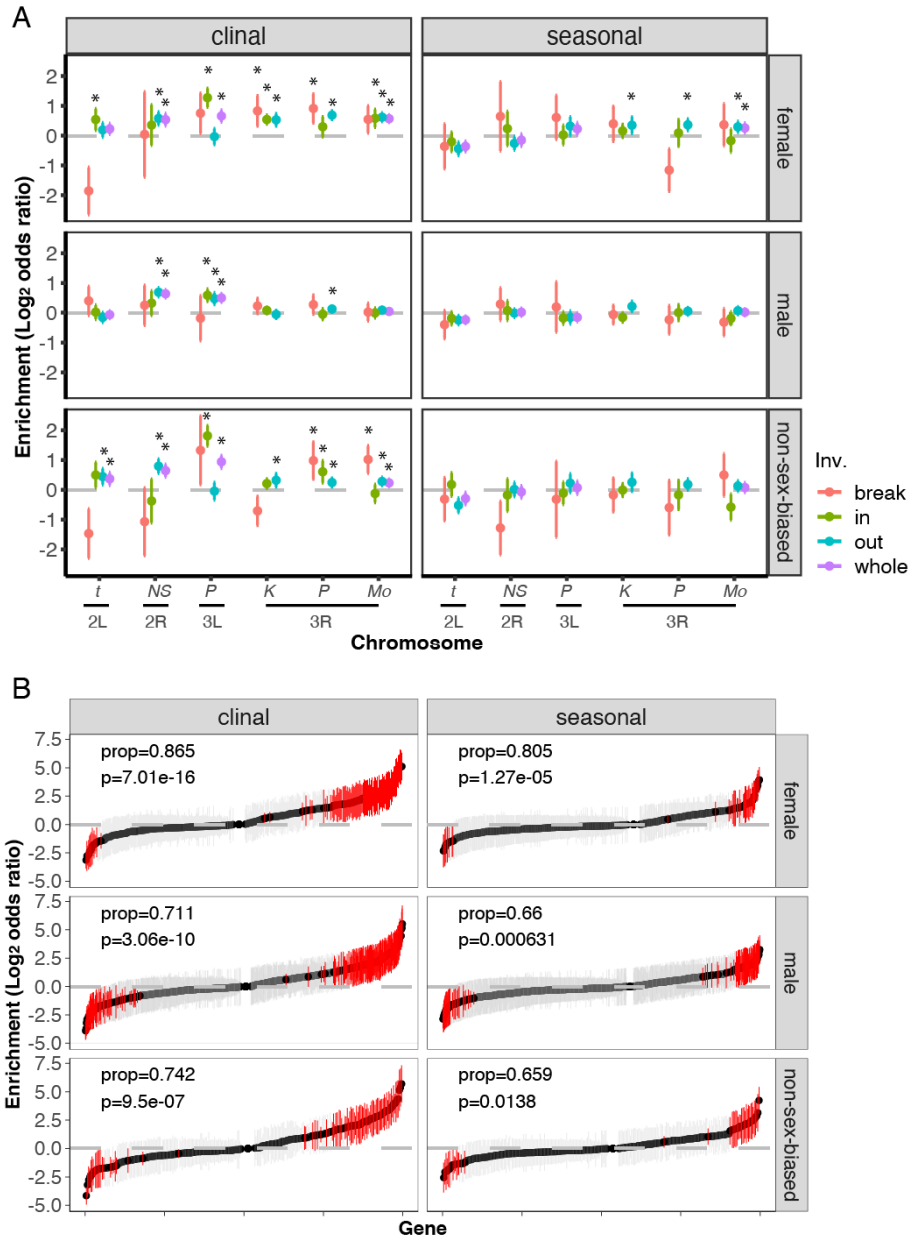

Figure 2. Enrichment of clinal or seasonal SNPs in female-, male, and non-sex-biased eQTLs on each chromosomal arm (whole) and inversion (breakpoints, inside, outside) regions (A), and in every gene identified with eQTL (B). (A) The x-axis is chromosomal arms. Error bars are confidence intervals, represented by 1.96 standard deviations of the mean over 1000 bootstraps. Asterisks indicate significant enrichment after Bonferroni correction for 22 tests (empirical $p \leq 0.002$). (B) The x-axis is genes identified with eQTLs. Genes are ranked by averaged $\log_2$ odds ratio within each analysis type (clinal or seasonal) and sex (female, male, non-sex-biased) combination panel. The y-axis is enrichment. Black dots represent average $\log_2$(odds ratio) over 1000 bootstraps. Red or grey error bars are 1.96

standard deviations of the mean over 1000 bootstraps for genes with significant or insignificant signals, respectively. Proportion (prop) represents the ratio between genes significantly enriched for clinal or seasonal eQTLs and the total number of genes with significant (enrichment and depletion) signals (empirical $p \leq 0.05$).

The directionality of eQTL frequency change across space and time. We tested whether eQTLs show concordant changes in allele frequency across the cline or between seasons. We show that female-, non-sex-biased eQTLs associated with latitudinal DE genes are more likely to change allele frequencies between clinal populations in a concordant way than controls are (CrossPop: *emp.p* < 0.001; FL-ME: *emp.p* < 0.001, Figure 3A). We also show discordant signal for female-eQTLs associated with latitudinal DE genes in the seasonal comparison (CrossPop: *emp.p* = 0.002), and concordant change for non-sex-biased eQTLs (*emp.p* = 0.002, Figure 3A). The significant cross-population signals at latitudinal DE genes are likely driven by *Hsc70-2* with ~1,000 female eQTLs (Figure S3A). eQTLs associated with *Hsc70-2* are strongly concordant across the latitudinal cline (~80%) but discordant (~25%) between seasons. The gene-specific concordance score also varies from gene to gene (Figure S3). In addition, the eQTLs associated with latitudinal DE genes do not always change allele frequencies in predicted directions in every paired spring-fall sample, suggesting that seasonal changes in selection pressure might not always be consistent between populations (Figure 3A).

Next, we evaluated the directionality of eQTL allele frequency change at environmental DE genes (Figure 3B). Consistent with our predictions, eQTLs associated with DE genes under starvation (CrossPop: *emp.p* < 0.001, FL-ME: *emp.p* < 0.001) and chill-coma (CrossPop: *emp.p* < 0.001, FL-ME: *emp.p* < 0.001) treatments show concordant change in clinal comparisons, suggesting plastic genes induced by these treatments could be adaptive. In contrast to our

prediction that northern flies are more “cold-adapted”, eQTLs associated with low-temperature treatment induced DE genes show discordant signal for clinal comparison (CrossPop: *emp.p* = 0.002). In seasonal comparisons, we show concordance for eQTLs associated with chill-coma and heat-shock (CrossPop: *emp.p* < 0.001) induced DE genes, consistent with our prediction that spring flies are more chill-coma resistant and less heat-shock resistant, but discordant signal for eQTLs affecting low-temperature induced DE genes (CrossPop: *emp.p* = 0.002). Like results for eQTLs associated with latitudinal DE genes, the gene-specific concordance score at environmental DE genes varies from one gene to another (Figure S3B) and the directionality for eQTLs allele frequency are inconsistent amongst Core20 spring-fall comparisons (Figure 3B).

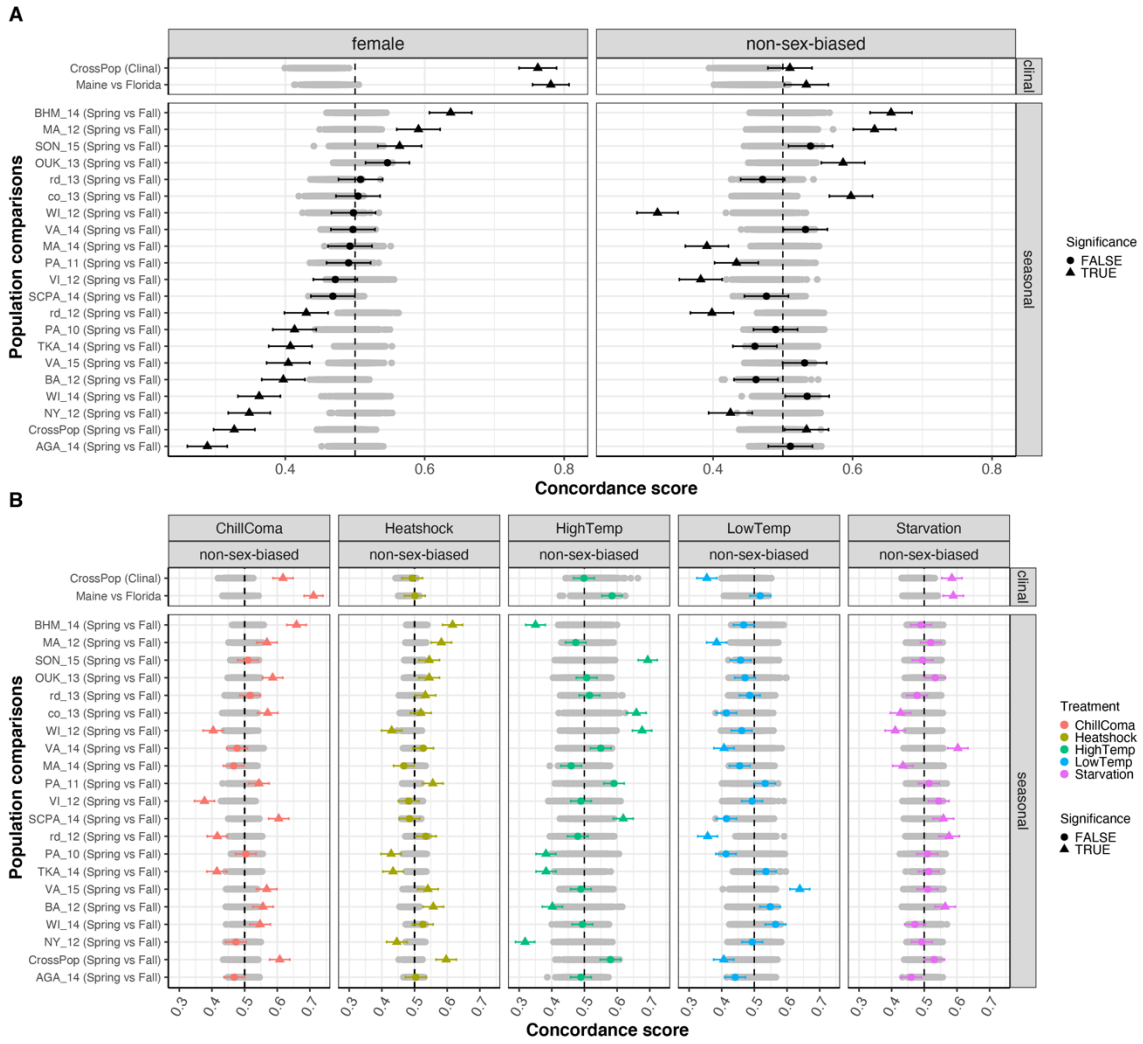

Figure 3. The directionality of allele frequency change for female-, non-sex-biased eQTLs associated with genes differentially expressed between high and low latitudinal populations (A), or for non-sex-biased eQTLs associated with genes differentially expressed under certain environmental treatments (B). The x-axis is the concordance score. The y-axis is clinal or seasonal population comparisons, with the assumption that gene expression patterns are similar between northern and spring populations, and similar between southern and fall populations. The cross-population comparison results were generated by using clinal or seasonal coefficients while other comparisons used allele frequencies of eQTLs from each sample pair. Black (A) or colored (B) dots (triangles and circles) are observed values of eQTLs. Grey circles are expected distributions generated by control SNPs, bootstrapped 1000 times. Black (A) or colored (B) triangles indicate observed concordance scores at eQTLs significantly deviate from

null distributions, with Bonferroni correction of empirical p values for 23 tests (empirical $p \leq 0.002$). Black (A) or colored (B) circles indicate non-significant deviations of observed eQTLs values from expected control distributions.

Discussion

Although there are well documented patterns of local adaptation across latitudinal clines and between seasons in *D. melanogaster*, we still have a limited understanding of the genetic architecture of this evolutionary change. Here, we show that the signal of clinal and seasonal adaptation differ at eQTLs, suggesting distinct evolutionary processes occur across space and time in this species. Our results rely on signals of enrichment and concordance that are calculated across eQTLs relative to controls. One caveat for our analyses is that eQTLs could be linked and less so for control SNPs. Thus, our analyses should be interpreted as a way to identify linked sets of SNPs that are enriched for functionality, as eQTLs, and spatial or temporal adaptive signals. This work provides novel insight into our understanding of spatial and temporal differentiation by identifying loci that are functionally and physically linked.

Enrichment. Gene expression has been shown as an important driver of local adaptation (López-Maury et al. 2008; Colicchio et al. 2020). Consistent with the expectation that gene expression variation contributes to clinal adaptation (Adrion et al. 2015), we show eQTLs are enriched for clinal SNPs (Figure 1). The levels of clinal enrichment observed are comparable to those observed for other functional categories in *Drosophila* (Machado et al. 2016) and other species (Ye et al. 2013; Mack et al. 2018). Such an enrichment agrees with growing evidence that spatial differentiation at eQTLs contributes to local adaptation across taxa (Fraser 2013; Gould et al. 2017; Mack et al. 2018; Phifer-Rixey et al. 2018; Kitano et al. 2019; Colicchio et al. 2020). We

also show the genome-wide enrichment signal is not driven by a few genes or solely due to linkage at a single locus (Figures 2B, S2), suggesting clinal adaptation at eQTLs is polygenic (Mateo et al. 2018).

We further show evidence that clinal differentiation at inversions may be driven by eQTLs. First, eQTLs near breakpoints or within inverted regions on chromosomal arms 2L, 3L, 3R are more 'clinal' than controls are in the same regions (Figure 2A). This result agrees with previous studies reporting latitudinal inversion clines in North America (Mettler et al. 1977; Kapun et al. 2014). Next, we observe clinal enrichment within inverted regions, but not near breakpoints, on chromosomal arms 2L and 3L (Figure 2A), suggesting that eQTLs, other than the chromosomal inversions, could be the target of selection. Such a result is consistent with the hypothesis that chromosomal inversions have little effect on gene expression and that natural selection acts on linked loci associated with the inversions, other than the structural variants themselves, in this species (Lavington and Kern 2017; Said et al. 2018). Thus, eQTLs might be the causal driver of allele frequency associated with the inversions on chromosomal arms 2L (Said et al. 2018) and 3L, forming the inversion clines (Kapun et al. 2016).

We hypothesized seasonal enrichment patterns at eQTLs should mirror clinal patterns (Rhomberg and Singh 1988; Machado et al. 2021). However, we do not observe enrichment of seasonal SNPs at eQTLs, like we do for clinal ones (Figures 1, 2A), either genome-wide or at inversions. Genome-wide, the lack of seasonal enrichment signal could be a result of polygenic adaptation at different alleles due to genetic redundancy (Barghi et al. 2019). For example, if different sets of eQTLs are under seasonal selection pressures in different geographic locations

or across years, we might not be able to observe significant overlap between eQTLs and seasonal SNPs identified from the cross-population model. Alternatively, lack of enrichment could be due to subtle changes of small effect eQTLs that are sufficient for seasonal adaptation collectively, but individually undetectable by the seasonal model.

For eQTLs located near breakpoints or within inversion regions, despite strong clinal enrichment, we do not observe any seasonal enrichment signal (Figure 2A). Previous studies have argued the importance of inversions underlying seasonal adaptation by showing seasonal inversion frequencies in various drosophilid species (Dobzhansky and Ayala 1973; Knibb 1986; Sanchez-Refusta et al. 1990) and enrichment of seasonal SNPs at inversion breakpoints or inside inverted regions in *D. melanogaster* genome (Machado et al. 2021). Our result does not contradict such arguments, but rather indicate that previously reported seasonal polymorphisms at these inversions may not be among our analyzed eQTLs (Kapun et al. 2016; Machado et al. 2021). Although the lack of seasonal enrichment patterns could be due to a possible lack of power in detecting seasonal SNPs (e.g., Bergland et al. 2014), it could also indicate that there is ecologically relevant idiosyncratic allele frequency changes between spring and fall amongst populations (see *eQTL directionality*, below).

eQTL directionality. Here we tested whether eQTLs associated with differentially expressed genes show predicted allele frequency change across a latitudinal cline and between seasons. eQTLs associated with previously identified latitudinal DE genes (Juneja et al. 2016) show concordance in allele frequency between Florida and Maine, and across a latitudinal cline in

North America in general (Figure 3A). Such concordant change suggests that these eQTLs are both functional and potentially underlie adaptive differentiation across spatial gradients.

Based on previous work (Bergland et al. 2014; Machado et al. 2021; Rodrigues et al. 2021), we predicted that alleles favored in high-latitude locales would be more common in the spring compared to the fall. Interestingly, we found the opposite pattern at female- eQTLs (Figure 3A), which is in contrast to previous results (Machado et al. 2021). For the directionality analysis of latitudinal DE genes, our results are strongly driven by a single gene, *Hsc70-2* (Figure S3A). This gene is associated with ~1,000 eQTLs that span almost 1Mb on chromosome 3R, that are likely in long-distance linkage as a consequence of a partial soft sweep at *Ace* (Garud et al. 2015). Therefore, our results show that the previously reported concordance of allele frequency through space and time (Machado et al. 2021; Rodrigues et al. 2021) is not consistent across the genome and may vary from gene to gene. Such gene-to-gene variation in concordance score is also reflected at environmental DE genes (Figure S3B). Thus, our results demonstrate that considering the inferred functional significance of clinal and seasonal polymorphisms at a gene level is important for interpreting whether shared selection pressures exist between latitudinal and seasonal gradients.

Interestingly, when we applied the directionality test to single population spring-fall comparisons, we show that some populations showed concordant signals, and some showed discordant signals at latitudinal and environmental DE genes. For example, 15% of comparisons show concordance at eQTLs, while 40% of comparisons show discordant signals when using eQTLs grouped by latitudinal DE genes (Figure 3A). Such idiosyncratic directionality patterns

amongst populations are similar to Erickson *et al.* (2020) that examined diapause associated SNPs. These results suggest that seasonal selection pressures might not always be consistent.

The use of environmental DE genes in our directionality tests is based on two critical assumptions. The first is that expression plasticity is adaptive. Whether this assumption is valid in general is an open question (Ghalambor et al. 2015), and evidence suggests that whether plasticity is adaptive or maladaptive varies between populations (Huang et al. 2022) and among traits (Huang and Agrawal 2016; Mallard et al. 2020). The tests also assume that the environment changes in a specific way through space and time. For instance, a signal of concordance assumes that flies collected in Maine are subject to more bouts of starvation than flies in Florida, and that flies collected in spring are more "cold-adapted" than those collected in the fall. Therefore, to interpret a signal of concordance as evidence of adaptation requires plasticity to be adaptive and that the direction of environmental change align with these assumptions. A signal of discordance could reflect maladaptive plasticity or environmental changes that are opposite to our expectations. Our work cannot test whether plasticity is adaptive or maladaptive, but it can shed light on the consistency of selection through space and time.

We propose several possible explanations for the idiosyncratic direction of allele frequency change among the paired spring-fall comparisons (Figure 3). First, selection pressures might not be consistent between spring and fall among different populations or years (Erickson et al. 2020; Machado et al. 2021). Therefore, eQTLs affecting a trait that has fitness advantage in one spring-fall population might not be favored in another, resulting in inconsistent allele frequency changes amongst populations. Second, seasonal adaptation could be achieved by a subset of common

eQTLs via combinations with other population-specific seasonal loci. It has been shown in previous studies that different combinations of genetic loci could evolve to adapt to the same selective condition while only a limited number of common loci is identified (Barghi et al. 2019). Such possibility could also explain the lack of genome-wide enrichment signals of seasonal SNPs in eQTLs (See *Enrichment*, above). Regardless, it is clear that signals of allele frequency change across latitudinal gradients and between seasons are not identical and thus suggest that the genetic architecture of clinal and seasonal adaptation might be different and that environmental changes across space and time might not reflect each other in ways previously identified.

Data availability: Data and scripts are available at Dryad with DOI: https://doi.org/10.5061/dryad.6m905qg32.

List of supplemental figures and tables

Supplemental Figure S1: Explanation chart for data and analysis.

Supplemental Figure S2: Enrichment of clinal or seasonal SNPs in female-specific, and male-specific and non-sex-biased eQTLs after block sampling.

Supplemental Figure S3: Gene-specific directionality of eQTLs grouped by latitudinal- or treatment-DE genes.

Supplemental Table 1: Populational information for the 5 clinal and Core20 (seasonal) populations used in our analysis.

Supplemental Table 2: Summary of genome-wide enrichment analysis for eQTLs.

Supplemental Table 3: Summary of gene-based enrichment analysis for eQTLs.

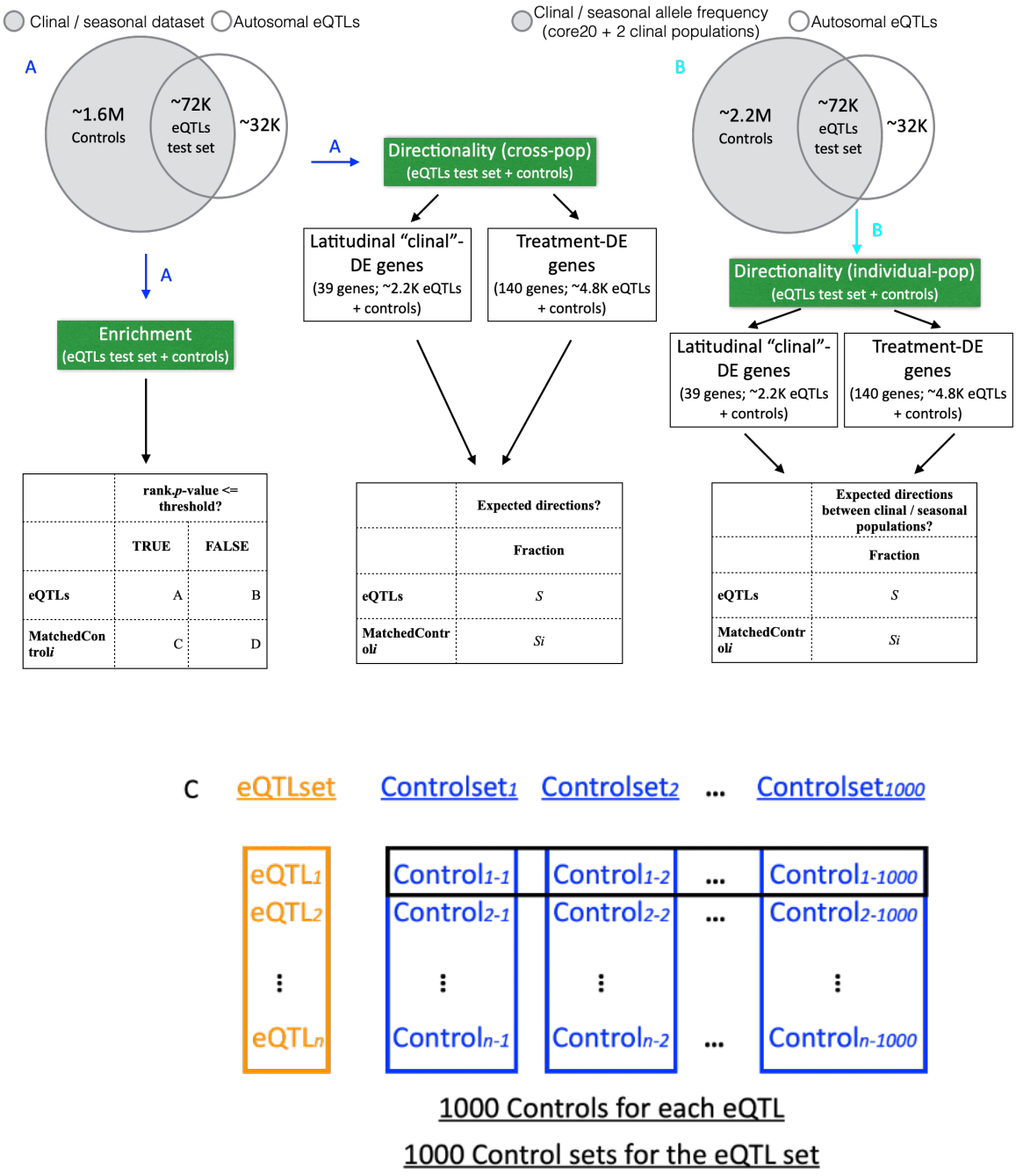

Figure S1. Explanation chart for data and analysis. (A). Clinal / seasonal dataset includes SNPs identified under generalized clinal or seasonal cross-population models reported in Machado *et al* (2021) and are used in enrichment and cross-population directionality tests (see Materials and Methods). (B). Clinal / seasonal allele frequency data includes allele frequency data from 20 "core20" and Florida and Maine

populations reported in Machado *et al* (2021). Autosomal eQTLs are identified by Everett *et al* (2020). We used non-eQTLs (~1.7M (A) or ~2.2M (B) controls) from Machado *et al* (2021) to draw matched controls for the test eQTLs datasets. MatchedControl*i* stands for the i^{th} set of controls, where i=1 to 1000. Threshold stands for ranked clinal or seasonal p-value thresholds (0.001 to 0.5). For enrichment tests, we calculated Odds Ratio (OR) = AD/BC. We $\log_2$ transformed odds ratio for every set and calculated confidence interval as 1.96 x standard deviation of the mean (1000 sets). For directionality tests, S and Si are the observed fraction of eQTLs and the fraction of the i^{th} (i=1 to 1000) set of controls changing allele frequency in the expected directions, respectively. Expected directions for directionality tests are clarified in Materials and Methods in the main text. For both enrichment and directionality tests, we calculated empirical p values using the formula described in "*Empirical p-values*" in Materials and Methods. (C). Explanation table for control sampling procedure. For each eQTL ($eQTL_1$-$eQTL_n$), 1000 matched controls were found based on descriptions in Materials and Methods (for example, $Control_{1\text{-}1}$-$Control_{1\text{-}1000}$ for $eQTL_1$); For the eQTL set being tested in our different analysis, 1000 control sets were sampled ($Controlset_1$-$Controlset_{1000}$).

