From leaves to defenders: how amount and dispersion of leaf damage affect extrafloral nectar production and ant-mediated protection in wild cotton (*Gossypium hirsutum*)

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Abstract

Extrafloral nectar (EFN) drives mutualistic interactions between plants and ants. However, EFN production is costly and its induction is predicted to vary with herbivory-related factors. This study examines how the amount and within-plant damage variability (i.e., damage uniformity within the plant canopy) affect EFN production and ant-mediated defence in wild cotton (*Gossypium hirsutum*). Previous work suggests that severe and concentrated damage is costliest to plants, leading us to hypothesize that such damage would induce greater EFN production and, consequently, stronger ant attraction and protection. We conducted a greenhouse experiment in which plants were subjected to one of the following mechanical damage treatments: control (no damage), low damage (two damaged leaves, 30% of area removed each), high concentrated damage (two damaged leaves, 60% each), and high dispersed damage (four damaged leaves, 30% each; i.e., same total area than concentrated). The day after treatments, we measured EFN volume and sugar content from one nectary per plant (a measure of per-nectary EFN response), and the number of secreting nectaries per plant (a proxy for whole-plant level EFN response). Subsequently, we performed a field experiment using the same treatments to evaluate EFN-mediated ant recruitment and ant-provided protection by placing caterpillars on plants. Both high-damage treatments increased EFN volume per nectary, whereas low damage did not. However, dispersed damage was the only treatment that increased the number of secreting nectaries. In the field, plants with dispersed damage recruited more ants and exhibited a higher probability of caterpillar attack by ants. Contrary to expectations, plants subjected to dispersed damage exhibited a greater total investment in EFN, presumably via local induction of more nectaries, enhancing ant-mediated defence. This study highlights the importance of herbivory intensity and uniformity patterns in shaping ant-plant interactions.

Keywords: ant-plant interactions, predation, mutualism, secondary defence, induced defence

Introduction

Extrafloral nectaries are specialised structures present in more than 100 families of plants (Weber and Keeler 2012) that secrete a sugary, energy-rich solution. This secretion can occur constitutively on leaves and stems but is also highly inducible in response to herbivory (Rico-Gray and Oliveira 2008, Heil 2015). Extrafloral nectar (EFN) serves as an important food source for ants and other insects, which often, in turn, protect the plant by deterring or attacking herbivores (Heil and McKey 2003, Rico-Gray and Oliveira 2008, Rosumek et al. 2009). However, EFN induction is highly context-dependent, shaped by both biotic and abiotic factors, and modulated by the balance of its benefits (via indirect defence) and costs to the plant across ecological conditions (Bronstein 1998, Bronstein et al. 2006, Kessler and Heil 2011, Marazzi et al. 2013). Addressing this context dependency is key for understanding the ecological relevance and evolutionary stability of EFN-mediated mutualisms.

Multiple herbivory-related factors, including the amount of herbivore damage, regulate the secretion of EFN. In many cases, EFN induction increases in a dose-dependent manner with the extent of herbivory (Rios et al. 2008, Xu et al. 2014, Huang et al. 2015, Calixto et al. 2021), suggesting that plants can fine-tune their defensive investment based on perceived herbivore pressure. For instance, higher levels of leaf damage often trigger increased EFN production, which in turn attracts more ant defenders to help mitigate further herbivory (Heil et al. 2001, Ness 2003, Nogueira 2025). However, this relationship is not always linear. Some studies have documented threshold effects, where EFN secretion significantly increases only after damage surpasses a certain level (Pimenta et al. 2025). Similar patterns have been shown for other plant defensive traits, such as chemical defences, where induction thresholds and saturation limits can constrain response intensity (e.g. Underwood 2010). These findings suggest that induction of

plant defences, including EFN production, operates within physiological or ecological limits that may plateau above certain thresholds. Research on EFN responses to varying levels of herbivory intensities has expanded in recent years (e.g., Xu et al. 2014, Abdala-Roberts et al. 2019, Calixto et al. 2021, Pimenta et al. 2025), yet further studies are needed to clarify how these patterns influence ant-plant interactions.

The uniformity of herbivore damage within a plant can also play a crucial role in shaping plant fitness and the induction of defensive traits (Wetzel et al. 2023). Early work suggests that plants tolerate dispersed damage better than concentrated damage, implying higher fitness costs associated with the latter (Marquis 1992, 1996, Mauricio et al. 1993). The reduced tolerance to concentrated damage likely stems from the modular nature of plants and their limited capacity for resource exchange between modules. When damage is concentrated on specific branches, resource limitations in those areas cannot easily be compensated for by other parts of the plant, leading to localised stress and reduced whole-plant level growth (Marquis 1996). In accordance, Optimal Defence Theory predicts that plants should induce stronger defensive responses under concentrated damage compared to dispersed damage, in order to offset the greater cost associated with more localised herbivory (Holland et al. 2009, Moreira et al. 2012, Calixto et al. 2021). However, most research to date has focused on tolerance mechanisms associated with sectoriality and resource reallocation to explain these patterns, rather than examining how damage uniformity affects the induction of resistance traits (but see Ohnmeiss et al. 1997). This knowledge gap is especially pronounced in the case of EFN, for which, to our knowledge, no study has explicitly tested how within-plant damage variability influences EFN induction and subsequent ant responses, or disentangled these effects from other herbivory-related factors (e.g., total damage amount).

Here, we used wild cotton (*Gossypium hirsutum*), an extrafloral nectary-bearing, myrmecophytic shrub distributed along the northern coastline of Yucatan (Mexico), as a study model to evaluate the effect of leaf damage amount and within-plant dispersion on the production of EFN. We also examined how these induction patterns influence ant recruitment, as well as deterrent or consumptive effects of ants on herbivores. Previous work with wild cotton has shown that EFN induction enhances ant recruitment and defensive responses against herbivores (Vázquez-Barrios et al. 2021, Díaz-Cruz under review), suggesting that EFN can provide fitness benefits via indirect defence, though not universally (Reyes-Hernández et al. 2022). Wild cotton plants also show limited ability to compensate (in terms of regrowth) after both high levels of damage and damage that is concentrated rather than dispersed (Quijano-Medina et al. 2021, Ramírez-Delgado et al. 2025), implying higher fitness costs under these herbivory scenarios. Based on this, we hypothesised that high and concentrated damage would elicit stronger EFN induction, leading in turn to greater ant recruitment and increased aggressive behaviour toward herbivores. By disentangling the effects of herbivory intensity and within-plant variability on EFN induction and ant-plant interactions, this study provides novel insights into how herbivory-related factors may shape indirect plant defence.

