

Specific impacts of two root herbivores and soil nutrients on plant performance and insect–insect interactions

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Soil-dwelling insects commonly co-occur and feed simultaneously on belowground plant parts, yet patterns of damage and consequences for plant and insect performance remain poorly characterized. We tested how two species of root-feeding insects affect the performance of a perennial plant and the mass and survival of both conspecific and hetero-specific insects. Because root damage is expected to impair roots' ability to take up nutrients, we also evaluated how soil fertility alters belowground plant–insect and insect–insect interactions. Specifically, we grew common milkweed *Asclepias syriaca* in low or high nutrient soil and added seven densities of milkweed beetles *Tetraopes tetraophthalmus*, wireworms (mainly *Hypnoides abbreviatus*), or both species. The location and severity of root damage was species-specific: *Tetraopes* caused 59% more damage to main roots than wireworms, and wireworms caused almost seven times more damage to fine roots than *Tetraopes*. *Tetraopes* damage decreased shoot, main root and fine root biomass, however substantial damage by wireworms did not decrease any component of plant biomass. With the addition of soil nutrients, main root biomass increased three times more, and fine root biomass increased five times more when wireworms were present than when *Tetraopes* were present. We detected an interactive effect of insect identity and nutrient availability on insect mass. Under high nutrients, wireworm mass decreased 19% overall and was unaffected by the presence of *Tetraopes*. In contrast, *Tetraopes* mass increased 114% overall and was significantly higher when wireworms were also present. Survival of wireworms decreased in the presence of *Tetraopes*, and both species' survival was negatively correlated with conspecific density. We conclude that insect identity, density and soil nutrients are important in mediating the patterns and consequences of root damage, and suggest that these factors may account for some of the contradictory plant responses to belowground herbivory reported in the literature.

Decades of research have yielded a broad understanding of interactions between plant shoots and aboveground insect herbivores. In contrast, our understanding of belowground herbivory remains limited, despite calls for greater attention to the impacts of root-feeding insects (Brown and Gange 1990, Blossey and Hunt-Joshi 2003, Rasmann and Agrawal 2008, Erb et al. 2012) and evidence that the effects of belowground herbivores are fundamentally different, and sometimes more detrimental, than the effects of aboveground herbivores (reviewed by Brown and Gange 1990, Zvereva and Kozlov 2011). Because belowground plant parts – including rhizomes, storage organs, vascular roots and root buds – constitute 50–90% of total plant biomass (Andersen 1987, Bazzaz et al. 1987), belowground herbivory can have important effects that extend well beyond the scope of tissue damage (reviewed by Zvereva and Kozlov 2011, Johnson et al. 2012). These include effects on individual plant chemistry, biomass and survival (Reichman and Smith 1991, Maron 1998, Rasmann et al. 2009, Coverdale et al. 2012), interactions with other trophic levels both above and belowground (Wäckers and Bezemer 2003, Rasmann et al. 2011), and plant distribution and

community structure (Brown and Gange 1989, Stein et al. 2010, Coverdale et al. 2012).

A recent meta-analysis confirmed that belowground herbivory causes negative impacts on root biomass and associated changes in plant performance; however, the magnitude of these effects depend on the plant part, morphological trait and function in question (Zvereva and Kozlov 2011). The impacts of belowground herbivory on plant performance have also been shown to vary with plant nutritional status (Brown and Gange 1990, Johnson et al. 2009), plant defenses (Bezemer and van Dam 2005, Rasmann and Agrawal 2008, van Dam 2009), root feeder identity (Müller 1989, Steinger and Müller-Schärer 1992, Würst and Van der Putten 2007), and root feeder density (Masters 1995, Strong et al. 1995, Onstad et al. 2006, Simelane 2010). Despite these foundational studies, questions about context-dependency in belowground plant–insect interactions remain.

Here, we report on an experiment using the perennial plant common milkweed *Asclepias syriaca*, (Asclepidaceae) and two types of root-feeding insects that naturally co-occur within its root system (Erwin unpubl.): generalist wireworms

(Elateridae) and specialist red milkweed beetles *Tetraopes tetraophthalmus* (Cerambycidae). By adding these insects at seven densities to plants growing in either low or high nutrient soil, we were able to ask the following questions: 1) what is the location and extent of root damage caused by each insect, and are these patterns influenced by insect density and soil nutrients? 2) How does damage influence plant root and shoot biomass? 3) How do conspecific and heterospecific interactions between insects affect their individual mass and survival, and are these interactions altered by soil nutrients?

In a previous study (Erwin unpubl.), we grew common milkweed from seed, transplanted seedlings to the field and added five red milkweed beetle larvae to half of the plants. During the harvest of this experiment, we observed that wireworms had naturally recruited to many plants' root systems (Erwin unpubl.). Although we did not test for whether wireworm abundance depended on the presence of *T. tetraophthalmus* larvae, their natural co-occurrence motivated us to investigate the ecology of these root-feeders in the milkweed rhizosphere.

In the present study, we predicted that 1) wireworms would predominantly damage fine roots, given their putatively higher nitrogen concentration (Gordon and Jackson 2000) and lower defenses (Griffith 1974, van Dam 2009); 2) *T. tetraophthalmus* would predominantly damage main roots, based on previous observations of their boring habit (Williams 1941, Chemsak 1963, Rasmann et al. 2011); 3) high soil nutrients would increase the plants' ability to compensate for damage, and this effect would vary by plant part and insect species. Finally, we predicted that 4) the performance of the generalist, omnivorous wireworms would decrease with intraspecific competition and increase with the presence of *T. tetraophthalmus*, a potential prey species. In contrast, we expected that performance of the specialist herbivore *T. tetraophthalmus* would not be negatively density-dependent (because eggs are laid in clutches and so larvae may be adapted to intraspecific competition), but would decrease in the presence of wireworms, which are potential predators. Despite expectations that insect identity, density, and soil nutrients are major factors shaping patterns of belowground herbivory, we currently have a limited understanding of their independent and interactive effects (Zvereva and Kozlov 2011).

