

## COSTS OF INDUCED RESPONSES AND TOLERANCE TO HERBIVORY IN MALE AND FEMALE FITNESS COMPONENTS OF WILD RADISH

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**Abstract.**—Theory predicts that plant defensive traits are costly due to trade-offs between allocation to defense and growth and reproduction. Most previous studies of costs of plant defense focused on female fitness costs of constitutively expressed defenses. Consideration of alternative plant strategies, such as induced defenses and tolerance to herbivory, and multiple types of costs, including allocation to male reproductive function, may increase our ability to detect costs of plant defense against herbivores. In this study we measured male and female reproductive costs associated with induced responses and tolerance to herbivory in annual wild radish plants (*Raphanus raphanistrum*). We induced resistance in the plants by subjecting them to herbivory by *Pieris rapae* caterpillars. We also induced resistance in plants without leaf tissue removal using a natural chemical elicitor, jasmonic acid; in addition, we removed leaf tissue without inducing plant responses using manual clipping. Induced responses included increased concentrations of indole glucosinolates, which are putative defense compounds. Induced responses, in the absence of leaf tissue removal, reduced plant fitness when five fitness components were considered together; costs of induction were individually detected for time to first flower and number of pollen grains produced per flower. In this system, induced responses appear to impose a cost, although this cost may not have been detected had we only quantified the traditionally measured fitness components, growth and seed production. In the absence of induced responses, 50% leaf tissue removal, reduced plant fitness in three out of the five fitness components measured. Induced responses to herbivory and leaf tissue removal had additive effects on plant fitness. Although plant sibships varied greatly (49–136%) in their level of tolerance to herbivory, costs of tolerance were not detected, as we did not find a negative association between the ability to compensate for damage and plant fitness in the absence of damage. We suggest that consideration of alternative plant defense strategies and multiple costs will result in a broader understanding of the evolutionary ecology of plant defense.

**Key words.**—Adaptive plasticity, compensation, herbivory, induced defense, male plant fitness, overcompensation, plant-insect interactions, *Raphanus raphanistrum*.

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Plant resistance is any trait that has a negative effect on the performance and/or preference of herbivores. A highly resistant plant may be unattractive to herbivores or may support low herbivore fitness; alternatively, a less resistant or susceptible plant may be preferentially consumed by herbivores or may be of superior food quality. In either case, the expectation has been that resistant plants should receive less damage than susceptible plants under natural conditions (Rausher 1992). It is also believed that genetic variation for resistance is, in part, maintained because of costs of resistance. In this case, resistant individuals are relatively more successful in the presence of herbivores and susceptible individuals are relatively more successful in the absence of herbivores. Fluctuating intensity of herbivory has been proposed as a mechanism maintaining genetic variation in resistance characters, assuming costs are present. However, evidence for genetic costs of resistance is equivocal (Parker 1992; Simms 1992; Bergelson and Purrington 1996). Costs of defense have always been measured in terms of growth and seed production, while no measures of male fitness components have been reported to our knowledge. If pollen and seed characters are differentially affected by herbivory or allocation to defense, then costs in terms of both male and female characters will have to be considered to fully under-

stand effects of herbivory and defensive traits on plant performance (see Mutikainen and Delph 1996; Strauss et al. 1996). In this paper we expand our view of constraints on the expression of plant resistance to include costs of induced responses and tolerance to herbivory. Induced responses and tolerance to herbivory do not fit into the typical framework of plant resistance and need to be explained from a cost-benefit perspective independently (Rhoades 1979; van der Meijden et al. 1988; Karban and Myers 1989; Tallamy and Raupp 1991; Trumble et al. 1993; Rosenthal and Kotanen 1994; Simms and Triplett 1994; Fineblum and Rausher 1995; Karban and Baldwin 1997).

Induced responses to herbivory deviate from the traditional view of resistance as follows: A plant that is initially susceptible in the field may change its defensive phenotype after being damaged through altered production of defensive chemicals or structures. If induced responses to herbivory truly benefit plants and increase fitness in the presence of herbivores, it is logical to ask why plants employ such facultative defense strategies. Why do plants not express maximal levels of resistance all of the time? Although induced resistance to herbivory may have many other benefits over constitutive resistance (Karbon et al. 1997), induction has largely been viewed as a cost-saving strategy: Plants that occur in environments without herbivory do not invest in unnecessary defenses. However, few studies have attempted to detect costs of induction (Brown 1988; Baldwin et al. 1990; Karban 1993; Gianoli and Niemeyer 1997; Zangerl et al.

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1997), although this is the primary method of addressing the assumption that inducibility saves the plant costs when not induced. Such demonstrations are important because phenotypically plastic traits, such as induced resistance, are thought to evolve as a mechanism for organisms to express adaptive phenotypes in variable environments (Bradshaw 1965; Thompson 1991; Gotthard and 1995; Kingsolver 1995; Dudley and Schmitt 1996; DeWitt et al. 1998; Kingsolver and Huey 1998). Demonstrating phenotypic benefits and costs of induction in environments with and without herbivory, respectively, confirms that inducible resistance is an adaptive trait across variable environments.

Tolerance to herbivory, or the ability to regrow and reproduce after damage, is an additional plant strategy that may have evolved in response to herbivore attack (Strauss and Agrawal 1999). We define tolerance as the proportional reduction in a fitness component of the plant for a given level of damage. As noted by Rausher (1992), tolerance can only be measured for a group of related plants because an individual plant is either damaged or not and tolerance is measured as the ratio of fitness of damaged and undamaged plants. A plant that appears to be highly susceptible because it has a great deal of natural herbivory may be highly tolerant and, in fact, not negatively affected by its herbivores. Here, at the genetic level, we ask why plants are not maximally tolerant. Costs of tolerance may reduce the fitness of genotypes in environments with relatively low levels of herbivory. Costs of tolerance can be determined by two methods: examining the relationship between tolerance and mean family fitness in the undamaged state or examining the relationship between family fitness means for damaged and undamaged plants. An unbiased negative correlation in either case demonstrates a cost of tolerance (Simms and Triplett 1994; Strauss and Agrawal 1999). Genotypic differences in the ability to tolerate damage are typically measured by looking for a statistical interaction between family effects and effects of damage on plant fitness.

We have chosen wild radish, *Raphanus raphanistrum*, as a study system because it is one of three systems for which clear fitness benefits of induced responses to herbivory have been reported (Agrawal 1998, 1999, unpubl.; Baldwin 1998). Only after detection of such benefits is it logical to proceed to ask which costs are associated with induced responses to herbivory. In this study we have attempted to manipulate induced plant responses to herbivory and to measure male and female reproductive costs associated with this trait along with costs of tolerance to herbivory. Our goal in undertaking such a project was to explore the evolutionary ecology of alternative plant defense strategies. Specifically, we determined the effects of foliar caterpillar damage on systemic concentrations of indole glucosinolates (mustard oil glycosides), estimated costs of induced responses to herbivory and costs of leaf tissue removal in terms of seed production and pollen characters, measured tolerance to herbivory using several correlates of male and female fitness, and evaluated fitness costs of tolerance.

