

Selection of floral traits by pollinators and seed predators during sequential life history stages

Diane R. Campbell^{1,2}, Mascha Bischoff², Robert A. Raguso³, Heather M. Briggs^{1,2}, Paula Sosenski^{1,2,4}

1. Department of Ecology and Evolutionary Biology, University of California, Irvine, California 92617; 2. Rocky Mountain Biological Laboratory, Crested Butte, Colorado 81224; 3. Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853; 4. CONACYT- Universidad Autónoma de Yucatan, Mérida, Mexico.

Keywords: correlational selection, floral scent, pollinator, seed predator, stabilizing selection, volatile organic compound

Number of words in text: 7905

Tables 1-3, figures 1-4

Expanded online addition: Table S1, Table S2, Table S3, Figure S1, Figure S2, Figure S3, Figure S4

Type: Article for focused topic

ABSTRACT: Organismal traits often influence fitness via interactions with multiple species.

That selection is not necessarily predictable from pairwise interactions, such as when interactions occur during different lifecycle stages. Theoretically, directional selection during two sequential episodes, e.g., pollination and seed survival, can generate quadratic or correlational selection for a set of traits that passes both selective filters. We compared strength of selection during pollination versus seed predation in the field and tested whether interactions with multiple species give rise to non-linear selection on floral traits. We planted common gardens with seeds of two species of *Ipomopsis* and hybrids in sites where pollination was primarily by hummingbirds or also included hawkmoths. We examined selection on six floral traits, including corolla width, sepal width, color, nectar, and two scent compounds. Female fitness (seeds) was broken down into fitness during (1) pollination (seeds initiated) and (2) seed predation (proportion seeds escaping fly predation). All traits showed evidence of selection. Directional and quadratic selection were stronger during seed initiation than seed predation. Correlational selection occurred mostly during seed initiation rather than arising from combining species interactions at two points in the lifecycle. These results underscore how multispecies interactions can combine to exert selection on trait combinations.

Introduction

Organisms often interact with multiple groups of other species, each of which can determine how traits influence fitness. Whereas most studies of natural selection in the wild focus on one kind of interaction, such as pollination or herbivory, traits can evolve in response to multiple interactions (Strauss and Whittall 2006). Moreover, selection due to interactions with multiple species is not necessarily predictable from examining pairwise interactions. A change in the pattern of selection exerted on one species by another when in the presence of a third species has been viewed as a signature of diffuse coevolution (Iwao and Rausher 1997). Several examples exist of such interactive effects, but typically these involve species interactions of one type, such as interactions with multiple species of herbivores (Juenger and Bergelson 1998; Lankau and Strauss 2008). Different species interactions often occur during different phases in the lifecycle, and theory suggests that multiple episodes of directional selection may give rise to new patterns of selection (McGlothlin 2010). If directional selection acts only on one trait early in the lifecycle and only on a second trait later in the lifecycle, the net effect can be correlational selection, defined as selection in which there are interactions (synergistic or antagonistic) between effects of traits on fitness, such that a combination of trait values is favored (Phillips and Arnold 1989). Whether this result generally holds in the wild is not known, as it is rare for investigators to measure non-linear selection at multiple points in the life cycle (McGlothlin 2010, but see Crean et al. 2010 for a study of a marine invertebrate). As a theoretical example, suppose flowers that are more nectar-rich receive more pollinator visits and initiate more seeds than flowers that are nectar-poor. Then suppose that during later seed maturation, low scent emission protects more of those seeds from consumption by seed predators, thereby enhancing the likelihood that a mature seed will be released. In this case, the net effect of pollinators and

predators on fitness would be non-additive, with weak scent increasing the overall number of viable seeds released by the plant more so for nectar-rich flowers. This correlational selection occurs because the first episode of selection (due to pollinators) changes the phenotypic variance-covariance matrix (Wade and Kalisz 1989), so that only nectar-rich flowers initiate seeds to offer seed predators, and the trait combination of nectar-rich, weakly scented flowers is the only one passing both selective filters. Thus plants that express the combination of traits enhancing pollination and escape from seed predation would have highest fitness (Herrera et al. 2002). In another scenario, if both organisms, such as pollinator and seed predator, select on the same trait but in the opposite direction, the combined result can be stabilizing selection for a compromise intermediate trait value, as seen for bract size in *Dalechampia* blossoms (Pérez-Barrales et al. 2013). Although these examples are couched in terms of floral traits that influence pollinators and seed predators, the same arguments would apply to other kinds of traits that have effects at multiple points in a lifecycle.

Whereas pollinators have long been considered to be the primary selective agents on floral traits (Darwin 1862; Harder and Johnson 2009), there is increasing recognition that natural enemies, including herbivores, florivores, seed predators, and nectar and pollen thieves, can also shape the evolution of floral traits, including color, size, and morphology (Strauss and Whittall 2006). In several examples, enemies respond to the same floral traits as pollinators (e.g. Galen and Cuba 2001; Frey 2004; Sletvold et al. 2015) and can even exert stronger selection on them, presumably because pollinator-mediated selection through female function can be diluted by resource limitation of seed set, whereas seed predators directly reduce female fitness (Cariveau et al. 2004). A recent meta-analysis revealed that pollinators on average exert stronger directional selection than do other biotic agents, including herbivores, on floral traits (Caruso et al. 2019),

but did not address whether that is also true for other forms of selection such as stabilizing, disruptive and correlational selection. Moreover, most studies of conflicting selection by pollinators and herbivores have evaluated floral color or morphology without considering how floral scent might respond to complex selective pressures (Kessler et al. 2013; Knauer and Schiestl 2017). Historical neglect of selection on floral scent is problematic because it cannot be assumed to respond to selection in the same way, given that scent bouquets are produced by independent biosynthetic pathways (Dudareva et al. 2004), and compounds can be emitted by flowers and leaves. Furthermore, the full spectrum of plant visitors, including at times herbivores and pathogens, can respond to floral scent compounds (Ramos and Schiestl 2019; Theis and Adler 2012). While at least one recent study has documented selection by pollinators and enemies on floral scent (Chapurlat et al. 2019), none have measured quadratic or correlational selection. So it remains unclear how selection at multiple points in the lifecycle shapes selection on fully integrated floral phenotypes.

Here we test how pollinators and seed predators combine to shape non-linear selection on scent as well as other floral traits. We take advantage of a well-studied system of *Ipomopsis* plants, including a wealth of previous experimental work over three decades demonstrating directional selection on a set of traits with demonstrated ecological functions (e.g., Campbell 1989, Campbell et al. 1996, Campbell and Powers 2015). This background information allows us to make predictions about how certain specific combinations of traits should be shaped by conflicting selection in the wild, which is not generally possible with most other systems. We also capitalize on common garden experiments planted in the field with artificially generated hybrids of *Ipomopsis* plants to expand the range of trait variation and make detection of selection more powerful. Using nine years of data, we address two general questions. 1. How do patterns

of selection (directional, quadratic, and correlational) on floral traits compare during pollination versus seed predation? The floral traits included aspects of shape, color, rewards, and scent, and we sought to determine whether (a) non-linear selection is stronger by pollinators and (b) floral scent compounds experience non-linear as well as directional selection in the wild. 2. Does combining the influence of species interactions at two points in the lifecycle generate quadratic or correlational selection not seen during either single selective episode?

Methods

Study System and Experimental Field Gardens

We employed both field common gardens and natural populations of *Ipomopsis* (Polemoniaceae) at Poverty Gulch, Gunnison County, CO, USA, where a natural hybrid zone occurs. Plants of *I. aggregata* ssp. *aggregata* (scarlet gilia) grow in the valley bottom at elevations up to around 2900 m, whereas plants of its close congener *I. tenuituba* ssp. *tenuituba* (slendertube skyrocket) grow on slopes above 3100 m, and natural hybrids are found at intermediate elevations (Campbell et al. 1997). Plants of both species are self-incompatible and monocarpic, almost always flowering during only one season (after 2-12+ years) before setting seed and then dying (Campbell et al. 2008). There is no seed bank (Campbell 1997).

The use of common gardens with second generation hybrids expanded the range of trait variation, increasing statistical power for detecting natural selection. The common gardens were started from seed in 2007 and 2008, as described elsewhere (see Campbell and Powers 2015). In 2007, we planted seeds consisting of *I. aggregata* (AA), *I. tenuituba* (TT), or hybrids (F_1 and F_2). Seeds were planted at three sites without removal of any existing vegetation: the *I. aggregata* site of parental origin (site L in Campbell et al. 1997), the *I. tenuituba* site (site C) and a site at the center of the natural hybrid zone (site I). In 2008, we planted additional seeds consisting of F_2

hybrids at the *I. aggregata* site. In this system, F₂ hybrids often reproduce in the wild, with no evidence for hybrid breakdown (Campbell et al. 2008). In the two years combined, we planted 4512 seeds, of which 718 emerged as seedlings. Seedlings typically emerge within 1 cm of the planted location (Campbell et al. 2008). Plants were censused for survival and flowering in each year following planting through 2018, with the first plants blooming in 2010. Floral traits were measured on the 192 plants that flowered by 2018 in the common gardens. An additional 15 plants survived from 2018 to 2019, but only 2 of those bloomed in 2019 and were not included in the dataset. The plants used in this study bloomed across nine years, including 27 AA, 16 TT, and 149 hybrids, although not every trait was measured in every year (see *Field Measurements of Traits and Fitness*). Starting in 2013, most plants at the *I. aggregata* and hybrid site gardens had bloomed and died, or died without blooming. So the gardens were supplemented with an additional 243 in situ plants over 2013-18 at those two sites, yielding a total of 435 flowering plants measured for traits and for female fitness.

At Poverty Gulch, both species of *Ipomopsis* are visited by Broad-tailed and Rufous hummingbirds (*Selasphorus platycercus* and *Selasphorus rufus*), hawkmoths (*Hyles lineata*), occasional swallowtail butterflies (Papilionidae), and rarely bees (Apoidea). Hummingbirds are more common visitors at the *I. aggregata* site than at the hybrid and *I. tenuituba* sites (Campbell et al. 1997). Hawkmoths are rarely seen at that low site, but are more common visitors at the hybrid and *I. tenuituba* sites (Aldridge and Campbell 2007; Campbell et al. 1997). Flies of the genus *Delia* sp. (Diptera: Anthomyiidae) are common pre-dispersal seed predators, with the percentage of fruits eaten ranging from 0 to nearly 80% across plants at these sites in a previous study (Campbell et al. 2002). A fly lays an egg (almost always a single egg) under the sepals of an elongated bud or flower. The larva typically consumes all of the seeds in the developing fruit

and exits as a pupa (Brody 1997). Occasional fruits are eaten by a moth caterpillar (*Heliothis* sp: Noctuidae), which is also a pre-dispersal seed predator (Juenger and Bergelson 1998).

Field Measurements of Traits and Fitness

We focused on six floral traits based on *a priori* evidence that they influence either pollinators or seed predators in *Ipomopsis*: corolla width, petal color, nectar production, sepal width, night time emission of indole (a nitrogenous volatile compound), and combined emission of α -pinene and β -pinene (monoterpene volatile compounds). Phenotypic manipulations of traits by modifying corollas, painting flowers and supplementing nectar demonstrated that plants with wider corollas, redder flowers and higher nectar production receive more hummingbird visits or higher pollination success in at least some years in this system (Campbell et al. 1991; Meléndez-Ackerman and Campbell 1998; Mitchell 1993). Plants with narrower corollas and higher nocturnal emission of indole receive more hawkmoth visits, with the effect of indole demonstrated through experimental supplementation of the compound (Bischoff et al. 2015; Campbell et al. 1997). Seed production is limited by low pollen transfer (Campbell 1991; Campbell and Halama 1993), and so lower pollen receipt associated with lower visitation is expected to lower seed production due to pollinator-mediated selection. Thus we identified four traits as under directional selection by pollinators that is not mediated by correlated traits: corolla width, petal color, nectar production rate, and indole emission. Wider corollas, along with wider sepals, are also associated with higher oviposition rate (Brody 1992; Campbell et al. 2002) by the seed predator *Delia* sp., and thus seed predators likely select for narrow corollas and narrow sepals. In addition to these five traits, we included a sixth trait, emission of pinene volatiles, for which selection has not yet been established. We did so because α -pinene is the largest constituent of volatile emissions by the flowers (Bischoff et al. 2014), and experimental

additions of pinene suggested that oviposition by *Delia* is influenced by levels of α - and β -pinene (Campbell and Bischoff, unpub.). This body of previous work on directional selection during one lifecycle stage at a time allowed us to make specific predictions about when correlational selection on a specific combination of two traits would be generated simply as a consequence of combining directional selection by pollinators and seed predators (Table 1A). As one example, when analyzing the combination of corolla width and sepal width, we predicted that the combination of directional selection by pollinators and seed predators would generate correlational selection for the combination of relatively wide corollas with narrow sepals because narrow sepals lower the chance that seeds from a successfully pollinated flower are eaten. In addition, we would predict stabilizing selection on corolla width, as wide corollas enhance pollination while narrow ones protect against seed predation.

Corolla width was measured with calipers as the diameter at the opening of the tube for two to ten flowers per plant. Petal color was measured for two to four flowers per plant using a reflectance spectrometer (methods in Campbell and Powers 2015). Redness of the flower was quantified as reflectance in the red region divided by reflectance in the green, as measured with an Ocean Optics Red Tide USB650 reflectance spectrometer with a LS-1 light source. Flowers of these species do not reflect in the ultraviolet (Meléndez-Ackerman 1997). Nectar production was measured over 48 hours and then converted to μL per 24 h for one to five flowers per plant (methods in Campbell and Powers 2015; Mitchell 1993). In a given year, all plants were measured on the same days, to the extent that flowers were available, to control for variation in water availability. These three traits were measured in all years of the study (2010-2018), which was originally designed and funded only to examine those traits. Sepal width was added for two

to four flowers per plant (methods in Campbell et al. 2002), in 2012, 2014, and 2015 . All four of these traits were averaged across the flowers on a plant prior to analysis.

