



Induced Floral and Extrafloral Nectar Production Affect Ant-pollinator Interactions and Plant Fitness

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ABSTRACT

Thousands of plant species throughout tropical and temperate zones secrete extrafloral nectar to attract ants, whose presence provides an indirect defense against herbivores. Extrafloral nectaries are located close to flowers and may modify competition between ants and pollinators. Here, we used Lima bean (*Phaseolus lunatus* L.) to study the plants interaction between ants and flower visitors and its consequences for plant fitness. To test these objectives, we carried out two field experiments in which we manipulated the presence of ants and nectar production via induction with jasmonic acid (JA). We then measured floral and extrafloral nectar production, the number of patrolling ants and flower visitors as well as specific plant fitness traits. Lima bean plants under JA induction produced more nectar in both extrafloral nectaries and flowers, attracted more ants and produced more flowers and seeds than non-induced plants. Despite an increase in floral nectar in JA plants, application of this hormone had no significant effects on flower visitor attraction. Finally, ant presence did not result in a decrease in the number of visits, but our results suggest that ants could negatively affect pollination efficiency. In particular, JA-induced plants without ants produced a greater number of seeds compared with the JA-treated plants with ants.

Abstract in Spanish is available with online material.

Key words: ant-pollinator conflict; indirect defenses; jasmonic acid; *Phaseolus lunatus*; seeds.

PLANT DEFENSES CAN BE BROADLY CLASSIFIED AS DIRECT OR INDIRECT (Karban & Baldwin 1997). Plant's direct defenses comprise chemical and mechanical traits that deter herbivores, reduce consumption, or decrease their survival. Indirect defenses, on the other hand, involve traits that provide shelter, reward, or information on herbivore presence to natural enemies (predators and parasitoids) that suppress herbivores and in turn, indirectly increase plant biomass and reproduction (Hairston *et al.* 1960, Schmitz *et al.* 2000, Romero & Koricheva 2011).

Some indirect defenses, such as the production of organic volatile compounds and extrafloral nectar, have been shown to mediate the interaction between herbivores and their natural enemies by increasing the likelihood of encounter or by increasing natural enemy density, both of which result in stronger herbivore suppression (Turlings *et al.* 1990, 1995, Heil 2008, Xiao *et al.* 2012). Such top-down control of herbivores by natural enemies can result in so called 'trophic cascades' and drive an increase in plant biomass (*i.e.*, 'the green world hypothesis', whereby predators reduce the abundance of herbivores allowing plants to thrive) (Hairston *et al.* 1960). In particular, extrafloral nectaries (EFN) have been shown to attract predatory ants that defend plants against herbivores and increase plant fitness (Heil & Mckey 2003, Rico-Gray &

Oliveira 2007, Heil 2008, Chamberlain & Holland 2009). Despite ample evidence for positive effects of predatory ants as an indirect defense for plants (reviewed by Marazzi *et al.* 2013), some previous studies have found that the presence of ants can also have negative consequences by reducing the number of pollinators visiting flowers (Wagner & Kay 2002, Ness 2006, Nicklen & Wagner 2006). For instance, ants can discourage pollination by robbing nectar from the flowers, by attacking pollinators, or simply by patrolling the plant (Galen 1999, Nicklen & Wagner 2006, LeVan *et al.* 2014). Such antagonistic interactions could result in trade-offs between indirect defenses and pollination (Heil 2002, 2008, Hernández-Cumplido *et al.* 2010, Ona & Lochman 2011).

Several mechanisms have been suggested to counteract this potential trade-off between pollination and indirect defense by ants (Lach 2008, Ona & Lochman 2011). Plants can produce specific odors that act as repellents against ants. For example, Ghazoul (2001) reported that two acacia-ant mutualists were repelled by floral tissue chemicals from their own host plant species as well as from 13 other plant genera, showing that ant repellents are widespread. Moreover, plants can also create chemical barriers that deter ants from visiting the flowers (Nicklen & Wagner 2006, Ballantyne & Willmer 2012). Ballantyne & Willmer (2012) documented that at least one-third of 49 plant species in a Costa Rican dry forest were ant-repellent, usually via repellent pollen. Finally, the interaction between ants and pollinators can