## MATERIALS AND METHODS

### Study System

*Raphanus raphanistrum* (Brassicaceae) is a widely distributed self-incompatible annual plant found in disturbed sites.

In natural populations approximately 15% of plants have 50% of their leaf tissue removed by herbivores (S. Strauss, unpubl. data). In laboratory and field trials, components of induced resistance to herbivory have been well characterized in this system (Agrawal 1998, 1999). Leaf damage results in increased concentrations of glucosinolates (see below) and increased densities of trichomes, both of which are likely to reduce herbivory compared to undamaged control plants. A broad array of common herbivores of wild radish are negatively affected by induction, including earwigs, grasshoppers, aphids, flea beetles, and lepidopteran larvae (Agrawal 1998, 1999).

Jasmonic acid (JA) is naturally found in plants and can be used as an exogenous elicitor to stimulate plant responses to herbivory (Bodnaryk 1994; Doughty et al. 1995; Baldwin 1996; Thaler et al. 1996). JA is produced as part of the octadecanoid defense pathway and increases in plants following natural herbivory (Farmer et al. 1992). Its increase in response to herbivory is thought to be one of the initial stages of the octadecanoid cascade of biochemical events relevant to induced resistance and the pathway is highly conserved among plant families (Karban and Baldwin 1997).

### Main Experiment

We used seeds that were from a second generation of greenhouse-grown plants to reduce potential maternal environmental effects. In January 1996, we planted approximately 20 seeds from each of 17 maternal families (full- or half-sib) of *R. raphanistrum* in a greenhouse. Originally, five seeds per 800-ml pot were sown in Sunshine Soil Mix #1 (Sun Grow Horticulture, Inc., Bellevue, WA). Natural light was augmented with sodium vapor lights on a 16:8 L:D cycle. One week later, all seedlings (in the cotyledonary stage) were individually transplanted to their own pots. Pots were haphazardly placed in trays (approximately eight pots per tray) and were watered from below using automated emitters. Each pot received 0.3 g of 17:9:13 N:P:K Osmocote slow release microfertilizer (Scotts-Sierra, Marysville, OH). To minimize the effects of temperature and light differences in different areas within our greenhouse, each tray was rotated (both within and among rows) every other day for the duration of the experiment.

At the four-leaf stage, each plant was randomly assigned to one of four treatments: (1) unmanipulated controls; (2) sprayed to run-off with 0.5 mM jasmonic JA (approximately 1 ml solution, or 0.1 mg JA, was delivered to each plant; JA treatment); (3) 50% of each leaf consumed by a caged *Pieris rapae* larva, a natural herbivore of *R. raphanistrum* (caterpillar treatment); (4) 50% of each leaf clipped and removed using scissors (clipping treatment; Fig. 1). Each of the 17 families had three to five individuals in each of the four treatments for a total of 280 plants.

We used a JA treatment to induce resistance in our plants without having the confounding factor of leaf tissue removal associated with damage by herbivores. Levels of JA were chosen that are known from tomato plants to induce similar phytochemical responses as insect herbivory without phytotoxic effects (Thaler et al. 1996). In wild radish plants, the JA treatment induced comparable levels of indole glucosi-

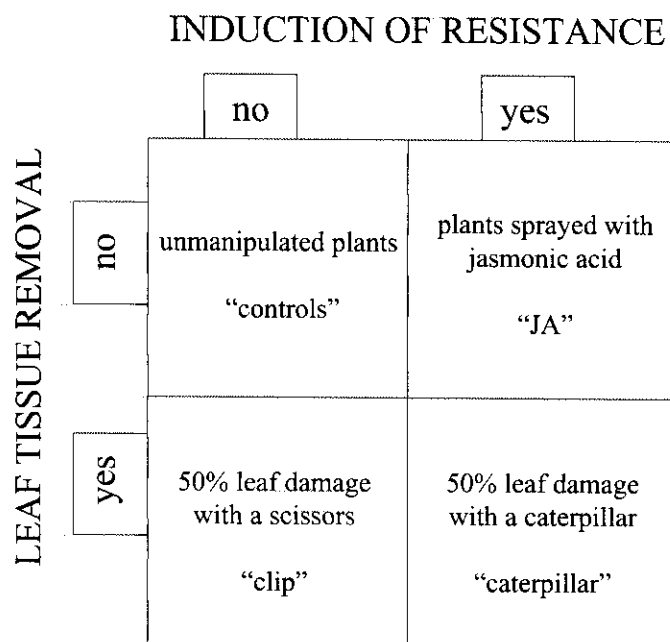


FIG. 1. Experimental design of the main experiment: a two-by-two factorial design with the main factors being with and without induced plant responses and with and without leaf tissue removal. Jasmonic acid is a natural product in plants that can be sprayed on leaves to stimulate systemic plant responses to herbivory without leaf tissue removal. Leaf tissue removal without induction was achieved by clipping leaves with scissors, which minimizes the amount of leaf tissue being damaged and without the other effects produced by herbivores.

nolates (and resistance to herbivores) to those produced in response to feeding by caterpillars (see Results; Agrawal 1999).

In the caterpillar treatment, 50% leaf area removed by *P. rapae* larvae was employed to naturally induce resistance in the plants while having a controlled level of leaf tissue damaged. Caterpillars (third to fifth instars) were contained in small "clip cages" made from the tops of petri dishes (5 cm) attached to either side of a hair clip. The cage structure was supported by a wooden stake, so as not to weigh down the leaves. The stakes were inserted into the pot's soil and displaced around to fit each of the leaves. Cages were placed adjacent to the midrib of a leaf, and caterpillars fed on tissue within the cages. After all leaf area within the cage was eaten (usually 12 h), we moved the cage along the leaf until the area on one side of the midrib was entirely consumed. These treatments were imposed continuously as plants produced new leaves. In other experiments (S. Y. Strauss, unpubl. data) wild radish plants with empty clip cages produced the same number of flowers as control plants without clip cages (mean  $\pm$  SE, control:  $279 \pm 15$ , clip caged:  $274 \pm 25$ ,  $P \gg 0.05$ ). In addition, empty clip cages were not found to induce resistance in wild radish plants (Agrawal 1999).