The volatile organic compounds were sampled in 2011-2018 using dynamic headspace methods. As the methods are described in detail elsewhere (Bischoff et al. 2014; Campbell et al. 2019), we give only an overview here. We sampled volatiles on 236 plants during the day (0900 to 1200 hours) and 195 plants at night (2000 to 2300 hours). At the time of sampling, a single flower was enclosed in an oven bag, headspace volatiles were allowed to equilibrate for 30 minutes and then pumped through a scent trap containing Tenax TA as the trapping agent for 15 minutes (yielding a total sampling time of 45 minutes), using a micro air sampler to create vacuum (Campbell et al. 2019). For control, air samples were taken in the same way but enclosing only air inside the bag. All day-time samples from a given site and year were taken on the same date, as were all night-time samples.

The scent traps were analyzed using thermal desorption GC-MS. Samples from 2011-2016 were analyzed on a Shimadzu GCMS-QP2010 at Cornell University (Arguello et al. 2013) and samples from 2017-18 on a Shimadzu GC-MS QP2020 at the Rocky Mountain Biological Laboratory (Campbell et al. 2019). Scent traps were thermally desorbed directly inside the injection port liner at Cornell and through a two-stage process using a Markes Unity-xr with a Markes Ultra autoloading system at RMBL. For both instruments, the GC temperature ramp increased 10 deg C per minute to 250 deg C and then at 30 deg C to 275C, with a final hold for 3 minutes. We used an Rtx-5MS column, with 30 m s 0.25 mm internal diameter, 0.25 μ m film thickness.

Volatiles were identified using Shimadzu GCMSsolutions “Postrun Analysis” software with retention times for indole and the pinenes confirmed by running authentic standards. Peak

areas were obtained through automatic integration for α - and β -pinene. Indole emissions can be biologically important at very low levels (Bischoff et al. 2015), so we also checked each chromatogram manually with fragment searches for the top ions (117 m/z and 90 m/z), and when found integrated the peak manually. Peak areas were converted to ng per hour by running authentic standards for α -pinene and indole at 4 dosages with 3-4 replicates per dosage and then also multiplying by 4/3 to account for a sampling interval of 45 minutes. We ran standards separately on the Cornell instrument and on the RMBL instrument in each year of analysis to account for potential variation over time in transfer efficiency from the Markes Unity-xr. Because indole is only emitted by *I. tenuituba* and hybrids, we did not analyze indole for plants from the natural populations at the *I. aggregata* site. For analyses involving pinene, we used the total amount of α - and β -pinene emitted by the plant averaged across the day and night sample. Emissions of these two compounds are highly correlated ($r = 0.90$, $P < 0.0001$, $N = 203$ plants), so we did not consider it possible to separate their influence. Plants analyzed for this study emitted an average of 112 ng of α - and β -pinene and 0.91 ng of indole per flower per hour.

For each plant, we obtained estimates of female fitness as number of viable seeds. On average, plants produced 56 flowers and 75 total seeds. Every 2 to 4 days we collected fruits just prior to dehiscence and the calyces from flowers that failed to make a fruit (i.e. aborted). Seed production by fruits that had already dehisced and spilled their seeds (4% of cases) was estimated as the average seeds per intact fruit for that plant. Fruits with a fly larva inside were assumed to make zero viable seeds, as were fruits with the much less common caterpillar damage to the calyces and ovary (fly or caterpillar damage occurred in 9% of all cases or 24% of the fruits). Flowers collected early for measurement (17%) were given values for seed production equal to the average for flowers that had been allowed to set seed on the plant. We divided

overall fitness (number of viable seeds produced by a plant) into two multiplicative components that represent fitness during pollination (number of seeds initiated whether eaten or not) and fitness during seed predation (proportion of fruits not attacked by the fly or caterpillar). This approach of dividing fitness is similar to an approach used to incorporate different trophic levels (Abdala-Roberts et al. 2014). We recognize that processes other than pollination also contribute to number of seeds initiated, however there is evidence for a strong link in this system. In *I. aggregata*, pollen receipt on stigmas increases with pollinator visitation rate (Engel and Irwin 2003; Price et al. 2005), seeds initiated increases with estimated pollen receipt on stigmas (Campbell 1991), and seed set is highly pollen-limited (Campbell 1991; Campbell and Halama 1993). Seeds initiated was determined by multiplying (a) total flowers produced by the plant by (b) fruits per flower (whether eaten or not) by (c) seeds per fruit for non-eaten fruits.

Statistical Estimation of Selection

Measurement of natural selection relied on multiple regression methods in which relative fitness is regressed on standardized trait values (Lande and Arnold 1983). To estimate selection gradients, we first standardized trait values to a mean of zero and standard deviation of one. Total fitness (seeds produced) and the two components (seeds initiated and proportion escaping seed predation) were each relativized by dividing by the mean and were analyzed separately. The study was originally setup only to measure selection on three traits, and logistical constraints requiring backpacking to remote sites for volatile sampling meant that we were unable to measure all six traits for many plants, generating missing data. To address question 1 about the overall strength of selection during pollination versus seed predation, we mainly used analyses with all six traits following multiple imputation of the missing data. To address question 2 about how selection across two lifecycle stages combines to produce non-linear selection, we tested

specific predictions about trait combinations using pairwise analyses of two (or three) traits at a time, allowing us to proceed with no missing data. Further rationale is provided below.

Question 1. To compare selection across lifecycle stages, using analysis of all six traits at once, we employed multiple imputation methods to infer missing data. Multiple imputation imputes missing values many times to create multiple partially imputed data sets. The analysis is then run on each imputed data set, and the results combined to obtain unbiased estimates and standard errors that properly reflect the uncertainty due to missing values (Newman 2014). We performed partial imputation of the data set five times, using procedure MI in SAS v9.3 and a model with all relative fitness components, all six standardized traits, and the auxiliary variables of site and type of plant (Newman 2014). To estimate directional selection, we then ran multiple regression of relative fitness on the six traits for each imputation and combined the partial imputations using procedure MIANALYZE in SAS. All analyses used Gaussian distributions, as least squares methods are robust to minor departures from normality, and data sets were not zero-inflated or multimodal (Figure S1).

For analysis of non-linear multivariate selection, an inherent difficulty is that inclusion of many traits quickly leads to a model with many parameters. The standard second-order polynomial regression model for measuring phenotypic selection is:

$$w = \alpha + \sum_{i=1}^n \beta_i X_i + \sum_{i=1}^n \frac{1}{2} \gamma_{ii} X_i^2 + \sum_{i=1}^n \sum_{j>i}^n \gamma_{ij} X_i X_j + \epsilon \quad \boxed{\text{Eqn. 1}}$$

where w = relative fitness, X_i are individual standardized traits, β_i are directional selection gradients, the quadratic terms γ_{ii} represent stabilizing (negative values) or disruptive selection (positive values), and the cross-product terms γ_{ij} represent correlational selection. (Phillips and Arnold 1989). Inclusion of six traits generates 27 parameters including linear and quadratic

coefficients for each trait and a cross-product term for each pair of traits, greatly diminishing statistical power. We approached that limitation in two ways. First, we employed a canonical analysis of the quadratic and cross-product terms (Phillips and Arnold 1989; Schluter and Nychka 1994; Blows and Brooks 2003). That procedure reduces the six dimensional trait space to two eigenvectors and is a more powerful way of detecting non-linear selection (Blows and Brooks 2003). We used procedure RSREG in SAS with the NOCODE option as variables were already standardized (Blows and Brooks 2003) to generate the canonical axes for each partially imputed data set. We then estimated the eigenvector coefficients for the two axes with largest absolute values for eigenvalues by averaging across imputations (Newman 2014). To test for significance of non-linear selection along these axes, we used those new canonical axes as traits, ran RSREG to obtain regression coefficients on them and combined results from multiple partial imputations with MIANALYZE.

Our second approach to the power limitation was to perform pairwise analyses of two traits at a time described below under “Question 2”, so that we could understand patterns of non-linear selection on specific trait combinations while retaining high statistical power. These analyses also allowed us to test explicitly for non-linear selection on floral volatile emissions. Question 2. Here our intent was to understand mechanistically how selection patterns during two lifecycle stages combine to generate patterns in overall fitness. We did so by analyzing fitness as a second order polynomial on each unique pair of traits, so that we could visualize selection on specific trait combinations at two life cycle stages combined. This procedure offered the advantage of not requiring imputation of missing trait values, and it avoided the power limitations inherent in the full model that caused us to reduce the six traits to canonical axes. Analyzing two traits at a time was further justified by the weak correlations between almost all

traits (absolute values of r ranged from 0.07 to 0.31 (Table S1). To our knowledge, no previous study has measured non-linear selection at multiple life history stages for a large number of traits simultaneously. As we have larger sample sizes for corolla width, petal color, and nectar production, for those traits only analyses were also performed separately for the (a) *I. aggregata* site (hummingbird dominant) and (b) the hybrid and *I. tenuituba* sites (hummingbirds and hawkmoths; hereafter referred to as the upper site for simplicity). To achieve sample sizes of approximately 100 plants or greater, for combinations involving volatile traits or sepal width, we analyzed data from the sites combined, after finding no statistical evidence for trait by site interactions on total seeds (ANCOVA on each trait, $P = 0.48, 0.62$, and 0.17). Analyses employed the GLM procedure in SAS to run multiple regression models with linear, quadratic and cross-product terms. The quadratic regression coefficients were then multiplied by 2 to obtain the quadratic selection gradients (Stinchcombe et al. 2008). For each quadratic gradient γ_{ii} we had five estimates and pooled those using inverse variance meta-analysis with function `rma` in the R package `metafor`. Since the five estimates for a given trait were not independent, we attributed statistical significance to the meta estimate only if $P < 0.01$ as in a Bonferroni correction. Separate analyses were performed for relative total seeds, relative seeds initiated, and relative escape from predation. For comparison, we also estimated selection based on fruits per flower and seeds per fruit for non-eaten fruits. This quadratic regression model is the standard way of analyzing phenotypic selection, facilitating comparison with hundreds of other studies (Kingsolver et al. 2012). Since it may not capture all elements of the selection surface (Schluter and Nychka 1994), we further explored complex cases by employing a generalized additive model (Morrissey and Sakrejda 2013) and smoothing via thin plate splines, as implemented in function `gam` of the R package `mgcv`.

Results

Question 1a: How do patterns of selection on floral traits compare during pollination versus seed predation? Analyzing all traits together, directional selection was stronger on each one of the six floral traits during seed initiation (and hence pollination) than during seed predation, as evidenced by larger absolute values for the directional selection gradients (Table 2). Absolute values for standardized directional selection gradients averaged 0.18 during pollination and only 0.03 during seed predation, compared with 0.21 across both parts of the life cycle (Table 2). Directional selection during pollination favored wider flowers ($P = 0.0042$), as was expected from previous work, and also favored higher emission of both volatile compounds, total pinene and indole ($\beta = 0.17$ and 0.28 , $P = 0.0265$ and 0.0017 ; Table 2). Directional selection during seed predation favored narrower sepals, also as expected. Furthermore, selection during seed predation favored higher emission of total pinene. Although we observed selection during both lifecycle stages for total pinene, directional selection on that volatile was still higher during pollination ($\beta = 0.17$) than during seed predation ($\beta = 0.05$), just as for all other measured traits (Table 2). We did not detect any directional selection for higher nectar production or any selection due to seed predation on corolla width, which had been reported before for *I. aggregata* (Campbell et al. 1991; Campbell et al. 2002; Mitchell 1993).

The canonical analysis with all six traits revealed a major axis favoring a combination of wide corollas with pale color, narrow sepals and high indole emission (Table S2). Quadratic regression on the two most important canonical axes revealed non-linear selection based on total seeds released on that axis (eigenvalue = 0.30, $P < 0.01$; Fig. 1A). The second canonical axis also showed evidence of non-linear selection (Fig. 1A). Selection based on seeds initiated was similar

in shape to that of net selection (Fig. 1B), with eigenvectors also very similar (Table S2), indicating that nearly all of the non-linear selection was generated during that portion of the lifecycle. Furthermore, the two greatest eigenvalues were nearly as large (absolute values = 0.26 and 0.15) as for total seeds, while those for escape from fly predation were small (0.03 and 0.04), generating a relatively flat fitness surface based on seed predation (Fig. 1C). Thus not only directional selection, but also non-linear selection was stronger during pollination than during seed predation.

That conclusion is supported by separate analyses of selection on particular combinations of traits. Examining non-linear selection for all pairwise combinations of traits, the absolute value of the selection gradient was larger during pollination than during seed predation for 20 out of 21 parameters (compare Table 1C and Table 1D). The sole exception was for correlational selection on nectar and total pinene emission, which was stronger during seed predation ($\gamma_{ij} = -0.07$ vs -0.00) and significantly different from zero ($P < 0.05$) only during seed predation. The differences in strength of selection between lifecycle stages can be explained in part by a higher opportunity for selection (Arnold and Wade 1984), as quantified by the variance in relative fitness for seeds initiated than for escape from seed predation (e.g. 1.79 vs. 0.10 at the *I. aggregata* site and 1.58 vs. 0.08 overall). Plants varied less in seed predation than seeds initiated despite the fact that 24% of fruits were attacked.

For three of the traits (width, color, nectar), sufficient data were available to analyze selection separately by site. At the *I. aggregata* site where hummingbirds dominate, net selection favored wide corollas ($\beta = 0.51 \pm 0.16$) and pale petal color ($\beta = -0.35 \pm 0.14$) with a disruptive component on color as well ($\gamma_{11} = 0.56 \pm 0.18$, Table 3). We also detected correlational selection in which wide flowers were favored more when flowers were pale in color (Table 3), as we had

for both sites together (Table 1B). Selection was generally weaker at the upper site, which is visited by both hummingbirds and hawkmoths than at the hummingbird-dominated *I. aggregata* site (Table 3). By adding the main effect of site and each site x trait interaction to our directional selection model for total seeds, we detected stronger selection for pale flowers at the *I. aggregata* site (interaction $P = 0.0114$) and marginally so for wider flowers ($P = 0.0652$), but no difference for nectar ($P = 0.5567$). After accounting for traits, the two sites did not differ detectably in total seeds per plant ($P = 0.2971$).

