DISSERTATION

# ADAPTATION TO NEW AND CHANGING ENVIRONMENTS:

# FROM EXPERIMENTS TO FIELD STUDIES

Submitted by

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# ABSTRACT

## ADAPTATION TO NEW AND CHANGING ENVIRONMENTS: FROM EXPERIMENTS TO FIELD STUDIES

In today's rapidly changing world, organisms are often exposed to new or challenging environments. Following environmental change, populations may experience one or more of the following outcomes: they may disperse to a new habitat, persist through phenotypic plasticity or adaptive evolution, or go extinct. Here, I focus on adaptive evolution as a mechanism for enabling populations to respond to different types of environmental change. Adaptation may be necessary in less mobile species, or in fragmented landscapes where dispersal corridors are not available. Insects serve as excellent models for studying the evolutionary dynamics of populations, as their life cycles are relatively short and multiple generations can occur within a few years, enabling rapid adaptation. In this dissertation, I explore the impacts of a change in phenology (Chapter 1), a challenging environment (Chapter 2), and the presence of a mountain range (Chapter 3) on the adaptation of insect populations in both the laboratory (Chapters 1-2) and in the field (Chapter 3).

To begin, I studied the effects of a phenology shift using experimental evolution. Climate change can affect the length and timing of seasons, which in turn can alter the time available for insects to complete their life cycles and successfully reproduce. Intraspecific hybridization between individuals from genetically distinct populations, or admixture, can boost fitness in populations experiencing environmental challenges. Admixture can particularly benefit small and isolated populations that may have high genetic load by masking deleterious alleles, thereby immediately increasing fitness, and by increasing the genetic variation available for

adaptive evolution. To evaluate the effects of admixture on populations exposed to a novel life cycle constraint, I used the red flour beetle (*Tribolium castaneum*) as a model system. Distinct laboratory lineages were kept isolated or mixed together to create populations containing 1 to 4 lineages. I then compared the fitness of admixed populations to 1-lineage populations while subjecting them to a shortened generation time. After an initial decline in fitness in the new environment, the admixed populations demonstrated significantly greater fitness compared to the 1-lineage populations after three generations. The timing of the increase in fitness suggests that adaptation to the novel environmental constraint occurred, as opposed to the masking of deleterious alleles.

In Chapter 2, I examined the effects of consistent immigration on the probability and timing of adaptation. Theory predicts that immigration can delay extinction and provide novel genetic material that can prevent inbreeding depression and facilitate adaptation. However, when potential source populations have not experienced the new environment before (i.e., are naive), immigration can counteract selection and constrain adaptation. This study evaluated the effects of immigration of naive individuals on adaptation in experimental populations of red flour beetles. Small populations were exposed to a challenging environment, and three immigration rates (0, 1, or 5 migrants per generation) were implemented with migrants from a benign environment. Following an initial decline in population size across all treatments, populations receiving no immigration gained a higher growth rate one generation earlier than those with immigration, illustrating the constraining effects of immigration on adaptation. After seven generations, a reciprocal transplant experiment found evidence for adaptation regardless of immigration rate. Thus, while the immigration of naive individuals briefly delayed adaptation, it did not increase extinction risk or prevent adaptation following environmental change.

For Chapter 3, I shifted my focus from laboratory populations to natural populations. In nature, elevation gradients provide a way to study populations across a range of environmental conditions within relatively small spatial scales. Adaptation to the local habitat might occur in

response to the unique selection pressures present at different elevations, resulting in distinct populations adapted to different environments. However, if there is high habitat connectivity, gene flow can slow or prevent adaptation while also maintaining genetic variation and large population sizes. In this chapter, I used genomics to investigate the interplay between selection and gene flow in butterfly populations collected from high (>2,500m) and low (<2,000m) elevations, both east and west of the Rocky Mountains. My study focused on the Rocky Mountain subspecies of the clouded sulfur butterfly (*Colias philodice eriphyle* Edwards). Weak, but statistically significant, patterns of population differentiation were apparent between butterflies collected east and west of the Rockies, and eastern butterflies harbored greater genetic diversity compared to those from the west. Additionally, $F_{ST}$ values close to zero suggest gene flow was high among the collection sites. I used a redundancy analysis to show that the observed east-west differentiation may be largely driven by greater precipitation east of the Rockies, and I identified over 16,000 putatively adaptive loci and 3,000 candidate genes associated with the environmental variables that may underly adaptive traits.

In summary, my dissertation research highlights that adaptation can occur with genetic mixing or immigration in less than 10 generations in the lab (Chapters 1-2) and that adaptation to different environments can be identified among well-connected populations in the field (Chapter 3). It is well documented that gene flow can help maintain genetic variation, particularly among fragmented populations. My results emphasize that gene flow does not impede natural selection (Chapters 2-3), and that mixing between distinct populations can even promote adaptation (Chapter 1). My findings, therefore, support the use of methods such as translocation and wildlife corridors to facilitate gene flow for sexually reproducing conservation targets.

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Lastly, thank you to my family for their unwavering support all of these years: to my moms, Ellen Durkee and Denise Cunningham, my sister June Durkee, and my partner Jake Jarník. None of this would be possible without you. To Jake, for being my everything: adventure partner, paddling buddy, butterfly catcher – you have been there every step of the way, and I could not imagine doing it all with anyone else.

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# CHAPTER 1: GENETIC MIXING FACILITATES ADAPTATION TO A NOVEL ENVIRONMENTAL CONSTRAINT[1]

## Introduction

Rapid adaptive evolution can enable plant and animal populations to persist through environmental change (Bell, 2013; Carlson et al., 2014). Given the strong selection pressures imposed by human actions in natural environments, a practical understanding of the factors that facilitate adaptation can help natural resource managers maintain healthy populations (Gonzalez et al., 2013). One process that has been shown to promote an increase in fitness in novel environments is admixture, or intraspecific hybridization between individuals from genetically distinct populations (Verhoeven et al., 2011).

Admixture tends to be most beneficial to small and isolated populations (Hardie & Hutchings, 2010), in which genetic drift, mutation, and inbreeding can lead to the loss of genetic variation and increase the frequency of deleterious alleles. Such populations are said to harbor high genetic load, meaning their fitness is reduced due to maladaptive or deleterious alleles relative to an ideal population without those alleles (Hedrick & Garcia-Dorado, 2016). Admixture can increase population fitness through two mechanisms: (1) it can directly and immediately increase fitness by reducing genetic load (Whiteley et al., 2015) and (2) it can increase genetic variation and contribute beneficial alleles to the population, which enhances the potential for adaptive evolution in response to natural selection over time (Agashe et al., 2011; Barrett & Schluter, 2008). However, admixture may also result in reduced fitness (i.e., outbreeding

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[1]Adapted from: Durkee, LF, Olazcuaga, L, Szymanski, R, Melbourne, BA & Hufbauer, RA (2023). Genetic mixing facilitates adaptation to a novel environmental constraint. *Ecological Entomology,* 48(4), 517-522. https://doi.org/10.1111/een.13242

depression) if the admixed populations are adapted to different environments or genetic incompatibilities exist (Lenormand, 2002).

Here, we evaluated the influence of admixture on populations exposed to a shortened and constrained generation time. Climate change and urbanization have altered the timing and length of seasons that have conditions suitable for the survival and reproduction of many organisms. These shifts in seasonality often require organisms to evolve life history traits that make use of favorable seasons, including shorter generation times (Bradshaw & Holzapfel, 2008, 2010). For our experiment, we used the red flour beetle (*Tribolium castaneum*) (Herbst) as a model system similarly to Hufbauer et al. (2015). We abruptly transitioned beetle populations from an unregulated life cycle with overlapping generations to non-overlapping, 5-week generations, with reproduction confined to a short period each generation. We created experimental populations with four levels of admixture, or combinations of one to four lineages. These populations evolved for three discrete generations to allow us to address the following questions: (1) How does the level of admixture affect fitness in the novel environment? (2) What mechanisms (reduction of genetic load, addition of genetic variation available for adaptation, or some combination) might underlie fitness differences among populations? By examining the effects of the level of admixture on population fitness for multiple generations, we can improve our understanding of how increased or restricted admixture might influence wild insect populations exposed to changes in phenology.

## Methods

*Colony maintenance*

We began with five lineages of *T. castaneum* ("Lab-S Rusty," "Estill," "Ga-1," "Z-2," and "Z-4") from a USDA stock center (Kansas, USA). These lineages were chosen because they were wild-type phenotypes originally collected from a relatively limited geographic range (midwestern and southeastern U.S.) and were therefore unlikely to have reproductive incompatibilities that can occur over large geographic distances, for example, across continents (Drury et al., 2011; Thomson et al., 1995). At the stock center, beetles were reared without gene flow and with continuous and overlapping generations, thus without constraints on the timing of reproduction. Upon arrival at our laboratory, the *T. castaneum* lineages were kept separately in small 4x4x6cm boxes, hereafter patches, containing 15g of standard medium (95% wheat flour, 5% brewer's yeast) and reared for a single generation prior to the start of the experiment.

*Experiment initiation and propagation*

To initiate the founding generation (G0), new experimental populations were created by outcrossing among the original lineages (Figure 1.1b: G0; Table A-1). Here, we define a population as a group of interbreeding individuals. Thus, 56 populations were initiated consisting of four different admixture levels (1, 2, 3, or 4 lineages; 14 populations each) across three temporal blocks. Across blocks, there was partial overlap of the genetic background of individuals used to create populations (Table A-1).

Each experimental population was founded with 39 or 40 adult beetles: 1-lineage populations contained 40 adults from a single lineage; 2-lineage populations contained 20 adults each from 2 lineages; 3-lineage populations contained 13 individuals from 3 lineages; and 4-lineage populations contained 10 individuals from 4 lineages. The beetles used to initiate these populations were about 5 weeks old (adults had eclosed within the week prior) and

individuals were chosen without regard to their sex. Leaving sex ratio to chance can lead to variation in offspring number and the amount of admixture between populations, which may reduce our ability to detect differences among admixture levels. However, a study by Wade (1984) found that the apparent population size in laboratory colonies of *T. castaneum* was representative of the effective number of breeding adults in small populations (10 or 20 individuals with varying sex ratios). This suggests that approximately equal numbers of each lineage reproduced in the initial generation in our experiment (Wade, 1984).

In the experimental populations made up of 1-4 lineages, the founding adults were allowed to mate with other individuals in their patch and oviposit for 24 hours. Adults were then discarded to allow the offspring to develop for another 34 days (Figure 1.1a). Thus, the generation time was constrained to 35 days in total. The abrupt shift from continuous generations to this strict life cycle regime, with oviposition occurring at 5 weeks old, likely imposed strong selection on these populations. Five weeks was used as a life cycle length to be comparable to other eco-evolutionary studies that use *T. castaneum* as a model organism in laboratory and mathematical modelling studies, for example, Hufbauer et al. (2015) (lab) and Melbourne and Hastings (2008) (lab and modelling).

With this design, the founding individuals eclosed as adults within their own lineage groups, so they were able to mate with other individuals within their lineage prior to founding the experimental populations. Therefore, the amount of outcrossing within populations containing multiple lineages in G0 was not known. However, two factors suggest that at least some offspring were the product of outcrossing: (1) inbreeding, such as was likely in long-term maintenance of lineages at the stock center, promotes increased mating frequency in female *T. castaneum* (Michalczyk et al., 2011), and (2) multiple mating is common in this species both within (Pai et al., 2007) and between lineages (Nilsson et al., 2003; Pai & Yan, 2002), with females mating with up to 12 males per hour (Pai et al., 2007).

To increase the sizes of our experimental populations and provide additional opportunities for outcrossing after the G0 mixing event, adults were allowed 72 hours for mating and oviposition, and an additional 34 days to develop in generation 1 (Figure 1.1b: G1). Thus, the generation time was constrained to 35 days in G0 and 37 days in G1. Generations 2 (G2) and 3 (G3) continued to constrain generation time, returning to a 24-hour reproductive period and 34 days for development. Adult beetles were censused at the end of G2 and G3 (Figure 1.1b). Based on the number of adults available per population and to increase replication, G1 was initiated with 20 adults, G2 with 40, and G3 with 20 (Table A-1). Individuals from each population were kept in 1-10 replicate patches in G2 and 1-20 patches in G3, depending on the size of the population.

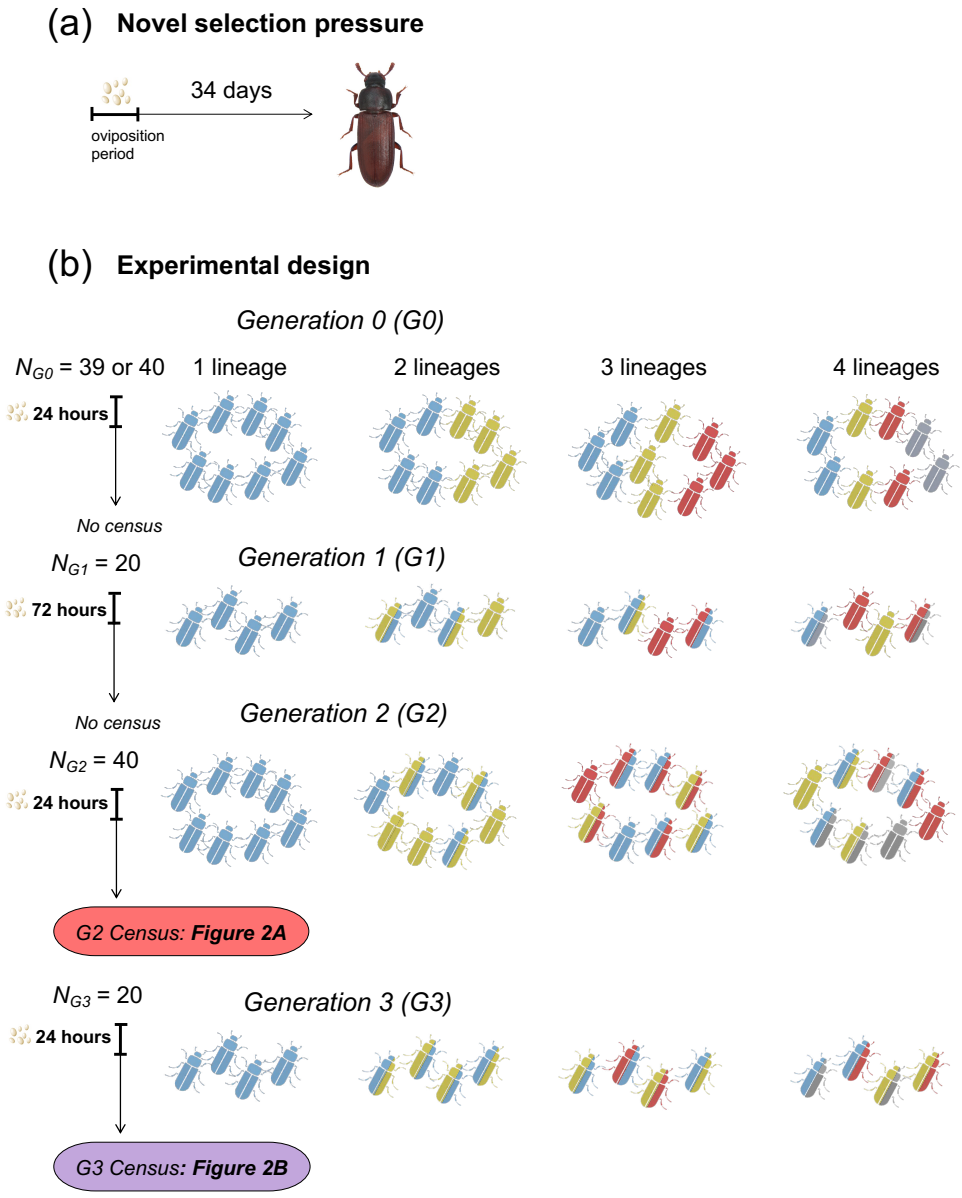


**Figure 1.1 (a)** The generation time and reproductive constraints imposed on the *Tribolium castaneum* populations in the experiment. **(b)** Experimental design showing how the four treatment groups were established through admixture over three generations. In generation 0 (G0), the 8 or 9 pictured beetles represent the 40 or 39 adults, respectively, that initiated each population. In G1, the 4 beetles represent the 20 adults that initiated the generation, in G2 the 8 beetles represent 40 adults, and in G3 the 4 beetles again represent 20. Lower density was used in G1 and G3 to enable replication even when fewer adults were available. The color changes represent admixture occurring, which likely increased over time. Photo by Tomasz Klejdysz/Shutterstock.com.

*Measuring fitness and statistical analysis*

We estimated fitness using population growth rate: the mean number of surviving adult offspring per individual, that is, population size at census ($N_t$) divided by the initial population size ($N_{t-1}$) or $\frac{N_t}{N_{t-1}}$. Fitness data were analyzed using two linear mixed effects models, one for each generation given the different values of $N_t$, and each were square root transformed for analysis. The models included the number of lineages per population (admixture level) as a fixed effect and temporal block and population identity (nested within block) as random effects. All analyses were performed in R (R Core Team, 2021) using the *lme4* (Bates et al., 2015) and *emmeans* packages (Lenth, 2021). Residual versus fitted values and quantile-quantile plots of residuals were used to check that the assumptions of equal variance and normality were satisfied, respectively.

## Results

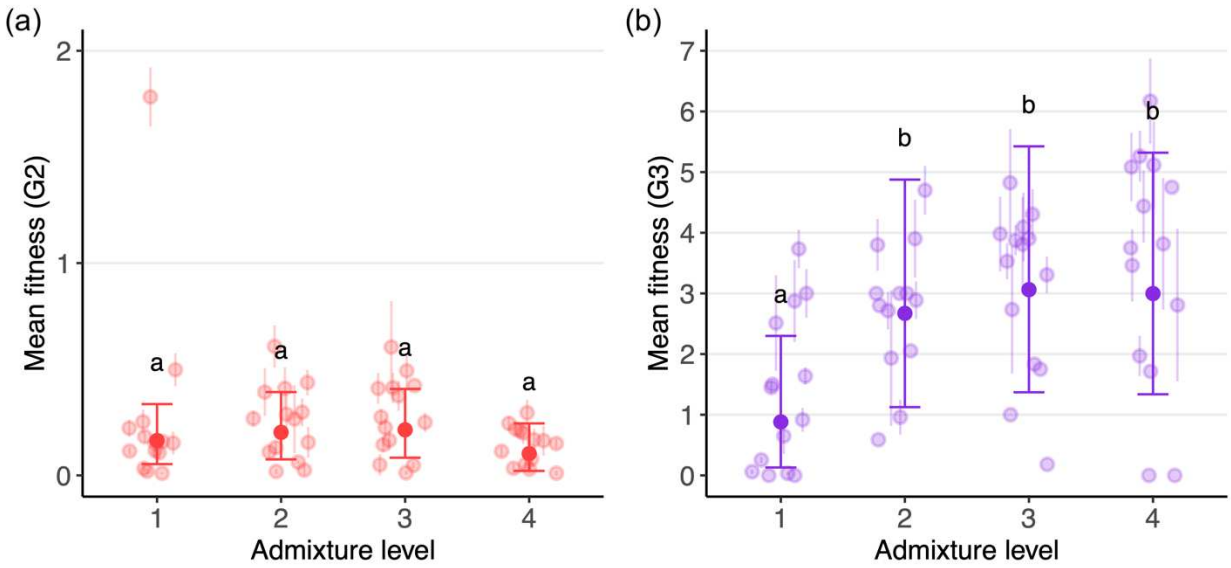


**FIGURE 1.2** Mean fitness among populations with different admixture levels in **(a)** generation 2 (G2; red points), which was initiated with 40 individuals, and **(b)** generation 3 (G3; purple points), which was initiated with 20 individuals. Each lighter point represents a population ±1 standard error generated from replicate patches (see Table A-1). Points are jittered for clarity. Solid points are overall means from the fitted model for each admixture level with 95% model-generated confidence intervals. Letters represent significant differences at $p < 0.05$ after controlling for multiple comparisons (Tukey) within each generation. Note the different scales of *Y*-axes.

In generation 2 (Figure 1.2a), population fitness was not significantly different between admixture levels (Figure 1.2a; ANOVA: $F_{3,49.94} = 1.24$; $p = 0.305$).

In generation 3 (Figure 1.2b), the pattern was strikingly different, with admixture level significantly influencing fitness (Figure 1.2b; ANOVA: $F_{3,48.86} = 7.92$; $p < 0.001$). The 1-lineage populations had the lowest mean fitness of 0.88 (95% confidence interval [CI] [0.13, 2.30]) when compared to the 2-4 lineage populations (2.67, 95% CI [1.13, 4.88]; 3.06, [1.37, 5.42]; 3.00, [1.34, 5.32], respectively for admixture levels 2-4), which were comparable regardless of the number of lineages (Figure 1.2b).

The apparent overall difference in fitness between G2 and G3 should not be over interpreted given the difference in adult density (40 vs. 20) that initiated the populations in these generations.

## Discussion

This study evaluated the impacts of admixture on population fitness under a shortened and constrained generation time, a life history shift that can be beneficial to insect populations experiencing changes in seasonality due to rapid climate change (Bradshaw & Holzapfel, 2010). Our results indicate that admixture (admixed vs. 1-lineage populations) increased fitness in populations exposed to this novel constraint in only three generations. Furthermore, we found that there was not a clear effect of increased admixture (2- vs. 3- vs. 4-lineage populations) on fitness, as the 2-lineage populations performed similarly to the 3- and 4-lineage populations in G3. This suggests that even a low degree of outcrossing can facilitate population persistence in novel environments. These findings agree with other studies in model systems, including *Arabidopsis* (Crawford & Whitney, 2010), *Daphnia* (Loria et al., 2022), and other work with *Tribolium* (Agashe et al., 2011). Mean differences in fitness between G2 and G3 are likely due to negative density dependence primarily from egg cannibalism (Melbourne & Hastings, 2008), which had greater effects in G2 (initiated with 40 adult beetles) compared to G3 (initiated with half as many individuals).

