

Strong evidence for positive and negative correlational selection revealed by recreating ancestral variation

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Abstract

The study of adaptation helps explain biodiversity and predict future evolution. Yet the process of adaptation can be difficult to observe due to limited phenotypic variation in contemporary populations. Furthermore, the scarcity of male fitness estimates has made it difficult to both understand adaptation and evaluate sexual conflict hypotheses. We addressed both issues in our study of two anther position traits in wild radish (*Raphanus raphanistrum*): anther exertion (long filament – corolla tube lengths) and anther separation (long – short filament lengths). These traits affect pollination efficiency and are particularly interesting due to the unusually high correlations among their component traits. We measured selection through male and female fitness on wild radish plants from populations artificially selected to recreate ancestral variation in each anther trait. We found little evidence for conflicts between male and female function. We found strong evidence for stabilizing selection on anther exertion and disruptive selection on anther separation, indicating positive and negative correlational selection on the component traits. Intermediate levels of exertion are likely an adaptation to best contact small bees. The function of anther separation is less clear, but future studies might investigate pollen placement on pollinators and compare species possessing multiple stamen types.

Keywords: correlational selection, stabilizing selection, disruptive selection, sexual conflict, pollination efficiency, heteranthery

Adaptation is the process by which organisms become better fitted to their environments through natural selection (Brandon, 2014). Because traits can also be shaped by non-adaptive processes such as genetic drift, determining the relationship between trait variation and fitness is critical for inferring adaptation in present-day populations (Stearns, 2014). However, such studies of adaptation are often limited by a lack of natural trait variation because past selection has removed less fit variants from the population (Grafen, 1988; Haller & Hendry, 2014). The most common method to deal with this problem is direct experimental manipulation of the putative adaptive trait (Sinervo & Basolo, 1996), but many traits cannot be directly manipulated, and appropriate controls can be difficult. For some traits and systems, a more feasible alternative method for overcoming this limitation is using artificial selection to expand the range of phenotypic variation in a trait of interest, ideally recreating at least some of the ancestral variation that may have been removed by past selection (Conner, 2003). Experimental populations can then be placed in the field and natural selection acting on the selected trait can be estimated.

Selection may also act on trait combinations, favoring either a higher (positive correlational selection) or lower

(negative correlational selection) product of the trait values. Quantitative genetic theory predicts that positive correlational selection on a pair of traits will increase the genetic correlation between those traits, while negative correlational selection will decrease the genetic correlation (Lande, 1980); a meta-analysis of empirical studies supported this prediction (Roff & Fairbairn, 2012). As for single traits, selection may rapidly deplete variance in multivariate trait space (Walsh & Blows, 2009), limiting our ability to study the evolution of functionally integrated traits.

Another limitation of past studies, particularly in plants, is that the majority of field-based measures of selection use only female fitness, ignoring half of the alleles contributed to the next generation. For example, selection through male fitness in plants was included in 0/44 studies reviewed by Geber and Griffen (2003), 10/37 studies reviewed by Kingsolver and Diamond (2011), and 0/36 studies reviewed by Caruso et al. (2019). Estimates of male fitness are expected to be especially important for studying male reproductive morphology in outcrossing species (Conner, 2006; Stephenson & Bertin, 1983). In some cases, selection through male and female functions may be conflicting or of differing magnitude (Ashman & Morgan, 2004). In plants,

selection through male fitness should increase as the supply of fertilizable ovules declines, increasing competition for those remaining, and selection through female fitness should increase with increasing pollen limitation of seed set (Burd & Callahan, 2000; Stephenson & Bertin, 1983; Wilson et al., 1994). Selection through male fitness should also increase with decreasing availability of pollination services, which are often distributed unevenly in time and space (Stanton, 1994).

For animal-pollinated plants, floral adaptations can be broadly grouped into those that influence pollinator attraction (e.g., petal size and shape, scent, color; Campbell et al., 2010; Huber et al., 2005), reward (e.g., nectar volume and concentration; Mitchell, 1993), or efficiency (e.g., corolla shape, placement of anthers and stigma; Campbell, 1991; Muchhala, 2007). Because pollination efficiency is partially dependent on how well pollinator bodies contact the pollen-dispensing anthers, the position of anthers within flowers might be adaptive, particularly for male function. While a great deal of research has examined the function of discrete variation in anther position in heterostylous species (reviewed in Barrett & Shore, 2008), the influence of quantitative variation in anther position on fitness is less clear.

One aspect of anther position within flowers is anther exsertion, defined as the difference in length between the filaments and the corolla tube (Figure 1). Comparisons across species suggest that greater exsertion is associated with flowers that are more generalized (Vaknin, 2009) and bird-pollinated rather than bee-pollinated (Castellanos et al., 2004). Within species, single-visit pollen removal was highest from highly exserted anthers in *Ipomoea trichocarpa* (Murcia, 1990) and intermediately exserted anthers in wild radish (*Raphanus raphanistrum* spp. *raphanistrum*; Conner et al., 1995).

To our knowledge, the fitness outcomes of variation in anther exsertion have only been studied in two species. In

Silene stellata, no significant selection gradients for exsertion through male or female fitness were found (Zhou et al., 2020). In wild radish, there was strong evidence for stabilizing selection through male fitness in 1 year, positive directional selection in a second year, and a combination of both stabilizing and directional selection in the third year (Morgan & Conner, 2001, a reanalysis of Conner et al., 1996b). In an experiment isolating individual pollinator taxa, small bees caused stabilizing selection on anther exsertion through both male and female fitness (Sahli & Conner, 2011), likely because small bees remove more pollen from intermediately exserted anthers in this species (Conner et al., 2009). Thus, there is inconsistent evidence for stabilizing selection on anther exsertion.

