Drosophila melanogaster pigmentation demonstrates adaptive phenotypic parallelism but genomic unpredictability over multiple timescales

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Abstract

Populations are capable of responding to environmental change over ecological timescales via adaptive tracking. However, the translation from patterns of allele frequency change to rapid adaptation of complex traits remains unresolved. We used abdominal pigmentation in *Drosophila melanogaster* as a model phenotype to address the nature, genetic architecture, and repeatability of rapid adaptation in the field. We show that *D. melanogaster* pigmentation evolves as a highly parallel and deterministic response to shared environmental gradients across latitude and season in natural North American populations. We then experimentally evolved replicate, genetically diverse fly populations in field mesocosms to remove any confounding effects of demography and/or cryptic structure that may drive patterns in wild populations; we show that pigmentation rapidly responds, in parallel, in fewer than ten generations. Thus, pigmentation evolves concordantly in response to spatial and temporal climatic gradients. We next examined whether phenotypic differentiation was associated with allele frequency change at loci with established links to genetic variance in pigmentation in natural populations. We found that across all spatial and temporal scales, phenotypic patterns were associated with variation at pigmentation-related loci, and the sets of genes we identified in each context were largely nonoverlapping. Therefore, our findings suggest that parallel phenotypic evolution is associated with an unpredictable genomic response, with distinct components of the polygenic architecture shifting across each environmental gradient to produce redundant adaptive patterns.

Significance Statement

Shifts in global climate conditions have heightened our need to understand the dynamics and pace of adaptation in natural populations. In order to anticipate the population-level response to rapidly changing environmental conditions, we need to understand whether trait evolution is predictable over short timescales, and whether the genetic basis of adaptation is shared or distinct across multiple timescales. Here, we explored parallelism in the adaptive response of a complex phenotype, *D. melanogaster* pigmentation, to shared conditions that varied over multiple spatiotemporal scales. Our results demonstrate that while phenotypic adaptation proceeds as a predictable response to environmental gradients, even over short timescales, the genetic basis of the adaptive response is variable and nuanced across spatial and temporal contexts.

Introduction

Characterizing the genetic basis of adaptation is a central goal of evolutionary biology. To better understand how populations may respond to environmental change, it is necessary to address whether evolution is repeatable at both the phenotypic and genomic level. This question has been explored across multiple scales, from macroevolutionary patterns and broad adaptive radiations (1) to controlled laboratory experiments detailing microbial evolution (2). Examining geographic clines in phenotypic and genotypic variation represents a classic approach to studying adaptation (3), and clines are often viewed through a macroevolutionary lens (4). Yet, both the exact timescale over which local adaptation occurs, and the respective roles of selection and demography in maintaining clines, are often obscured. Therefore, to understand how populations respond to the environment over ecological timescales, recent studies have leveraged high resolution temporal sampling as a powerful approach to decode the genetic changes that may contribute to adaptive evolution in the field (5-12). However, rarely have these approaches been combined to examine adaptation across multiple scales, and integrating spatial and temporal patterns provides an opportunity to address several fundamental questions about the dynamics of adaptation in natural populations. In particular, we can test whether patterns of adaptive change are parallel across multiple environmental gradients that arise over space and time, examine how allele frequencies for putatively causal loci covary with phenotypic patterns, and explore whether the genetic variation underlying phenotypic shifts is parallel or idiosyncratic across all scales.

Our understanding of whether parallel phenotypic adaptation is underpinned by predictable allelic variation at the same loci remains incomplete (13,14). While complex traits have been documented to evolve predictably in the field (7,15-17) signatures of genomic parallelism have been detected across populations in response to environmental shifts (7,10,12,16,18,19), we have yet to resolve whether the

same genetic changes tend to repeatedly encode polygenic trait adaptation. Similar phenotypes can arise through numerous combinations of redundant alleles (20); thus, polygenic trait adaptation may be characterized by modularity rather than predictability at the genomic level, in which variation across different sets of genes drives parallel phenotypic patterns (21). However, we may expect genetic variation to map more predictably to phenotypic patterns if the trait is regulated by fewer, large-effect loci (22-24), or if the presence of epistatic relationships between alleles limits genetic redundancy (13,25,26). Therefore, to test whether adaptation is underpinned by parallel or distinct patterns of genomic evolution, we explored the evolution of a complex trait across multiple timescales in *D. melanogaster*.

Drosophila species have been a classic model for exploring evolution over ecological timescales (27). *D. melanogaster* exhibits signatures of rapid evolution across a broad array of fitness-related phenotypes (28-33). Additionally, seasonally cycling polymorphisms have been detected in populations of *D. melanogaster,* representing genomic signatures of rapid evolution (10,12,34-36), and studies have begun to integrate rapid phenotypic adaptation with underlying patterns of genetic variation in this system (32,33). We thus aimed to probe for adaptive phenotypic and genomic parallelism across multiple scales using a complex, fitness-related trait with a well-characterized genetic basis. We selected *D. melanogaster* pigmentation as our model phenotype, and our motivation for studying pigmentation was threefold: it exhibits extensive phenotypic variation in wild populations (37), it is a putatively adaptive model trait (38,39), and it has a well-studied genetic architecture (40,41).

Drosophila pigmentation varies dramatically in natural environments both across and within species (37). Latitudinal clines for *D. melanogaster* pigmentation have been described in Europe, India, and Australia, in which melanization increases with latitude (42-47). Additionally, pigmentation has been shown to increase with altitude in Sub-Saharan Africa and India (38,39,44-46,48). These repeated clinal patterns suggest that abdominal pigmentation is likely an adaptive response to environmental conditions that vary predictably over latitudinal and altitudinal gradients, such as temperature (44,49). Crucially, the pathways underlying the biosynthesis of melanin and sclerotin in each abdominal tergite are also well-characterized (50-52), and genome-wide association studies (GWAS) have identified numerous major and minor effect loci associated with pigmentation in natural populations (40,41,53).

While pigmentation is highly polygenic (40,41), the regulation of the phenotype is influenced by major-effect genes including *tan*, *ebony*, *yellow, bab1*, and *bab2* (54-58). This raises the question of whether allelic variation at major-effect loci would also underpin phenotypic variation in wild populations, as was initially expected for the loci that regulate *D. melanogaster* bristle development (59) before further studies revealed a more complex genetic basis (60). Natural variation in pigmentation has been mapped to single nucleotide polymorphisms (SNPs) in major-effect pigmentation loci (40,41). Therefore, given that segregating variants for canonical pigmentation genes exist in natural populations of *D. melanogaster*, we hypothesized that adaptive shifts in pigmentation may be driven by repeated selection at these major-effect loci, similar to variation in the *Mc1r* and *Agouti* genes driving adaptive pigmentation patterns in wild populations of mice (8,61,62). However, the canonical *Drosophila* pigmentation genes are also highly pleiotropic (63,64), which may constrain selection over ecological timescales (65). In this case, pigmentation patterns may be driven by selection across numerous variants that are less subject to pleiotropic constraints (66). Thus, if parallel pigmentation patterns can arise from many combinations of alleles, we have no expectation for the same set of genes to evolve repeatedly in distinct populations across timescales.

We studied *D. melanogaster* pigmentation in wild populations to test several fundamental hypotheses about the genetic basis of adaptation. We sought to characterize whether phenotypic and genomic evolution is predictable across multiple scales, and to identify whether timescale modulates how patterns of genetic variation map to phenotype. We first examined whether pigmentation patterns are concordant across three environmental gradients with shared conditions, as would be expected if this trait adapts predictably to drivers such as temperature. After establishing parallelism in the phenotypic response across multiple spatiotemporal scales, we then explored whether predictable shifts in known, major- or minor-effect pigmentation genes consistently underpin the phenotypic patterns observed. This enabled us to determine whether repeated phenotypic adaptation is associated with a shared genetic architecture across each spatial and temporal scale, or instead with a distinct set of alleles that are incorporated

depending on the timescale. If the latter is true, we could then explore whether the genetic variation is completely idiosyncratic across each set of populations, or if a combination of shared and independent genes contribute to repeated phenotypic adaptation in a modular fashion.

Results

Abdominal pigmentation increases with latitude in North America

We first determined whether *D. melanogaster* pigmentation varies latitudinally in North America, a previously uncharacterized transect. We predicted that melanization would increase with latitude, consistent with documented latitudinal clines for abdominal pigmentation in *D. melanogaster* on other continents (43-47). We scored female abdominal pigmentation for six populations along the East Coast of the U.S. (see Methods; Fig. 1A,D), and our results were aligned with the previously established clines: melanization increased positively with latitude in North America ($F_{5,157} = 11.17$, $p < .0001$; Fig. 2A,D; Table S1A). These parallel and independent clines across continents suggest pigmentation evolves adaptively in response to environmental conditions that vary predictably with latitude (3). However, both demography and local adaptation can drive clinal patterns, and allele frequency clines in *D. melanogaster* appear driven by both local adaptation as well as admixture (67). Therefore, we next asked whether pigmentation evolves in response to seasonal environmental change in a focal orchard, and whether seasonal and latitudinal patterns are concordant based on shared putative environmental drivers.