Methods

Study species

Wild cotton (*Gossypium hirsutum*) is a myrmecophytic shrub native to Mexico, Central America, and the Caribbean, particularly abundant in the Yucatan Peninsula (Coppens d'Eeckenbrugge and Lacape 2014). Leaf chewers, such as grasshoppers and caterpillars, cause most of the damage to this species. However, leaf damage exhibits considerable variability —up to 3.5-fold difference among plants within populations, and up to three-fold variation in damage distribution

across leaves within individual plants (Abdala-Roberts et al. 2019a). In this context, high and concentrated damage appear to be more costly for wild cotton plants (Quijano-Medina et al. 2019, Ramírez-Delgado et al. 2025). Wild cotton secretes EFN from specialised nectaries located along the mid-vein and sometimes on secondary veins. Several studies have shown that EFN production in wild cotton increases primarily in response to mechanical leaf damage (Abdala-Roberts et al. 2019b, Vázquez-Barrios et al. 2021, Briones-May et al. 2023), with similar findings reported for domesticated cotton (Wäckers and Bonifay 2004, Llandres et al. 2019). A diverse ant community is associated with wild cotton in situ, including species of *Camponotus*, *Pseudomyrmex, Monomorium, Forelius, Solenopsis, Brachymyrmex*, *Dorymyrmex*, and *Crematogaster* (Vázquez-Barrios et al. 2021, S. Díaz-Cruz, unpublished data, data from this study). Many of these ant species consume EFN and can play a defensive role by deterring or consuming insect herbivores (Vázquez-Barrios et al. 2021, Díaz-Cruz et al. in review; data from this study). In a previous work, Abdala-Roberts et al. (2019b) found that higher levels of damage more strongly induced EFN, but only under recurrent damage events. To date, the effects of within-plant damage variability on EFN induction have not been tested or disentangled from the effects of damage amount.

Plant material and propagation

We collected seeds from seven adult plants (hereafter referred to as genotypes) located at a coastal scrubland site near the port of Sisal, Yucatán, Mexico (21°11'45.3" N, 89°57'18" W). We germinated seeds and transplanted them into 2-litre polyethene bags filled with a soil mixture of sand, native deciduous forest soil, and perlite (1:2:1). We maintained plants in a greenhouse at the Campus de Ciencias Biológicas y Agropecuarias of the Universidad Autónoma de Yucatán (20°51'57" N, 89°37'20" W). Plants were watered three times per week with 300 ml of water. At the time the experiments were conducted, the plants were four months old.

Greenhouse experiment: treatment effects on extrafloral nectar

We used 79 plants to evaluate the effects of leaf damage treatments on EFN induction. We placed plants 90 cm apart and subjected them to one of the following mechanical damage treatments using serrated scissors, with genotypes evenly represented across groups: a) control: no leaves were damaged; b) low damage: one side was removed from each of two leaves (leaves 2 and 4, counting from the apical meristem); c) high concentrated damage: two sides were removed from each of two leaves (2 and 4); and d) high dispersed damage: one side was removed from each of four leaves (2 to 5). Damage treatments were applied over two consecutive days. On the first day, we removed 15% of the area per leaf by cutting one side of each of two or four leaves per plant for low and high dispersed damage treatments, respectively or 15% of the area on both sides of each of two leaves per plant for high concentrated damage treatment. The following day, we removed an additional 15% from each previously damaged side of the same leaves. As a result, plants in the low and high dispersed damage treatments had two or four leaves damaged, respectively, with 30% area removed per leaf in both cases. In contrast, plants in the high concentrated damage treatment had two leaves damaged, each with 60% of the area removed. Thus, highly damaged plants experienced twice the total amount of damage compared to low-damaged plants. At the same time, the two high-damage treatments differed only in the uniformity of damage, not in total damage amount (Fig. S1, supplementary material). Comparisons between low- and high-damage treatments (which had the same total damage per plant) and between each of these and the control group provided insights into the effect of within-plant damage variability (Martín-Cacheda et al. 2025). It is important to note that in cotton, mechanical damage alone is sufficient to elicit EFN induction of a magnitude comparable to that caused by natural herbivory (Wäckers and Wunderlin 1999, J. Interian-Aguiñaga, unpublished data). This includes induction levels similar to those observed under

varying degrees of damage amount and within-plant damage dispersion caused by natural herbivory (Ramírez-Delgado et al. 2025). Thus, mechanical damage provides a realistic proxy for testing EFN defence induction, while offering greater precision and control over the amount and distribution of leaf damage (Abdala-Roberts et al. 2019b).

On the morning following the completion of damage treatments, between 06:00 and 07:00, we collected EFN from leaf 2 of each plant using 5-µL capillary tubes (micropipettes Blaubrand ® intraMARK, colour code white, Germany). Twenty-four hours prior to these nectar collections, we cleaned all nectaries with water and paper to remove any previously produced nectar, ensuring comparable measurements. We selected leaf two as it was the most apical leaf with damage, and EFN induction is typically strongest in younger leaves and at the site of damage (i.e., damaged vs. undamaged leaves on damaged plants; Abdala-Roberts et al. 2019b). After measuring the EFN volume collected in the capillary tubes, we determined sugar concentration (° Brix) using a refractometer (Atago Master, Germany). We then calculated the total sugar produced per sample as: total sugar = nectar volume × nectar sugar concentration (° Brix). One-degree Brix (1 ° Brix) is equivalent to 1 gram of sugar dissolved in 100 grams of solution (Kimball 1991). Finally, we recorded the number of active (secreting) nectaries per leaf by visual inspection. To ensure that nectaries classified as inactive were truly not secreting, we gently probed each one with a capillary tube to confirm the absence of EFN. Thus, in addition to per-nectary responses reflecting local induction (volume, sugars), the number of secreting nectaries provided a measure of whole-plant response, involving not only local but likely also systemic induction (EFN produced by non-damaged leaves).