We considered two mechanisms that can lead to increased fitness after admixture events: reduced genetic load and increased genetic variation for adaptation. In G2, all populations had statistically comparable fitness regardless of admixture level, suggesting that alleviation of genetic load did not occur in this generation. One possibility is that because we started with stock lineages that were likely highly inbred, deleterious alleles in those populations

could have been purged prior to the start of the experiment. While purging of lethal alleles likely did occur, mildly deleterious mutations were likely to have been maintained. At the stock center, the lineages were kept under benign conditions with adequate resources and little selection on development time, a setting that would allow mildly deleterious mutations to segregate, as well as alleles only deleterious in a more stressful environment (Fox & Reed, 2011). Results from a separate study (Olazcuaga et al., 2023) suggest that this is the case. Their experiment aimed to compare admixed populations, originally created by crossing the same *T. castaneum* lineages used in this experiment, that had experienced a bottleneck or not during their demographic history. After shifting them from a benign to a challenging environment, they found that fitness was lower in the bottlenecked populations than in the admixed populations (Olazcuaga et al., 2023, summarized in Figure A-1). This implies that there are segregated deleterious alleles in their admixed populations, and thus the lineages used in this experiment likely harbored some genetic load.

Here, we would expect fitness increases in admixed populations due to a reduction in genetic load to be apparent by G2, after two generations of outcrossing, which has been observed in animal populations in the field (Whiteley et al., 2015) and in *T. castaneum* populations in the lab (Stewart et al., 2017). However, fitness changes were not observed until G3, when we observed increased fitness in admixed populations relative to 1-lineage populations (Figure 1.2b). The three-generation time lag in the increase in fitness suggests that the dominant process was rapid adaptation to the shortened generation time, which occurred because selection for oviposition at 5 weeks of age acted on the increased genetic variation in the admixed populations. The relief of genetic load might have contributed to fitness increases as well, similar to the results of Hufbauer et al. (2015), who showed that adaptation and the reduction in inbreeding depression together increased fitness in declining populations.

Overall, this study provides experimental evidence for the benefits of admixture with as few as two lineages for promoting adaptation to a novel environmental constraint. More broadly,

when thinking about biological invasions, our findings support limiting admixture via multiple introductions to mitigate the range expansion of invasive populations (Ellstrand & Schierenbeck, 2000). When applied to conservation, our findings support facilitating admixture for isolated populations to increase the genetic variation available for adaptation in novel environments imposed by climate change (Kardos et al., 2021; Whiteley et al., 2015).

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# CHAPTER 2: IMMIGRATION DELAYS BUT DOES NOT PREVENT ADAPTATION FOLLOWING ENVIRONMENTAL CHANGE: EXPERIMENTAL EVIDENCE[2]

## Introduction

A critical goal of conservation biology is to establish how to effectively manage populations in human-altered habitats. Environmental changes such as pollution (Loria et al., 2019), deforestation (Foster et al., 2021), and fragmentation (Cheptou et al., 2017) can reduce habitat quality and connectivity, which can decrease the average fitness of residing populations. If fitness drops below the replacement rate, populations will decline in size and will approach extinction if the downward trend persists. Immigration, either through the maintenance of dispersal corridors or translocations, is one approach readily available to conservation practitioners to support declining populations, particularly in fragmented landscapes (Frankham et al., 2019).

Theoretical work within the conservation literature recommends an immigration rate of 1 to 10 migrants per generation for isolated populations (Frankham et al., 2019; Mills & Allendorf, 1996). More specifically, theory suggests a rate of one migrant per generation to offset population divergence and loss of heterozygosity that occurs through drift (Mills & Allendorf, 1996) and five migrants per generation to prevent damaging inbreeding depression and maintain adaptive genetic variation in changing environments (Frankham et al., 2019). Ideally, immigration can occur consistently through dispersal corridors, and if translocations are needed, consistent immigration should be sustained. However, these recommendations have been

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[2]Durkee, LF, Olazcuaga, L, Melbourne, BA & Hufbauer, RA (2024). Immigration delays but does not prevent adaptation following environmental change: experimental evidence. *Journal of Evolutionary Biology*, 37(6), 665-676. https://doi.org/10.1093/jeb/voae031

tested only partially. Laboratory experiments have demonstrated the benefits of immigration when comparing isolated populations to populations that received a single immigration event under scenarios of inbreeding (Spielman & Frankham, 1992) or low-diversity populations facing environmental change (Hufbauer et al., 2015). Only one study we know of, Newman and Tallmon (2001), tested immigration that occurred every generation. They assessed rates of 1 or 2.5 migrants per generation in recently isolated mustard plant populations and found that the rate of one migrant per generation was sufficient to maintain genetic variation and decrease inbreeding when compared to isolated populations (Newman & Tallmon, 2001). In this era of global change, it is important to also test these recommendations under more severe environmental conditions.

A separate but related body of literature focuses on how populations declining due to exposure to challenging environments can avoid extinction through adaptive evolution, a process called evolutionary rescue (Carlson et al., 2014; Gomulkiewicz & Holt, 1995). Examples include adaptation in weed or insect populations to pesticides, antibiotic resistance in bacteria, or invertebrate adaptation to metal pollution in streams (Bell, 2017). Experiments show that immigration or admixture can enhance the probability of evolutionary rescue when populations are exposed to a challenging environment (Durkee et al., 2023; Hufbauer et al., 2015). Furthermore, theoretical studies show that the probability of rescue can increase with immigration, primarily due to the added genetic variation, which increases the chance of beneficial alleles being present (Czuppon et al., 2021; Tomasini & Peischl, 2020). To contrast, other theoretical studies show that gene flow from maladapted immigrants can limit adaptation by swamping adaptive alleles in the recipient population, reducing fitness (i.e., outbreeding depression) (Brady et al., 2019; Lenormand, 2002; Slatkin, 1987).

To reduce the likelihood of outbreeding depression, using a source population from a habitat similar to the recipient population (i.e., habitat matching) is recommended by models (Edelaar & Bolnick, 2012) and in the context of conservation (Frankham et al., 2019). However,

this is often not possible, particularly under scenarios of novel environmental change (Aitken & Whitlock, 2013) or when the species of concern is rare (Frankham et al., 2019). Even when migrants are not adapted to their new habitat, for example, when individuals from a geographically distant population (e.g., Hedrick & Fredrickson, 2010) or from captivity (e.g., Crone et al., 2007) are used, every-generation immigration of 1 to 5 individuals is meant to be low enough to minimize outbreeding depression while still maintaining genetic diversity and adaptive potential of populations (Frankham et al., 2019; Newman & Tallmon, 2001). However, to our knowledge, no study has compared the recommended immigration rates of one and five migrants per generation and their impacts on adaptation in the context of severe environmental change.

Here, we evaluated how immigration rates recommended in the conservation literature (Frankham et al., 2019) impacted evolutionary rescue (Carlson et al., 2014). To do this, we first created experimental populations of red flour beetles (*Tribolium castaneum*) from a genetically admixed stock population (Durkee et al., 2023). We then introduced them to a challenging environment to mimic a situation where a large, diverse population experiences sudden habitat change that decreases population size and reduces habitat quality, for example, when forests are logged (Hillers et al., 2008). Each experimental population received either zero, one, or five migrants every generation from a large source population not adapted to the new environment. We observed the effects of this immigration on population persistence and growth. In habitats like these that have limited space and degraded resources, population recovery may be constrained by density-dependent processes like competition for food and space (Nordstrom et al., 2023; Osmond & de Mazancourt, 2013). Thus, we evaluated both density-dependent and density-independent population growth through time to tease apart the impacts of negative density dependence and adaptation. Our experiment was designed to address the following question: In populations experiencing severe environmental change, how do different

immigration rates affect (1) population size and fitness over time, (2) the timing of evolutionary rescue, and (3) adaptation to the challenging environment?

## Materials and methods

To evaluate the question posed in the introduction, we carried out an experiment where we subjected replicated, independent populations to a challenging environment that caused them to decline in size. This environment, therefore, would have led to extinction unless the populations were able to adapt sufficiently to the challenging conditions. Hereafter, we refer to this as the rescue experiment (Figure 2.1a and b).

*Rescue experiment initiation and propagation*

We created a genetically diverse *T. castaneum* stock population comprised of lineages described in Durkee et al. (2023). The stock population had been reared on standard media (19:1 ratio of wheat flour to brewer's yeast) and in standard environmental conditions (31 °C and 50%-80% humidity) in 4x4x6cm patches for nine, 35-day non-overlapping generations prior to the start of the rescue experiment. To start, we created 25 independent populations from the stock population comprised of 300 individuals each (Figure 2.1a). These populations were used to initiate replicated experimental populations and maintain populations to serve as a source of migrants (hereafter, source populations). These populations were divided at the start of the rescue experiment and therefore represent newly isolated populations with no history of divergence, similar to Newman and Tallmon (2001).

**Experimental populations** were each initiated with 50 adult beetles, 36 days old, and population sizes were allowed to fluctuate naturally in subsequent generations. Each population was placed onto a single patch of the challenging environment created using media containing

the pyrethroid insecticide deltamethrin (DeltaDust, Bayer). This event represents a sudden environmental change that reduced fitness and created replicated, isolated populations. The experimental patches each contained a concentration of deltamethrin that a dose-response curve created from preliminary experiments (Figure B-1) indicated would decrease the population growth rate to approximately 0.40 from a typical value of around 2. Without adaptation, this reduced growth rate should have led to extinction in 10 generations (based on simulations with the stochastic Ricker model developed for *T. castaneum* by Melbourne and Hastings, 2008). The experimental populations ($N_0$ = 50) were initiated in 25 groups of three, each group from one of the original 25 independent populations (Figure 2.1a).

**Source populations** were initiated with 150 adults, 36 days old, and each served as a source of migrants for a group of three experimental populations (Figure 2.1a). Thus, 25 source populations were initiated in total, each from one of the original 25 populations. We intended for the sources to represent larger populations, and so we used the large population size ($N$ = 150) from Hufbauer et al. (2015). Sources were maintained at 150 adults every generation, regardless of the number of offspring produced, to approximate populations with a carrying capacity of 150 individuals. Source populations were each maintained on a single patch containing standard dry media, a benign environment for *T. castaneum*. Thus, these individuals were not exposed to the challenging environment present in the experimental patches. Hereafter, we refer to these individuals as naive migrants, to refer to both evolutionary and behavioral naivety.

The adult beetles in the experimental and source patches were given 24 hours to mate and oviposit. Adults were then discarded, and the eggs developed for an additional 34 days. The first census event ($N_1$, Figure 2.1b) occurred on day 35. Subsequently, one of three immigration treatments was applied to each experimental population in every group: 0, 1, or 5 migrants. The migrants were moved from the source to each experimental patch manually using a paintbrush. Immigration occurred at the start of the following oviposition period. This

procedure (immigration, 24-hr oviposition, removal of adults, 34-day development period, census) was repeated each subsequent generation. The experiment was terminated after seven generations because nearly all the experimental populations had either gone extinct or their growth rates and population sizes had increased, indicative of evolutionary rescue (Figure 2.2).

In summary, there were 25 groups, each consisting of one source and three experimental populations (one of each immigration treatment). Thus, there were 25 replicates of each treatment, which we split across three temporal blocks (eight groups in blocks 1 and 2, nine in block 3). Within each group, treatments were set up sequentially and propagated for the first four generations in the following order to minimize opportunities for experimental error: 1-migrant, 5-migrant, and then the 0-migrant populations. As this was not random, we tested if this order mattered in a follow-up experiment and found no significant effect of treatment order on growth rate in the following generation (Figure B-2). During generations 4-7, the experimental protocol was well established, and so we randomized the order of set-up.

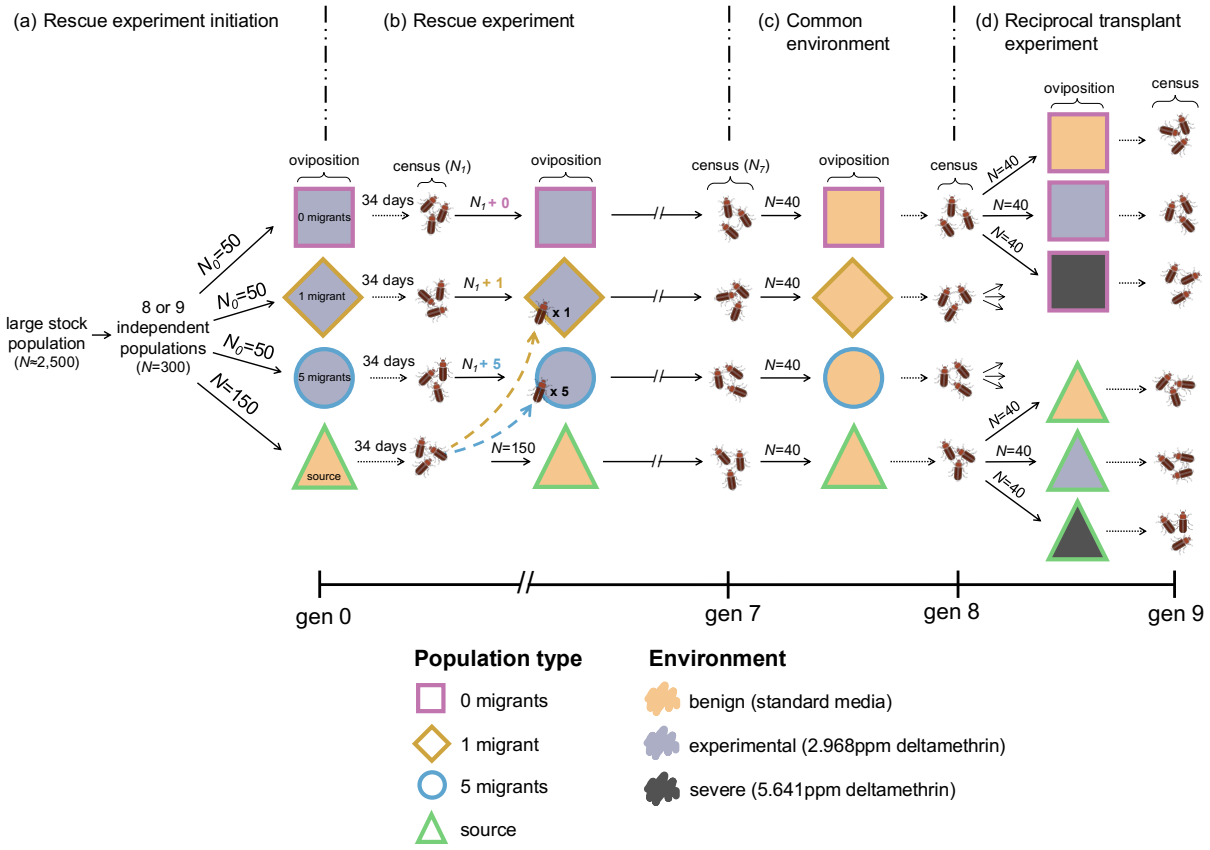


**FIGURE 2.1** Experimental design for the rescue, common environment, and reciprocal transplant experiments. **(a)** We began with a large stock population ($N \approx 2{,}500$), from which eight or nine independent populations were initiated ($N = 300$). Each of these populations was split into one source population, initiated and then maintained at $N = 150$ individuals, and three experimental populations, initiated with $N_0 = 50$ individuals, with population size allowed to fluctuate thereafter. The source populations were maintained in a benign environment (tan shading), and the experimental populations were maintained in a challenging environment containing the insecticide deltamethrin (light grey shading). **(b)** In the rescue experiment, census events (e.g., $N_1$ and $N_7$) are indicated with three cartoon beetles, and census occurred every generation on day 35, following a 24-hr oviposition period and 34 days of development (indicated with dotted arrows). Immigration from the source to the experimental populations (dashed arrows) occurred after each census event at the start of the oviposition period, and three treatments were implemented: 0 migrants (purple square outlines), 1 migrant (yellow diamond outlines), and 5 migrants per generation (blue circle outlines). **(c)** All extant populations were placed in a benign, common environment for a single generation following the census in generation 7 at a density of $N = 40$, with as many replicates created as possible. **(d)** In the reciprocal transplant experiment, all populations were split into groups of $N = 40$ and placed simultaneously and at constant densities in three different environments: benign, challenging, and severe (dark grey shading). This whole procedure (a-d) was repeated across three temporal blocks.

*Source population dispersal arrays*

The immigration recommendations we followed (one or five migrants per generation) are based on either natural dispersal or translocation. Thus, in order to approximate natural dispersal and make our results applicable to either case, we used migrants with higher demonstrated dispersal ability to represent individuals that would be more likely to leave the source patch and move to a new patch in the absence of translocation (Cote et al., 2022; Edelaar & Bolnick, 2012). Dispersal arrays were created to select dispersing individuals. On day 34, during generations 1-6, habitat patches housing source populations were each connected to a three-patch dispersal array with 2 mm holes, creating a four-patch dispersal array. Hereafter, we refer to the original source patch as patch 1 and the subsequent patches, in order, as patches 2-4, which contained media that had been previously used to rear beetles for 35 days to keep resource availability consistent throughout the array. Adult beetles were given 24 hours to disperse out of patch 1. On day 35, all adults were censused in each of the four patches of the dispersal array (Figure B-3). Individuals that dispersed to patches 2-4 were used as migrants (hereafter called dispersing migrants), with preference given to individuals that dispersed to the furthest patch (i.e., individuals were first chosen from patch 4, then patch 3, then 2).

In order to maintain the population size of the source populations at 150 to start every generation, growth rates needed to be above the replacement rate. However, growth rates were sometimes lower than expected, which likely occurred due to the effects of demographic stochasticity, which strongly influences the *T. castaneum* system (Melbourne & Hastings, 2008). When this happened, populations were supplemented with individuals from the stock population at the start of the oviposition period, after the dispersal period and immigration to the experimental populations occurred. After the first generation, two source populations were initialized with 110 individuals due to the limited availability of stock individuals.

*Common environment and reciprocal transplant experiment*

After seven generations, we terminated the rescue experiment, and all extant populations (58 experimental and 25 source populations) were placed at a constant density of 40 individuals on standard media for a single generation (hereafter, “common environment” generation; Figure 2.1c). All adults were kept within their experimental populations, i.e., no mixing occurred within or across treatments. In *T. castaneum*, maternal environmental effects can strongly affect growth rate (Hufbauer et al., 2015; Van Allen & Rudolf, 2013), and so this generation, where the environment and density were kept the same for all populations, served to standardize maternal effects for the subsequent reciprocal transplant experiment. With only a single generation, we did not standardize grand-maternal effects. However, inference from multi-generational studies suggests that grand-maternal effects are relatively weak in *T. castaneum* populations (Hufbauer et al., 2015; Van Allen & Rudolf, 2013).

In the ninth generation, a reciprocal transplant experiment was completed to assess adaptation to the challenging environment and potential costs to adaptation in the source environment (Figure 2.1d). We divided individuals from each population into three groups and randomly assigned them to one of three environments: standard media (0ppm, hereafter “source environment”), media containing the concentration of deltamethrin present in the challenging environment of the rescue experiment (2.968ppm, hereafter “experimental environment”), and media containing a higher concentration of deltamethrin (5.641ppm, hereafter “severe environment”). The dose-response curve (Figure B-1) indicated that the severe environment would reduce the growth rate of naive populations to near zero. Each replicate patch of these three environmental treatments received 40 beetles, which were allowed 24 hours for oviposition and 34 additional days for development prior to census. Replication within each population depended on the number of available individuals following the common environment generation (see Figure B-4).

*Data analysis*

All statistical analyses were performed in R version 4.1.2 (R Core Team, 2021). Population size at census, which occurred on day 35 during generations 1-7, was used to assess population dynamics throughout the rescue experiment. Populations were considered rescued if they grew to a population size >50, and maintained a growth rate >1 (the replacement rate) for at least two of the final four generations. This allowed for decreases in population size due to negative density dependence. Extinction risk and survival times were quantified using a survival analysis with the *ggsurvfit* function in the survival package (Therneau 2022). Extinct populations were defined as those with zero adults at census during the first 6 generations or one adult by generation 7. Overall extinction rates were calculated as raw percentages with confidence intervals around each percentage for each treatment.

Population growth rate was calculated as the mean number of surviving adult offspring per individual in the population, i.e., population size at census each generation ($N_t$) divided by the number of parents that produced those individuals (parent population size, $N_{t-1}$), or $\frac{N_t}{N_{t-1}}$. We evaluated growth rates through time using a linear mixed model with the *lme4* package (Bates et al., 2015). We included immigration treatment (0, 1, or 5 migrants per generation), generation (1-7, categorical), and parent population size ($N_{t-1}$, integers) as fixed effects, and temporal block (block 1, block 2, and block 3), source population (nested within block), and population identity (nested within source population) as random effects. Growth rate data were *log(x+1)* transformed to help satisfy the equal variance assumption. Diagnostic plots were visualized for all models using the *DHARMa* package (Hartig, 2022).

