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Plant chemical defense indirectly mediates aphid performance via interactions with tending ants

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Running head: Plant effects on ant-aphid interactions

Abstract: The benefits of mutualistic interactions are often highly context dependent. We studied the interaction between the milkweed aphid *Aphis asclepiadis* and a tending ant, *Formica podzolica*. While this interaction is generally considered beneficial, variation in plant genotype may alter it from mutualistic to antagonistic. Here we link the shift in strength and relative benefit of the ant-aphid interaction to plant genotypic variation in the production of cardenolides, a class of toxic defensive chemicals. In a field experiment with highly variable genotypes of the

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common milkweed (*Asclepias syriaca*), we show that plant cardenolides, especially polar forms, are ingested and excreted by the aphid proportionally to plant concentrations without directly affecting aphid performance. Ants consume honeydew, and aphids that excreted high amounts of cardenolides received fewer ant visits, in turn reducing aphid survival. On at least some plant genotypes, aphid numbers per plant were reduced in the presence of ants to levels lower than in corresponding ant-exclusion treatments, suggesting antagonistic ant behavior. While cardenolides thus appear ineffective as direct plant defenses against aphids, the multi-trophic context reveals an ant-mediated negative indirect effect on aphid performance and population dynamics.

Key words: *Asclepias syriaca*, cardenolides, multitrophic interactions, mutualism

Introduction

Mutualistic interactions among species are an important component of community dynamics, in which the abundance of a species may be significantly increased in the presence of a mutualist (Bruno et al. 2003). However, the rewards of engaging in mutualism are highly context dependent and vary greatly in time and space (Bronstein 1994). Even for pairwise species interactions that are generally mutualistic, extrinsic factors affecting the status of each partner can have strong impacts on the relative benefit of the mutualism, particularly if it involves costly rewards by one partner of the interaction (e.g., Heil et al. 2002, Yamawo et al. 2012).

Ants frequently engage in mutualistic interactions with aphids and other phloem-feeding insects, in which ants visit or ‘tend’ aphids to collect the aphid’s sugary excrement (a.k.a.,

honeydew), and in return may aggressively defend aphids against predators or provide other services (Banks 1962, Nielsen et al. 2010). Extrinsic factors such as nutrient availability may impact ant behavior (Petry et al. 2012), but ants can often offset costs associated with tending by consuming other herbivores or the aphid's natural enemies found on the plant (Styrsky and Eubanks 2007, Mooney and Agrawal 2008). At least some ant species facultatively consume aphids if they are not satisfied with the honeydew reward (Sakata 1994). Given the high mobility of ants and their often low degree of specialization or dependence on specific aphid species, the successful establishment of an ant-aphid mutualism should more strongly depend on the aphids, and is likely mediated by honeydew quality.

Aphids are phloem-feeding insects that excrete honeydew primarily as a waste product to cope with the excess of sugars in their diet (Dixon 1998). However, honeydew composition of tended and untended aphid species often differs significantly (Völkl et al. 1999, Fischer and Shingleton 2001, Mooney 2011), suggesting evolutionary adaptations by tended aphids to better attract ants and maintain mutualistic services. Both quantity and quality of honeydew are in part under the control of tended aphids (Takeda et al. 1982, Fischer and Shingleton 2001) and can mediate the degree of mutualistic behavior by ants (Völkl et al. 1999). However, there are likely physiological limits to this plasticity, and honeydew composition can have a fixed genetic component among individuals of a tended species (Mooney 2011). The composition of plant phloem, likely under genetic and environmental control, is therefore a key extrinsic factor for the determination of honeydew quality (Fischer and Shingleton 2001), and may have significant impacts on ant-aphid mutualisms.

The aphid *Aphis asclepiadis* Fitch feeds on the common milkweed *Asclepias syriaca* L. and is virtually always tended by ants in the field, with generally beneficial effects of tending on

aphid performance (Mooney and Agrawal 2008, Smith et al. 2008). However, natural variation among plant genotypes may alter this interaction from mutualistic to antagonistic (Mooney and Agrawal 2008). In a different system, Vrieling et al. (1991) demonstrated that aphids on the plant *Senecio jacobaeae* L. only established successful ant-tended colonies on plant individuals with low levels of defensive pyrrolizidine alkaloids. Alkaloids are present in the phloem and excreted at significant concentrations in aphid honeydew (Wink and Römer 1986), where they may act to repel mutualist ants.

