Floral Evolution Beyond Phenology: Adaptive Dynamics in Plant-Pollinator Interactions Under Global Change

by

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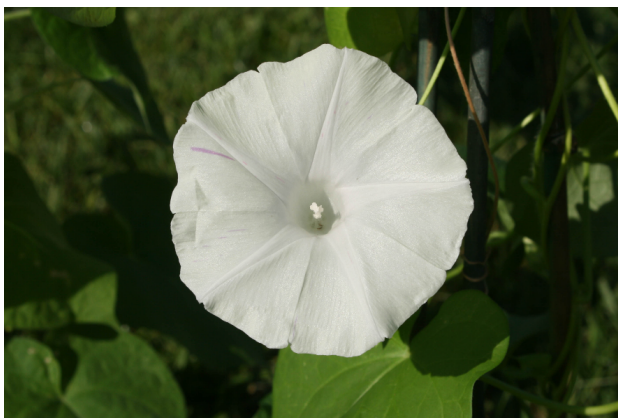   Associate Professor Rachel Spigler, Temple University

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Dedication

Finally, I owe a huge thank you to the many field and growth room technicians and assistants who contributed to plant maintenance and measurements.

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As my research has become more focused on the intersecting crises of climate change, habitat destruction, and other anthropogenically driven processes enmeshed in the oppressive structures of late stage capitalism, I have been grateful for the people who envision a more hopeful future and keep me grounded in methods of collective resistance. Specifically, I want to thank people in GEO, the Climate Action Movement at U-M, Science for the People, as well as a number of unnamed anti-authoritarian and liberatory struggles off-campus that I've been fortunate to learn from and be inspired by. I cannot name all those who have touched my life in

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Abstract

Global change generates rapid shifts in multiple environmental variables simultaneously, forming a multifactorial suite of pressures to which organisms must respond. To date, research regarding adaptive responses in plants has focused largely on flowering phenology, and little is known about other floral traits that may underlie adaptive response or how they interact with phenology to direct the evolutionary rate and trajectory of plant populations. Thus, my research addresses the overarching question: *Is there adaptive potential in floral traits beyond phenology to respond to selection from global change, and what are the implications for plant-pollinator interactions?*

Using an annual, mixed mating plant, *Ipomoea purpurea*, I conducted multiple resurrection experiments with seeds collected from the same populations in 2003 and 2012 to explore adaptation in floral traits to global change. Chapter 1 introduces the broad goals of the dissertation. In the first data chapter, Chapter 2, I investigate phenotypic evolution in a suite of pollination or mating system related traits and identify phenotypes underlying spatial and temporal adaptive responses. The findings from this study reveal that, beyond the well-documented shift to earlier flowering, corolla width, nectar sucrose content, and pollen count also play a role in adaptation to global change. I also find that changes in flowering phenology and corolla width are driven primarily by populations at more northern latitudes, and screen for signals of drift and migration to show that these changes are most likely adaptive.

In Chapters 3 and 4, I assess the potential for constraints on continued evolution in these traits and whether phenotypic evolution can be attributed to pollinators as an agent of selection in line with a hypothesis of an adaptive path that favors increased investment in pollinator attraction. The relationship between plant traits and fitness in ancestral (2003) and descendant (2012) populations revealed that descendant populations are more limited in their response to selection than ancestral populations, and correlative selection is present in descendant populations between flowering phenology and three other floral traits: corolla width, corolla length, and nectar sucrose content. I also show that the overall rate of evolution in these traits is constrained due to trait-trait covariances, and that the rate of evolution slows between 2003 and 2012, likely due to lowered variation. In Chapter 4, I find that pollinators select for large corolla width, high nectar sucrose content, and large ASD, but additional direct effects of corolla width and corolla length on fitness in descendant populations indicate that the constraint on the evolvability of flowering phenology I report in Chapter 3 is only partially explained by a mechanism of increased investment in pollinator attraction.

In brief, my dissertation provides novel evidence of floral traits beyond flowering phenology responding in real-time as a result of global change and highlights an adaptive pathway of increased investment in pollinator attraction. Furthermore, it represents the first study to use a resurrection framework to 1) assess differences in response to selection over time and show that adaptive potential in a plant-pollinator mutualism is declining, and 2) trace changes in adaptive potential in time to the selective mechanism and show that constraints on evolvability of flowering time are partially attributable to increased investment in pollinator attraction. While

responses to global change are expected to be species and region specific, collectively, this work

reveals that consideration of multivariate trait evolution provides important information

regarding expected rate and direction of adaptive response to global change.

Chapter 1 Introduction

Global climate change, over-exploitation, and habitat alteration are causing rapid, simultaneous, and sustained pressures on wild populations at rates that exceed those seen for millennia (IPCC 2022). Novel selective environments that involve changes in temperature, precipitation, photoperiod, CO_2 and N_2O levels, and shifting community composition and abundance of symbiotic partners form a multifactorial suite of pressures to which organisms must respond. Habitat fragmentation and destruction are limiting the ability of many species to spatially track their preferred environment, resulting in an increased need to respond plastically or adapt *in situ* to avoid extinction (Parmesan & Yohe, 2003; Razgour *et al.*, 2019). Theoretical projections of adaptive potential rely primarily on existing genetic variation and strength of selection to predict a population's response to novel environmental conditions (Visser, 2008). However, plasticity, genetic correlations between traits, indirect selection, and conflicting selection from multiple agents all contribute to significant, exigent discrepancies between these predictions and realized responses to selection in field settings (Lande & Arnold, 1983; Houle, 1991; Nussey *et al.*, 2007; Hadfield & Others, 2012). Changes in these factors hold the potential to alter evolutionary trajectories on a global scale and have been acknowledged as one of the main deficiencies in evolutionary forecasting models (Martin *et al.*, 2023), yet, despite this, we currently have very little understanding of how they may respond to global change or combine to limit adaptive ability in keeping pace with large scale climatic shifts. As such, my thesis uses a plant-pollinator system to investigate a suite of floral traits involved in mediating pollinator interactions to ask: What floral traits, if any, display phenotypic evolution over time reflective of a response to

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complex, naturally occurring selection in their natural environment? If floral traits are involved in an adaptive response, do those changes align with an evolutionary trajectory of increased self-pollination or increased pollinator attraction? Are there spatial differences in direction or degree of phenotypic evolution? Is response to selection (adaptive potential) changing over time? Is there evidence of trade-offs due to correlative selection acting on the interaction of focal traits? Is the rate of evolution constrained by trait correlation? If so, is the level of constraint on continued evolution changing over time? Is there plasticity in traits and, if so, does it differ between ancestral and descendant populations? Is the mechanism of selection underlying trait changes in line with a hypothesis of increased investment in pollinator attraction?

Plant Response to Global Change

 To date, the vast majority of research on plant responses to global change in natural, field settings has focused on changes in flowering time, showing that it is responsive to shifts in both temperature (Dai *et al.*, 2017; Cheptou *et al.*, 2022)and precipitation (Chand *et al.*, 2022), with a general trend toward earlier flowering (Parmesan, 2006; Schweiger *et al.*, 2010; Colautti *et al.*, 2017; Hamann *et al.*, 2021). This has raised concern over the possibility of temporal asynchrony between plants and pollinators, resulting in a disruption of the plant-pollinator mutualism (Hoover *et al.*, 2012; Thomann *et al.*, 2013; Robbirt *et al.*, 2014; Miller-Struttmann *et al.*, 2015; Gérard *et al.*, 2020). However, selection does not act on single traits in isolation, but rather on a multivariate composite of traits optimizing fitness and plant-pollinator interactions that are mediated by several floral traits beyond phenology, including floral morphology and rewards received via nectar and pollen (Glenny *et al.*, 2018; Parachnowitsch *et al.*, 2018; Sletvold, 2019; Chapurlat *et al.*, 2020; Descamps *et al.*, 2021). Experimental manipulations of single

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environmental variables or pollinator abundance demonstrate that multiple traits may experience selection from multiple sources that align with environmental shifts observed under global change, and thus may play a role in an adaptive response. For example, insect pollinators have been shown to select for high nectar concentration and low volume in *Rhododendron* communities (Basnett *et al.*, 2021), whereas reduced water selected for high nectar volumes in *Phlox drummondii (Suni et al., 2020)*. Similarly, flower size and number can decrease as temperature increases both plastically (Razanajatovo *et al.*, 2020) and through adaptation (Acoca-Pidolle *et al.,* 2023), but a reduction in pollinator abundance results in selection for increased floral size in *Lobelia siphilitica* and *Gentiana dahurica* (Hou *et al.*, 2019; Brown & Caruso, 2023). Pollinators can also have interacting effects with abiotic variables, such as bumblebees selecting for larger flowers in *Mertensia ciliata* under water stress, but toward an intermediate flower size when water is abundant (Gallagher & Campbell, 2017). Cumulatively, this raises the question of what traits are involved in adaptive responses in their natural, field setting, and how multiple, interacting sources of selection on multiple traits may constrain or facilitate evolution.

Resurrection Experiments

A resurrection approach involves growing ancestral and descendant genotypes sampled from the same location together in a common environment (Davis *et al.*, 2005; Franks *et al.*, 2008) and examining trait differences between years. By growing propagules together, this approach is an extension of the concept behind a spatial common garden set-up, allowing us to distinguish phenotypic evolution from phenotypic plasticity (Etterson *et al.*, 2016). Use of a resurrection approach avoids assumptions implicit in other common methods for understanding contemporary

evolution such as space-for-time substitution, which, though useful, accepts divergence among spatially separated populations as reflective of evolutionary changes over time, an assumption that is not always robust, particularly as we see increasingly novel combinations of environmental characteristics due to global change (Hoffmann & Weeks, 2007; Colautti & Barrett, 2013). If samples of ancestor and descendant populations are unbiased, the resurrection approach provides a direct and definitive assessment of change in phenotypes attributable to microevolutionary processes and can be combined with experimental manipulation or spatial sampling to simultaneously assess mechanistic underpinnings or spatial-temporal dynamics (Franks *et al.*, 2018; Vtipil & Sheth, 2020; Kooyers *et al.*, 2021)

The resurrection approach has been increasingly acknowledged as an especially pertinent approach to studying rapid environmental shifts such as those exemplified by global climate change, inspiring large-scale efforts such as Project Baseline (Etterson *et al.*, 2016), a cross-institution initiative to increase laboratory-stored seed banks that maintain maternal line structure for use in resurrection and quantitative genetics studies. Environmental change is happening at unprecedented rates (IPCC 2022), and the biological effects of these changes are widespread. As evidence for the rate of climate shifts exceeding the dispersal capacity of many species accumulates (Davis & Shaw, 2001), resurrection experiments play a critical role in assessing the question of if and how adaptive change will contribute to species persistence. One of the first studies to use a resurrection approach to showcase the potential for detecting rapid evolution found earlier flowering time within seven generations of *Brassica rapa* in response to drought (Franks *et al.*, 2007). Further studies have found shifts in flowering time in response to changes in precipitation patterns (Dickman *et al.*, 2019) and temperature, evolution of plasticity (Vahsen

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et al., 2023), and evolution of herbicide resistance (Kuester *et al.*, 2016). I use a resurrection approach in all three data chapters to investigate phenotypic evolution in floral traits. Populations are collected at two time points – once in 2003 and again in 2012, with a subset of two populations collected again in 2016. There are no known changes in land use in this region over that time, so, barring an unlikely incident of introduction of additional individuals to the region, detected changes in phenotype are reflective of evolutionary change in response to other global change variables, namely climatic shifts or declines in pollinator populations.

Study System

My experiments all use a resurrection approach with the annual, weedy vine, *Ipomoea purpurea*, also known as the common morning glory. *I. purpurea* is native to subtropical regions in the Americas but has been a popular ornamental plant in Europe since the sixteenth century due to its large, colorful floral display (Fang *et al.*, 2013). Introduced to North America by way of European exchange in the seventeenth century, *I. purpurea* is now naturalized across wide areas of the Southeast and Midwest United States and grows predominantly in disturbed habitats along roadsides or adjacent to agricultural fields (Baucom & Mauricio, 2008; Alvarado-Serrano *et al.*, 2019). Plants that grow at this agro-ecological interface hold a contested role as either problematic weeds or important reservoirs of insect biodiversity that provide critical ecosystem services such as pollination and pest control (Daily & Others, 1997; Egan & Mortensen, 2012). A review of weed species in southern states categorizes morning glory as one of the most troublesome weeds in common crops such as soybean, cotton, and corn fields (Webster 2010 & 2012). However, morning glories are also known to attract a variety of pollinators such as bees, lepidoptera, and hummingbirds with their wide, trumpet-shaped flowers, and they bloom until

the first frost, indicating that they could play an important ecosystem role in supporting local pollinator communities, especially late in the growing season (Defelice, 2001).

Plant species existing at the edge of agricultural fields are exposed to multiple, simultaneous processes associated with human-mediated global change including: climate changes, frequent land use shifts and habitat fragmentation, herbicide and pesticide use, and insect declines as a result of all three. As a result, *I. purpurea* is a powerful study system for exploring adaptive responses to global change and how they interact with the eco-evolutionary dynamics of plant-pollinator interactions.

In addition to existing at the agro-ecological interface, *I. purpurea* has a history of utility in the field of evolutionary and ecological study, contributing to theoretical frameworks regarding mating system evolution (Durbin *et al.*, 2003; Zufall & Rausher, 2003), molecular evolution (Streisfeld & Rausher, 2009), and herbicide resistance evolution (Baucom, 2019). As such, some basic natural history, trait characterization, and genomic resources for the species already exist, placing it at the cusp of becoming a model system for contemporary evolution. Populations across the southeast display high levels of inter-population gene flow such that strong forces of drift and isolation by distance are unlikely to be strong forces behind phenotypic differences (Alvarado-Serrano *et al.*, 2019), and high levels of floral trait variation are recorded in field settings (Fang *et al.*, 2013). Recently, a closely related species, *Ipomoea hederacea* has been used for multivariate trait-based approaches to highlight adaptive spatial clines that are difficult to detect with univariate trait measures (Stock *et al.*, 2014) and misalignment between selection and multivariate genetic variation (Henry & Stinchcombe, 2023), highlighting the potential

importance of multivariate considerations in evolvability and response to contemporary global changes. *I. hederacea*, however, is 92-94% selfing (Campitelli & Stinchcombe, 2014), whereas *I. purpurea* displays a 50-70% outcrossing rate (Epperson & Clegg, 1987a, 1987b; Kuester *et al.*, 2017), with some evidence that maintenance of preferential outcrossing and delayed self-pollination buffers against temperature changes (Liu *et al.*, 2020). Uncertainty over response to climate and other anthropogenically-induced changes is especially acute for mixed-mating species, where, in the face of global pollinator declines and shifting suites of abiotic variables, both selection for increased outcrossing (Bishop *et al.*, 2017) and selection for increased selfing (Jones *et al.*, 2013) are possible adaptive responses. As such, information generated through this work addresses multiple key questions regarding adaptation under global change by addressing a multivariate adaptive response within the context of a critical ecological mutualism, and sets this system up to serve as a model for continued investigation of the impact of ongoing anthropogenic climate change.

Thesis Overview

In chapter 2, I utilize data from multiple greenhouse resurrection experiments to investigate the phenotypic basis of contemporary adaptive responses to global change between the years of 2003 and 2012 in a mixed mating system plant. I compare measurements of six traits involved in regulating plant-pollinator interactions or mating system determination – corolla width, corolla length, anther-stigma distance, nectar sucrose content, pollen count, and the date of emergence of the first flower on a plant – between populations sampled at different time points (2003 and 2012). I find a significant temporal increase in corolla width and a shift to earlier flower emergence as well as some evidence of increased investment in floral rewards traits (*i.e.,* nectar

sucrose content and pollen count). Further, I identify latitudinal sensitivity in evolutionary response, with changes in corolla width and phenology both being driven primarily by populations at more northern latitudes. Finally, I screen for strong signals of drift and migration to infer that selection is likely acting to produce the observed spatial-temporal trait changes. Overall, this work identifies phenotypic traits involved in an evolutionary response to global change and indicates increased investment in pollinator attraction rather than self-pollinating capability.

In chapter 3, I focus on the potential for constraints on continued trait evolution. I perform a resurrection experiment in a field setting to determine whether covariance between traits is constraining the rate or direction of adaptation in the same set of traits investigated in chapter 2 and whether those constraints are reducing adaptive potential. I find that, despite the persistence of individual trait variation, descendant populations are more limited in their response to selection compared to ancestral populations, and, further, correlative selection (indicative of trait trade-offs) between corolla width, nectar quality, and phenology manifests in descendant, but not ancestral populations. I also use changes in the variance-covariance (**G**-) matrices to show that the overall evolutionary rate among these traits is constrained in both years and shows greater constraint in descendant compared to ancestral populations, indicating that the rate of adaptive change is slowing despite sustained selective pressures from global change. Finally, I compare trait values from a replicate resurrection experiment in a growth room to show that corolla width, corolla length, nectar sucrose content, and date of first flower are all highly plastic traits. Temporal trait changes are more extreme in a controlled growth room setting, however the

degree of difference between the field and growth room is reduced in descendant populations, additionally showing that plasticity in these traits is decreasing over time.

In chapter 4, I explore whether the adaptive changes identified above can be attributed to pollinators as an agent of selection in alignment with a hypothesis of increased investment in pollinator attraction as a driving selective force that constrains adaptive response in phenology. Here, I perform pollinator preference surveys and structural equation modeling to identify causal relationships between functional trait value, pollinator visitation, and plant fitness, and quantify differences in the structure of these relationships over time. I find that the effects of corolla width, nectar sucrose content, and anther-stigma distance (ASD) on fitness are entirely mediated through pollinator behavior in ancestral populations, whereas corolla width has an additional, direct effect on fitness in descendant populations that is not mediated by pollinator behavior, as does corolla length. Fitness benefits from earlier flowering, on the other hand, are not attributable to pollinators at all. Cumulatively, this work demonstrates that, although there is evidence of selection by pollinators, the constraint on flowering time evolution due to correlation with corolla width and nectar sucrose content is not entirely explained by a hypothesis of increased investment in pollinator attraction.

In Chapter 5, I synthesize results from all three of my data chapters and discuss future directions for addressing remaining gaps in our knowledge. Additionally, I include a series of supplemental figures and tables to accompany each chapter.

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Chapter 2 Not Just Flowering Time: A Resurrection Approach Shows Floral Attraction Traits Are Changing Over Time

2.1 Abstract

Contemporary anthropogenic changes in climate and landscape form a complex set of selective pressures acting on natural systems, yet, in many systems, we lack information about both whether and how organisms may adapt to these changes. In plants, research has focused on climate-induced changes in phenology and the resultant potential for disruption of plant-pollinator interactions, however, there remains a paucity of knowledge regarding how other pollinator-mediated traits may be involved in adaptive response. Here, I use resurrection experiments to investigate the phenotypic basis of adaptation in a mixed-mating system plant, the common morning glory (*Ipomoea purpurea*). Specifically, I measure temporal and spatial changes in traits grouped into three categories relevant to plant-pollinator interactions - floral morphology, floral rewards, and floral phenology. I show a significant temporal increase in corolla size and shift to earlier flowering times, as well as a potential for increased investment in floral rewards, all of which are driven primarily by populations at more northern latitudes. Additionally, I find evidence for directional selection on floral morphology and phenology, and evidence of balancing selection acting on anther-stigma distance. Overall, these results show an adaptive response in line with greater investment in pollinator attraction rather than self-pollination and fine-scale spatial differences in adaptive potential.

2.2 Impact Summary

Studies of global change in plants typically examine floral phenology due to the potential for pollinator and plant population mismatches. Several other under-investigated floral traits also play a critical role in mediating these interactions, however, and show potential to play an adaptive role in plant response to global change. In this work, I use a resurrection approach where I take seeds of the common morning glory collected from the same populations at two different time points and raise them together in a common garden to measure differences over time in multiple traits related to pollinator interactions and self-pollinating behavior. I find evidence of selection for increased corolla size and earlier flowering times as well as indication of increased investment over time in floral rewards. I also show that there are clear spatial differences in trait evolution within the relatively narrow range of the southeastern United States, with northern populations displaying greater phenotypic shifts. Overall, our work highlights an instance of evolutionary investment in traits that underlie pollinator attraction, and identifies phenotypes beyond phenology that may underlie adaptive responses in plants to the complex and rapid environmental changes associated with contemporary global change and biodiversity declines.

Keywords: floral traits, global change, rapid adaptation

2.3 Introduction

Global change factors such as climate and land use changes are causing rapid, simultaneous shifts in several environmental variables, consequently exposing communities to novel combinations of abiotic and biotic conditions. These changes include altered temperature, precipitation, photoperiod, CO_2 and N_2O levels, many of which display seasonally and geographically distinct patterns, and all of which together form a complex and multifactorial suite of selective pressures (IPCC 2022). For most species, we have very little understanding of which traits may underlie adaptive responses when exposed to the multivariate selective pressures typical of global change (Abatzoglou et al. 2020; Gallagher and Campbell 2021). In plant systems, the potential that climate or land use changes may disrupt plant-pollinator interactions is of particular concern. This is because many insect pollinators have faced significant global declines (Potts *et al.*, 2010; Winfree *et al.*, 2011; Thomann *et al.*, 2013; Hallmann *et al.*, 2017; Soroye *et al.*, 2020), and these declines have been accompanied by concomitant reductions in insect-pollinated plants (Biesmeijer *et al.*, 2006).

Much of the research investigating climate-induced disruptions of plant-pollinator interactions has focused on flowering phenology, with studies typically showing a general trend of earlier flowering across species (Byers, 2017; Renner & Zohner, 2018; Gérard *et al.*, 2020; Soares *et al.*, 2021). However, plant-pollinator interactions are not mediated by phenology alone, but by an array of interacting traits influencing both rate of pollinator visitation and pollinator effectiveness (Glenny *et al.*, 2018; Sletvold, 2019). For example, pollinator preference positively correlates with corolla size (Galen, 2000; Chapurlat *et al.*, 2020), and plant-pollinator interactions are often mediated by floral rewards received by pollinators in the form of nectar and pollen (Eckert *et al.*,

2010; Campbell & Powers, 2015; Descamps *et al.*, 2018, 2021; Parachnowitsch *et al.*, 2018). Although standing genetic variation of these floral traits is frequently high in field settings, demonstrating a potential for rapid evolutionary shifts (Thomann *et al.*, 2015), strikingly few studies have investigated changes in suites of pollinator-mediated traits beyond flowering phenology, such as corolla size and/or traits associated with floral rewards.

Traits related to self-pollination can also evolve given global shifts (Van Etten & Brunet, 2013), with some evidence pointing to increased selfing as climate change and/or pollinator declines associated with land use changes (Eckert *et al.*, 2010; Jones *et al.*, 2013; Cheptou, 2019). A generalized expectation for hermaphroditic plant species in this regard is a shift to smaller anther-stigma distances – *i.e.,* decreased distance between the anthers and stigmas within perfect flowers (Chang & Rausher, 1998) – since smaller anther-stigma distance is highly correlated with greater self-pollination (Chang & Rausher, 1998), as well as decreased investment in pollinator attraction traits such as flower size and nectar quality (Levin 2010). However, there is a major gap in our understanding of how traits that are crucial for plant-pollinator interactions may be evolving over time as a response to a changing climate, and a number of predictions could be made. Are traits responsible for plant-pollinator interactions evolving in light of pollinator decline, such that plants are evolving greater floral displays to attract pollinators? Or are traits that promote selfing like lower anther-stigma distance evolving to maintain populations in light of reduced pollinator presence?

In this work, I compare floral traits of populations of *Ipomoea purpurea* (common morning glory) stored as seed for a number of years to that of contemporary populations (*i.e.* a

resurrection approach) to examine the potential that traits responsible for plant-pollinator

interactions and self-fertilization may be evolving over time and in light of global change.

Specifically, I used three separate common garden greenhouse studies to compare floral traits of

populations sampled in 2003 to those of the same populations sampled nine years later in 2012.

Populations were located across a large range of the southeast and midwest US, such that I

examined floral morphology and flowering phenology trends across both different collection

times and spatial locations. I measured traits grouped into three classes relevant to plant-

pollinator interactions – floral morphology, floral phenology, floral rewards – and addressed the

following questions: (1) Is there evidence of variation in floral traits and do those traits exhibit

any changes between sampling years (2003 vs. 2012)? and (2) Are changes in phenotype likely

the result of adaptation through natural selection or neutral processes? Our expectation is that an

adaptive response toward greater selfing will result in selection for decreased anther-stigma

distance and smaller flower size, whereas positive changes in floral size and rewards indicate

greater investment in pollinator attraction. Cumulatively, our results fill critical gaps in our

understanding of plant responses to environmental change by highlighting adaptive changes in

floral traits beyond phenology and providing evidence of small-scale spatial heterogeneity in

adaptive potential.

2.4 Methods

Study System & Sampling History

Ipomoea purpurea (Convolvulaceae), or the common morning glory, is an annual, weedy vine

widely distributed across the eastern, midwestern, and southern United States (Tiffin & Rausher,

1999). It is frequently found along roadsides or in agricultural settings, often in areas of high

disturbance (Tiffin & Rausher, 1999). The species employs a mixed mating system as it

outcrosses ~50% of the time (Kuester *et al.*, 2017) and is typically pollinated by bees, syrphid flies, and wasps. *Ipomoea purpurea* germinates in late spring and typically begins flowering after 6-8 weeks of growth. Flowers are variable in color, with white, pink, and blue morphs of varying intensity and hue (Epperson & Clegg, 1988). Flowering continues until the first frost, and the fruits are dehiscent capsules that contain between one to six seeds.

In this resurrection study, I used replicate seeds sampled at two time points (2003 and 2012) from 23 different populations located within agricultural fields from Tennessee and North and South Carolina in the US (Figure 2-1). Details of the sampling are presented in (Kuester et al. 2016). Briefly, at each sampling time point, a 10-30 m transect was drawn and seeds from 10-20 flowers on a single plant were collected at 1-2 m intervals down the transect. Over this period, this region has seen climatic changes such as increased inland salinity from coastal sea level rise; an increase in the number of extreme rainfall events interspersed with more extreme drought; and rising temperatures, particularly rising minimum and nighttime temps (Reidmiller et al., 2019). Agricultural crops within sampled fields altered between soy and maize between 2003 and 2012 as is typical of crop rotation schemes, however GIS images indicated that no major changes in land-use occurred between sampling years. Thus, the collections from these populations represent time series data that capture environmental and phenotypic changes from the combination of climatic or agricultural regime changes.

Greenhouse Experiments

Floral Morphology - To assess floral morphology, I planted replicates of field-collected seeds from maternal lines sampled from 15 populations (Figure 2-1; Table S2-1) distributed from the

Cumberland Plateau of central Tennessee to the Coastal Plain region of North and South

Carolina and from two collection times (2003 and 2012). Specifically, I planted seeds from 6-18

maternal lines per population for 2003 (average = 14.67, median = 16) and 1-29 maternal lines

per population for 2012 (average = 15.8; median = 15; see Table S2-1 for the exact number of

maternal lines per population). Seeds were scarified and planted in 10.2-cm pots which were

arranged in a completely randomized design at the Matthaei Botanical Gardens (MBGNA) at the

University of Michigan (Ann Arbor, MI, USA). Plants were watered daily, with standard

conditions of 75°C and 12 hours of artificial sunlight. This experiment was performed in 2015.

Floral traits on an average of 6 flowers/plant were measured using digital calipers. Corolla width

was measured as the diameter of a fully open corolla, corolla length as the distance from the rim

of the corolla to where it fuses with the receptacle, and anther-stigma distance (ASD) as the

difference between the height of the pistil and the tallest stamen. Measurements were spread

across 17 sampling dates with an average of 2.3 flowers measured per plant on each date, such

that flowers from the 2003 and 2012 cohorts were always measured at the same time, ensuring

equi-aged flowers. In total, 2836 flowers were measured from 456 plants.

Floral Phenology - To assess floral phenology, I performed a separate common garden

experiment in 2013 at the University of Georgia Plant Biology Greenhouses (Athens, GA, USA)

using field collected seeds from 23 populations again from two different years (2003 and 2012).

A total of 451 plants were included in this study, with 2-16 plants per population (mean = 9.8,

median = 11). Thirteen of these populations overlapped with those included in the greenhouse

experiment at MBGNA assessing floral morphology (Figure 2-1). I recorded the first occurrence

of a fully open bloom as the date of first flower for all experimental individuals. To determine if there were size differences between plants from different sampling years, I counted the number of leaves of each individual, and dried plants at 70°C for three days and weighed each individual for an estimate of dried biomass. Germination of seeds in this experiment ranged from 50-98% across populations and varied between years, with more seeds germinating from the 2003 collections compared to the 2012 collections (87% vs 84%, $p < 0.001$; Kuester et al. 2016).

