

Herbivory reduces plant interactions with above- and belowground antagonists and mutualists

NICHOLAS A. BARBER,^{1,4} LYNN S. ADLER,¹ NINA THEIS,² RUTH V. HAZZARD,¹ AND E. TOBY KIERS^{1,3}

¹*Department of Plant, Soil, and Insect Sciences, University of Massachusetts, Amherst, Massachusetts 01003 USA*

²*Department of Natural Sciences, Mathematics, and Technology, Elms College, Chicopee, Massachusetts 01013 USA*

³*Department of Ecological Science, Faculty of Earth and Life Sciences, Vrije Universiteit Amsterdam, The Netherlands*

Abstract. Herbivores affect plants through direct effects, such as tissue damage, and through indirect effects that alter species interactions. Interactions may be positive or negative, so indirect effects have the potential to enhance or lessen the net impacts of herbivores. Despite the ubiquity of these interactions, the indirect pathways are considerably less understood than the direct effects of herbivores, and multiple indirect pathways are rarely studied simultaneously. We placed herbivore effects in a comprehensive community context by studying how herbivory influences plant interactions with antagonists and mutualists both aboveground and belowground. We manipulated early-season aboveground herbivore damage to *Cucumis sativus* (cucumber, Cucurbitaceae) and measured interactions with subsequent aboveground herbivores, root-feeding herbivores, pollinators, and arbuscular mycorrhizal fungi (AMF). We quantified plant growth and reproduction and used an enhanced pollination treatment to determine if plants were pollen limited. Increased herbivory reduced interactions with both antagonists and mutualists. Plants with high levels of early herbivory were significantly less likely to suffer leaf damage later in the summer and tended to be less attacked by root herbivores. Herbivory also reduced pollinator visitation, likely due to fewer and smaller flowers, and reduced AMF colonization. The net effect of herbivory on plant growth and reproduction was strongly negative, but lower fruit and seed production were not due to reduced pollinator visits, because reproduction was not pollen limited. Although herbivores influenced interactions between plants and other organisms, these effects appear to be weaker than the direct negative effects of early-season tissue loss.

Key words: *Acalymma vittatum*; *arbuscular mycorrhizal fungi*; *Cucumis sativus*; *indirect effects*; *pollinator*; *root*.

INTRODUCTION

Herbivory has well-known direct negative effects on plants, reducing growth and reproduction (Marquis 1984, Karban and Strauss 1993). However, plant–herbivore interactions take place in a larger community context (Miller and Travis 1996, Strauss and Irwin 2004). In addition to direct effects of tissue loss, herbivores may have indirect effects on other organisms through impacts on a shared host plant (Fig. 1, dashed arrows) (Wootton 1994). Variation in herbivore damage may alter the strength of plant interactions with other organisms and their effects on plant fitness (Fig. 1, solid arrows). These indirect pathways, which involve both antagonist and mutualist organisms, are considerably less understood (Strauss and Irwin 2004), and are rarely studied simultaneously. Additionally, mutualists and antagonists occur in both aboveground and below-

ground subsystems, but responses to herbivory on both sides of the soil surface rarely have been considered concurrently (Bardgett and Wardle 2003).

Herbivore damage often induces the expression of resistance traits in plants, which can reduce future herbivory and increase plant fitness (Agrawal 1998, Karban et al. 1999). Induced resistance traits may be expressed in different plant tissues than those that were damaged; for example, root defenses can be induced following leaf damage or vice versa (Bezemer and van Dam 2005, Kaplan et al. 2008). Thus, induced resistance across tissues can result in resistance against herbivores both temporally and spatially separated from the original inducers (Ohgushi 2005). This cross-system induction may benefit plants if damage to one tissue signals increased risk to other tissues (Karbon et al. 1999), such as when adults feed on leaves and larvae feed on roots (van Dam and Heil 2011).

The strength of interactions with mutualists, such as pollinators, may also be affected by herbivory. Numerous studies have shown that leaf damage can alter floral attractive traits that influence pollinator attraction (Adler 2007) such as flower number (Lehtila and Strauss 1997, Hambäck 2001), flower size (Aizen and Raffaele

Manuscript received 15 September 2011; revised 12 December 2012; accepted 25 January 2012. Corresponding Editor: C. V. Hawkes.

⁴ Present address: Department of Biological Sciences, Northern Illinois University, DeKalb, Illinois 60115 USA. E-mail: nbarber@niu.edu

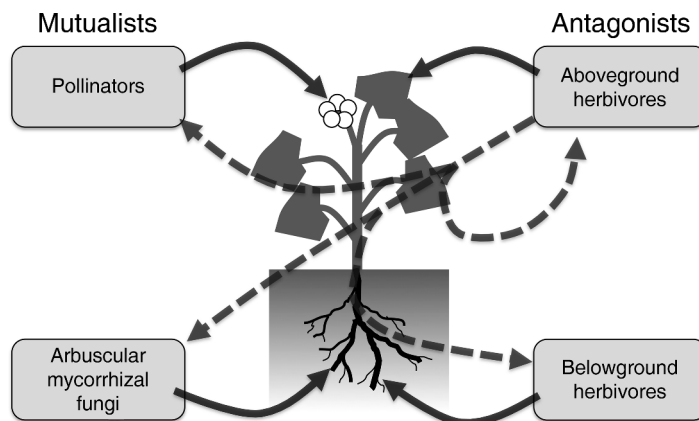


FIG. 1. Schematic illustrating direct and indirect effects of aboveground herbivory on mutualisms and antagonisms above- and below-ground. Dashed arrows indicate indirect effects of aboveground herbivores on other organisms (including other aboveground herbivores). Solid arrows indicate direct effects of organisms on the shared host plant.

1996, Strauss et al. 1996, Lehtilä and Strauss 1997, Mothershead and Marquis 2000), nectar production or quality (Adler et al. 2006, Samocha and Sternberg 2010), and floral volatiles (Kessler and Halitschke 2009, Theis et al. 2009). These changes can reduce pollinator attraction (Strauss et al. 1996, Lehtilä and Strauss 1997, Mothershead and Marquis 2000, Kessler and Halitschke 2009), with negative consequences for plant reproduction (Strauss and Murch 2004). For example, leaf herbivory in *Oenothera macrocarpa* (Onagraceae) resulted in smaller flowers that attracted fewer pollinators, reducing both fruit set and seed production as a result of pollen limitation (Mothershead and Marquis 2000). Nonetheless, plants may compensate for these negative effects in other ways, such as increasing male flower production and male fitness (Strauss et al. 2001).

