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Orthogonal fitness benefits of nitrogen and ants for nitrogen-limited plants in the presence of herbivores

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Abstract. Predictable effects of resource availability on plant growth-defense strategies provide a unifying theme in theories of direct anti-herbivore defense, but it is less clear how resource availability modulates plant indirect defense. Ant-plant-hemipteran interactions produce mutualistic trophic cascades when hemipteran-tending ants reduce total herbivory, and these interactions are a key component of plant indirect defense in most terrestrial ecosystems. Here we conducted an experiment to test how ant-plant-hemipteran interactions depend on nitrogen (N) availability by manipulating the presence of ants and aphids under different N fertilization treatments. Ants increased plant flowering success by decreasing the densities of herbivores, and the effects of ants on folivores were positively related to the density of aphids. Unexpectedly, N fertilization produced no changes in plant N concentrations. Plants grown in higher N grew and flowered more, but aphid honeydew chemistry stayed the same, and neither the density of aphids nor the rate of ant attraction per aphid changed with N addition. The positive effects of ants and N addition on plant fitness were thus independent of one another. We conclude that N was the plant's limiting nutrient and propose that addition of the limiting nutrient is unlikely to alter the strength of mutualistic trophic cascades.

Key words: ant-plant mutualism; Aphis nerii; Asclepias incarnata; *ecological stoichiometry;* Formica obscuripes; *honeydew; Law of the Minimum; resource availability; trophic cascade.*

Introduction

Nutrient availability drives tradeoffs between plant growth and defense (Coley et al. 1985, Herms and Mattson 1992). Although early studies of plant growth-defense tradeoffs focused on direct defense, recent studies have highlighted the importance, and potential complementarity, of indirect defense in holistic plant strategies (e.g., Mooney et al. 2010, Frederickson et al. 2013). Predators indirectly defend plants by consuming herbivores or by changing their behavior in so-called trophic cascades. At the scale of communities, nutrients can increase the strength of trophic cascades by increasing plant biomass or by changing plant chemistry; either mechanism can increase the densities of associated herbivores and predators per unit land area (Hunter 2016). At the scale of individual plants, nutrients can lead to stronger indirect defense when plants provide predators with direct (i.e., plant-based) food rewards whose concentration per plant

increases with nutrient availability (Heil et al. 2001). In addition, nutrient availability may also be able to change the strength of indirect defense when predators obtain food from plants only indirectly (i.e., via herbivores) (Stamp 2001). Given that plants can allocate additional nutrients in various ways, a key outstanding question is whether we can predict when, and in which direction, changes in nutrient availability will lead to changes in indirect defense (Agrawal 2011).

The effects of extensive anthropogenic nitrogen (N) deposition on trophic cascades remain little studied, despite the potentially significant ramifications for biodiversity and biological control (Chen et al. 2010). N limitation of primary productivity is widespread in terrestrial ecosystems (Vitousek and Howarth 1991). Heterotrophs are also widely predicted to be N-limited (White 1993), which may shape predator feeding strategies (Denno and Fagan 2003, but see Wilder and Eubanks 2010). Although increased N availability may increase predator attraction to plants by increasing the amino acids and carbohydrates available from herbivores (Baylis and Pierce 1991, Bi et al. 2001), additional

N can also change the concentration and composition of plant secondary metabolites, with the potential for complex effects that depend on the identity of the predator and on the nature of predator attraction and success (Dyer et al. 2004, Ballhorn et al. 2013).

Ants are frequently the dominant agents of plant indirect defense (Bronstein 1998). Ants feed on plant exudates, most often from phloem-feeding, honeydew-producing hemipteran insects. These ant-hemipteran interactions may or may not benefit the hemipterans (e.g., Züst and Agrawal 2017), but they contribute to indirect ant-plant mutualisms when ants attracted to plants by hemipterans reduce total herbivory (Styrsky and Eubanks 2007). Because hemipterans incur a direct cost, however, plants should benefit most from these interactions when ant to hemipteran ratios are high. The chemistry of hemipteran honeydew, and the composition of carbohydrates in particular, can affect the rate of ant attraction to hemipterans (Völkl et al. 1999). Honeydew chemistry can change with plant genotype (Douglas 1993) and nutrient status (Bi et al. 2001, Crafts-Brandner 2002). In particular, N fertilization can increase both the amino-acid and the sugar concentration of plant phloem, which can affect the composition and the production of hemipteran honeydew (Bentz et al. 1995, Bi et al. 2001).