Question 1b: Are floral scent compounds under non-linear as well as directional selection? In addition to the directional selection observed on both total pinene and indole (Table 2), total pinene was also under stabilizing selection during pollination and overall ($\gamma_{11} = -0.24 \pm 0.04$ for both fitness components, Table 1) such that very high levels led to a drop again in total seeds. As for the other floral traits, estimates of quadratic selection were stronger during seed initiation than during seed predation for both total pinene and indole (compare Tables 1C and 1D). The estimate of correlational selection between them was also higher during seed initiation, but was not statistically significant (Table 1C).

Question 2: Does combining the influence of species interactions at two points in the lifecycle lead to the generation of non-linear selection not seen during either single selective episode? We expected to see correlational selection on many pairs of traits based on previous studies of directional selection during those two lifecycle stages (Table 1A), but observed correlational selection based on total seeds for only two pairs of traits (Table 1B). First, correlational selection favored wide flowers more so if the flowers were pale in coloration ($\gamma_{12} = -0.22$, $P = 0.0172$, fig. 2A; see also gam model in fig. S2A). For the combination of corolla width and petal color, the pattern of selection based on total seeds was similar to the pattern based on

seeds initiated, with no contribution from selection during seed predation (fig. 2). Thus the correlational selection was not an emergent property of directional selection on different traits during two stages of the life cycle. The overall pattern of selection favored plants with wide, pale corollas. Second, we detected correlational selection for low nectar production with narrow sepals and high nectar production with wide sepals ($\gamma_{12} = 0.25$, $P < 0.05$, fig. 3A), again generated almost entirely by events during pollination and seed initiation (fig. 3B) rather than emerging from the combined effects of pollination and seed predation.

In addition to those two cases of pairwise correlational selection, we detected correlational selection for low nectar production and high emission of α - and β -pinene specifically during seed predation ($\gamma_{12} = -0.07$, $P < 0.05$, fig. 4). That correlational selection, however, was weak and had little influence on the overall pattern of selection based on total seeds. Net fitness was instead highest for plants with intermediate nectar production and intermediate emission of pinenes (fig. 4A), as evidenced by significant stabilizing selection on both nectar and total pinene (Table 1B). In this case, total seed production was maximized for total pinene emissions 1.35 SD higher than the mean, or an emission of 312 ng per hour per flower.

Fitting generalized additive models that are more flexible in shape of the fitness functions (fig. S2, S3, S4) gave similar results, with one exception. The gam model provided little support for correlational selection on nectar production and sepal width; instead selection based on total seeds was largely directional for plants with low nectar production and narrow sepals (fig. S3A).

Breaking seeds initiated down more finely into three multiplicative components revealed selection on five of the six traits based on either fruits per flower or seeds initiated per non-eaten

fruit (Table S3). Thus, selection based on seeds initiated was not due to correlations of traits with flower number. Indeed, out of the six focal traits, only corolla width correlated detectably with flower number at either site, and it did so weakly ($r = 0.18$ and 0.21 , $N = 212$ and 207 , uncorrected $P = 0.0082$ and 0.0027 , $P < 0.05$ after Bonferonni correction). For the three pairs of traits under correlational selection, plus the pair of volatiles (pinene and indole), the average absolute value of the standardized directional selection gradient was 0.07 for fruits per flower and 0.09 for seeds per fruit compared to only 0.04 for seed predation (Table S3). Similarly, the average absolute value of the non-linear selection gradients was 0.06 for fruits per flower and 0.11 for seeds per fruit compared to just 0.02 for seed predation. The opportunity for selection, as measured by the variance in relative fitness, was lower during seed predation than for any of these other fitness components (Figure S1).

Discussion

A major outcome of this study is that natural selection on floral traits is stronger during the process of pollination and seed formation than during pre-dispersal seed predation. That result holds for directional selection, in accordance with previous reports of stronger pollinator-mediated selection than selection due to other biotic factors (Caruso et al. 2019). We show here that it also holds for quadratic selection and correlational selection for these *Ipomopsis* plants, and that it does so regardless of whether we examine total seeds for a plant or fruit and seed production on a per flower basis. Moreover, it holds for understudied aspects of floral scent as well as other floral traits. In some systems, floral traits do have stronger effects on seed predation than pollination (Cariveau et al. 2004). In *Ipomopsis*, however, the larger absolute values for selection gradients during the earlier stage of the lifecycle can be explained by a higher variance in relative fitness (and thus higher opportunity for selection) for the component of seeds initiated

than for seeds escaping predation. The average directional selection intensity that we detected during pollination and seed initiation ($\beta = 0.18$) was relatively high but not out of line with averages reported in a comprehensive review (Harder and Johnson 2009).

The second major outcome is that correlational selection resulted from selection during one stage of the lifecycle rather than emerging from a combination of directional selection on different traits during pollination and seed predation, as we had predicted. We detected correlational selection through a canonical analysis of all six floral traits as well as specifically on pairs of floral traits, particularly on corolla width and petal color, and on nectar production and pinene emission. These results add to a handful of other studies that have also demonstrated correlational selection based on seed set for combinations of traits of individual flowers (e.g., Herrera 2001; Nattero et al. 2010; Reynolds et al. 2010). Correlational selection on corolla width and petal color was generated during seed initiation, with wide flowers especially advantageous when flowers were pale in color, as also seen in an earlier study (Campbell 2009). As hummingbird pollinators prefer wide and red flowers in this system (Campbell et al. 1997; Meléndez-Ackerman et al. 1997), we might have expected that trait combination instead to lead to the highest fitness. It is possible that pollinators treat the two traits as redundant, such that when the color is not attractive a hummingbird is attracted only when the flower is relatively wide so that it can insert its bill more deeply, as shown in (Campbell et al. 1996), and extract nectar more easily (Grant and Temeles 1992). One reason that we did not observe correlational selection as an emergent property may be that selection during seed predation was so weak compared with selection during pollination. Consider a numerical example in which a selection gradient during pollination and seed initiation on standardized trait 1 equals 0.18, and selection during seed predation on trait 2 equals 0.03 (the average absolute values observed here). Making

relative fitness equal to 1 when the standardized trait value = 0 and multiplying the two equations together ($1+0.18X_1$ and $1+0.03X_2$) yields a correlational selection gradient of only 0.0054.

In general, we would expect strong correlational selection to be generated by directional selection across two life cycle stages only when the opportunity for selection is strong during both stages. For floral traits, a meta-analysis of experimental manipulations of selection agents suggested that pollinator-mediated selection averages 1.5 times as strong as selection due to other biotic factors, including browsing and interspecific competition (Caruso et al. 2019). A study on the orchid *Gymnadenia conopsea* provided a rare example where selection due to both pollinators and herbivores was measured on the same reproductive traits, in this case finding similar strengths of directional selection during the two stages (Sletvold et al. 2015). For traits across all types of organisms, selection gradients during fecundity average approximately 1.8 times as large as selection during viability (Kingsolver et al. 2012). For both meta-analyses, those relative strengths are similar enough to suggest that there might be many other species in which correlational selection could still be generated by combining directional selection across stages. More tests of this idea would be welcome.

One limitation of our study is that we do not know the extent to which the variation in seeds initiated was determined by pollination level rather than resources for seed maturation, as could be done using experiments comparing selection of naturally pollinated and hand-pollinated plants (Caruso et al. 2019; Chapurlat et al. 2015; Sletvold 2019). It would be interesting to follow up to measure selection under factorial manipulations of supplemental hand-pollination and removal of fly eggs. Instead our results relied on the more common method of analyzing seed set of unmanipulated plants. In a comprehensive review of > 600 estimates of selection in unmanipulated plants (Harder and Johnson 2009), directional selection was higher when based

on seed set rather than pollination for the trait of flower color but not for flower size or nectar production. We are unaware of similar studies for floral scent. While we do not know the precise amount of selection that was due to pollination, we do know that pollination level relates strongly to seed set in *Ipomopsis* in the local area (Campbell 1991; Campbell et al. 2002; Campbell and Halama 1993). Indeed, previous studies in this system showed increases in fruits per flower with supplemental pollination but not with addition of nutrients (Campbell and Halama 1993), indicating that at least the selection based on fruit set (Table S2) was driven by pollinators. Supplemental pollination also increased seeds per fruit by nearly as much as fruits per flower (Campbell and Halama 1993). Furthermore, the selection surface that we obtained for corolla width and petal color (Fig. 2B) is strikingly similar to a previously published one on *Ipomopsis* using independent data from other years on pollinator visitation rate as the fitness component and thus more directly measuring selection due to pollinators (Fig. 2A in (Campbell 2009). Both studies found evidence for directional selection favoring wide flowers and correlational selection on the two traits during pollination. As for all such studies that did not manipulate traits, in our current study we cannot unambiguously attribute the effects to the measured traits rather than unmeasured ones. For corolla width, petal color, nectar production, and indole emission, however, we have independent information that pollinators respond behaviorally to manipulations of the trait (Bischoff et al. 2015; Campbell et al. 1997; Campbell et al. 1996; Campbell et al. 1991; Meléndez-Ackerman and Campbell 1998; Mitchell 1993).

A second caveat is that we combined data from nine years and two sites in order to generate sufficient sample sizes for powerful selection analysis. Selection on traits could vary with both factors, and we have previously demonstrated variation in selection on corolla width over years (Campbell and Powers 2015). The necessity for large sample sizes is one of the

challenges in moving beyond the estimation of directional selection to detect complex patterns of multivariate selection. This is especially true for chemical traits, which are intrinsically challenging to sample adequately for population-level studies and may include a very large number of biologically active compounds (Raguso et al. 2015)

Our study provides rare estimates of not only directional, but also non-linear selection, on volatile organic compounds emitted by flowers in the wild. We demonstrated selection for increased night-time emission of the nitrogenous compound indole and an intermediate level of pinene emission, both during the stage of pollination. We also demonstrated selection for increased pinene emission during seed predation. We had expected selection on indole to be driven by pollinators (Bischoff et al. 2015), and the selection on pinene to be driven by seed predators, yet selection on pinene was actually stronger during pollination than seed predation. We also saw that seed predation generated a pattern of weak correlational selection for low nectar combined with high levels of pinene emission, although that result was based on a relatively low sample size of 95 plants in the pairwise analysis only. It is possible that high emissions of α -pinene repels these fly seed predators, as it does in laboratory studies with house flies (Haselton et al. 2015). These are the first demonstrations of non-linear selection on floral scent in the wild, although a handful of previous field studies tested for directional selection on the specific floral scent compounds we investigated here. Pollinators favored reduced emission of indole in the orchid *Gymnadenia conopsea* at night when it is pollinated by moths on the island of Öland, Sweden (Chapurlat et al. 2019). In contrast, our populations of *Ipomopsis* plants experienced selection for increased nighttime emission of indole, which is consistent with prior work showing that application of 1 ng per flower of indole spurred the pollinator *Hyles lineata* to approach inflorescences of *Ipomopsis* (Bischoff et al. 2015). Although sphingid moths show

electroantennogram responses to indole (Raguso et al. 1996) and are pollinators in both *I. tenuituba* and *Gymnadenia conopsea*, the plant species differ greatly in average nighttime indole emission. Inflorescences of the orchid emitted 47 ng per hour, whereas *Ipomopsis tenuituba* and hybrids emitted only 8 ng per hour on a whole inflorescence level. Thus it is possible that overall sphingid moths prefer an intermediate dosage, and we did observe a drop off in seeds per fruit at very high levels of indole (see negative quadratic term $\gamma_{11} = -0.21$, $P < 0.05$ in Table S2). Alternatively, the difference in response between systems could reflect responses of other floral visitors, such as the noctuid moths on *G. conopsea*. For pinene, an intermediate emission rate led to the highest seed production in *Ipomopsis*, suggesting that behavioral responses could be dosage dependent, in which pollinators prefer to visit flowers with an intermediate emission. That finding of stabilizing selection on pinenes is reminiscent of the dosage dependent effects of 2-phenylethanol emission on both bumblebee visitation and flower-damaging ants in *Polemonium viscosum* (Galen et al. 2011). The few other studies of selection on pinenes have reported changes in plant reproduction with ordination axes that correlate with α -pinene emission, and the shape of the fitness function on the underlying trait is unknown (Gross et al. 2016; Schiestl et al. 2011). It would be valuable to test for more complex selection surfaces, along with directional selection, in future studies of floral volatiles. If there are dosage-dependent behavioral responses, volatiles may be more likely than other floral traits to be under non-linear selection.

As we did not specifically measure visitation by hawkmoths or hummingbirds in this study, we cannot attribute the effects of pinene on seed initiation specifically to one group of visitor. Hawkmoths can perceive a wide variety of volatile organic compounds (Raguso et al. 1996), and some moths have electroantennogram responses to α -pinene (Hull et al. 2004) and

can learn to preferentially visit rewarding flowers enhanced with that volatile (Cunningham et al. 2004). It is less clear whether hummingbirds respond to terpenoids, as they are thought to rely more on visual cues than scent, with hummingbird-pollinated species often scentless or weakly scented (Knudsen et al. 2004). Hummingbirds have rarely been tested for odor perception, but in one study, they were repelled by nicotine added to headspace of artificial flowers, indicating a response to odor, and also responded behaviorally to monoterpenes dissolved in nectar (Kessler and Baldwin 2006). In our study, we measured headspace emission of pinenes, but did not distinguish whether it could have come from nectar rather than other portions of the flower. Emissions did correlate most strong with sepal width (Table S1), and the trichomes on the sepals are a potential source of terpene emissions (Gonzales-Vigil et al. 2012). Future studies could manipulate the emission level of pinenes to match specifically the optimal level observed here versus other levels to test directly for a dose-dependent effect on pollinator visitation.

Conclusions

Not only directional selection but also non-linear forms of selection on floral traits were generally higher during pollination and seed maturation than during the later stage of seed predation. In the first such test of non-linear selection, floral volatiles experienced stabilizing as well as directional selection, suggesting that future studies of these traits should consider the dosage dependent effects of volatiles on pollinator behavior. Correlational selection favored some particular combinations of floral traits, but that correlational selection was generated primarily during pollination and seed initiation rather than as an emergent property of selection due to events during sequential life history stages.