Pigmentation varies predictably with environmental conditions over seasonal timescales in a wild orchard population

We explored the temporal dynamics of pigmentation patterns by examining flies collected from a single focal orchard in Media, Pennsylvania, across seasons in six successive years. Here, we tested the hypothesis that pigmentation evolves in response to conditions, such as temperature, that vary in parallel across latitude and season. Additionally, we aimed to establish the timescale over which pigmentation responds to environmental gradients. We collected flies in early June and early November from 2010-2015 and scored female abdominal pigmentation patterns following laboratory, common garden treatment (Fig. 1B,D). If pigmentation evolves in response to climate-associated environmental factors, we hypothesized that early-season flies ("winter-evolved") would have darker pigmentation similar to flies from higher latitudes. Likewise, we predicted that late-season flies ("summer-evolved") would exhibit lighter pigmentation in parallel to populations originating from lower latitudes. However, the evolution of pigmentation could be constrained over short timescales, or the environmental pressures driving variation in pigmentation may not be shared across spatial and temporal axes. In this case, we would expect to see seasonal patterns that are distinct from the latitudinal patterns.

We found that *D. melanogaster* females do evolve seasonally and become significantly less melanized in the fall relative to the spring ($F_{1,148} = 174.43$, $p < .0001$; Fig. 2B,D; Table S1B), and this seasonal pattern was reset and repeated across the six years recorded. The magnitude of pigmentation change varied with year ($F_{5,148} = 11.52$, $p < .0001$), with 2014 showing a dampened response. Thus, abdominal pigmentation evolves predictably and in parallel over the roughly 15 generations that span spring (June) to fall (November) in this species (17). The directionality of the seasonal change is consistent with the latitudinal patterns observed, supporting the hypothesis that environmental factors that vary in parallel over space and time (e.g., temperature) may be the primary drivers of shifts in phenotypic patterns. While this provided further evidence that pigmentation evolves adaptively in response to environmental heterogeneity, we still could not rule out effects of migration or cryptic population structure. Therefore, we utilized experimental evolution in field mesocosms to eliminate these confounding factors and directly examine the evolutionary dynamics of pigmentation over ecological timescales.

Replicate experimental populations of *D. melanogaster* exhibit highly rapid and parallel adaptation of pigmentation in field mesocosms

We characterized seasonal patterns of abdominal pigmentation in replicate experimental populations located in Philadelphia, Pennsylvania. The populations were housed in outdoor mesocosms to isolate the role of adaptation in driving phenotypic evolution, while maintaining a semi-natural environment (10,30,32,68,69). We seeded replicate mesocosms with an outbred founder population derived from spring collections in our focal orchard (Media, PA), ensuring our starting population had a similar genetic

background to local wild populations (see Methods; Fig. 1C). We then tracked the evolution of flies across early and late season phases, collected individuals from each mesocosm population at each timepoint, and then scored female abdominal pigmentation after laboratory, common garden treatment (Fig. 1D). If the variance in pigmentation among latitudinal and seasonal populations is driven by selection, we anticipated that we would observe the same pattern we did in wild orchard populations: melanization would decrease from spring to fall in the experimental orchard, similar to the wild-caught Pennsylvania flies. In accordance with our prediction, we found late season flies were indeed less melanized relative to early season flies across our replicate mesocosms ($F_{1,8}$ = 111.26, p < .0001; Fig. 2C,D; Table S1C), showing a magnitude and directionality of phenotypic change that was concordant with the wild populations. These parallel phenotypic patterns were observed across nine independent, replicate cages, strongly supporting our hypothesis that the observed shifts in melanization are adaptive.

Together, these findings indicate that pigmentation exhibits a strikingly parallel phenotypic response to environmental conditions that vary across all timescales examined, and characterizing these repeatable adaptive patterns set the stage for us to ask whether the underlying genomic architecture is also predictable across each spatiotemporal context. Therefore, we next studied evolutionary patterns at the genomic level in our three sets of populations by examining allele frequency shifts in genes known to be associated with pigmentation variation in natural populations. Our analyses addressed whether patterns of melanization are repeatedly associated with SNPs in the same set of genes, or if distinct genetic variation for pigmentation underlies parallel phenotypic adaptation in each context.

<u>Candidate pigmentation SNPs do not show enrichment for allele frequency change over space and time</u>

We designed our genomic analyses as a targeted method to ask how known pigmentation genes vary across space and time in our populations, and this allowed us to explore whether adaptive pigmentation patterns correlate with any parallelism at the genomic level. We compiled our list based on pigmentation-associated SNPs identified by GWAS in both natural *D. melanogaster* populations and inbred lines from the Drosophila Genetic Reference Panel (DGRP) (40,41). This list included polymorphisms in canonical genes of the biosynthetic pathway of sclerotin and melanin, as well as smaller-effect loci in genes outside of this core pathway, and it totaled 249 SNPs associated with ~110 genes.

We first asked whether the candidate pigmentation SNPs were enriched as a group for latitudinal allele frequency variation relative to the genomic average. We tested for group enrichment of pigmentation SNPs by comparing their distribution to permutated distributions of control SNPs, and we looked for a significant magnitude of allele frequency change. A minimum of 200 matched control SNPs were generated for each pigmentation SNP based on a set of shared characteristics (see Methods). We determined that our candidate pigmentation SNPs were not significantly more clinal relative to matched controls (asymptotic two-sample KS test, p = .5233; Fig. 3). We then repeated these analyses for our wild (p = .5174) and experimental (p = .8231) seasonal populations in the years corresponding to our phenotypic data, and similarly found that the pigmentation SNPs were not enriched as a group for seasonal allele frequency change above what would be expected by chance (Fig. 3). These results were not entirely surprising, as it is possible that the evolution of *D. melanogaster* pigmentation is being driven by selection across few loci, similar to other species (61,70). Therefore, we next analyzed allele frequency shifts in each individual pigmentation SNP across all spatiotemporal scales.

<u>Individual pigmentation SNPs show significant allele frequency shifts across latitudinal, seasonal, and experimental populations</u>

We compiled a list of matched control SNPs for each pigmentation SNP, and we then generated a null distribution for each pigmentation SNP from the allele frequency changes of its matched SNPs. We repeated this process across all loci in our latitudinal, seasonal, and experimental populations. This enabled us to assess whether pigmentation SNPs exhibited significant allele frequency shifts relative to control SNPs. We first identified SNPs that exhibited latitudinal clinality, and they were located in *tan, bab1, bab2,* a *bab1* binding site, *TrpA1, dally,* and *dpr10* (Fig. 4; Fig. S1). We next probed for seasonally evolving loci in the populations sampled from our focal orchard in Pennsylvania, cataloging a set of SNPs that (I) evolved rapidly over the course of ~15 generations, and (II) did so in a recurrent manner across 6 years. We observed significant, repeated allele frequency shifts between early- and late-season flies at several loci, including sites within or nearby *tan,* a *bab1* binding site, *Doc1, Doc2, CG1887,* and *CG9134*

(Fig. 4; Fig. S2). Finally, we analyzed genomic patterns in our experimental orchard. Here, we probed for SNPs that exhibited parallel shifts across all replicated populations, and our experimental design eliminated demographic confounds that may be present in the wild populations. The majority of pigmentation SNPs began at a low or intermediate starting frequency (Fig. S3), and we identified a small set of loci that exhibited detectable allele frequency shifts over the course of the experiment. These SNPs rapidly changed in parallel across all 9 mesocosms in less than 15 generations, and they included *tan*, *ZnT35C*, and possible binding sites for *inv* and *chinmo* (Fig. 4; Fig. S4).

While there was some overlap in the pigmentation loci that displayed significant allele frequency shifts, including SNPs in the major-effect gene *tan*, our genomic analyses indicated that a largely distinct set of pigmentation genes were associated with pigmentation patterns across each spatial and temporal context. Additionally, some of the significant SNPs in each context showed allele frequency shifts that were countergradient in direction to the expectation based on phenotypic patterns. Together, these findings suggest that genomic evolution is occurring in an unpredictable fashion between the latitudinal, seasonal, and experimental populations to produce adaptive phenotypic parallelism.