By changing the rate of ant attraction and the strength of indirect defense, changes in honeydew chemistry may have fitness consequences for plants, but, to our knowledge, this has not been shown. An early study of the effects of N on ant foraging found that N fertilization of the food plant of a honeydew-producing lycaenid caterpillar increased the rates of ant attraction to caterpillars (Baylis and Pierce 1991). Although honeydew chemistry was not measured in that study, fertilized plants had higher N concentrations, which may have affected both the sugar and the amino-acid composition of caterpillar honeydew. N fertilization can also decrease some plant secondary metabolites (Koricheva et al. 1998), and ants may prefer honeydew with less plant-derived toxins (Züst and Agrawal 2017).

Will anthropogenic N deposition thus tend to increase the strength of plant indirect defense via bottom-up effects on ant-plant-hemipteran trophic cascades? We addressed this question by conducting an experiment to manipulate the tritrophic interaction among swamp milkweed plants (*Asclepias incarnata*), aphids, and ants under different levels of N in a factorial design. Like many other plants, *A. incarnata* can increase proportional N uptake upon fertilization (Agrawal et al. 2008, Tao et al. 2014). However, levels of a major class of milkweed secondary metabolites, cardenolides, are low in this species and do not consistently change with N fertilization (Tao et al. 2014), decreasing the metabolic axes along which N fertilization is likely to act. We predicted that the presence of ants would increase plant fitness, and that ants would be more abundant, and thus have stronger effects on plants, in the presence of aphids. We also predicted that N fertilization would increase plant fitness directly, as well as

indirectly by increasing the strength of indirect defense via changes in the rates of ant attraction to aphids. Our results support all but this latter prediction. We propose that our plants were N-limited, and discuss the implications for plant allocation and indirect defense.

Methods

Study system

Milkweeds are frequent aphid hosts that have been used as models for studying the chemical mediation of ant-plant-hemipteran interactions (Mooney and Agrawal 2008, Pringle et al. 2014, Züst and Agrawal 2017). Milkweeds are also host to various other specialized insect herbivores that ants may attack, including the monarch butterfly (*Danaus plexippus*). *Aphis nerii* aphids are widespread and abundant milkweed specialists that are tended by several species of ants in non-specialized interactions throughout the range (Bristow 1991, Smith et al. 2008). *Aphis nerii* aphids excrete a variety of carbohydrates and amino acids in their honeydew when feeding on *A. incarnata*, but, so far, cardenolides have not been detected (Pringle et al. 2014).

Study location and experimental design

The study was conducted at the University of Michigan E.S. George Reserve (ESGR) in Livingston County, Michigan (42° 28′ N, 84° 00′ W). The site is a mixture of hardwood forests, wetlands, and open fields. *Formica obscuripes* Forel ants build thatch mounds in the open fields, where vegetation includes several species of milkweed (*Asclepias* spp.). Swamp milkweed, *A. incarnata* L., is found in the adjacent wetlands. *Aphis nerii* Fonscolombe aphids are common milkweed aphids at the site, and preliminary work showed that *A. nerii* are readily tended by *F. obscuripes* (Pringle, unpub data). Common lepidopteran milkweed folivores at the site include monarch butterfly (*D. plexippus* L.) and milkweed tussock moth (*Euchaetes egle* Drury) larvae.

Experimental *A. incarnata* plants were purchased as seeds from Seedsource (Junction, TX). Seeds were washed in 10% bleach in water and cold stratified at 4°C for 5 weeks. Seeds were germinated in Metro-Mix 380 potting soil (Sun Gro Horticulture) in a greenhouse in June 2014, and two-week-old seedlings were transplanted to 6-inch pots. Plants were fertilized with ammonium nitrate (NH_4NO_3) in water in three treatments at 3-week intervals to total levels of 0, 4, and 8 g m^{-2} N (hereafter "unfertilized," "intermediate N," and "high N"). The intermediate and high N levels represent presently observed and future predicted anthropogenic N deposition in the Midwest, respectively (Galloway et al. 2004). These levels produce changes in *A. incarnata* N concentrations, but not toxic cardenolides, in the lab (Tao et al. 2014). Plants were transplanted to the field at 5 weeks; plants received their final N treatment in situ in the field.