Acknowledgements

We are grateful to the many people who assisted in the field over the long gestation of this study: Laura Barbour, Amanda Barth, Kirsten Dales, Colleen Danaher, Rachel Dickson, Alexandra Faidiga, Michelle Forster, Christina Feng, Emily Graber, Victoria Luizzi, John Powers, Robert Schaeffer, and Gabe Trujillo. We also thank Ana Paula Assis, Paulo Guimarães, and anonymous reviewers for comments on the manuscript. GCMS equipment at RMBL was supported by NSF-DBI-1624073, and field research was supported by NSF-DEB-1654655 and NSF-DEB-0542876, RMBL Research Fellowships, and UC Irvine Research funds to DRC. GCMS equipment at Cornell was supported by NSF-DEB-0746106 and NSF-DEB-1342792 to RAR.

Statement of Authorship

D.R.C., M.B. and R.A.R. conceptualized and designed the study. D.R.C. and M.B. collected field data. All authors contributed to collection and processing of laboratory data. D.R.C. analyzed the data and wrote the manuscript, with all authors contributing to editing.

Data and Code Accessibility

Data are deposited in the Dryad Digital Repository: <https://doi.org/10.7280/D1KM49> (Campbell et al. 2021).

Literature Cited

- Abdala-Roberts, L., V. Parra-Tabla, D. R. Campbell, and K. M. Mooney. 2014. Soil fertility and parasitoids shape herbivore selection on plants. *Journal of Ecology* 102:1120-1128.
- Aldridge, G., and D. R. Campbell. 2007. Variation in pollinator preference between two *Ipomopsis* contact sites that differ in hybridization rate. *Evolution* 61:99-110.
- Arguello, J. R., C. Sellanes, Y. R. Lou, and R. A. Raguso. 2013. Can yeast (*S. cerevisiae*) metabolic volatiles provide polymorphic signaling? *PloS one* 8:e70219.
- Arnold, S. J., and M. J. Wade. 1984. On the measurement of natural and sexual selection: Theory. *Evolution* 38:709-719.
- Bischoff, M., A. Jürgens, and D. R. Campbell. 2014. Floral scent in natural hybrids of *Ipomopsis* (Polemoniaceae) and their parental species. *Annals of Botany* 113:533-544.
- Bischoff, M., R. A. Raguso, A. Jürgens, and D. R. Campbell. 2015. Context-dependent reproductive isolation mediated by floral scent and color. *Evolution* 69:1-13.
- Blows, M. W., and R. Brooks. 2003. Measuring nonlinear selection. *American Naturalist* 162:815-820.
- Brody, A. K. 1992. Oviposition choices by a predispersal seed predator (*Hylemya* sp). 1. Correspondence with hummingbird pollinators, and the role of plant size, density and floral morphology. *Oecologia (Berlin)* 91:56-62.
- Brody, A. K. 1997. Effects of pollinators, herbivores, and seed predators on flowering phenology. *Ecology* 78:1624-1631.
- Campbell, D. R. 1989. Measurements of selection in a hermaphroditic plant: variation in male and female pollination success. *Evolution* 43:318-334.
- Campbell, D. R. 1991. Effects of floral traits on sequential components of fitness in *Ipomopsis aggregata*. *American Naturalist* 137:713-737.

- Campbell, D. R. 1997. Genetic and environmental variation in life-history traits of a monocarpic perennial: a decade-long field experiment. *Evolution* 51:373-382.
- Campbell, D.R. 2009. Using phenotypic manipulations to study multivariate selection of floral trait associations. *Annals of Botany* 103:1557-1566.
- Campbell, D. R., M. Crawford, A. K. Brody, and T. A. Forbis. 2002. Resistance to pre-dispersal seed predators in a natural hybrid zone. *Oecologia (Berlin)* 131:436-443.
- Campbell, D. R., and K. Halama. 1993. Resource and pollen limitations to lifetime seed production in a natural plant population. *Ecology* 74:1043-1051.
- Campbell, D. R., and J. M. Powers. 2015. Natural selection on floral morphology can be influenced by climate. *Proceedings of the Royal Society B* 282:21050178.
- Campbell, D., M. Bischoff, R. Raguso, H. Briggs, and P. Sосenski. 2021. Data from: Selection of floral traits by pollinators and seed predators during sequential life history stages, American Naturalist, Dryad Digital Repository, <https://doi.org/10.7280/D1KM49>
- Campbell, D. R., P. Sосenski, and R. A. Raguso. 2019. Phenotypic plasticity of floral volatiles in response to increasing drought stress. *Annals of Botany* 123:601-610.
- Campbell, D. R., N. M. Waser, G. Aldridge, and C. A. Wu. 2008. Lifetime fitness in two generations of *Ipomopsis* hybrids. *Evolution* 62:2616-2627.
- Campbell, D. R., N. M. Waser, and E. J. Meléndez-Ackerman. 1997. Analyzing pollinator-mediated selection in a plant hybrid zone: hummingbird visitation patterns on three spatial scales. *American Naturalist* 149:295-315.
- Campbell, D. R., N. M. Waser, and M. V. Price. 1996. Mechanisms of hummingbird-mediated selection for flower width in *Ipomopsis aggregata*. *Ecology* 77:1463-1472.

- Campbell, D. R., N. M. Waser, M. V. Price, E. Lynch, and R. Mitchell. 1991. Components of phenotypic selection: pollen export and flower corolla width in *Ipomopsis aggregata*. *Evolution* 45:1458-1467.
- Cariveau, D., R. E. Irwin, A. K. Brody, L. S. Garcia-Mayeya, and A. Von der Ohe. 2004. Direct and indirect effects of pollinators and seed predators to selection on plant and floral traits. *Oikos* 104:15-26.
- Caruso, C. M., K. E. Eisen, R. A. Martin, and N. Sletvold. 2019. A meta-analysis of the agents of selection on floral traits. *Evolution* 73:4-14.
- Chapurlat, E., J. Ågren, J. Anderson, M. Friberg, and N. Sletvold. 2019. Conflicting selection on floral scent emission in the orchid *Gymnadenia conopsea*. *New Phytologist* 222:2009-2022.
- Chapurlat, E., J. Ågren, and N. Sletvold. 2015. Spatial variation in pollinator-mediated selection on phenology, floral display and spur length in the orchid *Gymnadenia conopsea*. *New Phytologist*.
- Crean, A. J., K. Monro, and D. J. Marshall. 2010. Fitness consequences of larval traits persist across the metamorphic boundary. *Evolution* 65:3079-3089.
- Cunningham, J., C. Moore, M. Zalucki, and S. West. 2004. Learning, odour preference and flower foraging in moths. *Journal of Experimental Biology* 207:87-94.
- Darwin, C. 1862, On the various contrivances by which British and Foreign Orchids are Fertilised by Insects, and on the Good Effects of Intercrossing. . London, John Murray.
- Dudareva, N., A. Klempien, J. K. Muhlemann, and I. Kaplan. 2013. Biosynthesis, function and metabolic engineering of plant volatile organic compounds. *New Phytologist* 198:16-32.

- Engel, E., and R. Irwin. 2003. Linking pollinator visitation rate and pollen receipt. *American Journal of Botany* 90:1612-1618.
- Frey, F.M. 2004. Opposing natural selection from herbivores and pathogens may maintain floral color variation in *Claytonia virginica* (Portulacaceae). *Evolution* 58:2426-2437.
- Galen, C., and J. Cuba. 2001. Down the tube: Pollinators, predators, and the evolution of flower shape in the alpine skypilot, *Polemonium viscosum*. *Evolution* 55:1963-1971.
- Galen, C., R. Kaczorowski, S. L. Todd, J. Geib, and R. A. Raguso. 2011. Dosage-dependent impacts of a floral volatile compound on pollinators, larcenists, and the potential for floral evolution in the alpine skypilot, *Polemonium viscosum*. *American Naturalist* 177:258-272.
- Gonzales-Vigil, E., D. E. Hufnagel, J. Kim, R. L. Last, and C. S. Barry. 2012. Evolution of TPS20-related terpene synthases influences chemical diversity in the glandular trichomes of the wild tomato relative *Solanum habrochaites*. *The Plant Journal* 71:921-935.
- Grant, V., and E. J. Temeles. 1992. Foraging ability of rufous hummingbirds on hummingbird flowers and hawkmoth flowers. *Proceedings of the National Academy of Sciences USA* 89:9400-9404.
- Gross, K., M. Sun, and F. P. Schiestl. 2016. Why do floral perfumes become different? Region-specific selection on floral scent in a terrestrial orchid. *Plos One*.
- Harder, L. D., and S. D. Johnson. 2009. Darwin's beautiful contrivances: evolutionary and functional evidence for floral adaptation. *New Phytologist* 183:530-545.
- Haselton, A. T., A. Acevedo, J. Kuruvilla, E. Werner, J. Kiernan, and P. Dhar. 2015. Repellency of α -pinene against the house fly, *Musca domestica*. *Phytochemistry* 117:469-475.

- Herrera, C. M. 2001. Deconstructing a floral phenotype: do pollinators select for corolla integration in *Lavandula latifolia*? *Journal of Evolutionary Biology* 14:574-584.
- Herrera, C. M., M. Medrano, P. J. Rey, A. M. Sánchez-Lafuente, M. B. García, J. Guitián, and A. J. Manzaneda. 2002. Interaction of pollinators and herbivores on plant fitness suggests a pathway for correlated evolution of mutualism- and antagonism-related traits. *Proceedings of the National Academy of Sciences USA* 99:16823-16828.
- Hull, C. D., J. P. Cunningham, C. J. Moore, P. Zalucki, and B. W. Cribb. 2004. Discrepancy between antennal and behavioral responses for enantiomers of α -Pinene: Electrophysiology and behavior of *Helicoverpa armigera* (Lepidoptera). *Journal of Chemical Ecology* 30:2701-2084.
- Iwao, K., and M. D. Rausher. 1997. Evolution of plant resistance to multiple herbivores: quantifying diffuse coevolution. *American Naturalist* 149:316-335.
- Juenger, T. E., and J. Bergelson. 1998. Pairwise versus diffuse natural selection and the multiple herbivores of scarlet gilia, *Ipomopsis aggregata*. *Evolution* 52:1583-1592.
- Kessler, D., and I. T. Baldwin. 2006. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *The Plant Journal* 49:840-854.
- Kessler, D., C. Diezel, D. G. Clark, T. A. Colquhoun, and I. T. Baldwin. 2013. *Petunia* flowers solve the defence / apparency dilemma of pollinator attraction by deploying complex floral blends. *Ecology Letters* 16:299-306.
- Kingsolver, J. G., S. E. Diamond, A. M. Siepielski, and S. M. Carlson. 2012. Synthetic analyses of phenotypic selection in natural populations: lessons, limitations and future directions. *Evolutionary Ecology* 26:1101-1118.

- Knauer, A. C., and F. P. Schiestl. 2017. The effect of pollinators and herbivores on selection for floral signals: a case study in *Brassica rapa*. *Evolutionary Ecology* 31:285-304.
- Knudsen, J. T., L. Tollsten, I. Groth, G. Bergstrom, and R. A. Raguso. 2004. Trends in floral scent chemistry in pollination syndromes: floral scent composition in hummingbird-pollinated taxa. *Botanical Journal of the Linnean Society* 146:191-199.
- Lande, R., and S. J. Arnold. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210-1226.
- Lankau, R. A., and S. Y. Strauss. 2008. Community complexity drives natural selection on a chemical defense of *Brassica nigra*. *American Naturalist* 171:150-161.
- Maad, J. 2000. Phenotypic selection in hawkmoth-pollinated *Platanthera bifolia*: targets and fitness surfaces. *Evolution* 54:112-123.
- McGlothlin, J. W. 2010. Combining selective episodes to estimate lifetime nonlinear selection. *Evolution* 64:1377-1385.
- Meléndez-Ackerman, E. 1997. Patterns of color and nectar variation across an *Ipomopsis* hybrid zone. *American Journal of Botany* 84:41-47.
- Meléndez-Ackerman, E. J., and D. R. Campbell. 1998. Adaptive significance of flower color and inter-trait correlations in an *Ipomopsis* hybrid zone. *Evolution* 52:1293-1303.
- Meléndez-Ackerman, E. J., D. R. Campbell, and N. M. Waser. 1997. Hummingbird behavior and mechanisms of selection on flower color in *Ipomopsis*. *Ecology* 78:2532-2541.
- Mitchell, R. J. 1993. Adaptive significance of *Ipomopsis aggregata* nectar production: observation and experiment in the field. *Evolution* 47:25-35.
- Morrissey, M. B., and K. Sakrejda. 2013. Unification of regression-based methods for the analysis of natural selection. *Evolution* 67:2094-2100.

- Nattero, J., A. N. Sérsic, and A. A. Cocucci. 2010. Patterns of contemporary phenotypic selection and flower integration in the hummingbird-pollinated *Nicotiana glauca* between populations with different flower-pollinator combinations. *Oikos* 119:852-863.
- Newman, D. A. 2014. Missing data: five practical guidelines. *Organizational Research Methods* 17:372-411.
- Pérez-Barrales, R., G. H. Bolstad, C. Pélabon, T. F. Hansen, and W. S. Armbruster. 2013. Pollinators and seed predators generate conflicting selection on *Dalechampia* blossoms. *Oikos* 122:1411-1428.
- Phillips, P. C., and S. J. Arnold. 1989. Visualizing multivariate selection. *Evolution* 43:1209-1222.
- Price, M. V., N. M. Waser, R. E. Irwin, D. R. Campbell, and A. K. Brody. 2005. Temporal and spatial variation in pollination of a montane herb: a seven-year study. *Ecology* 86:2106-2116.
- Raguso, R. A., D. M. Light, and E. Pickersky. 1996. Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. *Journal of Chemical Ecology* 22:1735-1766.
- Raguso, R. A., J. N. Thompson, and D. R. Campbell. 2015. Improving our chemistry: challenges and opportunities in the interdisciplinary study of floral volatiles. *Natural Product Reports* 32:893-903.
- Ramos, S. E., and F. P. Schiestl. 2019. Rapid plant evolution driven by the interaction of pollination and herbivory. *Science* 364:193-196.