We recently demonstrated that *A. asclepiadis* feeding on milkweed excretes honeydew with significant amounts of predominantly polar cardenolides (Züst and Agrawal 2016). Cardenolides are toxic defensive chemicals produced by milkweeds (Agrawal et al. 2012), but there is consistent genotypic variation in foliar cardenolides within species (Agrawal 2004). We thus hypothesized that plant variation is driving honeydew cardenolide content, which in turn modulates ant behavior and the extent of mutualistic benefits they provide to aphids. We test this hypothesis in a field experiment using genotypes from a broad sampling of *A. syriaca* genotypes known to vary in cardenolide content.

Materials and methods

Experimental design

Seeds of *A. syriaca* are sired by insertion of a single pollinium into a flower; hence all seeds from a fruit pod (follicle) represent a full-sibling genetic family, which we henceforth refer to as genotypes. In a previous study, multiple genotypes had been collected in each of 22 populations along a latitudinal gradient across eastern North America (Woods et al. 2012). Using information on their average foliar cardenolide content and diversity, we selected ten populations

(Table S1) that covered the range of intraspecific cardenolide variation and randomly picked one genotype from each population. For each genotype we grew 20 plants from seed. Seedlings were planted into plastic pots (10 cm diameter) filled with commercial potting soil (Metro-Mix, Sun Gro Horticulture, Canada CM Ltd.), fertilized once [N:P:K 21:5:20, 150 ppm N (mg/g)], and grown for six weeks in a growth chamber (16 h daylight, 26 °C day : 22 °C night). In August of 2013 we moved the plants to an old field in Tompkins Co, NY, USA, with abundant mounds of the native ant *Formica podzolica* Francoeur. The same field had been used in previous studies on ant-milkweed aphid interactions (Mooney and Agrawal 2008, Mooney 2011). *Formica podzolica* is a predator of many arthropods, but engages in tending with several aphid species (Mooney and Tillberg 2005). We cleared away all tall vegetation around eight mounds separated by at least 20 m, and placed 20 plants in 5-m-diameter circles around each mound (Fig. S1), transplanting them directly into the ground. Every genotype was represented by two randomly placed plants at each mound ($10 \times 2 \times 8 = 160$ plants total).

Plants were left to overwinter, and by early May 2014 a total of 96 healthy plants emerged. We assigned plants to either an ant-access or ant-exclusion treatment following a stratified-random design, resulting in 3-7 plants per genotype-by-treatment combination (4.8 on average). We excluded ants by burying pieces of PVC pipe (25 cm tall by 15 cm diameter) 15 cm deep into the soil around plants (leaving 10 cm above ground) and coating the outside surface with sticky paste (Tanglefoot, Grand Rapids, MI). This treatment successfully excluded ants for the duration of the experiment. For plants with ant access, we loosely placed a 10-cm-tall PVC ring around the plant stem and left it uncoated. From the end of May 2014 and running in parallel to our experiment, we carried out weekly surveys of *A. asclepiadis* abundance in a large natural population of *A. syriaca* at a separate site in Tompkins Co, NY, USA (Fig. S2).

Corresponding to the rapid rise in colonization of natural plants in mid-June (Fig. S2), we inoculated all experimental plants with five adult *A. asclepiadis* on June 23, using aphids from a laboratory colony established from a single individual collected earlier in the season.

Starting four days after the initial inoculation and continuing twice a week throughout the rest of the season, we counted aphid population densities on all plants and recorded the number of ants present on plants in the ant-access treatment. If the aphid population on a plant was lower than ten individuals on any census day, we supplemented that plant with adults up to a total count of ten using aphids from the same laboratory colony. On July 10, we began weekly honeydew collections, including all plants with more than 25 aphids at the time of collection. The first time a plant was included for honeydew collection, we removed two leaves and froze them immediately for subsequent foliar cardenolide analysis. If honeydew was collected on the same plant multiple times, no further leaves were removed to minimize damage to the plant. Honeydew was collected by fixing pre-weighed aluminum foil disks (\varnothing 5 cm) underneath the major aggregation of aphids for 48 hours (Mooney 2011). Cardenolides are relatively stable compounds and are unlikely to have degraded over this time. After 48 hours, foil disks were removed, frozen, and subsequently freeze-dried and re-weighed. Ant tending prevented aphids from dropping their honeydew, thus we temporarily excluded ants for the duration of collection by wrapping a piece of tape around the stem of plants and coating it with sticky paste. For one collection, plants were temporarily sheltered against sporadic rain by plastic canopies fixed 10 cm above each plant. The experiment ended on August 10 after ten population censuses and five honeydew collection series resulting in a total of 149 individual honeydew samples. Aphid populations had begun declining at this point and plant quality was visibly deteriorating due to

natural senescence, which was mirrored in the natural population dynamics of *A. asclepiadis* (Fig. S2).

