

## RESEARCH ARTICLE

# Effects of early-season insect herbivory on subsequent pathogen infection and ant abundance on wild cotton (*Gossypium hirsutum*)

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## Abstract

1. Plant-induced defences play an important role in mediating interactions between plants, insects and pathogens. Yet, the plant traits underlying these effects, the extended consequences for higher trophic levels (i.e. predators), and the implications for plant growth and reproduction have received little attention.
2. Here, we asked whether simulated early insect leaf damage on wild cotton (*Gossypium hirsutum*) affected subsequent damage by insect leaf chewers and pathogenic fungi, as well as ant abundance. To address the mechanisms behind such effects, we measured plant defensive traits induced by early leaf damage to determine which inducible traits might determine the effects on plant-associates. We also evaluated whether early damage influenced plant growth and flower production, and if such effects were mediated by subsequent insect herbivory or pathogen infection.
3. We show that simulated early leaf damage reduced damage by subsequent leaf-chewing insects, increased plant fungal infections, but did not affect ant abundance. Leaf defensive traits (lignins and pubescence) were significantly induced by early damage and were negatively associated with insect herbivory and infection severity, but did not account for the effects of early leaf damage on either of these subsequent attackers. In addition, ant abundance was not associated with (or accounted for) subsequent herbivory or infection, suggesting they did not confer plant protection. Finally, early leaf damage negatively affected plant growth and flower production and analyses suggested that the effect on the latter was, at least partly, mediated by increased fungal infections.
4. *Synthesis.* Overall, these findings show that early herbivory determines the outcome of cotton interactions with subsequent attackers, and such effects have an impact on plant growth and flower output.

## KEYWORDS

ant-plant interaction, induced defences, insect herbivory, pathogen infection, plant-mediated interaction, wild cotton

## 1 | INTRODUCTION

The induction of plant defences in response to attack is a widespread phenomenon and constitutes a principal mechanism of direct resistance against insect herbivores and pathogens (Agrawal, 2011; Karban & Baldwin, 1997). It is now widely recognized that the consequences of plant defence induction go beyond the direct effects between the plant and the eliciting attacker, and may have various indirect effects on other members of the community (Heil & Bostock, 2002; Strauss & Irwin, 2004). Notably, plants are under attack by multiple antagonists that vary in their phenology, setting the stage for plant defence responses to early-attacking enemies influencing subsequently arriving herbivores and pathogens (reviewed by Ohgushi, 2005; Stout, Thaler, & Thomma, 2006). Research has shown that these effects on subsequent attackers are important in shaping herbivore community structure (Poelman, Broaekgaarden, Loon, & Dicke, 2008; Utsumi, Ando, Roininen, Takahashi, & Ohgushi, 2013; Van Zandt & Agrawal, 2004), as well as influencing parasitoids and predators (Poelman & Dicke, 2014; Wimp & Whitham, 2007). A robust understanding of these indirect interactions via inducible plant defence responses is therefore essential if we wish to unravel the mechanisms of assembly and diversity maintenance of plant-dwelling arthropod communities.

A key realization about the nature of plant-mediated interactions between attackers is that they are strongly dependent on the attacker's feeding guild and that plant defensive responses are specific to each guild. For example, the jasmonic acid (JA) pathway is thought to be primarily associated with induced plant defences against attackers such as chewing insects and necrotrophic pathogens, whereas the salicylic acid (SA) pathway is mainly associated with induced plant defences against sucking herbivores and biotrophic pathogens (Pieterse, Schaller, Mauch-Mani, & Conrath, 2006; Thaler, Humphrey, & Whiteman, 2012). Attack by one guild is therefore expected to negatively influence subsequent attackers of the same guild because both are susceptible to the same plant defence reaction, whereas initial attackers are expected to positively influence subsequent attackers of a different guild due to interference between signalling pathways (reviewed by Moreira, Abdala-Roberts, & Castagnayrol, 2018; Thaler et al., 2012). However, most research on plant-mediated herbivore interactions has been conducted under laboratory or greenhouse conditions, and the fewer studies performed under natural conditions have frequently not measured plant phenotypic changes (e.g. gene expression or defence levels) to identify the mechanisms dictating these plant-mediated interactions (but see Hernández-Cumplido, Glauser, & Benrey, 2016; McArt, Halitschke, Salminen, & Thaler, 2013; Poelman et al., 2008). Hence, we have a good handle on the mechanisms by which plant defence responses mediate interactions with herbivores and pathogens under controlled conditions, but little is known about how this plays out in real communities, and the plant traits affecting these interactions.

There are additional important considerations about research on plant-mediated effects between attackers. One of these is that the influence of such interactions on higher trophic levels has usually

been neglected (Denno & Kaplan, 2007; Poelman & Dicke, 2014). In one of the few exceptions, Kessler and Baldwin (2004) found that previous feeding by mirid bugs on wild tobacco (*Nicotiana attenuata*) rendered subsequently attacking caterpillars more susceptible to predatory bugs. Similarly, Hernández-Cumplido et al. (2016) showed that early-season leaf-chewing beetles on lima bean (*Phaseolus lunatus*) reduced attack by subsequently arriving seed-eating beetles and this, in turn, influenced parasitoid attack on the latter (see also Poelman et al., 2011). In addition, Abdala-Roberts, Agrawal, and Mooney (2012) reported that initial caterpillar herbivory on milkweed leaves increased aphid abundance and this in turn increased the abundance of aphid-tending ants. Interestingly, this then led to a shift in ant effects on aphids from mutualistic to antagonistic. Another aspect to consider is effects involving plant pathogens, which have been neglected by most studies (Moreira et al., 2018; Stout et al., 2006). The few available studies on plant-mediated interactions involving pathogens have shown that plant pathogens strongly respond to as well as trigger profound changes in plant traits that directly or indirectly influence subsequently attacking herbivores (e.g. Rosa, Woestmann, Biere, & Saastamoinen, 2018) and their natural enemies (e.g. Desurmont, Xu, & Turlings, 2016). The documentation of these extended effects of plant-mediated interactions involving pathogens and upper trophic levels warrants further research to understand the role of induced plant defences within a community context.