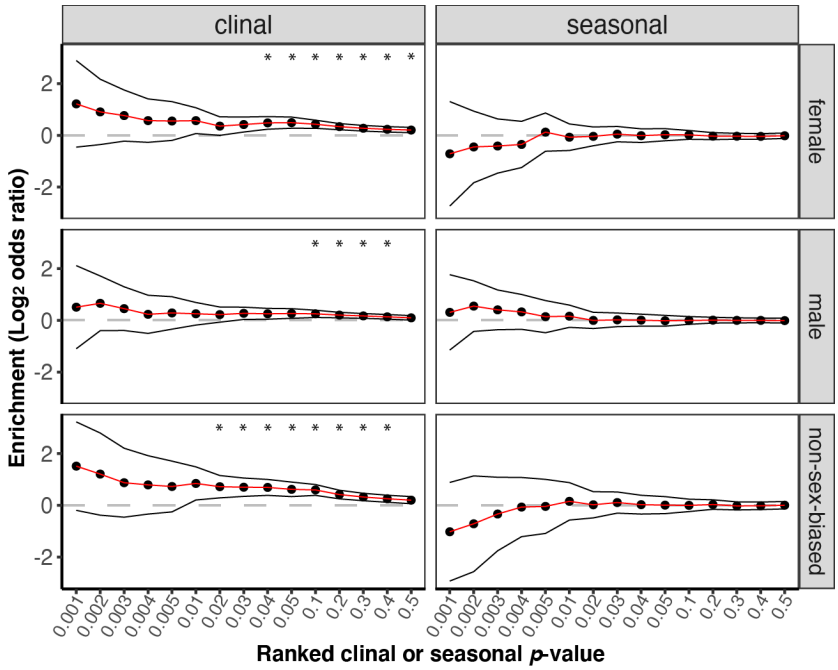

Figure S2. Enrichment of clinal or seasonal SNPs in female-, and male-, and non-sex-biased eQTLs after block sampling. The x-axis is ranked clinal (left) or seasonal (right) *p* value thresholds. The y-axis is enrichment, calculated as the $\log_2$ odds ratio of eQTLs having ranked clinal or seasonal *p* values below or equal to certain thresholds compared to controls. Female-, male, non-sex-biased eQTLs are down-sampled 100 times with only 1 random eQTL selected in every 10kb non-overlapping windows for each down-sampling. Black dots represent average $\log_2$ odds ratio across 100 samples of 1000 bootstraps each. Black lines are confidence intervals (1.96 standard deviation of the mean). Asterisks indicate significant enrichment after Bonferroni correction for 15 tests (empirical $p \leq 0.003$).

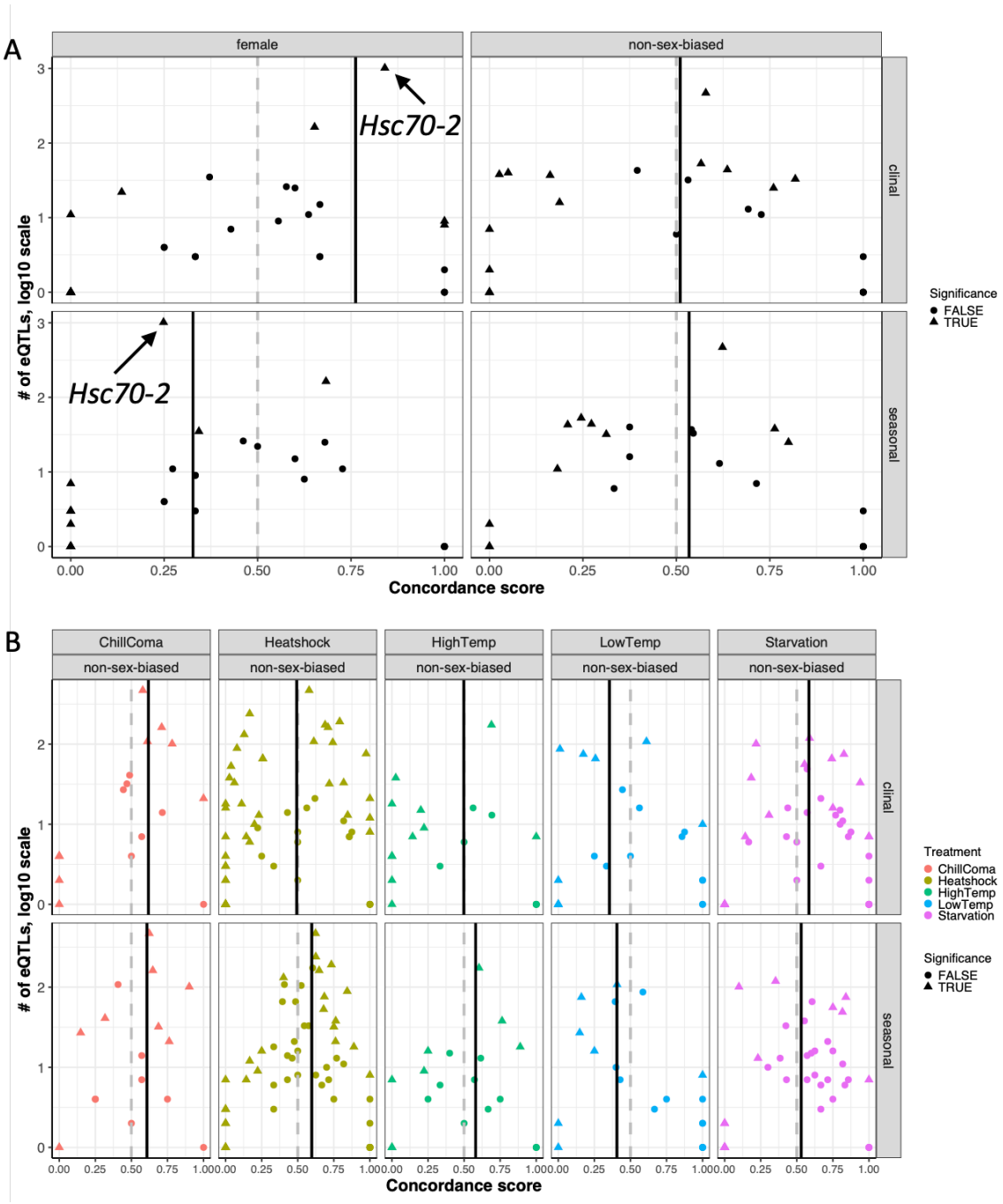

Figure S3. Gene-specific directionality of eQTLs grouped by latitudinal DE genes (A) and treatment DE genes (B). The x-axis is the concordance score. The y-axis is genes, which are ranked by the number of eQTLs (log_{10} scale) within each analysis type (clinal or seasonal) and sex (A) or treatment (B) combination panel. Triangles and circles indicate a significant or nonsignificant difference from the null

(centers around 0.5, dashed lines), respectively. Black lines represent average concordance score summing all the genes.

CHAPTER 2

Prevalent gene expression plasticity shows maladaptive signals across seasonal time points and temperature ranges in *Drosophila melanogaster*

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Keywords: Plasticity; gene expression; season; adaptive; maladaptive

Data availability: Data and scripts will be uploaded to online archive and are available upon request.

Abstract

Phenotypic plasticity in gene expression can be an important mechanism for populations to cope with environmental fluctuations across space and time. Expression plasticity can be adaptive or maladaptive, which can result in concordant or discordant signals between the direction of plastic response and that of gene expression evolution. In order to study the adaptive signatures of seasonal gene expression traits, we characterized genome-wide plastic gene expressions across 10 seasonal time points. We performed RNA sequencing experiments on F_1 *D. melanogaster* populations reared in an experimental orchard across seasons and modeled gene expression using linear models. We show that plasticity in gene expression is prevalent across seasonal time points and temperature ranges, and that plastically expressed genes are functionally enriched for metabolic and muscle processes. Interestingly, some ecologically important genes show "counter-gradient" patterns between the direction of plastic change and that of evolutionary change, indicating maladaptive plasticity signals. However, the "counter-gradient" signals for gene expression plasticity are trait specific. We further show that eQTLs associated with genes that show expression plasticity in our study are less likely to change allele frequencies across seasons than controls are, suggesting that plasticity and adaptive evolution likely have limited overlap at the eQTLs. Our study provides a valuable resource for understanding how populations cope with seasonality from a functional perspective as well as for testing putatively adaptive *vs.* maladaptive expression plasticity across seasons.

Introduction

Seasonality is characterized by cyclical environmental changes and can impose strong selection pressures on organisms (Dobzhansky and Ayala 1973). Deciphering the mechanisms for coping with seasonality can advance our understanding of local adaptation in general (Williams et al. 2017). Adaptive evolution and phenotypic plasticity are two widely studied mechanisms (Meyers and Bull 2002), which have been predicted to be non-overlapping processes (Botero et al. 2015). Adaptive evolution can result in cross generational genetic changes and could be the mechanism for short-lived organisms due to their short life spans relative to the scale of environmental change. Such a process of adaptation to seasonal environmental heterogeneity has been reasoned to mirror that of spatial adaptation to latitudinal gradients due to the commonality in selective pressures between space and time (Rhomberg and Singh 1988). Phenotypic plasticity, which is the production of variable phenotypes from single genotypes, allows organisms to respond within a single generation (Botero et al. 2015). Phenotypic plasticity in response to a new environment can be either adaptive (Kenkel and Matz 2016; Mallard et al. 2020; Josephs et al. 2021) or maladaptive (Levine et al. 2011; Ghalambor et al. 2015; Huang and Agrawal 2016). Plasticity is adaptive when it increases the performance and fitness (Rago et al. 2019). Adaptive plasticity predicts a concordant signal between the direction of plastic response and that of evolutionary changes. On the contrary, maladaptive plasticity is predicted to show discordant signals (i.e., “counter-gradient”) compared to evolved changes (Ghalambor et al. 2007), and may be deleterious to fitness (Huang et al. 2022). Therefore, such concordant or discordant signals can be used to test for adaptive or maladaptive plasticity based on the assumption that evolutionary changes shift toward the optimal phenotypic values in the selective environment.

Gene expression provides a great opportunity to study how populations cope with seasonality from a functional perspective. First, gene expression variation allows us to infer the physiological functions (Gracey 2007), such as biochemical processes, in response to environmental stimuli (Fraser et al. 2011) and uncover ecologically important phenotypes for adaptive evolution (e.g, Fraser et al. 2010; Nourmohammad et al. 2017). Plasticity of expression affecting seasonal migration (Johnston et al. 2016), reproductive status (Nakane and Yoshimura 2014), hibernation (Schwartz et al. 2013; Vermillion et al. 2015) and immune response (Dopico et al. 2015) have been shown to be important adaptions to seasonality in various species. Second, pairing plastic response direction with that of evolutionary change or *apriori* hypotheses of the direction of expression changes, functionally grouped genes could also be used to assess whether expression plasticity is adaptive (Ghalambor et al. 2007). In addition, knowledge of the identity of the expression quantitative trait loci (eQTLs), and their spatial and temporal allele frequency distributions can provide the opportunity to examine whether there is significant overlap between plasticity and adaptive evolution at the genetic level. For example, we can ask whether genes with plastic expression patterns (hereafter "plastic genes") are enriched or depleted for seasonally varying eQTLs, which are the putative genetic basis for seasonal adaptive evolution.

Despite the advantages of studying gene expression variation in response to environmental changes, a few challenges remain in investigating plastic expression changes and testing for adaptive plasticity across seasons. For example, seasonal environmental heterogeneity can be stochastic on a short time scale (Williams et al. 2017). Therefore, large sample sizes from multiple seasonal time points are required to gain insight into a more general scope of seasonal

gene expression variations. Moreover, it is difficult to test whether the plastic changes are adaptive when lacking *apriori* assumptions, either based on fitness consequences of expression differentiations or the direction of evolutionary change, about the direction of plasticity.

The model organism *Drosophila melanogaster* is a robust system to study plastic gene expression variation and test for adaptive plasticity across seasons. *D. melanogaster* is widely distributed across both latitudinal clines and seasons with documented genome-wide adaptive evolution in gene expression across latitudinal gradients (e.g., Zhao et al. 2015; Juneja et al. 2016) and environmental treatments (e.g., Sørensen et al. 2007). These evolved expression profiles can be viewed as proxies for seasonal evolutionary changes (e.g., in response to temperature oscillations) and can be used to test for adaptive plasticity in the absence of seasonal evolutionary profiles in gene expression. For example, in principle, the direction of gene expression plasticity between summer (high temperature) and fall (low temperature) should show concordant signal with evolved expression differences in selection for increased heat survival under the *a-priori* assumption that expression plasticity is adaptive. In addition, genome-wide eQTLs (Everett et al. 2020) and direct evidence of adaptive evolution at thousands of loci (i.e., seasonal SNPs), have been reported in this species (Bergland et al. 2014; Machado et al. 2021). Such genetic information provides an opportunity to examine whether eQTLs associated with plastic genes are evolving across seasons.

Genome-wide gene expression plasticity across seasonal time points or temperature ranges in *D. melanogaster* in the wild still remains to be investigated despite previously reported gene expression plasticity across temperature (Chen et al. 2015) or stress treatments (Zhou et al. 2012)

in the lab environments. The lack of effort to examine expression plasticity in wild seasonal environments in this species could be partially due to the difficulty in measuring plasticity in wild seasonal environments and the focus on studying extreme environmental treatments (Chen et al. 2015).

Here we examine gene expression plasticity across seasonal time points in an experimental orchard in the wild using genetically controlled F_1 *D. melanogaster* populations. We used F_1s instead of inbred lines to avoid inbreeding depression in gene expression. For example, inbreeding depression can cause gene expression differences that are enriched for functional categories that may also be ecologically important, such as stress and immune response (Vermeulen et al. 2013). Thus, using F_1s allows us to reduce the effect of inbreeding when inferring functions of seasonally plastic genes. We address three questions in this study: 1. Is plastic gene expression prevalent across seasonal time points? 2. What are the functional implications of the seasonal plastic genes and is there evidence for adaptive plasticity? 3. Are plastic genes enriched for seasonal varying eQTLs ?

Materials and Methods

Crossing design. We examined seasonal gene expression plasticity by doing whole genome bulk RNA sequencing (hereafter RNASeq) on pools of genetically controlled F_1 populations across 10 seasonal time points from May to October. We created two replicates of 21 experimental F_1 crossing panels in bottles of standard fly food using 29 inbred lines collected from 4 populations (Maine, North Carolina, Pennsylvanian spring, Pennsylvanian fall, Figure 1A) at approximately two-week intervals. There was a total of 84 F_1 genotype combinations. Our crossing design was

block randomized. We assigned 3 blocks covering 10 seasonal time points (May to July; July to August; August to October) such that in each block every 84 F_1s is selected only once. For each seasonal time point in every block, we randomly selected 21 F_1 genotypes (Figure 1B, Table S1). Although the genetic component for each of 10 seasonal time points is different, we reason that based on our block randomized design, the gene expression variation across seasonal time points can be considered as plastic changes.

Sample collections. After 24 hours of eggs being laid in the laboratory, we put out and reared the F_1s in an experimental orchard on Morven Farms (VA: 37.96°N, -78.47°W) from an embryo stage to 3-5 days after their eclosion throughout a growing season across 10 seasonal time points (referred to as 1^{st} to 10^{th}) in 2019 (May to October). The F_1s were kept in bottles of standard fly food in an experimental cage. Each bottle contained one replicate of an F_1 genotype. Temperature data was tracked at Morven Farms using *iButtons*® (iButtonLink, LLC). Adult flies 3-5-day-old were sampled, snap froze on-site with liquid nitrogen, and stored in -80°C freezer later.

RNAseq experiment. Whole body bulk RNA pooled from two female flies per sample was extracted and purified using the *Agencourt* Kit following the manufacturer protocol (Beckman Coulter, LLC). Four cDNA libraries (Table S2, L_{B1B4}: 90; L_{B2B3}: 88; L_{B5B7}: 92; L_{B6}: 60 samples) were prepared using 200 ng of input RNA from each sample with the *BRBSeq* Kit following the manufacturer protocol (Alpern et al. 2019). All four libraries were amplified with 15 polymerase chain reaction cycles and sequenced with NovaSeq6000 using a 2 x 150 bp protocol. All sequences have been archived online.

Data filtering and modeling. Sequenced reads were trimmed and mapped to the *D. melanogaster* 6.01 assembly using *STAR* (v.2.7.9a) (Dobin et al. 2013) following the recommended parameters (Alpern et al. 2019), resulting on average 1,296,655 unique molecular identifiers (read count) per sample (Table S2). We kept genes with read count value ≥ 16 and mapped in more than 80% (n = 260) of samples for further analysis. We then generated read count matrix from a filtered set of 5,198 genes using *edgeR* (v.3.28.1), including normalization steps (Robinson et al. 2010). To model plastic gene expression, we fitted linear models to measure gene expression changes using the seasonal time points (hereafter "seasonal model") or temperature (hereafter "temperature model") as independent variables. We grouped genes into significant expression categories (top1%, top5%, top10%, all-DE, non-DE) based on combined filtering on both ranked coefficient and ranked *p*-value quantiles identified in each of the models separately. Top10% differentially expressed genes, or "plastic genes" hereafter, (seasonal model: n = 170 genes; temperature model: n = 169 genes) from each model were used for GO, concordance, and consensus co-expression network analysis.

GO and concordance analysis. We performed GO enrichment analysis on the top 10% plastic genes identified in each model, comparing against the total data set of 5,198 genes using *GOStats* (v.2.52.0) (Falcon and Gentleman 2007). Significantly overrepresented GO categories were selected based on an FDR q value ≤ 0.01.

To test for putative adaptive plasticity in gene expression, we examined the directionality of gene expression in our data by comparing gene expression levels in summer or hot environment (July:

27.2°C) to fall or cold environment (October: 17.5°C). For this analysis, we incorporated four gene sets to generate *a-priori* hypotheses on the directionality of expression from summer to fall, and further tested whether plastic gene expression in our data show concordance with such assumptions.

The first gene set was the latitudinal differentially expressed genes (hereafter "latitudinal DE genes") identified by Juneja *et al* (2016) using females that are descendants of flies sampled from high and low latitudinal populations in both North America and Australia. These latitudinal DE genes thus represent genetically evolved expression differentiations between high and low latitudinal populations. Given the similarity in temperature between high latitudinal locales and fall as well as between low latitudinal locales and summer, we hypothesized that genes with higher expression in high latitudinal environments should also have higher expression in the fall. Such hypothesis is different from the commonly accepted assumption that high latitudinal locales is similar to the spring (end of winter) and that low latitudinal locales is similar to the fall (end of summer) (e.g., Machado et al. 2021). The difference arises from the different natures of the datasets: we examine plasticity within a generational time whereas Machado *et al* (2021) studied intergenerational genetic evolution. The second gene set we incorporated was the well-studied heat shock protein family genes (Logan and Cox 2020). For this data set, we hypothesized that heat shock proteins should be upregulated in the summer compared to fall. The third gene set was identified from a study examining female gene expression evolution under different environmental stresses (Sørensen et al. 2007), including heat survival (selection for increase in resistant to heat exposure in water bath), desiccation (selection for increase in desiccation resistance from 12h to 20h), and starvation treatments (selection for increase in starvation

resistance from 35h to 60h). We hypothesized that the direction of evolved gene expression change after selection for increased heat survival should show discordant signal compared to that of plastic change from summer to fall. On the contrary, the direction of evolved gene expression change after selection for starvation resistance and desiccation should show concordant signal compared to that of plastic change from summer to fall.

We then calculated concordance score as the proportion of the genes in our data that change in the expected direction as hypothesized from summer to fall for each of the four gene sets separately. We calculated binomial *p*-values for the concordance score with the null expectation of 50%. Plastic gene expression was considered to show concordant signal with evolutionary change if their concordance scores were significantly greater than 50%, discordant signal with concordance scores significantly lower than 50%, and neutral if concordance scores show non-significant results.

Consensus co-expression network analysis. To identify conserved co-expression networks across seasonal time points or temperature ranges, we used the top 10% plastic genes identified in each model and performed weighted gene co-expression network analysis in *WGCNA* (v.1.70.3) (Langfelder and Horvath 2008). We set the soft thresholding power as 6 and the minimum co-expression network size as 5.

Enrichment of seasonal SNPs in eQTLs. To tested if eQTLs associated with non-DE or DE genes show different patterns of seasonal allele frequency changes, we performed an enrichment analysis. We incorporated two other publicly available dataset for this analysis. The first dataset

reported the seasonal SNPs identified using 20 paired spring-fall samples (Machado et al. 2021; Nunez et al. 2021) from geographically distributed localities across two continents ("Core 20"). Machado *et al* (2021) modeled allele frequency change at each SNP across time using generalized linear models. The multi-population seasonal model used "spring" and "fall" labels as independent variables (hereafter "cross-population"). We used the output of this model to define "seasonal" polymorphisms based on *p*-value and used *p*-values for enrichment tests. The second dataset included eQTLs identified in standard lab conditions (Everett et al. 2020) that are also polymorphic among the seasonally sampled populations. Everett *et al* (2020) identified eQTLs using RNASeq data on pre-genotyped inbred DGRP lines against SNPs with > 0.05 allele frequency and < 25% missing phenotypes for both sexes (3-5 day mated, whole body). Thus, these eQTLs represent genetic variants affecting gene expression. For this enrichment analysis, we compared eQTLs to sets of matched control SNPs (hereafter "controls") that were not identified as eQTLs themselves. For each eQTL, we identified 1000 control SNPs matched for chromosomal arm, heterozygosity (binned by 0.05), and inversion status classified as "breakpoint" (±0.5Mb around known inversion breakpoints), "inside" the inversion region and excluding breakpoint regions, "outside" the inverted region and excluding break regions, of 6 cosmopolitan inversions (Corbett-Detig and Hartl 2012). Heterozygosity, ranging from 0 to 0.5 with an increment of 0.05, for each SNP was estimated from the DGRP.

We tested if eQTLs are enriched for seasonal SNPs relative to controls based on their ranked seasonal *p*-value quantiles for both eQTLs grouped by non-DE and DE genes from each model. For the test set of eQTLs or each of the 1000 sets of control SNPs, we used the counts of SNPs above and below a *p*-value quantile of 0.05 to calculate 1000 odds-ratios. We calculated the odds

ratio (OR) as AD/BC, where A and C are the counts of eQTLs (A) or controls (C) below or equal to a *p*-value quantile of 0.05, and B and D are the counts of eQTLs (B) or controls (D) above a *p*-value quantile of 0.05. We $\log_2$ transformed odds-ratio and calculated confidence interval as 1.96 x standard deviation of the mean (1000 sets).

We then calculated empirical *p*-values for the enrichment analysis. Let S be the observed value and S_0 be the expected distribution generated by 1000 sets of controls, N is the total number of tests, and defined

$$p = (1+\sum(S \geq S_0))/(N+1)$$

$$\text{emp.}p = 2 \text{ x min } (p, 1-p)$$

(Davison and Hinkley 1997)

Results

Differential expressions. We processed sequencing reads from RNASeq data on 330 samples across 10 seasonal time points (18 - 42 samples per seasonal time point). The average number of reads sequenced for each sample was ~6.54M, with the average of ~1.29M unique molecular identifier (UMI) counts and ~10,262 genes identified per sample (Table S2). After applying filtering step (see Materials and Methods), we used 5,198 genes expressed in 323 samples for downstream analysis (Table S3). By building time-series models in *edgeR*, we identified 4057 (78.05%) and 3712 (71.41%) of differentially expressed genes at an FDR $q < 0.05$ in the

seasonal and temperature model, respectively (Figure 1C). Of the differentially expressed genes, 50.65% (53.50%) of them increased and 49.35% (46.50%) decreased with seasonal time (temperature).