Chemical analyses

We randomly selected a subset of four leaf samples per plant genotype for HPLC analysis (see Appendix S1). Preliminary analyses revealed that the amounts of honeydew collected on single plants were often at or below the threshold of cardenolide detection by HPLC. For accurate quantification of cardenolides in honeydew we therefore pooled several samples within genotype and used an *in vitro* enzymatic assay described in Petschenka et al. (2013), which quantifies cardenolides at a higher sensitivity utilizing the biological activity of cardenolides on animal Na⁺/K⁺-ATPase (see Appendix S1).

Statistical analyses

All analyses were performed in R 3.2.2. We approximated aphid performance as the mean number of aphids found on a plant averaged across the ten census points. In addition, we estimated the average time for which a population persisted on a plant, and found that both metrics were highly positively correlated (see Appendix S1, Fig. S3), thus we focus on average population size for all subsequent analyses. We analyzed the average aphid counts per plant using a generalized linear mixed model with a negative-binomial error distribution and a log-link (function `glmmadmb`, `glmmADMB` package for R) (Skaug et al. 2015). To account for potential differences among ants of different mounds (e.g., workers per mound, reproductive or nutritional status), ant mound was treated as random effect. As this was part of the experimental design, we included it in all models and did not formally test for its significance. Ant treatment, plant genotype, and their interaction were fitted as fixed effects. Significance of fixed effects and

interactions was estimated by likelihood-ratio tests between models with an effect of interest and a nested model lacking that effect.

The number of ants observed on experimental plants was relatively low in our data and represents only ‘snapshots’ in time. To estimate robust rates of observed ants per aphids present on a plant (visitation rate hereafter), we summed ant and aphid observations for each plant across the whole season. We then analyzed the sums of observed ants as a function of aphid number using a generalized linear mixed model with negative-binomial error distribution as above (function *glmmadmb*). We included plant genotype and an interaction term with aphid number, but then simplified the model until only significant terms remained. From this most conservative model of the ant-aphid relationship, we extracted the genotype-specific regression coefficients to predict the number of ants visiting a population of the overall aphid population size average (Fig. S4).

Honeydew weights of individual samples (before pooling) were divided by the number of aphids present above the aluminum foil when collection was initiated and thus represents a rough estimate of per-capita honeydew production, not accounting for population growth during collection. This per-capita honeydew production was analyzed using a linear mixed effect model (function *lme* in R) with plant identity nested within ant mound as random effect to account for occasionally repeated measures on the same plant. Plant genotype and ant treatment were treated as fixed effect. Evaluation of residuals revealed large heteroscedasticity of variance among the ten genotypes, thus we included a *varIdent* weights term in the final model which estimates a unique variance term for each level of a factor (Zuur et al. 2009).

We analyzed the relationship between genotype means of ant visitation rate and honeydew cardenolides, and between honeydew cardenolides and foliar cardenolides of the host

plant. For the latter, we particularly focused on a comparison of polar cardenolides and the summed total cardenolides. Finally, we carried out a separate test for potential direct toxicity effects of foliar cardenolides on aphid performance. As the main analyses focused on genotype means, we had quantified foliar cardenolides only in a subset of leaf samples. We therefore analyzed plant-specific aphid counts as a function of the genotype averages of foliar cardenolide content, ant treatment, and an interaction term, using a linear mixed effect model with ant mound specified as a random effect.

Results

Effects of experimental ant exclusion on aphid population performance were strongly dependent on plant genotype (Fig. 1b, main effect of ant exclusion: Deviance $D = 1.76$, $\Delta df = 1$, $p = 0.18$; plant genotype by ant exclusion: $D = 23.23$, $\Delta df = 9$, $p = 0.006$), and ranged from positive to negative. Plant genetic effects were entirely mediated by ants, as the mean aphid population size differed 11-fold among plant genotypes when ants were present ($D = 28.49$, $\Delta df = 9$, $p < 0.001$), while the range of population size in the absence of ants was much more constrained, and not significantly impacted by plant genotype ($D = 9.17$, $\Delta df = 9$, $p = 0.422$). In a few cases, aphid population collapse on individual plants could be linked to fungal pathogens or predators, particularly syrphid and cecidomyiid larvae. However, these observations were too infrequent to be formally analyzed, and we can thus only speculate that beneficial effects of ant tending originated from interference with such antagonists.

