

# Above-ground herbivory by red milkweed beetles facilitates above- and below-ground conspecific insects and reduces fruit production in common milkweed

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## Summary

1. Initial herbivory and induced plant responses can influence subsequent above- and below-ground herbivore attack. When two life stages of the same herbivore damage different plant parts sequentially, there is strong potential for plants to respond with induced plant defence against the later attacker. Alternatively, the earlier attacker could manipulate the host plant to facilitate the later-feeding life stage.

2. We studied herbivory by foliage-feeding adults and root-feeding larvae of the red milkweed beetle (*Tetraopes tetraophthalmus*) on native common milkweed (*Asclepias syriaca*) in laboratory and field experiments. We applied factorial above- and below-ground herbivory treatments to test for induced responses, effects on later-feeding conspecific larvae, and damage by naturally colonizing herbivores, including adult *T. tetraophthalmus*.

3. We found that the inducibility of toxic cardenolides was systemic across the root–shoot barrier, with the highest concentrations in plants damaged both above- and below-ground. Initial above-ground herbivory increased root damage and larval survival, suggesting an increase in root quality following leaf herbivory. Initial below-ground herbivory did not affect the performance of later-feeding larvae, indicating limited importance of induced root cardenolides and competition between clutches of *T. tetraophthalmus*.

4. In a natural milkweed population, initial above-ground herbivory attracted conspecific adults and milkweed leaf beetles (*Labidomera clivicollis*) and ultimately reduced fruit production by 33%. Nonetheless, the probability of damage by monarch caterpillars (*Danaus plexippus*) was reduced on plants initially damaged by *T. tetraophthalmus* above-ground, likely due to reduced oviposition following induced plant responses.

5. *Synthesis.* Induced plant responses of common milkweed to above-ground damage by adult *T. tetraophthalmus* both facilitate further damage by adults and enhance the performance of their root-feeding larvae, most likely as a result of host plant manipulation. Although the same induction reduced monarch herbivory, the net effect of these interactions was negative for the plant as fruit production was substantially reduced. These results imply that host plant manipulation may be especially common by specialist herbivores that have sequential above- and below-ground life stages.

**Key-words:** cardenolide, common milkweed *Asclepias syriaca*, entomopathogenic nematodes, induced defence, monarch butterfly *Danaus plexippus*, plant–insect interactions, red milkweed beetle *Tetraopes tetraophthalmus*

## Introduction

It is well understood that insect herbivory can systemically alter a plant's physiology across the root–shoot boundary, yet

early work on plant-mediated species interactions focused almost entirely on foliage-feeding species (Karban & Baldwin 1997). More recently, it has become evident that herbivores sharing the same host plant but utilizing different subsystems (i.e. roots vs. shoots) may be linked via plant-mediated induced responses (Masters & Brown 1992; Wardle *et al.*

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2004a; van der Putten *et al.* 2009; Soler, Erb & Kaplan 2012). Many of the studies investigating above- and below-ground induced plant responses have neglected the natural phenology of herbivores when choosing species to attack the different subsystems (Masters & Brown 1992; Hunt-Joshi, Blossey & Root 2004; Rasmann & Turlings 2007). Arbitrary pairs of insect species have proven useful for developing a mechanistic understanding of induced responses across subsystems; however, they may not reveal representative adaptive strategies shaping plant–herbivore interactions (Kaplan *et al.* 2008; Van Dam & Heil 2011; Johnson *et al.* 2012). In contrast, interactions between herbivores linked by a natural phenological progression are likely to be shaped by natural selection on traits of the interacting species and allow for clear, testable predictions. Few studies have specifically addressed plant responses to sequential herbivory following a natural phenological progression (Van Zandt & Agrawal 2004a; Viswanathan, Narwani & Thaler 2005; Clark, Hartley & Johnson 2011), and while these studies revealed differences in the plant response to early- and late-season herbivores, no general pattern for their causes and consequences has emerged so far.

As a special case of above- and below-ground insect interactions, a number of herbivorous species complete their life cycle on a single host plant, but use different subsystems at different points in their ontogeny, for example, adults feeding on leaves and larvae feeding on roots (Rasmann & Agrawal 2008; Clark, Hartley & Johnson 2011). For plants attacked by such herbivores, the location and timing of damage in the two subsystems are likely to be highly predictable, and thus a coordinated response should be advantageous for the plant (Karban, Agrawal & Mangel 1997). We predict that the early-feeding life stage of an herbivore should induce resistance in the plant part predictably attacked by the later-feeding life stage, resulting in benefits to the plant (Clark, Hartley & Johnson 2011). Such an effect could be driven by the induction of direct resistance traits (e.g. toxins, antinutritive compounds) or by indirect resistance traits that attract enemies of the later-feeding stage. Alternatively, the herbivore might manipulate the plant to increase the performance of its progeny, in which case damage by the early-feeding life stage should increase the quantity or quality of the plant tissue that will be eaten by the later-feeding life stage (i.e. induced susceptibility or facilitation). In either scenario, initial herbivory may also have community-wide effects on other plant-associated insects due to local and systemic plant responses (Van Zandt & Agrawal 2004a; Viswanathan, Narwani & Thaler 2005; Poelman *et al.* 2010).

The red milkweed beetle *Tetraopes tetraophthalmus* Foster (Cerambycidae) is a univoltine specialist herbivore on common milkweed *Asclepias syriaca* L. (Apocynaceae), a clonal, perennial plant native to eastern North America. Early in the season, adult beetles are present above-ground, with females laying multiple clutches in mid-summer. Neonates find their way into the soil and feed on milkweed roots and rhizomes, with larvae and adults co-occurring in the peak of the growing season (Agrawal 2004). In response to above- and

below-ground attack by the community of milkweed herbivores ( $\approx 10$  species; Matter 2001; Van Zandt & Agrawal 2004a; Rasmann *et al.* 2009a), common milkweed induces a well-characterized suite of defences (Agrawal 2005). Thus, the *Tetraopes*–milkweed system represents an ideal model to examine how plants respond to sequential herbivory by adults and larvae of the same insect species.

