

EXPERIMENTAL MANIPULATION OF PUTATIVE SELECTIVE AGENTS PROVIDES EVIDENCE FOR THE ROLE OF NATURAL ENEMIES IN THE EVOLUTION OF PLANT DEFENSE

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Abstract.—Although biologists have long assumed that plant resistance characters evolved under selection exerted by such natural enemies as herbivores and pathogens, experimental evidence for this assumption is sparse. We present evidence that natural enemies exert selection on particular plant resistance characters. Specifically, we demonstrate that elimination of natural enemies from an experimental field population of *Arabidopsis thaliana* alters the pattern of selection on genetic variation in two characters that have been shown to reduce herbivore damage in the field: total glucosinolate concentration and trichome density. The change in pattern of selection reveals that natural enemies imposed selection favoring increased glucosinolate concentration and increased trichome density, and thus, supports one of the major assumptions of the coevolution hypothesis. We also demonstrate that a pattern of stabilizing selection on glucosinolate concentration results from a balance between the costs and benefits associated with increasing levels of this resistance character. This result provides direct confirmation of the appropriateness of cost-benefit models for characterizing the evolution of plant defenses.

Key words.—*Arabidopsis thaliana*, benefits, coevolution, costs, glucosinolates, herbivory, natural selection, resistance, trichome.

Received December 16, 1996. Accepted May 30, 1997.

Coevolution has been widely accepted as a paradigm for explaining the evolution of many features of both plants and their natural enemies (Thompson 1994). In particular, coevolution has been invoked to explain adaptive radiations in both plants and herbivorous insects (Ehrlich and Raven 1964; Berenbaum 1983), the evolution of diet specialization in herbivorous insects (Futuyma 1983; Rausher 1984), and the evolution of an incredibly diverse array of apparently defensive characters in plants, including natural chemical products, thorns and spines (Juniper and Southwood 1986; Rosenthal and Berenbaum 1991).

One of the key assumptions underlying our understanding of the process of coevolution is that natural enemies exert selection on plant populations. Such selection purportedly drives the evolution of characters that reduce or ameliorate the adverse effects of damage caused by herbivores and pathogens. However, this notion has proven controversial, in that (1) some workers have argued that selection imposed by natural enemies is generally too weak to account for the evolution of such traits (Hairston et al. 1960; Jermy 1976, 1984; Bernays and Graham 1988), and (2) evidence suggests that these characters serve other ecological and physiological functions (Muller 1969; Siegler and Price 1976; Bernays 1978; Jones 1979; Haslam 1986; Schmitt et al. 1995).

Although most biologists have nevertheless interpreted the principal function of such resistance characters to be the defense of the plant against its natural enemies (Fraenkel 1959; Whittaker and Feeny 1971; Levin 1973; Berenbaum 1983), the evidence for such selection is largely circumstantial. First, many ecological studies have demonstrated that elimination of herbivores or pathogens often increases average plant fitness, indicating the potential for natural enemies to exert

selection on their host plants (Burdon 1987; Alexander 1992; Marquis 1992). Second, plants possess characters that reduce the feeding rates, growth rates, and fitness of natural enemies, as is expected if coevolution drives the evolution of resistance characters (Feeny 1970; Cooper-Driver and Swain 1976; Berenbaum 1978; Blau et al. 1978; Rausher 1981; Rosenthal and Berenbaum 1991).

While consistent with the hypothesis that resistance characters evolve due to natural enemy-imposed selection (the “coevolution hypothesis”), this circumstantial evidence is also consistent with the alternative hypothesis (the “sequential evolution hypothesis”; Jermy 1984) that these characters evolve to serve other ecological or physiological plant function (e.g., drought tolerance, UV protection, nutrient storage) with natural enemies always evolving in response. The sequential evolution hypothesis predicts that natural enemies do not affect plant evolution.

Of course, simply demonstrating the potential for selection by natural enemies does not definitively indicate that such selection actually occurs, nor which characters such selection may favor (Jermy 1976, 1984). The common observation of the potential for natural enemy-imposed selection thus does not constitute strong evidence favoring the coevolution hypothesis. On the other hand, a reduction in herbivore damage by apparently defensive characters is easily explained by the sequential evolution hypothesis as incidental, pleiotropic effects of characters selected for other reasons (Jermy 1984; Rausher 1992a).

The fundamental distinction between these two hypotheses lies in the nature of the selective agents assumed to be responsible for the evolution of resistance characters. The coevolution hypothesis postulates these selective agents to be natural enemies, while the sequential evolution hypothesis postulates that those agents are something other than natural enemies. Consequently, one criterion that can be used to distinguish between these two hypotheses is the identity of the agents imposing selection on resistance characters.

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How does one rigorously determine whether natural enemies impose selection on plant resistance characters? This is a specific case of the more general question of how one determines whether any putative selective agent is actually exerting selection on a particular trait. We suggest that the most definitive way of addressing this question is to determine whether experimental removal of the putative selective agent causes a change in the pattern of selection on the traits of interest.