In addition to flowers varying in anther position relative to the corolla tube, some flowers possess multiple stamens whose anthers differ in position relative to each other. The term heteranthery is often applied to cases where stamens are both morphologically and functionally differentiated (Vallejo-Marín et al., 2010). Three main hypotheses to explain intrafloral stamen differentiation have been proposed. The “division of labor” hypothesis posits that short and brightly colored stamens are specialized for feeding pollinators, while long and cryptic stamens are specialized for pollen export; evidence supporting this hypothesis comes primarily from nectarless, bee-pollinated flowers (Jesson & Barrett, 2003; Luo et al., 2009; Müller, 1883; Vallejo-Marín et al., 2009). The second hypothesis is that intrafloral differences in stamen lengths represent specialization for outcrossing versus self-pollination due to differences in the position of the anthers relative to the stigma (Escaravage et al., 2001; Kudo, 2003). The final hypothesis is that intrafloral stamen differentiation is a mechanism for gradual pollen presentation (Conner et al., 2003; Kay et al., 2020). If pollen resources are limited, then male fitness depends on the deployment of pollen over time (Harder

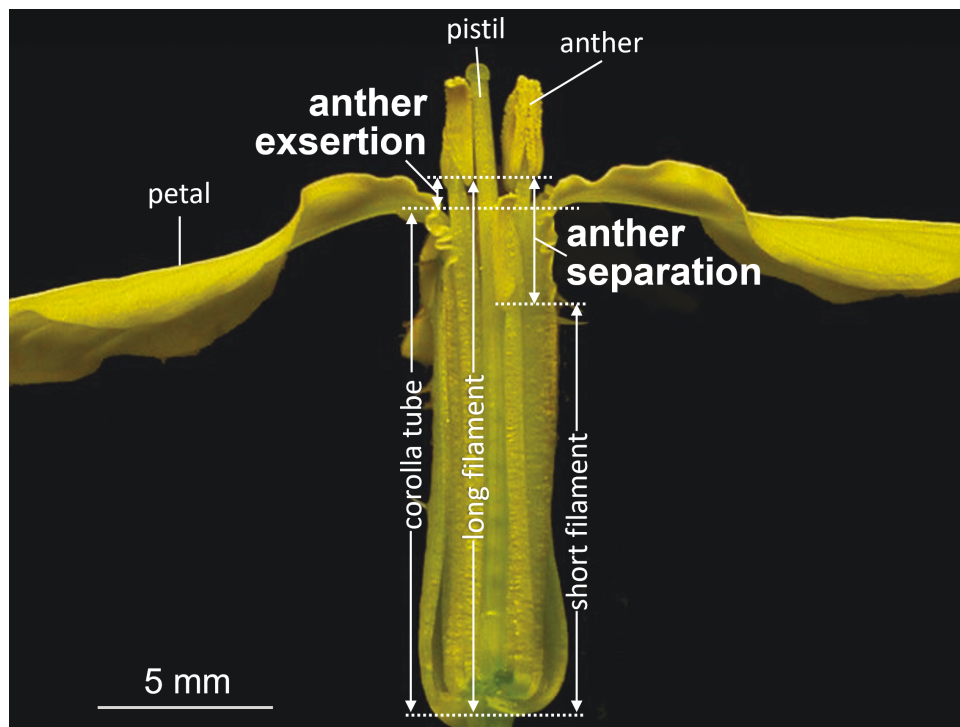


Figure 1. Floral anatomy of wild radish. The focal traits, anther exsertion and anther separation, are shown in bold.

& Thomson, 1989; Stanton, 1994), given that pollinators can rapidly remove pollen from virgin flowers (Kudo, 2003; Rush et al., 1995). When pollinators are abundant, restricting single-visit pollen removal is expected to increase male fitness because as the amount of pollen removed in a single visit increases, the percentage of that pollen deposited on stigmas decreases (Harder & Thomson, 1989), perhaps due to increased grooming behavior (Thomson, 1986).

This pollen presentation hypothesis is supported by the finding of staggered dehiscence or maturation of different anther types in *Clarkia*, *Digitalis*, and *Axinaea* (Dellinger et al., 2021; Kay et al., 2020; Percival & Morgan, 1965). Studies in *Brassica rapa* and wild radish have found that single-visit pollen removal from long anthers was higher than from short anthers (Conner et al., 1995, 2003; Kudo, 2003). Conner et al. (2003) also found that in wild radish, single-visit pollen removal increased with decreasing anther separation (Figure 1). We might expect the current population mean anther separation to reflect an optimal level of pollen removal. Field-based estimates of selection on this trait found stabilizing selection in 1 of 3 years (Conner et al., 2003), but only negative directional selection by honey bees in a cage experiment (Sahli & Conner, 2011).

Wild radish is a good study system because the species is a self-incompatible annual, allowing for straightforward measurements of lifetime male and female fitness. Furthermore, much of the limited prior work on the adaptiveness of quantitative variation in anther position has been done using this species, providing information on agents of selection (pollinators), the effects of anther position on pollen removal, and estimates of selection acting on anther position in natural populations and in expanded-variation populations with specific taxa (reviewed above). Previous estimates of selection on anther exertion and separation in wild radish have produced inconsistent and sometimes conflicting results, potentially due to the low variation in these traits caused by the very high phenotypic correlations between the three component traits (0.76–0.89; Conner & Via, 1993). To address this, in the present study, we measured selection through male and female fitness on the expanded variation that partially recreated the ancestral condition of both anther exertion and anther separation in wild radish. This expanded variation was accomplished by selecting for both higher and lower anther exertion (Conner et al., 2011), but only lower anther separation (Conner et al., in prep) to represent the inferred ancestral states of greater variation in anther exertion (Conner et al., 2009) and zero anther separation (six equal-height anthers in Capparaceae, sister group to Brassicaceae; Hall et al., 2002). Despite only selecting in one direction, anther separation variance was expanded nearly symmetrically around the original mean due to an increase in anther separation in one of the control lines owing to some combination of plasticity to greenhouse conditions across generations and drift (Conner et al., in prep). The present study improves our ability to make inferences about adaptation by measuring selection throughout the normal flowering season by all naturally occurring pollinators in plants with a recreated ancestral variation.

We specifically aimed to answer the following questions: (1) Is there evidence for conflicting selection on anther exertion and separation between male and female fitness? and (2) What is the form and magnitude of selection on anther exertion and separation? We predicted that the magnitude of selection on these anther position traits will be greater through

male fitness, but that there will be little conflict between functional sexes. This is consistent with prior studies finding selection through male but not female fitness for anther separation (Conner et al., 2003), selection through male fitness for anther exertion (Morgan & Conner, 2001), and selection through male but not female fitness for anther exertion in the cage experiment (Sahli & Conner, 2011). Based on prior functional studies (see above) and the fact that reduced variation is derived, we predicted that both anther traits will be under stabilizing selection in our expanded-variation populations, equivalent to positive correlational selection on the component trait pairs.