Using a series of manipulative experiments, we investigated the independent and interactive effects of above- and below-ground damage by *T. tetraophthalmus*. Specifically, we asked (i) how above- and below-ground herbivory affect cardenolide concentrations in shoot and root tissues, (ii) if above-ground herbivory by adults impacts below-ground herbivory by larvae and *vice versa*, and if above- and below-ground herbivory impact the growth and survival of later-feeding larvae, and (iii) if early-feeding above- and below-ground herbivory influences host plant use by later-feeding above-ground insects, soil-dwelling entomopathogenic nematodes (EPN, predators of beetle larvae) and plant performance.

## Materials and methods

### STUDY ORGANISMS

Common milkweed *A. syriaca* is found in disturbed areas and early successional habitats across eastern North America and has been introduced to south and central Europe. At our field site in central New York, USA, ramets typically emerge in late May and flower from mid-June through July. Common milkweed produces constitutive latex in shoots and numerous cardenolides in both shoots and roots; both traits can be induced upon damage by herbivores (Bingham & Agrawal 2010). Even though many specialist herbivores have evolved ways to cope with the otherwise toxic cardenolides in their diet (Agrawal *et al.* 2012a; Dobler *et al.* 2012), these adaptations are often dosage-dependent. Given that latex is absent from roots and Agrawal (2004) found no evidence for root-to-shoot induction of latex by root herbivory, we focus on cardenolides in the present study.

The red milkweed beetle *T. tetraophthalmus* is a monophagous herbivore of common milkweed. In central New York, USA, adult beetles emerge from the soil in late June and feed on flowers and foliage. Females oviposit clutches of 10–15 eggs in dry stems of grasses and forbs that are close to their food source (Gardiner 1961). *Tetraopes tetraophthalmus* dispersal is quite limited: previous work has demonstrated that individual adults may remain in a patch for several days under natural conditions (McCauley *et al.* 1981). Thus, females are likely to oviposit next to plants they choose for their own feeding. Larvae hatch after 6–10 days, drop to the ground, and begin feeding on common milkweed roots and rhizomes (Agrawal 2004). Because larvae are very small ( $< 0.25$  mg at first instar) and relatively immobile, we expect that most larvae feed on common milkweed plants that have been fed upon recently by adults. Adults and larvae typically co-occur on ramets from mid-July to late July. Previous observations have revealed that the root system of young *A. syriaca* grown in pots includes a persistent ‘main’ root (mean diameter  $4.5 \pm 1.1$  mm, usually 3rd order) and most below-ground herbivory by *T. tetraophthalmus* larvae occurs on this root type, rather than on ‘fine’ roots ( $0.5 \pm 0.25$  mm, 1st and 2nd order) (Erwin, Geber & Agrawal 2013).

In addition to *T. tetraophthalmus*, nine specialized herbivores of common milkweed are commonly observed at our field site: three

homopterans (*Aphis asclepiadis*, *A. nerii*, *Myzocallis asclepiadis*), two lepidopterans (*Danaus plexippus*, *Euchaetes egle*), two hemipterans (*Lygaeus kalmii*, *Oncopeltus fasciatus*), a coleopteran (*Labidomera clivicollis*), and a dipteran (*Liriomyza asclepiadis*).

#### INITIAL HERBIVORY TREATMENTS

All experiments utilized the same  $2 \times 2$  factorial design. Following an initial treatment of above- and/or below-ground herbivory, subsequent changes in the system were measured. Treatments consisted of an undamaged control (Con), *T. tetraophthalmus* adults added to impose above-ground herbivory (Abv), *T. tetraophthalmus* larvae added to impose below-ground herbivory (Blw), and both adults and larvae added to impose both types of herbivory (Abv + Blw). Adult *T. tetraophthalmus* were added to mesh bags enclosing whole plants; adults were thus able to move and feed freely, but not to leave the plant. An adult feeding event usually targets the tip of young leaves and results in a fairly consistent amount of leaf area removed ( $1.1 \pm 0.16 \text{ cm}^2$  per feeding event,  $n = 15$ ). The amount of initial above-ground herbivory was controlled by removing adults after they had eaten  $\sim 10\%$  leaf area (=6–9 leaf tips). Initial below-ground herbivory was imposed by digging 1-cm deep holes evenly spaced on a circle (5 cm diameter) around the plant stem and placing a single first-instar *T. tetraophthalmus* larva in each hole. We were unable to directly control the amount of below-ground herbivory; instead, we adjusted the number of larvae and time of exposure based on damage levels measured in a previous experiment (Erwin, Geber & Agrawal 2013) to achieve  $\sim 10\%$  of root damage. These levels of above- and below-ground herbivory are within the range commonly observed in the field.

Treatment damage was imposed by wild-caught adults and laboratory-reared larvae. Adults were collected from patches next to our field site and kept in ventilated containers (30 cm  $\times$  20 cm  $\times$  15 cm) under natural light at ambient humidity. Adults were provided with common milkweed leaves for food and dry grass stems as oviposition substrate. The oviposition substrate was removed every 2 days and incubated in the dark at 30 °C for 7–10 days (Rasmann *et al.* 2009a). First-instar larvae were kept without food on moist filter paper in petri dishes (10 cm diameter) for < 24 h before being transferred to experimental plants. Natural densities of milkweed beetles in the field are variable, but can be quite high (e.g. > 5 adult beetles  $\text{m}^{-2}$  in a milkweed patch, personal observations); as such, both our adult and larval treatments fall well within the range of naturally imposed attack.

#### EXPERIMENT 1: LOCAL AND SYSTEMIC CARDENOLIDE INDUCTION AND INSECT PERFORMANCE

To test for impacts of initial above- and below-ground herbivory on inducibility of cardenolides, interactions between above- and below-ground herbivory, and larval performance, in May 2009 plants were grown using seeds collected from 10 patches occurring in a single old-field in Tompkins Co., New York, USA. Seeds were cold stratified at 4 °C on moist filter paper for a week, scarified and germinated in the dark at 26 °C. Seedlings were then planted in commercial potting soil (Metro-Mix; Sun Gro Horticulture, Bellevue, WA, USA) in plastic pots (10 cm diameter) and grown in a growth chamber (12:12 D/N light, 26:20 °C D/N temperature). After 2 months, plants were moved to larger (3.8 L) plastic pots and placed outside for 2 years. In May 2011, plants of each family that were  $\geq 10$  cm tall were individually enclosed in spun polyester mesh bags to prevent natural colonization by other insects. Plants were watered as needed throughout the season.

