

PATTERNS AND MECHANISMS OF SELECTION ON A FAMILY-DIAGNOSTIC TRAIT: EVIDENCE FROM EXPERIMENTAL MANIPULATION AND LIFETIME FITNESS SELECTION GRADIENTS

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Abstract.—Plant traits that show little variation across higher taxa are often used as diagnostic traits, but the reason for the stasis of such traits remains unclear. Wild radish, *Raphanus raphanistrum*, exhibits tetradydynamous stamens (four long and two short, producing a dimorphism in anther height within each flower), as do the vast majority of the more than 3000 species in the Brassicaceae. Here we examine the hypothesis that selection maintains the stasis of dimorphic anther height by investigating the effects of this trait on pollen removal, seed siring success, and seed set in *R. raphanistrum* using both experimental and observational methods. Observational selection gradient analysis based on lifetime seed siring success provided evidence for an optimum dimorphism that was greater than zero in one of three years. In both experimentally manipulated and unmanipulated flowers, more pollen was removed in single visits from flowers with less dimorphism. There was no significant effect of anther dimorphism on female fitness (seed set). Therefore, there is some evidence to suggest that selection is maintaining anther dimorphism in wild radish, and that higher male fitness might result from restriction of single-visit pollen removal. We discuss these results in light of pollen presentation theory.

Key words.—Brassicaceae, family-diagnostic trait, floral morphology, pollen removal, *Raphanus raphanistrum*, selection gradients, stasis.

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Lack of phenotypic variability for some traits is common at the population or species level. Traits that vary little across higher taxonomic levels are less common, but potentially more interesting because they may shed light on important evolutionary processes. Traits that show little variation across higher taxa likely arose early in the evolutionary history of the group, and subsequently remained constant as the species diverged and radiated over evolutionary time. While often useful as a diagnostic trait for the taxonomic group, the reason for the stasis of such a trait is often not clear.

Evolutionary explanations for phenotypic invariability at a high taxonomic level include a lack of genetic variation for the trait, constraints due to genetic correlations or developmental processes, or stabilizing selection (Maynard Smith et al. 1985; Karoly and Conner 2000; Gould 2002). These three hypotheses are not mutually exclusive, and they may not be equally suitable for explaining stasis at different taxonomic levels. For example, some kind of constraint or stabilizing selection seems necessary to explain long-term stasis or lack of variance among higher taxa (Stearns 1994). Examples of stabilizing selection are surprisingly scarce in the literature despite its theoretical importance (Kingsolver et al. 2001); taxonomically conservative traits may be ideal places to uncover this form of selection in nature.

The vast majority of the approximately 3000 species in the mustard family, or Brassicaceae, exhibit the tetradydynamous stamen condition, in which there are four long medial stamens and two short lateral stamens in each flower (Fig. 1A). This condition leads to dimorphic anther height within each flower and is considered diagnostic for the family (Zomlefer 1994). Of the 331 genera in the family, 51 have some species that are classified as “subequal” or equal in filament length (Barnes 2001); that is, they lack a strong dimorphism. While

a rigorous comparative study of tetradydynamy has not been done, two genera that lack strong dimorphism (*Stanleya* and *Thelungiella*) are derived taxa in recent molecular phylogenies of the Brassicaceae (Price et al. 1994; Galloway et al. 1998; Barnes 2001). Karoly and Conner (2000) found evidence of genetic variation underlying tetradydynamous stamens in two closely related Brassicaceae, *Raphanus raphanistrum* and *Brassica rapa*, thus refuting the lack of variation hypothesis for these species. Our study was designed to test one of the other evolutionary explanations for conservation of dimorphic anther heights—stabilizing selection.

Multiple studies have found evidence suggesting that variation in floral morphology, including anther position, can affect pollen removal and deposition (e.g., Galen and Stanton 1989; Murcia 1990; Harder and Barrett 1993; Stone and Thomson 1994; Conner et al. 1995; Cresswell 2000), and others have shown that floral traits affect fitness (e.g. Galen 1989; Schemske and Horvitz 1989; Campbell et al. 1991; Stanton et al. 1991; Caruso 2000; Morgan and Conner 2001). In a literature survey of variation in floral traits in 151 species in 48 families, Cresswell (1998) noted that the least variable morphological traits were those that were potentially related to the fit between the pollinator and the flower. This supports the idea that pollinator-mediated selection can reduce floral variation. However, previous studies have rarely, if ever, examined selection on traits that are invariant at higher taxonomic levels.