Important plant mutualisms also occur belowground (van der Heijden et al. 2008). Arbuscular mycorrhizal fungi (AMF) are ubiquitous soil microbes that form symbiotic associations with host plants and assist in plant nutrient uptake while surviving on plant photosynthates (Smith and Read 2008). AMF can significantly benefit plant growth and reproduction (Maherali and Klironomos 2007, Smith et al. 2009), but these benefits may be modified by aboveground herbivores (Currie et al. 2011). Investigations of AMF responses to foliar damage have produced variable results, including both increases and decreases in fungal colonization (Gehring and Bennett 2009). A recent meta-analysis found that mycorrhizae responses to herbivory varied with treatment method and plant type, concluding that significant reductions in colonization are not widespread (Barto and Rillig 2010). Herbivory may also influence the allocation of fungal structures (i.e., number of arbuscules or vesicles) inside a host root, which can affect the benefits the host plant derives from the symbiosis (Wearn and Gange 2007).

Our aim was to place herbivory effects in a comprehensive community context by studying how herbivores influence plant interactions with organisms

that have the potential to exacerbate or ameliorate herbivore impacts. The studies discussed above suggest that herbivores frequently reduce the strength of interactions with other antagonists but may have more variable indirect effects on mutualists, perhaps with greater dependence on ecological context. By studying indirect effects aboveground and in the rhizosphere, we are responding to calls for empirical research further integrating indirect interactions with aboveground-belowground ecology (Ohgushi 2005, Kaplan et al. 2008, van Dam and Heil 2011).

METHODS

Study system

Cucumis sativus (cucumber, Cucurbitaceae) is a widely cultivated annual, monoecious herb reliant on pollinators to vector pollen between male and female flowers. Flowers are open for a single day and are visited by a variety of generalist pollinators including honey bees (*Apis mellifera*, Apidae; see Plate 1), bumble bees (*Bombus* spp., Apidae), a variety of solitary bees (e.g., Halictidae, Andrenidae), butterflies, and hover-flies (Syrphidae). *Cucumis sativus* is commonly colonized by AMF, and the symbioses have been shown to affect flowering, fruit production, photosynthesis rates, and disease resistance (Trimble and Knowles 1995, Valentine et al. 2001, Hao et al. 2005).

Acalymma vittatum (striped cucumber beetle, Chrysomelidae) is a common specialist herbivore and agricultural pest of Cucurbitaceae in the northeastern United States. Adult beetles feed on leaves, stems, and flowers, and oviposit near the soil surface. Larvae move underground after hatching to feed on roots for 10–20 days before pupation. In our region (Massachusetts, USA), larvae are present from mid-June until the end of the growing season. Feeding by *A. vittatum* is stimulated by cucurbitacins (Metcalfe et al. 1980), oxygenated tetracyclic triterpenes produced by Cucurbitaceae that act as feeding deterrents to other herbivores (Agrawal et al. 1999a). Adult *A. vittatum* also vector bacterial wilt

(*Erwinia tracheiphila*, Enterobacteriaceae), an economically important disease in cucurbit crops.

Experimental design and treatments

We germinated *C. sativus* seeds in the greenhouse and transplanted 248 seedlings into a 0.4-ha plot under organic management (site details in Appendix A) on 3 June 2009. We manipulated leaf herbivory (four treatment levels) and pollination (enhanced and natural) in a factorial design, for eight total treatment combinations in a randomized block design where blocks were rows of eight plants (31 rows total).

Herbivory treatment targets were 0, 10, 25, or 50% leaf area consumed, which are representative of natural variation in early-season herbivory (R. Hazzard, *personal observation*). Beginning on 10 June, we enclosed 0, 1, 2, or 3 adult *A. vittatum* in a small nylon mesh bag on the first fully expanded leaf of each plant depending on treatment assigned. Starting on 15 June, we doubled these numbers to 0, 2, 4, or 6 beetles per leaf to reach damage targets more quickly. Beetles were collected from local farms using handheld vacuums. Bags were checked every 1–2 weekdays, and missing or dead beetles were replaced. Beetles and bags were added to the second, third, and fourth leaves as soon as they were fully expanded. We removed bags when target damage was reached on each leaf and estimated total leaf damage using images of leaves with known damage levels. In some cases pre-treatment damage equaled or surpassed target damage, so we placed empty bags on these leaves. These empty bags and bags on control plants were removed when the last bag with beetles was removed for each leaf, with all bags removed by 13 July. Herbivory treatments were timed to mimic the first generation of *A. vittatum* in Massachusetts, which emerge in early June, peak in mid- to late-June, and decline in July while the second generation is feeding belowground. Treating the first four leaves was appropriate because these first leaves are the most heavily attacked by the first generation of *A. vittatum*. Because mean percent herbivory on each plant frequently differed from target damage level, we treated damage as a continuous variable, using the mean damage across the four treatment leaves (see *Statistical analyses*).

We manipulated pollination by hand-pollinating female flowers on plants assigned to the enhanced-pollination treatment. We collected male flowers from non-experimental donor plants and used pollen from these flowers to coat the stigmas of all open female flowers. We applied enhanced pollination treatments 5 days each week from 13 July to 3 September.

Response measurements

Plant growth, defenses, floral traits, and reproduction.—To measure aboveground plant growth, we counted fully expanded leaves on 13 July and 5 August and measured the length and width of the three most recently fully expanded leaves on a single runner. We

measured belowground growth on a subset of harvested plants (see *Belowground herbivores*) using dry biomass of harvested roots. We also recorded plants exhibiting symptoms of bacterial wilt. To determine if treatments affected cucurbitacins, we collected a leaf from each plant on 23–24 July (midway between the two aboveground herbivore surveys) by slicing the petiole with a razor. We collected roots from a subset of plants harvested during belowground herbivore surveys (see *Belowground herbivores*). We extracted cucurbitacins from leaves and roots and quantified concentration of cucurbitacin C, the main cucurbitacin produced by *C. sativus*, using high-performance liquid chromatography (HPLC; Appendix A).

Beginning on 6 July, when flowering started, we counted the number of male and female flowers on each plant 5 days each week. We assessed floral display by measuring length and width of a single petal on two male and two female flowers in mid-July and again in mid-August. To determine if treatments affected floral scent, we sampled floral volatiles from a single male flower on a subset of 82 plants concurrent with pollinator surveys in July and August (Appendix B).