Plant-mediated consumer interactions may ultimately have important implications for plants themselves. For example, studies have looked at how herbivores indirectly affect plant reproduction via effects on pollinators (reviewed by Bronstein, Huxman, & Davidowitz, 2007; Strauss & Irwin, 2004). Other studies have found increased plant reproduction due to induced responses that enhance predation on herbivores under natural conditions, particularly in ant-plant systems (Rico-Gray & Oliveira, 2007), and to a lesser extent tri-trophic interactions involving other carnivore groups (e.g. parasitoids, Schuman, Barthel, & Baldwin, 2012 and predatory bugs, Kessler & Baldwin, 2004). A few studies have also reported negative effects of early-season leaf herbivores on late-season flower herbivores or seed predators, which in turn lead to increased plant reproductive success (Hambäck et al., 2011; Hernández-Cumplido et al., 2016; McArt et al., 2013; Stam, Dicke, & Poelman, 2018). Finally, at least one study has looked at indirect interactions between insects feeding on vegetative tissues (e.g. leaves) and their effects on plant fitness correlates (Moreira et al., 2015). To date, however, most studies on plant-mediated interactions involving mutualistic or pathogenic microbes have generally overlooked effects on plant growth or reproduction (but see Barber et al., 2015). Whether plant-mediated insect-microbe (or microbe-microbe) interactions end up impacting plants is key since this will ultimately determine the persistence and structure of plant-associated arthropod communities on such host plants.

Wild cotton, *Gossypium hirsutum* L. (Malvaceae), possesses both direct (e.g. phenolics, terpenoids, trichomes) and indirect defences (e.g. inducible extrafloral nectar and volatile organic compounds),

and preliminary field observations (in Yucatan, Mexico) indicated that it is frequently attacked by leaf-chewing insects (mainly caterpillars and beetles), and to some extent also pathogenic fungi. The induction of cotton defences in response to enemy attack could play an important role in determining how subsequent interactions between plant attackers (insect herbivores and pathogens) and mutualists (ants) play out in natural cotton populations. To better understand these interactions and how they may ultimately determine plant growth and reproduction, we conducted a field common garden experiment to answer the following: (a) Does simulated insect leaf-chewer damage induce physical and chemical defensive traits in wild *G. hirsutum*? (b) Does this simulated leaf damage influence subsequent attack by leaf-chewing insects and infection by fungal pathogens, and are these effects correlated with direct or indirect (via ants) defensive traits? (c) Does simulated initial leaf-chewer damage affect the abundance of omnivorous/predatory ants, and which plant traits and mechanisms account for such effect? and (d) Does simulated leaf herbivory affect cotton growth and flower production, and are such effects associated with – and partially attributable to – changes in subsequent pathogen or insect attack? By addressing these questions, we aim to gain a more complete understanding of the community-level ecological mechanisms and consequences of plant responses to insect herbivory.

## 2 | MATERIALS AND METHODS

### 2.1 | Study species

Wild *Gossypium hirsutum* is a perennial shrub that grows up to 2 m tall under natural conditions (Oosterhuis & Jernstedt, 1999). It is native to Central America, Mexico and the Caribbean Basin (Oosterhuis & Jernstedt, 1999; Wendel, Brubaker, & Percival, 1992), and is thought to have originated in southeast Mexico (D'Eeckenbrugge & Lacape, 2014). Wild populations are common along the coasts of the Yucatan Peninsula (Mexico) growing in the coastal scrubland or sand dune vegetation (D'Eeckenbrugge & Lacape, 2014). Cultivated and feral populations of *G. hirsutum* are rare at the sampled sites as wild cotton distribution is isolated from inland populations and is restricted to the coastal vegetation strip (D'Eeckenbrugge & Lacape, 2014). Flowering occurs twice a year (May–June and December–January) and plants flower for the first time during the first or second year of life.

Throughout its coastal distribution, wild *G. hirsutum* is attacked by a diverse community of insect herbivores, among which the most important are leaf chewers belonging to Lepidoptera, Orthoptera and Coleoptera, whereas sap feeders (Hemiptera) are relatively less common (L. Abdala-Roberts, unpublished data). Under natural conditions, cotton is also attacked by several disease agents, including generalist fungi such as *Corynespora cassiicola* and *Alternaria solani* (L. Abdala-Roberts, pers. obs.), as well as other specialist species (e.g. *Glomera gossypii*). In particular, signs of infection by *C. cassiicola* and *A. solani* were observed over the course of this experiment and confirmed based on laboratory cultures of sampled leaves (E. Herrera, pers. comm.). Both

species are necrotrophs and attack a broad range of plant species, including numerous crops (López et al., 2018; Rahman, Oirdi, González-Lamonthe, & Bouarab, 2012). Insect leaf damage, particularly by beetles and caterpillars, peaks during the first months of the rainy season (June–July), whereas propensity for fungal infection may be greater under more humid conditions later in the season (September–October; L. Abdala-Roberts, pers. obs.). Ants commonly tend extrafloral nectaries and up to 30 morphospecies have been documented across different sites on wild cotton plants (L. Abdala-Roberts, unpublished data).

Research on chemical defences in *G. hirsutum* comes largely from studies with domesticated varieties (reviewed by Hagenbucher, Olson, Ruberson, Wäckers, & Romeis, 2013), though there have been a few studies using wild populations of other *Gossypium* species (Rudgers, Strauss, & Wendel, 2004). Both wild and cultivated *G. hirsutum* plants produce pigment glands throughout their surface which contain gossypol and other related terpenes with insecticidal effects (McAuslane & Alborn, 1997; Rudgers et al., 2004; Stipanovic, López, Dowd, Puckhaber, & Duke, 2006). In addition, leaves produce phenolic compounds which have been shown to provide herbivore resistance in this species (Mansour, Zohdy, ElGengaihi, & Amr, 1997; Nix, Paull, & Colgrave, 2017), as well as non-glandular trichomes which are thought to play a role in biotic (as well as abiotic) resistance in species of *Gossypium* (Rudgers et al., 2004). In addition, *G. hirsutum* also resorts to indirect defence by producing extrafloral nectar and volatile organic compounds (VOCs) which attract ants and parasitoids (McCall, Turlings, Loughrin, Proveaux, & Tumlinson, 1994; Rudgers et al., 2004; Wäckers & Bezemer, 2003). Most of cotton's defensive traits (e.g. gossypol glands and VOCs) are highly inducible in response to damage in both wild and cultivated *G. hirsutum* (Agrawal & Karban, 2000; Loughrin, Manukian, Heath, & Tumlinson, 1995; McAuslane & Alborn, 1997; McCall et al., 1994; Opitz, Kunert, & Gershenzon, 2008).