Floral Rewards - To measure floral rewards, I replanted a subset of four populations (Table S2-1) in a separate experiment at MBGNA in 2017 to quantify the number of pollen grains produced and the nectar sucrose content (°Brix), which we consider an important component of total nectar reward. I planted replicates of eight maternal lines for each of these populations, again sampled both in 2003 and 2012 (except for Duplin East from 2012 which included only 6 maternal lines). I measured a total of 1468 flowers from 213 plants, with an average of 26.6 plants per population and 6.89 flowers per plant.

To extract nectar from the flower, 10uL of reverse osmosis (RO) water was pipetted directly into the base of a flower, pushing the pipette tip past the base of the stamens and pipetting up and down to mix and extract nectar. I then quantified sucrose content of this nectar/water solution using a pocket refractometer to record percent mass sucrose (°Brix, hereafter nectar sucrose content). I counted pollen by removing the second tallest anther in each flower with forceps near the time of anthesis (i.e. early morning) when pollen was mature. The second tallest anther was collected because there is no significant difference in pollen count between the tallest and second tallest anther, and the second is easier to collect without disturbing the plant's ability to self-

pollinate. I then gently brushed the anther against all four corners of a basic fuchsin gelatin cube (Beattie, 1971). The cube was placed on a glass microscope slide, heated on a 180˚C hot plate until the cube completely melted, covered with a cover slip, and imaged with an iPhone camera affixed to a light microscope. I obtained a pollen count by analyzing pollen slides using the Analyze Particles function in ImageJ (Schneider *et al.*, 2012) with the default particle size setting (0-150).

2.5 Data Analysis

Temporal and Spatial Effects on Floral Traits

We first examined possible phenotypic evolution by comparing differences in trait distributions between collection years using a Kolmogorov-Smirnov test across all populations. To determine if the mean trait values were different between sampling years and spatial locations, I performed a linear mixed model using the lme4 package v. 1.1.29 (Bates *et al.*, 2015) in R (v. 4.2.0; R Core Team 2022) with year, latitude, and the interaction of year and latitude as fixed effects and population identifier as a random effect to control for longitudinal differences. Each phenotypic trait was used as the dependent variable in separate models of the following general form:

(1) $\qquad\qquad$ Trait ~ Year * Lat + (1|Pop),

and I used the bestNormalize v. 1.8.2 package (Peterson 2021) to determine the appropriate transformation for each trait to adhere to assumptions of normality. I assessed the significance of effects using the anova() function from lmerTest v. 3.1.3 (Kuznetsova *et al.*, 2017), which performs a type III ANOVA and uses the Satterthwaite method to determine the degrees of

freedom. All models were run as both unweighted and weighted to account for sampling differences across populations. Sampling differences did not impact model results for floral morphology or reward traits but are relevant for our analysis of phenology. I focus results below on the unweighted model for all traits except the date of first flower. Additionally, the day of first flowering showed a bimodal distribution (see Results); while most experimental individuals flowered in the first wave, a small group of individuals flowered for the first time in what I describe as a second wave. Due to the resulting bimodal distribution of first flowering dates (Figure 2-2), a normality transformation was not appropriate. I thus elected to model each flowering wave separately. Moving forward, I focus statistical analysis primarily on the first wave of flowering, as that captures information about flowering phenology for the majority of individuals in the experiment. However, I do describe differences between the first and second waves of flowering in the discussion for illustrative purposes.

The floral reward traits (pollen number and Brix) were measured on replicate maternal lines from four populations. Thus, I included a maternal line effect in our ANOVAs when testing for temporal changes in floral reward traits, and I used least square means to assess the potential for temporal changes within each population separately. For both traits, maternal line and population were included as random effects with year, latitude, and the interaction between year and latitude included as fixed effects:

(2)   Trait ~ Year*Lat + (1|Population/Maternal Line)

Data were again transformed to meet assumptions of normality and analyzed with a type III ANOVA.

Phenotypic Evolution

We next performed a screen to determine if the dominant evolutionary force influencing floral traits over time was selection, migration, or drift. To do so, I compared the change in trait value (δt; or 2012 – 2003) to the initial value in 2003 (t), and assessed potential changes in the variance of each trait, following (Goldberg *et al.*, 2020). Our expectations are presented in Table 2-1, but, briefly, the presence of selection influencing trait change would be evident by the following: a significant change in mean trait value from 2003 to 2012, a reduction in trait variation, and significant non-zero slope between t and δt. δt values either above or below zero would support positive or negative directional selection, respectively. If drift were driving phenotypic evolution, I would expect both an increase in trait variation across populations and a zero-slope relationship between $\delta t/t$. No net change in trait value or variance would suggest the presence of balancing or disruptive selection in traits; if values for δt were scattered both above and below zero, a significant linear regression between t and δt with a positive slope that intercepts with the line δt=0 would suggest disruptive selection (i.e. small t has a negative δt; large t has a positive δt), while a negative slope would indicate balancing selection (i.e. small t has a positive δt; large t has a negative δt). Finally, balancing selection can be differentiated from the homogenizing force of migration since migration would be expected to decrease trait variation.

To apply the (Goldberg *et al.*, 2020) framework to our system, I calculated trait variation as the phenotypic coefficient of variation (PCV; (standard deviation(x)/mean(x)) 100%; where x is the trait of interest). To test for temporal changes in PCV, I used the Coefficient of Variance with Confidence Intervals (cvcqv) package v. 1.0.0 in R (Beigy, 2019) and used bootstrap resampling

to obtain confidence intervals, then conducted a two-sided independent t-test for each trait. I used a linear regression assessed with a type II ANOVA and Pearson's correlation coefficient to determine if variation in the change in trait over time (δt) was explained by the initial trait value (t).

Finally, I revisited evidence for selection based on the relationship between t and δt by including latitude as a potential predictor of δt with the following linear model:

$$(3) \qquad\qquad \delta t \sim t * \text{Lat}$$

The predictions for $\delta t / t$ outlined above focus on detection of evolutionary forces that are consistent across populations, resulting in an overall dominant effect on the species under consideration. However, climatic changes can vary dramatically across latitude, resulting in different selective forces over space. Based on preliminary analysis, changes in corolla width appeared stronger in northern latitudes, with a significant latitude*year interaction when assessing mean changes in this trait. Thus, I also included a latitude effect for traits when examining the relationship between $\delta t / t$.

2.6 Results

Temporal and Spatial Effects

Patterns of trait change between collection years varied across floral morphology, phenology, and floral reward traits. The trait distribution for corolla width was significantly different between collection years (two-sample D = 0.157, p = 1.04e-14, Figure 2-2), and this difference

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was reflected in a change in mean value, with corollas becoming significantly wider over time (4.5 cm in 2003 vs 4.8 cm in 2012; F = 7.093, numDF = 1, denDF = 12.10, p = 0.020; Table S2-2). Although it appeared that corolla width increased across most populations (Figure 2-3), I found a highly significant interaction between latitude and year (F = 23.388, numDF = 1, denDF = 519.82, p = 1.75 x 10^{-6}; Figure 2-3, Table S2-2) and a highly significant effect of latitude (F = 16.850, numDF = 1, denDF = 2662.85, p = 4.167 x 10^{-5}; Table S2-2) such that the change in corolla width was greater in populations at more northern latitudes. No change from 2003 to 2012 was detected in plant biomass (t = 0.078, df = 54.289, p = 0.938) or in pre-flowering leaf count (t = 0.1865, df = 704.31, p-value = 0.8521), suggesting that increased corolla width detected here is not due to an overall increase in plant size.

Compared to corolla width, a smaller shift occurred in the distribution of corolla length (two-sided D = 0.057, p = 0.022; Figure 2-2), and I found evidence of a slight but significant increase in corolla length between collection years (5.43 cm in 2003 vs 5.47 cm in 2012; F = 10.472, numDF = 1, denDF = 11.77, p = 0.007; Table S2-2). There was no indication that corolla length differed across latitudes (F = 0.041, numDF = 1, denDF = 2781.94, p = 0.840; Table S2-2) nor was there a significant interaction between latitude and year (F = 0.580, numDF = 1, denDF = 923.79, p = 0.447; Figure 2-3, Table S2-2). Due to non-normality in the data even after correction, I checked for robustness of the year effect using a permutation test and again uncovered a nearly significant change in corolla length over time (p = 0.069).

For the final floral morphology trait I examined, anther-stigma distance, I found a nearly significant change in trait distribution (two-sided D = 0.049, p = 0.072; Figure 2-2), however no

evidence for a change in trait mean over time (F = 4.42, numDF = 1, denDF = 9.67, p = 0.659; Table S2-2), nor did I find evidence of a significant effect of latitude (F = 1.587, numDF = 1, denDF = 2468.51, p = 0.072; Table S2-2), or significant interaction between year and latitude (F = 2.633, numDF = 1, denDF = 1576.65 , p = 0.641; Figure 2-3, Table S2-2).

When assessing flowering phenology, I found that the start of flowering occurred in two waves (Figure 2-2). Using a weighted model for the first wave of flowering onset, I identified a significant effect of both collection year (F = 3.950, numDF = 1, denDF = 289.89, p = 0.048; Table S2-2) and interaction of collection year and latitude (F = 3.663, numDF = 1, denDF = 290.00, p = 0.048; Figure 2-3, Table S2-2) on the date of first flower. For the second wave of flowering onset, I found that the day of first flowering of the second wave differed according to latitude (F = 5.484, numDF = 1, denDF = 22. 10, p = 0.028; Table S2-2), but found no evidence for a collection year effect (F = 0.206, numDF = 1, denDF = 141.09, p = 0.651; Table S2-2) nor a significant interaction between collection year and latitude for this trait (F = 0.203, numDF = 1, denDF = 141.16, p = 0.653; Table S2-2).

Like floral morphology and flowering time, I found collection year and latitudinal differences in the floral reward traits. Similar to corolla width, the distribution of pollen grain number exhibited a significant shift in the distribution toward greater pollen grain number in 2012 (two-sided D = 0.100, p = 0.019; Figure 2-2). However, I did not find an overall effect on average pollen number between years (F = 0.028, numDF = 1, denDF = 1.90, p = 0.883; Table S2-2), nor was there a difference according to latitude (F = 2.187, numDF = 1, denDF = 163.41, p = 0.141; Table S2-2), or significant interaction between collection year and latitude (F = 2.180, numDF = 1, denDF =

33.25, p = 0.149; Figure 2-4, Table S2-2). For nectar sucrose content (°Brix), I found a significant interaction between year and latitude, such that the more northern populations exhibited increased °Brix over time (F = 4.59, numDF = 1, denDF = 60.45, p = 0.036; Figure 2-4, Table S2-2). There was no support for an overall year effect for this reward trait (F = 0.003, numDF = 1, denDF = 1.94, p = 0.961; Table S2-2) but there was a significant latitude effect (F = 5.877, numDF = 1, denDF = 200.63, p = 0.016; Table S2-2). Since population sampling was low, I also tested sensitivity of these results to population removal. For °Brix, the latitude and latitude by year effects appear to be largely driven by a single population at high latitude (population 9), as removal of this population from the analysis resulted in no significant trend for either latitude (F =1.0221, numDF = 1, denDF = 148.41, p = 0.313) or the interaction of year and latitude (F = 0.742, numDF = 1, denDF = 16.93, p = 0.401).

Adaptive Evolution

Using the framework of Goldberg et al 2020 (Table 2-1), I found that most of the morphology and flowering time traits examined (corolla width, corolla length, anther-stigma distance, and flowering time of the first flowering wave) appeared to be evolving under some form of selection. I did not include pollen number and °Brix in this analysis since four populations is insufficient for a regression analysis.

Corolla width displayed a significant, negatively sloped relationship (R = -0.55, p = 0.04) between the change in mean trait value (δt) and starting mean trait value in 2003 (*t*) after the removal of a single outlier population (Figure 2-5). There was likewise evidence for reduced variation in this trait over time (5.4% reduction in the phenotypic coefficient of variation (PCV),

t = 1.854, p = 0.059; Table S2-3). These two results together, along with the significant increase in trait mean over time, provide mixed evidence for either directional selection (*i.e.,* reduction in variation and change in mean) or balancing selection (*i.e.,* relationship between δt and *t*). However, including latitude as an explanatory effect for δt in an analysis of variance revealed a significant interaction between latitude and *t* (F = 6.058, numDF = 1, denDF = 11, p = 0.03, Table S2-2), corroborating previous evidence that latitude plays a strong role in determining changes over time in corolla width. Based on this model, the slope of δt/*t* for corolla width becomes positive above a latitude of 34.9; all populations except one above this latitude also demonstrate δt values greater than zero. It thus appears that populations at northern latitudes are responding to positive directional selection for increased corolla width over time. Corolla length displayed a non-significant negative relationship between δt and the starting mean trait value in 2003 (*t*) (R = -0.46, p = 0.083; Figure 2-5) and no indication that this relationship significantly changed following the inclusion of latitude in the model.

Finally, both the anther-stigma distance and flowering time of the first flowering wave showed a highly significant and negatively sloped relationship between the change in the mean value of the trait and the starting mean value (ASD: R = -0.81, p = 2.0 x 10⁴; first flowering wave: R = -0.81, p = 2.5 x 10-5). This relationship was significant regardless of whether latitude was included as an explanatory variable in an analysis of variance (ASD: F = 26.91, numDF = 1, denDF = 11, p = 3.004 x 10⁴, first flowering wave: F = 38.31, numDF = 1, denDF = 19, p = 6.022 x 10⁶). Neither trait showed evidence of reduced variation over time (Table S2-3). For ASD, δt values are evenly distributed around 0 whereas δt values for first flower are predominantly below zero (8 populations above, 15 populations below). This indicates that ASD is evolving under balancing

selection whereas the day of first flowering (first flowering wave), despite a non-significant reduction in variation, displays an evolutionary trajectory primarily driven by selection for earlier flowering dates (Table S2-3, Figure 2-5).

While I did not examine the $\delta t/t$ relationship for floral reward traits due to low sample size (N = 4 populations), I note that the percent change in the phenotypic coefficient of variation (PCV) values show a significant decrease in both floral reward traits between 2003 to 2012 (°Brix: t = 1.970, p = 0.05; pollen number: t = 2.399, p = 0.01; Table S2-3), in alignment with the idea that these traits are responding to selection.

2.7 Discussion

Global change encompasses both direct (abiotic) and indirect (biotic interactions) forces of selection, the effects of which can manifest in a range of growth and reproductive responses that maintain demographic performance despite substantial environmental change (Eckert *et al.*, 2010). Uncertainty over response to climate and other anthropogenically-induced changes is especially acute for mixed-mating species, where, in the face of global pollinator declines and shifting suites of abiotic variables, both selection for increased outcrossing (Bishop *et al.*, 2017) and selection for increased selfing (Jones *et al.*, 2013) are possible adaptive responses. Our results show *I. purpurea* is evolving broader corollas with some evidence for increased floral rewards. I found no indication that anther-stigma distance decreased over time across examined populations, which would be expected if populations were evolving higher rates of selfing. Overall, our findings are aligned with the expectation of increased investment in pollinator

attraction traits, especially at the northernmost populations, rather than increased rates of self-pollination.

Although patterns of trait change across each of the traits were compelling, corolla width showed the most dramatic overall increase in trait value from 2003 to 2012, as well as a decrease in the phenotypic coefficient of variation – together suggesting corolla width is responding to positive selection for increased size. An important nuance of this conclusion is that the evidence for such change in this trait was largely driven by the northernmost populations. Specifically, the use of a spatially explicit model for predicting the change in trait value over time(δt) showed that δt was better explained by the interaction between latitude and t rather than by t alone. Additionally, by removing the three southernmost populations in this regression, I found that the slope of $\delta t/t$ became positive (albeit $p = 0.14$), demonstrating again that corolla width changes are much larger in the north. Thus, the strongly significant spatial-temporal change in corolla width as well as decrease in variation for the trait are highly suggestive of directional selection occurring at northern latitudes. I found some indication of a temporal increase in corolla length, but believe this pattern is more likely due to the strong correlation between corolla width and length ($r =$ 0.61 in 2003 and 0.59 in 2012, $p < 2 \times 10^{-16}$, Table S2-4), rather than due to direct selection on corolla length. While corolla length plays an important role in pollination efficiency in some plant species with specialist pollinators (Naghiloo *et al.*, 2021; Faure *et al.*, 2022), *I. purpurea* is a generalist-pollinated plant, and the observed change in corolla length is so slight (increased 0.4 mm) that it is unlikely that this is a biologically significant effect or that pollinator efficiency is impacted by corolla length. Overall, our finding of a change in corolla width as a possible adaptive response to global change aligns with previous evidence that corolla width is responsive

to abiotic changes such as water availability and temperature, as well as changes in pollinator populations (Schueller, 2007; Campbell & Powers, 2015; Gallagher & Campbell, 2017). However, tracking floral traits over time remains rare, and, in contrast to our results, multiple other studies have suggested an increased investment in selfing in response to climate change and pollinator declines (Cheptou, 2019; Busch *et al.*, 2022) with rarer instances of increased outcrossing (Bishop *et al.*, 2017)

Our phenology and anther-stigma distance results are similar to responses across other species and previous work in *I. purpurea*, respectively. Phenology, measured here as the date of first flower, has repeatedly shown a shift to earlier flowering dates in a number of plant species (Bock *et al.*, 2014; Moore & Lauenroth, 2017; Wolf *et al.*, 2017; Büntgen *et al.*, 2022), and this is also the case in *I. purpurea*. I found some evidence of directional selection toward earlier flowering within the first wave of flower emergence, particularly at northern latitudes, with the exception of a reduction in trait variation. However, the bimodal distribution of first flowering dates demonstrates that earlier flowering is not captured fully within a single wave, rather, the mechanism underlying this shift is a greater proportion of individuals flowering in the first wave in 2012, rather than a shift of both peaks to earlier dates while retaining a bimodal nature. In fact, while all 23 populations have some individuals that flower in the first wave and some in the second in 2003, three of the populations flower entirely in the first wave in 2012. For anther-stigma distance, our results strongly point to balancing selection acting on this trait over time, a result corroborating previous empirical work in a single experimental population which showed that outcrossing success in *I. purpurea* is subject to a form of balancing selection, negative frequency dependent selection (Chang & Rausher, 1998). Our explicit spatial-temporal model for

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ASD showed no effect of latitude on ASD values, indicating that, unlike corolla width, selection is consistent across space.

While our data potentially indicate that both of the floral rewards – nectar sucrose content (°Brix) and pollen number – change over time (*i.e.,* significant reduction in variation between years for both traits; mean trait increase for nectar sugar in northernmost populations), due to a low number of populations examined in this study (N = 4), I cannot assess selection on them. I likewise did not examine the potential that such changes are correlated to, and perhaps evolving along with corolla width again due to sample size limitations. Furthermore, there is high sensitivity in the °Brix results to the exclusion of a single population, indicating that these results may change significantly if additional populations are included. Finally, both sucrose content and pollen count are only one component of potential rewards. In the case of nectar, volume and thus sugar concentration remain unaccounted for, while pollen count indicates little of pollen protein content. Nonetheless, it appears likely that there is a temporal increase in investment in pollinator attraction, and that this result is driven by populations at northern latitudes. Changes in floral rewards in response to global change also align with previous findings indicating that temperature and water availability, both of which are variables associated with climate change, can alter nectar volume and sugar content (Descamps *et al.*, 2018, 2021; Phillips *et al.*, 2018) as well as pollen count and viability (Bishop *et al.*, 2017; Descamps *et al.*, 2021).

This is the first paper to use the resurrection approach to examine the potential that traits responsible for plant-pollinator interactions may be evolving over time, concomitant to decreases in pollinator abundance and dramatic environmental changes due to changing climate and land

use regimes. While a unique feature of the resurrection approach is that it allows for comparisons of populations exposed to the multifactorial suite of selective pressures associated with global change in the field (Thomann *et al.*, 2013), the resurrection approach typically does not identify the causative agent(s) of selection, meaning that we will need to perform future direct manipulations of abiotic and biotic factors to determine which agents of selection are acting on corolla width and other floral traits. I also acknowledge that, while this framework for identifying selection allows for flexibility when direct fitness measurements are not feasible, phenotypic selection analysis should be conducted to verify putative signatures of selection identified here. However, with some notable exceptions (Inouye, 2008; Franks, 2011; Anderson et al., 2012; Thomann et al., 2015; Rauschkolb et al., 2022), relatively few studies investigating adaptation to climate or other global change factors capture adaptive responses from field settings, showcasing the power of the approach I have taken here. Additionally, while I measured changes in a number of traits relating to plant-pollinator interactions, this list is not exhaustive and, notably, does not include floral color. *I. purpurea* is highly polymorphic for color, with previous research showing that white flower morphs tend to be visited by pollinators less frequently when rare and self-pollinate more than darker colored morphs (Fehr & Rausher, 2004). I might expect that, in line with other changes indicating an increased investment in pollinator attraction, the frequency of white flowers may also decrease in these populations, however, preliminary analysis of changes in simple categorical estimates of floral color over time revealed no significant patterns.

Other important caveats to the resurrection approach are biases introduced through storage effects via the "invisible fraction" and through maternal effects (Franks et al., 2018). The

invisible fraction occurs when nonrandom mortality of stored seeds creates bias in measurements of phenotypic traits due to association between traits of interest and traits related to germination success (Weis, 2018). In this study, germination rates between 2003 and 2012 were very high and slightly higher in the older seeds (87% in 2003, 84% in 2012), such that germination failure is unlikely to be related to seed traits affecting storage survival and bias in trait measurements is expected to be trivial (Weis, 2018). Best practices for removing confounding maternal effects – when the growing conditions of mother plants contribute to the appearance of population differentiation in offspring traits – involve using a refresher generation and measuring traits of plants produced from F1 seeds (Franks et al., 2018). A refresher generation was not available for this study, so some trait changes observed may be attributable to maternal effects rather than evolution.

Despite this, our results are compelling in that they are in alignment with phenological shifts in other plant species (Byers, 2017; Renner & Zohner, 2018; Gérard *et al.*, 2020; Soares *et al.*, 2021) and a broad range of work showing northern populations tend to show more dramatic evolutionary responses to climate change due to spatially differential selective pressures, greater tolerance ranges, and/or standing genetic variation related to more extreme seasonal fluctuations at northern latitudes (Parmesan, 2007; Bonebrake *et al.*, 2010; Post *et al.*, 2018; Newbold *et al.*, 2020). This latitudinal trend has primarily been shown by associations between phenology, latitude, and land surface warming across hundreds of taxa (Parmesan, 2007, Post *et al.*, 2018), noting that warming alone still explains a relatively small portion of phenotypic variation. While I do not explore specific environmental associations here, our results nonetheless expand known

latitudinal trends beyond phenology and highlight additional spatial evolutionary trends in floral traits.

In summary, I show that, in addition to well-documented shifts to earlier flowering phenology, floral architecture and rewards can also play significant roles in the evolutionary response to contemporary environmental change. Populations of *I. purpurea* distributed across the southeast United States demonstrate a significant temporal increase in corolla size as well as potential for increased investment in floral rewards, all of which are driven primarily by populations at more northern latitudes. In addition, I show that the integration of phenotypic trait changes over time, measurement of variation, and spatial modeling can be used to detect signals of selection on phenotypic traits, notably, the presence of balancing selection on anther-stigma distance, and a probable instance of spatially divergent directional selection on floral architecture.

