DISSERTATION

EVOLUTION AT THE EDGE:
HOW HIBERNATION, HEAT WAVES, AND HYBRIDIZATION IMPACT A RANGE EXPANSION

Submitted by

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ABSTRACT

EVOLUTION AT THE EDGE:
HOW HIBERNATION, HEAT WAVES, AND HYBRIDIZATION IMPACT A RANGE EXPANSION

Evolutionary processes shape the diversity of life on earth. Over millennia, species diverge from one another, radiating out into the tree of life. The same processes of evolution are also acting in much shorter periods of time, selecting for traits, mixing genes across populations, and generating new mutations each generation. These rapid evolutionary processes interact with ecological processes, which are happening on similar time scales. Range expansions, or expansions of a population's geographic distribution, were once considered strictly ecological processes of populations interacting with other populations and the environment, unaffected by evolution. However, modern theory understands range expansions to be crucibles of rapid evolution. Rapid evolution shapes the process of range expansion itself, and is also integral to determining the outcomes of range expansion. During range expansions, ecological and evolutionary processes intertwine, combining to shape the dynamics of a range expansion, like where a population can establish, and how quickly the expansion moves. The study of evolution during range expansions has only just begun to make it out of the theory to be tested in wild populations in nature, so we don't yet know how common evolution during range expansion is, or how large its effects might be. Here, I explore how evolution impacts range expansions that are current and ongoing in natural systems in the wild. I focus on the tamarisk beetle (*Diorhabda spp.)*, deliberately introduced in the United States about two decades ago for biological control of a widespread invasive weed. Through its role as a biological control agent, the tamarisk beetle has expanded its range hundreds of kilometers along rivers, colonizing new areas of the invasive weed in environments very different from its original release habitat. The range expansion of the tamarisk beetle provides a unique opportunity to study evolution during an ongoing natural range expansion across an environmental gradient. Through the following four chapters, I document evolution of dispersal ability and life history traits (Chapter 1), evolution of seasonal dormancy and genetic variation of that trait (Chapter 2), evolution of phenotypic plasticity (Chapter 3), and the impacts of hybridization (Chapter 4). Throughout, I discuss the implications for biological control and the tamarisk beetle specifically, and more generally how these results improve our understanding of how evolution is caused by, enables, and alters natural range expansions over short time periods, even in natural range expansions.

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DEDICATION

For my parents

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CHAPTER 1: EVOLUTION OF REPRODUCTIVE LIFE-HISTORY AND DISPERSAL TRAITS DURING THE RANGE EXPANSION OF A BIOLOGICAL CONTROL AGENT[1]

1.1 Introduction

Ecological and evolutionary processes acting during range expansion (Bowler & Benton, 2005; Kokko & López-Sepulcre, 2006; Kubisch et al., 2014) are key to understanding the spread of invasive species (Hastings et al., 2005), potential success of biological control agents (Szűcs et al., 2019), and the ability of threatened species to track recent climate change (Mustin et al., 2009). The landscapes encountered by range expanding populations represent novel selective environments (Brown et al., 2013; Van Petegem et al., 2016), and simultaneously, the expansion itself can be a catalyst for evolution through spatial sorting and founder effects (Phillips et al., 2010b; Shine et al., 2011). Thus, range expansion can result in evolved differences in reproductive life-history and dispersal traits between individuals at the core of the range and the edge of the expansion front (Peischl et al., 2013; Phillips, 2015; Simmons & Thomas, 2004). We seek to evaluate such shifts occurring in a range expansion across a natural and heterogenous environment. Through documenting those patterns, and how they align with different theoretical predictions (**Figure 1-1**), we then can explore the possible evolutionary processes that may be acting on life-history and dispersal traits.

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Theory predicts that reproductive life-history traits, such as fecundity and age at first reproduction, can evolve in contrasting ways at the expanding edge relative to the core, depending upon whether selection or non-adaptive evolutionary processes are dominant at the expansion front (Phillips et al., 2010b). When selection is dominant, the edge of an expanding population is predicted to exhibit higher fecundity and earlier age at first reproduction than the core (Burton et al., 2010; Fronhofer & Altermatt, 2015). The stable, high-density core of a population generally exhibits density-dependent growth, where selection favors the ability to compete at high densities of conspecifics through having fewer, larger offspring (Phillips et al., 2010b). At the expansion front, population densities are low and competition is relaxed, so the edge generally exhibits density-independent growth (Altwegg et al., 2013; Burton et al., 2010) and selection favors high fecundity and early reproduction **Figure 1-1A**, solid arrow) (Brommer

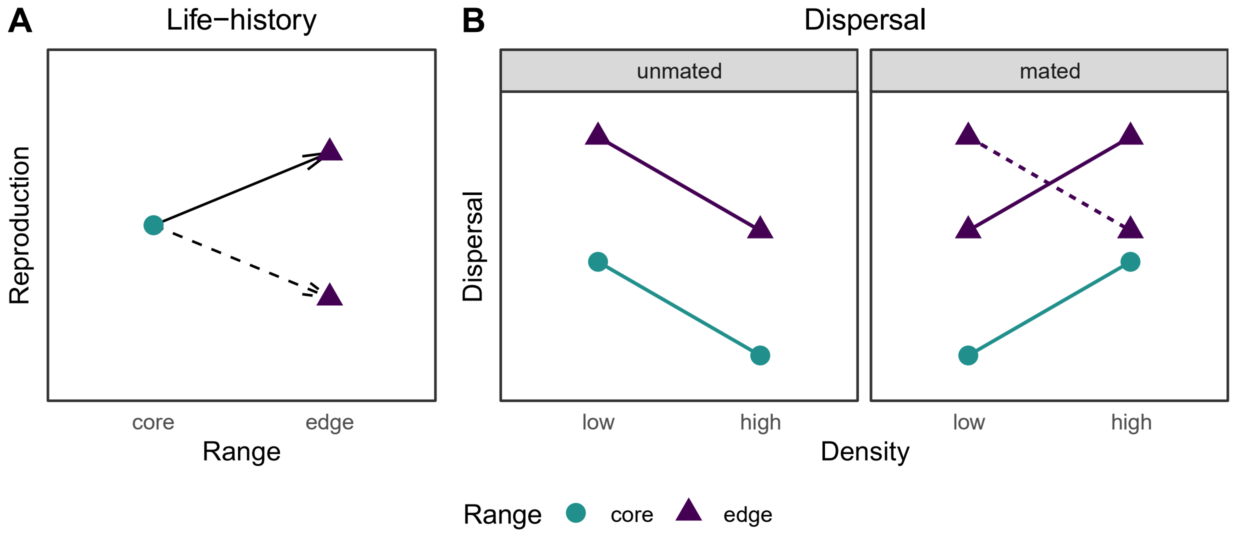

Figure 1-1. Life history and dispersal predictions

Reproductive life-history traits in edge populations may increase due to selection at low densities (A, solid line) or decrease due to expansion load (A, dashed line) compared to core. Dispersal is predicted to be informed by density and mating status and evolve upwards at the range edge (B). Density-dependence may also evolve at the edge (B, dashed line).

et al., 2002; Phillips et al., 2010b). Alternatively, when non-adaptive processes are dominant, the edge may experience reduced fitness, or expansion load, relative to the core (**Figure 1-1A**, dashed arrow) (Peischl et al., 2013; Peischl & Excoffier, 2015; Travis et al., 2007). This occurs when there are so few individuals at the edge that genetic drift can overwhelm selection, and deleterious alleles may 'surf' the wave of expansion during repeated founder events (Klopfstein et al., 2006). Additionally, fecundity at the edge may be reduced relative to the core due to trade-offs between dispersal, reproduction, and competitive ability (Burton et al., 2010; Fronhofer & Altermatt, 2015; Phillips et al., 2010b). There is model and experimental evidence for both the evolution of increased fecundity at the edge (Phillips, 2009; Siemann & Rogers, 2001) and the evolution of decreased fecundity at the edge (González-Martínez et al., 2017; Peischl et al., 2013). Insights from range expansions in natural rather than laboratory conditions will help elucidate which processes dominate in nature.

Theory predicts that dispersal will evolve at the expanding edge to increase relative to the core through the process of spatial sorting (Phillips et al., 2008; Shine et al., 2011; Travis & Dytham, 2002). Spatial sorting occurs when individuals with greater dispersal ability arrive at the range edge together and mate with each other. Since dispersal ability is heritable in many species (Saastamoinen et al., 2018), this spatially assortative mating among individuals at the edge produces offspring with even higher dispersal ability. Despite strong evidence for spatial sorting (e.g. Berthouly-Salazar et al., 2012; Hill et al., 2011; Lombaert et al., 2014; Merwin, 2019; Monty & Mahy, 2010; Phillips et al., 2006, 2010a), some factors may inhibit or weaken evolutionary shifts in dispersal between core and edge. For example, expansion over heterogenous landscapes (like latitudinal changes in climate or host plant genotype) and adaptation to novel environments could slow expansion speed and reduce spatial selection on dispersal (Andrade-Restrepo et al., 2019; Hillaert et al., 2015). Additionally, species that are unlikely to disperse from low-density patches may be less likely to evolve increased dispersal ability at the edge of the range expansion (Fronhofer et al., 2017a; Travis & Dytham, 2002).

Dispersal is a multi-faceted behavior that involves individual choices about whether and how far to move. Dispersal may be informed by intraspecific interactions such as the presence of relatives and population density (Bitume et al., 2013; Endriss et al., 2019), and factors internal to the organism such as body condition, sex, or mating status (**Figure 1-1B**) (Clobert et al., 2009; Schumacher et al., 1997). For many species, high population density can signal strong intraspecific competition, which may increase emigration (positive density-dependence) (Altwegg et al., 2013). Alternatively, species for which the benefit of living near conspecifics (e.g. mate availability, predator avoidance, reduced Allee effects) outweigh the cost of competition may decrease dispersal at high population densities (negative density-dependence) (Bowler & Benton, 2005). During range expansion, spatial selection increases dispersal even when population density is low, so density-dependent dispersal that is less strongly positive, or even negative, may evolve at the range edge (**Figure 1-1B**, dashed line) (De Bona et al., 2019; Fronhofer et al., 2017b; Travis et al., 2009).

Mating status may also influence dispersal decisions for sexually reproducing species that can disperse before and after mating (Clobert et al., 2009; Li & Kokko, 2019; Schumacher et al., 1997). Mated individuals may show positive density-dependent dispersal to reduce competition and reproduce in a low density environment where offspring might have a better chance of survival (**Figure 1-1B**, right), while unmated individuals may show negative density-dependent dispersal and disperse more from low density to increase the chances of finding a mate (**Figure 1-1B**, left) (Clobert et al., 2009).

We can infer the relative dominance of evolutionary processes during a natural range expansion by evaluating the patterns of key reproductive life-history and dispersal traits across the range. We use the range expansion of *Diorhabda carinulata,* an introduced biological control agent (hereafter, biocontrol agent) and examine patterns in life-history and dispersal traits to evaluate drivers of evolutionary change in range expansions in natural populations. Success of the biocontrol program has provided impetus for research, including this study, focused on the evolutionary and ecological processes enabling beetles to suppress a major invasive plant across western North America (Bean et al., 2012). This study contributes to a growing literature testing range expansion theory on natural populations (Phillips et al., 2006, 2010a; Wolz et al., 2020) and is the first test we know of in a modern biocontrol agent (Szűcs et al., 2019). Understanding the evolutionary dynamics of biocontrol agents is of particular interest for predicting future spread and improving efficacy and safety across the range of a target pest species (Stahlke et al., 2022; Szűcs et al., 2019; Van Klinken & Edwards, 2002; Wright & Bennett, 2018).

Diorhabda carinulata, the northern tamarisk beetle, was released in 2001 into the western United States for the biological control of invasive riparian shrubs in the genus *Tamarix*, or saltcedar or tamarisk (DeLoach et al., 2003). Beetles have dispersed southward from a few initial release sites (**Figure 1-2**) (Bean et al., 2012) following remote riparian corridors, which likely represent independent dispersal pathways. The *D. carinulata* range expansion provides an excellent study system for testing the predictions of range expansion theory because original release sites are precisely known, the range expansion has been monitored, and expansion along river corridors provides natural spatial replicates of expanding edge collection sites (Bean & Dudley, 2018). We use a common garden to evaluate the patterns of evolution of early fecundity, age at first reproduction, body mass, and dispersal of eight collections of *D. carinulata,* collected from the core and edge of its range in the western US.

Figure 1-1 summarizes how we can infer the evolutionary processes occurring during range expansion based on the pattern of trait values we find at the core and edge. Specifically, an increase in reproductive output of individuals from the edge relative to the core supports selection at low densities as the dominant process, while decreased reproductive output of edge individuals supports expansion load or trade-offs as the dominant process (**Figure 1-1A**). Increased dispersal among individuals from the edge indicates that spatial sorting is acting during this range expansion (**Figure 1-1B**), while if dispersal does not change between core and edge, other factors, such as adaptation to the new environment, may be limiting the opportunity for spatial sorting. We also expect that individuals' dispersal choices will be influenced by the contexts they experience, specifically density and mating status (**Figure 1-1B**). If density-dependence has evolved during the range expansion, we predict that dispersal will be less positively or negatively density-dependent for mated individuals from the range edge (**Figure 1-1B**, dashed line).

1.2 Materials and methods

Diorhabda carinulata are highly specialized leaf eating beetles that feed on plants in the genus *Tamarix*. The primary host plants (hybrids between *Tamarix ramosissima* and *Tamarix chinensis*) occupy riparian corridors throughout the area of the *D. carinulata* range expansion, providing abundant habitat for the beetle from range core to edge and beyond. During the summer, male *D. carinulata* emit a pheromone that attracts other beetles and likely assists in mate-finding (Bean et al., 2007b; Cossé et al., 2005). It is also common to observe a single beetle on a tree, especially when there are few beetles in an area. In the late summer, adults are cued by shortening daylengths to enter reproductive diapause for the winter (Bean et al., 2007b). The beetle has adapted to variation in daylength along latitude during this range expansion by shortening the daylength cue so that the timing of diapause remains synchronized with the season at southern latitudes (Bean et al., 2007a).

Eight collections of *Diorhabda carinulata* were made from across the introduced range of the species. At least 200 adult beetles were collected by hand at each site, with no more than five beetles being collected from a single tree where possible. Four collections were from well-established original introduction sites in the north of the range and represent the range core. Another four collections were from the newly established southern edge of the range (**Figure 1-2**, **Table A-1**; RiversEdge West, 2021).

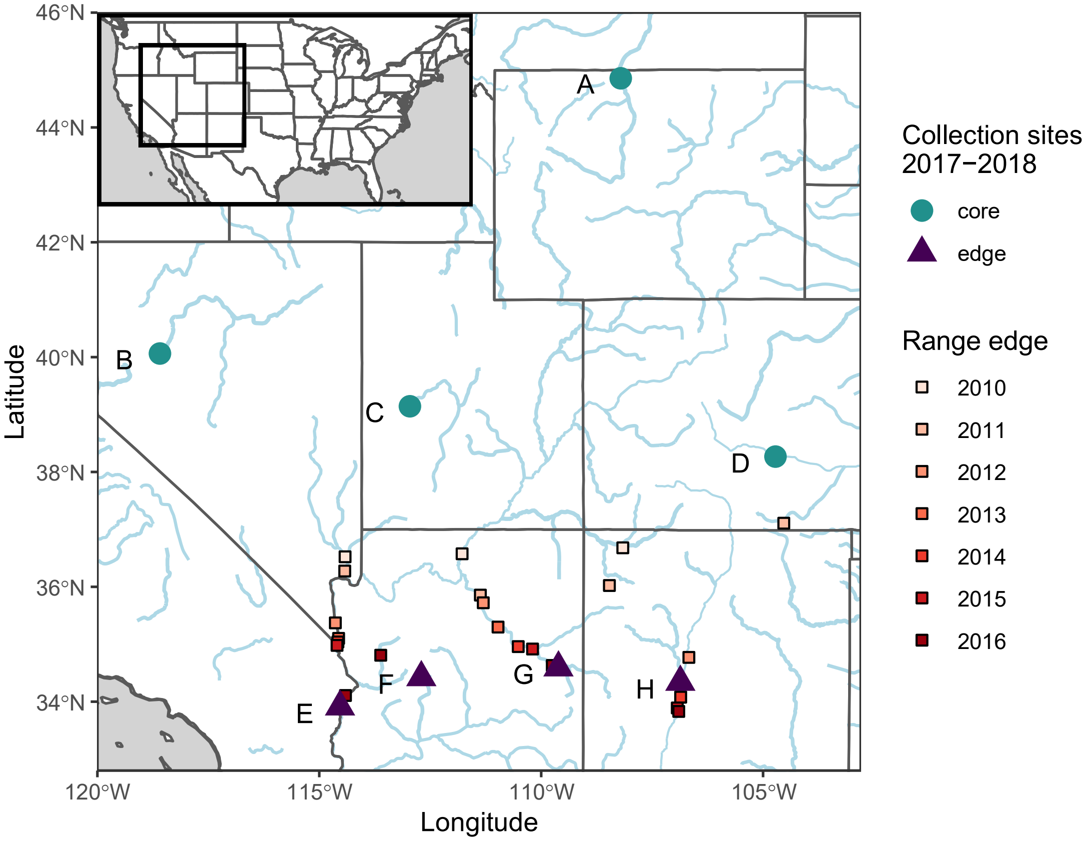

Figure 1-2 Range expansion and collection sites

D. carinulata range expansion along river corridors (squares show edge locations each year and were jittered to show overlapping points), and collection sites at the core and edge (circles and triangles). Letters correspond to ***Table A-1****. Range edge data are from the RiversEdge West Tamarisk Beetle Distribution Map, available at: https://arcg.is/1izOPW0 (RiversEdge West, 2021).*

One edge site (H in **Figure 1-2** and **Table A-1**) was collected slightly behind the edge in 2017 in order to avoid other *Diorhabda* species that were moving northward and overlapping the *D. carinulata* range (Ozsoy et al., 2019). Two sites (one core and one edge) were collected Fall 2017 and the first lab generations were put into reproductive diapause to reduce the number of lab generations before the start of the experiment. All other sites were collected in Summer 2018 and cultured in the lab for one generation to standardize maternal environment effects prior to starting experiments. All insects were reared in growth chambers with a 16h/8h light/dark cycle and 25°C/20°C day/night temperatures (reproductive conditions (Bean et al., 2007b)), and were fed fresh tamarisk as needed.

1.2.1 Life-history

Newly emerged adult females of the second lab generation were weighed before feeding and reared individually thereafter in 0.24 L plastic containers with mesh lids. Three days after eclosion, each female was paired with a male from the same collection site of about the same age and allowed to mate for 24 hours in the same containers as above before the male was removed. Presence of eggs in each container was assessed daily. All eggs that were laid on the first day of oviposition were counted to provide a measure of early fecundity and the age at first reproduction in days since adult emergence was recorded. Individuals that had not oviposited within seven days of emergence were recorded as non-layers (Lewis et al., 2003). We collected mass and egg count data from 130 core and 140 edge female *D. carinulata*, and age at first reproduction from 104 core and 118 edge females.

1.2.2 Dispersal

Dispersal ability was measured for only male *D. carinulata* since, in the field, males have been observed dispersing first and using pheromones to attract mixed-sex aggregations of reproductive adults (Cossé et al., 2005). After emergence as adults, males were randomly assigned to mating treatments and density. As described above, both mating status and density experienced can influence dispersal decisions as, for example, unmated beetles might fly in search of mates, or beetles that find themselves in higher densities of conspecifics might fly in search of low-competition sites. Males assigned to the mated treatment were paired with a female from the same collection site for 24 hours. The males were thereafter reared in 0.24 L plastic containers with mesh lids in groups of five (high density) or alone (low density). All males in each high-density container were of the same mating treatment. All containers received the same surplus amount of fresh tamarisk, regardless of how many beetles were in the container. Density conditions were designed to allow us to estimate how local social interactions will impact decisions to disperse and not intended to simulate density conditions in the field. Male beetles were between 6 and 23 days after eclosion during the dispersal trial and were weighed on the day of the dispersal trial.

We assessed dispersal of male beetles using tethered flight mills (reviewed in Minter et al., 2018), similar in design to Maes et al. (2014) (**Appendix A.2**). Each beetle was given one hour to take any number of flights on a flight mill. Data from each trial was converted into four dispersal elements: occurrence of at least one flight, number of flights, total flight distance, and average flight speed (**Appendix A.3**). Each dispersal element has different biological relevance (Stevens et al., 2013; Tung et al., 2017). Occurrence of flight and number of flights measure the probability and frequency of movement from the local patch. Total distance and average speed measure how far individuals disperse, after initiation. Spatial sorting may act on any one or combination of these elements. We collected dispersal data from 279 core males and 311 edge males, with 65 to 81 males in each density-mating treatment combination and at least 15 from each population. Average speed was calculated for 231 core and 266 edge males that took at least one flight, with 56 to 71 males in each density-mating treatment combination.

1.2.3 Statistical Analyses

The three life-history traits of female body mass, fecundity over 24 hours, and age at first reproduction were analyzed both individually and with a multivariate analysis of variance (MANOVA). In the MANOVA, only individuals that produced eggs during the experiment were included, since they were the only complete observations. The three life-history traits were the response variables, and range (core or edge), collection site, and eclosion date were fixed effects. No random effects were included in the MANOVA.

Mass of female beetles at adult emergence was analyzed individually with a linear mixed model, with range as a fixed effect and collection site as a random effect.

Since some females (17%) did not lay eggs within 10 days, the data on the numbers of eggs were split into two datasets, one including the number of eggs for laying individuals, and the other including the binary response (laying or non-laying) for all individuals, to assess whether probability of laying and fecundity differed between core and edge sites. To account for overdispersion, a negative-binomial mixed model was fit to the count data (excluding non-laying individuals) using the glmmTMB package version 1.0.2.1 (Brooks et al., 2017) with range, mass of beetle at emergence, and age at first reproduction as fixed effects and collection site as a random effect. A logistic mixed model was fit with the glmmTMB package to the binary dataset with range and mass as fixed effects and collection site as a random effect. Age at first reproduction could not be included as a covariate due to convergence issues.

Age at first reproduction was analyzed for individuals that reproduced during the experiment using a Conway-Maxwell Poisson mixed model which accounts for under-dispersion in this dataset (Brooks et al., 2019), with range and mass as fixed effects and collection site as a random effect. We complemented this analysis with a Kaplan-Meier survival analysis, which accounts for censoring of individuals that either did not reproduce during the experiment or died before reproducing. Since covariates and random effects cannot be added in Kaplan-Meier survival analyses, the analysis was run twice, once with collection site as the predictor to visualize the spread among sites and again with range as the predictor to estimate the total effect of range.

Mass of male beetles at the time of the dispersal trial was analyzed with a linear mixed model, with range, rearing density (low or high), mating status (unmated or mated), and interactions between those factors as fixed effects, age at time of weighing as a fixed covariate, and collection site as a random effect.

Each of the four dispersal elements (occurrence of flight, number of flights, total distance, and average speed) were analyzed separately. We chose not to do multivariate analyses on the dispersal traits since all but one variable was highly zero-inflated and skewed, which violates assumptions of multivariate tests such as MANOVA, and univariate tests could better incorporate sampling design and environmental covariates during the trials, which greatly improve model fit. For each dispersal element, the same factors were included. Range, density, mating status, and all interactions were fixed effects. Mass at the time of dispersal trial, age, mill friction (**Appendix A.2**), and air temperature were fixed covariates. Collection site and trial date were random effects.

The occurrence of at least one flight was analyzed with a binomial model with the packages lme4 version 1.1-26 (Bates et al., 2015) and lmerTest version 3.1-3 (Kuznetsova et al., 2017). The number of flights during the 1-hour trail was analyzed using both a negative binomial and a Poisson mixed model with the glmmTMB package. QQ plots of model residuals and residual vs. fitted plots from the DHARMa package version 0.3.3.0 were used to assess model fit (Hartig, 2020). The negative binomial mixed model best met assumptions of normality of residuals and was chosen as the final model. Total distance was a count of revolutions of the flight mill and thus a discrete variable. Six models were fit for total distance: a linear mixed model on log-transformed distance, a negative binomial mixed model, a generalized Poisson model (commonly used for highly right skewed data with a high frequency of low counts (Brooks et al., 2019; Joe & Zhu, 2005)), and a zero-inflated version of each of those, using the glmmTMB package. Based on the same residual diagnostics as above, the zero-inflated generalized Poisson model best met assumptions of normality of residuals and was chosen as the final model. Average speed during the 1-hr trial included only trials in which the beetle made at least one flight and was analyzed with a linear mixed model using lme4 and lmerTest.

In all dispersal models, the three-way interaction was dropped from the model if it was not statistically significant. Post hoc comparison of means was done with the emmeans package version 1.5.4 (Lenth, 2020). All analyses were done in R version 3.6.2 (R Core Team, 2022).

1.3 Results[2]

1.3.1 Life-History

The MANOVA showed statistically significant differences in reproductive life-history traits between core and edge, collection sites, and eclosion dates (range: Pillai–Bartlett statistic$_1$=0.093, p<0.001; collection site: Pillai–Bartlett statistic$_6$=0.133, p=0.046; eclosion date: Pillai–Bartlett statistic$_3$=0.359, p<0.001). Across all three traits, reproduction increased about 7% at the edge compared to core.

In the univariate analysis, mass of females at adult emergence ranged from 6.5 to 14.7 mg. Females from the edge of the range were larger than females from the core on average (core mass=9.65 mg SE=0.163; edge mass=10.30 mg SE=0.160; F$_1$=8.04, p=0.030; **Figure 1-3A**).

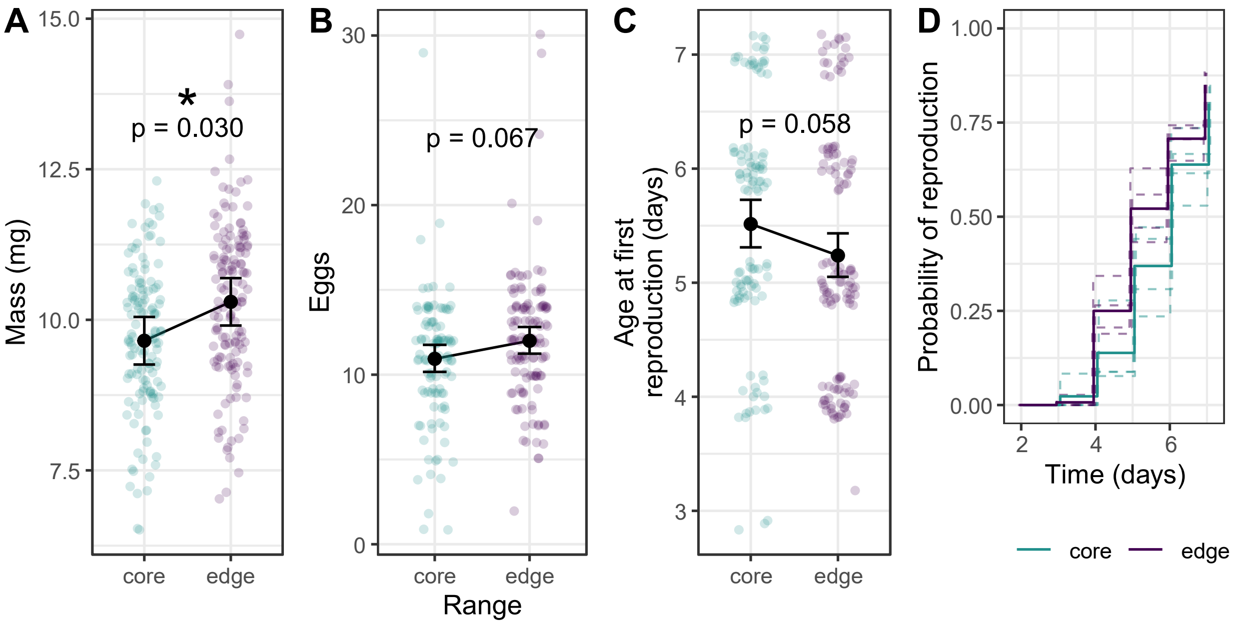

Figure 1-3. Life history results

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[2] Data from this study are archived at Ag Data Commons: https://doi.org/10.15482/USDA.ADC/1522916

Means and 95% confidence intervals of the life-history traits of female body mass (A), 24-hr fecundity (B), and age at first reproduction, analyzed with regression (C) and Kaplan-Meier survival analysis (D). Observations from laying individuals only are shown in B-C and are jittered to show individual points. In D, solid lines represent means of core and edge only and dashed lines are each population.

The number of eggs from the first day of reproduction ranged from 0 to 30. The proportion of beetles laying eggs during the study did not differ between the core and edge (core proportion=0.813, edge proportion=0.845, χ_1^2=0.46, p=0.499). Of those that oviposited during the study, edge beetles tended to be more fecund (core eggs=10.9, SE=0.40; edge eggs=12.0, SE=0.40; χ_1^2=3.39, p=0.067; **Figure 1-3B**). Age at first reproduction was not a predictor of the number of eggs laid (χ_1^2=0.01, p=0.916), but larger females laid more eggs (χ_1^2=5.79, p=0.016).

Age at first reproduction ranged from three to seven days after adult emergence. From the regression analysis, edge beetles tended to reproduce earlier than core beetles (core age=5.51 days, SE=0.11; edge age=5.24 days, SE=0.10; χ_1^2=3.64, p=0.058; **Figure 1-3C**). Mass at emergence was not a predictor of age at first reproduction (χ_1^2=1.92, p=0.166). Results were similar in the Kaplan-Meier survival analysis. Median age at first reproduction was 5 days (95% CI: 5, 6) for edge and 6 days (95% CI: 6, 6) for core (**Figure 1-3D**). The log-rank test for differences in survival curves indicated marginal differences between core and edge (χ_1^2=3.8, p=0.051).

The mass of males at the time of the dispersal trial (thus, after feeding *ad libitum*) ranged from 6.9 to 19.3 mg. There was no difference in mass of males between core and edge sites (F_1=0.67, p=0.445). Older beetles weighed more than younger beetles (F_1=46.55, p<0.001) and males reared at high density weighed more than those reared at low density (high mass=12.2 mg, SE=0.134; low mass=11.8 mg, SE=0.135; F_1=15.09, p<0.001).

1.3.2 Dispersal

All four dispersal elements were positively correlated with each other (Spearman rank-order correlation, all pairwise comparisons $p<0.05$). In the statistical models, the three-way interaction between range, density, and mate status was not statistically significant in any model, so it was removed from all models. Weight was significantly positively associated with all four dispersal elements (**Table A-2**). Results for covariates and random effects are in **Table A-2**.

