

ENEMIES OF HERBIVORES CAN SHAPE PLANT TRAITS: SELECTION IN A FACULTATIVE ANT–PLANT MUTUALISM

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Abstract. Predators and parasitoids of herbivores can play important roles in plant defense. Plant traits that attract the enemies of herbivores are presumed to have co-evolved with these enemies in obligate relationships such as ant–*Acacia* mutualisms. In facultative interactions, selection via herbivores' enemies also may shape plant traits, although no study has yet demonstrated all of the necessary criteria to document selection in a single tri-trophic system. Using manipulative experiments during a two-year study, I showed that predatory ants can act as agents of selection on extrafloral nectary (EFN) traits. Wild cotton (*Gossypium thurberi*) EFNs attract a number of generalist ant species that consume and disturb herbivores. Sire–offspring regression analyses demonstrated that both the proportion of leaves with EFNs and EFN size exhibit heritable variation in *G. thurberi*. A two-year insecticide experiment revealed that herbivory reduced plant growth and seed production in the field, independently of the effects of ants. Both ant abundance and EFN availability were manipulated in the field using a 2 × 2 factorial design. When ant visitation was reduced, plants supported more herbivores, experienced greater leaf damage, and produced fewer flowers and seeds. Furthermore, fewer ants visited plants with experimentally reduced EFNs; leaf damage was higher and seed number was lower compared to plants with ambient levels of EFN, indicating that EFNs mediate the benefits of ants. In addition, natural variation in EFN frequency correlated positively with seed production. This study provides the strongest support yet that casual ant associates can influence the evolution of extrafloral nectary traits and suggests that plant evolution in facultative mutualisms depends on community-level dynamics.

Key words: ant; *Bucculatrix thurberiella*; *Forelius pruinus*; *Gossypium thurberi*; indirect effect; mutualism; plant defense.

INTRODUCTION

Plants possess a number of strategies to defend against herbivory, including attracting the predators and parasitoids of their herbivores (Price et al. 1980). To a large extent, research on the evolution of plant defensive strategies has focused on traits involved in direct interactions between plants and herbivores (reviewed by Bergelson and Purrington 1996, Karban and Baldwin 1997, Strauss et al. 2002) and has given less consideration to the role of herbivores' enemies, and thus of indirect effects, in plant evolution. To demonstrate that the enemies of herbivores influence plant evolution, one must show (1) that these enemies increase plant fitness by reducing herbivory, (2) that there is heritable variation in the plant trait, and (3) that the plant trait mediates the benefit of herbivores' enemies (Marquis and Whelan 1996).

Several studies have confirmed that the enemies of herbivores can increase plant performance (reviewed by Schmitz et al. 2000, Halaj and Wise 2001). Many systems involve enemies that are ants (reviewed by

Huxley and Cutler 1991, Koptur 1992, Bronstein 1998). A classic example is the ant–*Acacia* interaction: ants live in modified thorns of *Acacia* species and deter both insect and mammalian herbivores; plants without ants rarely survive (Janzen 1966, Stapley 1998). Less specialized interactions, in which the absence of ants does not typically result in plant death, are also common in nature. Whether predators or parasitoids of herbivores currently influence the evolution of plant traits in such facultative associations is not known (Hare 1992, Marquis and Whelan 1996, Bronstein 1998). Selection via herbivores' enemies could be less likely to occur when interactions are facultative and generalized rather than obligate and specialized.

A number of plant species possess characters that attract herbivores' enemies, i.e., indirect defensive traits. Some plants have structures such as swollen thorns or domatia that house predatory arthropods (Janzen 1966, Norton et al. 2000). In other systems, damaged plants release volatiles that attract predators and parasitoids (Dicke and van Loon 2000, Turlings et al. 2002). In addition, some plants provide food resources to arthropod protectors (Fiala et al. 1994, Letourneau 1998). For example, extrafloral nectaries, which occur in more than 90 plant families worldwide (Koptur 1992), are commonly visited by herbivores' enemies,

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including ants, spiders, and even birds (Bentley 1977, Pemberton 1993, Taylor and Foster 1996).

When predators and parasitoids protect plants from herbivory in exchange for plant resources, the interaction often rewards both partners, resulting in a mutually beneficial association commonly called a protection mutualism. Despite the extensive literature on protection mutualisms (reviewed by Bronstein 1998, Stachowicz 2001, Bronstein and Barbosa 2002), few researchers have examined the genetic basis of traits important to these interactions (but see Loughrin et al. 1995, Wilkinson et al. 2000, Hoballah et al. 2002). While extrafloral nectary characters are heritable in cultivated cotton (Belcher et al. 1984) and in *Acacia koa* (Daehler et al. 1999), nectaries have not been shown to increase fitness in either species. Furthermore, scant information exists to address how intraspecific variation in plant traits such as extrafloral nectaries translates into variation in the costs and benefits of mutualistic interactions (Marquis and Whelan 1996, Bronstein 1998). For example, extrafloral nectaries may be costly in the absence of ants, particularly if nectar attracts herbivores (e.g., Henneberry et al. 1977, Beach et al. 1985). Considerations of intraspecific variation have recently improved models describing the evolution of mutualisms from parasitic interactions (reviewed by Hoeksema and Bruna 2000). Ultimately, deciphering the evolution of traits involved in protection mutualisms will require understanding the ecological costs and benefits of variation in the traits as well as the genetic basis underlying this variation.

I used a native plant–herbivore–predator system of the Sonoran Desert to investigate the evolution of an indirect defensive trait. *Gossypium thurberi* (wild cotton) receives damage from both folivores and predators of reproductive tissue. Extrafloral nectaries (EFNs) on plants attract a number of ant species that may interfere with and consume herbivores. I addressed the following specific questions to assess the importance of ants in shaping the evolution of EFNs within the context of this facultative interaction. (1) Do EFN traits exhibit heritable variation? (2) Does herbivory reduce *G. thurberi* fitness? (3) Does the ant assemblage benefit *G. thurberi* by reducing herbivory? (4) Do EFN rewards mediate the benefit of ants?

STUDY SYSTEM

Gossypium thurberi Todar (Malvaceae, wild cotton) is a perennial shrub native to canyon washes in the Sonoran Desert of Arizona, USA, and Mexico. Plants used in this study occurred naturally in Agua Caliente Canyon, Santa Rita Mountains, Amado, Arizona, USA (31°41'32.4" N, 110°57'22.7" W, elevation 1225 m). *G. thurberi* grow ~1–4 m tall and do not reproduce clonally (Hanson 1923). Seedlings germinate and leaves appear on established plants when monsoon rains begin in early July. Typically, fruits have matured and leaves have senesced by late December. *G. thurberi* bear EFNs

that occur singly along the midvein on the undersides of leaves and also below the bracts (Fryxell 1979). Unlike bract nectaries, which appear to be ubiquitous, foliar nectaries are not present on all leaves (Rudgers 2002).

Both folivores and predators of buds and seeds damage *G. thurberi*. Of the folivores, *Bucculatrix thurberiella* Busck (Lyonetiidae, cotton leaf perforator) inflicts the most damage, particularly late in the season (Karban 1993a, b, Karban and Adler 1996, Rudgers 2002). A specialist on *Gossypium*, *B. thurberiella* spends the first three instars as a leaf miner, then emerges as a caterpillar and feeds externally during the last two instars by skeletonizing leaves (Rejesus 1968, Smith and Flint 1977). One caterpillar consumes ~10–20% of one leaf during its lifetime (Karban 1993a, b). Adult *B. thurberiella* are nocturnal and subsist on nectar, including *G. thurberi* EFN (Rudgers 2002). Multiple generations of *B. thurberiella* are completed during a single season of *G. thurberi* growth (Rejesus 1968). Boll weevils are the most abundant bud and seed predators on *G. thurberi* at Agua Caliente Canyon. Adult weevils, as well as an unidentified caterpillar (Pyralidae), damage buds to feed on pollen; damaged buds and flowers are typically aborted. Boll weevil larvae and a seed bug (*Sphycorhis punctellus* Stal. Scutelleridae) consume developing seeds (Karban and Lowenberg 1992).