Material and methods

Study system

Common milkweed *Asclepias syriaca* is a perennial herb found in disturbed areas and early successional habitats across eastern North America (Wyatt 1996). In central New York, USA, ramets emerge in late May and flower from mid-June through July. Although ramets can produce multiple fruits that each contain up to ~200 seeds, reproduction is primarily asexual and occurs belowground via the production of new ramets (Woodson 1954). The root system of *A. syriaca* seedlings consists of a single 'main' root (mean diam. 4.5 ± 1.1 mm, usually third order) as well as numerous very 'fine' roots (mean diam. 0.5 ± 0.25 mm, first and

second order) (Erwin unpubl.). From visual inspection and in comparison to other species (Pregitzer et al. 2002, Guo et al. 2008), it is clear that the fine root system of young common milkweed plants is dominated by few, long, relatively unbranched individual roots rather than an intricately branched system of many short roots (Erwin unpubl.). Other belowground structures exist within milkweed root systems – including adventitious root buds and, in mature field plants, lateral branch roots and a rhizome – but these were not considered in the present study.

The red milkweed beetle *Tetraopes tetraophthalmus* Forster is a monophagous herbivore of *A. syriaca*. Adults emerge from the soil in late June and begin to feed on milkweed flowers and foliage (Matter 2001). Females oviposit clutches (10–15 eggs each) in dry stems of nearby grasses and forbs (Gardiner 1961, Agrawal 2004). Larvae hatch after approximately 6–10 days, drop to the ground, and begin to feed on milkweed roots and rhizomes (Agrawal 2004). Larvae overwinter in earthen cells as prepupae (Williams 1941, Chemsak 1963).

Wireworms (the juvenile stage of click beetles) are a species group of generalist omnivores that feeds on soil-dwelling insects, soil organic matter as well as the belowground parts of living plants from several botanical families, including weeds and crops (Griffith 1974). Wireworm neonates fail to survive in soils that do not contain plant roots or storage organs (Rawlins 1940). Second- and third- instar larvae are fairly tolerant of starvation, however growth slows and mortality is high when no belowground plant material is available for food (Rawlins 1940). Unlike univoltine *T. tetraophthalmus*, wireworms persist in the soil as larvae for 2–7 years, moving up and down in the soil profile as food availability, temperature and soil moisture change (Penev 1992, Benerfer et al. 2012). Adults emerge in late spring and remain active aboveground throughout the summer. A female adult may lay from 50 to 300 eggs, singly or in small clutches, 2.5–15 cm deep in the soil of grassy fields (Andrews et al. 2008, Kuhar and Alvarez 2008). An early survey of agricultural fields demonstrated that average abundance increases from 60 000 wireworms per acre in 1–3 year old grasslands to 230 000 wireworms per acre in grasslands older than 10 years (Fox 1961). Because of their long life cycle, larvae may persist in former agricultural areas (old fields), where common milkweed is typically found.

Plant and insect collection

In September 2009, we collected *A. syriaca* seeds from 40 discrete patches around Ithaca, NY, USA. In May 2010, we nicked each seed coat with a sterile blade, stratified the seeds on moist filter paper at 4°C for one week, and germinated them in a dark chamber at 28°C for several days (Rasmann et al. 2009).

Tetraopes tetraophthalmus adults were collected in June 2010 on naturally occurring *A. syriaca* patches around Tompkins County, NY, USA and kept in ventilated rearing boxes (30 × 20 × 15 cm) in the laboratory. Males and females were provided with fresh milkweed leaves and flowers for food, and dry grass stems as oviposition substrate (Rasmann et al. 2009). The substrate was removed from the rearing containers every third day and incubated in the dark at 27°C

for one week (Rasmann et al. 2011). Newly hatched larvae were kept without food in petri dishes (10 cm diam.) on moist filter paper for 24 h before being added to the roots of experimental plants (Rasmann et al. 2011). When added, individual *T. tetraophthalmus* fresh mass was on average 0.133 ± 0.005 mg ($n = 20$).

Wireworms were collected in June 2010 from soil below recently overturned sod in Steuben County, NY, USA. Larvae were kept at room temperature in ventilated containers ($30 \times 20 \times 15$ cm) filled with local field soil and placed in a dark area of the laboratory. Every third day, we moistened the soil and provided larvae with fresh potato pieces for food. Larvae were kept in this condition without food for 24 h before being added to experimental plants. When added, individual larvae had a mean fresh mass of 17.574 ± 1.440 mg ($n = 25$). A subsample of the wireworms used in our experiments was identified at the Cornell University Insect Diagnostic Laboratory, indicating that our collection was comprised of two generalist omnivores: *Hypnoides abbreviatus* (83%) and *Agriotes mancus* (17%).

Experimental set-up and data collection

Because of the relatively small spatial scale (< 1 m³) of rhizosphere dynamics (Van der Putten et al. 2001, Phillips et al. 2009), root–insect interactions can be studied semi-realistically in mesocosms. Here, we used plastic pots in a growth chamber. We lined each pot (10 cm diam.) with a layer of nutrient- and water-permeable fabric to discourage insects from exiting via pot drainage holes. We filled the pots with an equal volume of a 1:2 mixture of sand and a growing mix recommended for seed germination that included *Sphagnum* peat moss, perlite, gypsum and dolomitic limestone, but no nutrient charge. We incorporated sand to improve pot drainage and facilitate fine root recovery. We individually transplanted seeds into pots in a growth chamber (14:10 h day/night light and 26:16°C temperature) and watered ad libitum every other day. The seedlings grew slowly because micronutrients were not present in the soil mix. After two weeks, we completely randomized the plants and initiated the nutrient treatments. Plants in the high nutrient treatment received inorganic fertilizer (N:P:K 21:5:20 150 ppm N (g g^{-1}), Supplementary material Appendix A1 Table A7) once every week and plants in the low nutrient treatment received the same fertilizer once every other week. We chose these treatments based on a pilot study in which seedlings were grown under one of six fertilizer treatments that span the range of soil fertility found locally. After four weeks, stem height and leaf number differed between the two nutrient levels that we selected for the present study (stem height low: 17.80 ± 4.01 cm, high: 24.61 ± 4.97 cm; leaf number low: 5.87 ± 0.73 , high: 8.41 ± 1.69).