- Reynolds, R. J., M. R. Dudash, and C. B. Fenster. 2010. Multiyear study of multivariate linear and nonlinear phenotypic selection on floral traits of hummingbird-pollinated *Silene virginica*. *Evolution* 64:358-369.
- Schiestl, F. P., F. K. Huber, and J. M. Gomez. 2011. Phenotypic selection on floral scent: trade-off between attraction and deterrence. *Evolutionary Ecology* 25:237-248.
- Schluter, D., and D. Nychka. 1994. Exploring fitness surfaces. *American Naturalist* 143:597-616.
- Sletvold, N., K. K. Moritz, and J. Agren. 2015. Additive effects of pollinators and herbivores result in both conflicting and reinforcing selection on floral traits. *Ecology* 96:214-221.
- Sletvold, N. 2019. The context dependence of pollinator-mediated selection in natural populations. *International Journal of Plant Sciences* 180:934-943.
- Stinchcombe, J. R., A. A. Agrawal, F. Aneil, P. A. Hohenlohe, S. J. Arnold, and M. W. Blows. 2008. Estimating nonlinear selection gradients using quadratic regression coefficients: Double or nothing? *Evolution* 62:2435-2440.
- Strauss, S. Y., and J. B. Whittall. 2006. Non-pollinator agents of selection on floral traits in *L. Harder*, and S. Barrett, eds. *Ecology and Evolution of Flowers*. Oxford, UK, Oxford University Press.
- Theis, N., and L. S. Adler. 2012. Advertising to the enemy: enhanced floral fragrance increases beetle attraction and reduced plant reproduction. *Ecology* 93: 430-435.
- Wade, M. J., and S. Kalisz. 1989. The additive partitioning of selection gradients. *Evolution* 43:1567-1569.

Tables

Table 1. Predicted and observed values for non-linear selection based on total seeds.

A) Predicted						
Trait	Width	Color	Nectar	Sepal	Pinene	Indole
Width	Y	Y	Y	Y	Y	Y
Color		N	N	Y	Y	N
Nectar			N	Y	Y	N
Sepal				N	N	Y
Pinene					N	Y
Indole						N
B) Observed Total seeds						
Trait	Width	Color	Nectar	Sepal	Pinene	Indole
Width	0.18±0.04*	-0.22±0.09*	-0.07±0.13	0.03±0.08	-0.21±0.12	0.32±0.22
Color		0.12±0.01***	0.00±0.13	0.00±0.09	-0.05±0.06	-0.44±0.24
Nectar			-0.30±0.04*	0.25±0.11*	-0.07±0.13	0.40±0.29
Sepal				0.12±0.03	-0.17±0.11	-0.29±0.24
Pinene					-0.24±0.04*	0.05±0.10
Indole						-0.08±0.09
C) Seeds initiated						
Trait	Width	Color	Nectar	Sepal	Pinene	Indole
Width	0.09±0.06	-0.23±0.09**	-0.06±0.12	0.00±0.17	-0.19±0.11	0.24±0.21

Color	0.10±0.02*	0.00±0.12	-0.05±0.09	-0.03±0.06	-0.33± 0.23
Nectar		-0.31±0.04**	0.18±0.11	-0.00±0.12	0.38±0.27
Sepal			0.07±0.04	-0.20±0.11	-0.24± .24
Pinene				-0.24± .04*	0.06±0.10
Indole					-0.09± 0.08

D) Proportion escaped from seed predation

Trait	Width	Color	Nectar	Sepal	Pinene	Indole
Width	-0.04±0.01	0.00±0.02	0.01±0.03	0.03±0.05	0.02±0.03	0.01±0.04
Color		0.01±0.01	-0.00±0.02	0.02±0.02	-0.01±0.02	-0.04±0.04
Nectar			0.01±0.01	-0.02±0.03	-0.07±0.03*	0.01±0.04
Sepal				0.04±0.00**	-0.00±0.03	-0.02±0.06
Pinene					0.02±0.01	-0.01±0.03
Indole						0.02±0.01

Note. * P < 0.05. ** P < 0.01

Observed selection is from pairwise analyses of traits and expressed as $\gamma_{ij} \pm \text{SE}$. (A) Predictions for quadratic (diagonal) and correlational selection (off-diagonal) are based solely on how a combination of directional selection on one trait due to pollinators and on the other trait due to seed predators would together influence total seeds, as based on previous studies. Both pollinators and seed predators can exert directional selection on width, thus predicting stabilizing selection on that trait and correlational selection involving all of the other traits. (B-D) Observed quadratic and correlational selection based on total seeds, seeds initiated, and escape from seed predation. Bold indicates selection gradients that differ significantly from zero. Quadratic coefficients (and their standard errors) are doubled estimates from meta-analysis of all pairwise models, with associated P values adjusted using the Bonferroni method for 5 non-independent tests for a given trait.

Table 2. Estimates of directional selection gradients (β) and their standard errors for all six traits.

	Total seeds		Seeds initiated		Proportion escaped from predation	
Trait	β	SE	β	SE	B	SE
Width	0.287**	0.093	0.287**	0.087	-0.026	0.016
Color	-0.154*	0.067	-0.119	0.063	-0.009	0.017
Nectar	0.052	0.116	0.051	0.103	0.009	0.017
Sepal	-0.256*	0.111	-0.188	0.109	-0.060**	0.020
Pinene	0.231**	0.076	0.173*	0.074	0.052*	0.021
Indole	0.296**	0.080	0.284**	0.077	-0.018	0.026

Note. * $P < 0.05$. ** $P < 0.01$

Estimates are provided based on overall fitness using total seeds as the measure, and separately for both seeds initiated and proportion escaping seed predation. For total seeds the R^2 averaged across all partial imputations was 0.17.

Table 3. Estimates of directional and non-linear selection for three traits separately by site.

Site	Directional (β)	Non-linear (γ)		
		Width	Color	Nectar

I. aggregata

Width	$0.51 \pm 0.16^{**}$	0.16 ± 0.26	$-0.51 \pm 0.17^{**}$	0.01 ± 0.17
Color	$-0.35 \pm 0.14^*$		$0.56 \pm 0.18^{**}$	-0.05 ± 0.17
Nectar	0.06 ± 0.15			-0.34 ± 0.20

Upper site

Width	$0.29 \pm 0.12^*$	-0.16 ± 0.18	0.06 ± 0.16	-0.04 ± 0.19
Color	-0.02 ± 0.11		-0.04 ± 0.23	0.14 ± 0.15
Nectar	-0.05 ± 0.11			-0.36 ± 0.19

Note. * $P < 0.05$. ** $P < 0.01$

Error terms represent SE of the estimate. Directional terms were first estimated from regression on the three traits. The non-linear terms were estimated separately from the full model with linear, quadratic and cross-product terms. Quadratic coefficients (and their standard errors) were doubled to obtain the estimates of stabilizing/disruptive selection along the diagonal. N = 156 for *I. aggregata* site and N = 132 for upper site.

Figure Legends

Figure 1: Response surfaces showing regression on first two canonical analysis for all six traits combined. The curved surfaces illustrate overall correlational selection (A) generated mostly by selection based on seeds initiated (B). Panel (C) shows the response surface based on proportion of seeds that escaped from predation. All panels use the same scale for relative fitness. * $P < 0.05$. ** $P < 0.01$.

Figure 2: Selection surfaces for corolla width and petal color. The surfaces illustrate overall correlational selection (A) generated mostly by selection based on seeds initiated (B). Panel (C) shows the selection surface based on proportion of seeds that escaped from predation. All panels use the same scale for relative fitness and show the best fit to Eqn. 1 in the text. $N = 324$ plants. * $P < 0.05$. ** $P < 0.01$. * $P < 0.0001$.

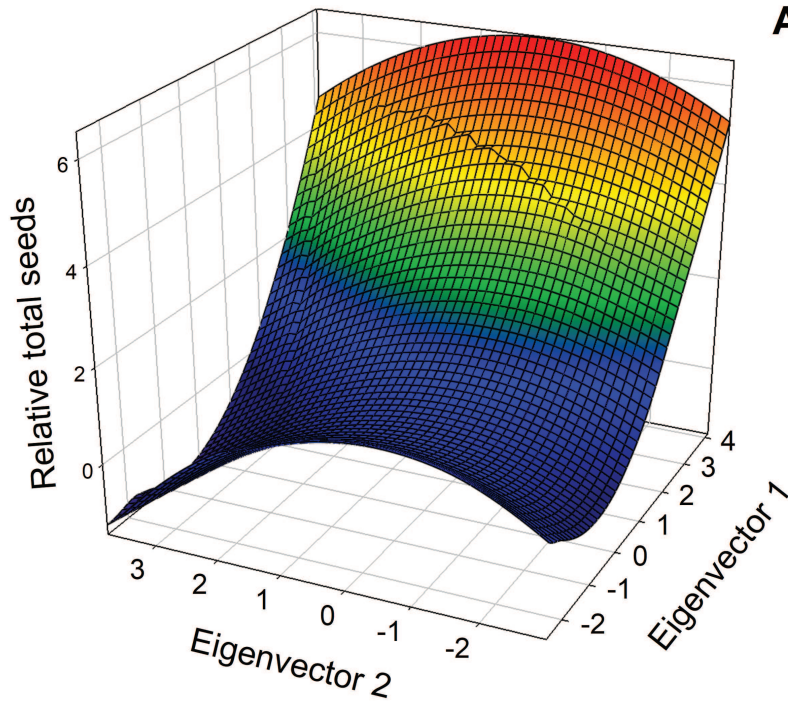
Figure 3: Selection surfaces for rate of nectar production and sepal width at all sites combined. Overall correlational selection was detected (A) in which wide sepals were advantageous for plants with high nectar production and narrow sepals for plants with low nectar production, due primarily to events during seed initiation (B) rather than seed predation (C). Surfaces are the best fit to Eqn. 1. $N = 95$ plants. * $P < 0.05$.

Figure 4: Selection surfaces for rates of nectar production and pinene emission at all sites combined. Correlational selection was detected due to seed predation (C) in which high pinene was advantageous for plants that produced little nectar but disadvantageous for plants that produced high nectar. The overall pattern of selection based on total seeds (A) showed directional and stabilizing selection only, due to more intense selection during seed initiation (B). Surfaces are the best fit to Eqn. 1. $N = 177$ plants. * $P < 0.05$.

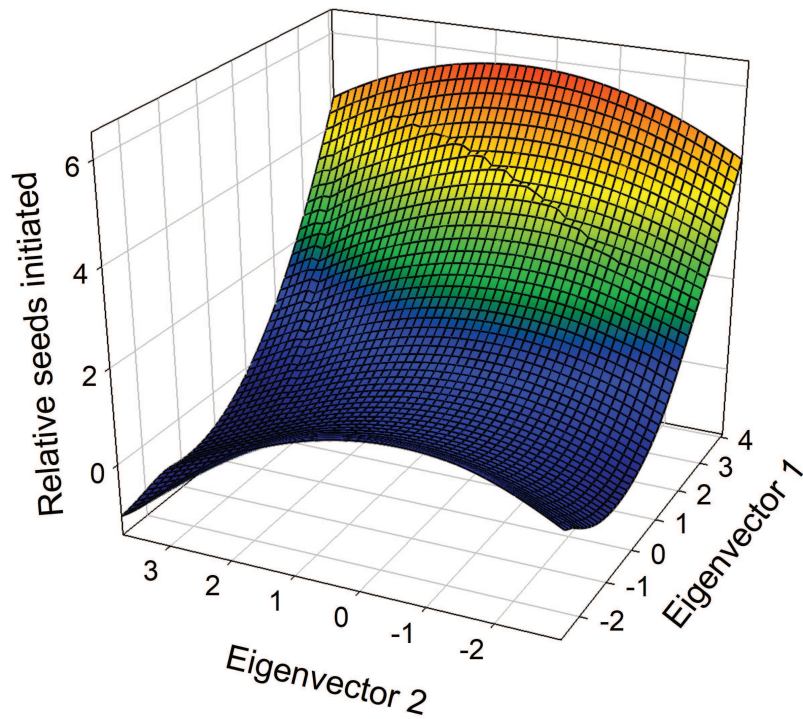
Figure 1

This is the author's accepted manuscript without copyediting, formatting, or final corrections. It will be published in its final form in an upcoming issue of The American Naturalist, published by The University of Chicago Press. Include the DOI when citing or quoting: <https://doi.org/10.1086/716740>
Copyright 2021 The University of Chicago Press.