I have recorded 12 species of ants visiting *G. thurberi* EFNs at Agua Caliente Canyon. *Forelius pruinosus* Roger (Formicidae) dominates the ant community at this site (Rudgers 2002; see Plate 1). This aggressive generalist bites humans encountering plants and attacks late instar *B. thurberiella* caterpillars after they have emerged from the leaf mining stage. Preliminary observations and experiments suggested that ants were effective in deterring caterpillars, but had little effect on boll weevils or seed bugs at Agua Caliente Canyon (Rudgers 2002). In addition to ants, parasitoids and predaceous insects, such as predatory wasps, also consume wild cotton EFN, although ants are the most common EFN visitors (*personal observation*).

METHODS

Do EFN traits exhibit heritable variation?

Crossing design.—I used sire–offspring regression analysis (Lynch and Walsh 1998) to estimate the heritability of three EFN traits: the proportion of leaves bearing nectaries, foliar nectary length, and foliar nectary width. Sire traits were measured in the field, and progeny traits were assessed in the greenhouse, allowing determination of a lower bound for the heritability estimate, a method superior to measuring heritability solely in the greenhouse (Riska et al. 1989). The crossing design maximized variation among sires (Hill and Thompson 1977) with sires representing extremes in EFN size and dams chosen at random. Plants used for crosses occurred naturally at Agua Caliente Canyon



PLATE 1. *Forelius pruinus* ants visiting a foliar extrafloral nectary on *Gossypium thurberi* (wild cotton). Photograph by Jennifer Rudgers.

and ranged in age from ~ 2 to ~ 10 years. Because *G. thurberi* are self-compatible (Fryxell 1979), dams were emasculated on the evening prior to flower opening (before anthers had dehisced), bagged in spun-bound polyester (Fibre-Aire, Kleen Test Products, Milwaukee, Wisconsin, USA), and hand pollinated on the following morning with a randomly assigned sire. No sires or dams were used in more than one sire \times dam combination, and multiple flowers were hand pollinated for each cross. Not all crosses produced viable seeds or progeny that survived the seedling stage (60 sire \times dam combinations were attempted). Therefore, final numbers of successful crosses using sires with large vs. small EFNs differed (large, $n = 18$; small, $n = 12$).

Extrafloral nectary traits.—Sire EFN traits were assessed in the field during 1–6 September 2000. I examined 30 randomly selected leaves per plant to determine the proportion of leaves bearing nectaries. I used digital calipers to measure the maximum length and width of foliar nectaries to the nearest 0.01 mm for 15 fully expanded leaves per plant (five randomly chosen leaves each from the bottom, middle, and top thirds of the plant). For the same 15 leaves, sire leaf area was estimated using a transparent grid of 1.0-cm² cells, by counting the number of cells with $>50\%$ leaf

coverage. To calibrate field measurements, actual EFN area was determined via image analysis (Scion Image 1998, Scion, Frederick, Maryland, USA) for a subset of leaves ($n = 50$) that were photographed with a digital camera on a dissecting stereoscope. EFN area was predicted from length and width with multiple regression analysis ($r^2 = 0.91$, $F_{2,47} = 228.69$, $P < 0.0001$; area = $0.54 \times \ln(\text{length}) + 1.47 \times \ln(\text{width}) - 0.38$; SAS Institute 1999).

I assessed progeny EFN traits in the greenhouse for 5.4 ± 2.7 (mean ± 1 SE) progeny per cross (range 2–12). Seeds from crosses were scarified with a scalpel and planted in 4 cm diameter pots (Ray Leach Container Nursery, Canby, Oregon, USA) on 24 June 2000. Progeny were initially kept in the field; however, seedlings grew poorly outdoors and were moved to a greenhouse in Davis, California during December 2000. The plants were grown in potting soil (Earthgro, Marysville, Ohio, USA) and watered daily with fertilizer (200 ppm N:100 ppm P:200 ppm K). During March–April 2001, all leaves on each progeny plant were assessed for the presence of nectaries (mean leaf number = 20 ± 0.5). I determined the size of EFNs on every fifth fully expanded leaf until at least five EFNs were measured per plant (mean number of nectaries per plant =

7 ± 0.1). I also measured maximum leaf length and width to the nearest 0.1 cm. Multiple regression analysis was used to predict leaf area from length and width for a randomly selected sample of leaves ($n = 50$; $r^2 = 0.92$, $F_{3,45} = 188.71$, $P < 0.0001$; $\text{area} = 0.28 \times \ln(\text{length}) + 0.14 \times [\ln(\text{length})]^2 + 0.94 \times \ln(\text{width}) - 0.43$; SAS Institute 1999). Mean values of EFN traits and leaf area were calculated for each plant.

EFN production was difficult to assess in the field because nectar volumes were small and variable. However, ants were regularly observed feeding at EFNs, and at minimum, they gained sugars, if not also water, from the nectaries. Due to the low volumes of nectar in the field, I determined the relationship between EFN size and EFN production in the greenhouse, where volumes were typically greater. On a random sample of leaves from 87 greenhouse-grown plants ($n = 453$ nectaries, mean nectaries per plant 11 ± 0.4), I quantified nectar volume by withdrawing standing nectar with a microcapillary tube. Nectar had accumulated since leaf expansion, and leaf ages varied. I measured the length of each nectary with digital calipers, as above. Regression analysis demonstrated that EFN length was positively correlated with nectar volume ($r^2 = 0.18$, $F_{1,204} = 44.79$, $P < 0.0001$; SAS Institute 1999), although considerable variation was unexplained and may derive from effects of leaf age and leaf position and from the imprecision associated with measuring standing nectar rather than the rate of nectar production.

Sire-offspring regression analyses.—The relevant question regarding EFN inheritance was whether genetically based variation was detectable, that is, whether estimates of heritability were significantly greater than zero. Heritabilities of EFN length and width were determined after removing variation due to leaf size (a correlated character) by using the residuals from regressions of these EFN traits on leaf area (per plant) in sire-offspring regressions. Using residuals makes the estimates conservative, as phenotypic variation in EFN size (upon which selection would act) would include variation due to the correlated character of leaf area. I used weighted least-squares sire-offspring regression analysis to estimate heritabilities (SAS Institute 1999). Weights were calculated using the Kempthorne-Tandon derivation and reiterated until weighted estimates of the regression coefficient converged (Lynch and Walsh 1998). Heritability was estimated as $2 \times$ the sire regression coefficient. A lower bound was calculated following Riska et al. (1989); the lower bound is conservative because the estimate of additive genetic variation from the greenhouse, which was used to calculate the lower bound, may be inflated by dominance, common environment, or maternal effects (Riska et al. 1989).

Does herbivory reduce G. thurberi fitness?

Reducing herbivory.—In July 1999, I randomly marked 60 naturally occurring *G. thurberi* plants at

Agua Caliente Canyon from among the subset of plants that showed no evidence of previous flowering and that were not seedlings. Plants were randomly assigned to an insecticide or control treatment. To reduce leaf and reproductive damage by the suite of herbivores affected by ants, I sprayed plants with an insecticide containing *Bacillus thuringiensis* var. *kurstaki*, which specifically targets Lepidoptera (Safer Caterpillar Killer, DIPEL, Ringer, Bloomington, Minnesota, USA; 1.25 mg/mL water; Flexner et al. 1986). Control plants were sprayed with water. Prior to treatment application, basal stem diameter was measured to estimate pretreatment plant size. I applied treatments every 7–10 d during the main leaf flush (July–November) for two consecutive seasons (1999 and 2000). *B. thuringiensis* insecticides have minimal effects on nontarget organisms, including predators of herbivores (Flexner et al. 1986).