In this study, we investigated the effects of dimorphic anther height on pollen removal, seed siring success, and seed set in *R. raphanistrum* using three datasets. The first measured pollen removal in single visits from flowers with both monomorphic and dimorphic anther heights created through experimental manipulation. Second, we reanalyzed the obser-

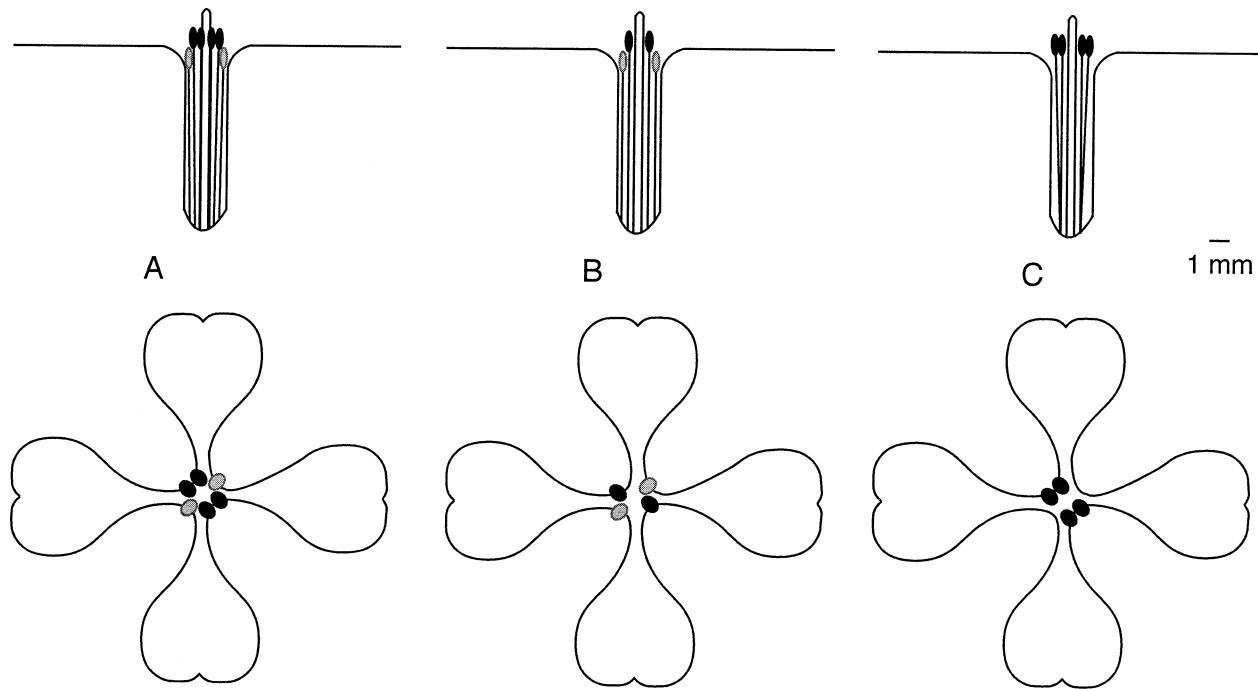


FIG. 1. Schematic drawings of wild radish flowers, showing the tetrodynamicous condition and the two experimental manipulations. Top, side views; bottom, top views. Short stamen anthers are shown in gray, long stamen anthers in black. Scale bar is approximate, and is based on average measurements of greenhouse-grown plants. Note that the side views depict the vertical positions (heights) of the anthers correctly, but not their horizontal positions. (A) Intact flower, (B) dimorphic manipulation, (C) monomorphic manipulation.

vational data from Conner et al. (1995) to determine the effect of natural variation in dimorphism on pollen removal in *R. raphanistrum*. Finally, we reanalyzed another observational dataset (Conner et al. 1996a; Morgan and Conner 2001) to measure selection on anther dimorphism through differences in lifetime male or female fitness (seed siring success and seed production, respectively).

