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Direct and indirect defense responses of Lima bean (*Phaseolus lunatus*) to herbivory: implications for tritrophic interactions

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"*Por mi raza hablará el espíritu*" (Jose Vasconcelos)

Introduction

Direct and indirect plant defenses against insect herbivores

During the last decade a recurrent question in the field of insect ecology has been how plant defense can affect not only the insects that are directly associated with a single plant species but also the community of organisms associated with that species. More recent work has also focused on exploring the ecological and evolutionary consequences of plant defense on insect community structure (Poelman et al. 2010, Stam et al. 2014). Plants respond to insect damage with a wide range of responses which can trigger cascade reactions that can affect not only conspecific herbivores that attack the same plant tissue, but also herbivores that arrive later on the season and attack different plant structures. This phenomenom can drive not only pairwise interactions between plants and insect herbivores but also can interfere in the entire community (third and fourth trophic level).

Herbivore-induced plant defenses

Historically plant defenses have been divided in direct and indirect responses against insect herbivory, direct defenses include those traits that affect plant susceptibility to insect attack (Kessler and Baldwin 2002) and indirect defenses include those traits that favor the attraction of natural enemies that will reduce the number of herbivores and as result, reduce the degree of herbivore damage (Heil 2008).

Direct Defenses

Plants are known to produce defensive compounds against insect herbivory (Karban et al. 1997). As soon as one herbivore start to feed on the plant, this triggers a series of defensive responses that result in the production and release of defensive compounds (Farmer and Ryan 1990, Karban et al. 1997, Agrawal 1999, Underwood and Rausher 2002). Several of these responses are very well studied and some of them include changes in the nutritional quality of the plant (Denno et al. 2000), the release of volatile substances that may serve as attractants for natural enemies (Turlings et al. 1990, Vet and Dicke 1992), as well as the production of physical defense structures such as thorns, spines and trichomes that may interfere with herbivore movement , and changes in plant architecture by compensatory regrowth and phenology which includes bud burst, leaf flush and flowering onset (Karban and Baldwin 1997, Oghushi et al. 2007).

All of the above factors are crucial in determining different food and habitat suitability for herbivores that share a host plant at the same time and at different times along the season. Evidence from several studies indicates that induced plant defenses can have positive or negative effects not only on the first events of herbivory, but also on those herbivores that subsequently utilize the same resource (Oghushi et al. 2007) and even on the natural enemies of these herbivores (Poelman et al. 2011). Ultimately, these interactions between plants and herbivores may provide the basis for the nature and structure of herbivore communities (Havill and Raffa 2000, Fornoni 2011, Stam et al. 2014). The aim of the first part of the current thesis is to add more and novel information to this growing field.

Indirect defenses

Indirect defenses include traits that increase the attraction of natural enemies (predators, parasitoids or ants) that will reduce the number of herbivores that attack a plant and thus reduce the degree of herbivore damage (Turlings et al. 1990, Heil 2008, Dicke and Baldwin 2012). Past and recent reviews have discussed numerous examples of indirect defense plant traits (Hairston et al. 1960, Schmitz et al. 2000, Romero and Koricheva 2011). For the specific case of the extrafloral nectaries (EFN) they have been shown to attract predatory ants that defend the plants against herbivores and positively affect plant reproduction (Heil and Mckey 2003, Heil 2004, Rico-Gray and Oliveira 2007, Heil 2008, Chamberlain and Holland 2009). Nevertheless there is evidence that ants also can affect plant reproduction by reducing the number of pollinators that visit the plants. For example, chasing away or attacking the pollinators, interfering with them or simply affecting their behavior only by being present while patrolling the entire plant (Galen 1999, Nicklen and Wagner 2006, LeVan et al. 2014). Although several hypotheses have been proposed to explain how plants solve this potential conflict, very little information is available (Raine et al. 2002, Wagner and Kay 2002, Galen 2005, Nicklen and Wagner 2006, Holland et al. 2010). I explored this question in two field studies that specifically examined the positive and negative effects of the interaction between a plant species, ants and flower visitors.

Study system

The Plant: Phaseolus lunatus

Phaseolus lunatus L. (Leguminosae) commonly known as Lima bean, has been used as a model plant in several studies on inducible plant defenses (e.g. Heil 2004, Ballhorn et al. 2008a, Heil 2008, Ballhorn et al. 2009). In nature, it occurs in natural populations along the pacific coast, from Mexico to South America. The phenology of Lima bean is seasonal; it germinates between June and July and starts to produce flowers at the beginning of October. Seeds are produced during November and December and are dispersed in January.

The Insects

Early in the season Lima bean plants are mainly attacked by two specialist herbivores, the leaf-chewing *Cerotoma ruficornis* Olivier (Coleoptera: Chrysomelidae) and the green-pod beetle *Apion Godmani* (Coleoptera: Curculionidae). *C. ruficornis* feeds on the lower surface of the leaves and maintaining the cuticule of the leaves without damage. In our study site, the life cycle of these species is synchronized with the phenology of Lima bean plants. Beetles start appearing around June-July which corresponds with the period when plants start to stablish in nature. Their densities start decreasing by mid-November, when most of the plants have already produced flowers and green pods start forming and when the plants start flowering the densities of these beetles start decreasing. The curculionid *A. godmani* oviposits in the newly formed pods and all its development takes place in the green pods throughout pod maturation, as soon as the seeds mature they come out from the pods and infest new green pods all this through late October and November. By Mid-December, when the pods are dry and start to open, the Mexican bean weevil *Zabrotes subfasciatus* enters the dry pods and glues its eggs on the seed coat of mature seeds. Second instar larvae of *Z. subfasciatus* are parasitized by the ectoparasitoid *Stenocorse bruchivora* from early January until the the end of the season, second to third week of February (Campan and Benrey 2006).

