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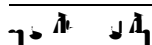


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Abstract Inducible anti-herbivore defenses are found within many plant taxa, but there are fewer examples of inducible indirect defenses that incorporate the third trophic level. This study links caterpillar foraging, herbivore-induced changes in extrafloral nectar production, and the attraction of ants to vulnerable leaves and plants. *Catalpa bignonioides* Walter (Bignoniaceae) uses extrafloral nectar to attract ant (*Forelius pruinosus* (Roger)) bodyguards in response to *Ceratonia catalpae* (Boisduval) (Lepidoptera: Sphingidae) herbivory. Ant density per leaf increased with the sugar content of extrafloral nectar excreted by sampled leaves, suggesting that increased nectar production could attract or retain beneficial arthropods. The masses of sucrose, fructose, glucose and all three sugars combined in the extrafloral nectar increased two- to three-fold on attacked leaves within 36 h of the experimental addition of caterpillars. Production rates for neighboring non-attacked leaves and non-attacked leaves on adjacent plants did not differ over the same time period. Ant attendance at caterpillar-attacked leaves increased two- to three-fold within 24 h of herbivory, relative to attendance at neighboring, undamaged leaves. These attacked leaves attracted the fewest ants prior to the onset of herbivory, suggesting the specialist caterpillar may avoid or be excluded from leaves with more bodyguards. The removal of leaf tissue with scissors did not alter ant attendance at damaged leaves. Mean ant attendance per leaf on attacked plants increased 6- to 10-fold after caterpillar introduction, relative to adjacent unattacked plants. The plant's biotic defense thus operates at two scales; the number of bodyguards (ant workers) on the plant increases after attack, and this increased workforce is biased towards

attacked leaves within plants. Fewer caterpillars remained on plants that attracted greater numbers of ants, suggesting these bodyguards benefit the plant.

Keywords Indirect plant defense · Induction · Mutualism · Rewards · Tritrophic interactions



Induced defenses are responses activated through a previous encounter with a consumer or competitor that confer some degree of resistance to subsequent attacks. Inducible defenses are distinguished from constitutive defenses by their activation in response to environmental stimuli (Harvell 1990). Induced defenses are observed within a range of taxa and frequently include the induction of new or increased physical and chemical defenses (e.g., trichomes, spines, and secondary compounds in foliage, see Karban and Baldwin 1997). These defenses are known as “direct defenses” because they act directly against the herbivore/consumer. The abundance and impact of herbivores can also be limited by higher

Agrawal and Rutter 1998). Some ants track seasonal and diurnal changes in extrafloral nectar production (Tilman 1978; Stephenson 1982; Gaume and McKey 1999), exhibit preferences for nectars or honeydews on the basis of sugar and/or amino acid composition (Lanza et al. 1993; V lkl et al. 1999), and stay longer in patches with more sugar (Bonser et al. 1998). Similarly, nectar-satiated parasitoids stay in herbivore-occupied patches longer and attack more herbivores (Stapel et al. 1997). These observations suggest that plants with increased nectar production could attract or retain more bodyguards, thereby receiving greater protection against herbivores.

Although changes in nectar production subsequent to leaf damage have been reported in seven plant species, the link between cause (herbivory) and effect (altered nectar production resulting in recruitment or retention of plant

extrafloral nectar. All crawling arthropods were then removed from the branches by hand. Leaves were not bagged, because (1) bags can alter sucrose production rates at the enclosed nectaries (Wyatt et al. 1992), and (2) ants were observed at nectaries much more frequently than were flying nectivores. After 36 h, extrafloral nectar was collected from the seven largest nectaries on each of the 60 leaves using 5 μ l microcapillary tubes. Viscous nectar was diluted with drops of distilled water prior to collection. As the addition of water made it impossible to estimate sugar concentrations or nectar volume, analyses focused on the mass of sugar produced over a 36-h period. Nectar was stored in the freezer in 0.5 ml of methanol to discourage post-collection microbial growth.