Discussion

Here, we provide the first demonstration that abdominal pigmentation in *D. melanogaster* adapts rapidly and predictably as a response to differential environmental conditions, and we uncover shifts in known pigmentation genes that are associated with this change in phenotype. We first found that melanization increases with latitude in North America, mirroring latitudinal clines for pigmentation on other continents (43-47) and providing further evidence that observed pigmentation shifts are an adaptive response to the environment. We then explored seasonal pigmentation patterns in a wild population, and we found that flies repeatedly evolved lighter pigmentation from spring to fall over six years. We also recapitulated these seasonal patterns in replicated experimental populations in the field, in order to eliminate any effects of demography while maintaining a natural context. The shifts we observed in our experimental populations were equivalent in both magnitude and direction to the patterns we observed in the field, and they were established across nine replicate populations in less than fifteen generations. These data indicate that *D. melanogaster* pigmentation is rapidly adapting in our experimental populations, and therefore also suggest that pigmentation is under selection in wild populations as a response to temperature or other ecological factors that co-vary with latitude and season. Thus, we found that pigmentation demonstrated concordant and highly predictable adaptive patterns across all spatiotemporal scales.

An important caveat to our phenotypic findings is that the effects of *Drosophila* pigmentation on fitness have not been established in natural populations; it is possible that pigmentation may be under indirect selection due to correlations with other traits (71), rather than direct selection on the degree of melanization itself. Thermoregulation, UV tolerance, desiccation tolerance, cuticle strength, and wound repair have been posited as fitness benefits associated with *Drosophila* pigmentation (49,72,73). However, opposite coloration patterns are observed across various species inhabiting regions with similar biotic and abiotic conditions (37), which obscures the adaptive significance of pigmentation phenotypes. Future experiments will examine if pigmentation is under direct selection by (I) manipulating the putative causative agents of selection (e.g., temperature) and observing consequent effects on pigmentation in our experimental orchard, and (II) testing genetic correlations of pigmentation and other fitness-related traits using a quantitative genetics framework.

After characterizing shifts in melanization, we then asked whether SNPs associated with pigmentation exhibited similarly predictable variation across contexts. The high levels of phenotypic parallelism we observed had raised the possibility that adaptive patterns would be coupled with clear and consistent allele frequency differences at major effect pigmentation loci across all timescales. This would mirror the genetic basis of pigmentation variation in natural populations across several taxa, which also experience selection at key loci for pigmentation biosynthesis (6,8,61,62,70,74-77). However, we found that nonoverlapping sets of loci were associated with pigmentation patterns at the latitudinal, seasonal, and experimental scales. These results suggest that numerous genes contribute to *D. melanogaster* melanization in an unpredictable and context-dependent fashion. While we did observe significant allele frequency shifts in the canonical *Drosophila* pigmentation genes *tan* and *bab1* in multiple contexts (Fig.

4), the magnitude of these shifts over short timescales appears insufficient to fully account for phenotypic evolution (Fig. S2; Fig. S4). Additionally, we did not identify significant patterns for alleles of other genes involved in pigmentation biosynthesis. This includes the gene *ebony*, which is known to be associated with altitudinal variation for melanization (38).

Therefore, our results suggest that allelic variation at major effect genes does not predictably underpin differences in pigmentation across wild populations of *D. melanogaster*. Many of the major-effect *Drosophila* pigmentation genes are highly pleiotropic (55,58,78-83), so we may not observe consistent variation in canonical genes due to pleiotropic constraints (84). Instead, we found that adaptive pigmentation patterns are likely driven by the combined effects of many loci, consistent with an omnigenic model of trait evolution (85). Interestingly, we detected significant shifts at a few low frequency alleles (Fig. S1; Fig. S2; Fig. S4), suggesting that rare alleles may contribute to phenotypic patterns in the field. This finding aligns with a growing body of work that indicates rare alleles play a key role in determining complex trait variation (86). We also observed that much of the allele frequency variation we measured over both space and time exhibited counter-gradient patterns, in which allele frequency differences did not match phenotypes observed (Fig. S1; Fig. S2; Fig S4). Counter-gradient patterns have been previously reported for numerous genetic polymorphisms in *D. melanogaster* (87-90), and while these findings contrast the simple prediction that all allele frequency shifts would be concordant with phenotypic gradients, they highlight the nuanced genetic basis of polygenic traits. Interactions among multiple combinations of alleles can produce redundant phenotypic patterns, and this can generate allele frequency shifts that conflict in directionality with phenotypic clines (91). Additionally, epistatic interactions strongly influence the effects of individual SNPs on quantitative trait variation across genetic backgrounds in *D. melanogaster* (92); thus, epistasis may modulate the effects of a given allele on phenotype across contexts (93,94). Altogether, our results underscore the complex genetic basis of *D. melanogaster* pigmentation, and future work can directly test the functional effects of variant alleles on adaptive patterns using reverse genetic techniques.

In conclusion, our findings illustrate that *D. melanogaster* abdominal pigmentation evolves as a deterministic response to predictable shifts in environmental conditions that vary over both space and time. Yet, while strikingly parallel pigmentation patterns arose across all spatiotemporal scales, the underlying associations between genotype and phenotype were distinct across all datasets, and the identification of causal variants may be increasingly difficult over shorter timescales. Therefore, our findings demonstrate that adaptive phenotypic patterns are produced in a predictable fashion over all timescales analyzed, but the genetic basis of complex trait evolution remains context dependent. This study contributes to our growing understanding of whether evolution is predictable in natural populations, and our findings will better inform our understanding of the mechanisms through which populations adapt to rapid environmental change.

Materials and Methods

Fly collection and maintenance

Wild orchard populations

D. melanogaster individuals were collected from six orchard populations located along the east coast of the United States by either direct aspiration or bait and sweep net methods. Gravid females were immediately sorted into isofemale lines in the field; once the resulting progeny eclosed, lines were typed to species. Populations sampled were: Lancaster, MA (42.45N, 71.87W); Media, PA (39.88N, 76.30W); Charlottesville, VA (38.02N, 77.47W); Athens, GA (33.95N, 83.66W); Jacksonville, FL (30.20N, 81.66W); and Homestead, FL (25.28N, 80.45W). Seasonal collections were done in Media, PA (39.88N, 76.30W) over six years (2010, 2011, 2012, 2013, 2014, and 2015). Early season collections were made over the first week of June, and late season collections were completed in early November (17,29,95). Long-term maintenance of all populations was done at 24°C, 12:12 light:dark photoperiod, with a generation time of approximately 21 days. Flies were maintained on standard cornmeal-molasses-agar medium.

Experimental orchard populations

We established an outbred founder population by pooling 10 virgin males and 10 females from density-controlled cultures of 80 inbred lines, and then expanding/recombining for four generations in the

laboratory (25°C, 12L:12D photoperiod). The inbred lines were derived from individuals collected from Linvilla Orchards in Media, PA, USA, on June 1, 2012. Wild-caught flies were inbred for 20 generations in laboratory conditions (25°C, 12:12 photoperiod) before being fully sequenced (SRA BioProject PRJNA722305). Constructing our outbred founder population by recombining these inbred lines enables us to use haplotype inference to yield high effective sequencing coverage for our experimental populations, as described by Rudman et al. (10).

On June 30, 2016, we founded 10 replicate, outdoor mesocosms with 500 males and 500 females from our outbred founder population. Each $8m^3$ mesocosm contains a dwarf peach tree, shelving units for *D. melanogaster* culture, and vegetative ground cover to provide a semi-natural environment for fly rearing. The experiment was conducted from June 30 to October 5, 2016, and each population was provided with 1 liter of standard cornmeal-molasses medium once per week. To assess evolutionary patterns, *D. melanogaster* eggs were sampled from each mesocosm at three timepoints (August 5, September 5, and October 5). DNA was sequenced at all three timepoints, and flies were preserved for pigmentation measurements at two timepoints (Aug. 5 and Oct. 5). 100 females were pooled from each mesocosm to conduct whole genome sequencing and analyses (using the same DNA extraction, sequencing and analysis pipelines as described in Rudman et al. (10) and Bitter et al. (12)). To measure evolved pigmentation patterns at each timepoint, additional flies sampled from each mesocosm were reared for 2 generations in laboratory, common garden conditions (25°C, 12L:12D photoperiod). To complete phenotyping for pigmentation, 5 day old F3 individuals corresponding to each mesocosm and timepoint were stored in 80% EtOH at -80°C for long term storage.