### 2.2 | Seed sources, experimental design and induction treatment

In July 2015, we collected seeds from 14 plants (i.e. maternal lines, “genotypes” hereafter) of wild cotton from a naturally occurring population located on the northwestern coast of the Yucatan Peninsula (20°58'30.2"N, 90°20'57"W), near the coastal town of Celestún (Yucatán, Mexico). Previous genetic work based on samples taken near this site indicated that populations in this area represent wild *G. hirsutum* (see D'Eeckenbrugge & Lacape, 2014). Distance between sampled plants was at least 2 m.

Seeds were germinated in June 2017 and kept under greenhouse conditions for 3 months. In early September 2017, 40–50 cm tall plants were transplanted to a 10 × 15 m common garden located at the Campus de Ciencias Sociales of the Universidad Autónoma de Yucatán (21°1'27"N, 89°33'15"W). We planted 7–8 plants per genotype ( $N = 100$ ) which were randomly allocated to nine spatial blocks ranging in size from 1 × 2 m to 2 × 4 m. Distance between plants within each block was 30–40 cm and distance between adjacent blocks was 1–1.5 m. At the start of the growing season, half of the

plants of each genotype were randomly assigned to simulated leaf damage whereas the other half were left undamaged (controls) (see Figure S1). Treatments were randomly assigned to plants of each genotype within each block and virtually all plants were free of fungal infection at the time of treatment application. The leaf damage treatment consisted in removing ca. 50% from a third of all the leaves of each plant using scissors, and then puncturing the central portion of the remaining leaf with an awl; insects (e.g. beetles, grasshoppers) feed on both new and old cotton leaves such that applying damage to all leaves throughout the plant canopy represented a realistic scenario. We then exposed the punctured area of most damaged leaves to regurgitant from third instar larvae of *Spodoptera frugiperda* by gently poking the abdomen of the caterpillar until it regurgitated on the leaf (Turlings, McCall, Alborn, & Tumlinson, 1993). We used two to three caterpillars per plant, depending on the number of leaves. Larvae were initially fed a wheat germ-based artificial diet and kept on cotton leaves for ca. 12 hr prior to treatment application. This insect exhibits extreme polyphagy and although it is usually not common in natural populations of wild cotton (T. Quijano-Medina, pers. obs.), it feeds on cultivated *G. hirsutum* (Brown & Dewhurst, 1975; Capinera, 2001), and has been shown to induce chemical defences in both wild and cultivated cotton (Chappuis & Egger, 2016). It was therefore considered good proxy for assessing cotton-induced responses to herbivory by a generalist insect, but at the same time we caution on inferences drawn from the results since *S. frugiperda* damage may not necessarily mimic responses to damage by native caterpillars. It should also be noted that applying mechanical damage and insect regurgitant is less realistic compared to natural insect damage, but that previous studies have demonstrated that exogenous application of herbivore regurgitant combined with mechanical damage provides an effective proxy of natural damage in several cultivated species (e.g. maize, Alborn et al., 1997; Turlings et al., 1993 and tobacco, Halitschke, Schittko, Pohnert, Boland, & Baldwin, 2001; McCloud & Baldwin, 1997), including wild cotton (Chappuis & Egger, 2016). Thus, our approach allowed us to precisely control the amount of leaf damage (relative to natural feeding) while achieving realistic levels of plant defence induction.

Field surveys indicate that the mean plant-level percent of leaf tissue removed or damaged by insects in natural populations of wild cotton is 23% (range: 9.4–53.2 across populations; L. Abdala-Roberts, unpublished data). Our treatment resulted in roughly 15%–20% of leaf damage which therefore fell within the natural range of herbivory observed in populations and approximated the mean value.

### 2.3 | Measurements

During the 2 months after treatment application, we conducted surveys of herbivory on new leaves by leaf-chewers, fungal infection and ant abundance. Fungal leaf damage was attributed to *C. cassicola* and *A. solani* and peaked 1.5 months after treatment application (B. Pérez-Niño, pers. obs.). Leaf herbivory and fungal infection were surveyed on weeks three and nine after treatment, ant abundance was surveyed at weeks three, six and nine, and the final

number of new leaves and flower number were recorded on week nine. Preliminary analyses indicated that treatment effects on each of these responses did not vary across surveys (see Table S1) and we therefore analysed the average (herbivory and infection) or cumulative (ant abundance) values across surveys. We also accounted for differences in initial plant size by recording the number of leaves 1 week after transplanting. Although this initial measure of plant size was somewhat distant from the leaf damage application, it was nonetheless assumed to be correlated with plant size subsequently in the study.

For leaf damage by subsequent leaf chewers, we visually estimated the percentage of area removed on new leaves (typically 5–10 new leaves per plant) by chewing insects by visually scoring damage of each leaf as follows: 0%, 1%–15%, 16%–30%, 31%–45%, 46%–60% and 61%–75% of leaf area removed. We used the midpoint of each category per leaf and averaged values across leaves and across surveys to obtain a single (mean) value per plant for statistical analysis (Castagneyrol, Giffard, Péré, & Jactel, 2013; Ríos, Marquis, & Flunker, 2008). Only new leaves were assessed for damage, as new tissues are expected to be more highly defended and exhibit higher levels of induced defences, and also because most of the damage by insect leaf chewers took place in new leaf tissues. We sampled leaves 2–4 starting from the apical meristem and moving downward in accordance previous work measuring cotton-induced leaf defences (Chappuis & Egger, 2016). For fungal attack, signs of infection are easily identified and consist of brown spots indicating necrosis. We estimated whole-plant disease severity by visually scoring each individual as follows: 0 (no apparent evidence of infection), 1 (<10% of the leaves with sign of infection, i.e. necrotic spots), 2 (ca. 50% of the leaves with sign of infection) and 3 (>50% of leaves with sign of infection). The number of leaves with sign of infection was usually correlated with the amount of infection per leaf, i.e., plants with a greater frequency of infected leaves tended to have more necrotic spots per leaf. We did not differentiate between the two species of fungi when scoring plants, though their incidence was roughly similar in both surveys (B. Pérez Niño, pers. obs.). Pathogen attack was recorded for all leaves (rather than only new leaves) since signs of infection were spread throughout the plant in both new and older leaves. Finally, surveys of ant abundance were conducted between 07:00 and 09:00 hr, when ant activity is highest, by examining stems and both sides of leaves starting from the apical meristem and moving down to the base of the plant.