The experiment began when plants were 7 weeks old and ran for 7 weeks (July to September 2014).

In a 12-ha old field at the ESGR, we chose 20 *F. obscuripes* nest mounds of similar size (109 ± 13 cm in diameter), light (33 ± 9% cover), and ant activity for the experiment. Each mound was surrounded by nine experimental plants, comprising three plants for each N treatment (0, 4, and 8 g m^{-2}), which were assigned to one of three ant-aphid treatments—"ants and aphids"; "ants, no aphids"; and "no ants, aphids"—in a factorial design. Plants were planted directly in field soil 1.5 m from the edge of the mound in random positions. We excluded one mammal-disturbed mound and retained 19 mounds (n = 171 total plants, 19 per treatment).

We manipulated ant and aphid presence/absence throughout the experiment. To manipulate ant access, we used rings of aluminum flashing (25 cm diameter) around each plant. To allow ants to access plants, we placed 15-cm-tall rings around the plant that rested on the soil surface. To exclude ants, we buried 20-cm-tall rings ~5 cm into the soil and painted Tanglefoot (Tanglefoot Co., Grand Rapids, MI, USA) around the top rim. To introduce aphids, we seeded aphid-present plants with five adult apterous *A. nerii* aphids derived from a single individual collected in Atlanta, Georgia in March 2014, and reared on *A. incarnata* for 1 month prior to the experiment. Every aphid-present plant was seeded twice, once on 25 July and again on 30 July. Aphids that colonized aphid-excluded, "ants, no aphids" plants were culled weekly and Tanglefoot on ant-excluded, "no ants, aphids" plants was maintained weekly.

Effects of experimental treatments on plant traits

Plant biomass, allometry, and reproduction.—To assess plant growth, we harvested aboveground biomass at the end of the experiment, dried it for >36 h at 60°C, and weighed it. We also measured the plants at the beginning and at the end of the experiment. For a subset of plants (n = ~22 per treatment), leaf lengths were measured to the nearest 0.1 cm, and leaf area was estimated by multiplying the number of leaves by the average leaf length per plant. It was noted when plants began to flower, and we counted total expanded plant flowers in all umbels.

Plant carbon:nitrogen ratios.—To test whether N treatments affected the stoichiometry of plant aboveground biomass, we measured % carbon (C) and N in ~0.5 g of dried and homogenized leaf and stem using a LECO TruMac determinator (LECO Co., St Joseph, MI).

Honeydew.—To test whether N treatments affected the chemical composition of *A. nerii* honeydew, we collected and analyzed honeydews. Previous measurements indicated that *A. nerii* honeydew from *A. incarnata* contains very low quantities of amino acids (Pringle et al. 2014). Our attempt to measure amino acids in these honeydews was unsuccessful, and we therefore focused on honeydew carbohydrates, which play a particularly important role in ant attraction (Völkl et al. 1999, Blüthgen and Fiedler 2004) and whose changes are likely to be correlated with those of amino acids (Bentz et al. 1995). Honeydew was collected in the field on aluminum disks for 48 h, 3 weeks after field measurements began. Disks were secured to the plant with tape, and accumulated honeydew was washed into 100 μL 9:1 water:methanol. Sugars were separated and detected using ultra-high performance liquid chromatography (UPLC, Waters Inc. Milford, MA) with a Luna Amide column and an ELS detector, then identified and quantified using a series of external standards. For more details, see Pringle et al. (2014).

Effects of experimental treatments on arthropods

Insect counts.—To investigate how treatments affected insect abundance and density, we counted ants and aphids on all plants every week for 7 weeks. Instantaneous counts of ants were made on each plant once per week between 10:00 and 14:00 h. The number of *A. nerii* aphids were counted on plant leaves and stems. The presence of leaf-eating herbivores, primarily lepidopteran larvae, was also noted weekly.