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be altered if plants provide rewards in temporal or spatial patterns that allow a separation between ant and pollinator activity (Raine *et al.* 2002, Wagner & Kay 2002, Galen 2005, Nicklen & Wagner 2006, Holland *et al.* 2010). Nicklen & Wagner (2006) observed that pollinators and ants associated with *Acacia constricta* visited plants at different times of the day. The ecological consequences resulting from ant-pollinator conflicts in some of these scenarios (temporal or spatial separation and chemical and physical barriers), appear to be a generally accepted paradigm in the context of indirect defense, and it has been named the ‘distraction hypothesis’ (Wagner & Kay 2002, Galen 2005). This hypothesis proposes that EFN maintain non-pollinator insects, such as ants or parasitoids, away from the flowers such that the disruption of pollination and plant reproduction is minimized (Wagner & Kay 2002, Chamberlain & Holland 2008, Holland *et al.* 2011). Nevertheless, very few studies have considered the role of EFN on pollinator behavior and even fewer on plant fitness (Ness 2006, Nicklen & Wagner 2006, Holland *et al.* 2011).

In a previous study, we manipulated the production of extrafloral nectar in wild plants of Lima bean *Phaseolus lunatus* (Fabaceae) by exogenous application of jasmonic acid (JA), and examined the consequences on the abundance of ants and pollinators (Hernández-Cumplido *et al.* 2010). Exogenous application of JA induces responses similar to those initiated following herbivore damage, and so it provides a tool with which to induce direct and indirect defense responses in plants (Heil 2004). We observed that JA-induced plants produced more extrafloral nectar than untreated control plants, and consequently attracted a higher number of ants (but no effects were observed on pollinators). However, in this previous study we did not examine if the production of floral and extrafloral nectar are subject to joint hormonal control such that, JA application also increases the production of floral nectar (*i.e.*, pollinator reward) and if this can affect plant fitness (*i.e.*, reproduction). Additionally, as we did not manipulate the presence of ants, we did not specifically test the effects of ants on the abundance of flower visitors.

Therefore, the goal of this study was to further explore the independent and interactive effects of predatory ants and increased production of floral and extrafloral nectar, ant, and flower visitor abundance, and plant reproductive success. Specifically, in this study we manipulated both ant presence and nectar secretion, to answer the following questions: (1) do floral and extrafloral nectar secretion and ant attraction increase after JA application? (2) How does increased nectar production in both flowers and extrafloral nectaries (EFN), as well as ant presence, affect ant–flower visitor (among these, potential pollinators) interactions throughout the season? And finally, (3) what are the effects of the interaction between ants and flower visitors on plant reproductive success? By addressing these questions, our work builds toward a better understanding on the overall consequences of ant-pollinator interactions for plant fitness.

METHODS

NATURAL HISTORY.—*Phaseolus lunatus* L. (Fabaceae), commonly known as Lima bean, occurs naturally throughout Meso- and

South America. This study was conducted in the coastal area of the state of Oaxaca, Mexico, 15 km northwest of Puerto Escondido (15°55.596'N, 97°9.118'W). At this site, *P. lunatus* plants in wild populations start to germinate in June–July. The first inflorescences appear in October–November and the seeds are produced in late December and early January. Plant phenology is synchronized with the weather, which is characterized by one main rainy season from June to October with a bimodal distribution of precipitation with peaks in July and September (Kost & Heil 2005). Like many species in the Fabaceae, Lima beans bear EFN located on their bracts or arranged pairwise at the stipules of the trifoliate leaves and on the petioles of the individual leaflets (Heil 2004).

EXPERIMENTAL DESIGN.—We conducted two field experiments. In February 2011, we collected seeds of *P. lunatus* from wild populations close to Puerto Escondido (Oaxaca, Mexico). In November 2011, we sowed plants in 5-L pots (20 plants for the first experiment and 40 for the second one) in the greenhouse at the experimental campus of the Universidad del Mar (Oaxaca, Mexico). Three weeks later (at the transition from the rainy to the dry season), we moved the potted plants to the field. To avoid undesirable herbivory, we grew plants in field cages for the first month until they started to produce flowers. We initiated the experiments when the inflorescences presented more than 50 percent of fully open flowers.