We harvested fruits when they matured (18 cm in length, although we delayed collecting some that were still visibly growing). Occasional fruits <18 cm were collected if it was apparent their growth had stopped. We recorded the fresh weight of every fruit collected and analyzed mean fruit weight per plant; to increase precision, plants that produced fewer than three fruits were excluded. We calculated fruit set for each plant, a measure of pollination success, as the total number of fruits divided by the total number of female flowers produced. We also measured seed production, another indicator of pollination success, for the first three fruits produced by each plant. We cut these fruits in half lengthwise, counted the number of developed seeds visible in each half, and summed these values. We limited seed analysis to plants that produced at least two fruits and calculated the average seed count rounded to the nearest whole number for use in Poisson models (see *Statistical analyses*). We estimated total seed production for each plant as the product of total fruits and mean seeds per fruit.

Aboveground herbivores.—We measured subsequent aboveground damage during leaf counts on 13 July and 5 August by estimating percent damage on the three most recently fully expanded leaves (which were also measured to assess leaf size).

Belowground herbivores.—Because it is difficult to accurately quantify tissue loss to root herbivores, we estimated root damage using the abundance of *A. vittatum* larvae on a subset of plants in the experiment. We harvested the roots of all plants in every third block by collecting a cylindrical soil core 15 cm in diameter and 15 cm deep. Roots were removed from the soil and dried to determine belowground growth and cucurbitacin C content (Appendix A). We placed the soil in

Berlese funnels and collected larvae in 70% ethanol. Each block was sampled and placed in funnels on a single day between 22 and 31 July and remained in funnels until the soil dried (17–24 days). Larvae were collected from alcohol, and identifications were verified under a dissecting microscope.

Pollinator visitation.—We observed pollinator behavior on 13 days for a total of 40.3 person-hours of observation. Observations took place between 10:00 and 15:00 hours, when pollinators were most active. We followed individual pollinators within the experimental plot and used handheld digital voice recorders to record pollinator taxon, number of visits to each plant, number of flowers probed per visit, and time spent per flower in seconds. Individual pollinators were followed as long as possible or until they left the plot. We calculated the proportion of flowers probed per visit as the total number of flower probes per visit on a given day divided by the number of flowers open on that day; this proportion was averaged across observation days. We analyzed number of visits and proportion of flowers probed for all pollinators combined and for honey bees and bumble bees separately because these were the most common pollinators (see *Results*). Probing time was analyzed only for honey bees and bumble bees because average probing time varies among pollinator species (Barber et al. 2011).

Mycorrhizal colonization.—We collected two soil cores (19 mm diameter, approximately 15 cm deep) from each plant 5 cm from the stem on 10 July. This collection date coincided with high host nutrient demand, and thus high mycorrhizal dependency. We rinsed cores in soil sieves to retrieve fine root fragments, which were stained with trypan blue and mounted on microscope slides (Appendix A). We quantified colonization using the magnified gridline intersect method (McGonigle et al. 1990).

Statistical analyses

We analyzed responses to treatments using generalized linear mixed models (GLMMs) in R (R Development Core Team 2010), using the functions `lme()` in the `nlme` package and `glmmPQL()` in the `MASS` package (Venables and Ripley 2002; `nlme` package, *available online*).⁵ In all models, herbivory (mean percent damage on the first four leaves), pollination treatment, and their interaction were fixed factors and block was a random factor. Sampling for AMF and *A. vittatum* larvae took place prior to or shortly after initiation of enhanced pollination, so we excluded pollination treatment from AMF colonization and larval abundance models.

For response variables that were counts (leaf and flower number, number of pollinator visits, fruit number, and seed count), we specified a Poisson error distribution and log link using `glmmPQL()`, which accounts for overdispersion when estimating model

parameters. Subsequent leaf damage, larval recovery, and plant death due to bacterial wilt were generally low, so we modeled presence of each using GLMMs with binomial errors and logit link. Continuous variables (root mass, petal measurements, probe time, proportion flowers probed, mycorrhizal colonization scores, fruit weight, and cucurbitacin content) were analyzed with Gaussian errors and identity link using `lme()` following appropriate transformations to normalize residuals. We used principal components analysis to describe concentrations of identified floral scent components (see Appendix B). The first two principal components, which described 63% of variation (see *Results*), were analyzed using `lme()`. Because one plant with >60% leaf damage was a potential outlier, we re-ran all models with this replicate excluded; there were no qualitative changes in results and parameter estimates were nearly identical.

RESULTS

Plant growth, defenses, floral traits, and reproduction

Herbivory significantly reduced the total number of leaves per plant in both July and August (Fig. 2A and B, Appendix C: Table C1), but there was no effect of enhanced pollination and no interaction. Herbivory also reduced leaf length and width in July, but the effect disappeared by August, when leaves were slightly longer on plants with enhanced pollination (Appendix C: Table C1). Herbivory significantly reduced belowground root biomass (Appendix C: Table C1), but there was no effect of enhanced pollination and no interaction. The probability of plants dying from bacterial wilt was not affected by either treatment (all $P > 0.3$). Leaf cucurbitacin C content was unaffected by treatments (all $P > 0.45$), but there was a marginally significant trend for root cucurbitacin C content to decline with herbivory (linear model coefficient estimate, β [mean \pm SE]; herbivory, -4139 ± 2098 , $t = -1.97$, $P = 0.053$). Other treatments did not affect root cucurbitacins (all $P > 0.4$).

Herbivory significantly reduced male and female total flower production, and enhanced pollination significantly increased female flower production (Fig. 2C and D, Appendix C: Table C2). Herbivory reduced the size of male flower petals in July but not in August. Female flower size was not affected by herbivory treatments. Enhanced pollination affected male petal width in August, and there was an interaction between herbivory and pollination treatments such that petals on enhanced-pollination plants were wider than on plants with natural pollination, but herbivory reduced this effect (Appendix C: Table C2).

The first floral scent principal component (PC1) was most strongly correlated with (E) furanoid linalool oxide and described 34.8% of the variation in volatiles. PC2 was correlated with α - and β -pinene and described 27.7% (Table B1). There was no effect of treatments or their interaction on PC1 or PC2 scores (all $P > 0.12$).