During week three after treatment application, we collected three to four fully expanded, healthy new leaves per plant for quantification of defensive traits, namely: density of gossypol glands, pubescence and levels of phenolic compounds (see ahead). We counted the number of glands and trichomes on two 0.20 cm<sup>2</sup> disks obtained from each of two leaves using a stereoscopic microscope. Trichome counts were conducted on the adaxial surface whereas glands were counted on the abaxial leaf surfaces. Values per disk were extrapolated to 1 per cm<sup>2</sup> to obtain the number of trichomes or glands/cm<sup>2</sup>, and these values were then averaged across disks to obtain a single value per plant for statistical analyses. Trichomes are

needle-like and may show ramifications (multiple needles per structure). In most cases, individual trichomes had one to three needles but in some cases they presented up to six needles (stellate morphology). We therefore counted the total number of needles rather than the number of trichomes because the former represented a better proxy of leaf pubescence (hereafter “leaf pubescence”).

## 2.4 | Chemical analyses

Upon collection, leaves were stored in a cooler and then transported to the laboratory where they were immediately dried at 45°C. We extracted phenolic compounds using 20 mg of dry plant tissue (pool of leaves per individual) with 1 ml of 70% methanol in an ultrasonic bath for 15 min, followed by centrifugation. These methanolic extracts were diluted (1:5 vol:vol) with the extraction solvent and transferred to chromatographic vials to perform phenolic profiling. We used ultrahigh-pressure liquid chromatography-quadrupole-time-of-flight mass spectrometry (UHPLC-QTOF-MS) to detect, identify and quantify phenolic compounds. The separation was carried out on a 50 × 2.1 mm Acquity UPLC BEH C18 column (Waters) thermostated at 25°C. Solvents were water +0.05% vol. formic acid (A), and acetonitrile +0.05% vol. formic acid (B). The gradient program was performed at a flow rate of 0.4 ml/min under the following conditions: 5%–30% B for 6 min, 30%–100% B for 2 min, holding at 100% B for 2 min followed by re-equilibration at 5% B for 2 min with an injection volume of 2 µl. The QTOF-MS was operated in  $MS^E$  negative mode over an m/z range of 85–1,200 Da with the following parameters: capillary voltage at –2.5 kV, cone voltage –25 V, source temperature 120°C, desolvation gas temperature 350°C, desolvation gas flow 800 L/hr. The instrument was internally calibrated by infusing a solution of leucine-enkephaline at 400 ng/ml at a flow rate of 15 µl/min through the Lock Spray™ probe. Phenolic compounds were tentatively identified on the basis of their molecular formula (as determined from exact mass measurements), fragment ions, and comparison with available databases such as the Dictionary of Natural Products (Chapman and Hall, CRC Informa, London; version 20.2) or ReSpec for Phytochemicals (Sawada et al., 2012). In total, 24 different phenolics were detected in cotton leaves, which were classified into different groups, namely: flavonoids, condensed tannins, hydrolysable tannins and lignins. We quantified flavonoids as rutin equivalents, condensed tannins as catechin equivalents, hydrolysable tannins as gallic acid equivalents, and lignins as ferulic acid equivalents. For the quantification of these phenolic compounds, we used calibration curves of standards at 0.2, 0.8, 2, 5 and 20 µg/ml. We expressed concentrations of each phenolic group in µg/g tissue on a dry weight basis. Hydrolysable tannins were virtually absent in the leaf samples and were therefore not considered in the statistical analyses.

## 2.5 | Statistical analyses

We ran general mixed models to test for effects of leaf induction treatment (fixed), cotton genotype (random) and their interaction (random) on direct defensive traits, namely: pigment gland density, leaf pubescence, flavonoids, condensed tannins and lignins. We next

ran general mixed models testing for the same effects on subsequent insect leaf herbivory (mean value across surveys), severity of fungal infection (mean value across surveys), ant abundance (proxy of indirect defence, sum across surveys), flower number, and total number of new leaves at the end of the experiment. Hereafter, we refer to these as “initial” models. All these models included initial leaf number (proxy of plant size) as a covariate to account for differences in plant size at the start of the experiment.

To investigate the traits and mechanisms mediating effects of defence induction on leaf damage inflicted by subsequent insects, infection susceptibility, and ant abundance, we ran again each model but now including covariates potentially associated with each response and accounting for the treatment effect (hereafter “mechanistic” models) (Abdala-Roberts et al., 2016; Moreira, Glauser, & Abdala-Roberts, 2017). For these mechanistic models, we started by including all covariates potentially associated with a given response variable and then followed a stepwise removal procedure of covariates based on model AIC values. If the effect of induction treatment was significant in the initial model but turned non-significant after accounting for the retained covariate(s) in the mechanistic model, this suggests that the treatment effect on the response was mediated by such covariate(s). If the treatment effect remained significant in the mechanistic model, this suggests that other (unmeasured) plant traits mediated the treatment effect. For fungal infection and insect herbivory, we included all leaf defensive traits and ant abundance (proxy of indirect defence) as covariates. Given that the causality of the association between leaf herbivory and infection is unclear as both took place simultaneously over the course of the experiment, and that preliminary analyses including leaf herbivory in the infection model and vice versa did not show significant effects of either covariate, we decided not to include these covariates in the models presented in the results. For ant abundance, we included subsequent insect herbivory, fungal infection severity and defensive traits because changes in leaf traits and subsequent attack may directly or indirectly (via some other unmeasured traits, e.g. changes in extrafloral nectar) influence ant attraction. Finally, we ran mechanistic models for the number of new leaves and flowers including infection severity, leaf damage by subsequent chewers and ant abundance as covariates to test whether induction effects on plant fitness-related traits were associated with (and potentially underlain by) subsequent enemies (leaf chewers and fungi) or ant defence. Initial plant size (leaf number) was not considered in the stepwise procedure and was thus retained in all the mechanistic models.