EXPERIMENT 1: EFFECT OF JA INDUCTION ON FLORAL AND EXTRAFLORAL NECTAR PRODUCTION.—We conducted a field experiment to determine the extent to which floral and extrafloral nectar secretions by wild plants of Lima bean are inducible by exogenous application of JA. We divided 20 potted plants into two groups. In the first group of ten plants, we sprayed all leaves with a solution of jasmonic acid (1 mM) applied with an atomizer. The other ten plants were used as controls and sprayed with distilled water. We repeated these treatments every 7 d during 4 wk. We covered both plant groups with mesh bags to protect them against EFN consumers, and we also applied tanglefoot (Tangletrap[®], The Tanglefoot Corporation, Grand Rapids, MI) around the base of the plant to deter ants from climbing the plants (Mooney & Agrawal 2008, Moreira *et al.* 2012). We treated plants at 0600 h and measured the nectar from EFN and flowers 4 h later as described by Heil (2004). For each plant we used five leaves and one flower. Nectar secretions were quantified as amount of secreted soluble solids using 5- μ L micropipettes (graduated with 1 μ L divisions to make a direct measurement of nectar volume) and a temperature-compensated portable refractometer (ATAGO hand refractometer, L. Kübler, Karlsruhe, Germany) for nectar concentration. To remove the nectar we used 5 μ L of distilled water applied into all nectaries. We repeated this procedure until we had concentrations of <1 percent. We summed values from all collections for the nectaries on one leaf to quantify leaf overall production of solid EFN compounds. The same procedure was followed for the flowers (see Stephenson 1982, Heil 2004).

EXPERIMENT 2: EFFECT OF ANT AND JA-INDUCTION TREATMENTS ON THE ABUNDANCE OF ANTS AND FLOWER VISITORS AND THEIR CONSEQUENCES FOR PLANT REPRODUCTIVE SUCCESS.—To test the effect of ant and JA-induction treatments on the interaction between ants and flower visitors and their consequences for plant reproduction, we carried out a two factorial field experiment with two ant treatments (presence vs. absence) and two treatments of defense induction (control and JA-induced plants). In total, there were 40 plants, corresponding to two ant treatments \times two induction treatments \times ten replicates.

We sprayed plants with either a solution of jasmonic acid (1 mM) or with distilled water as in the previous experiment, and excluded ants by applying tanglefoot around the base of the plant. For the treatments with JA, we treated plants twice a week in order to stimulate permanent induction. We always conducted induction at 0600 h to avoid wind-spreading of JA in the experimental plot.

We conducted a census three times per day (at 1000 h, 1200 h and 11400 h), for 21 d from January 29 to February 14. During this period, for each plant we counted the number of flowers, the number of flower visitor, and the number of patrolling ants (including ants visiting extrafloral nectaries and flowers). For statistical analyses, we averaged the number of flowers, the number of flower visitors, and the number of ants per day. Ninety percent of the insects approaching the flowers belonged to the orders Hymenoptera, 5 percent to the Diptera and the rest 5 percent included beetles and butterflies, but these never entered the flowers. We considered all hymenopterans and dipterans as 'flower visitors' when they entered the flowers and stayed more than 5 sec. Ants were counted only when seen patrolling along the plant and feeding was not recorded during observations. To avoid disturbance during observations, identification of ants was done with a sample collected at the end of the census. At the end of the experiment, we collected all mature and dry pods of each plant and counted the seeds.

STATISTICAL ANALYSES.—We analyzed extrafloral and floral nectar concentrations using generalized linear models (PROC GLM in SAS System, v. 9.2, SAS Institute, Cary, North Carolina, USA) (Littell *et al.* 2006). The main effect of induction treatment (control vs. JA) was considered as a fixed factor. Then, we compared the effect of induction treatment, structure (floral vs. extrafloral), and their interaction (fixed factors) using a mixed model (PROC MIXED in SAS). Plant was treated as a random factor to account for repeated measures taken on the same experimental unit.

We analyzed the counts of flower visitor abundance, and the number of flowers using generalized linear mixed models (PROC MIXED in SAS) (Littell *et al.* 2006). The main effects of ants (A), induction treatment (T), day (21 d) and their interactions were treated as fixed factors. The effect of plant was treated as a random factor to account for repeated measures taken on the same experimental unit. The effect of $A \times T \times \text{day}$ interaction was considered as a random factor in order to analyze the main factors A and T with the appropriate error term (Littell *et al.* 2006). The number of flowers was included as a covariate in the

analysis of flower visitors. Normality was achieved by log-transforming the original variables.