2.8 Acknowledgements

2.9 References

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2.10 Figures
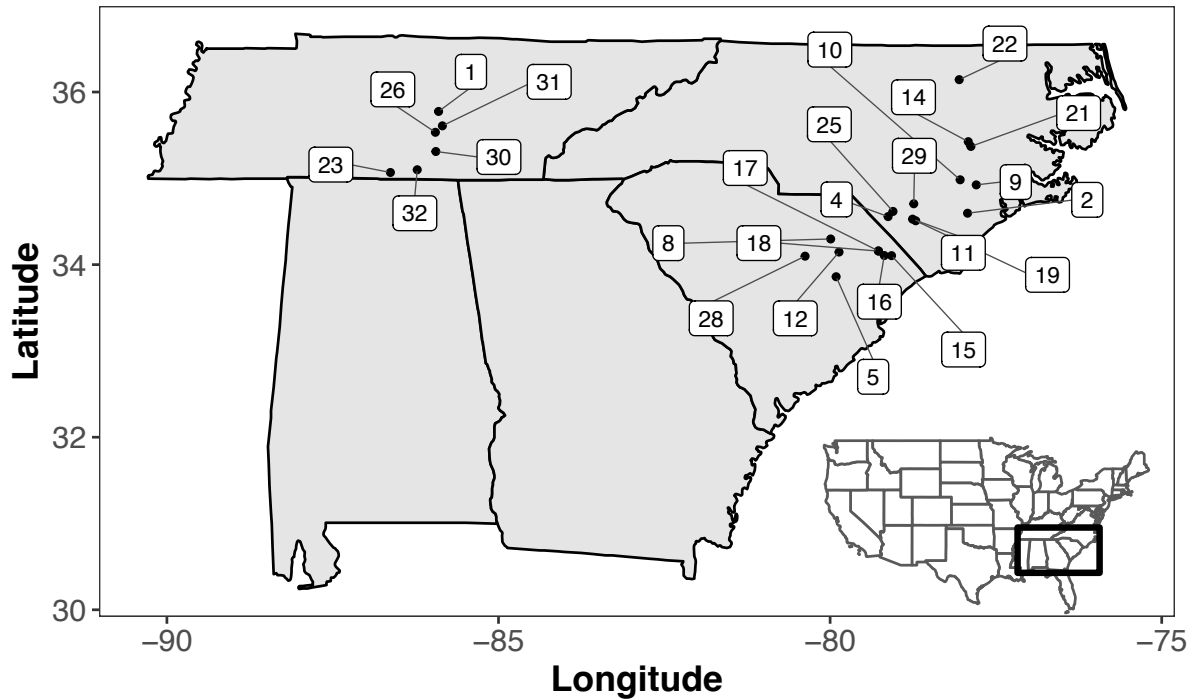
Figure 2-1. Distribution of sampling localities of *I. purpurea* labeled with population number. All populations were sampled from the edge of agricultural soy and maize fields. Fifteen populations were included in the resurrection experiment looking at floral morphology, twenty-three populations for phenology, and four populations with in-depth maternal line sampling were used to measure floral reward traits. Population representation for each resurrection experiment can be found in Supplemental Table 2-1.
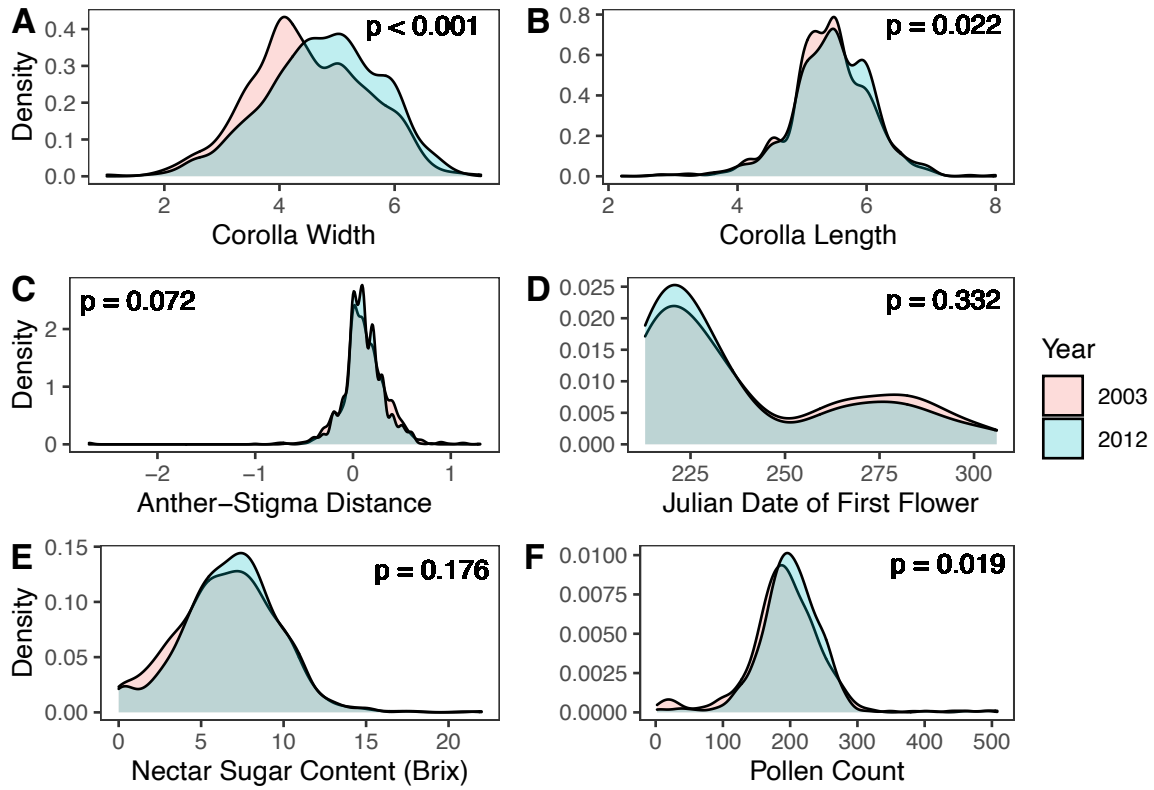
Figure 2-2. Distribution of trait values across all populations in 2003 and 2012 for (A) corolla width, (B) corolla length, (C) anther-stigma distance, (D) date of first flower, (E) °Brix, and (F) pollen count. P-values come from Kolmogorov-Smirnov tests for each trait.
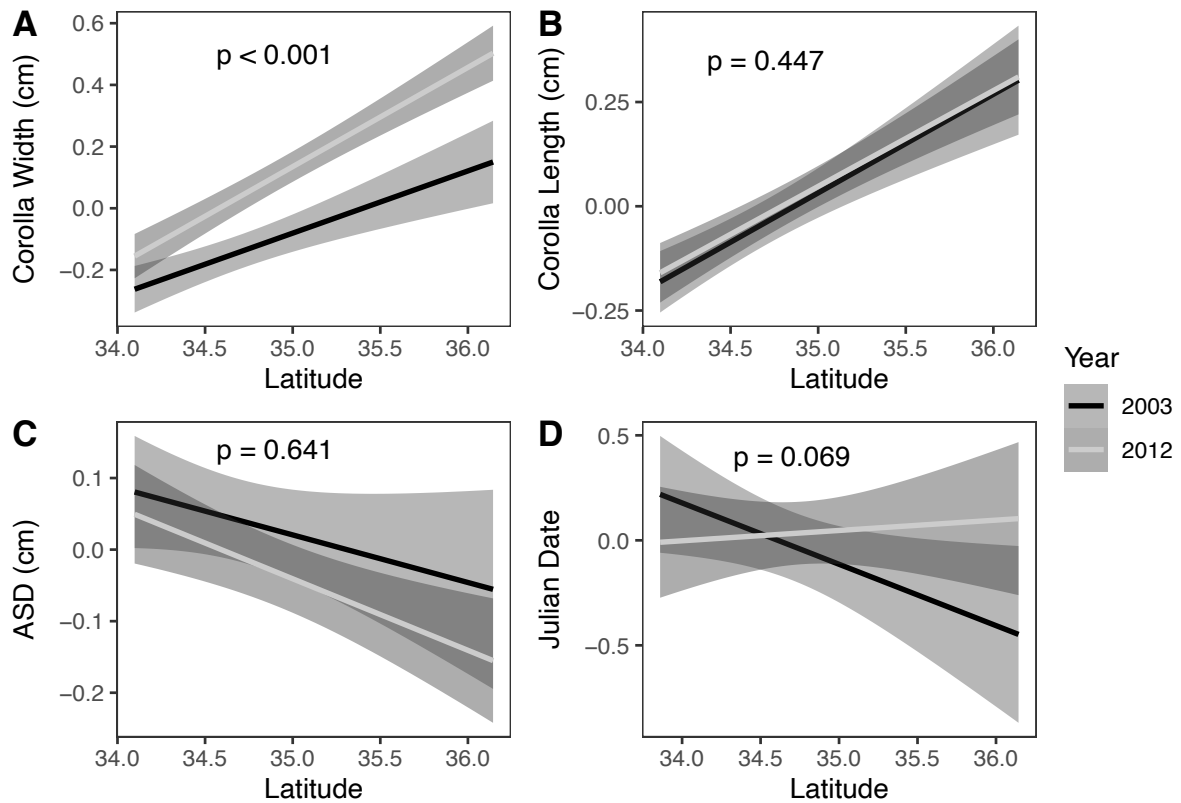
Figure 2-3. Linear mixed models for population means across latitude for (A) corolla width, (B) corolla length, (C) anther-stigma distance, and (D) the date of first flower for the first wave of flowering shown as the Julian date. Each line is plotted with a 95% confidence interval and p-values on plots refer to the year*latitude effect from a type III ANOVA of linear mixed model (1).
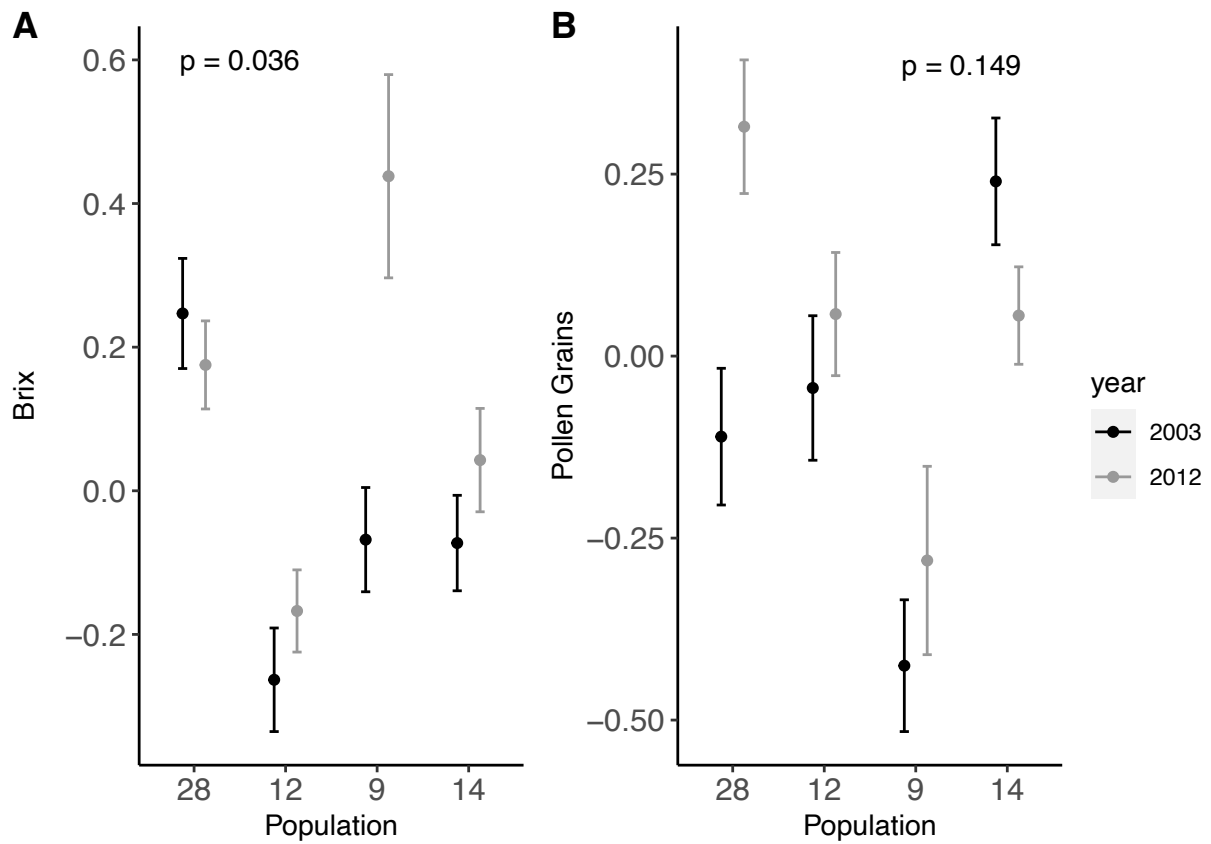
Figure 2-4. Per population changes in floral rewards from 2003 (black) to 2012 (grey) with populations ordered left to right from lowest to highest latitude. Mean and standard error for °Brix (A) and pollen count (B) are shown for the four measured populations and p-values refer to the year*latitude effect from a type III ANOVA of linear mixed model (1).
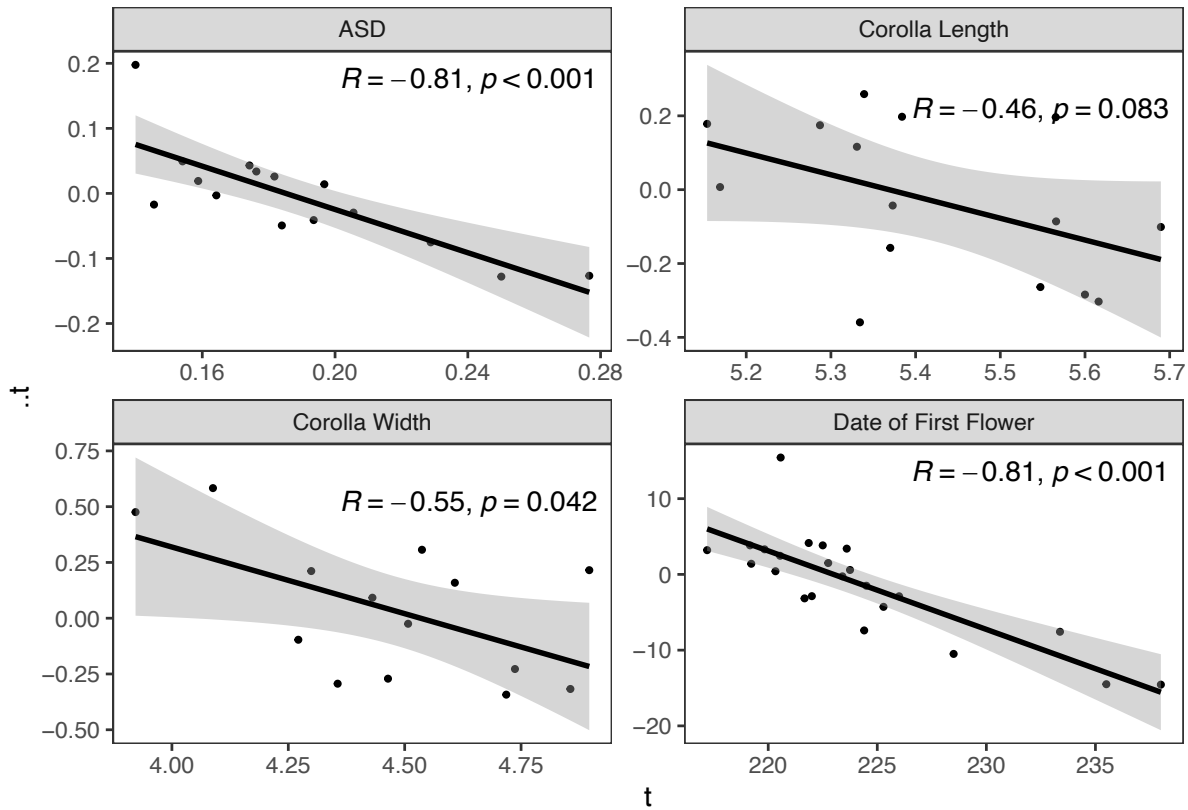
Figure 2-5. Linear regression showing the predictability of degree of change in trait value from 2003 to 2012 by the starting trait value in 2003 for (A) corolla width, (B) corolla length, (C) anther-stigma distance, and (D) the date of first flower. Each point in the regression is the mean trait value for a population, with date of first flower shown only for the first flowering wave. t represents the starting mean value in 2003, and δt shows the degree of change in mean value as the mean in 2003 subtracted from the mean in 2012.

2.11 Table

	δt	δt versus t	Variance in t
Drift	No net change	Slope = 0	Increase
Migration	No net change	δt distributed around 0	Decrease
Directional Selection (+)	Positive	$\delta t > 0$, positive slope	Decrease
Balancing Selection	No net change	δt distributed around 0, negative slope	No change
Disruptive Selection	No net change	δt distributed around 0, positive slope	No change

Table 2-1. A framework adapted from (Goldberg *et al.*, 2020) to differentiate between drift, migration, and selection on trait evolution between sampling years. t refers to the least squares mean for a trait value, δt is the difference in mean trait value from 2012-2003, and the PCV is used to assess variance in t. In addition to the expectations outlined by Goldberg *et al.,* 2020, for balancing and disruptive selection, I expect δt values to be distributed both above and below 0 such that the direction of change (increase or decrease) is dependent on the starting value, whereas for directional selection, the direction of change in δt will remain consistent regardless of starting value.

Chapter 3 Evolutionary Constraints on Adaptation in Floral Traits

3.1 Abstract

Adaptive evolution is a critical mechanism through which wild populations can respond to pressures from environmental shifts associated with anthropogenically-induced global change. Increasing theoretical and empirical evidence suggests that rapid adaptation commensurate with the rate of contemporary environmental change is possible. However, widespread examples of mismatch between expected and realized evolutionary rates persist, indicating an incomplete characterization of adaptation to global change. Particularly, there is a scarcity of research on the possibility for slowed evolutionary response due to trait-trait covariances and how that impacts the evolutionary potential of populations. In a field-based resurrection experiment, I investigate the possibility for constraints on continued trait evolution of the common morning glory (*Ipomoea purpurea*). Specifically, I assess univariate and correlative selection on a suite of pollination or mating system related traits – corolla width, corolla length, anther-stigma distance, nectar sucrose content, and date of first flower – and map patterns of selection to realized phenotypic change. I show that, despite the presence of genetic variation, descendant populations (2012) differ in their response to selection compared to ancestral populations (2003) in flowering phenology, whereas pollinator attraction traits, corolla width and nectar sucrose content, are under positive selection in both the ancestral and descendent populations. Additionally, I find evidence for a trade-off between corolla width and date of first flower such that shifts to earlier flowering are no longer favored in descendant populations. Finally, I find alignment between the

direction of selection and phenotypic change, but constraints on the rate of phenotypic change across traits. Overall, these results show that pollinator-attracting traits are evolving on a rapid time scale of less than ten years, but that genetic covariances between traits constrain the overall adaptive rate and evolutionary trajectory of flowering phenology.

3.2 Introduction

Understanding how biodiversity will respond to global change and whether evolutionary adaptation can keep pace with environmental shifts is a current focus of global change biology (Pujol *et al.*, 2018; Martin *et al.*, 2023). In general, species have three possible options to respond to rapid shifts in their environment associated with global change: geographic range shifts, plasticity, and *in situ* adaptation. As demonstrated in several pollinator species across North America (Potts *et al.*, 2010; Breed *et al.*, 2013; Hallmann *et al.*, 2017), large-scale habitat fragmentation and destruction can limit the potential for geographic range shifts (Lenoir *et al.*, 2020; Hamann *et al.*, 2021) . Similarly, compensatory response via plasticity demonstrates an upper bound in some species (Sgrò *et al.*, 2016; Cohen *et al.*, 2018). This leaves adaptive evolution as an important mechanism through which populations may respond to sustained climatic changes over time. A major unresolved question, however, is whether constraints on trait evolution will impact the adaptive capacity of wild populations in the face of contemporary pressures.

While increasing evidence suggests that adaptive evolution can occur on timescales commensurate with human-mediated environmental change (Geerts *et al.*, 2015; Padfield *et al.*, 2016; Logan & Cox, 2020; Mesas *et al.*, 2021), there are a number of examples where adaptation lags behind the pace of climatic shifts and other global changes (Kruuk *et al.*, 2008; Wilczek *et al.*, 2014; Mills *et al.*, 2015; Radchuk *et al.*, 2019). This lag can occur if one or both key components underlying adaptive potential (the presence of consistent natural selection and heritable genetic variation on which selection can act) are missing or limited. In the case of climate change, selection is not a smooth process of continuously increasing temperatures, but

rather is characterized by changes occurring over a broad range of temporal and spatial scales such that selection may fluctuate, possibly inducing lags in adaptation due to short-term climate extremes being out of sync with long-term climatic trajectories (Siepielski *et al.*, 2009, 2017). Conversely, consistent directional selection on a trait could be strong enough to decrease genetic variation to a degree that further evolution is not possible (Buckley & Bridle, 2014). However, analyses of contemporary selection from anthropogenic impacts show that it is frequently of sufficient magnitude and direction to promote rapid adaptation (Fugère & Hendry, 2018). Despite this, even in cases where adequate genetic variation remains in a population, mismatches between expected and realized evolution can occur (Pujol *et al.*, 2018).

It is this mismatch that has led to the widespread realization that our understanding of adaptation to global change is incomplete. In particular, the potential that trait-trait covariances – which are well documented to occur in natural populations – can lead to either a slowed evolutionary response (*i.e.,* an evolutionary constraint) or a more rapid evolutionary response (*i.e.,* evolutionary facilitation) has yet to be broadly examined. A constraint may occur if a genetic covariance between traits limits their capacity to respond independently to selection. More specifically, directional selection on genetically linked traits that is misaligned with the sign of the trait covariance may lead to adaptive shifts in one trait but induce nonadaptive shifts in the other (Walsh & Blows, 2009). Conversely, selection in the same direction as genetic covariance between traits can facilitate adaptation, generating rapid trait shifts. As anthropogenic impacts escalate and natural selection shifts with them (Anderson, 2016), re-alignment between the direction of selection and genetic covariances may become more common, and adaptive potential may shift over time due to changes in both variation and covariance structure. Thus, to predict

the long-term effects of global change, understanding how constraints on evolution manifest to alter evolutionary trajectories, and how adaptive capacity itself may be changing over time is critical.

Such questions are especially relevant to plant-pollinator dynamics, which present a system of critical ecological importance, yet where human-mediated environmental change is hypothesized to strengthen selection by lowering mean fitness and which also showcase high covariance between traits (Ashman & Majetic, 2006; Smith, 2016; Brown & Caruso, 2023). To date, however, research regarding adaptive responses to climate shifts in plant-pollinator systems has been largely univariate. In fact, from a review of the available literature with specific mention of global or climate change in plants, I find that most studies focus on analyses of shifts in phenology, or flowering time (Clarivate 2022), but less commonly examine other traits that are crucial to plant-pollinator interactions such as floral morphology and floral rewards (*i.e.,* nectar and pollen). Importantly, studies that explicitly investigate adaptive trade-offs between traits in the context of global change-induced shifts in phenology remain extremely rare, and while some demonstrate trade-offs between phenological shifts and growth (Lancaster *et al.*, 2017), size (Colautti & Barrett, 2010; Du & Qi, 2010; Ollivier *et al.*, 2020), and leaf traits (Ravenscroft *et al.*, 2014), with the exception of a single paper which finds a correlative selection with flower number (Chen *et al.*, 2017), I find no investigation of how global change impacts selective trade-offs between floral traits involved in mediating plant-pollinator interactions or changes in adaptive constraints among these traits over time. This highlights a glaring gap in research regarding the adaptive potential of floral traits that are crucial to maintaining relationships with pollinators and the eco-evolutionary consequences of those changes.

Here, I use a resurrection experiment to investigate the potential for constraints on continued trait evolution in the face of global change. Resurrection studies are a crucial tool for studying contemporary global change as they can be used to detect responses to complex field conditions over time, rather than simply using environmental proxies. Such studies can also control for the effects of plasticity, thus narrowing in on heritable, genetic changes (Etterson *et al.*, 2016; Franks *et al.*, 2018). However, they remain rare in part due to a lack of temporally collected populations and have yet to be leveraged to detect evolutionary constraints or to connect trait constraints to phenotypic change (or the lack thereof). Using replicate maternal lines from seven populations (Figure 3-1) sampled from three time points (2003, 2012, and 2016), I evaluate response to shared selection among a suite of pollinator-associated traits beyond phenology and identify trade-offs among traits that constrain evolutionary response. While selection is reflective of immediate environmental conditions, responses to that selection over multiple years shows changes in the overall evolutionary trajectory and marginalizes the effect of year-to-year fluctuations. Thus, using our temporally sampled populations I can qualitatively examine contemporary responses to selection in the field and determine how that does or does not align with realized phenotypic change over time. I ask: 1) Is there genetic variation in and selection on floral morphology, floral rewards, and floral phenology traits? 2) Are there trade-offs between floral traits suggesting constraints on evolution, and is the adaptive capacity of populations changing over time due to those constraints? 3) Are realized trait changes in a field setting in line with the direction of selection?

3.3 Results & Discussion

Cumulatively, our results assessing genetic variation, selection, and realized phenotypic change in the field demonstrate the potential for constraints on trait evolution that may slow adaptation in the face of global change. Evolutionary constraints are evident between focal traits when, despite the presence of selection and genetic variation in traits, adaptive response is more limited than expected. In this experimental set-up, I keep the selective environment constant in the form of a common garden, so differences between sampling years in the relationship between trait value and fitness are indicative of a change in how populations may respond to that selection, hereafter referred to as adaptive capacity. While changes to adaptive capacity can occur due to a lack of genetic variation, I find significant maternal line effects in a linear mixed model for all five floral traits investigated in populations sampled from both 2003 and 2012. This shows that populations have the necessary genetic variation to respond to novel selective pressures (Table S1).

Despite the presence of genetic variation, however, I found descendant populations (2012) to differ in their response to selection compared to ancestral populations (2003) in flowering phenology, in line with the hypothesis that the adaptive capacity of populations is changing over time. Specifically, the date of first flowering displays directional selection for earlier flowering in ancestral populations (S = -0.16, p = 0.03; β = -0.26, p = 0.04; Figure 3-2, Table S3-4, Table S3-5), whereas descendant populations show a pattern of selection indicating that intermediate flowering dates are favored (S = -0.10, p = 0.05; γ = -0.13, p = 0.005; Figure 3-2, Table S3-4, Table S3-5). I further confirm this change in adaptive capacity with an ANCOVA testing for a significant interaction between year and date of first flower on fitness (F = 9.08, p = 0.003 for

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linear selection; F = 2.83, p = 0.09 for quadratic selection; Figure 3-2, Table S3-5). These results indicate that despite the shared environment of the common garden, a continued shift toward earlier flowering is no longer favored in the descendant populations.

The adaptive capacity of the other four traits – corolla width, corolla length, nectar sucrose and anther-stigma distance – did not differ between ancestral and descendant populations in that patterns of response to selection across individuals from both sampling years were similar (Figure 3-2). Our results broadly indicate that pollinator attraction traits are under positive selection in both the ancestral and descendent populations. Selection differentials measuring the total selection on each trait show significant, positive directional selection on corolla width in both 2003 ($S = 0.11$, p < 0.01) and 2012 ($S = 0.12$, p < 0.01; Figure 3-2A, Table S3-4). Corolla length similarly displays a signature of positive, directional selection in 2003 ($S = 0.12$, p = 0.003) and 2012 ($S = 0.05$, p = 0.06; Figure 3-2A, Table S3-4) perhaps suggesting selection toward increased overall flower size. However, examination of selection gradients shows no significant selection on corolla length in 2003, and negative, direct selection in 2012 ($\beta = -0.37$, p = 0.03; Figure 3-2B, Table S3-5), indicating that the positive selection detected in the analysis of the selection differential is likely due to the highly correlated nature of corolla length with corolla width, and that corolla width is the more probable target of selection (Figure 3-3A). Nectar sucrose content displays significant disruptive selection in 2003 in both the differential ($S = 0.06$, p = 0.02, Figure 3-2A, Table S3-4) and gradient ($\beta = 0.22$, p = 0.01, Figure 3-2B, Table S3-4), and a weaker signal of disruptive selection in 2012 in the selection gradient ($\beta = 0.10$, p = 0.08), indicating that both high and low °Bx values confer a fitness advantage. No directional or quadratic selection is detectable on anther-stigma distance, indicating that there is no evidence of

an adaptive pathway emphasizing higher self-pollinating rates as has been hypothesized as a possible response to global declines in pollinator populations.

Strikingly, I found evidence that combinations of trait values were favored in descendent, but not ancestral populations. I identified correlative selection between the date of first flower and three other traits among populations sampled from 2012: corolla width ($\gamma = -0.34$, $p = 0.071$), corolla length ($\gamma = 0.39$, $p = 0.045$), and nectar sucrose content ($\gamma = -0.14$, $p = 0.067$, Figure 3-3B), but there was no evidence for correlative selection between these traits in the ancestral (2003) populations. This suggests the existence of a tradeoff between two traits previously under directional selection, corolla width and phenology, where, when there is sufficient combined variation of large flowers and early flowering, there is unrestricted evolution. However, strengthening of correlation between corolla width and date of first flower from 2003 ($r = 0.06$, $p = 0.48$) to 2012 ($r = 0.28$, $p < 0.0001$) causes constraints between the traits to manifest, favoring larger corolla size and restricting the response of date of first flower to more intermediate flowering times (Figure 3-3A). Correlative selection between nectar quality and flowering phenology favors a combination of intermediate flowering times and intermediate to high sucrose values. In this case, correlative selection between date of first flower and nectar quality favors the maintenance of a trait that increases the quality of nectar reward received by pollinators, despite the implication that there may be some energetic cost to high sucrose production as evidenced by the fitness advantage of plants with very low nectar sucrose content. Together, selection for increased corolla width and nectar sucrose content aligns with a path of reinforcement of plant-pollinator interactions, but at the cost of adaptive capacity of an earlier flowering phenology.

To further confirm whether genetic covariances are resulting in a decrease in adaptive response to selection from global change, I compare expected rates of evolution with and without genetic covariance, which will show if evolution occurs more slowly when covariances are considered than if each trait evolves independently. I use a multivariate statistic, R that defines rate of adaptation as the rate of increase in fitness of the mean phenotype and compares the expected rate of adaptation given a selection gradient, β, for all traits and variance-covariance matrix, G to the rate of adaptation with the same selection gradient, but a variance-covariance matrix with all trait covariances constrained to zero (Agrawal and Stinchcombe 2009). I expect that $R < 1$ demonstrates evolutionary constraint such that the rate of adaptation is slower than expected without covariance. Conversely, $R > 1$ would indicate evolutionary facilitation. I find that in both 2003 and 2012, the overall rate of adaptation across traits is constrained when genetic covariance is considered (95% bootstrap CI does not overlap with 1), though constraint is stronger in 2012 with R values ranging from $0.03 - 0.18$ compared to $0.21 - 0.96$ (*i.e.,* closer to 1) in 2003. A trait-specific approach also shows significant constraint on the rate of adaptation in corolla width and corolla length in both 2003 and 2012 (95% CI does not include 0 and is negative, Table 3-1). Intriguingly, the genetic covariance results in adaptive facilitation of date of first flower in 2003 (95% CI does not include 0 and is positive, Table 3-1) and adaptive constraint on date of first flower in 2012 (Figure 3-4).

Finally, our phenotypic data show that differences in adaptive capacity are altering evolutionary trajectory at least on a 4–9-year basis (Figure 3-5). This is an important consideration, because phenotypic selection is reflective only of immediate environmental conditions, and while the

above analyses test whether populations' response to selection in one year may differ in 2003

and 2012, it is possible that results for each year represent a transient phenomenon due to year-

to-year fluctuations in selection, such that detected constraints or facilitation do not represent a

consistent trajectory. Instead, I find general alignment between direction of selection and

phenotypic change over multiple years. From 2003 to 2012, I find a significant increase in

corolla width by an average of ~1mm (t-ratio = -2.53, p = 0.01) in line with a directional

response to selection in populations from 2003. I find no change in average corolla length,

anther-stigma distance, or nectar sucrose content, in line with either a lack of direct selection or,

in the case of nectar quality, the presence of quadratic selection. Including a third timepoint,

2016, for a subset of two populations to test whether reduced adaptive capacity in 2012 maps to

realized phenotypic change, I find a marginal (0.2mm), but insignificant increase in corolla width

in both populations. Further, and in alignment with a previous greenhouse study (Bishop et al

2022) I found that descendent (2012) populations flowered earlier than ancestral (2003)

populations (t-ratio = 1.78, p = 0.07; Figure 3-5, Table S3-3). Descendent populations sampled

from 2016 showed no change in flowering phenology from 2012 (t-ratio = -0.99, p = 0.32;

Figure 3-5, Table S3-3), aligning with a switch from directional to balancing selection, and

suggesting evolutionary constraints are altering the evolutionary trajectory away from shifts to

earlier flowering time.

A striking result across both this field study and the previous greenhouse study is that flowering

time in *I. purpurea* displays a bimodal distribution, with the onset of flowering occurring in two

waves: one with a peak number of plants initiating flowering at 56 days after sowing and one

with a peak at 65 days after sowing. Given the widespread evidence that flowering phenology is

a critical part of an adaptive response in plants to a changing climate, and that I find the ability of

I. purpurea to continue shifting toward earlier flowering dates may be limited, I elected to

analyze the two flowering waves separately to assess the possibility that they may each

contribute differently to overall phenotypic shifts. The first wave of flowering exhibited a pattern

similar to that of the overall dataset, with earlier flowering in 2012 compared to 2003 (t = 3.07, p

= 0.002), and a notable, but insignificant, increase in flowering date between 2012 and 2016 (t =

-1.54, p = 0.13; Figure 3-5, Table S3-3). The second flowering wave, however, differs.