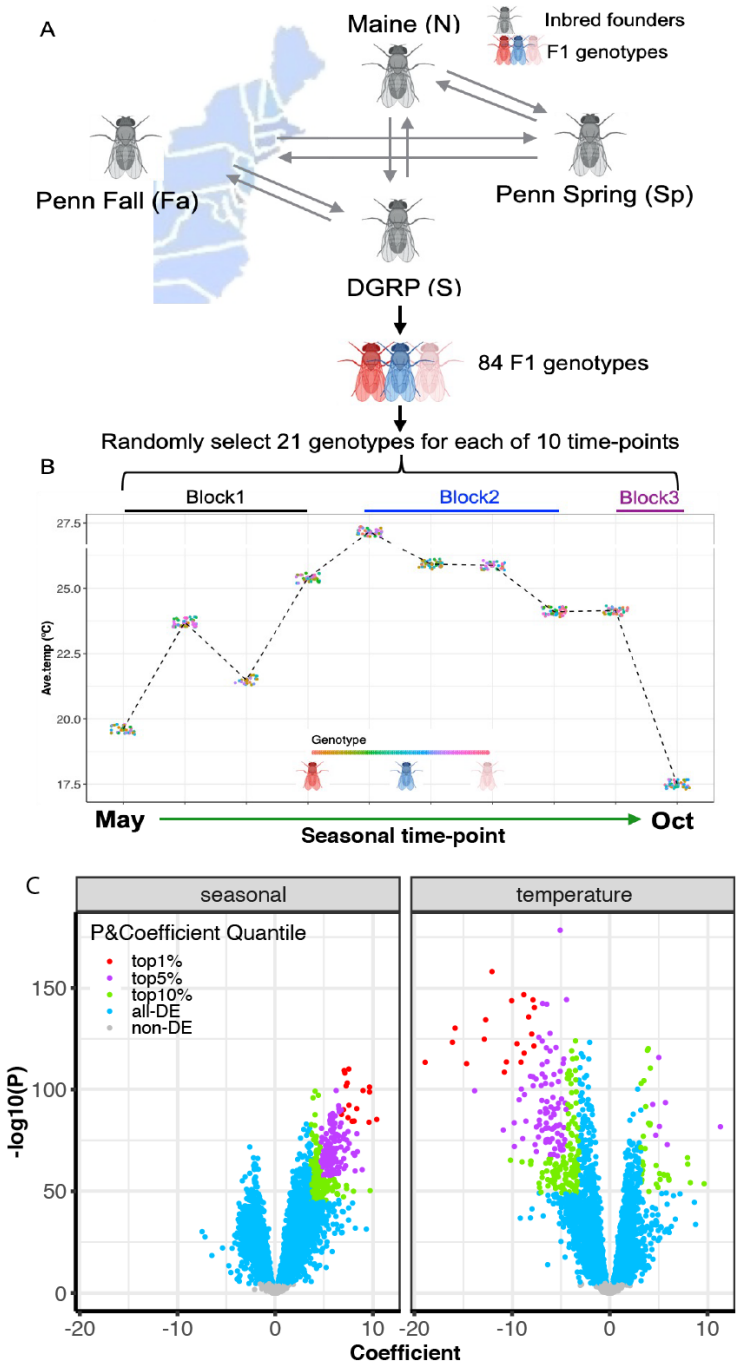

Figure 1. Experimental design (A-B) and differential expression under seasonal (left) or temperature (right) linear models (C). (A) 84 F_1 genotypes were created using 29 inbred founder lines from 4 populations. (B) In block randomized sampling processes, 21 F_1 genotypes were randomly selected for each of the 10 seasonal time points from May to October. In block1 and block2, 84 F_1s were non-repeatedly selected. In block3, 42 F_1s were non-repeatedly selected. The x-axis is the 1^{st} to 10^{th} seasonal experimental time point (May to Oct). The y-axis is the average temperature during each experimental period. Colored dots represent the F_1 genotypes. (C) The x-axis is the coefficient for gene expression under the linear models. The y-axis is the *p*-value of the model on a $-\log_{10}$ scale. Colors indicate level of differential expression, ranked by both *p*-value and coefficient identified by the models.

Functional implications of plastic genes. To assess the functions of the plastic genes, we performed GO analysis on the top 10% plastic genes in each model and identified overrepresented GO terms with a selection of an FDR $q \leq 0.01$. We identified 20 and 14 significantly overrepresented GO terms in seasonal and temperature model, respectively (Figure 2A). Six GO terms are shared between the two models, including four associated with metabolic processes and two associated with biosynthetic processes. The most overrepresented GO terms are "tricarboxylic acid cycle" ($\log_2(OR) = 3.488$, FDR $q = 3.48 \times 10^{-3}$) and "muscle contraction" ($\log_2(OR) = 4.723$, FDR $q = 1.56 \times 10^{-4}$) for seasonal and temperature model, respectively (Figure 2A). Of the plastic genes grouped by "tricarboxylic acid cycle" and "muscle contraction", 87% (binomial $p = 6.60 \times 10^{-5}$) and 100% (binomial $p < 0.01$) increase in expression level from summer to fall, respectively (Figure 2B).

Conserved co-expression networks. Next, we performed consensus network analysis in WGCNA using top10% plastic genes in each model separately. At a soft thresholding power of 6

and minimum gene size of 5, single co-expression networks are identified, suggesting conserved co-expression networks across seasonal time points or temperature ranges (Figure 2C).

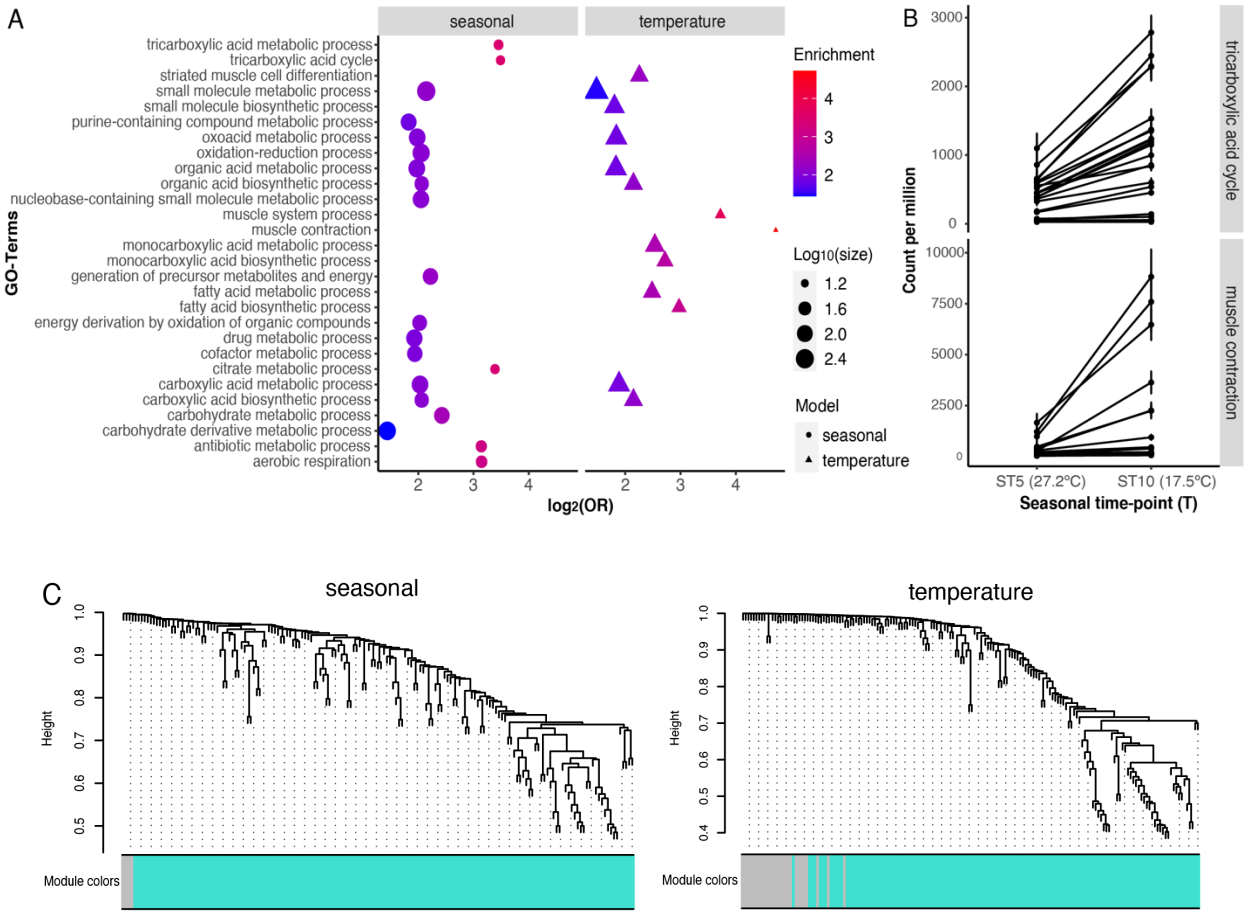

Figure 2. (A). GO terms enrichment levels for top10% plastic genes in each model with FDR q value ≤ 0.01. Enrichment level is represented by $\log_2$ (odds ratio). The size of the dots or triangles represent the gene sample size associated with the GO terms. (B). Expression levels between 5th (summer: 27.2°C) and 10th (fall: 17.5°C) seasonal time points for genes grouped by GO terms "tricarboxylic acid cycle" and "muscle contraction". Error bars represent 2 SEMs. (C). Consensus co-expression modules of top 10%

plastic genes under seasonal (left) or temperature (right) linear models. Color bars represent co-expression modules. Grey bars represent ungrouped genes.

Putative adaptive and maladaptive plasticity. To test whether plastic genes identified show adaptive plasticity in gene expression, we performed concordance analysis by incorporating gene sets with *apriori* hypotheses about the directionality of expression level differentiations (Figure 3). By comparing summer and fall gene expression levels, we show concordant signals for heat shock protein coding genes (marginal significance: binomial $p = 6.60 \times 10^{-2}$), suggesting gene expression plasticity could be adaptive. In contrast, we find discordant signal for plastic genes that are grouped by the previously identified latitudinal DE genes (binomial $p = 1.05 \times 10^{-13}$), desiccation genes (binomial $p = 1.86 \times 10^{-2}$), heat survival genes (binomial $p = 2.39 \times 10^{-5}$). Such discordant signal could suggest that either maladaptive plasticity in the expression differentiations or that selective pressures imposed by seasonal heterogeneity is different from our assumptions. In addition, we find neutral signal for starvation genes (binomial $p = 0.84$).

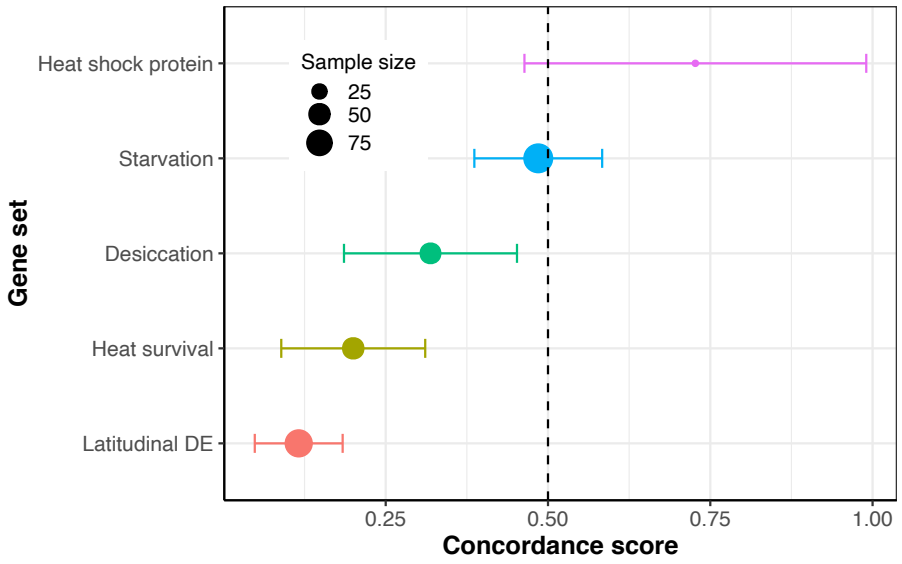

Figure 3. Concordance score of plastic genes grouped by four gene sets. Error bars represent binomial confidence intervals. Gene sample sizes are represented by the size of the dots.

eQTLs enrichment. To compare adaptive signals at eQTLs associated with both non-plastic and plastic genes, we calculated the enrichment of seasonal SNPs in eQTLs grouped by the two categories. We show that non-plastic genes are neither enriched nor depleted for seasonal SNPs (seasonal model: *emp.p* = 0.426; temperature model: *emp.p* = 0.274). However, we find marginal significance in depletion (*emp.p* = 0.088) and significant depletion signal (*emp.p* = 0.006) in plastic genes identified by the seasonal model and the temperature model, respectively (Figure 4). Such results suggest that eQTLs associated with plastic genes are less likely to change in allele frequencies across the season. It could also suggest that plasticity and genetic evolution of gene expression level could have different genetic bases or that plasticity and genetic evolution involve different sets of genes.

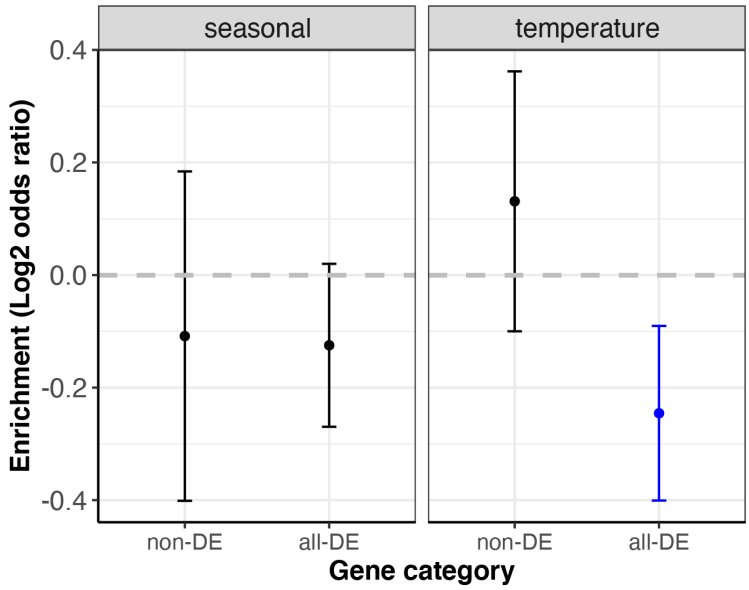

Figure 4. Enrichment of seasonal SNPs in eQTLs associated with genes under seasonal (left) or temperature (right) linear models. The x-axis represents gene category, including non-differentially expressed (non-plastic) and differentially expressed (plastic) genes under each model, respectively. The y-axis is the enrichment, represented by odds ratio on a $\log_2$ scale, of seasonal SNPs in eQTLs relative to matched controls.

Discussion

Our study on gene expression plasticity across seasonal time points and temperature ranges identified a large proportion of the fly genome being plastic. We show that the top plastic genes (top 10%) are functionally enriched, and form conserved co-expression networks across seasons. Interestingly, we observe discordant signal ("counter-gradient") between plastic response and evolutionary change for genes grouped by ecologically relevant traits or those identified with latitudinal differential expression patterns. The "counter-gradient" expression patterns suggest putatively maladaptive plasticity. We further show that these plastic genes have limited overlap with adaptive evolution at the eQTL level. Our study is one of the first datasets revealing prevalent seasonal plasticity in gene expression in *D. melanogaster* in wild environments and provides evidence that such plasticity possibly is maladaptive.

Prevalence of gene expression plasticity and functional implications. Here we show in our data that ~78% and ~71% of the analyzed genes are plastic in the seasonal and temperature model, respectively (Figure 1C). The observed proportion of genes with temperature induced plasticity is comparable to that measured in a previous laboratory study across four temperature ranges (Chen et al. 2015), but larger than a few other studies (Levine et al. 2011; Zhou et al. 2012). This

result could either due to the higher power in our study and the broader range of temperature surveyed (Chen et al. 2015) or that these changes could also be caused by other environmental factors that change co-linearly with temperature (Mathur and Schmidt 2017). Such a high level of plasticity may be important for adaptive evolution to novel seasonal environments in the future. For example, plastic response may decrease the probability for population extinction in rapidly changing seasonal environments by reducing the cost of selection and maintaining large population size, allowing for populations to adapt via standing genetic variation (Haldane 1957; Price et al. 2003). In addition, this high level of plasticity could prevent the deleterious novel mutations from being removed by purifying selection when expression traits are polygenic. Such accumulation and the later release of these mutations may play an important role in novel environments (Snell-Rood et al. 2010; Dayan et al. 2015).

Linking plasticity in expression to functional categories remains an important task in studying how organisms cope with environmental heterogeneity (Chen et al. 2015). By using the top10% plastic genes, we identified significantly overrepresented GO terms, including metabolic processes, biosynthetic processes, and muscle processes (Figure 2A). These overrepresented functions have been shown to be important physiological phenotypes in coping with the seasonal/temperature environments. For example, both ATP production (Colinet et al. 2017) and metabolic rates (Mallard et al. 2018) have been shown to decrease during cold stress evolution but increase after cold acclimations (Colinet et al. 2017). Muscle contraction affecting locomotor activity (Ormerod et al. 2022) may also be an important seasonal phenotype (MacMillan et al. 2016). Low temperature is known to suppress muscle force in insects (Langfeld et al. 1991) and therefore can affect their flying performances.

One interesting difference between our findings and some previous work is that we did not identify “ion channels”, whose conductance becomes lower in response to cold (Hoffmann and Dionne 1983), being an overrepresented GO category in relation to temperature response (e.g., Runcie et al. 2012). Such difference could be due to the fact that we performed GO analysis using the top10% plastic genes, either increasing or decreasing, whereas others identify GO terms by distinguishing expression into increasing and decreasing categories (Runcie et al. 2012).

Putative maladaptive plasticity. Plastic gene expression has long been associated with response to environmental shifts. Accumulating evidence on both adaptive and maladaptive plasticity exist (e.g., Huang et al. 2022). Adaptive plasticity predicts a concordant change between the direction of plasticity and that of evolution, whereas maladaptive plasticity predicts a discordant (“counter-gradient”) one. Here in our data, we show that seasonal expression plasticity shows “counter-gradient” signals compared to evolution, and thus is likely maladaptive. Such maladaptive signal is also trait specific.

Our results reveal maladaptive or neutral signals for genes grouped as latitudinal DE, heat survival DE, and desiccation DE genes (Figure 3). The maladaptive signal is consistent with several previous studies in both *D. melanogaster* (Levine et al. 2011; Huang and Agrawal 2016) and in other organisms (Dayan et al. 2015; Ghalambor et al. 2015). There could be several possible explanations for the maladaptive signal. First, due to rapid environmental shifts, the initial plastic response may be a maladaptive state (Leonard and Lancaster 2020; Huang et al.

2022), possibly due to genetic constraints on plasticity (Grether 2005). In such cases, adaptive evolution by selection can restore expression phenotypes to the optima by genetic compensation (Grether 2005). Thus, maladaptive plasticity can enforce genetic evolution (Gibert et al. 2019). Second, we may lack a fundamental understanding of the seasonal selective pressures. For example, we observe discordant signal for latitudinal DE genes under the assumption that the seasonal environmental changes mirrors that of spatial environmental heterogeneity (Figure 3). However, phenotypic adaptation to latitudinal and seasonal environmental heterogeneity could be fundamentally different processes and we might lack a general understanding of the ecological process of seasonal environmental changes (Yu and Bergland 2022). If seasonal selective pressures differ in directions in various geographical locations and therefore are population specific (Machado et al. 2021), we might observe such discordance due to the inconsistency between latitudinal and seasonal selective pressures (Yu and Bergland 2022).

We also note trait-specific patterns for the concordance signals in expression plasticity. For example, while both adaptive and maladaptive plasticity in starvation resistance have been shown on the phenotypic level (reviewed in (Rion and Kawecki 2007), plastic genes associated with starvation treatment show neutral concordance, suggesting they are changing in a stochastic way (Figure 3). Our result is possibly due to the fact that we kept flies in bottles on constant food supply, and they never suffered starvation. Thus, starvation tolerance and the associated expression plasticity may never have affected fitness in our study. Therefore, identifying the phenotypes under selection and the selection agent can be important steps for interpreting adaptive *vs.* maladaptive plasticity.

Our study generates new testable hypotheses in studying how populations cope with seasonality. For example, we can test whether metabolic rates differ across seasons and whether it influences fitness traits, such as heat survival. In addition, by incorporating physiological plasticity data (Chapter 3), it would be possible to underpin the plastic genes associated with adaptive physiological changes across space and time. However, in order to strictly test for adaptive *vs.* maladaptive seasonal gene expression plasticity, future work on evolved gene expression and the fitness consequences of gene expression evolution across seasons are needed.

eQTLs enrichment. Adaptive evolution and plasticity are predicted to be non-overlapping mechanisms for populations of organisms to cope with environmental heterogeneity. Transitioning from one mechanism to another could likely cause a population collapse when the predictability of environmental change remains the same (Botero et al. 2015). We assessed whether seasonal gene expression plasticity and adaptive evolution are non-overlapping processes by examining the enrichment of seasonal SNPs in eQTLs. We observe a depletion of seasonal SNPs in eQTLs in the plastic genes (Figure 4). Such a result suggests that the eQTLs associated with plastic genes are less likely to change allele frequencies across seasons.

We propose several possible explanations for the depletion of seasonal SNPs. First, adaptive plasticity could be a main mechanism for fly populations to cope with seasonality for gene expression traits. For example, plasticity can reduce the strength of selection on standing genetic variation (Price et al. 2003). Thus, eQTLs associated with plastic genes could show weak signals of evolution across seasons. Such a possibility can be assessed with evolution data on gene expressions or associating gene expression with performances and fitness (Hoffman and Parsons

1991). Second, the seasonal SNPs are identified by Machado *et al* (2021) from a multi-populational model. Seasonal allele frequency change could be unique from one seasonal population to another (Erickson et al. 2020; Yu and Bergland 2022). Thus, it is possible that the seasonal eQTLs associated with plastic genes in our study are omitted from the generalized multi populational model. Third, plasticity and evolution at the expression level could compose different sets of genes (Dayan et al. 2015). Therefore, allele frequency for eQTLs associated with plastic genes can be more stable than those associated with non-plastic or seasonally evolved genes.

List of supplemental tables

Supp. Table S1: Sample F1 genotype information table.

Supp. Table S2: Sequencing library information table.

Supp. Table S3: Grand Sample information table.