During the dispersal trials, 84.2% of all beetles took at least one flight. For occurrence of flight, the interaction between mating status and range was statistically significant (χ^2_1=4.14, p-value=0.042), indicating unmated edge beetles were more likely to fly than unmated core beetles (core probability=0.84, edge probability=0.93; z=-2.309, p=0.021), but there was no difference for mated beetles (core probability=0.86, edge probability=0.84; z=0.356, p=0.722; **Figure 1-4A**). The interaction between density and mate status was close to statistical significance (χ^2_1=3.01, p=0.083), such that unmated beetles tended to fly more than mated beetles at low density, but there was no difference between mated and unmated at high density (**Figure A-2A**).

The number of flights during the 1-hr trial ranged from 0 to 13 (median=2). The interaction between density and range was statistically significant (χ^2_1=4.93, p=0.026), indicating at low density, edge beetles took more flights than core beetles (core flights=1.68, SE=0.15; edge flights=2.13, SE=0.17; t_{576}=-2.064, p=0.040), but there was no difference at high density (core flights=2.03, SE=0.16; edge flights=1.93, SE=0.15; t_{576}=0.490, p=0.625; **Figure 1-4B**). The interaction between density and mate status was close to statistical significance, demonstrating that unmated beetles tended to take more flights than mated beetles at low density and the opposite at high density (χ^2_1=3.48, p=0.062) (**Figure A-2B**).

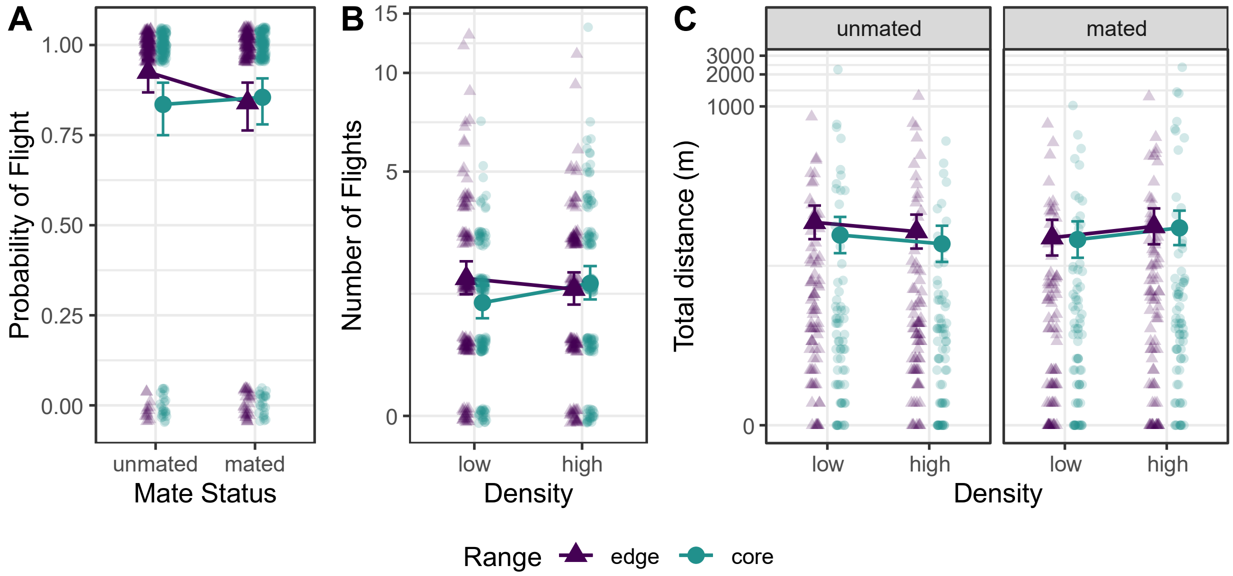

Figure 1-4. Dispersal results

Means and 95% confidence intervals of dispersal elements. Mating status-dependent dispersal evolved from core to edge for probability of flight (A), and density-dependent dispersal evolved from core to edge for number of flights (B). Flight distance is informed by mating status and density (C). Observations have been jittered to show individual points. Note log-scale y-axis in B and C.

Individuals flew up to 2.3 km (median=8 m), during the 1-hr trial. The interaction between density and mate status was statistically significant in the final model, such that unmated beetles flew further than mated beetles at low density, but the opposite at high density (χ_1^2=6.13, p=0.013; **Figure 1-4C**). The range main effect was also close to statistical significance, indicating edge beetles tended to fly further than core beetles (core distance=59.6 m, SE=10.01; edge distance=69.5, SE=11.45; χ_1^2=2.998, p=0.083), and this difference was most pronounced for unmated beetles at low density.

Average flight speed ranged from 0.138 m/s to 0.914 m/s, and did not differ by range, density, mate status or their interactions **(Figure A-2C**).

1.4 Discussion

Theory predicts that both life-history and dispersal traits will evolve during range expansion (e.g. Peischl et al., 2015; Peischl & Excoffier, 2015; Phillips et al., 2010b; Shine et al., 2011), with different evolutionary processes driving distinct patterns of evolution. It is unclear how well theoretical predictions hold up in complex natural systems including biocontrol agents, and which evolutionary processes appear to drive trait changes across range expansions (Szűcs et al., 2019). We studied the recent range expansion of the biocontrol agent *D. carinulata* in the western US and infer the dominant evolutionary processes acting during the range expansion by comparing life-history and dispersal traits from individuals at the core and edge of the range.

A trend towards higher fecundity and earlier age at first reproduction in individuals from the edge suggests that selection at low densities dominates over expansion load in driving evolution of reproductive life-history traits at the edge of the range. Females from the edge laid on average only one more egg than those from the core on the first day of reproduction. While this pattern was only weakly supported statistically, the biological effects could be large. If this difference persists throughout the multi-week lifespan of adults, it could sum to a substantial difference in fitness between core and edge individuals. Additionally, early fecundity in *D. carinulata* is a good predictor of lifetime fecundity (Bitume et al., 2017). Edge females also reproduced earlier than core beetles. Earlier age at first reproduction is a trait classically associated with "r" selection of low density environments (Stearns, 1992). Age at first reproduction is an often-overlooked trait that can be just as important for fitness as fecundity itself, since reproducing earlier can increase the total time available for an individual to reproduce and early offspring often have an advantage over later offspring because there are fewer other offspring to compete with (Stearns, 1976). Adults reaching reproductive maturity faster could allow more generations per year, which might allow acceleration of the range expansion from edge populations of *D. carinulata*, which are also less constrained by cold temperatures in the winter (Jamison et al., 2018). We used mass as an additional gauge of fecundity, since insect body size is often related to egg production and how many eggs females can carry (Berger et al., 2012). We found this association between mass and fecundity to hold in *D. carinulata* and that individuals from the edge were larger than those from the core. Higher fecundity, earlier age at first reproduction, and larger mass of females all suggest that selection has increased reproductive capacity at the edge of the range expansion. While the effect sizes are small for each individual trait, when viewed together, this provides strong evidence for a selection-driven shift in reproductive life-history traits across the range about 15 years after initial biocontrol releases.

These same phenotypic patterns provide evidence against the evolution of increased genetic load (or reduced fitness) in edge sites relative to core sites. Recent evidence of high genetic diversity along one *D. carinulata* expansion front (Stahlke et al., 2022) is consistent with no measurable genetic load, as the deleterious alleles responsible for genetic load are likely at low frequency in diverse populations. Low genetic load and high genetic variation among *D. carinulata* may also be explained by the pre-release history of this biocontrol agent. Specifically, they were collected from multiple source populations and population sizes were deliberately large to avoid reducing variation that could increase establishment in the field (Stahlke et al., 2022; Szűcs et al., 2017).

Dispersal is an inherently and notoriously variable behavioral trait (Bowler & Benton, 2005) and we found this to be true for *D. carinulata*, even when measuring dispersal in a controlled lab environment. Accounting for the density and mating context of dispersal decisions in our experiments allowed us to observe context-dependent evolution between core and edge more clearly and test hypotheses about the mechanisms behind the patterns we see. The occurrence of flight was affected by mating status along the range expansion such that unmated beetles from the edge flew more than those from the core. This implies that the response to being unmated has evolved between core and edge. Males on the edge may have evolved this behavior if mate availability is often low at the edge. For the number of flights, dispersal became negatively density-dependent at the edge, such that the number of flights increased in low density environments compared to core, while staying about the same in high density environments. This implies that the response to density has evolved during range expansion. Evolution of the response to density could be due to selection for increased dispersal at range edges at low density, as predicted by theory (De Bona et al., 2019; Fronhofer et al., 2017b; Travis et al., 2009). Estimates of beetle density in the field would be valuable for further exploring this mechanism. However, accurately measuring beetle density in the field is not without challenges, as density can vary within a collection site due to many factors, including beetle life stage, ephemeral weather patterns, and plant health (Henry et al., 2018), as well as range expansion.

Edge beetles flew further than core beetles across all density and mating treatments, though this pattern was most pronounced for unmated beetles at low density, as expected by condition-dependent dispersal theory. Unlike with occurrence of flight and number of flights, the relationship of distance flown with mating status and density did not change over the range, but we do find a weak signature of spatial sorting of dispersal ability. In this species, spatial sorting might primarily act on occurrence or frequency of flights rather than flight distance or speed if most dispersal flights driving the range expansion are comprised of multiple frequent flights to catch air currents, instead of long-distance flights. Future studies will be needed to explore how

D. carinulata disperse in nature and how spatial sorting acts on different dispersal elements in natural systems.

The effect of spatial sorting in the range expansion of *D. carinulata* system could be small because of maladaptation to novel environments on the edge of the range expansion that slow down range expansion and reduce assortative mating between dispersive individuals at the edge (Andrade-Restrepo et al., 2019; Hillaert et al., 2015). Early in its range expansion, *D. carinulata* was maladapted to photoperiod cues (Bean et al., 2007a, 2012) and possibly higher summer temperatures in southern latitudes (Herrera et al., 2005). Adaptation to photoperiod has limited the rate of southern range expansion in this beetle and thus may reduce the effect of spatial sorting of dispersal. Despite this, our results suggest that spatial processes during range expansion may be important to natural range expansions even over heterogenous environments.

In many species, dispersal evolves along with suites of traits, called dispersal syndromes (Ronce & Clobert, 2012), and in some cases, many life-history traits may correlate well with dispersal (Stevens et al., 2013). Trade-offs between dispersal and reproductive ability are widely hypothesized to be present due to allocation of finite resources (Bonte & Dahirel, 2017; Stearns, 1989) though support for such trade-offs during range expansion is mixed (e.g. Hughes et al., 2003; Jan et al., 2019; Kelehear & Shine, 2020; Tabassum & Leishman, 2018; Therry et al., 2015). In the *D. carinulata* range expansions, we do not see evidence of a trade-off between dispersal and life-history traits, though we were unable to measure all traits within the same individuals. One hypothesis for how this species has apparently increased dispersal and avoided genetic load is due to their ability to aggregate using a male-produced pheromone (Bean et al., 2007b; Cossé et al., 2005). While the expanding edge is led by individuals that are superior dispersers (thus allowing for spatial selection on dispersal), they can use pheromones to attract others to the area and population sizes can quickly increase, thus decreasing the negative effects of small population sizes. We measured dispersal for only male *D. carinulata*, but there are many reasons for dispersal to differ between the sexes (reviewed in Li & Kokko, 2019). There may also be trade-offs between other traits, such as lifespan or immune system development or function (reviewed in Chuang & Peterson, 2016).

Long-term success of the *Tamarix-Diorhabda* biocontrol program requires *D. carinulata* to continue its spread to cover the range of the target weed and to adapt to new environments. We show an increase in both reproductive output and dispersal ability in some contexts at the edge and low genetic load, which may enable an accelerating expansion front and will likely contribute to the establishment and persistence of *D. carinulata* populations at the edge (Phillips et al., 2010a). Evolution of these traits and others previously studied (Bean et al., 2012; Stahlke et al., 2022) suggests that there is sufficient genetic variation for populations to continue to adapt to novel environments during the expansion. As the first test of evolutionary theory of range expansions in a modern biocontrol agent, we show that these theoretical predictions can be applied to range expansions across heterogeneous environments, especially when the ecological context of individuals is included. We may expect to find selection at low densities to be the dominant evolutionary process over expansion load and for spatial sorting to act on other biocontrol agents that share many characteristics with *D. carinulata* (e.g. Bartelt et al., 2008; Muller-Scharer et al., 2014). Our results suggest that evolutionary processes impacting range expansions of natural populations can act simultaneously with adaptation to environmental gradients.

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CHAPTER 2: ADAPTATION AT THE EDGE: PATTERNS OF LOCAL ADAPTATION AND GENETIC VARIATION DURING A CONTEMPORARY RANGE EXPANSION[3]

2.1 Introduction

Understanding the factors that allow expansions of species' distributions is a major theme of ecology and evolutionary biology (Holt, 2003). Theory and model systems show that evolution during range expansion can lead to differences between populations at the core of a range and those at the expanding front, or edge (Miller et al., 2020). The process of range expansion itself can sort individuals spatially, based on dispersal ability, leading to an evolved increase in dispersal at the edge (Travis & Dytham, 2002), and repeated founder events and population bottlenecks can lead to spatial genetic drift and expansion load at the edge (Peischl et al., 2015). Theory and experimental work on these expansion dynamics usually focus on expansion across uniform environments, to which the populations are adapted. However, in nature, most range expansions occur into novel or patchy environments, or across environmental gradients, such as changing climates across latitudes. A few laboratory and theoretical studies show that novel or patchy environments influence expansion speed and success (Andrade-Restrepo et al., 2019; García-Ramos & Rodríguez, 2002; Gilbert et al., 2017; Polechová & Barton, 2015; Urquhart & Williams, 2021; Williams et al., 2016), but little is known about natural range expansions over environmental gradients and how adaptation over environmental gradients is influenced by spatial sorting and repeated founder events. Thus, a crucial area to

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advance research on range expansions is to understand expansion dynamics across natural environmental gradients.

Much research focuses on phenotypes expressed in populations well after range expansions have occurred, and often find evidence of adaptation to novel environments (Carbonell et al., 2021; Ittonen et al., 2022; Lancaster et al., 2015; Medley et al., 2019; Oduor et al., 2016; Urbanski et al., 2012). For example, a mosquito adapted to earlier winter onset in higher latitudes after its invasion in the United States (Medley et al., 2019; Urbanski et al., 2012). However, adaptation to local conditions is not ubiquitous, suggesting that populations may remain maladapted following range expansion or that other factors, like phenotypic plasticity, facilitate persistence across a range of environments (Ebeling et al., 2011; Ross et al., 2009; Zhao et al., 2013). These studies make important advances in understanding the power of adaptation, and patterns of adaptation following successful expansion, but are generally limited in the replication of the range expansion and are conducted well after the range expansion has already successfully taken place (but see Ittonen et al., 2022; Medley et al., 2019). Thus, in addition to studying range expansions across natural environmental gradients, an important next step is to understand the evolutionary dynamics occurring at the core and edge of ongoing natural range expansions.

In particular, in active range expansions across environmental gradients, the process of adaptation in response to selection in novel environments will be ongoing, providing a more dynamic perspective on evolution during range expansion. If sufficient quantitative genetic variation is present, we expect that populations that have been established for longer, near the core of the range will be locally adapted, but that populations that are newly established at the range edge may reflect their environment of origin, be in the process of adapting to their new environment, or show evidence of rapid adaptation to the new environment.

The outcome of selection in new environments will depend in part on the genetic variation available for adaptation, measured as heritability and evolvability, in the populations at the origin of the expansion. Low genetic variation in origin populations will hinder the response to selection in novel environments. Since trait values, and phenotypic and additive genetic variation estimated from them, depend on the environment in which they are measured, the environment will influence the amount of genetic variation expressed in a population (Hoffmann & Merilä, 1999; Rudin et al., 2019; van Heerwaarden et al., 2016; Winterhalter & Mousseau, 2007). A meta-analysis on wild populations suggests that stressful environments decrease heritability, either by directly reducing the additive genetic variance of a trait, or by increasing the total phenotypic variation of a trait thereby reducing the proportion of variability explained by additive effects (Charmantier & Garant, 2005).

In temperate climates with seasonally harsh conditions, organisms must synchronize life events like emergence, migration, and dormancy to the seasons [26]. Latitudinal clines in the timing of dormancy, specifically, are ubiquitous across many taxa (Bradshaw & Holzapfel, 2001; Ittonen et al., 2022; Urbanski et al., 2012), because the timing of harsh conditions and the seasonal cues that precede those conditions change across latitude (Bradshaw & Holzapfel, 2007). The timing of dormancy is under strong selection to synchronize an organism with climate, host plant, prey, and mutualist phenology, and the environmental cues that precede harsh conditions at their local site (Bradshaw & Holzapfel, 2007; Chuine, 2010; Grevstad et al., 2022; Grevstad & Coop, 2015; Iler et al., 2021; Van Asch & Visser, 2007). Thus, during a range expansion across latitude in a seasonal environment, a main constraint will be whether populations can adaptively respond to the seasonality of the new location.

Here, we study an active range expansion across an environmental gradient, to understand the evolution of seasonality across the range. We use the northern tamarisk beetle (*Diorhbada carinulata*) for this work. This beetle was introduced to the United States for biological control of a widespread invasive riparian plant. The beetle established only at release sites above the 38th parallel, because of mismatches between when diapause (insect dormancy) was initiated and the climate at sites below 38°N (Bean, Dudley, et al., 2007). After establishment, the photoperiod cue used for diapause initiation evolved, reducing the phenological mismatch and allowing the beetle to start expanding its range from north to south (Bean et al., 2012). This range expansion follows the target host, which grows along rivers, creating naturally separated expansion fronts that provide meaningful replicates to study how the cues for diapause are evolving. Genomic data has revealed that genetic diversity has largely been maintained during the southward range expansion (Stahlke et al., 2022) and phenotypic data has shown that males from edge populations are more dispersive and females are larger and more fecund than core populations, indicating both spatial sorting and selection at the edge (Clark et al., 2022). Here, we ask 1) how responses to daylength cues change between the core and recently colonized edge of the range and whether this pattern is consistent with local adaptation and 2) if adaptation to novel environments may be constrained by quantitative genetic variation expressed in novel environments.

2.2 Materials and Methods

2.2.1 Study system

The northern tamarisk beetle (*Diorhabda carinulata*: Coleoptera, Chrysomelidae, hereafter tamarisk beetle), was released as a biological control agent into the United States in 2001 to help manage the invasive woody shrub tamarisk (*Tamarix spp.*). In the field, the beetle has two to six generations per year, depending on location (Bean, Dudley, et al., 2007; Jamison et al., 2018).

Diapause in the tamarisk beetle is initiated in late summer or fall by a photoperiod cue, slightly influenced by temperature (Bean, Dudley, et al., 2007; Dalin et al., 2010). When reproductive individuals experience photoperiods shorter than a threshold value, they enter the process of diapause by resorbing reproductive organs and accumulating metabolic reserves within the fat body (Bean, Wang, et al., 2007; Koštál, 2006). All life stages, from larva to adult, are sensitive to the photoperiod cue, but larvae exposed to short daylengths will continue development until the adult stage before overwintering in leaf litter beneath the host plant (Bean, Wang, et al., 2007). The appropriate timing of diapause is important for fitness and is likely to be under strong selection (Chuine, 2010; Van Asch & Visser, 2007), since diapause too early in the season reduces individual fecundity, number of generations per season, increases the risks from predation and desiccation during the summer, and uses more metabolic reserves (Bean, Dudley, et al., 2007). Diapause too late in the season increases the risk of mortality as individuals are exposed to lethal temperatures and starvation after host plants senesce (Koštál, 2006). Because the host plant, tamarisk, senesces and loses its leaves each winter, even in areas with mild winters, diapause in the tamarisk beetle is still necessary to avoid starvation across our study sites (Friedman et al., 2011).

2.2.2 Diapause timing traits

While many studies compare diapause responses to photoperiod across populations using the critical daylength, which is the daylength that induces diapause in 50% of a population (e.g., Bean et al., 2012; Lankinen et al., 2013; Tanaka et al., 2015), genetic variance and heritability is best measured among individuals, not populations. We turn to a related aspect of diapause, the amount of time an individual takes to initiate diapause at a single, constant daylength, to quantify diapause responses in individuals. We call this trait days until diapause. We use this trait because insects store information about photoperiod across several days and initiate diapause when a threshold has been reached (Takeda & Suzuki, 2022). Days until diapause is related to the genetically encoded individual threshold and captures individual differences in sensitivity to a daylength. Previous experiments on the tamarisk beetle have also shown that adult females ceased oviposition 5 to 20 days after being placed in diapause-inducing daylengths, with fewer days required in shorter daylengths (Bean, Dudley, et al., 2007; Bean, Wang, et al., 2007), indicating variation in the trait among individuals and across daylengths. Days to diapause, while distinct from critical daylength, is importantly related to critical daylength, since the proportion of individuals from a population that eventually enter diapause indicates whether the population is above (less than 50% in diapause) or below their critical daylength (more than 50% in diapause).

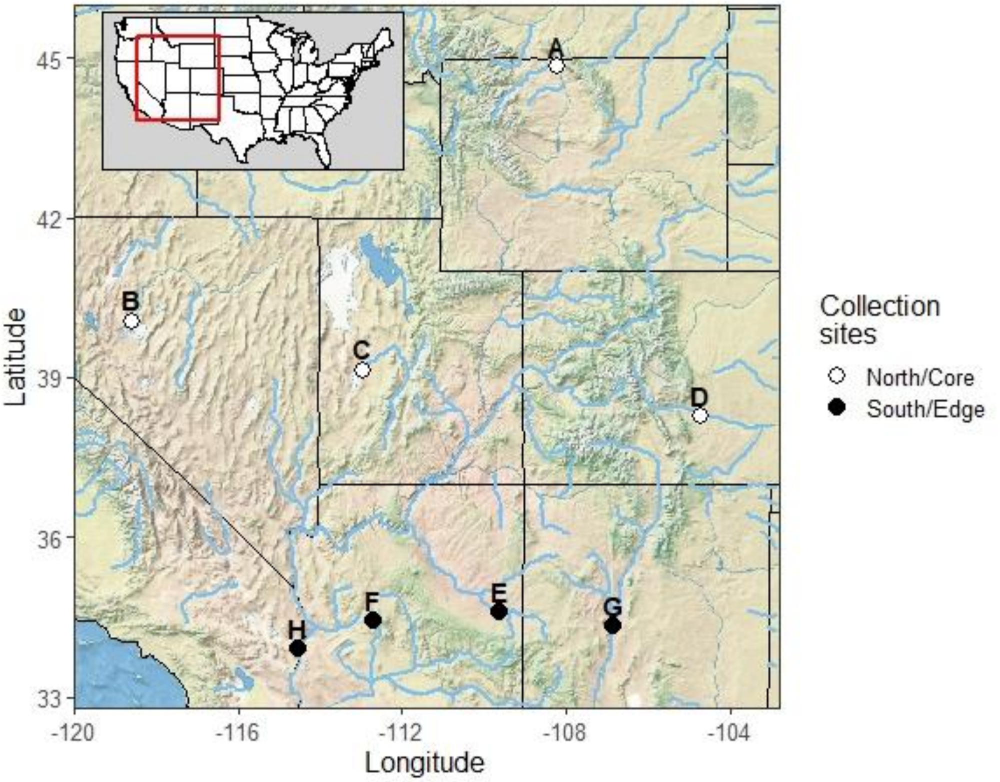

Figure 2-1 Collection Sites

Collection sites for the tamarisk beetle in the western United States. Letters refer to ***Table B-1****. Northern sites are original release sites of the tamarisk beetle and are in the range core. Southern sites were at the expanding range edge when collected. Genetic variation of diapause timing was only measured for Site C. All sites were used to examine the pattern of local adaptation of diapause timing.*

2.2.3 Reciprocal environment experiment to test for local adaptation

To evaluate local adaptation at the core and expanding edge of the range, we used the same collections as in Clark et al. (Clark et al., 2022). Briefly, adult tamarisk beetles were collected by hand from *Tamarix* spp. at eight sites at the core and edge of the range in the United States (**Table B-1 , Figure 2-1**) in Fall 2017 and Spring 2018. Northern/core sites were chosen to be original release sites from the biocontrol program (Carruthers et al., 2008), and the southern sites were chosen to be at the leading edge of the southward range expansion and had arrived within the previous year, except for population G (La Joya, New Mexico), which likely arrived four years prior to our collection, based on survey data (https://riversedgewest.org/documents/previous-annual-tamarisk-beetle-maps). We collected at site G to avoid collecting a sibling species expanding its range northward within the same river system. To standardize the effects of maternal environment, beetles were maintained in growth chambers under reproductive conditions of 16/8hr light/dark per day and 25/20°C day/night and fed fresh tamarisk as needed for one generation. For the reciprocal environment experiment, mated female adult beetles from the second lab generation were reared individually in 0.24 L plastic containers with mesh lids. Between 13 and 16 days after adult emergence, females were placed in growth chambers programmed to daylengths that represented diapause-inducing photoperiods from the north (14:20 hr:min of light per day) or south (12:40 hr:min of light per day). The sensitive stage for diapause initiation in the tamarisk beetle extends through adulthood, and thus diapause responses can be measured in adults switched from longer to shorter daylength environments. The daylength treatments were chosen to be near the critical daylength (daylength at which 50% of a population enters diapause) for Delta, Utah (site C, a northern core site), and Topock Marsh, Arizona (near site H, a southern edge site) in 2017 (unpublished data, Dan Bean). Four growth chambers in total were programmed to the two daylength environments and containers of beetles were rotated between the four chambers during the light period each day, keeping the daylength treatment experienced by each individual constant, to equalize growth chamber effects among treatments. Temperatures varied between light and dark periods as in the standard rearing procedure above, which was chosen to mimic natural conditions in the field.

We recorded diapause incidence and days until diapause for an average of 33 and minimum of 10 females per population per treatment, for a total of 521 individuals. Each day, we recorded whether each female laid any eggs in the previous 24 hours. When a female had not laid eggs in 7 consecutive days, that individual was scored as in diapause, starting from the first day with no eggs (Bean, Wang, et al., 2007). Days until diapause was the number of days it took a female to stop laying eggs after switching into the daylength treatment. Females were inspected for 43 days in the treatments, and females that were still laying eggs at that time were scored as reproductive (not in diapause). In our pilot experiments, there was no instance of a female that stopped oviposition for seven consecutive days starting to lay eggs again, so we consider this a reliable indicator that a female had started the process of entering diapause. Since the treatments were chosen to be close to the critical daylengths of northern and southern populations, a good fit to the environment is indicated by intermediate diapause incidence (e.g., 50%).

2.2.4 Measuring quantitative genetic variation

To understand how genetic variation in diapause timing and its expression may have impacted range expansion, we measured genetic variation in days until diapause and two morphological traits, body mass and thorax width. We focused on one collection site (site C, Delta, Utah) at the origin of the range expansion, which we collected in 2019 using the same methods as for the eight populations collected for the reciprocal environment experiment. Delta was an original tamarisk beetle release site in 2001 (Carruthers et al., 2008). The critical daylength of populations collected from Delta has been stable for many years and it is considered to be well adapted to the location (unpublished data, Dan Bean). This site is especially relevant to the southward range expansion because it was shown to be more genetically similar to the southern sites than the other northern populations (Stahlke et al., 2022).

Heritability (h^2) and evolvability (I^A) are metrics useful for understanding the genetic variation available for evolution of phenotypic traits (Hansen et al., 2011; Houle, 1992; Lynch & Walsh, 1998). Heritability scales additive genetic variation by total phenotypic variation of a trait, while evolvability scales additive genetic variation by the mean value of the trait and is interpreted as the proportion change in a trait over a generation of selection. Evolvability has been suggested to be the better measure of evolutionary potential, while heritability has been used historically, so we provide both (Hansen et al., 2011; Houle, 1992). Because heritability and evolvability are a function of the environment in which they are measured (Lynch & Walsh, 1998), we used two daylength regimes that simulated diapause-inducing daylengths from the northern (home) and southern (away) parts of the range. The northern treatment of 13:55 hr:min of light per day was chosen as the daylength when approximately 80% of individuals from Delta would enter diapause. We chose this rather than the critical daylength, because our trait, days until diapause, requires diapause to occur to be measured, so this daylength increased the number of individuals entering diapause on which we could measure days until diapause. The southern treatment of 13:26 hr:min of light per day was chosen as the daylength when all or nearly all individuals from Delta would enter diapause and represents the critical daylength of tamarisk beetles living near Lake Mohave, Nevada, about 480 km south of Delta. The temperature during these experiments was 28/20°C lights-on/lights-off for both treatments. Interactions between temperature and daylength in the diapause initiation cue have been shown to be minimal for this population (Dalin et al., 2010).

We used a paternal half-sibling breeding design (Lynch & Walsh, 1998) to estimate genetic variance components of days until diapause, body mass, and thorax width. Thirty-nine sires (males) of the second lab generation were each mated to seven or eight dams (females). Eggs were collected from each dam and reared in full-sibling families. When larvae were 3rd instars, density was standardized to 15 larvae per full-sibling family per 0.24 L container, to reduce environmental variation that might obscure additive genetic variation. When the offspring reached adulthood, we measured mass at adult emergence (before feeding) and thorax width on two females and one male per full-sibling family. We included these traits because female mass has increased at edge sites compared to core sites, while male mass has not clearly changed during the range expansion (Clark et al., 2022) and thus, we can investigate whether heritability hindered selection on these traits. These traits also provide a comparison of the heritability estimations within the same families and individuals as the diapause trait. After mating with non-siblings, the same two females from each full-sibling family were then split into the two daylength environments, reared individually for 43 days, and days until diapause was measured as above. We produced 238 full-sibling families from 38 sires and between 1 and 8 dams per sire. We measured days until diapause on 212-214 female adult offspring per daylength treatment, weight for 730 adults, and thorax width for 615 adults.

2.2.5 Statistical Methods

2.2.5.1 Reciprocal Transplant

Our goal from the reciprocal environment experiment was to determine if northern and southern sites (core and edge) had diverged in diapause timing during the range expansion, whether the patterns suggests local adaptation, and which environmental variables (latitude, elevation, and growing degree days) explain variation between sites within core and edge in their home environment.

To explore how diapause initiation was impacted by daylength across the range of the tamarisk beetle, diapause incidence was predicted by beetle origin (north or south), daylength treatment (northern or southern diapause-inducing daylengths), and their interaction as fixed effects in a logistic regression model with logit link function. Collection location (factor with eight levels) was a random effect. We aimed to examine how diapause incidence varied by collection site, however, since 100% of individuals from four collection locations entered diapause in the southern daylength treatment, we were unable to fit a standard logistic regression due to complete separation (which happens when a predictor variable or combination of predictor variables perfectly predict an outcome) (Gelman et al., 2008). To resolve this, we used a logistic regression with Cauchy priors, as suggested by Gelman et al. and implemented in the arm R package (Gelman et al., 2008; Gelman & Su, 2022), which can provide estimates even with complete separation. Diapause incidence was predicted by collection site, daylength treatment, and their interaction as fixed effects.