Scoring fly samples for abdominal pigmentation

Wild orchard populations

Abdominal pigmentation was scored in female flies using a lateral view of the abdomen, giving values from 0 (0% of the tergite is melanized) to 10 (100% of the tergite is melanized) for each of the seven abdominal tergites (1st –7th) as described by David et al. (96) (Fig. 1). Pigmentation scores were calculated by summing the value ('1' to '10') assigned to each tergite. To measure latitudinal patterns, a total of 17-30 isofemale lines derived from each population were selected, and ten individual females per line were scored for body pigmentation. To measure seasonal patterns, a total of 10-20 isofemale lines from Media, PA, for each year were scored for body pigmentation at early and late season timepoints. Five to ten individual females per line were scored for body pigmentation (5 individuals when 20 isofemale lines were used in 2010-2013, vs. 10 individuals when 10 isofemale lines were used in 2014-2015). We then calculated the mean pigmentation score of individuals for each isofemale line across seasons and years. Statistical analysis of latitudinal pigmentation patterns was assessed in R (v. 4.4.0) with a linear mixed effects model using the package 'nlme' (v. 3.1.164). Here, latitude was included in the model as a fixed effect and isofemale line was included as a random effect, according to the formula: lme(Pigmentation Scores ~ Latitude, random=~1|Isofemale Line). Similarly for the seasonal (Media, PA) populations, we examined pigmentation patterns with a linear mixed effects model where year, season (early or late), and their interaction were modeled as fixed effects, and isofemale line was included as a random effect: lme(Pigmentation Scores ~ Year*Season, random=~1|Isofemale Line). In this case, pigmentation scores were the mean scores of each isofemale line.

Experimental orchard populations

Abdominal pigmentation of *D. melanogaster* females was scored using the rubric described above (96). However, only the three most distal tergites were scored (#5-7), as they show the highest degree of variation and are sufficient in capturing pigmentation differences between populations. Pigmentation scores were calculated by summing the value assigned to each tergite. To obtain an average pigmentation score for the ancestral founder population, we scored 4 replicates of 20 females sampled from our founder population. For the August 5 and October 5 field-evolved timepoints, 20 individual females from each outdoor mesocosm were randomly selected and scored from the F3 samples stored at -80°C; we then calculated the mean pigmentation score across the individuals scored from each mesocosm. One of our initial 10 mesocosms was lost ("S2"), so we scored pigmentation patterns for 9 out of the original 10 mesocosms. We ran a linear mixed effects model in R (using the package 'nlme', v. 3.1.164) for pigmentation scores in each mesocosm between the summer and fall timepoints. Here, timepoint was included as a fixed effect and was modeled as an ordered categorical variable, and

mesocosm was included as a random effect nested within timepoint. We used the formula: lme(Pigmentation Scores ~ Timepoint, random=~1|Mesocosm/Timepoint). We included scores from our mid-season (Aug. 5) and late season (Oct. 5) timepoints in this model, but we excluded the founder population because we did not score pigmentation for the subsetted founder flies that seeded each cage.

Genomic Analyses of Sequencing Data

Selection of candidate SNPs

We based our candidate SNP selection on two previous GWAS studies of abdominal pigmentation (40,41). The studies were performed in different populations: Bastide et al. (40) collected wild samples in Austria and Italy, and Dembeck et al. (41) used the inbred lines of the Drosophila Genetic Reference Panel (DGRP). These GWAS also focused on different tergites: Bastide et al. looked at the pigmentation of the 7th abdominal tergite, and Dembeck et al. focused on the 5th and the 6th and the relationship between them. Since our pigmentation scoring considered the 5th, 6th, and 7th tergites, we included all significant SNPs from both studies (a total of 249 SNPs associated with 111 genes). We based our SNP coordinates on the *D. melanogaster* Release 5 (dm3) coordinate system, which required us to convert the SNP coordinates in the latitudinal and seasonal genomic datasets from Release 6 back to Release 5. From the initial paper describing Release 6 of the *D. melanogaster* genome (97), we downloaded the associated FlyBase file (http://flybase.org/reports/FBrf0225389.htm) that maps Release 5 to Release 6 and wrote a pipeline that would determine in which region a Release 5 SNP was located. We then applied the corresponding base pair location shift for that region in Release 6.

Calculating a metric of latitudinal clinality of candidate SNPs

To measure the degree of clinality for each SNP, we performed a linear regression (using the function 'lm' in R) on the SNPs' frequencies across latitudes. Our metric for clinality was the absolute value of the regression coefficient (the slope) of the resulting model. The latitudinal samples we analyzed were collected from Homestead, FL (2010), Atlanta, GA (2014), Charlottesville, VA (2014), Media, PA (2014) and Lancaster, MA (2014); however, we did not have genomic data available to include in our analyses for Jacksonville, FL. The genomic data for these samples was sourced from the DEST dataset (98), and the corresponding sample IDs were: "FL_ho_10_spring", "GA_at_14_spring", "VA_ch_14_spring", "PA_li_14_spring", and "MA_la_14_spring." We excluded any SNPs that were present in less than 3 of the 5 samples from this portion of the analysis.

Calculating a metric of seasonality of candidate SNPs in wild orchard populations

In considering a metric for seasonality, we determined that the allele frequency change from spring to fall and the repeatability of this shift were the key components. Therefore, we considered the periods where both a spring and a fall sample existed in a given year, and we calculated the magnitude of the allele frequency change between seasons. We then took the average across these spring-fall transitions to get the yearly seasonal change. The seasonal samples were all collected in Linvilla, PA between the years 2009-2015 (DEST dataset, (98)), and the corresponding sample IDs were: "PA_li_09_spring", "PA_li_09_fall", "PA_li_10_spring", "PA_li_10_fall", "PA_li_11_spring", "PA_li_11_fall", "PA_li_12_spring", "PA_li_12_fall", "PA_li_14_spring", "PA_li_14_fall", "PA_li_15_spring", and "PA_li_15_fall." Notably, we did not measure pigmentation patterns during the 2009 season, so this additional year is only represented in our genomic analyses; additionally, our genomic analyses did not include samples from 2013, which were included in our phenotypic analyses. We excluded SNPs with data in fewer than 3 out of a possible 6 spring-fall pairs.

Calculating a metric of seasonality of candidate SNPs in outdoor mesocosm populations

For experimental seasonal measurements, we performed a linear regression of the allele frequencies across the three timepoints collected during 2016. While we only had pigmentation measurements available for the August 5 and October 5 timepoints to include in our phenotypic analyses, DNA was sequenced from an additional timepoint (September 5); therefore, we included this additional timepoint in our genomic analysis. We designated the absolute value of the regression coefficient as the metric for seasonal change – in this broad analysis, we were looking for SNPs that changed in a large, concordant direction throughout the seasons. Given that there were only 3 timepoints, we excluded any SNPs with missing data in any of the samples.

Selection of matched SNPs

In order to establish a null model of the expected clinal and seasonal patterns of candidate SNPs, we selected a group of at least 200 matched SNPs for each of our candidate SNPs. Matched SNPs were always located on the same chromosome and further than 50KB from the candidate SNPs. Matched SNPs were in the same type of genomic region (exon, 5'UTR/3'UTR, intron, gene, or intergenic, in order of priority) and had the same relationship to the 6 major inversions that occur in wild North American populations (either in an inversion, within 500KB of a breakpoint, or further away than 500KB). After matching on all the above characteristics, SNPs with an average frequency across all samples within 0.01 of the average frequency of the candidate SNP were chosen as the matched SNP pool for that candidate SNP. If fewer than 200 SNPs were in the pool for a candidate SNP, we increased the possible difference in frequency until the pool reached at least 200 SNPs. However, no matched SNP had a frequency difference of more than 0.07 from its candidate SNP.

Tests of overall clinality and seasonality of the entire set of candidate SNPs

To determine the clinality and seasonality of candidate SNP frequency changes as a group, we leveraged these matched SNPs to create 1000 permuted groups equal in size to the number of candidate SNPs that passed the minimum threshold for each metric. Each matched group included one matched SNP drawn randomly from the pool of each candidate SNP with sufficient data. The averages of the clinal or seasonal metrics within each of the 1000 matched groups provided a null distribution of 1000 values, which was used to calculate the *p*-value of the average of the candidate group. By creating these bootstrap resamples that still matched the properties of the original candidate SNPs, we were able to determine if the candidate SNPs as a group were more likely to follow the clinal and seasonal patterns observed in the pigmentation phenotype, given enrichment for a significant magnitude of absolute change.

Tests of clinality and seasonality of individual candidate SNPs

To test if individual SNPs varied significantly more latitudinally or seasonally than expected, we returned to our pool of matched SNPs. For each candidate SNP, we drew a sample of 100 matched SNPs from the pool, and we repeated this 100 times. For each selection of 100 matched SNPs, we determined the proportion of matched SNPs with a metric greater than that of the candidate SNP. We considered a SNP significant if it was within the top 10% of matched SNPs in at least 90 trials and within the top 5% in at least half of the 100 trials, providing a significance cutoff of $\alpha=0.05 \pm 0.05$.

Data analysis and figures

All phenotypic and genomic analyses were conducted in R (version 4.4.0) using the packages tidyverse (version 2.2.0), reshape2 (version 1.1.4), readxl (version 1.4.3), and nlme (version 3.1.164). Figures were made using Keynote (version 8.1) and R (version 4.4.0) using the packages ggplot2 (version 3.5.1), emmeans (version 1.10.2), and plotrix (version 3.8.4), and Fig. 1 was illustrated by Dr. Rush Dhillon (https://www.rushstudio.ca/). Latitudinal and seasonal genomic data can be accessed via the DEST dataset, v.1 (98). Sequencing data for the mesocosm experiment used in this study, as well as raw data and scripts used in analyses, will be made available upon publication.

