

Conspecific and Heterospecific Aboveground Herbivory Both Reduce Preference by a Belowground Herbivore

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ABSTRACT Insect herbivores damage plants both above- and belowground, and interactions in each realm can influence the other via shared hosts. While effects of leaf damage on aboveground interactions have been well-documented, studies examining leaf damage effects on belowground interactions are limited, and mechanisms for these indirect interactions are poorly understood. We examined how leaf herbivory affects preference of root-feeding larvae [*Acalymma vittatum* F. (Coleoptera: Chrysomelidae)] in cucumber (*Cucumis sativus* L.). We manipulated leaf herbivory using conspecific adult *A. vittatum* and heterospecific larval *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) herbivores in the greenhouse and the conspecific only in the field, allowing larvae to choose between roots of damaged and undamaged plants. We also examined whether leaf herbivory induced changes in defensive cucurbitacin C in leaves and roots. We hypothesized that induced changes in roots would deter larvae, and that effects would be stronger for damage by conspecifics than the unrelated caterpillar because the aboveground damage could be a cue to plants indicating future root damage by the same species. In both the greenhouse and field, plants with damaged leaves recruited significantly fewer larvae to their roots than undamaged plants. Effects of conspecific and heterospecific damage did not differ. Leaf damage did not induce changes in leaf or root cucurbitacin C, but did reduce root biomass. While past work has suggested that systemic induction by aboveground herbivory increases resistance in roots, our results suggest that decreased preference by belowground herbivores in this system may be because of reduced root growth.

KEY WORDS specialist, generalist, induced defense, *Acalymma vittatum*, *Cucumis sativus*

Plants live simultaneously both above- and belowground, providing a link between organisms in both environments (Wardle et al. 2004). Antagonists such as insect herbivores attack both shoot tissues aboveground and root tissues belowground, providing a pathway for indirect effects transmitted by shoot–root signaling through a shared host plant (Bardgett and Wardle 2003, Erb et al. 2008). Although the indirect effects of leaf herbivores on other aboveground organisms are well-studied (Ohgushi 2005), knowledge of these indirect effects across the soil surface is comparatively limited (Van der Putten et al. 2001, Blossey and Hunt-Joshi 2003, Hunt-Joshi and Blossey 2005, Wang et al. 2014). Effects on belowground herbivores may be important because root herbivory can have strong direct impacts on plants and indirect effects on entire communities (Van der Putten et al. 2001, Wardle et al. 2004, Van Dam et al. 2005). Root damage can greatly reduce plant growth and fitness (Strong et al. 1995, Barber et al. 2011) and alter plant–pollinator interactions (Poveda et al. 2003, Barber et al. 2011,

Barber and Soper Gorden 2014). Furthermore, root damage can influence plant community dynamics (Brown and Gange 1989, Gange and Brown 2002) and ecosystem function (Bardgett et al. 2005).

Two potential mechanisms for indirect effects of aboveground herbivory on belowground herbivores are induced plant defense and reduced root biomass. Insect herbivore damage can induce a systemic resistance response, resulting in increased concentrations of defensive metabolites in undamaged plant parts and induced resistance to the subsequently feeding herbivores through reduced herbivore preference or performance (Karban and Baldwin 1997, Agrawal and Karban 1999, Karban et al. 1999, Bezemer et al. 2004, Kaplan et al. 2008). Root damage by belowground herbivores may increase resistance in aboveground plant tissues (Van Dam et al. 2003, Bezemer and van Dam 2005, Kaplan et al. 2008). However, the effects of aboveground herbivory on root-feeding herbivores have been studied much less frequently (Van Dam and Heil, 2011; but see Soler et al. 2007 and Tindall and Stout 2001). Aboveground herbivory may also influence root herbivore behavior by direct reductions in root biomass. Studies examining herbivory effects on plants consistently demonstrate that leaf damage reduces root growth rates and belowground plant biomass (Masters et al. 1993, Tindall and Stout 2001). If

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herbivores in a soil environment use root exudates as a cue to locate growing roots, a reliable food source, then reduced root growth may make plants less attractive (Bais et al. 2006).