In the manual clipping treatment, we used scissors to remove 50% of the leaf area, equal to the amount of leaf tissue removed in caterpillar-damaged plants, but with minimal induction (see below). The time and manner in which tissue is removed is the key distinction between the clipping and herbivore treatments. Leaf tissue removal with scissors has been

shown not to induce resistance in closely related *R. sativus*, as well as in two other species in the Brassicaceae (Mattiacci et al. 1995; Agrawal 1998, 1999, unpubl.). In addition, observations from previous experiments suggested that plant responses to damage by clipping were not the same as those to similar levels of caterpillar damage (S. Y. Strauss, unpubl. data). Induced responses are thought to be minimized by clipping with scissors because: (1) herbivore saliva is not present, and saliva can be an important component of the induction process (Turlings et al. 1990; Hartley and Lawton 1991; Mattiacci et al. 1995; Alborn et al. 1997); and (2) the number of cells that is damaged (rather than area removed) is virtually nil compared to the number damaged by very many small caterpillar bites (Haukioja and Neuvonen 1985; Hartley and Lawton 1987, 1991; Mattson and Palmer 1988; Baldwin 1990; Lin et al. 1990; Bodnaryk 1992).

Phytochemical measurements were taken (see below) to make sure that plants had (or did not have) the anticipated responses to our treatments. Plants induced with JA were treated once, whereas leaf area removal treatments, 50% damage by caterpillars, and clipping were all continuous treatments maintained in synchrony as long as the plants produced new leaves.

#### Reproductive Measurements

For each plant we recorded the number of days from germination to the opening of the first flower. Early season flowering is a disproportionately important component of fitness for wild radish plants in the field (Ashman et al. 1993), and thus phenological differences between treatments may have fitness consequences in a natural field context. One set of anthers was collected from the third flower of each plant to measure pollen production and size, which are components of male fitness (Wilson et al. 1994; Delph et al. 1997). Pollen size is positively correlated with seed siring ability in wild radish plants (Young and Stanton 1990a). Each morning before sunrise, plants were surveyed for flower buds that would open later that day. Three of the six undehiscent anthers (two long and one short) were collected from each target bud using forceps and deposited in a numbered centrifuge tube. Tubes were left open in an undisturbed cabinet for two weeks to allow the pollen to dry and then closed and stored for counting and size measurements with a particle counter (Elzone 180+, Particle Data, Inc., Elmhurst, IL; methods in Young and Stanton 1990b).

To allow seed set, plants were hand-pollinated every other day using a makeup brush. A brush loaded with pollen collected from many haphazardly chosen plants was dabbed across every open flower. Although families varied greatly in their length of flowering, when the plants were senescing and had ceased to produce new flowers, we stopped watering all of them. At this stage, plants had completed their initiation of lifetime seed production, and we harvested the experiment two weeks later. All fruits were removed from each plant and placed in a labeled paper bag. Seed number was recorded as the number of seed capsules; abortion rates were low (6%) and treatments did not differ in percent seeds aborted ( $P = 0.449$ ). Mean seed weight for each plant was calculated by

haphazardly sampling 10 seeds, one from each of 10 fruits from each plant.

#### *Phytochemical Measurements*

Four plants (one plant from each treatment) from each of 11 families were destructively sampled for glucosinolate analysis 10 days after treatments were initiated. The undamaged fifth true leaf was taken from each of these 44 plants designated for chemical analysis and was removed with a razor blade and immediately frozen in liquid nitrogen. Samples were then lyophilized and kept in a standard freezer until analyzed. The analytical procedure was modified from published procedures for determination of trimethylsilyl glucosinolate derivatives with capillary gas chromatography (GC) and flame ionization detection (FID; Brown and Mora 1995). The procedure starts with a methanolic extraction of approximately 0.2 g of lyophilized, ground plant material with addition of the internal standard (1 mM of benzyl glucosinolate), followed by separation of glucosinolates using an ion-exchange column containing Sephadex DEAE. After removing impurities, glucosinolates were desulfated using desulfatase enzyme and transferred into GC autosampler vials, where they were derivatized with a silylation mixture. Silylated samples were analyzed by capillary gas chromatography using an HP-5 (30 m, 0.25 mm ID, 0.25  $\mu$ m film; Hewlett-Packard, Wilmington, DE) with an injector temperature of 290°C and an FID temperature of 305°C, using the following oven temperature program: 260°C iso for 7 min, 8°C for 1 min, 300°C iso for 10 min.

Early peaks with retention times less than 8 min are usually sugar impurities, followed with peaks of silylated glucosinolates. Only peaks of glucosinolates larger than 1% of total glucosinolate peak area were evaluated. Retention indices (ratio peak retention time/standard [benzyl glucosinolate] retention time) were used for identification of known glucosinolates. Peak areas were normalized to the standard peak area and to the sample size using the following formula: (peak area  $\times$  1000)/(peak area of IS  $\times$  sample weight). No additional FID response factors were used. To estimate effects of leaf tissue removal and induction on glucosinolate levels, we report the micromolar-per-gram leaf concentrations of indole glucosinolates (glucobrassicins), which were identified by retention time. In the Brassicaceae, indole glucosinolates appear to be the dominant class of inducible glucosinolates with known biological effects (McDanell et al. 1988; Kortisas et al. 1991; Bodnaryk 1992, 1994; Gross 1993; Doughty et al. 1995).

#### *Statistical Analyses*

Costs of induced plant responses and leaf tissue removal were analyzed using a mixed-model multivariate analysis of variance. The experimental design was a two-by-two factorial with the main fixed effects being induced responses (with and without) and leaf tissue removal (with and without; Fig. 1). Family was the third factor and was treated as a random effect in the analyses. The *F*-statistics for the fixed main effects are calculated by using the treatment-by-family interaction degrees of freedom and mean square error values in the denominator (Zar 1996; appendix 6). We used the

Scheffé formulation of the mixed-model ANOVA, which interprets the family effect across treatment environments (Fry 1992). In this case, the *F*-statistics for the random main effects are calculated by using the error degrees of freedom and mean square error values in the denominator. We conducted a MANOVA to test for effects of our treatments on all five response variables (time to first flower, seed number, seed weight, pollen grain number, and pollen size) because response of these reproductive characters may be correlated; univariate analyses protected from Type I errors were only conducted when the MANOVA was significant (Barker and Barker 1984). Data for female fitness components approximated normal distributions and generally met the assumptions of ANOVA. Data for male fitness characters and leaf chemistry were ln-transformed to meet the assumptions of normality and homoscedasticity.

In the chemical analyses, we did not have replication within each family for each treatment; therefore the between-subjects (remainder) mean square error and degrees of freedom are used in the denominator terms to calculate the *F*-statistics (Zar 1996, p. 267).