In June 2011, plants were assigned to one of two groups, and factorial above- and below-ground herbivory treatments were applied to both groups. Using the first group of 56 plants (14 plants/treatment), cardenolide induction was measured in shoots and roots. To impose the below-ground herbivory treatment, five larvae were applied per plant. Two days after the introduction of larvae, one adult was applied per plant in the above-ground herbivory treatment, on average achieving  $\sim 10\%$  damage after  $6 \pm 0.5$  days, at which point each adult was removed. Ten days after the introduction of larvae, all plants were harvested. Below-ground material was washed in water and separated from soil. All plant material was oven-dried at 40 °C for 3 days, separated into shoots, main roots and fine roots and then ground to powder on a Wiley Mill (Thomas Scientific, Swedesboro, NJ, USA). Shoot and main root cardenolide concentrations were analysed via HPLC using 100 mg powder of each tissue (Rasmann *et al.* 2011). The concentration of cardenolides in fine roots was not analysed because *T. tetraophthalmus* do not cause substantial damage to this tissue (Erwin, Geber & Agrawal 2013).

In the second group of 80 plants (20 plants per treatment), we tested for interactions between above- and below-ground herbivory and conducted a bioassay to test for treatment effects on the survival and mass of later-feeding larvae. Treatments were the same as above, except that two (rather than five) larvae were applied per plant in the below-ground herbivory treatment to limit the impact on plant health. In this group, we did not control for the exact amount of leaf damage, but instead let treatment adults feed for 6 days to impose an equal treatment duration to all plants. Eight days after applying the treatment larvae (=6 days after applying treatment adults), we removed the bags and adults, moved all plants into a large mesh cage and applied 10 freshly hatched bioassay larvae to each plant. Bioassay larvae were added the same way as treatment larvae. Plants were allowed to grow for 10 more days and then were harvested. At harvest, we measured several traits (total ramet height, total number of leaves, number of damaged leaves, total length of the main root, damaged length of the main root and main root mass) as well as individual larval survival and mass. Two-way ANOVAs were used to test for the effects of initial above- and below-ground herbivory on plant traits and insect survival.

To test for direct interactions between initial above- and below-ground herbivory, we assessed the total vs. damaged tissue in each subsystem. Specifically, we counted the total and damaged numbers of leaves to estimate above-ground herbivory. We then separated above- and below-ground tissues, washed roots and separated main and fine roots. The total main root length and length of main root damage were measured to estimate below-ground herbivory following the methods in the study of Erwin, Geber & Agrawal (2013). Surviving *T. tetraophthalmus* larvae were counted, weighed and assigned to either the treatment or bioassay group (Fig. S1 in Supporting Information). Unrecovered larvae were presumed dead. We analysed the effect of below-ground larvae on the number of leaves damaged in the Abv vs. Abv + Blw treatments using ANCOVA, including total leaf number as a covariate. Correspondingly, we evaluated whether the effect of above-ground adults altered the length of main root damaged in the Blw vs. Abv + Blw treatments, including the total length of the main root as a covariate.

#### EXPERIMENT 2: LATER-SEASON ABOVE- AND BELOW-GROUND IMPACTS

To test for the impacts of above- and below-ground herbivory on colonization by the natural herbivore community, EPN, and plant per-

formance, we selected 20 patches of wild milkweed in the same field where seeds had been collected for Experiment 1. Patches were considered to be separate clones based on the proximity and density of the ramets as well as several morphological traits (A. Erwin, unpubl. data). In June 2011, we selected four similar, undamaged ramets in each patch and enclosed each ramet in a mesh bag to prevent damage by local herbivores. Selected ramets were  $\geq 5$  m apart, 100- to 120-cm tall and had between 3–5 umbels. We randomly assigned treatments to the four plants in each patch. Treatments were the same as above, except that we used 2–4 adults and 20 larvae because plants were quite large. In the above-ground herbivory treatment, applying 2–4 adults achieved our target damage level of  $\sim 10\%$  after  $2.5 \pm 0.5$  days, after which adults were removed. To simulate the herbivore's natural phenology, larvae were applied to each plant in two cohorts (10 larvae per 'clutch'), separated by 10 days (Fig. S2). First observations were made 10 days after the second cohort of larvae had been applied and mesh bags had been removed (Fig. S2).

Starting on 21 July 2011 (=21 days after the first application of larvae), we began weekly surveys of plants for leaf number, leaf damage and the abundance of all insect herbivores present on each plant. We were able to differentiate among leaf damage caused by *T. tetraophthalmus*, *L. clivicollis*, *E. egle*, *D. plexippus* and *L. asclepiadis* because these herbivores cause easily recognizable species-specific patterns of leaf damage. However, damage by *E. egle* and *L. asclepiadis* was too sparse for a meaningful analysis and therefore is not reported. Surveys were ended after 8 weeks, when plants began to senesce and insect populations declined. In early October, when fruits had fully matured, we counted the number of all fruits on each plant as an estimate of female sexual reproduction.

We estimated the leaf area consumed on experimental plants by generating a conversion relationship from discrete feeding bouts by the different major herbivores and the leaf area removed. Specifically, we collected and scanned leaves from plants growing next to our field site that had naturally received only one type of damage ( $n = 20$  leaves per damage type per species) using a Licor LI-3100 area metre. Scanned leaves were used to generate mean areas per damage type (*T. tetraophthalmus* tip: 1.08 cm<sup>2</sup>; *T. tetraophthalmus* side: 8.99 cm<sup>2</sup>; *D. plexippus* centre: 0.78 cm<sup>2</sup>; *D. plexippus* edge: 12.32 cm<sup>2</sup>), and weekly damage counts from the field were multiplied by these means.