We analyzed ant abundance using a linear mixed model (PROC MIXED in SAS) with the induction treatment and day as fixed factors and the plant as a random factor to account for repeated measures taken on the same experimental unit (Littell *et al.* 2006). The effect of $T \times \text{day}$ interaction was considered as a random factor in order to analyze the main factor T with the appropriate error term (Littell *et al.* 2006). Normality was achieved by log-transforming the original variable.

Finally, we analyzed the number of seeds using a generalized linear model (PROC GLM in SAS) (Littell *et al.* 2006). The main effects of ants (A), defense induction treatment (T) and their interaction were treated as fixed factors.

RESULTS

EXPERIMENT 1: EFFECT OF JA-INDUCTION TREATMENT ON EXTRAFLORAL AND FLORAL NECTAR PRODUCTION.—Exogenous application of JA significantly increased EFN and FN secretion (Fig. 1). The concentration of EFN and FN was 3.2-fold and 1.8-fold greater, respectively, in JA-induced plants than in untreated control plants (Fig. 1).

EXPERIMENT 2: EFFECT OF ANT AND JA-INDUCTION TREATMENTS ON THE ABUNDANCE OF ANTS AND FLOWER VISITORS AND THEIR CONSEQUENCES FOR PLANT REPRODUCTIVE SUCCESS.—Ant presence (vs. absence) did not significantly affect the abundance of flower visitors (Table S1, Fig. 2A). Exogenous application of JA significantly affected the abundance of ants, but not the abundance of flower visitors (Table S1). Specifically, we found that the number of ants was threefold greater in JA-induced plants than in control plants (Fig. 2B). There was no significant interaction between ant and JA treatments for the abundance of flower visitors (Table S1).

Ants patrolling the plants belonged to three species: *Solenopsis geminata*, *Camponotus novogranadensis*, and *Cephalotes minutus*. We observed no differences in the overall species identity among treatments. Ant presence significantly affected the number of flowers (Table S2), which was twofold greater in plants with ants than in ant-excluded plants (Fig. 3A). Ant presence did not significantly affect the total number of seeds (Table S2). Exogenous application of JA significantly affected the number of flowers and seeds (Table S2). Specifically, the number of flowers and seeds was threefold greater in JA-induced plants than in control plants, but this increase was only significant in ant-excluded plants (Fig. 3). We also observed that, independent of the presence/absence of ants, the time of production of flowers differed between treated and non-treated plants. Thus, we conducted an additional analysis to examine the number of flowers produced per plant during the first 6 d after the application of JA. Flower production per day during this period was 2.5-fold greater in plants treated with JA than in untreated control plants ($F_{1,20} = 32.29$; $P < 0.0001$; JA-treated plants: 6.85 ± 0.41 , Control plants: 2.25 ± 0.41).