In cases where the aboveground and belowground herbivores are different life stages of the same insect species, damage to one tissue type may be a reliable cue to the plant that the other tissue is likely to be attacked soon (Karban et al. 1999). For example, adults may feed on leaves and shoots before laying eggs that hatch into larval root feeders. Systemic induction would likely be adaptive for plants in this scenario because the aboveground damage would frequently indicate belowground damage in the near future. Conversely, damage by herbivores that feed on only one tissue may not induce defenses systemically if plants can distinguish and respond adaptively to different herbivore species (Delphia et al. 2007, Poelman et al. 2008, Ali and Agrawal 2012). However, from the insect perspective, it may be adaptive for herbivores that feed on both shoot and root tissue to suppress induction to maximize food quality for offspring. A meta-analysis found consistent negative effects of aboveground herbivory on root herbivore performance (Johnson et al. 2012), although only one of the included studies examined effects on a conspecific belowground herbivore (Clark et al. 2011). While this single study found a negative effect of leaf-feeding by adults on root-feeding larval development, supporting the idea that systemic plant responses are not species-specific, there have been no direct comparisons of aboveground conspecific and heterospecific effects on larval root preference or performance.

Here, we report on experiments in greenhouse and field settings to test the effects of leaf herbivory on root herbivores using *Cucumis sativus* L. (cucumber, Cucurbitaceae). Specifically, we asked: 1) does leaf herbivory affect root herbivore preference? 2) Do effects on root herbivores differ when leaf herbivory is by a conspecific or heterospecific? 3) Are responses of root herbivores related to defensive chemistry and root growth?

Methods

Study System. *C. sativus* is a widely cultivated vining plant that is attacked by aboveground and belowground herbivores, including both specialists and generalists. *C. sativus* and other members of Cucurbitaceae produce cucurbitacins, tetracyclic terpenoids, that act as defenses against generalist herbivores and are present in most tissues of the plants (Da Costa and Jones 1971, Gould 1978, Metcalf and Lampman 1989, Agrawal et al. 1999). The primary defensive compound of *C. sativus* is cucurbitacin C, an oxygenated tetracyclic triterpene that deters generalist herbivores (Metcalf 1992). An important herbivore of *C. sativus* throughout much of North America is *Acalymma vittatum* F. (Coleoptera: Chrysomelidae) (striped cucumber beetle), a specialist chewing herbivore that feeds on aboveground tissues (cotyledons, leaves, flowers, and

fruits) as an adult and on roots as a larva (Latin and Reed 1985). Adult female *A. vittatum* oviposit at the base of host plants, where larvae hatch and move into the soil (Necibi et al. 1992). Although Ellers-Kirk and Fleischer (2006) found that females laid ≤ 4 eggs per night in laboratory conditions, our field-collected beetles often laid >10 eggs per night (data not shown). Cucurbitacins are not effective defenses against *A. vittatum*, instead acting as kairomones that can attract beetles, which feed compulsively on cucurbitacin-containing tissues (Chambliss and Jones 1966, Metcalf and Lampman 1989, Deheer and Tallamy 1991). *A. vittatum* is an economically important pest of cucurbit crops, both for its direct herbivory effects and as a vector for *Erwinia tracheiphila*, a bacterium that causes bacterial wilt (Fleischer et al. 1999). The generalist herbivore *Spodoptera frugiperda* Smith (Lepidoptera: Noctuidae) also co-occurs in the study region (Hazzard et al. 2014), although *A. vittatum* is the most abundant herbivore on cucurbit crops, and other insect herbivores were nearly absent in previous studies at the field site used here (Barber et al. 2011, 2012, 2013).