Materials and methods

Study species

Wild radish is native to the Mediterranean but is now globally distributed as a major agricultural weed (Holm et al., 1997). Flowers of this species contain two short lateral stamens and four long medial stamens (tetradynamy), a diagnostic trait of the Brassicaceae family. The relative positioning of the stamens within a flower can be described using the two composite traits, anther exertion and anther separation (Figure 1). Wild radish is effectively pollinated by at least 15 genera of insects from at least three orders: Hymenoptera, Lepidoptera, and Diptera (Sahli & Conner, 2007). The wild radish plants in the present study are the weedy ecotype (Charbonneau et al., 2018), derived from the New York population used in our previous radish studies.

Artificial selection lines

Artificially selected lines were derived from two separate experiments: The first generated two lines selected for increased exertion (long filament length – corolla tube length), two lines selected for decreased exertion, and two control lines; the second generated two lines selected for low anther separation (long filament length – short filament length, Figure 1; syn. “stamen dimorphism” [Sahli & Conner, 2011] and “anther dimorphism” [Conner et al., 2003; Sapir et al., 2017]) and two control lines (see Conner et al., 2011, for further details on artificial selection). Artificial selection increased variance across lines by a factor of 3.3 for anther exertion and 1.8 for anther separation.

Following 5–6 generations of selection, four plants from each matriline (12 matriline × 6 selection lines for exertion; 20 matriline × 4 selection lines for anther separation) were grown in a common greenhouse environment at the W.K. Kellogg Biological Station (KBS; Hickory Corners, MI). Anther exertion and anther separation were measured on the third flower to open on each plant. One plant from each matriline was assigned to equal-sized groups, each with equal representation from each artificial selection line (three groups of 24 for exertion; four groups of 20 for anther separation). The groups were chosen to create the most uniform anther trait distributions possible. After the groups were chosen, each pot was assigned a unique identification number, so that the rest of the experiment was blind with respect to the selection line of origin. The first bud produced by each plant was collected and genotyped at four (2001) or eight (2002) microsatellite loci for paternity analysis. Pollen number per flower was measured from all anthers on the fourth flower to open (details in Rush et al., 1995). In the anther exertion experiment, 100–200 pollen grains from the long anther of one

flower were stained with fluorescein diacetate and pollen viability was defined as the percent of fluorescing pollen grains out of the total. In the anther separation experiment, experimental crosses between pollen donor plants and the same set of 10 randomly selected emasculated plants were carried out, and pollen viability was defined as the mean number of seeds produced by each pollen donor from all of its crosses.

Field trials

On 20 dry days from June through August 2001 (the core flowering period of wild radish in Michigan), one group of exsertion selection plants was placed in the field site at KBS. In the following year, this process was repeated on 27 dry days using the anther separation selection groups. Plants were spaced 1 m apart in a rectangular grid (4 × 6 for exsertion and 4 × 5 for anther separation). On each day, plants were exposed to natural pollinators for 6.6 hr on average (range = 3.5–12.0). Pollinators were primarily small native bees and syrphid flies (Sahli & Conner, 2007), as is typical in the Midwestern USA (Conner et al., 2009). Floral traits—long and short filament lengths, tube length, pistil length, petal width, petal length, and ovule number per flower—were measured on one recently opened flower on each plant each day and averaged across days to provide a single trait value per plant for selection analyses. Note that in this species, within-plant variation for flowers of similar age is lower than among-plant variation (Williams & Conner, 2001). The additional linear traits were included in a measure of overall flower size (see below), a trait that affects pollinator visitation and fitness in many species including wild radish (Conner & Rush, 1996; Conner et al., 2003; Morgan & Conner, 2001). Fruits were collected as they reached maturity. The flowers that were open at the time of each field trial were marked with a unique color of tape, so that seeds produced during each trial could be identified. The average pollinator visitation rate was calculated as the daily mean number of pollinator visits per flower per hour, based on 4–20 (mean = 19) plants observed per day in 10-min observational periods (previously described in Sapir et al., 2017).

This entire procedure was repeated daily with a different group of plants, cycled through sequentially so that each group was in the field for 6–7 days by the end of the season. Group composition remained constant throughout, but grid positions were re-randomized daily. This approach was taken (rather than leaving all plants in the field throughout the season) to restrict the number of potential fathers for the paternity analysis. At the conclusion of the field season, all fruits and seeds were counted and a random sample of seeds from each plant (~3% of total seeds produced, Figure S1A) was drawn from across collection days based on the proportions of total seeds produced by each plant on each day in the field. These offspring (total $N = 2,544$) were genotyped at the microsatellite loci and paternity was analyzed using Cervus 3.0 software (Kalinowski et al., 2007). In 2001, the most likely father was identified for 978 offspring, and these data were used for male fitness. With eight microsatellite loci in 2002, we were able to use a more stringent criterion, using the 1,220 offspring for which fathers were identified with 95% confidence, resulting in 2,198 total offspring assigned across the 2 years (Figure S1B; Kalinowski et al., 2007). Although plants were protected from mortality, seed production summed across field trials (mean = 540) was comparable

to seed production of plants from the same population grown in the field (Conner et al., 1996a).

Statistical analyses

Statistical analyses were conducted in JMP® Pro v. 14.3 (SAS, 2021) except where noted. All figures were created in R v. 4.0.3 (R Core Team, 2020) using the “ggplot2,” “patchwork,” “rsm,” and “plotly” packages (Carson, 2020; Hadley, 2016; Russell, 2009; Thomas Lin, 2020).

We conducted a set of selection gradient analyses, where relative fitness estimates were regressed on the artificially selected traits and other measured traits simultaneously in order to remove indirect selection resulting from trait correlations (Lande & Arnold, 1983). These analyses were conducted ignoring the selection lines that plants originated from, since our goal was to create new populations with increased phenotypic variation.