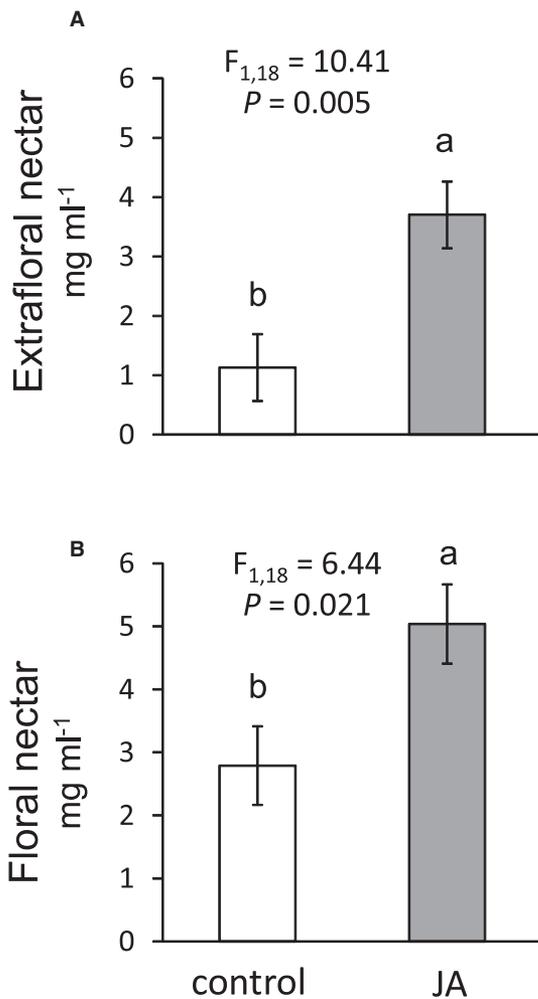


FIGURE 1. Effect of induction treatment (white bars for untreated control and gray bars for jasmonic acid) on the concentration of (A) extrafloral nectar and (B) floral nectar in *Phaseolus lunatus* plants. Nectar secretion was measured as mg of soluble solids secreted per g dry mass 4 h after treatments. Bars are means \pm SEM ($N = 10$). F -values, degrees of freedom, and associated significance levels (P) are shown. Different letters indicate significant differences between constitutive and induced conditions.

DISCUSSION

Our results showed that JA induction increased the amount of floral and extrafloral nectar produced by Lima bean plants in natural conditions. As a consequence of this increased nectar production, the attraction of ants increased markedly in JA-induced plants. Despite an increase in floral nectar in JA plants, application of this hormone had no significant effects on flower visitor's attraction. Finally, an increase in ant abundance did not result in a decrease in the number of visits by flower visitors, but our results suggest that ant presence may not be beneficial for the plant. In particular, JA-induced plants without ants produced a greater number of seeds compared with the JA-treated plants with ants, suggesting that ants could negatively affect pollination efficiency.

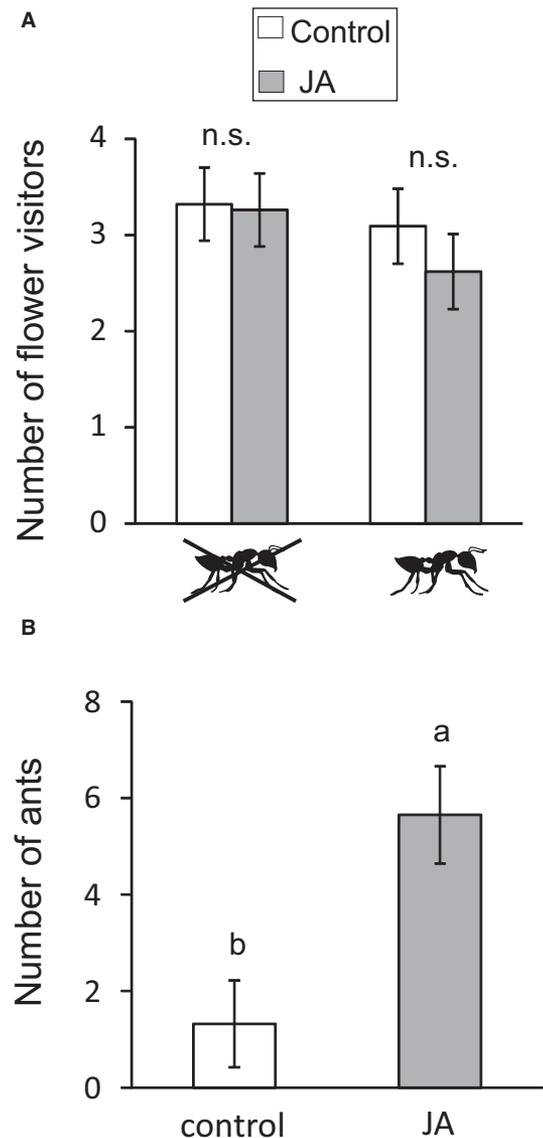


FIGURE 2. Effect of the presence of ants (two levels: presence or absence) and induction treatment (white bars for untreated control and gray bars for jasmonic acid) on flower visitor abundance in *Phaseolus lunatus* plants (A). Effect of induction treatment (white bars for untreated control and gray bars for jasmonic acid) on ant abundance (B). Measurements were carried out during 21 consecutive days. Bars are means \pm SEM ($N = 10$). ns in (A) indicates non-significant differences. Different letters in (B) indicate significant differences between constitutive and induced conditions.