Nectar composition (preliminary identification of sugars and amino acids) was determined using thin layer chromatography. Subsequent analyses focused on sugars, as they were found in much greater quantities within the nectar. Gas chromatographic analysis of the sugars was performed on a Hewlett-Packard 5890 Gas Chromatograph fitted with a DB-5 (25 m 0.32 mm i.d.) capillary column (0.52 μ m film thickness) (J.W. Scientific, Folsom, Calif.): injector 250 C, detector 350 C. The GC was operated in the splitless mode of injection. Phenylglucose (30.66 μ g in a methanol solution) was added to each sample as an internal standard. After mixing, a 250 μ l aliquot of each nectar solution was placed in an auto-injector vial, and water was removed under a stream of nitrogen. Two drops of acetonitrile were added to each sample to insure complete removal of water during the drying process. Sugars were analyzed as their trimethylsilylated derivatives. N,O-bis-(trimethylsilyl) trifluoroacetamide and dimethylforamide were added in the solution in a 1:1 ratio (usually 20 μ l each, although 100 μ l were added to the most concentrated nectars). This solution was heated at 75 C for 30 min, and a 1- μ l subsample was then injected into the GC. GC oven temperature increased from 100–320 C at 8 /min. Peaks corresponding to α - and β -glucose were summed for quantification, and total sugars consisted of summation of all peaks in the fructose, sucrose and glucose retention ranges of each chromatogram. Linear regressions were used to correlate mean ant attendance per leaf for the 36-h period prior to Tanglefoot application with the production of fructose, sucrose, glucose and all three sugars combined after Tanglefoot application, as 36-h production rates at undamaged leaves are consistent over short time periods (i.e. days, see below). The sugars were analyzed separately because they are differentially attractive to some ants (Koptur and Truong 1998). General Linear Models (GLM) compared production rates and ant attendance among branches and plants.

Caterpillars and nectar production

To test whether herbivory influenced extrafloral nectar production, caterpillars were added to 20 of the leaves described above (Nectar and ants) and nectar was collected from all 60 leaves 36 h later (19–20 September 2000). Four large trees and two seedlings were designated as caterpillar-attacked, and the remaining five seedlings as unattacked. A fifth instar caterpillar was added to two of the four focal leaves within each focal branch on the attacked plants. All 60 leaves remained encircled in Tanglefoot (see above), and the caterpillars were removed 24 h after their introduction. Late instars were used because they can cause considerable leaf damage within this short duration (e.g., 10–30% of total leaf area removed in 24 h). The change in 36-h production rates of fructose, glucose, sucrose, and all three sugars combined for each leaf were computed as follows:

$$D_{\text{production}} = \text{Production}_{\text{post-treatment mass}} - \text{Production}_{\text{pre-treatment mass}}$$

This design permits comparisons among caterpillar-damaged leaves, neighboring undamaged leaves, and undamaged leaves on unattacked control plants. One-sided nonparametric Wilcoxon tests, using individual leaves as replicates, tested the a priori hypotheses that damaged leaves increased production relative to undamaged leaves. Two-sided tests compared the changes in production among the two undamaged leaf groups, as attacked plants may exhibit a systemic response at undamaged leaves. The preceding analyses treat individual leaves and sugars as if they were independent data,

ignoring that some leaves are from the same plants and the physiological links between the sugars. To address these complications, an additional analysis included the treatment means from individual plants as replicates and total sugar production (all three sugars combined) as the dependent variable.

Caterpillars and ants: intra-plant comparisons

To test the hypothesis that ant attendance at caterpillar-damaged leaves differs from attendance at undamaged leaves, attendance at

experimental treatments. The caterpillars were added to randomly selected leaves, were unconfined, and were removed after 4 days. During each survey, the number of ants was counted on 25 randomly selected leaves (irrespective of caterpillar occupation or leaf damage). Plants were surveyed 7, 6, and 0 days prior to caterpillar addition to confirm that pre-treatment ant attendance levels were comparable between the control and experimental plants. The 30 plants were surveyed 24 and 36 h, and 2, 3, 4, 5, 6, 7, 8, 12, 15, and 17 days after caterpillar introduction. Pre- and post-treatment surveys were performed between 1700 and 1900 hours, from 3 September to 5 October 2000, with the second post-attack survey (36 h) performed at 0900 hours. All plants were surveyed during a 1-hour period.