After initiating the nutrient treatments, we allowed the plants to grow for an additional four weeks. At the six-week mark, plants received one of three insect treatments: 1) *T. tetraophthalmus*, 2) wireworms, or 3) both. The insect treatments were divided by density level such that plants with either single-insect treatment received 0, 1, 2, 3, 4, 5 or 6 larvae, and plants with the dual-insect treatment received 0, 1, 2 or 3 larvae of each insect (and thus had 0, 2, 4 or 6 total larvae). We chose to standardize insect treatments

according to density, acknowledging that this did not result in treatments with comparable mass. Densities fall within the range we observed in 2010 in a local field (Erwin unpubl.). With seven replicates per density treatment combination, this design yielded $n = 240$ plants. To apply the insect(s), we made a small hole (2.5 cm away from the plant stem, 1 cm deep) in the soil mixture with the end of a paintbrush, into which we gently placed the larva(e). Plants and insects were left to grow for an additional four weeks.

At harvest, we carefully loosened the soil mixture and collected surviving insects by hand. Insects were brushed to remove soil, counted, and weighed. Because initial weight was very consistent within a species (Methods) we did not weigh each individual larva before adding it to an experimental pot. Consequently, instead of calculating insect growth as final weight minus initial weight, we calculated growth on a per pot basis as the total mass of surviving larvae divided by the number of surviving larvae. This metric allows us to test for any effects of our treatments on mass without potentially confounding mass with survival. We calculated insect survival as the number of surviving larvae of a given species re-collected from a pot relative to the number that we added initially.

We then washed the roots and separated the main root from the fine roots using a sterile blade. We quantified main root length with a ruler. Damage to the main root (Fig. 1A) was visible to the unaided eye and characterized by direct consumption in the form of bore marks, i.e. ‘tunnels’ through an outer ‘shell’ of main root. Damage to all fine roots (Fig. 1B) was also readily apparent and characterized by the presence of black necrotic tissue at the site of feeding. The ends of some fine roots were probably consumed (as suggested by flat, rather than pointed, fine root tips), however the lengths of these consumed tissues were impossible to quantify because the tip had been removed and an outer ‘shell’ was not present. To the extent that this type of consumption occurred, our index of fine root damage is conservative. Our approach to quantifying damage – measuring individual sections of damaged tissue and summing over the entire root system – is considerably more detailed than other methods that are qualitative or based on a visual scale (Spike and Tollefson 1988, Agrawal 2004, Simelane 2010, Rasmann and Agrawal 2011, but see Oleson et al. 2005).

Since we did not recover a main root from some plants that received 4, 5 or 6 larvae, we did not measure damage to this root type. However, we did recover ‘orphaned’ fine roots (those no longer attached to the main root, but not yet decomposed) from these plants. Therefore, we are confident that the main root was absent due to consumption, rather than decomposition, because fine roots generally have higher decomposition rates than larger diameter roots. Therefore, we calculated the mean main root length for each treatment and substituted these values (Supplementary material Appendix A1 Table A1) where data were missing. We assumed that main root length equaled main root damage because the entire main root was consumed. We performed analyses with and without the substituted data and found that the direction and magnitude of the overall patterns and all statistical results remained unchanged. After quantifying root damage, all root and shoot material was oven-dried at 40°C for three days and weighed.

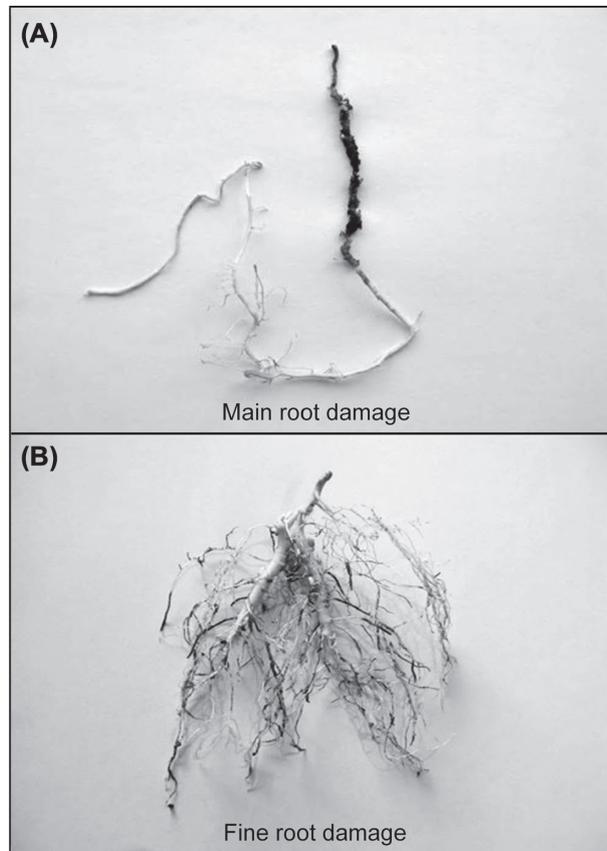


Figure 1. (A) A washed *Asclepias syriaca* root system in which three *T. tetraophthalmus* larvae had been feeding for four weeks. Fine roots, which had no herbivore damage, were removed to better show damage to the main root. Damage to the main root was measured as the length of the single section of black necrosis. (B) A washed *Asclepias syriaca* root system in which three wireworm larvae had been feeding for four weeks. Damage to fine roots was measured as the sum of the length of all necrotic sections ($n = 43$).

Statistical analyses

We performed analysis of covariance (ANCOVA) in JMP (ver. 8. SAS Inst.) to test for the fixed main effects of insect identity (discrete, factor), insect density (continuous, co-factor), and soil nutrient level (discrete, factor) and their interactions on damage to main and fine roots. Because we were interested in examining the full range of insect density (i.e. 1, 2, 3, 4, 5, 6), we excluded data from the dual-insect treatment because it lacked data at density levels 1, 3 and 5. We excluded the controls (density 0) because no damage was observed on their roots. We employed the same model to test for the effects on shoot, main root, and fine root biomass except that, here, we included the controls to determine potential change from an undamaged baseline. Data were log-transformed prior to the biomass analysis to meet assumptions of normality and homogeneity of variance.

To analyze insect mass, we separated the overall dataset (including all density levels except controls) into two subsets: 1) wireworm mass when alone and in the presence of *T. tetraophthalmus*, and 2) *T. tetraophthalmus* mass when alone and in the presence of wireworms. For each subset, we performed ANCOVA to determine how insect mass was

affected by insect identity, insect density, soil nutrient level, and their interactions.