Our results showing that exogenous JA application increased the amount of floral and extrafloral nectar confirm earlier observations on the same plant system in which JA was applied directly using tendrils from natural plants (Heil 2004, Hernández-Cumplido *et al.* 2010), as well as on other tropical systems (Heil *et al.* 2001). For example, Heil and colleagues observed that JA-induced plants of *Macaranga tanarius* (Heil *et al.* 2001) and *Phaseolus lunatus* (Heil 2004) produced higher concentrations of extraflo-

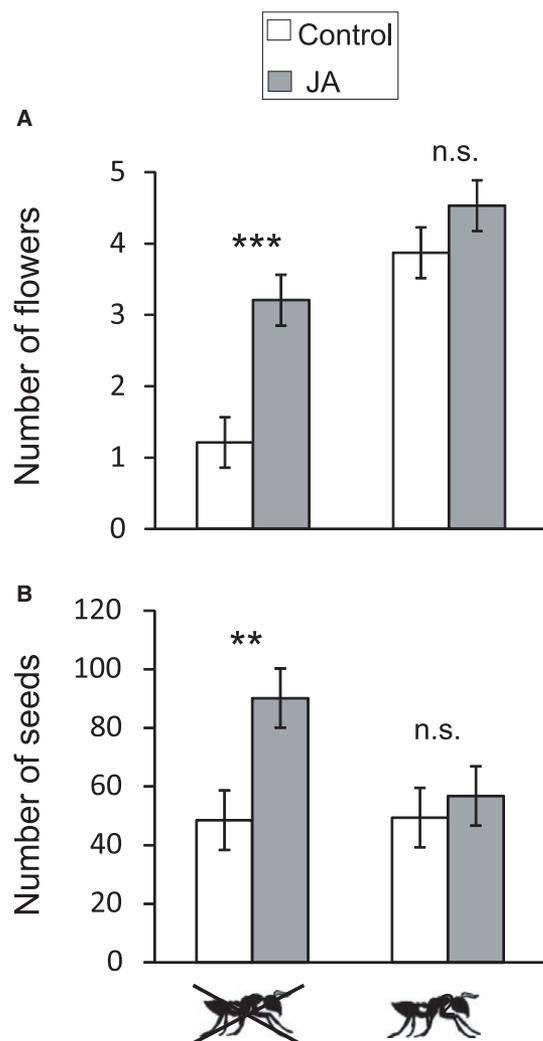


FIGURE 3. Effect of presence of ants (two levels: presence or absence) and induction treatment (white bars for untreated control and gray bars for jasmonic acid) on the number of (A) flowers and (B) seeds in *Phaseolus lunatus* plants. Measurements of flowers were carried out during 21 consecutive days. Bars are means \pm SEM ($N = 10$). Asterisks indicate significant differences between constitutive and induced conditions within each ant treatment at $P < 0.01$ (**) and $P < 0.001$ (***). ns indicates non-significant difference.

ral nectar than control plants. Heil (2011) also documented that JA induction markedly increased the amount of floral nectar, suggesting that nectar production in flowers and EFN of Lima beans might be subject to joint hormonal control.

Supporting previous observations in *P. lunatus* (e.g. Heil 2004, Hernández-Cumplido *et al.* 2010), we documented that increased floral and extrafloral nectar concentrations after JA application augmented ant attraction to JA-induced plants. These changes in nectar concentration and ant attraction should result in significant changes in the arthropod community and in plant fitness. However, in the present study we observed that flower visitors were not influenced by the JA-induction treatment. Previous research has reported contrasting effects of this hormone on