From this model, we generated estimates of both density-dependent and density-independent population growth rates using the predict function with different values for the parent population size ($N_{t-1}$) each generation. To estimate density-dependent growth rates, we allowed the values for $N_{t-1}$ to vary for each generation and treatment combination, matching the

observed mean population sizes at the start of each generation. This estimate was used to describe the population growth rate data and quantify the effects of immigration treatment in a finite habitat by considering the constraints of competition for resources and cannibalism that affected the populations in our experiment (Pointer et al., 2021). To estimate density-independent growth rates from the fitted model, we set $N_{t-1}$ to zero for every generation and treatment, which is equivalent to estimating the y-intercepts when plotting the model-estimated growth rate over $N_{t-1}$ (Figure B-5). These density-independent estimates allowed us to track adaptation to the environment by revealing the change in intrinsic fitness of individuals in the population unfettered by the effects of density. We expected the density-independent growth rates to be higher than the density-dependent estimates due to the known effects of negative density dependence in *T. castaneum* (Melbourne & Hastings, 2008). Confidence intervals for both density-dependent and density-independent growth rates were estimated by parametric bootstrap (percentile method) using the *bootMer* function with 5,000 simulations in the *lme4* package (Bates et al., 2015). Comparisons between treatments were made by estimating confidence intervals by parametric bootstrap (percentile method) for the difference in means between treatments within each generation using the *simpleboot* and *boot* packages with 5,000 simulations (Canty & Ripley, 2022; Peng, 2019).

The timing of evolutionary rescue was assessed in each treatment group using a generalized additive mixed model (Wood, 2017) similar to Olazcuaga et al. (2023). Only rescued populations were considered in this analysis. We modelled the population size of rescued populations using a negative binomial distribution to account for overdispersion using the package *mgcv* (Wood, 2004, 2011). To allow for the relationship between the population size and time to be non-linear, we modelled population size as a smooth function with the number of knots set to 4, which was chosen to maximize the adjusted R-squared value. Explanatory variables also included immigration treatment and the interaction of treatment and time as fixed effects, allowing for a smooth function to be generated for each treatment. The full

model included population identity and temporal blocks as random effects. The best-fit model was chosen by comparing the full model to reduced models using the *compareML* function from the *itsadug* package (van Rij et al., 2022).

We used data from the reciprocal transplant experiment to further evaluate adaptation to the experimental environment, as well as the potential fitness costs of adaptation in a benign environment. The growth rate in the reciprocal transplant experiment was $\sqrt{x}$ transformed and used as the response variable in a linear mixed effects model that included population type (source, 0 migrants, 1 migrant, and 5 migrants), test environment (source, experimental, and severe), and the interaction between population type and test environment as fixed effects. Population identity was included as a random effect nested within the temporal block. Comparisons within each environment and among immigration treatments were made using 95% bootstrapped confidence intervals (percentile method) for the difference in means, which were generated from 5,000 iterations.

## Results

*Population size and extinction*

During the rescue experiment, a majority of populations experienced evolutionary rescue (Figure 2.2, black lines), and extinction probability was similar for all three treatments (Figure 2.2, red lines). The difference in the extinction risk was statistically similar across treatments throughout the experiment (Figure B-6). Additionally, there was no clear difference in survival time among treatments ($\chi^2_2 = 0.1$, $p = 1.00$).

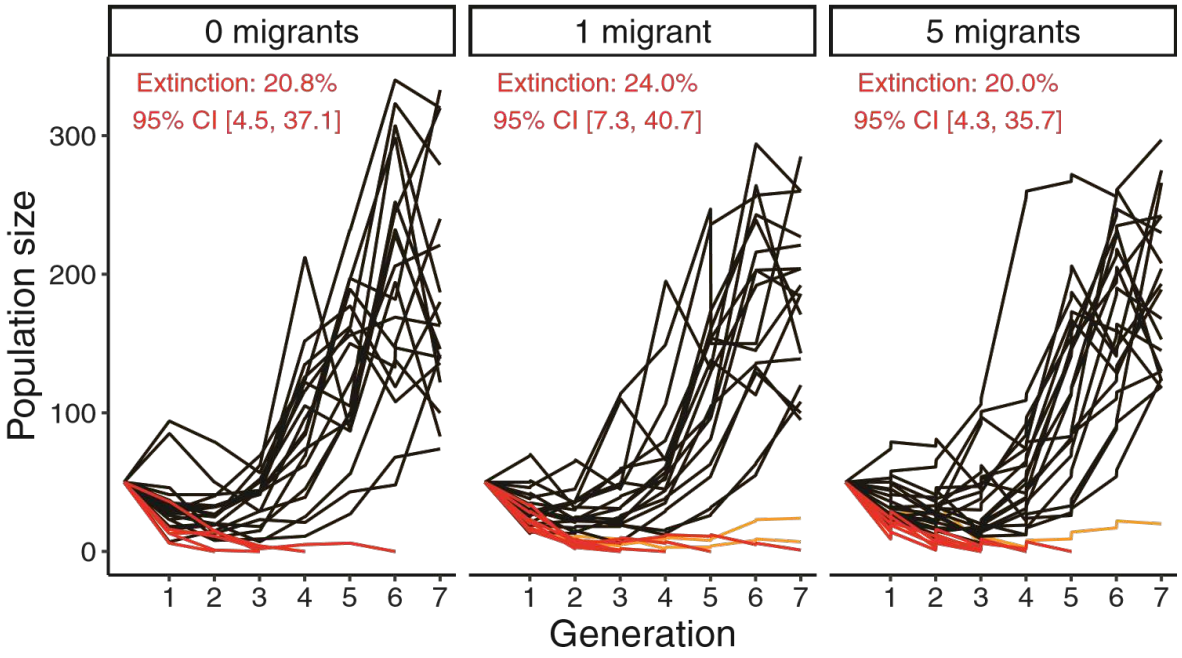


**FIGURE 2.2** Population size over time (in discrete generations) for the populations receiving 0, 1, or 5 migrants per generation during the rescue experiment. Population size represents the number of adult beetles present at census (day 35 each generation), except for generation 0, where the population size is the 50, the initial size for all experimental populations. Each black line represents a rescued population, each orange line represents an extant population that was not considered rescued, and each red line represents a population that went extinct or had a population size of one individual in generation 7. Immigration events are indicated by instantaneous increases in population size, which occurred at the start of generations 1-6 for the 1-migrant and 5-migrant populations. The percent of populations that went extinct and simple confidence intervals around each percent are provided.

*Growth rate through time*

Density-dependent growth rates increased through generation 4 or 5, then decreased again as population density increased (Figure 2.3a; Table B-1). Density-independent growth rates were below or close to the replacement rate (growth rate of 1) in generations 1-3 (Figure 2.3b; confidence intervals overlap with dashed line). This changed in generation four, when the 0-migrant treatment had a mean density-independent growth rate much larger than the replacement rate, illustrating rapid adaptation within those populations (Figure 2.3b). In generation 4, the density-independent growth rate in the 0-migrant populations was, on

average, 0.808 (95% CI [0.207, 1.453]) higher than the 1-migrant population and 0.969 [0.478, 1.573] higher than the 5-migrant populations. By generation 5, all treatments gained and then maintained a mean density-independent growth rate >1, demonstrating adaptation to the challenging environment. The comparison between the density-independent and density-dependent growth rates suggests that the decrease in the latter by generation 7 was likely due to the effects of negative density dependence, which increased as populations grew.

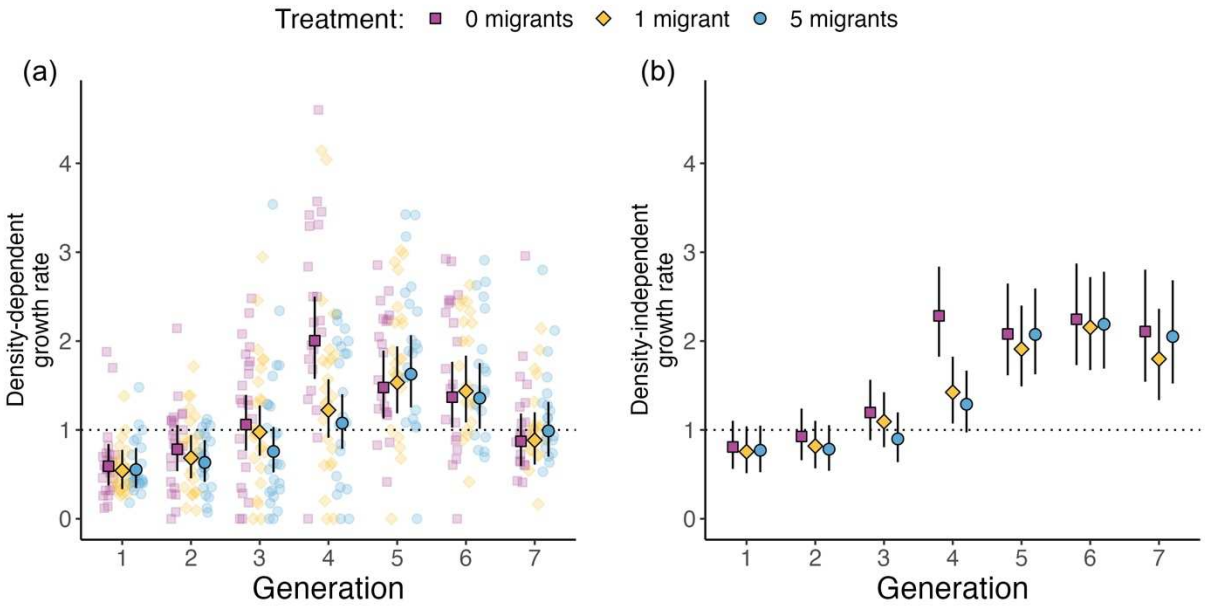


**FIGURE 2.3** Density-dependent growth rate **(a)** and density-independent growth rate **(b)** throughout the rescue experiment. Each point outlined in black is the predicted mean for each treatment (purple square = 0 migrants, yellow diamond = 1 migrant, and blue circle = 5 migrants). Error bars are bootstrapped 95% confidence intervals. Points without black outlines are raw growth rate values, and each point represents one population. The dotted line represents a growth rate of 1, the replacement rate. Two estimates for growth rate are presented: (a) the density-dependent estimates are predictions from the linear mixed model for each generation and treatment combination, used to incorporate the effects of negative density dependence and provide estimates that describe the raw data (shown), and (b) the density-independent estimates (i.e., predictions using $N_{t-1} = 0$), showing adaptation over time

*Timing of evolutionary rescue*

Among the rescued populations only (black lines in Figure 2.2), the analysis of population size with a generalized additive mixed model revealed a shallow U-shaped curve (Figure 2.4). The estimated curves from the best-fit model, which included treatment and the interaction of treatment and time as fixed effects and population identity as a random effect, are shown in Figure 2.4. The interaction between immigration treatment and time was significant ($\chi^2_3 > 800$, $p < 0.0001$), with the 0-migrant populations growing slightly faster than populations receiving migrants (Figure 2.4). The 1-migrant and 5-migrant treatments maintained similar population trajectories throughout the experiment.

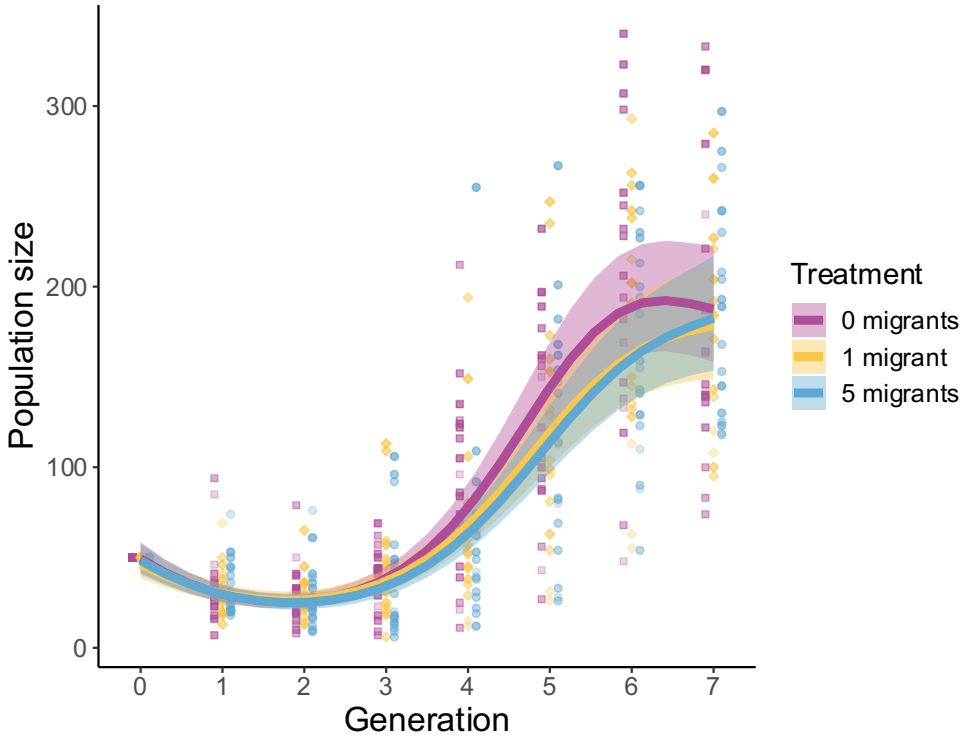


**FIGURE 2.4** Analysis of the timing of evolutionary rescue using a generalized additive mixed (GAM) model. Population size over time (in discrete generations) of rescued populations receiving 0 migrants (purple line, squares), 1 migrant (yellow line, diamonds), or 5 migrants (blue line, circles) per generation were estimated by the best-fit GAM model. Each point represents one population, and points are staggered to reduce overlap. The lines are estimates of the mean population size for the three immigration treatments, and the shaded region around each line is the 95% confidence interval given by the GAM model.

*Adaptation and associated costs*

The growth rate in the reciprocal transplant experiment was influenced by an interaction between environment and population type ($F_{6, 168.04} = 25.16$, $p < .0001$), in a pattern showing that adaptation to the insecticide was linked to reduced fitness in the benign source environment (Figure 2.5). In the benign environment, the experimental populations had lower growth rates on average compared to the source populations (confidence intervals are negative and do not overlap with zero: 95% $CI_{0\text{-migrant} - \text{source}}$ [-0.855, -1.628]; 95% $CI_{1\text{-migrant} - \text{source}}$ [-0.911, -1.748]; 95% $CI_{5\text{-migrant} - \text{source}}$ [-0.641, -1.482]), and a higher growth rate in the severe habitat (confidence intervals are positive and do not overlap with zero: 95% $CI_{0\text{-migrant} - \text{source}}$ [0.442, 0.171]; 95% $CI_{1\text{-migrant} - \text{source}}$ [0.424, 0.210]; 95% $CI_{5\text{-migrant} - \text{source}}$ [0.464, 0.216]). Additionally, the 5-migrant populations performed marginally better than the 0-migrant populations in the experimental environment (95% $CI_{0\text{-migrant} - 5\text{-migrant}}$ [-0.0675, 0.572]).

Unexpectedly, the experimental and source populations performed similarly in the challenging experimental environment (Figure 2.5, all confidence intervals of the differences overlap with zero: 95% $CI_{0\text{-migrant} - \text{source}}$ [-0.404, 0.209]; 95% $CI_{1\text{-migrant} - \text{source}}$ [-0.220, 0.403]; 95% $CI_{5\text{-migrant} - \text{source}}$ [-0.153, 0.452]). The experimental populations did not perform as well as expected based on their growth rates at the end of the rescue experiment (Figure 2.3). Our expectation for growth rates was based on predictions from the linear mixed model created for the rescue experiment with $N_{t-1} = 40$ (the number of parents used in the reciprocal transplant) and gen = 7 (the time closest to the start of the reciprocal transplant). Those predicted values were 1.80 (95% CI [1.32, 2.39]), 1.52 [1.12, 2.00], and 1.75 [1.31, 2.26] for the 0-migrant, 1-migrant, and 5-migrant treatments, respectively. The average growth rates we observed in the reciprocal transplant experiment in the experimental environment were, on average, 40% lower than these estimates, with means of 0.85 [0.64, 1.09], 1.04 [0.80, 1.32], and 1.10 [0.86, 1.35].

Furthermore, source populations did not perform as poorly as expected in the experimental environment. We expected them to perform similarly to the experimental populations in the first generation of the rescue experiment prior to adaptation. The prediction given $N_{t-1} = 40$ was 0.63 [0.38, 0.83] for the 0-migrant populations at the start of the rescue experiment, but the mean growth rate of source populations was about 46% higher at 0.92 [0.72, 1.15].

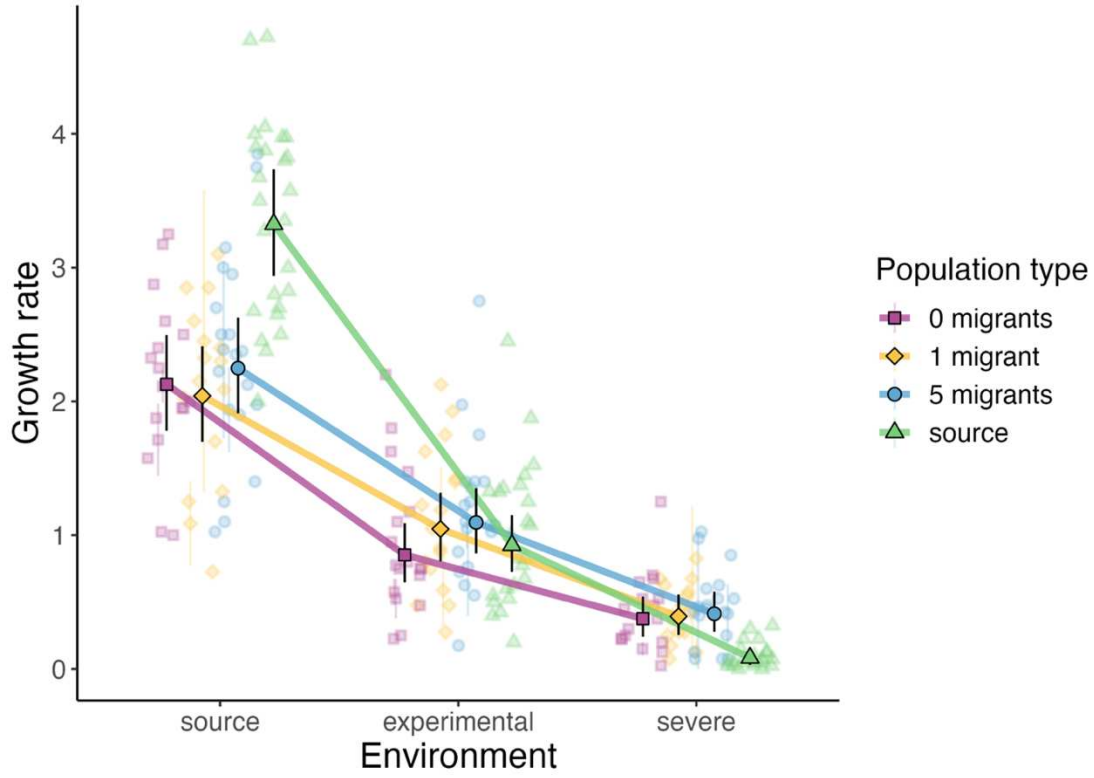


**FIGURE 2.5** Reciprocal transplant experiment results. The growth rate was measured in three environments: the source environment, the challenging experimental environment, and a severe environment containing a higher concentration of insecticide. Each point outlined in black is the model-estimated mean for each population type (purple squares = 0-migrant populations, yellow diamonds = 1-migrant populations, blue circles = 5-migrant populations, and green triangles = source populations). Black error bars are bootstrapped 95% confidence intervals; for the source populations in the severe environment (green triangles), the black error bars are not visible because they are too small to be seen. Thick lines connecting these points emphasize the population type-environment interaction. Each point without the black outline represents a single population, with the light error bars representing raw 95% confidence intervals across replicates; points without error bars have no replicates. Points are staggered and jittered to reduce overlap.

## Discussion

Our study evaluated the effects of different immigration rates on adaptation to a new, challenging environment using experimental populations of *T. castaneum*. We evaluated differences between initially declining populations receiving two immigration rates (one or five migrants per generation) and isolated populations that received no immigration. The migrants were not adapted to the challenging environment, allowing us to examine the degree to which immigration can limit adaptation (Savolainen et al., 2013). While the experimental populations declined in size initially, leading some populations to go extinct, around 80% of populations were able to persist for the duration of the seven-generation experiment. Most of these populations had growth rates above the replacement rate and had large population sizes, characteristic of evolutionary rescue. We also found that extinction rates were similar regardless of immigration. Notably, growth rates increased one generation sooner in the isolated populations compared to those with immigration. This delay was brief, as the following generation, growth rates were comparable across the different immigration treatments. We also tested for adaptation using a reciprocal transplant experiment. While some of the results from that experiment were unexpected, overall, it provided evidence that our experimental populations were adapted to the insecticide that created the challenging environment. There was also a cost to that adaptation, evident from the experimental populations having lower growth rates than the sources in the benign environment.

### *Observed delay in the increase in growth rate: Possible mechanisms*

Populations without immigration gained a much higher density-independent growth rate (more than doubling on average) compared to the populations with immigration (near or just above the replacement rate) in the fourth experimental generation. In the following generation, all populations grew at a similar rate regardless of immigration. The delay we observed may

have been due to (1) differences in density dependence, (2) phenotypic plasticity, or (3) outcrossing with naive migrants slowing adaptation to the challenging environment.