Phaseolus lunatus and all of the insects mentioned above are originally from Mexico and Central America where plants and insects have interacted for thousands of years and have shared a long evolutionary history (Benrey et al. 1998, Campan and Benrey 2004, Heil 2004, Alvarez et al. 2007, Aebi et al. 2008, Shlichta et al. 2014)

The Chemical Ecology of Lima bean Phaseolus lunatus

Several defensive traits have been evaluated in Lima bean plants. Heil (2004) studied the induction of two different indirect defenses in Lima bean using as a tool jasmonic acid and he found that eleven HIPVs at significantly higher amounts compared with control plants (herbivore induced plant volatiles) were released in plants under JA induction and only one (Methylsalicilate) at significant lower amounts than control plants. In addition, he found that extrafloral nectar production was increased in bracts (more than 20 times) and leaves (more than 30 times more)

Ballhorn and coauthors (Ballhorn et al. 2008a,b; 2009) measured cyanogenesis as a direct defense in leaves of Lima bean plants. When a herbivore damage the leaves triggers an hydrolysis reaction that results in the poisoning of the insects. This intoxication results from the endogenous release of hydrogen cyanide within the gastrointestinal tract (Ballhorn et al. 2005; 2007).

Recently Shlichta et al. (2014) examined the relationship between the concentration of cyanogenic compounds in seeds of 12 populations of wild Lima bean plants and the performance of the Mexican bean weevil *Zabrotes subfasciatus.* No relationship was found between concentrations of CNGs (linamarin and lotaustralin) and beetle performance. This lack of relationship between the amount of CNGs and the Mexican bean beetle performance is explained by the fact that seeds lack the amount of water necessary to allow the hydrolysis that activates the toxicity of these compounds.

The existing information on the chemical ecology of Lima bean make this plant species a great model to examine complex questions on the effect of plant defenses on multitrophic interactions.

Overview of the thesis

The main objective of this thesis was to use a multitrophic approach to examine the ecological consequences of some of the defensive traits that plants have developed to deal against herbivory and to test hypotheses concerning the way in which these traits can affect the abundance and performance of the insects associated with the plant. To explore this idea, I used a combination of field and laboratory experiments as well as chemical analyses and chose as model plant system wild plants of *Phaseolus lunatus* and its associated insects.

In this thesis I present empirical evidence that illustrates different direct and indirect ways in which plant defenses can affect multiple trophic levels. This thesis is divided in two sections. The first section comprises two chapters.

Chapter one presents a study in which we used as a tool the phytohormone jasmonic acid (JA) to induce defensive responses in the plant. We then examined the effects of this induction on the infestation of seeds by the last herbivore that attacks the plant along the season, the Mexican bean weevil and of its larval parasitoid. In laboratory experiments, the performance of the bean weevil and that of its parasitoid was examined on seeds produced by induced and non-induced plants. In a second experiment of this same chapter we explored the transgenerational effects of defense induction by using seeds produced by plants under JA induction. For the first experiment we found a negative effect of JA induction on plant traits and on the infestation rate of the Mexican bean weevil that oviposit on seeds, as well as the incidence of the parasitoids associated to the Mexican bean weevil larvae. We confirm these findings with the laboratory experiment; again JA-treatments reduced the quality of the seeds as a resource for the Mexican bean weevil and also negatively affected parasitoid performance. For the transgenerational experiment, we found that the effects of the JA induction remained on plant traits of the daughter plants but also affected negatively the Mexican bean weevil infestation, but not its parasitoid's abundance. The results from this study revealed that seeds from control plants had the highest germination rate, were larger and heavier, and the plants that reproduced, produced more seeds than plants treated with JA.

In chapter two, we present a field study in which we manipulated the presence /absence of different herbivore species associated with Lima bean which attack different plant tissues or structures throughout its lifespan. These include the first insect that arrive to the plant, the chrysomelid beetle *Cerotoma ruficornis* which as an adult feeds on leaf tissue and secondly, the curculionid beetle *Apion godmani* that attacks green pods. Our main question was how each insect species on its own and their interaction, affects the susceptibility and suitability of Lima bean seeds towards its seed predator, the Mexican bean beetle *Zabrotes subfaciatus* and of its parasitoid *Stenocorse bruchivora* in the field. Following a similar protocol as in the previous chapter, seeds obtained from this field experiment where used in laboratory experiments to examine the performance of both, the Mexican bean weevil and the parasitoid *S. bruchivora*. We found that early herbivory affected negatively the abundance and the performance of the Mexican bean weevil and of the parasitoid. For the plant traits we found that early herbivory decreased the production of flowers, pods and seeds in the field. In parallel, we also found that seeds produced by control plants contained higher quantities cyanogenic compounds associated with resistance against leaf herbivory, as well as higher protein content than seeds produced by plants that had been subjected to different types of herbivores.

Section B of the thesis focuses on one of the most important indirect defenses in *Phaseolus lunatus*, the attraction of ants via the production of extrafloral nectar. I include two studies that aimed to examine the interaction of ants and flower visitors in *Phaseolus lunatus* and the potential consequences of this interaction on plant fitness. It is well accepted that plants present a number of mechanisms that have evolved to defend themselves against herbivory. In the case of Lima bean, the plants have developed extrafloral nectaries (EFN), which normally attract ants to reduce herbivory a benefit the plant. However, an alternative hypothesis suggests that the function of EFN is mainly to distract ants from flowers. In the first chapter of this section we study the effect of induction mediated by jasmonic acid (JA) on the increase in nectar production and its effects on the interaction between ants and flower visitors. We found that both guilds co-occur on the plant at the same time and plants treated with JA increase the EFN secretion, which results in an increase of the intensity of indirect defense (number of ants patrolling the plant), but in a reduction of the number of flower visitors even though plants are producing more nectar rewards.

Finally in the last chapter of the thesis (chapter four), I present a follow-up study that examined further the fitness consequences of the interaction between ants and flower visitors for Lima bean plants throughout their phenology. We conducted two field experiments where the presence of ants and nectar production via JA induction were manipulated. We measured nectar production in extrafloral nectaries and in flowers, the number of patrolling ants and flower visitors as well as some plant traits. It was found that induction is linked in both structures (FN and EFN), and JA application had no significant effects on pollinator attraction. Finally, ant presence did not result in a decrease in the number of pollinator visits. Nevertheless, results suggest the existence of an ant-pollinator conflict by which plants under JA induction reproduced more than non-induced plants, but only when ants were excluded. In particular, JA-induced plants without ants produced more flowers and seeds than JA-treated plants with ants, suggesting that ants could negatively affect pollination efficiency.