the frequency of pollinator visits (Bruinsma *et al.* 2008, Radhika *et al.* 2010). For example, Bruinsma *et al.* (2008) observed that rates of flower visitation to *Brassica nigra* by honeybees and syrphid flies were similar in JA-induced plants and in untreated controls. In contrast, Radhika *et al.* (2010) found that *B. napus* plants induced by JA application showed increased pollinator visits, thus enhancing pollination efficiency. Our results showed that pollinator visits did not decrease in plants with ants present, and this seems to show an absence of conflict between ants and flower visitors. However, when we analyzed plant fitness traits, we observed that JA-treated plants without ants produced more seeds than JA-treated plants with ants (Fig. 3B), suggesting a negative interaction between ants and pollinators. Supporting our results, previous studies have observed that ants interfere with pollinators by driving them away from the flowers, or by reducing the time of pollinator visits (Ness 2006, LeVan *et al.* 2014), and this in turn could negatively affect pollination efficiency. For example, Ness (2006) observed that visitation rates by bees and the time that they spent on the flowers was only reduced by the presence of aggressive ant species. During our study we did not witness any aggressive behavior by ants, but they actively patrolled the entire plant, including the flowers (Hernández-Cumplido, personal observation). We cannot exclude other explanations for the fewer seeds produced by JA-induced plants with ants. Ants may, for instance, have directly affected the development and performance of the flowers. For example, some species of ants such as *Crematogaster dohrni* are known to castrate flowers by damaging them when the style and stamens are still folded (Gaume *et al.* 2005). Also, the increased secretion of nectar by JA-induced plants may have prompted the ants to constantly remove the nectar and act more as exploiters than mutualists, possibly resulting in reduced resource allocation toward reproduction (Gonzalez-Teuber *et al.* 2012).

Overall, the exogenous application of JA in the absence of ants had a positive effect on plant fitness (*i.e.*, the number of flowers and seeds), which indicates no reproductive costs associated with induction by JA. These findings contrast with those from several previous studies that have reported drastic reproductive costs (*e.g.*, decreases in the number of seeds, seed mass and seed germination) in a diverse array of plant systems (*e.g.*, Van Dam & Baldwin 2001, Cipollini 2007, 2010). Fitness costs associated with JA induction have usually been explained by emerging trade-offs between induced defenses and fitness traits (Cipollini 2010, Sampedro *et al.* 2011). Nevertheless, our results showing no apparent cost (or even positive effects) are also compatible with those of other studies in the same (Heil 2004) and different plant systems (Thaler 1999). For example, Heil (2004) observed that the number of fruits in *P. lunatus* plants treated with JA was threefold greater than in control plants.

An unexpected and noteworthy result from this study was that, independent of ant presence or absence, plants that were treated with JA produced flower considerably earlier than non-treated plants. This is further supported by another study in which Lima bean plants that were induced with JA and mechanically damaged to simulate herbivore-feeding, flowered earlier than

untreated plants (Hernandez-Cumplido *et al.* unpubl. data). Early flowering can have major consequences for plant fitness. For example, changes in the phenology of flower production can alter plant-pollinator interactions and influence patterns of seed production (Herrera 1995, Herrera *et al.* 2001, Elzinga *et al.* 2007, Munguía-Rosas *et al.* 2011, Lucas-Barbosa *et al.* 2013). For instance, Lucas-Barbosa *et al.* (2013) showed that oviposition by *Pieris brassicae* on plants of *Brassica nigra* accelerates flower and seed production. Our findings are highly relevant in the context of the Lima bean system, as pod and seed beetle cycles are synchronized with plant phenology, thus by producing flowers earlier the plants may escape seed predation.

In summary, we found that the interactive effects of JA induction and ant presence modify the production of nectar in both flowers and EFN, influence the arthropod community associated with Lima beans (*i.e.*, flower visitors and ants), and affect plant reproductive traits. In particular, Lima bean plants under JA induction produce more nectar in both EFN and flowers (suggesting joint hormonal control), attract more ants, and produce more seeds than non-induced plants when ants are excluded. A decrease in seed set in plants with ants suggests that ants may interfere with pollination, implying that, in situations of high ant abundance there may be an ant-pollinator conflict that affects the fitness of Lima bean plants. Future studies should examine the ant-created trade-off between the benefits of suppressing herbivory and the detrimental effects of interfering with pollinator, of which the outcome may vary under different ecological conditions and insect densities. Likewise, the mechanisms underlying the patterns observed in our study, such as the physiological processes that lead to increased nectar production and the specifically adapted chemical composition of the nectars, warrant further investigation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found with online material:

TABLE S1. Summary of mixed models analyzing independent and interactive effects of ant and induction treatments on the abundance of pollinators and ants.

TABLE S2. Summary of mixed models analyzing independent and interactive effects of ant and induction treatments on the number of flowers and seeds.

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