First, theory shows that reduced negative density dependence in a degraded habitat may enhance rescue (Czuppon et al., 2021; Uecker et al., 2014). In our system, population densities were low in the experimental populations in the initial generations, and thus, negative density-dependent processes were likely relatively weak. The added immigrants increased density slightly relative to the isolated populations, and this could have contributed to lower growth rates. However, if this were the case, the populations receiving five migrants each generation should have experienced further reductions in growth than the populations receiving one per generation, as their density was increasing by a greater amount (31% increase for the 5-migrant populations on average compared to a 7% increase for the 1-migrant populations). Thus, density dependence is an unlikely explanation for the one-generation delay in increased growth rates.

Second, the growth rate in the challenging environment could be influenced by phenotypic plasticity, which could have led to the lag in the increase in growth rates. Recently, it has been shown that plasticity in insects can be mediated by associated microbes (Kolodny & Schulenburg, 2020). Variation in the microbial communities in host insects is high (Lange et al., 2023), and the microbial community can evolve, which can facilitate persistence in new and challenging environments (Kolodny & Schulenburg, 2020). Microbes, therefore, can serve as an important source of adaptive phenotypic plasticity (Ghalambor et al., 2007; Kolodny & Schulenburg, 2020). Insect-associated microbes can be transmitted from parent to offspring (Lange et al., 2023), indicating that plasticity could be multi-generational. If the microbial community influences fitness in the presence of deltamethrin, it is possible that plasticity and adaptation in the microbiome helped facilitate the observed increase in growth rate during the rescue experiment. The delay in increase for populations with immigration could be due to the microbiome from the benign environment being continually re-introduced by the migrants. Third,

migration is well-known as a constraint to adaptation (Slatkin, 1987). For example, theory has shown that immigration between different habitat types can limit local adaptation through genetic swamping of adaptive alleles (Bolnick & Nosil, 2007; Kawecki & Ebert, 2004; Lenormand, 2002) and reduce the probability (Schiffers et al., 2013) or speed (Holt & Barfield, 2015) of evolutionary rescue because of maladaptation in the new environment. It seems likely that the immigration of naive individuals slowed adaptation to the novel environment, which may have been enhanced by the mating behavior of *T. castaneum*. In this species, males reared in high-quality environments have increased mating rates and insemination success compared to males reared in poor environments (Lewis et al., 2012). Thus, male migrants from the benign source environment may have successfully mated with multiple females in the experimental patches, enhancing the rate of gene flow of maladapted alleles. Female migrants would have also likely spread maladapted alleles, as a single female *T. castaneum* can lay over 10 eggs per day (Pray & Park, 1949). Thus, regardless of sex, outcrossing with the naive migrants likely contributed to the brief delay in growth rate increase. Given the strength of selection pressure, however, adaptation likely enabled growth rates to increase in the following generation, which we observed on average across all treatments.

We suggest that the increases in population size and growth rate, likely through adaptation and possibly aided by adaptive plasticity, can be described as evolutionary rescue. We further evaluate the effects of adaptation vs. plasticity when we discuss the reciprocal transplant experiment. The finding that rescue can occur with intermediate levels of immigration of not adapted individuals is supported by several modelling studies that assess evolutionary rescue in heterogenous, structured systems (Czuppon et al., 2021; Gomulkiewicz et al., 1999; Tomasini & Peischl, 2020; Uecker et al., 2014). This result has also been shown in a case study of Trinidadian guppies, where rescue was able to occur with gene flow from a maladapted source population (Fitzpatrick et al., 2015, 2020).

*Observed delay in rescue: Implications*

Our finding of only a single generation delay of adaptation has practical implications. Such delayed adaptation could be detrimental to populations at imminent risk of extinction due to small size (Gomulkiewicz & Holt, 1995; Melbourne & Hastings, 2008). However, for populations not at immediate risk of extinction, such a delay may be inconsequential and worth the potential increase in genetic diversity that occurs through immigration. Such increased diversity may prevent inbreeding depression from developing and improve the ability of populations to adapt to future environmental changes (Frankham et al., 2019).

Thus, these results suggest that naive migrants can be suitable to use in management efforts. Furthermore, they are often more feasible to obtain, either from captive rearing programs (Crone et al., 2007) or from large source populations living in a different habitat (e.g., different host plants in Bisschop et al., 2019) or geographic region (e.g., isolated guppy populations in Fitzpatrick et al., 2020), when habitat matching is not possible.

Additionally, our findings are consistent with refuge strategies to delay the evolution of pest resistance to toxins, such as in crops genetically modified to express *Bacillus thuringiensis* (*Bt*). The immigration of susceptible individuals from sections of fields planted in non-*Bt* crops or growing crops in areas with plentiful alternative host plants for the pest has proven to be highly effective (Carrière et al., 2012). In those situations, particularly with 50% or more of a pest population being susceptible, resistance can be successfully managed for multiple generations (Tabashnik et al., 2008). Given our much smaller proportion of susceptible individuals, it makes sense that the delay of the evolution of resistance was brief.

*Reciprocal transplant experiment: Interpretation of unexpected results*

The two most straightforward takeaways from the reciprocal transplant experiment are (1) the experimental populations had higher growth rates than the source populations in the

severe environment, suggesting they had adapted to the insecticide during the rescue experiment and (2) the experimental populations had lower growth rates in the benign environment than the source populations, suggesting that there was a cost associated with adaptation. However, the source populations had higher than expected growth rates in the challenging experimental environment, and the experimental populations had lower than expected growth rates in the experimental environment, which was inconsistent with the hypothesis of adaptation.

We next discuss (1) how experimental challenges might have affected the source populations, (2) how the common environment generation could have reduced growth rates by eliminating the effects of plasticity, and (3) how multiple pathways of adaptation in the experimental populations could explain their performance in the reciprocal transplant experiment.

First, the high performance of the source populations in the experimental environment could be due to challenges in implementing the experimental design. For instance, accidental contamination of the source populations with deltamethrin or accidental gene flow from the experimental to the source populations could have occurred during the rescue experiment. We took great care to avoid both forms of contamination, but if contamination nevertheless occurred, it could have allowed the source populations to adapt to low concentrations of deltamethrin. The source populations were not adapted to high concentrations of deltamethrin as evidenced by their performance in the severe environment.

A second consideration is that the common environment generation could have masked differences in growth rates if they were initially caused by plasticity. As described above, microbiome-mediated phenotypic plasticity could have contributed to the increase in growth rates in the rescue experiment (Lange et al., 2023). If microbiome-mediated plasticity drove performance, the generation in a common environment prior to the reciprocal transplant experiment would have alleviated selection pressure and reduced the ability of the experimental

populations to tolerate deltamethrin. This could help explain the similar performance of the experimental and source populations in the experimental environment. However, there does not appear to be strong evidence for the masking of phenotypic plasticity in the severe or benign environments, given that the experimental populations had a much higher growth rate than the sources in the severe environment and a lower growth rate in the benign. We suggest that those differences can be explained by our last point, adaptation, which is discussed below.

Finally, adaptation to deltamethrin likely explains the relative performance of the populations in the three environments. More specifically, it is possible that the experimental populations adapted through more than one mechanism during the rescue experiment. First, adaptation to detoxify the insecticide compound likely occurred, as rapid evolutionary responses to deltamethrin are well documented in pest populations of *T. castaneum* (Collins, 1998; Stuart et al., 1998) through the increased expression of cytochrome P450 enzymes (Kalsi & Palli, 2015). In other insect species, this mechanism of resistance to deltamethrin has a confirmed cost (Tchouakui et al., 2020), and such a cost is consistent with the reduced performance of experimental populations in the benign environment. The second way that populations could have adapted is by increasing the rate of egg cannibalism to supplement their diets, as cannibalism propensity is a genetically based trait that can evolve (Stevens, 1989) that has been shown to enhance fitness in challenging environments (Pointer et al., 2021; Via, 1999). These two potential pathways, the evolution of increased detoxification and increased cannibalism, are not mutually exclusive, and both may have facilitated increased population growth in the experimental environment during the rescue experiment. Then, in the reciprocal transplant experiment, the populations were initiated at lower densities (40 parents) in the three environments. In this lower-density environment, the number of eggs available for cannibalizing was reduced compared to what was available in the latter half of the rescue experiment (in generations 4-7, $N_{t11} > 150$ on average). Thus, the decreased density reduced the eggs available for cannibalism, limiting one of the adaptive pathways and reducing the overall growth

rates of the experimental populations in the experimental environment. In the severe environment, the growth rates of the experimental populations were low but still greater than the source populations, which can be explained primarily by their evolved ability to detoxify deltamethrin.

*Considerations for future experiments*

The bottleneck events that occurred during the rescue experiment, some as small as 10 individuals, would likely have longer-term consequences (Frankham et al., 1999; Olazcuaga et al., 2023). Census population size in a wide range of plant and animal taxa is almost always larger than the effective population size (the median $N_e/N_c$ ratio across taxa is approximately 0.1; for *T. castaneum*, it is estimated to be between 0.5 and 0.9) and bottlenecks of $N_e < 50$ can result in reductions in genetic variation and increases in inbreeding (Jamieson & Allendorf, 2012; Wade, 1980). For this reason, we suggest that future work examine the effects of demographic bottlenecks of this severity by propagating experiments like this for longer (i.e., more than eight generations). Longer-term experiments would be able to test the hypothesis that the benefits of immigration become more apparent over longer time scales. Such experiments would help shed light on the longer-term impacts of immigration for small and isolated populations, an important question when considering whether to implement translocations in declining natural populations (Frankham, 2015, 2016). Genomic methods would also provide important insights for monitoring the genetic contributions of migrants, the genetic differentiation between the experimental and source populations, and confirming the genes contributing to adaptation (Whiteley et al., 2015). Genomics would also allow us to differentiate between adaptation and plasticity more clearly (Koch & Guillaume, 2020).

*Conclusions*

This study explored the effects of immigration of naive individuals on large, outbred, and recently diverged populations following population decline due to sudden environmental degradation. To our knowledge, we are the first to experimentally test the effects of the recommended immigration rates of one compared to five migrants per generation on adaptation to a challenging environment. We found that though immigration slowed adaptation in the short term, extant populations with and without immigration were, on average, equally well adapted to the challenging conditions after seven generations. Thus, we confirm the findings of previous theoretical work (Czuppon et al., 2021; Tomasini & Peischl, 2020) by demonstrating experimentally that evolutionary rescue can occur both with and without immigration following severe environmental change.

## Data availability

Data and R scripts are publicly available on the Dryad Digital Repository (https://doi.org/10.5061/dryad.79cnp5j3c).


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# CHAPTER 3: ENVIRONMENTALLY-ASSOCIATED GENETIC VARIATION SUGGESTS ADAPTATION WITH GENE FLOW IN A GENERALIST BUTTERFLY[3]

## Introduction

Examining the genetic variation within and among populations for a species is a critical first step for conservation management in the modern era. The evolutionary processes of genetic drift and selection can lead to populations becoming differentiated genetically (Slatkin, 1987), particularly if populations are isolated (Allendorf, 1986; Hedrick & Garcia-Dorado, 2016) or exposed to different habitats (Funk et al., 2016). However, gene flow can slow or prevent differentiation by counteracting the effects of both selection and drift while also maintaining homogeneity among populations (Mills & Allendorf, 1996). When the interaction of selection, drift, and gene flow result in allele frequencies that differ among populations, this can be described as population genetic structure (Holsinger & Weir, 2009). Past studies have evaluated population genetic structure in declining populations (Allendorf 2017) and among fragmented habitat patches, such as sky islands (Halbritter et al., 2019) or across anthropogenic barriers such as roads (Trense et al., 2021). Population genetic analyses can be extended to define conservation or management units, and to assess the potential consequences and feasibility of using assisted migration to aid small, declining, or isolated populations in the wild (Whiteley et al., 2015).

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The interplay between gene flow and selection can impact adaptive evolution in heterogeneous environments (Durkee et al., 2024; Fitzpatrick et al., 2015; Lenormand, 2002). Scientists can study species that occupy environments that differ across space using environmental gradients (Reusch & Wood, 2007). Elevation gradients provide a way to study a species across a range of environmental conditions within relatively small spatial scales, as temperature tends to decrease as elevation increases (Branch et al., 2017; Keller et al., 2013). Organisms whose distributions span mountain ranges are exposed to changes in environmental conditions along elevation gradients as well potential barriers to dispersal (Machado et al., 2018). Thus, a focus on such organisms allows us to study the combined effects of adaptation to different environments and restricted gene flow that may occur across the mountains (Manel & Holderegger, 2013). Genomic analyses provide statistically powerful methods for identifying population differentiation driven by environmental variation (Allendorf, 2017; Capblancq & Forester, 2021; Cheek et al., 2022; Lou et al., 2021). More importantly, downstream analyses allow for the discovery of adaptive loci associated with the environment, helping us to identify the genetic basis of adaptation (Capblancq & Forester, 2021).

However, among animals, there is a bias in genomic studies towards vertebrates, and expanding analyses to include more invertebrate species has been recommended, particularly given recent insect declines (Kawahara et al., 2021; van Klink et al., 2024; Wagner et al., 2021; Webster et al., 2023). For example, the rusty-patched bumblebee, once common, has been lost from over 70% of its native range in the continental USA and is now a federally listed species (Colla et al., 2012; Mola et al., 2024). Recent efforts have focused on using genomics to monitor the genetic diversity and abundance of the bee to develop a plan for recovery (Mola et al., 2024). Preserving genetic variation has been shown to be critical for supporting the ability of populations to adapt to changing environments (Kardos et al., 2021), and insect populations have short life cycles, which can facilitate rapid adaptation following environmental change (Carlson et al., 2014). Recent studies have documented population genetic structure of rare and

declining insects (e.g., Czajkowska et al., 2020; Landmann et al., 2021). However, studies focusing on common species are also important, as insect losses may be driven by declines in formerly abundant species (van Klink et al., 2024). Studying insect populations that are common now, therefore, can inform effective and proactive management plans to prevent future declines.

Here, we examine the population genetics of the Rocky Mountain subspecies of the clouded sulfur butterfly (*Colias philodice eriphyle* Edwards) (Lepidoptera: Pieridae). Clouded sulfurs are native and widespread across the U.S. Mountain West region; they feed on legumes as larvae (Tabashnik, 1983) and are generalist flower visitors as adults (Ezzeddine & Matter, 2008; Watt et al., 1979). The subspecies occupies a wide range of elevations, from alfalfa fields and grasslands in the foothills of the Rocky Mountains (around 1,500m in elevation) to subalpine meadows (up to 3,000m), often at high densities (Tabashnik, 1980; Watt et al., 1979). As elevation increases, these butterflies exhibit increased melanization (black scales) relative to individuals from lower elevations, a signal of adaptation to elevation (Ellers & Boggs, 2002). Melanization increases the solar absorptivity of wings, which aids in thermoregulation during flight, as butterflies will only take flight when their muscles reach a specific temperature range (30-38°C) (Buckley & Kingsolver, 2019; Ellers & Boggs, 2002, 2004; Mattila, 2015). Darker wings at higher elevations are hypothesized to improve performance in the colder temperatures present with increasing altitude (Ellers & Boggs, 2004; Kingsolver & Buckley, 2017). Despite these findings, there have been no population-level genomic studies of *C. p. eriphyle* that address the genetic basis of these adaptive traits or population structure across habitat types. Genomic studies, therefore, can help further explain how this butterfly persists across such diverse habitats and aid in the development of future management plans.

Thus, we followed up on this past work by using population genomics to compare paired high- and low-elevation populations in the Rocky Mountains and foothills, both east and west of the North American continental divide. We conducted whole genome sequencing on 165 *C. p. eriphyle* individuals across 15 sites in Colorado and Wyoming. We used this methodology to

address the following question: How do the Rocky Mountains affect (1) genetic diversity, (2) gene flow, and (3) adaptation to different environments? We predicted several possible outcomes. First, genetic diversity could be similar among sites due to the small geographic region covered by the collection sites (~10,000km$^2$), enabling high gene flow and minimizing signals of adaptation. Second, high-elevation sites may be differentiated from low-elevation sites, due to environmental differences between foothills and subalpine habitats: in this case, we would expect differences in genetic diversity and adaptation due to potentially restricted gene flow across the elevation gradient up each side of the Rocky Mountains. Third, the Rockies may restrict gene flow, as the summit elevations (3,300 to 4,400m; Madole et al., 1987) are outside of the distribution of *C. p. eriphyle.* In this case, we would expect differences in genetic diversity and adaptation on either side of the continental divide.

## Methods

*Field collection*

In June through September 2022, we collected *Colias p. eriphyle* butterflies from seven low- and eight high-elevation sites on both sides of the continental divide in Colorado and southern Wyoming, USA (Figure 3.1). Sites ranged from 10m$^2$ to 1.8km$^2$ in area (Table 3.1). Low-elevation sites were less than ~2,000m in elevation and high-elevation sites were greater than ~2,500m in elevation on average. Sites were paired geographically, with one high and one low site identified that were geographically closer together than they were to other sites. The northernmost site (*Wy*, Figure 3.1) did not have a low-elevation pair due to geographic constraints in the surrounding area. All high-low site pairs had a difference in elevation of at least 1,000m. Up to 20 individuals were collected per site with mesh butterfly nets, and a GPS

coordinate was taken for every individual (except for individuals collected at *Wy*). We assessed the relative abundance of *Colias* species at each site using Pollard Walks (a standard measure of relative abundance of butterflies; Pollard 1977), and measured temperature and wind speed. Collections were only made when conditions for that day included an air temperature greater than 20°C and windspeed less than 20 kph, and when relative abundance was greater than 1. Successful field excursions resulted in at least five individuals netted and then placed in glassine envelopes. All collected individuals were transported to Colorado State University and were frozen alive or within 48hr of death at -80°C. Permission to collect individuals was obtained from the relevant agency (U.S. National Park Service permit no. ROMO-00257, U.S. Forest Service, City of Fort Collins, or private landowners) prior to the field season.

(a)

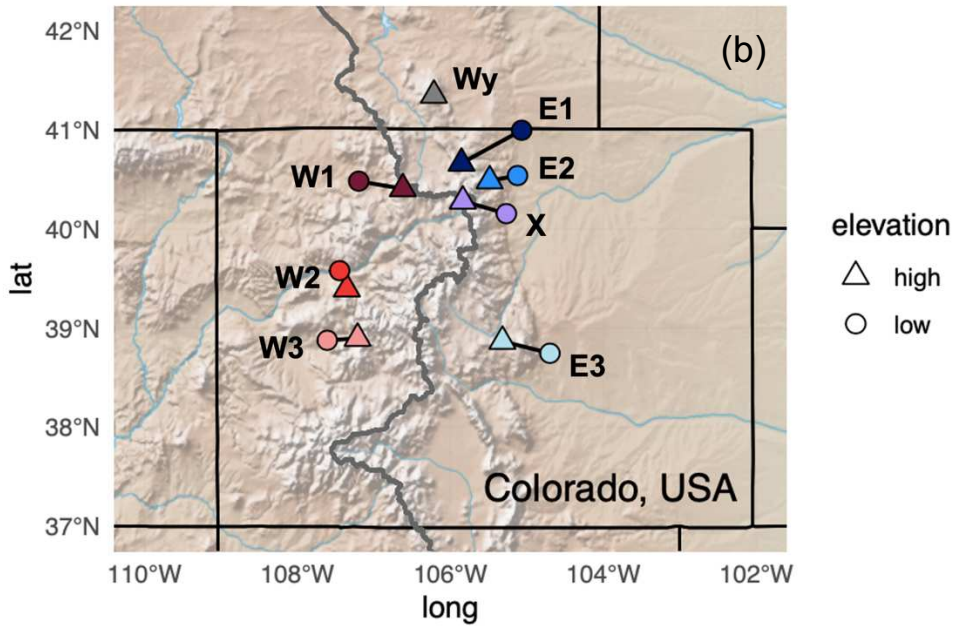


**FIGURE 3.1 (a)** Photo of a male *Colias p. eriphyle,* taken in Loveland, Colorado by LF Durkee. **(b)** Map of all sites sampled for *C. p. eriphyle* in Colorado and southern Wyoming. High (triangle) – low (circle) site pairs are indicated with black lines both east (E; blue shades) and west (W; red shades) of the continental divide, which is shown in gray. The X pair goes "across" the continental divide. The northernmost site, *Wy* (gray triangle), does not have a site pair.

*Sample preparation and DNA sequencing*

We extracted DNA from the thorax of up to 20 individuals per site using Qiagen DNeasy Blood and Tissue Kits. We assessed DNA yield using *qubit* assays and evaluated sample quality by running each on agarose gels. Library preparation was completed following Schweizer & DeSaix (2023) on up to 12 of the highest quality samples per site. In total, we sequenced 170 *C. p. eriphyle* individuals from 15 collection sites (6-12 individuals per site; Table 3.1) on three lanes of Novogene HiSeq 4000. Additionally, we included a related species, *Colias eurytheme,* the orange sulfur, which is known to hybridize with *C. p. eriphyle* and can be difficult to distinguish visually. We included six individuals of this species to assess hybridization or misidentification of our clouded sulfur individuals.

*Bioinformatic processing*

All bioinformatic processing and analyses were completed using the RMACC Alpine computing cluster managed by Colorado State University and University of Colorado, Boulder. Raw FASTQ files were trimmed to remove low-quality paired-end sequences using *TRIMMOMATIC* (Bolger et al., 2014; Lou & Therkildsen, 2022). We mapped the trimmed FASTQ files to the annotated clouded yellow butterfly (*Colias crocea*) reference genome (Ebdon et al., 2022) and removed PCR duplicates with *samtools* (Danecek et al., 2021). Individual coverage on average was 5.0x, with a wide range of read depth (1.3-14.7x) (Figure C-1). To remove potential coverage effects that can mask geographic signals, we downsampled the bam files to 4x coverage (Lou & Therkildsen, 2022). Three closely related individuals (based on the proportion of alleles identical by descent) were identified using *ngsrelate* (Korneliussen & Moltke, 2015), and removed from downstream analyses, as related individuals can skew assessments of population structure (Coleman et al., 2016). Exploratory analyses were then completed using genotype likelihoods in *ANGSD* (Korneliussen et al., 2014) using *pcangsd* to

create principal component analyses (Figure C-2). One individual was removed because it was misidentified; another was removed due to suspected hybridization with *C. euytheme*.