I end the thesis with a final discussion that integrates both sections and all findings of the various studies. Finally, I present an outline for the future directions of the research that could still be done in order to better understand the effects of plant-mediated interactions on insect communities. I argue for an integrative approach that should include biochemical and population and community ecology studies.

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One:

Inter-tissue and transgenerational effects of defense induction in Lima bean plants on natural tritrophic interactions

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ABSTRACT

Plants have evolved a vast array of mechanisms to defend themselves against insect herbivores. Herbivory-induced defense responses in plants typically involve the jasmonic acid (JA) pathway. The activation of the JA pathway leads to biochemical changes that may persist over time and can affect insects that attack other plant tissues later in the season and may even be transmitted to the plants' offspring. Yet, little is know about how these cascading consequences of defense induction affect plant and insect performance in nature. We tested for inter-tissue and transgenerational effects in a field study with wild Lima bean plants in their natural habitat near the Mexican pacific coast. Plants were grown from seeds obtained from four natural populations. A set of potted plants were grown in tents to prevent herbivory during the first three weeks, after they were transplanted into a common garden arrangement. There they were either left untreated, or their leaves damaged and treated with a JA solution. Towards the end of the season, a subset of these plants was damaged in the pods and treated with JA to simulate attack by green seed-feeding herbivores. At the end of the season, the occurrence of seed beetles and their parasitoids in the dried seeds of all plants was recorded and seeds were collected to measure their suitability for these insects in the laboratory. To evaluate effects of the treatments on the second generation of plants, seeds collected for one of the populations from three of the treatments were planted the following season in a random arrangement. Throughout this second season we recorded plant performance and natural insect infestation.

For the first season, we found a negative effect of JA-treatments on plant performance and on the incidence of infestation by the Mexican bean weevil that oviposit on mature bean seeds, as well as the incidence of parasitoids that attack the

Mexican bean weevil larvae. In laboratory assays it was further confirmed that the JA-treatments reduced the quality of the seeds as a resource for the Mexican bean weevil and also negatively affected parasitoid performance. For plants of the second generation, we found that the negative effect of the JA-treatments on plant performance still remained and negatively affected the Mexican bean weevil infestation, but not its parasitoid abundance. We argue that some of the observed effects may be due to plant maternal effects, but could also be adaptive if insect attacks are indicative of future attacks.

Keywords: beans, host plant-mediated interactions, induced plant defense, jasmonic acid, parasitoids, transgenerational effects, trophic cascades.

INTRODUCTION

Plant responses after damage by insect herbivory have been extensively documented (Karban and Baldwin 1997, Strauss et al. 2002, Underwood and Rausher 2002). One of the main phytohormones that has been shown to be involved in plant defense against chewing herbivores is jasmonic acid (JA) (Farmer and Ryan 1992, Stratmann 2003). This plant hormone is involved in the defensive signaling against necrotrophic pathogens and chewing herbivores (Thaler et al. 1996, Thaler 1999b). Basically, JA activates the genes encoding protease inhibitors that help protect plants from insect damage (Johnson et al. 1989), but it can also affect seed and pollen production (Feys et al. 1994, Benedetti et al. 1995), root growth (Berger et al. 1996) and tendril coiling (Ehret et al. 1994).

During the last two decades, exogenous application of JA to plants has been shown to increase plant resistance against herbivores in both herbaceous and woody plants (Thaler 1999b, Omer et al. 2000, Thaler et al. 2001, Traw and Bergelson 2003, Boughton et al. 2005, Moreira et al. 2012). For example, it is well-known that exogenous application of JA changes oviposition decisions (Bruinsma et al. 2008) and reduces the performance of insect herbivores (Omer et al. 2000), increases levels of polyphenol oxidase, and trichome density in herbaceous plants (Thaler 1999b, Traw and Bergelson 2003, Boughton et al. 2005, Oghushi et al. 2007), and reduces or increases the production of resin and phenolic compounds in conifer trees (see review by Moreira et al. 2012).

Although the positive effect of JA application on plant resistance has been shown, little information is available on its effects on herbivores that feed on systemic tissues and how this affects herbivore’s multitrophic interactions. In one of the few available examples Bruinsma et al. (2008), showed a differential effect of JA on different guilds of insect species that interact with the black mustard plants *Brassica nigra.* In particular, they found that the butterfly *Pieris rapae*, prefers to oviposit on leaves of control plants compared with JA induced plants (Bruinsma et al. 2008). In the case of the parasitoid *Cotesia glomerata*, the pattern was the opposite as parasitoids were more attracted to JA-treated plants compared with control plants. Finally, they did not detect significant differences in pollinator visits between induced and non-induced plants (Bruinsma et al. 2008). In another study, Thaler et al. (1999a) observed that JA application to tomato plants (*Lycopersicum esculetum*) induced the production of defensive proteins (proteinase inhibitor and polyphenol oxidase) which decreased the abundance of herbivore species from different feeding guilds (caterpillars, flea beetles, aphids and thrips) (Thaler 1999a).

Another aspect of the effect of early induction that still remains largely unexplored, is the extent to which changes triggered by JA induction can be transmitted to the plant's offspring "transgenerational effects" (Agrawal et al. 1999, Agrawal 2001, Herman and Sultan 2011, Holeski et al. 2012, Rasmann et al. 2012). In a recent review, Holeski et al. (2012) reported that up to now there are only 17 studies that provide empirical support for transgenerational induction of resistance against herbivores and pathogens using real herbivory and several biochemical treatments such as jasmonic and salicilyc acid. Rasmann et al. (2012) showed that induced resistance of *Arabidopsis thaliana* and *Solanum lycopersicum* offspring was associated with trangenerational priming of jasmonic acid-dependent defensive responses and this resulted in a 50% decreased in caterpillar growth (*Pieris rapae*) compared with control plants. In spite of this recent evidence, no information is available about how plant transgenerational effects on herbivore resistance can cascade upwards to the next trophic level and affect the performance of the natural enemies of herbivores.