For each experiment, the conservative full model included as predictors (all standardized) the artificially selected trait along with the other floral traits, either individually (pollen number, ovule number, pollen viability) or as a component of flower size (first principal component of petal length + petal width + pistil width + tube length + short filament length + long filament length). However, the nonartificially selected anther position trait was not included in the models because of either multicollinearity issues or reduced model fit (assessed using adjusted R^2 in all cases). The inclusion of all floral traits maximized model fit for the anther separation models; for exsertion models, including pollen number only slightly lowered R^2 -adj. (−0.01). The plant group was also included as a categorical variable. The number of open flowers during field trials was not included because it did not improve model fit, was not significant in all cases, and did not qualitatively change any results. The response variables (tested in separate models) were the relative total number of seeds sired (male fitness), the relative total number of seeds produced (female fitness), and the relative total hermaphroditic fitness: female fitness + (male fitness × [mean female fitness/mean male fitness]). In a response surface design, linear, cross-product, and quadratic terms were fit to test for directional, correlational, and variance selection (with doubled quadratic regression coefficients and error terms, Stinchcombe et al., 2008). Minima and maxima values of anther separation from the fitted curves and 95% confidence intervals were calculated with 10,000 bootstrapped samples using the “boot” package (Angelo & Ripley, 2020).

To more directly test for correlational selection, we ran similar models but replaced the artificially selected traits of anther exsertion and separation with their component traits (corolla tube and long and short filament lengths) as separate terms. However, these results should be interpreted with caution given the expected multicollinearity resulting from the strong trait correlations among the three component traits (variance inflation factor >34; but see Morrissey & Ruxton, 2018). Given that all of the above models included a high ratio of terms to sample sizes, we also ran two different sets of reduced models, removing either all cross-products or traits with no significant linear or nonlinear selection gradients (see Tables S5–S8 for model terms); removed traits were not highly correlated with retained traits (Figure S2). Results from the reduced models were qualitatively similar to the full models, with any significant deviations discussed in the results.

To test if traits had significantly different effects based on male versus female fitness, we used a linear mixed effects model with an unstructured covariance structure that included plant ID as the random subject variable and sex as the repeated variable, plus all of the traits and interactions in the full models above.

Results

Selection through male versus female fitness components

Variance in relative fitness (opportunity for selection, I) was greater for male than female function (Table S1), but not significantly so (exsertion experiment: $F = 1.53$, $df = 71$, $p = .08$; separation experiment: $F = 1.17$, $df = 77$, $p = .49$). For both artificially selected anther traits, quadratic selection through male fitness was ~52% stronger and more

significant than through female fitness (Tables S2 and S3), resulting in a slightly flatter selection gradient through total fitness (Figure 2B, D) compared with male fitness (Figure 2A, C). However, there was no significant interaction between sex and either of the anther trait quadratic terms (exsertion \times exsertion \times sex, $p = .51$; separation \times separation \times sex, $p = .54$; Table S4). In the exsertion experiment, the only trait with different effects on male versus female fitness was pollen viability ($\beta = -0.08$, $p = .04$; Table S3), with nearly significant selection for increased viability through male fitness ($\beta = 0.15$, $p = .06$; Table S2) but negligible selection through female fitness ($\beta = -0.02$, $p = .79$; Table S2). In the anther separation experiment, the only trait with significantly different effects on male versus female fitness was ovule number ($\beta = 0.10$, $p < .001$; Table S4), where high ovule number significantly increased female fitness ($\beta = 0.12$, $p = .008$; Table S3) but nearly significantly decreased male fitness ($\beta = -0.09$, $p = .07$; Table S3). Across all traits and terms