MATERIALS AND METHODS

Wild radish, *Raphanus raphanistrum*, is a member of the family Brassicaceae and exhibits the tetrodynamicous stamen condition. Anthers on long stamens are placed just outside the opening of the corolla tube in wild radish, while anthers on short stamens are placed just inside the corolla tube (Fig. 1A). These relationships do not vary much because the short filaments, long filaments, and corolla tube are highly correlated with each other (Conner and Via 1993). The position of the stigma relative to the anthers is more variable, but on average it is placed above the long filament anthers as depicted in Figure 1. Wild radish is self-incompatible and pollinated by a variety of insects, including cabbage butterflies (*Pieris rapae*), honeybees (*Apis mellifera*), small bees, and syrphid flies (Conner and Rush 1996). On wild radish flowers cabbage butterflies feed exclusively on nectar, honeybees on nectar and pollen, small bees almost exclusively on pollen, and syrphids entirely on pollen. The experimental study was conducted from July to September 1999 and June to July 2000 at the W.K. Kellogg Biological Station in Hickory Corners, Michigan. Plants used in this study were grown from field-collected seeds (Conner and Via 1993) in a largely pollinator-free greenhouse. We added 1/2 teaspoon of Osmocote

Plus 15-9-12 fertilizer (Grace-Sierra, Marysville, OH) to each 10-cm pot at planting.

We manipulated flowers to produce monomorphic and dimorphic anther heights. We removed two anthers from each flower, leaving four in each manipulation type (Fig. 1); note that this reflects an unnatural condition because flowers in the Brassicaceae have six anthers. However, this allowed us to produce both monomorphic and dimorphic flowers with the same number of anthers. In flowers receiving the dimorphic manipulation, we removed two anthers on long stamens, one from each pair of adjacent long stamens, diagonal from each other (Fig. 1B). In flowers receiving the monomorphic manipulation, we removed the two anthers on short stamens, leaving four anthers of one height (Fig. 1C). Each plant received both manipulations but on separate days. The order of the manipulations performed on each plant was randomly assigned.

Each manipulation was performed on two adjacent flowers. Pollinators were allowed to visit only one of these flowers (see below), and the difference in pollen counts between the visited and unvisited flowers is our estimate of number of pollen grains removed in a single visit. To test this method before the experiment began, we performed each manipulation type (monomorphic and dimorphic) in the greenhouse on 10 pairs of adjacent flowers. We then counted the pollen in these 40 unvisited flowers. The results indicated that pollen count varied much more among plants than among adjacent flowers within plants (monomorphic manipulation, 84% of total variance was among plants, $F = 11.43$, $P = 0.0004$; dimorphic short stamen anthers, 90% of total variance, $F = 18.42$, $P < 0.0001$; dimorphic long stamen anthers, 82% of

total variance, $F = 10.11$, $P = 0.0006$). Because the two adjacent manipulated flowers had similar postmanipulation pollen counts, the difference in pollen count between the two flowers after one of them received a pollinator visit was an accurate estimate of the number of pollen grains removed in that visit (Harder and Barrett 1993; Conner et al. 1995).

Each day, we transported a number of experimental plants from the greenhouse to the pollinator-free field laboratory. We then performed one set of manipulations. Immediately after the anther manipulation, the plant was carried outdoors to the field plot and placed in the middle of a patch of flowering *R. raphanistrum*. The plant was observed until a pollinator visited one of the manipulated flowers. After leaving one flower, the pollinator was prevented from visiting the second manipulated flower, which became the control.

In the field lab, we collected the anthers from both flowers in clean vials. For the flowers receiving the monomorphic manipulation, the four remaining anthers from each flower were placed in one vial. During the summer of 1999, the four remaining anthers from each dimorphic flower were collected in the same manner as for the monomorphic flowers. In the summer of 2000, anthers on long stamens were collected separately from the anthers on short stamens for both visited and unvisited flowers. This enabled us to compare the amount of pollen removed between short and long stamen anthers. After anther collection, we removed all flowers on the plant with the exception of the two manipulated flowers in 2000 (see below). This was done to insure that when the plant was used on a subsequent day for the other treatment, all of the flowers were newly opened and therefore unvisited.