The aim of this study was to examine the effect of exogenous application of JA to two different plant structures (leaves and pods) of wild Lima bean (*Phaseolus lunatus*) on plant performance and on seed susceptibility to attack by the last herbivore that attacks the plant. Parallel, we evaluted if thise effects affect the weevil's main natural enemy, an hymenopteran ectoparasitoid. We further investigated if early-induction effects, positive or negative are trasmited to the plant's offspring. Exogenous application of JA has been previously shown to induce direct (Heil 2004) and indirect (Heil 2004, Ballhorn et al. 2009) defenses in Lima bean. We applied JA to leaves and pods of Lima bean plants early in the season and then we examined plant performance and the abundance of a late-season seed beetle and its parasitoid in the field. In addition, using seeds collected from JA-treated and control plants we conducted a laboratory experiment to test the effect of early-induction on beetle performance. In the following field season we conducted a second experiment with seeds produced by the plants subjected to the induction treatments (JA in pods, in leaves and control) and investigated their offspring's susceptibility to the seed beetle, as well as the effects on the beetle's main parasitoid. Finally, a sample of seeds from the two field experiments was used to quantify two cyanogenic glycosides (CNGs) known to be associated with resistance of Lima bean plants against leaf herbivores (Ballhorn et al. 2009). Thus, by using this combination of field and laboratory experiments and chemical analyses, we tested the hypothesis that prior induction of plant defenses early in the season will result in increased resistance against late-season herbivores and their parasitoids, negatively affecting their performance. In addition, we examined the extent to which these effects can be maintained in the plant’s offspring. To our best knowledge there is no previous study of plant transgenerational effects on the third trophic level.

MATERIAL AND METHODS

Study System

Field experiments were conducted at the experimental campus of the Universidad del Mar located 15 km northwest of the town Puerto Escondido (Oaxaca, Mexico, 15°55.596’N, 97°9.118’W). The site is surrounded by agricultural fields, as well as bordering wild Lima bean plants (Heil, 2004, Hernandez et al. 2010, Shlichta et al. 2014). At this site, Lima bean phenology is synchronized with the regional weather, which is characterized by one main rainy season from June to October. This seasonality follows a bimodal distribution peaking in July and September. Seeds start to germinate during June and July. The first inflorescences appear in October or November and the seeds are produced at the end of December and early January. Lima bean plants show an explosive seed dispersal mechanism; once seeds are mature the seedpods pop open ejecting their seeds away from the plant. Previous studies have shown considerable variation in chemical composition among different populations of wild Lima bean plants (Vargas et al. 2000, Ballhorn et al. 2008b, Shlichta et al. 2014). To account for potential treatment differences due to natural variation in plant quality, experiments were conducted using plants from 4 different populations.

At our study site, Lima bean plants are attacked by several insect species. Two of the most abundant leaf herbivores are the chrysomelid species *Cerotoma ruficornis* (Olivier) and *Ginandrobrotica guerreroensis* (Jacoby), (Heil and Silva

Bueno 2007). Adult beetles feed on the lower surface of the leaves causing the upper surface to dry which results in a skeletonized appearance. Upon the production of pods, the first herbivore that attacks the seeds is the bean pod weevil *Apion godmani* (Curculionidae). Adults appear in the field before or at flowering time, and oviposition takes place in newly formed pods. The female chews a small hole in the mesocarp of the pod where it oviposits and larvae feed on the young developing seeds (Mc Kelvey 1950). Later in the season, when the pods are dry and start to open, females of the Mexican bean weevil, *Zabrotes subfasciatus* (Chrysomelidae: Bruchinae) colonize the bean plants and enter the dry pods. Females glue their eggs on the seed coat and upon emergence, larvae tunnel and develop within the seed where they complete their development and emerge as adults (Benrey et al. 1998). Second instar larvae of *Z. subfasciatus* are parasititized by the solitary ectoparasitoid *Stenocorse bruchivora* (Campan and Benrey 2006). The performance of this parasitoid species is dependent on host quality mediated by the plant (Benrey et al. 1998, Campan and Benrey 2004, Campan and Benrey 2006) also they are selective parasitizing fewer hosts and mainly in seeds in were they perform better. Thus, we would expect that the foraging behaviour of adult wasps will be affected by early-induced chemical changes in the plant.

Experimental set-up and design

First field experiment (2011-2012)*: Effect of early JA-induction on plant traits and infestation by the bean weevil and its parasitoid.*

Seeds of Lima bean plants were obtained from four wild populations: three populations from Oaxaca State near the coast: Km. 103 (Km; N15°57.742

W97°20.503), San Martin Caballero (SMC; N15°56.850 W97°19.339) and Cruz Azul (CA; N15°54.271 W°97 07.131), and a fourth population from the center of Mexico: Yautepec, Morelos (Y; N18 55.191 W99 02.397). One hundred and twenty plants were grown in 5 L pots. After sowing the seeds, pots where placed under field tents (4x4x2 m) for a period of 3 weeks to avoid any unwanted foliar damage.

On the second week of November, groups of 40 plants (10 plants per population, in total 40 plants) were subjected to 3 different treatments: 1) leaves treated with 1 mM JA in H_2O (JAL), 2) pods treated with JA (JAP) and 3) untreated control plants (C). For the first treatment, 10 leaves of each plant, were damaged by piercing the entire surface with a metal brush (±30% damage) and each damaged leaf was sprayed with water containing jasmonic acid (1 mM JA in H_2O). This was repeated once a week for 4 weeks, but on different leaves (resulting in 40 damaged leaves per plant). This treatment simulated damage by leaf herbivores. For the second treatment, pods were treated in a similar manner as the leaves, but 45 days later in the season. Again, 40 plants (10 from each population) were treated once a week for 4 weeks, by damaging all immature pods with pins by poking each pod 10 times and spraying them with 1 mmol litre−1 aqueous solution of JA. This treatment simulated the attack of the bean pod weevil, *Apion godmani.* Finally, the third treatment served as a control (plants were only sprayed with water).

Plants were randomly arranged in a common garden in the experimental field. Plants were monitored once a week for a period of two months and each time we counted the number of leaves per plant, and the number of newly formed and mature pods. Plants were examined for pod and seed maturation until the end of the season. Two weeks after the last treatment and when pods had dried and showed signs that they were ready to shed seeds, they were harvested and stored in plastic containers.