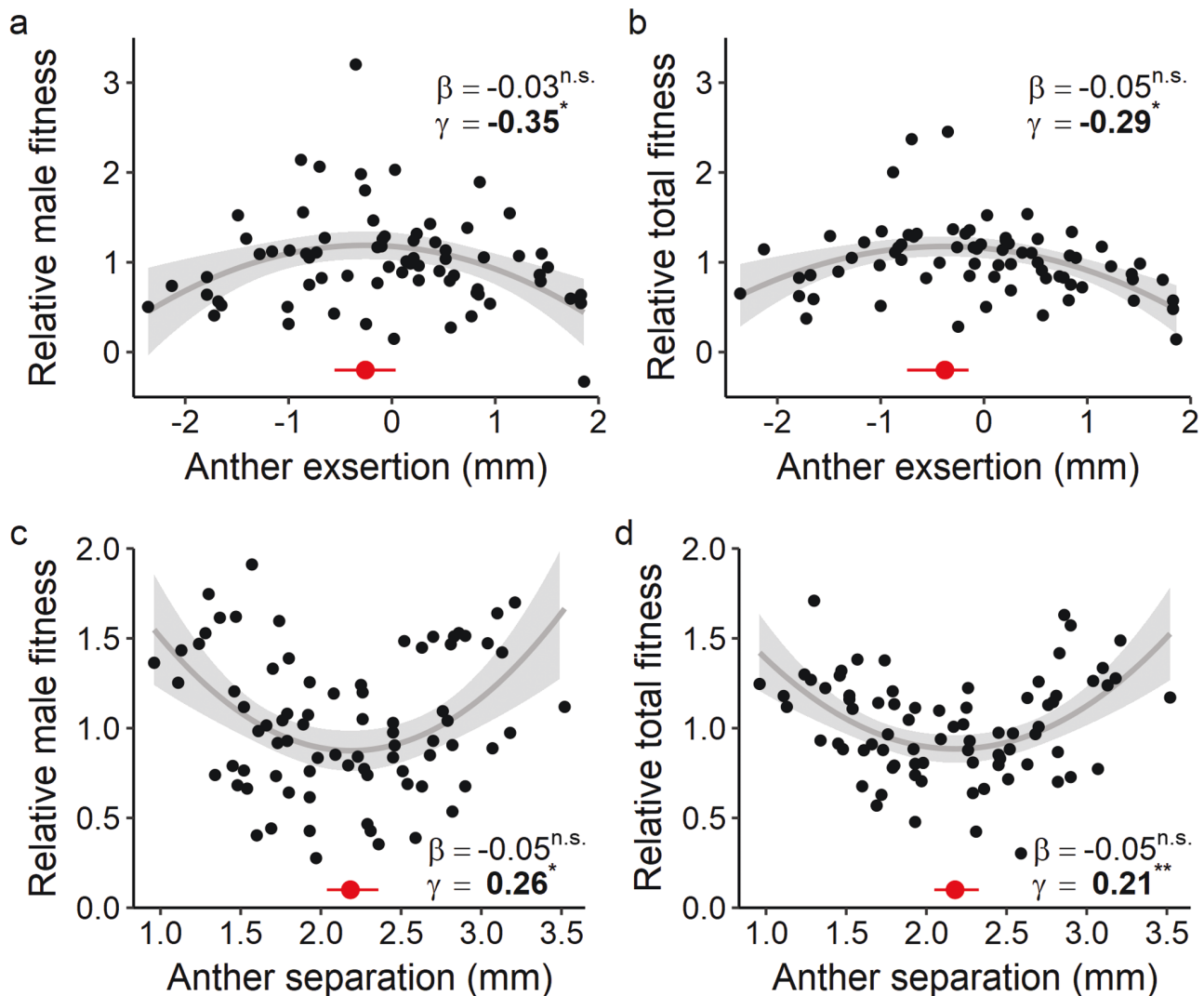


Figure 2. Selection gradients for anther exsertion (A and B) and anther separation (C and D) based on relative male (A and C) and total (B and D) fitness, after adjusting for the effects of the other floral traits using the model residuals and model-fitted values extracted from the predict() function. This visualization can be considered a “predictor effect display,” combining an effect plot with partial residuals (Fox & Weisberg, 2018). Beta and gamma coefficients for the effect of either anther exsertion or separation on fitness are shown (see Tables 1, S2, and S3 for full regression output, including for female fitness). Quadratic regression lines (dark gray) and 95% confidence bands (light gray) are shown. Bootstrapped minima and maxima x-values of the fitted curves (red points) and 95% confidence intervals (red lines) are shown at the bottom. Black points are individual plants. Note different scales for anther exsertion and separation. The gamma coefficients are still significant after excluding the topmost point in (A) and the top three points in (B).

in the models, there was no difference in the average magnitude of selection gradients for male versus female fitness in either the exertion experiment (male–female, paired $t = 0.41$, $df = 19$, $SE = 0.02$, $p = .69$) or the anther separation experiment (male–female, paired $t = 1.23$, $df = 19$, $SE = 0.01$, $p = .24$). Based on these results, we focus primarily on results from total fitness.

Selection on expanded variation in anther exertion

There was strong evidence for stabilizing selection on the expanded variation in anther exertion based on lifetime male (Figure 2A) and total fitness (Figure 2B; Table 1)—the quadratic terms in the regressions were significantly negative, the linear terms were not significant, and there were intermediate maxima in both. These results were highly consistent in both reduced models (Table S5). This relationship also represents positive correlational selection on tube length and long filament length, that is, selection to increase the correlation between these traits. Although not significant in the full model, the tube length \times long filament length cross-product was significantly positive in the first reduced model (Table S6). The fitness surface shows a ridge, with high fitness in flowers with similar filament and tube lengths and low fitness with either small tubes and large long filaments or large tubes and small long filaments (Figure 3A, Figure S1A). Collapsing the data along the ridge rotates the

3D surface to show the stabilizing selection acting on the difference between the traits—another exertion (see Brodie, 1992).

The maximum of the total fitness function for anther exertion occurred at -0.38 mm (95% CI = $[-0.74, -0.15]$). This value is smaller than the means of both the nonselected control group (0.03 mm, 95% CI = $[-0.28, 0.19]$) and the greenhouse-grown first generation of plants from the source population (-0.26 mm, 95% CI = $[-0.32, -0.19]$, $N = 340$; Conner & Via, 1993), but all three confidence intervals overlap.

Based on total fitness, there was a positive correlational selection on exertion and flower size (Table 1, Figures 3B and S1B). The fitness surface forms a ridge, with higher fitness where both flower size and exertion have similar relative magnitudes and lower fitness with large exertion combined with small flowers and vice versa. There was also a negative correlational selection on exertion and pollen viability (Table 1, Figures 3C and S1C). The fitness surface forms a saddle, where fitness is maximized with high values of one trait and low values of the other and minimized with high or low values of both traits.

Selection on expanded variation in anther separation

There was strong evidence for disruptive selection on the expanded variation in anther separation based on lifetime

Table 1. Selection gradients for the artificially selected trait (anther separation or anther exertion), ovule number, flower size, pollen number, and pollen viability through total fitness.

Term	Anther exertion				Anther separation			
	$R^2 =$	0.481	$R^2_{Adj} =$.248	$R^2 =$	0.562	$R^2_{Adj} =$.375
	Estimate	Std error	t-Ratio	p	Estimate	Std error	t-Ratio	p
Artificially selected trait	−0.05	0.06	−0.92	.361	−0.05	0.04	−1.36	.180
Ovule number	0.04	0.06	0.70	.484	0.02	0.04	0.46	.645
PC1 flower size	0.05	0.06	0.70	.485	0.02	0.04	0.51	.610
Pollen number	0.01	0.06	0.10	.920	0.02	0.04	0.62	.540
Pollen viability	0.07	0.06	1.14	.259	0.03	0.05	0.65	.518
Artif sel trait \times Artif sel trait	−0.29	0.11	−2.58	.013*	0.21	0.07	3.05	.004**
Ovule # \times Ovule #	−0.07	0.09	−0.87	.391	0.08	0.06	1.41	.164
Flwr size \times Flwr size	−0.20	0.13	−1.46	.152	−0.04	0.07	−0.51	.610
Pollen # \times Pollen #	−0.05	0.10	−0.47	.640	0.06	0.05	1.21	.230
Pollen viab \times Pollen viab	0.15	0.11	1.35	.185	−0.04	0.07	−0.59	.558
Artif sel trait \times Ovule #	−0.02	0.09	−0.23	.818	−0.11	0.04	−2.82	.007**
Artif sel trait \times Flwr size	0.26	0.08	3.24	.002**	−0.06	0.05	−1.18	.243
Ovule # \times Flwr size	0.06	0.10	0.58	.567	0.05	0.05	1.00	.321
Artif sel trait \times Pollen #	−0.06	0.07	−0.90	.370	0.04	0.04	1.03	.309
Ovule # \times Pollen #	0.09	0.09	0.96	.342	−0.06	0.04	−1.55	.126
Flwr size \times Pollen #	−0.06	0.10	−0.60	.549	−0.04	0.04	−0.97	.339
Artif sel trait \times Pollen viab	−0.18	0.07	−2.68	.010**	−0.03	0.04	−0.83	.410
Ovule # \times Pollen viab	0.00	0.08	−0.01	.990	−0.10	0.04	−2.52	.015*
Flwr size \times Pollen viab	0.00	0.07	0.02	.982	0.07	0.04	1.56	.125
Pollen # \times Pollen viab	0.08	0.08	1.03	.309	−0.05	0.04	−1.28	.207

Note: The plant group was included in the models but is not presented. Linear terms are from separate models including only linear terms. Estimates are β coefficients for linear terms and doubled γ coefficients (and doubled SE) for quadratic terms. Bolded rows are significant at $p < .05$. * $p < .05$, ** $p < .01$.