To investigate whether above- and below-ground herbivory and plant patch affected the abundance of EPN, an agent of indirect plant defence, we buried cages containing EPN sentinel larvae in the field as described in Ali *et al.* (2012). Each cylindrical cage (7 cm length  $\times$  3 cm diameter) was filled with autoclaved sand (10% moisture) plus one late instar larva of the greater wax moth *Galleria mellonella* L. (Pyralidae) (GrubCo<sup>®</sup>, Fairfield, OH, USA). One cage was buried 15 cm below the base of each experimental plant in 12 of the 20 patches used in this experiment, resulting in 12 replicate cages per treatment. After 4 days, we recovered cages, rinsed larvae and placed them on moistened filter paper in individual Petri dishes. We confirmed EPN infection (by infective juvenile emergence) and recorded EPN-inflicted larval mortality 0–48 h after removal from soil. We used chi-square tests of independence to examine the effects of above- and below-ground herbivory and plant patch (block) on *G. mellonella* mortality.

All other analyses of the field experiment were fit using the statistical program R (R Core Team 2013) and the packages *nlme* 3.1-104 and *lme4* 0.999999-0 (Bates *et al.* 2012; Pinheiro *et al.* 2012). Damage by the different herbivore species was analysed using a set of mixed-effects models with identical structure. Plant identity was treated

as random effect to account for repeated measures over 8 weeks and was nested within patch. In each full model, above-ground herbivory, below-ground herbivory, week, and all interactions were fitted as fixed effects, and leaf number was fitted as a covariate. The *lme4* package used for non-normal data does not provide *F*-tests (Gelman & Hill 2007). Therefore, we followed a model simplification approach to identify the most parsimonious model for each herbivore by comparing nested models using Akaike's information criterion (AIC). For each herbivore species, we selected the model with the lowest AIC, favouring simpler models when pairs of nested models were tied with AICs within two units per difference in the number of parameters. To test for significance of treatment differences, we constructed 95% confidence intervals (CIs) for parameter estimates (Gelman & Hill 2007). We present these parameter estimates with their 95% CIs and an approximate *P*-value, based on resampling of the posterior distribution (Gelman & Hill 2007).

Within the mixed-model framework, we selected the models most appropriate for the types of data. Damage caused by *T. tetraophthalmus* and *D. plexippus* was analysed in two steps: first, we analysed probability (presence or absence) of damage using models with a binomial error structure (function *glmer* in *lme4*), and second, we removed all zeros and analysed the log-transformed data using Gaussian models (function *lme* in *nlme*). The number of damage marks caused by *L. clivicollis* was analysed using a model with a Poisson error structure (function *glmer* in *lme4*), which is appropriate for count data.

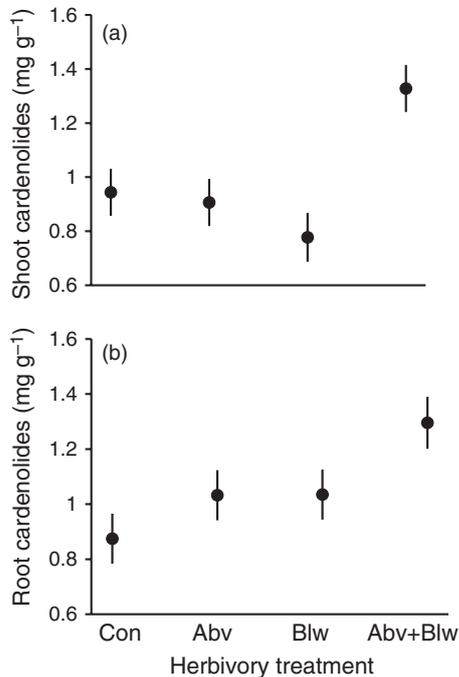
## Results

### EXPERIMENT 1: LOCAL AND SYSTEMIC INDUCED RESPONSES

Plants damaged both above- and below-ground showed the strongest increase in shoot cardenolides, producing 40% more cardenolides compared to controls (Fig. 1a). Interestingly, both single damage treatments had little to no effect, and thus this increase resulted from a significant interaction term (Abv:  $F_{1, 51} = 8.51$ ,  $P = 0.005$ ; Blw:  $F_{1, 51} = 2.10$ ,  $P = 0.154$ ; Abv  $\times$  Blw:  $F_{1, 51} = 11.192$ ,  $P = 0.002$ ); note that the significant main effect of above-ground herbivory is not interpretable because of the significant interaction term (see Fig. 1a). Above- and below-ground herbivory each increased root cardenolides by 18% compared to controls, and their effects were additive in the dual-damage treatment (Fig. 1b, Abv:  $F_{1, 51} = 5.19$ ,  $P = 0.027$ ; Blw:  $F_{1, 51} = 5.32$ ,  $P = 0.025$ ; Abv  $\times$  Blw:  $F_{1, 51} = 0.32$ ,  $P = 0.579$ ).

### EXPERIMENT 1: INDIVIDUAL PLANT AND INSECT PERFORMANCE

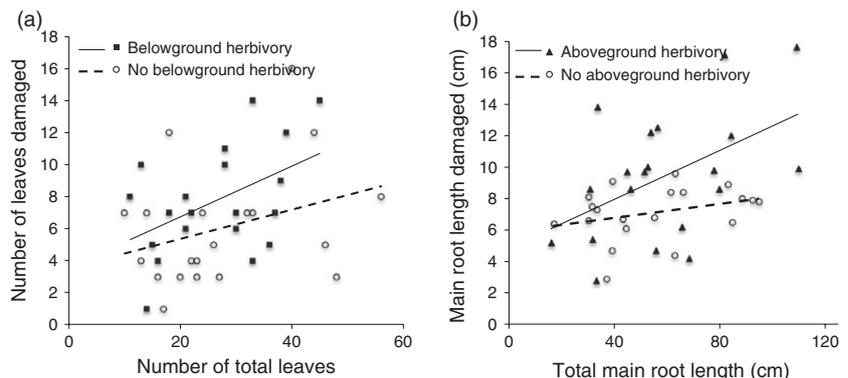
Initial herbivory treatments did not significantly impact total ramet height (full model,  $F_{1, 75} = 0.94$ ,  $P = 0.428$ ), but did reduce below-ground root biomass (full model,  $F_{1, 73} = 3.62$ ,  $P = 0.017$ ). Below-ground herbivory reduced main root mass by 31% (Blw:  $F_{1, 73} = 4.51$ ,  $P = 0.037$ ), and main root mass also tended to be lower with above-ground herbivory, although this effect was marginal (Abv:  $F_{1, 73} = 2.94$ ,  $P = 0.091$ ; Abv  $\times$  Blw:  $F_{1, 73} = 3.43$ ,  $P = 0.068$ ).