Herbivory significantly reduced total fruit production and mean fruit mass, but pollination did not have a

⁵ <http://cran.r-project.org/web/packages/nlme/>

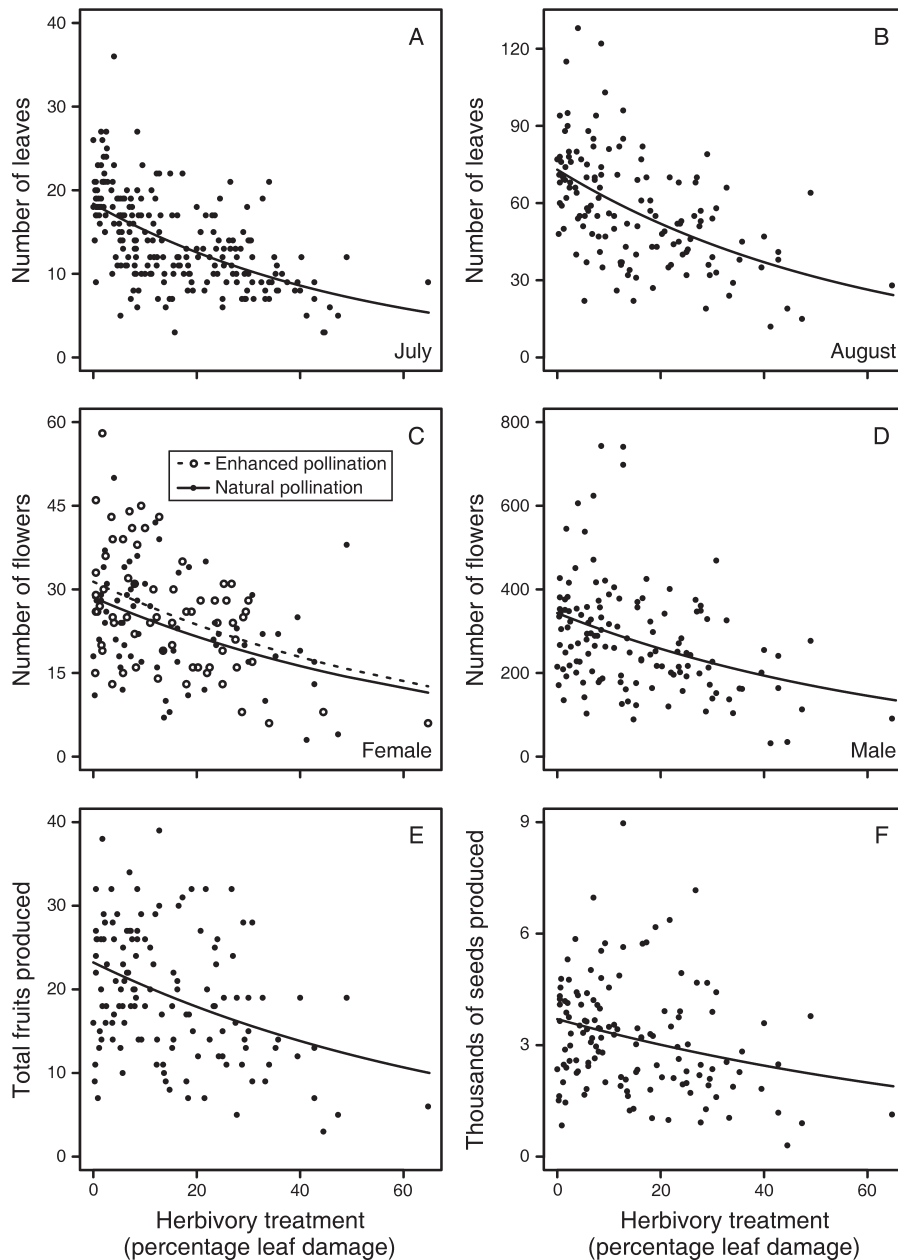


FIG. 2. Effects of herbivory treatment on plant growth and reproduction. Herbivory reduced number of leaves per plant in (A) July and (B) August and total number of (C) female and (D) male flowers. Plants with enhanced pollination treatments also produced more female flowers. Herbivory also reduced (E) the number of fruits per plant and (F) estimated total seed production (product of total number of fruits per plant and average number of seeds per fruit). Note that data are untransformed and do not account for block effects. Fitted lines represent significant effects of treatments (statistical results in Appendix C: Tables C1–C3).

significant effect, and there was no interaction (Table C3, Fig. 2E). Fruit set, the proportion of female flowers that developed into fruits, was not affected by treatments or their interaction (Table C3). Seed counts tended to increase through the season such that fruits collected later in the season had more seeds than those collected earlier, likely due to observer bias. To account for this, we included as fixed factors the mean day of

year (with 1 January = day 1) of fruit collection for each plant and the interaction between mean collection date and herbivore damage. Both of these terms were significant (β date, 0.020 ± 0.006 , $t = 3.19$, $P = 0.002$; β date \times herbivory, -0.001 ± 0.000 , $t = -4.02$, $P < 0.001$). After controlling for the influence of date, herbivory still had a highly significant, positive effect on seed production per fruit (Table C3). Enhanced

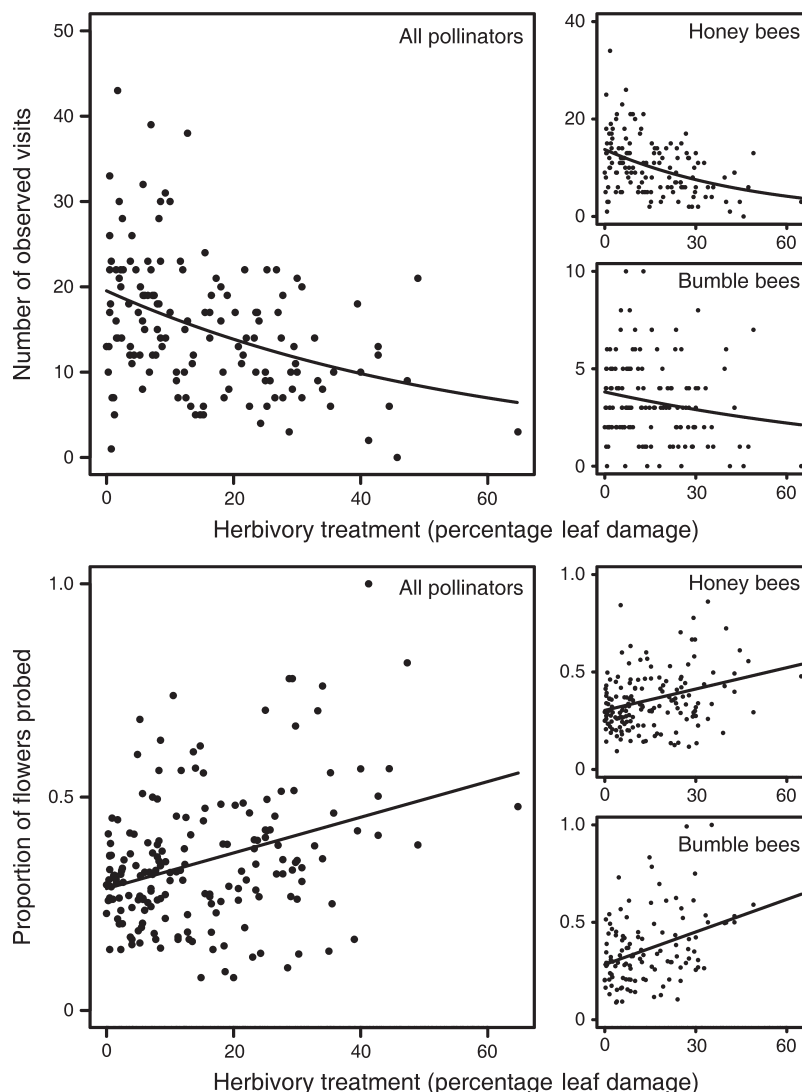


FIG. 3. Effects of herbivory treatment on pollinator interactions. Herbivory reduced the total number of pollinator visits, honey bee visits, and bumble bee visits per plant (top three panels). The proportion of flowers probed increased with herbivory for total pollinators, honey bees, and bumble bees (bottom three panels). Note that data are untransformed and do not account for block effects. Fitted lines represent significant effects of treatments (statistical results in Appendix C: Table C4).