Behavioral experiment.—To test whether ant aggressive behavior varied with plant N treatment and the number of aphids, we conducted a behavioral experiment to assay *F. obscuripes* agression towards another ant species, *Camponotus pennsylvanicus*. We used ants in order to have sufficient individuals to introduce multiple, novice individuals in each trial. Behavioral trials of ant aggression were conducted once per plant that received the "ants and aphids" experimental treatment for all N treatments (n = 57 plants, 19 per N treatment) between 9:00 and 17:00 h on 2 d in late August. Individual behaviors by *F. obsurcipes* (ignore, touch, prolonged touch, avoid, aggression, fight) were scored for 5 min and aggregated into a single aggression index (for more details, see Appendix S1).

Leaf herbivory.—To test whether the experimental treatments affected leaf herbivory, we measured herbivory in weeks 1, 3, and 5. A single investigator categorized percent leaf area eaten for all leaves on each plant using visual estimates to place leaves in the following categories: 0 = 0%, 1 = >1–6%, 2 = >6–12%, 3 = >12–25%, 4 = >25–50%, 5 = >50–100%. We calculated an herbivory index per plant as: $\left(\sum_{i=0}^{5} n_i \times i\right)/N$, where i is the herbivory category.

Statistical analyses.—Statistical analyses were conducted in R 3.2.3 (R Core Team 2015).

For plant traits, multiple comparisons among nitrogen treatments were conducted using ANOVA with post-hoc Tukey HSD. Allometric relationships were determined by comparison of sample-size-corrected Akaike information criteria (AIC$_c$) of the possible regression

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relationships, which were then compared by ANCOVA. Multivariate comparisons of honeydew sugars were conducted in the *manyglm* package (Wang et al. 2015) using a Tweedie distribution with a log-link and a variance power of 1.5 (Dunn 2014). We used a log-likelihood ratio test with 999 bootstraps and PIT-trap resampling to determine whether the model with nitrogen as a factor differed from the nested intercept model.

Insect count data were analyzed using generalized mixed effects models (GLMMs) to account for the overdispersed count data, the repeated sampling of individuals over time, and the nested effect of ant mound. We found the optimal model using backward selection (Zuur et al. 2009). GLMMs were analyzed in the *glmmADMB* package (Skaug et al. 2016) or the *lme4* package (Bates et al. 2015) with a negative binomial, binomial, or Tweedie distribution. Ant attraction to aphids was assessed by both ant counts and a presence/absence score. Plants with zero aphids were excluded. For count models, the deviance explained by experimental treatments was assessed with type II tests in the *car* package (Fox and Weisberg 2011).

For the behavioral experiments, we used quasi-GLM models (log link) with a dispersion parameter ϕ ($\sigma^2 = \mu \times \phi$). The overall fit of each model was assessed with a chi-squared test, and F statistics were calculated by type I tests. For leaf herbivory, values from the three surveys were averaged because some leaves were completely eaten, and we tested the global effect of ant-aphid and N treatments using a GLM with a Tweedie distribution (log-link, var power = 1.5). Analysis of deviance was conducted with a type II test. We also analyzed the effect of ants and aphids within each N treatment using Tweedie-distributed GLMs. Direct and indirect effects on flowering were analyzed by path analysis in the *lavaan* package (Rosseel 2012). Path model fit was assessed using robust estimators to account for non-normal distributions of the residuals.

RESULTS

Effects of experimental treatments on plant traits

Plants grew larger when fertilized with more N (Fig. 1a). The allometric relationship between leaf area and plant height at the end of the experiment was similar among N treatments (Fig. 1b). Plant stoichiometry was homeostatic under different N treatments; C:N ratios and nutrient content did not differ among treatments (Fig. 1c; Appendix S2: Fig. S1). The sugar composition
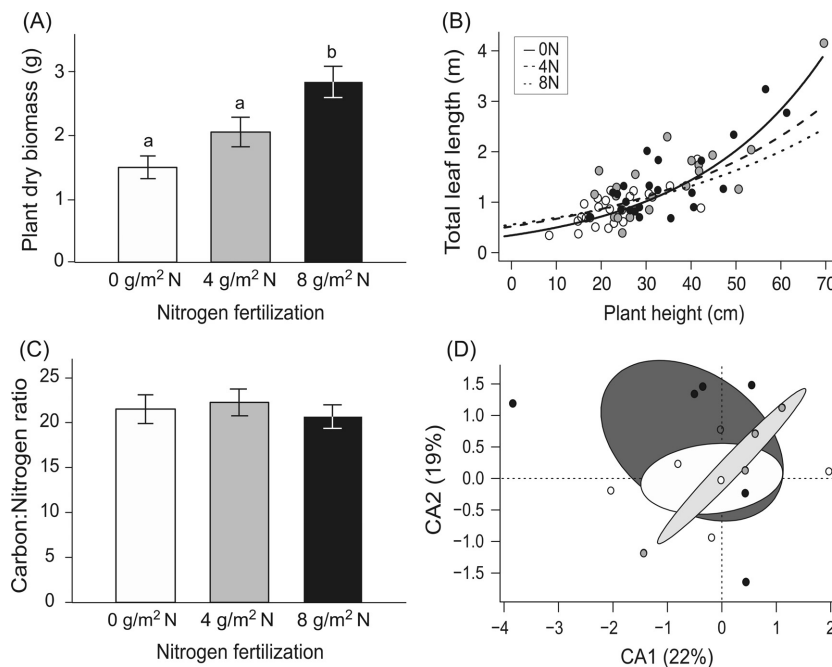