We used a Coulter Counter to count the number of pollen grains in each vial (for details, see Rush et al. 1995). The total sample was 127 plants; 72 from 1999 and 55 from 2000. Each plant received two pollinator visits, one per manipulation treatment.

It is possible that anther position could affect pollen deposition on stigmas by changing the body position of pollen feeders. In 2000 only, we measured seed set to see if the anther dimorphism affects pollen deposition and thus female fitness. To do this, we marked the visited flowers and left them on the plant. We allowed the flowers to develop fruit in the greenhouse, and the number of seeds produced by each flower was recorded.

Data Analysis

Experimental manipulation of dimorphism

The statistical software JMP version 3.2.1 (SAS Institute 1994) was used for statistical analyses of pollen removal data. The data for the floral manipulations contained data from both summers, with a total of 254 observations; two per plant. These data were analyzed using a least-squares linear model with the number of pollen grains removed in a visit as the response variable. The predictor variables were type of manipulation, pollinator taxon, and year. The number of pollen grains in the control flower was used as a covariate to account for natural variation in amount of pollen produced. All possible two-, three-, and four-way interactions among these main effects were included in an initial model. None of the interactions with the covariate were significant (i.e., slopes

were not significantly different), so these interactions were excluded from the model reported here.

The data on seed set from manipulated plants in the summer of 2000 had 108 total observations; two per plant. Data from one plant (two observations) were discarded because one of the control flowers was accidentally damaged. The response variable was the difference in the number of seeds formed by the visited and control flowers on each plant. This difference was used because four of the 108 control flowers set one to three seeds, due to incomplete incompatibility (Stanton et al. 1989) or accidental pollination. Predictor variables were type of manipulation, pollinator taxon, and the interaction between them.

Observational study of pollen removal

The first observational study was from a reanalysis of data on filament lengths and pollen removal from Conner et al. (1995). In this study and the selection analysis described below, one long and one short filament was measured on each flower, because there is very little variation among filaments of the same length within flowers of greenhouse-grown plants (Conner, unpubl. data). We calculated a natural dimorphism ratio for each plant by dividing the long filament length by the short filament length (larger ratio = greater natural dimorphism). This ratio is uncorrelated with long filament length ($r = -0.06$, $P = 0.34$) due to the strong correlation between the long and short filament lengths. We then created another least-squares linear model, with pollen removed as the response variable and natural dimorphism, long stamen anther exertion (affects pollen removal; Conner et al. 1995), pollinator taxon, and the number of pollen grains on an unvisited adjacent control flower (similar to the control flower in the anther removal experiment) as predictor variables. The two-way interaction between pollinator taxon and control pollen (covariate) was included in an initial model, but it was not significant so was dropped from the final model.

Observational selection gradient analysis

Similarly, the effect of natural dimorphism ratio on seed siring success and seed production was examined with a reanalysis of the three years of lifetime fitness data from Conner et al. (1996a,b). Natural dimorphism ratio was calculated as described above, and linear and quadratic selection gradients (Lande and Arnold 1983) were estimated for this trait plus flower size, long stamen anther exertion (male fitness only), stigma exertion (female fitness only), and lifetime flower production. Male fitness gradients were calculated using the methods of Morgan and Conner (2001). Confidence intervals on the 1991 dimorphism optimum (see Results) were obtained by analyzing 1000 bootstrapped datasets formed by resampling progeny within families. The optima calculated from bootstrapped datasets were ranked, and confidence intervals defined by excluding the upper and lower 2.5 percentiles.

RESULTS

Syrphid flies were the most frequent pollinators in 1999 and 2000 (35% of visits), with small bees (26%), honeybees

TABLE 1. Results of least-squares linear model analysis of number of pollen grains removed from experimentally manipulated flowers. Whole model $R^2 = 0.24$, $P < 0.0001$, $N = 254$.

Effect	df	Sum of squares	F	P
Manipulation	1	2.19×10^9	7.55	0.006
Pollinator taxon	3	3.97×10^9	4.56	0.004
No. pollen in control flower	1	1.03×10^{10}	35.53	<0.0001
Year	1	2.22×10^8	0.76	0.38
Manipulation × pollinator taxon	3	1.48×10^9	1.70	0.17
Manipulation × year	1	3.01×10^7	0.10	0.75
Pollinator taxon × year	3	4.00×10^7	0.04	0.99
Manipulation × pollinator × year	3	1.59×10^9	1.83	0.14

(16%), and cabbage butterflies (11%) accounting for most of the rest of the visits. The short stamen anthers produced more pollen grains per anther than the long stamen anthers (1.7×10^4 versus 1.4×10^4 respectively; paired $t = 5.43$, $P < 0.0001$). These results agree with earlier results for this species (Conner et al. 1995; Conner and Rush 1996).