Individuals sampled from 2012 exhibit later flowering than individuals from 2003, albeit the

difference was nonsignificant (Figure 3-5). Selection in 2012 highlights the ways in which the

bimodal nature of flowering time may impact continued evolution in these populations. As

above, selection analyses of flowering phenology in individuals sampled in 2012 shows a pattern

of balancing selection when considering the full dataset (i.e., both waves of flowering time), but

different patterns of selection across each wave. The first wave shows evidence for selection

toward later flowering dates (β = 0.22, p = 0.026), and the second wave of flowering exhibits

selection for earlier flowering dates (β = -0.44, p = 0.024, Table S3-5), cumulatively resulting in

the signature of balancing selection. It is notable that I see persistent genetic variation in wave

one in 2016, but a lack of genetic variation in the second wave (Table S3-1). This is further

complemented by an anecdotal, but repeated, pattern from both this data and the greenhouse

study that proportionately more individuals flower in the first wave in 2012 than in 2003.

Together, the 1) positive selection on wave one, the 2) indication that evolutionary potential for

change in flowering phenology may reside primarily in individuals that flower earlier in the

season, and an 3) overall increase, albeit insignificant, in date of first flower from 2012 to 2016

(Figure 3-5) indicates that the bimodal nature of flowering phenology may contribute to further

changes in evolutionary trajectory such flowering dates may begin to shift toward later in the season.

Multivariate pressures from global change including climate shifts, land use, chemical application, and species abundance or composition changes are expected to have wide-ranging and drastic implications for biodiversity. One of the primary ways in which populations can respond to these pressures in a sustained manner is through adaptive evolution. However, despite indications that evolutionarily-informed models yield more accurate predictions for the impact of global change on wild populations (Martin *et al.*, 2023), we lack information about a critical part of how evolution will function under global change – namely, how genetic covariances between traits impact adaptive potential, and if adaptive capacity itself is changing over time. Our central finding that pollinator-attracting traits are evolving on a rapid time scale of less than ten years, but that genetic covariances between traits may also constrain the overall adaptive rate of flowering phenology, points to the necessity of understanding the role that multivariate trait constraints play in observed lags in adaptation.

Even when rapid adaptation in plant systems occurs, two major hypotheses have been suggested regarding potential adaptive pathways. The first is that plants may respond to decreased pollinator abundance by evolving increased self-pollination which may serve as temporary rescue of a population, but ultimately result in loss of critical genetic variation that buffers against future environmental change (Jones *et al.*, 2013). The second is that adaptation to climatic shifts through shifting phenology will result in temporal mismatch between plants and pollinators, disrupting this critical ecological interaction (Freimuth *et al.*, 2022). I highlight a

third possible adaptive pathway, increased investment in pollinator-attracting traits at the cost of evolvability in flowering time, and discuss its potential implications below.

Results from our previous greenhouse work and the present work suggest that increased corolla width in *Ipomoea purpurea* descendent populations may have occurred due to plants experiencing selection for larger, showier displays to attract pollinating insects. Our findings of direct selection on corolla width, as well as correlative selection that favors intermediate to high nectar quality, provides evidence of a potential adaptive investment in pollinator attraction. Despite this, I found evidence of constraint on the evolution of corolla width in that response to selection with covariance included (2003: $\Delta z_i = 0.016$, 2012: $\Delta z_i = 0.072$; Figure 3-4, Table 3-1) is significantly reduced compared to response to selection without trait covariance (2003: $\Delta z_{nci} = 0.442$, 2012: $\Delta z_{nci} = 0.446$). This indicates that the potential for rapid adaptation to selective pressures such as declining pollinator abundance via a pathway of increased investment in pollinator attraction is limited by trait covariances and will display an evolutionary lag behind that which is predicted by trait variation and selection strength alone. The rate of adaptation in corolla width may be further affected by high levels of plasticity I have measured in this trait (Figure S3-1) which, in some instances, can circumvent trait trade-offs (Yi & Dean, 2016). More specifically, when I compare our field results to a second resurrection experiment with the same populations but conducted in a growth room, I find a much larger effect size of year in the growth room plants compared to plants grown in the field (Figure S3-1). While I do not test directly for it here, our qualitative comparisons between growth room and field-grown plants shows evidence of decreasing plasticity over time, which would indicate decreasing capacity for buffering of costs incurred from trait trade-offs via plasticity (Table S3-7). Furthermore, while I

do not rule out the possibility that ASD and nectar sucrose content may be plastic under different

conditions, our results imply that there is little potential for a plastic response to increase

autonomous selfing or to improve the nectar reward received by pollinators (Table S3-7).

In addition to constraints on the rate of adaptation apparent in corolla width, I find constraint on

both the rate (Figure 3-4) and direction (Figure 3-3B) of adaptation of date of first flower.

Specifically, I see that the ability of descendant populations to respond to selection by shifting to

earlier flowering dates (*i.e.* adaptive capacity of flowering phenology) is constrained by trade-

offs with corolla width and nectar sucrose content, such that this decrease in adaptive capacity is

attributable to correlative selection. This evolution of decreased adaptive capacity in flowering

phenology functions in direct opposition to projected climatic shifts that predict continuing and

increasing shifts in environmental variables such as temperature and precipitation, both of which

have been identified as drivers of adaptive change in flowering phenology (Yu *et al.*, 2016;

Chand *et al.*, 2022). Given its well-documented role in the adaptive response of plants to climate

change, constraints occurring on or because of phenology hold the potential to impact a

population's ability to respond to global change in major ways and trade-offs with other

pollinator-mediated traits, specifically, can indicate ways in which different mechanisms of

selection may interact to maintain or disrupt plant-pollinator interactions.

Finally, it is important to note that global change exposes populations to a myriad of selection

pressures simultaneously. Although I do not test for the mechanism of selection in this work, our

results point to the importance of future work investigating selective mechanisms and how two

multivariate systems – selective agents and traits upon which they may act – interact to direct

evolutionary response. Our result of correlative selection on two traits directly involved in pollinator attraction and successful pollinator interactions, corolla width and nectar sucrose content, is in line with research in *Lobelia siphilitica* suggesting that plants may invest more in pollinator attraction traits under decrease in pollinator abundance (Brown & Caruso, 2023). I also see evidence in our previous work for decreasing variation in corolla width over time (Bishop *et al.*, 2023), perhaps pointing to an eventual process of canalization of large floral size. Suppression of phenotypic variation among individuals in a population can occur when environmental conditions require a specific trait range to ensure survival. For example, in regions with short growing seasons, variation in phenology for different life history stages can be quite low, whereas in regions with longer growing seasons, variation in phenology persists (Gaudinier & Blackman, 2020). Similarly, if pollinators are a driving agent of selection, the investment in pollinator attraction I observe may result in a narrowing of variation in pollinator-attracting traits to ensure reproductive survival. In this case, correlative selection indicates that a narrowing of variation in corolla width may also result in the fixing of intermediate trait values for date of first flower such that there is greater vulnerability to climatic shifts that would otherwise favor survival and reproductive success of earlier flowering individuals. Alternatively, the relative importance or impact of different selective agents may change under different environmental conditions, as is seen in *Mertensia ciliate* where pollinators select for larger flowers under drought stress, but intermediate flower sizes when water is abundant (Gallagher & Campbell, 2017). In this scenario, the observed evolutionary constraints I record in 2012, but not in 2003, may indicate that pollinator-driven selection on floral traits, and consequent selection on correlated traits, is becoming stronger with time not due to declines in pollinator abundance, but due to interactions with climatic conditions. Additionally, contemporary changes in adaptive

capacity may not be driven by pollinator-mediated selection interacting with climate-driven selection at all, but rather by shared selective pressures, cumulatively highlighting a need for further investigation into which underlying causes of selection are inducing evolutionary constraints.

Generally, our study lays the groundwork for more theoretically robust investigations of how adaptive constraints may slow adaptation to global change in a critical eco-evolutionary system, and highlights the importance of incorporating changing adaptive capacity into our predictions of the impact of global change on biodiversity.

3.4 Methods

Study System

Ipomoea purpurea (Convolvulaceae), or the common morning glory, is an annual, weedy vine native to the subtropical Americas. *I. purpurea* employs a mixed mating system and has a large, showy floral display with marked variation in floral traits, indicating the importance of phenotypic selection in understanding maintenance of variation and evolution in this system (García *et al.*, 2023). It is pollinated by generalist species, though most typically visited by bees, syrphid flies, and wasps and offers nectar as a reward to pollinators (Galetto & Bernardello, 2004). The species is an opportunistic colonizer of disturbed environments, and is widely distributed across the eastern, midwestern, and southern United States in areas of high disturbance such as roadsides and agricultural settings.

Resurrection Experiment Design

To investigate evolution in floral traits in response to global change, I planted two separate resurrection experiments, one in a field environment, and one in a growth room with seeds from seven populations distributed across South Carolina, North Carolina, and Tennessee collected at two time points, 2003 and 2012. An additional time point of 2016 was included for a subset of two populations from North Carolina and Tennessee. Prior to planting in the field, a refresher generation was planted to control for maternal line effects in wild-collected seeds. Three replicates of each line were grown in 4-inch pots in a growth room with daily watering and a 12-hr light cycle. Plants were allowed to naturally self-pollinate, and all seeds were collected.

In May 2021, I planted 1,536 scarified seeds from 2-8 maternal lines per population in a randomized block design with three spatial blocks in a common garden field setting at the Matthaei Botanical Gardens in Ann Arbor, Michigan. Each year was randomized within a block and four replicate plants per maternal line were present within each year/block combination. Plants were staked to provide structural support and watered daily until formation of the first true leaves, then as needed throughout the season. 98% of seeds germinated, but intensive levels of vole herbivory in the field resulted in a final sampling of 762 plants. These same populations and maternal lines were re-planted in a growth room in 4-inch pots in Winter 2021 to assess plastic differences in trait response between the field and growth room environments.

Phenotypic Measurements

In a field setting, we measured a total of five traits involved in mediating pollinator interactions and mating system, including three floral morphology traits: corolla width, corolla length, and anther-stigma distance; the floral reward received by pollinators as the sucrose content of the

nectar in °Brix; and phenology as the date of first flower. I also measured early growth rate and plant size to include as covariates in models of selection and phenotypic change. Early growth rate was calculated as the difference in leaf number on two separate days, divided by the number of intervening days. Leaf count surveys were conducted 25 days post-sowing when most plants had produced at least one true leaf, and then again one week later. A final leaf count was conducted at the initiation of flowering and used as an estimate for plant size in data analysis.

We recorded the date of flowering onset for each plant and measured floral morphology and reward traits for four flowers per plant. Corolla width, corolla length, and anther-stigma distance were measured using digital calipers (precision: ±0.01mm), with anther-stigma distance representing the difference in height between the stigma and the tallest anther. To extract nectar from the flower, 10uL of reverse osmosis (RO) water was pipetted directly into the base of a flower, pushing the pipette tip past the base of the stamens and pipetting up and down to mix and extract nectar. I then quantified sucrose content of this nectar/water solution using a pocket refractometer to record percent mass sucrose (°Brix, hereafter nectar sucrose content) in flowers protected with a mesh cover the night before to control for nectar removal. Previous research indicates that the proportion of white flowers in a population may also impact pollinator behavior and plant fitness (Epperson & Clegg, 1987), so I also recorded floral color, but due to sampling limitations and the collapsing of color data down to a proportion in the population, I lack power to analyze its effect and preliminarily find that it is not under selection or changing over time, so it is omitted from further reference. Finally, I collected and counted the number of seeds produced by each plant and used the total seed set as our estimate of fitness.

3.5 Data Analysis

Variation in Floral Traits

To first determine if there is potential for adaptive evolution in floral traits in our populations, I tested for the two primary criteria required for adaptive evolution to occur: selection, and genetic variation in traits. To test for genetic variation underlying floral traits, I fit the following model for each trait:

$$Trait \sim Pop:ML + (1|Block)$$

where each trait is tested separately for each year, with Pop (the geographic location of the wild population from which that seed was collected) and ML (maternal line) as fixed effects, and possible spatial variation in environment across the common garden site, represented as block, as a random effect. I determined if there was evidence of genetic variation for each floral trait by performing a likelihood ratio F-test to estimate F for maternal line using the lmerTest package in R (Bates *et al.,* 2015).

Phenotypic Selection

To determine whether selection in a shared environment manifests differently in plants collected in 2003 compared to 2012, I used phenotypic selection analysis to estimate both selection differentials and selection gradients (Lande & Arnold, 1983). I report differentials and gradients so I can assess both the total (indirect and direct) selection acting on a trait, as well as isolate which traits are directly under selection, and are thus the causal drivers of evolution in this set of pollination-related traits. Focal traits corolla width, corolla length, anther-stigma distance, nectar sucrose content, and the date of first flower were all standardized to a mean of zero and standard deviation of one across all data, and relative fitness was measured as the total seed set for an

individual plant divided by the mean seed count for that year. The onset of flowering occurred in two waves, one with a peak at 56 days after sowing and one with a peak at 65 days after sowing. As such, I assessed selection on date of first flower in two ways: first, on the full dataset to assess the total effect of selection on flowering phenology, and second on the two flowering waves separately to see if selection differs between earlier and later flowering individuals.

We estimated selection differentials (S) using a regression of relative fitness on each trait separately in a model containing linear and non-linear terms for the trait of interest and controlling for block by using block as a random effect:

$$\text{Relative Fitness} \sim \text{Trait} + \text{Trait}^2 + (1|\text{Block})$$

Selection gradients were calculated by performing multiple regression of relative fitness on all phenotypic traits together in an extension of the model above containing linear terms (β), quadratic terms with coefficients doubled to estimate non-linear selection (γ), cross-product terms of all focal traits, and leaf count as a proxy for plant size.

We used an analysis of covariance (ANCOVA) to determine whether collection year altered patterns of selection on plant traits. To do this, I included a year and year x trait interaction term for each trait in the full, multivariate regression model. For all these analyses, a significant linear effect of a trait indicates directional selection, a significant quadratic effect indicates either balancing or directional selection, and a significant interaction between a trait and year indicates that selection gradients differed between years.

Evolutionary Constraints

Correlations are common among phenotypes involved in the facilitation of plant-pollinator interactions, so I explored the possibility of evolutionary constraint or facilitation between traits in two ways: first through the presence of correlative selection indicating that selection is acting on the phenotypic covariance between traits, and second using a G-matrix to compare expected evolutionary outcomes with and without trait covariances. I include analysis of potential constraints between all terms; however, I am particularly interested in trade-offs involving phenology.

To assess correlative selection, coefficients of cross-product terms were extracted from the multivariate regression described above. Significance of interactions between traits were evaluated separately for 2003 and 2012, where significance indicates that selection favors a combination of focal traits. I visualized fitness surfaces for significant correlational selection by performing a thin-plate spline nonparametric regression approach, using the Tps function in the R fields package (Nychka *et al.*, 2015). Smoothing parameters for each spline were chosen to minimize generalized cross-validation score.

To test for the effect of genetic correlations on the evolution of traits, I follow a procedure described in Agrawal and Stinchcombe (2009) to measure the impact of genetic covariances on the rate of adaptation. I first calculated two variance-covariance matrices. The first, $\mathbf{G}$, is composed of variances and covariances of maternal line means, whereas the second, $\mathbf{G}_{nc}$, has the genetic covariances manually constrained to zero such that the diagonal of the matrix contains trait variances, but all other terms in the matrix are set to zero. Vectors of predicted response to selection, $\Delta\bar{\mathbf{z}}$ and $\Delta\bar{\mathbf{z}}_{nc}$ were obtained by multiplying the selection gradient, β by $\mathbf{G}$ and $\mathbf{G}_{nc}$,

respectively. Defining the rate of adaptation as the rate of increase of fitness of the mean phenotype, and assuming fitness can be approximated by a quadratic function, I use the following equation for change in fitness of the mean phenotype (Agrawal & Stinchcombe, 2009):

$$\Delta W(\bar{z}) = \Delta \bar{z}^T \boldsymbol{\beta} + 0.5\, \Delta \bar{z}^T \boldsymbol{\gamma}\, \Delta \bar{z}$$

We then use $\Delta W(\bar{z})$ and $\Delta W_{nc}(\bar{z})$ to compute a single, multivariate statistic, $\boldsymbol{R} = \frac{\Delta W(\bar{z})}{\Delta W_{nc}(\bar{z})}$. This provides an intuitive metric for quantifying the rate of adaptation with covariances relative to the expected rate without them. If $\boldsymbol{R} = 0$, then there is an absolute constraint on adaptation. If $\boldsymbol{R} < 1$, then evolutionary constraint is present; for example, if $\boldsymbol{R} = 0.5$ the fitness of the mean phenotype is increasing only 50% as quickly as if the traits were genetically independent. Conversely, if $\boldsymbol{R} > 1$, then genetic covariances are facilitating evolution and accelerating the pace of adaptation.

We used bootstrap resampling to test whether the difference between evolutionary response for each trait individually with ($\Delta \bar{z}_i$) and without ($\Delta \bar{z}_{nci}$), taking into account covariance structure, was significant. For each of 2000 iterations, I resampled hierarchically with replacement first by maternal line, and then by individual within maternal line. I then estimated $\boldsymbol{\beta}$, $\mathbf{G}$, and $\mathbf{G_{nc}}$ for each bootstrap sample and calculated $\Delta \bar{z}$, $\Delta \bar{z}_{nc}$, and $\boldsymbol{R}$. If 95% or more of the calculated $\boldsymbol{R}$ values were <1, I concluded that there is significant evolutionary constraint acting among traits. Similarly, for each individual trait if $|\Delta \bar{z}_i|$ - $|\Delta \bar{z}_{nci}|$ <0 for 95% or more of the resampled populations, then I concluded that genetic covariances acted to constrain evolution. Each step of this analysis was performed separately for plants collected in 2003 and 2012. Since the selective environment of the common garden is constant between 2003 and 2012, a comparison of years shows us how the capacity of populations to respond to shared selection is changing over time.

Phenotypic Change Over Time

To assess whether trait values change between 2003 to 2012 in alignment with our expectations given the pattern of selection and genetic covariances, I test for realized phenotypic change between 2003 to 2012 for all populations and between 2012 to 2016 for a subset of populations. To do this, I first perform a linear mixed model using the lme4 package (Bates *et al.*, 2015) in R with year, population, and the interaction of year and population as fixed effects and block as a random effect to control for spatial differences across the common garden. All phenotypic traits were log-transformed to adhere to assumptions of residual normality. Each trait was analyzed in a separate model of the following general form:

$$\text{Trait} \sim \text{Year*Population} + (1|\text{Block})$$

We assessed the significance of differences in trait values between sampling years and whether responses differed between sampling populations, using a posthoc analysis of estimated marginal means for each trait in each year. To do so, I used the emmeans package in R (Lenth, 2022) and performed a t-ratio test separately for 2003 to 2012 and 2012 to 2016.

Plasticity

To assess the possible contribution of plasticity to responding to environmental shifts caused by global change, I compare phenotypic shifts from 2003 to 2012 in the field-based common garden used for all other analyses to phenotypic shifts between years in a second common garden set-up in a controlled growth room environment. I use linear models with year, population, and garden as fixed effects, within-garden block as a random effect, and phenotypic traits as the response variable. Garden represents either the field common garden or the growth room common garden. First, I assess the presence of plasticity in traits by looking for significant garden or garden ×

population effects indicating that trait values for the same populations differ significantly depending on the common garden environment. Second, I test for differences in the degree of plastic response between years by looking for a significant year × garden effect. I further verify the direction of this effect using bootstrap resampling and comparison of the mean difference in plasticity between gardens for each year. If 95% or more of the resampled populations show a consistent direction of change from growth room to field in 2012 compared to 2003, I conclude that there is a significant decrease or increase in plasticity, depending on the direction of that change.

3.6 Acknowledgements

3.7 References

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3.8 Figures
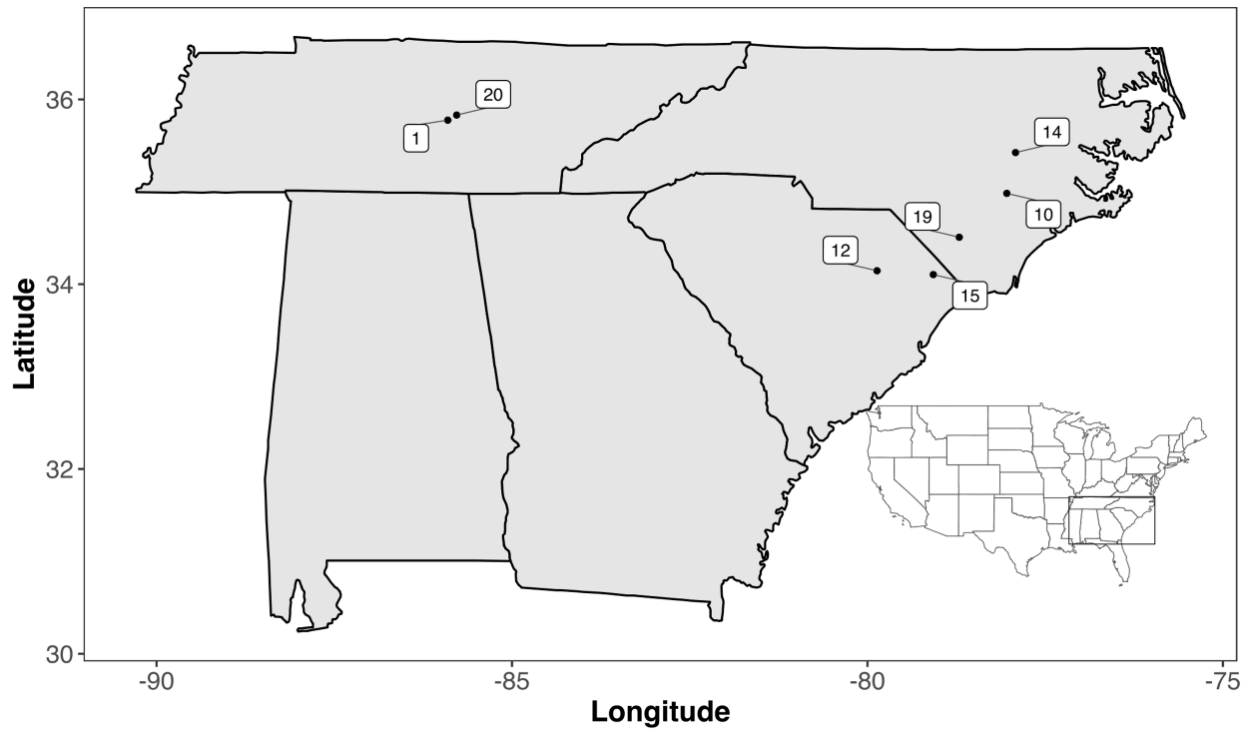
Figure 3-1. Distribution of seven sampling localities of *I. purpurea* labeled with population number. All populations were sampled from the edge of agricultural soy and maize fields.
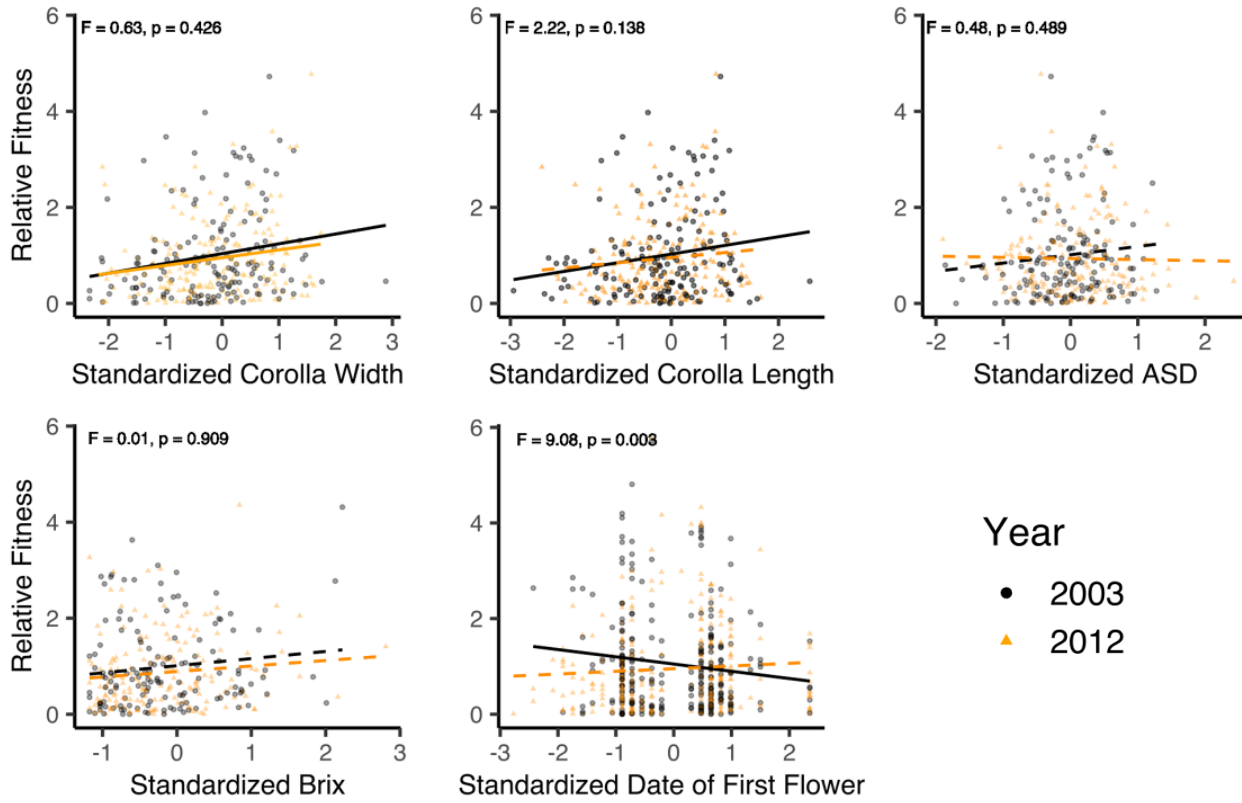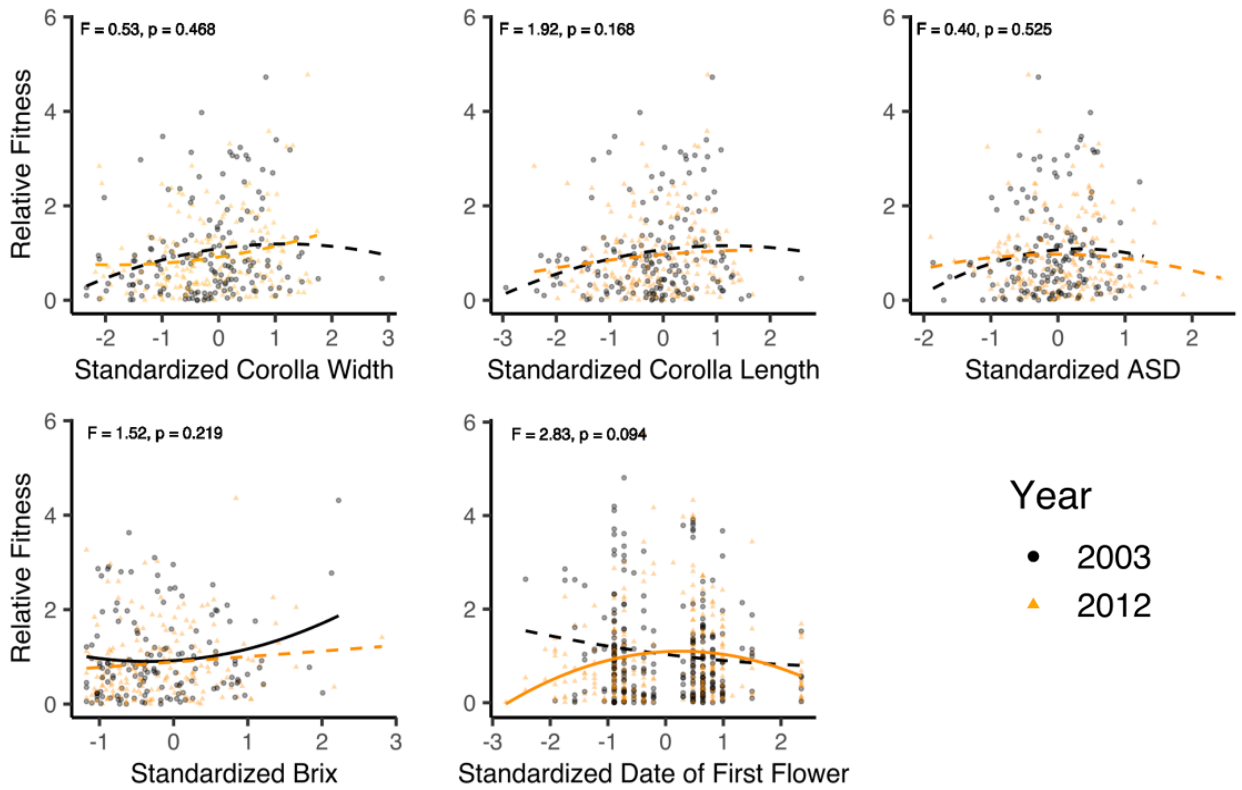
Figure 3-2. Linear (A) and nonlinear (B) relationships between relative fitness and phenotypic traits: corolla width, corolla length, anther-stigma distance, nectar sugar content (°Brix), and the date of first flower. Each trait is standardized to a mean of zero and standard deviation of one. Solid lines represent significant selection differentials for each trait, and F- and p-statistics show the year by trait interaction from an ANCOVA, with $p < 0.05$ indicating a significant difference in selection between 2003 (black) and 2012 (orange).