Assessing herbivore damage.—Different methods were used during each year to estimate herbivore damage. In 1999, numbers of externally feeding (late instar) *B. thurberiella* caterpillars were counted (14 October). In 2000, leaf damage was assessed by placing a transparent grid comprised of 1.0-cm² cells over a leaf and counting the number of grid squares containing >50% leaf tissue. Where leaf tissue had obviously been removed by herbivores, missing leaf area was visually estimated. Leaf damage was estimated to the nearest 0.25 cm² using the grid and assigned to early instar *B. thurberiella* mines, late instar *B. thurberiella* perforations (caused by externally feeding caterpillars), or to other herbivores. Damage was estimated four times during the 2000 season (from ~15 August to ~15 November) for 15 randomly selected leaves per plant. In 2000, I also assessed bud and flower predation by boll weevils and Pyralid caterpillars. Aborted buds and flowers remain connected to the pedicel; these were collected weekly and classified as damaged (when a puncture or frass was visible) or undamaged. Proportional bud and flower predation was calculated by dividing the total number of damaged buds and flowers by the total number of buds produced per plant.

Estimating plant fitness.—At the end of each season, I assessed plant growth and seed production. For growth, I measured the length from the base of the plant to the apex of the main stem to the nearest cm (plant height). In addition, I collected all fruits and counted the number of filled, undamaged seeds.

Nontarget effects on ants.—I monitored ant abundance for insecticide-treated and control plants to determine whether the insecticide had detrimental effects on ants. Numbers of ants per plant were counted once each year near the peak of ant visitation (14 October 1999 and 28 August 2000).

Statistical analyses.—Response variables were combined into a single multivariate analysis of covariance (MANCOVA) including the fixed effect of insecticide treatment with pretreatment stem diameter (log-transformed) as a covariate (SAS Institute 1999). Propor-

tional damage estimates were angular-transformed, and the remaining responses were log-transformed to meet assumptions of MANCOVA. When the effect of the insecticide treatment was statistically significant in the multivariate model, subsequent analyses were performed in a stepwise procedure to decompose effects on single responses. First, all herbivory responses were combined into a single MANCOVA. Then, ANCOVA models were used to test the effect of insecticide on the number of externally feeding *B. thurberiella* caterpillars (1999) and on bud and flower predation (2000), and a profile analysis (i.e., repeated measures MANCOVA; von Ende 2001) assessed effects on leaf damage over the four census dates (SAS Institute 1999). Effects on plant height, seed production, and ants (1999, 2000) were examined with repeated measures ANCOVA (SAS Institute 1989) with year as the repeated factor. Throughout, I present Pillai's trace statistic for multivariate analyses following Scheiner (2001).

Does the ant community benefit G. thurberi by reducing herbivory, and do EFN rewards mediate the benefit of ants?

I addressed these questions in concert using a factorial design that manipulated the presence of ants and the availability of EFNs. Naturally occurring plants chosen along two transects were randomly assigned to treatments in a 2×2 design with an ant treatment (ant access or ant exclusion) and an EFN treatment (ambient nectar or nectar reduction). Transects followed the floor of a canyon on either side of an access road; as they delimited two distinct regions of the canyon (one more shaded and wet than the other), transect was considered a fixed effect. Sample sizes for ant access were 28 with ambient nectar ("EFN") and 30 with nectar reduction ("EFN reduction") and, for ant exclusion, 25 with EFN and 27 with EFN reduction. Under the factorial design, if ants benefit plants and EFN mediates this benefit, both a significant main effect of the ant treatment and a significant ant \times EFN interaction are expected. In other words, ants would have an overall positive effect on *G. thurberi* by increasing plant performance. And, importantly, if mediated by EFNs, this benefit would depend on the availability of EFN such that only in the presence of ants would EFN reduction alter plant responses.

Experimental treatments.—I excluded ants from plants using a sticky barrier (Tanglefoot, Grand Rapids, Michigan, USA). In 1999, a 10-cm-wide band of triple layer masking tape was applied to the stem \sim 10 cm above the ground, and Tanglefoot was spread over a 2.5 cm wide band of the masking tape. Control plants in 1999 received masking tape only. In 2000, because plants were larger, plants were protected with triple-layered Tangleguard (Tanglefoot, Grand Rapids, Michigan, USA). Again, ant exclusion plants received a 2.5 cm wide band of Tanglefoot. However, control plants

in 2000 also had Tanglefoot but were outfitted with ant bridges constructed from branches of dead *G. thurberi* plants (25 cm long). In both years, I tied back (with twine) or trimmed vegetation surrounding both ant exclusion and control plants to deter ants from using vegetation bridges. Tanglefoot was reapplied as needed and removed at the end of each season.

I reduced EFN production by covering each nectary with a drop of waterproof glue (Aleene's OK to Wash It, Aleene's, Buellton, California, USA). Glue generally prevented visible nectar secretion for the lifetime of a nectary. Control plants received the same amount of glue placed <1 cm away from nectary along the midvein of leaves or on the bracts. Glue was applied to new leaves and bracts as they were produced.

Predator, herbivore, and plant responses.—I assessed ant visitation, herbivory, and components of plant fitness in both years. I counted ant visitors on plants every two weeks during the season (July–December), between 0700 and 1900 hours, when ants were most active (*unpublished data*). While ants were identified to species, only the total number was used in analyses. Every two weeks in 1999, I also counted spiders (Dictynidae, Oxyopidae, Thomisidae), the most abundant other predators on *G. thurberi*. For flying predators (e.g., coccinellids, predatory wasps) and parasitoids, responses to EFN were monitored indirectly. If EFN alters the effects of these enemies on herbivory, then EFN reduction is expected to increase herbivore damage in the absence of ants. In both years, I assessed bud and flower predation (as in the insecticide experiment), and I counted *B. thurberiella* mines (early instars) every four weeks and externally feeding *B. thurberiella* caterpillars (late instars) every two weeks (July–December). I estimated leaf damage five times during the 2000 season following methods described in the insecticide experiment. During both years, flower production and seed number were determined, and final plant height was recorded at the end of the experiment. Because I manipulated naturally occurring plants, on 5 July 1999, I measured initial plant height (in centimeters) and stem diameter (in millimeters), counted all leaves, and summed these variables to create an index of pretreatment plant size, a covariate.

Statistical analyses.—Fixed effects in all analyses included the ant treatment, the EFN treatment, the interaction of ant \times EFN, and transect. Also, when the ant \times EFN interaction was significant, I used a single planned contrast to examine the difference between the ambient EFN and EFN reduction treatments for the subset of plants that had ants. A significant contrast would indicate that EFNs mediated ant–*G. thurberi* interactions. Response variables in three subsystems were analyzed with MANCOVA (Huberty and Morris 1989), using pretreatment plant size (log transformed) as a covariate: (1) effects on ant visitation, (2) effects on herbivore abundance, and (3) effects on plants. Ant responses were examined with a profile analysis that

included the repeated effects of census date (nested within year) and year. The herbivore MANCOVA included mean numbers of *B. thurberiella* mines as well as numbers of externally feeding caterpillars for each year (log transformed to meet the normality of errors assumption). Effects on plants were examined with MANCOVA combining plant height, flower number, and seed number (all log transformed to meet the assumption of normality of errors) in addition to bud and flower predation and mean leaf damage (over five censuses), both of which were angular transformed. If effects in the MANCOVA were statistically significant, subsequent univariate analyses were conducted. When appropriate, these analyses incorporated the repeated measures design (census date and/or year). Lastly, I evaluated effects of the treatments on spider abundance in 1999 with ANOVA.