Pollen Removal and Seed Set—Manipulation Effects

Overall, the flowers with the monomorphic anther height manipulation had a significantly greater amount of pollen removed (after correcting for pollen production differences) in one visit than did the flowers with the dimorphic anther height manipulation ($1.97 \times 10^4 \pm 2.12 \times 10^3$ and $1.18 \times 10^4 \pm 1.91 \times 10^3$ respectively, least-squares means \pm SE; Table 1). We found no evidence of differential pollen removal from the two floral manipulations by different pollinator taxa (Table 1; manipulation by pollinator taxon interaction). Pollinators differed in the number of pollen grains removed in a single visit (Table 1; pollinator taxon effect), with honeybees removing the most, syrphid flies and small bees an intermediate amount, and butterflies removing the least (data not shown). We found no evidence of differences in seed set between the two manipulations (whole model $P = 0.36$).

Pollen Removal—Natural Dimorphism Effects

The observational data from Conner et al. (1995) indicate that the number of pollen grains removed (after correcting for pollen production) decreased with increasing anther dimorphism and increased with increasing anther exertion (Ta-

ble 2). There is no evidence of differential pollen removal over the natural range of dimorphism ratios by the different pollinator taxa (Table 2; natural dimorphism by pollinator taxon interaction). The pollinator taxa differed in the proportion of pollen removed in a single visit (Table 2; pollinator taxon effect), in the same rank order as in the manipulation data (Conner et al. 1995). All these results are consistent with the manipulation experiment.

Pollen Removal within Dimorphic Flowers

In both the undisturbed flowers from Conner et al. (1995) and the experimental flowers with dimorphic anther height in 2000, we found a greater percentage of pollen removal from the long stamen anthers compared to the short stamen anthers for each taxon of pollinators. The difference in the percent pollen removed (long minus short) ranged from 18 to 45% for the different pollinator taxa across the two years, and overall this difference was highly significant (paired $t = 6.2$, $P < 0.0001$, $N = 224$).

Selection through Lifetime Male and Female Fitness

In 1991, both the linear and quadratic selection gradients for anther dimorphism through seed siring success were significantly negative (Table 3), indicating an optimum slightly less than the current mean (Fig. 2). There was no significant selection on dimorphism through male fitness in 1992 and 1993 (Table 3), nor through female fitness in any year (P -values for dimorphism selection gradients ranged from 0.34 to 0.95).

TABLE 2. Results of least-squares linear model analysis of number of pollen grains removed from intact flowers. For the continuous variables (natural dimorphism, long stamen exertion, and no. pollen in control flower), we present the corresponding slope and standard error. The squared terms for the first two variables are quadratic terms testing for curvature in the relationship between these dependent variables and the number of pollen removed. For the categorical variable (pollinator taxon) and the interaction term (natural dimorphism × pollinator taxon), we present the sum of squares and the F-ratio. The slopes and sums of squares tend to be large because the number of pollen grains removed (the dependent variable) ranged up to 80,000. Whole model $R^2 = 0.56$, $P < 0.0001$, $N = 237$. Data from Conner et al. (1995).

Effect	df	Sum of squares, or slope	F or SE	P
Natural dimorphism	1	-27244	11663	0.02
(Natural dimorphism) ²	1	31915	55717	0.57
Long stamen exertion	1	3732	1378	0.007
(Long stamen exertion) ²	1	903	1218	0.46
Pollinator taxon	3	7.6×10^9	13.7	<0.0001
No. pollen in control flower	1	0.71	0.06	<0.0001
Natural dimorphism × pollinator taxon	3	1.1×10^8	0.19	0.90

TABLE 3. Standardized selection gradients for the floral traits based on lifetime male seed siring success. Distance is the distance between the sire and the plant producing the seed; in 1993 plants were in pots whose positions were re-randomized each day. Squared terms are quadratic gradients measuring curvature in the fitness function. The significance levels given are from simulations; parametric significance levels based on the chi-square distribution were very similar in all cases. For details see Morgan and Conner (2001); data are from Conner et al. (1996a).