Seeds were transferred to the laboratory and placed in an incubator (Percival incubator model: 1-36VL) under controlled conditions (11 h at 27°C /13h at 25°C, 11L/13D, ~80% relative humidity and irradiance 200µmol m–2 s –1) until beetle and parasitoid emergence. We counted the number of individuals that emerged from all the seeds of each plant. Parasitism rate was calculated as a proportion: number of parasitoids / number of bean weevils + number of parasitoids per plant. Finally, a set of uninfested seeds (N=30) from each plant-treatment was randomly chosen and their size (Toolzone 150mm (6") Electronic Digital Vernier Caliper)) and weight (balance Mettler AE163) were recorded.

Mexican bean weevil performance in the laboratory

Seeds produced by plants at the end of the first field season were used in a lab experiment to determine if seeds from the different induction treatments differed in their suitability to bean weevils. Treatments consisted of seeds produced by: 1) untreated plants, 2) plants treated with JA on leaves and 3) plants treated with JA on pods. Five seeds randomly selected from each induction-treatment/ population were placed in small plastic cups (28x23x5mm, Semadeni AG, A4686), with a couple of recently emerged *Z. subfasciatus* individuals (*i.e.* one male and one female). All the weevils originated from a lab colony that has been maintained for over two years on cultivated seeds of *Phaseolus vulgaris.* The set-up was repeated five times for each treatment; three treatments x 4 populations, 5 cups per induction treatment/population combination (N=60). Cups were randomly arranged in an incubator under controlled temperature and humidity conditions (11 h at 27°C /13h at 25°C, 11L/13D, ~80% relative humidity and irradiance 200µmol m–2 s –1). Beetles were left in the cup for 5 days, and each day we inspected for the presence of eggs on each seed. Seeds that carried eggs were removed from the cup and individually stored in 2 mL Eppendorf tubes until weevil emergence. Only one egg was left on each seed, additional eggs were removed with a fine brush. Upon emergence, we recorded the development time (time from oviposition until emergence), number of beetles that emerged per seed and sex and weight of each individual. We also estimated the probability of seeds to receive *Z. subfasciatus* eggs, calculated as the number of seeds that had eggs / total number of seeds available in each cup. Because of the limited number of uninfested seeds recovered from each plant-treatment, we were not able to test parasitoid performance with these seeds. However, we can infer by using the information available from numerous previous studies with this and other *Phaseolus* species, which have consistently shown that parasitoid performance is positively correlated with beetle performance. This latter one is highly influenced by host plant quality. Such that lower quality seeds will carry beetle hosts of lower quality for parasitoids negatively affecting their performance (Benrey et al. 1998, Campan and Benrey 2004, Zaugg et al. 2013 Hernandez-Cumplido et al. and Moreira et al. unpublished data).

Second field experiment (2012-2013) *(F1))*: *Transgenerational effects of early JA-induction on seed susceptibility to the bean weevil and its parasitoid.*

For this experiment we used seeds from only one population, Km103 that were produced in the first field experiment. We chose to do this because in the previous experiments we did not find significant effects of population on the seed's susceptibility to the attack of bean weevils and their parasitoids. Plants from the three treatment groups in the previous year (details above) were sown in 5 L pots. During the field season of September 2012 and February 2013, we conducted a common garden experiment with the following plant treatments: 1) plants grown from seeds produced by plants mechanically damaged at the leaf stage and sprayed with JA, 2) plants grown from seeds produced by plants mechanically damaged at the pod stage and sprayed with JA, 3) plants grown from seeds produced by undamaged plants sprayed with water (control). In total we had 54 plants, 18 per treatment and randomly distributed in a 60 square meters plot. Plants were exposed to natural herbivore damage.

As in the first field experiment, plants were examined throughout the season and we counted the number of leaves, flowers, pods and seeds produced per plant. Here, we also recorded seed germination rate, calculated as the number of germinated seeds of each treatment group. Upon pod maturation seeds were harvested and stored in plastic containers, transferred to the laboratory until beetle and parasitoid emergence. Seeds produced by these plants (F2) were also measured and weighed.

Chemical analysis (Quantification of Cyanogenic glycosides):

A subset of uninfested seeds from both years corresponding to the three different treatments was used to quantify the two dominating CNGs, linamarin and lotaustralin, using liquid chromatography coupled to mass spectrometry (LC/MS).

Eight samples per treatment (3 treatments) and per field experiment (2 experiments) were used (N=48). To prepare samples for quantification of CNGs, 5-10 beans per sample were submerged in liquid nitrogen and ground with a mortar and pestle to obtain a fine powder for each sample following the methodes described in Shlichta et al. (2014). Approximately 0.020 g of prepared bean powder per sample was stored in a 1.5 mL screw-top plastic tube. Samples were kept cold using liquid nitrogen throughout grinding and weighing of the sample before storing them at -80° C degrees. We added 1mL of ice cold 70% methanol to each sample and the samples were immediately placed on a heating block at ~90° C for 10 minutes. Tubes were removed from the heating block and allowed to cool on ice. Samples were then placed in a Branson 2210 ultrasonic shaker for 10 minutes and centrifuged at 8000rpm for 3 minutes. The supernatant was carefully removed from the tubes, avoiding any particulates at the bottom of the tube. Supernatant was stored in a 1.5mL plastic tubes at -80°C until analysis. The supernatant was diluted 1:50 with 70% methanol before analysis. CNGs were analyzed using an Acquity UPLC system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, USA) controlled by Masslynx 4.1. Separation was performed on a Waters Acquity BEH C18 column (50x2.1 mm i.d., 1.7 µm particle size) thermostated at 25°C. Mobile phases consisted of water + 0.05% formic acid 0.05% (solvent A) and acetonitrile + 0.05% formic acid (solvent B). The following gradient was applied: 2-30% B in 1.5 min, 30-100% B in 1.0 min, holding at 100% B for 2.0 min, and re-equilibrating at 2% B for 1.0 min. The flow rate was set to 400 µL/min. Under these conditions, linamarin eluted at 0.83 min and lotaustralin at 1.10 min (Online Resource1). The injection volume was 1 µL. Detection was performed in electrospray negative ionization mode using the [M+HCOO]- ion. Extracted ion chromatograms at m/z 292.113 ± 0.02 Da and 306.119 ± 0.02 Da were generated for quantification of linamarin and lotaustralin, respectively

Statistical analyses

Because our data did not meet the assumptions of normality, plant traits and beetle and parasitoid abundance in the field were analyzed using a generalized linear mixed model (PROC GLIMMIX with a Poisson distribution in SAS, SAS 9.2 System, SAS, Cary, NC) (Littell et al. 2006). The main effect of treatment was treated as a fixed factor. The effects of population and population × treatment were considered as fixed factors. For the analysis of bean weevil abundance, the total number of seeds collected per plant was used as a covariate.