Observed ant visitation was strongly affected by aphid population size ($D = 29.72$, $\Delta df = 1$, $p < 0.001$), and this relationship significantly differed among the ten genotypes (Fig. S4, $D = 21.91$, $\Delta df = 9$, $p = 0.009$). Six genotypes revealed a positive relationship between ant visits and

aphid number, while four genotypes revealed a negative relationship (Fig. S4). The standardized genotype-specific ant visitation rate was strongly positively correlated with the relative effect of the ant treatment on aphid performance (Fig. 1c, $F_{1,8} = 9.01$, $p = 0.017$). Aphids on plant genotypes with a high ant visitation rate were thus more likely to benefit from the presence of ants, while aphids on plants with low visitation gained no benefit or suffered from ant visitation relative to aphids on the same genotype with ant exclusion.

The amount of excreted honeydew sugars varied over 3-fold among different plant genotypes ($F_{9,53} = 2.15$, $p = 0.041$), and ranged from 10.59 to 32.96 μg per aphid during 48 hours. Ant treatment had no effect on the amount of excreted honeydew sugars ($F_{1,75} = 0.19$, $p = 0.666$, but note that ants were temporarily excluded during honeydew collection) and there was no interaction with plant genotype ($F_{9,66} = 1.40$, $p = 0.207$). The amounts of cardenolides per unit honeydew sugars (quantified by the Na^+/K^+ -ATPase assay) varied 4-fold for aphids feeding on different plant genotypes ($F_{9,29} = 3.72$, $p = 0.003$) but was unaffected by ant treatment ($F_{1,29} = 0.02$, $p = 0.891$) or an interaction with plant genotype ($F_{9,20} = 1.04$, $p = 0.443$). Per-capita sugar excretion on different plant genotypes varied independently of aphid number ($n = 10$ plant genotypes, Spearman's $r_S = 0.24$, $p = 0.514$) and of honeydew cardenolide content ($n = 10$, $r_S = -0.22$, $p = 0.348$). Variation in honeydew cardenolides affect ant behavior, with a marginally significant negative effect of ln-transformed honeydew cardenolides content on ant visitation rate (Fig. 1d, $F_{1,8} = 4.17$, $p = 0.076$), while there was no similar effect on ant behavior for per-capita honeydew amount ($F_{1,8} = 0.03$, $p = 0.867$).

Honeydew cardenolide concentrations quantified by the Na^+/K^+ -ATPase assay and foliar cardenolides quantified by HPLC were positively correlated (Fig. 2a, $F_{1,8} = 5.23$, $p = 0.052$, $r^2 = 0.249$), but mean total foliar cardenolide content did not differ significantly between the ten

genotypes ($F_{9,30} = 1.71$, $p = 0.129$). However, we found a much stronger correlation between honeydew cardenolides and the most polar cardenolide (determined by HPLC retention time) present in leaves (Fig. 2b, $F_{1,8} = 10.09$, $p = 0.013$, $r^2 = 0.503$). This compound contributed an average of 10.7% to the total foliar cardenolide content of plants, and the ten plant genotypes differed 17-fold in its amount, from 1.9% to 23.6% ($F_{9,30} = 4.92$, $p < 0.001$). There was no evidence of direct cardenolide toxicity to aphids (Fig. 3, t-test on model coefficient: $t = 1.68$, $p = 0.096$), but the presence of ants reversed the direction of cardenolide effects, resulting in a significant interaction between foliar cardenolides and ant treatment ($F_{1,85} = 4.27$, $p = 0.042$). This result was more pronounced if just the most polar cardenolide compound was considered (Fig. 3, $F_{1,85} = 7.67$, $p = 0.007$).

Discussion

Plant genetic variation had no direct impacts on aphid performance, but affected the quantity and toxin content of honeydew produced by aphids. When ants were allowed to tend aphids, high cardenolide levels in honeydew reduced ant visitation, which in turn lowered aphid population size and persistence. Aphids are not generally susceptible to cardenolides (Züst and Agrawal 2016), thus while plant cardenolides appear ineffective as direct plant defenses against aphids, the multi-trophic context reveals their potential defensive benefits via an indirect ant-mediated negative effect of cardenolides on aphid performance.

We demonstrated that high honeydew cardenolide content had a negative effect on the visitation rate of *F. podzolica*, while the per-aphid honeydew quantity had no effect on visitation in the field. Reports on the ability of ants and other hymenopteran species to detect cardenolides vary and may be highly species-specific, with reported responses ranging from deterrence

(Bristow 1991 , Mooney et al. 2008, Jones and Agrawal 2016) to indifference (Pringle et al. 2014), even though ants may have suffered negative effects from cardenolide consumption in the latter case (Pringle et al. 2014). Our results suggest that *F. podzolica* is able to detect cardenolides in honeydew and to adjust its tending behavior in response to this unfavorable resource. Ants that are dissatisfied with a honeydew reward will likely neglect aphids, thus allowing natural enemies of aphids to become more devastating. Interestingly, aphid performance on the four plant genotypes with low ant visitation was improved when ants were excluded. This suggests that in addition to neglect, other mechanisms such as direct harassment by ants may be driving differences in aphid performance among plant genotypes. Unfortunately we currently lack close behavioral observations that would be necessary to evaluate this hypothesis.