Table S1. Sample F_1 genotype information table. "time" represents 10 seasonal time points CT1 (ST1) to CT10 (ST10). "date" represents the start date of the assays for each of the 10 seasonal time point. "actual.geno" is the genotype of the F_1s formatted as "maternal inbred line id" x "paternal inbred line id". "geno" represents the genotype identifier in the RNA sequencing experiment. "replicate #" is the number of replicate samples for each genotype at each seasonal time point.

time	date	actual.geno	geno	replicate #
CT1	5/3/19	12LN10_32x12LN6_6	Cr1A	1
CT1	5/3/19	dgrp_29660x12BME10_270	Cr1B	1
CT1	5/3/19	dgrp_28191x12LN6_6	Cr1C	1
CT1	5/3/19	12LN6_28x12LN10_67	Cr1E	2
CT1	5/3/19	12LN10_35x12BME10_211	Cr1F	2
CT1	5/3/19	12LN6_19xdgrp_28261	Cr1G	1
CT1	5/3/19	12BME10_204x12LN10_76	Cr1H	1
CT1	5/3/19	12LN6_19x12LN10_22	Cr1I	1
CT1	5/3/19	dgrp_29660x12BME10_229	Cr1J	1
CT1	5/3/19	12BME10_204xdgrp_25744	Cr1K	2
CT1	5/3/19	12LN6_28x12LN10_76	Cr1L	2
CT1	5/3/19	12LN6_16x12LN10_67	Cr1M	2
CT1	5/3/19	dgrp_28189x12BME10_211	Cr1N	1
CT1	5/3/19	12LN6_12xdgrp_28261	Cr1O	2
CT1	5/3/19	12BME10_218xdgrp_25744	Cr1P	2
CT1	5/3/19	12BME10_212x12LN10_76	Cr1Q	2
CT1	5/3/19	12BME10_130xdgrp_28241	Cr1R	1
CT1	5/3/19	12LN6_28x12LN10_95	Cr1S	2
CT1	5/3/19	12LN10_14x12LN6_6	Cr1T	2
CT2	5/24/19	12BME10_130x12LN10_22	Cr2A	2
CT2	5/24/19	dgrp_28189x12BME10_270	Cr2B	2
CT2	5/24/19	12LN6_12xdgrp_28241	Cr2C	2
CT2	5/24/19	dgrp_28189x12BME10_229	Cr2D	2
CT2	5/24/19	12LN6_16xdgrp_28241	Cr2E	2
CT2	5/24/19	12LN10_35x12LN6_84	Cr2F	2
CT2	5/24/19	dgrp_28191x12BME10_229	Cr2G	2
CT2	5/24/19	12LN10_13x12LN6_6	Cr2H	2
CT2	5/24/19	12LN10_32x12LN6_84	Cr2I	2
CT2	5/24/19	12LN10_32x12LN6_46	Cr2J	2

CT2	5/24/19	12LN6_16x12LN10_22	Cr2K	2
CT2	5/24/19	12LN10_14x12LN6_84	Cr2L	2
CT2	5/24/19	12LN10_13x12BME10_270	Cr2M	2
CT2	5/24/19	dgrp_28191x12BME10_270	Cr2N	2
CT2	5/24/19	12LN6_19x12LN10_95	Cr2O	2
CT2	5/24/19	dgrp_28191x12LN6_46	Cr2P	2
CT2	5/24/19	12LN10_32x12BME10_270	Cr2Q	2
CT2	5/24/19	dgrp_28191x12BME10_104	Cr2R	2
CT2	5/24/19	12BME10_204xdgrp_28261	Cr2S	2
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CT2	5/24/19	dgrp_28189x12LN6_6	Cr2U	2
CT3	6/3/19	12BME10_204x12LN10_67	Cr3B	1
CT3	6/3/19	12LN6_16xdgrp_28261	Cr3E	1
CT3	6/3/19	12BME10_212xdgrp_28261	Cr3F	2
CT3	6/3/19	12BME10_204x12LN10_22	Cr3G	1
CT3	6/3/19	12BME10_218x12LN10_95	Cr3H	2
CT3	6/3/19	12LN6_16x12LN10_76	Cr3I	2
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CT3	6/3/19	12BME10_212xdgrp_28241	Cr3P	1
CT4	6/19/19	12LN10_35x12BME10_229	Cr4A	1
CT4	6/19/19	12LN10_14x12BME10_211	Cr4B	1
CT4	6/19/19	12LN6_16x12LN10_95	Cr4E	2
CT4	6/19/19	12BME10_218x12LN10_67	Cr4F	2
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CT4	6/19/19	12BME10_130xdgrp_28261	Cr4H	1
CT4	6/19/19	12LN6_28xdgrp_25744	Cr4I	2
CT4	6/19/19	12LN6_19x12LN10_67	Cr4J	1
CT4	6/19/19	12BME10_218xdgrp_28261	Cr4L	2
CT4	6/19/19	12LN10_14x12BME10_229	Cr4M	2
CT4	6/19/19	12LN10_35x12LN6_46	Cr4N	2
CT4	6/19/19	dgrp_29660x12BME10_104	Cr4O	1
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CT4	6/19/19	dgrp_28191x12LN6_84	Cr4Q	2
CT4	6/19/19	12LN10_14x12LN6_46	Cr4R	2
CT4	6/19/19	12BME10_218xdgrp_28241	Cr4S	2

CT4	6/19/19	dgrp_28189x12BME10_104	Cr4Z	2
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CT5	7/5/19	12LN10_32x12BME10_270	Cr5B	1
CT5	7/5/19	12BME10_130xdgrp_25744	Cr5C	1
CT5	7/5/19	12BME10_212xdgrp_28261	Cr5D	1
CT5	7/5/19	dgrp_29660x12LN6_46	Cr5E	2
CT5	7/5/19	12LN10_35x12LN6_84	Cr5F	2
CT5	7/5/19	dgrp_28191x12LN6_6	Cr5G	2
CT5	7/5/19	dgrp_29660x12BME10_229	Cr5H	2
CT5	7/5/19	dgrp_28189x12BME10_104	Cr5I	2
CT5	7/5/19	12LN10_32x12LN6_84	Cr5J	2
CT5	7/5/19	12LN6_28x12LN10_22	Cr5K	2
CT5	7/5/19	dgrp_29660x12LN6_6	Cr5L	2
CT5	7/5/19	12BME10_218x12LN10_67	Cr5M	1
CT5	7/5/19	12LN6_28x12LN10_76	Cr5N	2
CT5	7/5/19	dgrp_28189x12BME10_229	Cr5O	2
CT5	7/5/19	12LN10_14x12LN6_84	Cr5P	2
CT5	7/5/19	12LN10_13x12BME10_270	Cr5Q	2
CT5	7/5/19	12LN6_28xdgrp_25744	Cr5R	2
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CT6	7/20/19	12LN6_16xdgrp_28261	Cr6B	1
CT6	7/20/19	12LN6_12xdgrp_28241	Cr6C	2
CT6	7/20/19	12BME10_218x12LN10_76	Cr6D	2
CT6	7/20/19	12LN6_19xdgrp_28261	Cr6E	2
CT6	7/20/19	dgrp_28191x12BME10_104	Cr6F	2
CT6	7/20/19	12BME10_204x12LN10_76	Cr6G	2
CT6	7/20/19	12BME10_212xdgrp_28241	Cr6H	2
CT6	7/20/19	12LN6_12x12LN10_22	Cr6I	2
CT6	7/20/19	12LN10_13x12LN6_46	Cr6J	2
CT6	7/20/19	12LN6_16x12LN10_95	Cr6K	2
CT6	7/20/19	12BME10_130xdgrp_28241	Cr6L	2
CT6	7/20/19	12LN6_16xdgrp_28241	Cr6M	2
CT6	7/20/19	12LN6_16x12LN10_67	Cr6N	2
CT6	7/20/19	12BME10_218xdgrp_28261	Cr6O	1
CT6	7/20/19	12BME10_212xdgrp_25744	Cr6P	2
CT6	7/20/19	12LN6_16x12LN10_22	Cr6Q	2
CT6	7/20/19	12LN10_14x12LN6_6	Cr6R	2

CT6	7/20/19	12LN10_13x12BME10_229	Cr6S	1
CT6	7/20/19	12LN10_32x12LN6_6	Cr6T	1
CT6	7/20/19	12BME10_204xdgrp_25744	Cr6U	2
CT7	8/7/19	12LN6_19x12LN10_67	Cr7B	2
CT7	8/7/19	dgrp_28189x12BME10_211	Cr7C	2
CT7	8/7/19	12LN10_32x12BME10_229	Cr7D	2
CT7	8/7/19	dgrp_28189x12LN6_6	Cr7E	1
CT7	8/7/19	dgrp_28189x12BME10_270	Cr7F	2
CT7	8/7/19	12BME10_218xdgrp_25744	Cr7G	2
CT7	8/7/19	12BME10_204xdgrp_28261	Cr7H	2
CT7	8/7/19	12BME10_212x12LN10_76	Cr7I	2
CT7	8/7/19	12LN6_19x12LN10_22	Cr7J	2
CT7	8/7/19	12LN6_28x12LN10_67	Cr7K	2
CT7	8/7/19	12LN10_32x12LN6_46	Cr7L	2
CT7	8/7/19	dgrp_29660x12BME10_270	Cr7M	2
CT7	8/7/19	12LN6_28xdgrp_28261	Cr7P	1
CT7	8/7/19	12LN6_12xdgrp_28261	Cr7Q	1
CT7	8/7/19	12BME10_130x12LN10_22	Cr7S	1
CT7	8/7/19	dgrp_28191x12LN6_46	Cr7V	1
CT7	8/7/19	12LN10_35x12LN6_6	Cr7W	1
CT7	8/7/19	dgrp_28191x12BME10_211	Cr7Z	2
CT8	8/22/19	12LN10_14x12LN6_46	Cr8A	2
CT8	8/22/19	12LN6_12x12LN10_95	Cr8B	2
CT8	8/22/19	dgrp_28191x12LN6_84	Cr8C	2
CT8	8/22/19	12BME10_218xdgrp_28241	Cr8D	2
CT8	8/22/19	12LN6_12x12LN10_76	Cr8E	2
CT8	8/22/19	dgrp_29660x12BME10_211	Cr8F	2
CT8	8/22/19	12LN10_35x12LN6_46	Cr8G	2
CT8	8/22/19	12LN6_12x12LN10_67	Cr8H	2
CT8	8/22/19	12BME10_218x12LN10_95	Cr8I	2
CT8	8/22/19	12LN6_28xdgrp_28241	Cr8J	2
CT8	8/22/19	12BME10_204xdgrp_28241	Cr8K	2
CT8	8/22/19	dgrp_28191x12BME10_270	Cr8L	1
CT8	8/22/19	12LN6_19x12LN10_76	Cr8M	2
CT8	8/22/19	dgrp_29660x12BME10_104	Cr8N	2
CT8	8/22/19	dgrp_28189x12LN6_84	Cr8O	2
CT8	8/22/19	12LN10_14x12BME10_229	Cr8P	2
CT8	8/22/19	12LN6_16x12LN10_76	Cr8Q	2
CT8	8/22/19	dgrp_29660x12LN6_84	Cr8R	2

CT8	8/22/19	12LN10_14x12BME10_211	Cr8S	2
CT8	8/22/19	dgrp_28191x12BME10_229	Cr8W	1
CT8	8/22/19	12BME10_204x12LN10_22	Cr8Z	1
CT9	9/11/19	12LN10_35x12LN6_46	Cr9A	1
CT9	9/11/19	12LN6_28x12LN10_95	Cr9B	1
CT9	9/11/19	12BME10_204x12LN10_22	Cr9C	2
CT9	9/11/19	dgrp_28191x12BME10_104	Cr9D	1
CT9	9/11/19	12LN10_35x12LN6_84	Cr9E	2
CT9	9/11/19	12LN10_13x12LN6_84	Cr9F	1
CT9	9/11/19	dgrp_29660x12LN6_46	Cr9G	2
CT9	9/11/19	12BME10_218x12LN10_95	Cr9H	1
CT9	9/11/19	dgrp_29660x12BME10_211	Cr9I	1
CT9	9/11/19	dgrp_29660x12LN6_84	Cr9J	1
CT9	9/11/19	dgrp_29660x12BME10_270	Cr9K	2
CT9	9/11/19	dgrp_28191x12BME10_270	Cr9L	2
CT9	9/11/19	12BME10_212xdgrp_28261	Cr9M	2
CT9	9/11/19	12LN6_12xdgrp_28241	Cr9N	2
CT9	9/11/19	12LN10_32x12LN6_84	Cr9O	2
CT9	9/11/19	12LN10_32x12LN6_46	Cr9Q	2
CT9	9/11/19	12LN10_35x12BME10_211	Cr9R	2
CT9	9/11/19	12LN6_28x12LN10_76	Cr9V	2
CT9	9/11/19	12LN6_12xdgrp_28261	Cr9W	1
CT9	9/11/19	12BME10_130x12LN10_22	Cr9Z	2
CT10	10/2/19	dgrp_29660x12BME10_229	Cr10A	2
CT10	10/2/19	dgrp_29660x12BME10_104	Cr10B	2
CT10	10/2/19	12BME10_204x12LN10_76	Cr10C	2
CT10	10/2/19	12BME10_212xdgrp_25744	Cr10D	2
CT10	10/2/19	dgrp_28191x12LN6_6	Cr10E	2
CT10	10/2/19	12LN6_16xdgrp_28261	Cr10F	2
CT10	10/2/19	12LN6_19x12LN10_22	Cr10G	2
CT10	10/2/19	12LN10_32x12BME10_270	Cr10I	1
CT10	10/2/19	12LN10_32x12LN6_6	Cr10J	2
CT10	10/2/19	12LN10_35x12BME10_229	Cr10K	2
CT10	10/2/19	12BME10_204xdgrp_28241	Cr10L	1
CT10	10/2/19	12BME10_218xdgrp_28261	Cr10M	1
CT10	10/2/19	12LN6_19x12LN10_95	Cr10N	2
CT10	10/2/19	12BME10_218x12LN10_76	Cr10O	2
CT10	10/2/19	dgrp_28189x12BME10_229	Cr10P	2
CT10	10/2/19	12LN6_19xdgrp_28261	Cr10Q	2

CT10	10/2/19	12LN6_12x12LN10_95	Cr10R	2
CT10	10/2/19	12LN6_19x12LN10_76	Cr10S	2
CT10	10/2/19	12LN10_13x12LN6_6	Cr10T	1
CT10	10/2/19	12LN6_12x12LN10_22	Cr10U	1

Table S2. Sequencing library information table. “Library” are 4 sequenced libraries. “# Sample” represents the number of samples in each library. “Mean UMI per sample” is the average number of unique molecular identifiers detected for each sample. “Mean gene per sample” is the average number of genes detected for each sample. “Total genes” is the total number of genes detected in each library.

Library	# Sample	Mean UMI per sample	Mean gene per sample	Total genes
B1B4	90	1,377,446	10,298	14,119
B2B3	88	1,079,960	10,128	14,125
B5B7	92	1,453,418	10,368	14,240
B6	60	1,252,919	10,259	13,806

Table S3. Grand Sample information table. “Lib” are 4 sequenced libraries. “Actual.geno” are the founder line ids for each F_1 sample formatted as “maternal id x paternal id”. “nUMIs” is the number of unique molecular identifiers used in the analysis for each sample. “nGenes” is the number of genes included in the analysis for each sample. “Time” represents the 10 seasonal time points from CT1 (ST1) to CT10 (ST10). “Date” represents the start dates of each of the 10 seasonal time points.

Lib	Name	barcode	actual.geno	geno	SID	rep	nUMIs	nGenes	time	date
B1B4	A01	CTCGAGTAGCAG	dgrp_28191x12LN6_6	Cr1C	Cr1C-1	1	2420793	10451	CT1	5/3/19
B1B4	A02	CAGCACACGTCA	12LN10_35x12BME10_211	Cr1F	Cr1F-1	1	1628851	10406	CT1	5/3/19
B1B4	A03	ACAGCGATCGAC	12LN6_19x12LN10_95	Cr2O	Cr2O-1	1	1880726	10711	CT2	5/24/19
B1B4	A04	TAGTGTACGACA	12LN10_14x12LN6_46	Cr4R	Cr4R-1	1	1348464	10459	CT4	6/19/19
B1B4	A05	TAGTCGTCTAGC	12BME10_204xdgrp_25744	Cr6U	Cr6U-1	1	1073836	10480	CT6	7/20/19
B1B4	A06	CATCAGCTGCAC	12LN6_12xdgrp_28261	Cr1O	Cr1O-1	1	1969819	10304	CT1	5/3/19
B1B4	A07	TAGTAGCACGCA	12BME10_204x12LN10_67	Cr6A	Cr6A-1	1	1052595	10310	CT6	7/20/19
B1B4	A08	CAGTCAGCTGAC	12BME10_130x12LN10_22	Cr7S	Cr7S-1	1	2027220	10576	CT7	8/7/19
B1B4	A09	CAGCAGTCTACG	12BME10_218x12LN10_95	Cr8I	Cr8I-1	1	1550754	10555	CT8	8/22/19
B1B4	A10	CAGCTAGAGCAC	dgrp_28191x12LN6_84	Cr8C	Cr8C-1	1	1150640	10266	CT8	8/22/19
B1B4	A11	CTAGCATGACGA	12LN6_28x12LN10_95	Cr1S	Cr1S-2	2	1758318	10265	CT1	5/3/19
B1B4	A12	ACTCTACGCGAC	12LN6_12xdgrp_28261	Cr1O	Cr1O-2	2	1401491	10081	CT1	5/3/19
B1B4	B01	CTGTCGAGCTGA	dgrp_29660x12BME10_229	Cr1J	Cr1J-1	1	2396930	10827	CT1	5/3/19
B1B4	B02	ACAGACGAGTCA	dgrp_28189x12LN6_6	Cr2U	Cr2U-1	1	1847735	10375	CT2	5/24/19
B1B4	B03	CTATGATCTACG	12LN10_32x12LN6_46	Cr2J	Cr2J-1	1	1451613	10445	CT2	5/24/19
B1B4	B04	CTCAGAGCAGAC	12BME10_218x12LN10_67	Cr5M	Cr5M-1	1	1221619	10376	CT5	7/5/19
B1B4	B05	ACAGAGACTACG	12LN10_13x12BME10_229	Cr6S	Cr6S-1	1	1384575	10124	CT6	7/20/19
B1B4	B06	CTCTGCACTAGC	dgrp_28189x12BME10_211	Cr1N	Cr1N-1	1	868014	10054	CT1	5/3/19
B1B4	B07	ACTAGTGACGAC	12BME10_204x12LN10_76	Cr6G	Cr6G-1	1	988981	10204	CT6	7/20/19
B1B4	B08	TACGATGCGTAC	12LN10_32x12BME10_229	Cr7D	Cr7D-1	1	984572	10102	CT7	8/7/19
B1B4	B09	ACGAGACATCAC	12LN10_14x12LN6_46	Cr8A	Cr8A-1	1	1593556	10426	CT8	8/22/19
B1B4	B10	CATCACTGCACA	dgrp_29660x12BME10_211	Cr8F	Cr8F-1	1	1284280	10414	CT8	8/22/19
B1B4	B11	CTGACATCACAG	12LN10_14x12LN6_6	Cr1T	Cr1T-2	2	880753	9750	CT1	5/3/19

B1B4	B12	TAGTACGACTAC	12BME10_218x12LN10_67	Cr4F	Cr4F-1	1	1257613	10222	CT4	6/19/19
B1B4	C01	CACGCAGAGTCA	dgrp_29660x12BME10_270	Cr1B	Cr1B-1	1	1242232	9928	CT1	5/3/19
B1B4	C02	CACACGCATAGC	dgrp_28191x12BME10_104	Cr2R	Cr2R-1	1	1162449	10248	CT2	5/24/19
B1B4	C03	ACGTATGTCTAG	12LN10_32x12LN6_84	Cr2I	Cr2I-1	1	1199129	10466	CT2	5/24/19
B1B4	C04	CATCTCACTAGA	12LN10_13x12BME10_270	Cr5Q	Cr5Q-1	1	803294	9858	CT5	7/5/19
B1B4	C05	ATCGTCATACGA	12LN10_14x12LN6_6	Cr6R	Cr6R-1	1	1129601	10388	CT6	7/20/19
B1B4	C06	TCTAGCACGTGC	12BME10_218xdgrp_25744	Cr1P	Cr1P-1	1	1272047	10356	CT1	5/3/19
B1B4	C07	TCAGACTGTCAC	12BME10_212xdgrp_28241	Cr6H	Cr6H-1	1	1071386	10242	CT6	7/20/19
B1B4	C08	TCAGTAGTCTAC	12LN6_19x12LN10_67	Cr7B	Cr7B-1	1	1670668	10613	CT7	8/7/19
B1B4	C09	TACTGACACGAC	12LN10_35x12LN6_46	Cr8G	Cr8G-1	1	950441	10023	CT8	8/22/19
B1B4	C10	CATACTCATGAG	12BME10_204x12LN10_22	Cr9C	Cr9C-1	1	1213523	10490	CT9	9/11/19
B1B4	C11	CACATGCAGTCG	12LN10_14x12LN6_6	Cr1T	Cr1T-1	1	1232102	10049	CT1	5/3/19
B1B4	C12	CACAGATCGAGC	12LN6_16x12LN10_95	Cr4E	Cr4E-1	1	1142561	10124	CT4	6/19/19
B1B4	D01	TCGTGACTCAGC	12BME10_204x12LN10_76	Cr1H	Cr1H-1	1	1116280	10398	CT1	5/3/19
B1B4	D02	CAGCACGATAGC	dgrp_28191x12BME10_270	Cr2N	Cr2N-1	1	1179150	10137	CT2	5/24/19
B1B4	D03	CTACAGCACACG	12LN10_32x12BME10_270	Cr2Q	Cr2Q-1	1	1138028	10275	CT2	5/24/19
B1B4	D04	CTGTACGCATGC	12LN10_14x12LN6_84	Cr5P	Cr5P-1	1	954964	9903	CT5	7/5/19
B1B4	D05	CTACACACAGCG	12LN10_32x12LN6_6	Cr6T	Cr6T-1	1	1115814	10182	CT6	7/20/19
B1B4	D06	ACTCGTGCGAGA	12BME10_204xdgrp_25744	Cr1K	Cr1K-2	2	1703952	10477	CT1	5/3/19
B1B4	D07	TAGCGCATGCAC	12BME10_218x12LN10_76	Cr6D	Cr6D-1	1	1265148	10325	CT6	7/20/19
B1B4	D08	ACGAGCATCGCA	dgrp_28189x12BME10_211	Cr7C	Cr7C-1	1	1368174	10157	CT7	8/7/19
B1B4	D09	CTCGCGTACACA	12LN6_12x12LN10_67	Cr8H	Cr8H-1	1	1068835	10104	CT8	8/22/19
B1B4	D10	CTGTAGCATCGC	12LN10_35x12LN6_46	Cr9A	Cr9A-1	1	1198454	10319	CT9	9/11/19
B1B4	D11	TCTACGTATCGC	12LN6_28x12LN10_95	Cr1S	Cr1S-1	1	1263563	9887	CT1	5/3/19
B1B4	D12	CTGAGCTGTACA	12LN10_35x12BME10_229	Cr4A	Cr4A-1	1	880733	9986	CT4	6/19/19
B1B4	E01	CATGCACACAGA	12LN6_19x12LN10_22	Cr1I	Cr1I-1	1	1438958	10233	CT1	5/3/19
B1B4	E02	TCATCGACATAG	dgrp_28191x12LN6_46	Cr2P	Cr2P-1	1	1597362	10459	CT2	5/24/19
B1B4	E03	CACACACTGCGA	12LN10_13x12BME10_270	Cr2M	Cr2M-1	1	1541732	10389	CT2	5/24/19
B1B4	E04	CACTGCTAGACA	12LN6_28x12LN10_76	Cr5N	Cr5N-1	1	649913	9813	CT5	7/5/19
B1B4	E05	ACGCGTAGCTAC	12LN6_16x12LN10_22	Cr6Q	Cr6Q-1	1	1444824	10113	CT6	7/20/19
B1B4	E07	CAGCATACACGC	12LN6_12xdgrp_28241	Cr6C	Cr6C-1	1	1753425	10468	CT6	7/20/19
B1B4	E08	CACAGTCGACAC	dgrp_28189x12BME10_270	Cr7F	Cr7F-1	1	1283318	10098	CT7	8/7/19