A.
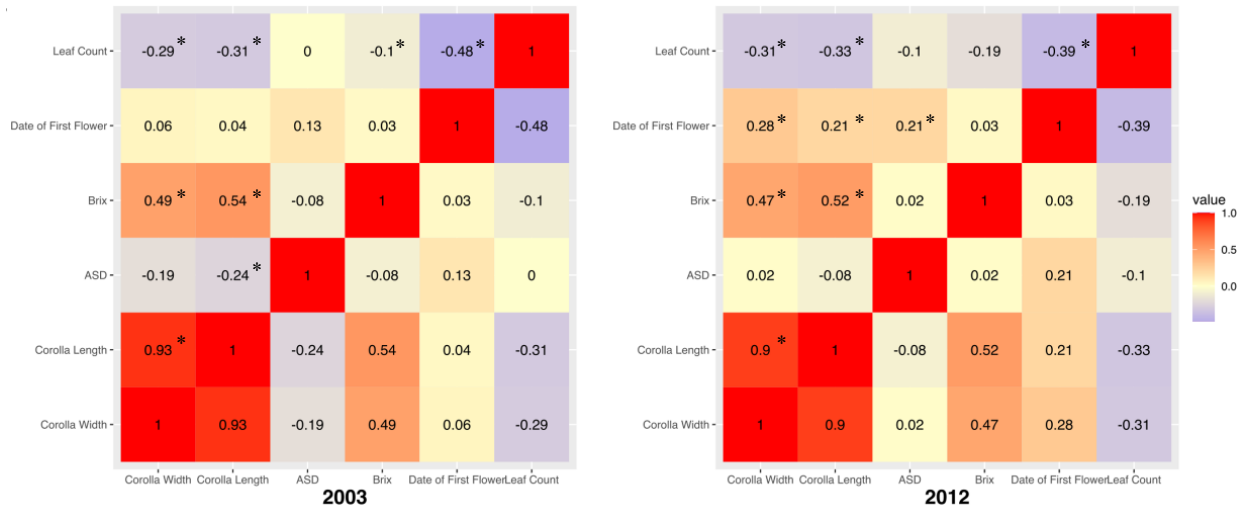
2003

2012

B.
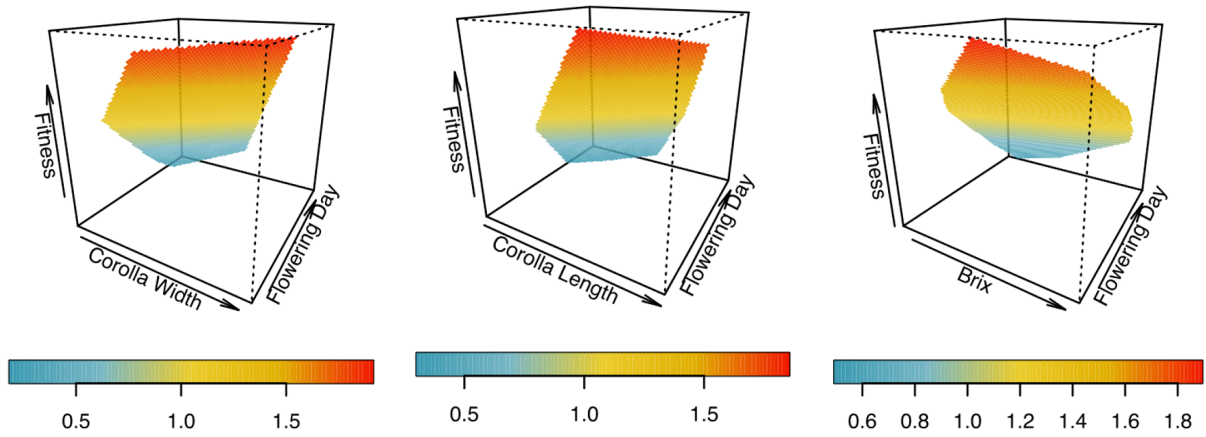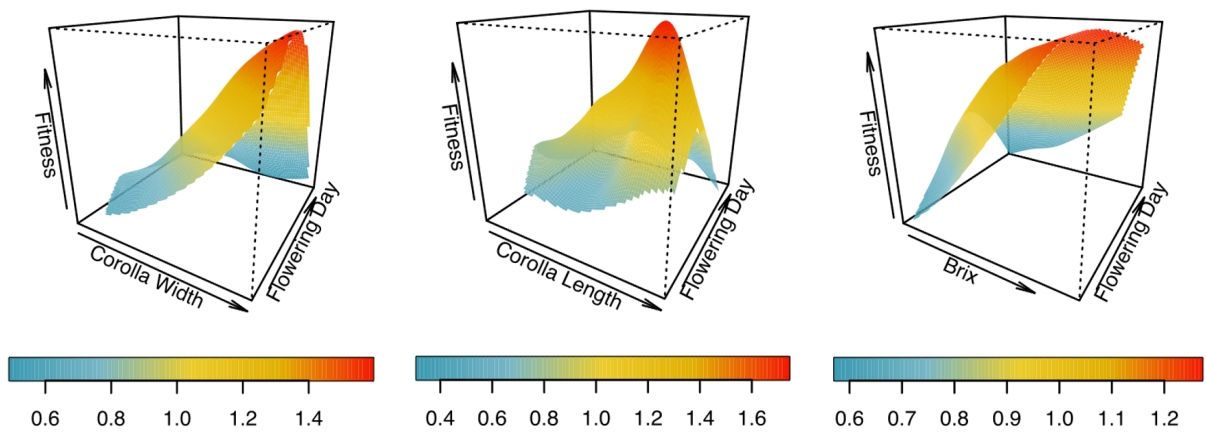
2003

2012

Figure 3-3. A) Heat map showing trait correlations in 2003 and 2012. Correlation ranging from zero to one is displayed in each tile where one (red) is the highest possible positive correlation and negative one (purple) is the lowest possible negative correlation. Significance from a Pearson's correlation test is marked with an asterisk above the diagonal. B) Fitness surface for correlative selection in 2003 and 2012 acting upon date of first flower and three other floral traits tested using selection gradients including all linear, non-linear, and interacting terms (multivariate selection). Relative fitness is depicted by the color gradient with red being the highest fitness, yellow intermediate fitness, and blue lowest fitness. In 2003, no indication of correlative selection was present. All traits were included in the selection gradient, only those with selection gradients indicative of correlated selection in 2012 are shown: corolla width (γ = -0.34, p = 0.071), corolla length (γ = 0.39, p = 0.045), and °Brix (γ = -0.14, p = 0.067).
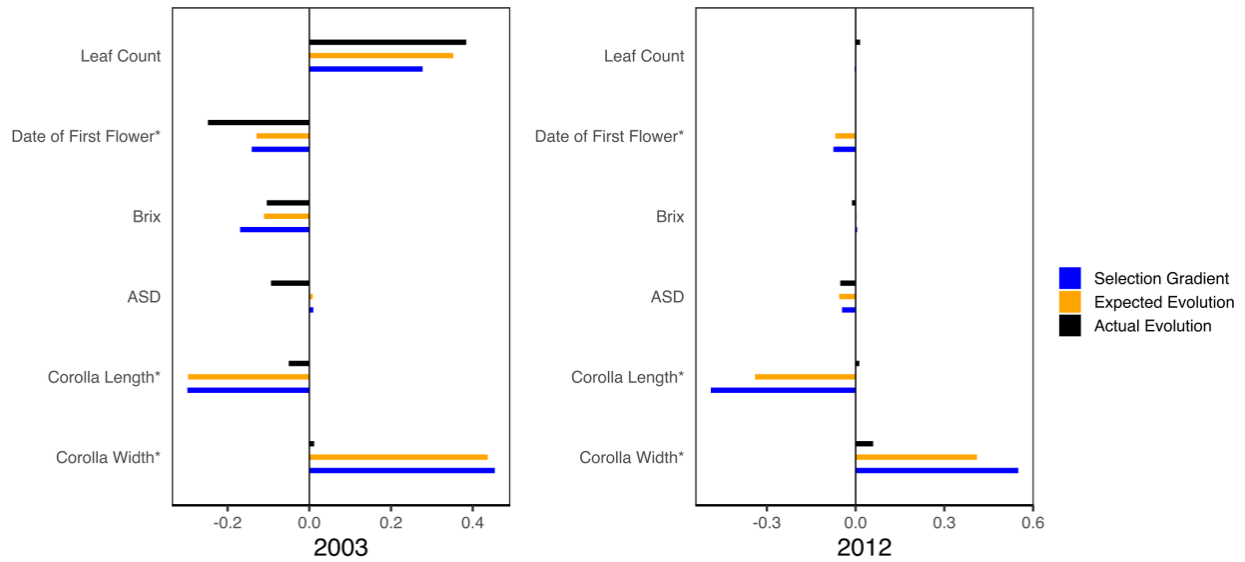
Figure 3-4. For each trait, I show the strength and direction of selection from the selection gradient (blue); Δz_{nci}, the expected response to selection without trait covariances (orange), and Δz_i, the response to selection with trait covariances (black). Traits with an asterisk had a significant difference between Δz_i and Δz_{nci} based on bootstrap resampling, indicating either evolutionary facilitation if the expected response to selection is smaller than the actual response, or constraint if the expected response to selection is larger than the actual response.
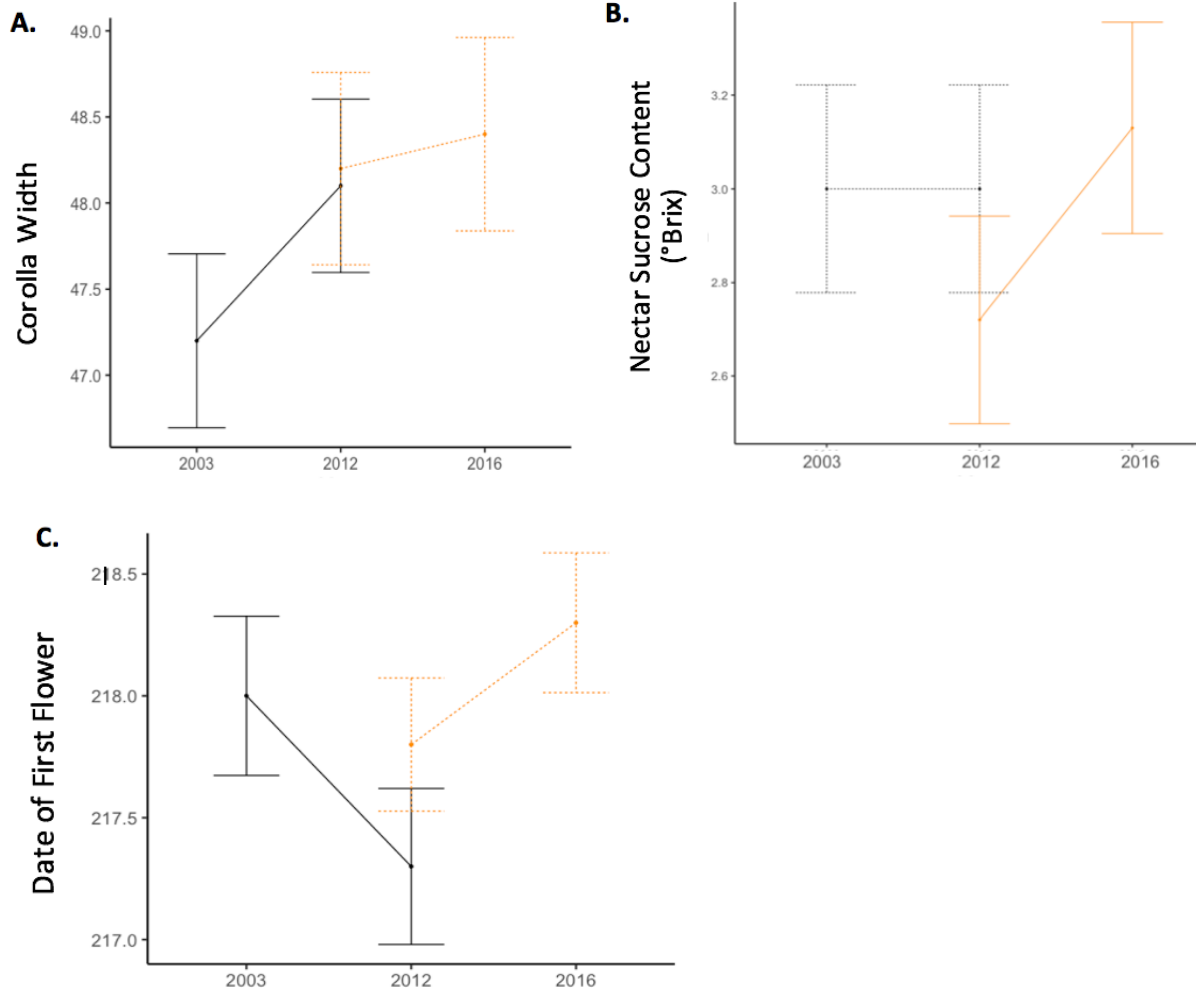
Figure 3-5. Estimated marginal means for floral traits that show significant response to selection in 2003, 2012 and 2016 plotted with standard deviation. Significant difference of estimated marginal means between years is indicated with a solid line. For the comparison of trait values in 2003 to 2012 (black), seven populations are used. For the comparison of trait values in 2012 to 2016 (orange), a subset of two populations is used.

3.9 Table

	2003			2012		
	Mean z	Mean znc	Significance	Mean z	Mean znc	Significance
All	0.182	0.318	1	0.036	0.464	1
Corolla Width	0.016	0.442	1	0.072	0.446	1
Corolla Length	-0.048	-0.302	1	0.014	-0.368	1
ASD	-0.080	0.008	0.928	-0.056	-0.057	0.532
Brix	-0.102	-0.116	0.643	-0.002	0.004	0.645
Date of First Flower	-0.249	-0.129	1	-0.009	-0.074	0.998
Leaf Count	0.388	0.354	0.88	0.017	-0.002	0.843

Table 3-1. For each trait, I show the mean Δz_i and Δz_{nci} and significance of the difference between them. Significance is defined as the percent of bootstrap samples where $\Delta z_i - \Delta z_{nci}$ is either above or below zero, depending on the expected difference calculated from the actual data. If > 95% of the samples are consistently above or below zero, I accept that difference as significant. Traits that show significant evolutionary constraint are highlighted in green. I also show significance for multivariate constraint in all traits combined into a single, multivariate index, as the proportion of sampled values of W that fall below 1.

Chapter 4 Investment in Pollinator Attraction Partially Explains Adaptive Trends

4.1 Abstract

Disruption of biotic interactions is one of the major threats to biodiversity in the face of global change, and can arise from spatial, phenological, morphological, or physiological shifts that occur at different rates between interacting partners. Studies linking morphological or physiologically induced changes under global change to their impact on mutualist interactions, however, remain rare. Here, I investigate whether phenotypic evolution in floral traits identified in Chapters 2 and 3 is driven by adaptive investment in pollinator attraction. Specifically, I use a pollinator visitation survey and structural equation modeling to assess whether the fitness costs and benefits of trait values are mediated through pollinator visitation frequency (an indirect effect on fitness) or impact fitness through some other, uncharacterized selective mechanism. Finally, I ask whether these direct and indirect fitness effects are changing between ancestral and descendant populations. I show that pollinator preference drives fitness benefits of large corolla width, high nectar sucrose content, and large ASD in ancestral populations, whereas earlier flowering phenology induces a fitness advantage unrelated to pollinator behavior. Additionally, I show that pollinator mediated fitness effects are reduced in descendant populations and flowering phenology no longer impacts plant fitness. Overall, these results show that investment in pollinator attraction underlies the adaptive trajectory in ancestral populations, but only partially explains predicted evolutionary potential in descendant populations, highlighting a need for further investigation into underlying selective causes of phenotypic shifts.

4.2 Introduction

One of the major threats to biodiversity in the face of global change is the potential that crucial biotic interactions, such as plant-pollinator mutualisms, are disrupted as organisms respond to abiotic stressors (Thomann *et al.*, 2013; Johnson *et al.*, 2022). Insect pollinator populations have seen declines since the 1950s ((Potts *et al.*, 2010)) with particularly precipitous die-offs in the past few decades (Winfree *et al.*, 2011; Thomann *et al.*, 2013; Hallmann *et al.*, 2017; Soroye *et al.*, 2020) and concomitant declines in insect-pollinated plants (Biesmeijer *et al.*, 2006), generating widespread alarm regarding the potential vulnerability of both wild and agricultural plant-pollinator populations. Human-mediated environmental change can contribute to this vulnerability by causing selection on traits that mediate species interactions (Brown & Caruso, 2023), potentially leading to life history asynchrony between mutualistic partners and their ability to interact. While this life history asynchrony can arise from multiple sources including spatial, temporal or morphological mismatch (Gérard *et al.*, 2020), studies that integrate multiple drivers of mismatch in natural populations remain rare.

The potential for temporal mismatch is a widely studied phenomenon in studies of plant-pollinator interactions. Temporal mismatches may occur when there are shifts in the timing of life history events such that there is little or no seasonal overlap between mutualistic partners despite continued spatial coexistence (Morton & Rafferty, 2017; Maglianesi *et al.*, 2020). Instances of field-based shifts in phenology are now commonly documented in both plants (Menzel *et al.*, 2006) and insect pollinators (Roy & Sparks, 2000), and such phenology shifts have also been documented in resurrection experiments, which are designed specifically to compare traits between populations sampled at different time points (Hamann *et al.*, 2021).

Evidence of a disruption of plant-pollinator interactions because of phenological shifts, however, is mixed (de la Torre Cerro & Holloway, 2021). For example, temporal tracking has been found in ten species of generalist bees and their host plants in North America (Bartomeus *et al.*, 2011), but multiple other studies have found reduced or eliminated overlap between plant and pollinator species (Kudo & Ida, Memmott).

Conversely, the impact of changes in morphological or physiological traits that shape the costs and benefits of plant-pollinator interactions on the maintenance or break-down of those interactions under global change remains largely theoretical. Multiple studies involving direct manipulation of temperature and/or water stress have highlighted the possibility of altered floral traits, especially those involved in pollinator attraction or rewards [e.g. reduction in flower size and number ((Saavedra *et al.*, 2003; Hoover *et al.*, 2012; Descamps *et al.*, 2021; Kuppler & Kotowska, 2021)) and decreased pollen viability and nectar sugar content (Pacini *et al.*, 2003; Descamps *et al.*, 2018)]. However, with a few exceptions (see: (Peralta *et al.*, 2020; de Manincor *et al.*, 2023) a link between these trait changes to pollinator behavior remains rare, and relating potential changes identified in controlled environments to realized changes in field settings where populations are exposed to a multifactorial suite of environmental changes simultaneously is difficult ((Anderson, 2016; Chen *et al.*, 2017)). For example, pollinators can also act as agents of selection, sometimes in opposing directions to abiotic shifts. Declines in pollinator abundance may result in an increase in attraction-based traits such as flower size and nectar production (Bishop *et al.*, 2023), in direct opposition to decreases in those same traits under temperature stress. The relative role of different selective agents can be further confounded by interdependence between them such as selection by pollinators for larger flowers only under

water stress and not water abundance conditions (Gallagher & Campbell, 2021). As such, there is a need to understand the relative role of selective agents in driving evolutionary effects, and particularly the role of pollinators as agents of selection under a changing climate.

In the previous chapters I report changes in corolla width, nectar sucrose content, and phenology highlighting trait change over time in wild populations. Here, I investigate the potential underlying selective mechanism of these trait changes by evaluating the hypothesis that changes in functional traits are driven by increasing investment in pollinator attraction. I specifically conduct a pollinator visitation survey and perform structural equation modeling to ask 1) What potential floral traits, if any, exhibit an indirect effect on fitness mediated by pollinator visitations, and 2) Does the relative role of pollinators (*i.e.* strength of indirect effects on fitness mediated through pollinator behavior vs. direct effect of traits on fitness that are not mediated through pollinator behavior) differ between 2003 and 2012? I use this evidence to infer whether changes in functional traits are driven pollinator-based selection and whether observed evolutionary constraints due to correlations between traits are attributable to investment in pollinator-attracting traits constraining the response to other possible selective agents.

4.3 Methods

Pollinator Observations

Using the same resurrection set-up described in Chapter 3, pollinator observations were conducted once a week during the months of August, September, and early October, always between the hours of 10AM – 1PM. In total, a field team observed pollinator visitation of 277 plants distributed across nine plots, each with 28-33 plants. Each observation round had a

duration of twenty minutes, and eight rounds per plot were conducted in total. Each week, we

rotated the plot observed at 10AM to balance the influence of time of day across plots. To

standardize across different observers and varying levels of insect taxonomic expertise, we

identified pollinators to the level of order and then morphospecies based on size. The types of

pollinators we looked for were: large (10-15mm) and extra-large (>15mm) social bees in the

family Apidae; small (<5mm) and medium (5-10mm) solitary bees in the families Halictidae or

Apidae, large (>10mm) solitary bees in the families Andrenidae or Megachilidae; bee flies

(family Bombyliidae), small (<10mm) and large (^{3}10mm) syrphid flies in the family Syrphidae;

other flies (families Muscidae and Calliphoridae), Lepidoptera, Coleoptera, Orthoptera,

Hemiptera, wasps (family Vespidae), ants, and other. We recorded pollinator approach as a

pollinator flying up to a flower, but not contacting the stigma or anthers. Pollinator foraging was

recorded as a pollinator directly contacting the anthers and/or stigma. Five functional traits –

corolla width, corolla length, anther-stigma distance, nectar sucrose content (°Bx), and flowering

phenology (Julian date of first flower) as well as a plant size covariate were measured as

described in chapter 3 and recorded for each plant used in the pollinator behavior survey.

4.4 Data Analysis

Pollination Frequency and SEM Model Selection

For each plant, I sum all observed approaches and forages to get individual approach and forage

frequencies and sum both numbers to get an overall visitation (approach + forage) frequency.

Since the length of observation period and number of rounds of observation were held constant

across all plots, this represents a time-standardized metric of insect pollinator visitation to each

plant. Differences in the frequency of pollination visitation between years were tested for using

estimated marginal means and a t-ratio test. I calculated relative fitness as the total seed set for an

individual plant divided by the mean seed count for either 2003 or 2012, depending on the

collection year of origin for that plant. For corolla width, corolla length, ASD, and nectar sucrose

content, I use the mean of four randomly selected flowers distributed over the growing season as

the trait value for each plant, whereas date of first flower is a single value and is represented as a

Julian date. For all phenotypic traits and relative fitness I see strong plot effects, so I performed a

linear regression of plot on trait and extracted residuals from the model which I then

standardized and used in all further analyses. I do not see evidence of plot effects on approach or

forage frequency.

To determine if changes in trait values over time are mediated by pollinator behavior, I use

structural equation modeling (SEM) to evaluate seven a priori hypotheses exploring the

relationship between phenotype, pollinator visitation, and fitness. Structural equation modeling

assesses causal relationships between variables, both direct and indirect, by taking two inputs: 1)

qualitative causal assumptions (i.e. some a priori biological hypothesis about how parameters

interact), and 2) empirical data, to then derive two logical conclusions: a statistical measure of fit

for the model that describes the implications of the assumptions (i.e. do your assumptions about

causality between parameters adequately describe the covariance structure of the data), and

coefficients representing the strength and significance of causal relationships between parameters

(Bollen & Pearl, 2012). Even if a model contains significant coefficients, poor model fit casts

doubt on the assumptions included in the model structure. An accepted model does not prove

causal assumptions (i.e. model-reality consistency); however it does indicate higher plausibility

of estimated relationships by demonstrating model-data consistency through variance-covariance

structure. As such, I first assess model fit for each of our seven models that represent hypotheses about how plant traits influence fitness either directly or indirectly via pollinators.

These hypotheses include five nested models A-E (i.e. removal and inclusion of directional relationships with no change in the causal order of relationships), and two additional non-nested models, F and G (Table 4-1). Models A-C test the degree to which the impact of floral traits on fitness is mediated through pollinators. Model A proposes that the fitness effect of all traits other than plant size are fully mediated through pollinator interactions. In turn, model B proposes that the fitness impact of floral traits is only partially mediated by pollinators, and model C proposes that the impact of floral traits on fitness is not mediated through pollinators at all. Removing the causal link between plant size and fitness tests the assumption that fitness is limited by internal resources (Model D) and removing direct links from functional traits to pollinator foraging tests whether all pollinator foraging choice occurs prior to entering the corolla such that fitness impacts are mediated by signaling cues that can be perceived from a distance (Model E). Finally, I test two non-nested models: Model F proposes that variation in traits is all mediated through plant size, adding a causal relationship between leaf count and all other traits, and Model G assesses the possibility that relationships between traits and pollinator behavior are correlated, but not causal (i.e. a bi-directional rather than uni-directional relationship).

I use piecewiseSEM in R, which allows for more flexible data assumptions, namely that the data is not required to be multivariate normal, and generalized linear models can be fit for non-Gaussian data types such as count (Lefcheck, 2016). piecewiseSEM implements a log-likelihood based goodness of fit measure that produces a χ^2 statistic comparing model-implied variance-

covariance relationships with actual variance-covariance relationships in the data. A significant

p-value (< 0.05) in this case indicates a significant difference between the relationships proposed

by the model and those present in the data, meaning the model is not an appropriate fit to the

data. Conversely, a non-significant χ^2 comparison indicates that the model does fit the data. In

one case (model B), the model I wish to assess does not have any degrees of freedom, so I use

Akaike's information criterion (AIC) to compare it to a similar model assuming plant size

influences only pollinator approach frequency, not pollinator foraging frequency, as it is unlikely

that plant size influences pollinator foraging choice after the pollinator has already approached

the plant. I find that the AIC is significantly (>2 AIC units) lower for this second model, so

proceed with model selection using a modified model B that does not include plant size as an

effect on pollinator foraging frequency.

Causal Relationships

For the model that provided best fit for the observed data, I perform multigroup analysis in

piecewiseSEM to calculate direct and indirect effects and compare differences in path

coefficients between ancestral (2003) and descendant (2012) populations. Direct effects

represent standardized partial regression coefficients, and indirect effects of traits are calculated

by multiplying direct effect coefficients along any given path from trait to pollinator behavior to

plant fitness.

Pollinator-Specific Effects and Power Analysis

Observations in the field indicated that the pollinator class of "extra large social bee" included

carpenter bees. Carpenter bees are nectar robbers, so may respond to floral attraction cues such

as large flower size and high nectar concentration without providing any fitness benefit to the plant. As such, I re-ran the model with extra large social bees removed from the analysis to test whether the loss of effect of foraging rate on fitness in 2012 could possibly be attributed to foraging by carpenter bees. To do this, I first test for goodness of fit with extra large social bees removed, then use multigroup analysis to compare a model with the full dataset and a model with the subset data to verify that they result in different structures, and finally re-test direct and indirect effects of by-year comparisons. Finally, I assess the statistical power of all our models to assess the likelihood that any differences detected between years in the multigroup analysis that involve the lack of a significant relationship in one year is a true difference, not a lack of power to detect a relationship. I use pwrSEM to perform a power analysis for each relationship within the SEM (Wang & Rhemtulla, 2021) and do this separately for each year.