We then proceeded with a *GATK* pipeline and used the *ANGSD* analysis as a check of the validity of subsequent analyses with called genotypes. We processed bam files to call genotypes using GATK4 *HaplotypeCaller* to create GVCF files with mean *QUAL* score >20 (Miller et al., 2023; Poplin et al., 2018), and we used two different minor allele frequency (MAF) and minor allele count (MAC) filters: (1) MAF = 5-95% for population structure and genetic diversity analyses, and (2) MAC=1, which removes singletons, instead of a MAF filter for Tajima's D, a demographic statistic that measures the frequency of rare alleles (Hemstrom et al., 2024; Lou et al., 2021). For both pipelines, we created genomic databases using *GenomicsDBImport*, genotyped the GVCFs using *GenotypeGVCFs,* and removed systematic errors from the VCF file using *VariantFiltration* and *SelectVariants* (Auwera et al., 2013; McKenna et al., 2010; Poplin et al., 2018). We then filtered the called genotypes allowing only biallelic sites, < 80% missing data per site, and a *QUAL* score >30 (Auwera et al., 2013; Lou et al., 2021). *NA* values were marked as missing using *bcftools* (Danecek et al., 2021). For the MAF-filtered file only, imputation was completed using *beagle v4.1* (Browning & Browning, 2016)*,* as imputed genotypes were required for some downstream analyses (Forester et al., 2018). Lastly, we conducted linkage disequilibrium (LD) pruning using *plink* (Purcell et al., 2007) to reduce redundancy as recommended for analyses with *ADMIXTURE* (Alexander et al., 2009).

*Bioinformatic analyses: Genetic diversity*

Among the individuals that passed our processing steps (*N* = 165), we used *vcftools* (Danecek et al., 2011) to calculate window-based nucleotide diversity ($\pi$) with a window size of 100kb (Martin et al., 2016). Nucleotide diversity is a measure of genetic variation, and it is estimated as the average number of pairwise nucleotide differences within each window in the

genome (Allendorf et al., 2021). We averaged over the windows to create an estimate of π for each collection site. Next, using the VCF file without MAF filters, we calculated window-based Tajima's D with a window size of 100kb, an estimate that can be used to infer departures from neutrality, with negative values of Tajima's D indicating an excess of rare alleles, and positive values indicating balancing selection against rare alleles (Oostra et al., 2018; Tajima, 1989). For π and Tajima's D, we tested for differences between east vs. west sites, high vs. low sites, and across latitude using simple linear models and the *emmeans* package in R (Lenth, 2021).

*Bioinformatic analysis: Gene flow*

Exploratory analyses of population structure were completed using principal component analyses (PCA) with the R package *vegan* (Oksanen et al., 2024). We then evaluated the generated PCAs using *F*-statistics generated by multivariate analysis of variance (MANOVA) tests using the principal components (PC1 and PC2) as response variables and site-specific variables as predictors (e.g., [PC1, PC2] ~ site) similar to Flanagan et al. (2016). We identified principal components explaining significant levels of genetic structure using broken stick criterion (Jackson, 1993; Forester et al., 2018). We then used $F_{ST}$ to assess differentiation among groups.

We tested for isolation by distance (IBD) by evaluating the correlations between pairwise genetic distances and geographic distance. To do this, we calculated pairwise $F_{ST}$ using *plink* and estimated geographic distance between each population (using the *geosphere* package in R, which provides Haversine values, in units of meters) (Hijmans, 2022; Mikheyev et al., 2013; Trense et al., 2021). Slatkin's D was calculated as $\frac{F_{ST}}{1-F_{ST}}$ (Slatkin 1991). We also completed a Mantel test to evaluate the correlation between genetic distance and geographic distance matrices using *vegan* (Cheek et al., 2022; Manel & Holderegger, 2013). Lastly, we completed a maximum likelihood analysis of ancestry using *ADMIXTURE* to test for the number of ancestral

populations (*K*) that maximized the likelihood of the data. We evaluated *K*=1 through 10 using cross-validation on the LD-pruned VCF file in *plink* format.

*Bioinformatic analysis: Adaptation*

To identify genetic variation correlated with environmental and climate variables, we used a redundancy analysis (RDA), a multivariate genotype-environment association method that identifies genomic signatures of selection related to environmental variables (Capblancq & Forester, 2021; Forester et al., 2018). The individuals from population *Wy* (*N* = 9) were excluded from this analysis because individual GPS coordinates were not taken in the field. For all other individuals (*N* = 156), average monthly precipitation (mm), maximum temperature (°C), daily solar radiation (kJ/m$^2$), and wind speed (m/s) data were extracted for each individual's GPS coordinate from *WorldClim* raster files for monthly values at 30s (~1km$^2$) spatial resolutions (Fick & Hijmans, 2017). Most of our collection sites were smaller than 1km$^2$, so individuals within sites generally had either the same or very similar climatic variables. We then took the average values for the summer months (June-August), because this is when most of the collection was completed (13 of 14 sites; Table 3.1) and when *Colias* flights are most likely to occur (Watt et al., 1979). Elevation (in meters) associated with each individual were obtained using the *elevatr* package in R for a greater resolution than provided by *WorldClim* (Hollister et al., 2023). We included elevation as a variable in the model to account for other environmental shifts that occur along elevation gradients, for example temperature and snowpack, as these variables are known to be correlated with elevation (Rebetez, 1996). To avoid including collinear predictors, we removed highly correlated (Pearson's $r > 0.8$) climate variables (Dormann et al., 2013) resulting in three retained predictors: elevation, precipitation, and solar radiation (Figure C-3). We chose precipitation and solar radiation over wind speed because precipitation has

been shown to shape insect distributions (Kellermann & Van Heerwaarden, 2019) and solar radiation is important for thermoregulation in *Colias* butterflies (Kingsolver & Buckley, 2017).

We identified SNPs associated with the multivariate environment by identifying those with constrained ordination axis loadings in the tails of the distribution using a standard deviation cutoff value of 3 (equivalent to $p < 0.0027$) along one or more of the RDA axes (Forester et al., 2018). To identify genes associated with each candidate loci, we used the *closest* function in *bedtools* (Quinlan & Hall, 2010) to intersect the SNP positions with the annotation of the reference genome, and kept loci found within 25kb of each gene, an estimated distance before linkage disequilibrium breaks down (Backström et al., 2006). We further analyzed the function of identified SNPs using the program *SnpEff* (Cingolani et al., 2012), which predicts the effects of genetic variants on gene function by classifying them by their impact (low, moderate, high, or intergenic/modifier) on protein-coding regions and regulatory genomic elements.

## Results

Our bioinformatic processing and variant detection with GATK4 resulted in 165 *Colias p. eriphyle* individuals being included in our downstream analyses from 15 sites (Figure 3.1), comprised of 1,454,624 SNPs and 31 scaffolds from the alignment with the reference genome of sister species *Colias crocea.* Scaffolds 1-30 represent autosomal chromosomes, followed by the sex chromosome *Z.*

### *Genetic diversity*

Nucleotide diversity ($\pi$), a measure of genetic variation, was low (around 0.001) across sites. Notably, there was a significant difference between sites east (average $\pi = 0.001075$) and

west (π = 0.001058) of the continental divide (t-test: $t = 4.332$, $p < 0.001$). Nucleotide diversity also increased with longitude (t-test: $t = 2.658$, $p = 0.02$) (Figure 3.2; Table 3.1). Mean values for Tajima's D were -0.91 on average, which signals that these individuals possess more rare alleles than expected, suggesting recent population expansion (Oostra et al., 2018; Tajima, 1989). There was no significant difference in Tajima's D between east-west (t-test: $t = 0.933$, $p = 0.3678$) or high-low sites ($t = 0.965$, $p = 0.3520$).

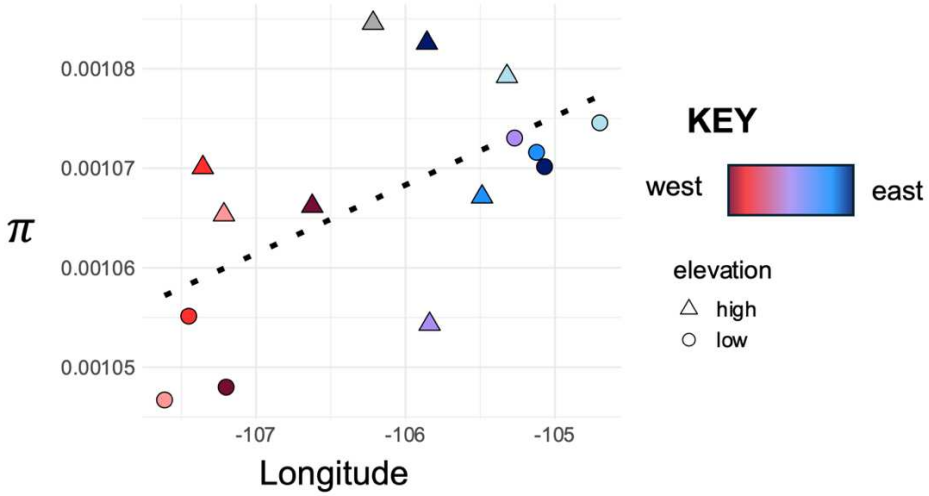


**FIGURE 3.2** Nucleotide diversity (π) over longitude, indicating that there is greater diversity in the east (blue shaded points) compared to the west (red shaded points). The positive, linear trend is shown with the dotted line.

**TABLE 3.1** Collection site information is displayed, including site names, locations, collection date (month/day in 2022) and area ($km^2$), average elevation (elev, m) from *elevatr,* as well as average monthly precipitation (precip, mm) and average daily solar radiation (srad, $kJ/m^2$) for the summer months (June-August). Sample and diversity measures are also shown, including the number of butterflies from each site that were included in analyses (no. seq) along with the mean of two genetic diversity indices: window-based π (100kb window) and window-based Tajima's D (100kb window).

| Site | Longitude | Latitude | Date | Area ($km^2$) | Elev. (m) | Precip. (mm) | Srad. ($kJ/m^2$) | No. seq | $\pi_{100kb}$ | Tajima's $D_{100kb}$ |
|---|---|---|---|---|---|---|---|---|---|---|
| W1-high | -106.6236 | 40.40549 | 6/29 | 0.5664 | 2,958 | 35.33 | 24188 | 12 | 0.00101 | -0.993 |
| W1-low | -107.1999 | 40.48171 | 7/12 | 0.0148 | 2,005 | 53.08 | 23333 | 12 | 0.00099 | -0.951 |
| W2-high | -107.3554 | 39.39437 | 8/7 | 1.7593 | 2,693 | 37.18 | 23798 | 11 | 0.00101 | -0.929 |
| W2-low | -107.4500 | 39.57932 | 8/8 | 0.0023 | 1,745 | 30.33 | 24618 | 12 | 0.00100 | -0.970 |
| W3-high | -107.2145 | 38.89614 | 8/9 | 1.9654 | 2,711 | 38.12 | 23882 | 11 | 0.00101 | -0.924 |
| W3-low | -107.6115 | 38.87611 | 8/8 | 1.06e-5 | 1,759 | 26.33 | 24719 | 12 | 0.00099 | -0.935 |
| X-high | -105.8385 | 40.28101 | 6/27 | 0.0093 | 2,648 | 50.67 | 22829 | 12 | 0.00100 | -0.956 |
| X-low | -105.2697 | 40.15433 | 7/13 | 0.0004 | 1,716 | 45.33 | 23380 | 12 | 0.00102 | -0.948 |
| Wy | -106.2166 | 41.34944 | 7/11 | *n/a* | 2,997 | 57.00 | 23039 | 9 | 0.00103 | -0.828 |
| E1-high | -105.8564 | 40.66277 | 7/9 | 0.0011 | 2,644 | 49.33 | 23214 | 12 | 0.00102 | -1.042 |
| E1-low | -105.0693 | 40.99582 | 7/15 | 0.0003 | 1,924 | 53.33 | 22555 | 12 | 0.00102 | -0.994 |
| E2-high | -105.4875 | 40.48606 | 6/11 | 0.0028 | 2,497 | 44.00 | 23473 | 6 | 0.00101 | -0.416 |
| E2-low | -105.1233 | 40.53881 | 9/18 | 0.0060 | 1,567 | 59.33 | 23980 | 11 | 0.00101 | -0.924 |
| E3-high | -105.3211 | 38.86602 | 8/10 | 0.0031 | 2,704 | 62.67 | 23188 | 11 | 0.00103 | -0.994 |
| E3-low | -104.7001 | 38.74506 | 7/27 | 0.0175 | 1,755 | 35.33 | 24188 | 11 | 0.00102 | -0.828 |

*Gene flow: PCA, ADMIXTURE, and IBD*

The PCA showed minimal clustering by collection site (Figure 3.3) and none of the PC axes exceeded the broken-stick criterion, suggesting weak population structure across the study area. The multivariate analysis of variance (MANOVA) suggested that collection site was a

significant predictor of PC1 and 2 (MANOVA: $F_{14,150}$ =7.982, $p$ < 0.001), which suggests that though the population structure among collection sites was weak, it was still greater than expected by chance. Additionally, we used *F*-statistics generated by the MANOVA and weighted $F_{ST}$ to assess differentiation between groups of sites. For the east vs. west sites, the MANOVA indicated strong differentiation ($F_{1,163}$ = 74.63), while the weighted, global $F_{ST}$ = 0.0017. For high vs. low sites, the MANOVA indicated weaker differentiation than between east-west sites ($F_{1,163}$ = 9.62) and the weighted $F_{ST}$ was also lower (0.00045). Both $F_{ST}$ values were very close to zero, confirming that gene flow was likely high among sites, however the significant MANOVA tests suggest that the population structure was not completely panmictic. Additionally, the *F*-statistic and weighted $F_{ST}$ values for the east-west comparison were 8X and 4X greater than the high-low comparison, respectively, suggesting differentiation was driven more by restricted gene flow over the continental divide than between high and low sites.

Alongside these findings, the *ADMIXTURE* analysis revealed that the best-supported number of ancestral populations (*K*) by cross-validation (CV) was $K$ = 1 ($CV_{K=1}$ = 0.483 vs. $CV_{K=2\text{-}10}$ = 0.498-0.665). This suggests that the butterflies in our study likely descend from a single ancestral population and represent a single genetic cluster, providing additional evidence for a relatively continuous population structure.

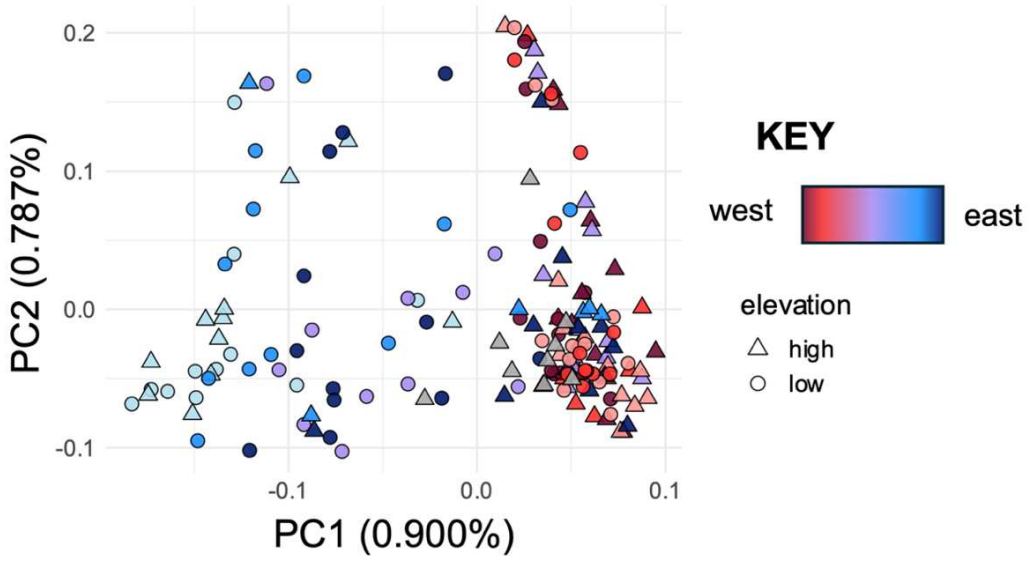


**FIGURE 3.3** Principal component analysis of all individuals (*N* = 165) across all sites (*N* = 15). Each point represents an individual butterfly: red shades represent butterflies from the west, blue shades represent butterflies from the east, triangles represent high-elevation sites, and circles represent low-elevation sites. The colors and shapes match the scheme in the map of the collection sites (Figure 3.1).

Lastly, our investigation of pairwise $F_{ST}$ between sites suggests that there is evidence of isolation by distance (IBD), which indicates that collection sites farther apart are more genetically differentiated than sites nearby (Figure 3.4). The Mantel test indicated that the two distance matrices (Slatkin's D and Haversine) are significantly correlated ($r$ = 0.4692, $p$ = 0.001). This is a strong indication of IBD among the collection sites. Furthermore, we found a stronger signal of IBD in eastern sites ($r$ = 0.600, $p$ = 0.011) compared to western sites ($r$ = 0.3914, $p$ = 0.032).

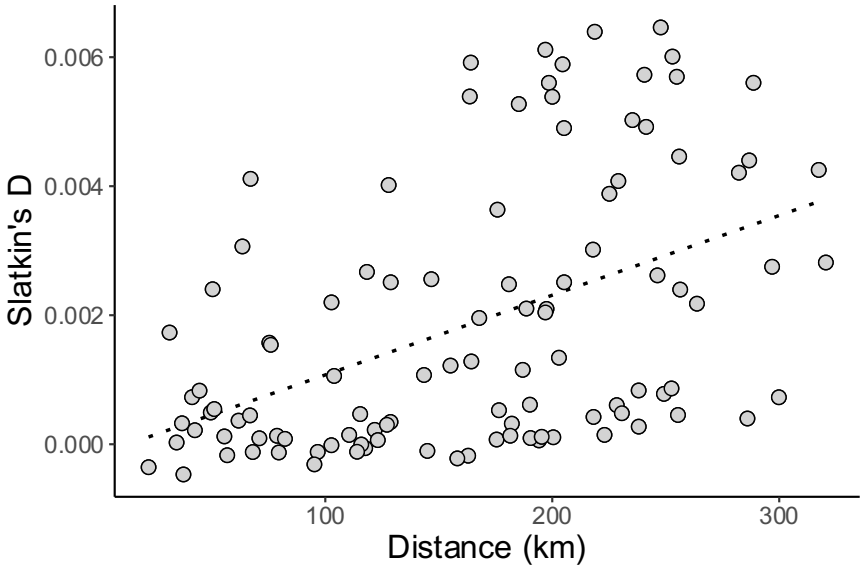


**FIGURE 3.4** Distance among site pairs (Haversine distance, km) is positively correlated with Slatkin's D, a measure of genetic distance ($\frac{F_{ST}}{1-F_{ST}}$ ). The dashed line shows the fitted linear regression model.

*Adaptation: Redundancy analysis (RDA)*

Because of the relatively weak population structure, we did not include a correction for it in the RDA, as this can decrease power to detect loci under selection (Forester et al., 2018). The redundancy analysis indicated that elevation, summer monthly precipitation, and daily solar radiation were significantly associated with genetic variation ($F_{3, 31005}$ = 1.107; $p$ < 0.001). Precipitation appears to load most strongly on RDA1 based on the angle of each vector with the x- and y-axis (Figure 3.5a), solar radiation loads most strongly on RDA3 (Figure 3.5b), and elevation may load on all three axes (see loading values for a subset of SNPs in Table C-2). The spread of values for elevation, precipitation, and solar radiation for each site can be found in Figure C-4; average values of all three variables per site are shown in Table 3.1.

The distribution of butterflies in the ordination plots shows that individuals (points, circles or triangles) arranged in space relative to their relationship with the environmental predictors (purple arrows, Figure 3.5). We observe three notable patterns: first, butterflies collected from

high-elevation sites have SNPs that are correlated with increasing elevation, suggesting adaptation to high altitude and the associated environmental variables (Figure 3.5a-b, triangles); second, increased solar radiation and decreased elevation are correlated with butterflies collected from lower altitude habitats (Figure 3.5b, circles); and third, eastern and western populations appear to be separating across RDA1, a division driven by differences in precipitation (Figure 3.5, red vs. blue points). More specifically, butterflies from the southeast (Figure 3.5a: light blue points) appear most associated with higher amounts of precipitation, where precipitation amounts are known to be the highest of all sites (Figure C-4). Interestingly, high and low site pairs do not cluster together despite being separated by 45km on average.