B1B4	E09	ACGACGCGTAGA	12LN6_12x12LN10_76	Cr8E	Cr8E-1	1	1527767	10319	CT8	8/22/19
B1B4	E10	ATGCGACAGACG	12LN6_28x12LN10_95	Cr9B	Cr9B-1	1	1147105	10316	CT9	9/11/19
B1B4	E11	ATCTACTAGTGC	12BME10_218xdgrp_25744	Cr1P	Cr1P-2	2	1510094	10408	CT1	5/3/19
B1B4	F01	CATCATAGTGAC	12LN6_19xdgrp_28261	Cr1G	Cr1G-1	1	1185517	10212	CT1	5/3/19
B1B4	F02	CTCGACAGCTCA	12BME10_204xdgrp_28261	Cr2S	Cr2S-1	1	940043	10177	CT2	5/24/19
B1B4	F03	ACGCTATCGAGC	12LN10_14x12LN6_84	Cr2L	Cr2L-1	1	1626009	10272	CT2	5/24/19
B1B4	F04	CATGCGTCTCAG	12LN6_28xdgrp_25744	Cr5R	Cr5R-1	1	525716	9774	CT5	7/5/19
B1B4	F05	ACTGTAGACAGC	12LN10_32x12LN6_46	Cr7L	Cr7L-1	1	950198	10088	CT7	8/7/19
B1B4	F06	TCGCTGCATAGC	12BME10_130x12LN10_22	Cr9Z	Cr9Z-1	1	1452753	10648	CT9	9/11/19
B1B4	F07	CAGTAGCGTGAG	12LN6_16xdgrp_28261	Cr6B	Cr6B-1	1	1452930	10322	CT6	7/20/19
B1B4	F08	ACTGTCTGTGCA	dgrp_28189x12LN6_6	Cr7E	Cr7E-1	1	1292249	10141	CT7	8/7/19
B1B4	F09	ACGACAGCATGC	12LN6_12x12LN10_95	Cr8B	Cr8B-1	1	1510358	10371	CT8	8/22/19
B1B4	F10	CTCTACATAGAC	12LN6_28x12LN10_76	Cr1L	Cr1L-2	2	1098254	9913	CT1	5/3/19
B1B4	F11	CAGCGAGTGACA	12LN10_35x12BME10_211	Cr1F	Cr1F-2	2	1245748	10341	CT1	5/3/19
B1B4	G01	TCATGACGAGAG	12LN6_28x12LN10_67	Cr1E	Cr1E-1	1	1842525	10559	CT1	5/3/19
B1B4	G02	ACATCGTAGTCG	12BME10_218x12LN10_76	Cr2T	Cr2T-1	1	1495085	10307	CT2	5/24/19
B1B4	G03	CTAGAGATGCGA	dgrp_28189x12BME10_104	Cr4Z	Cr4Z-1	1	1824300	10524	CT4	6/19/19
B1B4	G04	TATCAGACATCG	dgrp_28189x12BME10_229	Cr5O	Cr5O-1	1	1757659	10788	CT5	7/5/19
B1B4	G05	ACACTATGCACA	dgrp_29660x12BME10_270	Cr7M	Cr7M-1	1	916593	10233	CT7	8/7/19
B1B4	G07	CATGACTAGTCG	12LN6_19xdgrp_28261	Cr6E	Cr6E-1	1	1074918	10143	CT6	7/20/19
B1B4	G08	CTACGTATATGC	12BME10_204xdgrp_28241	Cr8K	Cr8K-1	1	1350179	10309	CT8	8/22/19
B1B4	G09	CTACGCTCGTAG	12LN6_28xdgrp_28241	Cr8J	Cr8J-1	1	1512897	10543	CT8	8/22/19
B1B4	G10	CTCGTAGCTAGA	12LN6_16x12LN10_67	Cr1M	Cr1M-2	2	1571001	10426	CT1	5/3/19
B1B4	G11	CTACTAGACGCA	12LN6_28x12LN10_67	Cr1E	Cr1E-2	2	1472971	10570	CT1	5/3/19
B1B4	H01	ACACGTAGTGCA	12LN10_32x12LN6_6	Cr1A	Cr1A-1	1	2318610	10515	CT1	5/3/19
B1B4	H02	ACTGAGCAGCGA	12LN6_16x12LN10_22	Cr2K	Cr2K-1	1	2112489	10636	CT2	5/24/19
B1B4	H03	TCACGCGTAGCA	dgrp_28191x12LN6_84	Cr4Q	Cr4Q-1	1	1833539	10373	CT4	6/19/19
B1B4	H04	TCATGCACTGCG	dgrp_29660x12LN6_6	Cr5L	Cr5L-1	1	1588160	10766	CT5	7/5/19
B1B4	H05	ACTGCTATCTCG	12LN6_12x12LN10_67	Cr4P	Cr4P-1	1	1756649	10773	CT4	6/19/19
B1B4	H07	ACACGCGATACG	dgrp_28191x12BME10_104	Cr6F	Cr6F-1	1	1475695	10547	CT6	7/20/19
B1B4	H08	TCGACGATGACA	12BME10_218xdgrp_28241	Cr8D	Cr8D-1	1	2089607	11082	CT8	8/22/19
B1B4	H10	CACATCACTGAC	12BME10_212x12LN10_76	Cr1Q	Cr1Q-2	2	1605771	10485	CT1	5/3/19

B1B4	H11	TCAGCATACTCA	12LN6_16x12LN10_67	Cr1M	Cr1M-1	1	1888597	10457	CT1	5/3/19
B2B3	A01	CTCGAGTAGCAG	12BME10_204x12LN10_22	Cr3G	Cr3G-1	1	1246193	10272	CT3	6/3/19
B2B3	A02	CAGCACACGTCA	12LN10_32x12LN6_84	Cr5J	Cr5J-1	1	768119	10212	CT5	7/5/19
B2B3	A03	ACAGCGATCGAC	12BME10_212x12LN10_76	Cr7I	Cr7I-1	1	1745507	10635	CT7	8/7/19
B2B3	A04	CTCTCTACAGCA	12BME10_218x12LN10_95	Cr9H	Cr9H-1	1	897249	10236	CT9	9/11/19
B2B3	A05	TAGTCGTCTAGC	12BME10_212xdgrp_25744	Cr10D	Cr10D-1	1	724951	10016	CT10	10/2/19
B2B3	A06	CATCAGCTGCAC	dgrp_29660x12BME10_104	Cr10B	Cr10B-1	1	1397730	10448	CT10	10/2/19
B2B3	A07	TAGTAGCACGCA	dgrp_28189x12BME10_229	Cr2D	Cr2D-1	1	1219300	10308	CT2	5/24/19
B2B3	A08	CAGTCAGCTGAC	12BME10_212xdgrp_25744	Cr3M	Cr3M-1	1	1203868	10044	CT3	6/3/19
B2B3	A09	CAGCAGTCTACG	12BME10_218xdgrp_28261	Cr4L	Cr4L-1	1	1137985	10300	CT4	6/19/19
B2B3	A10	CAGCTAGAGCAC	12LN6_16x12LN10_67	Cr6N	Cr6N-1	1	1046787	10320	CT6	7/20/19
B2B3	A11	ACAGCAGCGTAG	12LN6_12xdgrp_28241	Cr9N	Cr9N-1	1	1334237	10486	CT9	9/11/19
B2B3	A12	ACTCTACGCGAC	12LN6_19x12LN10_76	Cr10S	Cr10S-1	1	972991	10264	CT10	10/2/19
B2B3	B01	CTGTCGAGCTGA	12BME10_212xdgrp_28261	Cr3F	Cr3F-1	1	2105922	10601	CT3	6/3/19
B2B3	B02	ACAGACGAGTCA	12LN10_35x12LN6_84	Cr5F	Cr5F-1	1	1893703	10588	CT5	7/5/19
B2B3	B03	CTATGATCTACG	12BME10_218xdgrp_25744	Cr7G	Cr7G-1	1	1238804	10261	CT7	8/7/19
B2B3	B04	CTCAGAGCAGAC	12LN10_13x12LN6_84	Cr9F	Cr9F-1	1	1062151	10168	CT9	9/11/19
B2B3	B05	ACAGAGACTACG	12LN10_32x12LN6_6	Cr10J	Cr10J-1	1	706087	9908	CT10	10/2/19
B2B3	B06	CTCTGCACTAGC	dgrp_29660x12BME10_229	Cr10A	Cr10A-1	1	491896	9570	CT10	10/2/19
B2B3	B07	ACTAGTGACGAC	dgrp_28189x12BME10_270	Cr2B	Cr2B-1	1	1062403	9974	CT2	5/24/19
B2B3	B08	TACGATGCGTAC	12BME10_212xdgrp_28241	Cr3P	Cr3P-1	1	879261	9568	CT3	6/3/19
B2B3	B09	ACGAGACATCAC	12LN10_35x12LN6_46	Cr4N	Cr4N-1	1	1259251	10501	CT4	6/19/19
B2B3	B10	CATCACTGCACA	12LN6_16x12LN10_95	Cr6K	Cr6K-1	1	921327	10172	CT6	7/20/19
B2B3	B11	CTGACATCACAG	dgrp_28191x12BME10_270	Cr9L	Cr9L-1	1	818420	10263	CT9	9/11/19
B2B3	B12	TAGTACGACTAC	12LN6_19x12LN10_95	Cr10N	Cr10N-1	1	1144927	10383	CT10	10/2/19
B2B3	C01	CACGCAGAGTCA	12BME10_218x12LN10_95	Cr3H	Cr3H-1	1	1332213	10251	CT3	6/3/19
B2B3	C02	CACACGCATAGC	12LN6_28x12LN10_22	Cr5K	Cr5K-1	1	1012751	10042	CT5	7/5/19

B2B3	C03	ACGTATGTCTAG	12LN6_19x12LN10_22	Cr7J	Cr7J-1	1	934147	10109	CT7	8/7/19
B2B3	C04	CATCTCACTAGA	12LN10_35x12LN6_84	Cr9E	Cr9E-1	1	869724	10202	CT9	9/11/19
B2B3	C05	ATCGTCATACGA	12LN10_32x12BME10_270	Cr10I	Cr10I-1	1	719831	9981	CT10	10/2/19
B2B3	C06	TCTAGCACGTGC	dgrp_29660x12LN6_46	Cr9G	Cr9G-1	1	921641	9991	CT9	9/11/19
B2B3	C07	TCAGACTGTCAC	dgrp_28191x12BME10_229	Cr2G	Cr2G-1	1	1323996	10260	CT2	5/24/19
B2B3	C08	TCAGTAGTCTAC	12LN6_12x12LN10_22	Cr3K	Cr3K-1	1	1239477	9881	CT3	6/3/19
B2B3	C09	TACTGACACGAC	12LN10_14x12BME10_229	Cr4M	Cr4M-1	1	584903	9752	CT4	6/19/19
B2B3	C10	CATACTCATGAG	12LN6_16xdgrp_28241	Cr6M	Cr6M-1	1	798286	9920	CT6	7/20/19
B2B3	C11	CACATGCAGTCG	dgrp_29660x12BME10_270	Cr9K	Cr9K-1	1	887841	10084	CT9	9/11/19
B2B3	C12	CACAGATCGAGC	12LN6_19xdgrp_28261	Cr10Q	Cr10Q-1	1	1165118	10286	CT10	10/2/19
B2B3	D02	CAGCACGATAGC	dgrp_28189x12BME10_104	Cr5I	Cr5I-1	1	1071335	10274	CT5	7/5/19
B2B3	D03	CTACAGCACACG	12LN6_28x12LN10_67	Cr7K	Cr7K-1	1	997539	10029	CT7	8/7/19
B2B3	D04	CTGTACGCATGC	dgrp_28191x12BME10_104	Cr9D	Cr9D-1	1	1360354	10433	CT9	9/11/19
B2B3	D05	CTACACACAGCG	12LN10_35x12BME10_229	Cr10K	Cr10K-1	1	845352	10193	CT10	10/2/19
B2B3	D07	TAGCGCATGCAC	12BME10_130x12LN10_22	Cr2A	Cr2A-1	1	974678	10030	CT2	5/24/19
B2B3	D08	ACGAGCATCGCA	12LN6_16x12LN10_76	Cr3I	Cr3I-1	1	902429	9779	CT3	6/3/19
B2B3	D09	CTCGCGTACACA	12BME10_130xdgrp_28241	Cr6L	Cr6L-1	1	861411	10034	CT6	7/20/19
B2B3	D10	CTGTAGCATCGC	12LN10_14x12BME10_211	Cr8S	Cr8S-1	1	955376	10030	CT8	8/22/19
B2B3	D11	TCTACGTATCGC	12BME10_218xdgrp_28261	Cr10M	Cr10M-1	1	1197450	10484	CT10	10/2/19
B2B3	D12	CTGAGCTGTACA	dgrp_28189x12BME10_229	Cr10P	Cr10P-1	1	1065903	10345	CT10	10/2/19
B2B3	E01	CATGCACACAGA	12BME10_218xdgrp_28241	Cr4S	Cr4S-1	1	717925	9904	CT4	6/19/19
B2B3	E02	TCATCGACATAG	dgrp_28191x12LN6_6	Cr5G	Cr5G-1	1	857957	9924	CT5	7/5/19
B2B3	E03	CACACACTGCGA	dgrp_28191x12BME10_211	Cr7Z	Cr7Z-1	1	1723551	10390	CT7	8/7/19
B2B3	E04	CACTGCTAGACA	dgrp_29660x12BME10_211	Cr9I	Cr9I-1	1	1105324	10198	CT9	9/11/19
B2B3	E05	ACGCGTAGCTAC	12LN6_16xdgrp_28261	Cr10F	Cr10F-1	1	1017718	10166	CT10	10/2/19
B2B3	E07	CAGCATACACGC	12LN6_12xdgrp_28241	Cr2C	Cr2C-1	1	1411396	10575	CT2	5/24/19
B2B3	E08	CACAGTCGACAC	12LN6_28x12LN10_22	Cr3L	Cr3L-1	1	1035027	9846	CT3	6/3/19

B2B3	E09	ACGACGCGTAGA	12BME10_212xdgrp_25744	Cr6P	Cr6P-1	1	658586	9893	CT6	7/20/19
B2B3	E10	ATGCGACAGACG	12LN6_16x12LN10_76	Cr8Q	Cr8Q-1	1	942427	10150	CT8	8/22/19
B2B3	E11	ATCTACTAGTGC	12BME10_218x12LN10_76	Cr10O	Cr10O-1	1	869174	10187	CT10	10/2/19
B2B3	E12	ATCATGAGCAGA	12LN10_32x12LN6_84	Cr9O	Cr9O-1	1	686247	10183	CT9	9/11/19
B2B3	F01	CATCATAGTGAC	12LN6_19x12LN10_67	Cr4J	Cr4J-1	1	753245	9929	CT4	6/19/19
B2B3	F02	CTCGACAGCTCA	dgrp_29660x12BME10_229	Cr5H	Cr5H-1	1	457364	9375	CT5	7/5/19
B2B3	F03	ACGCTATCGAGC	12LN10_14x12BME10_229	Cr8P	Cr8P-1	1	1371146	10031	CT8	8/22/19
B2B3	F04	CATGCGTCTCAG	dgrp_29660x12LN6_84	Cr9J	Cr9J-1	1	578159	9710	CT9	9/11/19
B2B3	F05	ACTGTAGACAGC	12LN6_19x12LN10_22	Cr10G	Cr10G-1	1	488129	9761	CT10	10/2/19
B2B3	F07	CAGTAGCGTGAG	12LN6_16xdgrp_28241	Cr2E	Cr2E-1	1	1735218	10450	CT2	5/24/19
B2B3	F08	ACTGTCTGTGCA	12LN6_28xdgrp_28261	Cr3O	Cr3O-1	1	872874	9853	CT3	6/3/19
B2B3	F09	ACGACAGCATGC	12BME10_218xdgrp_28261	Cr6O	Cr6O-1	1	874188	9968	CT6	7/20/19
B2B3	F10	CTCTACATAGAC	dgrp_28189x12LN6_84	Cr8O	Cr8O-1	1	768956	9792	CT8	8/22/19
B2B3	F11	CAGCGAGTGACA	12LN10_13x12LN6_6	Cr10T	Cr10T-1	1	912712	10204	CT10	10/2/19
B2B3	G01	TCATGACGAGAG	12LN6_28xdgrp_25744	Cr4I	Cr4I-1	1	891020	10064	CT4	6/19/19
B2B3	G02	ACATCGTAGTCG	dgrp_29660x12LN6_46	Cr5E	Cr5E-1	1	925340	10161	CT5	7/5/19
B2B3	G03	CTAGAGATGCGA	12LN6_19x12LN10_76	Cr8M	Cr8M-1	1	1307856	10393	CT8	8/22/19
B2B3	G04	TATCAGACATCG	12BME10_204xdgrp_28241	Cr10L	Cr10L-1	1	1089217	10478	CT10	10/2/19
B2B3	G07	CATGACTAGTCG	12LN10_35x12LN6_84	Cr2F	Cr2F-1	1	1358325	10267	CT2	5/24/19
B2B3	G08	CTACGTATATGC	12LN6_28xdgrp_28241	Cr3N	Cr3N-1	1	1386240	10171	CT3	6/3/19
B2B3	G09	CTACGCTCGTAG	12LN10_13x12LN6_46	Cr6J	Cr6J-1	1	1454155	10102	CT6	7/20/19
B2B3	G10	CTCGTAGCTAGA	dgrp_29660x12LN6_84	Cr8R	Cr8R-1	1	1504172	10348	CT8	8/22/19
B2B3	G11	CTACTAGACGCA	12LN6_12x12LN10_22	Cr10U	Cr10U-1	1	775420	10074	CT10	10/2/19
B2B3	H02	ACTGAGCAGCGA	12BME10_204xdgrp_28261	Cr7H	Cr7H-1	1	1645226	10616	CT7	8/7/19
B2B3	H03	TCACGCGTAGCA	dgrp_29660x12BME10_104	Cr8N	Cr8N-1	1	1216501	10334	CT8	8/22/19
B2B3	H04	CACTCGAGTCAC	12BME10_204x12LN10_76	Cr10C	Cr10C-1	1	1806239	10705	CT10	10/2/19

B2B3	H05	ACTGCTATCTCG	dgrp_28191x12LN6_6	Cr10E	Cr10E-1	1	1347497	10351	CT10	10/2/19
B2B3	H07	ACACGCGATACG	12LN10_13x12LN6_6	Cr2H	Cr2H-1	1	1454334	10269	CT2	5/24/19
B2B3	H08	TCGACGATGACA	dgrp_29660x12BME10_104	Cr4O	Cr4O-1	1	1147381	10502	CT4	6/19/19
B2B3	H09	ATACATCGACGA	12LN6_12x12LN10_22	Cr6I	Cr6I-1	1	2009980	10453	CT6	7/20/19
B2B3	H10	ATCAGACTACAG	12BME10_212xdgrp_28261	Cr9M	Cr9M-1	1	2021545	10578	CT9	9/11/19
B2B3	H11	TCAGCATACTCA	12LN6_12x12LN10_95	Cr10R	Cr10R-1	1	1106471	10442	CT10	10/2/19
B5B7	A02	CAGCACACGTCA	12LN10_13x12BME10_270	Cr2M	Cr2M-2	2	954522	10093	CT2	5/24/19
B5B7	A03	ACAGCGATCGAC	12LN6_28x12LN10_76	Cr5N	Cr5N-2	2	1163380	10383	CT5	7/5/19
B5B7	A04	TAGTGTACGACA	dgrp_28191x12LN6_46	Cr7V	Cr7V-2	2	1471935	10623	CT7	8/7/19
B5B7	A05	TAGTCGTCTAGC	dgrp_29660x12BME10_104	Cr8N	Cr8N-2	2	1105504	10548	CT8	8/22/19
B5B7	A06	CATCAGCTGCAC	dgrp_28191x12BME10_270	Cr9L	Cr9L-2	2	1678944	10633	CT9	9/11/19
B5B7	A07	TAGTAGCACGCA	dgrp_28189x12BME10_229	Cr2D	Cr2D-2	2	1701876	10590	CT2	5/24/19
B5B7	A08	CAGTCAGCTGAC	12BME10_218x12LN10_76	Cr2T	Cr2T-2	2	1939186	10352	CT2	5/24/19
B5B7	A09	CAGCAGTCTACG	12LN6_12x12LN10_22	Cr3K	Cr3K-2	2	1905693	10055	CT3	6/3/19
B5B7	A10	CAGCTAGAGCAC	dgrp_29660x12BME10_229	Cr5H	Cr5H-2	2	539185	9909	CT5	7/5/19
B5B7	A11	CTAGCATGACGA	12LN10_14x12LN6_46	Cr8A	Cr8A-2	2	2216023	10800	CT8	8/22/19
B5B7	A12	ACTCTACGCGAC	dgrp_28191x12LN6_84	Cr8C	Cr8C-2	2	1919597	10770	CT8	8/22/19
B5B7	B02	ACAGACGAGTCA	12LN10_14x12LN6_84	Cr2L	Cr2L-2	2	1517906	10431	CT2	5/24/19
B5B7	B03	CTATGATCTACG	12LN6_28xdgrp_25744	Cr5R	Cr5R-2	2	534093	9779	CT5	7/5/19
B5B7	B04	CTCAGAGCAGAC	12BME10_204x12LN10_22	Cr8Z	Cr8Z-2	2	1169786	10384	CT8	8/22/19
B5B7	B05	ACAGAGACTACG	dgrp_29660x12LN6_84	Cr8R	Cr8R-2	2	1283833	10379	CT8	8/22/19
B5B7	B06	CTCTGCACTAGC	dgrp_29660x12BME10_270	Cr9K	Cr9K-2	2	1034551	10166	CT9	9/11/19
B5B7	B07	ACTAGTGACGAC	dgrp_28189x12BME10_270	Cr2B	Cr2B-2	2	1361232	10504	CT2	5/24/19
B5B7	B08	TACGATGCGTAC	12LN6_16xdgrp_28241	Cr2E	Cr2E-2	2	1397703	10326	CT2	5/24/19
B5B7	B09	ACGAGACATCAC	12LN6_16x12LN10_76	Cr3I	Cr3I-2	2	1814329	10363	CT3	6/3/19
B5B7	B10	CATCACTGCACA	dgrp_29660x12LN6_46	Cr5E	Cr5E-2	2	1641180	10354	CT5	7/5/19
B5B7	B11	CTGACATCACAG	12LN10_35x12LN6_46	Cr8G	Cr8G-2	2	1095138	10368	CT8	8/22/19
B5B7	B12	TAGTACGACTAC	dgrp_29660x12BME10_211	Cr8F	Cr8F-2	2	1899840	10166	CT8	8/22/19
B5B7	C01	CACGCAGAGTCA	dgrp_28189x12LN6_6	Cr2U	Cr2U-2	2	728827	10026	CT2	5/24/19
B5B7	C03	ACGTATGTCTAG	12BME10_218xdgrp_25744	Cr7G	Cr7G-2	2	1059952	10282	CT7	8/7/19

B5B7	C04	CATCTCACTAGA	12BME10_218x12LN10_95	Cr8I	Cr8I-2	2	775808	10127	CT8	8/22/19
B5B7	C05	ATCGTCATACGA	12BME10_130x12LN10_22	Cr9Z	Cr9Z-2	2	1085760	10345	CT9	9/11/19
B5B7	C06	TCTAGCACGTGC	dgrp_29660x12LN6_46	Cr9G	Cr9G-2	2	813817	9898	CT9	9/11/19
B5B7	C07	TCAGACTGTCAC	dgrp_28191x12BME10_104	Cr2R	Cr2R-2	2	1140946	10412	CT2	5/24/19
B5B7	C08	TCAGTAGTCTAC	12LN10_32x12BME10_270	Cr2Q	Cr2Q-2	2	1154194	10360	CT2	5/24/19
B5B7	C09	TACTGACACGAC	12LN6_16xdgrp_28261	Cr3E	Cr3E-2	2	1135959	10121	CT3	6/3/19
B5B7	C10	CATACTCATGAG	dgrp_29660x12LN6_6	Cr5L	Cr5L-2	2	1268561	10283	CT5	7/5/19
B5B7	C11	CACATGCAGTCG	12LN6_12x12LN10_76	Cr8E	Cr8E-2	2	1649420	10228	CT8	8/22/19
B5B7	C12	CACAGATCGAGC	12BME10_204xdgrp_25744	Cr1K	Cr1K-1	1	913215	10200	CT1	5/3/19
B5B7	D01	TCGTGACTCAGC	12LN6_12xdgrp_28241	Cr2C	Cr2C-2	2	995960	10201	CT2	5/24/19
B5B7	D02	CAGCACGATAGC	12BME10_212xdgrp_28261	Cr5D	Cr5D-2	2	1080459	10148	CT5	7/5/19
B5B7	D03	CTACAGCACACG	12LN10_32x12BME10_229	Cr7D	Cr7D-2	2	1027363	10250	CT7	8/7/19
B5B7	D04	CTGTACGCATGC	12LN10_14x12BME10_211	Cr8S	Cr8S-2	2	1557098	10640	CT8	8/22/19
B5B7	D05	CTACACACAGCG	12BME10_212xdgrp_28261	Cr9M	Cr9M-2	2	1107000	10364	CT9	9/11/19
B5B7	D06	ACTCGTGCGAGA	12BME10_204x12LN10_76	Cr10C	Cr10C-2	2	1398273	10416	CT10	10/2/19
B5B7	D07	TAGCGCATGCAC	dgrp_28191x12BME10_229	Cr2G	Cr2G-2	2	1290970	10278	CT2	5/24/19
B5B7	D08	ACGAGCATCGCA	12LN10_35x12LN6_84	Cr2F	Cr2F-2	2	1561932	10473	CT2	5/24/19
B5B7	D09	CTCGCGTACACA	12LN6_28xdgrp_28261	Cr3O	Cr3O-2	2	1585409	10050	CT3	6/3/19
B5B7	D10	CTGTAGCATCGC	12LN6_28x12LN10_67	Cr7K	Cr7K-2	2	1503367	10340	CT7	8/7/19
B5B7	D11	TCTACGTATCGC	12LN6_12x12LN10_95	Cr8B	Cr8B-2	2	2344071	10631	CT8	8/22/19
B5B7	D12	CTGAGCTGTACA	12BME10_130xdgrp_28241	Cr1R	Cr1R-1	1	1074242	9982	CT1	5/3/19
B5B7	E01	CATGCACACAGA	12LN6_16x12LN10_22	Cr2K	Cr2K-2	2	1535706	10323	CT2	5/24/19
B5B7	E02	TCATCGACATAG	12LN10_13x12BME10_270	Cr5Q	Cr5Q-2	2	1587849	10389	CT5	7/5/19
B5B7	E03	CACACACTGCGA	12LN10_35x12LN6_6	Cr7W	Cr7W-2	2	1649456	10482	CT7	8/7/19
B5B7	E04	CACTGCTAGACA	12LN10_14x12BME10_229	Cr8P	Cr8P-2	2	1202178	10393	CT8	8/22/19
B5B7	E05	ACGCGTAGCTAC	12LN10_35x12LN6_84	Cr9E	Cr9E-2	2	1595584	10810	CT9	9/11/19
B5B7	E06	CACGTCTATCGC	12BME10_218x12LN10_76	Cr10O	Cr10O-2	2	1173097	10396	CT10	10/2/19
B5B7	E07	CAGCATACACGC	dgrp_28191x12BME10_270	Cr2N	Cr2N-2	2	1797537	10649	CT2	5/24/19
B5B7	E08	CACAGTCGACAC	12LN10_13x12LN6_6	Cr2H	Cr2H-2	2	1344817	9825	CT2	5/24/19