FIG. 1. Effects of nitrogen fertilization on plant traits. (A) Plant dry biomass at the end of the experiment under different levels of N fertilization ($F_{2,168} = 9.23$, $P < 0.0002$). Different letters represent significant differences by Tukey's HSD ($P < 0.04$). (B) Exponential allometric relationships between plant height and leaf area at plant harvesting, approximated by total leaf length ($F_{5,57} = 9.16$, $P < 0.0001$; height $P < 0.0001$, nitrogen $P = 0.4$, height × nitrogen $P = 0.6$). (C) Carbon:nitrogen ratios in plant leaf and stem tissue at the end of the experiment ($F_{2,58} = 0.31$, $P = 0.7$). (D) Relationship between the first two unconstrained axes (eigenvalues: CA1 = 0.12, CA2 = 0.10) in a constrained correspondence analysis of the 15 sugars detected in aphid honeydew (the axes constrained by nitrogen treatment explained only 9% and 5% of the variation, respectively, and are not shown). Elipses represent 95% confidence intervals of the SE. In all panels, white represents 0 g m^{-2} N, gray represents 4 g m^{-2} N, and black represents 8 g m^{-2} N fertilization treatments. In (a) and (c), error bars represent SE.

of aphid honeydew also did not differ across N treatments (Σ LR = 23.0, df = 2, P = 0.7; Fig. 1d; Appendix S3: Table S1).

Effects of experimental treatments on arthropods

Insect counts.—The number of aphids on plants changed over time, but the number of aphids per plant was increased by nitrogen addition and reduced by ants overall (Appendix S4: Fig. S1, Table S1, Table S2). We did not observe direct predation of aphids by ants, but our result is consistent with previous reports that ants can reduce the population sizes of tended aphids on milkweeds, apparently in the absence of direct predation (Smith et al. 2008, Züst and Agrawal 2017). Aphid number was not affected by the interaction between N addition and ants (Appendix S4: Fig. S2; at a representative survey date, AIC: 1485.1 with the interaction term; 1462.8 without the interaction term; Appendix S4: Table S1, Table S2).

Ants were present more often on plants with more aphids (Fig. 2a). The attraction of ants to aphids depended on N treatment (Appendix S5: Table S1), but this interaction was driven by the larger range in the number of aphids on fertilized plants than on unfertilized plants, not by differences among N treatments in the number of ants attracted to a plant per aphid (χ^2 = 0.18, df = 2, P = 0.9).

Caterpillars of either *D. plexippus* or *E. egle* were found eating plants in all plant surveys beginning in the second week and continuing to the experiment's end (4 Aug – 11 Sept).

Behavioral experiment.—Aggressive behavior of *F. obscuripes* ants towards intruders increased when plants had more total aphids (global χ^2 = 11.41, df = 5, P < 0.05; number of aphids: incr. $F_{1,55}$ = 5.17, P < 0.03; Fig. 2b) but did not vary with plant N treatment (N: incr. $F_{2,53}$ = 1.09, P = 0.3; number of aphids × N treatment: incr. $F_{2,51}$ = 0.10, P = 0.9).