To estimate tolerance to herbivory and costs of tolerance, we conducted a separate analysis employing a mixed-model ANOVA using only control and caterpillar-damaged plants (50% herbivory). Treatment was considered as a fixed effect and family was treated as a random effect. Here we used the Hocking formulation of the mixed-model ANOVA that examines the family effect as a genetic correlation between treatment environments (Fry 1992). In this case, the *F*-statistic for the random main effect (family) is calculated by using the treatment-by-family interaction mean square error values in the denominator with their degrees of freedom. We calculated whether there were significant differences in tolerance among our 17 families by looking at the treatment by family interaction for each of the fitness components measured. Because all families received the same proportion of herbivore damage, significance of such an interaction suggests that families differ in their level of tolerance or ability to compensate for damage.

Costs of tolerance were examined by two methods. First, the family effect in the Hocking formulation of the mixed-model ANOVA is an estimation of the genetic correlation between family fitness means for damaged and undamaged plants. A negative correlation of fitness components between damaged and undamaged plants demonstrates a cost of tolerance (Simms and Triplett 1994). In such a case where costs of tolerance are found, plant families that have low fitness when damaged (i.e., not tolerant) have relatively higher fitness when undamaged; highly tolerant families will have relatively high fitness when damaged, but low fitness when undamaged. Second, the same relationship between family fitness means for damaged and undamaged plants was examined by a more conservative linear regression approach (Fry 1992).

## RESULTS

### *Phytochemical Induced Responses*

Analysis of leaf chemistry revealed that several types of glucosinolates are present in *R. raphanistrum*. Total indole

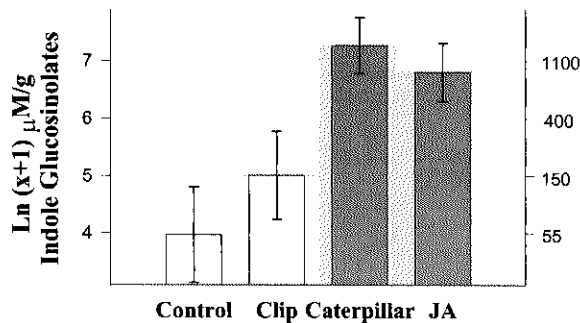


FIG. 2. The effects of treatments on systemic concentrations of glucosinolates in wild radish plants as determined by gas chromatography analysis. Treatment labels are explained in Figure 1. Undamaged leaves were assayed. Bars are mean  $\pm$  SE, and back transformed values are given on the right side of the y-axis. Leaf tissue removal alone (clipping) did not affect glucosinolate concentrations.

glucosinolates were unaffected by 50% leaf tissue removal using scissors. In contrast, both caterpillar damage and exogenous application of JA had the same result: a 10- to 20-fold increase in indole glucosinolate levels in the plants (Fig. 2, Table 1). The primary indole glucosinolates included: 3-methyl-indolyl, 4-hydroxy-3-methyl-indolyl, 4-methoxy-3-methyl-indolyl, methoxy-3-methyl-indolyl, and three unidentified peaks. These results support our assumption that clipping causes minimal induction of glucosinolates and that clipping can be used as a control for leaf area removal.

#### Costs of Induced Responses and Leaf Tissue Removal

MANOVA revealed significant effects of induction, leaf tissue removal, and family on all five fitness components (Table 2). The design of this experiment (Fig. 1) allowed us to tease apart the fitness costs of induced responses (in the absence of leaf tissue removal) and leaf tissue removal (in the absence of induction). Importantly, there was no significant induction-by-leaf removal interaction (Table 2), suggesting that costs of induction and leaf tissue removal are independent and that our JA treatment elicited similar induction effects as did natural herbivory.

Both induced responses to herbivory and 50% leaf tissue removal significantly reduced fitness of plants compared to unmanipulated controls when all fitness measures were considered together in the MANOVA (Table 2). There was an overall significant induction-by-family interaction, indicating that families varied in the degree to which induction affected their fitness parameters. The leaf tissue removal-by-family interaction was marginally significant. Following this analysis we proceeded to conduct univariate ANOVAs on each of the fitness parameters (Table 3). Figure 3 shows the effects of our treatments on time to first flower, seed production, pollen production, and pollen size.

We found significant effects of induction on time to first flower (a 6% or two-day delay in phenology) and number of pollen grains produced (6% reduction; Fig. 3). We did not detect costs of induction on seed number, seed size, or pollen size, when we considered these traits individually. Families varied in whether induction affected lifetime seed production (i.e., there was an induction-by-family interaction; Table 3).

TABLE 1. Analysis of variance for the effect of induction and leaf tissue removal on systemic concentrations of indole glucosinolates. Significant *P*-values ( $< 0.05$ ) are in bold.

Source	df	MS	<i>F</i>	<i>P</i>
Induction (I)	1	32.636	6.598	<b>0.016</b>
Leaf removal (L)	1	12.496	2.526	0.123
I*L	1	3.151	0.637	0.431
Family	10	4.675	0.945	0.509
Error	29	4.946		

Leaf tissue removal significantly affected one of the two female fitness components (seed number: 15% reduction), time to first flower (a 6% or two-day delay in phenology), which was considered both a male and female fitness component, and one of the male fitness components measured (pollen size: 2% reduction; Fig. 3). The number of pollen grains produced was also reduced by 1.5%, but this difference was not statistically significant (Table 3). Mean seed weight was not affected by induction or leaf tissue removal treatments.

On average, induction reduced fitness components by 3% and leaf tissue removal reduced fitness components by 4%. Plants experiencing both induction and leaf tissue removal (i.e., caterpillar herbivory) exhibited a 7% reduction in fitness components, again indicating that costs associated with induction and leaf tissue removal are additive. All fitness parameters measured showed significant differences among families.

#### Costs of Tolerance to Herbivory

To estimate tolerance we considered only caterpillar-damaged plants and undamaged controls in a separate analysis. Plants with 50% of each leaf consumed by a caterpillar showed significant reductions in fitness measures when they were considered together using MANOVA (Wilks'  $\lambda = 0.789$ ,  $F_{5,89} = 4.760$ ,  $P = 0.001$ ). This analysis considered joint effects of induced responses and leaf tissue removal. Univariate analyses revealed that changes in number of pollen grains per flower, pollen size, and days to first flower were significantly negatively affected by herbivore damage compared to undamaged controls (Table 4). Tolerance was calculated using family means for each trait by dividing the measure for damaged plants by undamaged plants. Across fitness measures there was little observed fitness decrement due to herbivory (Table 4).

Despite the fact that seed production showed no overall

TABLE 2. Multivariate analysis of variance for the overall effects of induction, leaf tissue removal, and family on five plant fitness components. Significant *P*-values ( $< 0.05$ ) are in bold.