B5B7	E09	ACGACGCGTAGA	12LN6_28xdgrp_28241	Cr3N	Cr3N-2	2	1657465	10376	CT3	6/3/19
B5B7	E10	ATGCGACAGACG	dgrp_28189x12BME10_270	Cr7F	Cr7F-2	2	1727757	10365	CT7	8/7/19
B5B7	E11	ATCTACTAGTGC	12LN6_19x12LN10_76	Cr8M	Cr8M-2	2	1355044	10226	CT8	8/22/19
B5B7	E12	ATCATGAGCAGA	12LN6_28x12LN10_76	Cr1L	Cr1L-1	1	1800201	10482	CT1	5/3/19
B5B7	F01	CATCATAGTGAC	12LN6_19x12LN10_95	Cr2O	Cr2O-2	2	1244526	10317	CT2	5/24/19
B5B7	F02	CTCGACAGCTCA	12LN10_14x12LN6_84	Cr5P	Cr5P-2	2	917496	9803	CT5	7/5/19
B5B7	F03	ACGCTATCGAGC	12LN6_19x12LN10_22	Cr7J	Cr7J-2	2	1249067	10193	CT7	8/7/19
B5B7	F04	CATGCGTCTCAG	12LN6_12x12LN10_67	Cr8H	Cr8H-2	2	1385904	10403	CT8	8/22/19
B5B7	F05	ACTGTAGACAGC	12LN6_12xdgrp_28261	Cr9W	Cr9W-2	2	1130138	9947	CT9	9/11/19
B5B7	F06	TCGCTGCATAGC	12LN6_19x12LN10_76	Cr10S	Cr10S-2	2	1314449	10510	CT10	10/2/19
B5B7	F07	CAGTAGCGTGAG	dgrp_28191x12LN6_46	Cr2P	Cr2P-2	2	2281107	10560	CT2	5/24/19
B5B7	F08	ACTGTCTGTGCA	12BME10_204x12LN10_67	Cr3B	Cr3B-2	2	1566386	10204	CT3	6/3/19
B5B7	F09	ACGACAGCATGC	12BME10_130xdgrp_25744	Cr5C	Cr5C-2	2	1143724	10693	CT5	7/5/19
B5B7	F10	CTCTACATAGAC	dgrp_29660x12BME10_270	Cr7M	Cr7M-2	2	1170962	10257	CT7	8/7/19
B5B7	F11	CAGCGAGTGACA	12LN6_28xdgrp_28241	Cr8J	Cr8J-2	2	1370667	10078	CT8	8/22/19
B5B7	F12	CACGACATCAGC	12BME10_212x12LN10_76	Cr1Q	Cr1Q-1	1	1277703	10135	CT1	5/3/19
B5B7	G01	TCATGACGAGAG	12LN10_32x12LN6_46	Cr2J	Cr2J-2	2	1034030	10230	CT2	5/24/19
B5B7	G02	ACATCGTAGTCG	12LN10_35x12BME10_211	Cr5A	Cr5A-2	2	570612	10077	CT5	7/5/19
B5B7	G03	CTAGAGATGCGA	dgrp_28189x12BME10_211	Cr7C	Cr7C-2	2	1525621	10607	CT7	8/7/19
B5B7	G04	TATCAGACATCG	12LN6_16x12LN10_76	Cr8Q	Cr8Q-2	2	1862056	10918	CT8	8/22/19
B5B7	G05	ACACTATGCACA	12LN6_12xdgrp_28241	Cr9N	Cr9N-2	2	1141993	10302	CT9	9/11/19
B5B7	G06	CAGAGTAGTGCG	12LN6_19xdgrp_28261	Cr10Q	Cr10Q-2	2	1261395	10653	CT10	10/2/19
B5B7	G07	CATGACTAGTCG	12BME10_130x12LN10_22	Cr2A	Cr2A-2	2	1610707	10515	CT2	5/24/19
B5B7	G08	CTACGTATATGC	12BME10_212xdgrp_28261	Cr3F	Cr3F-2	2	2160015	10868	CT3	6/3/19
B5B7	G09	CTACGCTCGTAG	12LN10_35x12LN6_84	Cr5F	Cr5F-2	2	2634428	10864	CT5	7/5/19
B5B7	G10	CTCGTAGCTAGA	12BME10_204xdgrp_28241	Cr8K	Cr8K-2	2	1654929	10731	CT8	8/22/19
B5B7	G11	CTACTAGACGCA	dgrp_28189x12LN6_84	Cr8O	Cr8O-2	2	2656588	10746	CT8	8/22/19
B5B7	H01	ACACGTAGTGCA	12LN10_32x12LN6_84	Cr2I	Cr2I-2	2	1379401	10412	CT2	5/24/19

B5B7	H02	ACTGAGCAGCGA	12LN6_19x12LN10_95	Cr5W	Cr5W-2	2	1464779	10705	CT5	7/5/19
B5B7	H03	TCACGCGTAGCA	dgrp_28191x12BME10_211	Cr7Z	Cr7Z-2	2	1804965	10645	CT7	8/7/19
B5B7	H04	TCATGCACTGCG	dgrp_28191x12BME10_229	Cr8W	Cr8W-2	2	1933011	10941	CT8	8/22/19
B5B7	H05	ACTGCTATCTCG	12LN6_28x12LN10_76	Cr9V	Cr9V-2	2	1461885	10416	CT9	9/11/19
B5B7	H06	CAGACTCTGACG	dgrp_28189x12BME10_229	Cr10P	Cr10P-2	2	1302654	10595	CT10	10/2/19
B5B7	H07	ACACGCGATACG	12BME10_204xdgrp_28261	Cr2S	Cr2S-2	2	3999874	11118	CT2	5/24/19
B5B7	H08	TCGACGATGACA	12BME10_218x12LN10_95	Cr3H	Cr3H-2	2	2870547	10561	CT3	6/3/19
B5B7	H09	ATACATCGACGA	12LN6_28x12LN10_95	Cr5Z	Cr5Z-2	2	349782	9416	CT5	7/5/19
B5B7	H10	CACATCACTGAC	12BME10_218xdgrp_28241	Cr8D	Cr8D-2	2	2595644	11047	CT8	8/22/19
B5B7	H11	TCAGCATACTCA	dgrp_28191x12BME10_270	Cr8L	Cr8L-2	2	2112997	10661	CT8	8/22/19
B6	A01	CTCGAGTAGCAG	dgrp_28189x12BME10_104	Cr4Z	Cr4Z-2	2	1729489	10360	CT4	6/19/19
B6	A02	CAGCACACGTCA	12LN6_28xdgrp_25744	Cr4I	Cr4I-2	2	1209056	10227	CT4	6/19/19
B6	A03	ACAGCGATCGAC	dgrp_28189x12BME10_104	Cr5I	Cr5I-2	2	1594999	10463	CT5	7/5/19
B6	A04	TAGTGTACGACA	12BME10_212xdgrp_28241	Cr6H	Cr6H-2	2	1757676	10439	CT6	7/20/19
B6	A05	TAGTCGTCTAGC	12LN6_16x12LN10_95	Cr6K	Cr6K-2	2	1013362	10297	CT6	7/20/19
B6	A06	CATCAGCTGCAC	12LN6_19x12LN10_67	Cr7B	Cr7B-2	2	1700667	10595	CT7	8/7/19
B6	A07	TAGTAGCACGCA	12LN6_19x12LN10_95	Cr10N	Cr10N-2	2	2032759	10855	CT10	10/2/19
B6	A08	CAGTCAGCTGAC	12LN10_32x12LN6_46	Cr9Q	Cr9Q-1	1	1573562	10620	CT9	9/11/19
B6	B01	CTGTCGAGCTGA	dgrp_28189x12LN6_84	Cr4G	Cr4G-2	2	1773805	10360	CT4	6/19/19
B6	B02	ACAGACGAGTCA	12LN10_35x12LN6_46	Cr4N	Cr4N-2	2	1937513	10534	CT4	6/19/19
B6	B03	CTATGATCTACG	dgrp_28189x12BME10_229	Cr5O	Cr5O-2	2	1332258	10053	CT5	7/5/19
B6	B04	CTCAGAGCAGAC	12BME10_218x12LN10_76	Cr6D	Cr6D-2	2	1049356	9992	CT6	7/20/19
B6	B05	ACAGAGACTACG	12LN6_16xdgrp_28241	Cr6M	Cr6M-2	2	1097504	10278	CT6	7/20/19
B6	B06	CTCTGCACTAGC	12LN6_28xdgrp_28261	Cr7P	Cr7P-2	2	701743	9918	CT7	8/7/19
B6	B07	ACTAGTGACGAC	dgrp_28191x12LN6_6	Cr10E	Cr10E-2	2	2430210	10894	CT10	10/2/19
B6	C01	CACGCAGAGTCA	dgrp_28191x12LN6_84	Cr4Q	Cr4Q-2	2	1067932	10297	CT4	6/19/19
B6	C02	CACACGCATAGC	12LN10_14x12LN6_46	Cr4R	Cr4R-2	2	875895	10216	CT4	6/19/19

B6	C03	ACGTATGTCTAG	dgrp_28191x12LN6_6	Cr5G	Cr5G-2	2	1334634	10210	CT5	7/5/19
B6	C04	CATCTCACTAGA	12LN10_13x12LN6_46	Cr6J	Cr6J-2	2	1352276	9852	CT6	7/20/19
B6	C05	ATCGTCATACGA	12LN6_19xdgrp_28261	Cr6E	Cr6E-2	2	705857	10017	CT6	7/20/19
B6	C06	TCTAGCACGTGC	12BME10_212xdgrp_25744	Cr10D	Cr10D-2	2	1423252	10379	CT10	10/2/19
B6	C07	TCAGACTGTCAC	dgrp_29660x12BME10_104	Cr10B	Cr10B-2	2	1617936	10515	CT10	10/2/19
B6	C08	TCAGTAGTCTAC	12BME10_204x12LN10_22	Cr9C	Cr9C-2	2	1457907	10653	CT9	9/11/19
B6	D01	TCGTGACTCAGC	12BME10_130xdgrp_28261	Cr4H	Cr4H-2	2	718756	9987	CT4	6/19/19
B6	D02	CAGCACGATAGC	12LN10_14x12BME10_211	Cr4B	Cr4B-2	2	796333	10037	CT4	6/19/19
B6	D03	CTACAGCACACG	12BME10_130xdgrp_28241	Cr6L	Cr6L-2	2	787030	10046	CT6	7/20/19
B6	D04	CTGTACGCATGC	12LN10_14x12LN6_6	Cr6R	Cr6R-2	2	1229646	10403	CT6	7/20/19
B6	D05	CTACACACAGCG	dgrp_28191x12BME10_104	Cr6F	Cr6F-2	2	1277122	10003	CT6	7/20/19
B6	D06	ACTCGTGCGAGA	12LN10_32x12LN6_6	Cr10J	Cr10J-2	2	1149660	10352	CT10	10/2/19
B6	D07	TAGCGCATGCAC	dgrp_29660x12BME10_229	Cr10A	Cr10A-2	2	956661	10230	CT10	10/2/19
B6	D08	ACGAGCATCGCA	12LN10_35x12BME10_211	Cr9R	Cr9R-1	1	1185669	10435	CT9	9/11/19
B6	E01	CATGCACACAGA	12BME10_218xdgrp_28261	Cr4L	Cr4L-2	2	1031667	10133	CT4	6/19/19
B6	E02	TCATCGACATAG	12LN10_14x12BME10_229	Cr4M	Cr4M-2	2	729369	9939	CT4	6/19/19
B6	E03	CACACACTGCGA	12BME10_204xdgrp_25744	Cr6U	Cr6U-2	2	744238	10083	CT6	7/20/19
B6	E04	CACTGCTAGACA	12LN6_12x12LN10_22	Cr6I	Cr6I-2	2	1147287	9918	CT6	7/20/19
B6	E05	ACGCGTAGCTAC	12BME10_204xdgrp_28261	Cr7H	Cr7H-2	2	1128719	10236	CT7	8/7/19
B6	E06	CACGTCTATCGC	12LN10_35x12BME10_229	Cr10K	Cr10K-2	2	1368409	10434	CT10	10/2/19
B6	E07	CAGCATACACGC	12LN6_28x12LN10_76	Cr9V	Cr9V-1	1	1752317	10626	CT9	9/11/19
B6	F01	CATCATAGTGAC	12BME10_218xdgrp_28241	Cr4S	Cr4S-2	2	648989	9835	CT4	6/19/19
B6	F02	CTCGACAGCTCA	12LN10_32x12LN6_84	Cr5J	Cr5J-2	2	734614	10032	CT5	7/5/19
B6	F03	ACGCTATCGAGC	12BME10_204x12LN10_67	Cr6A	Cr6A-2	2	561334	9744	CT6	7/20/19
B6	F04	CATGCGTCTCAG	12LN6_12xdgrp_28241	Cr6C	Cr6C-2	2	808702	9732	CT6	7/20/19
B6	F05	ACTGTAGACAGC	12BME10_212x12LN10_76	Cr7I	Cr7I-2	2	948123	9929	CT7	8/7/19
B6	F06	TCGCTGCATAGC	12LN6_12x12LN10_95	Cr10R	Cr10R-2	2	759736	10142	CT10	10/2/19

B6	F07	CAGTAGCGTGAG	12LN10_32x12LN6_84	Cr9O	Cr9O-2	2	450258	9306	CT9	9/11/19
B6	G01	TCATGACGAGAG	12BME10_218x12LN10_67	Cr4F	Cr4F-2	2	1080075	10477	CT4	6/19/19
B6	G02	ACATCGTAGTCG	12LN10_32x12BME10_270	Cr5B	Cr5B-2	2	1308581	10250	CT5	7/5/19
B6	G03	CTAGAGATGCGA	12BME10_204x12LN10_76	Cr6G	Cr6G-2	2	1005463	10145	CT6	7/20/19
B6	G04	TATCAGACATCG	12LN6_16x12LN10_22	Cr6Q	Cr6Q-2	2	2080603	10366	CT6	7/20/19
B6	G05	ACACTATGCACA	12LN10_32x12LN6_46	Cr7L	Cr7L-2	2	903222	10039	CT7	8/7/19
B6	G06	CAGAGTAGTGCG	12LN6_16xdgrp_28261	Cr10F	Cr10F-2	2	1186430	10478	CT10	10/2/19
B6	G07	CATGACTAGTCG	12LN10_32x12LN6_46	Cr9Q	Cr9Q-2	2	1278278	10503	CT9	9/11/19
B6	H01	ACACGTAGTGCA	12LN6_16x12LN10_95	Cr4E	Cr4E-2	2	1160204	10296	CT4	6/19/19
B6	H02	ACTGAGCAGCGA	12LN6_28x12LN10_22	Cr5K	Cr5K-2	2	1613607	10635	CT5	7/5/19
B6	H03	TCACGCGTAGCA	12BME10_212xdgrp_25744	Cr6P	Cr6P-2	2	857121	10097	CT6	7/20/19
B6	H04	TCATGCACTGCG	12LN6_16x12LN10_67	Cr6N	Cr6N-2	2	1374248	10326	CT6	7/20/19
B6	H05	ACTGCTATCTCG	12LN6_12xdgrp_28261	Cr7Q	Cr7Q-2	2	2373568	10630	CT7	8/7/19
B6	H06	CAGACTCTGACG	12LN6_19x12LN10_22	Cr10G	Cr10G-2	2	1946626	10748	CT10	10/2/19
B6	H07	ACACGCGATACG	12LN10_35x12BME10_211	Cr9R	Cr9R-2	2	1747075	10701	CT9	9/11/19

CHAPTER 3

Plasticity in fitness-related traits is mediated by seasonally varying developmental conditions in *Drosophila melanogaster*

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Data availability: Data and scripts will be uploaded to online archive and are available upon request.

Abstract

Spatial and temporal environmental heterogeneity induce selection on populations of short-lived organisms. Previous studies have shown genetic evolution for fitness traits in *Drosophila melanogaster* populations across latitudinal clines and between seasons, and plasticity of those traits in response to thermal conditions in standard laboratory environments. However, we lack an understanding of whether thermal induced plasticity is adaptive in wild seasonal environments. Moreover, we have limited knowledge in whether plasticity alters genetic correlation patterns across seasons. Here in this study, we made F_1 crosses using inbred lines from four different populations and examined seasonal plasticity in an experimental orchard as well as in controlled lab environments for three fitness-related traits: eclosion time, fecundity, and thorax length. Our findings show that all three traits show clear patterns of plastic responses, and that the direction of seasonal plastic change in eclosion time and thorax length are concordant with clinal and seasonal evolutionary changes in those traits. In addition, seasonal temperature regimes have significant effects on the measured plastic traits: eclosion time is negatively correlated with temperature while thorax length and fecundity changes non-linearly with temperature. Moreover, we show that phenotypic line mean correlations between fitness traits are environmental specific. Taken together, our results suggest that temperature mediated seasonal phenotypic plasticity are likely adaptive and could shape population size dynamics in the wild.

Introduction

Identifying the mechanisms of how populations respond to environmental changes remains a key question in evolutionary biology (Orr 2005). Recent studies across a wide range of taxa have shown that populations of organisms can cope with environmental shifts through two major mechanisms, including adaptive evolution (Simons 2011) and phenotypic plasticity (Bradshaw 1965; Murren et al. 2015; Fox et al. 2019). Plasticity is characterized by the production of various environmentally induced phenotypes (Scheiner 1993), thus it allows the organisms to respond quickly within a generational time in rapidly changing environments (Tufto 2015; Fox et al. 2019). However, phenotypic plasticity can be either adaptive or maladaptive (Ghalambor et al. 2007; Huang et al. 2022).

Adaptive plasticity occurs when plastic changes increase the performance and fitness in response to specific environments (Via 1993)**.** Often times, adaptive plasticity is shown by using *a-priori* assumptions based on knowledge of the environmental heterogeneity and fitness. For example, populations sampled from temperate environments exhibit more robust plastic response to cold exposure than those sampled from comparatively more stable tropical environments (Mathur and Schmidt 2017), and thus plasticity is considered adaptive. Additionally, adaptive plasticity can be inferred from the concordance of the direction between plastic changes and that of known evolutionary changes (Huang et al. 2022). Maladaptive plasticity is characterized by the "counter-gradient" pattern between plastic response and adaptive evolution (e.g., Ghalambor et al. 2007). Whether phenotypic plasticity is adaptive in oscillating environments remains an open debate.

Phenotypic plasticity can also lead to environment-specific genetic correlation patterns (Via and Lande 1985). Genetic correlations between fitness traits determines the multivariate response to selection and are means to evaluate the evolutionary potentials and constraints between traits of shared genetic basis (Wood and Brodie 2015; Logan and Cox 2020). For example, the negative genetic correlations between life history traits are usually interpreted as evolutionary constraints, possibly due to resource allocation tradeoffs (Reznick et al. 2000). However, growing evidence suggests that environmentally induced phenotypic plasticity can alter, or even change the sign of, genetic correlations and therefore affect evolutionary potentials of associated traits even in the short term (Pigliucci 2005; Wood and Brodie 2015). Changes in signs of genetic correlations between temperature regimes or between lab and field environments are prevalent and could be the result of the expression of new genes, difference in environmental sensitivity of plasticity amongst traits, or possible resource limitations (Sgrò and Hoffmann 2004). Therefore, it is important to study how plasticity affects environment-specific genetic correlation patterns. However, estimating genetic correlations is difficult without information about relatedness, especially in natural environments.

Drosophila melanogaster is a robust system to study adaptive *vs*. maladaptive plasticity in fitness traits and genetic correlations. For example, evolutionary changes in fitness traits across latitudinal clines (James et al. 1997; Hoffmann et al. 2002) and seasons (Schmidt et al. 2005; Schmidt and Paaby 2008; Chown and Gaston 2010; Behrman et al. 2015; Rudman et al. 2022) are pervasive in this species. In principle, the evolutionary change across seasons should mirror that across the latitudinal clines due to the common selective pressures across space and time

(Rhomberg and Singh 1988; Yu and Bergland 2022). Therefore, we can take advantage of the well documented clinal and seasonal phenotypic evolutionary differentiations to assess whether plasticity shows adaptive or maladaptive signals by examining the concordance in direction of phenotypic changes. In addition, phenotypic plasticity in many fitness traits is also well characterized. For example, consistent with *temperature-size rule*, lower temperature in the lab environment results in longer developmental time (Gebhardt and Stearns 1993) and larger body size (Nunney and Cheung 1997). Moreover, given the large population size, genetic correlations between traits can be measured. Estimating genetic correlations between traits can help us understand the genetic constraints and gain insight into their evolutionary potentials (Ackermann et al. 2001).

Despite the advantages of using *D. melanogaster* to study plasticity, some issues remain to be addressed. First, although phenotypic plasticity has been extensively studied in fruit flies, so far, only limited effort has been made to measure plasticity in fitness traits across seasons (Mathur and Schmidt 2017). Such a lack of effort could be partially due to the difficulty in measuring plasticity in this species in the wild. Second, identifying the environmental factors that elicit seasonal phenotypic plasticity in the wild can be a challenging task. Temperature regimes could be an important environmental factor that can elicit plastic response in fitness-related traits. However, most previous work examining the effects of temperature on plasticity was conducted in lab environments with fixed temperature regimes (e.g., Ackermann et al. 2001). Thus, they often omit the fact that temperature is only a single component of environmental heterogeneity (Stillwell et al. 2007; Mathur and Schmidt 2017), and that temperature fluctuates in nature as opposed to being fixed under lab conditions. As a consequence, results from the lab and the wild

might differ (Kristensen et al. 2008; Vanin et al. 2012). Therefore, whether temperature has the same effect in the wild seasonal environments remains to be examined. Third, estimating genetic correlations across seasonal environments remains challenging due to the scale of sampling and the difficulty in measuring genetic relatedness. Thus, we still have a limited understanding of whether genetic correlation is the same between lab and wild seasonal environments and whether it changes across seasons in this species.

In this study, we used pools of genetically controlled F_1 *D. melanogaster* populations reared in both lab conditions and in an experimental orchard to study the phenotypic plasticity of developmental time, fecundity, and body size across 10 seasonal time points. We used F_1s to avoid inbreeding depression in fitness traits and to reduce the effect of inbreeding on measuring genetic correlation. For example, inbreeding can affect developmental time (Kristensen et al. 2011) and can also change the sign of genetic correlations in unpredictable ways (Phillips et al. 2001). Thus, using F_1s allows us to measure traits that represent variable genetic background and have more accurate estimates of genetic correlations. We address three questions in this study. First, we tested whether the measured traits exhibit plastic responses between lab and orchard reared flies across seasons, and whether plasticity shows adaptive signals. Second, we examined whether seasonal temperature regimes are associated with these traits as well as the effects of temperature. Third, we asked if the plastic responses in fitness traits lead to environment-specific genetic correlations in wild seasonal environments.

Materials and Methods

Crossing design. We created two replicates of 21 experimental F_1 crossing panels in bottles (20 pierced holes on each) of standard fly food at approximately two-week intervals (Supplemental Table S1) using 29 inbred lines collected from 4 populations (Maine, North Carolina, Pennsylvanian spring, Pennsylvanian fall). There were in total 84 F_1 genotype combinations. Our crossing design is block randomized. We assigned 3 blocks (May to July; July to August; August to October) covering 10 seasonal time points such that in each block every 84 F_1s is selected only once. For each seasonal time point in every block, we randomly selected 21 F_1 genotypes (Supplemental Figure S1). Although the genetic component for each of 10 seasonal time points is different, we reason that based on our block randomized design, the phenotypic changes amongst seasonal time points in the wild can be considered as plastic changes. After 24 hours of eggs being laid, we put out and reared one replicate of the F_1s on an experimental orchard at Morven Farms (VA: 37.96°, -78.47°) and another replicate in the lab from an embryo stage to 2 days after their eclosion throughout a growing season across 10 seasonal time points (referred to as 1^{st} to 10^{th}) in 2019 (May to October). The flies were kept in bottles with standard fly food supply inside an experimental cage. Temperature data was tracked both at Morven Farms and in-lab using *iButtons*® (iButtonLink, LLC).

Eclosion time assay. Fly samples were checked at 3:30 pm daily during their development for time to eclosion. An F_1 is recorded as eclosed if more than three adult flies were observed in the bottle. Field live samples were brought back to lab condition in their bottles 2 days after their eclosion day at 3:30 pm and sorted for fecundity assay.