Bean weevil performance traits in the laboratory were also analyzed using a generalized linear mixed model (PROC GLIMMIX with a Poisson distribution in SAS) (Littell et al. 2006). The main effect of treatment was treated as a fixed factor. The effects of cup (to account for repeated measures taken on the same experimental unit), population and population × treatment were considered as well as fixed factors.

For the second experiment, analyses of beetle and parasitoid abundance and of the number of leaves, flowers, pods and seeds were carried out using a generalized linear model (PROC GLM in SAS System, version 9.2, SAS Institute, Cary, NC) (Littell et al. 2006). The main effect of induction treatment was considered as a fixed factor. The number of seeds was used as a covariate for bean weevil abundance. Finally, a generalized linear model (PROC GLM) was also used to analyze the effect of induction treatment on seed size and weight.

Cyanogenic glycosides were analyzed by using a generalized linear model (PROC GLM in SAS) (Littell et al. 2006), we conducted a separate analysis for each field experiment.

RESULTS

First field experiment (2011-2012)*: Effect of early JA-induction on plant traits and infestation by the bean weevil and its parasitoid*

We found a significant interaction between the induction treatment and population on the number of leaves, pods and seeds produced by Lima bean plants (Table 1). Despite this significant effect on this interaction, specific patterns can be detected. Firstly, the performance of plants from the Yautepec population was considerably lower for all plant traits, (fewer leaves, pods and seeds) compared to plants from the other three populations. And secondly, plants from the 4 populations that were induced with JA at the leaf stage produced overall more leaves at the end of the season than plants that were induced at the pod stage and control plants (Fig 1a, b and c).

Induction treatment had a significant effect on the size and weight of the seeds produced by the plants ($F_{(2,73)}$=31.84, P<0.0001; $F_{(2,73)}$= 42.47, P<0.0001). Seeds produced by control plants were heavier (Mean±EE, C=0.082±0.0028 grams.) and larger (C= 7.18 ± 0.0115) than seeds produced by plants subjected to the JA-induction treatments (Weight: JA-leaves=0.0528±0.0027, JA-pods=0.00571±0.0278 and size: JA-leaves=5.784±0.1152, JA-pods=6.009±0.1132) (Fig. 2 a, b first year).

Results from the GLM analysis on insect abundance showed no significant effect of plant population and of the interaction between induction treatment and population ($F_{(2,107)}$=10.54 P<0.0001). However, JA application on leaves and green pods influenced the susceptibility of mature seeds to the attack by bean weevils and their parasitoids. Significantly more weevils emerged from seeds of non-induced control plants than from seeds of plants induced at the leaf and pod stage effect of induction treatment on seed infestation (C= 1.805±0.1189, JA-leaves=0.738±0.1187, JA-pods=0.905±0.188. Similar trend (although not statistically significant; P=0.08) was found for the parasitoids (Fig. 3 a and b for the first year). In both cases we did not detect an effect of population or an interaction between population x treatment (P=0.4738).

Effect of JA induction on beetle performance in the laboratory

We found a significant effect of the induction treatment on the number of eggs laid on the seeds (Table 2, Fig 4a). Female beetles laid more eggs on seeds that came from control plants than on seeds from JA induction treated plants. In the case of the weight of the adults, we detect a marginal effect of the treatment, weevils that emerged from seeds of control plants were slightly larger than beetles that emerged from seeds of plants under JA induction in pods (Table 2, Fig 4b). We did not find significant differences between treatments for developmental time (Table 3, Fig. 4c). No significant effects of population and of treatment x population interaction on any of the performance parameters measured (Table 2). Finally, exogenous application of JA on leaves and pods of Lima bean (vs. control) had significant effects on the sex ratio of adult bean weevils that emerged from the seeds (Table 2, Fig. 4d). More male betles emerged from seeds produced by plants induced at the pod stage.

Transgenerational effects of JA application in the field on plant performance and seed infestation by bean weevils and their parasitoids

Early plant induction with JA significantly affected the performance of the plant's offspring. Germination rate was significantly lower for seeds whose mothers were treated with JA on leaves and pods than for seeds whose mothers remained untreated (, $F_{(2,50)}$= 4.86, P= 0.011; Fig. 5a). No significant transgenerational effects of JA application were found for the number of flowers and pods produced by progeny plants (Fig. 5b, c). Finally, progeny plants produced significantly fewer seeds when their mothers were treated with JA at the pod stage than plants whose mothers were untreated ($F_{(2,50)}$= 26.95, P<0.001; Fig. 5d).

We also found transgenerational effects on the susceptibility of seeds to bean weevil attack consistent with the pattern found for seeds of the first generation ($F_{(2,36)}$= 12.53, P<0.001). Specifically, the number of beetles that attacked seeds on plants whose mothers were subjected to leaf or pod damage and treated with JA, was significantly lower than on plants whose mothers were untreated (Fig. 3c). However, for this second generation of seeds, we did not find any specific trends on the effects of JA application on the mother plant on parasitoid attack rates on seeds produced by the progeny ($F_{(2,17)}$= 0.23, P=0.79, Fig. 3d).

The analyses on the CNGs in seeds produced by plants from the mother and progeny generations revealed significant differences in the concentration of linamarin between seeds from the second year JA-induced and non-induced (control) plants, but only for the second year ($F_{(2,21)}$= 4.42, P=0.025). Linamarin concentration in control seeds was significantly higher than in seeds from the JA-induction treatments (Fig 6.)