Source	Wilks' $\lambda$	df	<i>F</i>	<i>P</i>
Induction (I)	0.917	5, 180	3.244	<b>0.008</b>
Leaf removal (L)	0.918	5, 180	3.224	<b>0.008</b>
Family (F)	0.253	80, 870	3.603	<b>&lt; 0.001</b>
I*L	0.997	5, 180	0.112	0.990
I*F	0.579	80, 870	1.309	<b>0.041</b>
L*F	0.593	80, 870	1.248	0.077
I*L*F	0.646	80, 870	1.034	0.401

TABLE 3. Analyses of variance for effects of induction (fixed effect), leaf tissue removal (fixed effect), and family (random effect) on each of five plant fitness components. Significant *P*-values (< 0.05) are in bold.

Source	df	Time to first flower		Total seeds		Seed weight		Pollen size		Pollen number	
		MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>	MS	<i>P</i>
Induction (I)	1	137.674	<b>0.037</b>	1,506.494	0.761	< 0.001	0.934	0.014	0.173	0.343	<b>0.019</b>
Leaf removal (L)	1	175.128	<b>0.020</b>	76,307.181	<b>0.025</b>	< 0.001	0.954	0.019	<b>0.022</b>	0.088	0.272
Family (F)	16	125.780	< <b>0.001</b>	59,399.080	< <b>0.001</b>	< 0.001	< <b>0.001</b>	0.009	<b>0.009</b>	0.139	<b>0.010</b>
I*L	1	15.178	0.399	2,358.282	0.638	< 0.001	0.977	0.001	0.595	0.001	0.924
I*F	16	26.710	0.274	15,687.078	<b>0.046</b>	< 0.001	0.199	0.007	0.103	0.050	0.728
L*F	16	26.094	0.296	12,557.902	0.157	< 0.001	<b>0.020</b>	0.003	0.691	0.068	0.430
I*L*F	16	20.252	0.563	10,249.885	0.337	< 0.001	0.687	0.004	0.669	0.078	0.288
Error	*	22.348		9,131.977		< 0.001		0.004		0.066	

\* df = 210 for time to first flower, 192 for total seeds and seed weight, 200 for pollen size, 201 for pollen number.

response to caterpillar damage treatment, we did find a significant family effect for seed production. Under the Hocking interpretation of the mixed-model ANOVA, this effect indicates that families generally exhibit similar relative fitness across environments (with and without herbivory). More importantly, however, we found a significant family-by-treatment interaction for seed production, indicating there may be genetic variation for tolerance to herbivory (Table 4). Of the 17 families, six had a 0–25% reduction in seed set with half leaf area defoliation, five had a 25–51% reduction, and 6 appeared to overcompensate and produced 100–136% more seeds when damaged. We did not detect overall effects of herbivory on seed traits in caterpillar-damaged plants because

family differences in ability to compensate for damage were balanced, with approximately equal numbers of families over- and undercompensating and with similar magnitudes of compensation in both directions. Thus, this family-by-treatment interaction was due to rank changes in fitness of families across the two environments.

To examine the cost of tolerance, we plotted the average family values of seed production for damaged plants against undamaged plants; a negative correlation of fitness components between damaged and undamaged plants suggests a cost of tolerance (Simms and Triplett 1994). We found no significant association between seed production in damaged and undamaged plants when we took the conservative re-

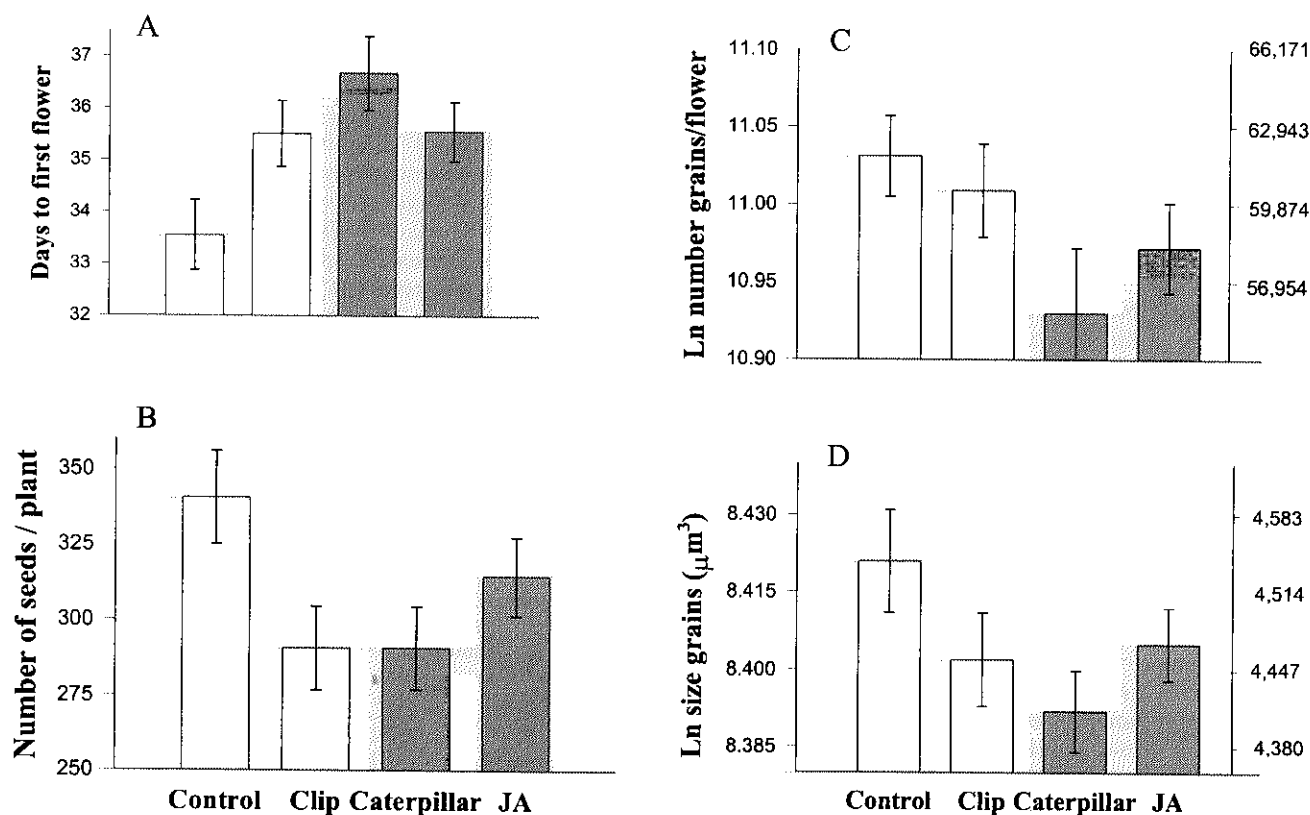


FIG. 3. The effect of treatments on (A) the number of days from seed germination to the opening of the first flower; (B) lifetime seed production; (C) pollen production per flower; and (D) mean pollen size. Treatment labels are explained in Figure 1. Bars are mean  $\pm$  SE. Where ln-values are shown (C and D), back transformed values are given on the right side of the y-axis.