**Fig. 1.** Impact of initial herbivory to *Asclepias syriaca* by adult *Tetraopes tetraophthalmus* (above-ground, Abv) and their larvae (below-ground, Blw) on the mean  $\pm$  1 SE concentration of cardenolides in (a) shoots and (b) main roots. 'Con' indicates undamaged controls.

Comparing plants in the Abv and the Abv + Blw treatment only, we found weak evidence for a facilitative effect of below-ground herbivory on damage by adults ( $F_{1, 36} = 3.03$ ,  $P = 0.090$ ; Fig. 2a). Plants with more leaves suffered more damage (Tot. lvs:  $F_{1, 36} = 6.66$ ,  $P = 0.014$ ; Fig. 2a), but this effect was independent of the herbivory treatment (Tot. lvs  $\times$  Blw:  $F_{1, 36} = 0.48$ ,  $P = 0.492$ ). Comparing plants in the Blw and the Abv + Blw treatments, above-ground herbivory by adults facilitated below-ground damage by larvae ( $F_{1, 36} = 5.64$ ,  $P = 0.023$ ; Fig. 2b), and plants with longer roots also suffered more damage (Tot. length:  $F_{1, 36} = 7.34$ ,  $P = 0.010$ ; Fig. 2b), but again, this latter effect was independent of the herbivory treatment (Tot. length  $\times$  Abv:  $F_{1, 36} = 2.27$ ,  $P = 0.141$ ).

**Fig. 2.** (a) Impact of below-ground herbivory to *Asclepias syriaca* roots by larvae of *Tetraopes tetraophthalmus* on the number of leaves damaged by later-feeding conspecific adults in relation to the total number of leaves. (b) Impact of above-ground herbivory to *A. syriaca* leaves by *T. tetraophthalmus* adults on damage to main roots by later-feeding conspecific larvae in relation to main root total length.

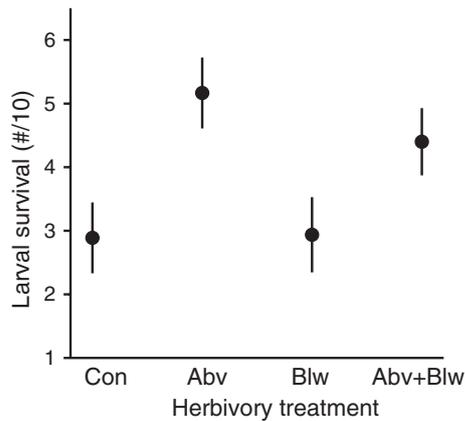


We recovered 26.3% of the treatment larvae and 35.0% of the bioassay larvae; the two groups could be easily distinguished (Fig. S1). These recovery rates are similar to or higher than those reported in other studies of this system (Rasmann & Agrawal 2011; Rasmann *et al.* 2011; Erwin, Geber & Agrawal 2013). Above-ground herbivory did not impact the survival of treatment larvae (Abv:  $F_{1, 17} = 0.05$ ,  $P = 0.822$ ), yet the survival of bioassay larvae was 64.2% higher on plants that had been previously damaged above-ground; previous below-ground damage had no effect (Abv:  $F_{1, 68} = 11.20$ ,  $P = 0.001$ ; Blw:  $F_{1, 68} = 0.41$ ,  $P = 0.523$ ; Abv  $\times$  Blw:  $F_{1, 68} = 0.53$ ,  $P = 0.468$ ; Fig. 3). Treatments had no impact on the mass of the surviving larvae (Whole model:  $F_{1, 65} = 1.76$ ,  $P = 0.164$ ).

#### EXPERIMENT 2: LATER-SEASON ABOVE-GROUND IMPACTS

Above-ground herbivory by *T. tetraophthalmus* adults affected subsequent damage by several naturally colonizing insect species (Table S1). Damaged plants were 19.8% more likely to be damaged further by colonizing *T. tetraophthalmus* adults (parameter estimate for the treatment effect relative to control,  $\beta_{\text{Abv}} = +8.01$ , CI = 2.77–13.15,  $P = 0.002$ ; Fig. 4a). Essentially all plants with previous adult damage received more adult damage (Fig. 4a). In the subset of plants that had some later-season leaf damage (excluding damage caused by treatment), initial above-ground herbivory was associated with 125% more damage by *T. tetraophthalmus* adults (Abv:  $F_{1, 50} = 106.95$ ,  $P < 0.0001$ ; Fig. S3). Plants exposed to initial above-ground herbivory by *T. tetraophthalmus* also had 80.4% more leaf damage by *L. clivicollis* than plants that were previously undamaged above-ground ( $\beta_{\text{Abv}} = +0.59$ , CI = 0.23–0.91,  $P = 0.004$ ; Fig. 4b, Table S1).

In contrast, plants that were initially damaged above-ground by *T. tetraophthalmus* adults were 41.6% less likely to receive subsequent leaf damage by *D. plexippus* caterpillars during the first half of the experiment (weeks 1–4,  $\beta_{\text{Abv}} = -1.13$ , CI = -1.93 to -0.32,  $P = 0.006$ ; Fig. 4). In the second half of the experiment (weeks 5–8), 100% of plants received some level of damage by *D. plexippus*, but there was no effect of treatment on the amount of leaf damage (Abv:  $F_{1, 46} = 0.41$ ,  $P = 0.527$ ; Fig. S3). Overall, there



**Fig. 3.** Impact of initial herbivory to *Asclepias syriaca* plants by adult *Tetraopes tetraophthalmus* (above-ground, Abv) and their larvae (below-ground, Blw) on mean  $\pm$  1 SE survival of conspecific bioassay larvae feeding on roots. 'Con' indicates undamaged controls.

was no indication of an effect of initial below-ground herbivory by *T. tetraophthalmus* larvae on later-season leaf damage, and no support for treatment interactions (Table S1).