A linear on eigenvector 1**
non-linear on eigenvector 1*
non-linear on eigenvector 2*



B linear on eigenvector 1*
non-linear on eigenvector 1*
non-linear on eigenvector 2*



C

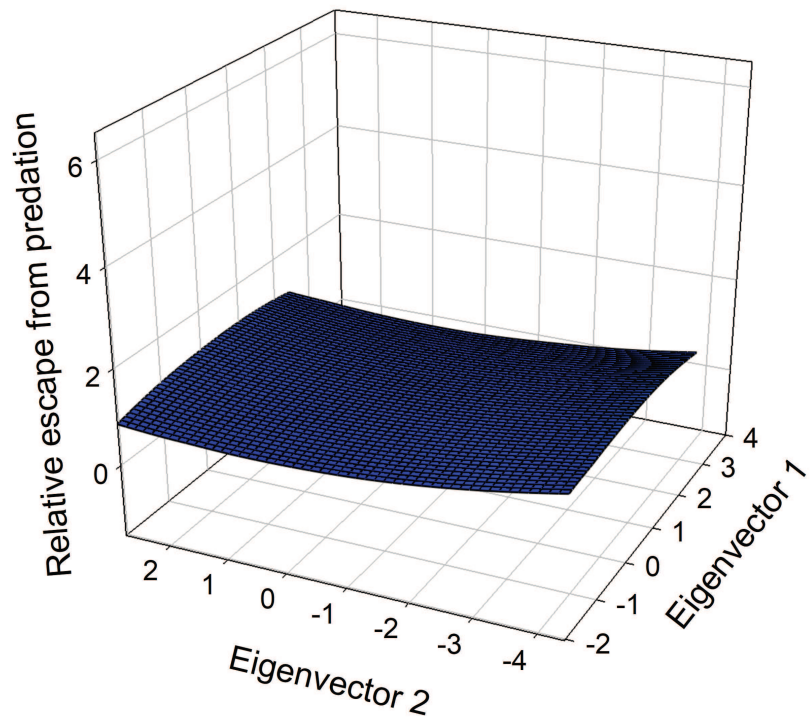
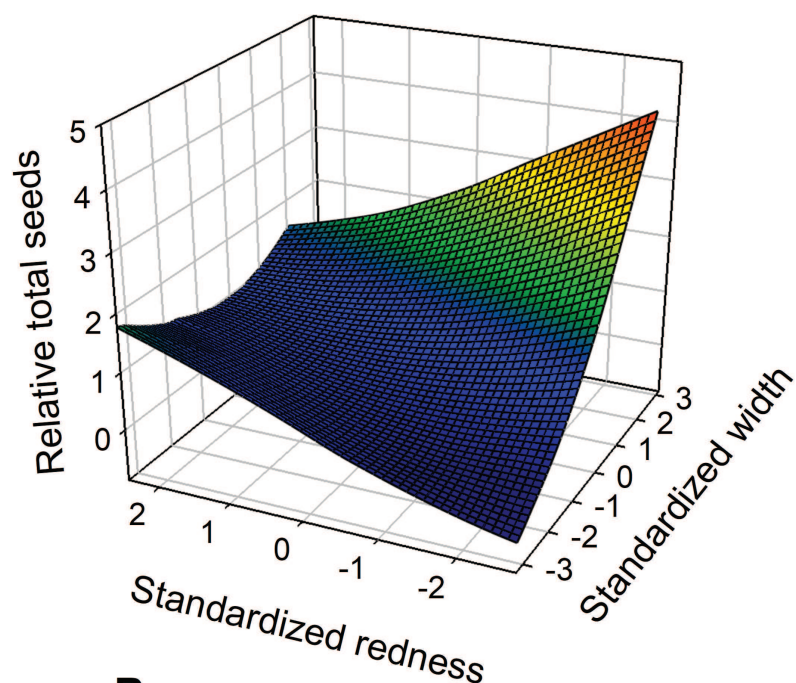


Figure 2

This is the author's accepted manuscript without copyediting, formatting, or final corrections. It will be published in its final form in an upcoming issue of The American Naturalist, published by The University of Chicago Press. Include the DOI when citing or quoting: <https://doi.org/10.1086/716740>
Copyright 2021 The University of Chicago Press.

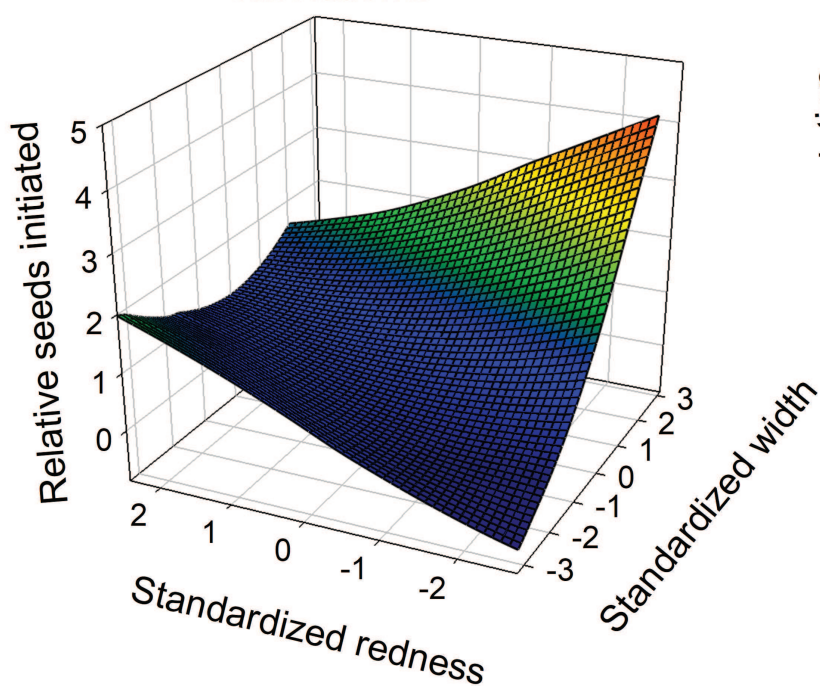
A

*directional on width*****
*directional on color**
*quadratic on width**
*correlational**



B

*directional on width*****
*quadratic on width**
*correlational***



C

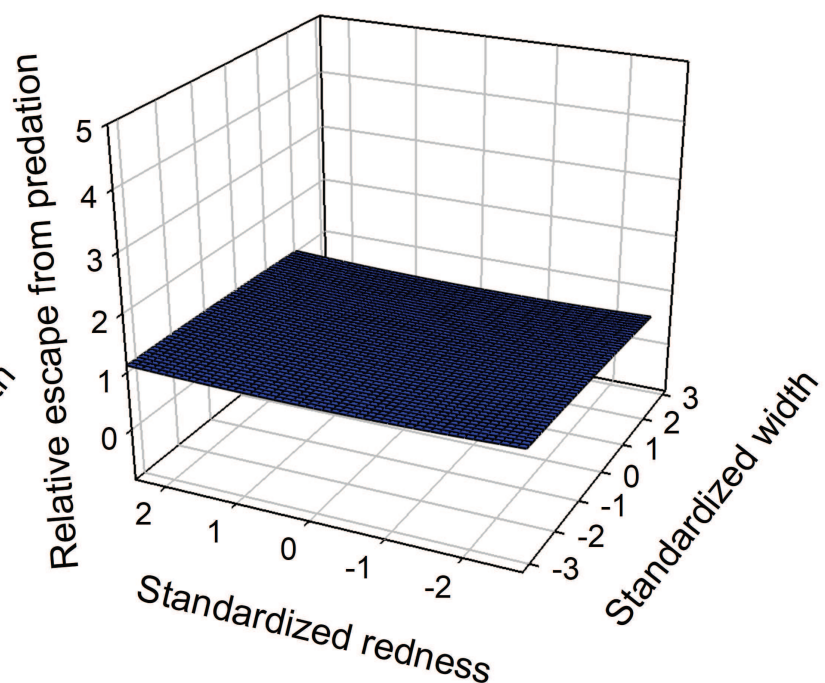


Figure 3

This is the author's accepted manuscript without copyediting, formatting, or final corrections. It will be published in its final form in an upcoming issue of The American Naturalist, published by The University of Chicago Press. Include the DOI when citing or quoting: <https://doi.org/10.1086/716740>
Copyright 2021 The University of Chicago Press.

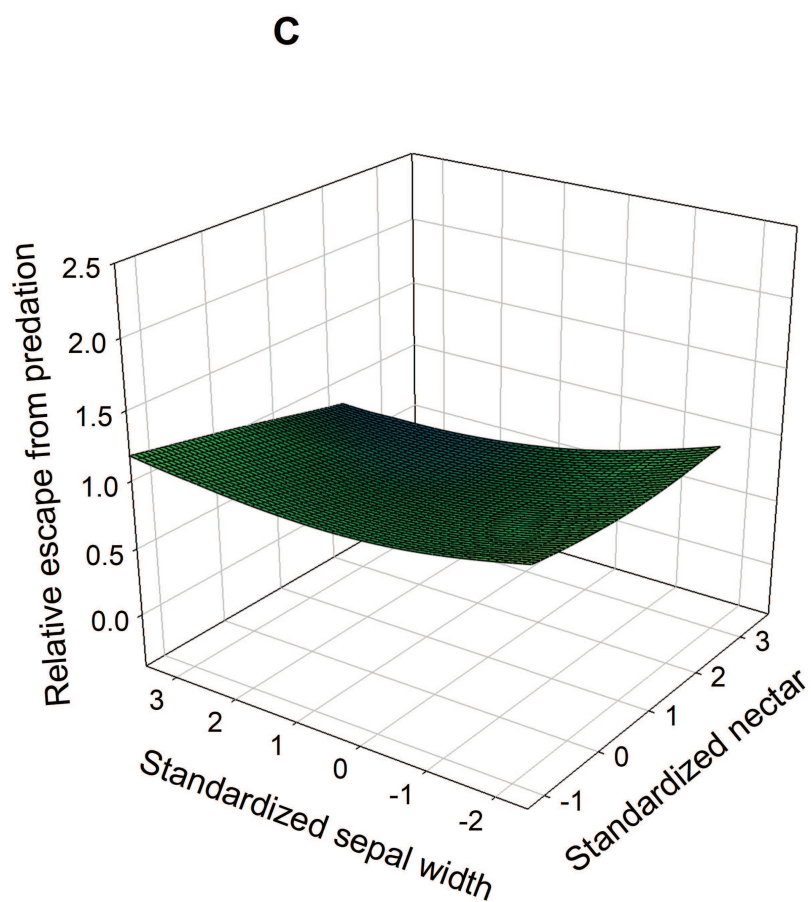
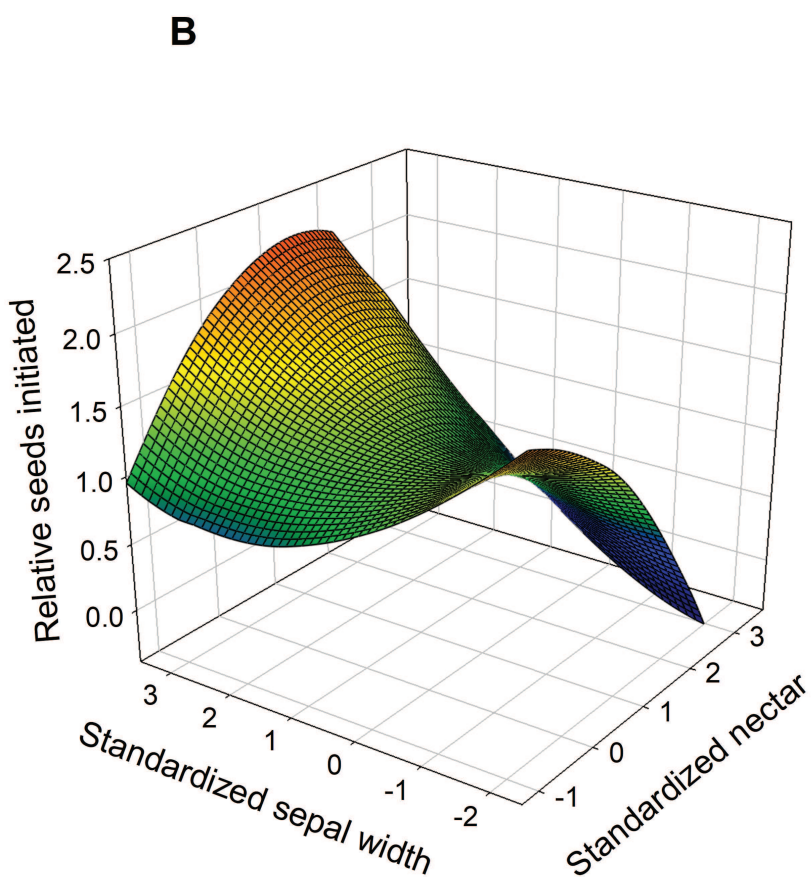
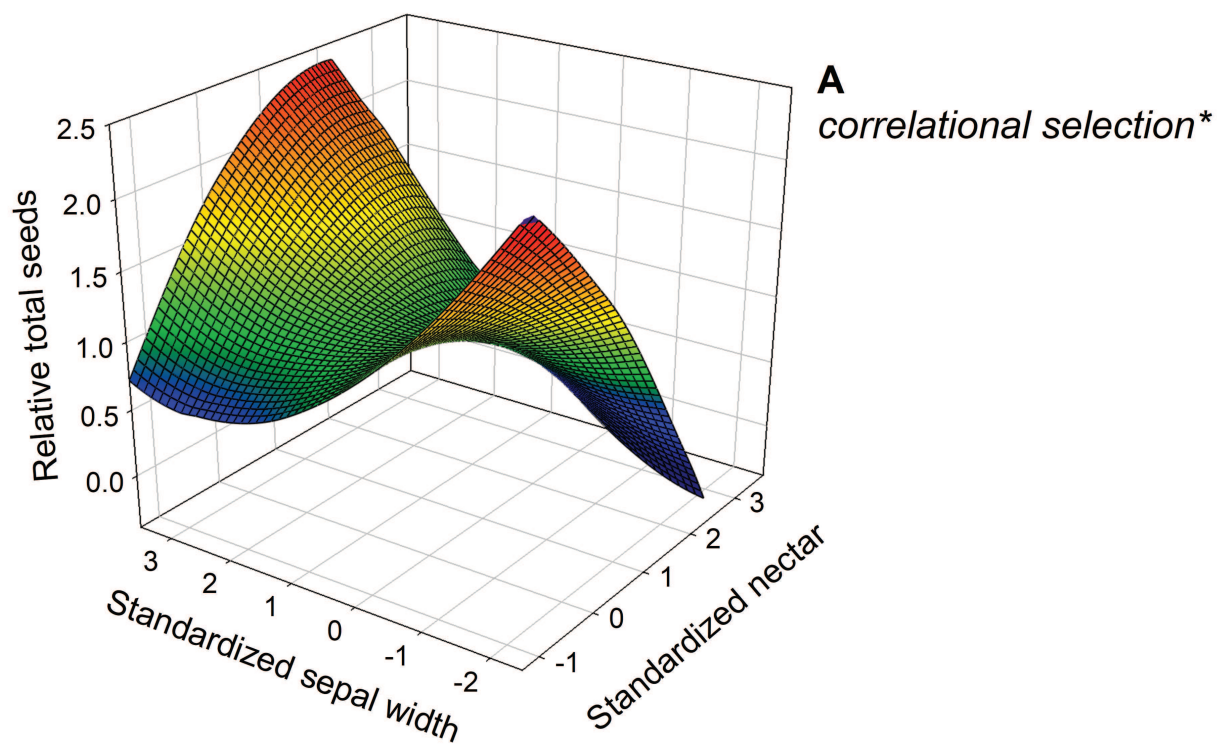
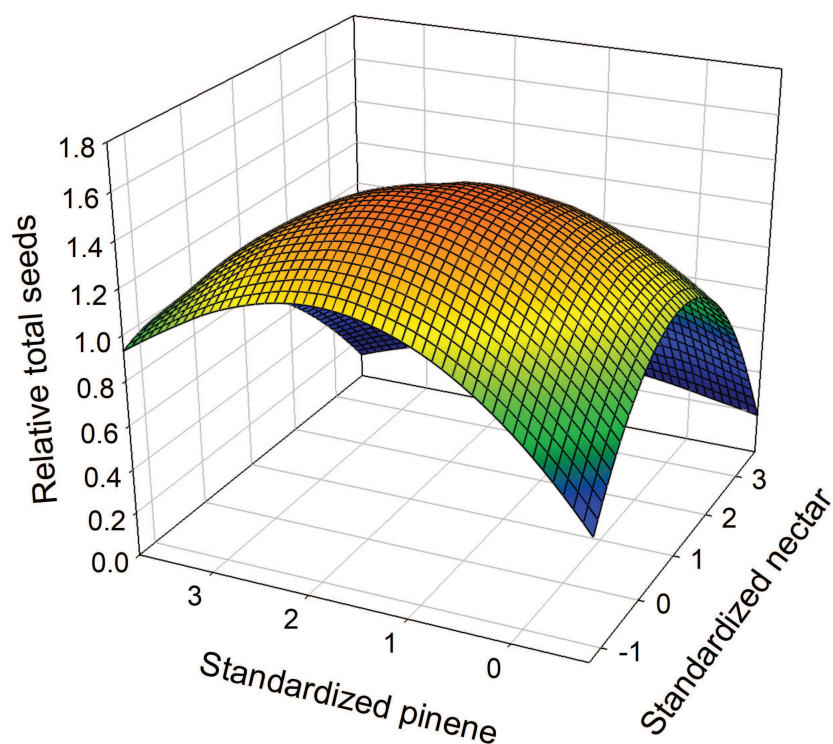