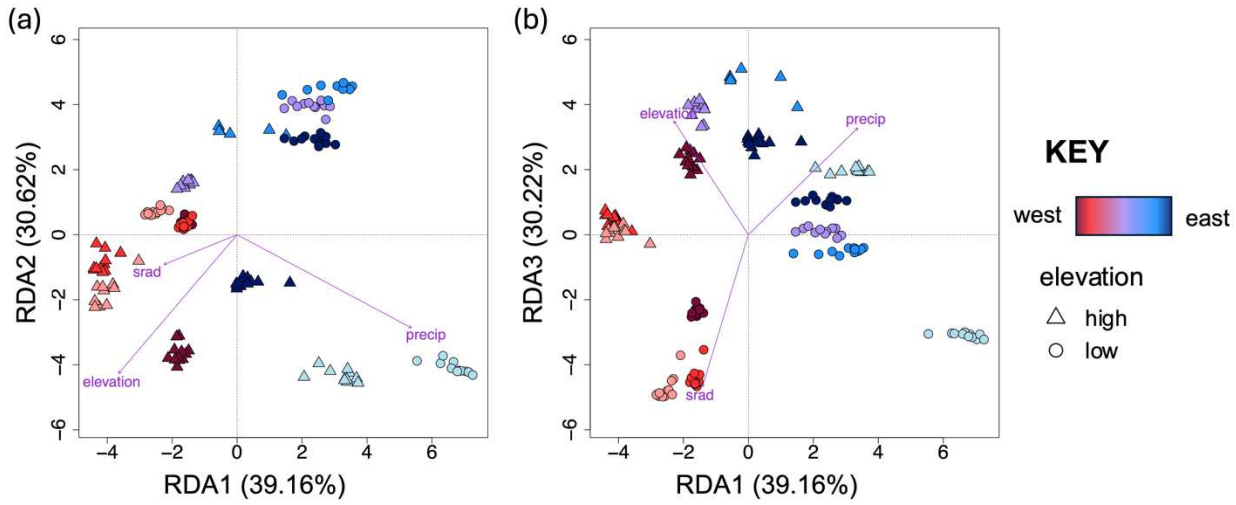


**FIGURE 3.5** Redundancy analysis (RDA) ordination plots showing environmentally-associated genetic divergence. **(a)** Shows the plot of RDA2 vs. RDA1, and **(b)** shows RDA3 vs. RDA1. Each point represents an individual butterfly: red shades represent butterflies from the west, blue shades represent butterflies from the east, triangles represent high-elevation sites, and circles represent low-elevation sites (Figure 3.1). Each purple vector represents the environmental predictors. The arrangement of the points represents their relationship with the ordination axes, which are linear combinations of the environmental variables.

Next, we investigated significant SNPs that were correlated with each environmental predictor, and we explored overlap between the significant loci and the reference genome to

identify candidate genes. Instead of examining each candidate (there were over 3,000 identified; Figure 3.6), we first searched for genes that have been documented to be involved with adaptation to high altitude, varying precipitation, and solar radiation or temperature, in insects (Kellermann & Van Heerwaarden, 2019). Additionally, we searched for genes involved in the insect melanization pathway, which is involved in adaptation to elevation in *C. p. eriphyle* (Ellers & Boggs 2002). We identified three notable genes: *ABCG4,* which has been shown to affect thermal adaptation (You et al., 2024), *PTPB1,* which is involved in adaptation to hypoxia and high altitude (Ding et al., 2018), and *TH,* which contributes to insect melanization (Zhang et al., 2019).

Subsequently, we identified how SNP variants may be involved in gene function using SnpEff: 11.6% were predicted to have moderate effect on downstream protein function (nonsynonymous variants), 31.2% had low effect (synonymous and splice region variants), and 57.2% were identified as intergenic or modifiers (Table C-1). The 20 genes with moderate effect that were most strongly correlated with elevation, precipitation, and solar radiation are displayed in Table C-2. We suggest that future studies explore the potential biological function or adaptive significance of these candidate genes (Montejo-Kovacevich et al., 2022).

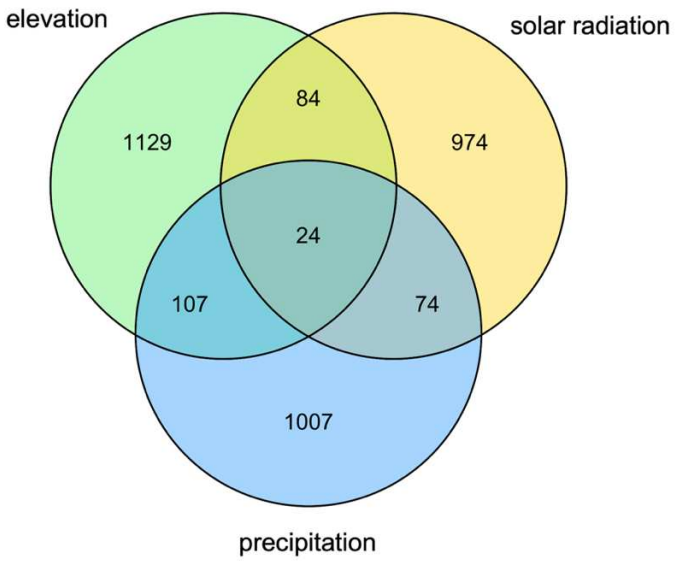


**FIGURE 3.6** The number of genes associated with each environmental variable (elevation [m], solar radiation [kJ/m$^2$/day], and monthly precipitation [mm]) assessed in the redundancy analysis. Some genes were found to be associated with multiple variables, and 24 genes were found to be associated with all three.

## Discussion

Clouded sulfur butterflies (*Colias p. eriphyle*) were sampled from fifteen sites in Colorado and Wyoming, USA, within a relatively small geographic area (roughly 10,000 km$^2$). The sampling area encompassed both low (<2,000m) and high (>2,500m) elevation sites, located both east and west of a potential barrier to dispersal, the Rocky Mountains. We conducted whole genome sequencing on 165 individuals to evaluate how elevation (low vs. high) and geographic position (east vs. west of the mountain range) affected genetic diversity, gene flow, and adaptation to the local environment. We expected that (1) genetic diversity and signals of adaptation could be similar among collection sites due to high gene flow (i.e., *panmictic population structure*), (2) high-elevation sites could be differentiated from low-elevation sites due to environmental differences and reduced gene flow between foothills and subalpine habitats

(i.e., *differentiation with increased elevation*), or (3) the Rockies could restrict gene flow and result in differences in genetic diversity and adaptation among sites east vs. west of the mountain range (i.e., *differentiation across the divide*).

*Genetic diversity trends*

Overall, we found that nucleotide diversity was low (~0.001), about 10x lower than the values observed from a whole genome analysis of *Heliconius* (Martin et al., 2016). Additionally, nucleotide diversity was significantly lower in the sites west of the Rockies compared to eastern sites, suggesting that the Rockies may somewhat restrict gene flow; in a panmictic population, we would expect statistically similar values across all sites (Allendorf et al., 2021). Past studies with *C. p. eriphyle* suggest that the genetic diversity present is likely driven by exposure to environmental variation (Watt et al., 1979), and differences in diversity could be influenced by hybridization with *C. eurytheme* (Wheat & Watt, 2008; Dwyer et al., 2015; Cordeiro et al., 2020). The range of *C. eurytheme* overlaps with *C. p. eriphyle* in the Rocky Mountain region, and future analyses could compare the amount of introgression present to see if that contributes to the increased genetic diversity observed in eastern sites. Additionally, we did not find large disparities in genetic diversity between high and low sites, suggesting dispersal from the foothills up the mountains on either side of the divide likely occurs, preventing any one site from becoming isolated (Manel & Holderegger, 2013).

Additionally, Tajima's D values were negative (-0.91 on average), suggesting the presence of more rare alleles than expected. This could imply that the populations of *C. p. eriphyle* in the Colorado Rocky Mountain region have experienced recent population expansion, which has been observed in agricultural alfalfa (Tabashnik, 1980; Wheat & Watt, 2008; Dwyer et al., 2015). The lack of difference in Tajima's D among sites suggests that the demographic

history of the butterflies, regardless of collection site, is likely similar. This was further supported by the ADMIXTURE analysis, which found evidence for a single ancestral population.

In summary, based on the patterns of genetic diversity, we see support for the differentiation across the continental divide expectation, along with consistent patterns of rare genetic variants among sites. These patterns can be further explained by gene flow, which is discussed below.

*Gene flow: Genetic differentiation and isolation by distance*

The principal component and $F_{ST}$ analyses showed that while differentiation was relatively weak overall, it was strongest between east and west sites, providing additional support for the differentiation across the divide expectation. A similar and stronger pattern of differentiation has been found in orange sulfur (*C. eurytheme*) populations analyzed with AFLP data on either side of the High Sierras in California and Nevada (Dwyer et al., 2015). Our results may suggest that *C. p. eriphyle* populations may be starting to experience similar patterns of differentiation in the Rockies. Furthermore, the Mantel test suggests that there is a positive and significant association between genetic distance and geographic distance leading to a pattern of isolation by distance (IBD), which has been found in other butterfly species studied in small spatial scales (Lebigre et al., 2022; Trense et al., 2021). Notably in our study, we found IBD to be stronger in eastern populations when compared to western populations.

We believe patterns of IBD at this spatial scale are likely explained by the natural history of *C. p. eriphyle.* Clouded sulfurs live in meadows and grasslands containing host plants from the Fabaceae (pea) family (Watt et al., 1979). Dispersal distances of clouded sulfurs are less than 1km on average (Watt et al., 1977), with populations described as “sedentary” when host plants are at high density (Tabashnik, 1980). Personal field observations and previous studies have documented the patchiness of meadows in subalpine habitats, with forest stands acting as

barriers to dispersal in butterflies (Keyghobadi et al., 1999). In the lower elevation areas in the eastern foothills and western slope on either side of the Rocky Mountains, dispersal barriers additionally include roads and housing developments present in these more urban areas (Rochat et al., 2017; Trense et al., 2021). Thus, patterns of genetic differentiation with increased distance are likely driven by the short flight distances and barriers to dispersal present across the sampling region. Additionally, the increased urbanization in eastern Colorado compared to the western slope may help explain why IBD is stronger in the east. Eighty-eight percent of the population of Colorado resides in the Front Range (HRSA, 2022), which comprises the urban centers of Denver, Boulder, Fort Collins, and Colorado Springs. All of the eastern collection sites were located in this region, and the increase in urbanization and human activity likely contributes to the increase in IBD in the east.

Overall, we suggest that the population structure of *C. p. eriphyle* is comprised of relatively continuous sites, connected via intermediate populations. Dispersal between these intermediate populations creates relatively high gene flow overall. Together, a higher density of anthropogenic dispersal barriers in eastern Colorado and some restricted gene flow across the continental divide both lead to the observed differentiation between eastern and western butterflies.

*Adaptation to local environments*

Genome-wide genetic variation is critical for adaptation to changing environments (Kardos et al., 2021). Redundancy analysis (RDA) is a preferred method of discovering genetic variation associated with the environment, which can aid in identifying genetic signatures of adaptation (Forester et al., 2016). We present evidence that clouded sulfurs harbor genetic variation that is associated with precipitation, solar radiation, and elevation (which itself is correlated with and represents numerous additional climate variables including temperature and

snowpack) (Rebetez, 1996). We used RDA to identify loci associated with each of these variables, and we identified over 16,000 environmentally-associated, putatively adapted SNPs and over 3,000 candidate genes, with more than 40% predicted to have an effect on downstream protein function. Our finding that clouded sulfur genetic variation is associated with precipitation, solar radiation, and elevation, which intentionally varied in our sampling design, suggests that we have a strong signal of adaptation driven by environmental differences between our collection sites.

To help explain these patterns, we examined the differences between precipitation and solar radiation both between high- vs. low-elevation sites and east vs. west of the continental divide using the *WorldClim* dataset (Figure C-4). As we investigated the differentiation with increased elevation expectation, we found significantly greater average monthly precipitation (50mm vs. 41mm) and reduced solar radiation (which may be due to increased cloud cover) at higher elevations. Numerous other environmental variables also correlate with elevation that we did not include in this analysis, including temperature (Figure C-3). These environmental differences between high and low sites likely underly the signals of adaptation to elevation that we observed in the RDA. Furthermore, we found significantly greater precipitation (53mm vs. 39mm) in the eastern sites and greater solar radiation in the west in these data. Solar radiation has been shown to affect insect behavior and adaptation, particularly relating to thermoregulation (Somme, 1989; Kingsolver & Buckley, 2017), and precipitation patterns during the summer months impact the survival of *C. p. eriphyle* specifically (Watt et al., 1979). Thus, we suggest that reduced solar radiation and greater precipitation in the east, which is supported by historic trends (Schumacher, 2024), largely drive the observed patterns of population adaptation and differentiation across the divide in Colorado.

We identified a few genes of interest that have documented functions relating to the environmental variables we investigated. We found candidate genes associated with thermal acclimation in a moth species (*ABCG4*, You et al., 2022) and adaptation to high altitudes in a

locust (*PTPB1,* Ding et al., 2018). Another gene that produces the enzyme tyrosine hydroxylase (*TH*) has been found to be associated with melanization in Hemiptera (Zhang et al., 2019). This is notable because wing melanization has been studied extensively in clouded sulfurs, and it is a key factor in thermoregulation during flight (Ellers & Boggs, 2002, 2004; Kingsolver & Buckley, 2017; MacLean et al., 2016). Overall, the large number of SNPs and candidate genes significantly correlated with environmental variation suggests that adaptive genetic variation is present and plentiful in these populations. This likely enables *C. p. eriphyle* to live across a wide range of elevations and habitats within the area we studied, and it may enable their persistence through future environmental changes.

*Implications for management*

The clouded sulfur is not currently in decline, however insect species that were once common have been shown to decline quickly (e.g., the rusty-patched bumble bee) (Wagner et al., 2021; Mola et al., 2024; van Klink et al., 2024). Thus, it is important to conduct genetic assessments on common species to inform conservation measures that can prevent future declines (Webster et al., 2023). In our study of the clouded sulfur butterfly, a common species, we found nucleotide diversity to be low compared to species with similar ecology and life history (Junker et al., 2021; Martin et al., 2016). We also found that genetic diversity was higher east of the continental divide, suggesting that there is some differentiation between the eastern and western populations in this region. Given their reduced genetic diversity, western populations may harbor greater genetic load (Allendorf et al., 2021), and therefore may be more susceptible to future declines. Additionally, negative values of Tajima's D suggest that the populations may have recently expanded, resulting in a higher frequency of rare alleles from recent mutations that remain at low frequency. This would be consistent with observations that clouded sulfur

populations have been increasing in size on agricultural alfalfa, similar to the orange sulfur, which is considered a pest on alfalfa in the western USA (Tabashnik, 1983).

Given the comparable values of genetic diversity and low $F_{ST}$ that we observed between high and low sites, we suggest that agricultural populations of the clouded sulfur could be sustaining higher-elevation populations. Due to the low dispersal capabilities of this subspecies, movement across the elevational gradient separating low and high sites likely takes several generations. This would enable populations to adjust to the lower temperatures present at high elevations through adaptation, which has been documented extensively in Colorado populations of *C. p. eriphyle* (Ellers & Boggs, 2002, 2004; Kingsolver & Buckley, 2017). Our study suggests a gene associated with elevation in insects, the *TH* gene, may underlie melanization in this subspecies, and future studies could further examine the function of this gene in high-elevation populations of *Colias* specifically.

The clouded sulfur butterfly has been used as a model system in the past to study insect host evolution and adaptive responses to elevation (Buckley & Kingsolver, 2019; Ellers & Boggs, 2004; Kingsolver, 1983; Kingsolver & Buckley, 2017; Tabashnik, 1983). Here, we add a genomic component to the study of *C. p. eriphyle*, suggesting the genetic basis of adaptation to local climates is strongly associated with precipitation, solar radiation, elevation, and other correlated variables. These genetic signatures of adaptation persist despite high gene flow between low- and high-elevation sites, and moderate gene flow over the Rocky Mountains. Driven by environmental differences, these adaptive signatures may foreshadow shifts in population structure under future climate change. Continued monitoring of *C. p. eriphyle*, particularly given the increases of drought, wildfire, and heat waves in the region (Liu et al., 2015; McCoy et al., 2022), will be important for creating effective management plans and determining potential conservation units (Barbosa et al., 2018; Webster et al., 2023) for the clouded sulfur in the coming years.

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# APPENDIX A: CHAPTER 1

**TABLE A-1. Initiation of the experimental populations.** The crossed lineages included in each experimental population and the replicate patches of each in generation 2 (G2) and generation 3 (G3). Populations are defined as individuals that are interbreeding each generation. Some crossed lineages (including single lineage groups with no admixture) were replicated temporally and thus had population representation in more than one block.

| Admixture level | Population name (Lineages present and block) | Replicate patches in G2, $N_0$=40 | Replicate patches in G3, $N_0$=20 |
|---|---|---|---|
| 1 | Estill Block 1 | 10 | 2 |
| 1 | Estill Block 2 | 10 | 4 |
| 1 | Estill Block 3 | 10 | 1 |
| 1 | Ga-1 Block 1 | 10 | 1 |
| 1 | Ga-1 Block 2 | 10 | 1 |
| 1 | Lab-S Block 1 | 10 | 20 |
| 1 | Lab-S Block 2 | 10 | 3 |
| 1 | Lab-S Block 3 | 10 | 10 |
| 1 | Z-2 Block 1 | 10 | 4 |
| 1 | Z-2 Block 2 | 10 | 3 |
| 1 | Z-2 Block 3 | 10 | 3 |
| 1 | Z-4 Block 1 | 10 | 2 |
| 1 | Z-4 Block 2 | 7 | 4 |
| 1 | Z-4 Block 3 | 10 | 2 |
| 2 | Ga-1, Lab-S Block 2 | 10 | 3 |
| 2 | Lab-S, Estill Block 3 | 10 | 8 |
| 2 | Z-2, Ga-1 Block 2 | 2 | 1 |
| 2 | Z-2, Ga-1 Block 3 | 2 | 0 |
| 2 | Z-2, Lab-S Block 2 | 10 | 8 |
| 2 | Z-2, Lab-S Block 3 | 10 | 8 |
| 2 | Z-4, Estill Block 2 | 4 | 1 |
| 2 | Z-4, Ga-1 Block 1 | 2 | 1 |
| 2 | Z-4, Ga-1 Block 3 | 4 | 1 |
| 2 | Z-4, Lab-S Block 1 | 10 | 10 |
| 2 | Z-4, Lab-S Block 2 | 10 | 5 |
| 2 | Z-4, Lab-S Block 3 | 10 | 6 |
| 2 | Z-4, Z-2 Block 1 | 3 | 1 |
| 2 | Z-4, Z-2 Block 2 | 4 | 1 |

| | | | |
|---|---|---|---|
| 3 | Ga-1, Estill, Z-4 Block 1 | 6 | 1 |
| 3 | Ga-1, Estill, Z-4 Block 2 | 2 | 1 |
| 3 | Ga-1, Lab-S, Z-4 Block 2 | 10 | 3 |
| 3 | Ga-1, Lab-S, Z-4 Block 3 | 10 | 8 |
| 3 | Z-2, Estill, Lab-S Block 1 | 10 | 3 |
| 3 | Z-2, Estill, Lab-S Block 2 | 10 | 1 |
| 3 | Z-2, Ga-1, Lab-S Block 1 | 10 | 6 |
| 3 | Z-2, Ga-1, Lab-S Block 2 | 10 | 8 |
| 3 | Z-2, Ga-1, Lab-S Block 3 | 5 | 6 |
| 3 | Z-2, Ga-1, Z-4 Block 2 | 1 | 1 |
| 3 | Z-2, Ga-1, Z-4 Block 3 | 10 | 10 |
| 3 | Z-2, Lab-S, Z-4 Block 1 | 10 | 5 |
| 3 | Z-2, Lab-S, Z-4 Block 2 | 10 | 5 |
| 3 | Z-2, Lab-S, Z-4 Block 3 | 10 | 8 |
| 4 | Ga-1, Lab-S, Estill, Z-4 Block 1 | 10 | 4 |
| 4 | Ga-1, Lab-S, Estill, Z-4 Block 2 | 7 | 1 |
| 4 | Ga-1, Lab-S, Estill, Z-4 Block 3 | 10 | 4 |
| 4 | Z-2, Ga-1, Estill, Z-4 Block 1 | 4 | 1 |
| 4 | Z-2, Ga-1, Estill, Z-4 Block 2 | 3 | 1 |
| 4 | Z-2, Ga-1, Estill, Z-4 Block 3 | 8 | 3 |
| 4 | Z-2, Ga-1, Lab-S, Estill Block 1 | 10 | 3 |
| 4 | Z-2, Ga-1, Lab-S, Estill Block 2 | 6 | 1 |
| 4 | Z-2, Ga-1, Lab-S, Z-4 Block 1 | 10 | 3 |
| 4 | Z-2, Ga-1, Lab-S, Z-4 Block 2 | 9 | 2 |
| 4 | Z-2, Ga-1, Lab-S, Z-4 Block 3 | 10 | 6 |
| 4 | Z-2, Lab-S, Estill, Z-4 Block 1 | 10 | 5 |
| 4 | Z-2, Lab-S, Estill, Z-4 Block 2 | 10 | 1 |
| 4 | Z-2, Lab-S, Estill, Z-4 Block 3 | 10 | 2 |

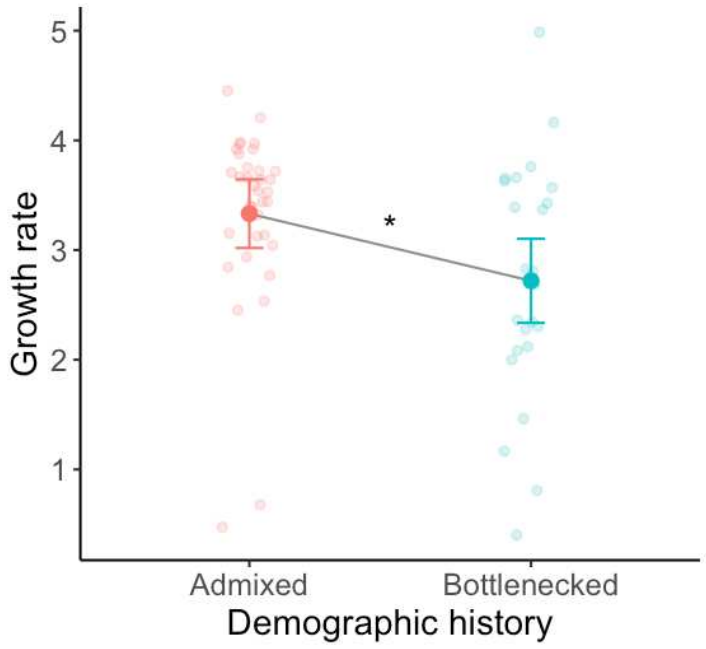


**FIGURE A-1. Comparison of growth rates between admixed and bottlenecked populations** after experiencing a new challenging environment (modified from Olazcuaga *et al.,* 2023). The admixed populations corresponded to replicated populations from a population created by crossing the five lineages used in this experiment, which was maintained for approximately ten generations at a population size of ~10,000 individuals. The bottlenecked populations were created from this admixed population but had experienced a bottleneck event (reduction in population size to one pair of siblings for one generation) during their demographic history. These data represent the first generation of exposure to a challenging environment (corn flour) after several generations of rearing in a benign environment (wheat flour). Growth rate is measured as $\frac{N_t}{N_{t-1}}$ where $N_{t-1} = 100$. Olazcuaga *et al.* observed lower growth rates in the bottlenecked populations when compared to the admixed populations in this first generation in the challenging environment. Points represent population means and error bars represent 95% confidence intervals generated by a linear model (non-bottlenecked: 95% CI [3.02, 3.64], bottlenecked: 95% CI [2.34, 3.10]).