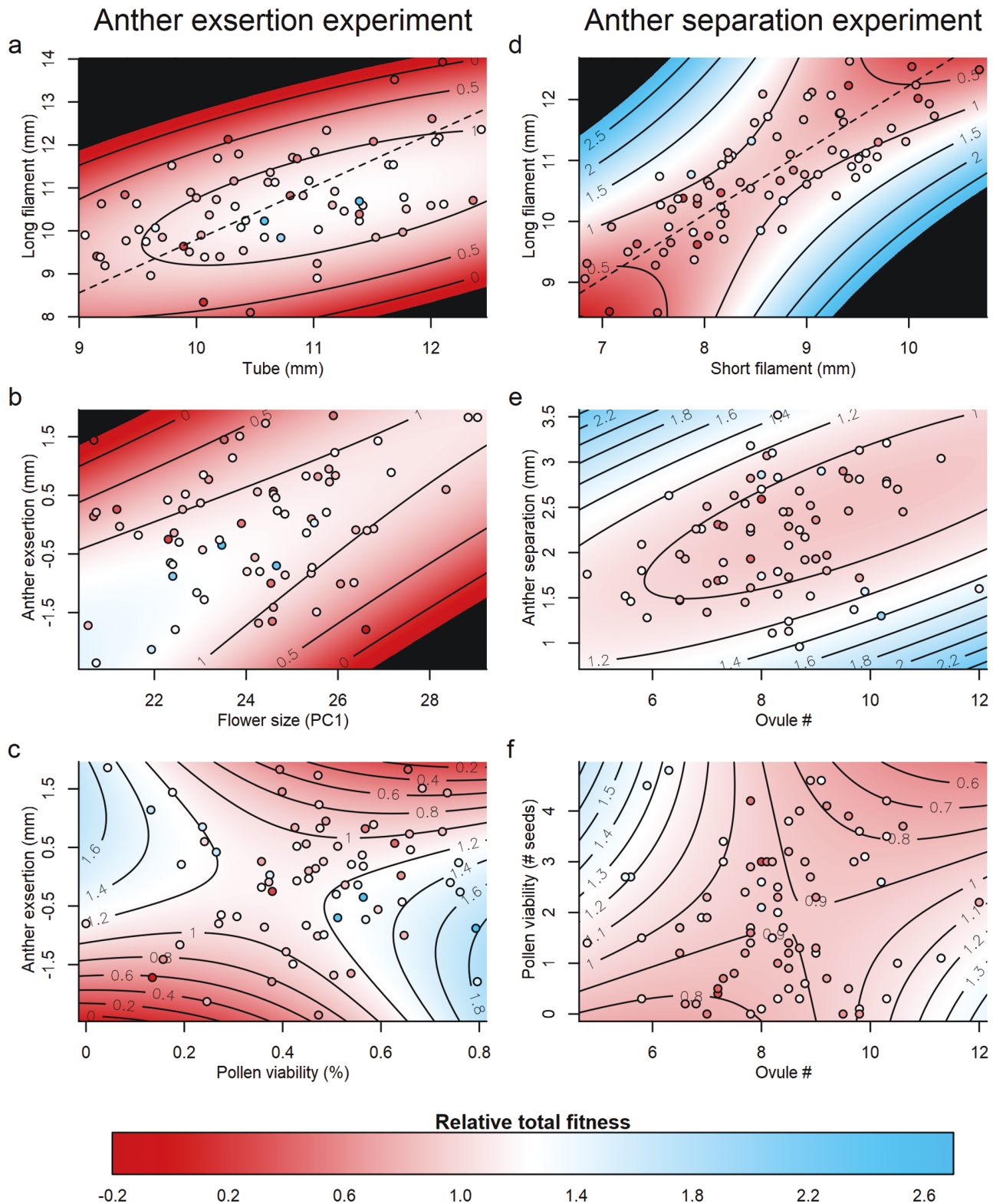


Figure 3. Fitness surface plots for traits with significant correlational selection on pairs of floral traits in anther exsertion experiment (A–C) and anther separation experiment (D–F). Relative total fitness surfaces from selection gradient models are depicted using contours (where numbers are relative total fitness) and a color gradient; areas in black are outside the range of the fitness data so they were not estimated. Dashed lines in (A and D) depict the major axis of the bivariate distribution. Points are individual plants, filled with the same color gradient to represent relative total fitness after adjusting for the effects of the other traits in the model; therefore, points that are perfectly predicted by the modeled surface are the same color as the background. Traits are shown in their original scales.

male (Figure 2C) and total fitness (Figure 2D, Table 1)—the quadratic terms in the regressions were significantly positive, the linear terms were not significant, and there were

intermediate minima in both. These results were highly consistent in both reduced models (Table S7). This relationship represents negative correlational selection on long and short

filament lengths, that is, selection to reduce the very high correlation between them. Using a separate model with short and long filament lengths (see Methods), the fitness surface can be visualized as a valley, with the lowest fitness at the mean difference between long and short filament lengths (short filament \times long filament, $\beta = -0.55$, $p = .01$; Table S8, Figures 3D and S1D). Collapsing the data along the valley rotates the 3D surface to show the disruptive selection acting on the difference between the traits—anther separation.