Days until diapause can only be measured if individuals enter diapause, but many individuals did not enter diapause during the experiment. Thus, we ran two Poisson models, one on the full dataset and one on only the individuals that entered diapause. For the full Poisson model, non-diapausing beetles were given a value of 43 days until diapause, because this was the last day on which we collected data. We included non-diapausing individuals in this analysis because they represent biologically important variation within the data and assigning 43 days to these individuals is a conservative way to include them in the dataset, since these individuals will likely never enter diapause at the treatment daylengths. Including all samples inflates estimates of days until diapause for those that did enter diapause, but gives a more complete picture of diapause timing across a whole population. Excluding non-diapausing samples accurately estimates days until diapause for those that did enter diapause. In both Poisson models, days until diapause was predicted by beetle origin (north or south), daylength treatment, and their interaction as fixed effects in a Poisson model with a log link function. Collection location was included as a random effect. To examine the pattern of days until diapause across individual populations, we fit separate Poisson models on the full and diapausing-only data subsets. For both models, days until diapause was predicted by collection site, daylength treatment, and their interaction as fixed effects.

We also analyzed days until diapause across the range and between populations with a survival regression model. While this model has the benefit of accounting for censoring in the data, it makes the assumption that non-diapausing individuals would eventually enter diapause, which is not likely to be a good assumption. Methods for this analysis are presented in **Appendix B.2.2**.

To determine how the responses of individual populations were influenced by environmental factors that vary across the range expansion, like latitude, elevation, climate, and growing season length. We approximated climate and growing season length using the total cumulative growing degree days for the tamarisk beetle (calculations of all site characteristics are in **Appendix B.1**). We performed these analyses on the days until diapause response of the four northern populations in the local northern daylength treatment and the four southern populations in their local southern daylength treatment separately. We chose to analyze populations in their local environment, since this will more accurately represent the variation present in the field. Because all three environmental predictors were highly collinear (Variance Inflation Factor >5), we performed separate linear regressions for each site characteristic for the northern and southern data subsets (i.e., six total models, where days until diapause in the northern or southern subset of data was predicted by latitude, elevation, or degree days). Poisson models were fit with the GLMMTMB R package (Brooks et al., 2017) and survival models in the *survival* R package (Therneau, 2020). We visually assessed model fit with Q-Q and residual vs. fitted plots in the DHARMa package (Hartig, 2020). Statistical significance of effects was determined with Wald Chi-square tests in the car package (Fox & Weisberg, 2019). Post-hoc tests of differences between marginal means were done using the emmeans package (Lenth, 2020). Analyses were performed in R version 4.2.0 (R Core Team, 2022).

2.2.5.2 Components of genetic variation

To include all individuals in the estimation of genetic variation, non-diapausing individuals were assigned a value of 43 days until diapause, a conservative approach, as described above in the days until diapause analysis. Days until diapause in the two daylength treatments were taken to be different traits and thus analyzed separately. Variance components were estimated using restricted maximum likelihood (REML). We used a half-sibling design with sire as a random effect (Lynch & Walsh, 1998). Variance due to dam could not be estimated due to the half-sibling breeding design (we assume maternal variance to be similar to sire variance). Additive genetic variance was estimated to be $V_A = 4*V_{sire}$ (based on the paternal half-sibling breeding design) and total phenotypic variance $V_P = V_{sire} + V_{resid}$ (Lynch & Walsh, 1998). Narrow-sense heritability (h^2) was calculated as $h^2 = V_A/V_P$. Evolvability (I_A), or the expected proportional change in a trait under a unit strength of selection, was calculated as $I_A = V_A/m^2$, where m is the trait mean (Hansen et al., 2011; Houle, 1992). Likelihood ratio tests were used to determine the significance of the sire variance component (V_{sire}). Standard error and confidence intervals around V_A, V_P, h^2, and I_A were calculated with a bootstrap method, following Houde & Pitcher (2016). In short, samples of sires were drawn from the observations with replacement up to the original sample size and the variance components, heritability, and evolvability were calculated as above for each sample. Variance, standard error, and confidence intervals were calculated from the distribution of 1000 iterations.

For body mass at emergence and thorax width, analyses were separate for males and females, since tamarisk beetle females are generally larger than males (Lewis et al., 2003). Only one male per full-sib family was measured, so we used a half-sibling design with sire as a random effect and all variance components were calculated as above (Lynch & Walsh, 1998). Two females per full-sib family were measured, so we used a full-sibling design with two random effects: sire and dam nested within sire. For females, additive genetic variance was estimated to be $V_A = 4*V_{sire}$, as above, but total phenotypic variance was estimated as $V_P = V_{sire} + V_{dam(sire)} + V_{resid}$ (Lynch & Walsh, 1998). Heritability, evolvability, and standard deviations of all variance components were calculated with the same bootstrap method.

2.3 Results

2.3.1 Diapause responses shift across the range

We find clear shifts in an adaptive trait, diapause timing, across the range expansion. In each daylength treatment, beetles from core and edge sites diverged in their diapause responses, both in the proportion of beetles that entered diapause and the average number of days to diapause. The three analyses of days until diapause yielded similar results, so we present below the results for the two Poisson models. Results from the survival analysis on days until diapause are included in the Appendix (**Figure B-2**).

In the northern daylength environment (longer daylength), 57% of core beetles entered diapause, compared to only 9% of edge beetles (comparison Z=2.40, *P*=0.0164) (**Figure 2-2A**). Similarly, of those that entered diapause, core beetles took 10.64 days to enter diapause, compared to 21.01 days for edge beetles (comparison Z=-3.72, *P*=0.0002) (**Figure B-1**). The difference between core and edge in days to diapause was not statistically clearly different when all diapausing and non-diapausing beetles were considered (core=23.8 days, edge=35.2; Z=-1.45, *P*=0.1471), though the direction of the response is the same as when only diapausing beetles were included (**Figure 2-2B**).

In the southern environment (shorter daylengths), 99% of core beetles entered diapause, compared to only 76% of edge beetles (comparison Z=2.45, *P*=0.0142) (**Figure 2-2A**). Considering only those that entered diapause, core beetles took 4.13 days to enter diapause, compared to 6.90 days for edge beetles (comparison Z=-2.83, *P*=0.0046) (**Figure B-1**). For all diapausing and reproductive beetles, core beetles took 4.47 days to enter diapause, compared to 16.99 days for edge beetles (comparison Z=-4.88, *P*=<0.0001) (**Figure 2-2B**).

These comparisons in each environment show that beetles from the core and edge of the range expansion have diverged in their responses to daylength cues and the differences are genetically based, as revealed by the common garden design. At long daylengths, southern populations require more days at that daylength to start the diapause process compared to northern populations, while, at short daylengths, northern populations require very few days to enter diapause, compared to southern populations.

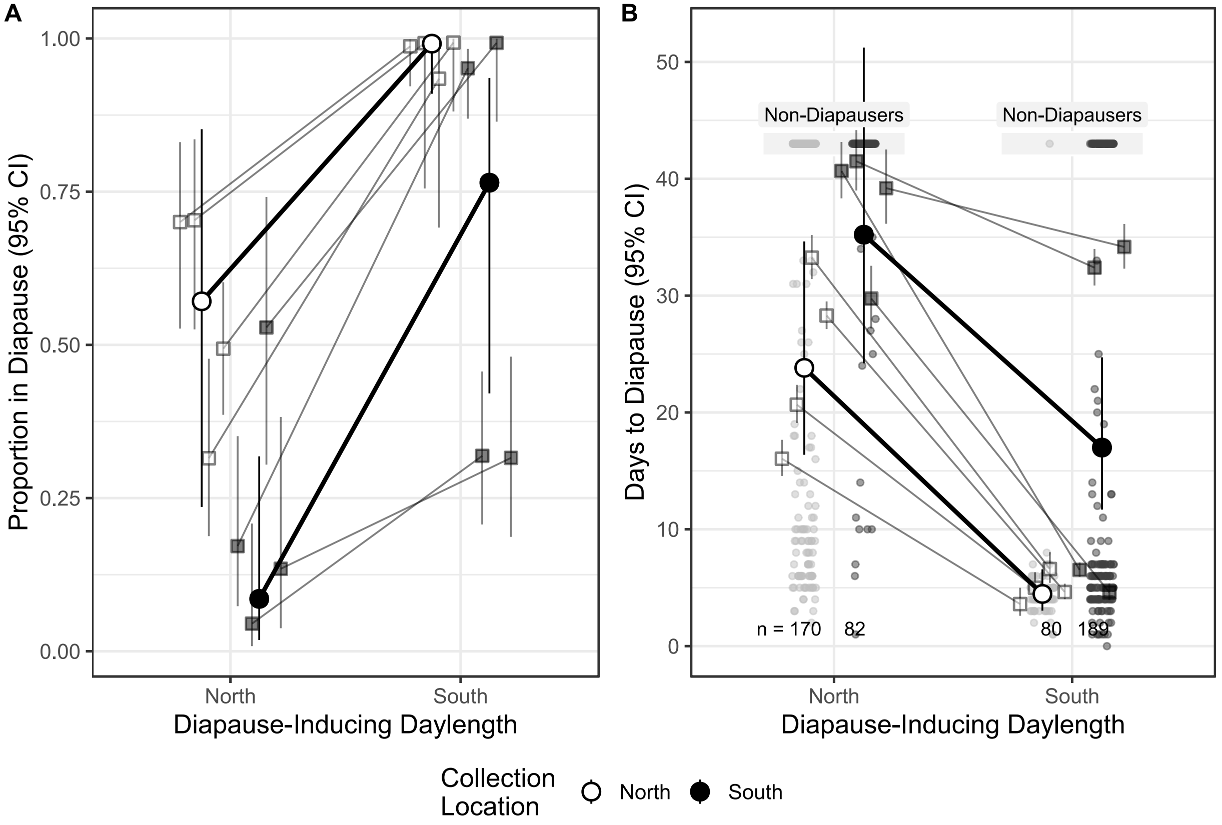

Figure 2-2 Diapause timing estimates

Model estimates of ***A)*** *proportion of beetles in diapause and* ***B)*** *days until diapause in northern and southern diapause-inducing daylength treatments. In both panels, large white and black circles indicate means of core and edge, respectively. Light and dark grey squares indicate means for individual core and edge populations, respectively. In panel B, points in the background indicate days until diapause for each beetle measured. Points are not present in panel A, since all responses were at 0 or 1. In panel B, non-diapausing beetles are included at a value of 43 days to diapause in calculations of means and those points are indicated in the boxes at the top of the figure.*

2.3.2 Some populations show a pattern of local adaptation

The evolution we observe in response to daylength between the core and edge sites is likely the result of adaptive evolution. We can infer the fitness consequences of diapause timing in each environment based on the well-documented relationship between fitness and phenology in the tamarisk beetle (Bean et al., 2012) and many other taxa (Chuine, 2010; Iler et al., 2021; Urbanski et al., 2012). Because the daylength treatments were chosen to be close to the critical daylength of collections from the north and south, based on previous data (Bean et al., 2012), populations that are adapted to the daylength treatment or a daylength close to it are expected to have close to 50% of individuals enter diapause. This percent is approximate because the populations are rapidly evolving (Bean et al., 2012), and limits on the number of growth chambers required that we pick only two daylengths to represent daylengths across the range.

Northern populations in the longer, northern daylength all show proportions in diapause close to 50%, while the southern populations have very low proportions in diapause, meaning in a northern environment, southern populations would delay diapause and likely have reduced fitness when they are unprepared for winter freezes. Although, one southern site, Site G, appears to be adapted to the northern daylength treatment, since about 50% entered diapause. In the shorter, southern daylength, the southern populations were split in their responses with two appearing to be adapted to more northern daylengths, while two appear to be adapted to more southern daylengths. All northern populations appear maladapted to the southern daylength treatment, since much more than 50% enter diapause. Northern populations in general appear well adapted to their local environment and maladapted to a novel environment, as would be expected for locally adapted populations. The southern populations vary in their responses to the southern treatment, but are generally maladapted to the northern treatment, indicating they have diverged from the northern populations, but based only on daylength, may not be adapted to their current environments. Though, selection on these populations could be occurring based on environmental factors other than daylength.

2.3.3 Site characteristics explain some variation among populations

We also sought to understand how environmental factors influenced the specific response to daylength of each population, since populations may be adapting to multiple aspects of the physical environment during range expansion. We approximated these effects with latitude, elevation, and degree days. For northern sites, latitude and elevation were the strongest predictors of days until diapause, explaining 7% and 8% of the variation in days until diapause in the data, respectively (**Figure 2-3A**). Cumulative degree days explained less variation in days until diapause (3%), indicating that northern populations in general are less sensitive to temperature and climate than they are to the physical relationship between daylength and latitude.

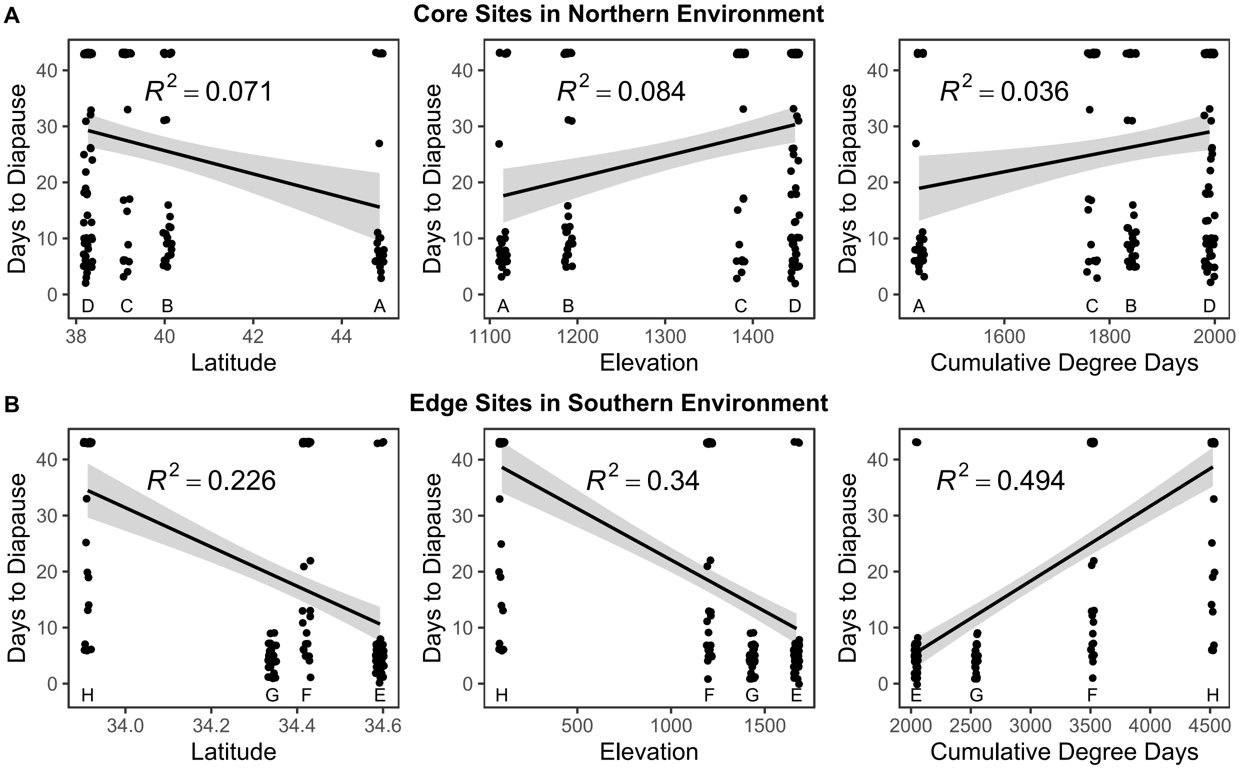

Figure 2-3 Diapause timing across environments

*Linear regressions for days until diapause with latitude, elevation, and cumulative degree days for **A**) core sites in the northern environment, and **B**) edge sites in the southern environment. All regressions were modeled separately, due to high collinearity between all predictors. Letters indicate populations, as in **Table B-1**.*

For the southern populations, cumulative degree days was the strongest predictor of days to diapause, explaining nearly 50% of variation in days until diapause. Latitude and elevation also explained substantial portions of variation (22% and 34%, respectively) (**Figure 2-3B**). These three variables were much more correlated with days until diapause for edge populations than they were for core populations, though it should be noted that the available latitudes of edge populations were constrained, and all were within a single degree of latitude. The optimal timing of diapause appears to be much more sensitive to temperature and site characteristics for edge populations than core populations.

2.3.4 Genetic variance of days until diapause

We estimated variance components, heritability, and evolvability of days until diapause for individuals from northern site C in two daylength environments, because site C was an original release site and is more genetically similar to the edge of the range expansion than other northern sites (Stahlke et al., 2022). Family means for days until diapause were more variable in the northern (home) environment (10-26 days) than the southern (away) environment (7-8 days) (**Figure 2-4**). Genetic variance components depended on the environment in which they were measured. Variance due to sire was highly statistically significant in the northern environment (LRT_1=10.93, P=0.0009), but not statistically significant in the southern environment (LRT_1=0.41, P=0.52). Total phenotypic variance and additive genetic variance were reduced in the southern environment compared to the northern environment (**Figure 2-5A, Table B-2**). Heritability of days until diapause was estimated at 0.76 (95% CI 0.13, 1.42) in the northern environment and 0.12 (95% CI 0, 0.52) in the southern environment (**Figure 2-5B, Table B-2**). Evolvability of days until diapause was significantly positive in the northern environment

(I_A=0.50), but not significantly different from zero in the southern environment (I_A=0.04, **Figure 2-5C, Table B-2**).

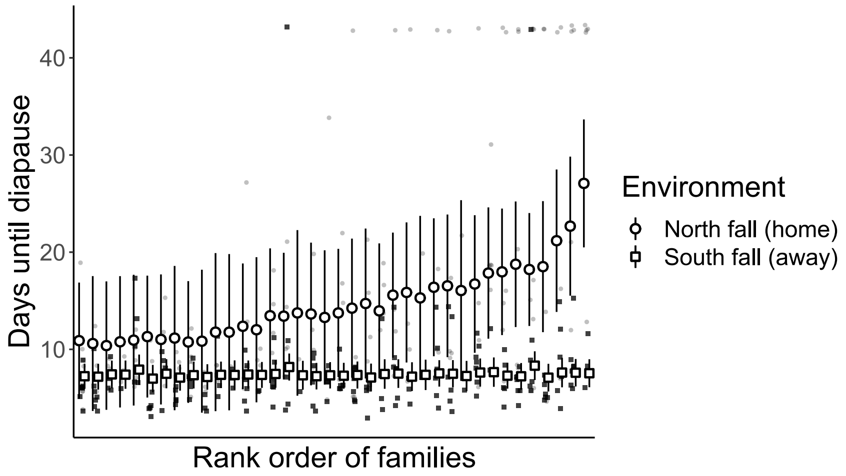

Figure 2-4 Variation in days until diapause across families

Variation in days until diapause among half-sibling families from site C in northern (home) and southern (away) daylength environments. Family estimates (95% CI) are ordered along the x-axis by increasing mean days until diapause in the home treatment. Grey circles and black squares show days until diapause for individual females in the northern and southern environments, respectively.

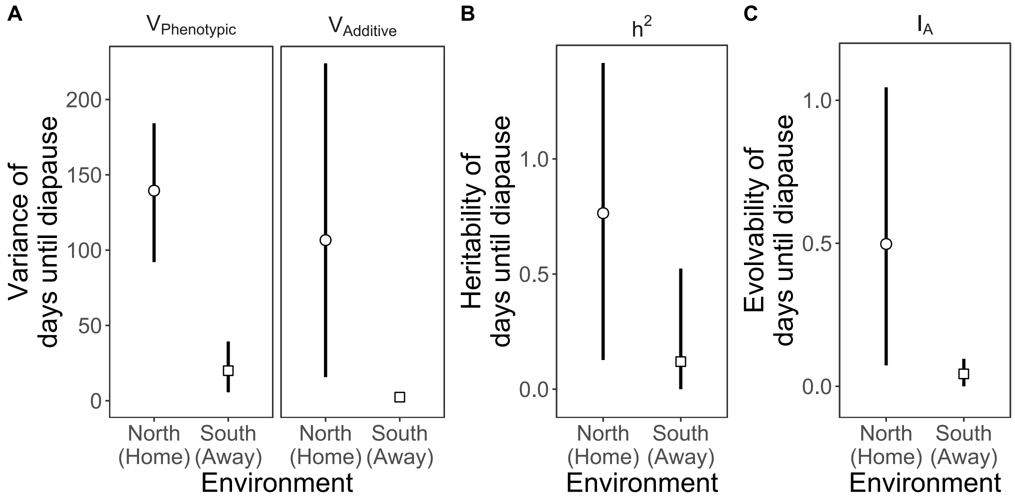

Figure 2-5 Genetic variance estimates

Estimates of components of genetic variation (bootstrap 95% CI) for days until diapause for females from site C in two daylength environments. ***A)*** *Total phenotypic and additive genetic variation.* ***B)*** *Heritability.* ***C)*** *Evolvability.*

2.3.5 Genetic variance of body mass and thorax width

We measured body mass and thorax width on all female individuals from site C prior to their entering the two daylength treatments, and an additional male per full-sibling family. For body mass, only heritability for females (0.53; 95% CI 0.11-0.87) was statistically clearly greater than zero, but evolvability was not statistically clearly different from zero (**Figure B-3**, **Table B-3**). The patterns were similar for thorax width, though variance and heritability were smaller (**Figure B-4, Table B-4)**. Full results are in **Appendix B.4** and **Appendix B.5**.

2.4 Discussion

2.4.1 Local adaptation

We found evidence of evolution of diapause timing in the tamarisk beetle across 10.9 degrees of latitude in the introduced range in the United States. In a striking example of rapid evolution, the newly established southern populations have diverged from the northern populations in their responses to the environments that they dispersed from only 20-30 generations (10 years) ago. Furthermore, we find that local populations respond to diapause-inducing light regimes in ways that would likely maintain higher mean fitness than non-local populations, suggesting local adaptation to latitude across the range expansion.

Southern sites were generally more variable in their diapause incidence and timing than the northern populations. While the four northern sites have been established since 2001 (Carruthers et al., 2008), the four southern sites used in this study had recently arrived at the edge of the range expansion at the time of collection. Newly colonized southern sites may require additional time to adapt more optimally to the seasonality of their locations, if genetic variation has been retained during the range expansion. Additionally, the unexpected diapause responses of site G on the Rio Grande may be due to different source populations colonizing that river basin compared to the southern sites further east on the Colorado River and its tributaries, which could impact available genetic variation in the trait. Repeated sampling in these locations could provide additional insight to ongoing adaptation after colonization.

The variation among southern sites in this experiment may also be due to adaptation of beetles to different climate and seasonality of their local environments, such as timing and variability of winter onset and host plant senescence, and cumulative degree days. For example, the southern sites La Joya, NM and Little Colorado, AZ (E and G) appeared to be maladapted to the southern daylength regime and adapted to more northern fall daylengths, but these sites are also at higher elevations and have earlier winter onset than the other two southern sites (**Table B-1**). Indeed, our analyses show that latitude, elevation, and degree days are all more predictive of diapause timing (days until diapause at a constant daylength) than they are for northern populations. For southern populations, earlier diapause (fewer days until diapause) was associated with higher latitudes and elevations, and fewer cumulative degree days. Temperature in the fall may be one factor that is especially important in matching diapause timing with a local environment and weather in any particular year. In the tamarisk beetle, there is some evidence that diapause is delayed if temperatures are warm in the fall, especially in southern sites where winters are milder (Bean, Dudley, et al., 2007; Dalin et al., 2010). The role of temperature-sensitivity in diapause timing or the quality of the host plant as food has not been well explored but would further illuminate how adaptation and adaptive phenotypic plasticity facilitate establishment in novel environments (Dalin et al., 2010; Lankinen et al., 2013; Winterhalter & Mousseau, 2007). The outcome of selection in newly established range edges will depend on how long a population has been in an area, gene flow between populations, the strength of selection and heritable genetic variation in the new environment, and the role of adaptive plasticity at each location.

Our results suggest that the patterns we observe here are due to adaptation, rather than drift or phenotypic plasticity. An advantage of the tamarisk beetle system is its range expansion along river corridors, tracking its host plant, which provide the replicate populations used in this study. Although we were only able to simulate two environments representative of northern and southern diapause-inducing daylengths, populations in their local environments exhibit the expected trends by latitude (more northern populations enter diapause earlier and faster), which we would not expect if the pattern was generated by genetic drift. Additionally, the reciprocal rearing in common gardens eliminates the possibility that these results are due to phenotypic plasticity or environmental factors in the collection locations.

2.4.2 Quantitative genetic variation

We found heritable variation in body mass and thorax width of female tamarisk beetles, which coincides well with recent evidence that female body mass increased at the edge of the tamarisk beetle range expansion, but male body mass did not (Clark et al., 2022), indicating that low heritability in males may have hindered selection on body size during range expansion. We found that there is substantial heritability and evolvability of days until diapause at the origin of the expansion at a photoperiod near its critical photoperiod but not at a photoperiod that occurs only 13 days later in the field. This shows that there is ample underlying genetic variation in diapause related traits, but that expression of this variation depends on the environment. In the southern environment, beetles exhibited reduced variation overall, both additive and total phenotypic variation. Our results align with diapause being a threshold trait, which is a trait with discrete phenotypes (e.g., diapause and non-diapause) underlain by a continuous variable with a threshold (Roff, 1996; Tanaka & Murata, 2017). Because nearly all individuals reached the threshold for diapause induction in the southern environment, there was little variation in the trait and no heritability. This provides insight into why early releases of the tamarisk beetle failed below 38°N but were able to establish there years later. In the early releases, northern-adapted individuals mistimed diapause in southern locations by entering diapause too early (Bean, Dudley, et al., 2007), a response we still see in northern populations in this experiment. However, the genetic variation that could have fueled adaptation of northern-adapted individuals and matched diapause initiation with the climate in the southern environment was not expressed in this environment. The high heritability and evolvability we observed when individuals were close to the home environment suggests that adaptation can be rapid, if the environmental gradient is gradual or movement across it is relatively slow so that heritability and evolvability can be maintained (Polechová & Barton, 2015). The tamarisk beetle's range expansion since about 2010 was enabled by evolution of induction of diapause, with beetles initiating diapause at shorter daylengths at southern latitudes (Bean et al., 2012; Benning et al., 2023). That evolution was likely made possible by the maintenance of heritability of diapause timing during gradual movement southward, with few human translocations.

The trait days until diapause allowed us to track individual variation in the developmental response to photoperiod. It might also provide information needed to determine the molecular basis of dormancy in insects. Despite the importance of photoperiodism in determining the response to changing environments (Bradshaw & Holzapfel, 2007; Emerson et al., 2009), the integration of photoperiod sensors, summation of photoperiod information, and hormonal signaling of diapause remain poorly understood (Saunders et al., 2004). Experimental work shows that the sum of information gained over several light cycles determines whether insects follow continuous development or switch to diapause (Saunders, 2002; Takeda & Suzuki, 2022). This may proceed like a molecular bucket filling with a molecule when days are short, with diapause initiated when the bucket fills up. Shorter days fill the bucket faster than longer days. We find that photoperiod summation varies between individuals (e.g., variation in bucket size or rate of accumulation at different photoperiods), is heritable, and evolves. This information may provide additional insight into the molecular mechanism and evolution of the photoperiodic cue that is not evident from population-level critical daylength measurements. Future genomic studies may be able to use this trait to examine the genes that underlie individual variation in diapause timing.

2.4.3 Implications for range expansions

During the tamarisk beetle range expansion, dispersal ability has increased in edge populations compared to core populations, especially at low densities, and females from the edge are more fecund compared to those from the core (Clark et al., 2022), showing that spatial sorting, evolution of density dependent dispersal, and selection on reproductive ability have occurred during this range expansion. The current results show that adaptation to novel environments has occurred alongside these spatial processes. The need for diapause timing to adapt along the latitudinal gradient of photoperiod has likely slowed down the range expansion and reduced the magnitude of dispersal evolution (Benning et al., 2023).

Our results suggest that introductions or human translocations of biological control agents or species of conservation concern to ecologically distinct sites done with good intentions may actually hinder adaptation and establishment if genetic variation is not expressed in those novel environments. Indeed, most biological control agents fail to establish after release (Heimpel & Mills, 2017; Schulz et al., 2019) and assisted migration of species facing human-caused climate change is also frequently unsuccessful (Butt et al., 2021; Schäfer et al., 2020). Many hypotheses to explain these failures focus on the role of ecological interactions between the agent and biotic or abiotic features of its environment, while others emphasize the role of depleted genetic variation and lack of adaptation (Forsman, 2014; Heimpel & Mills, 2017; Schäfer et al., 2020; Schulz et al., 2019). Our research reveals another alternative: underlying allelic variation may be adequate for selection to act on, but it may not be expressed as heritable trait variation in new environments after release. Here we show that diapause timing is a highly heritable trait in local environments, but even a relatively small mismatch with the environment depleted the heritability of the trait. Studies of heritability and evolvability of ecologically important traits in the relevant environments prior to release may be beneficial in predicting both suitability for the environment and ability of an agent to adapt post-release.

Traits closely related to fitness are often expected to have lower heritability than traits less closely related to fitness, theoretically because traits related to fitness are under strong stabilizing selection and many loci underly these traits (Mousseau & Roff, 1987), though this may also be caused by a related increase in phenotypic variability (Houle, 1992). We found high heritability and evolvability of a diapause timing trait that should be closely related to fitness (Bradshaw & Holzapfel, 2007). Variable selection over time and adaptation to a variety of other environmental factors could have maintained high heritability and genetic variation in this population, even in the face of stabilizing selection on this trait (Hunt et al., 2007). Our results contrast with the common observation that life history traits have lower heritability than morphological traits (Postma, 2014).

The genetic paradox of invasions posits that introduced species will have low genetic variation because of small population sizes and repeated population bottlenecks (Dlugosch et al., 2015; Estoup et al., 2016). The paradox is, then, how do invasive species persist, expand their ranges, and ultimately thrive in introduced environments? Some have argued that for many invasions, the assumptions of the paradox are faulty – quantitative genetic diversity remains high after introduction, and that variation can be selected on to match the population with its introduced environment (Dlugosch & Parker, 2008; Estoup et al., 2016). For the tamarisk beetle in North America, additive genetic variation for an ecologically important trait, diapause timing, remains high, and can evolve quickly. Biological control agents may particularly fall into this category, especially if multiple source populations are collected and population sizes are maintained through quarantine and mass rearing (Szűcs et al., 2019).