While this approach has been advocated previously (e.g. Lande and Arnold 1983; Mitchell-Olds and Shaw 1987; Wade and Kalisz 1990), it has seldom been adopted in investigations on natural systems (Endler 1986, but see Rausher and Simms 1989; Simms and Rausher 1989; Dudley 1996). In the context of natural enemies as putative selective agents on plant resistance traits, this approach involves the manipulation of the abundance of natural enemies in order to determine if the pattern of selection acting on the traits depends on the presence or absence of natural enemies. A finding that it does would constitute definitive evidence that natural enemies are, in fact, contributing to selection acting on the characters of interest.

In this study, we adopted this approach to test the hypothesis that natural enemies are the primary selective agents influencing the evolution of two resistance characters in the annual plant, *Arabidopsis thaliana*. Natural populations of *A. thaliana* exhibit genetic variation for fitness and for two characters that have been shown to reduce damage by natural enemies in the field: total glucosinolate concentration and trichome density (Mauricio 1998). Therefore, *A. thaliana* should be capable of responding to selection imposed by natural enemies. To examine whether natural enemies impose such selection, we assessed whether removal of these suspected selective agents altered the overall pattern of selection acting on the two resistance characters. We measured and compared selection gradients for the resistance characters in both the presence and absence of natural enemies. Any difference between the gradients would indicate that natural enemies exert selection on these resistance characters.

MATERIALS AND METHODS

Natural History

Arabidopsis thaliana (L.) Heynh. (Brassicaceae) is a winter annual plant that typically grows in disturbed habitats (Baskin and Baskin 1972). It is found throughout North America, although its center of origin is thought to be eastern Europe (Ratcliffe 1961; Lawrence 1976). We obtained a conservative estimate of the date of introduction of *A. thaliana* into the United States using information from four herbaria with early U.S. collections: the Academy of Natural Sciences, Philadelphia (ANS); the Harvard University Herbaria (HUH); the New York Botanical Garden Herbarium (NYBG); and the Smithsonian Institution. The earliest dated specimens of *A. thaliana* were collected in Pennsylvania in 1843 (ANS), although there exist earlier, but undated collections (HUH and NYBG). The earliest dated collection of *A. thaliana* from North Carolina was taken in 1874 (HUH).

In North Carolina, seeds of *A. thaliana* germinate in mid-autumn and plants grow through the winter, flower in early

spring, and complete fruiting and senescence by late spring. Most studies have concluded that outcrossing in natural populations of *A. thaliana* is rare (Jones 1971; Snape and Lawrence 1971; Abbott and Gomes 1987; Trnená et al. 1987), although significant levels of genetic variation have been found in natural populations for both allozymes (Gomes and Abbott 1987; Kilian and Maluszynski 1987) and life-history characters (Kilian et al. 1985; Mauricio 1998). In the field, we have frequently seen several species of flies (Syrphidae), small bees (Apidae), and skippers (Hesperiidae) actively visit and probe flowers and transport pollen of *A. thaliana*.

We have observed leaf damage on many individuals of *A. thaliana* in the field, as well as insects feeding on the plants, including several species of beetles, lepidopteran larvae, leaf miners, and aphids. The most common herbivores we have collected on *A. thaliana* in Durham County, North Carolina are two species of flea beetles, *Psylliodes convexior* LeConte (Chrysomelidae) and *Phyllotreta zimmermani* Crotch (Chrysomelidae). Several fungal and bacterial pathogens are reported to occur on natural populations of *A. thaliana* (Morgan 1971; Koch and Slusarenko 1990; Dangl et al. 1992; Tsuji and Somerville 1992).

Arabidopsis thaliana possesses two resistance characters, one chemical and one morphological, which have been shown to reduce damage by herbivores in the field (Mauricio 1998). First, the leaves and seeds of *A. thaliana* contain glucosinolates, a class of natural products found in all plants of the Brassicaceae and that have been shown to be toxic or unpalatable to a variety of insect and mammalian herbivores (Vaughan et al. 1976; Blau et al. 1978; Hogge et al. 1988). Second, small hairs, or trichomes, cover the leaves and stems of *A. thaliana*. Trichomes have also been shown to reduce damage by herbivores in a variety of plants (Levin 1973; Johnson 1975; Lamb 1980; Juniper and Southwood 1986).

Experimental Design

In the spring of 1992, we collected seeds from 144 plants growing in Durham County, North Carolina. Since *A. thaliana* is primarily selfing, seeds from each plant essentially form an inbred line, or family. Differences among these families represent both genetic differences and effects of common (maternal) environment. To minimize these environmentally derived differences, we propagated the families used in this experiment in a common growth chamber for three generations.

On December 7, 1993, we transplanted 1728 young plants (144 families, 12 replicates per family) to a field site in Durham County, North Carolina, where *Arabidopsis thaliana* grows naturally. The distance between planted individuals, approximately 10 cm, reflected the density of plants found in the field. Vegetation within the experimental area was not disturbed.