DISCUSSION

The present study demonstrates that exogeneous application of JA on leaves and pods of Lima bean plants, triggers chemical changes that affect the suitability of the seeds for bean weevils and their parasitoids. Transgenerational effects of this induced resistance were also revealed by the lower insect infestation rates of Lima bean seeds produced by progeny plants whose mothers were subjected to JA application.

Effects of early-season JA application on the abundance and performance of late-season seed beetles and their parasitoids

Independent of the population of origin of the plant, seeds from plants that were induced early in the season suffered lower attack by the Mexican bean weevil and parasitism rates although not significant (*P*=0.08), followed a clear similar trend. Most likely chemical changes in the plants as a result of triggering the JA pathway resulted in the production of seeds of lower quality. We found similar results for the laboratory performance experiments.

Female of the Mexican bean weevil laid approximately 50% fewer eggs on seeds from treated plants (on average 3.25±0.57 in JAL and 3.6±0.58 in JAP) than on seeds of untreated plants (6.25±0.58), either as a result of being repelled by induced toxic allelochemicals in the seeds or by detecting a lower quality resource.

Most of the studies that have examined the effects of JA-induction on herbivores that attack the plant after a previous attack, report negative effects for the subsequent herbivores (Bartlet et al. 1999, Thaler 2002, van Dam and Oomen 2008, War et al. 2013). For example, Bartlet et al. (1999) found that JA induction in oilseed rape plants (*Brassica napus*) increased the glucosinolate concentrations which influenced the feeding behavior of the cabbage stem flea beetle, *Psylliodes chrysocephala* even to the extent to arrest their feeding on JA-induced plants. Similarly, War and collaborators (2013) found that following JA-induction on groundnut plants (*Arachis hypogaea*), female moths of *Helicoverpa armigera* laid fewer eggs on these plants (War et al. 2013).

This negative effect of JA-induction in *Phaseolus lunatus* on beetle performance could be the result of induced toxic allelochemicals in the seeds or a lower nutritional quality that influences beetle oviposition choices. Several studies with bruchid beetles have shown variation in oviposition patterns as a result of host quality and availability (Kawecki 1995, Fox 1997, Fox et al. 1997, Benrey et al. 1998, Campan and Benrey 2004, Or and Ward 2004, Takakura 2004, Gonzalez-Teuber et al. 2008, Zaugg et al. 2013). For instance, Fox et al. (1997) found great variation in egg size of the bruchid beetle *Stator limbatus* as a result of host plant quality. Females laid large eggs on seeds of the good quality host, *Cercidium floridum* and small eggs on seeds of the bad quality host, *Acacia greggii*. When females were offered the “good quality” host and then forced to oviposit on the “bad quality host” and vice versa, they were able to change the size of the oviposited eggs accordingly. We have not seen any evidence for this type of plasticity in egg size for *Zabrotes sufasciatus*. However we found that the proportion of males that emerged from infested seeds was higher in plants treated with JA in pods. Males of *Zabrotes subfasciatus* are in general smaller than females (Campan and Benrey 2004, 2006). If seeds produced by induced plants are smaller and/or of lower quality than those of non-induced plants, it could be that females do not have enough resources to successfully complete their development, resulting in a male-biased sex ratio. To confirm this idea, we measured and weighed seeds produced by plants subjected to the different treatments and as expected, control plants produced larger and heavier seeds than seeds produced by JA-treated plants (their size was 20% bigger and their weight was 30% heavier than the other treatments). Thus, it appears that beetles are selecting seeds based on their size and seed size could be used by the beetles as an indicator of quality. As shown by the results from the chemical analyses, CNGs do not appear to be an important component of seed quality, at least for these beetles. Linamarin was higher in control seeds but only for plants of the second generation. For all other seeds no significant differences were found on the concentrations of both linamarin and lotaustralin between induced and non-induced treatments. Although CNGs in Lima bean have been implicated in plant resistance against leaf herbivores (Ballhorn et al 2009), a recent study on the seeds showed no correlation between CNGs concentrations in wild seeds and beetle performance (Shlichta et al 2014). The explanation offered by this latter study is that most likely the seeds do not contain enough water that is required for the release of β-glucosidase that triggers the activity of the cyanogenic compounds transforming them into toxic cyanide (Selmar et al. 1988). Thus, it appears that for seeds of lima bean plants, even though they contain potentially toxic CNGs, other factors, chemical and/or nutritional, may be responsible for the higher beetle abundance and better performance on seeds produced by plants not treated with JA.

At the beginning of this study we predicted lower parasitism rates on *Z. subfasciatus* attacking seeds of JA-treated plants. This idea was based on the assumption that chemical-induced changes in the plant may negatively influence the host location and/or acceptance behavior of female parasitoids (Benrey et al. 1998, Turlings and Benrey 1998, Gols 2008, Chen et al. 2015). Moreover, due to the lower quality of the beetle hosts inside the seeds of induced plants, parasitoid survival should be lower (Turlings and Benrey 1998). However, in the field experiment we did not find enough evidence to support this notion. Although we see a trend of lower parasitism rates on beetle-infested seeds of treated plants, it is not statistically significant. It may be that, sample sizes are not large enough to detect any potential treatment effects. Alternatively, in order to locate and parasitize their host, parasitoids may rely on cues associated with the presence or absence of the beetle inside the seed and not with seed (and host) quality. Host quality may play a role once the host is parasitized and will be reflected on parasitoid performance (Turlings and Benrey

1998). Unfortunately due to limited sample size of non-infested seeds we were not able to conduct parasitoid performance experiments in the laboratory. But we have abundant evidence to support this idea from numerous studies with this bean and other *Phaseolus* species. In previous studies we have repeatedly found lower parasitism rates and lower parasitoid performance for parasitoids that attack bruchid-infested seeds of lower quality (Benrey et al. 1998, Campan and Benrey 2004, 2006, Zaugg et al. 2013, Laurin-Lemay et al. 2013, Hernandez-Cumplido et al.,Moreira et al. and Quijano et al. unpublished data).

Transgenerational effects of JA-exogeneous application on seed susceptibility to beetles and parasitoid attack

We found that beetle infestation was higher on seeds produced by plants whose mothers where not treated at the plant stage, indicating that JA- induction results in higher resistance for offspring plants.