Natural phenotypic correlations.—During 2000, the proportion of leaves with nectaries (EFN frequency) and EFN length were assessed for control plants (ant accessible plants with ambient EFN, $n = 28$), as described in the experiment above. EFN frequency was determined for all leaves per plant every six weeks beginning on 15 July. EFN length was measured on 15 randomly chosen leaves per plant during 1–7 July and 1–7 October, as described in the *Methods* for heritability. Mean trait values per plant were used in multiple regression analysis to determine partial correlation coefficients between plant traits and seed production (SAS Institute 1999). Mean ant number per plant over all censuses conducted during the year 2000, mean leaf area per plant, and initial plant size (1999) were also included in the model. The data did not exhibit significant multicollinearity and were normally distributed, with the exception of EFN frequency (SAS Institute 1999). Angular transformation normalized this variable but did not qualitatively alter the results; thus, untransformed values were presented for ease of interpretation.

RESULTS

Do EFN traits exhibit heritable variation?

Both the proportion of leaves with nectaries and EFN size expressed genetic variation. Heritability in EFN frequency was significantly greater than zero: $h^2 \pm 95\%$ CI = 0.62 ± 0.38 (mean slope ± 1 SE = 0.32 ± 0.095 ; $h^2_{\text{lower bound}} = 0.014$), and sire explained 7% of the variation in progeny EFN frequency ($r^2 = 0.07$; $F_{1,149} = 11.38$; $P = 0.0009$). The length of EFNs also had a genetic basis with $h^2 \pm 95\%$ CI = 0.48 ± 0.44 (mean slope ± 1 SE = 0.24 ± 0.111 ; $h^2_{\text{lower bound}} = 0.025$), but sire explained only 3% of the offspring variance in nectary length ($r^2 = 0.03$; $F_{1,151} = 4.67$; $P = 0.0322$). EFN width was not detectably heritable, $h^2 \pm 95\%$ CI = 0.28 ± 0.67 (mean slope ± 1 SE = 0.14 ± 0.169 ; $h^2_{\text{lower bound}} = 0.001$; $r^2 = 0.004$; $F_{1,151} = 0.66$; $P = 0.42$).

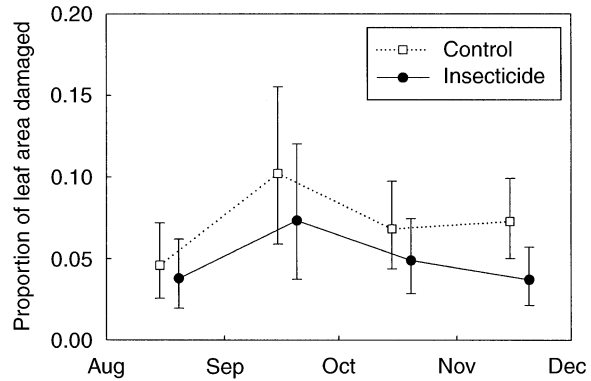


FIG. 1. Effects of the insecticide treatment on proportion of leaf area damaged in 2000. Back-transformed least-squares means are plotted with 95% CI. Symbols are slightly offset to show confidence intervals.

*Does herbivory reduce *G. thurberi* fitness?*

The comprehensive MANCOVA demonstrated a strong effect of the insecticide treatment (Pillai's trace = 0.44; $F_{12,44} = 2.83$; $P = 0.006$) as well as the covariate, pretreatment stem diameter (Pillai's trace = 0.46; $F_{12,44} = 3.10$; $P = 0.003$). Subsequent analyses partitioned responses into herbivory, plant fitness, and nontarget ant components.

Herbivory.—The insecticide reduced herbivory on *G. thurberi*, measured as numbers of herbivores (1999), levels of bud and flower predation, and leaf damage (2000) combined (Pillai's trace = 0.21; $F_{6,50} = 2.27$; $P < 0.05$). Specifically, the insecticide reduced the mean number of externally feeding *B. thurberiella* caterpillars per plant by 59% compared to controls (back-transformed least-squares means, with 95% CI: insecticide = 0.5, CI = 0.2–0.9; control = 1.3, CI = 0.8–1.9; $F_{1,57} = 6.57$; $P = 0.01$). Pretreatment stem diameter contributed positively to the variation in caterpillar number ($F_{1,57} = 5.95$; $P = 0.02$). Proportional bud and flower predation was also reduced by the insecticide treatment with 50% lower predation on insecticide plants than on controls, although the effect was marginally significant (back-transformed least-squares means with 95% CI: insecticide = 0.05, CI = 0.02–0.08; control = 0.10, CI = 0.06–0.14; $F_{1,57} = 3.66$; $P = 0.06$). Stem diameter did not influence bud and flower predation ($F_{1,57} = 0.71$; $P = 0.40$). Most importantly, leaf damage was substantially lower for insecticide-treated plants than for controls (Fig. 1; $F_{1,55} = 4.05$; $P < 0.05$). On average, insecticide-treated plants experienced 32% less leaf damage than controls (Fig. 1). Neither census date (Pillai's trace = 0.06, $F_{3,53} = 1.22$, $P = 0.31$; census \times insecticide Pillai's trace = 0.04, $F_{3,53} = 0.74$, $P = 0.53$) nor stem diameter ($F_{1,55} = 0.05$, $P = 0.48$; census \times stem diameter $F_{3,53} = 1.04$, $P = 0.38$) affected leaf damage.

Plant fitness.—Plants sprayed with insecticide grew larger and produced more seeds than control plants. In

1999, insecticide plants grew 16% taller (back-transformed least-squares means with 95% CI: insecticide = 108.5, 99.3–118.6 cm; control = 93.7, 85.7–102.4 cm) and produced 168% more seeds per plant than controls (insecticide = 1.49, 0.53–3.05; control = 0.56, –0.04–1.53). In 2000, insecticide plants were 11% taller (insecticide = 131.4, 121.3–142.4 cm; control = 118.5, 109.3–128.4 cm) and yielded 86% more seeds per plant (insecticide = 611.2, 400.4–932.5; control = 329.0, 215.4–502.2). Univariate models showed that the insecticide significantly benefited plants through both growth and seed production (height $F_{1,55} = 4.75$, $P = 0.03$; seeds $F_{1,55} = 6.16$, $P = 0.02$). Year also had a strong effect on height ($F_{1,55} = 18.58$, $P < 0.0001$) and seed number ($F_{1,55} = 9.08$, $P = 0.004$), but no year \times insecticide interaction occurred (height $F_{1,55} = 2.93$, $P = 0.09$; seeds $F_{1,55} = 0.11$, $P = 0.74$). The covariate, stem diameter, influenced both responses (height $F_{1,55} = 5.93$, $P = 0.02$; seeds $F_{1,55} = 6.19$, $P = 0.02$) and for plant height interacted with year such that the effects of the covariate were stronger in the first year (height $F_{1,55} = 6.67$, $P = 0.01$; seeds $F_{1,55} = 0.34$, $P = 0.56$). Two major factors likely influenced the greater number of seeds produced in 2000 compared to 1999: older plants were larger, and fewer boll weevils damaged buds in 2000 (Rudgers 2002).