To analyze insect survival, we again separated the overall dataset into two subsets. Based on previous work in this system (Agrawal 2004, Rasmann et al. 2009, 2011), we generated a priori a set of nine candidate models that represent survival as a function of the main effects and/or interaction terms. We used generalized linear modeling to fit our data to these models according to a binomial error distribution with logit as link function (Quinn and Keough 2002). Our data conformed to variance assumptions based on binomial distributions (wireworm data subset: $c = 1.07$; *T. tetraophthalmus* data subset: $c = 0.99$; Burnham and Anderson 2002). We evaluated the fit of each candidate model using the Akaike's information criterion corrected for small sample sizes, AICc (Akaike 1973), and associated statistics: delta AICc (Δ_i), Akaike weight (w_i), and log likelihood values with standard cutoffs (Burnham and Anderson 2002). We also calculated model-averaged coefficients and unconditional confidence intervals (CI) based on Akaike weights for each parameter. Finally, we calculated relative importance (RI) values to estimate the relative importance of each parameter in each data subset. RI values are calculated as the sum of all weights from models that include that parameter; the higher the RI value, the more important that parameter is relative to others (Burnham and Anderson 2002). Survival analyses were conducted in R ver. 2.13.0.

Note that in our analyses of plant traits, the insect identity term refers to the difference between the effects of wireworms and *T. tetraophthalmus* and so a significant identity-by-density interaction would indicate that the effect of increasing density on plant damage or biomass depended on 'which insect' was present. In contrast, in our analyses of insect traits, the insect identity term refers to the presence of the other insect on the performance of the focal insect. In these cases, a significant identity-by-density interaction would indicate that the effect of increasing density on a focal insect's mass or survival depended on 'whether heterospecifics' were present, e.g. wireworms alone versus wireworms in the presence of *T. tetraophthalmus*.

Results

The damage to *Asclepias syriaca* main roots (Fig. 1A) depended on insect identity and density (Table 1). Averaged across all density and nutrient levels (but not including undamaged controls), *Tetraopes tetraophthalmus* damaged 6.31 ± 0.63 cm of each plant's main root, which is 59% more damage than wireworms caused (Supplementary material Appendix A1 Table A2). Damage to main roots was positively correlated with overall insect density, and increased with increasing density of *T. tetraophthalmus*, but not wireworms (Fig. 2). Under high soil nutrients, mean damage to main roots by *T. tetraophthalmus* increased by 44% and by wireworms by 2% (Supplementary material Appendix A1 Table A2), but neither the effect of nutrients nor the insect identity-by-nutrients interaction were significant (Table 1). Damage to main roots increased with insect density under high, but not low soil nutrients, as shown by a significant density-by-nutrients interaction (Table 1).

Table 1. Analysis of covariance (ANCOVA) of insect identity, insect density, soil nutrient level and their interactions as predictors of *Asclepias syriaca* main root (MR) and fine root (FR) damage (cm), shoot (SH), MR and FR biomass (mg), and mass of wireworm and *Tetraopes tetraophthalmus* larvae (mg). Numbers in parentheses below column headers refer to DF. Numbers in table are F-values with asterisks indicating significant differences (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). For plant damage and biomass, the Identity term refers to which species was present. For insect mass, however, the Identity term refers to whether heterospecifics were present.

Factor	Plant damage		Plant biomass			Insect mass	
	MR (1, 145)	FR (1, 151)	SH (1, 178)	MR (1, 179)	FR (1, 179)	Wireworms (1, 63)	<i>T. tetraophthalmus</i> (1, 37)
Identity	7.14**	28.18***	35.43***	276.31***	74.00***	0.29	0.77
Density	10.35**	19.55***	5.93*	57.58***	21.91***	10.04**	0.88
Nutrients	1.80	2.15	130.27***	6.46*	16.45***	4.93*	12.05**
Identity × Density	8.80**	18.97***	1.54	56.93***	11.40***	0.44	0.51
Identity × Nutrients	1.54	1.07	2.25	4.15*	8.20***	1.07	7.22*
Density × Nutrients	3.97*	1.14	1.60	0.74	0.12	2.07	0.47
Identity × Density × Nutrients	0.12	1.57	0.64	0.50	1.03	0.85	0.56

Like damage to main roots, damage to fine roots (Fig. 1B) was affected by insect identity and density (Table 1). Averaged across all density and nutrient levels (excluding controls), wireworms damaged 3.75 ± 0.43 cm of each plant's fine roots, which is almost seven times more damage than *T. tetraophthalmus* caused (Supplementary material Appendix A1 Table A2), and this difference was especially large at high insect densities (Fig. 2). Damage to fine roots was 54% higher under high nutrients overall (Supplementary material Appendix A1 Table A2), but neither the effect of nutrients nor the interactions of other factors with nutrients were significant (Table 1).

Insect identity and density also influenced final above and belowground plant biomass (Table 1). Averaged across all density and nutrient levels (including controls), mean main root biomass was 180% lower when *T. tetraophthalmus* were present than when wireworms were present (Supplementary material Appendix A1 Table A2). This result is consistent with the insect-specific patterns of main root damage, noted above. *Tetraopes tetraophthalmus* caused more modest, but still highly significant decreases in mean fine root and shoot biomass (36% and 15%, respectively) compared to wireworms (Table 1, Supplementary material Appendix A1 Table A2). All components of plant biomass were affected by insect density (Table 1), but reductions

in biomass were most pronounced in fine roots and especially main roots exposed to *T. tetraophthalmus* (Fig. 3). Indeed, when we excluded the *T. tetraophthalmus* data and again tested for the effects of density, nutrients, and their interaction on plant biomass, we found that only nutrients remained significant (Supplementary material Appendix A1 Table A4). This result indicates that the significant effects of density, identity-by-density, and identity-by-nutrients in the full model (Table 1) are driven by the overwhelming influence of *T. tetraophthalmus*.