Field experiment: treatment effects on ant recruitment and their anti-herbivore responses

A week after the greenhouse experiment, we transported a separate group of 101 plants (of the same age as those used in the greenhouse experiment) to the coastal shrubland near Sisal,

Yucatán. We placed plants at a site nearby where the wild cotton mother plants had been originally collected. Plants were spaced 1 meter apart in an open area surrounded by natural vegetation. We assigned plants randomly to the same leaf damage treatments described above, using identical methodology, with genotypes evenly represented across treatment groups. Throughout the experiment, we watered the plants twice a week. Two days after transplantation, we applied the mechanical damage treatments. The following morning, we surveyed ant abundance between 9:00 and 11:00 AM over two consecutive days. We observed each plant for two minutes, and the number of ants present was recorded. When needed, we collected ant specimens for taxonomic identification in the laboratory. The day after the second ant survey, we conducted ant attack bioassays using caterpillars over two consecutive days. Each morning at 9:00 AM, we placed a freeze-killed third- or fourth-instar caterpillar of the specialist *Alabama argillacea* on each plant, gently securing it to an apical leaf with a metal clip. The use of dead caterpillars allowed us to standardise their position and eliminate behavioural and chemical variability associated with live caterpillars. However, this approach also removed dynamic interactions that are typical of live prey (Nagy et al. 2020). We observed no saprophagous insects on the plants during the bioassays. We inspected each plant 100 minutes after caterpillar placement to determine whether the caterpillar had been attacked (scored as 1) or not attacked (scored as 0). An attack was defined as any instance in which one or more ants made direct contact with the caterpillar and exhibited aggressive behaviour or attempted to remove it. Cases in which the caterpillar was missing for unknown reasons (< 6% of observations) were excluded from statistical analyses.

Statistical analyses

We used generalised linear mixed models (GLMMs) to analyse nectar volume, total sugar production, the proportion of active nectaries (number of leaves with active nectaries relative to

the total number of leaves per plant), the number of patrolling ants, and the probability of ant attack. In all models, we included treatment as the main fixed effect and plant height as a covariate. We included plant genotype as a random effect to account for genetic or maternal effects. For the models analysing ant abundance and probability of attack, we also included survey day and plant ID as random factors to account for repeated measures taken on the same plants across sampling days. For nectar volume and total sugar production, we used a Tweedie distribution with a log link function, which is suitable for continuous, non-negative, and right-skewed data that include zeros (Hasan and Dunn 2015). The proportion of active nectaries was analysed using a beta distribution with a logit link function. We modelled ant abundance using a negative binomial distribution with a log link function to account for overdispersion. Finally, we modelled the probability of ant attack using a binomial distribution with a logit link function.

All statistical analyses were performed in R 4.4.2 (R Core Team 2024). We used the "glmmTMB" package (Brooks et al. 2017) to fit the GLMMs, and the "DHARMa" package (Hartig 2024) to evaluate models fit. To test for main effects, we employed the *Anova*() function from the "car" package (Fox and Weisberg 2019), using a Wald $\chi 2$ tests. When treatment effects were significant, we used the "emmeans″ package (Lenth 2024) to perform Tukey pairwise comparisons between groups and to extract back-transformed estimated marginal means (EMMs) with their 95% confidence intervals (95% CIs). We visualised these results using the "ggplot2" package (Wickham 2016). Model estimates are reported throughout the Results section as EMMs with 95% CI, in the format: EMM [2.5% lower CI, 97.5% upper CI].

Results

Extrafloral nectar

Results from the greenhouse experiment showed that leaf damage significantly influenced EFN volume (Table 1). Both high damage treatments—concentrated (0.589 [0.409, 0.850] μL) and dispersed (0.694 [0.479, 1.01] μL)—resulted in significantly greater EFN volume produced compared to the control group (0.197 [0.107, 0.36] μL) (Fig. 1a). The low damage treatment (0.457 [0.3, 0.697] μL) did not differ significantly from either the control or the high damage treatments (Fig. 1a). Leaf damage also had a significant effect on total sugar production (Table 1). In this case, all damage treatments—high concentrated (0.140 [0.097, 0.2] mg), high dispersed (0.162 [0.113, 0.233] mg), and low (0.115 [0.077, 0.172] mg)—produced significantly more sugar than the control group (0.036 [0.019, 0.069] mg), with no significant differences among the damage treatments themselves (Fig. 1b). Finally, the proportion of secreting nectaries was also significantly affected by leaf damage (Table 1). However, only the high dispersed damage treatment (0.527 [0.454, 0.599]) differed significantly from the control (0.288 [0.226, 0.359]), low (0.378 [0.312, 0.449]), and high concentrated (0.355 [0.294, 0.421]) damage treatments (Fig. 1c). Statistics for Tukey pairwise comparisons for all nectar responses are presented in Table S1 A-C, of the supplementary material.

Ant recruitment and anti-herbivore defensive response

We identified 10 ant species on experimental cotton plants, with *Monomorium ebeninum*, *Brachymyrmex australis*, and *Forelius pruinosus* being the most abundant (Table S2, Supplementary Material). The leaf damage treatment significantly affected ant abundance (Table 1). Specifically, the high dispersed damage treatment attracted significantly more ants (5.45 [3.86, 7.7]) than the control (2.33 [1.61, 3.35]) and high concentrated damage (2.45 [1.69, 3.55]) treatments (Fig. 2a; Table S1). This pattern extended to defensive behaviours. Caterpillars on plants with high dispersed damage had the highest probability of being attacked by ants (0.886 [0.713, 0.961]), which was significantly greater than the probability of attack in the control (0.442 [0.27,

0.629]) and low damage (0.499 [0.293, 0.705]) treatments. The attack probability for the high concentrated damage treatment (0.629 [0.429, 0.794]) was intermediate and did not differ significantly from any other treatment group (Fig. 2b; Table S1).