In all cases, the genotype by treatment interaction was not significant. We therefore present results from models excluding this effect and testing only for main effects of induction treatment and cotton genotype. All models included a block effect (random) to account for biotic or abiotic spatial heterogeneity. Plant trait and herbivory data were square-root transformed to achieve normality of residuals (normal distribution, identity as link), whereas severity of infection and final plant height were normally distributed and did not require transformation. New leaf number and flower number were analysed with a generalized mixed model using a Poisson

distribution (log link), whereas for ant abundance we used a negative binomial distribution (log link) which controlled for overdispersion in these data. Variance components of random effects (i.e. cotton genotype) for models with a normal distribution were estimated with restricted maximum likelihood. The statistical significance of the genotype effect was assessed using likelihood ratio tests where the difference in  $-2$  times the log-likelihood of models including and excluding the random effect is distributed as one-tailed chi-squared test with one degree of freedom (Littell, Milliken, Stroup, Wolfinger, & Schabenberger, 2006). In the case of Poisson and negative binomial models, we used Wald tests to estimate variance components and assess the significance of the genotype effect (Bolker et al., 2009). In all cases, we chose to report raw means and standard errors as these were virtually the same as model least-square means and standard errors. All analyses were performed with SAS ver. 9.4 (SAS, 2014) using PROC GLIMMIX.

### 3 | RESULTS

#### 3.1 | Treatment and plant genotype effects on leaf traits

The induction treatment had a significant positive effect on the concentration of leaf lignins (Table 1), with induced plants exhibiting a 42% greater mean value than controls (control:  $124.80 \pm 20.14$ ; induced:  $176.60 \pm 19.98$   $\mu\text{g/g}$ ). In contrast, the induction treatment had no significant effect on flavonoids (control:  $1,385.97 \pm 60.79$ ; induced:  $1,347.77 \pm 60.19$   $\mu\text{g/g}$ ) or condensed tannins (control:  $26.62 \pm 4.56$ ; induced:  $29.51 \pm 4.54$   $\mu\text{g/g}$ ) (Table 1). In addition, there was no significant treatment effect on leaf gland density (Table 1; control:  $382.95 \pm 17.90$ ; induced:  $390.05 \pm 21.75$  glands/ $\text{cm}^2$ ), whereas for leaf pubescence we found a significant negative

**TABLE 1** Results from general linear mixed models testing for effects of induction treatment (mechanical leaf damage +caterpillar regurgitant) on leaf defensive traits in wild cotton (*Gossypium hirsutum*), namely: gossypol gland density (number of glands/ $\text{cm}^2$ ), pubescence (number of trichome needles/ $\text{cm}^2$ ), and the concentration ( $\mu\text{g/g}$  d.w.) of flavonoids, condensed tannins and lignins. All models included plant genotype (see statistics in Supporting Information) and initial plant size (results not shown).  $F$ -values with degrees of freedom (numerator, denominator), and associated significance levels ( $p$ ) are shown. Significant ( $p < 0.05$ )  $p$ -values are typed in bold. Signs in parenthesis next to each response variable indicate whether the induction treatment increased (+) or decreased (-) the mean value for given trait relative to control plants

Response	Induction treatment	
Gland density	$F_{1,67} = 0.07$	$p = 0.791$
Leaf pubescence (-)	$F_{1,68} = 4.84$	$p = \mathbf{0.031}$
Flavonoids	$F_{1,67} = 0.55$	$p = 0.466$
Condensed tannins	$F_{1,67} = 0.82$	$p = 0.365$
Lignins (+)	$F_{1,67} = 5.20$	$p = \mathbf{0.025}$

treatment effect (Table 1), with induced plants exhibiting a 26% lower mean value than controls (control:  $791.25 \pm 98.75$ ; induced:  $588.05 \pm 120.15$  trichome needles/ $\text{cm}^2$ ). With respect to plant genotype, we found significant differences for leaf pubescence and condensed tannins (Table S2), with the former ranging from 0 to  $1,361.20 \pm 249.60$  trichome needles/ $\text{cm}^2$  and the latter ranging from  $11.92 \pm 3.77$  to  $65.74 \pm 11.81$   $\mu\text{g/g}$  among genotypes. All other traits did not exhibit significant variation among cotton genotypes (Table S2).

#### 3.2 | Effects of leaf damage and plant genotype on subsequent herbivory, fungal infection and ant abundance

The initial model for herbivory indicated a significant negative effect of the induction treatment on percent herbivory in new leaves (Table 2a), with induced plants showing a 27% lower mean value relative to controls (control:  $6.61 \pm 0.67$ ; induced:  $4.80 \pm 0.56$  leaf consumed), suggesting higher resistance for induced plants than controls (Figure 1a). The effect of plant genotype on herbivory was not significant (Table S2). After running the mechanistic model, the only covariate retained was flavonoid concentration, which was significantly negatively associated with herbivory (Table 2b). However, the treatment effect remained significant after including this trait (Table 2b), suggesting that flavonoids did not play a major role in explaining differences in herbivory and that other (unmeasured) traits were responsible for the induced resistance in damaged plants.

The initial model for fungal infection indicated a significant positive effect of the induction treatment on the severity of infection (Table 2a), with induced plants showing a 24% greater mean value relative to controls (control:  $1.09 \pm 0.14$ ; induced:  $1.41 \pm 0.13$  infection score) (Figure 1b). There was also a significant effect of plant genotype (Table S2), with up to 2.6-fold variation among cotton maternal lines ( $0.91 \pm 0.20$  to  $2.36 \pm 0.26$ ). Leaf pubescence and condensed tannins were the only traits retained in the mechanistic model, both being significantly negatively correlated with infection severity (Table 2b). However, the treatment effect remained significant after accounting for these plant traits (Table 2b).

With respect to ants, the initial model indicated no significant effect of the induction treatment on ant abundance (control:  $13.21 \pm 1.29$ ; induced:  $11.15 \pm 1.28$  ants) (Table 2a; Figure 1c). There was no significant plant genotypic variation in ant number (Table S2). Leaf flavonoids, condensed tannins, severity of infection and herbivory were retained in the mechanistic model. Among these covariates, only severity of infection and flavonoids were significantly (negatively) associated with ant abundance (Table 2b).