TABLE 4. Tolerance to herbivory reflected in five fitness components. Data given are mean  $\pm$  SE per plant across families except as noted. Percent tolerance (damaged/undamaged) and ANOVA are given. Significant  $P$ -values ( $<0.05$ ) are given in bold.

Fitness measure	Damaged	Undamaged	Tolerance	Source	df	F	P
Seed number	290.68 $\pm$ 13.66	340.60 $\pm$ 15.43	85.3	Treatment	1, 16	2.915	0.107
				Family	16, 16	2.439	<b>0.042</b>
				Treatment * family	16, 96	1.775	<b>0.046</b>
Seed weight (g)	0.003 $\pm$ 0.001	0.003 $\pm$ 0.001	100	Treatment	1, 16	0.001	0.975
				Family	16, 16	1.184	0.370
				Treatment * family	16, 96	1.980	<b>0.022</b>
Pollen grains <sup>1</sup>	55,585 $\pm$ 2058	63,091 $\pm$ 1556	92.9	Treatment	1, 16	5.510	<b>0.037</b>
				Family	16, 16	1.403	0.253
				Treatment * family	16, 100	0.922	0.547
Pollen size ( $\mu\text{m}^3$ ) <sup>1</sup>	4418 $\pm$ 35	4555 $\pm$ 47	97.0	Treatment	1, 16	4.655	<b>0.047</b>
				Family	16, 16	1.211	0.354
				Treatment * family	16, 99	1.549	0.098
Days to first flower <sup>2</sup>	36.69 $\pm$ 0.72	33.55 $\pm$ 0.68	91.4	Treatment	1, 16	9.208	<b>0.008</b>
				Family	16, 16	2.724	<b>0.027</b>
				Treatment * family	16, 104	1.276	0.227

<sup>1</sup> Actual values are given; ANOVA performed on ln-transformed data.

<sup>2</sup> Tolerance for this trait was calculated as undamaged/damaged because damaged plants were expected to have increased values, not decreased values as in all the other traits.

gression approach (Fig. 4). However, under the Hocking interpretation of the mixed-model ANOVA, the significant family effect for seed production (Table 4) indicates that the relationship in Figure 4 has a slope significantly different from zero and probably less than one. This demonstrates that for our system, set of experiments, and analyses, tolerance was not costly, and in fact, there was a trend for families that produced the most seeds in the damaged state also to produce the most seeds in the undamaged state.

## DISCUSSION

### Induced Responses

Induced responses to herbivory in the wild radish system included increased levels of mustard oil glycosides (glucosinolates), a diverse class of sulfur-containing compounds. Induction of glucosinolates following herbivory or treatment of plants with JA has also been reported in several related *Brassica* species (Kortisas et al. 1991; Bodnaryk 1992, 1994;

Griffiths et al. 1994; Doughty et al. 1995). Glucosinolates have been studied extensively and have been shown to have negative effects on vertebrates, invertebrates, and microorganisms (Chew 1988a,b; Louda and Mole 1992). Other induced responses of wild radish plants included increased densities and total numbers of setose trichomes on newly formed leaves of damaged plants compared to trichomes on undamaged controls (Agrawal 1999). Trichome density has also been shown to reduce herbivory by *P. rapae* larvae in other brassicaceous plants (Ågren and Schemske 1993). In field experiments, these induced responses resulted in increased resistance to several herbivores, although clipping damage did not induce resistance to these herbivores (Agrawal 1998, 1999).

For plant responses to be considered defensive, they must increase plant fitness compared to plants without that response (Karban and Myers 1989). In experiments with wild radish in field environments with natural levels of herbivory, induced plants suffered less herbivory and had higher seed set than uninduced control plants (Agrawal 1998, 1999). Therefore, we were interested in measuring the costs of this defense to better understand why it may be expressed facultatively as an induced response. In our analysis we found that induced plant responses reduced plant fitness when five male and female fitness components were considered together in the greenhouse without herbivores. However, individually, costs of induction were only detected in two traits: time to first flower and the number of pollen grains produced per flower. Time to first flower and pollen characters are important components of male fitness in wild radish plants (Young and Stanton 1990a; Ashman et al. 1993). Our study is the first to demonstrate that induced plant responses (and plant resistance in general) may be costly in terms of male reproductive function (see also Biere and Antonovics 1996). These costs would have been missed using only conventional measures of female fitness (i.e., seed production).

The process of natural herbivory involves both leaf tissue removal and induced plant responses. We were able to separate costs associated with each of these factors because of

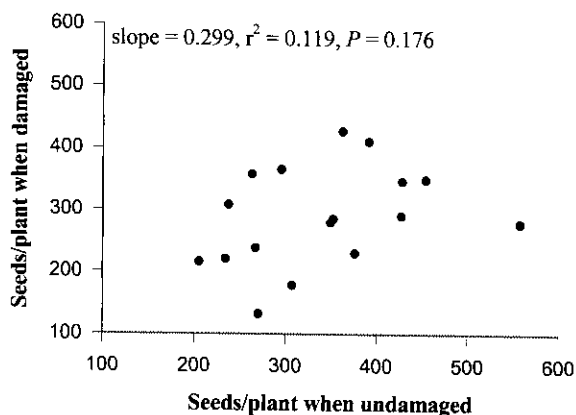


FIG. 4. The relationship between mean family female fitness (seed production) between damaged (caterpillar) and undamaged (control) plants. Each point represents the average of three to five damaged and three to five undamaged plants within a single family. A negative association would indicate a cost of tolerance.

our factorial design. We found that both leaf tissue removal and induction reduced fitness of *R. raphanistrum* and that these effects were additive. In contrast to costs of induction, costs of leaf area removal were expressed as a decrease in both male and female function. It is interesting that costs of these two aspects of leaf herbivory should be manifested in different reproductive traits. Previous work on wild radish demonstrated that pollen characters and corolla size are more sensitive to damage by caterpillars than are ovule characters (Lehtilä and Strauss 1999). Mazer and Schick (1991) showed that intraspecific competition between wild radish plants results in decreased ovule size and a trend for decreased number of pollen grains, compared to plants growing in less competitive regimes. It appears that the effects of intraspecific competition result in consequences for the plant that are more similar to the effects of leaf area removal than to the effects of induced responses. Leaf area removal results in a loss of photosynthate and photosynthetic capacity that could act as a similar type of stress as resource limitation imposed by intraspecific competition. In contrast, induced responses which include the production of plant defenses, may have different consequences for the plant, drawing more on other limiting resources and thus only affecting male fitness characters.