Discussion

Effects of damage amount on EFN induction by wild cotton were relatively weak and limited to EFN volume. Only plants subjected to high damage treatments, but not those subjected to low damage, differed from control plants (Fig. 1a). We did not find a significant difference between low and high damage treatments. In contrast, differences in within-plant dispersion of damage had a more apparent effect, particularly on the proportion of secreting nectaries; only plants in the high dispersed damage group differed significantly from controls and all other treatment groups. Consistently, these plants also recruited more ants and exhibited higher ant attack rates on caterpillars. Overall, our findings suggest that within-plant variability in damage variability, rather than total damage amount, is the key driver of ant recruitment and indirect defence via EFN induction. These results highlight a previously underappreciated role of variability in herbivore damage in shaping ant-plant mutualisms and call for greater attention to how herbivory patterns influence the induction of plant defences

Extrafloral nectar

Leaf damage amount has been shown to correlate positively with EFN production in other systems (Rios et al. 2008, Xu et al. 2014, Calixto et al. 2021), suggesting that plants optimise their defensive strategies in response to perceived herbivore threats (e.g., as a function of herbivory severity). Our findings provide partial support for such patterns: both high damage treatments, but not the low damage treatment, resulted in significantly greater EFN volume

compared to controls. However, this effect was relatively weak, as there was no significant difference between the low and high damage treatments. Moreover, all three damage treatments significantly increased total sugar production relative to controls, indicating no clear influence of herbivory amount on sugar output. Similar results have been observed for the shrub *Banisteriopsis malifolia*, where leaf damage increases sugar secretion, but the response remains constant regardless of damage severity (Pimenta et al. 2025). Interestingly, sugar production did not decrease despite the higher EFN volumes observed under high damage treatments Fig. 1b). This suggests active secretion of additional carbohydrates in response to damage, preventing a dilution effect (Abdala-Roberts et al. 2019b). Nonetheless, the induction levels may not have been strong enough to produce a measurable difference in sugar content between low and high damage treatments.

Previous work on wild cotton found no effect of damage amount on EFN induction (Abdala-Roberts et al. 2019b). However, methodological differences, such as the use of overall lower damage levels and the combination of mechanical damage with oral secretions from *Spodoptera frugiperda* (a generalist herbivore), warrant caution when comparing results. Elicitor compounds in *S. frugiperda* oral secretions may have triggered different plant responses. Importantly, a recent study using a similar experimental design but applying natural herbivory by the specialist *Alabama argillacea* found that dispersed herbivory induced more EFN on damaged leaves than either low or high concentrated herbivory (Ramírez-Delgado et al. 2025). Collectively, findings across both natural and mechanical damage systems and across varying levels of leaf damage suggest that the amount of mechanical damage alone is not a strong predictor of EFN induction. That said, further experimentation is needed to better assess the role of herbivory amount in this system. Specifically, studies incorporating a broader and more finely graduated

range of damage levels could help clarify potential herbivore density-dependent responses in EFN induction.

Our results revealed that within-plant variability in damage significantly affected EFN induction, specifically by altering the proportion of secreting nectaries (Fig. 1c). Plants subjected to high dispersed damage were the only treatment group that differed significantly from the control and other damage treatments in this respect. This effect on EFN production appears to operate at the whole-plant level, potentially through two mechanisms. First, high dispersed damage may have locally induced a greater number of nectaries (i.e., on more damaged leaves), while maintaining similar EFN production per nectary compared to other damage treatments (Wäckers et al. 2001). Second, dispersed damage may have triggered a stronger systemic response, leading to increased nectar production in undamaged leaves. Supporting the latter, we observed that all plants treated in the high dispersed damage treatment exhibited nectar secretion in non-damaged leaves.

These findings suggest that dispersed leaf damage patterns provide a more reliable or stronger risk signal throughout the plant, triggering a more robust and sustained investment in EFN production. Several mechanisms could explain this response, including inter-plant communication via volatile compounds (Hernández-Zepeda et al. 2018) or signal transport through the xylem, which may reach undamaged leaves and upregulate nectar production (Chatt et al. 2021). However, these explanations remain speculative at this stage. Future research that records quantitative data per nectary to estimate total EFN investment would provide valuable insights into the complexity of systemic EFN responses to damage variability. Additionally, targeted experiments are needed to evaluate the proposed mechanistic hypotheses and clarify their role in systemic EFN induction.

Ant abundance and anti-herbivore response

The ant species recorded on wild cotton during the field experiment have been previously reported on the species, though some differences in composition and relative abundance were observed. For example, Reyes-Hernández et al. (2022) reported high abundances of *Crematogaster* on wild cotton in a nearby tropical forest, while Vázquez-Barrios et al. (2021) found *Camponotus planatus* and *Paratrechina longicornis* dominating in a coastal shrubland. In contrast, our study recorded high abundances of *Monomorium ebeninum*, *Brachymyrmex australis*, and *Forelius pruinosus*. These small-bodied species are not strictly predatory (Díaz-Castelazo et al. 2004). However, their persistent presence on plants may discourage herbivores through disturbance or harassment, potentially leading to reduced overall damage (Bentley 1977, Rudgers et al. 2003). Larger, more aggressive species, including predatory ants in the genera *Pseudomyrmex* and *Camponotus*, were much less abundant but have been previously documented on wild cotton (Vázquez-Barrios et al. 2021). Habitat ant competitive dynamics and the availability of alternative plant resources or prey may influence ant community composition (Byk and Del-Claro 2011, Nogueira et al. 2015) and warrant further examination across multiple wild cotton populations.

Mirroring the treatment effects on the proportion of secreting nectaries, ant abundance was highest on plants subjected to high dispersed damage (Fig. 2a). This was likely due to an increased availability of secreting nectaries per plant under this treatment, which enhanced ant recruitment and patrolling activity. Consistent with this finding, attack rates on caterpillars were highest on plants subjected to dispersed damage, suggesting increased ant abundance led to higher attack rates, i.e., a density-mediated effect (Abdala-Roberts et al. 2012). At the same time, it is also possible that dispersed damage increased ant aggressiveness or foraging efficiency (Ness 2003, Ness et al. 2006, Shenoy et al. 2012), independently of the numerical response observed (i.e., a trait-mediated effect; Abdala-Roberts and Mooney 2015). Therefore, a key challenge for future work will be to disentangle these two potential mechanisms to better understand

the interaction mechanisms and the evolution of ant-plant interactions (EFN induction), as well as the effects on top-down control of herbivores on wild cotton.

Mechanism for cotton defence evolution and future work

Our results should be interpreted within the broader context of cotton's extensive defensive arsenal, including its direct chemical defences. Concentrated herbivory typically imposes greater fitness costs on plants (Marquis 1992, Wetzel et al. 2023), a notion that is consistent with our findings for wild cotton (Ramírez-Delgado et al. 2025). We predict that direct chemical defences (e.g., phenolics, terpene aldehydes) should be more strongly induced under such attacks. In theory, this would not only reduce herbivory but also promote its spread from leaf-to-leaf, mitigating the high costs of localised damage (Marquis 1996, Karban and Yang 2020). This dynamic could create a positive feedback loop: selection for direct chemical defences to counter concentrated damage becomes linked with selection for increased EFN allocation under dispersed damage. However, we speculate that physiological trade-offs, particularly under abiotic stress (Quijano-Medina et al. 2021), could weaken this linkage. Current research is aimed at testing the effects of herbivory variability on both chemical defences and EFN, as well as assessing fitness costs of different herbivory patterns (Ramírez-Delgado et al. 2025). This will allow us to expand and evaluate these responses across populations that experience different ecological conditions (e.g., varying ant composition, herbivore pressure, and abiotic stress).