Fecundity assay. We used CO_2 to anesthetize the flies and collected 25 females and 25 males from each bottle and put them on agar plates capped with clear beakers. If fewer than 25 flies from each sex were alive, we collected as many flies as possible for each sex. The medium was prepared following the recipe described in (Nouhaud et al. 2018). We took photos with two settings for each plate (2 photos for ISO 200 & 1 photo for ISO 3200) every 12 hours for 36 hours using a Canon Camera (Canon EOS 750D with an 18-55mm lens). The ISO 200 photos were processed with *Fiji* (Schindelin et al. 2012) and the ISO 3200 photos were used as manual checks if automated software returned with error. Immediately after photo-taking, the photos were uploaded from the camera to our online archive.

The software *Fiji* (Schindelin et al. 2012) was used to automate the egg-counting process on the photos with the *Droso egg counter* plugin (Nouhaud et al. 2018). We adopted the default settings in the plugin, with the additional selection of the "Smooth" functionality. No region of interest (ROI) settings was changed initially. Once the Fiji plugin was run on all photos, the resulting data were analyzed using *produce_counts_from_tables.R* (Nouhaud et al. 2018). The egg counts produced from the R script were recorded in our online spreadsheet, and any plates that reported over 50% error were noted.

Plate quality, plate position, lighting, and camera angle were all kept as consistent as possible, but due to slight natural variations in each, the plugin was not able to analyze every photo successfully. In this case, the program was run again on just the photos that produced errors: first with the default settings with a manual threshold of 38; then with the default settings, manual threshold of 38, and "Smooth." If the two reruns produced comparable results, the average of the

two values was recorded. If the reruns still produced errors, the multi-point tool in Fiji was used to manually count the eggs using the ISO 3200 photo. We also replaced auto-counts with manual counts for the photos with results of zeros, since they are likely to generate relatively larger errors (Nouhaud et al. 2018). Fecundity is estimated as egg count per female capita every 12 hours.

Thorax length assay. Fly samples were collected on CO_2 immediately following the fecundity assay and kept frozen in 70% ethanol at -80°C for thorax length assay. We thawed flies on ice for 20 minutes and collected 10 females and 10 males from each sample. We then measured their thorax length using a micrometer under the dissecting microscope with a 10X magnification (Bergland et al. 2008). If fewer than 10 flies from each sex were available, we collected as many flies as possible for each sex.

Statistical analysis. To test whether temperature had significant effects on the measured phenotypes, we built mixed effect models with *lmer4* function in *R* (Bates et al. 2015), including null models and temperature models. Based on our observed data, we built linear temperature models for eclosion time and quadratic temperature models for fecundity and thorax length separately for each phenotype measured on both orchard and lab reared flies:

$$\beta_{eclosion\ null} = y_i \sim 1 + (1|t_s) + \varepsilon$$

$$\beta_{fecundity\ null} = y_i \sim 1 + (1|t_s) + \varepsilon$$

$$\beta_{thorax\ null} = y_i \sim 1 + (1|t_s) + (1|g_i) + \varepsilon$$

$$\beta_{eclosion\ T} = y_i \sim \bar{T} + (1|t_s) + \ \varepsilon$$

$$\beta_{fecundity\,T} = y_i \sim \bar{T} + \bar{T}^2 + (1|t_s) + \varepsilon$$

$$\beta_{thorax\,T} = y_i \sim \bar{T} + \bar{T}^2 + (1|t_s) + (1|g_i) + \varepsilon$$

Where y_i stands for the observed phenotypic value for each F_1 genotype (eclosion time and egg count per capita) or each individual (thorax length), t_s is the seasonal time point, and $\bar{T}$ is the average temperature during each seasonal experimental time points in a given environment, g_i is the genotype of the F_1 cross, and 1 is the model intercept.

We then used χ^2 statistics to compare the null versus temperature models using *ANOVA*.
To further understand how temperature affects phenotypic variances, we used *ANOVA* function in *car* package (Fox and Weisberg 2019) to assess effects of temperature using the temperature models.

Line mean correlations. To test whether genetic correlations between fitness traits show environment-specific patterns, we first calculated phenotypic line mean correlations (here after "line mean correlations") between all possible trait combinations for orchard and lab reared flies separately using Pearson product-moment correlations by treating phenotypic measurements on the same genotypes from multiple seasonal time points as independent (hereafter "cross-time model"). The line mean correlation in our study is analogous to the family mean correlation (Roff and Preziosi 1994), which is a close estimate of the genetic correlation when the heritability of the traits are reasonably large (Sgrò and Hoffmann 2004). Next, to assess whether extreme seasonal conditions, in terms of temperature, affect patterns of genetic correlation, we calculated line mean correlations between traits in both orchard and lab reared flies in high

temperature environment ("hot season"; 5th : 27.2°C) and low temperature environment ("cold season"; 10th: 17.5°C), respectively.

To compare the difference between orchard and lab estimates of line mean correlations and account for the possible bias generated by repeated measurements from the same genotypes across seasonal time points in the cross-time model, we built linear mixed effect models:

$$\beta_{corr} = y_i \sim x_i * location + (1|g_i) + \varepsilon$$

Where y_i and x_i stand for the observed line mean phenotypic values for two separate traits for each F_1 genotype conditional on being sampled at the same seasonal time point, g_i is the genotype of the F_1 cross, and 1 is the model intercept. A significant response variable x location interaction term therefore indicates a significant difference between orchard and lab estimates of line mean correlations for the cross-time model.

Results

Seasonal environments elicit plasticity in fitness traits. We assessed whether seasonal conditions in the orchard can induce plastic responses for eclosion time, fecundity, and thorax length. We compared the phenotypic values of these traits between orchard- and lab-reared flies for every seasonal time point independently (Supplemental table S2, *t* tests, adjusted $p \leq 0.005$). We observed plastic responses in eclosion time (May to October), in fecundity (July and October), and in thorax length for females (May to October) and males (May to July). These

results suggest that wild environment induced plastic responses are prevalent in these traits (Figure 1).

To study the direction of seasonal plastic phenotypic changes, we compared seasonal end points (in May and in October) to the seasonal middle point of the growing season (5^{th} time point in July) for each trait separately. We show that eclosion time for orchard flies decreased from May to July (t = -28.81, p = 2.2 x 10^{-16}), and increased from July to October (t = 13.16, p = 2.1x10^{-10}), compared to an opposite trend for lab reared flies (May – July: increase, t = 2.36, p = 0.02; July – October: decrease, t = -15.97, p = 2.2 x 10^{-16}). The phenotypic changes across seasons in lab reared flies suggest either there are genetically determined differences or that subtle environmental fluctuations in lab environments can induce phenotypic plasticity. The difference in the direction of seasonal plasticity in eclosion time between orchard and lab flies suggests that plasticity plays an important role affecting the seasonal phenotypic changes in the orchard (Figure 1A). Fecundity is relatively constant from May to July and from July to October for both orchard and lab reared flies (Figure 1B). Thorax length for both sexes decreased from May to July in the orchard and lab (orchard female: t = -7.14, p = 3.2x10^{-11}; lab female: t = -2.17, p = 0.031; orchard male: t = -7.37, p = 6.8x10^{-12}; lab male: t = -2.38, p = 0.018). While orchard reared flies increased in thorax length in both sexes from July to October (orchard female: t = 8.10, p = 2.1x10^{-14}; orchard male: t = 6.90, p = 5.9x10^{-11}), lab reared flies were constant (Figure 1C).

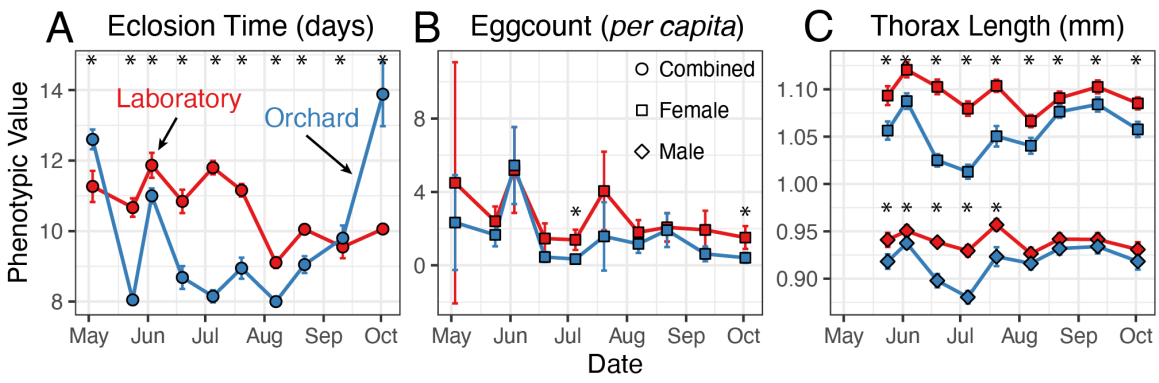

Figure 1. Plastic responses and seasonal trend for eclosion time (A), fecundity (B), thorax length (C). The x-axis is the start date of every seasonal experimental time point (1st in May to 10th in Oct). The y-axis is phenotype value; the units are days to eclosion from 24-hour embryos (eclosion time); number of eggs per female capita every 12 hours (fecundity); mm (thorax length). Error bars represent one standard error of the mean. Asterisks indicate significant difference between orchard and lab reared flies on measured phenotype at certain experimental time points (*t* tests; corrected $p \leq 0.005$).

Developmental temperature affects plastic traits. Average temperature increased from May to July and decreased from July to October in the orchard and stayed relatively constant in lab (Figure 2A). To test whether developmental temperature affects fitness-related traits, we built null and temperature mixed effect models for each trait. We first tested whether temperature models fits better than null models for each trait (see Materials and Methods). We show significant differences between temperature and null models for eclosion time and thorax length in both orchard and lab reared flies (Table 1, $p < 0.05$), as well as marginal significance for fecundity in orchard flies ($p = 0.067$). Eclosion time is linearly correlated with temperature, whereas fecundity and thorax length show non-linear correlations with temperature (Figure 2, B-D, Table 2).

Next, we assessed the effects of temperature on the measured traits. The quadratic terms of temperature have significant effects on fecundity and thorax length for orchard reared flies, but not for fecundity in lab. The linear term of temperature has significant effects on all traits for both orchard and lab reared flies (Table 2). Orchard reared flies have the largest thorax lengths (female: 1.088 mm; male: 0.938 mm) and fecundity (5.442 eggs per capita) at 21.5°C (3rd seasonal time point in June), showing an increase in thorax length and fecundity from 17.5°C to 21.5°C and a decrease from 21.5°C to 27.2°C. Eclosion time decreases from 17.5°C to 21.5°C to 27.2°C.

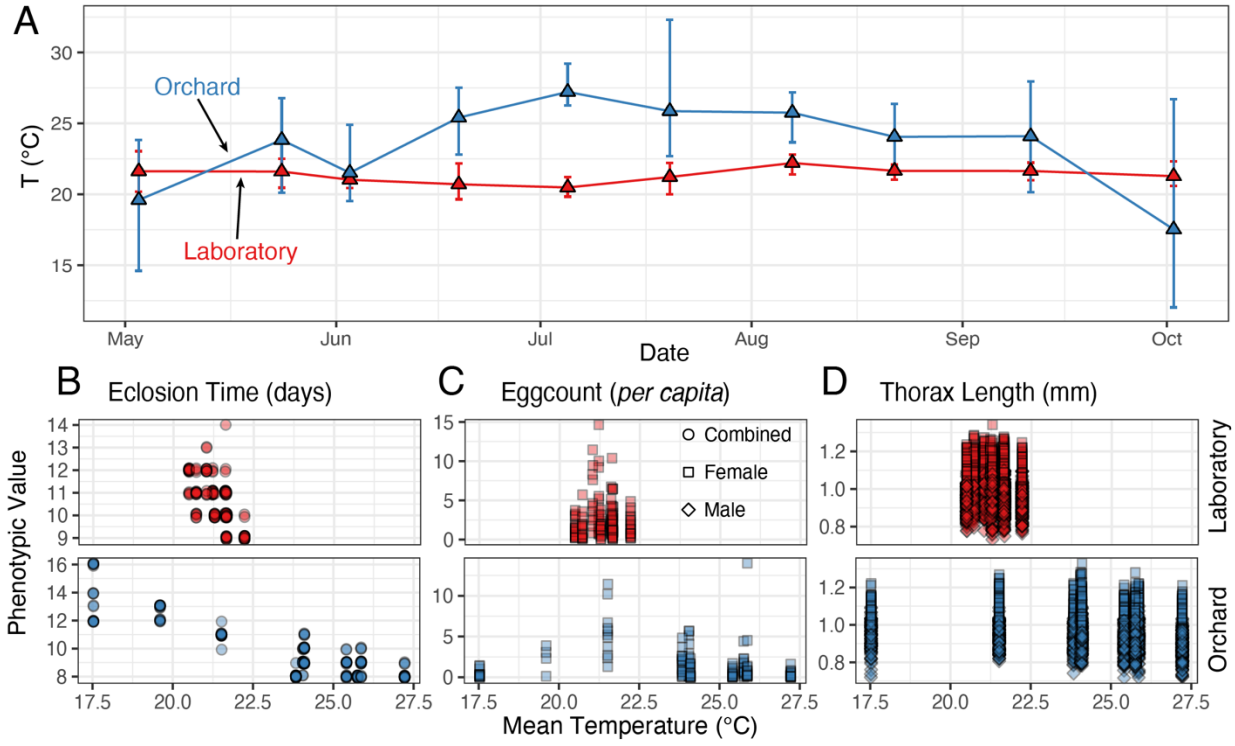

Figure 2. Mean, max and min temperature in orchard and lab during the experiments for each of 10 seasonal experimental time point (A). Correlation between eclosion time (B), fecundity (C), thorax length

(D) and average temperature for their developmental environments. Each dot represents individual phenotype value (thorax length) or the phenotypic value (fecundity and eclosion time) for an F1 genotype, colored by the environment it was reared in and shaped by sex.

Eclosion				**Fecundity**				**Thorax L.**							
Combined				*Female*				*Female*				*Male*			
orchard		lab		orchard		lab		orchard		lab		orchard		lab	
AIC	χ^2	AIC	χ^2	AIC	χ^2	AIC	χ^2	AIC	χ^2	AIC	χ^2	AIC	χ^2	AIC	χ^2
436.63	**22.47*****	325.79	**8.23****	622.74	5.38	691.27	2.59	-3953.39	**16.52*****	-4666.73	**7.77***	-4098.27	**15.57*****	-4708.59	**7.79***

Table 1. Comparison of temperature mixed effect models to the null models for eclosion time, fecundity and thorax length for orchard and lab reared flies. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Line mean correlations show environment-specific patterns. To test if genetic correlations differ between orchard and lab estimates, we first calculated line mean correlations (as an estimate of genetic correlations) for lab and orchard reared flies separately using a cross-time model. While we show positive correlations for both orchard and lab estimates, we do not observe significant difference between orchard and lab correlations for the same comparisons (Table 3). In addition, we show a positive correlation ($p \leq 0.05$) between eclosion time and thorax length, indicating that longer developmental time results in larger body size, for orchard reared flies in the hot season (5^{th}), but not for lab reared flies. Such a positive correlation ($p \leq 0.01$) between eclosion time and thorax length is also observed for orchard reared flies in the cold season (10^{th}) with an opposite sign ($p \leq 0.05$) observed for lab reared flies (Table 3), which is likely caused by an outlier. After outlier removal, we do not observe a significant correlation in the cold season. Interestingly, the line mean correlation between fecundity and thorax length is

negative ($p \leq 0.01$) in the cold season but not in the cross-time model or the hot season (Table 3). The negative correlation suggests a tradeoff between the two fitness traits. Such results suggest that line mean correlations between traits do not differ between wild seasonal environments and lab conditions across seasons but show seasonal environment-specific correlation patterns at hot and cold seasons separately.

Source	**Eclosion**		**Fecundity**		**Thorax L.**			
	Combined		*Female*		*Female*		*Male*	
	orchard	lab	orchard	lab	orchard	lab	orchard	lab
$\bar{T}$	**83.69*****	**12.80*****	1.39	0.003	**22.07*****	1.26	**20.50*****	0.44
$\bar{T}^2$	-	-	**5.80***	2.94	**28.60*****	**11.49*****	**28.87*****	**11.76*****

Table 2. Effects of temperature on eclosion time, fecundity and thorax length for orchard and lab reared flies. The numbers reported represent χ^2 statistics. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

	Cross-time			**5th (27.2°C)**			**10th (17.5°C)**		
	Eclosion	*Fecundity*	*Thorax L.*	*Eclosion*	*Fecundity*	*Thorax L.*	*Eclosion*	*Fecundity*	*Thorax L.*
Eclosion		$0.088_{\text{n.s.}}$	$\mathbf{0.294}^{***}_{n.s.}$		-0.069	**0.469***		-0.459	**0.666****
Fecundity	**0.174***		$\mathbf{0.238}^{**}_{n.s.}$	-0.266		-0.065	-0.109		**-0.662****
Thorax L.	**0.174***	0.146		-0.235	-0.015		**-0.586***	-0.308	

Table 3. Line mean correlations for the measured traits. The numbers reported represent Pearson product-moment correlation. The numbers above the diagonal represent orchard estimate and the numbers below represent lab estimate. * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$. The subscripts n.s. stand for non-significant differences between orchard and lab estimates of the line mean correlations in the cross-time model.

Discussion

Whether phenotypic plasticity is adaptive across seasons and whether it results in seasonal environment-specific genetic correlations between fitness traits remains an open debate (Williams et al. 2017). In this study, we assessed how eclosion time, fecundity and thorax length change plastically across a growing season in flies. Our results show that plasticity in these fitness traits is likely adaptive across seasons. Such adaptive plastic responses should be considered when estimating the strength of seasonal selection pressures on fitness traits. We further show that temperature is an important environmental factor affecting the plastic responses of these traits and that both fecundity and thorax length changes non-linearly with temperature while ecolsion time show a linear association with temperature. In addition, we show that while there is non-significant difference between orchard and lab estimates of genetic correlations in general, phenotypic plasticity in certain seasons (hot or cold) can result in environment-specific patterns. Such seasonal-specific correlation pattern highlights the importance of assessing how plasticity affects the interpretations of genetic architecture underlying fitness traits, and studying genetic correlations in an environment-dependent context (Via and Lande 1985).

Putative adaptive plasticity across seasons. In our study, we show clear patterns of plasticity elicited in the orchard compared to lab reared flies and provide evidence that the seasonal phenotypic plasticity in the orchard is likely adaptive (Figure 1). First, the direction of seasonal plasticity in eclosion time in the orchard show concordance with the evolved differences across seasons. Lab reared descendants of flies sampled across the seasons show an evolved increase in developmental time from July to October (Behrman et al. 2015). Such an increase is also

observed in our eclosion time plasticity data (Figure 1). Second, the direction of seasonal plastic change observed in our data are also reflected across a latitudinal cline. For example, it is hypothesized that the higher latitudinal environments are winter-like and that lower latitudinal environments are summer-like due to the shared abiotic environmental factors, such as temperature (Rodrigues et al. 2021). Therefore, flies sampled in cold seasons should exhibit traits similar to those sampled from higher latitudinal locales. Accordingly, flies sampled in the hot seasons should exhibit traits similar to those sampled from lower latitudinal locales. Previous studies have shown that flies living in higher latitudinal locales experiencing colder environments are larger (Gilchrist and Partridge 1999) and develop slower (Folguera et al. 2008) than those from lower latitudinal locales. We show that the observed seasonal plastic change in thorax length and eclosion time mirror the clinal differences as if they were caused by shared selective pressures due to environmental similarities (Figure 1). Together, we suggest that plasticity in these traits in response to seasonal heterogeneity are likely adaptive.

Adaptive plasticity may bias estimates of strength of seasonal selection pressures (Kingsolver et al. 2001). Seasonal selection is annually cyclical (Mathur and Schmidt 2017; Nagano et al. 2019). However, rapid seasonal environmental shift often occurs within the matter of days (Denlinger et al. 2017), which is shorter than the lifespan of *D. melanogaster*. Adaptive plasticity could allow the organisms to adjust quickly to the shifting fitness optima without invoking adaptive evolution between generations. Therefore, the identification of seasonal loci using seasonal end-point data (e.g., Machado et al. 2021) could be underpowered and the strength of seasonal selection in a short period of time could be underestimated.

In addition, the seasonal plastic changes observed in our data are consistent with predictions of life history theory (Roff 1981). For example, longer developmental time may lead to larger body size (Kivelä et al. 2011). Larger body size then gives an advantage in competing for resources (Hoffmann 1987), mating success (Partridge et al. 1987), and larger flies usually have higher fecundity (Anderson 1994). Empirical studies have shown support for life history theory in both *D. melanogaster* (Flatt 2020) and other species (Roff 2000), but see (Klingenberg and Spence 1997).

Temperature effect. We also show developmental temperature is an important environmental factor affecting the plastic responses of these traits in wild seasonal environments (Figure2, Table 1), and that temperature has similar effects in the wild environments as that measured in the lab conditions. For example, our results agree with the temperature-size rule (i.e., increased body size at lower developmental temperature within a certain range (Atkinson 1994), as well as with previous studies on plasticity in response to temperature in lab environments (Forster et al. 2012). It has been shown that thorax length for both female and male flies is largest between 18°C - 22°C, and smaller as temperature decreases to below 18°C or rises to 25°C and above (Karan et al. 1999). Eclosion time has also been shown to decrease from 18°C to 28°C (Trotta et al. 2006). These results are reflected in our findings (Figure 2). In addition, we observe a positive genetic correlation between fecundity and thorax length (Figure 3). Both traits change non-linearly with temperature, and peak at around 21.5°C. This result agrees with the optimum developmental temperature hypothesis (Dell et al. 2011), which posits that there is a thermal opitma for fitness traits at intermediate temperatures (Klepsatel et al. 2019). Consistent with our findings, previous studies in *Drosophila* on other fitness related traits, such as reproduction

(Klepsatel et al. 2019), life span (Zwaan et al. 1992) and male competition (Zamudio et al. 1995) have also shown support for the optimum developmental temperature theory.

Implications on population dynamics in the wild. Temperature induced plasticity in eclosion time and fecundity, in addition to selection on thermal tolerance or starvation resistance traits (e.g., Rudman et al. 2022), could shape seasonal population size and age dynamics (Carey et al. 2008; Behrman et al. 2015). Drosophilids are overwintering species that experience severe seasonal bottlenecks across the growing season (Cogni et al. 2014). As a consequence, the drosophilids population size increases from the start of growing season (Tauber et al., 1986) and decreases in late fall (Rudman et al. 2022). Age structure also changes across seasons in wild fly populations: overwintering descendants sampled in June are uniformly younger than those sampled in October or November (Behrman et al. 2015). Here in our data, we show that when temperature is intermediate (~ 21.5°C) at the start of growing season, flies develop at an intermediate speed, but fecundity is highest (Figure2). Therefore, the increase in population size in the wild could be due to higher fecundity. In the middle of summer, as temperature increases (~ 27.2°C), flies lay fewer eggs but develops faster (Figure2). The constant large population size in the hot seasons could be maintained by the fast developmental rate. Such fast development could also lead to shorter generational gaps and therefore increasing the chance of sampling younger flies in the population. In the late growing season (~17.5°C), both fecundity and developmental rate decrease with temperature, and the reduction in these traits together (Figure 2) could result in reduced population size as well as longer generational gaps, which could be the cause of a more heterogeneous age structure.

Environment-specific line mean correlations. Genetic correlations are often the means to study genetic constraints and to infer the evolutionary potentials of life history traits (Sgrò and Hoffmann 2004). Consistent with previous findings (e.g., Windig 1994; Kause and Morin 2001), our results suggest that line mean correlations between traits are environmentally dependent in both hot and cold seasons. For example, the line mean correlations between eclosion time and thorax length in both two environments are positive for orchard estimates compared to non-significant lab estimates (Table 3). The positive correlation between the two traits is also observed in the cross-time model, suggesting that longer developmental time leads to larger body size across seasons and in extreme temperature environments. Thus, temperature induced plasticity may not have affected the genetic architecture between these traits in seasonal environments (Stearns et al. 1991). However, line mean correlations do not show significant difference between orchard and lab estimates in the cross-time model, indicating that we cannot predict how genetic correlation changes from one environment to another and that estimating environment-specific genetic correlation is an empirical issue (Wood and Brodie 2015).

Interestingly, we observe a negative correlation between fecundity and thorax length, which is only present in the cold season (Table 3). Such negative correlation suggests a tradeoff between reproduction and self-maintenance based on the resource allocation theory (Reznick et al. 2000). However, the food supply was consistent across seasons in our experiment even in such cold season. Therefore, the tradeoff induced by plasticity could suggest that under extreme seasonal temperature regimes certain stress related genes of large effect may alter the correlation between traits (Shirley and Sibly 1999), or even by affecting a third trait (Clark 1987; Pavlicev et al. 2011), such as energy production. Thus, by examining the genetic correlations between fitness

traits in seasonal environments, we can generate hypothesis of the ecologically relevant trait being selected across seasons.