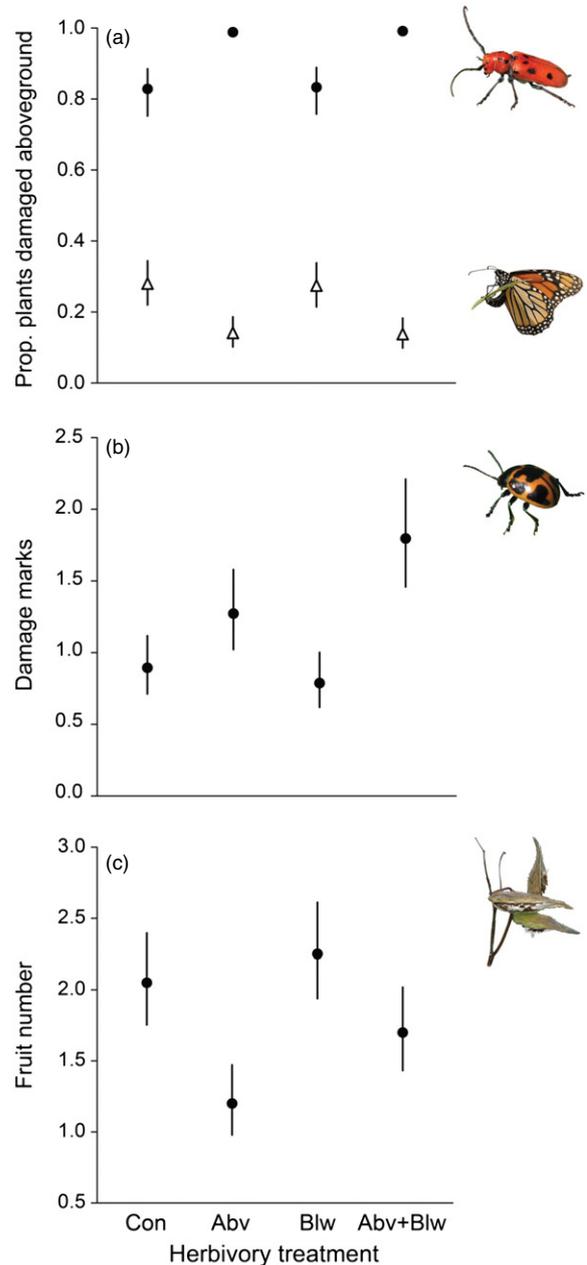
We observed individuals of *T. tetraophthalmus*, *E. egle*, *D. plexippus*, *L. asclepiadis*, *L. kalmii*, *O. fasciatus*, *A. nerii*, *A. asclepiadis* and *M. asclepiadis* (but never *L. clivicollis*) during the growing season. Of the herbivores that were present in sufficient numbers to be analysed, none deviated from a random distribution among treatments (*L. kalmii*:  $\chi^2 = 2.28$ , d.f. = 3,  $P = 0.513$ ; *T. tetraophthalmus*:  $\chi^2 = 4.41$ , d.f. = 3,  $P = 0.220$ ; pooled aphid numbers:  $\chi^2 = 3.471$ , d.f. = 3,  $P = 0.325$ ; *E. egle*:  $\chi^2 = 0.769$ , d.f. = 3,  $P = 0.857$ ; *L. asclepiadis*:  $\chi^2 = 4.12$ , d.f. = 3,  $P = 0.249$ ).

We tested for differences in the mortality of *G. mellonella*, a sentinel for EPN in the soil. We recovered 100% of the 48 *G. mellonella* sentinels we added (1 per plant), and 42% were infected by EPN. Infection was caused exclusively by *Steinernema carpocapsae*, a species native to the USA. Infection resulted in variable mortality among treatments (Con: 33%; Abv: 58%; Blw: 33%; Abv + Blw: 42%), but these rates were not significantly different ( $\chi^2 = 2.057$ , d.f. = 3,  $P = 0.56$ ). Nonetheless, sentinel mortality marginally differed among plant patches ( $\chi^2 = 18.706$ , d.f. = 11,  $P = 0.067$ ), indicating spatial variation in EPN within the field.

The net effects of above- and below-ground herbivory were negligible on leaf number (Abv:  $F_{1, 57} = 0.05$ ,  $P = 0.812$ ; Blw:  $F_{1, 57} = 1.01$ ,  $P = 0.319$ ; Abv  $\times$  Blw:  $F_{1, 57} = 1.03$ ,  $P = 0.315$ ). Nonetheless, fruit production was reduced 32.5% in the above-ground herbivory treatment (Abv: Deviance = 5.48, d.f. = 76,  $P = 0.019$ ; Fig. 4c) and was unaffected by below-ground herbivory (Blw: Deviance = 1.36, d.f. = 76,  $P = 0.243$ ; Abv  $\times$  Blw: Deviance = 0.56, d.f. = 76,  $P = 0.456$ ).