Of the full range of foliar cardenolides present in *A. syriaca*, the most polar cardenolide correlated most strongly with honeydew cardenolides. It is interesting to note that Malcolm et al. (1989) identified the polar cardenolide aspecioside as a highly abundant compound in *A. syriaca*, which is a likely candidate for the unknown most polar compound detected in our samples. Polar cardenolides are excreted by aphids and may never enter the aphid's body cavity, while less polar cardenolides typically enter the aphid's body and are retained or modified (Züst and Agrawal 2016). Even though apolar cardenolides are generally considered more toxic than polar forms (Agrawal et al. 2012), the low level of control over the excretion of the latter by aphids thus makes plant variation in polar cardenolides a strong extrinsic factor affecting ant-aphid mutualisms.

Throughout the experiment, we monitored population dynamics of *A. asclepiadis* in a natural population and found that at the beginning of the season up to 40% of natural plants are

colonized by individual aphids, but more than two thirds of these colonies failed to persist for more than a few weeks (Fig. S2). Ant assistance is likely crucial for successful aphid population persistence, thus success of natural establishment may well be linked to low polar cardenolide levels in the field. Ants tending aphids on milkweed have been shown to quickly remove neonate monarch caterpillars from the plant (Mooney and Agrawal 2008), which could provide a sufficient selective force to maintain both high- and low-defended plants within a population. As low control over excretion of plant toxins seems to be general among aphids and ant preference for toxin-free honeydew is widespread, ant-aphid interactions may be an important mechanism for the maintenance of plant genotypes with low defense in many systems (Vrieling et al. 1991, Ando and Ohgushi 2008).

Plant genotypic variation can clearly have direct effects on aphid performance and can explain substantial variation in natural aphid distribution patterns (Wimp et al. 2005, Johnson 2008), but the underlying drivers are not always known. Indirect effects of genotypic variation that involve interactions with other biotic agents are emerging to be at least as important as direct effects (Smith et al. 2008), and are key drivers of natural selection on plants. Mutualistic interactions are important components of plant-insect community dynamics. Where mutualisms successfully establish they can dramatically alter species interactions, for example through alterations of the relative dominance of strong competitors (e.g., Scheublin et al. 2007). The present work shows how plant secondary metabolites impact a mutualism, and demonstrates tipping points at which interactions change from mutualistic to neutral or even antagonistic.

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Figure legends

Fig. 1. a) Tending of *Aphis asclepiadis* by *Formica podzolica* in the field. Photo credit: Ellen C. Woods. b) Effects of ant exclusion on aphid population size. Each line connects the genotype averages of aphid population size for ant exclusion (0) and ants access treatments (1). Solid black lines highlight significant differences between treatments, and dashed black lines indicate marginal significance ($p < 0.1$), while grey lines indicate no significant difference. c) Effect of ant visitation rate on the relative benefit of ant tending. Each point is a plant genotype average and the solid line represents the fit of the linear model between means of the two variables. The standardized ant visitation rate is the genotype-specific model prediction of observed ant visits for an aphid population at the overall average of 234 aphids. Relative benefit of ant tending is calculated from the difference in aphid population size between ant access and ant exclusion treatments, and corresponds to the slopes of the reaction norms in panel b). d) Relationship

between ant visitation rate and honeydew cardenolide content. The dashed line represents the marginally significant model fit using ln-transformed cardenolides values.

Fig. 2. a) Relationship between total foliar plant cardenolides and cardenolide content of aphid honeydew feeding on these plants. Each dot represents the mean value of a plant genotype. The dashed line represents the marginally significant linear regression on the genotype means with the 95% confidence intervals given by the grey areas. b) This relationship becomes significant if only the most polar foliar cardenolide compound is considered (solid line). Note that due to differences in quantification methods, absolute values of honeydew and foliar cardenolides are not directly comparable.

Fig. 3. Effects of foliar concentrations of the most polar cardenolide compound on aphid population size. Each symbol represents a per-plant average of aphid population size and the corresponding genotype mean of cardenolide concentration. Solid lines are the fits of a model testing interactive effects of honeydew cardenolides and ant treatment on aphid population size, and the shaded areas are the 95% confidence intervals around each fit. Green colors and triangles correspond to aphid populations in the absence of ant, while blue colors and circles are populations in the presence of ants.