Nontarget effects on ants.—During both years, the insecticide had no detectable influence on the number of ants per plant (back-transformed least-squares means with 95% CI, 1999: insecticide = 5.0, 2.8–8.5; control = 3.6, 1.9–6.3; 2000: insecticide = 11.0, 7.1–16.9; control = 14.0, 9.0–21.5; $F_{1,56} = 0.01$; $P = 0.92$). Stem diameter explained some variation, with more ants visiting larger plants ($F_{1,56} = 7.20$; $P = 0.01$). There were no strong effects of year ($F_{1,56} = 0.18$; $P = 0.67$) or interactions with year (year \times insecticide, $F_{1,56} = 1.92$, $P = 0.17$; year \times stem diameter, $F_{1,56} = 0.01$, $P = 0.92$).

Does the ant community benefit G. thurberi by reducing herbivory, and do EFN rewards mediate the benefit of ants?

Ant visitation.—The experimental sticky barrier effectively excluded ants (Fig. 2, Table 1). Not only were ants strongly deterred by the barrier, but they also responded to EFN rewards (Fig. 2, ant \times EFN, $P = 0.04$, Table 1). When ants had access to plants, gluing EFNs reduced peak ant abundance per plant by 85% in 1999 and by 67% in 2000 relative to plants with ambient EFN (planned contrast: ants, EFN vs. ants, EFN reduction, $F_{1,96} = 14.31$, $P = 0.0003$).

Herbivore abundance.—Ant exclusion increased mean herbivore abundance when mines and externally feeding caterpillars were considered collectively (Pillai's trace = 0.16; $F_{4,93} = 4.47$; $P = 0.002$). However, EFN reduction (Pillai's trace = 0.03; $F_{4,93} = 0.80$; $P = 0.53$) and the interaction between ant exclusion and EFN reduction (Pillai's trace = 0.02; $F_{4,93} = 0.58$; P

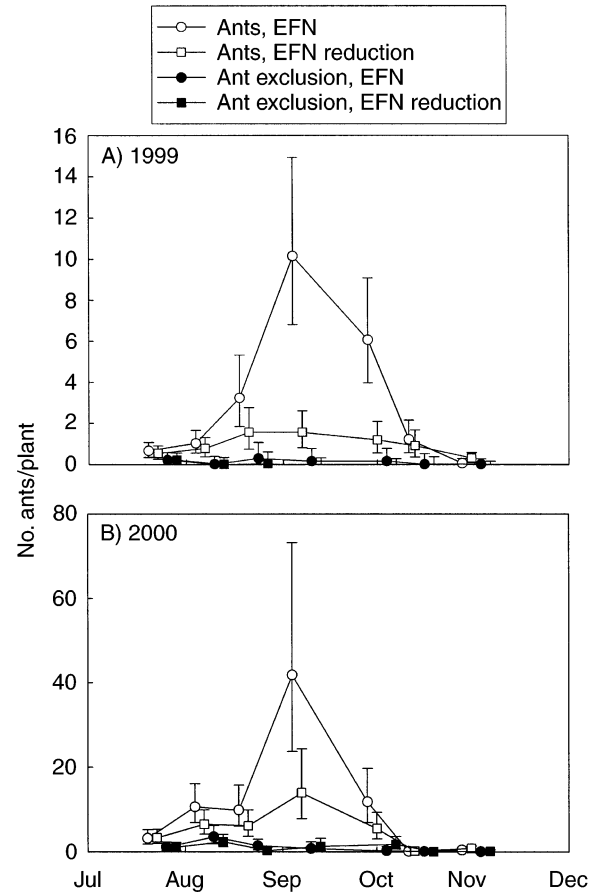


FIG. 2. Effects of the ant treatment (ants or ant exclusion) and the extrafloral nectar treatment (EFN or EFN reduction) on the number of ants per plant in (A) 1999 and (B) 2000. Symbols represent back-transformed least-squares means \pm 95% CI. Symbols are slightly offset and different scales are used to highlight treatment effects.

= 0.68) had no effect. Transects did not differ in herbivore number (Pillai's trace = 0.01; $F_{4,93} = 0.27$; $P = 0.90$), and initially larger plants supported greater numbers of herbivores (Pillai's trace = 0.13; $F_{4,93} = 3.50$; $P = 0.01$).

Ant exclusion did not affect the number of *B. thurberiella* leaf mines, suggesting that ants do not interfere strongly with adult moths, eggs, or early instar larvae (Fig. 3A; ant treatment, $F_{1,96} = 0.30$, $P = 0.58$; ant \times EFN, $F_{1,96} = 0.54$, $P = 0.47$). However, plants with nectaries glued had, on average, 16% fewer mines than controls in 1999 and 21% fewer in 2000 (Fig. 3A), but this effect was only marginally significant ($F_{1,96} = 3.17$; $P = 0.078$).

Although ant exclusion did not affect the leaf mining (early instar) stage, externally feeding *B. thurberiella* caterpillars were more abundant on plants without ants (Fig. 3B, C; $F_{1,96} = 8.57$, $P = 0.004$). On average, plants without ants had 22% more caterpillars than plants with ants in 1999 and 59% more in 2000 (Fig.

TABLE 1. Results of profile analysis examining the effects of the ant treatment (ant access or exclusion), the extrafloral nectar treatment (ambient or reduced EFN), ant \times EFN, transect, and pretreatment plant size (size) on the abundance of ants per plant for multiple census dates during 1999 and 2000.

Treatment	Number of ants		
	df	<i>F</i>	<i>P</i>
Ant	1, 96	153.94	<0.0001***
EFN	1, 96	8.07	0.0055**
Ant \times EFN	1, 96	4.31	0.0406*
Transect	1, 96	2.32	0.1313
Size	1, 96	2.43	0.1221
Year	1, 96	0.52	0.4722
Year \times ant	1, 96	5.95	0.0166*
Year \times EFN	1, 96	1.55	0.2154
Year \times ant \times EFN	1, 96	0.00	0.9965
Year \times transect	1, 96	7.57	0.0071**
Year \times size	1, 96	1.07	0.0329*
Census	6, 91	0.40	0.8789
Census \times ant	6, 91	23.56	<0.0001***
Census \times EFN	6, 91	3.64	0.0028**
Census \times ant \times EFN	6, 91	4.79	0.0003***
Census \times transect	6, 91	5.40	<0.0001***
Census \times size	6, 91	0.49	0.8158
Year \times census	6, 91	2.63	0.0215*
Year \times census \times ant	6, 91	6.59	<0.0001***
Year \times census \times EFN	6, 91	1.89	0.0901
Year \times census \times ant \times EFN	6, 91	0.55	0.7651
Year \times census \times transect	6, 91	9.46	<0.0001***
Year \times census \times size	6, 91	2.84	0.0139*

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

3B), and caterpillar densities at the end of the season were 37% and 64% greater in 1999 and 2000, respectively (Fig. 3C). The effect of ants on externally feeding caterpillars did not depend on EFN, in contrast to expectations (ant \times EFN, $F_{1,96} = 0.03$, $P = 0.87$; EFN treatment $F_{1,96} = 1.05$, $P = 0.31$).

Effects on plants.—Plant fitness and damage measures were, collectively, affected by both the ant treatment (Pillai's trace = 0.22, $F_{6,90} = 4.13$, $P = 0.001$) and the interaction of ant \times EFN (Pillai's trace = 0.13, $F_{6,90} = 2.28$, $P = 0.04$). EFN reduction had no overall effect (Pillai's trace = 0.08, $F_{6,90} = 1.39$, $P = 0.23$), but initial size (Pillai's trace = 0.37, $F_{6,90} = 8.70$, $P < 0.0001$) and transect explained variation in plant responses (Pillai's trace = 0.16, $F_{6,90} = 2.93$, $P = 0.01$).