#### 3.3 | Effects of leaf damage and plant genotype on plant growth and flower number

The initial models for number of new leaves and flower number indicated significant negative treatment effects in both cases

**TABLE 2** Results from general or generalized linear mixed models testing for effects of induction treatment on insect herbivory on new leaves (% leaf area eaten or damaged), ant abundance and fungal attack (score of infection severity) in wild cotton (*Gossypium hirsutum*). (a) Models were ran initially testing only for the effect of induction treatment, and then (b) including covariates to assess the mechanisms behind treatment effects. Leaf traits measured were: gossypol gland density (number of glands/cm<sup>2</sup>), pubescence (number of trichome needles/cm<sup>2</sup>) and the concentration (µg/g d.w.) of flavonoids, condensed tannins and lignins. Both initial and mechanistic models also included plant genotype (see statistics in Supporting Information) and initial plant size (results not shown). Removed = covariate was removed based on stepwise forward elimination; NT = covariate not tested in the model. Significant ( $p < 0.05$ ) results are typed in bold, and marginally significant results ( $0.10 > p > 0.05$ ) are in italics

	Response					
	Leaf herbivory		Infection severity		Ant abundance	
(a) Initial model						
Induction treatment	$F_{1,70} = 4.32$	<b><math>p = 0.040</math></b>	$F_{1,69} = 5.87$	<b><math>p = 0.018</math></b>	$F_{1,69} = 0.32$	$p = 0.574$
(b) Mechanistic model						
Induction treatment	$F_{1,67} = 6.86$	<b><math>p = 0.011</math></b>	$F_{1,64} = 6.56$	<b><math>p = 0.012</math></b>	$F_{1,63} = 0.24$	$p = 0.628$
Gland density	Removed	Removed	Removed	Removed	Removed	Removed
Pubescence	Removed	Removed	$F_{1,64} = 13.82$	<b><math>p = 0.0004</math></b>	Removed	Removed
Flavonoids	$F_{1,67} = 10.09$	<b><math>p = 0.002</math></b>	Removed	Removed	$F_{1,63} = 8.81$	<b><math>p &lt; 0.0001</math></b>
Condensed tannins	Removed	Removed	$F_{1,64} = 4.47$	<b><math>p = 0.038</math></b>	$F_{1,63} = 3.64$	$p = 0.061$
Lignins	Removed	Removed	Removed	Removed	Removed	Removed
Ant abundance	Removed	Removed	Removed	Removed	NT	NT
Infection severity	NT	NT	NT	NT	$F_{1,63} = 4.28$	<b><math>p = 0.042</math></b>
Leaf herbivory	NT	NT	NT	NT	$F_{1,63} = 0.60$	$p = 0.442$

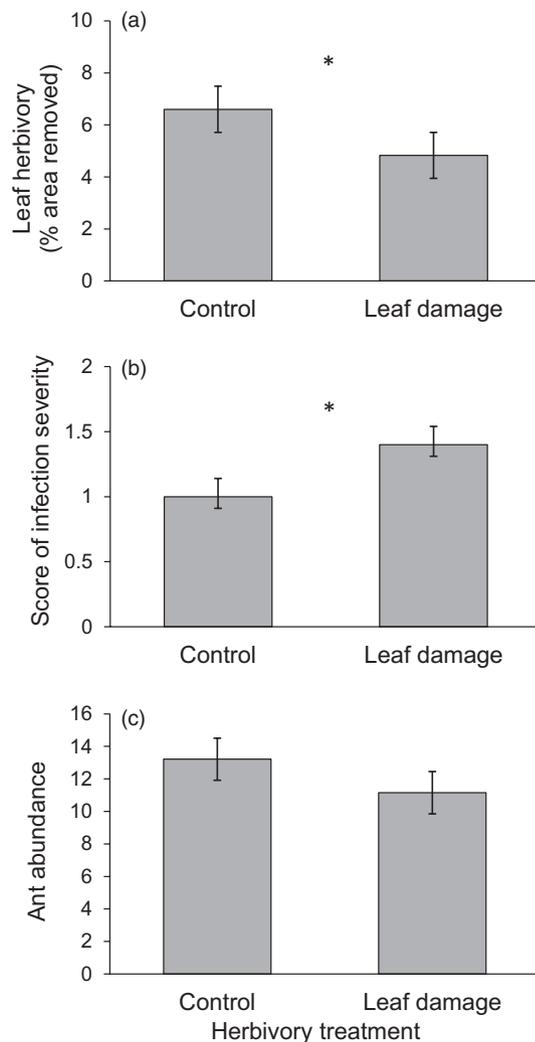
(Table 3a), with induced plants exhibiting 42% and 46% lower mean values than controls, respectively (control:  $14.38 \pm 1.21$ ; induced:  $8.36 \pm 0.81$  leaves; control:  $10.17 \pm 1.43$ ; induced:  $5.50 \pm 1.17$  flowers) (Figure 2a,b). In addition, we found significant variation among plant genotypes in both leaf (fivefold;  $4.14 \pm 1.37$  to  $21.00 \pm 3.94$ ) and flower (10-fold;  $2.14 \pm 0.88$  to  $22.16 \pm 6.83$ ) number (Table S2). Infection severity and ant abundance (but not subsequent leaf damage) were retained in the mechanistic models for both variables, with infection severity being significantly negatively associated with both leaf number and flower number whereas ant abundance was positively associated with both variables (Table 3b). The treatment effect on leaf number remained significant after accounting for these covariates, whereas for flower number it turned marginally significant ( $p = 0.051$ ) (Table 3b). We ran follow-up models for flower number including one covariate at a time, and the treatment effect turned non-significant only when infection severity was included ( $p = 0.056$ ). This suggests that the reduction in flower output for treated plants was due, at least in part, to an increase in severity of infection.