The biomass of all plant parts was influenced by soil nutrient level (Table 1), but increases were more pronounced in shoots (a 30% mean increase overall) than in either root type (a 16% mean increase in both main and fine roots overall, Supplementary material Appendix A1 Table A2). Soil nutrient level affected aboveground plant biomass, regardless of which insect was added (Table 1). In plants without insects (i.e. controls) and plants exposed to wireworms or *T. tetraophthalmus*, shoot biomass increased 39%, 33% and 29%, respectively (Supplementary material Appendix A1 Fig. A1A). However, the pattern for belowground biomass was insect-specific, as indicated by a significant identity-by-nutrients interaction (Table 1). In plants exposed to wireworms, the mean biomass of main roots and fine roots increased under high nutrients by 20% and

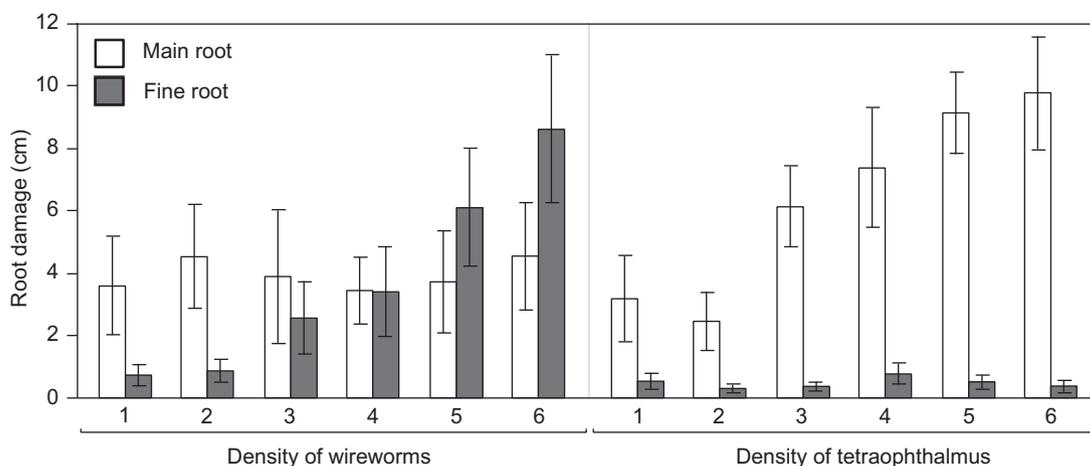


Figure 2. Mean \pm SE damage to the main and fine roots of *Asclepias syriaca* fed upon by different densities (1 to 6 larvae per plant) of wireworms or *Tetraopes tetraophthalmus*. Control (0 larvae) plants were excluded because no damage was observed on their roots.

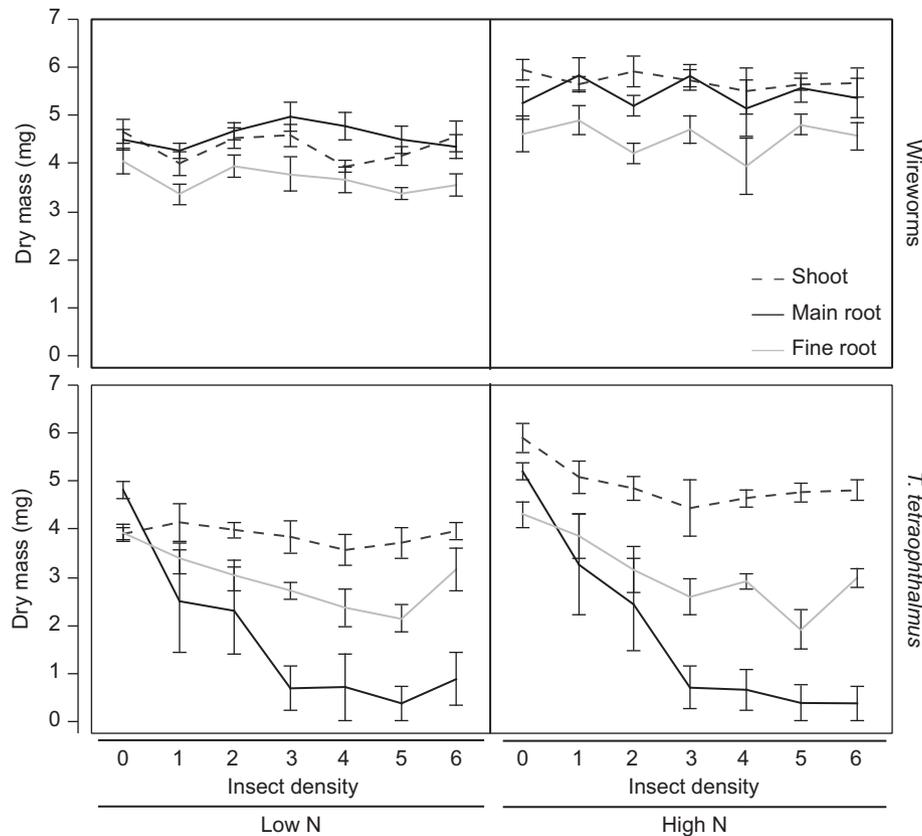


Figure 3. Mean \pm SE shoot, main root, and fine root dry mass of *Asclepias syriaca* plants fed upon by different densities of wireworms or *Tetraopes tetraophthalmus* larvae and grown in either low or high nutrient soil.

26% respectively (Supplementary material Appendix A1 Fig. A1B–C), whereas in plants exposed to *T. tetraophthalmus*, increases in mean main root and fine root biomass were much smaller (5% and 4%, respectively) and not significant (Supplementary material Appendix A1 Fig. A1B–C). In plants without insects, we did not detect significant increases in belowground biomass in response to added nutrients, however belowground biomass was generally higher than when *T. tetraophthalmus* were present (Supplementary material Appendix A1 Fig. A1B–C). That plants are less able to increase biomass under high nutrients in the presence of *T. tetraophthalmus* compared to wireworms is consistent with the result that root damage by *T. tetraophthalmus*, but not wireworms, reduces plant biomass.