Greenhouse Study. Experimental Design. To address questions 1 and 2, we manipulated leaf damage with *A. vittatum* and *S. frugiperda* and measured root herbivore preference. We germinated *C. sativus* seeds (Marketmore 76, Southern Exposure Seed Exchange, Mineral, VA) in Fafard Canadian Growing Mix No. 2 (Conrad Fafard Inc., Agawam, MA) in early July 2009, allowed them to grow for 3 wk under natural light, and transplanted them at the one-leaf stage into 3.79-liter pots with the same soil. Each pot contained three plants near the pot edge, arranged in a triangle, and we randomly assigned plants in each pot to one of the three treatments: conspecific leaf damage (adult *A. vittatum*), heterospecific leaf damage (*S. frugiperda* caterpillars), or undamaged control. There were 60 plants in total among 20 pots.

We collected adult *A. vittatum* from farms near Amherst, MA, and purchased third-instar *S. frugiperda* (Benzon Research Inc., Carlisle, PA). We applied treatments by placing a mesh bags over each of the first three fully expanded leaves on each plant as they reached full size, placing four insects in each bag. Leaves of control plants were contained in empty bags to control for bag effects. Leaf 1 treatments began on the day plants were transplanted ("day 1"), with all plants in a pot initiated on the same day; leaf 2 treatments began on days 2 and 3; and leaf 3 treatments began on days 4–7, depending on plant growth. We removed insects and bags after 1 wk or earlier if all leaf area was consumed, and plants were in the three- to five-leaf stage during treatments. There was an average of 30 and 37% area removed per leaf in *A. vittatum* and *S. frugiperda*-treated plants, respectively, calculated from visual damage estimates. These values are well within the range of damage experienced by young cucumber plants in the field, where >50% of leaf area can be removed from the first three leaves on young plants (Barber et al. 2012).

We applied *A. vittatum* eggs to all pots on day 3, while plants were being damaged by leaf herbivores. This simulates a typical field situation, where eggs are being oviposited at plant bases while adult beetles are still consuming leaves (Ellers-Kirk and Fleischer 2006). We obtained eggs by mating field-collected pairs of adult beetles in 30-ml plastic cups containing moistened Kimpack paper and cotyledons of winter squash (*Cucurbita maxima* Duchesne: Cucurbitaceae). We rinsed eggs from the Kimpack every 2 d and stored them on moistened filter paper in petri dishes at 11°C. We suspended eggs in agar solution and counted 10 10-ml samples to estimate egg density (mean 3.4 eggs per ml, range: 2.8–4.1). We applied 22 ml of agar containing ~75 eggs to the center of each pot, which is comparable with the number of eggs that may be deposited on a plant in a night (Necibi et al. 1992).

We expected larvae to take ~5 d for eclosion and an additional 15 d to reach the final instar before pupation (Ellers-Kirk and Fleischer 2006). On days 20–23 (17–20 d after egg application), we collected soil and roots by dividing the pot into thirds and placing each section into a Berlese funnel. Samples remained in funnels until 4 September, and we stored collected larvae in 90% ethyl alcohol for identification and counting.

Statistical Analysis. To analyze larval counts, we used a generalized linear mixed model, treating pot as a random effect. This allows comparisons between treatments to be performed within each pot, which accounts for pot-level variation in factors including egg hatching success or larval mortality among pots. We assumed a Poisson distribution and log link function, and fit individual-level random effects models to account for overdispersion. We evaluated treatment as a fixed factor using a likelihood ratio test as compared to a chi-square distribution. Because this test was significant (see Results), we performed two orthogonal contrasts to answer questions 1 and 2 using Wald z tests. The first compared undamaged plants to damaged plants (both damage treatments combined), and the second compared conspecific- and heterospecific-damaged plants. Here and below, analyses were carried out in R (R Development Core Team 2012) using the lme4 package (Bates et al. 2012).