## 4 | DISCUSSION

Results indicated that simulated early-season caterpillar damage on leaves of wild cotton had significant effects on subsequent attackers, namely insect herbivores and pathogenic fungi, but not ants, and that these effects presumably in turn influenced *G. hirsutum* flower production. We show that early leaf damage drove changes in leaf

defensive traits, increasing the concentration of lignins but decreasing leaf pubescence, and also affected subsequent attackers by reducing damage on new leaves by subsequent insects and increasing infection by pathogenic fungi. Mechanistic models indicated that one or more defensive leaf traits, including pubescence, flavonoids and condensed tannins were negatively associated with subsequent attackers, but did not explain the effect of early leaf damage on subsequent herbivory or fungal infection. Counter to expectations, ant abundance was not associated with either subsequent leaf damage or pathogen infection, suggesting that they did not serve a defensive role in wild cotton. Finally, early leaf damage had negative effects on both cotton growth and flower number, and reduced flower output appeared to be mediated, at least partly, by an increase in severity of fungal infection on early damaged plants. Overall, these findings indicate that early insect herbivory affects subsequent plant–herbivore and plant–pathogen interactions and that such altered interactions may ultimately influence cotton flower production.

Early leaf damage reduced herbivory by subsequent insect leaf chewers, which is in accordance with the prediction that early herbivory induces resistance in plants that negatively affects late herbivores of the same guild (Moreira et al., 2018; Thaler et al., 2012). Although levels of late herbivory were low (~6%) and the decrease in leaf damage in previously induced plants was small (2%–3%) relative to controls, this reduction may be important for new leaves and could also be indicative of an initial effect that gives way to larger differences once herbivory levels build up over the growing season. In addition, leaf flavonoid concentrations were negatively associated with the amount of subsequent leaf damage, but this trait (as well



**FIGURE 1** Effects of simulated early leaf damage on subsequent insect leaf herbivory (a), severity of fungal infection (b), and ant abundance (i.e. number of ants) (c) on wild cotton, *Gossypium hirsutum*. Values are raw means and standard errors. \* $p < 0.05$

as the others) presumably did not account for the observed reduction of subsequent herbivory in early damaged plants, as treatment differences in leaf damage remained significant after including these predictors. Thus, further work measuring other defensive traits as well as plant hormones (e.g. JA and SA) is necessary to explain this result. Our results also indicated no significant correlation between ant abundance and subsequent leaf herbivory or fungal infection (ant abundance was dropped from both mechanistic models), suggesting that ants were not effective in providing indirect defence against attackers on wild cotton over the course of the experiment. This runs counter to previous work implying a defensive role of ants in *G. hirsutum* (e.g. Wäckers & Bezemer, 2003; Wäckers, Zuber, Wunderlin, & Keller, 2001), as well as studies demonstrating their mutualistic effects on other species of wild cotton (e.g. *G. thurberi*; Rudgers, 2004). Although ants were observed actively feeding on extra-floral nectaries (EFNs) during the study, their protective effects remain to be formally tested (experimentally) under natural conditions to conclusively determine their role as mutualists of wild cotton.

Opposite to the effect on subsequent insect herbivores, we found a significant positive effect of early leaf damage on attack by pathogenic fungi. *Corynespora cassiicola* and *A. solani* are both considered necrotrophic pathogens which presumably target the JA plant signalling pathway (Moreira et al., 2018; Thaler et al., 2012). Thus, our expectation was that early damage by leaf chewers (inducing JA) would instead increase resistance to these fungi (and therefore lower infection severity). Further work is necessary to determine whether insect herbivory is in fact influencing JA levels as well as the effects of these fungi on both JA and SA. Previous studies have in fact shown that necrotrophic pathogens manipulate the SA plant signalling pathway to increase plant susceptibility. For example, a study by Rahman et al. (2012) with tomato showed that necrotrophic fungi (one of which was *A. solani*) suppress plant JA-dependent genes by upregulating the SA pathway. In a recent meta-analysis, Moreira et al. (2018) also reported that plant-mediated effects of initial on subsequent attackers are far more variable than previously thought, and that studies involving responses (or effects) of pathogens are less common compared to studies with insect herbivores. Results from studies conducted thus far suggest that some pathogen groups exhibit responses that do not always follow predictions of antagonism between the JA and SA signalling pathways (Moreira et al., 2018). More studies addressing plant-mediated effects involving pathogens and their underlying mechanisms are clearly needed.

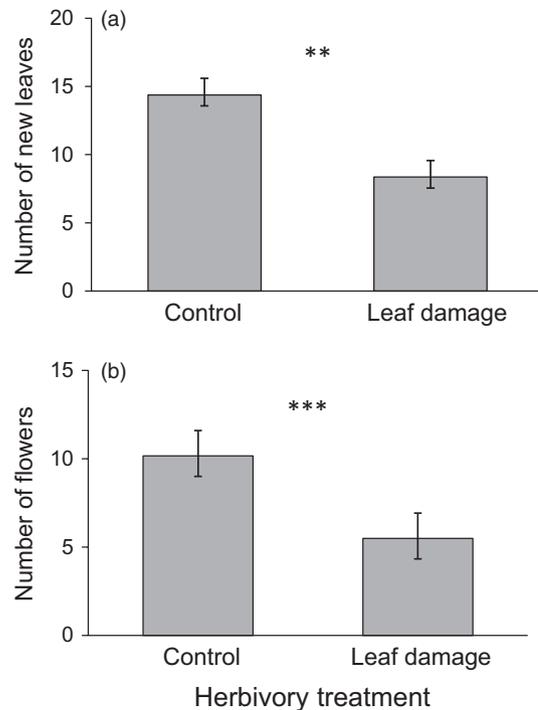
We found no evidence that simulated insect herbivory affected ant abundance in cotton plants. This result runs counter to previous work reporting increased ant attraction to plants in response to herbivory (reviewed by Rico-Gray & Oliveira, 2007), mainly through increased production of extrafloral nectar (Wäckers & Bezemer, 2003; reviewed by Heil, 2015) or VOCs (e.g. Rasmann et al., 2014). Interestingly, we found a negative association between infection severity and ant abundance, which, combined with the fact that early damaged plants suffered increased infection severity, would have presumably led to reduced (rather than increased) ant numbers on induced plants. For example, Desurmont et al. (2016) recently reported that infection with powdery mildew (*Erysiphe cruciferarum*) reduces VOC emissions in *Brassica rapa*, and this in turn reduces parasitoid attraction to leaf chewers (see also Martini, Pelz-Stelinski, & Stelinski, 2014). Similarly, work by LeVan and Holway (2015) found that plant fungal infection negatively affected ant visitation conceivably through a reduction in flower output. Although early leaf damage did not affect ant abundance precluding a test of these types of indirect effects of early plant damage on ants, the fact that previous work has shown that leaf damage induces volatiles and EFN in *G. hirsutum* and here we found that it reduces flower output (ants frequently visit cotton flowers in natural populations; L. Abdala-Roberts, pers. obs.), suggests ample potential for effects of plant-mediated herbivore-pathogen interactions on ants. To date, few studies have reported on the extended effects of these plant-mediated interactions on higher trophic levels (Denno & Kaplan, 2007; Hernández-Cumplido et al., 2016), particularly in cases involving plant pathogens. Increased attention to the multi-trophic consequences of