	1991		1992		1993	
	β	P	β	P	β	P
Dimorphism	-0.25	<0.001	0.07	0.17	0.04	0.12
Dimorphism ²	-0.72	0.006	-0.06	0.28	-0.04	0.29
Flower size	0.10	0.08	-0.03	0.36	0.10	<0.001
Flower size ²	0.14	0.14	-0.58	<0.001	0.03	0.46
Anther exsertion	-0.07	0.13	0.04	0.29	0.04	0.10
Anther exsertion ²	-0.15	0.40	-0.17	0.001	-0.03	0.44
Flower production	1.26	<0.001	0.63	<0.001	0.10	<0.001
Distance	-0.19	<0.001	-0.27	<0.001	—	—

DISCUSSION

Our reanalysis of phenotypic selection data based on lifetime seed siring success shows selection for dimorphic anther heights in one of three years. The 1991 selection gradient data show an adaptive peak at a filament length ratio of 1.24 (Fig. 2), suggesting that dimorphism led to increased siring success. However, the lack of significant selection on dimorphism in 1992 and 1993 suggests that the standing variation in dimorphism was neutral in those years. The difference in selection among years cannot be explained by the mean or variance in dimorphism being greater in 1991, because the mean dimorphism ratio was 1.3 in all three years, with coefficients of variation of 5.4, 7.7, and 4.0 in the three years chronologically. A possible explanation for the difference among years is that the pollinator composition was dominated by approximately equal proportions of small bees and syrphid flies in 1992 and 1993, but in 1991 honeybees made the majority of visits, with small bees making up most of the rest (Conner et al. 1996a). Given the low natural variation in dimorphism and differences in pollinators among years, it is possible that the population was at a broader adaptive plateau for dimorphism in 1992 and 1993 compared to 1991 and there was therefore inadequate variation to demonstrate selection with the broad plateau (cf. Schlüter 1988).

Regarding the mechanisms for this selection, our results clearly showed that dimorphic anther heights led to reduced single-visit pollen removal. More pollen was removed (after correcting for pollen production) from flowers with experimentally monomorphic anther height than those with dimorphic anther height (Table 1). Similarly, there was a significant negative relationship between the natural dimorphism ratio and pollen removal (Table 2). The agreement between these two types of studies, one relying on experimental manipulation and the other on natural variation, makes our conclusion that two anther heights reduces overall pollen removal very robust (Waser 1983; Conner and Rush 1996). It also suggests that our manipulated flowers with only four anthers still produced results relevant to the natural six-anther condition. Previous studies have found increasing pollen removal with increasing anther exsertion within one anther level (Murcia 1990; Conner et al. 1995), as well as maximum pollen removal from long-level anthers in a tristylous species (Wolfe and Barrett 1989; Harder and Barrett 1993). We found

significantly greater pollen removal from long stamen anthers (which are more exserted) than short stamen anthers. Thus, the increased removal from monomorphic flowers is likely due to increased pollinator contact with the four long stamen anthers in these flowers, compared to only two long stamen anthers in the dimorphic treatment.

The relationship between single-visit pollen removal and seed siring success depends on a number of factors, including the timing of flower opening and ovule availability (Stanton et al. 1992; Stanton 1994), pollen longevity and diversity of pollinator efficiency (Thomson and Thomson 1992), and frequency and predictability of visitation and the rate of pollen