Repeated measures analysis was used to evaluate the treatment effects on a plant's mean ant attendance per leaf over time. A t-test was used to confirm that the mean ant attendance of plants in the two groups did not differ prior to the experiment.

Ant density and caterpillar deterrence

To test the hypothesis that plants with greater densities of ants per leaf are better protected against herbivores, caterpillar disappearance rates (mortality + emigration) were compared among plants. Seventeen second-instar larvae were added to each of 30 seedlings on 6 July 2000, and all plants were surveyed for caterpillars 48 h later. Larvae molted to the third instar during this period, and this density is within the range observed in naturally occurring aggregations for these instars (mean aggregation size and SD: second instar = 17.2€16.1 (n=20); third instar = 6.2€6.2 (n=38)). Caterpillars were added to randomly-selected leaves and the main stem. Plants were inspected from top to bottom, and the total number of larvae remaining on each plant was recorded. The numbers of ants on 15 randomly-selected leaves were also counted on each plant at that time (1900 hours). Using these ant counts, the 30 plants were divided into three groups: low (mean ant density per leaf <0.07, n=10), intermediate (mean ant density per leaf €SE=0.28€03, range: 0.13–0.53 ants leaf⁻¹, n=15) and high ant density (mean: 1.09€15; range: 0.8–1.67, n=5). A General Linear Model (GLM) was used to compare caterpillar disappearance rates among these three groups.

Fifth instar larvae were not used in this experiment because they abandon the host plant to pupate (Baerg 1935), and count data could not distinguish this absence from predation. Third instars originally damage leaves with low ant attendance, and ant attendance at damaged leaves and plants increases within 24 h after attack (Ness 2001). These responses are qualitatively similar to those of, and elicited by, later instars (see below).

Nectar and ants

The amount of sugar produced in the extrafloral nectar varied greatly among leaves (mean € SD=259€522 g/36h, range: 0–2,495 g), as did the mean number of ants observed on each leaf (mean € SD=0.95€1.23, range: 0–6.28). Glucose, fructose and sucrose accounted for 31%€10, 19%€10, and 23%€14 (mean € SD), respectively, of the total sugars by mass. The remaining sugars included rutinose (sporadically) and a variety of unidentified trisaccharides. Thin layer chromatography suggested that trace amounts of alanine, glycine, glutamine, serine, threonine, valine, and aspartic acid were also present in the nectars. Mean ant attendance per leaf among all leaves was positively correlated with production of total sugars ($r^2=0.45$, $n=60$, $P<0.0001$), sucrose ($r^2=0.43$, $P<0.0001$),

fructose ($r^2=0.40$, $P<0.0001$) and glucose ($r^2=0.28$, $P<0.0001$). Ants were approximately twice as abundant in the late afternoon as at noon, although the relative abundance of ants among individual leaves was consistent. There was a strong positive correlation between the number of ants observed on a leaf in any one survey with the mean number of ants observed on that leaf during the other surveys (mean r^2 €SD=0.63€0.12, range: 0.43–0.76, $P<0.0001$ in all cases), suggesting the “snap-shots” provided an accurate estimate of relative ant attendance over longer time periods.

Comparisons among all sampled leaves may be misleading, because sugar production rates per leaf varied among branches (GLM, $F=4.56$, $df=14$, 37 , $P<0.0001$) and plants ($F=2.32$, $df=10$, 42 , $P=0.028$) and branches differ in their proximity to ant colonies. Within the 15 branches, mean ant attendance at the leaves with higher total sugar production rates was significantly greater than that of the leaves with lower rates (paired t-test: $t = 3.03$, $df=14$, $P<0.005$). The difference in total combined sugar production rates per leaf between seedlings and large trees, however, was not statistically significant (two-sided t-test: $t = 1.52$, $df=3,6$, $P=0.61$).