## Discussion

We demonstrated that above- and below-ground herbivory by different life stages of the same insect can have distinct



**Fig. 4.** (a) Impact of initial herbivory to *Asclepias syriaca* plants by adult *Tetraopes tetraophthalmus* (above-ground, Abv) and their larvae (below-ground, Blw) on the probability of a plant to be subsequently damaged above-ground by naturally colonizing adult *T. tetraophthalmus* (black circles) and *Danaus plexippus* caterpillars (white triangles). Values are back-transformed whole-season means  $\pm$  1 SE. 'Con' indicates undamaged controls. (b) Average number of damage marks caused by *Labidomera clivicollis*. Values are back-transformed means  $\pm$  1 SE for weeks 5–8 after initial damage by *T. tetraophthalmus*. Despite marginally higher damage in the combined Abv + Blw treatment, there was no support for an interaction term with below-ground herbivory (Table S1). (c) Impact of initial herbivory treatments on the number of fruits produced by *A. syriaca*. Values are back-transformed means  $\pm$  1 SE.

consequences for induced plant responses. Specifically, damage by early-feeding above-ground adult *T. tetraophthalmus* facilitated the performance of the later-feeding below-ground

larvae of the same species, altered host plant use by other species, and the net effect of these interactions decreased *A. syriaca*'s reproductive output in the field. Early-season below-ground herbivory did not influence damage by, or the survival of, later-feeding larvae despite induction of cardenolides in roots. These results suggest that the observed interactions between *A. syriaca* and *T. tetraophthalmus* might not be mediated by induction of cardenolides, but rather that *T. tetraophthalmus* is able to manipulate other host plant traits to maximize its own fitness.

For the plant responses we measured (i.e. cardenolides in shoots and roots), both were at the highest concentrations in plants damaged both above- and below-ground. While shoot cardenolides appeared to be unaffected by herbivory to the roots alone, root cardenolides were substantially induced by herbivory to the shoot and to a comparable level as initial root herbivory. There is mixed evidence in the literature for systemic effects of herbivory in one subsystem on induction in the other. For example, Ali, Alborn and Stelinski (2011) found that above-ground beetle damage by *Diaprepes abbreviatus* (L.) adults failed to induce indirect below-ground defence cues that were induced by root-feeding larvae of the same species. In *Brassica nigra*, below-ground feeding by *Delia radicum* larvae induced glucosinolates locally in roots as well as systemically in shoots (van Dam & Raaijmakers 2006). Based on a meta-analysis, Kaplan *et al.* (2008) concluded that foliar herbivory generates strong above-ground induced responses, but much weaker below-ground responses; conversely, root herbivory tends to elicit responses in leaves and roots of roughly equal magnitude. These conclusions were largely based on studies of above- and below-ground herbivory by different insect species and are in stark contrast to our own findings. Further work is required to address whether the differential patterns we observed will be general for herbivores with above- and below-ground life stages.

#### ABOVE- AND BELOW-GROUND INTERACTIONS BETWEEN LIFE STAGES OF A SINGLE INSECT SPECIES

Above-ground herbivory is known to affect soil biota via plant changes (Bardgett & Wardle 2010) and can vary among both plant and herbivore species (Wardle *et al.* 2004b). Although a classic conceptual model (Masters, Brown & Gange 1993) proposed negative effects of above-ground herbivores on the performance of below-ground herbivores, evidence for this pattern has been mixed (Johnson *et al.* 2012). Our bioassay and field experiments showed that initial above-ground herbivory by *T. tetraophthalmus* adults had positive effects on below-ground larvae, indicating induced susceptibility (i.e. increased root damage and individual survival). Some plants respond to herbivory by altering growth patterns to compensate for damage (Orians *et al.* 2011). In the present experiment, we speculate that facilitation of *T. tetraophthalmus* larvae by initial above-ground herbivory was achieved via increased root quality (nutrition) rather than quantity (biomass) because we observed a marginal decrease in main root biomass following above-ground herbivory. Induced resource

sequestration has been proposed to denote the process of herbivores inducing plant signals that result in increased concentrations of primary metabolites in storage tissues (Orians *et al.* 2011). Enhanced carbon or nitrogen storage may be beneficial to plants under some conditions, and thus, adult *T. tetraophthalmus* may be hijacking this response to facilitate root-feeding of their larvae. A recent study of short-term allocation in *A. syriaca* revealed that following 2 days of above-ground herbivory by monarch caterpillars or below-ground herbivory by *T. tetraophthalmus* larvae, plants immediately increased nitrogen allocation to stem tissues (Tao & Hunter 2013). It is yet unclear whether adult *T. tetraophthalmus* feeding causes the same response, and whether this reallocation would continue or change in the subsequent days. Nonetheless, shunting of resources may also have contributed to the dramatic reduction in fruit production of plants receiving relatively minimal above-ground damage.

Masters, Brown and Gange (1993) initially predicted positive effects of below-ground herbivores on the performance of above-ground herbivores, but most subsequent tests of their predictions found evidence for the opposite relationship, especially for chewing herbivores (Kaplan *et al.* 2008; Johnson *et al.* 2012). In contrast, the results of our bioassay experiment would support the prediction of Masters, Brown and Gange (1993): leaf damage by *T. tetraophthalmus* adults was higher on plants exposed to initial below-ground herbivory by larvae, and this was coincident with a trend for lower shoot cardenolides. Other studies have demonstrated the same pattern for inter-specific species pairs (Gange & Brown 1989; Masters, Jones & Rogers 2001; Poveda *et al.* 2004), although it is certainly not universal (Moran & Whitham 1990; Salt, Fenwick & Whittaker 1996; Bezemer *et al.* 2003). Surprisingly, these same patterns did not hold in our field experiment: subsequent leaf damage by *T. tetraophthalmus* adults was found to be independent of initial below-ground herbivory. Weak or undetectable effects of initial below-ground herbivory on subsequent leaf damage in the field may reflect a greater ability of established field plants to tolerate attack. We note, however, that we did not measure larval survival or root damage in the field, and the intensity of our below-ground herbivory treatment may have been less than intended due to predation or poor establishment of larvae.

#### EFFECTS ON PLANT PERFORMANCE

We found that typical levels of above-ground herbivory ( $\leq 10\%$ ) decreased fruit production, a component of plant fitness. Common milkweed's fitness is determined by investment in both vegetative propagation and sexual reproduction, typically over many years. Thus, the potential impact of reduced fruit production for the long-term persistence of genets and populations may be modest. Nonetheless, the reduction in fruit production may shape future interactions between shoots and *T. tetraophthalmus* adults if it translates to altered future investment in sexual reproduction and, specifically, the availability of flowers, which are important attractants for adults (Reigel, Ginzel & Hanks 2002; Agrawal 2004).