Herbivore numbers provide surrogate estimates of herbivory, but the best measures are actual damage levels. The importance of ants in reducing leaf damage varied with census date (census \times ant, $P = 0.0002$; Fig. 4A, Table 2); responses were stronger later in the season. At peak damage, ant exclusion increased leaf damage by 61% relative to ant access (Fig. 4B). Importantly, the effect of ants also depended on the availability of EFN (ant \times EFN, $P = 0.01$, Table 2). In the presence of ants, plants with reduced EFN received significantly more damage (up to 22% more) than plants with ambient EFN (Fig 4; planned contrast: ants, EFN vs. ants, EFN reduction; $F_{1,96} = 5.73$, $P = 0.02$). Damage caused exclusively by externally feeding (late instar) *B. thurberiella* caterpillars was, in part, responsible for this increase in damage in the EFN reduction

treatment (Table 2; planned contrast, $F_{1,96} = 4.25$, $P = 0.04$). Despite effects on leaf damage, the treatments did not alter bud and flower predation (Table 3) or seed damage (Rudgers 2002) in either year.

Both the ant treatment and the interaction of ant \times EFN significantly affected components of plant fitness. Flower number for ant exclusion plants was 77% of that for controls in 1999 and 33% in 2000 (Fig. 5A, B; Table 3). Similarly, seed production was lower when ants were excluded, by 86% in 1999 and by 45% in 2000 (Fig. 5C, D; Table 3). Ant exclusion had no significant influence on final plant height in cm (back-transformed least-squares means with 95% CI: ants, EFN = 123.6 cm, CI = 116.7–130.9 cm; ants, EFN reduction = 121.14 cm, CI = 114.6–128.0 cm; ant exclusion, EFN = 119.7 cm, CI = 111.8–128.1 cm; ant exclusion, EFN reduction = 114.4 cm, CI = 107.4–122.0 cm). The benefits of ants for seed production were contingent upon the availability of EFN (ant \times EFN, $P = 0.02$, Table 3). Nectar reduction strongly decreased seed production when plants had ants, by 92% in 1999 and by 42% in 2000 (planned contrast: ants, EFN vs. ants, EFN reduction, $F_{1,104} = 4.17$, $P = 0.04$; Fig. 5C, D). Thus, in the presence of ants, plants that had more EFN benefited through increased reproduction.

Nontarget effects on spiders and other enemies.—Excluding ants from plants did not strongly influence the numbers of spiders per plant (least-squares means ± 1 SE: ants = 0.86 ± 0.02 , ant exclusion = 0.90 ± 0.02 ; $F_{1,113} = 3.31$, $P = 0.07$). The exclusion barrier

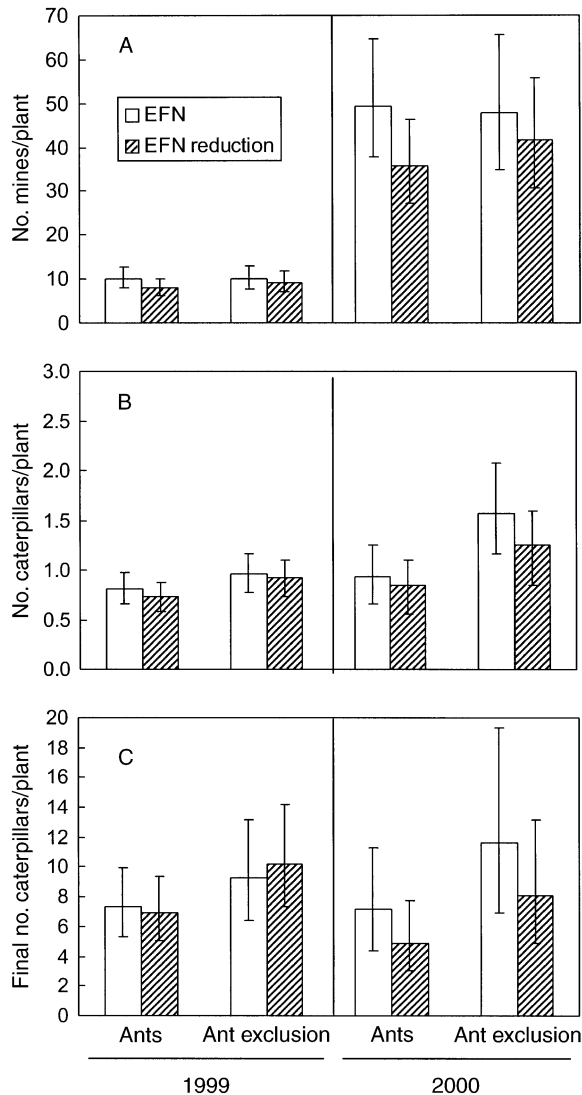


FIG. 3. Effects of the ant treatment (ants or ant exclusion) and the extrafloral nectar treatment (EFN or EFN reduction) on (A) *B. thurberiella* mines (early instars) per plant, averaged over the season; (B) externally feeding *B. thurberiella* caterpillars (late instars) per plant, averaged over the season; and (C) end-of-season externally feeding *B. thurberiella* caterpillars per plant. Data are given for 1999 and 2000. Bars represent back-transformed least-squares means \pm 95% CI.

clearly did not prevent spider colonization; in contrast, plants without ants had marginally greater spider abundance. Therefore, the experimental test of ants' effects on herbivores was conservative. EFN reduction did not affect spiders ($F_{1,113} = 1.27$, $P = 0.26$), and the ant and EFN treatments did not strongly interact ($F_{1,113} = 2.63$, $P = 0.11$). In addition, EFN reduction did not increase herbivory in the absence of ants (Fig. 4, Tables 2 and 3), which suggests that EFNs did not mediate the effects of parasitoids and flying predators (which could access EFN despite ant barriers) on herbivory.

Natural phenotypic correlations.—Natural variation in EFN frequency was positively correlated with seed production (partial correlation coefficient $r = 0.47$, $P = 0.03$). In addition, despite substantial environmental variation in ant abundance, the mean number of ants per plant was marginally correlated with seed production ($r = 0.37$, $P = 0.09$). Initial plant size also contributed positively to seed number ($r = 0.79$, $P < 0.0001$), but mean leaf area per plant did not ($r = 0.07$, $P = 0.76$). In opposition to predictions, EFN size was negatively correlated with seed production ($r = -0.66$, $P = 0.001$), which may indicate a trade off in allocation between EFN size and frequency.

DISCUSSION

Variation in traits mediating protection mutualisms

This study provides the first evidence demonstrating genetic variation in EFN characters in a system where EFNs are known to influence components of plant fitness. Significant heritabilities were detected for the proportion of leaves bearing nectaries and for EFN length, indicating that these traits have the potential to respond to selection. However, the low proportion of offspring variance attributable to sire suggested a high degree of plasticity in EFN traits. In other protection mutualisms, genetic variation in rewards has been infrequently investigated (Bronstein 1998, but see Loughrin et al. 1995, Daehler et al. 1999, Wilkinson et al. 2000, Hoballah et al. 2002), even though such work is critically important for understanding the evolution of mutualisms.

Intraspecific variation in indirect defenses may also derive from phenotypic plasticity in response to herbivory (reviewed by Karban and Baldwin 1997). Substantial evidence suggests that plants increase EFN secretion following damage by herbivores (induction; reviewed by Agrawal and Rutter 1998, see also Heil et al. 2000). Induced EFN production occurs in cultivated cotton, *G. barbadense* and *G. herbaceum* (Mound 1962, Wackers and Wunderlin 1999, Wackers et al. 2001), and therefore may also play a role in the defensive strategies of the related *G. thurberi*. Preliminary data from the *G. thurberi* system indicate that EFN investment increases when plants are experimentally, mechanically damaged (M. C. Gardener and J. A. Rudgers, *unpublished data*). Plasticity would allow plants to adjust allocation to EFN in ecological time but would not necessarily constrain the evolution of non-inducible traits, such as nectary size or frequency (e.g., Zangerl and Berenbaum 1990).