# APPENDIX B: CHAPTER 2

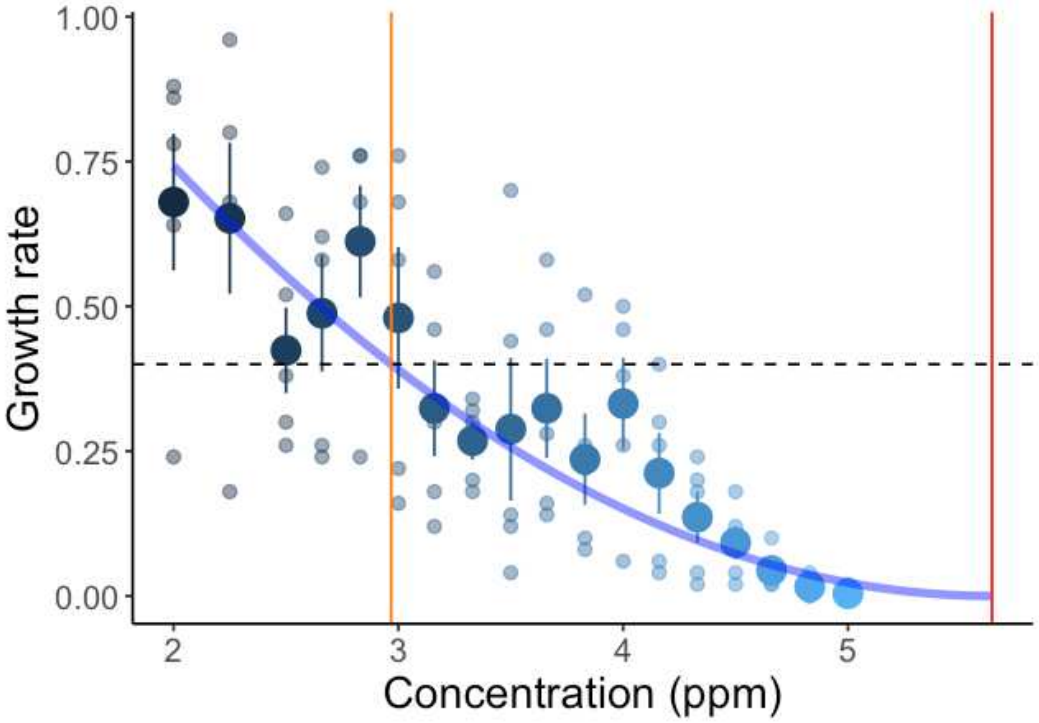


**FIGURE B-1. The dose-response curve** for *Tribolium castaneum* using different concentrations of deltamethrin (DeltaDust, Bayer). The orange line is the experimental concentration chosen based on a goal of growth rate of 0.4 (dashed line). The red line is the concentration used in the severe environment of the reciprocal transplant experiment.

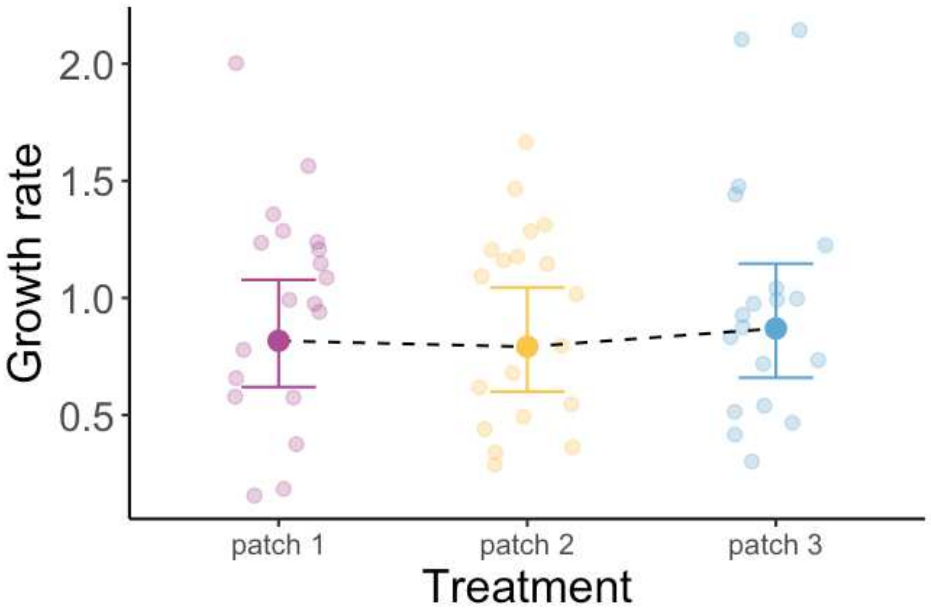


**FIGURE B-2. Order experiment results.** Results of the experiment testing the effects of patch order on growth rate after one generation. Error bars show 95% confidence intervals generated from a linear mixed model including treatment (patch order: position 1-3) as a fixed effect and patch array (19 total across 3 temporal blocks) as a random effect.

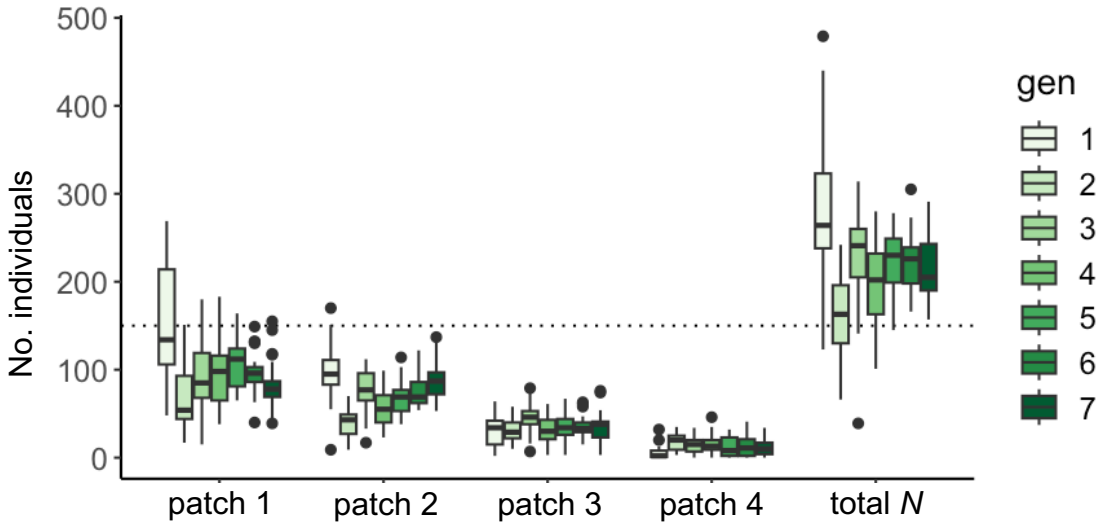


**FIGURE B-3. Source population dispersal array** census throughout the rescue experiment. Distribution of adult beetles among the four patches of the source dispersal arrays (patch 1-4) and the total number across all patches (total *N*) for each generation (indicated by darkening shades of green). The dashed line is the initial population size (*N* = 150) that was used each generation. Box and whisker plots show the median and interquartile range, while whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range from the box, and points are data more extreme.

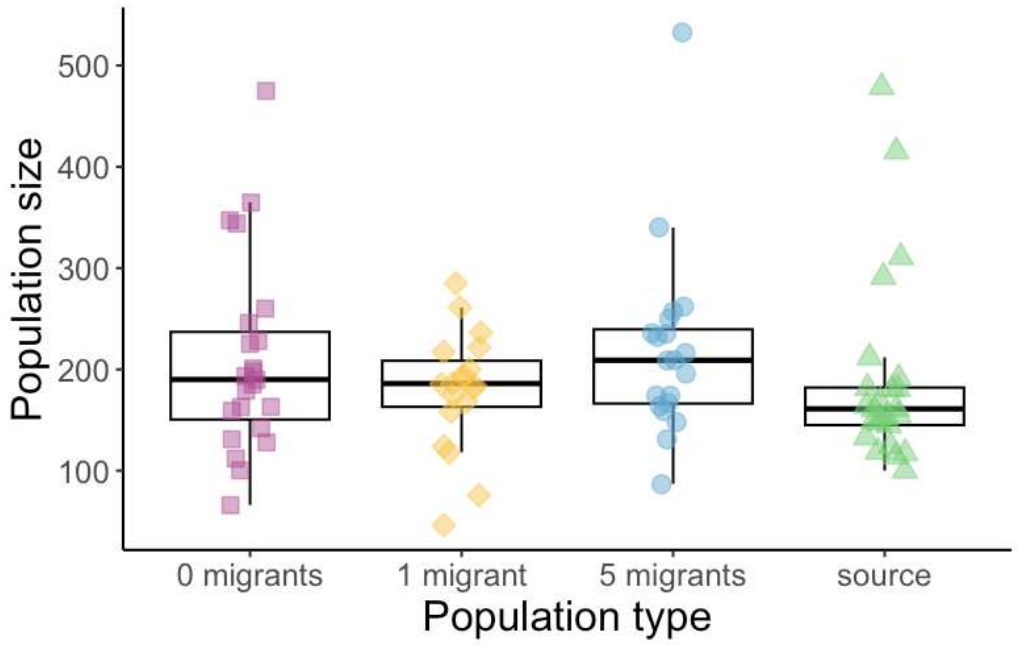


**FIGURE B-4. Common environment generation census.** Total size of each population after the common environment generation (generation 8), in the standard, benign conditions. Each point is the population size for a single population. Box and whisker plots show the median and interquartile range, while whiskers extend to the most extreme data point that is no more than 1.5 times the interquartile range from the box. Between 2-4 replicates were initiated for each population, and some of these populations were allowed to reproduce twice over consecutive days to increase population sizes. We kept the proportion of these double set ups consistent within each treatment. All replicates were initiated with 40 individuals, unless populations were small ($N < 50$) at the conclusion of the rescue experiment; three such populations were initiated with fewer than 40 individuals. The individuals produced in this generation were used to initiate the reciprocal transplant experiment in the subsequent generation. During the reciprocal transplant, populations were split into three groups of 40 individuals, each exposed to a different environment. Additional replication occurred when enough individuals (*i.e.,* 160 or more) were available. In total across all three environments, there were 23-24 replicates of populations from the 0-migrant populations (average replication of 1.1/population/environment), 19-20 replicates (average replication of 1.2/population/environment) from the 1-migrant populations, 22 replicates for the 5-migrant treatment (average replication of 1.1/population/environment), and 24-25 replicates for the source populations (average replication of 1/population/environment).

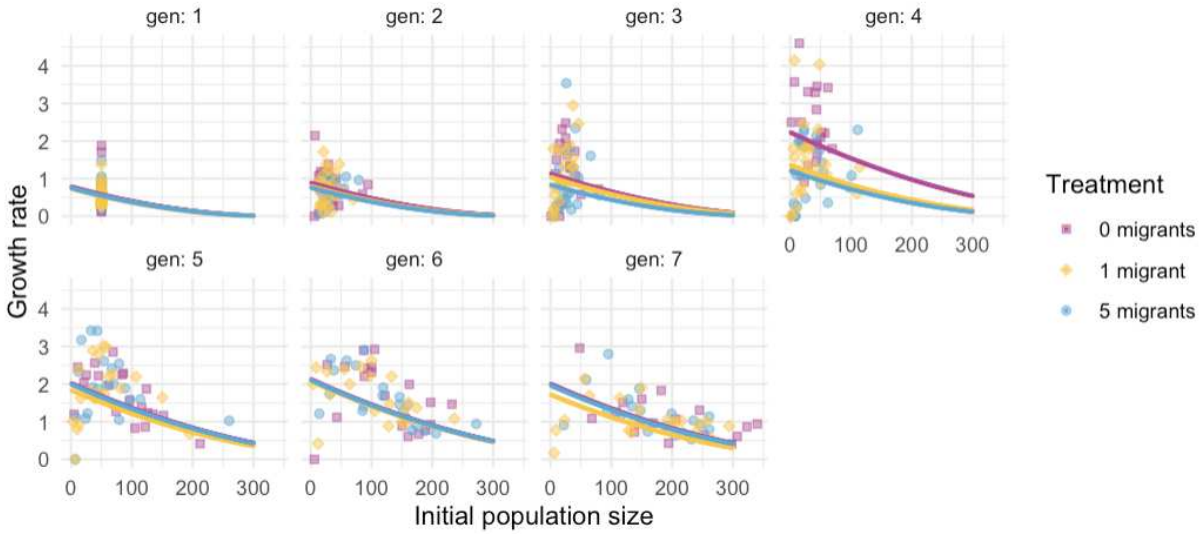


**FIGURE B-5. Growth rate predictions and model fit.** Predictions from the growth rate model across the values of initial population size for each generation during the rescue experiment. The density-independent growth rates were estimated at $N_0 = 0$, which is the Y-intercept for each generation and treatment combination.

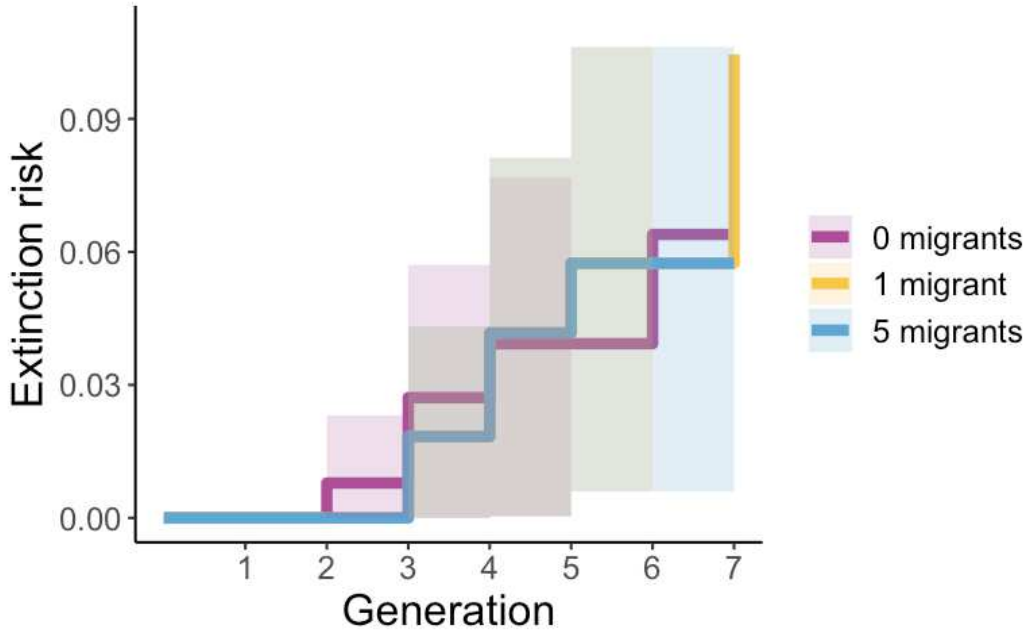


**FIGURE B-6. Extinction risk analysis.** The overall extinction risk for each treatment throughout the rescue experiment using a survival analysis (*ggsurvfit* package in R).

**TABLE B-1. Growth rate comparison through time using different methods.** The density-dependent estimates are predictions from the linear mixed model using average initial population size for each generation and treatment combination, and the density-independent estimates use an initial population size of 0.

| Treatment | Gen | *N* | Density dependent mean + 95% boot CI | Density independent mean + 95% boot CI |
|---|---|---|---|---|
| *0 migrants per gen* | 1 | 24 | 0.578 (0.351, 0.867) | 0.787 (0.511, 1.137) |
| | 2 | 24 | 0.746 (0.481, 1.072) | 0.888 (0.601, 1.247) |
| | 3 | 23 | 0.998 (0.684, 1.371) | 1.130 (0.790, 1.523) |
| | 4 | 21 | 1.978 (1.519, 2.501) | 2.232 (1.738, 2.804) |
| | 5 | 20 | 1.452 (1.052, 1.924) | 2.030 (1.533, 2.629) |
| | 6 | 20 | 1.318 (0.931, 1.769) | 2.131 (1.595, 2.758) |
| | 7 | 19 | 0.838 (0.540, 1.211) | 2.005 (1.417, 2.709) |
| *1 migrant per gen* | 1 | 25 | 0.533 (0.313, 0.818) | 0.736 (0.476, 1.062) |
| | 2 | 25 | 0.670 (0.416, 0.978) | 0.794 (0.522, 1.134) |
| | 3 | 25 | 0.924 (0.628, 1.277) | 1.035 (0.725, 1.405) |
| | 4 | 23 | 1.170 (0.820, 1.580) | 1.356 (0.981, 1.794) |
| | 5 | 21 | 1.477 (1.085, 1.947) | 1.839 (1.376, 2.380) |
| | 6 | 20 | 1.410 (1.020, 1.863) | 2.081 (1.574, 2.670) |
| | 7 | 20 | 0.847 (0.546, 1.207) | 1.716 (1.209, 2.329) |
| *5 migrants per gen* | 1 | 25 | 0.544 (0.322, 0.831) | 0.749 (0.480, 1.076) |
| | 2 | 25 | 0.616 (0.379, 0.912) | 0.760 (0.488, 1.079) |
| | 3 | 25 | 0.705 (0.452, 1.022) | 0.834 (0.554, 1.164) |
| | 4 | 23 | 1.009 (0.683, 1.391) | 1.210 (0.853, 1.625) |
| | 5 | 21 | 1.586 (1.171, 2.059) | 1.995 (1.516, 2.538) |
| | 6 | 20 | 1.328 (0.943, 1.773) | 2.109 (1.569, 2.738) |
| | 7 | 20 | 0.959 (0.637, 1.353) | 1.959 (1.416, 2.616) |

# APPENDIX C: CHAPTER 3

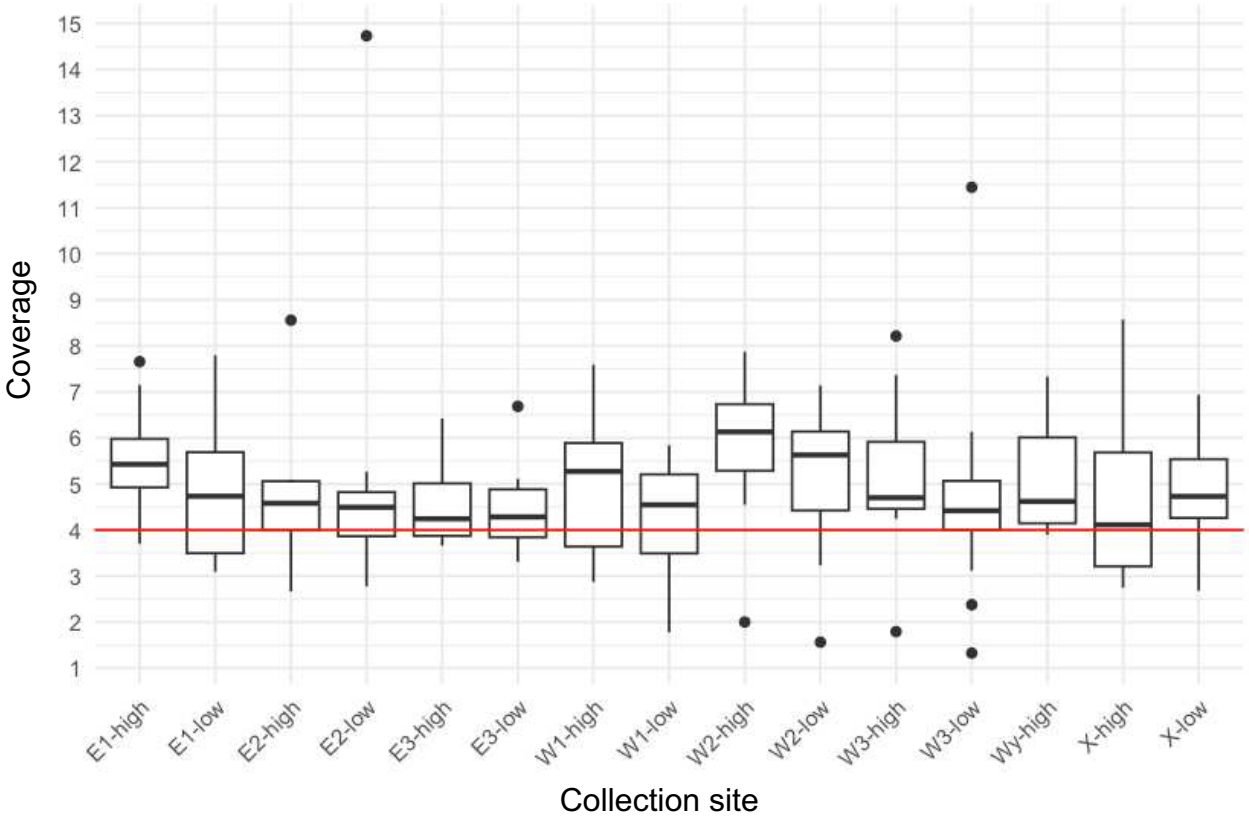


**FIGURE C-1. Variation in read depth coverage among collection sites.** Due to the variation in coverage, we downsampled to 4X coverage (red line).