Half the plants were exposed to natural enemies at natural densities and the other half were sprayed at approximately two-week intervals through the entire field season in order to remove herbivores and fungal pathogens (Mauricio 1998). Plants were sprayed with the following pesticides: a synthetic pyrethroid insecticide (ASANA® XL, esfenvalerate), a benzimidazole fungicide (Benlate®, benomyl), a benzenoid fun-

gicide (Ridomil®, metalaxyl), and an organochlorine fungicide (Bravo®, chlorothalonil). This spraying regime eliminated virtually all visible leaf damage from the treatment plants (Mauricio 1998). In a separate experiment conducted in a growth chamber, this regime of pesticide application was shown not to affect the seed production of *A. thaliana* (Mauricio 1998). Because of the possibility of contamination of controls by pesticides, plants were arranged in a split plot design, with three spatial blocks each consisting of two control and two sprayed subplots. One individual from each of the 144 families was randomly assigned to each treatment subplot.

In mid-March 1994, four leaves were collected from each plant in the field. All sampled leaves were fully expanded and approximately the same age. Total glucosinolates, expressed as the concentration of glucose (mg) released by enzymatic hydrolysis of glucosinolates (1 glucose = 1 glucosinolate) per mg leaf (wet weight), were assayed using the micro-column method of Heaney and Fenwick (1981). Trichome density was estimated as the total number of trichomes within a 2.4 mm² area of the upper central area of the adaxial (upper) side of the leaf.

From April 9–12, 1994, plants in the field were examined for signs of damage by herbivores. To facilitate the measurement of damage in the field, for each plant we measured total number of leaf damage holes made by herbivores (Mauricio et al. 1997). Damage holes were roughly uniform in size, with an average area of 1.26 mm² and a standard deviation of 1.25. Average area of a single leaf, by contrast, was 40.5 mm² with a standard deviation of 32.3.

Plants were harvested after senescence in mid-May, 1994. We used the total number of fruits to estimate fitness. Total fruit number is an excellent predictor of total seed number in field-collected *A. thaliana* (seed number = 40.5 × fruit number - 72.0; $r = 0.98$; $n = 50$ plants). We assume that since *A. thaliana* is a selfing annual, fruit number represents both the total female and male reproductive effort and is a reasonable predictor of lifetime fitness. For analysis, we calculated relative fitness by dividing the total number of fruits by the maximum fruit number observed in both treatment groups.

Analyses

Our level of genetic analysis is the family (Arnold 1981; Via 1984). Because each family represents, in a selfing plant like *A. thaliana*, an inbred line, any among-family variance or covariance detected in this experiment represents total, rather than additive, genetic variance or covariance. Estimates of total genetic variance and covariance are more appropriate for this species, however, for two reasons. First, in a population of selfing organisms, natural selection acts on the total genetic variation and not exclusively on the additive genetic variation as in the case of outcrossing species (Roughgarden 1979). Second, the crosses that would be used to partition total genetic variation into additive and nonadditive components would generate offspring that would be artificially highly heterozygous, and thus be of questionable relevance to evolution in natural populations of *A. thaliana*.

Analyses of variance were performed using the GLM pro-

cedure in SAS (version 6.09) and Pearson correlation coefficients were calculated using the CORR procedure. To compare the damage levels in 1995 with damage experienced by the control plants in 1994, we used a Dunnett's T test ($\alpha = 0.05$). This test controls the type I experimentwise error for comparisons of a number of treatments to a common control (SAS Institute 1989).

Measurement of Selection

We used the partial regression analysis described by Rausher (1992b) to measure selection acting on resistance characters. Rausher's (1992b) approach to the measurement of selection on quantitative traits is similar to that of the phenotypic analysis of Lande and Arnold (1983), but accounts for biases due to environmental correlations between characters and fitness by examining selection acting directly on genotypic values rather than on phenotypic values (Mauricio and Mojonner 1997). Here, we measured selection acting on family means, which serve as estimates of parental genotypic values.

In this regression analysis, partial regression coefficients are used to estimate selection gradients which represent selection that acts directly on a character (Lande and Arnold 1983; Rausher 1992b). An estimate of the directional selection gradient is obtained from the partial regression coefficient of a linear regression of relative fitness on the character (a measure of the slope). The sign of the gradient indicates the direction of evolutionary change expected from selection acting directly on the character. An estimate of the stabilizing/disruptive selection gradient is obtained from the second-order coefficient of a quadratic regression of relative fitness on the character (a measure of the curvature). The sign of the gradient indicates whether the fitness function is concave downward (negative = stabilizing selection) or concave upward (positive = disruptive selection).

The selective effect of natural enemies on resistance was determined by comparing the slope (directional selection gradient) or the curvature (stabilizing/disruptive selection gradient) of the fitness functions for the resistance characters between the two treatments (natural enemies eliminated [spray treatment] versus the presence of the assemblage of natural enemies present in the field [control]). If the slopes do not differ (no significant treatment by resistance interaction) and the curvature is the same (no significant treatment by resistance² interaction), then natural enemies are not exerting selection on resistance. Conversely, a significant treatment by resistance interaction for fitness indicates that genetic variation in fitness and the intensity of selection depend on the presence of natural enemies. This implies that natural enemies impose selection on resistance (Simms and Rausher 1989).