The intermediate total fitness minimum for anther separation was 2.18 mm (95% CI = [2.05, 2.33]; Figure 2D), which is in the third quartile for the low treatment (1.91–2.29 mm), the first quartile for the control treatment (1.93–2.45 mm), and slightly higher than the mean of the greenhouse-grown first generation of plants from the source population (1.96 mm, 95% CI = [1.91, 2.01]; Conner & Via, 1993).

Based on total fitness, there was a negative correlational selection on anther separation \times ovule number with the fitness surface forming a valley (Table 1, Figure 3E and S1E), and on ovule number \times pollen viability with the fitness surface forming a saddle (Table 1, Figures 3F and S1F). This means that for flowers with few ovules, fitness was maximized with both high pollen viability and anther separation; on the other hand, for flowers with many ovules, fitness was maximized with both low pollen viability and filament separation.

Discussion

Little evidence for conflict between male and female functions

The selection was somewhat stronger on the anther position traits through male rather than female fitness, in line with our hypothesis. However, selection gradients through these two fitness components were in the same direction and not significantly different from each other. Because the artificially selected traits are both aspects of male floral morphology, stronger selection through male fitness is expected (Conner, 2006). However, anther traits can influence not only pollen export but also pollen deposition on stigmas (Kudo, 2003; Murcia, 1990). Therefore, the similarity in selection operating through male and female functions may indicate that variation in anther position results in positively correlated effects on pollen removal and deposition.

Two main hypotheses have been proposed to explain how selection differs between sexual functions. First, Bateman's principle predicts that selection should be stronger through male fitness if female fitness is more limited by resources (Bateman, 1948). Second, the gender-balance hypothesis uses an equilibrium quantitative genetic model to propose a selection of equal magnitude but opposing signs between sexes (Morgan, 1992; Morgan & Schoen, 1997). Our results do not support either hypothesis. When considering all traits, there was no difference in the strength of selection through male versus female fitness and the majority of selection gradients for male and female fitness were of the same sign (30/40; Tables S2 and S3), with only one marginal case for conflict (ovule number in the anther separation experiment). This is in line with prior meta-analyses of both observational and manipulative studies measuring male and female fitness in plants, along with several more recent studies (Ashman & Morgan, 2004; Delph & Ashman, 2006; La Rosa & Conner, 2017; Sahli & Conner, 2011). Overall, individual plant traits may affect sexual function

fairly independently, with fewer tradeoffs between male and female functions than traditionally predicted (similar to the trait specialization hypothesis in Sahli & Conner, 2011). It has also been suggested that the lack of evidence for sexual conflict is due to spatial and/or temporal variation in agents of selection (Ashman & Morgan, 2004; Connallon & Hall, 2016).

Evidence for stabilizing selection on anther exertion

Our results provide support for the hypothesis that intermediate levels of anther exertion are the most adaptive in wild radish. Pollinators exerted stabilizing selection on this trait, based on measures of total hermaphroditic fitness across the growing season in a population with an artificially expanded range of variation in exertion, partially recreating the ancestral condition (Figure 2A and 2B). To our knowledge, this study and our related prior study (Sahli & Conner, 2011) are the first to use this promising approach for studying adaptation. This result is consistent with 2 of 3 years of selection on natural exertion variation (Morgan & Conner, 2001) and represents positive correlational selection on the two-component traits of anther exertion—long filament length and corolla tube length (Figure 3A). If this correlational selection acted in the past, then it would explain the derived high phenotypic and genetic correlations in the wild radish between these traits (Conner & Via, 1993; Conner et al., 2009). This aligns with a meta-analysis of 22 studies finding a significant positive association between the correlational selection gradient and genetic or phenotypic correlation for a given trait pair (Roff & Fairbairn, 2012). However, this pattern may not hold in the five of these studies that examined floral traits: nearly the same number of correlational selection and trait correlation estimates were of mismatching (33/68) as matching signs (35/68). For example, Caruso (2000) found very strong positive correlational selection on corolla width and length in one population of *Ipomopsis aggregata* and very strong negative correlational selection in another, despite positive phenotypic correlations between these traits in both populations. Future studies investigating genomic signatures of correlational selection (Svensson et al., 2021) may help explain the apparent disconnect between theory and evidence. Anther exertion in wild radish provides a promising system for such studies; note that previous work showed that the very high genetic correlations among the filaments and corolla tube in wild radish were caused by pleiotropy or tight linkage (Conner, 2002).

The most likely mechanism driving selection on anther exertion is differential pollen placement on pollinators. Consistent with this hypothesis, Newman and Anderson (2020) found that differences in anther exertion among three *Pelargonium* species altered pollen placement on pollinator bodies. This explains why optimal levels of exertion seem to differ among pollinators and species. In wild radish, small bees exerted stabilizing selection on exertion and removed the most pollen from flowers with anther exertion close to the mean, whereas bumblebees exerted positive directional selection (Conner et al., 2009; Sahli & Conner, 2011). It is therefore likely that the pattern of stabilizing selection found in the present study can be explained as an adaptation to make the best contact with small bee bodies. Future studies quantifying such expected differences in anther contact would help confirm this hypothesis.

Evidence for disruptive selection on anther separation

Contrary to expectations, we found strong evidence for disruptive selection on anther separation, with an intermediate fitness minimum near the mean for the trait in the natural population (Figure 2C and 2D). Previous work based on natural variation found evidence for stabilizing selection over 3 years, significant in 1 year (Conner et al., 2003). Both disruptive and stabilizing selection maintain the current population mean but have opposite effects on the variance and the correlation between the component traits. Disruptive selection would decrease the correlation between the lengths of the short and long filaments, while stabilizing selection would increase this correlation. In natural populations of wild radish, long and short filament lengths are highly phenotypically and genetically correlated ($r = 0.89$ and 0.91 , respectively; Conner & Via, 1993). This tight correlation indicates that negative correlational selection on these traits was likely relatively rare in the evolutionary history of the species. Artificial negative correlational selection has been found to significantly reduce the correlation between flower size in males and females in only three generations (Delph et al., 2011). Below, we offer three nonmutually exclusive explanations for our finding of disruptive selection on anther separation.