As many species expand their ranges across environmental gradients, it is vital to understand the processes of local adaptation and maintenance of genetic variation in newly established habitat at the range edge. The appropriate timing of dormancy will be crucial for many species in temperate climates to persist in novel environments. For invasive species, biological control agents, and natural populations facing rapid environmental changes caused by climate change, understanding patterns of adaptation and expressed genetic variation will help to predict future movement and establishment in novel environments.

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CHAPTER 3: EVOLUTION OF PLASTICITY IN THE TIMING OF DIAPAUSE DURING RANGE EXPANSION INCREASES FITNESS OF A BIOLOGICAL CONTROL AGENT[4]

3.1 Introduction

Geographic range expansions are a frequent outcome of climate change and biological invasion. When expanding into new areas, populations typically face novel environments to which they are not adapted. An important question in evolutionary ecology is, thus, how do expanding populations adapt to these novel environments? This is especially relevant during range expansion, since adaptation may be hampered by reduced genetic variation caused by serial founder events and bottlenecks (Slatkin & Excoffier, 2012; Travis et al., 2007). Performance can be maintained in novel environments through rapid adaptation of trait means, or through adaptive plasticity, which is the ability of a genotype to produce a phenotype that improves fitness in a given environment (Fox et al., 2019). However, maladaptive plasticity can also arise, leading to phenotypes that have low fitness in the environment. Theory suggests that selection will lead to adaptive plasticity in range expansions, particularly when gene flow is high or environments are unpredictable (Chevin & Lande, 2011; Lande, 2015; Usui et al., 2023), yet there is little empirical evidence of the evolution of adaptive plasticity during range expansions. There is, in contrast, evidence for maladaptive plasticity in populations in novel environments but in the situations examined, the conditions did not necessarily favor the evolution of adaptive plasticity (Campbell-Staton et al., 2021; Davidson et al., 2011; Ghalambor et al., 2015; Leonard

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& Lancaster, 2020). To advance the field, these theoretical predictions should be tested more fully.

Many of the best examples of adaptive phenotypic plasticity come from organisms in seasonal climates that time life-history events such as dormancy, spring emergence, or migration to avoid harsh conditions and take advantage of favorable conditions (Kelly, 2019; Winterhalter & Mousseau, 2007). Because the process of entering or breaking dormancy and migration take time, these events are usually initiated by an environmental cue (such as daylength), rather than the environmental conditions that necessitate the behaviors (such as temperature) (Bradshaw & Holzapfel, 2007; Koštál, 2006). Daylength is used as a reliable cue by many organisms because it does not vary across years but is correlated with seasons and temperature. However, during a range expansion into a novel environment, both the appropriate timing of seasonal life-history events and the relationship between environmental cues and the weather shift. For example, during a range expansion to lower latitudes, winters are shorter and milder than at higher latitudes and the daylength cues that previously signaled the onset of winter will occur earlier in the year and will no longer be an accurate cue for the start of winter (Bean et al., 2012; Bradshaw & Holzapfel, 2007; Clark et al., 2023). Therefore, to persist in novel environments, the cues and responses to those cues must evolve, which can happen by shifting the existing environmental cue to be a more appropriate, or the environmental cue may become sensitive (i.e., plastically respond) to additional factors which can modulate the response to the present conditions. In more mild climates, we hypothesize that the photoperiod cue for diapause will be more plastic. There are many examples of a photoperiod cue shifting during range expansion (Ittonen et al., 2022; Lehmann et al., 2015; Tanaka et al., 2015; Urbanski et al., 2012), but there are no examples of plasticity in dormancy cues evolving during range expansion.

We seek to understand evolution of plasticity of dormancy cues in the northern tamarisk beetle, an insect biological control agent that has undergone a range expansion from north to south of more than six degrees of latitude since the early 2010s (Bean et al., 2012; Bean, Wang, et al., 2007). While spatial processes inherent in range expansion, like spatial sorting and increased reproductive ability at the edge have occurred (Clark et al., 2022), genetic variation has also been maintained at the edge (Stahlke et al., 2021), and the daylength used to cue diapause (insect winter dormancy) has evolved (Bean et al., 2012; Clark et al., 2023). Tamarisk beetles at the newly established southern range edge enter diapause later in the season and at a shorter daylength than beetles from northern sites (Bean et al., 2012). Early studies found that the timing of diapause in the tamarisk beetle was slightly sensitive to temperature (Dalin et al., 2010), though later studies suggested that diapause of some populations might be influenced by temperature (Bean et al., 2012). Sensitivity in the timing of diapause to temperature may be adaptive if it increases the time available for reproduction while temperatures are warm in the fall, but still allows populations enough time to prepare for the onset of winter and freezing temperatures (Herrera et al., 2005). We specifically ask **1)** Has sensitivity to temperature evolved in the diapause cue during range expansion? And **2)** Is temperature sensitivity adaptive?

3.2 Materials and Methods

3.2.1 Study system and insect collections

The northern tamarisk beetle (*Diorhabda carinulata*, Coleoptera: Chrysomelidae, hereafter tamarisk beetle), was released in North America in 2001 for control of the invasive shrub *Tamarix spp.*, and established well in areas with temperate climates and cold winters, similar to their original collection locations in Fukang, China and Chilik, Kazakhstan (Bean et al., 2012). Since about 2010, the beetle's range has expanded southward along rivers infested with *Tamarix* to areas with subtropical climates with mild winters and few freezes (RiversEdge West, 2021).

We collected tamarisk beetles from four sites along one expansion front on the Virgin and Colorado Rivers (**Figure 3-1, Table C-1**). The most northern site, Delta, was first established in 2001 and is cooler throughout the year than the most southern site, Cibola, which first established in 2018 and rarely freezes in the winter (RiversEdge West, 2021) (**Figure C-1**).

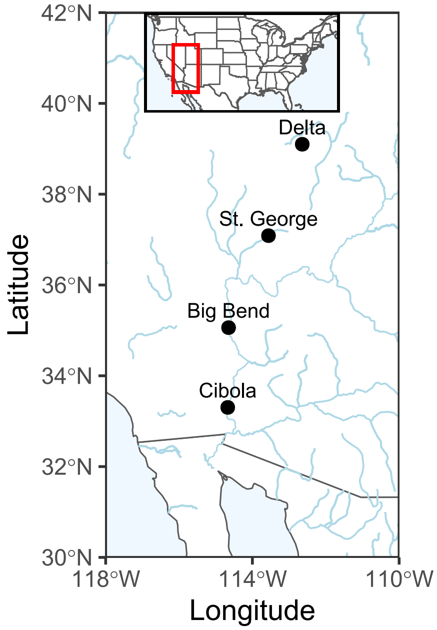

Figure 3-1 Plasticity collection sites

Tamarisk beetle collection sites at four latitudes in the southwestern United States. Delta, St. George, and Cibola were collected in both 2020 and 2022, while Big Bend was only collected in 2022.

In 2020, beetles from Delta, St. George, and Cibola were collected. In 2022, the same sites and also Big Bend were collected. At each site in each year, 150-300 live adults were collected by hand from tamarisk trees, with third instar larvae supplementing if not enough adults were present. Beetles were reared for one or two generations in the lab prior to the experiments to standardize maternal environmental effects. Beetles were maintained at the

Colorado Department of Agriculture Insectary in Palisade, Colorado at ambient indoor temperatures (25-27°C) with 16 hours of light per day and were fed fresh tamarisk as needed.

3.2.2 Diapause response to temperature experiments

To test whether temperature sensitivity in diapause timing has evolved during the range expansion into warmer climates, we compared the diapause behavior (diapause initiated or not) of adult female beetles under warm and cool temperatures across a set of daylengths chosen to induce diapause in different proportions of beetles, from 0 to 100%, from each of the sites. The sensitive stage for diapause initiation for the tamarisk beetle extends through all life stages, including adults, and thus we can measure diapause responses using only adult beetles. In 2020, we used six daylength treatments ranging from 10:30 hr:min to 15:05 hr:min in 55 minute intervals under both cool and warm temperature regimes (cool: 28/13°C high/low; warm: 38/23°C high/low). Each of the twelve temperature x daylength treatments occupied a single growth chamber. Temperature increased at lights on, reached the maximum after 3 hours, then cooled after lights off, reaching the minimum temperature after 3 hours. The temperature treatments were chosen to represent typical temperatures during the late summer and fall at the collection sites, and the 15°C diurnal cycle is typical at all collection sites (**Figure C-1**). In 2020, 40 pairs of beetles from each population were assigned a single growth chamber treatment (total of 1,440 pairs of beetles).

In 2022, we repeated the experiment with new collections from all four sites, and adding more daylength treatments to more accurately estimate diapause timing. Because of constraints on the number of growth chambers available, we ran the 2022 experiment in two blocks. The first block contained six daylength treatments from 10:20 to 13:15 hr:min of light per day in 35 minute intervals replicated at the same warm and cool temperatures used in 2020. In the second block, the six daylength treatments ranged from 13:15 to 14:55 hr:min of light per day in 35 minute intervals at the same warm and cool temperatures. Since it was impossible to run all daylength and temperature combinations at a single time due to constraints on the number of growth chambers available, we included the 13:15 hr:min treatment in both blocks to ensure the block design did not significantly influence the diapause responses. Across both blocks, 2,497 pairs of beetles were used in 2022. In the first block, we subjected forty pairs of beetles from Big Bend and Cibola, 15 pairs from Delta, and between 15 and 40 pairs from St. George to each treatment combination. In the second block, forty pairs of beetles from Delta and St. George and 15 pairs from Big Bend and Cibola were subjected to each treatment combination. In a few treatments, not enough beetles were available, so beetles were first allocated to treatments where we expected an intermediate proportion of beetles to enter diapause, based on the 2020 data. In both blocks, we used fewer than forty replicates from each population when we expected that close to 0 or 100% of beetles would diapause at that daylength, since additional replicates in these treatments would not improve estimation of diapause response to daylength.

Female beetles were scored as in diapause when they stopped laying eggs for seven consecutive days and reproductive if they continued to lay eggs through the end of the experiment (20 days) (Bean, Wang, et al., 2007; Clark et al., 2023). The cessation of oviposition has been used as a reliable indicator that the process of diapause has been initiated (Bean, Wang, et al., 2007; Clark et al., 2023), but there are no other external indicators of diapause in the tamarisk beetle. To verify individuals' diapause status, we dissected a subset of individual females from Delta, from treatments where diapause was initiated in an intermediate proportion of beetles, following Bean et al. (Bean, Wang, et al., 2007). Individuals were considered to be in diapause if they had a fat body and did not have mature reproductive structures. They were considered not in diapause if the fat body was absent, and eggs were evident inside mature reproductive structures.

3.2.3 Statistical analysis of diapause experiments

Analyses were done in R (version 4.2.0) (R Core Team, 2022). To determine the effect of temperature and daylength on diapause timing across the populations, we fit a generalized linear model with logit link function and binomial error distribution on the proportion of beetles that were reproductive at the end of the experiment, with daylength (continuous, in hours), temperature (warm or cool), population (four levels), and all interactions as fixed effects and year (2020 or 2022) as a covariate. Interactions between year and the other variables could not be included because Big Bend was only included in 2022, leading to some coefficients being inestimable. The fitted logistic curve was estimated using the predict.glm function and 95% confidence intervals were computed from the standard error on the logit scale and back-transformed to the probability scale.

We also used critical daylength to further analyze the effect of temperature on diapause timing and as a summary of the logistic curves. Critical daylength is a population-level statistic that describes the daylength that induces diapause in 50% of a population, like LD50 in a dose-response curve (Batz et al., 2020; Bean et al., 2012). Longer critical daylengths indicate that diapause will start earlier in the year, while shorter critical daylengths indicate later diapause. To estimate critical daylengths, we fit a generalized linear model with binomial error distributions with diapause (yes/no) as the response variable and daylength as a continuous predictor variable. We fit separate models for each population, temperature regime, and year. We then calculated critical daylength and its standard error with the *dose.p* function in the MASS package (Venables & Ripley, 2002). 95% confidence intervals were calculated using the Wald method, as suggested by Landes et al. (2013) and Landes et al. (2019). Means of the critical daylengths were compared using the 95% confidence intervals.

3.2.4 Fitness consequences of plasticity in diapause timing

To understand whether the evolution of temperature sensitivity was adaptive during this range expansion, we estimated the timing of diapause relative to the available growing season and onset of winter at each site using the temperature-sensitive daylength cue measured in the experiments and a daylength-only diapause cue, from previous data (Dan Bean, unpublished data). If the temperature-sensitive cue is adaptive, the time available to a population for reproduction should increase compared to a daylength-only cue, and diapause would be initiated before harsh conditions. Detailed methods are available in **Appendix C.2**.

3.3 Results

3.3.1 Temperature impacts diapause timing and the effects vary by population

The proportion of individuals entering diapause statistically clearly varied at the P=0.05 level across daylengths, populations, temperatures, and years of the experiment. Diapause was initiated in fewer individuals at shorter daylengths in southern versus northern populations, indicating the daylength cue has shifted during range expansion (**Figure C-2**). The logistic curve was sensitive to temperature in all populations: warm temperatures flattened the curve, while cool temperatures steepened the curve (**Figure C-2**).

We used critical daylength to simply quantify the logistic curves to further explore temperature sensitivity across the range expansion. Critical daylengths decreased at lower latitudes regardless of temperature (**Table C-3, Figure 3-2**) and were similar to or slightly lower than previous measurements of critical daylength for these collection locations (Dan Bean, unpublished results).

The effects of temperature on the diapause cue varied by population (**Figure 3-2, Figure C-2**). For the most northern site, Delta (39°N), the critical daylength increased by 30 minutes in 2020 and 1 hour in 2022 in warm temperatures compared to cool temperatures (**Figure 3-2, Table C-3**), contrary to expectations. Dissections of reproductive and non-reproductive beetles from Delta indicated that all non-reproductive beetles, including those in the warm temperature treatment, had resorbed their reproductive organs and grown their fat body, as is typical for diapausing beetles.

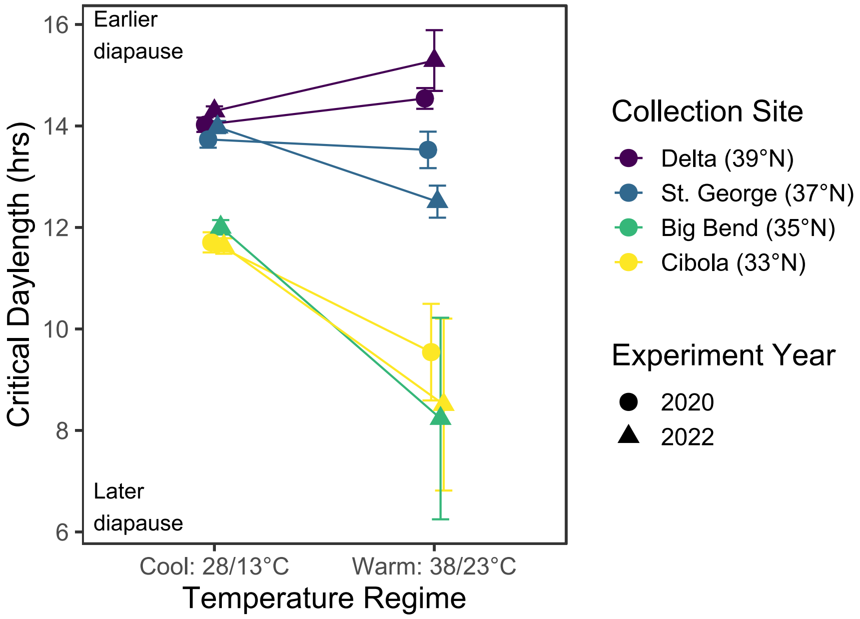

Figure 3-2 Plasticity reaction norms

Reaction norm for critical daylength (daylength when 50% are in diapause) at each site in warm and cool temperatures. Error bars represent 95% confidence intervals. The Big Bend site was only used in 2022.

For the three more southern sites, critical daylengths decreased in warm temperatures compared to cool temperatures, with the more southern locations having larger differences between the two treatments, indicating more plasticity. Critical daylength decreased in warm temperatures compared to cool temperatures by 12 minutes in 2020 and 1.5 hrs in 2022 for St.

George, almost 2 hours in 2022 for Big Bend, and 2 hours in 2020 and 3 hours in 2022 for Cibola (**Figure 3-2, Table C-3**). The critical daylengths of Big Bend and Cibola in the warm treatment are below the minimum daylength that occurs at those latitudes. Thus, diapause in the southern latitudes has evolved to require both short daylengths and low temperatures.

3.3.2 Temperature-sensitivity is mostly stable between 2020 and 2022

Critical daylength estimates were stable (as evidenced by overlapping confidence intervals) between 2020 and 2022 for all populations, except St. George, where critical daylength decreased in warm conditions in 2022 compared to 2020, showing increased plasticity in 2022 compared to 2020.

3.3.3 Phenotypic plasticity is adaptive

Temperature sensitivity in the diapause cue significantly changed the date of diapause initiation compared to a daylength-only diapause cue, though the effect on expected fitness differed by population. An adaptive shift in diapause timing should increase calendar and degree days available for reproduction and diapause should occur before freezing conditions. For Delta on average, the temperature-sensitive cue reduced the time available for reproduction by 11 calendar days or 171 degree days each year compared with a daylength-only cue (**Figure 3-3A**, **Table C-4**), indicating this response may not be adaptive for this population. For St. George, Big Bend, and Cibola, the temperature-sensitive cue extended the growing season compared to a daylength-only cue, increasing the average time available for reproduction at each site by 194 to 485 degree days and 10 to 35 calendar days each year, with bigger gains at more southern sites (**Figure 3-3A, Table C-4**). Tamarisk beetle development from egg to reproductive adult requires about 550 degree days, so these gains represent significant increases in the time available for development at the end of the season, where all life stages are present simultaneously. While temperature sensitivity extended the growing season, all populations are still estimated to enter diapause at least one month before the first frost (temperature at or below 0°C) (**Figure 3-3B**). For the three more southern populations, temperature sensitivity in the diapause cue is highly adaptive according to our model because it extends time available for reproduction, while still avoiding harsh conditions.

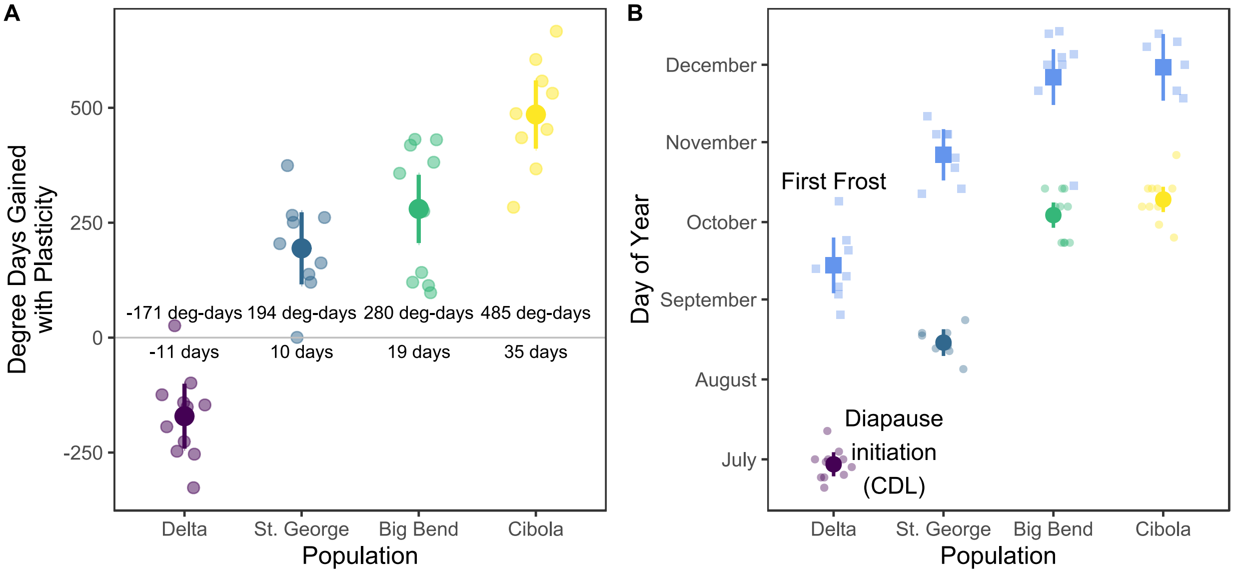

Figure 3-3 Fitness consequences of diapause timing

***A)** Degree days gained (or lost) due to temperature sensitivity for each population. Large points with error bars indicate the average degree days gained over 10 years (95% CI) and smaller points indicate the actual degree days gained each year. **B)** Diapause timing relative to first frost. Light blue squares with error bars indicate the average day with a low temperature at or below 0°C (95% CI) and smaller squares indicate the day of first frost each year. Colored circles and error bars indicate the average day when the critical daylength would occur over 10 years (95% CI), assuming temperature sensitivity estimated in the experiment, and small colored points are the actual day of the critical daylength each year.*

3.4 Discussion

Phenotypic plasticity is a major way that populations expanding their ranges can adjust their phenotypes to be able to establish and persist in novel environments (Fox et al., 2019). Adaptive plasticity is often seen as a way that selection pressures on organisms are reduced (Ghalambor et al., 2007; Gibert et al., 2019). Contrary to that paradigm, we show that increased adaptive plasticity, in the form of temperature sensitivity, could itself be an outcome of selection. Here, we assessed temperature sensitivity in the timing of diapause across an expansion front of the tamarisk beetle, from a well-established original release site to recently colonized sites. We show that plasticity in the diapause cue increased during the range expansion from beetles being mostly unresponsive to temperature in cooler northern climates to being dependent on cold temperature to initiate diapause in the warmer southern sites. The evolution of increased plasticity in the timing of diapause provides more time for reproduction and development, while still enabling diapause to occur before the first frosts. This rapid evolution of plasticity will likely allow the populations to continue the range expansion and allow the beetle to better control the target weed that it was released to manage.

The evolution of increased plasticity in thermal tolerance during range expansion has been documented in several cases. For example, plasticity in thermal tolerance increased in damselflies at newly colonized range edges in Europe, while long-established damselfly populations were adapted to their local temperature regimes and exhibited less plasticity in thermal tolerance (Swaegers et al., 2023). In another study, a horned beetle in its introduced range evolved increased thermal plasticity along a latitudinal cline (Rohner & Moczek, 2020). Although theoretically predicted (Usui et al., 2023), examples of the evolution of increased plasticity during range expansion are scarce, especially in phenological traits, like diapause timing. This study adds to a growing body of evidence that the evolution of plasticity may be an increasingly important component of population responses to changing environments, including range expansions (Gibert et al., 2019; Kelly, 2019; Merilä & Hendry, 2014).

In the Delta site in the present study, the plasticity we observed did not lead to phenotypic changes that seem adaptive. In this site, warm temperatures hastened diapause, instead of delaying it. We believe this result is not an experimental fluke since we found the same result in two separate years. Furthermore, this response was not simply a temporary cessation of reproduction in stressful conditions since dissection revealed physiology typical of diapause. The photothermographs indicate that the plastic response we observe in this population may be a maladaptive response to high temperature, since it reduces the degree days available for reproduction. However, this pattern may be adaptive if it decreases mortality during extreme heat waves at this site. Summer diapause (aestivation) has not been reported in the northern tamarisk beetle, but it has been documented in the closely related larger tamarisk beetle (*Diorhabda carinata*) (Knutson et al., 2019; Tracy & Robbins, 2009). Our results suggest that aestivation may occur in the tamarisk beetle in Delta, but not at similar temperatures during the range expansion to the south. In other taxa, like fish, reptiles, some mammals, and other invertebrates, aestivation is induced by environmental cues other than daylength, such as temperature and drought, while diapause is more typically linked with daylength (Lajus & Alekseev, 2019; Wilsterman et al., 2021). We may have captured a similar phenomenon evolving in this population. Climate change may select for aestivation or other approaches to avoiding or persisting through high temperatures and desiccation across many taxa.

There are several possible reasons for why temperature sensitivity is adaptive in only the southern part of the range. We hypothesize that in the northern part of the range, the possibility of sudden freezing temperatures makes a daylength cue that is consistent between years more advantageous than a fluctuating cue that could leave beetles vulnerable. In southern sites in subtropical climates, that risk is largely removed, and a plastic diapause cue that changes based on weather allows more time for reproduction. Additionally, high temperatures in the winter, as in the south of the range, are metabolically stressful and reduce survival for beetles in diapause

(Irwin & Lee, 2003). So, selection should favor a diapause cue that reduces the amount of time in diapause. The evolution of diapause cues is further constrained by their effect on spring emergence timing, as the diapause cue should not initiate diapause early in the spring when photoperiods are still short. The evolution of plasticity that we document appears to be adaptive for this range expansion.

The data from this study and the wealth of information published on the tamarisk beetle and its range expansion in North America (Bean & Dudley, 2018) suggest possible hypotheses for how plasticity was able to evolve in less than a decade across about 6 degrees of latitude. Between 2013 and 2015, the southward range expansion along the Colorado River halted near Big Bend, AZ (35°N), which was considered to be the southern extent of the tamarisk beetle's range. However, in 2016, the range expansion continued southward (RiversEdge West, 2021). Similar to the lag phase in invasions, this pause in expansion may have provided the necessary time for selection to act on standing genetic variation and new mutations, allowing beetles to survive at more southern latitudes (Bock et al., 2015). Diapause timing has been a main limiting factor in the tamarisk beetle's survival below the 38th parallel (Bean, Dudley, et al., 2007), so evolution of plasticity in diapause timing may have been a critical adaptation during this lag period that helped improve fit with the environment.

Interestingly, only the St. George population exhibited a change in temperature sensitivity between 2020 to 2022, increasing over the two years. The photothermographs indicate that plasticity is beneficial in this population and that greater plasticity may still evolve, since available degree days after diapause initiation is still high for this population, compared to more southern populations. This population may have evolved increased plasticity over two years from gene flow from populations further south in the range expansion. Although range expansions are generally studied in the overall direction of movement to previously uninhabited regions, actual movement of individuals is not unidirectional and gene flow can occur in all directions. A recent review found little support that asymmetrical gene flow from the range core to the edge is a general phenomenon (Kottler et al., 2021). Similarly, directionality of dispersal had not evolved during the range expansion of the cane toad, but that toads disperse in random directions (Brown et al., 2015).

Our study focused on a single range expansion front along the Virgin and Colorado Rivers, because there are few independent expansion fronts of the tamarisk beetle that do not contain beetles from three other closely related species that are also present in North America. However, finding consistent results in independent experiments in two years gives us confidence in the robustness of our results.

The evolution of plasticity that we observe could have important implications for the tamarisk biological control program. The longer time available for reproduction and development for three beetle populations could increase the efficacy of control the target weed, whereas possible summer aestivation may decrease efficacy of control in northern areas. Phenological mismatching was a major reason for failed establishment early in this biocontrol program (Bean, Dudley, et al., 2007) and has been an important consideration in many others (Wepprich & Grevstad, 2021). In this system, range expansion was facilitated by local adaptation, evident in shifts in the daylight regime that triggers diapause (Bean et al., 2012; Clark et al., 2023) and phenotypic plasticity in response to daylength and temperature. For this species, this is also a cautionary tale, since the beetle was originally predicted to not be able to survive at these latitudes and would not interfere with the endangered southwestern willow flycatcher, which sometimes nests in tamarisk (Dudley & Bean, 2012). Rapid evolution in diapause timing and plasticity of diapause cues has enabled range expansion into that territory less than 20 years after the original releases. In a future with more variable environments, increased plasticity could potentially modulate phenology based on temperatures of the current year and could increase both persistence and efficacy of the biocontrol program. Understanding the phenological responses of species to warming climates will continue to be important for biological control, vulnerable native species, and range expanding populations (Schilthuizen & Kellermann, 2014; Tougeron, 2019; Williams et al., 2017).

Much of the literature has focused on how plasticity can help populations survive in the short term and ‘buy time’ for selection to take place to better adapt populations to their environments (Fox et al., 2019; Usui et al., 2023). The ability to respond plasticly to environmental cues is itself a trait that can be an adaptation that can enhance the match of populations to their environments. Ongoing climate change, invasive species, and growing habitat degradation necessitate a better understanding of the role of phenotypic plasticity in adaptation in novel environments.

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CHAPTER 4: FITNESS AND HOST USE REMAIN STABLE IN A BIOLOGICAL CONTROL AGENT AFTER MANY YEARS OF HYBRIDIZATION[5]

4.1 Introduction

Hybridization can drive both ecological and evolutionary change. For example, it can increase invasiveness (Hovick & Whitney, 2014; Mesgaran et al., 2016), alter which environments a population can use (Rieseberg et al., 2003), and influence interactions between species through changes in traits such as plant secondary chemicals (Orians, 2000; Whitney et al., 2006). Given these kinds of effects, hybridization may have important consequences for classical biological control (hereafter, biocontrol), in which natural enemies are intentionally introduced for the control of invasive pest species (Heimpel & Mills, 2017; Szűcs et al., 2021; Wright & Bennett, 2018). In classical biocontrol programs, multiple ecotypes (Lowry, 2012) or even distinct species may be released (Van Driesche & Bellows, 1996) and hybridization among them may affect fitness, which influences the efficacy of control, and host use, which affects the safety of non-target species.

Hybridization may directly improve fitness through masking of deleterious recessive alleles in heterozygotes (Drake, 2006). Additionally, hybridization can facilitate adaptation to the environment by increasing phenotypic and genetic diversity (Ellstrand & Schierenbeck, 2000; Hovick & Whitney, 2014; Schierenbeck & Ellstrand, 2009). Hybridization can also reduce

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fitness if it disrupts local adaptation or creates deleterious gene combinations (i.e., incompatibilities). A challenge in understanding such outbreeding depression (reduction in fitness after hybridization) is that it may take several generations to manifest (Edmands, 2002, 2007). The effects of hybridization on fitness may also change over time, for instance fitness may increase in early generations through hybrid vigor, then subsequently decrease for several generations due to outbreeding depression, and then increase again as selection acts on the increased genetic variation. The fitness outcomes of hybridization are crucial to biocontrol agent efficacy, as populations of highly fit agents can grow rapidly, enabling them to better control the target pest.