Field Study. Experimental Design. We repeated the greenhouse experiment in a field setting and measured plant traits to assess whether root herbivore responses might be related to defensive chemistry or root growth. We germinated *C. sativus* seeds (Marketmore 76 untreated organic, Johnny's Selected Seeds, Winslow, ME) in organic potting soil (Fort Light organic compost-based potting soil, Vermont Compost Company Co., Montpelier, VT) in May 2010 in an organic greenhouse with natural light. After 21–22 d of growth, we transplanted 120 plants on 10 and 11 June (days 1 and 2) to a field at the University of Massachusetts–Amherst Crop Research and Education Center in South Deerfield, MA. The field has fine sandy loam soil and was planted with *Cucurbita moschata* Duschene ex Poirette (butternut squash, Cucurbitaceae) the previous year and with *Trifolium pratense* L.

(red clover, Fabaceae) for three consecutive prior years.

We planted *C. sativus* at the four- to six-leaf stage in pairs in 60 plastic compartments (50 by 19 cm, 12 cm in depth with bottoms removed) that were buried in the soil. The compartments provided room for plants to grow separately, so the roots from the two plants did not overlap, but both root masses were contained within a single compartment. We arranged plants in 10 rows of six compartments with 3 m spacing between all compartments.

We randomly assigned plants in each compartment to either damage or undamaged control treatments. Because the greenhouse experiment found no differences in effects of *A. vittatum* and *S. frugiperda* herbivory, we only used *A. vittatum* for the damage treatment. We collected *A. vittatum* for damage treatments and egg production as in the greenhouse experiment and applied damage in the same way except that we treated the first four fully expanded leaves because some plants lost their first leaf after transplanting. We initiated damage treatments on day 7 and treatments lasted until the first four leaves had expanded and been damaged. Thus, the end date of the damage period varied by plant, with the last treatments concluding by day 33. On average, each leaf was exposed to bagged beetles for 5.8 ± 0.2 d. Additional natural herbivory by wild herbivores was minimal because control leaves were enclosed in mesh bags during the treatments.

We added ~75 eggs to the center of each compartment using the same methods as in the greenhouse experiment. However, because of the compacted field soil, we applied eggs to a small hole dug ~10 cm in depth at the center of each compartment that was filled with moistened potting soil. This was intended to help eggs establish more easily and reduce the likelihood of desiccation. We added eggs between days 22 and 33, so leaf damage treatments and egg application overlapped, again simulating field conditions. We collected soil and root masses from each compartment between days 38 and 54 (16–21 d after applying eggs in each replicate) by dividing the compartment in half and removing all soil to a depth of 12 cm, which included the majority of each plant's root mass. We placed soil and roots in Berlese funnels for at least 15 d to collect *A. vittatum* larvae. Fifteen compartments were excluded from the study because one or both plants died.

To measure plant defense (cucurbitacin C content) and growth responses, we collected the four most recently fully expanded leaves from each plant at the time of soil collections. We collected and cleaned roots from Berlese funnels after larval collections were completed, and dried both root and leaf samples at 45°C. We weighed roots to determine belowground dry biomass. Cucurbitacins are heat resistant and remain stable at high temperatures (Fenwick et al. 1990). Adapting the methods of Gorski et al. (1986) and Balkema-Boomstra et al. (2003), we ground dried leaf and root samples, extracted 0.3 g of each sample in 3 ml of methanol, and sonicated for 10 min. After centrifugation, we distilled the supernatant 1:1 with deionized water and filtered it through a 0.45-μm nylon syringe