In summary, our study provides evidence supporting the hypothesis that plasticity in fitness traits across seasons shows adaptive signal and that temperature is an important factor eliciting such plastic response in the wild. In addition, we show that the cross seasonal line mean correlations cannot be used to predict those either in the hot or cold seasons. Thus, our study highlights the importance of considering adaptive plasticity in studying the strength of seasonal selection pressures or population dynamics and estimating seasonal-specific genetic correlation pattern from an empirical perspective.

List of supplemental figures and tables

Supp. Figure S1: Crossing design.

Supp. Table S1: Sample information.

Supp. Table S2: T test summary statistics for plasticity.

Acknowledgement. We thank Yasmin Khodaei for helping with collecting thorax length data.

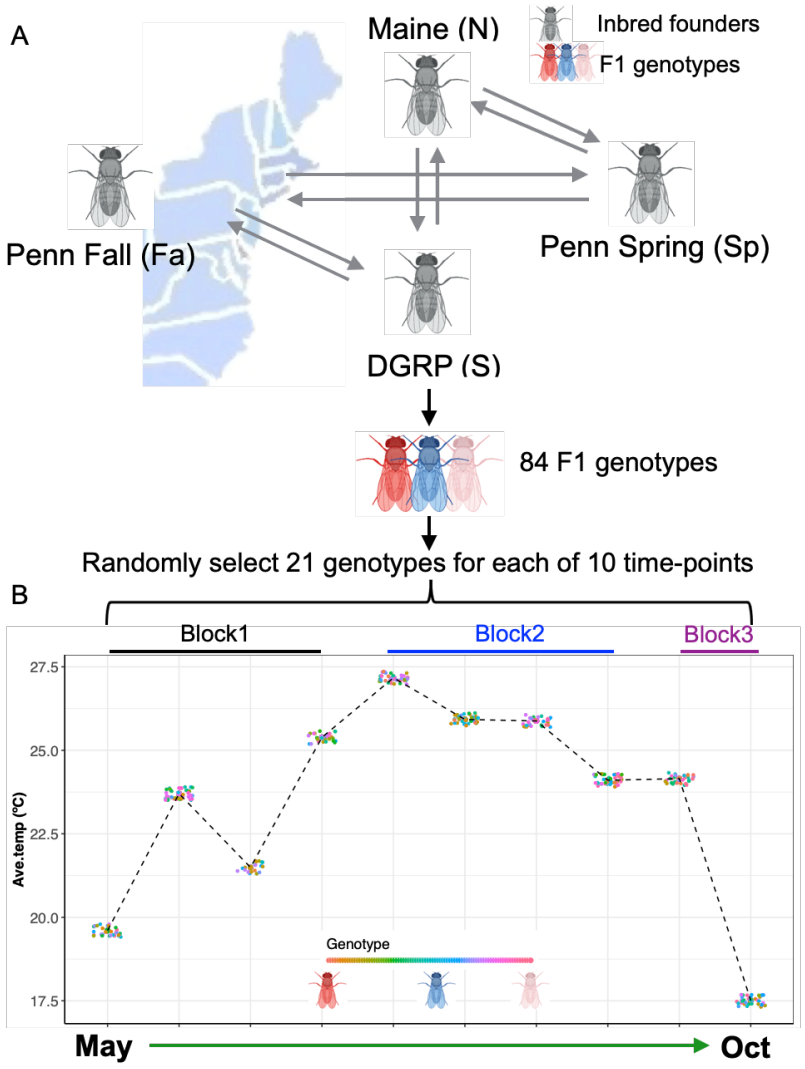

Figure S1. Crossing design. (A) 84 F_1 genotypes were created using 29 inbred founder lines from 4 populations. (B) In block randomized sampling processes, 21 F_1 genotypes were randomly selected for each of the 10 seasonal time points from May to October. In block1 and block2, 84 F_1s were non-repeatedly selected. In block3, 42 F_1s were non-repeatedly selected.

Table S1. Sample information. "time" represents the 10 seasonal time points from first to tenth. "Genotype" is the F_1 genotypes, represented by the founder line ids formatted as "maternal id x paternal id". The following columns are the four phenotypes measured, where "yes" and "no" indicate whether certain F_1 genotypes are used in the analysis.

time	Genotype	thorax.length_female	thorax.length_male	eggcount.per.cap_female	eclosion.time_combined
first	12BME10_130xdgrp_28241	no	no	no	yes
first	12BME10_204x12LN10_76	no	no	yes	yes
first	12BME10_204xdgrp_25744	no	no	no	yes
first	12BME10_212x12LN10_76	no	no	no	yes
first	12BME10_218xdgrp_25744	no	no	no	yes
first	12LN10_14x12LN6_6	no	no	no	no
first	12LN10_32x12BME10_229	no	no	no	no
first	12LN10_32x12LN6_6	no	no	no	no
first	12LN10_35x12BME10_211	no	no	no	yes
first	12LN6_12xdgrp_28261	no	no	no	yes
first	12LN6_16x12LN10_67	no	no	no	yes
first	12LN6_19x12LN10_22	no	no	no	yes
first	12LN6_19xdgrp_28261	no	no	yes	yes
first	12LN6_28x12LN10_67	no	no	yes	yes
first	12LN6_28x12LN10_76	no	no	no	yes
first	12LN6_28x12LN10_95	no	no	no	yes
first	dgrp_28189x12BME10_211	no	no	no	yes

first	dgrp_28191x12LN6_6	no	no	no	no
first	dgrp_29660x12BME10_229	no	no	yes	yes
first	dgrp_29660x12BME10_270	no	no	no	no
first	dgrp_29660x12LN6_84	no	no	no	no
second	12BME10_130x12LN10_22	no	no	yes	yes
second	12BME10_204xdgrp_28261	no	no	yes	yes
second	12BME10_218x12LN10_76	no	no	yes	yes
second	12LN10_13x12BME10_270	yes	yes	yes	yes
second	12LN10_13x12LN6_6	no	no	no	yes
second	12LN10_14x12LN6_84	no	no	yes	yes
second	12LN10_32x12BME10_270	no	no	yes	yes
second	12LN10_32x12LN6_46	no	no	yes	yes
second	12LN10_32x12LN6_84	yes	yes	yes	yes
second	12LN10_35x12LN6_84	no	no	no	yes
second	12LN6_12xdgrp_28241	yes	yes	yes	yes
second	12LN6_16x12LN10_22	yes	yes	yes	yes
second	12LN6_16xdgrp_28241	no	no	yes	yes
second	12LN6_19x12LN10_95	yes	yes	yes	yes
second	dgrp_28189x12BME10_229	no	no	yes	yes
second	dgrp_28189x12BME10_270	no	no	yes	yes
second	dgrp_28189x12LN6_6	no	no	yes	yes
second	dgrp_28191x12BME10_104	yes	yes	no	yes
second	dgrp_28191x12BME10_229	no	no	yes	yes

second	dgrp_28191x12BME10_270	yes	yes	yes	yes
second	dgrp_28191x12LN6_46	yes	yes	yes	yes
third	12BME10_130xdgrp_25744	no	no	no	no
third	12BME10_204x12LN10_22	yes	yes	yes	yes
third	12BME10_204x12LN10_67	yes	yes	yes	yes
third	12BME10_212xdgrp_25744	yes	yes	yes	yes
third	12BME10_212xdgrp_28241	yes	yes	yes	yes
third	12BME10_212xdgrp_28261	yes	yes	yes	yes
third	12BME10_218x12LN10_95	yes	yes	yes	yes
third	12LN10_13x12BME10_229	no	no	no	no
third	12LN10_13x12LN6_84	no	no	no	no
third	12LN10_35x12LN6_6	yes	yes	yes	yes
third	12LN6_12x12LN10_22	yes	yes	yes	yes
third	12LN6_12x12LN10_95	yes	yes	yes	yes
third	12LN6_16x12LN10_76	yes	yes	yes	yes
third	12LN6_16xdgrp_28261	yes	yes	yes	yes
third	12LN6_19x12LN10_76	no	no	no	yes
third	12LN6_28x12LN10_22	no	no	no	no
third	12LN6_28xdgrp_28241	yes	yes	no	yes
third	12LN6_28xdgrp_28261	yes	yes	no	yes
third	dgrp_28191x12BME10_211	no	no	no	yes
third	dgrp_29660x12BME10_211	no	no	no	no
third	dgrp_29660x12LN6_6	no	no	no	no

fourth	12BME10_130xdgrp_28261	yes	yes	yes	yes
fourth	12BME10_204xdgrp_28241	yes	yes	yes	yes
fourth	12BME10_218x12LN10_67	yes	yes	yes	yes
fourth	12BME10_218xdgrp_28241	yes	yes	yes	yes
fourth	12BME10_218xdgrp_28261	yes	yes	yes	yes
fourth	12LN10_13x12LN6_46	no	no	no	yes
fourth	12LN10_14x12BME10_211	yes	yes	yes	yes
fourth	12LN10_14x12BME10_229	no	no	yes	yes
fourth	12LN10_14x12LN6_46	yes	yes	yes	yes
fourth	12LN10_35x12BME10_229	no	no	yes	yes
fourth	12LN10_35x12LN6_46	yes	yes	yes	yes
fourth	12LN6_12x12LN10_67	yes	yes	yes	yes
fourth	12LN6_12x12LN10_76	no	no	yes	yes
fourth	12LN6_16x12LN10_95	no	no	no	yes
fourth	12LN6_19x12LN10_67	no	no	yes	yes
fourth	12LN6_28xdgrp_25744	yes	yes	yes	yes
fourth	dgrp_28189x12BME10_104	yes	yes	yes	yes
fourth	dgrp_28189x12LN6_84	no	no	no	yes
fourth	dgrp_28191x12LN6_84	no	no	no	no
fourth	dgrp_29660x12BME10_104	yes	yes	yes	yes
fourth	dgrp_29660x12LN6_46	no	no	no	no
fifth	12BME10_130xdgrp_25744	yes	yes	yes	yes

fifth	12BME10_130xdgrp_28261	yes	yes	yes	yes
fifth	12BME10_212xdgrp_28261	no	no	no	no
fifth	12BME10_218x12LN10_67	yes	yes	yes	yes
fifth	12LN10_13x12BME10_270	yes	yes	yes	yes
fifth	12LN10_14x12LN6_84	no	no	no	yes
fifth	12LN10_32x12BME10_270	yes	yes	yes	yes
fifth	12LN10_32x12LN6_84	yes	yes	yes	yes
fifth	12LN10_35x12BME10_211	yes	yes	yes	yes
fifth	12LN10_35x12LN6_84	yes	yes	yes	yes
fifth	12LN6_19x12LN10_95	yes	yes	yes	yes
fifth	12LN6_28x12LN10_22	yes	yes	yes	yes
fifth	12LN6_28x12LN10_76	yes	yes	yes	yes
fifth	12LN6_28x12LN10_95	yes	yes	yes	yes
fifth	12LN6_28xdgrp_25744	yes	yes	yes	yes
fifth	dgrp_28189x12BME10_104	yes	yes	yes	yes
fifth	dgrp_28189x12BME10_229	yes	yes	yes	yes
fifth	dgrp_28191x12LN6_6	yes	yes	yes	yes
fifth	dgrp_29660x12BME10_229	yes	yes	yes	yes
fifth	dgrp_29660x12LN6_46	yes	yes	yes	yes
fifth	dgrp_29660x12LN6_6	yes	yes	yes	yes
sixth	12BME10_130xdgrp_28241	yes	yes	yes	yes
sixth	12BME10_204x12LN10_67	no	no	no	no

sixth	12BME10_204x12LN10_76	yes	yes	yes	yes
sixth	12BME10_204xdgrp_25744	yes	yes	yes	yes
sixth	12BME10_212xdgrp_25744	yes	yes	yes	yes
sixth	12BME10_212xdgrp_28241	yes	yes	yes	yes
sixth	12BME10_218x12LN10_76	yes	yes	yes	yes
sixth	12BME10_218xdgrp_28261	yes	yes	yes	yes
sixth	12LN10_13x12BME10_229	yes	no	no	yes
sixth	12LN10_13x12LN6_46	no	no	no	no
sixth	12LN10_14x12LN6_6	yes	no	no	yes
sixth	12LN10_32x12LN6_6	yes	no	no	yes
sixth	12LN6_12x12LN10_22	yes	yes	yes	yes
sixth	12LN6_12xdgrp_28241	yes	yes	yes	yes
sixth	12LN6_16x12LN10_22	yes	yes	yes	yes
sixth	12LN6_16x12LN10_67	yes	yes	yes	yes
sixth	12LN6_16x12LN10_95	yes	yes	yes	yes
sixth	12LN6_16xdgrp_28241	yes	yes	yes	yes
sixth	12LN6_16xdgrp_28261	yes	yes	yes	yes
sixth	12LN6_19xdgrp_28261	yes	yes	yes	yes
sixth	dgrp_28191x12BME10_104	yes	yes	yes	yes
seventh	12BME10_130x12LN10_22	yes	yes	yes	yes
seventh	12BME10_204xdgrp_28261	yes	yes	yes	yes
seventh	12BME10_212x12LN10_76	yes	yes	yes	yes

seventh	12BME10_218xdgrp_25744	yes	yes	yes	yes
seventh	12LN10_13x12LN6_6	no	no	no	no
seventh	12LN10_13x12LN6_84	no	no	no	no
seventh	12LN10_32x12BME10_229	yes	yes	yes	yes
seventh	12LN10_32x12LN6_46	yes	yes	yes	yes
seventh	12LN10_35x12BME10_229	yes	yes	yes	yes
seventh	12LN10_35x12LN6_6	yes	yes	yes	yes
seventh	12LN6_12xdgrp_28261	yes	yes	yes	yes
seventh	12LN6_19x12LN10_22	yes	yes	yes	yes
seventh	12LN6_19x12LN10_67	yes	yes	yes	yes
seventh	12LN6_28x12LN10_67	yes	yes	yes	yes
seventh	12LN6_28xdgrp_28261	yes	yes	yes	yes
seventh	dgrp_28189x12BME10_211	yes	yes	yes	yes
seventh	dgrp_28189x12BME10_270	yes	yes	yes	yes
seventh	dgrp_28189x12LN6_6	yes	yes	yes	yes
seventh	dgrp_28191x12BME10_211	yes	yes	yes	yes
seventh	dgrp_28191x12LN6_46	yes	yes	yes	yes
seventh	dgrp_29660x12BME10_270	yes	yes	yes	yes
eighth	12BME10_204x12LN10_22	yes	yes	yes	yes
eighth	12BME10_204xdgrp_28241	yes	yes	yes	yes
eighth	12BME10_218x12LN10_95	yes	yes	yes	yes
eighth	12BME10_218xdgrp_28241	yes	yes	yes	yes

eighth	12LN10_14x12BME10_211	yes	yes	yes	yes
eighth	12LN10_14x12BME10_229	yes	yes	yes	yes
eighth	12LN10_14x12LN6_46	yes	yes	yes	yes
eighth	12LN10_35x12LN6_46	yes	yes	yes	yes
eighth	12LN6_12x12LN10_67	yes	yes	no	yes
eighth	12LN6_12x12LN10_76	yes	yes	yes	yes
eighth	12LN6_12x12LN10_95	yes	yes	yes	yes
eighth	12LN6_16x12LN10_76	yes	yes	yes	yes
eighth	12LN6_19x12LN10_76	yes	yes	yes	yes
eighth	12LN6_28xdgrp_28241	yes	yes	yes	yes
eighth	dgrp_28189x12LN6_84	yes	yes	yes	yes
eighth	dgrp_28191x12BME10_229	yes	yes	yes	yes
eighth	dgrp_28191x12BME10_270	yes	yes	yes	yes
eighth	dgrp_28191x12LN6_84	yes	yes	yes	yes
eighth	dgrp_29660x12BME10_104	no	no	yes	yes
eighth	dgrp_29660x12BME10_211	yes	yes	yes	yes
eighth	dgrp_29660x12LN6_84	no	no	no	no
ninth	12BME10_130x12LN10_22	yes	no	no	yes
ninth	12BME10_204x12LN10_22	yes	yes	yes	yes
ninth	12BME10_212xdgrp_28261	yes	yes	yes	yes
ninth	12BME10_218x12LN10_95	yes	yes	yes	yes
ninth	12LN10_13x12LN6_84	no	no	no	yes
ninth	12LN10_32x12LN6_46	yes	yes	yes	yes
ninth	12LN10_32x12LN6_84	yes	yes	yes	yes

ninth	12LN10_35x12BME10_211	yes	yes	yes	yes
ninth	12LN10_35x12LN6_46	yes	yes	yes	yes
ninth	12LN10_35x12LN6_84	yes	yes	no	yes
ninth	12LN6_12xdgrp_28241	yes	yes	yes	yes
ninth	12LN6_12xdgrp_28261	no	no	no	no
ninth	12LN6_28x12LN10_76	yes	yes	yes	yes
ninth	12LN6_28x12LN10_95	yes	yes	yes	yes
ninth	dgrp_28189x12BME10_104	no	no	no	yes
ninth	dgrp_28191x12BME10_104	yes	yes	yes	yes
ninth	dgrp_28191x12BME10_270	no	no	yes	yes
ninth	dgrp_29660x12BME10_211	yes	yes	yes	yes
ninth	dgrp_29660x12BME10_270	yes	yes	yes	yes
ninth	dgrp_29660x12LN6_46	yes	yes	yes	yes
ninth	dgrp_29660x12LN6_84	yes	yes	yes	yes
tenth	12BME10_204x12LN10_76	yes	yes	yes	yes
tenth	12BME10_204xdgrp_28241	yes	yes	yes	yes
tenth	12BME10_212xdgrp_25744	yes	yes	yes	yes
tenth	12BME10_218x12LN10_76	yes	yes	yes	yes
tenth	12BME10_218xdgrp_28261	yes	no	no	yes
tenth	12LN10_13x12LN6_6	no	no	no	no
tenth	12LN10_32x12BME10_270	yes	yes	yes	yes
tenth	12LN10_32x12LN6_6	yes	yes	yes	yes

tenth	12LN10_35x12BME10_229	yes	yes	yes	yes
tenth	12LN6_12x12LN10_22	yes	yes	yes	yes
tenth	12LN6_12x12LN10_95	yes	yes	yes	yes
tenth	12LN6_16xdgrp_28261	no	no	no	no
tenth	12LN6_19x12LN10_22	yes	yes	yes	yes
tenth	12LN6_19x12LN10_76	yes	yes	yes	yes
tenth	12LN6_19x12LN10_95	yes	yes	yes	yes
tenth	12LN6_19xdgrp_28261	no	no	no	yes
tenth	12LN6_28xdgrp_28241	no	no	no	yes
tenth	dgrp_28189x12BME10_229	no	no	no	no
tenth	dgrp_28191x12LN6_6	yes	no	no	no
tenth	dgrp_29660x12BME10_104	yes	yes	yes	yes
tenth	dgrp_29660x12BME10_229	yes	yes	yes	yes

Table S2. T test summary statistics for plasticity. The tests are performed between orchard and lab reared flies. “phenotype” represents the phenotype being tested. “time” represents the 10 seasonal time points from first to tenth.

phenotype	time	sex	*t*.value	*p*
thorax.length	second	Female	-5.32	4.04×10^{-7}
thorax.length	third	Female	-5.69	3.69×10^{-8}
thorax.length	fourth	Female	-15.11	2.09×10^{-36}
thorax.length	fifth	Female	-12.41	2.58×10^{-29}
thorax.length	sixth	Female	-8.37	1.13×10^{-14}
thorax.length	seventh	Female	-4.88	1.66×10^{-6}
thorax.length	eighth	Female	-3.03	2.62×10^{-3}
thorax.length	ninth	Female	-3.61	3.58×10^{-4}
thorax.length	tenth	Female	-5.32	2.45×10^{-7}
thorax.length	second	Male	-4.25	3.94×10^{-5}
thorax.length	third	Male	-3.11	2.08×10^{-3}
thorax.length	fourth	Male	-9.57	3.40×10^{-18}
thorax.length	fifth	Male	-10.85	1.40×10^{-23}
thorax.length	sixth	Male	-5.69	5.23×10^{-8}
thorax.length	seventh	Male	-2.29	0.023
thorax.length	eighth	Male	-2.29	0.022
thorax.length	ninth	Male	-1.49	0.139
thorax.length	tenth	Male	-2.14	0.033
eggcount.per.cap	first	Female	-0.97	0.386
eggcount.per.cap	second	Female	-1.52	0.137

eggcount.per.cap	third	Female	0.18	0.862
eggcount.per.cap	fourth	Female	-2.47	2.35×10^{-2}
eggcount.per.cap	fifth	Female	-3.77	1.05×10^{-3}
eggcount.per.cap	sixth	Female	-1.86	0.073
eggcount.per.cap	seventh	Female	-1.62	0.115
eggcount.per.cap	eighth	Female	-0.27	0.788
eggcount.per.cap	ninth	Female	-2.48	2.24×10^{-2}
eggcount.per.cap	tenth	Female	-3.51	2.54×10^{-3}
eclosion.time	first	Combined	5.46	1.36×10^{-5}
eclosion.time	second	Combined	-19.45	7.49×10^{-17}
eclosion.time	third	Combined	-4.52	1.59×10^{-4}
eclosion.time	fourth	Combined	-9.79	1.11×10^{-11}
eclosion.time	fifth	Combined	-29.67	1.18×10^{-27}
eclosion.time	sixth	Combined	-13.28	5.41×10^{-14}
eclosion.time	seventh	Combined	-15.28	9.46×10^{-12}
eclosion.time	eighth	Combined	-8.03	1.65×10^{-8}
eclosion.time	ninth	Combined	1.09	0.284
eclosion.time	tenth	Combined	8.86	1.08×10^{-7}

Conclusions

Deciphering how populations respond to temporal environmental heterogeneity across seasons has long been a key interest of evolutionary biology (Dobzhansky 1955; Haldane and Jayakar 1963). Adaptation to seasonal selection pressures has been assumed to reflect that across latitudinal clines for short-lived organisms (Rhomberg and Singh 1988; Rodrigues et al. 2021). However, by performing computational analyses in chapter 1, I show that seasonal adaptation at eQTLs in *D. melanogaster* is distinct from that of clinal adaptation, which challenges the previous assumptions (Rhomberg and Singh 1988; Rodrigues et al. 2021). Such a result highlights the importance of understanding seasonal adaptation from a functional perspective. I also show seasonal adaptive signal at eQTLs is idiosyncratic across populations and is weak, suggesting the possibility that plasticity in gene expression could be an important mechanism for seasonal adaptation. Indeed, in chapter 2, I show evidence that seasonal gene expression plasticity is prevalent for flies reared in the experimental orchard. Interestingly, such seasonal plasticity in gene expression shows maladaptive signals for ecologically important traits. In addition, I show that plasticity and genetic evolution may compose different sets of genes. These findings suggest that plasticity and adaptive evolution could both be important mechanisms for fly populations to cope with seasonal environmental heterogeneity. Therefore, the theoretical prediction that transitioning from one mechanism to another likely causes a population collapse requires further testing with empirical data (Botero et al. 2015). Finally, I show that seasonal developmental temperature can elicit plastic response in fitness traits in the wild and that phenotypic line mean correlations show seasonal specific patterns in chapter 3. Such results further highlight the importance of considering the effects of plasticity when studying seasonal adaptation in wild populations. In general, my dissertation work can further advance our

understanding of seasonal adaptation from multiple levels, including genetics, gene expression, and fitness related traits.

My dissertation work can shed light on future research for a more comprehensive understanding of seasonal adaptation from the functional perspective. For example, one interesting question needs to be addressed is the functional implication of the seasonally varying SNPs (Bergland et al. 2014; Machado et al. 2021). I have shown that those seasonal SNPs are less likely to be eQTLs and that they are less likely to be associated with seasonal plastic gene expression variation. Future studies could gain insight into the functions of those seasonal SNPs by incorporating multidisciplinary approaches, such as by doing GWAS, to understand which fitness traits are affected by the seasonal SNPs. In addition, we still lack the direct evidence of whether seasonal gene expression plasticity is adaptive. Such a question can be addressed with seasonal evolution data on gene expression and measurements of fitness (Ghalambor et al. 2007; Rago et al. 2019). Finally, we still lack the understanding of the relative importance between adaptive evolution and plasticity to seasonal adaptation for short-lived organisms (Botero et al. 2015). One first step to decipher the issue could be by assessing whether plasticity and adaptive evolution compose different sets of genes and revealing the associated functions. For example, we can ask whether plastic gene expression across seasons is more likely to affect physiology or performances whereas adaptive evolution is more likely to be associated with morphological traits.

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