The potential effects of hybridization on host use are complex (Wright & Bennett 2018). Host use of highly specialized phytophagous insects, such as those used in the biocontrol of weeds, seems to be quite canalized and not prone to evolving rapidly or forming extreme phenotypes (i.e., transgressive segregation) (Hardy et al. 2020). Indeed, there are no examples of evolutionary shifts in the fundamental host range of biological control agents of invasive weeds, where the fundamental host range is defined as all possible hosts that can be used by an agent (Van Klinken & Edwards 2002; Wright & Bennett 2018; Hinz et al. 2019). However, shifts in preference for or performance on hosts already within the fundamental host range have occurred in a few agents (Hoffmann et al. 2002; Thomas et al. 2010; Fukano et al. 2016; Bitume et al. 2017), often in conjunction with hybridization. For example, two biotypes of the scale insect *Dactylopius tomentosus* have distinct host preferences for target cactus species in the genus *Cylindropuntia*, but their hybrids have higher fitness than parental biotypes and no longer exhibit differences in host preference (Mathenge et al. 2010). In another example, the effects of hybridization between two biotypes of another scale insect, *Dactylopius opuntiae*, which also prefer distinct *Opuntia* cactus species, depended on generation: F1 hybrids could develop on both *Opuntia* species, but half of F2 hybrids reverted to being host specific (Hoffmann et al. 2002). These studies suggest that hybridization between introduced biological control agents needs further study as it may present an opportunity for true shifts in host use patterns (Szűcs et al., 2019).

Most research on hybridization of biocontrol agents relies on F1 and sometimes F2 crosses generated in the lab (e.g. Bitume et al., 2017; Szűcs et al., 2012). However, the consequences of hybridization for both fitness and host use may not be evident within that time frame. Furthermore, later generation crosses or crosses among more than two species can occur when hybridization is ongoing in natural environments. Selection and drift can also act on the new hybrid combinations, increasing the frequency of some allelic combinations over others (Szűcs et al., 2011). To characterize fitness and host use of natural hybrids of complex ancestry, high-resolution estimates of ancestry (which species, or mix of species, are represented in an individual's genome) are needed for individuals sampled from the field. These estimates are now possible with modern genomic tools (Andrews et al., 2016; Stahlke et al., 2022).

Here, we study the consequences of hybridization among four species of *Diorhabda* (Coleoptera: Chrysomelidae), which are biocontrol agents with similar host preferences introduced to control invasive *Tamarix* spp. (Caryophyllales: Tamaricaceae) in North America (Milbrath & DeLoach, 2006b). Hybrids between three *Diorhabda* species are common in the introduced range (Stahlke et al., 2022), and previous studies indicate that shifts in host preference are possible (Thomas et al., 2010) and hybridization may play a role in host choice (Bitume et al., 2017). Two non-target plant species in the introduced range are palatable to *Diorhabda* and it is important to understand the potential impacts of biological control on them and whether that risk has changed since original host testing. We ask the following questions: **1)** In locations where species overlap, what hybrid individuals (with mixed ancestry) are found? **2)** How does hybridization affect body size and fecundity? **3)** How does hybridization affect palatability and adult feeding preferences on target and non-target hosts? **4)** How does hybridization affect larval performance on non-target hosts? We synthesize these results to assess the role of hybridization in risk and efficacy for *Tamarix* biocontrol.

4.2 Materials and Methods

4.2.1 Study system

The *Tamarix-Diorhabda* biocontrol system provides a unique opportunity to examine the effects of hybridization. Four species in the genus *Diorhabda* were released in the United States. Hybrids between *D. carinata* (Faldermann), *D. elongata* (Brullé), and *D. sublineata* (Lucas) are viable and have been found in the field since 2013, or at least 16 generations (Bean et al., 2013; Knutson et al., 2019; Stahlke et al., 2022). The fourth species, *D. carinulata* (Desbrochers), does not readily hybridize (Bean et al., 2013). All four species have very similar external morphology and are only distinguishable through dissection (Tracy & Robbins, 2009) or genetic analysis (Bean et al., 2013), the latter of which was done in this study.

All four *Diorhabda* species have similar host preferences (Milbrath & DeLoach, 2006b) and survival is highest and oviposition is preferred on *Tamarix chinensis Lour.* × *Tamarix ramosissima Ledeb.* hybrids (DeLoach et al., 2003; Lewis et al., 2003a; Milbrath & DeLoach, 2006b). *T. chinensis* × *T. ramosissima* hybrids (hereafter, target host or tamarisk) are the most common genotype of invasive tamarisk in North America (Gaskin & Kazmer, 2009), but introgression between the two species varies by latitude, with *T. ramosissima* dominant in northern areas and *T. chinensis* dominant in southern areas (Gaskin & Kazmer, 2009; Williams et al., 2014). Tolerance of and resistance to herbivory also varies with tamarisk introgression, with *T. ramosissima* being more tolerant of and less resistant to herbivory (Williams et al., 2014). All *Diorhabda* species can feed and complete development on the two non-target species used in this experiment. *Tamarix aphylla* (L.) H. Karst. (hereafter, athel) is a non-native species common in the southwest USA (Gaskin & Shafroth, 2005) and is chosen over tamarisk by *Diorhabda* in feeding preference trials about 33% of the time (Bitume et al., 2017; DeLoach et al., 2003; Herr et al., 2014; Lewis et al., 2003a; Milbrath & DeLoach, 2006b, 2006a; Moran et al., 2009). *Frankenia salina* (Molina) I.M. Johnst. (hereafter, Frankenia) is a native species distributed in California and Nevada, USA and is chosen in feeding preference trials 1-2% of the time (Herr et al., 2014; Lewis et al., 2003a; Milbrath & DeLoach, 2006b; Moran et al., 2009).

4.2.2 Insect and plant material

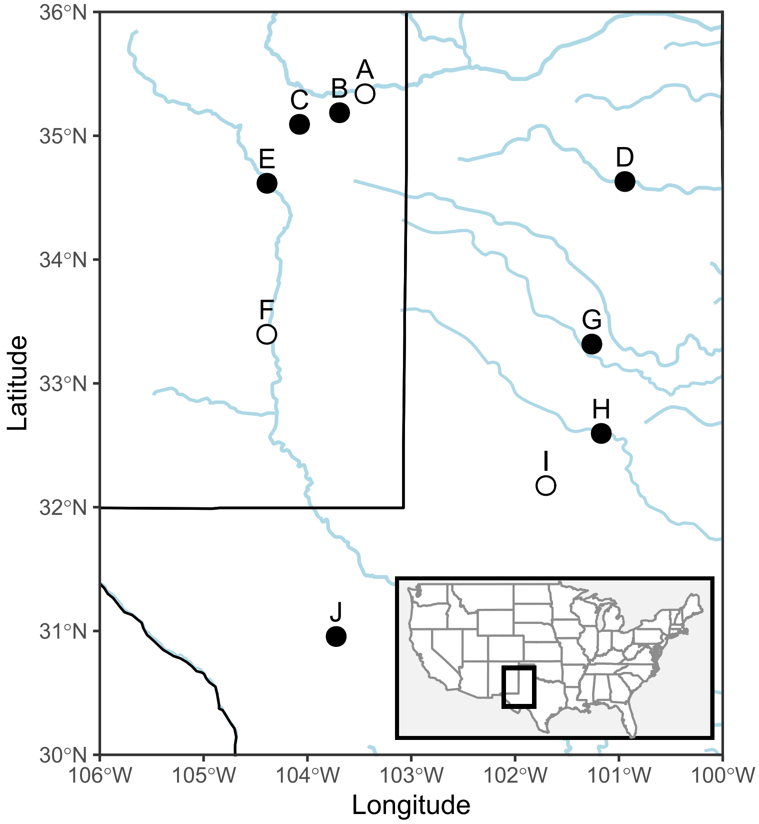

Figure 4-1. Map of hybrid zone

Diorhabda collection sites, labeled A-J from north to south, corresponding to ***Table D-1****. Unfilled points represent sites from which first-lab generation adults were genotyped following phenotyping. Most of the larval performance data came from site A. Filled points represent sites from which field individuals were genotyped.*

Diorhabda were sampled off of the target host (tamarisk) at ten sites across eastern New Mexico and western Texas, USA from 16-19 Sept. 2019 (**Figure 4-1, Table D-1**, sites labeled A-J from north to south), a region where hybridization between the three species had been confirmed (RiversEdge West, 2021; Stahlke et al., 2022). At seven sites, at least 20 adults were collected for direct genotyping. The remaining three sites were chosen for both phenotyping and genotyping, as previous research suggested they should contain hybrids across a gradient of ancestries (Stahlke et al., 2022). From these three sites, 200 adult Diorhabda were collected, brought to the lab, and maintained in growth chambers with a 16:8hr light:dark regime at 28°C:20°C day:night (Bean et al., 2007). Diorhabda were allowed to reproduce on the target host for one generation in the lab to reduce maternal effects prior to collecting phenotypic data on correlates of fitness and host use. At the time of collection and for all experiments, ancestry, including hybrid status, was unknown for all individuals, as those data could only be collected through lethal sampling after experiments ended.

The target host plant (*T. chinensis* × *T. ramosissima*) was collected as vegetative cuttings from Bonny Reservoir, Colorado, USA in March 2019. Since the level of introgression between *T. chinensis* and *T. ramosissima* impacts *Diorhabda* herbivory (Williams et al., 2014), we genotyped many cuttings and used only cuttings that were 30-44% *T. ramosissima* for all rearing and host use testing. This tamarisk genotype was chosen as it represents ancestry commonly found in the hybrid zone. AFLP markers were used to determine the level of introgression of each cutting following Gaskin et al. (2012). Genomic DNA was extracted from approximately

20 mg of silica-dried material using a modified CTAB method (Hillis et al., 1996). The AFLP method followed Vos *et al.* (1995) with modifications as in Gaskin *et al.* (2012) using the two polymorphic primer pairs *Mse*I + CAT/*Eco*RI + ACC and *Mse*I + CTA/*Eco*RI + ACC. Loci were initially scored by the fragment analyzer software GeneMapper v 4.0 (Applied Biosystems). These bins were then manually screened, making this a semi-automatic scoring method, as suggested by Papa et al. (2005). Average estimated admixture coefficient (Q, or assignment value) of each plant to *T. chinensis* and *T. ramosissima* was determined using Structure v 2.3.3 (Falush et al., 2003, 2007; Pritchard et al., 2000). Prior population information was included for 100 native Asian *Tamarix* plants included for reference (but not the plants collected from the USA), admixture was assumed to be possible, allele frequency was considered to be independent in each population and 50,000 burn-in and 50,000 run lengths were used.

Athel was collected near Yuma, Arizona, USA and Frankenia was collected near Lone Pine, California and Goleta, California, USA and neither were standardized by genotype. All three host species were grown in the Insectary Greenhouse at Colorado State University during the experiments and all plants were watered and fertilized regularly.

4.2.3 Genomic analysis of ancestry

We generated genomic data to determine ancestry for 20 adult *Diorhabda* collected from 7 sites and all female adults from 3 additional sites that were phenotyped in choice and no-choice host use trials (see section 2.5). As noted above, ancestry was unknown at the time of the host use trials, because our genomic approach used whole head and thoraxes, and thus any phenotyping had to occur prior to DNA extraction. All individuals were frozen at -80°C either after field collection or after completion of host use trials to protect high molecular weight DNA. Genomic data and ancestry analyses were performed following Stahlke et al. (2022). Briefly, DNA was extracted from the heads and thoraxes of individuals using a Qiagen DNeasy Blood and Tissue Kit following the manufacturer's protocol and treated with 4µL RNase A (Qiagen) to eliminate RNA contamination. DNA sample concentration was quantified for each individual by fluorometric quantification (Qubit 2.0 HS DNA assay; Invitrogen, Life Technologies). We prepared a total of 321 individually barcoded RADseq samples, including two individual replicates per plate, across four single-digest RADseq libraries using the 8-bp restriction enzyme *Sbf*I following the protocol described by Ali et al. (2016). Adapter-ligated libraries were multiplexed to achieve approximately 47.83 million 150 bp paired-end reads per library sequenced on an Illumina NovaSeq 6000 (University of Oregon).

Genotyping, filtering, and Bayesian clustering analyses were generally performed as previously described (Cerca et al., 2021; Rochette et al., 2019; Stahlke et al., 2022). For these analyses we included 40 RADseq samples previously identified as pure individuals of each of the four *Diorhabda* species derived from previous work to serve as a reference panel (Ravagni et al., 2021; Stahlke et al., 2022). This reference panel included individuals from all source populations introduced to North America, sampled from both laboratory cultures and field-caught individuals from the native and introduced ranges (data available at: Clark et al., 2022b). Briefly, all samples were aligned to the *D. carinulata* reference genome (Genbank Accession GCA 020975425.1) with bowtie2 version 2.2.9 (Langmead & Salzberg, 2012) and sorted with SAMtools version 1.9 (Li et al., 2009). Genotyping was performed including the reference panel with gstacks, using the default 'maruki_low' model, which incorporates population allele frequencies (Maruki & Lynch, 2017). We performed an iterative procedure to mitigate the effect of allele dropout and missing data within and among populations (Cerca et al., 2021). Through this process, samples with < 4x effective coverage, as well as individuals or loci with more than 50% missing data within and across populations were discarded from further analysis.

Using a single SNP from each RAD locus and the program Structure version 2.3.4 (Pritchard et al., 2000), we identified species-specific ancestry clusters based on the reference panel of known single-species individuals. As previously validated (Stahlke et al., 2022), we used the uncorrelated allele frequency model and allowed the alpha parameter to be inferred for each population (Falush et al., 2003). We executed 10 independent runs for each K from 1 to 10, allowing a burn-in period of 10,000 steps and 10,000 Markov chain Monte Carlo replicates, and printed the estimation of 90% credible intervals. After inspection of likelihood values for each K, we used the clustering results of K=4, corresponding to each of the four species, to infer ancestry across all field-collected and experimental individuals (Stahlke et al., 2022) (**Figure D-1**). We examined the confidence intervals across independent runs to conservatively identify the threshold at which operational pure ancestry could be confidently inferred for diagnostic individuals for each species, q = 0.024 (i.e., the lower bound of the 90% credible interval), below which pure identification could be unreliable and due to technical biases (Caniglia et al., 2020). Thus, by including reference samples of known identity, individuals with ancestry from more than one species (mixed ancestry) could be identified as having a hybrid origin resulting from secondary contact in North America.

4.2.4 Fitness

Body size and fecundity were used as fitness proxies. For insects in general (Berger et al., 2012) and *Diorhabda* specifically (Clark et al., 2022a), body size is a good indicator of fitness since it is associated with competitive ability and fecundity. These fitness proxies were measured on individuals reared in a common environment in the lab and thus differences among individuals from different collection sites or with different ancestry indicate evolutionary shifts and not plastic effects caused by differing field conditions. Adults from the first lab generation were weighed at emergence. Eggs laid during the 24-hour choice test (see **4.2.5**) were counted as a measure of fecundity.

4.2.5 Palatability of host plants and preference of adults

We measured palatability of each host plant through no-choice tests in which adults were presented with only one host plant and feeding was measured. We measured adult feeding preference through choice tests between all three host plants, in which adults could choose which plant to feed when presented with multiple options. All females were paired with a male from the same collection site shortly after eclosion and kept in pairs until the host use trials. After all females were laying eggs (about four days after emergence), each female was randomly assigned to a no-choice test with a single host plant or a choice test between all three host plants. Trials were started daily for five days from 13-17 November 2021. Choice and no-choice tests were similar to those conducted by DeLoach et al. (2003) and are briefly described here. Bouquets of each plant were made from 10-15 cm cuttings of fresh foliage with the cut end in a vial of water. For no-choice tests, a single bouquet of one plant was placed on a 5x10 cm piece of weighing paper at the center of a 15.25 cm diameter petri dish. In choice tests, separate pieces of weighing paper were evenly spaced in a petri dish and a bouquet of each plant was placed on each paper. In both types of tests, a single female beetle was placed at the center of the dish, away from any one plant, and allowed to eat and oviposit for 24 hours. We quantified feeding behavior by measuring frass (insect excrement) left on and under each plant after 24 hours (Bitume et al., 2017; DeLoach et al., 2003). At the end of 24 hours, frass was swept off each bouquet with a paintbrush onto the corresponding weighing paper and any plant material was removed from the paper. For each trial, the weighing paper with frass was photographed with a ruler for scale. Photographs were analyzed by first reducing shadows in Windows Live Photo Gallery editor to improve photo quality, then area of frass under each plant (in mm^2) was determined with the Analyze Particles function in ImageJ (Abràmoff et al., 2004). All females from no-choice and choice trials were frozen at -80°C for genomic analysis after completion of the trials.

4.2.6 Performance of larvae

Eggs were collected from the adults that were assigned to the choice test prior to the 24-hour trial and during the choice test if eggs were laid on the target host. Due to space and foliage constraints, most replicates came from site A (n=32), with only a subset of individuals from sites F (n=6) and I (n=4). On the day larvae hatched, larvae from each full-sibling family were randomly assigned in groups of 6-10 to develop on a single host plant (target host, athel, or Frankenia). Larvae from each full-sibling family were assigned to all three hosts as they hatched, over a 7-day period from 20-26 November 2021. Bouquets of each plant were replaced every 1-2 days, as needed. Twelve days after hatching, the number of surviving larvae in each container was recorded. All surviving larvae were frozen at -20°C and then dried at 70°C for 24 hours before weighing each individual to the nearest 0.01 mg. Survival and weight were recorded at 12 days in order to obtain a standardized measure of development before the fastest growing larvae reached the pupa stage.

During the experiments, an infestation of leaf hoppers and spider mites attacked the target host in the greenhouse, so availability of high-quality target host material decreased during the larval performance tests. Since plant quality can influence host performance (Lewis et al., 2003a), the data from larval performance on the target host have been excluded from analyses since they reflect low plant quality, not true performance. As our scientific question focuses on performance on non-target hosts, this exclusion restricts our ability to compare to the target host, but does not compromise our ability to address our question. Larvae were not genotyped.

4.2.7 Statistical analysis

To test for relationships between ancestry and individual phenotypes of fitness and host use, ancestry was described with both categorical scores and continuously. The categorical scoring of genotypes among the phenotyped individuals, produced six categories of hybridization: pure *D. carinulata* (where "pure" here indicates q > 0.976 for a single species, based on the confidence of the ancestry assignment), pure *D. carinata*, pure *D. sublineata*, hybrids of *D. carinata* × *D. sublineata* (where "hybrid" here indicates q > 0.024 for more than one species), hybrids of *D. carinata* × *D. elongata*, and hybrids of *D. carinata* × *D. sublineata* × *D. elongata*. We used this broad classification of pure species and hybrids that captures any amount of introgression above the 0.24 threshold because we are interested in the current field populations rather than in early generation crosses (which are reported on in Bitume et al. 2017). To ensure the classification threshold did not impact the results of the host specificity analyses, we classified hybrids using a more conservative q > 0.1 threshold, and found no qualitative differences in the results of any analysis. Additionally, we visualized individual genotypes with principal components analysis (PCA) using adegenet version 2.1.8 (Jombart, 2008) as a complementary inspection of hybridization classifications. We found that individuals identified as hybrids clustered together on the analysis, between the two parental species, indicating that our classification of hybrid categories is robust (**Figure D-2B**).

Hybrids of *D. carinata* × *D. elongata* were excluded from further analyses due to very low realized sample sizes (n=4). Pure *D. carinulata* beetles were also excluded because they rarely hybridize with the other species and are not relevant to the questions of this study. Thus, following genotyping, we could compare phenotyping results from two pure species (*D. carinata* and *D. sublineata*), *D. carinata* × *D. sublineata* hybrids (hereafter, 2-way hybrids), and *D. carinata* × *D. sublineata* × *D. elongata* hybrids (hereafter, 3-way hybrids).

Ancestry was also classified continuously. Proportion *D. sublineata* and *D. carinata* ancestry were tightly correlated because most individuals in our samples were either *D. sublineata*, *D. carinata*, or a hybrid of the two. Proportion *D. carinata* ancestry was chosen for the statistical models because it was a slightly better predictor in most models and in no models were results qualitatively different between the two.

Adult weight was analyzed using models with Gaussian error distribution, with ancestry (separate models for categorical (factor with 4 levels) and continuous (proportion *D. carinata*) ancestry) and collection site as fixed effects, and emergence date as a random effect. Both ancestry and collection site were included in the model in order to estimate the effect of each after accounting for the other. For fecundity, models with negative binomial error distribution and zero-inflation were fit to egg counts, with ancestry (categorical and continuous) and collection site as fixed effects, weight as a fixed covariate, and test date as a random effect. The significance of model effects here and below were tested with Wald χ^2 tests with degrees of freedom denoted by subscripts. Pairwise comparison of means was performed with t-tests with degrees of freedom denoted by subscripts in the emmeans package (Lenth, 2020).

Palatability of each plant was estimated from no-choice tests. A mixed model was fit to frass area (mm^2) with gaussian error distribution with ancestry (categorical and continuous), plant treatment (3 levels: target host, athel, Frankenia), and their interactions as main effects, weight as a covariate, and trial date as a random effect. Sample size per ancestry group per treatment ranged from 3 to 13 (average = 7). Additional models with collection site as a fixed or random effect were fit and compared using AIC.

Feeding preference for each host plant was estimated from the choice tests. We modeled total frass area under each plant and proportion of total frass under each plant separately. For frass area, a small constant of 0.01 was added to the frass area under each plant in a choice arena before log transformation in order to fit the assumptions of a linear model. Models with gaussian error distribution were fit with ancestry (categorical and continuous), plant in the choice arena, and their interaction as main effects, weight as a covariate, and date of trial and individual beetle as random effects. We also fit additional models with site as a fixed or random predictor. To account for differences in variance across predictors, additional models were fit that included a dispersion component (Brooks et al., 2017). AIC was used to compare between models and results are reported from the model with the lowest AIC. The proportion of frass under each plant was calculated by dividing frass area under one plant by the total frass area for each individual. Proportion frass was then transformed with the formula, $(x(n-1)+0.5)/n$, where x is the proportion and n is the sample size, so the data were between 0 and 1 to fit the assumptions of a beta regression (Douma & Weedon, 2019). A separate beta regression model was fit for each of the three plants, with ancestry (categorical and continuous) as a main effect, weight as a covariate, and trial date as a random effect. Additional models with dispersion components and collection site effects were fit and compared using AIC.

For larval survival, a model with binomial error distribution was fit to proportion survival for each family, with ancestry of mother (categorical and continuous), plant (2 levels: athel, Frankenia), and all interactions as main effects, weight, date of hatching (correlated with plant quality), and collection site as fixed covariates, and mother as a random variable, to account for the nested design. For larval growth, a model with gaussian error distribution was fit to dry weight of larvae at 12 days of development with ancestry of mother (categorical and continuous), plant, and all interactions as main effects, and weight and hatch date as fixed covariates. To account for the design of the experiment, mother and the interaction between mother and treatment were random effects. Additional models with dispersion components were fit and compared with AIC.

Host specificity can be reinforced by adult individuals preferring hosts that increase the performance of future generations (Poisot et al., 2011). This preference-performance association can be an important indicator for how host specificity may evolve. We tested this association by calcuating Pearson correlation statistics between mother's preference (proportion of total frass under one plant in a choice test) and larval performance (proportion survival on one plant).

4.3 Results[6]

4.3.1 Ancestry of Diorhabda

We determined the ancestry composition of at least 20 individuals per site from the field from seven sites across the region and 59 to 63 per site females from the first lab generation from three additional sites that were also phenotyped in the lab. After read-processing and aligning to the existing reference genome for *D. carinulata* (Stahlke et al., 2022), effective per-sample coverage across the 38,641 loci had a mean of 40x and standard deviation of 20.4x. Structure analysis was performed on 1,838 SNPs. At *K* = 4, change in likelihood values plateaued (i.e., the

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[6] Raw sequence reads for each demultiplexed individual in this study are deposited on NCBI SRA. Ancestry assignment and phenotypic data are available at Ag Data Commons (https://doi.org/10.15482/USDA.ADC/1528155).

Ln''(Δ*K*) method; **Figure D-1**) and matched species identities for the reference panel of four species. Structure results for K=5-10 were the same as those for K=4 (I.e., no additional clusters were recovered with increasing values of K)*,* so we only present K=4.We found three of the four possible parental species (finding no *D. elongata*) across our collection sites. Across the entire region, 25% of individuals were assigned to be pure *D. sublineata*, 9% pure *D. carinata*, and 3% pure *D. carinulata* (*D. carinulata* is expected to be rare in the region sampled). 62% were assigned to be hybrids, with hybrids of *D. carinata* × *D. sublineata* dominating (69% of hybrids) (**Figure 4-2**). We found evidence of one hybrid with *D. carinulata* (out of 400 individuals genotyped), a cross that is expected to be rare (Bean et al., 2013; Stahlke et al., 2022). Ancestry composition differed between the three phenotyped sites (A, F, and I) (**Table 4-1, Figure D-2A**), but it was overall similar to the distribution of ancestry throughout the region (**Figure 4-2**). For example, phenotyped site A is similar to sites B and D with *D. carinata* ancestry highly represented among the individuals, phenotyped site F is similar to sites C, E, and G with *D. sublineata* highly represented, and phenotyped site I is similar to site H, with a high proportion of 3-way hybrids.

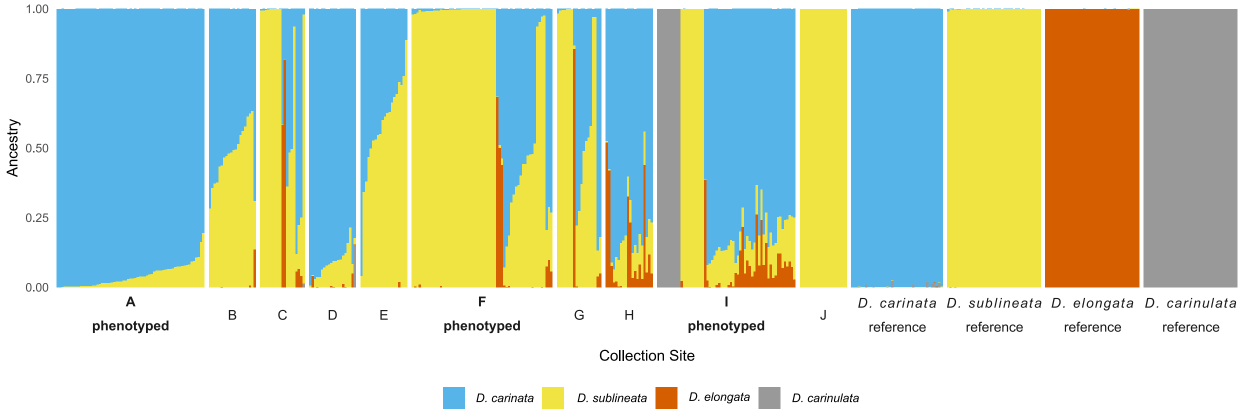

Figure 4-2. Ancestry assignments

Diorhabda ancestry assignment for individuals from 10 sites (A-J, from north to south) and samples of pure species used for reference. Each vertical bar represents a single individual and *the proportion of their genome that was confidently assigned (q > 0.024) to each parental species. Sites are labeled as on* ***Figure 4-1****,* ***Table 4-1****, and* ***Table D-1****. Individuals from bolded sites (A, F, and I) were from the first lab generation and phenotyped in the lab. Individuals from the other 7 sites were collected from the field.*

Table 4-1. Ancestry by collection site.

Distribution of ancestry in each phenotyped collection site. D. carinata × D. elongata hybrids and D. carinulata were excluded from fitness and host-use analyses due to low sample sizes and lack of hybridization, respectively.

	Collection Site		
	A	**F**	**I**
D. carinulata	-	-	10 (17%)
D. carinata	28 (44%)	-	-
D. sublineata	-	36 (60%)	10 (17%)
D. carinata × *D. sublineata* (2-way hybrids)	35 (56%)	18 (30%)	12 (20%)
D. carinata × *D. elongata*	-	3 (5%)	1 (2%)
D. carinata × *D. sublineata* × *D. elongata* (3-way hybrids)	-	3 (5%)	26 (44%)
Total	**63 (100%)**	**60 (100%)**	**59 (100%)**

4.3.2 Fitness

Results of fitness and host-testing experiments are reported only for the four ancestry groups that had sufficient sample size to analyze: *D. carinata*, *D. sublineata*, 2-way hybrids (*D. carinata* × *D. sublineata*), and 3-way hybrids (*D. carinata* × *D. sublineata* × *D. elongata*). Average adult body size ranged from 9 to 22.5 mg. Collection site was a statistically significant predictor of size (χ^2_2= 40.59, $P < 0.001$), while ancestry (categorical) was not (χ^2_3 = 2.96, P = 0.398). On average, when measured in a common lab environment, individuals from sites I (mean = 16.8 mg, SE = 0.38) and A (mean = 16 mg, SE = 0.37) were larger than individuals from site F (mean = 13.7 mg, SE = 0.364; pairwise tests $P < 0.001$), but not different from each other (pairwise test $P > 0.05$) (**Figure 4-3A**). Within hybrid beetles, proportion *D. carinata* ancestry was not significantly associated with adult weight (χ^2_1 = 0.33, P = 0.566). Average fecundity for each ancestry group ranged from 17.8 to 20.0 eggs in 24 hours. There were no significant differences between ancestry groups or collection sites in fecundity (**Figure 4-3B**).

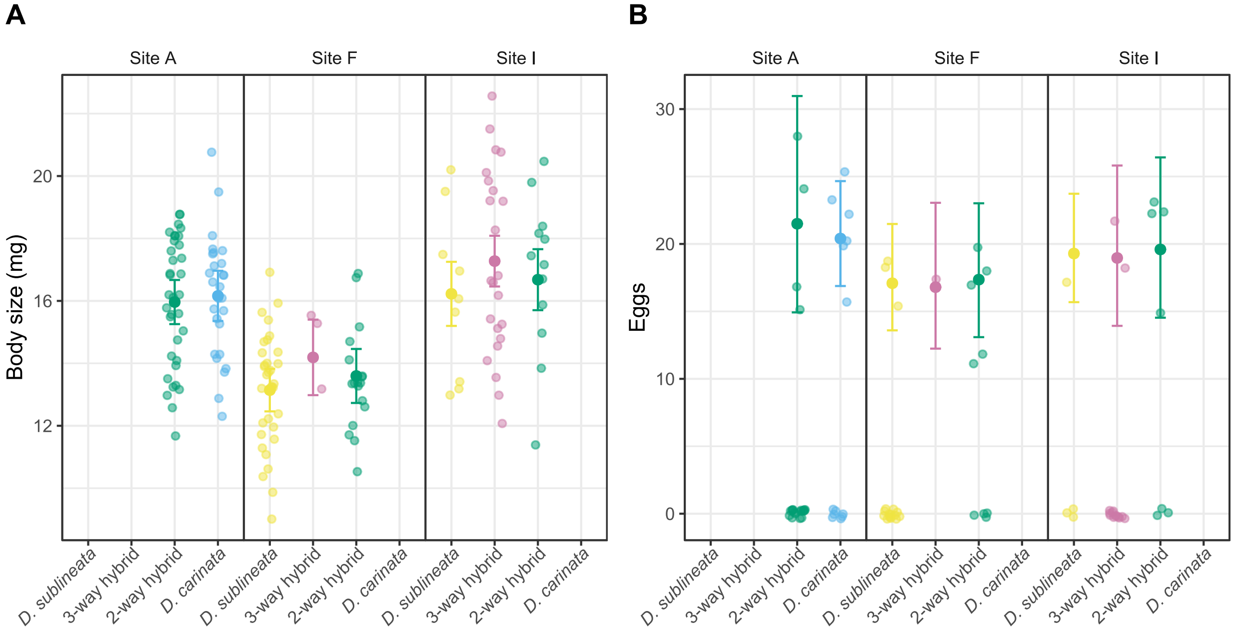

Figure 4-3. Body size and fecundity of hybrids

Body size (A) and fecundity (B) of female adult Diorhabda of different ancestry from three collection sites reared in a common environment. Solid points and lines represent means and 95% CI, transparent points are all observations. Ancestry groups without data were not represented in that site.