Visualization of Fitness Surfaces

Although the statistical analysis of selection indicates whether the general pattern of selection differs when natural enemies are included or removed, it does not show how the pattern differs in detail. To determine more precisely the pattern of selection that natural enemies exert on resistance, we require some method for visualizing the selection surface

under different treatments. For example, it is possible to use the regression coefficients from the selection analysis to construct selection surfaces for visualization. However, Schluter and Nychka (1994) have shown that more accurate portrayals of the selective surface are obtained by the use of a non-parametric procedure, the spline. This procedure has been advocated as a means to visualize selective surfaces, although it is subject to many of the same caveats as selection analysis (Mauricio and Mojonier 1997). Since the sampling distributions of these fitness surfaces are unknown, it is difficult to estimate a "confidence interval" for a surface, although resampling statistical procedures can be used (Schluter and Nychka 1994). Thus, the estimation of selective surfaces is best thought of as an exploratory procedure.

We used the G3GRID procedure of SAS to generate selective surfaces. This procedure fits a smoothed estimate of a bivariate spline to the data (SAS Institute 1988). Selective surfaces were generated for each treatment using a grid spacing of 5 for trichome density and 0.5 for glucosinolate concentration. To obtain a visual representation of the pattern of selection imposed by natural enemies, the selective surface for the spray treatment was subtracted from that of the control treatment. Specifically, for each of the grid points, the value of fitness for the spray treatment was subtracted from the value of fitness for the control treatment, yielding a difference in fitness for each grid point. These fitness measures were used to plot both three-dimensional surfaces and fitness contour plots.

Field Survey of Spatial and Temporal Variation in Herbivore Damage

To determine the spatial and temporal variation in herbivory, we compared the levels of damage experienced by the plants in the experiment with levels of damage in another year (1995). One hundred plants were collected from each of 10 locations and the total number of damage holes made by herbivores were measured. These locations included sites from which the experimental plants were originally collected.

RESULTS

Statistical Analysis of Selection

Natural selection acts on both trichome density and total glucosinolate concentration. There is a significant genetic relationship between total glucosinolate concentration and relative fitness as well as a significant genetic relationship between trichome density and relative fitness. In other words, genotypes that differ in resistance differ in relative fitness. Estimated directional selection gradients indicate that in both treatments, selection favored a decrease in trichome density and a decrease in glucosinolate concentration (Table 1). For trichome density, in neither treatment was the quadratic selection gradient significantly different from zero (Table 1). However, significant stabilizing selection on total glucosinolate concentration occurred in the spray treatment (Table 1).

Both resistance characters were included in all our statistical models since we found a genetic relationship between the two resistance characters: there is a significant positive

genetic (family-mean) correlation between total glucosinolate concentration and trichome density (Pearson correlation coefficient, $\rho = 0.381$; $P < 0.001$). This correlation is not an artifact of correlations of each character with plant size because there was no significant correlation between plant size and either resistance character. In addition, when we recalculated the correlation using the residual values of glucosinolate concentration and trichome density after removing the effects of plant size, the significant positive correlation remained ($\rho = 0.259$; $P = 0.0017$).

We attempted to detect whether natural enemies exerted selection on glucosinolate concentration and trichome density by determining whether the general pattern of selection on these characters differed between the control treatment, in which natural enemies were present, and the sprayed treatment, in which natural enemies were absent. The pattern of selection was quantified by using the directional and stabilizing selection gradients on these characters (Table 1). The stabilizing selection gradients differed significantly between treatments for both characters (Table 2B, treatment \times character² interactions), indicating that natural enemies modify the pattern of stabilizing selection on these characters. By contrast, natural enemies did not detectably modify the pattern of directional selection for trichomes and glucosinolate concentration, as indicated by the absence of a significant difference between treatments in the directional selection gradients (Table 2A, treatment \times character interactions).

Visualization of Fitness Surfaces

While the partial regression analysis of selection indicates that natural enemies alter the general shape of the selective surface, it provides little insight into the specific details of how the surface is changed. In addition, even though both resistance characters are included in the statistical model, the estimates of selection acting on a single character are calculated by holding the other character constant at a mean trait value. To see how natural enemies modify the entire selective surface, which includes axes for the two resistance traits and relative fitness, we examined the fitness surfaces corresponding to the two treatments in the experiment.

The selective surface for trichome density and total glucosinolate concentration in control plants represents the net effect of all selective forces acting on the resistance traits (Fig. 1A,B). Along the direction of the trichome axis, the surface decreases with very little curvature, producing the directional selection favoring a decline in trichome density to zero. By contrast, along the direction of the glucosinolate axis, there appears to be a concave downward curvature, especially at low trichome densities. Together, these patterns produce a single fitness peak corresponding to genotypes with no trichomes and a glucosinolate concentration of 3.5 mg glucosinolates/L/mg leaf. The population is currently not at this equilibrium, although it will presumably evolve to reach it eventually, assuming the fitness surface remains constant over time. When it does so, it should experience stabilizing selection on glucosinolates but directional selection to minimize trichome density.