**TABLE 3** Results from general or generalized linear mixed models testing for effects of induction treatment (mechanical leaf damage + caterpillar regurgitant) on the number of flowers and new leaves produced by wild cotton (*Gossypium hirsutum*). (a) Models were ran initially testing only for the effect of induction treatment, and then again (b) including covariates to assess the mechanisms behind treatment effects on these response variables. Mechanistic models included severity of fungal infection and ant abundance, and were both retained after applying a stepwise removal procedure. Both initial and mechanistic models also included plant genotype (see statistics in Supporting Information) and initial plant size (results not shown). Significant ( $p < 0.05$ ) results are typed in bold, and marginally significant results ( $0.10 > p > 0.05$ ) are in italics

	Response			
	Leaf number		Flower number	
(a) Initial model				
Induction treatment	$F_{1,69} = 10.40$	$p = 0.002$	$F_{1,68} = 13.80$	$p = 0.0004$
(b) Mechanistic model				
Induction treatment	$F_{1,68} = 5.69$	$p = 0.019$	$F_{1,66} = 3.95$	$p = 0.051$
Ant abundance	$F_{1,67} = 4.33$	$p = 0.041$	$F_{1,66} = 23.58$	$p < 0.0001$
Infection severity	$F_{1,67} = 6.89$	$p = 0.010$	$F_{1,66} = 11.68$	$p = 0.001$
Subsequent herbivory	Removed	Removed	Removed	Removed

changes in plant indirect defensive traits (e.g. EFNs, VOCs) due to plant-mediated herbivore and pathogen interactions is therefore needed.

Finally, there were strong negative effects of early leaf damage on cotton growth and flower production. For flower production, results further suggest that this pattern was not caused exclusively by direct effects of leaf damage on the plant, but was also (indirectly) due to increased severity of fungal infection. Ant abundance, on the other hand, was positively correlated with flower output, but did not explain the effect of early leaf damage on this plant reproductive measure, suggesting that ants did not mediate effects of herbivory on flower production (but see LeVan & Holway, 2015). Compared with previous studies reporting on indirect effects of early herbivory on plant reproductive output via changes in pollinator visitation (e.g. Rusman, Lucas-Barbosa, & Poelman, 2018), flower-visiting ants (e.g. LeVan & Holway, 2015), or seed predator attack (e.g. McArt et al., 2013; Hernández-Cumplido et al., 2016), our results suggest that early insect herbivory indirectly affects plant flower output through increased susceptibility to pathogens. In one of the few studies addressing indirect interactions between herbivory and pathogen infection, Barber et al. (2015) found that root herbivory by beetle larvae on cucumber (*Cucumis sativus*) caused a significant decrease in plant reproduction despite reducing above-ground infection by a biotrophic fungi, thus implying a strong negative direct effect of herbivory on plant fitness over and above the (indirect) positive effect of reduced pathogen infection. We must note, however, that our experiment did not allow to quantify the independent contributions of direct and indirect effects of early insect herbivory on plant flower production, as this would require testing for herbivory effects in the presence and absence of subsequent attackers (e.g. see Hambäck et al., 2011). In addition, because our study focused on short-term effects, it is not possible to determine whether leaf damage delayed rather than reduced reproductive output, in which case plant-pollinator



**FIGURE 2** Effects of simulated early leaf damage on the number of new leaves (a) and flowers (b) produced by wild cotton, *Gossypium hirsutum*. Values are raw means and standard errors. \*\* $p < 0.01$ , \*\*\* $p < 0.001$

interactions may have been affected but not necessarily plant fitness (e.g. seed set). The extent to which these short-term plant growth and flower production responses translate into actual differences in plant fitness remains to be determined following longer term measurements.

Together, these findings indicate that plant-mediated interactions between insect herbivores and pathogens can lead to unexpected effects on host plant growth and flower output which may

ultimately determine the persistence and complexity of plant-associated consumer communities. The meta-analysis by Moreira et al. (2018) found only 9 out of 108 studies (8.3%) on plant-mediated interactions between attackers that tested for effects of early insect herbivores on subsequently attacking pathogens. A greater number of studies testing for these interactions are necessary to gain a better handle on the mechanisms involved in plant-mediated interactions between insect herbivores and plant pathogens, as well as determine their impacts on plant fitness. In addition, while our work extends the conventional approach of plant-mediated interactions at two trophic levels by incorporating pathogens and higher trophic levels (ants), the next step is to fully embrace the complexity of plant-associated arthropod and microbe communities (Kaplan, 2012; Poelman & Kessler, 2016). In this sense, current approaches such as network analyses can help bridge classic work on the ecology of plant-mediated interactions with community-level dynamics involving direct and indirect mutualistic and antagonistic interaction networks (Eubanks & Finke, 2014; Guimarães, Pires, Jordano, Bascompte, & Thompson, 2017). Although evidence from this study suggests that ants do not protect wild cotton against herbivores and pathogens, experimental manipulations of ant abundance and plant enemies are needed to formally test this and gain a better understanding of community-level effects of plant-mediated interactions and potential feedbacks on plant fitness via indirect defence.

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## AUTHORS' CONTRIBUTIONS

L.A.-R. formulated the idea of the manuscript; L.A.-R. and X.M. designed the experiment; L.A.-R. and B.P.N. performed the experiment; L.G. and G.G. performed the chemical analyses; T.T. and G.G. contributed reagents/materials/analysis tools; L.A.-R. analysed the data; L.A.-R. wrote the first draft of the manuscript; X.M., T.T., B.B., V.P.-T. and B.P.N. contributed critically to the writing.

## DATA ACCESSIBILITY

Data available from the Dryad Digital Repository: <https://doi.org/10.5061/dryad.d796p97> (Abdala-Roberts et al., 2019).

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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