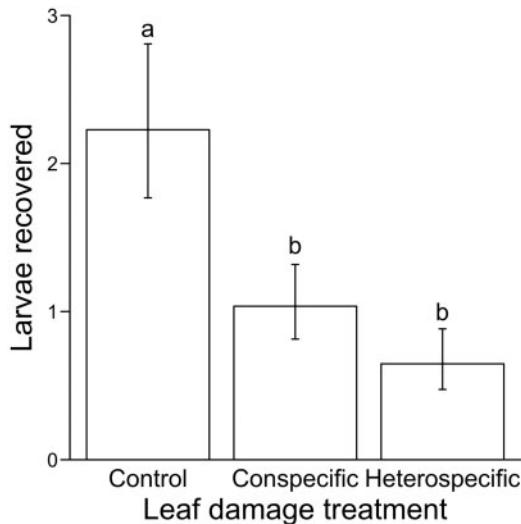


Fig. 1. Larvae recovered from roots of plants whose leaves were undamaged (control) or damaged by conspecific (*A. vittatum*) or heterospecific (*S. frugiperda*) herbivores in the greenhouse experiment. Values are means \pm 1 SE, and letters above bars indicate significant differences ($P < 0.05$).

filter. We analyzed content using high-performance liquid chromatography (HPLC; Perkin-Elmer: series 200, Shelton, CT) with a diode array detector at 254 nm, Altima C₁₈ column (250 by 4.6 mm, Grace Davison, Deerfield, IL), and a 55% acetonitrile/45% H₂O mobile phase at 1 ml/min. We identified the peak centered between 3.1 and 3.3 min as cucurbitacin C, based on Gorski et al. (1986), and used peak area per milligram dry sample as proxy for cucurbitacin C content because we did not have access to a cucurbitacin C standard.

Statistical Analysis. We used paired *t*-tests to analyze differences in larval abundance, leaf and root cucurbitacin C content, and root dry biomass between damaged and undamaged plants paired by compartment. As in the greenhouse experiment, paired comparisons account for variation in conditions among compartments. For larval abundance analyses, we excluded compartments from which no larvae were recovered.

Results

In the greenhouse experiment, leaf damage treatment significantly explained root larval abundance ($\chi^2_2 = 8.84$; $P = 0.012$; Fig. 1). There were significantly fewer larvae present on the roots of plants with damaged leaves than those with undamaged leaves (62% reduction, Wald $z = 2.934$; $P = 0.003$), but there was no difference between conspecific- and heterospecific-damaged plants (Wald $z = 0.916$; $P = 0.360$).

In the field experiment, there were again significantly fewer larvae present on the roots of plants with damaged leaves than undamaged leaves (89% reduction, $t_{18} = 2.144$; $P = 0.0459$; Fig. 2A). Leaf damage