4.3.3 Palatability of host plants and preference of adults

No-choice tests allow us to assess the palatability of each host plant, or the amount an adult will feed when presented with no other options. In no-choice tests, the target host and athel received more frass than Frankenia (both pairwise *P*-values < 0.01), but there was no difference between the target host and athel (t_{60}=0.58, *P*=0.833) (**Figure 4-4**). Ancestry (categorical) nor its interaction with host plant were significantly associated with palatability (ancestry: χ_3^2 = 2.75, *P* = 0.432; interaction: χ_6^2 = 3.63, *P* = 0.726). Larger beetles left more frass than smaller beetles (χ_1^2

= 5.63, $P = 0.018$). Results were qualitatively the same when collection site of origin was included in the model.

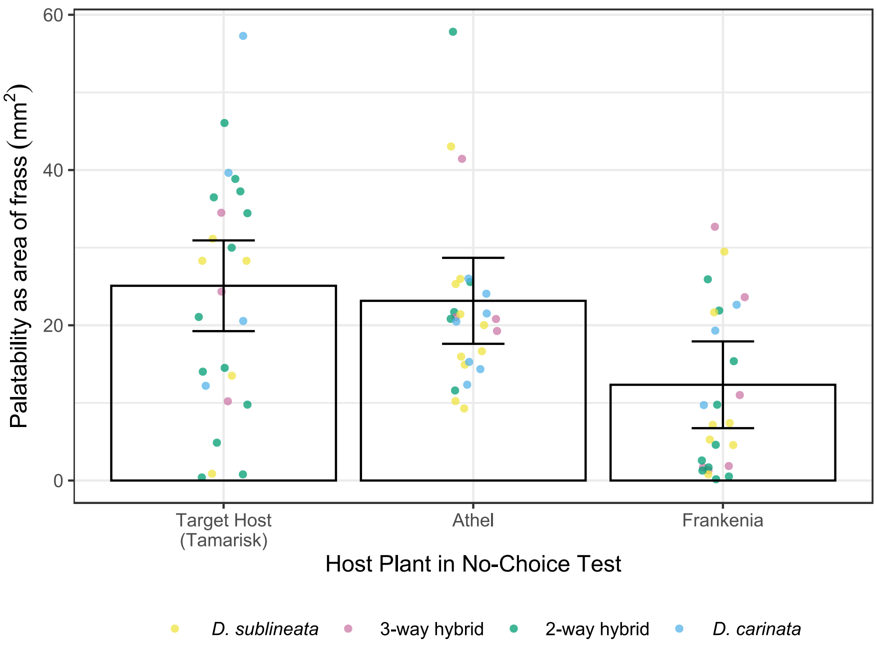

Figure 4-4. Palatability of host plants

Area of frass in mm2 under each host plant in a no-choice test with adult Diorhabda. Error bars are 95% confidence intervals. Colors indicate which ancestry group each individual was assigned.

Total frass area under each plant in a choice test allows us to assess preference and potential feeding damage for each host plant in absolute terms and to compare preference for host plants within each ancestry group. The best fitting model modeled dispersion of errors independently for each plant (see section **4.2.7**) and conclusions did not differ when collection site of origin was included in the model, so it was excluded. In general, *Diorhabda* preferred the target host over non-target athel and Frankenia, but the effect differed by ancestry (**Figure 4-5**). *D. sublineata*, 3-way hybrids, and 2-way hybrids, all preferred the target host over both athel and Frankenia (all pairwise contrasts between plants $P < 0.01$). For *D. carinata*, there was no difference in preference between the target host and athel ($t_{231} = 0.48$, $P = 0.629$), but both were preferred over Frankenia (P-values < 0.001).

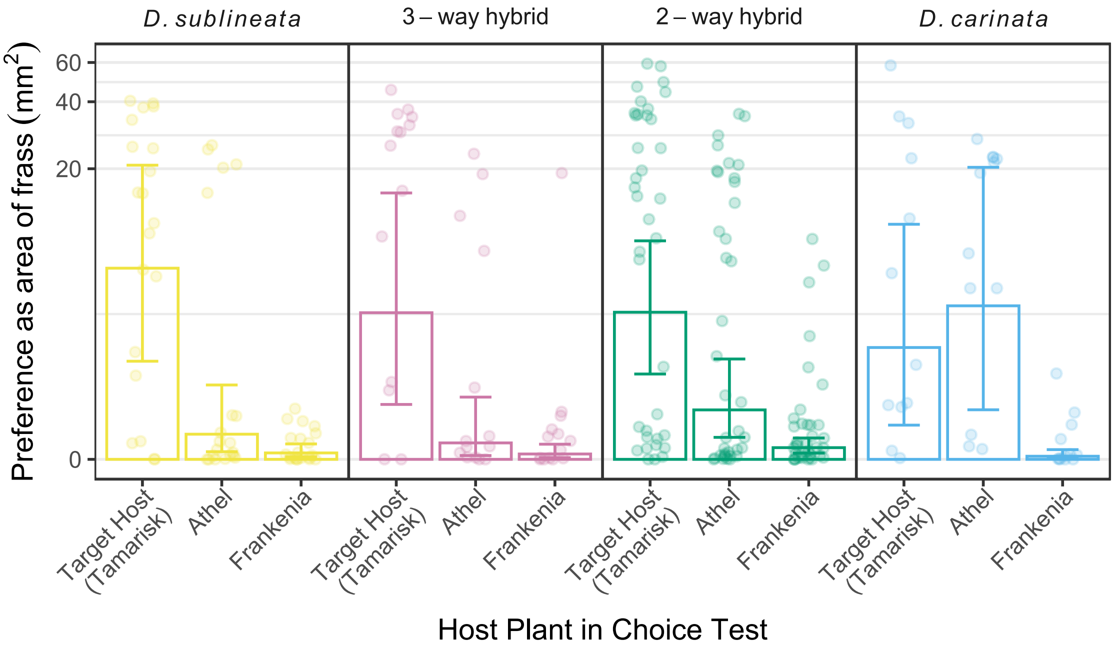

Figure 4-5. Preference for host plants as area of frass

Preference, measured as area of frass in mm^2 under each host plant, of female Diorhabda of different ancestry in choice tests with the three host plants. Error bars show 95% confidence intervals. Note log-scale y-axis.

Proportion frass under each plant for each trial in a choice test allows us to assess preference for each host plant relative to the other plants in the choice arena and estimate the effects of ancestry on preference for one host at a time. Including collection site of origin in any model was not preferred by AIC and did not change any conclusions, so it was excluded. Preference for the target host was above or not significantly different from 50% for all ancestry groups, but *D. carinata* tended to have lower preference for the target host than *D. sublineata* (pairwise comparison $t_{76} = 1.98$, $P = 0.051$) (**Figure 4-6A**). *D. carinata* had preference for athel of 56%, which was significantly stronger than *D. sublineata*’s preference of 29% for athel (pairwise comparison $t_{76} = 2.14$, $P = 0.035$) (**Figure 4-6B**). Preference of both classifications of hybrids was intermediate between parental groups and did not differ from either parental group at the 0.05 significance level on either the target host (hybrid preference of about 54%) or athel

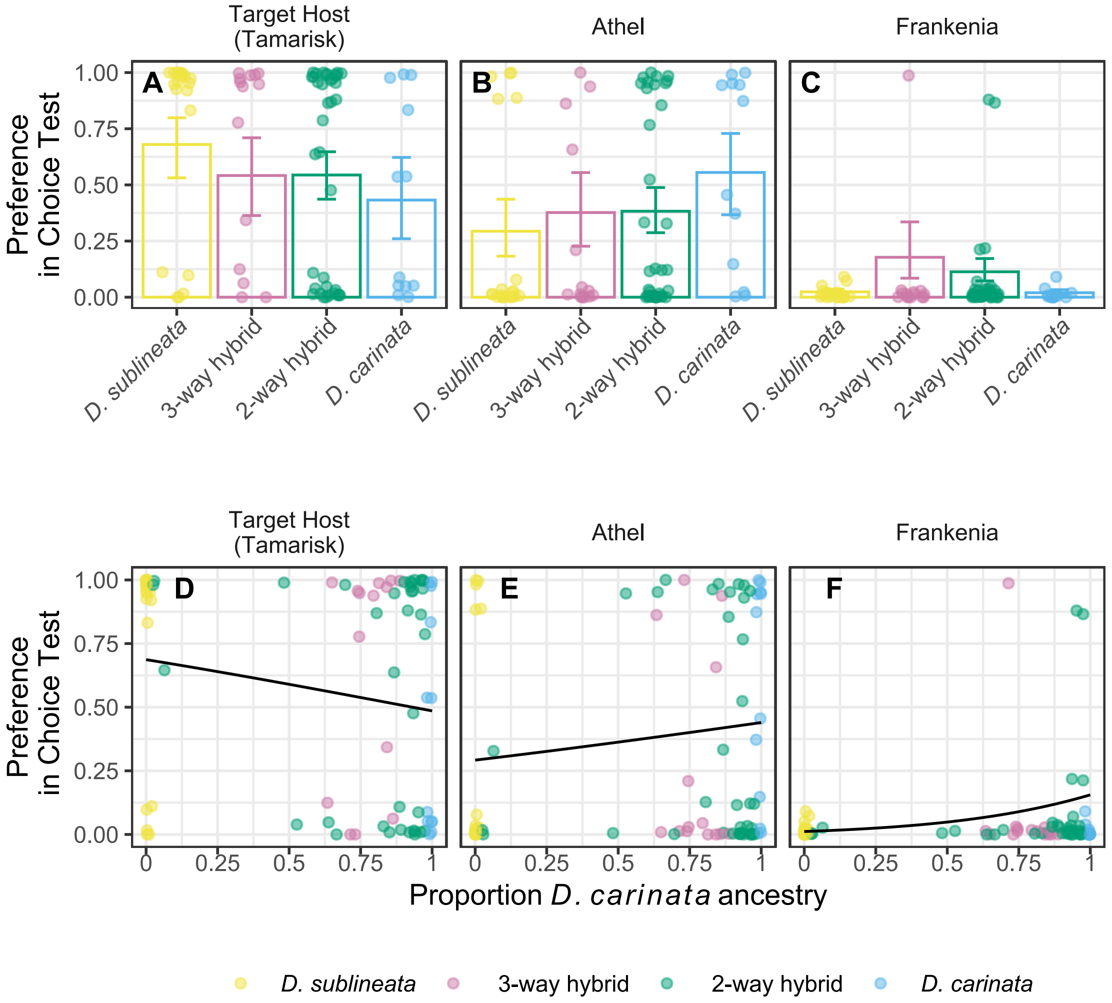

Figure 4-6. Preference for host plants as proportion of frass

Preference of female Diorabda in choice tests as indicated by the proportion of frass under a plant for each of the three host plants by ancestry group (A-C) and proportion D. carinata ancestry (D-F). In A-C, bars and error bars represent means and 95% CI, with individual observations as transparent points. In D-F, colors indicate which hybrid group each individual was assigned. Trend lines are predicted from the model fit.

(hybrid preference of about 38%). Preference for Frankenia was best modeled with separate dispersion parameters for each ancestry group (ΔAIC = 42.53). Preference for Frankenia was strongest in hybrids, which had significantly stronger preference for Frankenia than either *D. carinata* or *D. sublineata* at the 0.001 level. Two 2-way hybrids (5% of 2-way hybrids) and one 3-way hybrid (7% of 3-way hybrids) had preference for Frankenia over 75%, while no *D. carinata* or *D. sublineata* individuals had preference for Frankenia over 12% (**Figure 4-6C**).

Higher proportion *D. carinata* ancestry was associated with a decrease in preference for the target host ($\chi_1^2 = 4.35$, $P = 0.037$) (**Figure 4-6D**). However, when pure species were excluded from the analysis, the trend was in the same direction, but no longer significant ($\chi_1^2 = 0.69$, $P = 0.405$). This indicates that the trend is driven by the differences between pure *D. sublineata* and *D. carinata*, rather than introgression within the hybrids. Preference for athel tended to increase with proportion *D. carinata* ancestry, but the trend was not significant ($\chi_1^2 = 2.62$, $P = 0.106$) (**Figure 4-6E**). Preference for Frankenia was best modeled by accounting for uneven dispersion (ΔAIC = 38.14). Preference for Frankenia significantly increased with proportion *D. carinata* ancestry ($\chi_1^2 = 68.13$, $P < 0.001$) (**Figure 4-6F**). When pure species were excluded from this analysis, the trend was still highly significant ($\chi_1^2 = 14.61$, $P < 0.001$), indicating that *D. carinata* ancestry within hybrids is associated with increased preference for Frankenia.

4.3.4 Performance of larvae

Performance to 12 days of development on the non-target hosts allows us to estimate the ability of larvae to feed and develop on the non-target hosts, if adults oviposited on the non-targets. Larval survival was higher on athel than on Frankenia across all ancestry groups (athel mean = 0.929, SE = 0.0259; Frankenia mean = 0.566, SE = 0.059; $\chi_1^2 = 7.40$, $P = 0.007$). Larval survival differed by ancestry of the mother on both non-target host plants. Larvae from 3-way hybrid mothers had the highest survival overall and it was significantly higher than survival of larvae from 2-way hybrid mothers ($P < 0.05$), marginally higher than survival of *D. carinata* mothers ($P < 0.1$), but not significantly different from survival of larvae from *D. sublineata* mothers ($P = 0.26$) on both athel and Frankenia (**Figure 4-7A**). However, 3-way hybrids were represented by only three families in larval performance tests.

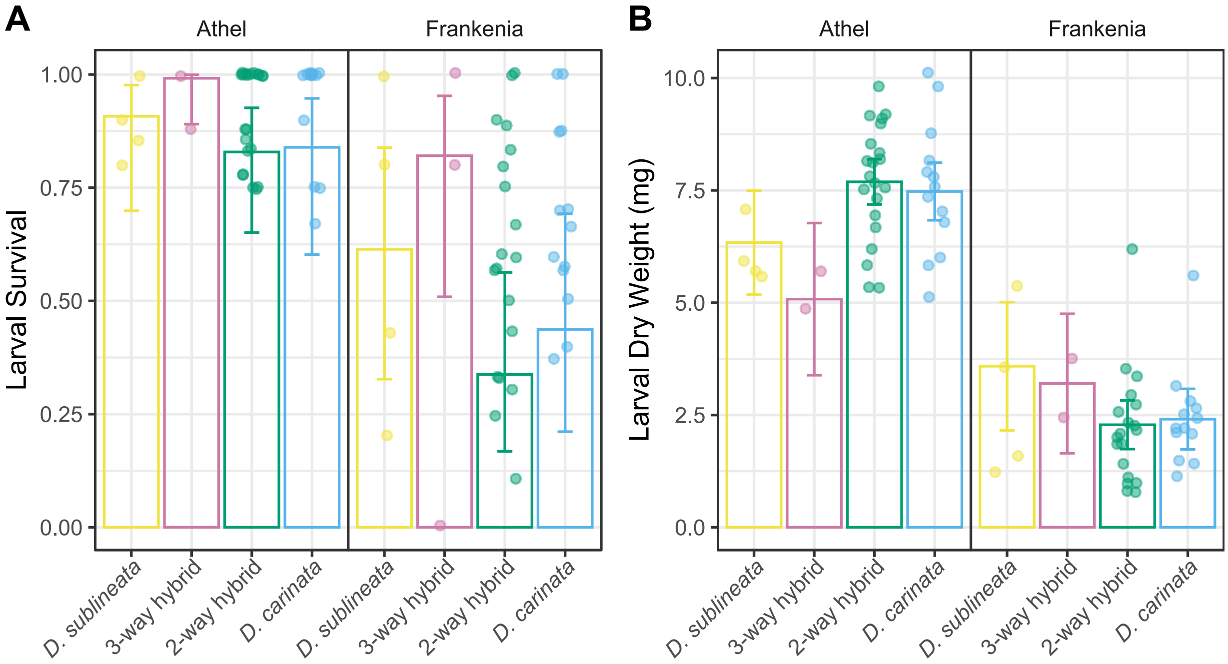

Figure 4-7. Larval performance on non-target hosts

Larval performance as survival (A) and weight (B) on non-target plants athel and Frankenia. Bars represent means for ancestry group, point represents the mean value of one full-sibling family, and error bars show 95% confidence intervals. Ancestry was determined for only the mother of each family.

Weight of larvae after 12 days of development on a non-target plant was best modeled with additional dispersion parameters for ancestry of the mother, treatment, and their interaction. There was a significant interaction between ancestry of the mother and treatment ($\chi^2_3 = 15.06$, $P = 0.002$), such that larval weight significantly differed by ancestry group on athel, but not on

Frankenia (**Figure 4-7B**). On athel, larvae from *D. carinata* and 2-way hybrid mothers were larger than larvae from *D. sublineata* and 3-way hybrid mothers. Weight of mothers (χ_1^2 = 1.52, *P* = 0.218) and hatch date of larvae (χ_1^2 = 0.01, *P* = 0.913) were not significantly associated with larval weight.

4.3.5 Preference-performance correlation

There was no significant trend between maternal preference and larval performance on either athel or Frankenia (**Figure D-3)**.

4.4 Discussion

Hybridization of biocontrol agents after introduction may have consequences for fitness and host use of hybrid individuals (Ellstrand & Schierenbeck, 2000; Szűcs et al., 2019). Here, we examine the effects of multiple generations of hybridization on fecundity, body size, and host use of *Diorhabda* using both genomic and phenotypic data. Overall, we find that hybridization has largely not impacted body size or fecundity, and that host use of hybrid individuals is similar to that of the parents, with a few exceptions. We discuss our findings in the context of evolutionary theory and the implications for biocontrol of *Tamarix* and for biocontrol programs generally.

The host use of specialized phytophagous insects is often very stable (Hardy et al., 2020). Our results indicate that both pure species and hybrid *Diorhabda* prefer the target host, tamarisk, in choice tests of adult feeding preference. Individuals of pure *D. sublineata* ancestry chose the target host 68% of the time, which matches well with previous studies, where preference for the target host ranged from 55-80% with an average of 68% across all studies (standardized for comparison between the target host and athel only) (Bitume et al., 2017; Milbrath & DeLoach, 2006b, 2006a) (previous studies summarized in **Table D-2** and **Figure D-4**). In this study, preference for the target host of individuals of pure *D. carinata* ancestry was not significantly different from 50%, somewhat lower than previous studies, which ranged from 54-71%, with an average preference of 63% (Bitume et al., 2017; Milbrath & DeLoach, 2006a, 2006b). The low preference for the target host by *D. carinata* resulted in a negative trend between *D. carinata* ancestry and preference for the target host and a positive trend for preference for athel. The differences in preference of *D. carinata* between this and previous studies could be explained by two non-mutually exclusive mechanisms. First, *D. carinata* could have evolved to be less host specific since previous testing. Second, quality or genotype of the target host used in this study could impact host preference. A spider mite outbreak on the target host during the choice tests could have altered concentrations of secondary compounds that *D. carinata* were more sensitive to than *D. sublineata* or hybrids. Plant genotype may also impact our results. We standardized the genotype of the target host plant to reflect 30-44% *T. ramosissima* ancestry, which is representative of common genotypes in the hybrid zone, but is not as palatable as genotypes with more *T. chinensis* ancestry (Williams et al., 2014). Here, in this study, both types of beetle hybrids we tested chose the target host at a rate intermediate between both parental species, indicating that novel genetic combinations present in hybrids have not significantly altered preference for the target host. Our results are in agreement with previous host-testing studies showing that non-target effects are possible on both athel and Frankenia, and that hybridization does not worsen potential effects. Previous work has shown that hybrids of *D. sublineata* × *D. elongata* had reduced preference for tamarisk compared to pure parental species (Bitume et al., 2017). This cross was not detected in any of the ten sites surveyed in 2019, so risk of non-target effects from this hybrid combination is very low in this region.

Frankenia was the least preferred host plant by all ancestry groups. The parental species *D. carinata* and *D. sublineata* chose Frankenia on average 1-2% of the time, which is very similar to original host testing of all *Diorhabda* species (Herr et al., 2014; Lewis et al., 2003a; Milbrath & DeLoach, 2006b; Moran et al., 2009) (**Table D-2** and **Figure D-4**). However, three individual adults deposited over 85% of their frass on Frankenia, whereas all other individuals deposited less than 12% of frass on Frankenia. Those three individuals were categorized as hybrids. One was assigned 71% *D. carinata*, 7% *D. sublineata*, and 22% *D. elongata*, making it a three-way hybrid. The other two individuals were of predominately *D. carinata* ancestry (95-97.5%), with some *D. sublineata* ancestry (2.5-5%). For those individuals, frass was also observed under at least one other plant, indicating they explored the arena during the 24-hour trial. Feeding on Frankenia increased with *D. carinata* ancestry in hybrid individuals. This indicates that hybrids may have genetic variation associated with feeding on Frankenia, and this variation could be selected on in the future. However, offspring of the individuals that preferred Frankenia performed better on athel than Frankenia and exhibited no increase in survival or growth on Frankenia relative to the offspring of mothers that did not prefer Frankenia. The lack of a correlation between maternal preference and larval performance suggests that preference for Frankenia will not be reinforced (Gripenberg et al., 2010). Frankenia is primarily distributed in California, which does not currently overlap with the hybrid zone of *Diorhabda.* Based on the total evidence from this study, we believe that the risk to Frankenia from *Diorhabda* remains relatively low, but should be monitored in light of ongoing hybridization.

We found that all three host plants tested here were palatable in no-choice tests to adult *Diorhabda*, which was expected, based on previous host testing (DeLoach et al., 2003; Lewis et al., 2003a). Our results support previous work showing that feeding stimuli (e.g., plant volatiles) from Frankenia are reduced compared to the target host and athel (DeLoach et al., 2003; Milbrath & DeLoach, 2006a, 2006b). The utility of no-choice tests has been debated in the literature, since no-choice environments are rare in the field and may overestimate non-target impacts (Milbrath & DeLoach, 2006b; Schaffner, 2001). However, others have argued that these tests are useful, particularly in the *Diorhabda* system because *Diorhabda* can quickly defoliate areas of the target host, leaving non-target hosts in what is essentially a no-choice situation in the field (Herr et al., 2009). Previous work on non-target impacts on Frankenia from *Diorhabda* show that in field situations, the risk to Frankenia is much lower than in artificial lab conditions, as were used in this study (Lewis et al., 2003a; Moran et al., 2009). Larval performance tests here only measured larval development to 12 days after hatching, which could over-estimate the probability of survival to adulthood. Our results also show that risk to Frankenia already present from the biocontrol program is not increased by hybridization of *Diorhabda*, with a few exceptions noted above.

In a meta-analysis, hybridization among introduced species can increase fecundity and size, though these effects vary by taxon (Hovick & Whitney, 2014). Our results show that body size (which is related to fecundity and fitness in insects) measured in a common environment did not vary by ancestry. This is in partial agreement with a previous study showing no differences in fecundity or hatching rate between most lab-created hybrids and pure species of *Diorhabda* (Bitume et al., 2017). In that study, however, lab-created crosses between *D. carinata* and *D. sublineata* did exhibit some hybrid vigor (Bitume et al., 2017). The results of the present study do not support either fixed hybrid vigor or outbreeding depression in hybrids from the field, based on body mass and fecundity. Fecundity in this study was measured only over 24 hours (during the choice tests), which likely explains the high proportion of females that did not lay any eggs. *Diorhabda* often lay eggs in clusters of 10-30 every 1-3 days (Lewis et al., 2003b). Body size did significantly vary by collection site when measured in a common lab environment, which indicates that there are genetically-based differences between collection sites. Collection site reflects multiple processes that may be occurring at each site, including introduction history of different species, population bottlenecks, and selection to different environmental factors. Future research will be needed to investigate this trend and determine if conditions present at each site or other factors are driving this pattern.

Secondary contact between species may have several long-term evolutionary outcomes, from complete isolation of species, to formation of stable hybrid zones, to complete admixture between them, depending on the isolation history of the hybridizing species, extent of genetic incompatibilities, and selection on the hybrid phenotypes in the local environment (Fischer et al., 2015; Sánchez-Guillén et al., 2016). While other studies have examined host use of lab-created crosses of biocontrol agents (Bitume et al., 2017), and change in proportions of hybrid biocontrol agents through time (Fischer et al., 2015), this study is the first to examine fitness and host use of a hybridizing biocontrol agent with hybrids collected from the field after many generations of hybridization. Our field-collected samples included beetles with pure ancestry of two species and variation in the amount of introgression between all three hybridizing *Diorhabda* species. These crosses are difficult to create in the lab but crucial for our understanding of adaptation across the range and the risk of host shifts in the field.

Hybrid and pure species beetles were found across the sampled region of New Mexico and Texas. Hybrids have been known to be in these locations since at least 2010 (Knutson et al., 2019), and the distribution of ancestry groups at each site have varied through time, indicating ongoing evolution across the region (Knutson et al., 2019; Stahlke et al., 2022). For example, in three sites that were sampled in this study (F, G, and H) and also by Stahlke et al. (2022), the abundance of individuals of pure ancestry has declined and abundance of hybrids has increased.

At site F (Roswell, New Mexico), *D. sublineata* dominated from 2014-2017 (Stahlke et al., 2022), but in 2019 the site was primarily composed of *D. carinata* × *D. sublineata* hybrids. Similarly, at site G (Post, Texas), *D. carinata* was most abundant in 2014 (Stahlke et al., 2022), but in 2019, *D. sublineata* and *D. carinata* × *D. sublineata* hybrids were most abundant. Finally, at site H (Lake J. B. Thomas, Texas), *D. elongata* was previously abundant (Stahlke et al., 2022), but no *D. elongata* were found in 2019, and instead there were many 2- and 3-way hybrids. Interestingly, although many releases of *D. elongata* have been made in the region (Deloach et al., 2011) and their establishment was confirmed both through genomic (Stahlke et al., 2022) and morphological (Knutson et al., 2019) characterization, we found no pure *D. elongata* individuals in 2019. We also found *D. carinulata* to be less abundant in the region than in previous studies and rarely forming hybrids with the other three species (Knutson et al., 2019; Stahlke et al., 2022). Introduction history, migration, genetic drift, and selection have likely all influenced the current distribution of hybrids through the landscape. A time series analysis of local ancestry and hybridization in these sites could further characterize evolution across the region (Gompert et al., 2017).

Releasing multiple ecotypes or species of biocontrol agents that may be adapted to different environments in the native range has been suggested for many years to increase efficacy of an agent or control of the target pest through transient heterosis or increasing adaptive genetic variation (but see Clarke & Walter, 1995), though worries remain about hybridization leading to agents host-switching to non-target species (Szűcs et al., 2019; Van Driesche & Bellows, 1996). Introgression has been beneficial for several biocontrol agents (Wright & Bennett, 2018), including the biocontrol agent of tansy ragwort *Longitarsus jacobaeae* (Szűcs et al., 2012), *Dactylopius tomentosus*, the biocontrol agent of cacti in the genus *Cylindropuntia* (Mathenge et al., 2010), and the egg parasitoid wasp *Trichogramma chilonis* Ischii (Benvenuto et al., 2012). Hybridization was detrimental to biocontrol efficacy of *Dactylopius opuntiae*, the biocontrol agent of cacti in the genus *Opuntia* (Hoffmann et al., 2002), but did not lead to host switching to non-target species. The present study corroborates previous work that shows that hybridization of *Diorhabda* seems to have no negative effect on fitness and may in some cases increase fitness (Bitume et al., 2017). It remains unknown how much hybridization may have facilitated long-term establishment of *Diorhabda* in the United States, but the widespread presence of hybrids over several years and increase in abundance of hybrids at some sites suggests that there may be selection for hybrids. Some have suggested that recent population contractions of *Diorhabda* in the hybrid zone could be due to hybridization (Knutson et al., 2019). Our results showing increased frequency of hybridization over time and similar fitness of hybrids and pure species do not support hybrid breakdown in this region. The ecological conditions that select for hybrid genotypes, their interaction with release history in the area, and consequences for establishment and control of tamarisk need to be further explored (Stahlke et al., 2022).

In this study, we opted for a two-generation experiment of host use after only one generation in the lab to standardize maternal environment to most directly understand how contemporary field populations behave. Future work should explore if strong selection for feeding on Frankenia imposed over multiple generations will be able to increase preference for or performance on Frankenia, given our finding that some individuals prefer the non-target plant. Our study suggests that variation may be present for feeding and development on Frankenia, but that selection pressure may be lacking in the field. A multi-generation selection experiment would be able to assess rapid evolutionary change in host use and future risk under different scenarios of plant and insect range shifts (Müller-Schärer et al., 2020).

4.4.1 Conclusions

Hybridization between closely related species is hypothesized to be an avenue for increased genetic diversity that can influence fitness and host range of herbivorous insects. We find that fitness and host preference of hybrid *Diorhabda* biocontrol agents from the field is quite stable. We demonstrate the utility of genomic methods for understanding the effects of hybridization in the field between more than two species and the importance of using field-collected individuals to more fully understand the risks posed by hybridizing biological control agents. Our work supports the prediction and current evidence that hybridization among closely related species rarely generates transgressive phenotypes or incompatibilities, which suggests that hybridization is safe and even beneficial when the fundamental host range does not differ among parental species.

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APPENDICES

Appendix A – Chapter 1

Appendix A.1 Collection site information

Table A-1 Collection site coordinates

Collection sites for the eight populations used in the life history and dispersal studies.