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Figures

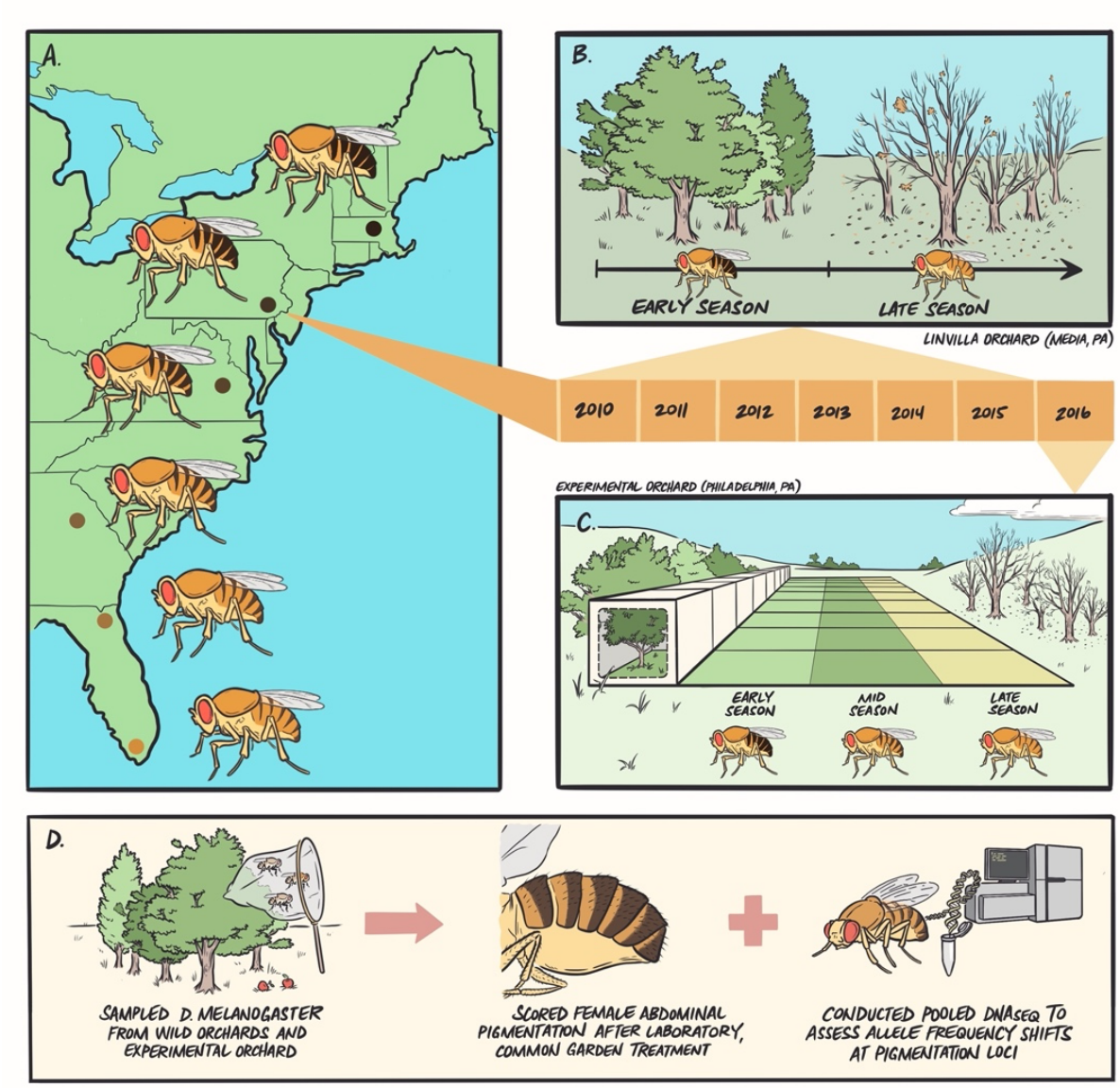

Fig. 1. Experimental overview. (A) We sampled flies from six wild orchard populations ranging from Homestead, FL, to Lancaster, MA, and established isofemale lines in the laboratory. (B) We returned to a focal orchard in Media, PA, at early- and late-season timepoints and collected flies to capture evolutionary patterns following winter and summer conditions. (C) We then seeded outdoor mesocosms (N=9) with an outbred population originating from early-season collections in Media, PA, and sampled flies at the end of summer (mid-season) and fall (late-season) to determine if seasonal patterns are recapitulated in experimental populations controlled for migration, drift, and cryptic population structure. (D) Across each wild or experimental context, we sampled flies, established lines in the lab, completed common garden treatment to remove environmental effects, and scored females for abdominal pigmentation. We also conducted pooled DNA sequencing on additional flies sampled from each population to map genomic patterns for candidate pigmentation SNPs.

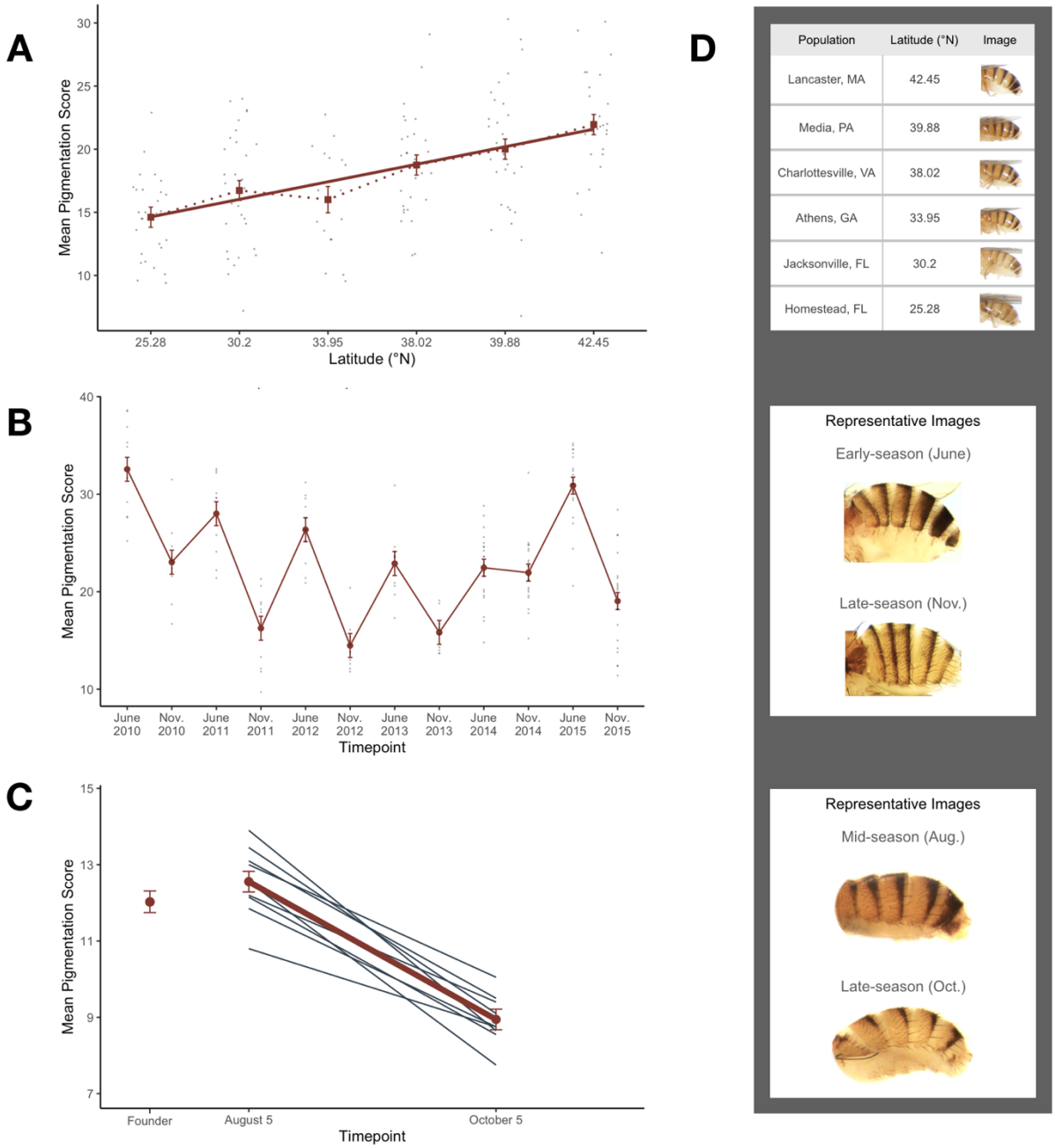

Fig. 2. Phenotypic patterns and representative images for *D. melanogaster* populations in each natural context. (A) *D. melanogaster* pigmentation increases significantly with latitude. (B) A population of flies sampled from Media, PA, exhibit predictable and significant decreases in melanization from June to November over six years. (C) Decreases in pigmentation over seasonal time are recapitulated in semi-natural mesocosms (N=9) located in Philadelphia, PA, and female flies adapt to exhibit significantly lighter pigmentation between August 5th and October 5th. The average pigmentation score of our spring-derived ancestral population ("Founder") is also pictured. In all plots, mean pigmentation score and standard error are depicted alongside either points representing isofemale line means (A,B) or individual mesocosm means (C). (D) Representative images corresponding to each population (from top to bottom): across latitudes, across seasons in wild populations, and across seasons in experimental populations.