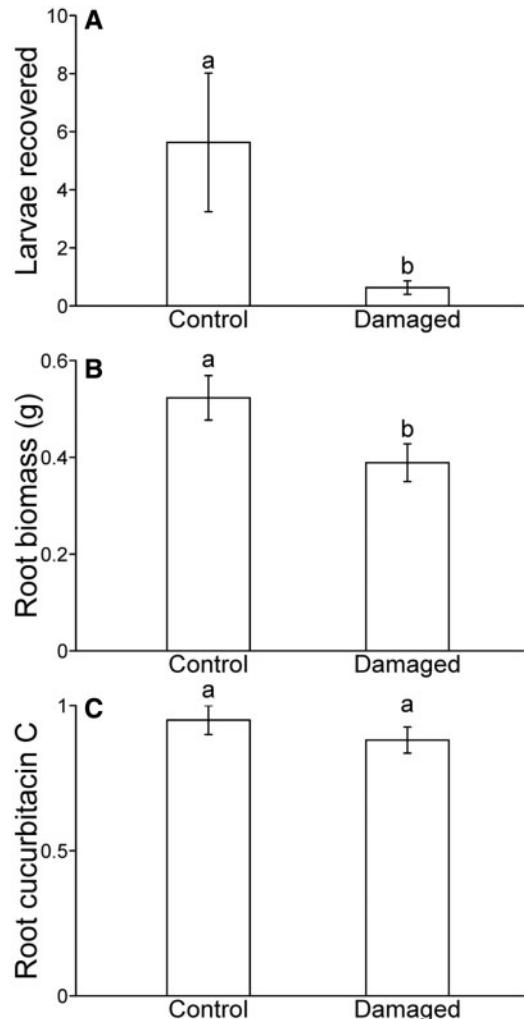


Fig. 2. Larval recovery, root biomass, and root cucurbitacin C levels of plants whose leaves were undamaged (control) or damaged by the conspecific herbivore (*A. vittatum*) in the field experiment. (A) Larvae recovered, (B) root dry biomass, and (C) relative cucurbitacin C content in root tissue of plants in field experiment. Values are means \pm 1 SE, and letters above bars indicate significant differences ($P < 0.05$). Cucurbitacin C values are unitless because they are relativized HPLC peak areas.

significantly reduced root dry biomass (26% reduction, $t_{31} = 2.876$; $P = 0.007$; Fig. 2B). Cucurbitacin C content did not differ between treatments in roots ($t_{25} = 1.197$; $P = 0.243$; Fig. 2B) or leaves ($t_{24} = 0.138$; $P = 0.892$).

Discussion

The aim of this study was to examine the effects of aboveground herbivory on belowground herbivores. We used leaf herbivore manipulations in the greenhouse and field to demonstrate that root herbivores preferred to feed on roots of undamaged plants. This pattern did not differ between conspecific- and

heterospecific-damaged plants. Although leaf herbivory manipulations significantly reduced belowground growth, they did not alter levels of a defensive chemical, cucurbitacin C, in roots or leaves.

In both greenhouse and field experiments, there were fewer *A. vittatum* larvae feeding on roots of damaged plants than undamaged plants. Because both experiments were set up as choice experiments, where larvae have the option of moving toward damaged or undamaged plants, this indicates greater preference for roots of plants without leaf herbivory. Our design mimics conditions in agricultural fields, where *C. sativus* plants are planted in close proximity, so that larvae can choose among adjacent plants. Larval recovery rates were generally low, as expected given the high egg and larval mortality rates observed in *A. vittatum*, particularly during summer heat (Ellers-Kirk and Fleischer 2006). Our ability to detect the same significant pattern across two separate experiments despite high larval mortality indicates the robustness of our results. The results are generally consistent with similar experiments (Johnson et al. 2012). In rice (Tindall and Stout 2001) and maize (Erb et al. 2011), root herbivores also had reduced preference for plants that suffered aboveground herbivory by *S. frugiperda*. Work in *Brassica nigra* L. (Brassicaceae) also found reduced root herbivore performance owing to leaf damage, either as reduced herbivore growth or increased mortality, which was attributed to the induction of secondary metabolites (Soler et al. 2007).

Root tissue damage may cause severe reductions in plant growth and fitness (Strong et al. 1995). Root damage creates water stress in plants (Blossey and Hunt-Joshi 2003), which may cause lodging, and the loss of root tissue usually results in a reduced ability by plants to obtain water and nutrients from soil (Brown and Gange 1990). In *C. sativus*, root damage reduced aboveground growth, flower and fruit production, fruit size, and seed production (Barber et al. 2011). If plants' responses to aboveground damage make them less preferred by or less susceptible to belowground herbivores, these responses may be adaptive. In the case of *C. sativus*, this may be beneficial to the plant given that feeding by *A. vittatum* aboveground is likely to be followed by root damage from larvae if adult females, which oviposit at the base of host plants (Necibi et al. 1992), lay eggs at the same plants on which they feed.