The final mass of both insects was independent of the presence of heterospecifics, as shown by a non-significant identity term (Table 1). Conspecific insect density influenced the mass of individual wireworms, but not the mass of individual *T. tetraophthalmus* (Table 1). Soil nutrient level influenced the mass of both insects (Table 1), but in opposite directions: wireworm mass decreased by 23% under high nutrients whereas *T. tetraophthalmus* mass increased by 114% (Supplementary material Appendix A1 Table A3). This result is consistent with the result that *T. tetraophthalmus* has greater impacts on belowground biomass than wireworms, especially under high nutrients. We did not detect a significant interaction between the presence of heterospecifics and soil nutrients for wireworm mass (Table 1, Fig. 4A). In contrast, we did detect an

identity-by-nutrients interaction for *T. tetraophthalmus* (Table 1), whose mass increased by 20% under high nutrients when it occurred alone, and by almost three-fold when it occurred with wireworms (Supplementary material Appendix A1 Table A3, Fig. 4B).

The best model for predicting wireworm survival included insect identity (indicating the presence of heterospecifics) and conspecific insect density ($AIC_{c_{best}}$: 289.15, Supplementary material Appendix A1 Table A5). The confidence intervals around these two coefficients did not overlap zero (CI_1 : 0.09, 1.62; CI_D : -0.35 , -0.01 , Supplementary material Appendix A1 Table A6). In addition, the relative importance values for insect identity and density were high (0.89 and 1.0, respectively, Supplementary material Appendix A1 Table A6), lending further support to the inference that wireworm survival decreased when it occurred with *T. tetraophthalmus* and with increasing conspecific density (Fig. 5A). The impact of conspecific density on wireworm survival did not appear to depend on the presence of *T. tetraophthalmus* because the identity-by-density interaction did not appear in the best model.

The best model for predicting *T. tetraophthalmus* survival included insect density only ($AIC_{c_{best}}$: 172.98, Supplementary material Appendix A1 Table A5). The CI around the density coefficient excluded zero (CI_D : -0.73 , -0.17 , Supplementary material Appendix A1 Table A6). Moreover, the relative importance of density for predicting *T. tetraophthalmus* survival was 1.00, showing very strong support for the inference that *T. tetraophthalmus* survival was

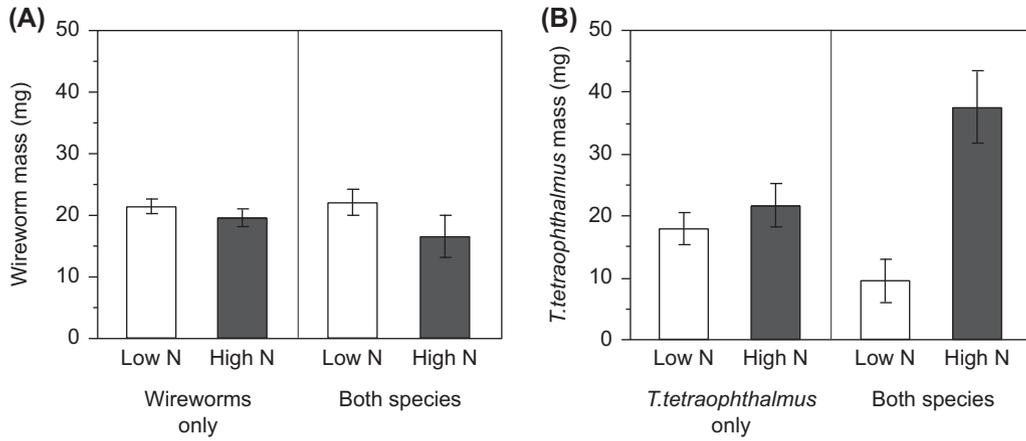


Figure 4. Mean \pm SE mass of individual (A) wireworms and (B) *Tetraopes tetraophthalmus* larvae when co-occurring with conspecifics only or conspecifics plus heterospecifics under low or high nutrient soil. Total insect density was controlled in all comparisons.

influenced negatively by conspecific density (Supplementary material Appendix A1 Table A6, Fig. 5B).

Discussion

A continuing challenge in ecology is to predict the specificity and abiotic context-dependency of species interactions (Agrawal et al. 2007). We tested how belowground plant–insect and insect–insect interactions were influenced by insect identity, insect density and soil nutrients. We showed that two types of root-feeding beetles differed in the location and severity of damage they imposed on common milkweed roots and in the consequences of that damage for plant root biomass and the mass and survival of individual insects. Additionally, we demonstrated that insect density and soil nutrients play a role in mediating root–herbivore interactions: the density of conspecifics was negatively correlated with the survival of both insects, but the presence of heterospecifics was important for only one insect; nutrients affected plant and insect mass, but not root damage. These

results add to the growing appreciation of belowground herbivory as an inconspicuous yet critically important factor shaping plant and insect performance.

Species-specific impacts of belowground herbivory

We detected specific effects of root damage on below- and aboveground plant biomass. Considerable damage – mostly to fine roots, as predicted – by high densities of wireworms did not reduce belowground biomass, indicating that compensatory growth occurred. Plant compensation for belowground damage (imposed by herbivores or mechanically) has been previously reported, especially in agricultural systems (reviewed by Andersen 1987, Brown and Gange 1990) and there is also widespread evidence of compensation and overcompensation to aboveground herbivory (reviewed by Trumble et al. 1993). In contrast to damage by wireworms, extensive damage by *Tetraopes tetraophthalmus* – almost exclusively to main roots, as predicted – decreased the biomass of both main roots and fine roots, indicating little to