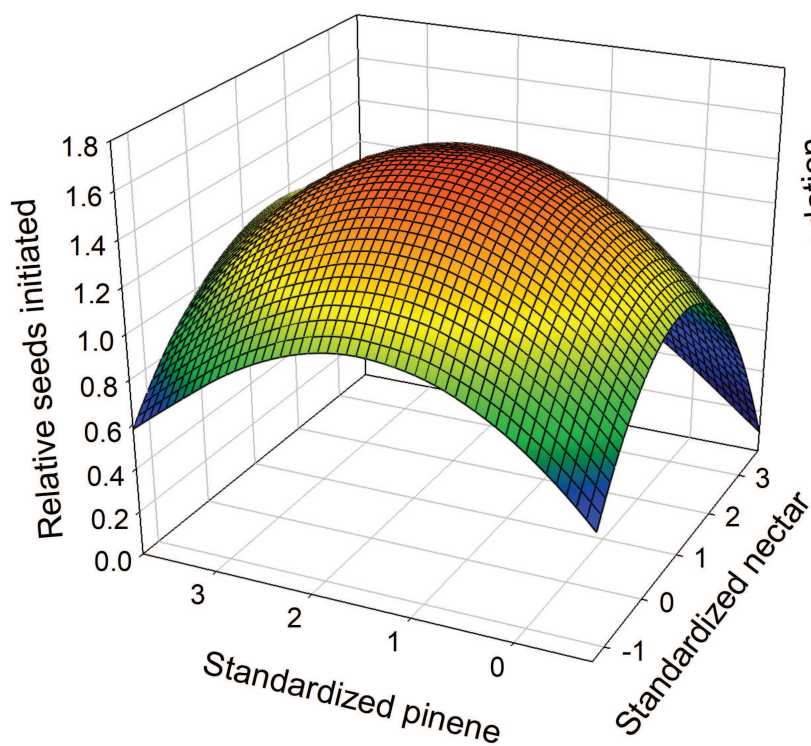
Figure 4

This is the author's accepted manuscript without copyediting, formatting, or final corrections. It will be published in its final form in an upcoming issue of The American Naturalist, published by The University of Chicago Press. Include the DOI when citing or quoting: <https://doi.org/10.1086/716740>
Copyright 2021 The University of Chicago Press.

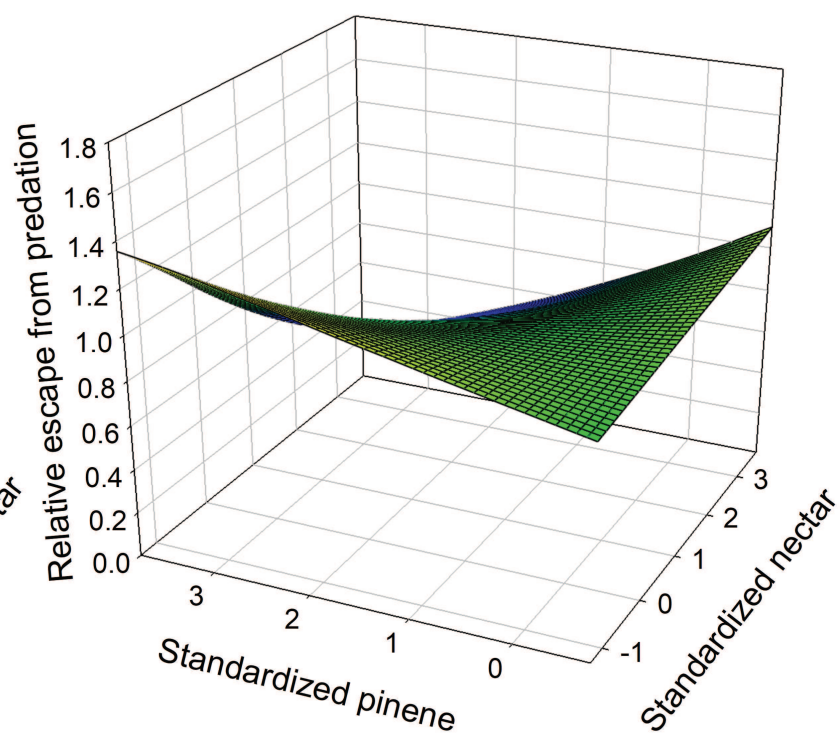


A
*directional on pinene**
*stabilizing on nectar**

B
*directional on pinene**
*directional on nectar**
*stabilizing on nectar**



C
*correlational**



Selection of floral traits by pollinators and seed predators during sequential life history stages

Diane R. Campbell^{1,2}, Mascha Bischoff², Robert A. Raguso³, Heather M. Briggs^{1,2}, Paula Sosenki^{1,2,4}

1. Department of Ecology and Evolutionary Biology, University of California, Irvine, California 92617

2. Rocky Mountain Biological Laboratory, Crested Butte, Colorado 81224

3. Department of Neurobiology and Behavior, Cornell University, Ithaca, New York 14853

4. CONACYT- Universidad Autonoma de Yucatan, Merida, Mexico.

*Corresponding author; email: drcampbe@uci.edu.

American Naturalist

Supplemental Material

Tables S1 – S3, Figures S1 – S4

Table S1. Pearson correlation coefficients between floral traits at all sites combined (with sample size of plants in parentheses). Values significant at $P < 0.05$ after sequential Bonferroni correction are bolded.

	Width	Color	Nectar	Sepal width	Pinene
Color	0.25 (342) P < 0.0001				
Nectar	0.31 (274) P < 0.0001	0.30 (272) P < 0.0001			
Sepal width	0.12 (108) P = 0.2113	-0.08 (104) P = 0.3950	-0.08 (96) P = 0.4620		
Pinene	0.15 (199) P = 0.0322	0.09 (195) P = 0.2301	-0.14 (180) P = 0.0625	0.28 (93) P = 0.0066	
Indole	0.12 (170) P = 0.1347	-0.19 (166) P = 0.0166	-0.13 (154) P = 0.1096	0.22 (95) P = 0.0306	0.07 (129) P = 0.4096

Table S2. Eigenvalues and eigenvectors from canonical analysis. Only the two vectors with largest absolute value for the eigenvalue are shown. Eigenvalues (λ) in bold are significantly different from zero ($P < 0.05$) based on 2nd order polynomial regression of relative fitness on the two eigenvector axes.

			Eigenvector coefficients					
Fitness component	Canonical axis	λ	Width	Color	Nectar	Sepal	Pinene	Indole
Total seeds	1	0.30	0.46	-0.35	-0.06	-0.57	0.28	0.36
	2	-0.17	-0.02	0.06	-0.25	0.12	-0.24	0.32
Seeds initiated	1	0.27	0.38	-0.36	-0.07	-0.55	0.26	0.32
	2	-0.15	-0.04	0.07	-0.24	0.08	-0.23	0.34
Prop. escaped predation	1	-0.04	-0.29	0.15	0.66	-0.01	0.24	0.08
	2	0.03	0.25	0.31	0.09	-0.09	0.06	0.09

Table S3. Further breakdown of selection gradient estimates for the pairwise trait combinations for which correlational selection was detected and for the combination of volatile emissions.

Estimates are provided for directional selection gradients (β_1 and β_2 for the two traits in the order written), quadratic selection gradients (γ_{11} and γ_{22}), and correlational selection (γ_{12}). Directional gradients were obtained from a model with just the linear terms. Quadratic selection gradients were obtained by doubling the quadratic regression coefficients in the full second order regression (Stinchcombe et al. 2008). Bold typeface indicates estimates that differed significantly from zero at $P < 0.05$. N = number of plants. P value is for the full second order regression. Selection estimates are based on flower number, fruits per flower, and seeds per fruit as three multiplicative components of seeds initiated. For comparison, selection estimates based on seeds initiated and escape from predation are included.

Traits	Fitness component	β_1	β_2	γ_{11}	γ_{22}	γ_{12}	N	P
Width, Color	Flowers	0.15	-0.05	0.02	0.13	-0.08	342	0.0180
	Fruits/flower	0.11	-0.04	-0.02	0.00	-0.06	333	0.0178
	Seeds initiated/fruit	0.18	0.03	0.15	0.02	-0.04	305	<0.0001
	Seeds initiated	0.42	-0.15	0.28	0.15	-0.23	327	<0.0001
	Prop. escaped predation	-0.03	-0.01	-0.05	0.02	0.00	307	0.5284
Nectar, Sepal	Flowers	-0.00	-0.05	-0.36	0.05	-0.01	96	0.4570
	Fruits/flower	-.13	-0.00	0.05	0.00	0.13	96	0.1217
	Seeds initiated/fruit	-0.02	0.15	0.12	0.04	0.14	89	0.0004

	Seeds initiated	-0.15	0.04	-0.27	0.09	0.18	95	0.2094
	Prop. escaped predation	-0.07	-0.05	0.04	0.04	-0.02	89	0.0759
Nectar,	Flowers	0.07	0.13	-0.14	-0.13	0.13	180	0.0175
Pinene	Fruits/flower	-0.04	-0.04	-0.06	-0.03	-0.09	180	0.5199
	Seeds initiated/fruit	0.06	0.09	-0.04	-0.02	-0.01	172	0.0473
	Seeds initiated	0.07	0.11	-0.26	-0.22	-0.00	177	0.0693
	Prop. escaped predation	-0.01	0.03	0.00	0.00	-0.07	174	0.2003
Pinene,	Flowers	0.05	0.09	-0.10	0.11	0.22	128	0.1580
Indole	Fruits/flower	0.05	0.13	-0.13	0.09	-0.10	128	0.0352
	Seeds initiated/fruit	0.15	0.04	-0.13	-0.21	-0.03	123	<0.0001
	Seeds initiated	0.17	0.21	-0.38	0.03	0.06	126	0.0018
	Prop. escaped predation	0.02	-0.11	0.04	-0.05	-0.01	123	0.1376

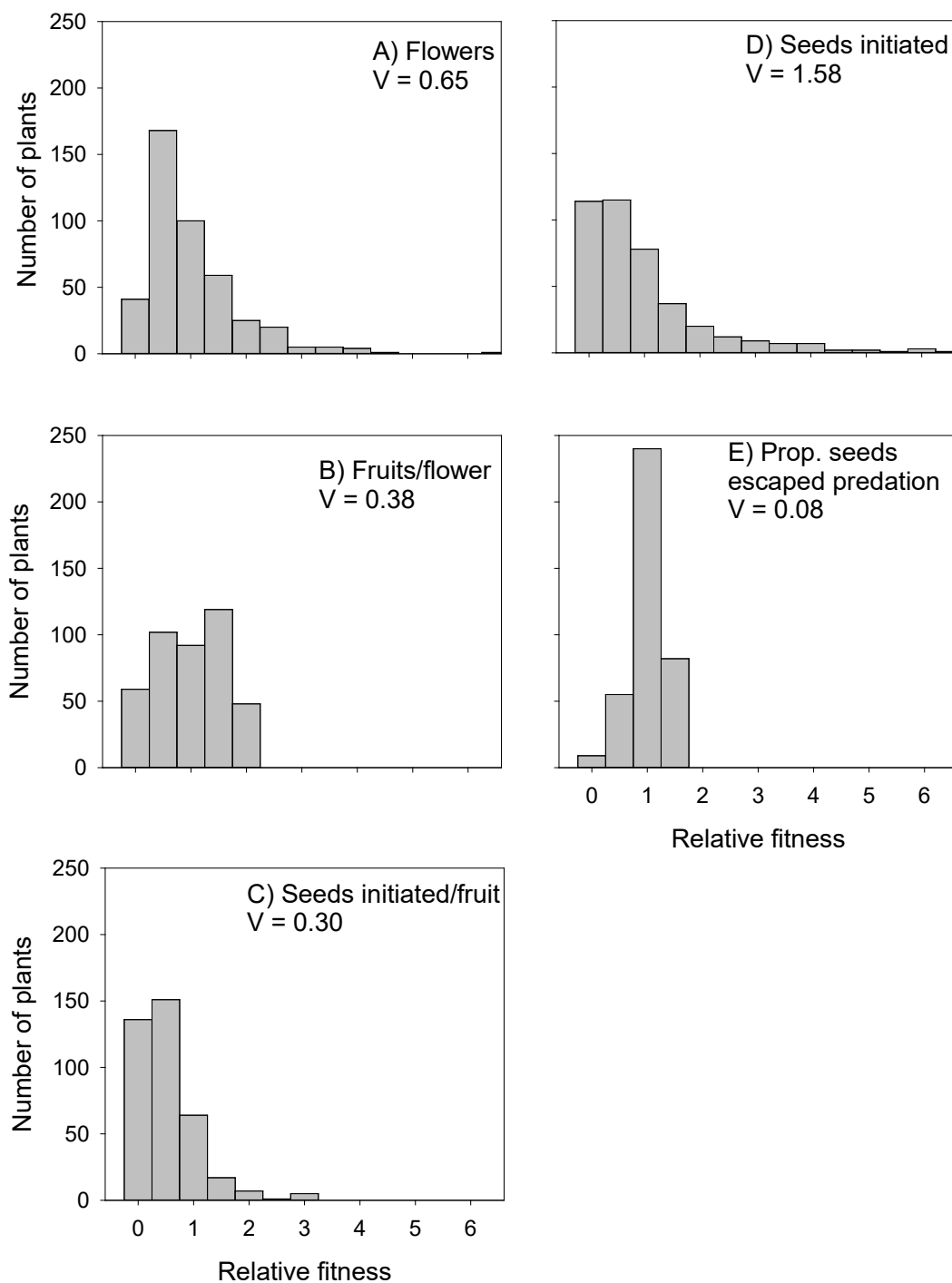


Figure S1: Histograms showing distributions of relative fitness components. The panels on the left (A, B, C) show the individual fitness components that multiply to generate seeds initiated. The panels on the right (D, E) show a direct comparison of the distributions for relative seeds initiated and relative proportion escaped predation. V: Variance in relative fitness (opportunity for selection).