Although we predicted that effects of aboveground feeding would be transmitted across the soil surface in response to conspecific feeding (*A. vittatum*), but not heterospecific feeding (*S. frugiperda*), the effects of these two treatments on larval preference did not differ. That is, larvae avoided roots of all damaged plants in favor of undamaged plants, suggesting there were no herbivore-specific damage cues. Our treatment herbivores also represented a cucurbit specialist (*A. vittatum*) and a generalist (*S. frugiperda*), which may have different inductive effects on host plants. Specialists may suppress the induced response as compared with generalists (Sirvent et al. 2003, Voelckel and Baldwin 2004, Kaplan et al. 2008), although a recent review found that this pattern is inconsistent and may be

explained better by herbivores' feeding guilds (Ali and Agrawal 2012). Both of the aboveground herbivores in our greenhouse experiment are leaf-chewers, which tend to elicit stronger responses than phloem-feeding insects. Given the similarity of responses to both leaf damage treatments, the mechanism by which larval preference was reduced may be the same for both the conspecific specialist and the heterospecific generalist, and the response by *C. sativus* may be a general response to chewing damage. Future work addressing the potential role of specialist- versus generalist-induced effects will need to test multiple species in each diet breadth group while controlling for feeding guild (Ali and Agrawal 2012).

In the field experiment, we found no effect of leaf damage on root cucurbitacin C. While reduced larval abundance on damaged plants is consistent with a previous study in *C. sativus* (Barber et al. 2012), that study also found that leaf herbivory tended to reduce root cucurbitacin C. Because we did not measure root defenses in the absence of *A. vittatum* larvae, it is possible that root feeding increased cucurbitacin C levels and erased any reduction due to leaf damage. Despite the role of cucurbitacins as phagostimulants in the larvae of a related species (*Diabrotica undecimpunctata* L.; Deheer and Tallamy 1991), the effects of cucurbitacins on *A. vittatum* larvae are unknown. Systemic induction in roots in response to leaf herbivory is expected if leaf herbivory serves as a reliable cue for future herbivory belowground (Karban et al. 1999, Kaplan et al. 2008). If cucurbitacins are a phagostimulant for larval *A. vittatum*, as they are for adult *A. vittatum* and *D. undecimpunctata* larvae (Metcalfe et al. 1980, Deheer and Tallamy 1991), then suppressed or reduced induction may be an adaptive plant response. Although cucurbitacin C is the best studied defensive chemical in *C. sativus*, it is also possible that other defensive chemicals or physical defenses not considered in this study were induced, and are responsible for reduced larval preference on damaged plants.

Alternatively, root herbivore preference for undamaged plants could be explained by root biomass and nutrient content rather than defensive chemistry. Leaf herbivory often reduces root growth and biomass, especially when the damage is extensive (Masters and Brown 1992, Bardgett et al. 1998, Hunt-Joshi and Blossey 2005, Hummel et al. 2009). Root herbivory was reduced in this experiment, matching other studies of *C. sativus* (Barber et al. 2011, 2012) and a confamilial cucurbit (Hladun and Adler 2009). *A. vittatum* may seek more vigorously growing plants because their roots represent a more reliable food source than plants with reduced belowground growth (Clark et al. 2012) or because they have higher nutrient content. For example, *Centaurea maculosa* Lamarck (Asteraceae) allocates nitrogen to leaves in response to root damage (Newingham et al. 2007); the opposite effect could have occurred in our study. This explanation is also consistent with the similar effects of conspecific and heterospecific damage on root larval preference we have shown here, given that both herbivore species removed very similar amounts of leaf area on average

and so were likely to have similar impacts on root growth and nutrient status. A clear understanding of the mechanism driving larval host preference will require more detailed analyses of root growth and content of primary and secondary metabolites.

This study demonstrates indirect effects of herbivory transmitted through a shared host plant, complementing the growing body of research linking above- and belowground interactions (Bardgett and Wardle 2010). The response of belowground herbivores did not depend on the identity of the leaf herbivore, suggesting that the mechanism was the same for both conspecific and heterospecific herbivores. Although past research has focused on the role of induced secondary metabolites as mediators of indirect effects across the soil surface (Van Dam et al. 2004, Kaplan et al. 2008), the purported primary defensive chemical of *C. sativus* was unchanged in roots, and the preference of root feeders may be a result of differences in belowground plant growth.

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