pollination did not increase per-fruit seed production, and there was no treatment interaction. Estimated total seed production decreased with herbivory and was not affected by other factors (Table C3, Fig. 2F).

Aboveground herbivores

Plants with low initial herbivory were more likely to receive subsequent damage in July (β herbivory, -0.023 ± 0.011 , $t = -2.21$, $P = 0.028$) but not August (-0.004 ± 0.024 , $t = -0.158$, $P = 0.875$).

Belowground herbivores

There was a trend of decreased probability of recovering larvae from a plant with increasing herbivory (β , -0.037 ± 0.022 , $t = -1.69$, $P = 0.096$).

Pollinator visitation

Herbivory reduced the total number of visits by all pollinators combined, and both honey bees and bumble bees alone (Table C4; Fig. 3). Enhanced pollination had no effect on flower visits. The probe time for honey bees and bumble bees was unaffected by either herbivory or pollination treatments. The proportion of flowers probed increased significantly with herbivory for all pollinators, honey bees, and bumble bees (Table C4), indicating that individual flowers were more likely to be probed per visit on high-damage plants than low-damage plants.

Mycorrhizal colonization

Herbivore damage significantly reduced total arbuscular mycorrhizal colonization (β , -0.210 ± 0.088 , $t =$



PLATE 1. Honey bee (*Apis mellifera*) probing a *Cucumis sativus* flower. Photo credit: Paul CaraDonna.

-2.38 , $P = 0.019$). However, there was no effect of damage on specific structures, such as arbuscular colonization (β , -0.088 ± 0.070 , $t = -1.26$, $P = 0.211$).

DISCUSSION

Plant–herbivore interactions occur in a complex community context, in which herbivores have both direct effects on plants and indirect effects on other community members via changes in plant traits. Furthermore, the net impacts of herbivores on plants include both direct damage and the effects of these altered subsequent community interactions on the shared host plant. We manipulated aboveground herbivory to determine the indirect effects of herbivores on plant mutualists and antagonists in aboveground and belowground environments. This research unites two subfields of ecology that have generally been studied separately: aboveground–belowground linkages and indirect interactions among antagonists and mutualists.

Early-season herbivory negatively affected interactions with both antagonists (above- and belowground herbivores) and mutualists (AMF and pollinators). Despite the potential benefit of reducing subsequent damage, the net effects of early herbivory on plant growth and reproduction were strongly negative. This

suggests that the indirect benefits of reduced subsequent antagonisms were much weaker than the direct negative effects of early damage. Although treatments significantly reduced plant–pollinator interactions, enhanced pollination had no influence on plant reproduction, indicating that the direct impacts of herbivores were also more detrimental for plant reproduction than indirect costs of deterring pollinators from damaged plants. Below we discuss in detail first the indirect effects of herbivores on antagonists and mutualists, and then how these may have contributed to the net effects of herbivory on plant growth and reproduction.

Indirect effects on antagonists

Early leaf herbivory decreased subsequent leaf damage by *A. vittatum* (the only leaf-chewing herbivore commonly observed on the plants). Increased beetle damage in June reduced the probability of leaf damage in July, such that a 10% increase in leaf area consumed corresponded to a 2–3% decrease in the probability of later attack. A similar trend occurred belowground, where root herbivores were marginally less likely to be recovered from the roots of high-damage plants. These patterns suggest that early herbivory induced resistance in *C. sativus* that lasted at least several weeks before

relaxing in August, when evidence of induction was no longer found.

This is the first examination of defense induction in response to *A. vittatum*, for which cucurbitacins, the putative primary defensive compounds of cucurbits, are phagostimulants (Metcalf et al. 1980). Although induction of cucurbitacin C in *C. sativus* has been demonstrated in leaves following attack by generalist spider mites (Agrawal et al. 1999a), in our study we found no change in cucurbitacin C concentrations in leaves. That we found a reduction in herbivore attack aboveground, without an effect on cucurbitacins, indicates *A. vittatum* may induce other, unknown mechanisms of resistance or alter leaf nutrient status (Barrett and Agrawal 2004). Response of *C. sativus* to spider mites may differ from response to chewing insects like *A. vittatum* (Pozo and Azcón-Aguilar 2007); other research suggests that attack by the chewing herbivore *Spodoptera exigua* may also induce other defensive compounds in *C. sativus* (Barrett and Agrawal 2004).

Belowground cucurbitacin C content in roots tended to decline as herbivory increased. This may explain the pattern of reduced *A. vittatum* larval occurrence in high-herbivory treatments since larvae would be less attracted to damaged plants with low root cucurbitacins. Declines in root defensive chemistry following leaf damage or application of plant hormones that induce defense responses have been found in other plant species (Hol et al. 2004, van Dam et al. 2004), although both of these studies focused on alkaloids with well-known anti-herbivore effects. Belowground induction is expected to occur when foliar damage is a reliable signal that roots are more likely to be attacked (Karban et al. 1999, Bezemer et al. 2004, Kaplan et al. 2008). In cucumber, adult *Acalymma vittatum* feed on aboveground tissues and larvae subsequently consume roots. Thus, systemic induction may be adaptive for the host plant, whether the mechanism is by decreasing phagostimulant concentration or increasing other resistance traits.