The selective surface for the plants in the spray treatment represents the net effect of all selective forces acting on re-

TABLE 1. Directional (β) and stabilizing/disruptive (γ) selection gradients from selection analysis. Standard errors are in parentheses. The model and error sums of squares (and degrees of freedom) for the linear models were 0.05746 (2) and 0.28259 (141) for natural enemies present, and 0.13447 (2) and 0.47855 (141) for natural enemies absent. The model and error sums of squares (and degrees of freedom) for the quadratic models were 0.06690 (5) and 0.27314 (138) for natural enemies present, and 0.13743 (5) and 0.43868 (138) for natural enemies absent.

Resistance character	Natural enemies present (control)			Natural enemies absent (sprayed)		
	β	γ		β	γ	
		Trichome density	Glucosinolate concentration		Trichome density	Glucosinolate concentration
Trichome density	-0.00317** (0.00081)	0.00009 (0.00014)	-0.00101 (0.00129)	-0.00469*** (0.00086)	-0.00017 (0.00010)	0.00116 (0.00078)
Glucosinolate concentration	-0.01012* (0.00524)		-0.00396 (0.00505)	-0.00640* (0.00337)		-0.00329** (0.00111)

* $P = 0.05$; ** $P = 0.01$; *** $P = 0.0001$.

sistance except those due to natural enemies (Fig. 1C,D). The major difference between this surface and the one for control plants is the shift in the fitness peak to a position corresponding to genotypes with no glucosinolates and no trichomes. Thus, the shape of this surface indicates that, in the absence of natural enemies, there is selection acting to decrease both trichome density and total glucosinolate concentration, i.e., there is a fitness cost to both traits (Mauricio 1998).

TABLE 2. Analysis of variance for relative fitness of *A. thaliana* grown in the field. The treatments were plants exposed to the natural assemblage of herbivores and pathogens or sprayed with pesticides. All effects are considered fixed. The type III sums of squares are designed to remove the effect of all the other effects in the model before testing the effect in question (SAS Institute 1989). A. Analysis of directional selection. Only linear terms are included because adding quadratic terms would result in biased estimates of directional selection gradients. B. Analysis of stabilizing/disruptive selection. Both linear and quadratic terms are included.

A.				
Source of variation	df	Type III sums of squares	F	P
Family	143	0.55800	2.67	0.0001
Treatment	1	0.02410	16.49	0.0001
Glucosinolates	1	0.00017	0.12	0.7349
Trichome density	1	0.00470	3.22	0.0751
Treatment \times glucosinolates	1	0.00043	0.29	0.5905
Treatment \times trichome density	1	0.00221	1.51	0.2207
Error	139	0.20313	—	—
B.				
Source of variation	df	Type III sums of squares	F	P
Family	143	0.53347	2.75	0.0001
Treatment	1	0.03254	23.96	0.0001
Glucosinolates	1	0.00653	4.81	0.0300
Trichome density	1	0.00002	0.02	0.8988
Glucosinolates ²	1	0.00301	2.22	0.1388
Trichome density ²	1	0.00048	0.35	0.5545
Glucosinolates \times trichome density	1	0.01074	7.91	0.0056
Treatment \times glucosinolates ²	1	0.00727	5.35	0.0222
Treatment \times trichome density ²	1	0.00969	7.14	0.0085
Treatment \times glucosinolates \times trichome density	1	0.00775	5.71	0.0182
Error	135	0.18334	—	—

The actual pattern of selection imposed by natural enemies is revealed by subtracting the selective surface for the spray treatment from that of the control treatment (Fig. 1E,F). High points on the resulting selective surface represent combinations of glucosinolates and trichomes for which natural enemies have relatively little detrimental impact on fitness, whereas low areas represent combinations for which natural enemies decrease plant fitness greatly. The most prominent feature of this surface is a valley that runs diagonally from the origin (zero glucosinolate concentration and zero trichome density). Because the origin is part of this valley, the detrimental impact of natural enemies is large for genotypes that produce little glucosinolates and have few trichomes. On either side of the valley are two fitness peaks, corresponding to, on the one hand, maximal trichome density and no glucosinolates, and, on the other hand, maximal glucosinolates and no trichomes. It thus appears that, as might be expected, damage by natural enemies favors increasing levels of both resistance characters.

However, there seems to be an inhibitory interaction between the protective effects of glucosinolates and trichomes. Genotypes with intermediate levels of both traits experience a greater fitness depression by natural enemies than genotypes with comparable levels of one resistance trait only. This interaction is reflected in the fitness valley separating the two peaks, and results in a combination of both resistance traits being less effective in reducing the detrimental effects of natural enemies than either trait alone.

Spatial and Temporal Variation in Herbivore Damage

Our field census of herbivore damage in 1995 indicated that the amount of herbivore damage experienced by *A. thaliana* can vary both in location and year (Table 3). Comparison of damage experienced by plants in 1995 with damage experienced by the experimental plants in 1994 showed that the damage levels observed in the experiment were not significantly different from those experienced by plants in eight of 10 locations sampled. However, in the remaining two locations, the mean level of damage was significantly higher than in the experimental population.