One explanation is that multiple episodes of conflicting linear selection combined into overall nonlinear selection (McGlothlin, 2010). This phenomenon might arise from the combination of multiple fitness components (e.g., survival and fecundity; Wadgymar et al., 2017), multiple selective agents (e.g., different pollinators; Schemske & Bradshaw, 1999), or multiple time points (e.g., in response to changing climate; Campbell & Powers, 2015). Kingsolver and Diamond (2011) propose that this may account for why the literature contains just as many cases of disruptive as stabilizing selection. However, prior studies of selection (Sahli & Conner, 2011) and pollen removal (Conner et al., 2003) did not find evidence for conflicting selection by different pollinators in wild radish, and it is difficult to see how anther separation would be affected by fitness components other than reproduction. Thus, the conflicting selection hypothesis seems unlikely to explain nonlinear selection on anther separation.

The predictions of pollen presentation theory (Harder & Thomson, 1989) could also theoretically account for disruptive selection if high and low pollinator visitation rates favor different trait extremes. Given that pollen removal rates increase linearly with decreased anther separation (Conner et al., 2003), we might expect positive directional selection for anther separation at high visitation and negative directional selection at low visitation. Estimates of selection across our different measurement days, which can be thought of as selective episodes {Arnold, 1984 #130}, suggest the opposite pattern (Figure S5). While the pollen presentation theory has received support in flowers with anthers that dehisce at different times, we hypothesize that it is less relevant in explaining flowers with anther types that differ in height but not dehiscence time, such as wild radish (Conner, unpublished data). Future work might test this by measuring pollen removal and selection on anther separation in manipulated pollinator visitation treatments.

The third functional explanation for disruptive selection on anther separation is a division of labor between pollination and feeding anthers. It is possible that flowers with greater

anther separation have higher fitness due to pollinators making better contact with long anthers or stigmas as they feed on short anthers, while low-separation flowers achieve high fitness through maximizing pollen removal or pollinator visits. However, pollen from short anthers is only slightly and not significantly less viable than pollen from long anthers ($p = .08$, linear mixed effects model; Conner, unpublished data), whereas sterile pollen is often found in feeding anthers (Vallejo-Marín et al., 2009). Furthermore, pollen-feeding insects actually seem to feed on long anthers more often than short anthers in wild radish, based on a preliminary analysis of 30 slow-motion videos (28 long-anther vs. 15 short-anther feedings observed, $\chi^2 = 3.93$, $p = .05$, N. Bhandari and R. Waterman, unpublished data). Despite the lack of clear feeding anthers, it remains possible that greater separation of anther heights favorably manipulates the body position of pollen foragers, causing pollen to be more frequently deposited on pollinator bodies or specifically on sites less likely to be groomed. Alternatively, anther separation may affect the placement of pollen on the bodies of pollinators foraging for nectar. During nectar foraging, all or part of the pollinator body enters the corolla tube, causing the short anthers to be more likely to contact the pollinator than the long anthers (personal observation, R. Waterman). Finally, the marginally significant disruptive selection of anther separation through female fitness suggests that separation affects pollinator contact with stigmas.

On balance, it seems most likely that different functional explanations account for the fitness benefits of possessing flowers with either high or low anther separation. For highly separated anthers, we propose increased pollen removal and possibly deposition by manipulating the body position of pollen foragers and/or making better contact with nectar foragers. On the other hand, we propose that minimally separated anthers attract more pollinators through their greater visibility (Sapir et al., 2017) and/or maximize the amount of pollen removed during pollen foraging.

Although the functional consequences of variation in anther separation remain unclear, this study's finding that anther separation impacts relative fitness even after correcting for the effects of other floral traits, together with the prior finding that this trait impacts pollen removal rates (Conner et al., 2003), support the hypothesis that tetradynamy in the Brassicaceae evolved as an adaptation. Despite the high correlation between long and short filament lengths, the rapid evolution of decreased anther separation in the selected lines and reversion of at least two Brassicaceae genera to equal-height anthers (*Stanleya* and *Thelungiella*; Barnes, 2001) makes it implausible that evolution has been constrained by a lack of variation in the appropriate direction. It is also possible that current levels of anther separation evolved nonadaptively due to correlations with other traits under selection. This too seems unlikely, given that none of the other measured floral traits in our study had significant linear or quadratic selection gradients, phenotypic correlations between anther separation and the other floral traits not involving filament length were generally very low, and phenotypic correlations between floral and vegetative traits are lower on average than correlations among floral traits (Berg, 1960; Conner et al., 2014).

Conclusions

We measured selection on two anther traits through male and female fitness in populations of wild radish that were

artificially selected to have ancestral expanded phenotypic variation. We found strong evidence for stabilizing selection on anther exertion, indicating positive correlational selection on the component traits, tube length and long filament length. This matches previous work using natural variation showing this same form of selection in some years and that intermediate levels of anther exertion increase pollen removal by the plant's primary pollinator. In contrast, we found strong evidence for disruptive selection on anther separation, indicating negative correlational selection on the component traits, short filament length and long filament length, in contrast to previous work on natural variation showing positive correlational selection in 1 year. Strong evidence for stabilizing and disruptive selection (not just significant quadratic selection gradients) and especially negative correlational selection are uncommon in the literature. Both stabilizing and disruptive selection maintain current trait means, supporting adaptive explanations for the derived high filament–corolla tube correlation in some Brassicaceae and tetradynamy across most of the family. However, the functional significance of anther separation remains unclear and the high correlation between long and short filament lengths suggests that disruptive selection is relatively rare. Future studies testing whether variation in anther separation affects pollinator body position and resulting pollen placement on pollinator bodies could be useful. Our results also highlight the usefulness of using an expanded variation to study adaptation, particularly when ancestral populations are known to possess greater variation for the focal trait. Our previous estimates of selection on the anther position using natural variation alone were weaker and sometimes in different directions compared with estimates using an expanded variation. Future studies using this approach in other species could also be illuminating.

Supplementary material

Supplementary material is available online at *Evolution* (<https://academic.oup.com/evolut/qpac001>).

Data availability

Data are archived at Dryad (doi:10.5061/dryad.02v6wwq6g, Waterman et al. 2022).

Author contributions

J.K.C. and K.K. designed the experiments, H.S., J.K.C., K.K., and V.A.K. carried out the experiments, J.K.C. and R.W. conducted data analysis, and R.W. and J.K.C. drafted the manuscript. All authors reviewed and approved the manuscript.

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