We acknowledge that our study captures the short-term consequences of herbivory on ant-mediated protection, rather than longer-term ecological dynamics. EFN induction can be rapid but transient (e.g., Wäckers et al. 2001; Jones and Koptur 2015; Yamawo and Suzuki 2018; Calixto et al. 2023), and the typically low and variable herbivory pressure at sites with wild cotton (Abdala-Roberts et al. 2019a) could destabilise this mutualism over time (Rudgers and Strauss 2004). Therefore, future research should extend over longer durations and across

multiple sites to evaluate these interactions robustly. Such work must integrate responses of both EFNs and ants, direct chemical defences, and ambient herbivory rates, while also dissecting the underlying mechanisms (e.g., trait- vs. density-mediated effects).

In conclusion, our results highlight the critical role of within-plant herbivory variation in shaping plant defensive strategies and mutualistic interactions. To the best of our knowledge, this is the first study to demonstrate that differences in within-plant uniformity damage affect EFN induction, with significant consequences for ant recruitment and indirect defence. These findings underscore the complexity of plant-insect multitrophic interactions in natural ecosystems and emphasise the need to understand how plants optimise their defensive investments across varying herbivory contexts in order to maximise the benefits derived from mutualistic interactions with predators.

Significance statement

We feel that our manuscript, "From leaves to defenders: how leaf damage amount and within-plant variability influence extrafloral nectar production and ant-mediated defence in wild cotton (*Gossypium hirsutum*)," is a god fit for *Oikos* because it presents a significant conceptual advance in understanding the ecological mechanisms governing plant-insect mutualisms. We challenge a central paradigm in induced defence research by demonstrating that the spatial pattern of herbivory within a plant can be a more critical driver of indirect defence than the total amount of damage. This work builds directly upon previous research by our group and others, which established that wild cotton suffers higher fitness costs from concentrated damage and that extrafloral nectar can mediate defence. However, the link between damage patterns and extrafloral nectar induction remained an explicit knowledge gap. By addressing this gap, our study offers a more nuanced perspective on plant defensive strategies, aligning with *Oikos*'s mission to publish research that substantially improves our understanding of ecological patterns and processes.

Data archiving statement

The data and analysis scripts for this study have been deposited in the Zenodo repository and are available at [DOI]

Conflict of interest statement

The authors declare no conflict of interest.

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References

Abdala-Roberts, L. and Mooney, K. A. 2015. Plant and herbivore evolution within the trophic sandwich. - In: Hanley, T. and La Pierre, K. (eds), Trophic interactions: bottom-up and top-down interactions in aquatic and terrestrial ecosystems. Cambridge University Press, pp. 340–364.

Abdala-Roberts, L., Quijano-Medina, T., Moreira, X., Vázquez-González, C., Parra-Tabla, V., Berny Mier Y Terán, J. C., Grandi, L., Glauser, G., Turlings, T. C. J. and Benrey, B. 2019a. Bottom-up control of geographic variation in insect herbivory on wild cotton (*Gossypium hirsutum*) by plant defenses and climate. - Am. J. Bot. 106: 1059–1067.

Abdala-Roberts, L., Reyes-Hernández, M., Quijano-Medina, T., Moreira, X., Francisco, M., Angulo, D. F., Parra-Tabla, V., Virgen, A. and Rojas, J. C. 2019b. Effects of amount and recurrence of leaf herbivory on the induction of direct and indirect defences in wild cotton. - Plant Biol. 21: 1063–1071.

Bentley, B. L. 1977. Extrafloral nectaries and protection by pugnacious bodyguards. - Annu. Rev. Ecol. Syst. 8: 407–427.

Briones-May, Y., Quijano-Medina, T., Perez-Nino, B., Benrey, B., Turlings, T. C. J., Bustos-Segura, C. and Abdala-Roberts, L. 2023. Soil salinization disrupts plant-plant signaling effects on extra-floral nectar induction in wild cotton. - Oecologia 202: 313–323.

Bronstein, J. 1998. The contribution of ant plant protection studies to our understanding of mutualism. - Biotropica 30: 150–161.

Bronstein, J. L., Alarcon, R. and Geber, M. 2006. The evolution of plant-insect mutualisms. - New Phytol. 172: 412–428.

Brooks, M. E., Kristensen, K., Benthem, K. J. van, Magnusson, A., Berg, C. W., Nielsen, A., Skaug, H. J., Maechler, M. and Bolker, B. M. 2017. glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. - R J. 9: 378–400.

Byk, J. and Del-Claro, K. 2011. Ant–plant interaction in the Neotropical savanna: direct beneficial effects of extrafloral nectar on ant colony fitness. - Popul. Ecol. 53: 327–332.

Calixto, E. S., Lange, D., Bronstein, J., Torezan-Silingardi, H. M. and Del-Claro, K. 2021. Optimal defense theory in an ant–plant mutualism: extrafloral nectar as an induced defence is maximized in the most valuable plant structures. - J. Ecol. 109: 167–178.

Calixto, E. S., Del-Claro, K., Lange, D. and Bronstein, J. 2023. Time course of inducibility of indirect responses in an ant-defended plant. - Ecol. 104: e4029.

Chatt, E. C., Mahalim, S.-N., Mohd-Fadzil, N.-A., Roy, R., Klinkenberg, P. M., Horner, H. T., Hampton, M., Carter, C. J. and Nikolau, B. J. 2021. Nectar biosynthesis is conserved among floral and extrafloral nectaries. – Plant Physiol. 185: 1595–1616.

Coppens d'Eeckenbrugge, G. and Lacape, J.-M. 2014. Distribution and differentiation of wild, feral, and cultivated populations of perennial upland cotton (*Gossypium hirsutum* L.) in Mesoamerica and the Caribbean. - PLoS ONE 9: 1–19.