Herbivory reduces plant fitness

Application of insecticide established that herbivore damage reduced *G. thurberi* growth and reproductive success. Average levels of 32% greater leaf damage for control plants resulted in 11–16% reductions in plant growth and 86–168% reductions seed production

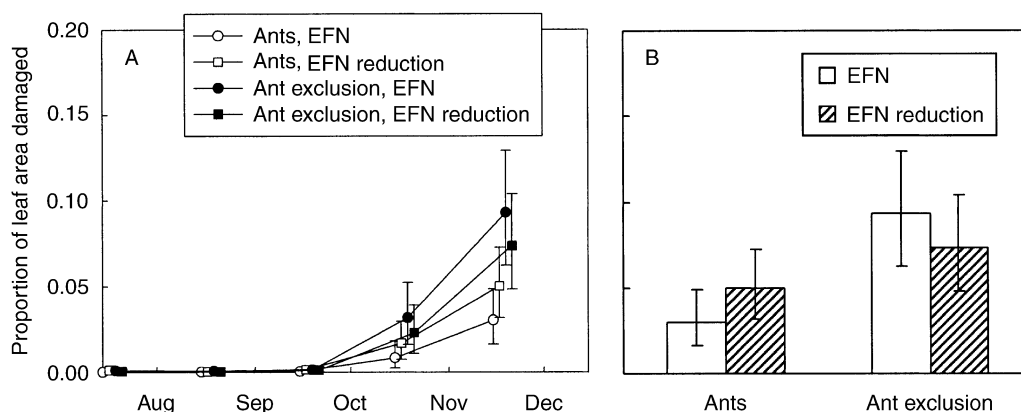


FIG. 4. Effects of the ant treatment (ants or ant exclusion) and the extrafloral nectar treatment (EFN or EFN reduction) on all types of leaf damage, measured as proportion of leaf area damaged during (A) five censuses in 2000 and (B) the final census in 2000. Symbols represent back-transformed least-squares means \pm 95% CI and are slightly offset to show confidence intervals.

as compared to insecticide-treated plants. This result is not new: insect herbivores have often been shown to reduce plant survival, biomass, and reproduction (Marquis 1992, but see Strauss and Agrawal 1999). However, studies of indirect defenses in plants have rarely examined the impact of herbivory except via direct manipulations of herbivores' enemies (but see Louda 1982a, b, van Loon et al. 2000, Turlings et al. 2002). Targeted insecticide experiments can reveal the fitness consequences of herbivory without the potentially confounding effects of modifying other trophic levels (e.g., changes in apparent competition or anti-predator behavior of herbivores). In *G. thurberi* herbivory has been shown to reduce plant fitness independently of the effects of ants.

Ants as indirect plant defenses

G. thurberi experienced 33–77% lower flower production and 45–86% lower seed production when ants were experimentally excluded from plants. For plants

with ants excluded, numbers of *Bucculatrix thurberiella* caterpillars were 22–59% greater while the percentage of leaf damage was up to 61% higher than in the presence of ants. By contrast, ants had no influence on numbers of *B. thurberiella* mines or on damage by bud and flower predators, including the boll weevil, *Anthonomus grandis* var. *thurberiae*. Previous studies of Mexican boll weevils, *A. grandis* var. *grandis*, have suggested that ants can be important predators of both larvae and adults (Sterling 1978, Sturm and Sterling 1990, Fernandes et al. 1994). Boll weevils and other bud/flower predators appear to be less susceptible to predation by ants visiting *G. thurberi*, perhaps because the ant assemblage does not include the same species as in prior investigations. In *G. thurberi*, predation and parasitism by non-ant taxa may also reduce damage by herbivores and contribute to indirect resistance in this system. Although the effects of enemies other than ants (e.g., spiders, predatory wasps, and parasitoids) on herbivory did not appear to be mediated by EFN in the

TABLE 2. Results of profile analysis for the effects of the ant treatment (ant access or exclusion), the extrafloral nectar treatment (ambient or reduced EFN), ant \times EFN, transect, and pretreatment plant size (size) on leaf damage for five censuses during 2000.

Treatment	All leaf damage			Caterpillar damage		
	df	F	P	df	F	P
Ant	1, 96	5.55	0.0205*	1, 96	9.87	0.0022**
EFN	1, 96	0.37	0.5448	1, 96	0.22	0.6424
Ant \times EFN	1, 96	6.34	0.0135*	1, 96	4.95	0.0284*
Transect	1, 96	0.18	0.6703	1, 96	2.30	0.1330
Size	1, 96	0.02	0.9017	1, 96	0.91	0.3429
Census	4, 93	3.02	0.0218*	4, 93	0.78	0.5386
Census \times ant	4, 93	6.01	0.0002***	4, 93	3.15	0.0177*
Census \times EFN	4, 93	1.51	0.2070	4, 93	1.49	0.2129
Census \times ant \times EFN	4, 93	0.76	0.5519	4, 93	0.86	0.4882
Census \times transect	4, 93	6.26	0.0002***	4, 93	1.33	0.2643
Census \times size	4, 93	2.31	0.0634	4, 93	0.52	0.7243

Note: "Caterpillar damage" indicates damage caused exclusively by externally feeding (late-instar) *B. thurberiella* caterpillars.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

TABLE 3. Results of ANCOVA for the effects of the ant treatment (ant access or exclusion), the extrafloral nectar treatment (ambient or reduced EFN), ant \times EFN, transect, and pretreatment plant size (size) on bud/flower predation, final plant height (cm), flower number, and seed number.

Treatment	Bud/flower predation			Plant height			Number of flowers			Number of seeds		
	df	F	P	df	F	P	df	F	P	df	F	P
Ant	1, 89	0.81	0.3702	1, 96	2.24	0.1379	1, 104	6.79	0.0105*	1, 104	7.52	0.0072**
EFN	1, 89	0.20	0.6523	1, 96	1.09	0.2989	1, 104	0.22	0.6377	1, 104	0.23	0.6341
Ant \times EFN	1, 89	0.01	0.9305	1, 96	0.19	0.6670	1, 104	2.78	0.0982	1, 104	5.31	0.0232*
Transect	1, 89	0.26	0.6101	1, 96	3.94	0.0500	1, 104	0.65	0.4208	1, 104	1.12	0.2933
Size	1, 89	0.31	0.5807	1, 96	49.32	<0.0001***	1, 104	11.43	0.0010**	1, 104	7.77	0.0063**
Year	1, 89	0.64	0.4244	1, 104	2.64	0.1072	1, 104	2.04	0.1557
Year \times ant	1, 89	1.05	0.3083	1, 104	0.05	0.8324	1, 104	0.01	0.9398
Year \times EFN	1, 89	0.00	0.9650	1, 104	1.63	0.2041	1, 104	1.83	0.1794
Year \times ant \times EFN	1, 89	0.23	0.6351	1, 104	0.18	0.6689	1, 104	0.00	0.9612
Year \times transect	1, 89	0.00	0.9551	1, 104	12.59	0.0006***	1, 104	5.98	0.0162*
Year \times size	1, 89	1.65	0.2013	1, 104	5.42	0.0219*	1, 104	4.34	0.0398*

Note: Analyses were repeated-measures ANCOVA models, with the exception of that for plant height.