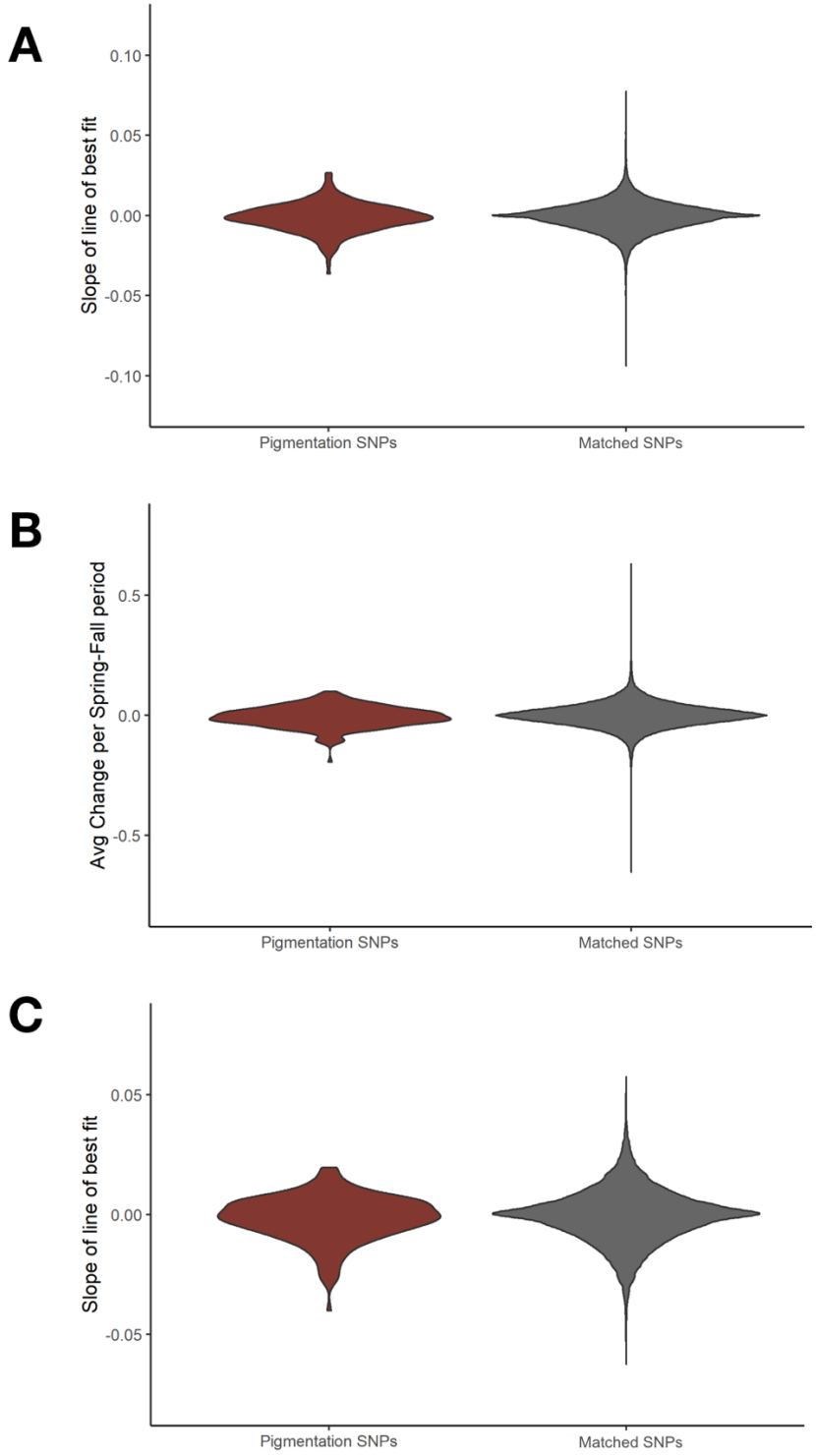

Fig. 3. Distributions of candidate pigmentation SNPs vs. bootstrap resamples of matched SNPs over space and time. Candidate pigmentation SNPs (maroon) are not enriched as a group relative to matched controls (gray) across (A) latitudinal, (B) seasonal, or (C) experimental populations. Candidate SNPs were selected based on associations with pigmentation from GWAS of natural populations, and enrichment of candidate pigmentation SNPs was determined relative to groups of matched, control SNPs.

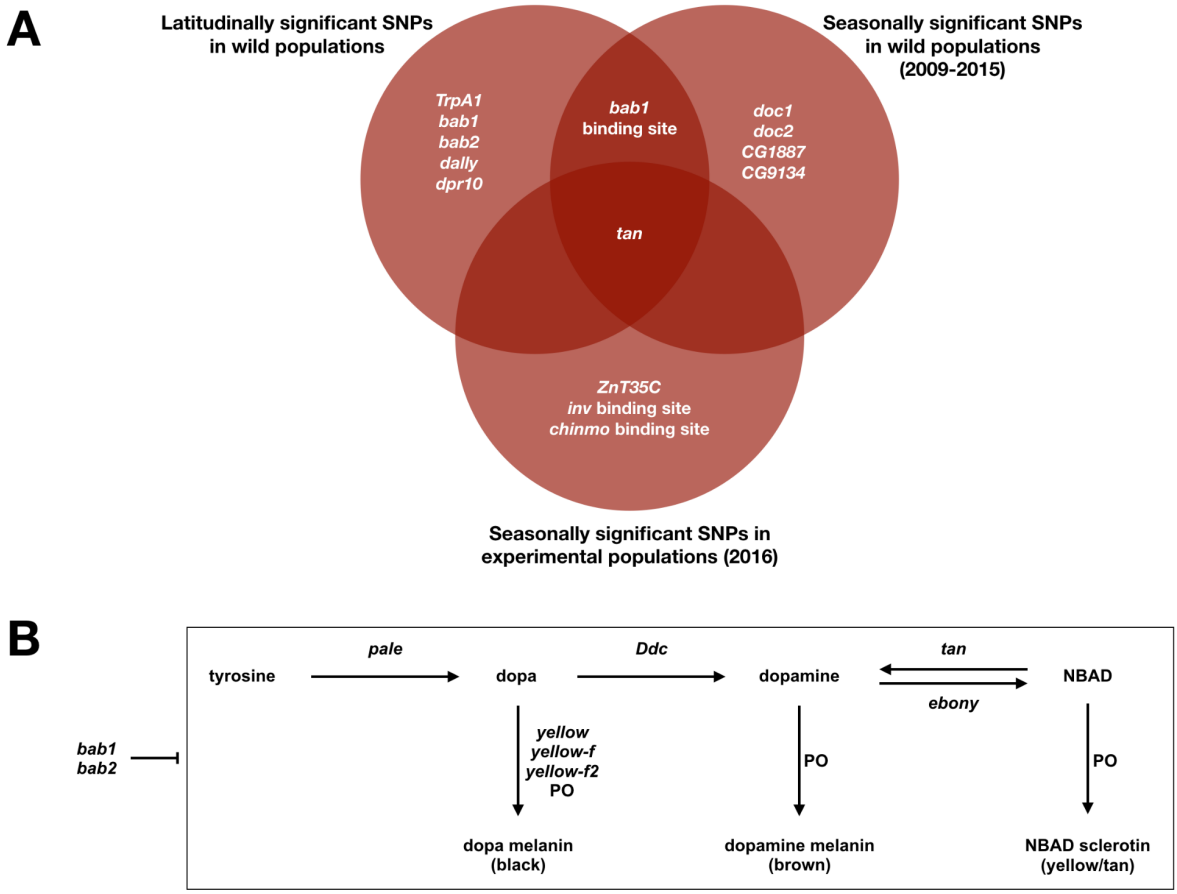

Fig. 4. Genes harboring SNPs with significant allele frequency shifts over latitude, seasons in wild populations, and seasons in experimental populations. (A) Diagram of pigmentation genes containing significant SNPs from populations in each natural context. Significance of candidate pigmentation SNPs was determined relative to matched SNPs, and concordant genes across contexts are displayed. The directionality of allele frequency shifts was co-gradient with observed phenotypic patterns for some SNPs, while other SNPs had countergradient allele frequency shifts. (B) Simplified biosynthetic pathway displaying the canonical pigmentation genes, as well as the relationship between *bab1* and *bab2* to the pathway. *Bab* modulates the pathway by repressing *tan* and *yellow* (99,100), well as *Ddc* (101).