Díaz-Castelazo, C., Rico-Gray, V., Oliveira, P. and Cuautle, M. 2004. Extrafloral nectary-mediated ant-plant interactions in the coastal vegetation of Veracruz, Mexico: Richness, occurrence, seasonality, and ant foraging patterns. - Écoscience 11: 472–481.

Díaz-Cruz, S., Turlings, Ted. C. J., Solís-Rodríguez, U., Interian-Aguiñaga, J., Ramírez-Delgado, V. H., Clancy, M. V., Mamin, M., Wendel, J. F., Grover, C. E., Arick II, M. A., Hsu, C.-Y., Pechanova, O., Thrash, A., Peterson, D. G., Bustos-Segura, C. and Abdala-Roberts, L. Tri-trophic consequences of plant-to-plant volatile signaling and its contingency on plant relatedness in wild cotton. - Under review.

Fox, J. and Weisberg, S. 2019. An R Companion to Applied Regression. - Sage.

Hartig, F. 2024. DHARMa: Residual Diagnostics for Hierarchical (Multi-Level / Mixed) Regression Models.

Hasan, M. M. and Dunn, P. K. 2015. Seasonal rainfall totals of Australian stations can be modelled with distributions from the Tweedie family. - Int. J. Climatol. 35: 3093–3101.

Heil, M. 2015. Extrafloral nectar at the plant-insect interface: a spotlight on chemical ecology, phenotypic plasticity, and food webs. - Annu. Rev. Entomol. 60: 213–232.

Heil, M. and McKey, D. 2003. Protective ant-plant interactions as model systems in ecological and evolutionary research. - Annu. Rev. Ecol. Evol. Syst. 34: 425–553.

Heil, M., Koch, T., Hilpert, A., Fiala, B., Boland, W. and Linsenmair, K. E. 2001. Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius*, is an induced, indirect, defensive response elicited by jasmonic acid. - Proc. Natl. Acad. Sci. 98: 1083–1088.

Hernández-Zepeda, O. F., Razo-Belman, R. and Heil, M. 2018. Reduced responsiveness to volatile signals creates a modular reward provisioning in an obligate food-for-protection mutualism. - Front. Plant Sci. 9: 1076.

Holland, J. N., Chamberlain, S. A. and Horn, K. C. 2009. Optimal defence theory predicts investment in extrafloral nectar resources in an ant–plant mutualism. - J. Ecol. 97: 89–96.

Huang, W., Siemann, E., Carrillo, J. and Ding, J. 2015. Below-ground herbivory limits induction of extrafloral nectar by above-ground herbivores. - Ann. Bot. 115: 841–846.

Jones, I. M. and Koptur, S. 2015. Dynamic extrafloral nectar production: The timing of leaf damage affects the defensive response in *Senna mexicana* var. *chapmanii* (Fabaceae). - Am. J. Bot. 102: 58–66.

Karban, R. and Yang, L. H. 2020. Feeding and damage-induced volatile cues make beetles disperse and produce a more even distribution of damage for sagebrush. - J. Anim. Ecol. 89: 2056–2062.

Kessler, A. and Heil, M. 2011. The multiple faces of indirect defences and their agents of natural selection: Multiple faces of indirect defences. - Funct. Ecol. 25: 348–357.

Kimball, D. A. 1991. Brix and soluble solids. - In: Springer, Dordrecht, pp. 7–33.

Lenth, R. V. 2024. emmeans: Estimated Marginal Means, aka Least-Squares Means.

Llandres, A. L., Verdeny-Vilalta, O., Jean, J., Goebel, F.-R., Seydi, O. and Brévault, T. 2019. Cotton extrafloral nectaries as indirect defence against insect pests. - Basic Appl. Ecol. 37: 24–34.

Marazzi, B., Bronstein, J. L. and Koptur, S. 2013. The diversity, ecology and evolution of extrafloral nectaries: current perspectives and future challenges. - Ann. Bot. 111: 1243–1250.

Marquis, R. J. 1992. A bite is a bite is a bite? Constraints on response to folivory in *Piper arieianum* (Piperaceae). - Ecology 73: 143–152.

Marquis, R. J. 1996. Plant architecture, sectoriality and plant tolerance to herbivores. - Vegetatio 127: 85–97.

Martín-Cacheda, L., Briones-May, Y., Bustos-Segura C., Turlings, T. C. J., Ramírez-Delgado, V. H. and Abdala-Roberts, L. 2025. Effects of herbivory amount and within-plant variability by a specialist caterpillar on volatile emissions mediating inter-plant signalling in wild cotton. - Ecol. Entomol. een.13455: 1–12.

Mauricio, R., Bowers, M. D. and Bazzaz, F. A. 1993. Pattern of leaf damage affects fitness of the annual plant *Raphanus sativus* (Brassicaceae). - Ecology 74: 2066–2071.

Moreira, X., Zas, R. and Sampedro, L. 2012. Differential allocation of constitutive and induced chemical defenses in pine tree juveniles: a test of the optimal defense theory. - PLoS ONE 7: e34006.

Nagy, R. K., Schellhorn, N. A. and Zalucki, M. P. 2020. Fresh, frozen or fake: A comparison of predation rates measured by various types of sentinel prey. - J. Appl. Entomol. 144: 407–416.

Ness, J. 2003. Catalpa bignonioides alters extrafloral nectar production after herbivory and attracts ant bodyguards. - Oecologia 134: 210–218.

Ness, J. H., Morris, W. F. and Bronstein, J. L. 2006. Integrating quality and quantity of mutualistic service to contrast ant species protecting *Ferocactus wislizeni*. - Ecology 87: 912–921.

Nogueira, A., Rey, P. J., Alcántara, J. M., Feitosa, R. M. and Lohmann, L. G. 2015. Geographic mosaic of plant evolution: extrafloral nectary variation mediated by ant and herbivore assemblages. - PLoS ONE 10: e0123806.

Nogueira, R. R. 2025. Mutualistic interactions between plants with extrafloral nectaries and ants: ecological impacts, ant sharing among neighboring plants, and the effects of environmental conditions. - Community Ecol. 26: 437–447.

Ohnmeiss, T. E., McCloud, E. S., Lynds, G. Y. and Baldwin, I. T. 1997. Within-plant relationships among wounding, jasmonic acid, and nicotine implications for defence in *Nicotiana sylvestris*. - New Phytol. 137: 441–452.