4.5 Results

Field Observations

Over the season, I recorded 1,116 pollinator visits, all of which were insect pollinators except for a single instance of hummingbird foraging. Survey plots included a total of 267 plants, 211 of which were involved in at least one pollinator interaction during our observing bouts, with a maximum number of 14 interactions for a single plant. The pollinators recorded fall primarily into the large or extra-large social bees categories with 536 and 363 interactions, respectively, the bulk of which were bumblebees (Figure 4-1). Inconsistency across observers in the categorization of bumblebees as large or extra-large renders the distinction between these two categories minimal, though it is worth noting that honeybees fall into the category of large bees, whereas carpenter bees were consistently categorized as extra-large.

The functional traits measured do not display high levels of correlation, apart from a strong

positive correlation between corolla width and corolla length, which have a Pearson's correlation

coefficient of 0.83, p < 0.001 (Table 4-2). I opt to retain both variables in the model, however, as

they represent different functions in pollination biology. Corolla width plays a role in pollinator

attraction whereas corolla length plays a role in pollination efficiency and has also been shown to

respond to pressure from nectar robbers (Tie *et al.*, 2023), such that differing mechanisms or

strength of selection on the traits could result in a change in floral proportion as opposed to

overall size (Bishop *et al.*, 2023). I do not find any difference in pollinator approach (2003 total

= 145, 2012 total = 121, p = 0.412) or forage (2003 total = 464, 2012 total = 386, p = 0.324)

frequency between years (Figure 4-1, Table 4-2).

Model Selection

Of the seven models I assessed, the only model with a reliable fit to the observed data was Model

B, which tested the hypothesis that the effect of floral traits on fitness is partially mediated

through pollinator behavior (Model B, c^2 = 0.246, p = 0.619, Table 4-3). All other models failed

to explain the correlation matrix of the data (p <0.05). This refutes hypotheses of either full or

negligible mediation of traits through pollinator behavior (Models A and C). I also reject

competing hypotheses that plant fitness has no internal resources limitation (Model D), that

variation in floral traits is mediated entirely through plant size (Model F), that relationships

between traits and pollinators are merely correlated rather than causal (Model G), and finally, I

reject a hypothesis that visitation decisions are made strictly using traits that signal at a distance

(e.g. corolla width, Model E).

Direct and Indirect Effects of Functional Traits on Fitness

Using Model B as the best-supported model, I assessed the relative strength of direct and indirect effects of traits on fitness in ancestral and descendant populations and evaluated differences between the two years. Direct effects are significant relationships between floral traits and fitness that are not explained by the influence of a trait on pollinator behavior, whereas indirect effects are fitness benefits or costs that are mediated through pollinator visitation rate. The effects of floral traits on pollinator behavior are largely conserved between years, whereas differences are primarily detected in the direct effects of floral traits on fitness and in the relative role pollinator behavior plays in overall fitness. I find a conserved, positive effect of corolla width on frequency of pollinator approach (2003: $r = 0.242$, $p = 0.02$; 2012: $r = 0.265$, $p = 0.02$) and foraging (2003: $r = 0.286$, $p = 0.004$; 2012: $r = 0.296$, $p = 0.004$; Table S4-1, Figure 4-2) in both years, such that increased flower size results in increased frequency of pollinator interaction. I also find a positive relationship between nectar sucrose content on pollinator approach (2003: $r = 0.149$, $p = 0.002$, 2012: $r = 0.218$, $p = 0.002$), and ASD on pollinator foraging (2003: $r = 0.213$, $p = 0.0001$; 2012: $r = 0.269$, $p = 0.0001$; Table S4-1, Figure 4-2), meaning that high sucrose content in nectar attracts more pollinators to a flower and high conversion of approach to foraging corresponds with greater distance between the stigma and tallest anther.

However, while there is a positive relationship between approach frequency and foraging frequency in both years (2003: $r = 0.366$, $p < 0.001$; 2012: $r = 0.346$, $p < 0.001$) and a positive relationship between pollinator foraging and plant fitness in ancestral populations ($r = 0.173$, $p = 0.032$), I find that fitness in descendant populations is not influenced by pollinator foraging ($p =$

0.226). This means that while there are indirect effects of corolla width, nectar sucrose content, and ASD on plant fitness in ancestral populations, there are no indirect effects of any floral traits on fitness in descendants.

Additional differences between ancestral and descendant populations are apparent largely in the direct, non-pollinator mediated effects of floral traits on fitness. Specifically, corolla width has a large, direct positive effect on fitness in 2012 ($r = 0.455$, $p = 0.001$), resulting in a much stronger total effect of corolla width on plant fitness than that seen in 2003, which was due to indirect effects alone (total effect of $r = 0.455$ in 2012 compared to a total effect of $r = 0.065$ in 2003; Figure 4-2, Table S4-1). I find an additional direct negative effect of corolla length on plant fitness in 2012 ($r = -0.798$, $p < 0.001$; Figure 4-2, Table S4-1), indicating that there is some unmeasured mechanism by which corolla architecture is influencing plant fitness independent of its influence on pollinator attraction. Finally, I see a significant difference between ancestral and descendant populations in the role of flowering phenology on fitness. In 2003, I find a direct relationship between the date of first flower and fitness ($r = 0.321$, $p = 0.005$) such that earlier flowering conveys a fitness advantage, but that advantage is not present in the 2012 descendant populations ($p = 0.144$). To ensure that differences between years are not a statistically spurious result, a power analysis of relationships within the SEMs show there is statistical power to detect the effect of phenology ($P = 1$) and foraging ($P = 0.99$) on fitness in 2012 and direct effects of corolla width ($P = 1$) and corolla length ($P = 1$) on fitness in 2003, indicating that differences in these relationships between years are biological, not statistical (Table S4-2A).

Predicted Effects of Nectar Robbing Removal

I next tested whether the removal of extra-large social bees, which includes instances of nectar robbing by carpenter bees, impacts the relationship between functional traits and visitation frequency or visitation frequency and plant fitness. The goodness of fit of Model B is robust to the removal of extra-large social bees such that I retain good model fit to test relationships between traits, pollinator visits, and fitness with this group of pollinators removed (Table 4-3). I performed a multigroup comparison (*i.e.*, testing path coefficient differences) of an SEM with all pollinators included to an SEM with extra-large social bees extracted from the dataset. This allows us to see if there are significant differences in the relationship between traits, visitation, and fitness strictly due to the exclusion of this pollinator class. From this, I found that removal of Extra-Large Social Bees results in a significant changes to paths for 2012, but not for 2003.

I examined the effect of this class of insects, specifically, to assess whether the lack of relationship between pollinator visitation and plant fitness in 2012 is due to a breakdown of pollinator fitness advantage, or more likely due to an artifact of the field methods by which nectar robbing visitations were mixed with visits by true pollinators. An SEM of 2012 data with extra-large social bees removed retains the same direct effects of size and flowering phenology on fitness as well as the relationship between pollinator approach and pollinator foraging. Notably, a positive, direct effect of pollinator foraging on fitness is now significant in 2012 ($r = 0.164$, $p = 0.047$), reintroducing the possibility of indirect, pollinator-mediated effects. While the effect of ASD and nectar sucrose content on pollinator foraging and approach, respectively, remain unchanged from the full dataset, I no longer detect a significant impact of corolla width on pollinator behavior. However, statistical power for these relationships is quite low in the subset data ($P < 0.30$ for corolla width to approach and foraging in both 2003 and 2012), so the

lack of relationship between corolla width and pollinator behavior may be a factor of reduced power due to lower sampling size. As such, to perform a rough comparison of indirect effects in 2003 and 2012, I predict the indirect effects if the influence of carpenter bees is removed by multiplying the standardized coefficient between trait and pollinator behavior from the full model by the standardized coefficient for the effect of foraging rate on fitness for a model with carpenter bees removed (Table S4-1, Figure S4-2). I find an indirect effect of nectar sucrose content mediated through pollinator approach of $r = 0.01$ in 2003 and $r = 0.01$ in 2012, and an indirect effect of ASD mediated through pollinator foraging of 0.05 in 2003 and 0.03 in 2012. For corolla width, the indirect and total effect in 2003 is $r = 0.07$, whereas the indirect effect in 2012 is $r = 0.04$, however, the total effect of corolla width on fitness ($r = 0.48$) is notably stronger in 2012 due to the additional, direct effect of corolla width on fitness.

4.6 Discussion

The goal of this study was to determine if pollinator preference behavior drives the evolution of *Ipomoea purpurea* floral traits. More specifically, I sought to determine if the increased corolla size and nectar sucrose content over time that I previously reported in this species (Bishop *et al.*, 2023) could be due to increased investment in pollinator attraction. I was likewise interested in determining how other floral traits impact overall plant fitness and whether that occurs indirectly *via* pollinator visitations or through a direct impact on fitness not mediated by pollinators. Using field observations from a resurrection experiment and structural equation modeling, I found significant pollinator-mediated effects of corolla width, nectar sucrose content, and anther-stigma distance on plant fitness in the ancestral and possibly descendant populations such that increases in any of these traits results in increased pollinator foraging and overall plant fitness. I further

found a direct, non-pollinator mediated fitness advantage associated with earlier flowering in ancestral but not descendant populations, and a strong direct effect of corolla width and length on fitness unrelated to pollinator behavior, but only in descendant (2012) populations. Our findings expand current knowledge of the role pollinators play in influencing the effect of trait values on fitness under global change, and thus driving adaptive changes in floral traits.

Adaptation Through Pollinator Attraction

Although SEM detects some differences in the relationships between phenotypic values and plant fitness between ancestral (2003) and descendant (2012) populations, the influence of corolla width, nectar sucrose content, and ASD on pollinator visitation behavior are typically conserved through time in both direction and magnitude. Simply, this means that flowers with wider corollas are approached and foraged in more frequently. A larger distance between the stigma and tallest anther corresponds with higher foraging but does not influence the initial rate of pollinator approach, whereas high sucrose levels in the nectar results in higher approach rates such that increased foraging is an indirect result of increased attraction from a distance. While there is a link between floral traits, pollinator foraging, and fitness in ancestral populations, providing evidence for investment in pollinator attraction, there is no such connection between pollinator foraging and overall plant fitness in descendant populations.

We test whether the loss of this relationship is biologically meaningful in two ways – first whether it is a statistically spurious result due to low power and second whether it is an artifact relating to lack of fine-scale resolution in field-based insect identification. Power analysis of this relationship shows that there is adequate statistical power to detect the influence of foraging rate

on fitness, implying that the breakdown of the relationship between pollinator activity and plant fitness in the descendent populations may be a true signal. Notably, statistical power to detect a direct relationship between pollinator approach and fitness is quite low ($P = 0.16$ in 2003 and $P = 0.18$ in 2012). However, given that an approach does not involve pollinator contact with anthers or stigma, and instead the influence of approach rate on fitness relies on the conversion of approach to successful foraging (which shows a positive relationship in both years, $r = 0.366$, $p < 0.001$; Figure 4-2), it is biologically unlikely to find a direct link between approach rate and fitness such that this lack of power does not explain the deteriorated relationship between pollinator activity and fitness. Visitation surveys in the field failed to distinguish between very large bumblebees (true pollinators) and carpenter bees (nectar robbers). While nectar robbers may still respond to floral cues of attraction, especially high nectar sucrose content, they do not provide the mutualistic service of pollination (Willmer & Corbet, 1981; Wang *et al.*, 2013; Tie *et al.*, 2023), meaning that the presence of nectar robbers in the dataset could explain the existence of a relationship between floral traits and visitation, without conveying a fitness advantage (*i.e.* the break-down of a connection between recorded foraging and fitness). I find that removing nectar robbers from the dataset does result in a positive relationship between foraging rate and fitness, reinstating the indirect influence of ASD and nectar sucrose content. With this removal, however, I lose a relationship between corolla width and pollinator activity. Power to detect a relationship between corolla width and pollinator behavior in the subset data is low, however, so while this may be a true loss indicating that the change in the role of pollinators in 2012 is that corolla width is no longer a strong attracting factor, I cannot confidently state that the isolated effect of pollinators would maintain a connection between larger corolla widths, increased pollination, and increased fitness.

A notable second point of evidence that there may be biological significance to a deteriorating signal of pollinator driven selection on corolla width is that overall approach and foraging rates did not differ between ancestral and descendant populations. If investment in increasing corolla widths due to pollinator attraction is continuing to convey a fitness advantage strong enough to drive adaptive changes, I would expect to see that larger corolla widths in 2012 would coincide with higher rates of foraging. However, I see no indication that descendant populations are attracting more pollinators. Though pollinator populations have been declining for decades, thus generating a potential selective force for increased attraction (Winfree *et al.*, 2011; Thomann *et al.*, 2013; Hallmann *et al.*, 2017), a recent study highlighted a previously unseen escalation in die-offs in more recent decades, with unprecedentedly high declines of bumblebee species in North America specifically between the years of 2000-2014 (Soroye *et al.*, 2020), the same years within which I record a possible loss of increased pollinator-driven selection on corolla width. While I do not directly test any mechanism here, it is possible that large corolla widths simply are not sufficiently attractive under conditions of severely low pollinator abundance and that pollinators are instead selecting plants based on more immediate cues of gain, such as nectar volume or sucrose content, or factors that maximize conservation of energy such as proximity. There is also evidence that pollinator preference varies under different climatological conditions (García *et al.*, 2023), meaning that pollinator preference for large corolla widths may not be persisting as climate shifts occur.

This uncertainty about the adaptive role of shifts in corolla width indicates a need for further exploration of 1) how *I. purpurea* may be accounting for a loss of fitness gains from previous

investment in pollinator attraction as well as 2) a need to better understand what selective mechanisms are continuing to drive changes in this trait. One possible route through which *I. purpurea* might account for a loss of fitness advantage from pollinator attraction is through increased self-pollination. While I do not detect decreases in ASD indicative of higher rates of increased self-pollination, ASD does not fully capture the probability of selfng. There is some indication in *I. purpurea* that, instead, environmental stress is accounted for by preferential outcrossing early in the day, followed by temporally delayed self-pollination as the closing of a bloom creates a cooler microclimate more suitable for fertilization (Liu *et al.*, 2020). As such, other traits such as duration of flowering may be more likely to capture likelihood of self-pollination and a switch away from reliance on pollinators.

Given that I focus only on pollinators in this analysis, remaining fitness effects not attributable to pollinator preference may be due to a number of possible other selective agents. In descendant populations, I see a strong, direct relationship between increased corolla width and fitness. This is in line with our previous results of continued directional selection on corolla width in 2012, but it is not attributable to pollinators, indicating that some other selective force is at play. Evidence of increases in floral size due to climatic variables is scarce in the literature, with the vast majority of studies indicating that both temperature and water stress induce fewer and smaller blooms (Saavedra *et al.*, 2003; Hoover *et al.*, 2012; Descamps *et al.*, 2021). However, these studies are in no way comprehensive to all possible abiotic shifts, so it remains possible that floral architecture is influenced by climatic changes in some unknown way. Notably, there is one study that found that increases in temperature resulted in a shift from selfing to outcrossing in *Vicia faba*, thereby generating a possibility for increased genetic diversity and consequently

resilience by accelerating the selection for more stress-tolerant genotypes (Bishop *et al.*, 2017).

While this study explicitly measured increased fitness advantages of outcrossed pollen, it is

unclear whether this is tied to increased pollinator activity at higher temperatures or shifts in any

floral traits, highlighting a greater need for studies relating changes in floral traits, pollinator

selection, and climatic variables. In addition to climate variables, another possible agent of

selection in the field is herbivory. While I do observe both insect herbivory and florivory in the

field, most commonly from the invasive Japanese beetle, there is little work on the relative

selective roles of herbivory and pollinators under global change, with preliminary indication that

pollinators may outweigh selective impacts of herbivores (Chen *et al.*, 2017). Perhaps most

intriguing as a possible additional source of selection in this system is the presence of nectar

robbery, in part because I see higher visitation of flowers with large corollas when nectar robbers

are included in the dataset, but not without them, and in part because there is evidence in the

literature suggesting a negative relationship between nectar robbing intensity and corolla length

(Tie *et al.*, 2023), possibly accounting for some of the negative relationship between corolla

length and fitness I observe in 2012 populations .

Implications for Constraints on Flowering Phenology

In support of our results of directional selection (Chapter 3), as well as broad evidence in the

literature (Roy & Sparks, 2000; Menzel *et al.*, 2006; Hamann *et al.*, 2021), our structural

equation model shows that the date of first flower has a direct, negative impact (*i.e.* earlier

flowering is favored) on fitness in ancestral populations. Notably, this means that flowering

phenology in this system does not appear to be under selection by pollinators. This aligns with

evidence in the literature showing that climatic factors such as temperature and precipitation are

strong drivers of flowering phenology (Chand *et al.*, 2022; Cheptou *et al.*, 2022). However, some authors have found that pollinators also act as direct agents of selection on flowering phenology, thereby helping maintain temporal overlap between mutualist partners (Chen *et al.*, 2017). Instead, selection by pollinators on traits related to attraction and pollinator reward, combined with a lack of direct selection by pollinators on flowering phenology, suggests an adaptive path in which *I. purpurea* may be responding to decreased pollinator abundance from either population decline or reduced temporal overlap by increased investment in attraction.

In contrast to ancestral populations, I find no relationship between flowering phenology and fitness in descendant populations. Instead, I uncovered strong direct effects of corolla width and corolla length on fitness. Due to the important role flowering phenology can play in response to climatic shifts, and the observed evolutionary constraints I find in Chapter 3, I am interested in whether pollinator driven selection on nectar sucrose content and corolla width may be the driving cause of limits on the evolvability of flowering phenology. Our results show that while constraint on date of first flower due to nectar sucrose content is entirely due to investment in pollinator attraction, constraint due to correlation with corolla width is not entirely explained by a hypothesis of increased pollinator attraction. Instead, I see that, in relation to the indirect effect of corolla width on fitness mediated by pollinators, the direct effect of corolla width on fitness is substantially stronger, and there remains an unexplained driver behind the strong selection for increasing corolla width (Figure 4-3).

Caveats and Summary

While this study addresses a critical question of how different traits may respond to different agents of selection in a complex field setting, there are a couple key caveats to the approach taken here. Specifically, although I am inferring selection based on causal pathways detected in a structural equation modeling approach, this is not the same as direct detection of differential fitness between outcrossing pollination and self-pollination through the use of a control group in the field (Emel *et al.*, 2017). As such, I cannot comment as robustly on the importance of pollinators to overall fitness. Additionally, though the resurrection approach allows us to isolate the effects of adaptation from plasticity and identify different causal links to fitness when exposed to a shared selective environment, plants in this experiment were grown outside of the range from which they were collected. Therefore, some aspects of the selective environment, such as the presence of nectar robbing by carpenter bees, may not be reflective of their natural conditions. Finally, in-depth analysis of the differential role of different pollinators or other insect interactions is limited by the lack of specificity in insect identification. While the disruptive effect of carpenter bees on the link between pollinating behavior and fitness is removed by removing the category of extra large social bee, so, too, are some bumblebee pollinators, meaning that I lose signal to detect effects of corolla width on pollinator behavior and, as such, the indirect effects of corolla width are inferred rather than directly measured.

In summary, I show that, while there is selection by pollinators on pollinator-attracting traits across time, a hypothesis of increased investment in pollinator attraction only partially explains the adaptive trajectory in this system and the observed evolutionary constraints between flowering time, corolla width and nectar quality. Our results indicate that evolution in flowering phenology is due to selection from non-pollinator agents, likely climatic variables such as

temperature precipitation, and that pollinator-driven selection is the primary source of constraint between nectar sucrose content and flowering phenology, indicating that a need to invest in reproductive success via pollination is, at least to some extent, constraining the possibility of response to climatic shifts through changing flowering phenology. This result is caveated by low statistical power to detect non-pollinator mediated effects of nectar on fitness, but is corroborated by the correlative selection found in Chapter 3 that favors intermediate to high values of nectar sucrose. Furthermore, the effect size for which there is low power is relatively small ($r = 0.11$), and I find adequate power ($P > 0.80$), but still no significant effect at a coefficient threshold of r $= 0.15$. Research on global pollinator declines has found that, although there are climate impacts on insect survival and reproductive capacity, much of global insect decline can be attributed to habitat loss. As such, the adaptive pathway highlighted here – increased pollinator attraction at the cost of evolvability in flowering phenology – could still result in a 'stranded assets' phenomenon wherein pollinator populations collapse to an extent where increased attraction capabilities are insufficient for population rescue, and plant populations are also reduced in their capacity to respond to climatic shifts due to decreased variation in climate responsive traits such as flowering phenology.

It is possible that this is already occurring and partially responsible for the lack of relationship between corolla width, pollinator visitation, and plant fitness in descendant populations. Though there is pollinator selection on corolla width in ancestral populations, the direct effect of corolla width on fitness in descendant populations is much stronger than the indirect effect through pollinators, highlighting a need for further exploration of selective agents to evaluate sources beyond pollinator attraction that are driving changes in this trait and possibly slowing the

evolutionary rate between corolla width and flowering phenology. Despite not knowing the source of external selection on corolla width and length in descendant populations, it is noteworthy that the direction of selection on corolla width aligns with that of pollinator preference, possibly indicating an adaptive pathway that simultaneously favors pollinator attraction and response to other, as yet unknown environmental shifts.

4.7 Acknowledgements

4.8 References

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4.9 Figures
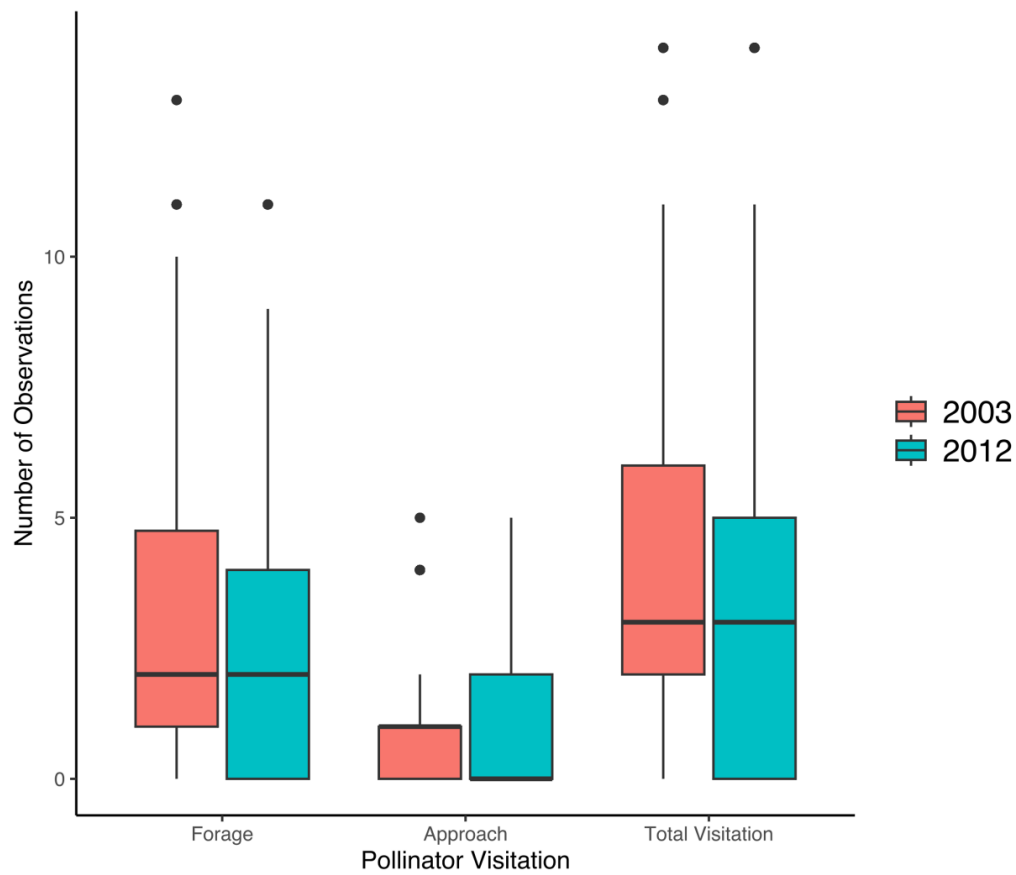
Figure 4-1. Box plots showing mean frequency of pollinator foraging, approach, and total visitation (sum of approaches and forages). No significant differences between ancestral (2003) and descendant (2012) populations are present for any category of visitation.
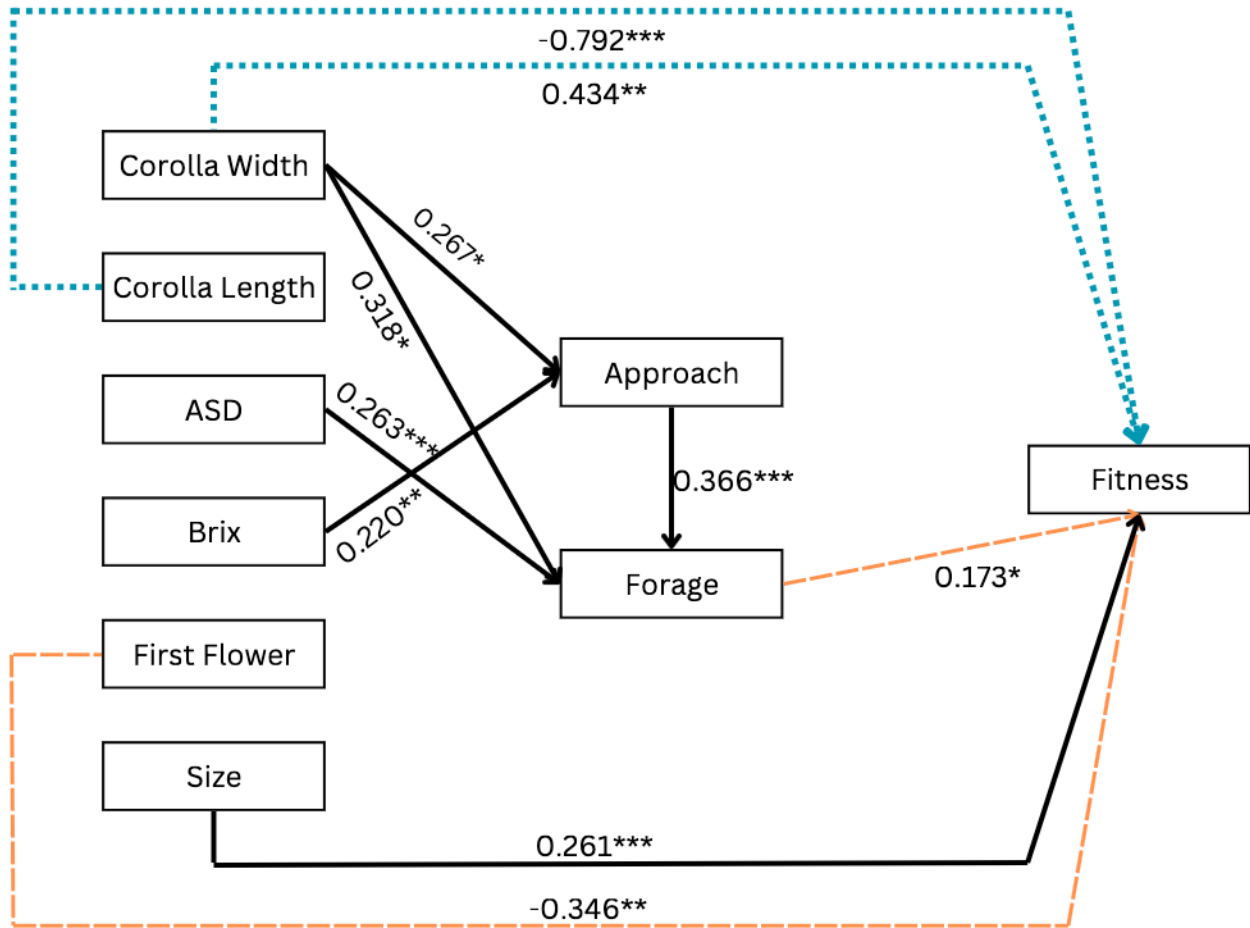
Figure 4-2. Best-fit SEM showing the direct and indirect effects determining pollinator visitation rate and fitness. Effects that are conserved in both direction and magnitude between 2003 and 2012 are indicated by solid, black lines. Relationships that only exist in 2003 are shown with a dashed orange line whereas relationships that are only present in 2012 are shown with a dashed blue line. Standardized path coefficients are shown for each significant path where * indicates a p-value <0.05, ** indicates p < 0.01, and *** indicates p < 0.001. See Table S4-1 for exact indirect and direct path values.
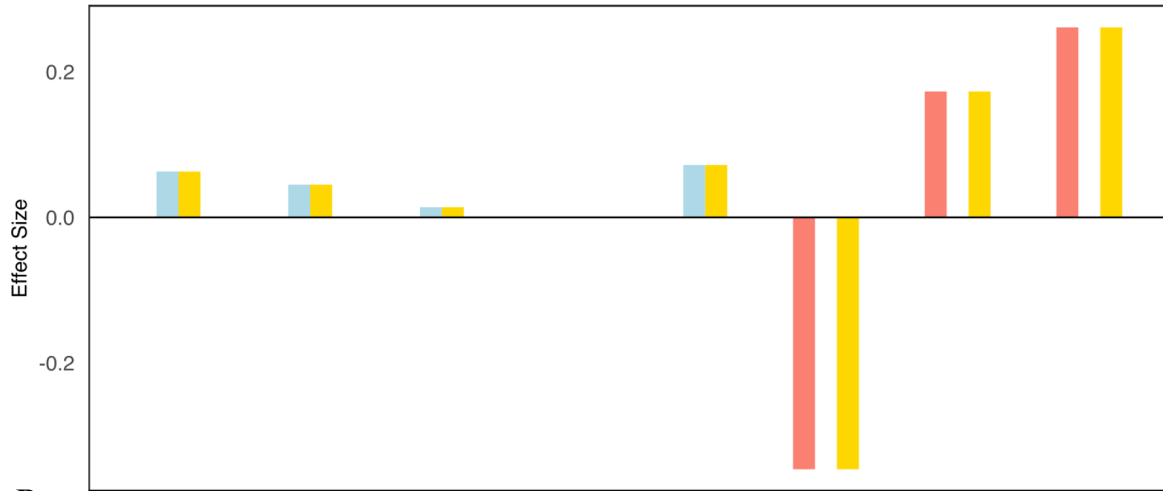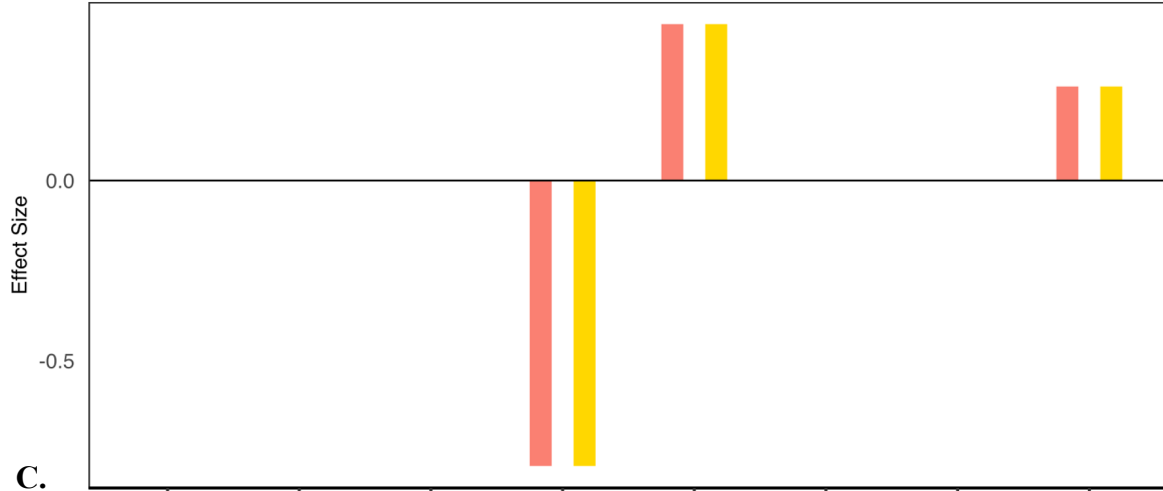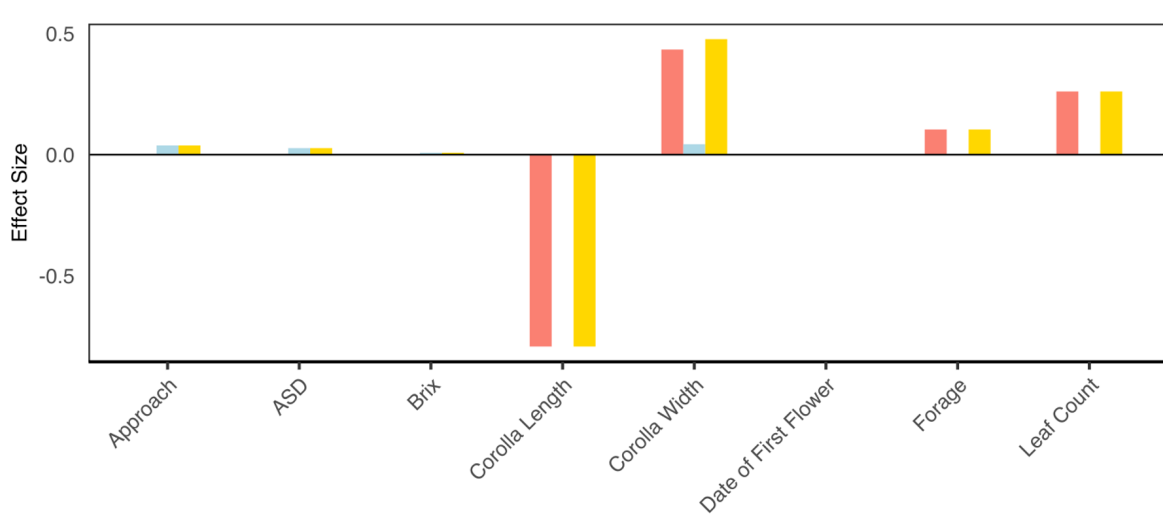
Figure 4-3. Indirect (blue), direct (red), and total (yellow) effect sizes of each trait on fitness for Model B shown for A) 2003 and B) 2012. Panel C represents predicted indirect effects if the influence of carpenter bees is removed where the magnitude of indirect effects are calculated by multiplying the standardized coefficient between trait and pollinator behavior from the full model by the standardized coefficient for the effect of foraging rate on fitness for a model with carpenter bees removed (see supplemental Figure 4-2 for full path coefficients of this model alone).