Many previous studies have failed to detect allocation costs of induced plant responses (Brown 1988; Simms 1992; Karban 1993; Gianoli and Niemeyer 1997; Karban and Baldwin 1997). Although lack of documentation of costs may be a result of lack of statistical power or appropriate experimental design, some have speculated that the studies may not be measuring the appropriate factor (see Karban et al. 1997). Baldwin et al. (1990) were able to detect costs of nicotine induction in wild tobacco plants that may have been due to an allocation trade-off. However these costs were only detected when plants were damaged just before flowering, and costs were not detected when damage was imposed early in the season. Although Baldwin et al. (1990) measured effects of induction on female fitness, induction may also have had effects on components of male fitness. Strauss (1997) has recently demonstrated that foliar wounding to wild tobacco, in the absence of leaf tissue removal, resulted in reduced corolla size of the flowers. Future studies of costs and constraints on plant defenses will have to consider multiple fitness measures that encompass male and female reproductive function.

The belief among evolutionary ecologists that plant defense traits must have resource-based costs is changing. Recently, our view of costs of defense has expanded to include nonresource based "ecological" trade-offs, such as those that involve trade-offs between susceptibility to different plant attackers or relationships with other plant associates (Parker 1992; Simms 1992; Karban 1993; Adler and Karban 1994; Mole 1994; Rausher 1996; Strauss 1997; Agrawal and Karban 1999). For example, in the Brassicaceae and Cucurbitaceae, secondary compounds implicated in defense against generalist herbivores attract specialist herbivores (Carroll and Hoffman 1980; Chew 1988a,b; Tallamy and McCloud 1991; Giamoustaris and Mithen 1995; reviewed in Agrawal and Karban 1999). Induction of glucosinolates in *Brassica* spp. was associated increased resistance to several species; how-

ever, it was also associated with attraction of (or susceptibility to) herbivorous flies (Baur et al. 1996) and flea beetles (Vaughn and Hoy 1993). High constitutive expression of such compounds may be detrimental to plants because of attraction of such specialized herbivores. In addition, the role of defensive compounds in attracting and repelling pollinators has received little attention (Strauss 1997).

#### *Adaptive Plasticity and the Evolution of Induced Defense*

Induced responses of wild radish appear to be beneficial in the presence of herbivores but costly in the absence of herbivores. Such phenotypic costs and benefits demonstrate adaptive plasticity. Matched phenotypes in variable environments have relatively higher fitness than alternative phenotypes (Gotthard and Nylin 1995; Kingsolver 1995; Dudley and Schmitt 1996; DeWitt et al. 1998; Kingsolver and Huey 1998). Although empirical data supporting this reasoning have rarely been reported for an induced plant response, cost-benefit models have dominated the thinking on the consequences of induction and on the evolution of inducible and constitutive defenses (Rhoades 1979; Fagerström et al. 1987; Simms and Rausher 1987; Harvell 1990a,b; Karban and Baldwin 1997; Agrawal and Karban 1999). If resistance is costly in terms of diverting resources away from growth and reproduction and is only beneficial in environments with herbivory, then variation in resistance traits could be mediated by variation in herbivore pressure. Most previous studies of costs of resistance were conducted on plants that varied genetically in their level of resistance. Demonstrations of genetic costs of resistance provide evidence that costs may constrain maximal levels of plant resistance. Production (phenotypic) costs of inducible defense are relevant as a constraint on the evolution of plant defense only if phenotypic correlations are good estimates of genetic correlations, as is thought to be the case for many systems (Cheverud 1988). Although our results are consistent with theoretical predictions of costly plant resistance (e.g., Herms and Mattson 1992; Zangerl and Bazzaz 1992), we do not know the cause of the cost of defense in our system. Phenotypic costs associated with induction could have resulted from a reallocation from growth and reproduction to resistance traits or from other factors such as autotoxicity of secondary compounds (e.g., Chew and Rodman 1979; Baldwin and Callahan 1993).

Natural selection can act on traits for which there is heritable variation that affects fitness. We detected an overall induction-by-family interaction for fitness of wild radish plants (Table 2). This result indicates either there was genetic variation in inducibility (and thus its effects on fitness) or there was simply genetic variation for the effects of induction on fitness. Our interpretation of this result is that either induced defenses or costs of induced defenses are subject to natural selection in this system. Elsewhere (Agrawal 1998, 1999) we have also reported evidence for genetic variation in induced resistance of wild radish to aphid herbivores. There is currently a paucity of other ecological genetic data on induced plant defense (Karbon and Baldwin 1997).



### Tolerance

Most studies of costs of defense do not consider multiple antiherbivore strategies that plants may employ. In this study, both costs of induced responses and tolerance to herbivory were considered. If resistance mechanisms are ineffective or have been overcome by herbivores, damaging herbivores may select for tolerance to herbivory in plants. Removal of 50% leaf area by *P. rapae* larvae should generate both costs of induction and leaf area loss, and this treatment reduced plant fitness when the five fitness measures were considered together. Reductions in individual traits were only detected for pollen grain production and size and time to first flower. These results are consistent with those of a previous study that showed effects of caterpillar herbivory on pollen characters, but not on ovule or seed characters measured over the entire lifetime of *R. raphanistrum* plants (Lehtilä and Strauss 1999). Overall, damaged plants were able to tolerate 50% leaf herbivory with only a 7% reduction in the fitness components measured. The high level of tolerance to half defoliation observed in this study may be a result of past selection by herbivores that are unaffected by the resistance mechanisms of wild radish. Specialist herbivores such as cabbage worms (*Pieris* spp.), diamondback moth larvae (*Plutella xylostella*), and flea beetles (*Phyllotreta* spp.) are often undeterred by foliar glucosinolates (Blau et al. 1978; Chew 1988a,b; Bodnaryk and Palaniswamy 1990; Louda and Mole 1992). These specialized herbivores may have been important in the evolution of tolerance to herbivory in wild radish.

Despite the fact that the average reduction in fitness due to herbivory was 7%, in fact, families varied markedly in the degree to which seed production was affected by herbivory. Fitness of some families was reduced by 50%, whereas other families appeared to overcompensate to almost the same degree. In other fitness traits where overall effects of herbivory were detected, families did not differ in their proportional tolerance to herbivory. As an important methodological issue, we note that most current studies of tolerance to herbivory utilize herbivory simulations (reviewed in Strauss and Agrawal 1999). However, it is clear that patterns and types of damage have very different consequences for plant responses to herbivory in terms of growth (Capinera and Roltsch 1980; Detling and Dyer 1981), induction of phytochemicals (reviewed in Baldwin 1990; Karban and Baldwin 1997), and effects on fitness (Agrawal 1998, 1999, unpubl.). Figure 5 illustrates mean family tolerance estimates from our 17 families as estimated by caterpillar damage and clipping damage. Tolerance to clipping damage was a poor indicator of tolerance to real caterpillar herbivory.