Indirect effects on mutualists

Leaf herbivory significantly reduced pollinator visits, and this pattern was consistent for honey bees and bumble bees independently. Pollinator visitation may be influenced by flower number and size, and herbivory reduced the number of both male and female flowers, and the size of male flowers. Leaf damage to wild radish (*Raphanus raphanistrum*) similarly reduced flower production and size, although the effects persisted for only a week after treatments were applied (Lehtilä and Strauss 1997). We saw a longer-term attenuation of herbivory effects on flower size. Male flowers were significantly smaller in July (about a month after treatments), but the effect disappeared by August. Female flower size was never significantly affected by herbivory. In a high-stress environment (e.g., high herbivory), monoecious plants may reduce resource allocation to male reproductive function to maintain female reproduction (Thomson et

al. 2004, Theis et al. 2009). This contrasts with hermaphroditic wild radish, in which paternity analyses showed that herbivory increased male function (Strauss et al. 2001). Herbivory may change other floral traits that influence pollinator attraction and behavior. Recent experiments have shown that floral volatile blends can change following herbivore damage (Theis et al. 2009), sometimes with consequences for pollinator behavior (Kessler and Halitschke 2009). However, timing of damage may be critical (Effmert et al. 2008), and we found no effect of herbivory on floral volatiles. Herbivory may change nectar constituents such as concentrations of secondary metabolites, which can influence pollinator preferences (Adler 2000). We did not quantify nectar production because these flowers produce very low volumes that are difficult to quantify accurately, and bagging flowers to measure production in the field precludes pollinator visitation. However, we found no effect of herbivory on probe duration; if nectar quality or quantity was affected by leaf damage, we might expect changes in foraging time per flower (Biernaskie et al. 2002, Irwin and Adler 2008). Of the floral traits we measured, flower number was likely the most important trait affecting visitation. The effects of herbivory on flower size were transient, but plants were unable to compensate for reduced flower numbers. High-damage plants produced fewer flowers than plants with little or no damage throughout the growing season (data not shown), providing the most likely mechanism by which herbivory could consistently reduce pollination. The increases in female flower production and male petal width due to enhanced pollination are surprising but suggest plants may be able to allocate resources to flowers in response to high pollen receipt. However, enhanced pollination did not increase fruit or seed production or increase pollinator visitation.

Herbivory also reduced interactions with AMF. Total AMF colonization declined as leaf damage increased, although the trend was somewhat weak. These results mirror those of Gange et al. (2002), who found decreased colonization of the host *Plantago lanceolata* as insect herbivory accrued across their experiment. They attributed this pattern to decreases in available carbon allocated to roots. We documented a similar relationship between degree of defoliation and fungal colonization, although the variation in leaf herbivory occurred in a short period at the beginning of the growing season in our experiment. Given the strong net effect of herbivores on both above- and belowground plant growth in our study (see *Net indirect effects on plants*), reduced photosynthate availability is a likely explanation for reduced colonization (Klironomos et al. 2004), suggesting that short- as well as long-term herbivory may have consistently negative effects on AMF. There may be negative feedback from this effect if reduced AMF further limit the growth of damaged plants. Although the abundance of arbuscules, the putative site of nutrient exchange in plant-AMF

associations, has been shown to be influenced by both mammalian herbivory (Wearn and Gange 2007) and clipping (Klironomos et al. 2004) in grasses, we found no difference with herbivory treatments.

When multiple species interact with a focal species, their effects may be nonadditive. We were able to test for such an interaction by manipulating two groups of organisms (aboveground herbivores and pollinators). Additional interactions between other groups (e.g., between above- and belowground pollinators; Barber et al. 2011) are possible but beyond the scope of this study, as they would require additional factorial manipulations. Nonetheless, such experiments manipulating multiple interactors will be necessary in the future to quantify the contributions of indirect feedbacks to net plant effects as well as to address the possibility of nonadditive effects among community members (Morris et al. 2007).

Net indirect effects on plants

Although early-season herbivory reduced subsequent *A. vittatum* damage, the net effect of herbivory treatments was still a reduction in plant above- and belowground growth and reproduction. A net positive effect of induced defense (increased growth or fitness due to reduced herbivory) has been demonstrated in other annual plant systems (Agrawal 1998, 1999a, Baldwin 1998), but this did not occur in our experiment. It is possible that herbivore attack lessened the direct effects of subsequent herbivores, particularly belowground given the strong negative effects of root damage on *C. sativus* growth and reproduction (Barber et al. 2011), but this indirect effect was insufficient to overcome the direct negative impacts of early herbivore damage. It is also possible that the costs associated with induction outweighed the benefit of reduced herbivory (Agrawal et al. 1999b). The amount of leaf area lost to *A. vittatum* in mid- and late-summer was small compared to early season damage. The benefit of induction likely would be greater if the beetles were more abundant later in the summer, because the cost:benefit ratio of induction decreases as local herbivory levels increase (Baldwin 1998).

Even though herbivory strongly reduced pollinator visitation, this did not affect plant reproduction. Increased herbivory reduced fruit production, fruit size, and total seed production per plant, but this was not driven by pollen limitation. If plants had been pollen limited, we would have seen significant positive effects of enhanced pollination on fruit production of high-damage plants. Instead we found no effect of the pollination treatment on fruit or seed production, despite a small increase in female flower production. Although several studies have combined leaf damage and pollinator manipulations in diverse plant species (e.g., Lehtilä and Syrjänen 1995, Juenger and Bergelson 1997, Mothershead and Marquis 2000, Hladun and Adler 2009), only Strauss and Murch (2004) found that

herbivory increased pollen limitation. Enhanced pollination increased neither number of fruits nor fruit set (the proportion of female flower that developed into fruits) in our study. This suggests that pollinators may have been abundant enough to supply all plants with sufficient pollen and that fruit production was limited by resources as a direct result of herbivory rather than by reduced pollination services. Similarly, enhanced pollination did not increase number of seeds per fruit, which surprisingly increased with herbivory treatment. The positive relationship between herbivory and the proportion of flowers probed (Fig. 3) does not explain this increase in seed production because, again, enhanced pollination would have erased the pattern. Increasing the number of seeds per fruit may be a mechanism by which small plants with limited resources maximize their potential fitness with the few fruits they can produce. Despite the net positive effect of herbivory on seeds per fruit, estimated total seed production was still significantly reduced by herbivory, indicating that plants were unable to overcome the direct negative effects of early leaf damage on flower and fruit production.

Our design did not manipulate AMF to demonstrate their direct fitness effects or interactive effects with herbivory on plants. However, the reduction of AMF colonization by leaf damage suggests that any indirect effects of herbivores on plants via AMF would be negative. The fungal structures we quantified likely represented multiple species, some of which could have responded positively to herbivory treatments, while others declined. Growth and defense benefits conferred to plants may vary depending on the AMF species (Bennett and Bever 2007), making it difficult to make predictions based on total colonization measurements.