4.10 Tables

Model	Path Alterations	Biological Hypothesis
A	No direct paths from traits (except plant size) to fitness	Effect of floral traits on fitness is entirely mediated through pollinator behavior, fitness is limited by internal resources
B	Indirect and direct paths from traits to fitness	Effect of floral traits on fitness is partially mediated through pollinator behavior
C	No indirect paths from traits to fitness	Effect of floral traits on fitness is not mediated through pollinator behavior at all
D	No causal link between plant size and fitness	Fitness is not limited by internal resources
E	No paths from traits to pollinator foraging	All pollinator foraging choice occurs prior to entering corolla such that fitness impacts are mediated by signaling cues that can be perceived from a distance
F	Additional path between leaf count and all other traits	Variation in functional traits is mediated through plant size
G	Bi-directional instead of uni-directional paths between traits and pollinator visitation/foraging	Relationships between traits and pollinator behavior are correlated, not causal

Table 4-1. Seven hypothesized models of the direct and indirect effects that determine pollinator visitation frequency and plant fitness. Models A – E are nested models testing the degree to which trait-based effects on fitness are moderated through pollinator behavior. Models F and G are non-nested and test whether trait effects are reducible to plant size or are equally well explained by correlative rather than causative relationships. All models include bi-directional paths accounting for correlations between functional traits.

	Corolla Width	Corolla Length	ASD	Brix	Date of First Flower	Size	Approach	Forage	Fitness
Corolla Width	1.00								
Corolla Length	0.83	1.00							
ASD	-0.28	-0.41	1.00						
Brix	0.15	0.18	-0.13	1.00					
Date of First Flower	0.23	0.09	-0.02	-0.03	1.00				
Size	-0.17	-0.20	0.12	0.00	-0.47	1.00			
Approach	0.22	0.15	-0.01	0.20	0.07	0.05	1.00		
Forage	0.24	0.12	0.19	0.07	0.13	-0.03	0.41	1.00	
Fitness	-0.15	-0.23	0.17	0.06	-0.07	0.26	0.10	0.08	1.00
Mean	52.29	56.87	1.37	3.67	217.60	33.88	0.96	3.08	1.22
Standard Deviation	6.10	5.81	1.58	1.87	5.37	17.71	1.19	3.25	1.11

Table 4-2. Pearson's correlation coefficients among morphological characters, approach rate, forage rate, and relative fitness are shown below the diagonal. Mean and standard deviation displayed below correlations.

Model	Dataset	χ^2	df	p-val	AIC
A	Full	16.244	6	0.013	3062
B	Full	0.246	1	0.619	3056
C	Full	32.248	6	0	2209
D	Full	12.831	2	0.002	3066
E	Full	32.248	6	0	3078
F	Full	404.652	11	0	10135
G	Full	497.33	15	0	12477
B.2	Extra-large social bees removed	2.103	1	0.147	2022

Table 4-3. A comparison of goodness of fit for alternative path diagrams (Figure 4-2) using structural equation modeling. Nonsignificant χ^2 values suggest that the models do not deviate significantly from the observed data. AIC is Akaike's information criterion and df is degrees of freedom in the model. Models that show nonsignificant χ^2 and minimize AIC provide the most reliable fit to the observed data.

Chapter 5 Conclusions and Future Directions

Research Summary

The objective of this dissertation is to expand our understanding of how floral traits involved in mediating plant-pollinator interactions respond to global change. First, I identified traits beyond the commonly studied trait of flowering phenology that are involved in mating system determination and exhibit phenotypic evolution between ancestral populations in 2003 and descendant populations in 2012. I then explored how trait-trait covariances might result in evolutionary constraints and whether adaptive potential is declining over time. Finally, I tested whether phenotypic evolution and evolutionary constraints are explained by a hypothesis of adaptation through increased investment in pollinator attraction. The results stemming from this thesis provide novel insight into the evolutionary trajectory of floral traits involved in a plant-pollinator mutualism in an annual mixed-mating system plant, *Ipomoea purpurea*, and is the first body of work to use resurrection experiments to directly study adaptive potential and identify declines in adaptive rate in response to global change due to changes in trait covariance structure. Overall, this research demonstrates that the incorporation of a multi-trait approach and an assessment of evolutionary constraints can aid predictions for how biological communities may respond to the multivariate selective pressures of global change.

Adaptive Resilience & Future Directions

Three possible adaptive routes to maintain reproductive success in plants under global change have been highlighted by research in global change biology. First, an earlier shift in the timing of life history events will help plants avoid experiencing temperature or water stress that occur later in the season (Gérard *et al.*, 2020). The second is a path of increased self-pollination both in response to decreases in pollinator abundance as well as in response to acute temperature stress (Van Etten & Brunet, 2013; Razanajatovo *et al.*, 2020; Cheptou *et al.*, 2022). Third, plants may instead show adaptation and reproductive insurance through increased attraction of pollinators (Brown & Caruso, 2023) which, as I highlight in this system, may incur costs through evolutionary constraint on other traits such as flowering phenology.

Each possible route has potential consequences as an adaptive strategy, especially in light of sustained and escalating environmental change. In the case of shifting phenology, research has repeatedly highlighted the possibility of disrupted mutualisms due to interacting partners either responding to different cues (Simmonds *et al.*, 2019) or to the same cues at different rates (Kharouba *et al.*, 2018; Visser & Gienapp, 2019), such that, even in instances where rapid adaptation matches the pace of environmental change, critical ecological services may disappear. Increased self-pollination as a strategy provides temporary rescue in the face of acute environmental stress, however, as a long-term strategy will likely lead to dramatic decreases in genetic variation, rendering populations more vulnerable to the environmental perturbations characteristic of climate change (Busch *et al.*, 2022). My dissertation reveals two major vulnerabilities as well as insight regarding the third adaptive pathway – increased pollinator attraction. First, I show that pollinator-related traits beyond phenology are changing over time, but represent a highly constrained system due to trait-trait covariances. Adaptive potential in

traits is decreasing over time with adaptation occurring at 84% the expected rate, were genetic

covariances removed, in samples collected in 2003 but at only 6% the expected rate were genetic

covariances removed in samples from 2012. This shows that, although rapid adaptation is

occurring, and I find significant trait changes in <10 years, if rate of adaptation continues to

slow, the ability to keep pace with environmental change is a substantial concern. Another

possible vulnerability with this adaptive path is an outcome of 'stranded assets' adaptation

wherein plants end up having invested heavily in attracting pollinators that are simply no longer

present in the ecosystem, while concurrently having reduced genetic variation with which to

respond phenologically to climate shifts and possibly even shifting to later-season flowering

(Chapter 3) due to correlative selection.

Climate science has shown that there is a temporal lag on the scale of approximately 10-40 years

between when emissions are released into the atmosphere and when biologically perceptible

shifts in climate occur (Hansen *et al.*, 2005; Samset *et al.*, 2020). I observe trait evolution in less

than a quarter of that time, indicating that, even if carbon emissions are drastically reduced

immediately, we will see climate shifts that influence the evolution of plant systems for the next

half century even in the most optimistic scenarios of social and political action. There is broad

indication that evolvability in flowering phenology plays a critical role in plants' response to

climate, such that variation that is maintained in this trait is important for an adaptive response to

sustained pressures. Pollinator declines, however, while partially attributable to climate change

(Soroye *et al.*, 2020), are heavily driven by other global change pressures from habitat

destruction (Potts *et al.*, 2010; Raven & Wagner, 2021) and widespread chemical application

(Hallmann *et al.*, 2017). As such, this work reinforces the idea that resilience in biodiverse

systems to one factor of global change – climate – is highly dependent on taking action to prevent habitat loss and further insect decline. Habitat fragmentation has already been highlighted as a limiting factor in response to global change through range shifts and reduction of gene flow. I show that identifying driving agents of selection can also help identify ways in which global change factors interact to limit co-adaptation to multiple pressures.

This research also provides a framework for using resurrection experiments to study adaptive potential to global change and identifies one mechanism by which constraints may occur. However, it is limited in a few crucial ways that highlight important future directions. First, the common garden set-up that I used falls outside the geographic range of collected populations. Within-range common gardens and reciprocal transplant studies will help determine realized fitness and pollination levels in naturally occurring populations. I also saw that these traits are highly plastic, such that, though evolution is occurring, it is unclear what trait values will manifest in different field settings more reflective of wild conditions. Second, I explored only pollinators as an agent of selection, but global change is multifactorial, and elucidating the selective basis of other trait changes will provide important insight into the adaptive trajectory of populations. Notably, there is selection on corolla width in 2012 that contributes strongly to evolutionary constraints and is unexplained by pollinator preference. Correlative selection between flowering phenology and corolla width points to an importance of identifying the driving selective force to this change as it may play a key role in inadvertently facilitating the maintenance of plant-pollinator mutualisms as well as constraining the evolvability of flowering phenology. Fortunately, Project Baseline, which began storing seeds in 2013 for use in resurrection experiments to study global change, will make continued study in this area more

accessible and less ad hoc based on fortuitous historical collecting than it has been thus far.

Finally, identifying gene regions underlying adaptive change and constraints will provide insight

into adaptive dynamics and the interaction of ecological process and genomic architecture.

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Appendices

Appendix A: Supplemental Figures and Tables for Chapter 2

ID	Population Name	Year	State	Latitude	Longitude	Number of Plants			Number of Flowers		Number of Maternal Lines
						Morphology	Phenology	Rewards	Morphology	Rewards	Rewards
1	billings	2003	TN	35.77524	-85.90342	10	NA	NA	55	NA	NA
1	billings	2012	TN	35.77524	-85.90342	22	NA	NA	191	NA	NA
2	bergaw1	2003	NC	34.59571	-77.92748	13	14	NA	68	NA	NA
2	bergaw1	2012	NC	34.59571	-77.92748	15	2	NA	84	NA	NA
4	chicken road	2003	NC	34.55667	-79.12560	16	12	NA	74	NA	NA
4	chicken road	2012	NC	34.55667	-79.12560	17	12	NA	60	NA	NA
5	clarendon1	2003	SC	33.85988	-79.90907	NA	13	NA	NA	NA	NA
5	clarendon1	2012	SC	33.85988	-79.90907	NA	1	NA	NA	NA	NA
8	darlington2	2003	SC	34.29720	-79.99126	16	10	NA	90	NA	NA
8	darlington2	2012	SC	34.29720	-79.99126	12	11	NA	46	NA	NA
9	duplin east	2003	NC	34.92404	-77.79617	NA	13	26	NA	179	8
9	duplin east	2012	NC	34.92404	-77.79617	NA	4	8	NA	65	6
10	duplin west	2003	NC	34.98316	-78.03931	NA	9	NA	NA	NA	NA
10	duplin west	2012	NC	34.98316	-78.03931	NA	2	NA	NA	NA	NA
11	grimsley	2003	NC	34.52714	-78.75670	NA	5	NA	NA	NA	NA
11	grimsley	2012	NC	34.52714	-78.75670	NA	16	NA	NA	NA	NA

12	florence	2003	SC	34.14581	-79.86531	18	12	28	78	195	8
12	florence	2012	SC	34.14581	-79.86531	13	11	31	83	222	8
14	hare road	2003	NC	35.42476	-77.91712	16	14	32	106	208	8
14	hare road	2012	NC	35.42476	-77.91712	17	11	31	97	213	8
15	horry1	2003	SC	34.10421	-79.07373	15	10	NA	94	NA	NA
15	horry1	2012	SC	34.10421	-79.07373	12	13	NA	53	NA	NA
16	horry2	2003	SC	34.10535	-79.18323	13	9	NA	101	NA	NA
16	horry2	2012	SC	34.10535	-79.18323	29	11	NA	177	NA	NA
17	marion1	2003	SC	34.15915	-79.27291	15	11	NA	97	NA	NA
17	marion1	2012	SC	34.15915	-79.27291	18	12	NA	96	NA	NA
18	marion2	2003	SC	34.15659	-79.27027	NA	11	NA	NA	NA	NA
18	marion2	2012	SC	34.15659	-79.27027	NA	13	NA	NA	NA	NA
19	mckinnon	2003	NC	34.50819	-78.70899	17	13	NA	135	NA	NA
19	mckinnon	2012	NC	34.50819	-78.70899	20	8	NA	195	NA	NA
21	new hope	2003	NC	35.36982	-77.87731	NA	13	NA	NA	NA	NA
21	new hope	2012	NC	35.36982	-77.87731	NA	11	NA	NA	NA	NA
22	oldkenley	2003	NC	36.14360	-78.05342	18	12	NA	125	NA	NA
22	oldkenley	2012	NC	36.14360	-78.05342	27	10	NA	279	NA	NA
23	snakes	2003	TN	35.06791	-86.62955	NA	3	NA	NA	NA	NA
23	snakes	2012	TN	35.06791	-86.62955	NA	11	NA	NA	NA	NA
25	starlight	2003	NC	34.61636	-79.05167	17	12	NA	89	NA	NA
25	starlight	2012	NC	34.61636	-79.05167	1	5	NA	4	NA	NA
26	spears soy	2003	TN	35.53341	-85.95190	NA	3	NA	NA	NA	NA
26	spears soy	2012	TN	35.53341	-85.95190	NA	9	NA	NA	NA	NA
28	sumter2	2003	SC	34.09792	-80.37771	16	9	25	62	187	8

28	sumter2	2012	SC	34.09792	-80.37771	13	9	29	60	199	8
29	tarheal	2003	NC	34.70513	-78.73890	14	12	*NA*	63	*NA*	*NA*
29	tarheal	2012	NC	34.70513	-78.73890	10	9	*NA*	65	*NA*	*NA*
30	willis corn	2003	TN	35.31105	-85.94500	*NA*	11	*NA*	*NA*	*NA*	*NA*
30	willis corn	2012	TN	35.31105	-85.94500	*NA*	9	*NA*	*NA*	*NA*	*NA*
31	vervilla	2003	TN	35.60848	-85.84638	*NA*	10	*NA*	*NA*	*NA*	*NA*
31	vervilla	2012	TN	35.60848	-85.84638	*NA*	10	*NA*	*NA*	*NA*	*NA*
32	walnut grove	2003	TN	35.09936	-86.22551	6	*NA*	*NA*	57	*NA*	*NA*
32	walnut grove	2012	TN	35.09936	-86.22551	10	*NA*	*NA*	52	*NA*	*NA*

Table S2-4. Sampling localities included in the phenotypic analyses including geographic coordinates, the number of plants included for each of the three greenhouse experiments, the number of flowers measured for the floral morphology and floral rewards experiments, and the number of maternal lines used in the floral rewards experiment. Abbreviations: TN: Tennesses, NC: North Carolina, SC: South Carolina

		Corolla Width	Corolla Length	ASD	First Flowering Wave	Second Flowering Wave	°Brix	Pollen Count
Trait ~ Year*Latitude + (1|Population)								
Year	numDF	1	1		1	1	1	1
	denDF	12.10	11.77	9.67	289.89	141.09	1.94	1.90
	F	7.093	10.472	4.42	3.950	0.206	0.003	0.028
	p	0.0205**	0.007**	0.659	0.048**	0.651	0.961	0.883
Latitude	numDF	1	1	1	1	1	1	1
	denDF	2662.85	2781.94	2468.51	20.98	22.10	200.63	163.41
	F	16.850	0.041	1.587	0.121	5.484	1.94	2.187
	p	4.167e-05**	0.840	0.072	0.731	0.029**	0.016**	0.141
Year*Latitude	numDF	1	1	1	1	1	1	1
	denDF	519.82	923.79	1576.65	290.00	141.16	60.45	33.25
	F	23.388	0.580	2.633	3.950	0.203	4.59	2.180
	p	1.747e-06**	0.447	0.641	0.048**	0.653	0.036**	0.149
δt ~ t*Latitude								
t	numDF	1	1	1	1	1	NA	NA
	denDF	11	11	11	19	16	NA	NA
	F	2.600	3.507	26.905	38.307	6.291	NA	NA

	p	0.14	0.088*	3.004e-4**	6.022e-6**	0.023**	NA	NA
Latitude	numDF	1	1	1	1	1	NA	NA
	denDF	11	11	11	19	16	NA	NA
	F	2.654	1.277	3.055	0.789	1.157	NA	NA
	p	0.13	0.283	0.108	0.386	0.298	NA	NA
t*Latitude	numDF	1	1	1	1	1	NA	NA
	denDF	11	11	11	19	16	NA	NA
	F	6.058	0.671	0.345	3e-4	0.792	NA	NA
	p	0.032**	0.430	0.569	0.987	0.387	NA	NA

Table S2-5. Linear mixed models showing the influence of latitude on temporal changes in trait value and the predictability of δt by t. The first model shows significance of latitude, sampling year, and the interaction of the two on all trait values, reported as a truncated p-value based on Satterwaite's degrees of freedom method for a type III ANOVA. For floral morphology and phenology, latitude is included as a fixed effect while population is used as a random effect to control for longitude. The model for floral rewards additionally uses maternal line as a nested random effect within population (Trait ~ Year*Latitude + (1|Pop/ML)). The second model shows the interactive effect of latitude and starting population mean trait value on the degree of change in mean trait value for a population. Significance ($p<0.05$) is marked with a double asterisk** and relationships trending significant ($0.05 < p < 0.1$) are marked with a single asterisk*.

	Year	Range	Mean	SD	PCV (%)	% Change PCV	Significance (p)
Corolla Width	2003	1.8-7.5	4.503	0.993	21.976	NA	NA
	2012	1-7.1	4.764	0.993	20.849	5.406	0.063*
Corolla Length	2003	2.8-8	5.431	0.616	11.463	NA	NA
	2012	2.2-7	5.474	0.6165	11.259	1.812	0.642
ASD	2003	-2.7-1.3	0.116	0.256	212.296	NA	NA
	2012	-1-1.15	0.108	0.201	186.923	13.57	0.387
Flowering Wave 1	2003	213-240	223.2	8.112	3.629	NA	NA
	2012	214-241	223	7.466	3.343	8.555	0.211
Flowering Wave 2	2003	256-305	277.8	13.82	4.959	NA	NA
	2012	257-307	278.3	14.26	5.103	-2.822	0.753
Nectar Sucrose Content	2003	0-21	6.524	3.056	46.838	NA	NA
	2012	0-22	6.805	2.903	42.66	9.794	0.048**
Pollen Count	2003	3-508	191.5	59.25	30.925	NA	NA
	2012	2-493	200.8	49.92	24.855	24.42	0.016**

Table S2-6. Range, least squares mean, and standard deviation of floral trait values are shown for each year. Floral morphology traits are measured in centimeters, flowering dates use a Julian calendar, nectar sucrose content is measured as °Brix, and pollen count is a total number of pollen grains found on a single anther. Also shown is the phenotypic coefficient of variation (PCV) determined using a bootstrapped resampling procedure with 10000 draws, percent change of PCV from 2003 to 2012, and the p-value associated with a two-sided independent t-test comparing levels of phenotypic variation in 2003 to 2012 for each trait. Significance ($p<0.05$) is marked with a double asterisk** and relationships trending significant ($0.05 < p < 0.1$) are marked with a single asterisk*.

	CW:CL		CW:ASD		CL:ASD		CW:FF		CL:FF		ASD:FF	
	2003	2012	2003	2012	2003	2012	2003	2012	2003	2012	2003	2012
Correlation Coefficient (r)	0.611	0.596	0.035	-0.030	-0.055	-0.129	-0.237	-0.008	-0.188	0.081	0.364	-0.174
Significance (p)	<2.2e-16**	<2.2e-16**	0.223	0.253	0.047**	3.681e-07**	0.245	0.970	0.359	0.695	0.068*	0.394

Table S2-7. Pearson's correlation coefficients and associated p-values for floral morphology traits and phenology. Brix and pollen count excluded due to low population sampling. Since floral traits and phenology are not paired data, correlation is calculated with population means, otherwise correlation coefficients and p-values are calculated from raw measurements. Repeating a Pearson's correlation test on floral traits using population means yields a higher correlation between floral traits. Abbreviations: CW = Corolla Width, CL = Corolla Length, ASD = Anther Stigma Distance, FF = date of first flower. Significance (p<0.05) is marked with a double asterisk** and relationships trending significant (0.05 < p < 0.1) are marked with a single asterisk*.
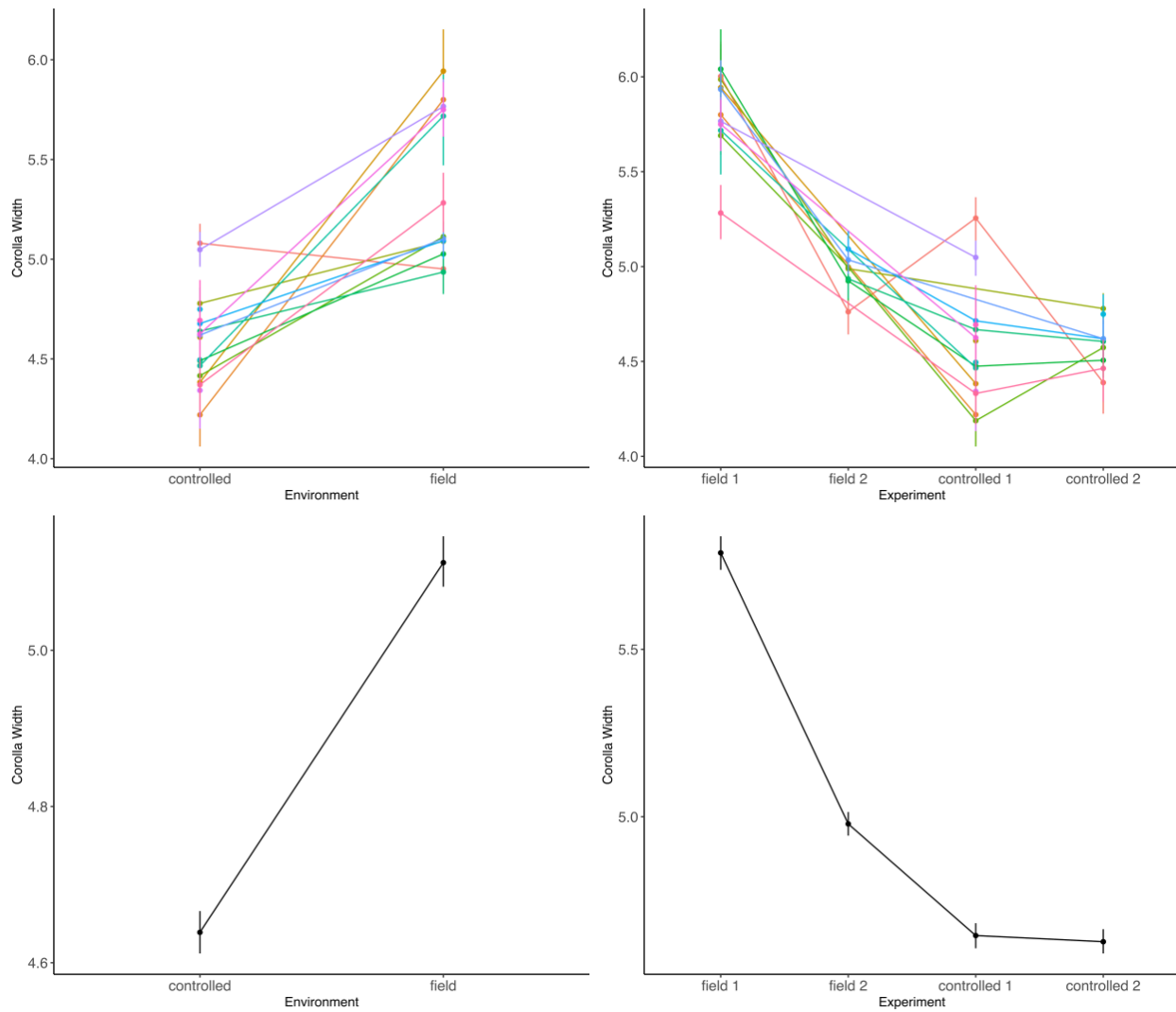
Figure S3-4. Average corolla width across four different experiments using the same populations showing significant plasticity in the trait. Two field experiments and two controlled-environment experiments in a greenhouse and growth room are compared. Different populations are displayed in color. Effect pooled across all populations is shown in black.