DISCUSSION

The idea that selection imposed by natural enemies on plants causes the evolution of resistance characters is central

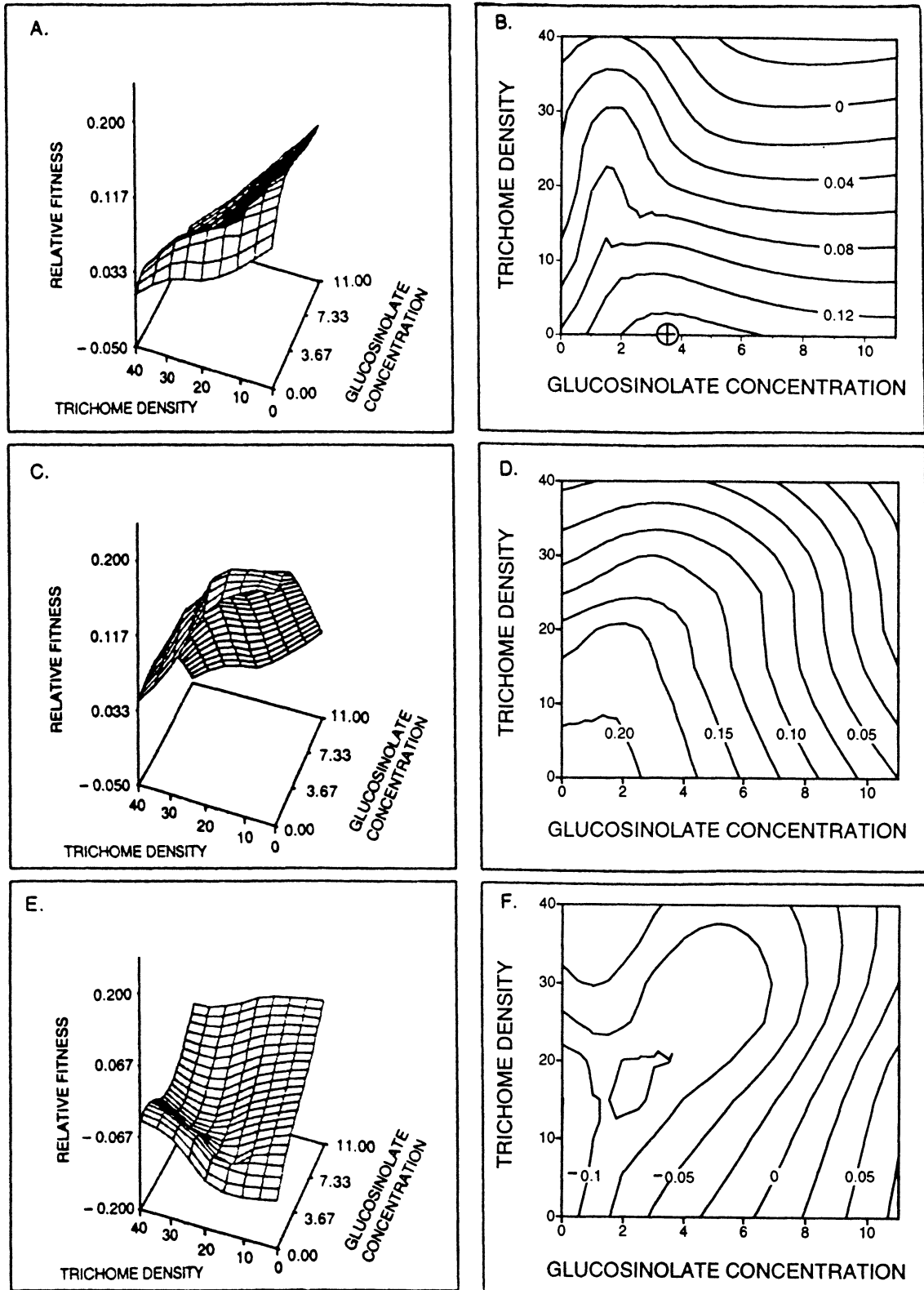


FIG. 1. Dissection of fitness surfaces for total glucosinolate concentration (mg glucosinolates/L/mg leaf) and trichome density (number of trichomes per 2.4 mm²). The fitness surface, which reflects the pattern of selection on the two characters, represents the relationship between relative fitness (relative to the maximum measured fitness), trichome density and glucosinolate concentration. For each treatment, and their difference, the fitness surface is portrayed as both a 3-D graph and a contour plot. (A,B) Overall pattern of selection (control plants). Circled cross in (B) indicates approximate position of expected evolutionary equilibrium. (C,D) Pattern of selection in absence of natural enemies (sprayed plants). Decreasing fitness contours along each axis reflect costs associated with trichome density and glucosinolate concentration. (E,F) Difference between fitness surfaces in control and sprayed treatments, which reflects the pattern of selection imposed by natural enemies.

TABLE 3. Mean damage levels in 1995. Standard errors are in parentheses. Damage is measured as the total number of holes made by insect herbivores. Damage levels marked with a * are significantly different from the experimental damage levels at the 0.05 level. $N = 100$ for each location and $N = 864$ for the experimental population.