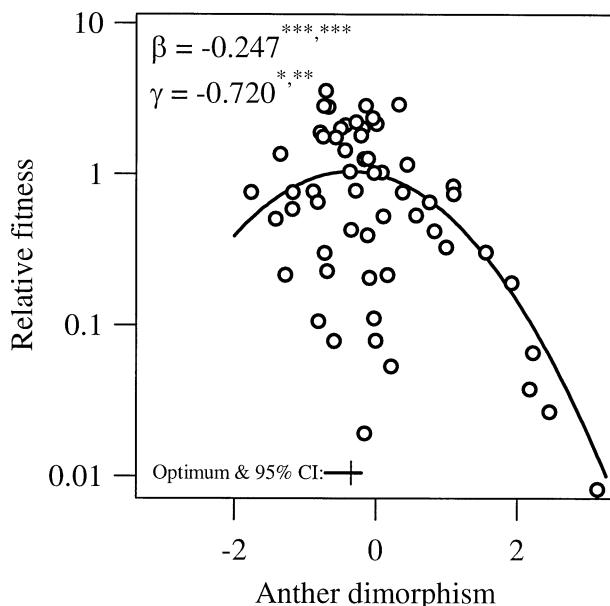


FIG. 2. Standardized quadratic fitness function for natural variation in anther dimorphism based on lifetime male fitness in 1991. Linear (β) and quadratic (γ) selection gradients are shown, with asterisks denoting statistical significance from parametric and simulation tests respectively (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$). Anther dimorphism is standardized (mean = 0, SD = 1), and is thus presented in standard deviation units. The optimum and 95% confidence interval from simulation are shown at the bottom of the figure. In absolute units, the optimum dimorphism ratio is 1.24, with the 95% CI ranging from 1.21 to 1.25, and the current population mean is 1.3. For details see Table 3.

loss (Harder and Thomson 1989; Kobayashi et al. 1999). Wild radish flowers open mainly early in the day, flowers are heavily visited, depleting pollen rapidly, and ovules are quickly fertilized, in both *R. sativus* and *R. raphanistrum* (Stanton et al. 1992; Rush et al. 1995; J. Conner, unpubl. data). These conditions produce intense competition for ovules, and select for traits that maximize rates of pollen removal (Stanton 1994).

On the other hand, variation in effectiveness among pollinators combined with abundant and reliable pollinator service and long-lived pollen should select for restriction of removal (Thomson and Thomson 1992). While wild radish has a diversity of pollinators that differ in efficiency, the two main groups in the small populations we study, small bees and syrphid flies, have roughly similar efficiencies (Conner et al. 1995). To the best of our knowledge, pollen longevity has not been systematically studied in wild radish, although anecdotal evidence suggests pollen remains viable for up to two weeks in vitro (J. Fant, pers. comm.).

In spite of the increased pollen removal from long stamen anthers, we found that the short stamen anthers produced a larger number of pollen grains than their counterparts on long stamens. Previous studies have reported similar results (Wolfe and Barrett 1989; Harder and Barrett 1993; Conner et al. 1995; but see Escaravage et al. 2001). Harder (1990) suggests that the cost of producing more pollen can be alleviated if this pollen is packaged or has a dispersal mechanism that can limit the number of grains removed at one time. Taken together, the evidence suggests that the short anthers in Brassicaceae could be a mechanism to limit single-visit removal, but considerably more research is necessary to strongly support this hypothesis. Alternatively, single-visit pollen removal might not be a strong determinant of male fitness in wild radish (Harder and Thomson 1989; Kobayashi et al. 1999).

The reasons for multiple anther positions within flowers are well studied in the special case of heterostylous species (Barrett 1992) but not in other species. Escaravage et al. (2001) found evidence that short stamens function for reproductive assurance in a nonheterostylous species, likely performing autogamous selfing. This is certainly not the case in wild radish, because it is self-incompatible and because it is the long stamen anthers that are usually closest to the stigma.

There are at least three hypotheses to explain the invariability of the tetrodynamic stamen condition in the Brassicaceae: lack of genetic variation for the trait, constraints due to genetic correlations or developmental processes, or stabilizing selection. Karoly and Conner (2000) presented evidence refuting the lack of genetic variation hypothesis for two species, and this study explored the selection hypothesis. Our results provide some evidence that selection may be maintaining dimorphism in *R. raphanistrum*. Clearly, work that combines increased variation in dimorphism with measurements of male fitness are needed to settle this question, as well as studies addressing the third hypothesis, constraints. Current work is addressing both these issues. Just as clearly, stamen dimorphism needs to be studied in more species. The family Brassicaceae exhibits extensive diversity in flower size, flower shape, and mating systems (from autogamous selfing to obligate outcrossing; Preston 1986). Therefore, re-

search on additional species in the Brassicaceae is needed to uncover the reasons behind the evolutionary stasis of dimorphic anther height in this large family.

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