Supplemental Materials

Table S1. Summary table of linear mixed effects model statistics for phenotypic patterns. (A) We determined whether pigmentation varies significantly over latitude by running a linear mixed effects model using the function lme (R package 'nlme', v. 3.1.164). Here, latitude was the fixed effect and isofemale line was included as a random effect. (B) To examine whether pigmentation shifts significantly across seasons in the wild populations (Media, PA), we similarly ran a linear mixed effects model where year, season, and their interaction were fixed effects, and isofemale line was a random effect. (C) Finally, we probed for significant pigmentation patterns across timepoints in our experimental orchard (Philadelphia, PA) by running a linear mixed effects model where timepoint was the fixed effect and mesocosm was a random effect nested within timepoint.

A

Experiment: latitudinal populations
Formula: Pigmentation Scores ~ Latitude, random=~1|Isofemale Line

	numDF	denDF	*F*-value	*p*-value
(Intercept)	1	157	2909.3350	< .0001
Latitude	5	157	11.1707	< .0001

B

Experiment: seasonal wild populations
Formula: Pigmentation Scores ~ Year*Season, random=~1|Isofemale Line

	numDF	denDF	*F*-value	*p*-value
(Intercept)	1	148	5606.354	< .0001
Year	5	148	13.931	< .0001
Season	1	148	174.433	< .0001
Year:Season	5	148	11.517	< .0001

C

Experiment: seasonal experimental populations
Formula: Pigmentation Scores ~ Timepoint, random=~1|Mesocosm/Timepoint

	numDF	denDF	*F*-value	*p*-value
(Intercept)	1	8	2658.3068	< .0001
Timepoint	1	8	111.2574	< .0001

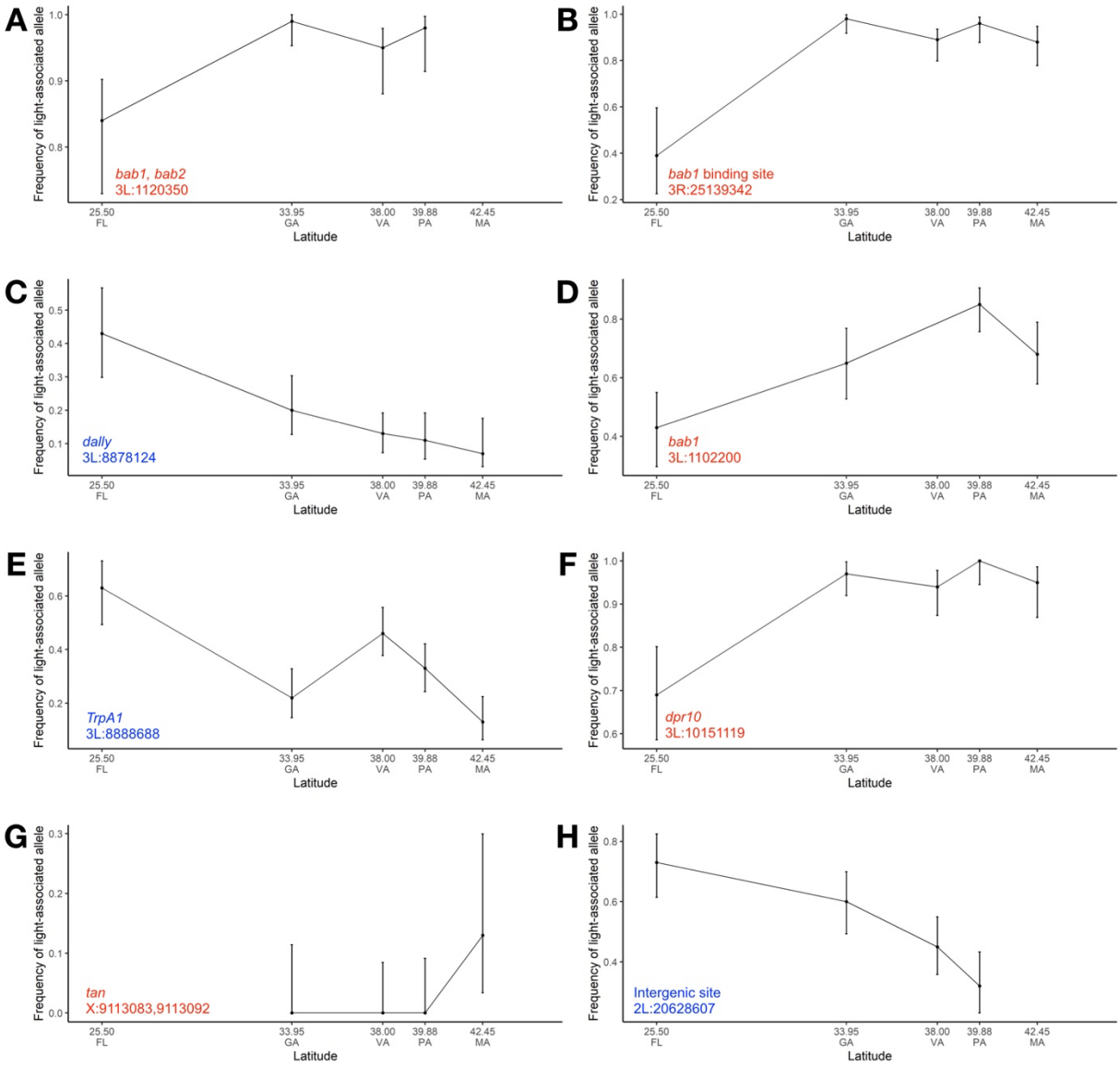

Figure S1. Individual pigmentation SNPs with significant allele frequency shifts across the North American latitudinal cline. Significant SNPs were identified by performing a linear regression of the allele frequencies across latitudes, and then determining if the regression slope for each candidate pigmentation SNP was in the tail of the null distribution created from the slopes of its matched SNPs. The allele frequency of the light-associated allele across latitudes is plotted to illustrate latitudinal differences in SNP frequencies, and error bars represent the 80% confidence interval. Some allele frequency clines were concordant with phenotypic clines (blue text), while other SNPs demonstrated reverse clines (red text).