Correspondingly, the reduction in fruit production could potentially influence future root–larvae interactions if plants reallocate resources away from sexual reproduction above-ground and towards below-ground storage and/or vegetative propagation (Tao & Hunter 2013).

#### SPECIES-SPECIFIC IMPACTS ON COMMUNITY MEMBERS

Our experiment on naturally occurring field plants showed that the community-wide effects of early-season herbivory by *T. tetraophthalmus* foliar-feeding adults were stronger than those of root-feeding larvae and affected subsequent leaf damage. Specifically, we showed that the effects of early-season above-ground herbivory facilitated subsequent damage by *T. tetraophthalmus*. Although we did not determine the sex of adult beetles, sex-specific behaviour of adults provides a clear hypothesis for why above-ground damaged plants received more damage: males prefer to feed on leaves with latex deactivation by females, which provides latex-free meals without requisite trenching (Gontijo 2013). Such feeding may then lead to the retention of beetles on plants and a greater number of leaves being eaten. Above-ground herbivory facilitated subsequent damage by another beetle, *L. clivicollis*, that might benefit from the same latex deactivation caused by *T. tetraophthalmus*. In a previous experiment, monarch damage also induced susceptibility (increased attack) to *T. tetraophthalmus* adults (Van Zandt & Agrawal 2004a). Thus, deactivated laticifers and associated herbivory may be a general cue for *T. tetraophthalmus* in mate finding and feeding (Gontijo 2013).

In contrast to the attractant effect on conspecifics, we found that above-ground herbivory by *T. tetraophthalmus* adults decreased the probability of subsequent damage by *D. plexippus* larvae. Thus, the plant-mediated interactions between monarchs and *T. tetraophthalmus* are highly asymmetrical. Within the subset of plants that received some *D. plexippus* damage, the total amount of damaged area was not affected by *T. tetraophthalmus* herbivory. This result appears to be a consequence of avoidance of host plants with previous leaf damage by ovipositing adult *D. plexippus* butterflies. The strength of this discriminant behaviour is most likely mediated by the abundance of undamaged host plants: in the second half of the field experiment, the discriminant behaviour completely disappeared as monarch populations dramatically increased (Fig. S3). Indeed, *D. plexippus* appears to be quite sensitive to prior damage, as damage by various chewing herbivores has been repeatedly shown to substantially decrease the growth of larvae feeding later on the same host plants (Van Zandt & Agrawal 2004a,b; Agrawal 2005; Rasmann, Johnson & Agrawal 2009b; Agrawal *et al.* 2012b). Initial above- and below-ground herbivory did not impact subsequent damage by several other species we surveyed (i.e. three aphids, leaf miners, two species of seed predators), although these were generally too rare for our results to be conclusive.

The only naturally occurring organisms we surveyed in soil were EPN. Although we did not quantify the number of EPN

attracted to specific treatments, our previous work showed that root herbivory induced volatile organic compounds in the roots that attract EPN in the laboratory and field (Rasmann *et al.* 2011). Nonetheless, we found that the mortality of sentinel larvae by EPN was independent of treatment in the current experiment. The lack of an effect of early-season below-ground herbivory could have been due to EPN-inflicted mortality of our *T. tetraophthalmus* larvae used to impose the treatment. We detected a marginal difference in sentinel mortality among patches, which is an indication of the patchy distribution typical of EPN populations (Stuart & Gaugler 1994). Future work should address the survival and persistence of early-season *T. tetraophthalmus* feeding in the field and its ecological impacts through above- and below-ground induced responses.

#### Conclusions and speculation

Although induced defences are widely acknowledged to be effective and adaptive plant responses to herbivory, we found that induced responses of *A. syriaca* to early-feeding above-ground herbivory by *T. tetraophthalmus* adults caused increased preference and performance of their own species, increased damage by another beetle species and reduced fruit production, with only a modest repellent effect against a third herbivore. The strongest evidence for induced plant resistance in the milkweed system comes from work on monarchs, with damage by various species reducing the performance of subsequently feeding caterpillars (Van Zandt & Agrawal 2004b; Agrawal *et al.* 2012b). Nonetheless, for *T. tetraophthalmus*, adults appear to manipulate the plant to increase the performance of their root-feeding larvae (induced susceptibility), a response that should be maladaptive for the plant. We expect that ‘runaway consumption’ induced by adult *T. tetraophthalmus* feeding is unlikely in the field, as there are trade-offs associated with resistance to monarchs, and high beetle densities on roots may also result in other ecological effects, for example, attraction of natural enemies and other induced plant changes.

Based on our findings, we speculate that distinct patterns (facilitation vs. resistance) of above- below-ground induced responses may emerge from intra-specific comparisons, where different life stages of the same herbivore attack different plant subsystems, compared to inter-specific comparisons. We encourage tests of the effects of the natural timing of damage, that is, how initial above-ground, initial below-ground or simultaneous attack affect the strength and direction of above-below-ground interactions (Erb *et al.* 2011; Johnson *et al.* 2012). Specifically, we expect it to be revealing whether a short lapse between damage types (as is the natural pattern for many univoltine insect species) has different impacts on plant and insect performance than a long lag (as is common among species with long larval stages). Such an approach would contribute to a broader understanding of when and how plant-mediated interactions between above- and below-ground insects shape community dynamics.

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## Data accessibility

Raw data are available from the factorial manipulations of above- and below-ground herbivory by the red milkweed beetle on root and shoot chemistry, beetle performance, insect abundances in the field, leaf damage in the field and fruit production in the Dryad Digital Repository (Erwin *et al.* 2014).

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## Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Histograms of individual larval masses (Experiment 1), separated into treatment (upper panel) and bioassay (lower panel) larvae.

**Figure S2.** Timeline of the field experiment (Experiment 2).

**Figure S3.** Amount of damage caused by *Tetraopes tetraophthalmus* (a) and *Danaus plexippus* (b) throughout the field season.

**Table S1.** Results of model selection using Akaike's information criterion (AIC) to identify terms with a significant effect on later-season leaf damage to *Asclepias syriaca* field plants for models with non-normal data.