Caterpillars and nectar

$c^2=8.336$, $P=0.002$; $c^2=10.182$, $P=0.0007$, respectively). The changes in sugar production in undamaged leaves on attacked and unattacked plants were not significantly different (glucose: $c^2=0.023$, $P=0.879$; fructose: $c^2=0.101$, $P=0.749$; sucrose: $c^2=0.1809$, $P=0.671$). The relative ratios of these three sugars did not change appreciably in any of the three treatment groups.

Results were similar when the masses of three sugars were summed and the treatment means from individual plants used as replicates (Fig. 1). The mean change in production was greater in damaged leaves (mean \pm SE=222 \pm 141 g, $n=5$) than in undamaged leaves on attacked plants (-145 \pm 81 g, $n=6$) and undamaged leaves on unattacked plants (-27 \pm 47 g, $n=5$). Comparisons of damaged leaves with undamaged leaves on attacked and unattacked plants indicated these differences were significant (one-sided Wilcoxon test: $c^2=4.82$, $P=0.014$ and $c^2=3.15$, $P=0.038$, respectively), although the latter groups did not differ ($c^2=0.536$, $P=0.464$).

Caterpillars and ants: intra-plant comparisons

sucrose increased on caterpillar-damaged leaves ($n=14$), a response not observed in neighboring undamaged leaves on attacked plants ($n=19$) (one-sided Wilcoxon test: $c^2=10.04$, $P=0.0007$; $c^2=8.07$, $P=0.003$; $c^2=8.705$, $P=0.002$, respectively). Fig. 1 hints that increased sugar excretion at damaged leaves could come at the expense of decreased excretion at neighboring undamaged leaves. The mean changes in sugar production on damaged leaves within a branch, however, were not significantly correlated with mean changes in production in neighboring undamaged leaves on those same branches (regression analyses; sucrose: $F=1.54$, $n=10$, $P=0.261$; glucose: $F=1.23$, $P=0.309$; fructose: $F=0.598$, $P=0.469$). The non-significant trends were positive for all three sugars (i.e., the greatest increases in production at damaged leaves were associated with increases at neighboring leaves, rather than decreases). The increases in glucose, fructose and sucrose production rates at damaged leaves also differed from those observed in undamaged leaves on undamaged plants ($n=18$) (one-sided Wilcoxon test: $c^2=11.43$, $P=0.00035$;

Caterpillars fed upon, and were found on, slightly less than half the marked leaves within individual plants (mean \pm SD: 0.47 \pm 0.12% of marked leaves). These leaves had lower levels of ant attendance prior to caterpillar introduction (day 0, Fig. 2). Ant attendance on leaves previously damaged by caterpillars increased within 24 h of the arrival of caterpillars, and at that point exceeded the attendance observed on unoccupied leaves. RA was significantly <1 prior to caterpillar occupation (day 0), and >1 on days 1, 2, and 5 ($P<0.05$ in all 4 cases). The

at scissor-damaged versus control leaves (RA) over the six post-treatment surveys was 1.19 ± 0.24 (mean \pm SE)

plants over this long time period (weeks), as nectar production at damaged and undamaged leaves was only measured over a 36-h duration (and on other plants). Herbivory induces increases in nectar production at both damaged and undamaged leaves in cotton, although the latter response was delayed (Wickers et al. 2001). Induction in *Catalpa bignonioides* may occur in a similar fashion, albeit with a delay greater than 36 h after herbivory.

Fewer herbivores remained on plants that attracted more ants. Smaller caterpillars (1–3 instars) are attacked by *F. pruinosa* ants and carried back to the nest, although later instars (4–5) are harassed but rarely killed (J. Ness, personal observation). Plant protection and bodyguard density have been linked in both ant-plant systems (Rocha and Bergallo 1992; Madden and Young 1992; Heil et al. 2006).

Stephenson AG (1982) The role of extrafloral nectaries of *Catalpa speciosa*