The results of our attempt to detect costs of tolerance contrasted with our results for costs of induced responses; tolerance did not appear to be a costly trait for wild radish plants. We found a positive relationship between seed production in damaged and undamaged plants. This result is contrary to theoretical predictions of tolerance as a costly trait (see discussions in Rosenthal and Kotanen 1994), although we may not have measured the appropriate factors to detect costs. Strauss and Agrawal (1999) show that correlations of mean family fitness of damaged and undamaged plants may not be the best method to detect costs of tolerance because high

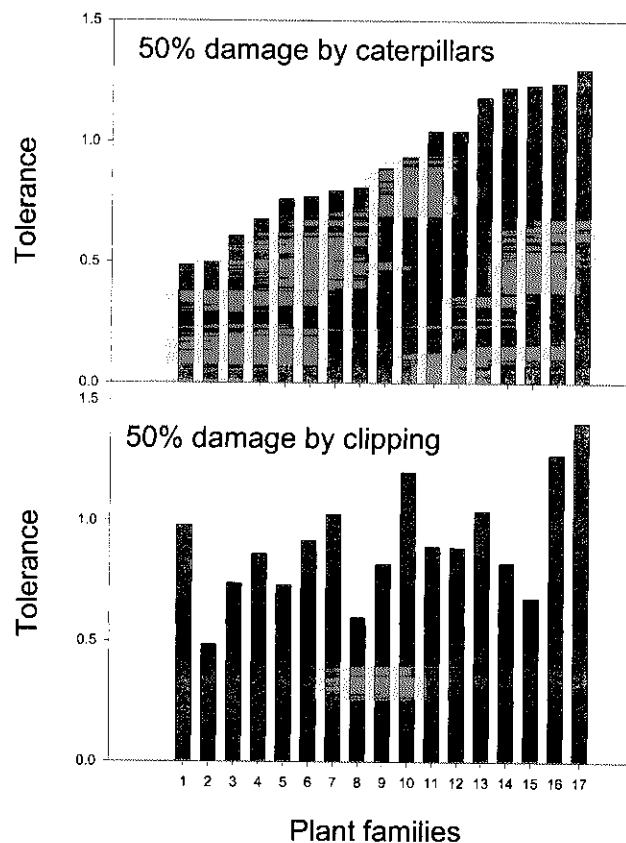


FIG. 5. Differences in plant tolerance to herbivory in *Raphanus raphanistrum* using caterpillar damage and manual clipping. Plant families are ranked based on their ability to tolerate 50% leaf area removal by *Pieris rapae* butterfly larvae (top panel); there is a significant herbivory-by-family interaction indicating familial variation in tolerance to herbivory ( $P = 0.046$ ). The bottom panel shows the same families and their ability to tolerate the same percent leaf area removal imposed by clipping with scissors, as opposed to caterpillar damage (no significant herbivory-by-family interaction,  $P = 0.226$ ). Several families have very different tolerance responses to the two types of damage (e.g., families 1 and 14). Had we only used simulated herbivory, we might have concluded that there was not evidence for a genetic basis to tolerance in this species.

across-family variance in mean fitness can obscure detection of costs. The apparent fitness benefit of tolerance, at no cost, could result in directional selection for increased tolerance (see also Mauricio et al. 1997).

There have been some suggestions that plant resistance and tolerance should be negatively correlated traits (van der Meijden et al. 1988; Rosenthal and Kotanen 1994; Simms and Triplett 1994; Fineblum and Rausher 1995). Because resistant plants do not experience much herbivory, there should not be selection for tolerance within resistant lineages (van der Meijden et al. 1988). Likewise, plants that remain susceptible in an environment with herbivores should evolve tolerance. van der Meijden et al. (1988) have found evidence for such a trade-off among five species of plants that naturally co-occur in the Netherlands. Fineblum and Rausher (1995) and Stowe (1998) have found evidence for an intraspecific negative genetic correlation between resistance and tolerance to herbivory. It has been argued that the costs of resistance may not lie in an observable trade-off between defense and

fitness components because resistance may negatively covary with tolerance to herbivory (Fineblum and Rausher 1995). If resistance and tolerance are both costly, then this negative correlation may obscure measures of costs when only one factor is considered. Because of our limited chemical analysis, we had little statistical power to assess patterns between constitutive and induced glucosinolates and levels of tolerance between families. No significant relationships were found in our preliminary analyses. Although the data are few, a negative association between resistance and tolerance does not appear to be universal (Rosenthal and Kotanen 1994; Simms and Triplett 1994; Rosenthal and Welter 1995; Rausher 1996; Abrahamson and Weis 1997; Mauricio et al. 1997; Strauss and Agrawal 1999). Future studies should incorporate measures of tolerance with measures of constitutive and inducible resistance to examine the nature of the resistance-tolerance relationship.

#### Conclusion

The path to an understanding of the evolutionary ecology of plant defense is likely to be achieved through a pluralistic consideration of multiple defenses and alternative constraints on their expression. Male fitness characters have traditionally been neglected in plant-herbivore studies (see reviews by Mutikainen and Delph 1996; Strauss et al. 1996; Delph et al. 1997; Strauss 1997). Measurement of male fitness components is especially important because male reproductive characters may be differentially affected depending on the particular stress that is imposed on the plant. In addition there may be a trade-off between male and female function itself (Eckhart and Chapin 1997). The next step will be to consider costs not only in terms of male and female reproductive success, but also with regard to other parasites and mutualists associated with plants. If certain plant traits present an ecological trade-off between resistance and susceptibility to different herbivores (e.g., Giamoustaris and Mithen 1995; Mithen et al. 1995; Van Dam and Hare 1998) or a trade-off between herbivory and pollination (Strauss 1997), allocation costs may not be necessary for the maintenance of variation in defense (Carroll and Hoffman 1980; Linhart 1991; Parker 1992; Simms 1992; Mole 1994; Karban et al. 1997; Agrawal and Karban 1999).

The results of this study indicate that there is genetic variation for plant tolerance to herbivory, but no costs of tolerance were detectable. In contrast, the expression of inducible defenses imposes a fitness cost for annual wild radish plants. The cost is largely in male reproductive characters, which have not traditionally been studied. Our detection of costs of induction relied on novel techniques to elicit induced defenses in the absence of herbivory and to remove leaf tissue without the associated induction. Induced defenses may be favored because they are only beneficial in environments with herbivory.

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