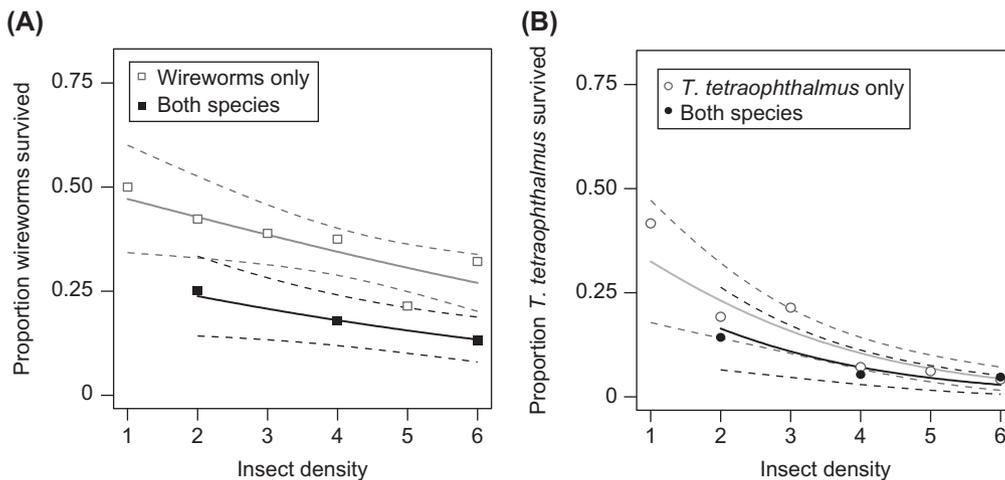


Figure 5. Proportion of insects that survived when co-occurring with conspecifics only or conspecifics plus heterospecifics. Total insect density was controlled in all comparisons. Solid lines indicate the predicted proportion insect survival based on logistic regression for plants to which a single species was added (grey lines) or both species were added (black lines). Dashed lines indicate 95% confidence intervals for each predicted fit. Symbols indicate the mean proportion survival at a given density level.

no compensation as well as 'downstream' effects on fine root biomass. Although neither insect interacted directly with plant shoots, root damage by *T. tetraophthalmus* reduced root and, in turn, shoot biomass, as has been reported in other systems (Morón-Ríos et al. 1997, Wardle et al. 2004). In contrast, root damage by wireworms reduced neither root nor shoot biomass.

Species-specific impacts of root-feeding insects on plant performance have been reported in other systems (reviewed by Andersen 1987, Brown and Gange 1990, Blossey and Hunt-Joshi 2003). For example, Sheppard et al. (1995) showed that when *Carduus nutans* (Asteraceae) plants were exposed to root-feeding larvae of *Hadroplontus trimaculatus* (Curculionidae) and *Cheilisia corydon* (Syrphidae), damage by the weevil resulted in more stems being produced whereas damage by the syrphid fly reduced seed production. More recently, Wurst and Van der Putten (2007) demonstrated that wireworms (*Agriotes* spp., Elateridae) decreased total root biomass and led to compensatory shoot growth in *Plantago lanceolata* (Plantaginaceae), however plant parasitic nematodes had no effect on root or shoot biomass.

We suggest that the location of root damage is a key factor in explaining specific impacts of belowground herbivory on root and shoot biomass. Compensation may be more common when belowground damage is concentrated on fine roots, which are generally less costly to produce, less well defended, and often have a high turnover rate (Eissenstat and Yanai 1997, van Dam 2009, Hodge et al. 2009). In addition, main roots, if undamaged, can potentially mobilize stored resources to maintain fine root and shoot biomass (Trumble et al. 1993). Steinger and Müller-Schärer (1992) showed that specific impacts of two root-feeding insects, the moth *Agapeta zoegana* (Tortricidae) and the weevil *Cyphocleonus achates* (Curculionidae), on host plant *Centaurea maculosa* (Asteraceae), biomass were produced not by a difference in the amount of tissue consumed, but by the consumption of different tissue. Feeding by the moth in the lower taproot did not affect *C. maculosa* biomass likely because new root growth above the feeding site compensated for the putative reduction in water and nutrient uptake caused by herbivory. On the other hand, feeding by the weevil in the vascular tissue reduced shoot biomass likely because compensation above the feeding site was anatomically constrained by the destroyed xylem vessels (Steinger and Müller-Schärer 1992). In the present study, we did not observe an increase in main root biomass in plants exposed to fine root-feeding wireworms, indicating that common milkweed responses to fine root damage differ from the pattern of compensation mentioned above. However, our result that main root-feeding by *T. tetraophthalmus* decreases shoot biomass is consistent with Steinger and Müller-Schärer's inference about destroyed transport cells.

These patterns highlight the relevance of studying belowground herbivory at a finer spatial scale than has typically been investigated. Using split-pot experiments, Robert et al. (2012a) demonstrated both localized and systemic induced responses within root systems of maize *Zea mays* (Poaceae) plants exposed to belowground herbivory by *Diabrotica virgifera virgifera* (Chrysomelidae) larvae. This study, together with the results presented here, suggests a need for increased attention to individual root types and within-root system

responses, instead of treating belowground plant material as a single mass of undifferentiated tissue. While our method of assessing root damage – measuring the length of each necrotic section – is more accurate than some traditional methods such as slant boards and visual scales, it, too, has limitations. Because main and fine roots differ in mean diameter, a centimeter of damage to a main root usually reflects the removal of more tissue than the same length of damage to a fine root. To avoid the limitations of length-based approaches, future work could employ newer techniques, such as acoustic detection, mini-rhizotrons, and x-ray microtomography, which provide non-destructive means of observing belowground insect behavior and quantifying the amount of tissue consumed (Mankin et al. 2008).

Specific impacts of root-feeding insects also may be explained by the physiological consequences (and associated impacts on up- and downstream tissue) of different types of feeding. Boring damage to main roots often removes (Erwin unpubl.) or compromises the function of the vasculature, putatively reducing transport to shoots, and explaining the decrease in shoot biomass reported here. External damage to fine roots is less likely to produce such a pattern; common milkweed fine roots have smaller vascular bundles than main roots (Erwin unpubl.). Thus, we suggest that different physiological impacts of different types of damage may be another factor explaining why main root feeding by *T. tetraophthalmus* decreased shoot biomass whereas fine root feeding by wireworms did not.

A recent meta-analysis demonstrated that the magnitude of the effect of belowground herbivory on root biomass is predicted by insect guild: overall, external chewers cause greater root losses than borers (Zvereva and Kozlov 2011). We detected different impacts of a chewing versus a boring insect, but our results are not consistent with this general pattern. Damage by wireworms did not reduce any component of plant biomass whereas damage by *T. tetraophthalmus* reduced main root and fine root biomass. One consideration is that not all wireworms feed exclusively by chewing. *Agriotes* spp. bite the root and then suck at the point of damage. Seventeen percent of our wireworm collection was comprised of *Agriotes mancus*. If this mode of feeding is common in *H. abbreviatus* (83% of the collection), then this may explain why our findings do not follow the conclusion of the meta-analysis (Zvereva and Kozlov 2011).