Herbivory.—Leaf herbivory was significantly reduced by ants ($F_{2,162}$ = 6.00, P < 0.004; Fig. 3) but unaffected by N ($F_{2,162}$ = 0.43, P = 0.6; Fig. 3; Appendix S6: Fig. S1). The best model to explain leaf herbivory included only the ant-aphid treatment (Appendix S6: Table S1, Table S2).

Effects on plant fitness

N fertilization and the presence of ants increased plant flowering, whereas the presence of aphids decreased it (Fig. 3; Appendix S7: Fig. S1). Ants indirectly increased the number of flowers per plant by decreasing herbivore densities, whereas N fertilization did not affect the densities of herbivores but did have a direct, positive effect on the total number of flowers in all umbels (Fig. 3; Appendix S7: Table S1, Table S2, Text S1).
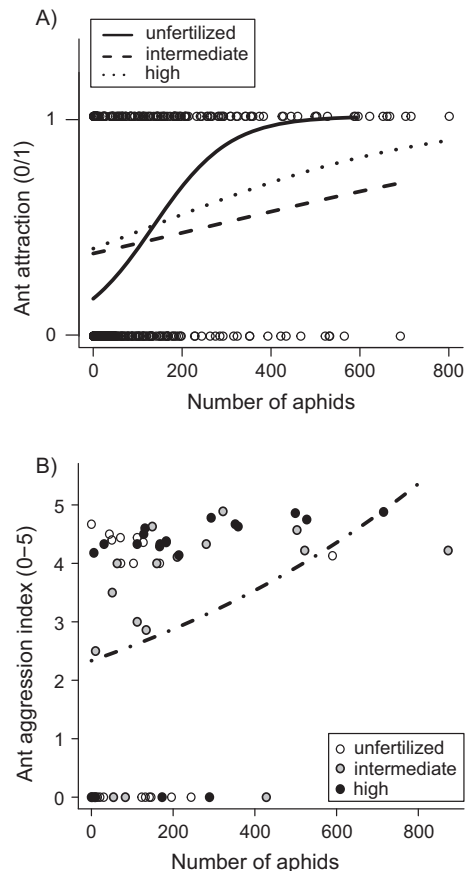

FIG. 2. (A) Ant attraction to aphids on plants with different N fertilization treatments. Points represent all N treatments at all time points, and lines represent logistic regressions of each N treatment (see also Appendix S5: Table S1). Post-hoc tests showed that a model binning the fertilized plants explained the data as well as the model with three fertilization levels (Appendix S5). (B) Relationship between *Formica obscuripes* ant aggression toward intruder ants and the number of aphids on a plant. Points are colored by N treatment. N treatment was not a significant predictor, so the line represents the predicted polynomial fit of a quasi-GLM model with a dispersion parameter (see Methods) over all N treatments.

DISCUSSION

Here we tested whether and how N deposition could affect the strength of mutualistic trophic cascades. We found that the fitness benefits of indirect defense were actually orthogonal to those of N fertilization. Our results show that a facultative ant-aphid interaction increased flowering in *A. incarnata* milkweeds. Contrary to prediction, however, N fertilization did not alter the fitness effects of these interactions for plants. Although *A. incarnata* milkweeds are capable of taking up proportionally more N in high-N environments (Tao et al. 2014), they did not do so here. Instead, plants simply used additional N to grow larger. Densities of predators per plant area determine the strength of defensive mutualism (Duarte-Rocha and Godoy-Bergallo 1992). In our system, N
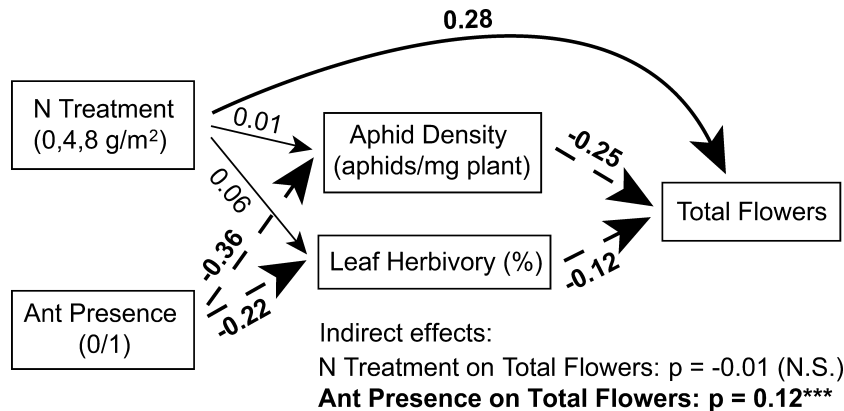
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Fig. 3. Path analysis of direct and indirect effects of experimental treatments on the total number of flowers as a proxy for plant fitness. The test statistic, which tests against the null hypothesis that the model represents an ideal description of the data, indicated that our model was a good fit ($\chi^2 = 0.85$, df = 2, $P = 0.65$; RMSEA <0.0001; Appendix S7: Table S1). Solid arrows indicate positive effects; dashed arrows indicate negative effects (Appendix S7: Table S2). Numbers along the arrows and for indirect effects indicate the sign and magnitude of standardized path coefficients (p). Arrows and numbers in bold are significant at the $P < 0.02$ level.