2003   **2012**
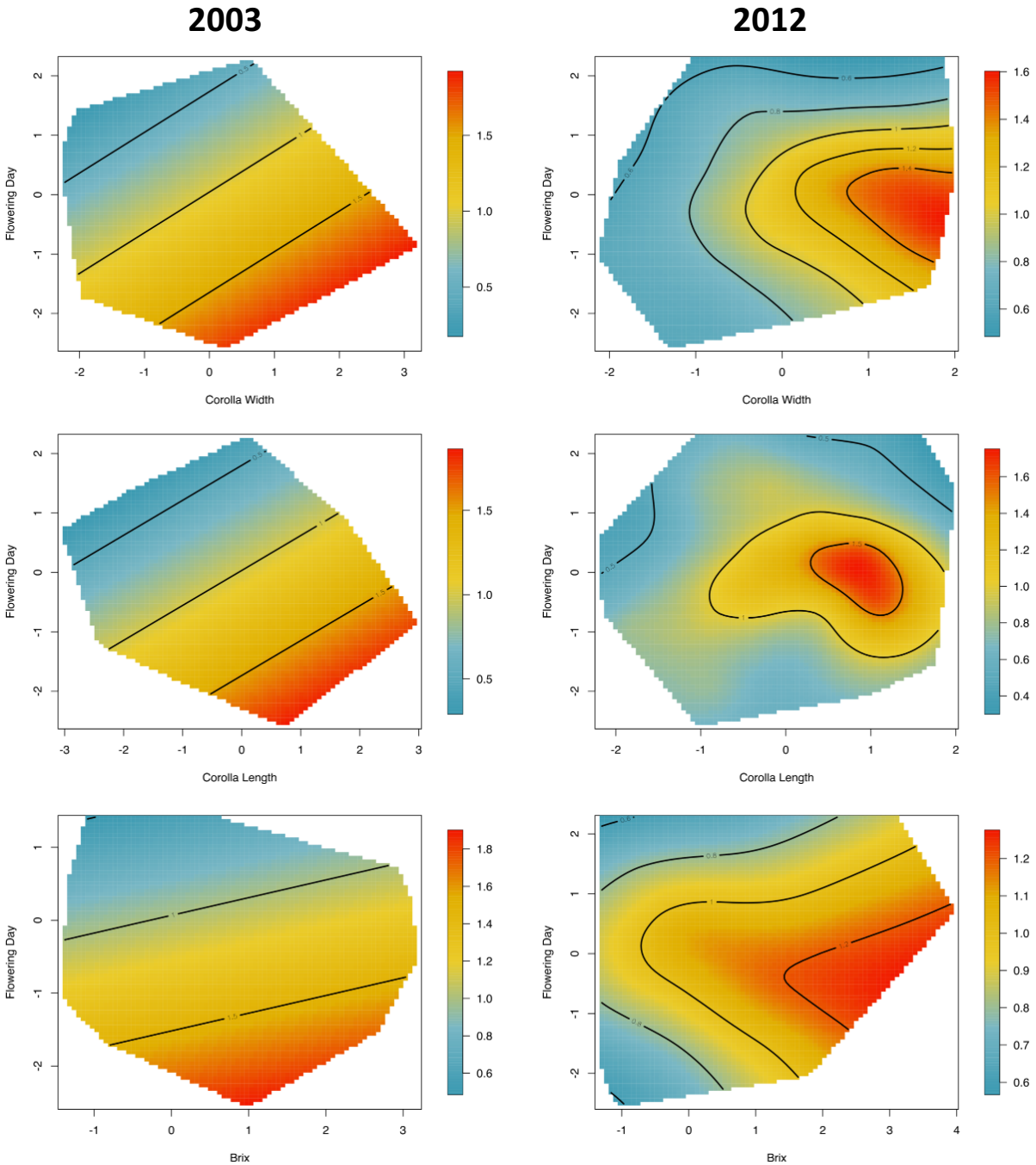
Figure S3-5. Fitness contour plots for correlative selection in 2003 and 2012 acting upon date of first flower and three other floral traits tested using selection gradients including all linear, Quadratic, and interacting terms (multivariate selection). Relative fitness is depicted by the color gradient with red being the highest fitness, yellow intermediate fitness, and blue lowest fitness. In 2003, no indication of correlative selection was present. All traits were included in the selection gradient, only those with selection gradients indicative of correlated selection in 2012 are shown: corolla width ($\gamma = -0.34$, p = 0.071), corolla length ($\gamma = 0.39$, p = 0.045), and °Brix ($\gamma = -0.14$, p = 0.067). This plot contains the same data as Figure 2-4B, but displayed as contours instead of surfaces.

	2003			2012			2016		
	df	F-value	p-value	df	F-value	p-value	numDF	F-value	p-value
Corolla Width	36, 1010	6.5	<0.0001	42, 1172	5.98	<0.0001	15, 261	1.76	0.04
Corolla Length	36, 1008	6.17	<0.0001	42, 1169	3.55	<0.0001	15, 261	1.38	0.15
ASD	36, 993	3.08	<0.0001	42, 1158	4.41	<0.0001	15, 255	3.03	0.0002
Brix	36, 738	3.38	<0.0001	42, 930	3.67	<0.0001	15, 145	2.45	0.003
Date of First Flower	1, 381	5.27	0.02	1, 417	11.17	0.001	1, 113	1.43	0.23
Date of First Flower (wave 1)	1, 191	2.67	0.1	1, 226	6.86	0.01	1, 53	4.18	0.046
Date of First Flower (wave 2)	1, 184	11.22	0.001	1, 190	3.33	0.07	1, 58	1.57	0.22

Table S3-8. Maternal line variation present for each trait in 2003, 2012, and 2016 tested for by a linear mixed model: Trait ~ Pop:ML + (1|Block) per year. Reported is the nested effect of maternal line within population.

		Corolla Width	Corolla Length	ASD	Brix	Date of First Flower (wave 1)	Date of First Flower (wave 2)	Date of First Flower (full)
Mean	2003	47.2[a]	52.7[a]	0.44[a]	3[a]	213.5[a]	222.4[a]	218[a]
	2012	48.1[a] 48.2[b]	53[a] 52.4[b]	0.59[a] 0.74[b]	3[a] 2.72[b]	212.7[a] 213.1[b]	222.6[a] 222.3[b]	217.3[a] 217.8[b]
	2016	48.4[b]	54.0[b]	0.30[b]	3.13[b]	213.9[b]	222.3[b]	218.3[b]
Standard Error	2003	3.03[a]	3.17[a]	0.73[a]	0.61[a]	0.20[a]	0.15[a]	0.49[a]
	2012	3.02[a] 3.35[b]	3.17[a] 3.17[b]	0.73[a] 0.65[b]	0.61[a] 0.61[b]	0.20[a] 0.39[b]	0.14[a] 0.28[b]	0.48[a] 0.41[b]
	2016	3.37[b]	3.18[b]	0.66[b]	0.62[b]	0.36[b]	0.28[b]	0.43[b]
t-ratio	2003-2012	-2.53	-0.85	-1.08	-0.02	3.07	-1.51	1.78
	2012-2016	-0.34	-2.84	2.05	-1.74	-1.54	0.34	-0.99
Degrees Freedom	2003-2012	1888	1885	1860	1432	363	320	799
	2012-2016	681	681	671	477	116	125	257
p-value	2003-2012	0.01	0.40	0.28	0.98	0.002	0.13	0.074
	2012-2016	0.73	0.005	0.04	0.08	0.13	0.73	0.32

Table S3-9. Phenotypic change from 2003 to 2012 and 2012 to 2016 using estimated marginal means. Significance was assessed with trait data standardized to a mean of 0 and a standard deviation of one, however effect sizes here are shown with non-transformed data such that the scale of change is maintained. Values marked with an "a" include all seven populations, values marked with a "b" include a subset of two populations for comparability to 2016. Significant differences of trait means between years is shown with a t-ratio and p-value.

Trait		2003			2012			F-values from ANCOVA
		S	SE	p-value	S	SE	p-value	
Corolla Width	Linear	0.11	0.04	0.001	0.12	0.03	<0.0001	0.27
	Quadratic	-.04	0.02	0.08	0.02	0.02	0.20	4.59*
Corolla Length	Linear	0.12	0.04	0.003	0.05	0.03	0.06	1.83
	Quadratic	-0.02	0.02	0.30	-0.01	0.02	0.56	0.28
ASD	Linear	0.03	0.04	0.53	0.03	0.02	0.24	0.97
	Quadratic	-1e-04	0.01	0.99	-0.007	0.004	0.066	0.67
Brix	Linear	-0.03	0.05	0.519	0.05	0.03	0.122	0.04
	Quadratic	0.06	0.03	0.022	0.003	0.02	0.902	3.40~
Date of First Flower (wave 1	Linear	-0.25	0.12	0.048	0.28	0.09	0.003	8.65**
	Quadratic	-0.04	0.07	0.62	0.07	0.04	0.093	1.79
Date of First Flower (wave 2	Linear	-12.24	10.26	0.236	-32.97	12.16	0.008	1.24
	Quadratic	0.03	0.02	0.238	0.07	0.03	0.008	1.20
Date of First Flower (full	Linear	-0.16	0.08	0.033	0.06	0.06	0.345	5.39*
	Quadratic	0.03	0.09	0.763	-0.10	0.05	0.053	1.18

Table S3-10. Total linear and nonlinear selection (S) on floral traits (corolla width, corolla length, ASD, °brix, and date of first flower) in 2003 and 2012. Shown are selection differential values, standard errors, and p-values for traits in each year. The F-value indicates the year by trait interaction from an ANCOVA, and significant effects are indicated with an asterisk.

Trait		2003			2012			F-values from ANCOVA
		β	SE	p-value	β	SE	p-value	
Corolla Width	Linear	0.32	0.25	0.197	0.45	0.16	0.007	0.63
	Quadratic	-0.37	0.45	0.408	0.44	0.26	0.089	0.53
Corolla Length	Linear	-0.11	-.25	0.672	-0.37	0.17	0.03	2.22
	Quadratic	-0.39	0.44	0.379	-0.22	0.24	0.352	1.92
ASD	Linear	0.08	0.12	0.458	-0.004	0.06	0.954	0.48
	Quadratic	-0.07	0.07	0.358	-0.04	0.04	0.317	0.40
Brix	Linear	-0.15	0.13	0.225	0.01	0.09	0.884	0.01
	Quadratic	0.22	0.08	0.012	0.10	0.06	0.089	1.52
Date of First Flower (wave 1	Linear	-0.37	0.29	0.198	0.22	0.09	0.026	3.32~
	Quadratic	-0.03	0.12	0.814	0.02	0.06	0.670	0.16
Date of First Flower (wave 2	Linear	-0.13	0.28	0.649	-0.44	0.19	0.024	0.07
	Quadratic	0.27	0.37	0.475	0.09	0.08	0.263	1.27
Date of First Flower (full	Linear	-0.26	0.13	0.042	-0.05	0.06	0.389	9.08**
	Quadratic	0.10	0.14	0.474	-0.13	0.04	0.005	2.83~

Table S3-11. Direct (multivariate) selection acting on floral traits corolla width, corolla length, ASD, °brix, and date of first flower in 2003 and 2012. Shown are linear and quadratic gradient values, standard errors, and p-values for each year. F-values are from the ANCOVA analysis testing the effect of year on selection gradients. Significant effects are indicated with an asterisk.

		Year	Exp	Pop	Year:Exp	Year:Pop	Exp:Pop	Year:Exp:Pop
Corolla Width	F-value	3.19~	4.08*	4.26*	4.05*	4.29*	6.07*	5.98*
	df_{num}, df_{den}	1, 3333	1, 3333	1, 3333	1, 3333	1, 3333	1, 3336	1, 3336
Corolla Length	F-value	5.99*	7.38**	5.55*	7.45**	5.57*	9.66**	9.58**
	df_{num}, df_{den}	1, 3324	1, 3324	1, 3324	1, 3324	1, 3324	1, 3331	1, 3331
ASD	F-value	0.004	0.005	0.601	0.009	0.593	0.787	0.758
	df_{num}, df_{den}	1, 3300	1, 3300	1, 3300	1, 3300	1, 3300	1, 3289	1, 3289
Brix	F-value	1.04	0.60	5.26***	0.60	5.34***	1.87~	1.87
	df_{num}, df_{den}	1, 2793	1, 2793	1, 2793	1, 2793	1, 2792	1, 2791	1, 2791
Day of First Flower	F-value	6.73**	8.62**	4.99*	7.14**	5.02*	0.11	0.11
	df_{num}, df_{den}	1, 1121	1, 1121	1, 1121	1, 1121	1, 1121	1, 1120	1, 1120

Table S3-12. Phenotypic plasticity assessed using linear models with the interactions of year, population and experiment as fixed effects and within-garden block as a random effect. F-statistics from a type III ANOVA are marked with an asterisk for p<0.05, two asterisks for p<0.01, and three asterisks for p<0.001. Significant experiment or experiment x population effects indicate a significant plastic response in at least some populations. A significant year x experiment effect indicates a difference in plasticity between years.

	Year	Mean difference in trait value from field to growth room	Absolute difference in plastic response from 2003 to 2012	Proportion of simulations with a different sign (+/-) from the difference in plastic response
Corolla Width	2003	4.11	NA	NA
	2012	3.09	1.02	0.045
Corolla Length	2003	5.34	NA	NA
	2012	4.06	1.28	0.011
ASD	2003	2.12	NA	NA
	2012	1.63	0.49	0.265
Nectar Sucrose Content (°Brix)	2003	0.63	NA	NA
	2012	0.31	0.32	0.049
Date of First Flower	2003	-10.44	NA	NA
	2012	-10.16	0.28	0.291

Table S3-13. Phenotypic plasticity represented as the difference in average trait values between field and growth room resurrection experiments using the same populations and maternal lines. A positive absolute difference in plastic response from 2003 to 2012 indicates that plasticity is decreasing from 2003 to 2012 (i.e. the change in trait value from field to growth room in 2003 is greater than in 2012). Significance of the increase or decrease in plasticity from 2003 to 2012 is shown as the proportion of bootstrap resampled simulations with the same sign as the absolute difference in plastic response.
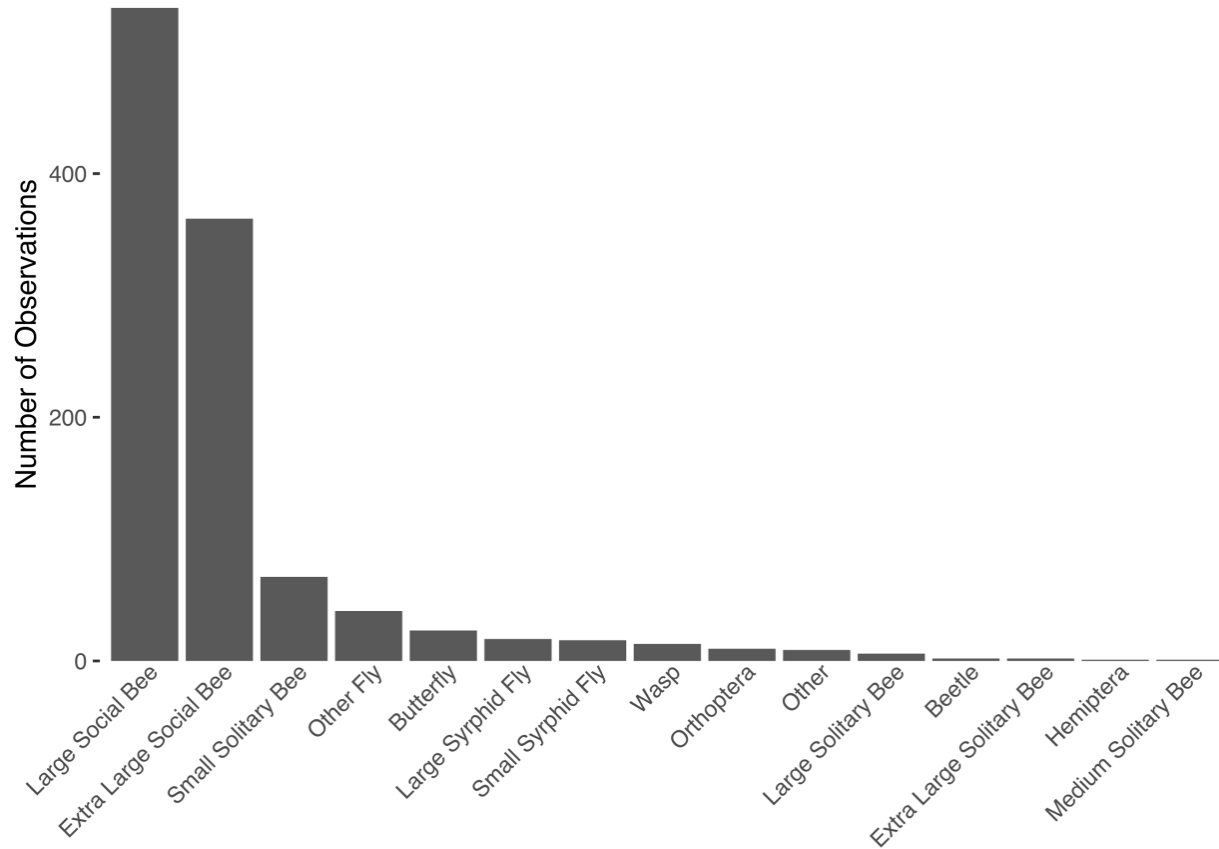
Figure S4-6. Number of observed visitations (approach + forage) for each category of pollinators. There was a total of 1,1116 visitations observed, the vast majority of which were bumblebees falling into the category of either large or extra large social bees.
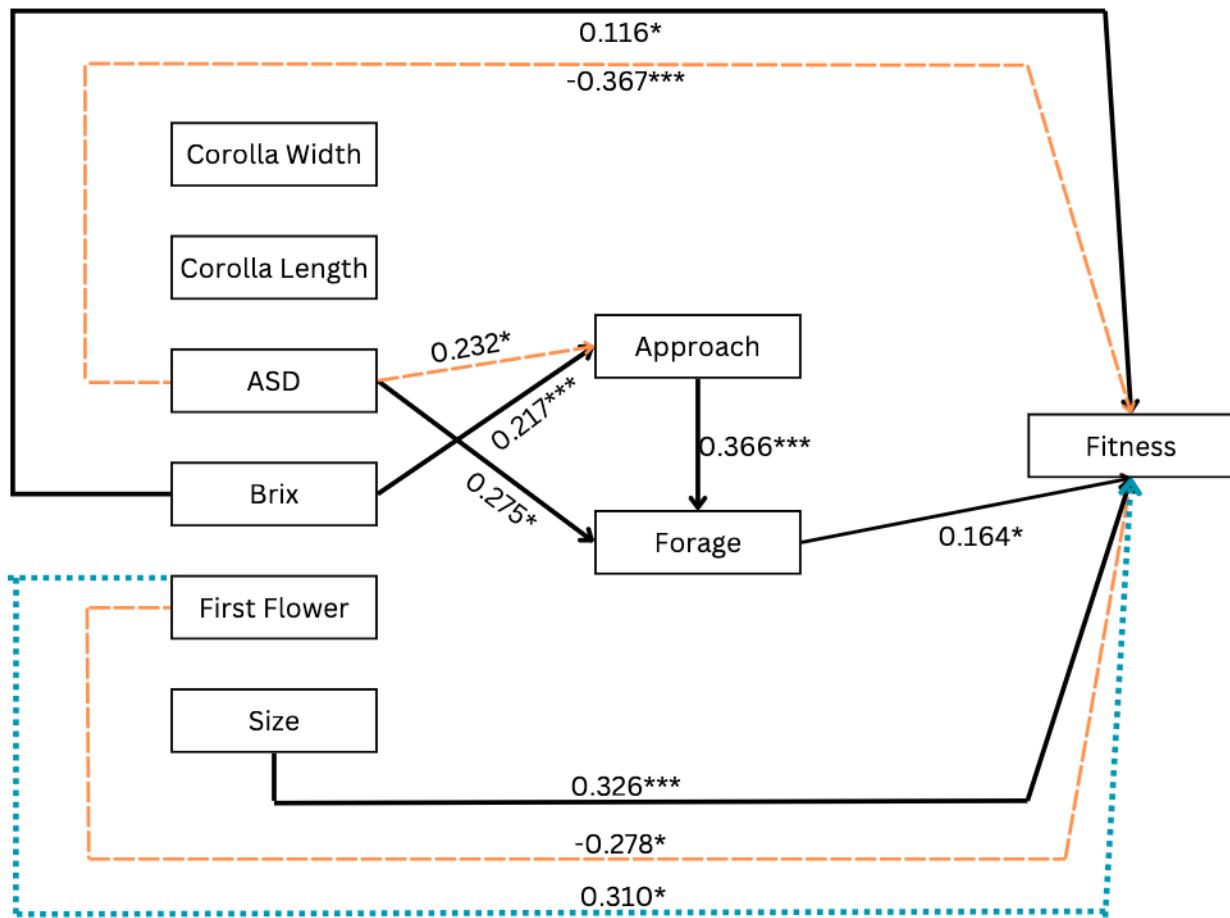
Figure S4-7. SEM for model B with extra-large social bees removed, showing the direct and indirect effects determining pollinator visitation rate and fitness. Effects that are conserved in both direction and magnitude between 2003 and 2012 are indicated by solid, black lines. Relationships that only exist in 2003 are shown with a dashed orange line whereas relationships that are only present in 2012 are shown with a dashed blue line. Standardized path coefficients are shown for each significant path where * indicates a p-value <0.05, ** indicates p < 0.01, and *** indicates p < 0.001.

Trait	Year	Approach	Forage	Fitness		
		DE	DE	DE	IE	TE
Corolla Width	2003	0.242	0.286	NA	0.065	0.065
	2012	0.265	0.296	0.434	NA (0.043)	0.434 (0.477)
Corolla Length	2003	NA	NA	NA	NA	NA
	2012	NA	NA	-0.792	NA	-0.792
ASD	2003	NA	0.213	NA	0.045	0.045
	2012	NA	0.269	NA	NA (0.028)	NA (0.028)
Brix	2003	0.149	NA	NA	0.014	0.014
	2012	0.218	NA	NA	NA(0.008)	NA (0.008)
Date of First Flower	2003	NA	NA	-0.321	NA	-0.321
	2012	NA	NA	NA	NA	NA
Leaf Count	2003	NA	NA	0.256	NA	0.256
	2012	NA	NA	0.210	NA	0.210
Approach	2003	NA	0.366	NA	0.063	0.063
	2012	NA	0.346	NA	NA (0.036)	NA (0.036)
Forage	2003	NA	NA	0.173	NA	0.173
	2012	NA	NA	NA (0.104)	NA	NA (0.104)

Table S4-14. Direct, indirect, and total effects of floral characteristics and pollinator approach and forage frequency on plant fitness for Model B (Figure 4-2). Direct effects are standardized partial regression coefficients from the SEM. Indirect effects are the product of all direct effects along a given path. If multiple indirect paths between trait and fitness exist, coefficients of the independent paths are summed to get a total indirect effect on fitness. Total effects are the sum of indirect and direct effects, and non-significant paths are marked with NA. Coefficients in parentheses represent a predicted effect if the influence of carpenter bees is removed where the magnitude of indirect effects are calculated by multiplying the standardized coefficient between trait and pollinator behavior from the full model by the standardized coefficient for the effect of foraging rate on fitness for a model with carpenter bees removed (see supplemental Figure 4-2 for full path coefficients of this model alone).

A)

Parameter	2003			2012		
	Coefficient	Median Coefficient	Power	Coefficient	Median Coefficient	Power
relFit ~ Forage	0.17	0.17	1	-0.12	-0.12	0.99
relFit ~ Approach	0.07	0.07	0.16	0.08	0.08	0.18
relFit ~ CWresid	-0.3	-0.3	1	0.45	0.45	1
relFit ~ CLresid	0.19	0.19	1	-0.8	-0.8	1
relFit ~ ASDresid	0.06	0.05	0.13	0.08	0.08	0.31
relFit ~ Bresid	0.07	0.07	0.19	0.11	0.11	0.63
relFit ~ FFresid	-0.32	-0.32	1	0.13	0.13	1
relFit ~ LCresid	0.26	0.26	1	0.21	0.21	1
Approach ~ CWresid	0.24	0.24	1	0.26	0.26	1
Approach ~ CLresid	-0.06	-0.06	0.81	-0.06	-0.06	0.71
Approach ~ ASDresid	0.04	0.04	0.09	0.05	0.05	0.16
Approach ~ Bresid	0.15	0.15	0.58	0.22	0.22	0.99
Approach ~ FFresid	0.2	0.2	1	-0.14	-0.14	1
Approach ~ LCresid	0.12	0.12	1	0.09	0.09	1
Forage ~ CWresid	0.29	0.29	1	0.3	0.3	0.98
Forage ~ CLresid	-0.09	-0.09	0.38	-0.08	-0.08	0.26
Forage ~ ASDresid	0.21	0.22	0.2	0.27	0.27	0.38
Forage ~ Bresid	0.01	0	0.06	0.01	0.01	0.06
Forage ~ FFresid	0.21	0.21	0.93	-0.12	-0.12	0.54
Forage ~ Approach	0.37	0.36	0.87	0.35	0.34	0.52

B)

	2003			2012		
Parameter	**Coefficient**	**Median Coefficient**	**Power**	**Coefficient**	**Median Coefficient**	**Power**
relFit ~ Forage	0.16	0.16	0.99	0.1	0.1	0.39
relFit ~ Approach	0.14	0.14	0.25	0.13	0.12	0.18
relFit ~ CWresid	-0.11	-0.11	0.99	-0.11	-0.11	0.99
relFit ~ CLresid	-0.2	-0.2	1	-0.2	-0.2	1
relFit ~ ASDresid	-0.37	-0.37	0.98	0.09	0.09	0.23
relFit ~ Bresid	0.12	0.11	0.33	0.17	0.17	0.68
relFit ~ FFresid	-0.28	-0.28	1	0.31	0.31	1
relFit ~ LCresid	0.33	0.33	1	0.18	0.18	1
Approach ~ CWresid	0.02	0.02	0.23	0.03	0.03	0.28
Approach ~ CLresid	0.05	0.05	0.66	0.06	0.06	0.71
Approach ~ ASDresid	0.23	0.24	0.87	-0.12	-0.12	0.56
Approach ~ Bresid	0.22	0.22	0.91	0.34	0.34	1
Approach ~ FFresid	0.09	0.09	0.99	0.05	0.05	0.8
Approach ~ LCresid	0.13	0.13	1	0.15	0.15	1
Forage ~ CWresid	-0.02	-0.03	0.08	-0.04	-0.04	0.17
Forage ~ CLresid	0.05	0.05	0.13	0.08	0.08	0.41
Forage ~ ASDresid	0.27	0.28	0.24	0.57	0.57	1
Forage ~ Bresid	-0.06	-0.06	0.08	-0.14	-0.14	0.33
Forage ~ FFresid	0.23	0.23	0.96	-0.18	-0.18	1
Forage ~ Approach	0.14	0.14	0.23	0.2	0.2	0.45

Table S4-15. Power analysis: A) 2003 and 2012 for the full dataset and B) 2003 and 2012 with extra-large social bees removed. Coefficient is the estimated coefficient from the SEM, Median Coefficient is the median coefficient estimate of 5000 simulations, and power is the percent of 5000 simulations with a parameter estimate not significantly different than the coefficient provided ($p < 0.05$). Yellow highlight indicates where lower power and lack of a significant signal coincide, indicating that there may be a significant relationship, but I lack the power to detect it.