CONCLUSIONS

We found that leaf herbivores had indirect negative effects on both antagonists and mutualists above- and belowground, which suggests the potential for both positive and negative indirect feedbacks on plants. However, the net result of herbivory on *C. sativus* was unequivocally negative. Damage treatments, which mimicked the natural timing of *A. vittatum* feeding in this system, may have occurred so early in the plants' development that the resulting resource loss was more than the plants could tolerate. In some cases, mutualists can mitigate the negative impacts of plant enemies such as herbivores (Morris et al. 2007), although this did not occur with *C. sativus* pollinators. In agricultural systems, cultivating mutualisms has been proposed as a method to increase yields and counteract the negative effects of plant damage (Kremen et al. 2002, Strauss and Murch 2004). Our results suggest that this approach will only be successful if impacts of damage are not too severe. Future work in both natural and managed systems will need to address how the strength of interactions between plants and community members influences other com-

munity interactions to ultimately determine plant performance.

ACKNOWLEDGMENTS

We thank the many assistants who helped with this project, H. Mashayekhi, F. Halaweish, and the staff at the UMass Center for Agriculture. The manuscript benefited from comments by members of the Adler lab. This work was supported by USDA NRI 2008-02346 to L. S. Adler, E. T. Kiers, and R. V. Hazzard, NSF DEB-0542819 to N. Theis, and a Vidi and Meervoud grant from the Dutch Science Foundation (NWO) to E. T. Kiers.

LITERATURE CITED

- Adler, L. S. 2000. The ecological significance of toxic nectar. *Oikos* 91:409–420.
- Adler, L. S. 2007. Selection by pollinators and herbivores on attraction and defense. Pages 162–173 in K. J. Tilmon, editor. *Specialization, speciation and radiation: the evolutionary biology of herbivorous insects*. University of California Press, Berkeley, California, USA.
- Adler, L. S., M. Wink, M. Distl, and A. J. Lentz. 2006. Leaf herbivory and nutrients increase nectar alkaloids. *Ecology Letters* 9:960–967.
- Agrawal, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 279:1201–1202.
- Agrawal, A. A., P. M. Gorski, and D. W. Tallamy. 1999a. Polymorphism in plant defense against herbivory: constitutive and induced resistance in *Cucumis sativus*. *Journal of Chemical Ecology* 25:2285–2304.
- Agrawal, A. A., S. Y. Strauss, and M. J. Stout. 1999b. Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53:1093–1104.
- Aizen, M. A., and E. Raffaele. 1996. Nectar production and pollination in *Alstroemeria aurea*: responses to level and pattern of flowering shoot defoliation. *Oikos* 76:312–322.
- Baldwin, I. T. 1998. Jasmonate-induced responses are costly but benefit plants under attack in native populations. *Proceedings of the National Academy of Sciences USA* 95:8113–8118.
- Barber, N. A., L. S. Adler, and H. L. Bernardo. 2011. Effects of above- and belowground herbivory on growth, pollination, and reproduction in cucumber. *Oecologia* 165:377–386.
- Bardgett, R. D., and D. A. Wardle. 2003. Herbivore-mediated linkages between aboveground and belowground communities. *Ecology* 84:2258–2268.
- Barrett, R. D. H., and A. A. Agrawal. 2004. Interactive effects of genotype, environment, and ontogeny on resistance of cucumber (*Cucumis sativus*) to the generalist herbivore, *Spodoptera exigua*. *Journal of Chemical Ecology* 30:37–51.
- Barto, E. K., and M. C. Rillig. 2010. Does herbivory really suppress mycorrhiza? A meta-analysis. *Journal of Ecology* 98:745–753.
- Bennett, A. E., and J. D. Bever. 2007. Mycorrhizal species differentially alter growth and response to herbivory. *Ecology* 88:210–218.
- Bezemer, T. M., and N. M. van Dam. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends in Ecology and Evolution* 20:617–624.
- Bezemer, T. M., R. Wagenaar, N. M. van Dam, W. H. van Der Putten, and F. L. Wäckers. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *Journal of Chemical Ecology* 30:53–67.
- Biernaskie, J. M., R. V. Cartar, and T. A. Hurlly. 2002. Risk-averse inflorescence departure in hummingbirds and bumble bees: could plants benefit from variable nectar volumes? *Oikos* 98:98–104.
- Currie, A. F., P. J. Murray, and A. C. Gange. 2011. Is a specialist root-feeding insect affected by arbuscular mycorrhizal fungi? *Applied Soil Ecology* 47:77–83.
- Effmert, U., C. Dinse, and B. Piechulla. 2008. Influence of green leaf herbivory by *Manduca sexta* on floral volatile emission by *Nicotiana suaveolens*. *Plant Physiology* 146:1996–2007.
- Gange, A., E. Bower, and V. Brown. 2002. Differential effects of insect herbivory on arbuscular mycorrhizal colonization. *Oecologia* 131:103–112.
- Gehring, C., and A. Bennett. 2009. Mycorrhizal fungal–plant–insect interactions: the importance of a community approach. *Environmental Entomology* 38:93–102.
- Hamback, P. A. 2001. Direct and indirect effects of herbivory: feeding by spittlebugs affects pollinator visitation rates and seedset of *Rudbeckia hirta*. *Ecoscience* 8:45–50.
- Hao, Z. P., P. Christie, L. Qin, C. X. Wang, and X. L. Li. 2005. Control of fusarium wilt of cucumber seedlings by inoculation with an arbuscular mycorrhizal fungus. *Journal of Plant Nutrition* 28:1961–1974.
- Hladun, K. R., and L. S. Adler. 2009. Influence of leaf herbivory, root herbivory, and pollination on plant performance in *Cucurbita moschata*. *Ecological Entomology* 34:144–152.
- Hol, W. H. G., M. Macel, J. A. van Veen, and E. van der Meijden. 2004. Root damage and aboveground herbivory change concentration and composition of pyrrolizidine alkaloids of *Senecio jacobaea*. *Basic and Applied Ecology* 5:253–260.
- Irwin, R. E., and L. S. Adler. 2008. Nectar secondary compounds affect self-pollen transfer: implications for female and male reproduction. *Ecology* 89:2207–2217.
- Juenger, T., and J. Bergelson. 1997. Pollen and resource limitation of compensation to herbivory in scarlet gilia, *Ipomopsis aggregata*. *Ecology* 78:1684–1695.
- Kaplan, I., R. Halitschke, A. Kessler, S. Sardaneli, and R. F. Denno. 2008. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* 89:392–406.
- Karban, R., A. A. Agrawal, J. S. Thaler, and L. S. Adler. 1999. Induced plant responses and information content about risk of herbivory. *Trends in Ecology and Evolution* 14:443–447.
- Karban, R., and S. Y. Strauss. 1993. Effects of herbivores on growth and reproduction of their perennial host, *Erigeron glaucus*. *Ecology* 74:39–46.
- Kessler, A., and R. Halitschke. 2009. Testing the potential for conflicting selection on floral chemical traits by pollinators and herbivores: predictions and case study. *Functional Ecology* 23:901–912.
- Klironomos, J. N., J. McCune, and P. Moutoglis. 2004. Species of arbuscular mycorrhizal fungi affect mycorrhizal responses to simulated herbivory. *Applied Soil Ecology* 26:133–141.
- Kremen, C., N. M. Williams, and R. W. Thorp. 2002. Crop pollination from native bees at risk from agricultural intensification. *Proceedings of the National Academy of Sciences USA* 99:16812–16816.
- Lehtilä, K., and S. Y. Strauss. 1997. Leaf damage by herbivores affects attractiveness to pollinators in wild radish, *Raphanus raphanistrum*. *Oecologia* 111:396–403.
- Maherali, H., and J. N. Klironomos. 2007. Influence of phylogeny on fungal community assembly and ecosystem functioning. *Science* 316:1746–1748.
- Marquis, R. J. 1984. Leaf herbivores decrease fitness of a tropical plant. *Science* 226:537–539.
- McGonigle, T. P., M. H. Miller, D. G. Evans, G. L. Fairchild, and J. A. Swan. 1990. A new method which gives an objective measure of colonization of roots by vesicular arbuscular mycorrhizal fungi. *New Phytologist* 115:495–501.
- Metcalf, R. L., R. A. Metcalf, and A. M. Rhodes. 1980. Cucurbitacins as kairomones for Diabroticite beetles. Pro-