Location	Damage
1	2.33 (0.22)
2	2.39 (0.23)
3	5.86 (0.55)*
4	2.43 (0.24)
5	5.60 (0.54)*
6	2.46 (0.24)
7	2.09 (0.20)
8	2.85 (0.27)
9	3.33 (0.31)
10	3.06 (0.31)
Experimental	2.34 (0.08)

to the theory of coevolution (Ehrlich and Raven 1964; Janzen 1980; Berenbaum 1983). Although, in accordance with this theory, it has long been asserted that natural enemies such as insect herbivores and pathogens are the primary selective agents on plant resistance characters, experimental evidence for this assertion has been weak. This lack of evidence has led several workers to question whether natural enemies are responsible for the evolution of apparently defensive characters (Muller 1969; Jermy 1976, 1984; Siegler and Price 1976; Bernays 1978; Jones 1979; Haslam 1986; Jones and Firn 1991; Schmitt et al. 1995).

Any demonstration that a particular environmental factor or agent is having an evolutionary impact on a specific character must meet three criteria. First, there must be genetic variation for the character, a requisite for an evolutionary response to selection. Mauricio (1998) has shown that there is genetic variation for both total glucosinolate concentration and trichome density in natural populations of *A. thaliana*. This criterion seems to be commonly met by resistance characters since many studies have demonstrated that genetic variation for resistance to natural enemies exists in natural plant populations (Dirzo and Harper 1982; Berenbaum et al. 1986; Simms and Rausher 1987).

Second, individuals with different values of the character must have differential fitness, i.e., natural selection must be acting. The statistical analysis of selection reported here revealed that both resistance characters were subject to selection. Several studies on other systems have shown a similar pattern of selection acting on plant resistance characters (Dirzo and Harper 1982; Berenbaum et al. 1986; Parker 1990; Simms and Rausher 1993; Ågren and Schemske 1994; Núñez-Farfán and Dirzo 1994).

The first two criteria for an evolutionary impact of natural enemies on plants thus seem to be commonly met. However, the simple demonstration that selection operates on a resistance character is not sufficient evidence for concluding that natural enemies are the cause of that selection. Rather, a third criterion for demonstrating an evolutionary impact must also be satisfied: the putative selective agent, in this case natural enemies, must be shown to contribute to the observed selection. Demonstrating that a system meets this third condition normally requires assessing the effect of experimental

manipulation of the suspected selective agent on the pattern of selection.

Using such experimental manipulation in this study, we obtained evidence indicating that natural enemies do exert selection on both a chemical and a morphological resistance character: total glucosinolate concentration and trichome density. When we eliminated the putative selective agent, natural enemies, from plants under natural conditions, we observed a significant change in the pattern of selection on the resistance characters. Therefore, all three criteria for demonstrating an evolutionary impact of natural enemies on these resistance characters have been met in *A. thaliana*. Our study thus provides conclusive evidence for the view that natural enemies can impose selection on plant resistance characters.

Our experimental design does not allow us to identify the particular natural enemies that are the agents of selection. We used broadly acting pesticides in the hopes that we would eliminate all or most of the natural enemies in the treatment plots. A finer scale experimental manipulation of herbivores and pathogens should allow for the identification of the particular agents of selection on these defensive characters.

We are aware of only one other study that has examined all three criteria for demonstrating an evolutionary impact of the natural assemblage of herbivores and pathogens on resistance in natural plant populations. Simms and Rausher (1989) found that, for the morning glory, *Ipomoea purpurea*, in the presence of herbivores, damage by capsule-feeding lepidopteran larvae was subject to selection, manifested as a genetic covariance with fitness. When herbivores were removed by spraying, however, selection on resistance was eliminated. Although their results indicated that herbivores were exerting some sort of selection on resistance to lepidopteran larvae in morning glories, their study is ambiguous because they did not identify any particular resistance traits. Nevertheless, the two studies that have manipulated natural enemies have detected an effect on the pattern of selection acting on resistance, or on specific resistance traits. These results suggest that natural enemies may often exert such selection on resistance characters and support the general belief that coevolution between plants and their natural enemies is common.

Balance between Costs and Benefits

The pattern of selection in the absence of natural enemies suggests that there are fitness costs associated with the production of both glucosinolates and trichomes (Mauricio 1998). In particular, the fitness peak in the absence of natural enemies corresponds to absence of both resistance characters. The existence of these costs validates a primary assumption of models of the evolution of defensive characters (Rhoades 1979; Gulmon and Mooney 1986; Fagerström et al. 1987; Simms and Rausher 1987).

These models also predict that benefits associated with resistance can offset these costs and yield stabilizing selection that produces an intermediate level of resistance. Our results for glucosinolate concentration appear to represent the first direct experimental confirmation of this prediction: the combination of decreasing detrimental effects of natural enemies with increasing cost as glucosinolate concentration in-

creases leads to weak stabilizing selection on glucosinolates. Interestingly, in most models it is generally assumed that cost increases linearly with allocation to defense, while benefits plateau. We found, however, that the opposite occurred; benefits increased approximately linearly within the range of observed variation in glucosinolate concentration, while costs increased slowly at first, then with an increasing rate (Fig. 1).