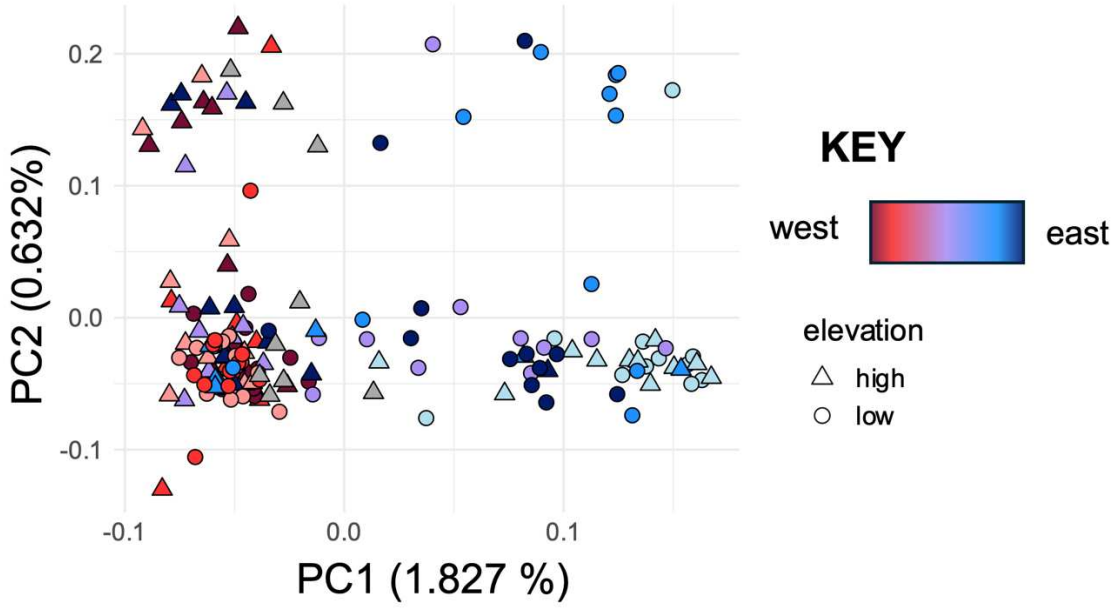


**FIGURE C-2. Principal component analysis generated using genotype probabilities** with *pcangsd*. This was used to compare against the analysis done with GATK4.

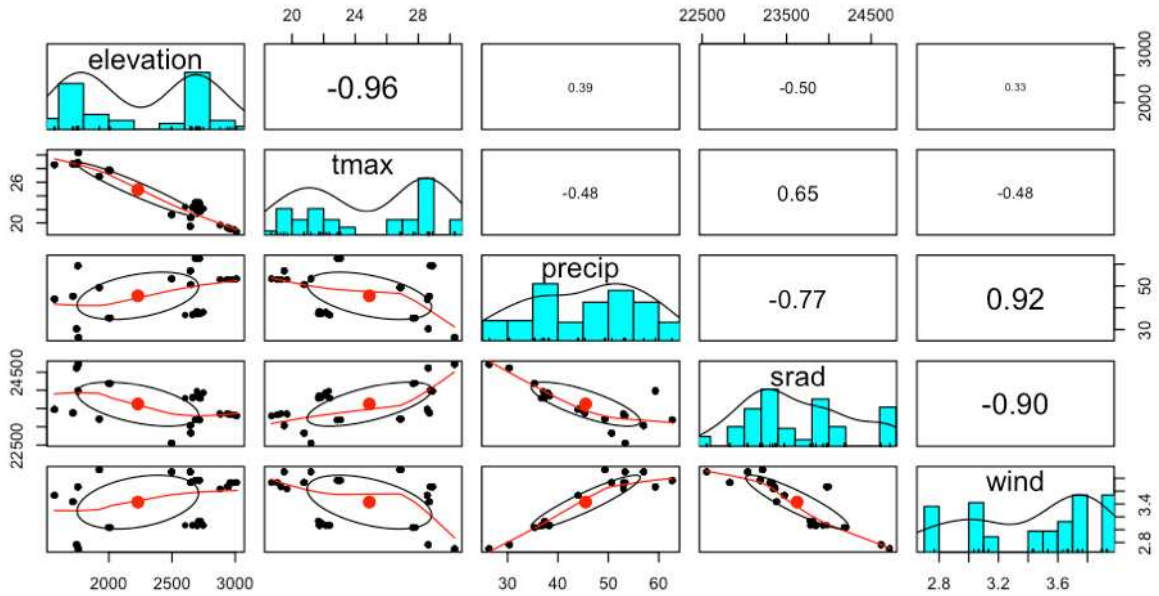


**FIGURE C-3. Pearson correlation coefficient (r)** for the environmental variables considered for the redundancy analysis. We included variables with |r| < 0.80, and therefore selected elevation (m), precipitation (precip, mm/month), and solar radiation (srad, kJ/m$^2$/day). We eliminated max temperature (tmax, °C), which was highly correlated with elevation ($r$ = -0.96) and wind (m/s), which was highly correlated with precipitation and solar radiation ($r$ = 0.92 and -0.90, respectively).

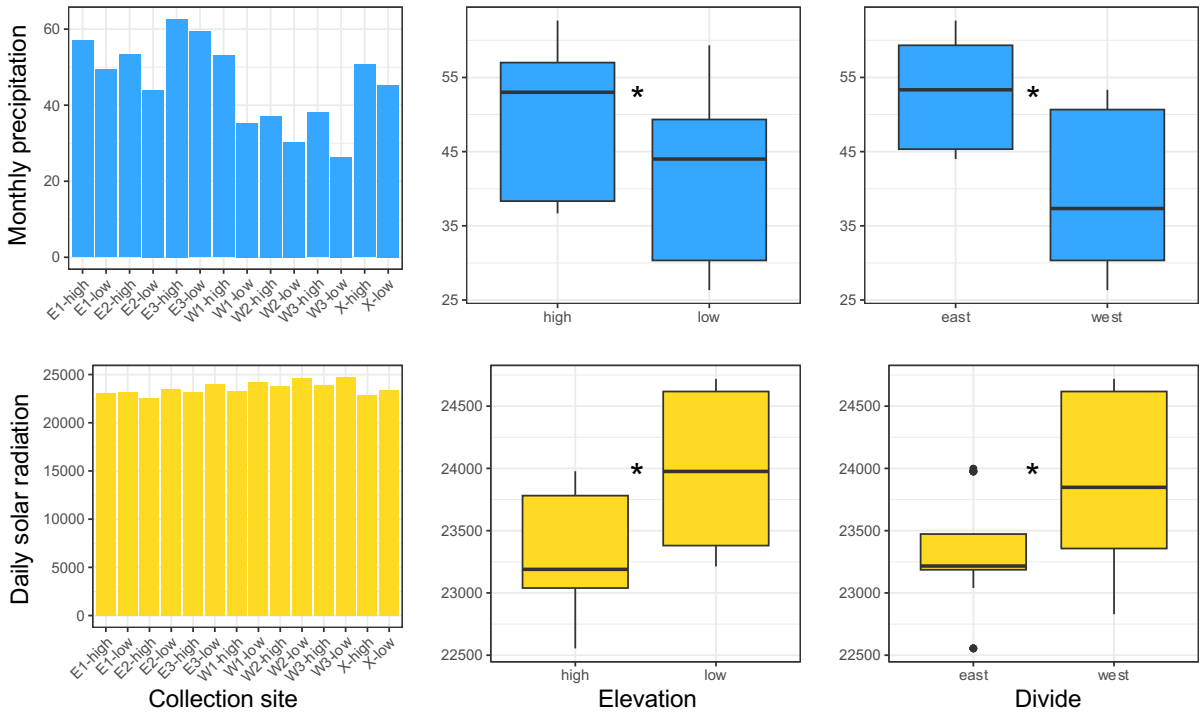


**FIGURE C-4. Summary of the environmental variables** included in the redundancy analysis: average monthly precipitation (mm; top row) and daily solar radiation (kJ/m$^2$; bottom row) among site pairs extracted from *BioClim* for the summer months (June-August). Comparisons are shown between high (>2,500m) vs. low (<2,000m) sites and sites east vs. west of the continental divide. Asterisks (*) indicate that the difference in means was significantly greater than zero (t-test, $p < 0.001$).

**TABLE C-1. SnpEff summary output** describing the number of SNPs and the percent that make up each category (variant type and effect size).

| Variant type | Effect size | No. SNPs | % |
|---|---|---|---|
| Synonymous variant | Low | 9966 | 22.312 |
| Intron variant | Modifier/intergenic | 9850 | 22.053 |
| Upstream gene variant | Modifier/intergenic | 5850 | 13.097 |
| Missense variant | Moderate | 4927 | 11.031 |
| Downstream gene variant | Modifier/intergenic | 3816 | 8.543 |
| 3’ UTR variant | Modifier/intergenic | 3381 | 7.57 |
| 5’ UTR variant | Modifier/intergenic | 2669 | 5.975 |
| Splice region variant & intron variant | Low | 2330 | 5.216 |
| 5’ UTR premature start codon gain variant | Low | 987 | 2.21 |
| Splice region variant & synonymous variant | Low | 605 | 1.354 |
| Missense variant & splice region variant | Moderate | 203 | 0.454 |
| Start retained variant | Low | 38 | 0.085 |
| Stop gained | High | 33 | 0.074 |
| Splice region variant | Low | 8 | 0.018 |
| Stop retained variant | Low | 3 | 0.007 |

**TABLE C-2. Significant genes with moderate effect**. SNP name is comprised of the chromosome, position, and allele. The gene name is from the reference genome of *Colias crocea*; the axis and loading refer to which RDA axis the SNP loaded most strongly; the predictor is the environmental variable most strongly correlated with the SNP; and the correlation coefficient (*r*) gives the correlation of the SNP with the predictor. The entries are sorted in descending order by the correlation. Finally, *d* refers to the distance the SNP is from the gene, and the product is the protein given by the reference genome (Ebdon et al., 2022).

| SNP name | Gene name | Axis | Loading | Predictor | *r* | *d* | Product |
|---|---|---|---|---|---|---|---|
| NC_059544.1. 7177668_A | LOC123694002 | 1 | 0.045 | elevation | 0.390 | 0 | acyl-CoA Delta-9 desaturase 2C transcript variant X2 |
| NC_059544.1. 7122945_G | LOC123694041 | 1 | 0.034 | elevation | 0.330 | 0 | homeobox protein goosecoid-like |
| NC_059544.1. 7177358_A | LOC123694002 | 1 | 0.046 | elevation | 0.313 | 0 | acyl-CoA Delta-9 desaturase 2C transcript variant X1 |
| NC_059544.1. 7315111_C | LOC123693984 | 1 | -0.038 | elevation | 0.307 | 0 | glutamyl aminopeptidase 2C transcript variant X2 |
| NC_059555.1. 5345599_T | LOC123700535 | 2 | 0.030 | elevation | 0.303 | 0 | luciferin 4-monooxygenase-like |
| NC_059556.1. 1200185_C | LOC123700764 | 3 | -0.026 | elevation | 0.301 | 0 | calcineurin-binding protein cabin-1-like |
| NC_059544.1. 7239708_C | LOC123693996 | 1 | -0.043 | elevation | 0.299 | 0 | acyl-CoA Delta-9 desaturase-like |
| NC_059553.1. 5419035_T | LOC123698951 | 3 | 0.027 | elevation | 0.298 | 0 | thrombospondin type-1 domain-containing protein 4-like |
| NC_059545.1. 5814484_A | LOC123694281 | 3 | 0.027 | elevation | 0.297 | 0 | pupal cuticle protein C1B-like |
| NC_059545.1. 7159493_C | LOC123694498 | 3 | -0.024 | elevation | 0.295 | 0 | DNA replication factor Cdt1 |
| NC_059544.1. 7316997_G | LOC123693984 | 1 | -0.032 | elevation | 0.290 | 0 | glutamyl aminopeptidase 2C transcript variant X3 |
| NC_059568.1. 6672404_T | LOC123705008 | 3 | -0.030 | elevation | 0.286 | 0 | protein Jumonji 2C transcript variant X2 |
| NC_059546.1. 10421035_A | LOC123694962 | 2 | -0.027 | elevation | 0.285 | 0 | 50S ribosomal protein L1 |
| NC_059542.1. 11596275_G | LOC123692468 | 3 | -0.028 | elevation | 0.285 | 0 | esterase FE4-like |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| NC_059541.1.3492498_T | LOC123691609 | 1 | -0.031 | elevation | 0.282 | 0 | post-GPI attachment to proteins factor 2-like |
| NC_059553.1.7972618_C | LOC123698839 | 3 | -0.025 | elevation | 0.280 | 0 | aldehyde dehydrogenase 2C dimeric NADP-preferring 2C transcript variant X4 |
| NC_059540.1.6401150_A | LOC123690947 | 1 | -0.031 | elevation | 0.276 | 0 | allantoinase-like |
| NC_059568.1.6672422_T | LOC123705008 | 3 | -0.029 | elevation | 0.276 | 0 | protein Jumonji 2C transcript variant X4 |
| NC_059547.1.7502564_G | LOC123695851 | 2 | -0.032 | elevation | 0.267 | 5275 | Down syndrome cell adhesion molecule-like protein Dscam2 |
| NC_059552.1.4619265_G | LOC123698540 | 3 | 0.031 | elevation | 0.262 | 0 | furin-like protease 1-2C transcript variant X4 |
| NC_059544.1.4714561_C | LOC123693833 | 1 | 0.058 | precip | 0.414 | 230 | ATP-binding cassette sub-family G member 4-like 2C transcript variant X2 |
| NC_059568.1.3080983_C | LOC123705403 | 1 | 0.057 | precip | 0.407 | 0 | calcitonin gene-related peptide type 1 receptor-like |
| NC_059544.1.4700421_G | LOC123693833 | 1 | 0.053 | precip | 0.406 | 0 | ATP-binding cassette sub-family G member 4-like 2C transcript variant X1 |
| NC_059543.1.9887011_G | LOC123693159 | 1 | 0.047 | precip | 0.396 | 0 | aquaporin-4-like |
| NC_059544.1.4684487_G | LOC123693832 | 1 | 0.060 | precip | 0.395 | 1102 | ATP-binding cassette sub-family G member 1-like |
| NC_059568.1.4545277_T | LOC123705435 | 1 | 0.063 | precip | 0.379 | 0 | carboxyl-terminal PDZ ligand of neuronal nitric oxide synthase protein 2C transcript variant X1 |
| NC_059568.1.1794266_A | LOC123705594 | 1 | 0.053 | precip | 0.376 | 0 | RUS family member 1-2C transcript variant X3 |
| NC_059568.1.1780820_T | LOC123705594 | 1 | 0.054 | precip | 0.375 | 0 | RUS family member 1-2C transcript variant X4 |
| NC_059568.1.1794571_T | LOC123705594 | 1 | 0.051 | precip | 0.370 | 0 | RUS family member 1-2C transcript variant X |
| NC_059568.1.1780792_A | LOC123705594 | 1 | 0.054 | precip | 0.368 | 0 | RUS family member 1-2C transcript variant X1 |
| NC_059568.1.1772804_T | LOC123705596 | 1 | 0.047 | precip | 0.362 | 0 | peptidoglycan-recognition protein LE-like 2C transcript variant X1 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| NC_059568.1.1772799_G | LOC123705596 | 1 | 0.047 | precip | 0.362 | 0 | peptidoglycan-recognition protein LE-like 2C transcript variant X3 |
| NC_059568.1.1774283_C | LOC123705596 | 1 | 0.048 | precip | 0.342 | 0 | peptidoglycan-recognition protein LE-like 2C transcript variant X2 |
| NC_059552.1.5309278_T | LOC123698663 | 3 | -0.030 | precip | 0.341 | 0 | histone-lysine N-methyltransferase ash1-2C transcript variant X3 |
| NC_059544.1.4591516_G | LOC123693652 | 1 | 0.054 | precip | 0.337 | 0 | limbic system-associated membrane protein-like |
| NC_059543.1.5347307_T | LOC123693042 | 1 | 0.037 | precip | 0.335 | 0 | aminopeptidase N-like 2C transcript variant X2 |
| NC_059552.1.5307161_C | LOC123698663 | 3 | -0.031 | precip | 0.330 | 0 | histone-lysine N-methyltransferase ash1-2C transcript variant X1 |
| NC_059544.1.4761959_A | LOC123693698 | 1 | 0.043 | precip | 0.330 | 0 | protein retinal degeneration B |
| NC_059568.1.4477622_C | LOC123705119 | 1 | 0.049 | precip | 0.330 | 0 | endoplasmic reticulum mannosyl-oligosaccharide 1-2C2-alpha-mannosidase 2C transcript variant X2 |
| NC_059544.1.7447292_C | LOC123693992 | 2 | -0.025 | precip | 0.320 | 0 | pyrokinin-1 receptor-like 2C transcript variant X2 |
| NC_059552.1.5307855_A | LOC123698663 | 3 | -0.031 | srad | 0.346 | 0 | histone-lysine N-methyltransferase ash1-2C transcript variant X2 |
| NC_059548.1.10084949_C | LOC123696375 | 3 | 0.035 | srad | 0.320 | 0 | muscle M-line assembly protein unc-89 2C transcript variant X3 |
| NC_059548.1.4188388_T | LOC123696216 | 3 | -0.032 | srad | 0.318 | 0 | ATP-dependent DNA helicase MER3 homolog |
| NC_059543.1.2095919_A | LOC123693421 | 3 | -0.028 | srad | 0.313 | 0 | bifunctional purine biosynthesis protein ATIC 2C transcript variant X1 |
| NC_059549.1.8664573_A | LOC123696964 | 3 | -0.024 | srad | 0.305 | 0 | BTB/POZ domain-containing protein KCTD12 |
| NC_059554.1.4956410_A | LOC123699446 | 3 | -0.032 | srad | 0.302 | 0 | claspin-like |
| NC_059547.1.5601688_A | LOC123695320 | 3 | 0.027 | srad | 0.302 | 0 | mucin-22-like |
| NC_059538.1.2018113_T | LOC123705179 | 3 | -0.028 | srad | 0.300 | 0 | UDP-xylose and UDP-N-acetylglucosamine transporter-like |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| NC_059552.1.8087020_G | LOC123698722 | 3 | -0.031 | srad | 0.300 | 0 | carbonyl reductase [NADPH] 1-like |
| NC_059550.1.9778263_A | LOC123697330 | 3 | 0.030 | srad | 0.297 | 0 | PR domain zinc finger protein 5-like 2C transcript variant X1 |
| NC_059545.1.10737092_G | LOC123694402 | 3 | 0.029 | srad | 0.296 | 0 | RNA-binding region-containing protein 3-like 2C transcript variant X1 |
| NC_059545.1.10737094_C | LOC123694402 | 3 | 0.029 | srad | 0.296 | 0 | RNA-binding region-containing protein 3-like 2C transcript variant X2 |
| NC_059547.1.6779289_T | LOC123695857 | 3 | -0.027 | srad | 0.296 | 0 | lysophospholipid acyltransferase 7-like |
| NC_059543.1.5904523_T | LOC123693351 | 3 | -0.030 | srad | 0.296 | 0 | centrosomal protein 20 |
| NC_059553.1.7972734_A | LOC123698839 | 3 | 0.028 | srad | 0.294 | 0 | aldehyde dehydrogenase 2C dimeric NADP-preferring 2C transcript variant X1 |
| NC_059560.1.5943549_C | LOC123702698 | 3 | -0.029 | srad | 0.291 | 0 | serine/arginine repetitive matrix protein 2-like |
| NC_059556.1.5332528_C | LOC123700798 | 3 | -0.026 | srad | 0.288 | 0 | glycoprotein 3-alpha-L-fucosyltransferase A-like |
| NC_059548.1.10084947_A | LOC123696375 | 3 | 0.032 | srad | 0.286 | 0 | muscle M-line assembly protein unc-89 2C transcript variant X2 |
| NC_059553.1.3844322_C | LOC123698996 | 3 | -0.027 | srad | 0.286 | 0 | peptidyl-prolyl cis-trans isomerase FKBP8 2C transcript variant X2 |
| NC_059557.1.182909_T | LOC123701463 | 3 | -0.032 | srad | 0.284 | 0 | prolyl 3-hydroxylase sudestada1 |