	Population	**Range**	**Latitude**	**Longitude**	**Collection Year**
A	Lovell, Wyoming	Core	44.856	-108.207	2017
B	Humboldt, Nevada	Core	40.063	-118.590	2018
C	Delta, Utah	Core	39.144	-112.958	2018
D	Pueblo, Colorado	Core	38.268	-104.721	2018
E	Blythe, California	Edge	33.912	-114.533	2018
F	Wickenburg, Arizona	Edge	34.422	-112.701	2018
G	Little Colorado River, Arizona	Edge	34.593	-109.611	2018
H	La Joya, New Mexico	Edge	34.342	-106.864	2017

Appendix A.2 Dispersal trial methods

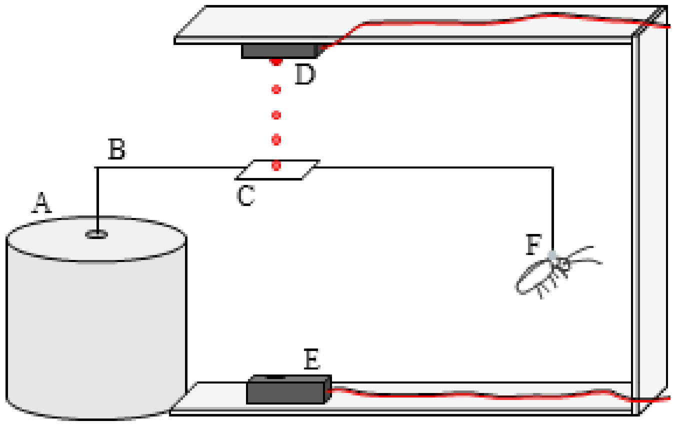

Figure A-1 Flight mill diagram

Diagram of flight mills used in dispersal trials. A. Low friction plastic base with tiny hole for wire to freely rotate. B. Teflon-coated wire. C. Paper flag to block sensor each rotation. D. Infra-red light emitter. E. Infra-red light sensor. F. Poster putty beetle attachment. Each flight mill was also equipped with a light source and a heat source.

Flight activity in *D. carinulata* has been observed in the field to occur primarily in the afternoon and at high temperatures, so trials were conducted between 7 and 11 hours after lights on and heating cables were used to increase the air temperature during flight trials. Actual air temperature during each trial was recorded using HOBO sensors.

For the dispersal trials, a beetle was attached by the pronotum to the wire of the flight mill with a small piece of poster putty. Beetles started the trial holding on to a small strip of paper to act as a support. The strip of paper was returned to the beetle between flights during the trial. The paper was dropped when the beetle initiated a flight, so it did not impact the weight of the beetle during flight.

Differences in friction between the 24 flight mill set-ups were evaluated by applying a standardized force to the wire of each flight mill and calculating the ratio of the duration of the first rotation to the duration of the second rotation. A higher ratio indicates less friction in the flight mill.

Appendix A.3 Python script for flight mill sensor analysis

We analyzed the raw sensor output to generate summary statistics for each trial, including number of flights, total distance, and average flight speed. Code is available at https://onlinelibrary.wiley.com/doi/full/10.1111/eva.13502

Appendix A.4 Dispersal statistical results

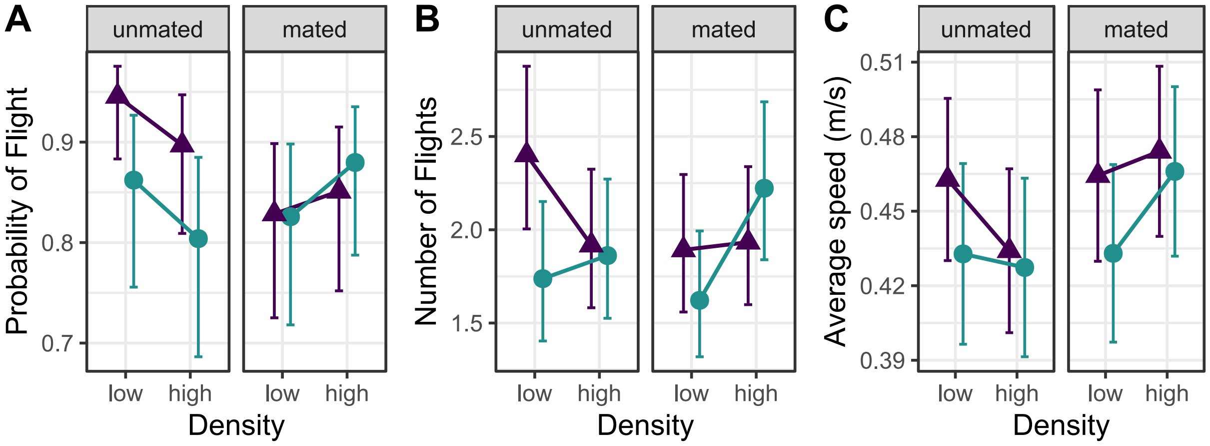

Figure A-2 Additional dispersal results

Results of all two-way interactions between range, density, and mating status for occurrence of flight (A), number of flights (B), and average flight speed (C).

Table A-2 Dispersal model results

Results from the final model for each of the four dispersal parameters. Shown are the variance of random effects, and X^2 or F-value and p-value for all main effects, interactions, and covariates.

	Probability of flight			**Number of flights**		
Type of model:	Binomial			Negative binomial		
	Var	X^2	p	Var	X^2	p
Density		1.01	0.315		0.33	0.567
Mate status		0.44	0.507		0.32	0.574
Range		4.61	**0.032**		5.94	**0.015**
Density*Mate		3.01	0.083		3.48	0.062
Density*Range		0.30	0.582		4.93	**0.026**
Mate*Range		4.14	**0.042**		1.63	0.202
Weight		13.55	**0.000**		11.64	**0.001**
Age		1.31	0.253		1.11	0.293
Mill friction		1.77	0.184		1.16	0.281
Temperature		9.70	**0.002**		5.11	**0.024**
Population (R)	0.00	0.00	1.000	0.01	0.00	1.000
Trial date (R)	0.10	1.72	0.189	0.00	0.05	0.829
	Flight distance			**Flight speed**		
Type of model:	Generalized Poisson			Linear		
	Var	X^2	p	Var	F	p
Density		1.38	0.240		0.03	0.867
Mate status		0.36	0.550		2.55	0.111
Range		3.00	0.083		2.14	0.196
Density*Mate		6.13	**0.013**		2.39	0.123
Density*Range		0.00	0.956		0.88	0.348
Mate*Range		1.69	0.193		0.00	0.956
Weight		21.73	**0.000**		12.26	**0.001**
Age		0.11	0.736		10.10	**0.005**
Mill friction		3.12	0.077		0.84	0.359
Temperature		0.48	0.489		0.57	0.449
Population (R)	0.00	0.16	0.687	0.00	0.00	1.000
Trial date (R)	0.04	8.73	0.003	0.00	0.38	0.536

Appendix B – Chapter 2

Appendix B.1 Calculations of site characteristics

Elevation data are from USGS TNM Elevation Tool. Degree days were calculated by the phenology model for the tamarisk beetle at USPest.org with temperature thresholds of 11.1°C and 36.7°C, averaged for four to ten years before collections (2008-2017), depending on availability of weather station data. Average first frost day was calculated as the first day after the summer with a daily low temperature below 0°C and averaged for the same 10-year period. Daylength at first frost was calculated using standard daylength tables for each site and are presented to show how the appropriate photoperiod cue for diapause will change based on latitude and winter onset. All temperature data were retrieved from National Oceanic and Atmospheric Administration for the closest available station to the collection location.

Table B-1 Site characteristics

Characteristics of tamarisk beetle collection sites.

		Site	**Lat.**	**Long.**	**Elevation (m)**	**Average Cumulative Degree Days**	**Average First Frost Date**	**Daylength at first frost (hrs)**
North	A	Lovell, Wyoming	44.856	-108.207	1115.35	1438	Oct. 2	11.73
	B	Humboldt, Nevada	40.063	-118.590	1189.62	1841	Sep. 30	11.87
	C	Delta, Utah	39.144	-112.958	1386.43	1767	Oct. 8	11.54
	D	Pueblo, Colorado	38.268	-104.721	1448.19	1990	Oct. 15	11.27
South	E	Little Colorado River, Arizona	34.593	-109.611	1669.56	2043	Oct. 13	11.45
	F	Wickenburg, Arizona	34.422	-112.701	1204.74	3516	Nov. 18	10.32
	G	La Joya, New Mexico	34.342	-106.864	1433	2547	Oct. 29	10.91
	H	Blythe, California	33.912	-114.533	96.9	4524	Dec. 12	9.94

Appendix B.2 Additional analyses of days until diapause

B.2.1 Days until diapause estimates excluding non-diapausing samples

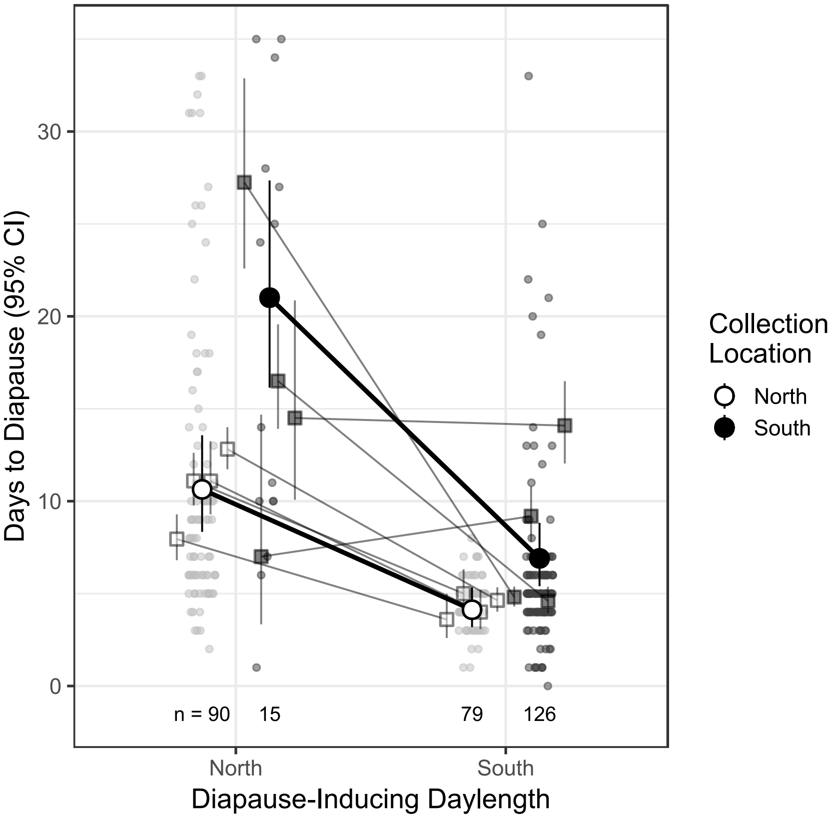

Figure B-1 Days until diapause alternate analysis without non-diapausers

Model estimates of days until diapause in northern and southern diapause-inducing daylength treatments for only those individuals that entered diapause during the 43-day experiment. Large white or black circles indicate means of core and edge, respectively. Light and dark grey squares indicate means for individual core and edge populations, respectively. Points in the background indicate days until diapause for each beetle measured.

B.2.2 Days until diapause estimates from a survival regression analysis

We analyzed days until diapause using a survival regression analysis (survival package in R), which takes into account individuals that either died before entering diapause or did not enter diapause during the experiment. We ran two analyses, one to estimate the means of core and edge, the second to estimate the means of each population. For the range estimates, the diapause response was predicted by range origin (core or edge), treatment, their interaction, with population as a covariate. For the population estimates, the diapause response variable was predicted by population, treatment, and their interaction.

We found the results to be qualitatively very similar to the Poisson analyses presented in the main text and the Appendix and the conclusions drawn are the same, though this analysis is not favored because of the unrealistically high estimates of days until diapause for groups with low diapause incidence (e.g., southern populations in the northern environment). These estimates of days until diapause are much higher than would be realistic, since these individuals will likely never enter diapause at these daylengths.

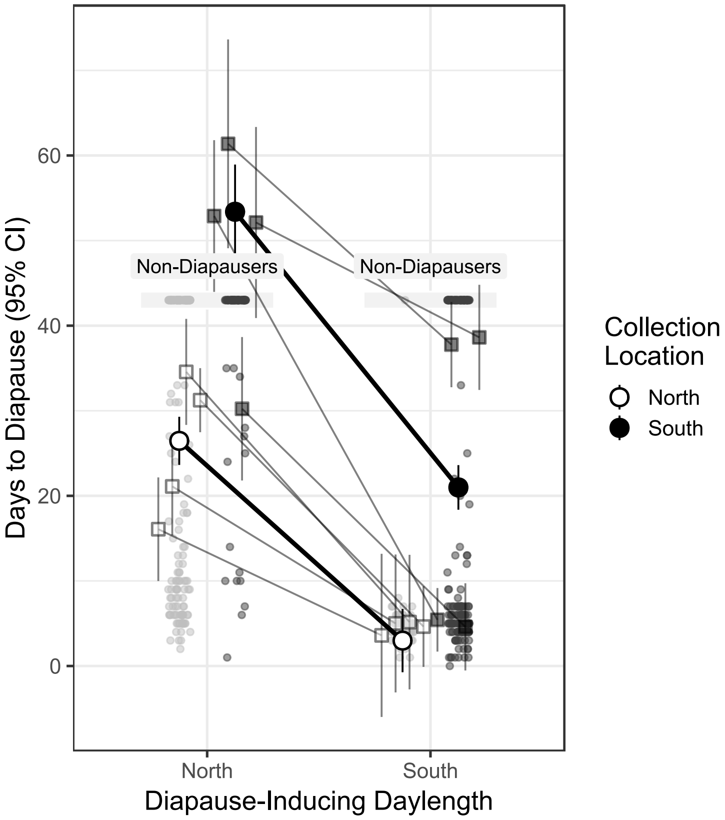

Figure B-2 Days until diapause survival regression analysis

Estimates of days to diapause in two different fall environments from a survival regression analysis. Large white and black points connected by think lines are means of core and edge, respectively (+/- 95% CI). Transparent light and dark grey squares connected by lines are means *of individual core and edge populations, respectively. Light and dark grey points are raw data points of days to diapause.*

Appendix B.3 Heritability days until diapause

Table B-2 Variance components of days until diapause

Variance components ($V_{Additive}$ and $V_{Phenotypic}$), heritability (h^2), and evolvability (I_A) for days until diapause measured on females in two daylength environments. Standard deviations (SD) estimated from bootstrap procedure.

		$V_{Additive}$	$V_{Phenotypic}$	h^2	I_A
North (Home)	Estimate	107.00	139.00	0.76	0.50
	Bootstrap SD	53.54	24.10	0.34	0.25
	Bootstrap 95% CI	(15.69, 224.03)	(92.07, 184.25)	(0.13, 1.42)	(0.07, 1.05)
South (Away)	Estimate	2.39	20.00	0.12	0.04
	Bootstrap SD	1.54	8.87	0.14	0.03
	Bootstrap 95% CI	(0, 5.29)	(5.62, 39.37)	(0, 0.52)	(0, 0.10)

Appendix B.4 Heritability of body mass

Body mass and thorax width are positively and statistically clearly correlated with each other (Pearson correlation=0.64). For body mass, both phenotypic variance and additive genetic variance were higher in females than males. The sire variance component was marginally significant for males (likelihood ratio=3.553, df=1, P=0.05944), while for females it was highly significant (likelihood ratio=20.283, df=1, P=6.679e-06). Heritability of females was estimated to be 0.53 (95% CI 0.11-0.87) and 0.31 (95% CI 0-0.59) for males (**Figure B-3**). Only the heritability estimate for females was statistically clearly greater than zero. Evolvability of body mass for both males and females were close to zero and not statistically clearly different from zero (**Table B-3**).

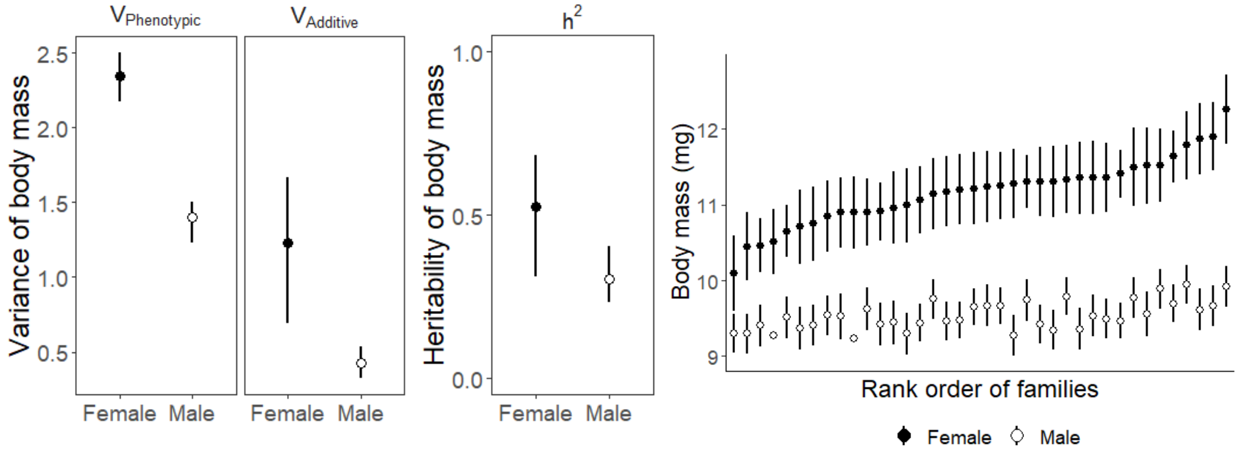

Figure B-3 Variance components of body mass

Total phenotypic and additive genetic variation and heritability of body mass at eclosion of males and females. The right panel shows variation in body mass for males and females by family, ordered on the x-axis by family mean of body mass for females.

Table B-3 Variance components of body mass

Variance components, heritability, and evolvability for body mass of females and males. Standard deviations (SD) estimated from bootstrap procedure.

		$V_{Additive}$	$V_{Phenotypic}$	h^2	I_A
Female	**Estimate**	1.23	2.34	0.53	0.01
	Bootstrap SD	0.51	0.17	0.20	0.00
	Bootstrap 95% CI	(0.23, 2.25)	(2.03, 2.66)	(0.11, 0.87)	(0.00, 0.02)
Male	**Estimate**	0.43	1.40	0.31	0.00
	Bootstrap SD	0.21	0.12	0.15	0.00
	Bootstrap 95% CI	(0, 0.82)	(1.16, 1.64)	(0, 0.59)	(0, 0.01)

Appendix B.5 Heritability of thorax width

The patterns were similar for thorax width as for body mass, though variance and heritability were smaller. Both phenotypic and additive genetic variance were higher in females than males, leading to a heritability estimate of 0.36 (95% CI 0.07-0.61) for females and 0.05

(95% CI 0-0.29) for males (**Figure B-4**). The sire variance component was statistically significant for females (likelihood ratio=8.4198, df=1, P=0.003712), but not for males (likelihood ratio=0.13062, df=1, P=0.7178). Evolvability of thorax width was estimated to be zero for both males and females (**Table B-4**).

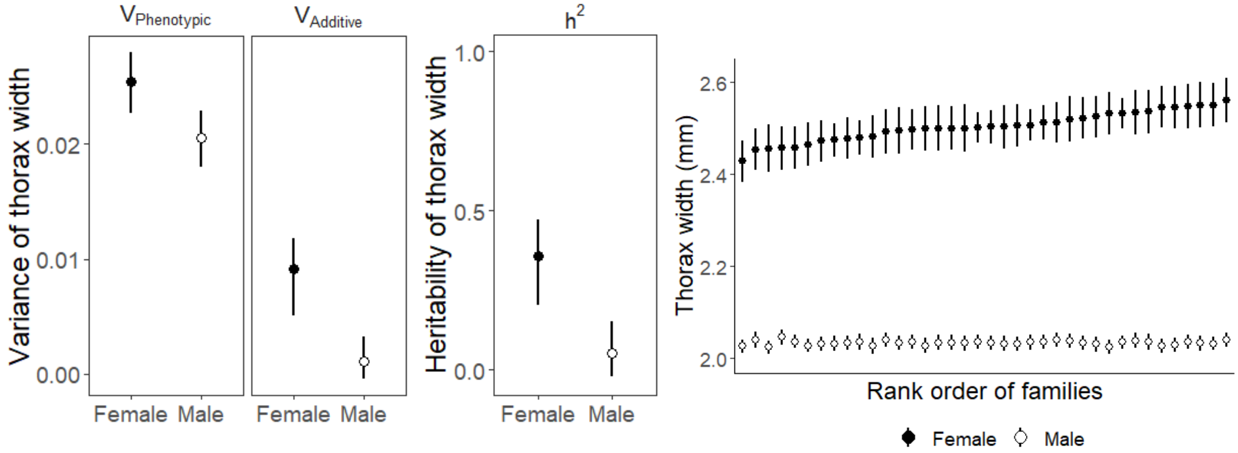

Figure B-4 Variance components of thorax width

Total phenotypic and additive genetic variation and heritability of thorax width of males and females. The right panel shows variation in thorax width for males and females by family, ordered on the x-axis by family mean of thorax width for females.

Table B-4 Variance components of thorax width

Variance components, heritability, and evolvability for thorax width of females and males. Standard deviations (SD) estimated from bootstrap procedure.

		$V_{Additive}$	$V_{Phenotypic}$	h^2	I_A
Female	**Estimate**	0.01	0.03	0.36	0.00
	Bootstrap SD	0.00	0.00	0.14	0.00
	Bootstrap 95% CI	(0, 0.02)	(0.02, 0.03)	(0.07, 0.61)	(0, 0)
Male	**Estimate**	0.00	0.02	0.05	0.00
	Bootstrap SD	0.00	0.00	0.09	0.00
	Bootstrap 95% CI	(0, 0.01)	(0.02, 0.03)	(0, 0.29)	(0, 0)

Appendix C – Chapter 3

Appendix C.1 Collection sites and conditions

Table C-1 Coordinates of collection sites in 2020 and 2022

Population	**Latitude**	**Longitude**
Delta	39.10004	-112.641
St. George	37.08671	-113.562
Big Bend	35.06076	-114.65
Cibola	33.30337	-114.672

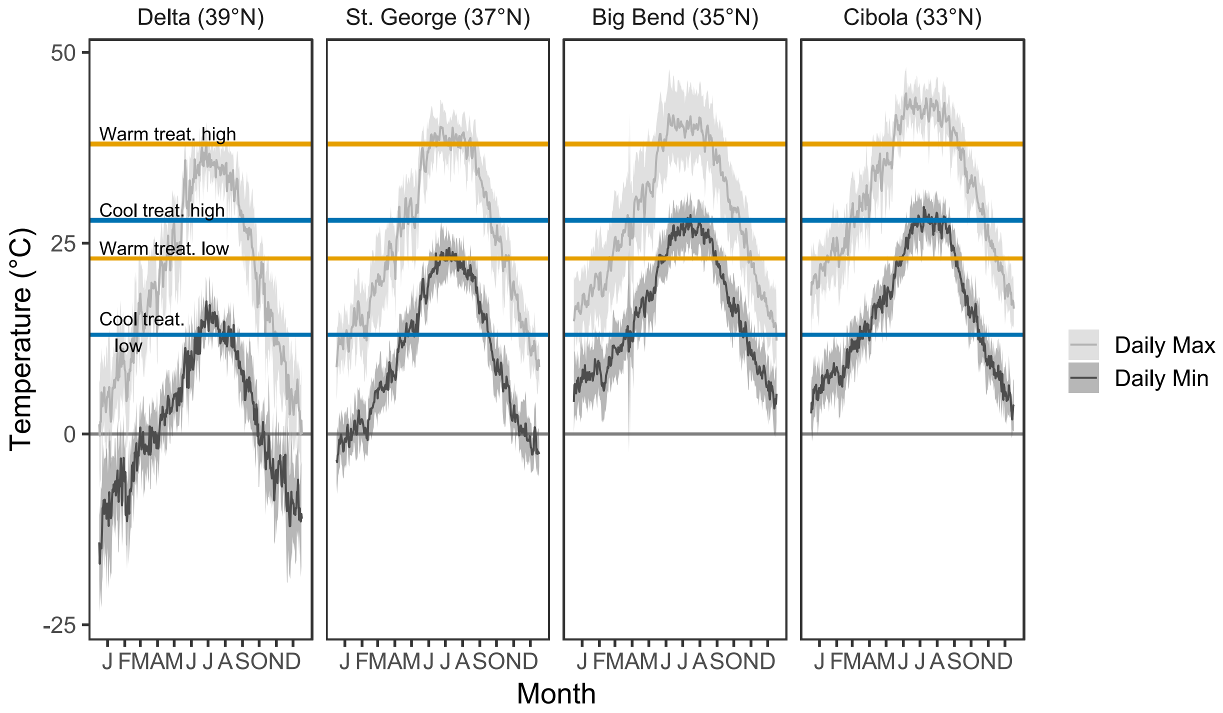

Figure C-1 Site temperatures

Temperatures at each of the four study sites throughout the year. Daily temperature data were from NOAA for the town or weather station closest to each point. Ten years (from 2012 to 2021) of daily maximum and minimum temperatures were averaged for each day of the year. The solid line shows the average temperature and the ribbon shows +/- one standard deviation. Colored horizontal lines indicate the high and low temperatures used in the warm and cool treatments of the phenology experiment.

Appendix C.2 Phenology modeling methods

We estimated the timing of diapause using photothermographs (Beck & Apple, 1961; Grevstad & Coop, 2015), which shows the relationship between daylength and cumulative degree days over a year. Degree days are a continuous measure indicating the amount of time that temperatures are above a thermal threshold, and are commonly used in agriculture and to predict insect pest population development (Pruess, 1983). On a photothermograph, insects are predicted to be in diapause when the daylength at a site is less than the critical daylength of that population. When temperature and daylength cue diapause together, the critical daylength will change throughout the season based on the temperature and insects are expected to be in diapause when the daylength is less than the temperature-informed critical daylength. We estimated diapause timing at each site for each of 10 years using the daylength and temperature responses we observed in the diapause experiment and previously measured critical daylengths (calculated at a single, standard temperature) (unpublished results, Dan Bean). We calculated degree days and calendar days gained or lost due to plasticity and compared diapause timing with the day of first frost for each year. Our goal was to assess whether plasticity was likely to beneficial to fitness, which here, we take to mean provide additional time in the growing season for reproduction, while still entering diapause before freezes.

C.2.1 Degree Days.

We calculated degree days for the tamarisk beetle for all years 2012-2022 at each study site using the tamarisk beetle phenology model on the Oregon State University IPM Phenology and Degree-Day models available online (USPest.org, Deloach et al 2011). We used the single-sine method and the default temperature thresholds (lower: 11.1°C, upper: 36.7°C) to calculate degree days. Some sites were missing data for some years, so in total, there were 9 or 10 years of data for each site. We chose the weather station that had sufficient data to reliably calculate degree days closest to the collection site (**Table C-2**). The phenology model interpolates missing weather data from nearby weather stations or previous years and has accurately predicted tamarisk beetle phenology in the field. We calculated the daylength on each day at the latitude of the site using the *daylength* function in the geosphere package in R (Hijmans 2022). These points are plotted in shades of blue on the photothermographs (**Figure C-3**).

C.2.2 Critical daylength.

We calculated critical daylengths based on the temperature-sensitivity found in the diapause experiments. Because of the temperature-sensitivity, critical daylength changes throughout the year, based on the slope of the response of each population. We estimated these temperature-sensitive critical daylengths each week of the year, to avoid noise from daily fluctuations in temperature. We assumed that critical daylength varied linearly with temperature, since we had only two temperature treatments in the diapause experiment. We estimated the slope and intercept coefficients with a linear model of the critical daylength measured in this experiment with rearing temperature in each treatment as the continuous predictor variable of each site (**Figure 3-2**). We fit the linear model with only the daily high temperature of the treatments (cool: 28°C; warm: 38°C) since the average daily fluctuation in temperature in the field was similar to the condition in the lab (around 15°C). We estimated the weekly plastic critical daylength for each population according to the formula: $CDL_{weekly} = temp_{weekly\ average\ high} * slope + \ intercept$, where $temp_{weekly\ average\ high}$ is the average high temperature for a single week and $slope$ and $intercept$ were the slope and intercept coefficients estimated above. We calculated weekly plastic critical daylengths for each week for the 9 to 10 years of available data for each site. These points (CDL on y-axis, accumulated degree days on the x-axis) are plotted in shades of black on the photothermographs (**Figure C-3**). Non-temperature-sensitive critical daylength estimates are flat lines on photothermographs, because their value does not fluctuate with temperature. Estimates of non-plastic critical daylength were taken from Bean et al. (2023 *in prep*) and are plotted as horizontal black lines in the photothermographs (**Figure C-3**).

C.2.3 Diapause timing.

We estimated the day and degree day of diapause initiation for the 9-10 years of available data for each site. The timing of diapause initiation can be seen visually on the photothermograph as the point when the critical daylength line (black) intersects the daylength line (blue). The non-temperature-sensitive critical daylength occurs on the same day each year, when the daylength is below the critical daylength. The temperature-sensitive critical daylength will occur on a different day each year, depending on the temperature of that year. We calculated the day of diapause initiation as the first day after July when the daylength was less than the weekly critical daylength calculated above.

C.2.4 Days and degree-days gained/lost with plasticity.

Next, we wanted to see how the available time for reproduction changed between a daylength-only cue and a temperature-sensitive cue. This difference can be seen visually on the photothermograph, as the distance between when the intersection of the non-sensitive CDL line and the daylength line and the temperature-sensitive CDL line and the daylength line. we calculated the difference in the degree-day and calendar day of diapause initiation for each cue type. To quantify this, we subtracted the temperature-sensitive calendar day and degree day of diapause initiation from the same quantities for the non-sensitive diapause timings for each year.

C.2.5 Assumptions.

This approach makes several assumptions. First, we assume that the relationship between critical daylength and temperature is linear. Because we could only include two temperature treatments (28 and 38°C) in the experiment, this is the best assumption for this dataset, but at temperatures far below our treatments, the relationship may not be accurate. However, because diapause initiation occurs during the time of year when temperatures are near this range, this assumption should not have a large impact on our results. However, the weekly critical daylengths estimated during the winter, when temperatures are much lower than our treatments, should be interpreted with caution.

Table C-2 Weather stations for temperature data

Site	Weather station	Years
Delta	CMP08; DLTU1	All, except 2019 2019
St. George	KSGU	All except 2012 and 2013
Big bend	KIFP	All
Cibola	CBRA3	Not 2013

Appendix C.3 Supplemental Results

Table C-3 Critical Daylength

Critical daylength, standard error, and Wald 95% confidence interval for critical daylength in each temperature regime in two years of the experiment.