Pimenta, I. C. D. O., Calixto, E. S. and Del-Claro, K. 2025. Impacts of leaf damage intensity on ant–plant protection mutualism and plant fitness. - Plants 14: 837.

Quijano-Medina, T., Covelo, F., Moreira, X. and Abdala-Roberts, L. 2019. Compensation to simulated insect leaf herbivory in wild cotton (Gossypium hirsutum): responses to multiple levels of damage and associated traits. - Plant Biol. 21: 805–812.

Quijano-Medina, T., Turlings, T. C. J., Sosenski, P., Grandi, L., Cervera, J. C., Moreira, X. and Abdala-Roberts, L. 2021. Effects of soil salinity on the expression of direct and indirect defences in wild cotton *Gossypium hirsutum*. - J. Ecol. 109: 354–368.

R Core Team 2024. R: A language and environment for statistical computing.

Ramírez-Delgado, V. H., Briones-May, Y., Sánchez-Durán, J., Martín-Cacheda, L., Moreira, X., Turlings, T. C. J. and Abdala-Roberts, L. 2025. Effects of varying amounts and distribution of herbivory on resistance- and tolerance-related responses in wild cotton. - bioRxiv 2025.07.14.664844.

Reyes-Hernández, M., Angulo-Pérez, D., Quijano-Medina, T., Moreira, X., Parra-Tabla, V., Vásquez-Bolaños, M. and Abdala-Roberts, L. 2022. An experimental test of ant effects on herbivory and pathogen infection on wild cotton (*Gossypium hirsutum* L.). - Arthropod-Plant Interact. 16: 77–86.

Rico-Gray, V. and Oliveira, P. S. 2008. The ecology and evolution of ant-plant interactions. - University of Chicago Press.

Rios, R. S., Marquis, R. J. and Flunker, J. C. 2008. Population variation in plant traits associated with ant attraction and herbivory in *Chamaecrista fasciculata* (Fabaceae). - Oecologia 156: 577–588.

Rosumek, F. B., Silveira, F. A. O., De S. Neves, F., De U. Barbosa, N. P., Diniz, L., Oki, Y., Pezzini, F., Fernandes, G. W. and Cornelissen, T. 2009. Ants on plants: a meta-analysis of the role of ants as plant biotic defenses. - Oecologia 160: 537–549.

Rudgers, J. A., Hodgen, J. G. and White, J. W. 2003. Behavioral mechanisms underlie an ant-plant mutualism. - Oecologia 135: 51–59.

Rudgers, J. A. and Strauss, S. Y. 2004. A selection mosaic in the facultative mutualism between ants and wild cotton. - Proc. R. Soc. Lond. B Biol. Sci. 271: 2481–2488.

Shenoy, M., Radhika, V., Satish, S. and Borges, R. M. 2012. Composition of extrafloral nectar influences interactions between the myrmecophyte *humboldtia brunonis* and its ant associates. - J. Chem. Ecol. 38: 88–99.

Underwood, N. 2010. Density dependence in insect performance within individual plants: induced resistance to *Spodoptera exigua* in tomato. - Oikos 119: 1993–1999.

Vázquez-Barrios, V., Boege, K., Sosa-Fuentes, T. G., Rojas, P. and Wegier, A. 2021. Ongoing ecological and evolutionary consequences by the presence of transgenes in a wild cotton population. - Sci. Rep. 11: 1959.

Wäckers, F. L. and Wunderlin, R. 1999. Induction of cotton extrafloral nectar production in response to herbivory does not require a herbivore-specific elicitor. - Proc. 10th Int. Symp. Insect-Plant Relatsh.: 149–154.

Wäckers, F. L. and Bonifay, C. 2004. How to be sweet? Extrafloral nectar allocation by *Gossypium hirsutum* fits optimal defense theory predictions. - Ecology 85: 1512–1518.

Wäckers, F. L., Zuber, D., Wunderlin, R. and Keller, F. 2001. The effect of herbivory on temporal and spatial dynamics of foliar nectar production in cotton and castor. - Ann. Bot. 87: 365–370.

Weber, M. G. and Keeler, K. H. 2012. The phylogenetic distribution of extrafloral nectaries in plants. - Ann. Bot. 111: 1251–1261.

Wetzel, W. C., Inouye, B. D., Hahn, P. G., Whitehead, S. R. and Underwood, N. 2023. Variability in plant–herbivore interactions. - Annu. Rev. Ecol. Evol. Syst. 54: 451–474.

Wickham, H. 2016. ggplot2: Elegant graphics for data analysis. - Springer-Verlag New York.

Xu, F. F., Chen, J. and Husson, J. 2014. Leaf area lost, rather than herbivore type, determines the induction of extrafloral nectar secretion in a tropical plant (*Clerodendrum philippinum*). - Arthropod-Plant Interact. 8: 513–518.

Yamawo, A. and Suzuki, N. 2018. Induction and relaxation of extrafloral nectaries in response to simulated herbivory in young *Mallotus japonicus* plants. - J. Plant Res. 131: 255–260.

Tables

Table 1. Wald χ^2 tests for the GLMMs assessing the effects of leaf damage treatments and plant height on EFN (volume, total sugar, proportion of active nectaries) and ant activity (patrolling, and attack on caterpillars).