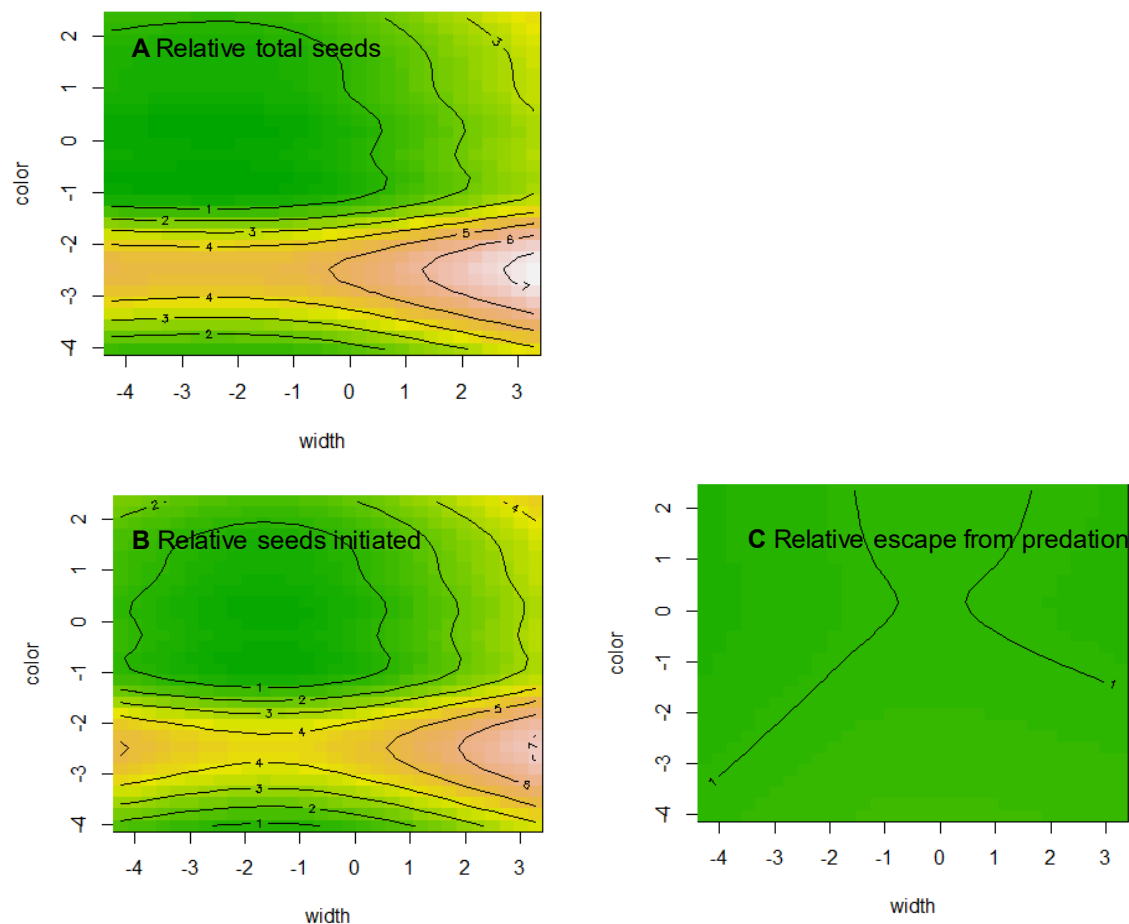


Figure S2: Selection surfaces for corolla width and petal color at the *I. aggregata* site. Contour lines for fitness indicate (A) relative total seeds, (B) relative seeds initiated, or (C) escape from seed predation. Surfaces were fit using the function `vis.gam` to plot the results of a generalized additive model in R function `mgcv` using a thin plates regression spline smooth. Color and width are standardized to a mean of 0 and SD of 1. Both color and width significantly influenced relative total seeds and relative seeds initiated (all $P < 0.05$ in the gam model) but not relative escape from predation. Total seeds was highest for plants with wide flowers and relatively low petal color. Correlational selection is evident in (A) and (B); for example, the effect on fitness of

increasing width from 1 to 3 is high for plants with a color value near -2.5 but low for plants with a color value near -1.5. Non-linear selection on both width and color can be seen in (B).

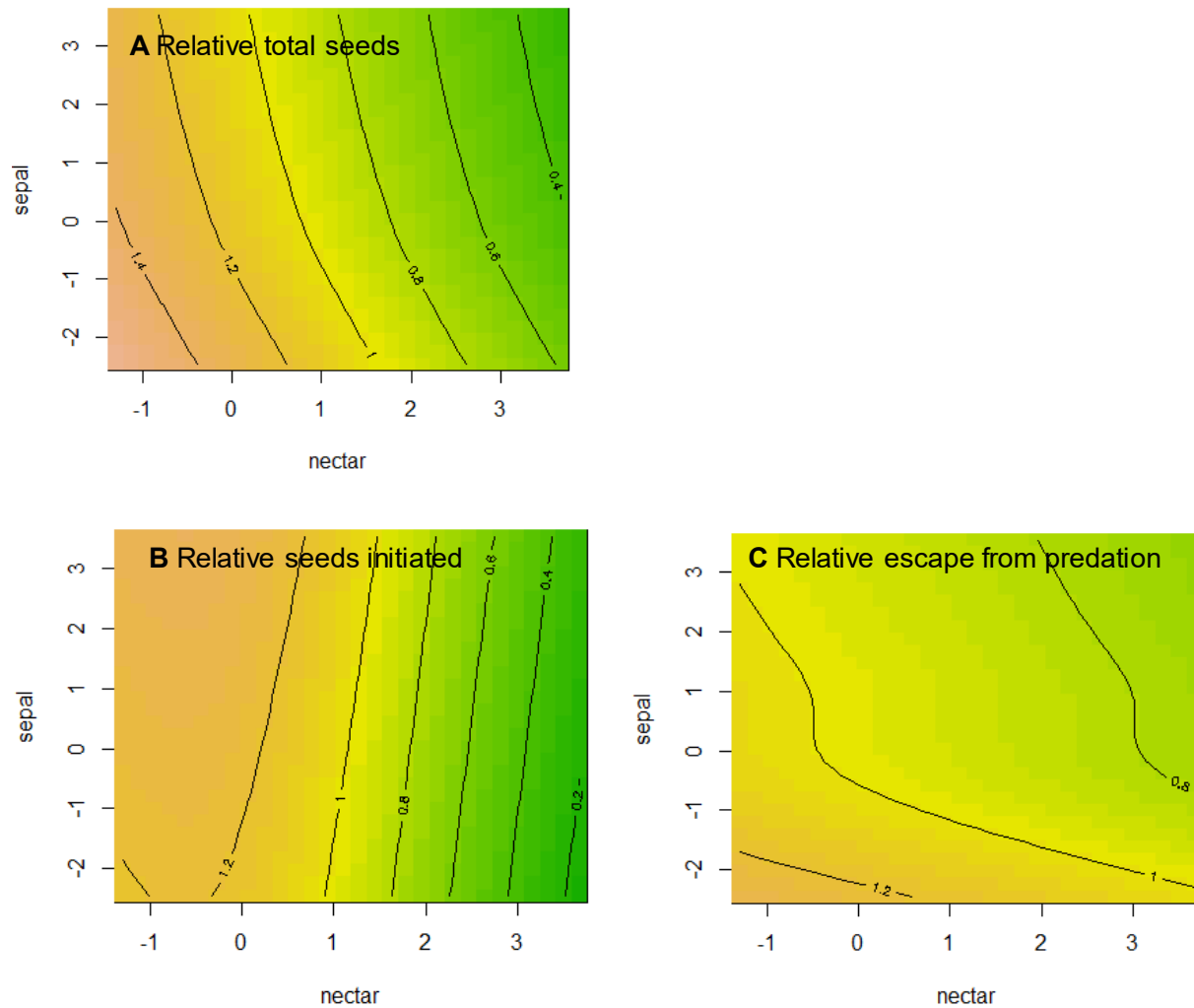


Figure S3: Selection surfaces for nectar production and sepal width at all sites combined. Contour lines for fitness indicate (A) relative total seeds, (B) relative seeds initiated, or (C) escape from seed predation. Surfaces were fit using the function `vis.gam` to plot the results of a generalized additive model in R function `mgcv`. Nectar and sepal are standardized to a mean of 0 and SD of 1. Total seeds was highest for plants with narrow sepals and low nectar production.

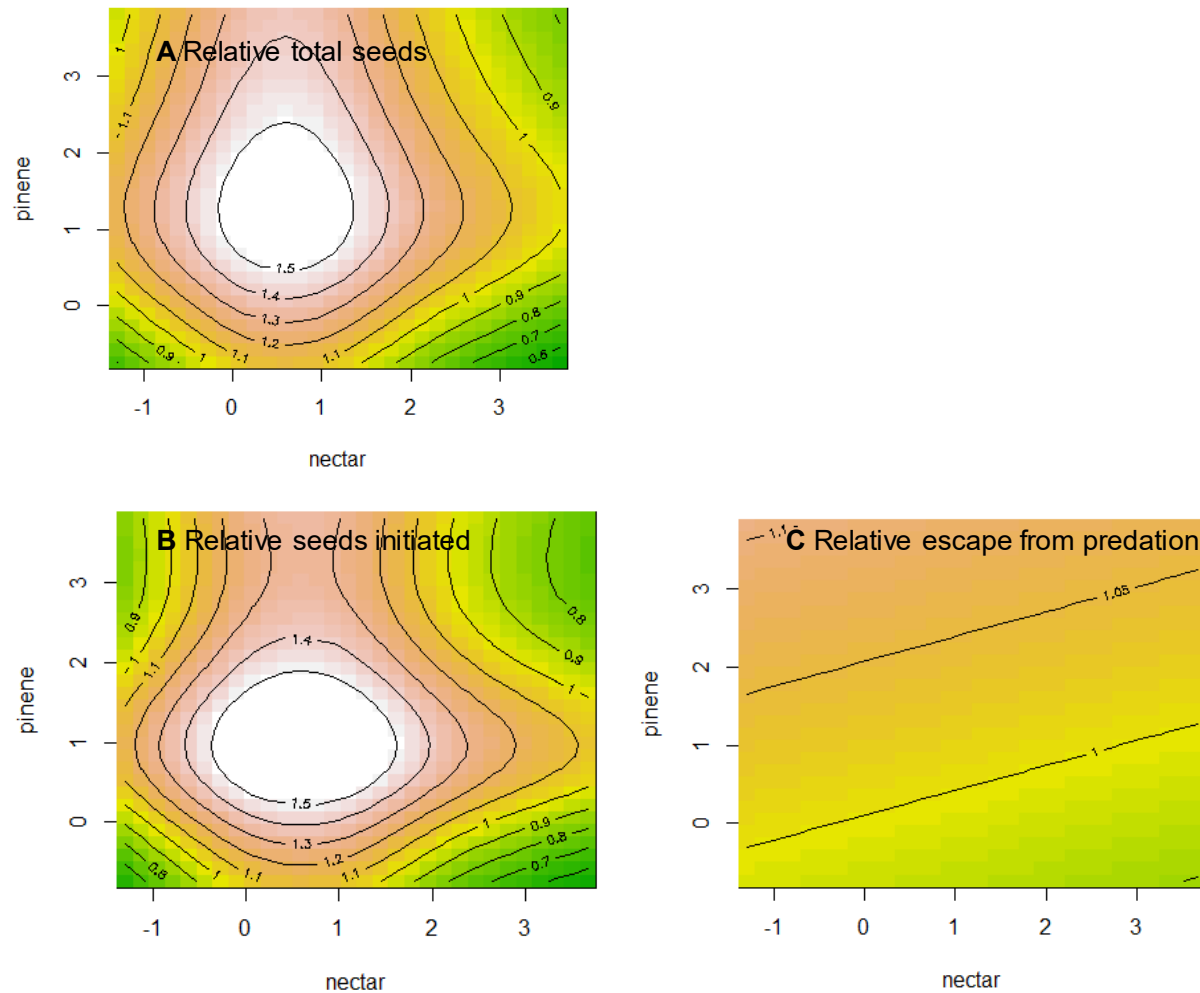


Figure S4: Selection surfaces for nectar production and pinene emissions at all sites combined. Contour lines for fitness indicate (A) relative total seeds, (B) relative seeds initiated, or (C) escape from seed predation. Surfaces were fit using the function `vis.gam` to plot the results of a generalized additive model in R function `mgcv`. Nectar and pinene are standardized to a mean of 0 and SD of 1. Total seeds was highest for plants with intermediate levels of pinene emission and nectar production.