Population	Year	Temp. Treatment	Critical Daylength (hr)	SE	95% CI
Delta (39°N) Shortest day ~9.5 hrs	2020	Cool	14.02	0.072	(13.88, 14.17)
	2022	Cool	14.30	0.047	(14.20, 14.39)
	2020	Warm	14.54	0.104	(14.34, 14.75)
	2022	Warm	15.29	0.305	(14.69, 15.89)
St. George (37°N) Shortest day ~9.5 hrs	2020	Cool	13.73	0.083	(13.57, 13.90)
	2022	Cool	13.98	0.061	(13.86, 14.10)
	2020	Warm	13.53	0.183	(13.17, 13.89)
	2022	Warm	12.51	0.161	(12.19, 12.82)
Big Bend (35°N) Shortest day ~9.75 hrs	2022	Cool	12.00	0.073	(11.86, 12.15)
	2022	Warm	8.23	1.013	(6.25, 10.22)
Cibola (33°N) Shortest day ~10 hrs	2020	Cool	11.71	0.101	(11.51, 11.91)
	2022	Cool	11.64	0.078	(11.48, 11.79)
	2020	Warm	9.54	0.486	(8.59, 10.50)
	2022	Warm	8.51	0.864	(6.82, 10.20)

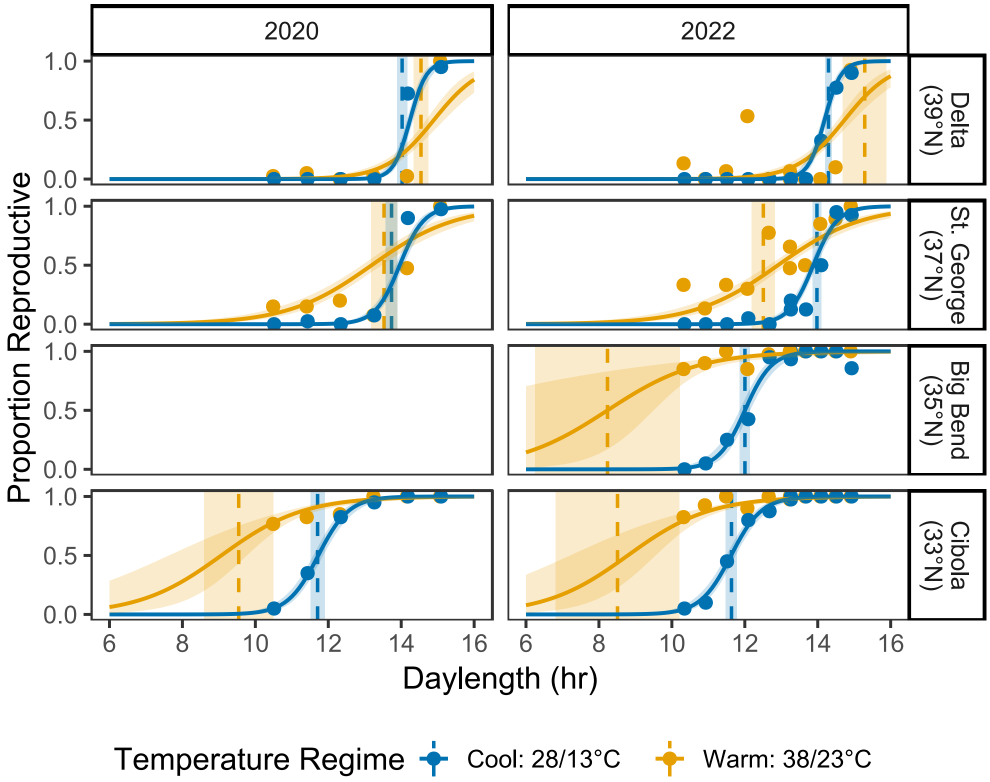

Figure C-2 Diapause response curves

Diapause response of four populations to daylength and temperature regimes in two years. The Big Bend site was only used in 2022. Solid lines and shaded areas indicate the predicted proportion reproductive (not in diapause) from a logistic regression and 95% confidence interval. Points show actual proportion of reproductive individuals. Vertical dashed lines and shaded areas show the estimated critical daylength (50% diapause) and 95% confidence interval. Temperature sensitivity is shown by divergent orange and blue lines and by non-overlapping confidence intervals around the estimated critical daylengths.

Table C-4 Diapause timing

The timing of diapause when critical daylength (CDL) is plastic or non-plastic. The non-plastic CDLs are from Bean et al. (unpublished results, Dan Bean) and plastic CDLs are estimated from the experimental data. Degree days or calendar days gained with plasticity shows how many extra days per season are available for tamarisk beetle reproduction when diapause timing depends on temperature. The figures are negative for Delta, since plasticity in that population reduces the days available for reproduction.

Population	CDL type	Day of CDL	Remaining degree days	Degree days gained with plasticity (SE)	Calendar days gained with plasticity (SE)
Delta	Temp. sens.	195.1 (Jul 15)	1025	**-170.9** (34.83)	**-10.9** (2.30)
	Daylen. only	206 (Jul 25)	854		
St. George	Temp. sens.	242.3 (Aug. 31)	767	**194.3** (38.67)	**10.3** (2.56)
	Daylen. only	232 (Aug 20)	958		
Big Bend	Temp. sens.	291.7 (Oct 20)	433	**280.0** (36.60)	**18.7** (2.42)
	Daylen. only	273 (Sep 30)	714		
Cibola	Temp. sens.	297.8 (Oct 26)	334	**485.4** (36.61)	**34.8** (2.42)
	Daylen. only	263 (Sep 20)	814		

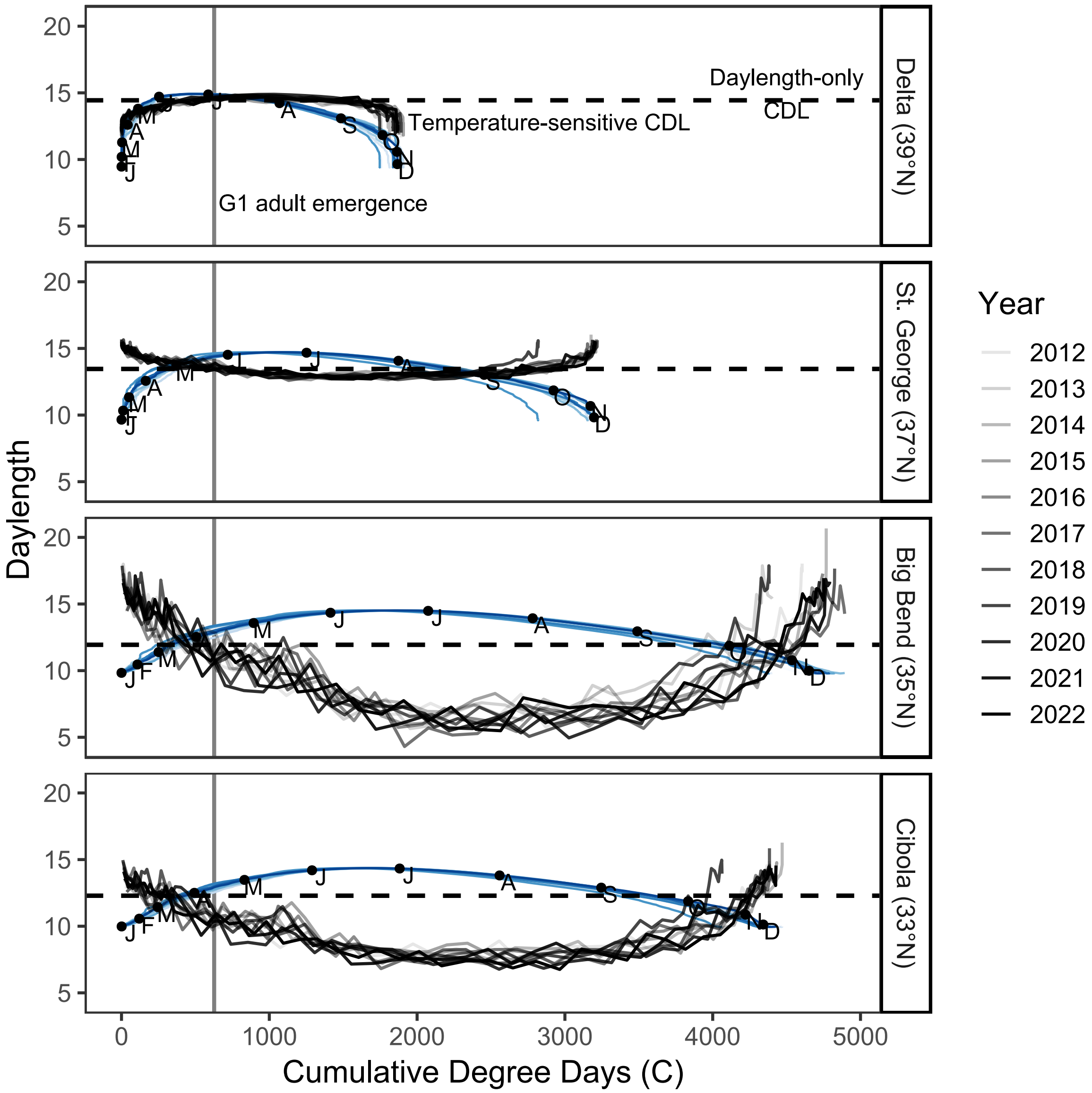

Figure C-3 Photothermographs

Photothermographs for the four sites. The blue curves show the daylength across annual cumulative degree days for 2012-2022. Points and letters indicate the first day of each month. The black horizontal dashed line indicates the daylength-only critical daylength (CDL). The solid black curves indicate the temperature-sensitive critical daylength calculated for each week based on the average high temperature and slope in ***Figure 3-2*** *(see* ***Appendix C.2****). Tamarisk beetles will be reproductive when the critical daylength line (solid or dashed) is below the blue line for a particular year. The vertical grey line indicates the approximate timing of adult emergence for the first generation. Each subsequent generation requires about 550 degree days, though generations overlap later in the season.*

Appendix D - Chapter 4

Appendix D.1 Supplemental Figures and Tables

Table D-1 Hybrid zone collection sites

Diorhabda collection sites with letters corresponding to ***Figure 4-1****. Individuals from bolded sites (A, F, and I) were phenotyped in the lab.*

	Site	Latitude	Longitude	Collection Date
A	**Ute Lake, NM**	35.3391667	-103.44528	9/16/2019
B	Tucumcari Lake, NM	35.1861111	-103.69083	9/16/2019
C	Montoya, NM	35.09236	-104.07638	9/17/2019
D	Prairie Dog Town Fork Red River, TX	34.630144	-100.94195	9/19/2019
E	Lake Sumner, NM	34.616136	-104.38936	9/17/2019
F	**Roswell, NM**	33.396303	-104.39174	9/17/2019
G	NE of Post, TX	33.31729	-101.2609	9/19/2019
H	Lake J. B. Thomas, TX	32.59574	-101.17	9/19/2019
I	**Big Spring, TX**	32.17443	-101.703	9/18/2019
J	Balmorhea, TX	30.955456	-103.72306	9/18/2019

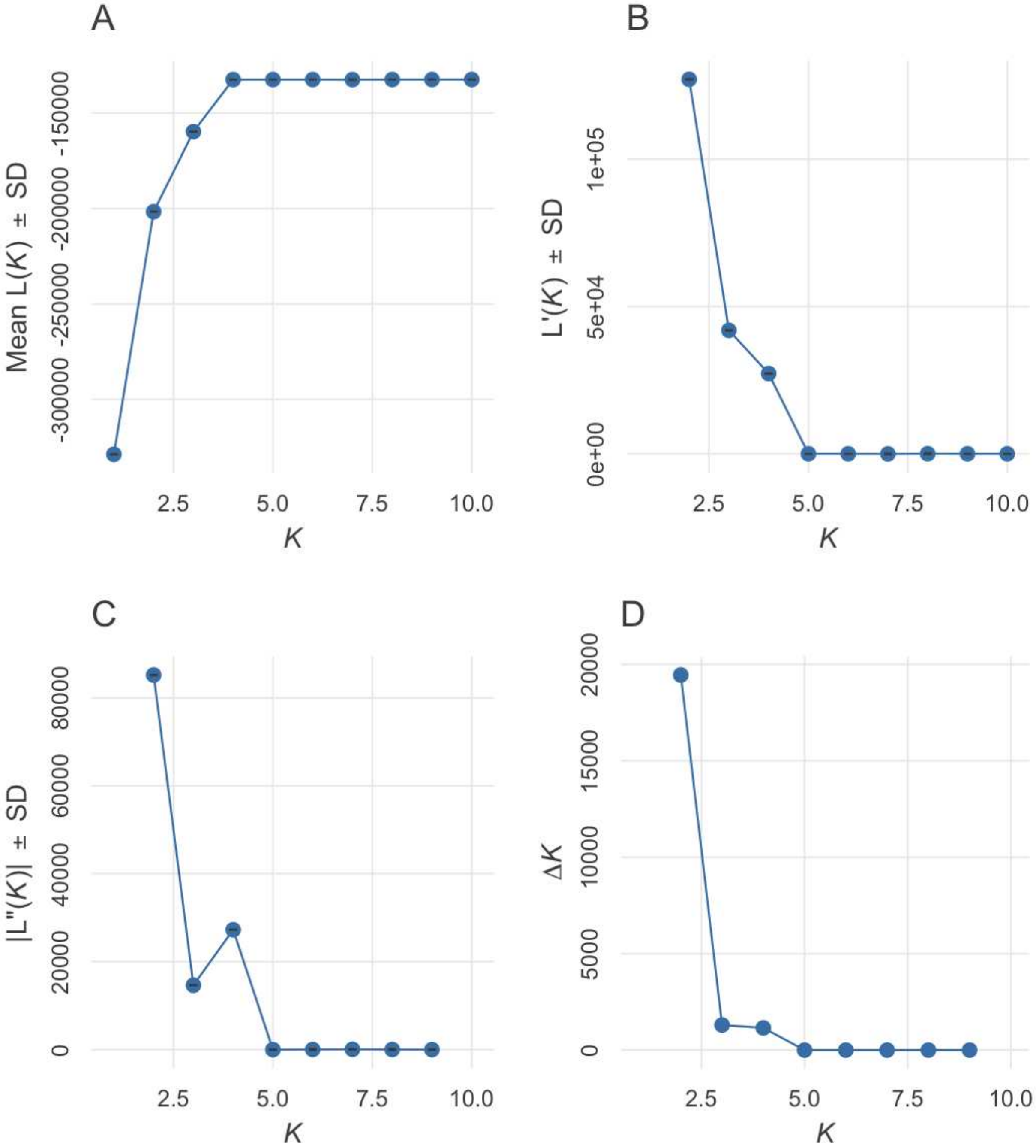

Figure D-1 Likelihood values from Structure

Change in likelihood values from Structure results, visualized according to four methods (A-D) for values of K = 1 to K = 10.

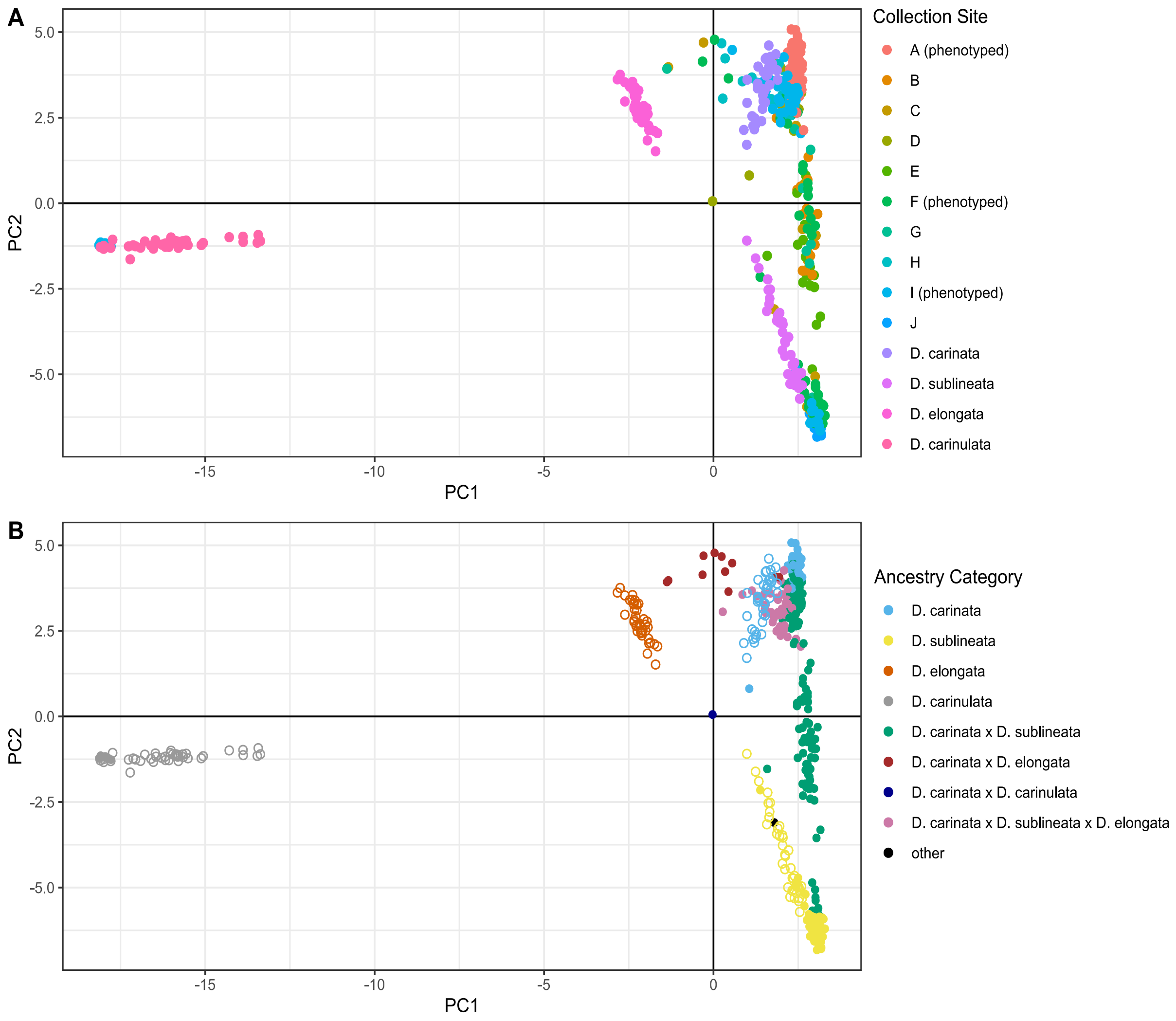

Figure D-2 Principal coordinate analysis of hybrids

Principal coordinate analysis of all field, experimental, and reference individuals. In A, points are colored by collection site of origin or reference individuals of known pure ancestry. In B, points are colored by ancestry category, as described in ***Section 4.2.7****. Unfilled points are the reference individuals.*

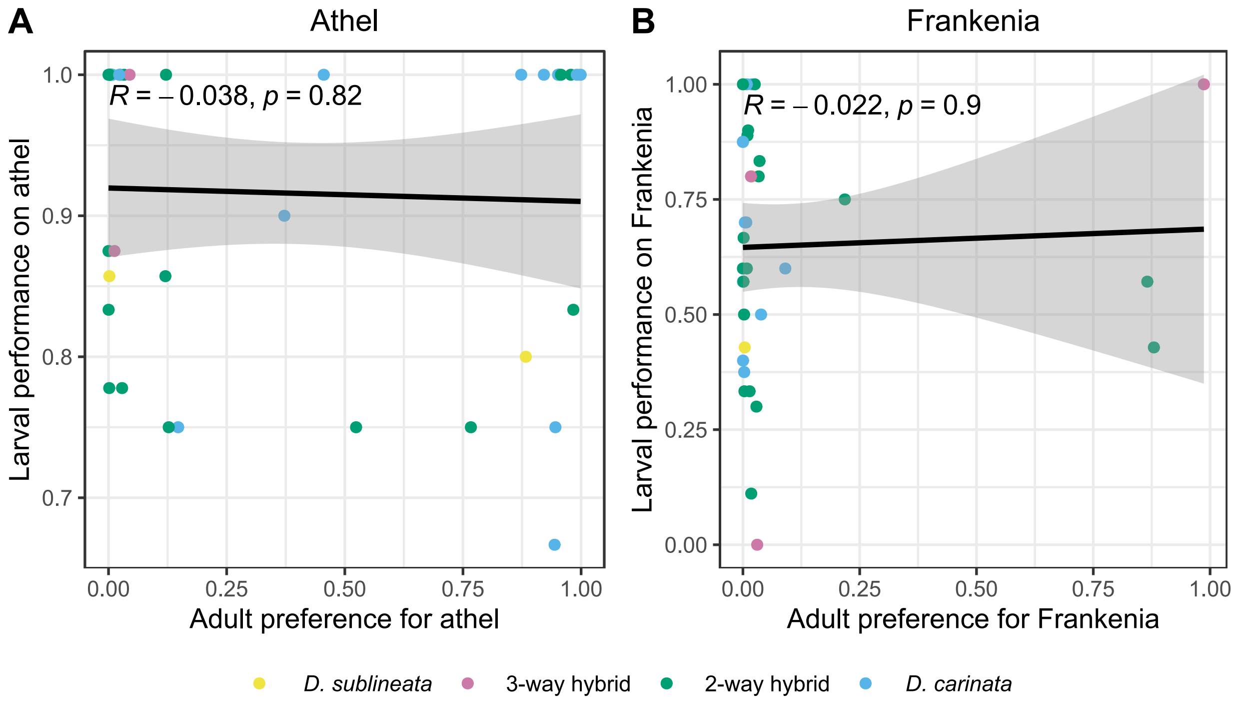

Figure D-3 Preference-performance correlations

Correlation between maternal preference and larval performance (survival) on athel (A), and Frankenia (B). Points show preference of mothers and average larval performance of her offspring on each plant. Colors show the ancestry group the mother was assigned. R and p values show Pearson correlation between x and y variables and significance of correlation, respectively.

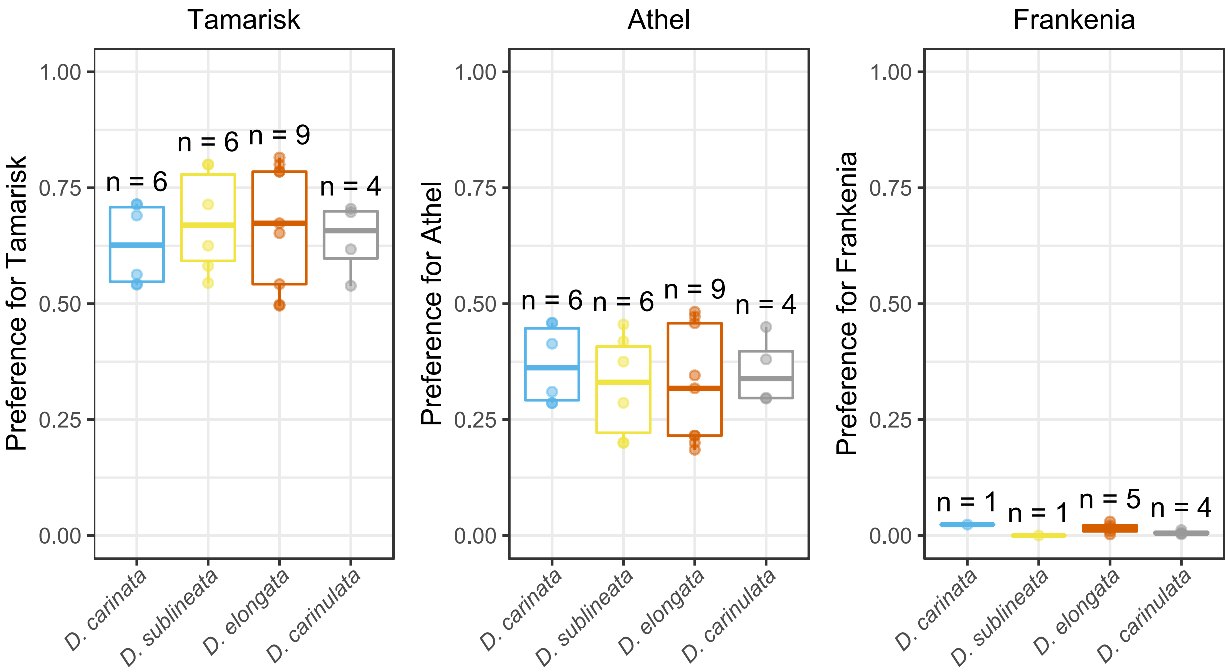

Figure D-4 Host preference from previous studies

*Mean preference for tamarisk (target host), athel, and Frankenia for four Diorhabda species as described in previous studies (**Table D-2**). Studies where athel was not included as part of a choice test were excluded from calculations of mean preference for tamarisk. Numbers above boxplots indicate the number of studies that tested each species in a choice test including that plant.*

Table D-2 Host testing in previous studies

Results from previous host preference testing studies from 2003 to 2017 of Diorhabda spp. on tamarisk (Tamarix spp. target host), athel (T. aphylla), and Frankenia (F. salina). Each line in the table is a separate study on one of the four Diorhabda species or a hybrid. The values for Athel, Frankenia, and Tamarisk are means or totals from a single study on that plant. Cells are blank if that plant was not included as a choice in that study. Multiple choice studies often included hosts other than those reported here, so data was standardized for each study so comparisons could be made for only these three host species. Standardization was done by dividing the score for each plant by the total for each row. Standardized values were used in calculating the results in ***Figure D-4****. Studies where athel was not included as part of a choice test were excluded from calculations of mean preference for tamarisk.*

Reference	**Data Source**	**Species or Cross**	**Response**	**Athel**	**Frankenia**	**Tamarisk**	**Total**	**Standardized Athel**	**Standardized Frankenia**	**Standardized Tamarisk**	**Study Details**
(Milbrath and DeLoach, 2006a)	Table 8	*D. carinata*	% adults	15.8	0.9	21.5	**38.2**	0.41	0.02	0.56	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 2	*D. carinata*	# of adults	3.2		8	**11.2**	0.29		0.71	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 5	*D. carinata*	# of adults	9.4		23.5	**32.9**	0.29		0.71	multiple choice test
(Bitume et al., 2017)	Table 1	*D. carinata*	% frass	45.8		54.2	**100**	0.46		0.54	two-way choice test
(Bitume et al., 2017)	Table 1	*D. carinata*	% frass	31		69	**100**	0.31		0.69	two-way choice test
(Bitume et al., 2017)	Table 3	*D. carinata*	% frass	45.9		54.1	**100**	0.46		0.54	two-way choice test
(Milbrath and DeLoach, 2006a)	Table 8	*D. sublineata*	% adults	14.8	0	17.7	**32.5**	0.46	0	0.54	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 2	*D. sublineata*	# of adults	5.4		9	**14.4**	0.38		0.63	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 5	*D. sublineata*	# of adults	15.2		21.1	**36.3**	0.42		0.58	multiple choice test
(Bitume et al., 2017)	Table 1	*D. sublineata*	% frass	20		80	**100**	0.2		0.8	two-way choice test
(Bitume et al., 2017)	Table 1	*D. sublineata*	% frass	28.6		71.4	**100**	0.29		0.71	two-way choice test
(Bitume et al., 2017)	Table 2	*D. sublineata*	% frass	20		80	**100**	0.2		0.8	two-way choice test
(Milbrath and DeLoach, 2006a)	Table 5	*D. elongata*	% adults	14	0.4	29.7	**44.1**	0.32	0.01	0.67	multiple choice test
(Milbrath and DeLoach, 2006a)	Table 5	*D. elongata*	% adults		0.4	24.95	**25.35**		0.02	0.98	multiple choice test
(Milbrath and DeLoach, 2006a)	Table 7	*D. elongata*	% adults	39.2	2.5	41.3	**83**	0.47	0.03	0.5	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 2	*D. elongata*	# of adults	2.8		10.2	**13**	0.22		0.78	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 4	*D. elongata*	# of adults	2.8		10.2	**13**	0.22		0.78	multiple choice test
(Milbrath and DeLoach, 2006b)	Table 5	*D. elongata*	# of adults	17.4		20.6	**38**	0.46		0.54	multiple choice test

(Moran et al., 2009)	Table 6	*D. elongata*	# of adults	590	4	1114	**1708**	0.35	0	0.65	field test
(Herr et al., 2014)	Figure 4	*D. elongata*	% adults	39.12	1.77	40.18	**81.06**	0.48	0.02	0.5	field test
(Bitume et al., 2017)	Table 2	*D. elongata*	% frass	20		80	**100**	0.2		0.8	two-way choice test
(Bitume et al., 2017)	Table 3	*D. elongata*	% frass	18.5		81.5	**100**	0.19		0.82	two-way choice test
(DeLoach et al., 2003)	Table 5	*D. carinulata*	% frass	13.5		32.2	**45.7**	0.3		0.7	multiple choice test
(Lewis et al., 2003)	Table 6	*D. carinulata*	% adults	26.9	0.19	43.7	**70.79**	0.38	0	0.62	multiple choice test
(Milbrath and DeLoach, 2006a)	Table 5	*D. carinulata*	% adults	10.3	0.2	24.2	**34.7**	0.3	0.01	0.7	multiple choice test
(Milbrath and DeLoach, 2006a)	Table 5	*D. carinulata*	% adults		0.2	43.7	**43.9**		0	1	multiple choice test
(Milbrath and DeLoach, 2006a)	Table 7	*D. carinulata*	% adults	34.4	0.9	41.2	**76.5**	0.45	0.01	0.54	multiple choice test
(Bitume et al., 2017)	Table 3	*D. carinata* × *D. elongata*	% frass	45.7		54.3	**100**	0.46		0.54	two-way choice test
(Bitume et al., 2017)	Table 1	*D. carinata* × *D. sublineata*	% frass	46.2		53.8	**100**	0.46		0.54	two-way choice test
(Bitume et al., 2017)	Table 1	*D. carinata* × *D. sublineata*	% frass	38.2		61.8	**100**	0.38		0.62	two-way choice test
(Bitume et al., 2017)	Table 3	*D. elongata* × *D. carinata*	% frass	54.4		45.6	**100**	0.54		0.46	two-way choice test
(Bitume et al., 2017)	Table 2	*D. elongata* × *D. sublineata*	% frass	50		50	**100**	0.5		0.5	two-way choice test
(Bitume et al., 2017)	Table 1	*D. sublineata* × *D. carinata*	% frass	23.1		76.9	**100**	0.23		0.77	two-way choice test
(Bitume et al., 2017)	Table 1	*D. sublineata* × *D. carinata*	% frass	49.8		50.2	**100**	0.5		0.5	two-way choice test
(Bitume et al., 2017)	Table 2	*D. sublineata* × *D. elongata*	% frass	61.5		38.5	**100**	0.62		0.39	two-way choice test