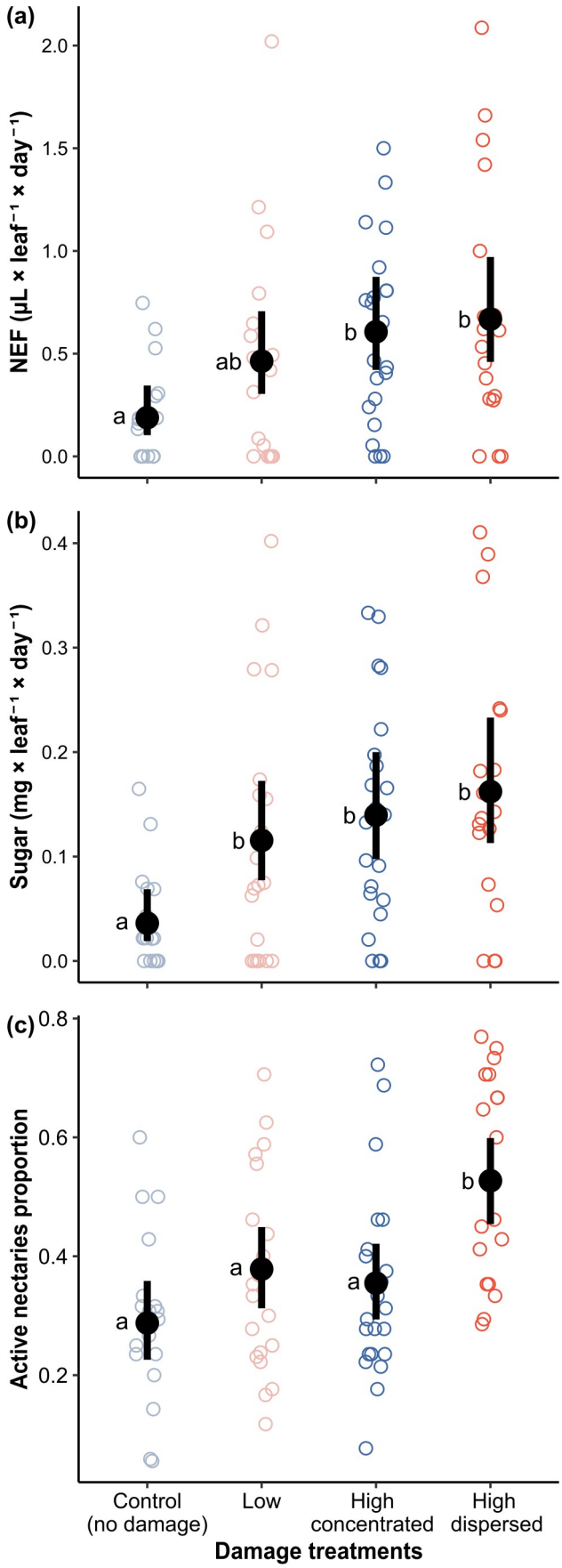
Response variable	Effect	df	χ^2	*P*
EFN volume	Damage treatment	3	13.69	**0.0003**
	Plant height	1	1.41	0.235
Sugar production	Damage treatment	3	16.95	**0.0007**
	Plant height	1	0.21	0.6394
Proportion of nectaries active	Damage treatment	3	23.39	**< 0.0001**
	Plant height	1	1.246	0.2643
Patrolling ants	Damage treatment	3	14.99	**0.0018**
	Plant height	1	3.12	0.0773
Attack on caterpillars	Damage treatment	3	10.92	**0.0121**
	Plant height	1	1.53	0.2454

Figures legends

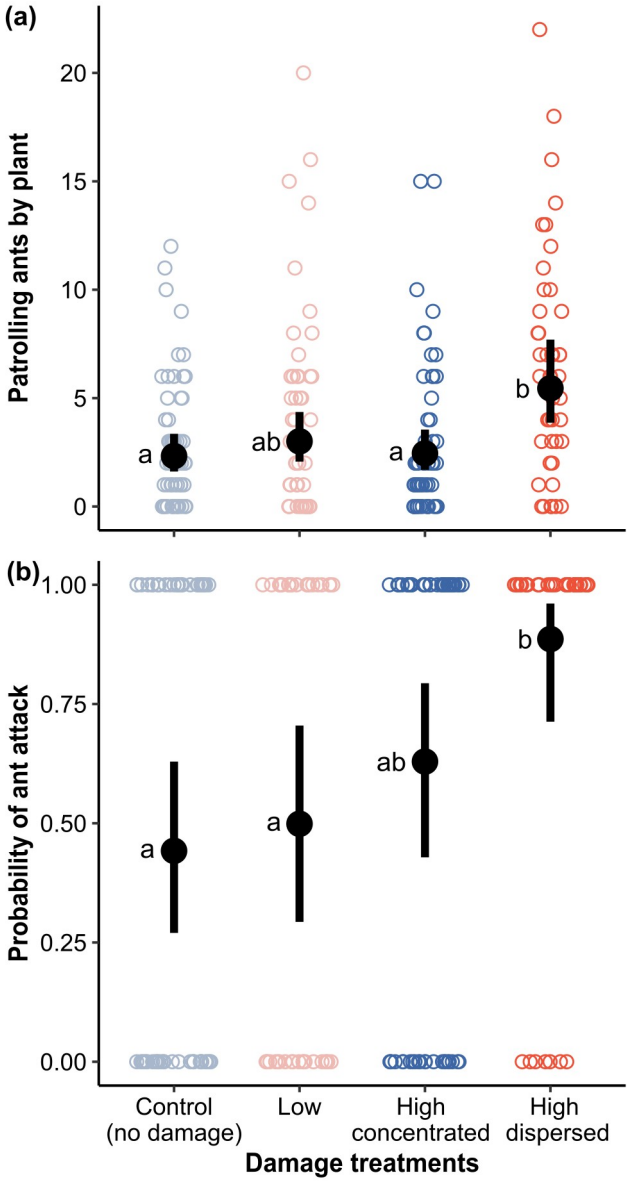
Figure 1. EFN volume production and sugar content in damaged and undamaged leaves of *Gossypium hirsutum* plants under different damage treatments. (a) EFN volume, (b) Sugar content in EFN, (c) Proportion of active nectaries per plant. Black dots and error bars indicate EMMs and 95% CIs based on GLMMs. Single values are plotted (open color circles). Different letters indicate significant differences between treatments according to Tukey's post hoc test ($p < 0.05$).

Figure 2. Ant activity on *Gossypium hirsutum* plants under different damage treatments. (a) Mean number of patrolling ants per plant. (b) Probability of ant attack on caterpillars (defence bioassays). Black dots and error bars indicate EMMs and 95% CIs based on GLMMs. Single values are plotted (open color circles). Different letters indicate significant differences between treatments according to Tukey's post hoc test ($p < 0.05$).

607 Figure 1


(a)
NEF (µL × leaf⁻¹ × day⁻¹)
2.0
1.5
1.0
0.5
0.0
a
ab
b
b
(b)
Sugar (mg × leaf⁻¹ × day⁻¹)
0.4
0.3
0.2
0.1
0.0
a
b
b
b
(c)
Active nectaries proportion
0.8
0.6
0.4
0.2
a
a
a
b
Control (no damage)
Low
High concentrated
High dispersed
Damage treatments

608 Figure 2


(a)
Patrolling ants by plant
20
15
10
5
0
a
ab
a
b
(b)
Probability of ant attack
1.00
0.75
0.50
0.25
0.00
a
a
ab
b
Control
(no damage)
Low
High
concentrated
High
dispersed
Damage treatments