addition may exert bottom-up effects at a community scale because larger plants hosted more aphids that fed more ants, but the density of ants *per plant area* did not change with N addition, and the effects of indirect defense on plant fitness thus stayed the same.

According to Liebig's Law of the Minimum, addition of the limiting nutrient increases plant biomass (Liebig 1855). Midwestern grasslands are often N-limited, and our results indicate that *A. incarnata* was strongly N-limited in this Michigan field. We also found that plants maintained consistent N concentrations regardless of N fertilization treatment, suggesting that *A. incarnata* regulated C:N ratios when experiencing low N and used additional N to grow at the same stoichiometric ratio. Vascular plants may in fact exhibit greater stoichiometric homeostasis than is typically appreciated. C:N ratios of field-collected plants often exhibit phylogenetic signal (Elser et al. 2010), and C:N ratios in *A. incarnata* are heritable (Agrawal et al. 2008).

Our results are in contrast to the significant increases in plant N concentrations reported in a recent meta-analysis of N-fertilization experiments (Bracken et al. 2015), which may indicate that plants usually become limited by other, non-N nutrients (e.g., phosphorus) in such experiments (Bracken et al. 2015). Variability in plant nutrient concentrations may in fact usually result from "excess" uptake of non-limiting nutrients (Chapin 1980). Excess N uptake under limitation by other nutrients could also explain the contrast between our results and previous findings of decreases in *A. incarnata* C:N ratios with fertilization in growth chambers (Agrawal et al. 2008, Tao et al. 2014). Laboratory conditions may introduce other limiting resources directly (e.g., light, soil space) or indirectly by precluding interactions that naturally occur in the field, e.g. between plants and soil microbes. Our results suggest that the N levels necessary to increase

A. incarnata N concentrations in the field will be above those of anthropogenic N deposition in the Midwest, but future studies should assess how this perennial plant responds to increased N over multiple growing seasons.

Excess uptake is of course very likely to serve an adaptive function for the plant, be it storage (Chapin 1980) or increased defense (Bryant et al. 1983). Given that N uptake must be converted by the plant (Bracken et al. 2015) and that herbivores typically prefer to consume plant tissue with higher nutritive N (White 1993), storage and defense may need to go hand-in-hand. Plants like *A. incarnata* that convert resources rapidly into growth and invest little in direct defenses (Mooney et al. 2010) may be particularly well poised to exploit indirect defenses whose strength can be mediated by primary metabolites. This hypothesis deserves further study.

If addition of the limiting nutrient rarely alters plant stoichiometry, it is also unlikely to change the strength of mutualistic trophic cascades. The few other studies that have examined how indirect defense changes with nutrient availability have usually added mineral nutrients with broad (e.g., NPK) fertilizers (but see Trager and Bruna 2006). Nevertheless, we found hints in other studies that addition of non-limiting nutrients may be more likely to cascade up. For example, light-limited tropical plants appear to produce higher concentrations of secondary metabolites when fertilized (Dyer et al. 2004, Trager and Bruna 2006), which may increase parasitoid success (Smilanich et al. 2009). We propose that the Law of the Minimum, previously suggested to be a useful predictor of plant-mycorrhizal mutualism (Johnson et al. 2015), may also help to predict the outcomes of plant defensive mutualism. As studies of the effects of nutrient addition on plant mutualisms mount (e.g., Tamburini et al. 2017), it will be useful to consider the effects of such additions on the chemistry of plants and of their mutualistic rewards.

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