- ceedings of the National Academy of Sciences USA 77:3769–3772.
- Miller, T. E., and J. Travis. 1996. The evolutionary role of indirect effects in communities. *Ecology* 77:1329–1335.
- Morris, W. F., R. A. Huffbauer, A. A. Agrawal, J. D. Bever, V. A. Borowicz, G. S. Gilbert, J. L. Maron, C. E. Mitchell, I. M. Parker, A. G. Power, M. E. Torchin, and D. P. Vázquez. 2007. Direct and interactive effects of enemies and mutualists on plant performance: a meta-analysis. *Ecology* 88:1021–1029.
- Mothershead, K., and R. J. Marquis. 2000. Fitness impacts of herbivory through indirect effects on plant–pollinator interactions in *Oenothera macrocarpa*. *Ecology* 81:30–40.
- Ohgushi, T. 2005. Indirect interaction webs: herbivore-induced effects through trait change in plants. *Annual Review of Ecology, Evolution, and Systematics* 36:81–105.
- Pozo, M. J., and C. Azcón-Aguilar. 2007. Unraveling mycorrhiza-induced resistance. *Current Opinion in Plant Biology* 10:393–398.
- R Development Core Team. 2010. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.
- Samocho, Y., and M. Sternberg. 2010. Herbivory by sucking mirid bugs can reduce nectar production in *Asphodelus aestivus* Brot. *Arthropod–Plant Interactions* 4:153–158.
- Smith, F. A., E. J. Grace, and S. E. Smith. 2009. More than a carbon economy: nutrient trade and ecological sustainability in facultative arbuscular mycorrhizal symbioses. *New Phytologist* 182:347–358.
- Smith, S. E., and D. J. Read. 2008. *Mycorrhizal symbiosis*. Third edition. Academic Press, San Diego, California, USA.
- Strauss, S. Y., J. K. Conner, and K. P. Lehtila. 2001. Effects of foliar herbivory by insects on the fitness of *Raphanus raphanistrum*: damage can increase male fitness. *American Naturalist* 158:496–504.
- Strauss, S. Y., J. K. Conner, and S. L. Rush. 1996. Foliar herbivory affects floral characters and plant attractiveness to pollinators: implications for male and female plant fitness. *American Naturalist* 147:1098–1107.
- Strauss, S. Y., and R. E. Irwin. 2004. Ecological and evolutionary consequences of multispecies plant–animal interactions. *Annual Review of Ecology, Evolution, and Systematics* 35:435–466.
- Strauss, S. Y., and P. Murch. 2004. Towards an understanding of the mechanisms of tolerance: compensating for herbivore damage by enhancing a mutualism. *Ecological Entomology* 29:234–239.
- Theis, N., K. Kesler, and L. S. Adler. 2009. Leaf herbivory increases floral fragrance in male but not female *Cucurbita pepo* subsp. *texana* (Cucurbitaceae) flowers. *American Journal of Botany* 96:897–903.
- Thomson, V. P., A. B. Nicotra, and S. A. Cunningham. 2004. Herbivory differentially affects reproductive traits of *Cucumis sativus*. *Plant Biology* 6:621–628.
- Trimble, M. R., and N. R. Knowles. 1995. Influence of vesicular–arbuscular mycorrhizal fungi and phosphorus on growth, carbohydrate partitioning and mineral nutrition of greenhouse cucumber (*Cucumis sativus* L.) plants during establishment. *Canadian Journal of Plant Science* 75:239–250.
- Valentine, A. J., B. A. Osborne, and D. T. Mitchell. 2001. Interactions between phosphorus supply and total nutrient availability on mycorrhizal colonization, growth and photosynthesis of cucumber. *Scientia Horticulturae* 88:177–189.
- van Dam, N. M., and M. Heil. 2011. Multitrophic interactions below and above ground: en route to the next level. *Journal of Ecology* 99:77–88.
- van Dam, N. M., L. Witjes, and A. Svatoš. 2004. Interactions between aboveground and belowground induction of glucosinolates in two wild brassica species. *New Phytologist* 161:801–810.
- van der Heijden, M. G. A., R. D. Bardgett, and N. M. van Straalen. 2008. The unseen majority: soil microbes as drivers of plant diversity and productivity in terrestrial ecosystems. *Ecology Letters* 11:296–310.
- Venables, W., and B. Ripley. 2002. *Modern applied statistics with S*. Springer, New York, New York, USA.
- Wearn, J. A., and A. C. Gange. 2007. Above-ground herbivory causes rapid and sustained changes in mycorrhizal colonization of grasses. *Oecologia* 153:959–971.
- Wootton, J. T. 1994. The nature and consequences of indirect effects in ecological communities. *Annual Review of Ecology and Systematics* 25:443–466.

SUPPLEMENTAL MATERIAL

Appendix A

Methodological details of plant propagation, field site, cucurbitacin analyses, and mycorrhizal staining (*Ecological Archives* E093-139-A1).

Appendix B

Volatile sampling methods, analyses, and results (*Ecological Archives* E093-139-A2).

Appendix C

Tables of statistical analyses (*Ecological Archives* E093-139-A3).