By contrast, the balance between costs and benefits did not produce any indication of stabilizing selection on trichomes. Instead, when both costs and benefits are present, absence of trichomes is favored. For this character, the costs were simply too high to be offset by benefits of reduced damage. The difference in net pattern of selection between trichomes and glucosinolates thus illustrates how the balance between costs and benefits may be tipped in different ways, depending on the relative magnitudes of the costs and benefits of resistance.

The failure of benefits to outweigh the costs of trichomes raises the question of why trichomes have not been eliminated from local populations of *A. thaliana*. One possible explanation is that trichome density is not at evolutionary equilibrium. Because *A. thaliana* is not native to North America, the abundance of natural enemies may not be as high as that of the native guild of natural enemies in the original habitat of *A. thaliana*. Thus, while natural enemies may have caused sufficient damage to compensate for the costs of trichome production in that habitat, damage in North Carolina may be insufficient to do so. Consequently, we might expect trichomes eventually to be eliminated from our local populations.

Alternatively, although *A. thaliana* has been growing in North Carolina for at least 120 years, natural enemy abundance itself may currently not be at a steady state and may gradually increase to levels that will sustain trichomes. This outcome might be expected because many Eurasian plants were introduced to North America along with their native Eurasian natural enemies. Since all plants in the Brassicaceae possess glucosinolates (Vaughan et al. 1976), and glucosinolates can serve as both feeding and oviposition stimulants for herbivorous insects (Blau et al. 1978; Nielsen 1978; Renwick and Radke 1983; Traynier and Truscott 1991), we would expect both native crucifer-feeding and introduced natural enemies to eventually incorporate *A. thaliana* into their diets (Thomas et al. 1987), although this may be an ongoing process.

Finally, we note that natural enemy abundance varies spatially in North Carolina. The data from the 1995 damage census indicates that two locations had significantly more damage than eight other sites in 1995 and the experimental plants in 1994. At such locations, selection imposed by natural enemies may be sufficient to balance the costs of trichome production. If such sites are common enough, they may account for the continued maintenance of trichomes in North Carolina populations.

Conclusion

Previous investigations have demonstrated that purported defensive characters are subject to selection under natural conditions. Because these investigations have not manipu-

lated natural enemy abundance, however, it has not been possible to determine whether herbivores and other natural enemies cause that selection. The results of these studies are also consistent with the hypothesis that either these defensive characters, or pleiotropic effects of the genes that produce them, are subject to selection imposed by ecological factor(s) other than natural enemies. In fact, several other functions, such as nutrient storage (Wink and Witte 1985), prevention of water loss (Ehleringer et al. 1976; Ehleringer and Björkman 1978; Meinzer and Goldstein 1985) and protection against ultraviolet radiation (Rhoades 1977; Karabourniotis et al. 1992), have been specifically ascribed to the resistance characters under study here: trichomes and secondary compounds.

Our manipulations of the abundance of natural enemies permit us to distinguish between these alternatives and indicate that selection imposed by natural enemies is favoring both increased trichome density and increased glucosinolate concentration. We have not, of course, ruled out the possibility that other selective forces may contribute to favoring these characters, since costs associated with these characters may be large enough to mask other beneficial effects in the absence of natural enemies. However, as in the case of natural enemies, experimental manipulation of these other putative selective agents must be used to test any hypotheses concerning their role in selection on these characters. In addition, we have no information on the historical pattern of selection acting on these resistance characters. It is certainly possible that these characters evolved for reasons other than defense against natural enemies and are only now subject to selection from herbivores and pathogens. Nevertheless, our results support the predominant view that herbivores and other natural enemies are a major, if not the primary, selective agent molding the evolution of apparently defensive characters in plants, i.e., that the "co" in "coevolution" is often appropriate for describing the interactions between plants and natural enemies.

ACKNOWLEDGMENTS

We are grateful to A. Powell for generously providing the field site for this research. We immensely appreciate the people who volunteered many hours of assistance in the field and lab: K. Bright, M. Butler, S.-M. Chang, M. Gustafson, D. Hall, A. Pfrimmer Hensley, B. Inouye, K. Iwao, S. Paulsen, S. Mille Comitre, P. Murphy and N. Underwood. We thank D. Burdick for statistical advice and K. Iwao, L. Mojonier, I. Shonle and N. Underwood for comments on earlier drafts of this manuscript. T. Mitchell-Olds and D. Siemens shared their time and expertise in glucosinolate analysis. N. Gillham, C. Laurie and the Duke University Morphometrics Laboratory gave us access to needed equipment. The Ciba-Geigy and Dupont corporations donated the pesticides. J. Barber, D. Boufford, P. Holmgren, E. Schuyler and V. Funk provided information on collections of *Arabidopsis thaliana* in their respective institutions. Financial support was provided by National Science Foundation Grant Dissertation Improvement Grant, DEB 93-22462, Sigma Xi Grants-in-Aid of Research, the North Carolina Academy of Science, and the Duke University Department of Zoology. Seeds from the

plant families used in this experiment have been deposited in the Arabidopsis Biological Resource Center at Ohio State University, stock numbers CS8180 through CS8323.

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Corresponding Editor: J. Mallet