G. thurberi system, these enemies may provide indirect defenses that are mediated by plant traits other than EFN (e.g., volatiles released from damaged plants, Dicke and van Loon 2000).

Ant defense may have application to agricultural systems. Prior studies have shown that ants can reduce herbivore damage and enhance plant fitness components in several natural systems (reviewed in Bentley 1977, Huxley and Cutler 1991, Koptur 1992, Agrawal and Rutter 1998, Bronstein 1998). As long as 2000 years ago, weaver ants were used to reduce herbivory

in citrus (Huang and Yang 1987), and, in cashew, sapodilla, and other crops, ants may provide herbivore resistance (reviewed by Way and Khoo 1992, see also Peng et al. 1999, van Mele and Cuc 2001). Less attention has been paid to ants as biological control agents in cotton crops (but see Eubanks 2001; and weevil studies including Sterling 1978, Sturm and Sterling 1990, Fernandes et al. 1994). Work on wild cotton, *G. thurberi*, suggests that the addition of ants to cultivated cotton systems, particularly those susceptible to *B. thurberiella*, could prove beneficial. However, poten-

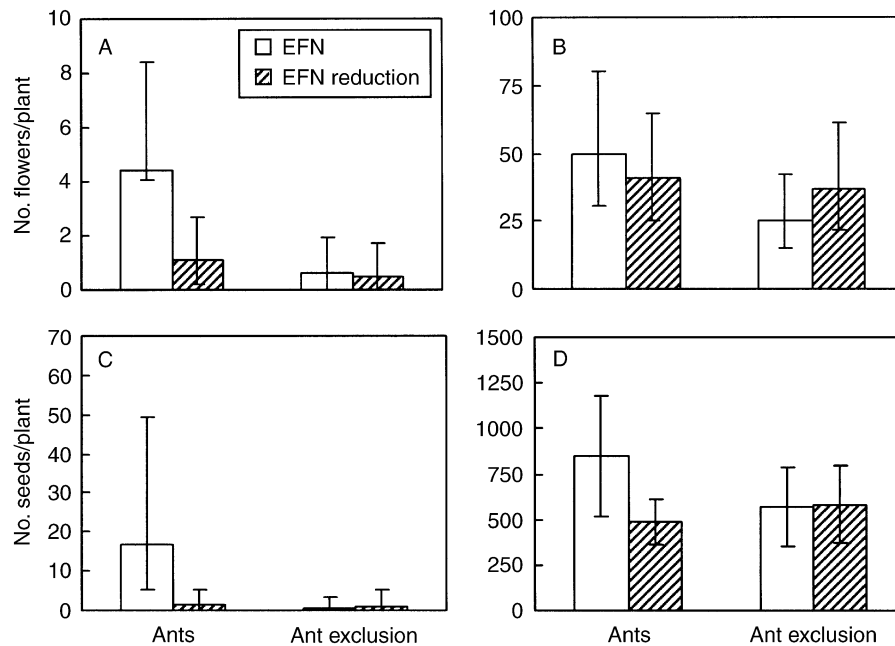


FIG. 5. Effects of the ant treatment (ants or ant exclusion) and the extrafloral nectar treatment (EFN or EFN reduction) on plant performance: number of flowers per plant (A) in 1999 and (B) in 2000 and number of seeds per plant (C) in 1999 and (D) in 2000. Bars represent back-transformed least-squares means \pm 95% CI. Different scales are used to highlight treatment effects.

tially negative effects of ants via the tending of homopterans or via intraguild predation deserve consideration (Way and Khoo 1992, Eubanks 2001).

*Trade-offs between attracting ants
and attracting herbivores*

In *G. thurberi*, experimental reductions of EFN marginally reduced the abundance of *B. thurberiella* mines ($P = 0.0787$), with, on average, 16% fewer mines in the EFN reduction treatment than in controls in 1999 and 21% fewer in 2000. This result is consistent with the hypothesis that low-nectar plants are less attractive to nectar-feeding *B. thurberiella* adults, and thus receive less oviposition. Although I did not monitor adult moth abundance in this study, evidence from cultivated cotton suggests that EFNs can attract herbivores, particularly in agricultural fields where resident ants may be excluded by the heavy use of pesticides or by routine tillage operations. In fact, cotton cultivars lacking EFNs have been created via hybridization because cotton with EFNs often experiences increased herbivory (Meyer and Meyer 1961). For example, adult *B. thurberiella* survived longer on cotton cultivars with EFNs than on cultivars without EFNs (Benschoter and Leal 1974), and fewer *B. thurberiella* as well as less caterpillar damage occurred in plots of nectariless cotton than in plots of nectaried cotton (Henneberry et al. 1977). Additionally, both oviposition by adult soybean loopers and unspecified fruit damage was greater on cotton that had EFNs (Adjei-Maafa et al. 1983, Beach et al. 1985). Thus, *Gossypium* species may face trade-offs between attracting beneficial predators and subsidizing herbivores that consume nectar. In *G. thurberi*, during the two years of this study, the benefits of EFN outweighed the costs. However, the potential ecological cost of EFNs in attracting herbivores (as well as the direct cost of nectar production) could function to constrain the evolution of this indirect defense. Recent evidence supports the idea that ecological trade-offs are important components of the costs of defenses in other plant-herbivore systems (reviewed by Strauss et al. 2002).

Selection on indirect defensive strategies

To demonstrate that enemies of herbivores currently act as selective agents on plant traits, the plant trait must be shown to mediate the benefits of these enemies. Experimental manipulation of plant traits (in addition to manipulation of the agents of selection) allows one to ascertain the current fitness consequences of these traits. In addition, positive correlations between natural trait variation and fitness suggest that natural variation is important. This study is distinguished by the use of experimental manipulation to determine the importance of plant rewards to components of fitness. In the presence of ants, plants with reduced EFN were less attractive to ants (67–85% fewer ants than controls), experienced up to 22% more leaf herbivory, and had 42–

92% lower seed production. As EFN reduction did not affect plant fitness correlates in the ant exclusion treatment, it is reasonable to conclude that ants were responsible for differences in *G. thurberi* reproductive success. Furthermore, a significant positive correlation was found between natural EFN frequency and seed production; this result suggests that even small differences in EFN phenotypes could have fitness consequences.

Several other researchers have manipulated EFN (Bentley 1976, Koptur 1979, Beckman and Stucky 1981, McLain 1983, Tempel 1983, Rashbrook et al. 1992, Apple and Feener 2001). In those studies that blocked EFN secretion, blocked plants attracted fewer ants than plants with ambient nectar (Beckman and Stucky 1981, Apple and Feener 2001), indicating that EFN mediates ant visitation in these systems. Only two studies have examined the ultimate fitness consequences of EFN for plants, however. Tempel (1983) blocked nectaries on bracken fern, *Pteridium aquilinum*, which reduced ant visitation by ~50%, but neither ant exclusion nor nectar reduction affected herbivory in this system. McLain (1983) removed EFNs from *Passiflora incarnata*. In this study, plants without EFNs had significantly lower ant visitation, greater leaf damage, and less fruit production than unmanipulated plants (McLain 1983). While this result is consistent with benefits of EFN, the lack of control for nectary removal prohibits assignment of causality. In the remaining studies, either the abundance of ants and other predators was not recorded (Koptur 1979) and thus differences among treatments could not be attributed exclusively to ants, or plant fitness was not assessed. Therefore, the *G. thurberi* system provides the best demonstration thus far that EFN availability alters the benefits afforded by ants. Combining this result with evidence that variation in EFN traits has a genetic basis, we expect that the enemies of herbivores shape plant evolution in the *G. thurberi*-ant association. With further investigation, we may find evidence for selection by herbivores' enemies in many other unspecialized and facultative mutualisms.

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