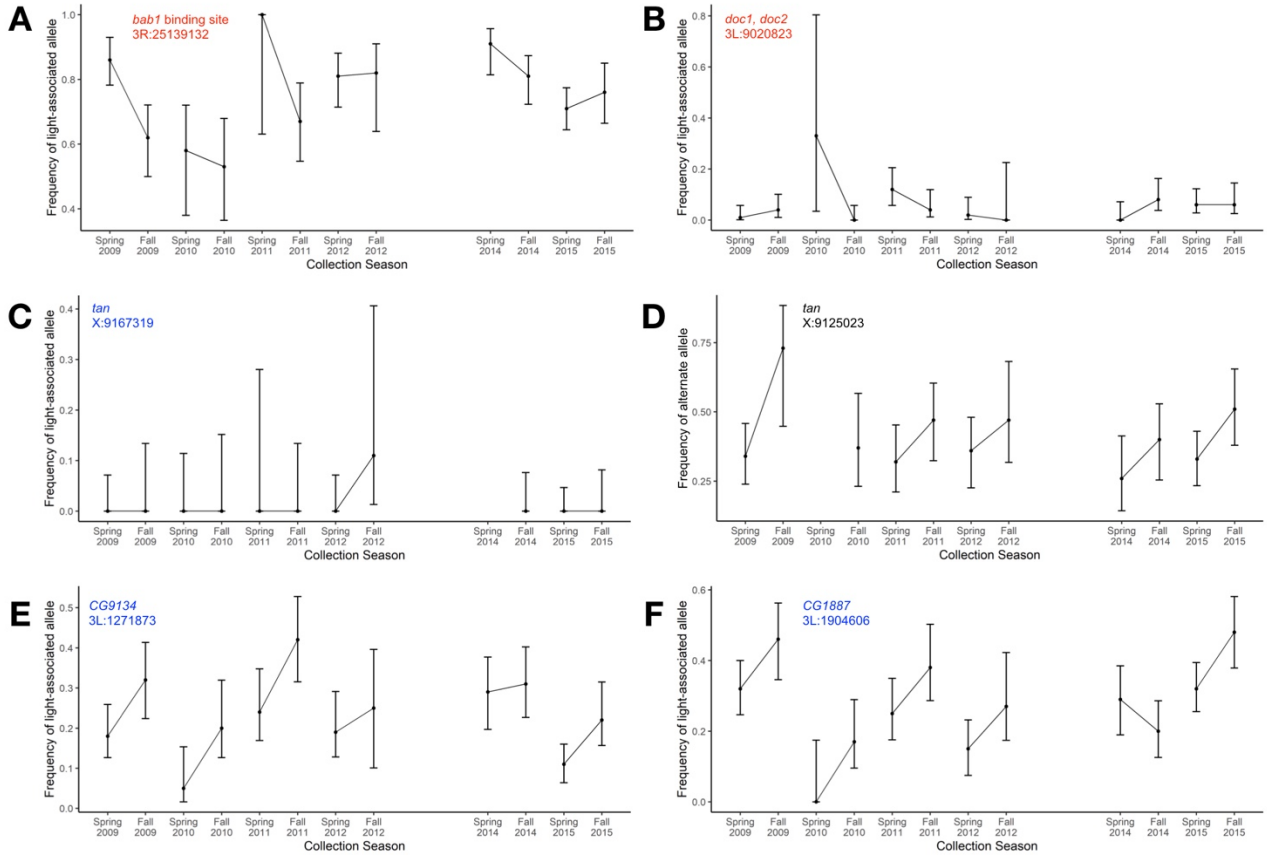

Figure S2. Individual pigmentation SNPs with significant allele frequency fluctuations from spring to fall across multiple years. We determined whether repeated allele frequency shifts were significant by calculating the allele frequency changes from spring to fall in each year and determining the average shift per year for each SNP. We then measured whether the shifts for each candidate pigmentation SNP were in the tail of the null distribution generated from their matched SNPs. The allele frequency of the light-associated allele is plotted across collection seasons to show parallel shifts in SNP frequencies across seasons in multiple years, and error bars represent the 80% confidence interval. Some SNPs exhibited more consistent patterns than others across years, and SNPs showed both co-gradient (blue text) and counter-gradient (red text) patterns with the phenotypic patterns observed. The directionality for one of the *tan* SNPs (X:9125023) was unspecified in the Bastide et al. (2013) dataset (black text).

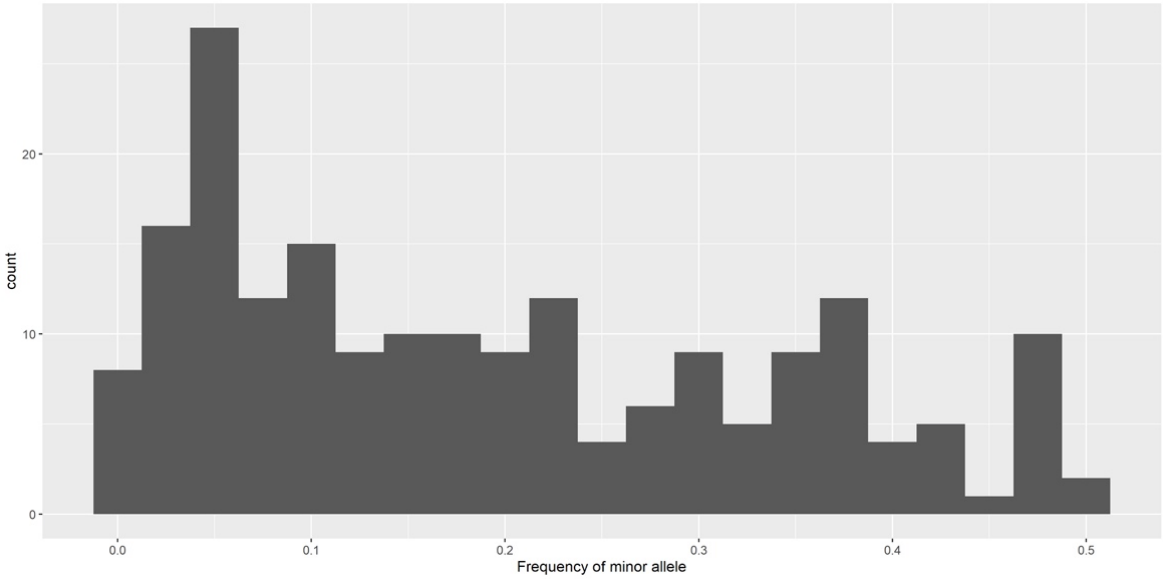

Figure S3. Folded site frequency spectrum displaying the starting frequency of pigmentation SNP alternate alleles in the experimental orchard. The majority of pigmentation SNPs had low or intermediate minor allele frequencies in the baseline population for the experimental orchard, with the largest proportion of initial frequencies falling between 0 and 0.1.

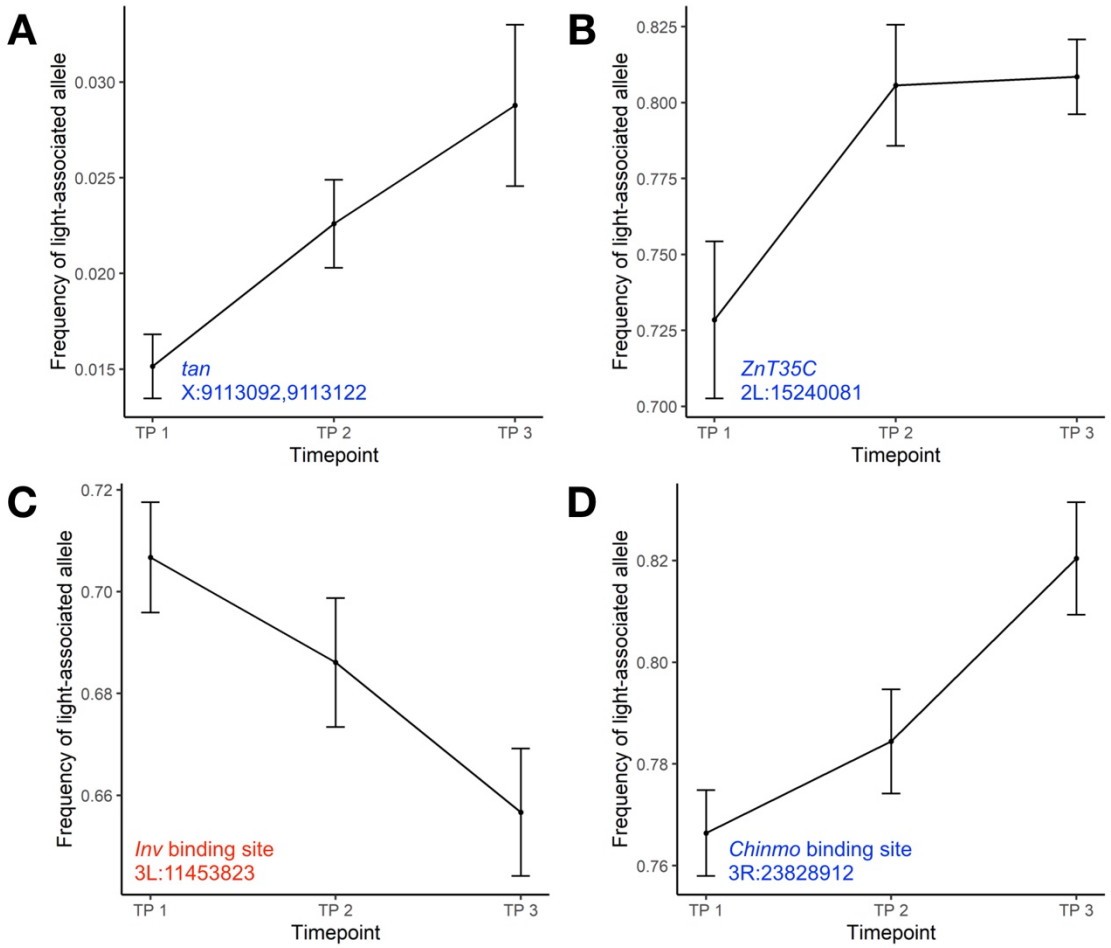

Figure S4. Individual pigmentation SNPs with significant allele frequency shifts across seasons in replicate experimental orchard populations. Significance was determined by first performing a linear regression of the allele frequencies across timepoints, and then identifying if the regression slope for the candidate SNP was in the tail of the null distribution created from the slopes of the matched SNPs. The allele frequency and standard error of the light-associated allele is plotted here to show the movement of SNP frequencies over time. The directionality of allele frequency shifts was concordant with observed phenotypic patterns for some SNPs (blue text), while other SNPs had countergradient allele frequency shifts (red text). (N=10 cages. TP1=Aug. 5, TP2=Sept. 5, TP3=Oct. 5).