Finally, diet breadth may play a role in explaining the species-specific pattern of root damage reported here. Robert et al. (2012b) recently showed that specialist root-feeding larvae (*Diabrotica virgifera virgifera*) preferred and grew best on the nutrient rich, yet well-defended crown roots of maize whereas two generalist herbivores (*Diabrotica balteata* Chrysomelidae and *Spodoptera littoralis* Noctuidae) were deterred from feeding on these tissues.

Effects of insect density and soil nutrients

The density of root-feeding insects may influence patterns of belowground herbivory via effects on host location, feeding behavior, growth rates, sex ratios and survival (Brown and Gange 1990, Whittaker 2003, Onstad et al. 2006, Robert et al. 2012a). We tested how insect density influences root damage under typical (non-outbreak) conditions to

determine whether density dependence attenuates the negative effects of herbivores on plants. We found that damage to main roots was positively correlated with overall insect density, and increased with increasing density of *T. tetraophthalmus*. Damage to fine roots was also positively correlated with overall density, and increased with the density of wireworms. Previous experiments (Spike and Tollefson 1988) have demonstrated an interactive effect of insect density and soil nutrients on root damage, yet our study is one of the first to show that the interaction itself may depend on root type; here, damage increased with insect density under high, but not low, soil nutrients for main roots only.

Increasing insect density decreased all components of plant biomass (shoots, main roots and fine roots) overall, but reductions were most pronounced in main roots exposed to *T. tetraophthalmus*. Indeed, when we excluded the *T. tetraophthalmus* data and again tested for the effects of density, nutrients, and their interaction on plant biomass, we found that only nutrients remained significant. This result indicates that the effects of density, identity-by-density, and identity-by-nutrients on plant damage and biomass were driven by the overwhelming influence of *T. tetraophthalmus*.

The level of nutrients in the soil may mediate patterns of belowground herbivory by influencing the quantity (e.g. production, lifespan) and quality (e.g. defenses, nitrogen content) of plant roots (Stevens et al. 2007, Johnson et al. 2009). We predicted that high soil nutrients would increase overall plant biomass as well as plants' ability to maintain biomass when damaged, and that these effects would vary by plant part and insect species. All components of plant biomass increased under high nutrients, as expected. Damage by *T. tetraophthalmus*, but not wireworms, constrained the ability of main and fine roots to increase biomass in response to added soil nutrients. Moreover, high nutrients affected the mass of individual wireworms and *T. tetraophthalmus* larvae that were feeding belowground. This result potentially suggests a plant-mediated indirect effect of soil nutrients on insect performance, however it is important to note that because fertilized soil and root systems were contained in the same pots, we cannot distinguish between direct and plant-mediated effects of the low and high fertilizer treatments. Split pot experiments would facilitate the identification of treatment- versus plant-mediated effects. This experimental approach is difficult in common milkweed because most genotypes produce a single main root (Erwin unpubl.), which cannot be subdivided.

Belowground herbivory may alter resource allocation between different types of roots, potentially affecting the performance of larvae that feed on these different tissues. Studies using radioisotopes have shown resource allocation away from the site of damage and to distal tissues that are not easily accessible to herbivores (reviewed by Orians et al. 2011). This process, termed induced resource sequestration, may be effective as a 'safeguarding' strategy, but may also be associated with ecological costs (Orians et al. 2011). For example, such allocation may make enriched tissues increasingly vulnerable to attack by other species. Here, we demonstrate that *T. tetraophthalmus* larval mass increased under high nutrients significantly more in the presence of wireworms than in the presence of only conspecifics. If in common milkweed photosynthates are diverted away from

the site of damage (e.g. fine roots) to other undamaged (fine and main root) tissues, then fine root attack by wireworms may have led to increases in main root quality and, in turn, the observed increase in *T. tetraophthalmus* larval mass. That we did not detect an increase in wireworm mass in response to *T. tetraophthalmus* feeding (regardless of nutrient level) may possibly be explained by structural limitations in signal transport, i.e. vascular flow may be limited to one direction.

Insect–insect interactions

Because plant roots are often simultaneously attacked by different insects, belowground intra- and interspecific interactions may be common. In this study, conspecific density was a strong negative predictor of the mass of wireworms, but did not affect the mass of the specialist *T. tetraophthalmus*, as predicted. The survival of both insects was negatively correlated with conspecific density, in contrast to a recent study on *Diabrotica virgifera* that demonstrated a hump-shaped correlation between larval density and performance (Robert et al. 2012a). The shape of density dependence was species specific, as shown in a comparison of the western corn rootworm *Diabrotica virgifera virgifera* and the northern corn rootworm *Diabrotica barberi* (Onstad et al. 2006). We also found that the presence of the herbivore *T. tetraophthalmus* decreased, rather than increased, the survival (but not mass) of omnivorous wireworms. The decrease in wireworm survival when the two insects co-occurred potentially could be explained by 1) the significant reduction in the predominant food source (i.e. fine roots) of wireworms in response to main root feeding by *T. tetraophthalmus*, and/or 2) a stronger induction of chemical defenses (cardenolides) in *A. syriaca* roots by *T. tetraophthalmus* damage than by wireworm damage. Furthermore, *T. tetraophthalmus* survival and mass were not reduced in the presence of wireworms. One explanation for the lack of evidence of intraguild predation is that *T. tetraophthalmus* larvae sequester root cardenolides (Malcolm 1991, Agrawal et al. 2012). If *A. syriaca* roots have lower concentrations of cardenolides than *T. tetraophthalmus* larvae, then roots may provide omnivores with a more palatable food resource than herbivores. An alternative explanation is that *T. tetraophthalmus* feed internally (Erwin unpubl., Williams 1941), a habit that may provide physical protection from predators.

We have demonstrated that insect identity, density, and soil nutrients strongly influence patterns of belowground herbivory in common milkweed. Species-specific root damage had differential impacts on below- and aboveground plant biomass. Density dependence was generally important, affecting damage and the mass of both plants and root-feeding insects, whereas heterospecific interactions between insects appear to have lesser influence. Soil nutrients mediated the impacts of insect identity and density, rather than acting as an independent driving force.

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Supplementary material (available as Appendix oik-00434 at <www.oikosoffice.lu.se/appendix>). Appendix A1.