Several studies have focused on the mechanistic basis of transgenerational defense induction and on how plants transmit to their offspring the resistance factors. Most of these studies have been conducted using bacteria, pathogens or simulated herbivory under laboratory conditions (Lucht et al. 2002, Kathiria et al. 2010, Slaughter et al. 2012) and have used homologous recombination, methylation, gene expression and virulence against pathogens as resistance proxies. But only a handful of studies have approached this question with an ecological perspective.

In a first study, Agrawal (1999) found that leaf damage on plants of *Raphanus raphanistrum* by larvae of the cabbage butterfly *Pieris rapae*, not only induced resistance to subsequent herbivory, but also the plant’s offspring was more resistant than undamaged plants (Agrawal et al. 1999). Offspring of damaged plants had a higher concentration of hidroxylated glucosinolates. Similarly, with the same biological system but using jasmonic acid as an elicitor, Agrawal (2002) observed that herbivory by *Pieris rapae* in the maternal generation of plants of *Raphanus rapahanistrum* affected the growth of progeny and seed mass which was found to be highly correlated with early plant growth. More recent studies like the one of ter Horst & Lau (2012), showed a decrease in survival and fecundity of the exotic Egyptian alfalfa weevil *Hypera brunneipennis* on plants of *Lotus wrangelianus* that came from seeds produced by plants that had been damaged by this herbivore species compared to undamaged plants (terHorst and Lau 2012). Slaughter et al. (2012) documented that *A. thaliana* seedlings primed with BABA (β-aminobutyric acid) and inoculated with *Pseudomonas syringae* promotes enhanced resistance in the next generation to both *P. Syringae* and the oomycete *Hyaloperonospora Arabidopsis* (Slaughter et al. 2012).

Also recently, Rasmann and collaborators (2012) documented that induced resistance of tomato and *Arabidopsis* plants was closely related to the jasmonic acid pathway. In particular, they found that larvae of *P. rapae* perform better in control plants than in induced plants and even further these responses remain for two generations. Moreover, they also used mutant plants of *Arabidopsis* that were deficient in jasmonate perception (coronatine insensitive1) and demonstrated that these plants did not show inherited resistance. It appears that MeJA induction in the previous generation can prime progeny to display enhanced resistance (Rasmann et al. 2012). We found that JA induction in Lima bean affects negatively seed production but also the germination success of seeds from the second generation. Germination was higher for seeds from control plants compared to those from plants under jasmonic acid induction. Moreover, plants from both generations; treated mothers and their naturally damaged progeny produced larger and heavier seeds than plants control. However, beetle infestation was higher on these seeds (control plants), indicating that induction with jamonic acid results in higher resistance for offspring plants, which is compatible with the studies cited above (Agrawal et al. 1999, Agrawal 2001, Rasmann et al. 2012). Neverhetless the mechanistic basis of the trangenerational effect of induced resistance operating in the Lima bean system remains to be investigated.

Effects of early defense induction on plant traits

As it is commonly the case for field experiments where there are numerous sources of temporal and spatial variation, we found differences in the effects of JA application on plant traits among the 4 native populations of Lima bean. Overall and regardless of the induction treatment to which they were subjected, plants from Yautepec had lower performance than plants from the other three populations. This population is located in the central plane of Mexico in localities with much cooler and dryer temperatures than those found on the coast of the state of Oaxaca, from where the other three plant populations originated and were field experiments were conducted. The extreme conditions between their original environment and the environment in which they were grown for the experiments, may explain in part the differences between plants from this and the other three plant populations. Differences among plant populations may also reflect variation in defense-induced responses. Previous studies have documented great variation in Phaseolin content (Vargas et al. 2000) and concentrations of cyanogenic glycosides (Shlichta et al. 2014) in seeds of wild lima bean populations, as well as population differences in cyanogenesis capabilities in leaves (Ballhorn et al. 2006, Ballhorn et al. 2007, Ballhorn et al. 2008a). In addition, local plant populations could have evolved or coevolved with the local populations of the other trophic levels (Campan et al 2005).

Based on the existing evidence we expected negative effects of induction on plant traits. Some previous studies have documented that JA negatively affects flowering time and seed and flower production (Thaler 1999b, a, Agrawal et al. 2000, Redman et al. 2001, Cipollini 2010). For example, Thaler (1999) found that JA-induced tomato plants produced fewer flowers than control plants, but this did not affect their yield (Thaler 1999a). Similarly, Cipollini (2010) observed that total seed mass and seed number in *Arabidopsis thaliana* was lower in JA-induced plants than in controls(Cipollini 2010). The latter result (i.e. positive effect of JA on leaf production) was unexpected regarding previous literature. In this sense, several authors have reported that JA induction increased leaf senescence (Creelman and Mullet 1997). However, and supporting our findings, other authors have observed that plants with intermediate levels of herbivory can fully compensate and even overcompensate for herbivore damage by maintaining or increasing foliar biomass (i.e. Rosenthal and Kotanen 1994, Strauss and Agrawal 1999, Núñez-Farfán et al. 2007, Fornoni 2011), and in some cases they can produce more seeds than non-damaged plants (Tiffin 2000), but this was not the case in our study. The only clear and highly significant effect of JA application on plant traits was for seed weight and size. Seeds from untreated plants were larger and heavier than seeds from treated plants (Weight C=0.082 ± 0.002, JAL= 0.052± 0.002 and JAP= 0.057± 0.002; Size C=7.18 ± 0.11, JAL= 5.78 ± 0.11 and JAP= 6.00 ± 0.11). In this regard, there is ample evidence that shows that seeds size is highly correlated with a successful germination and establishment not only in beans (Zhang 1993, Leishman and Westoby 1994, Lima et al. 2005), but also in plants of many other families (Westoby et al. 1992, van Mölken et al. 2005).

According with the optimal defense theory in plant herbivore interactions, plants have to pay a cost of producing secondary compounds to increase herbivore resistance which in turn will affect reproduction or growth (McKey 1979, Rhoades 1979, Karban & Baldwin 1997, Strauss et al. 2002). From our data we infer that there is a trade off between reproduction and the transmition of resistance to the plant's offspring. Plants under induction seem to have paid a cost in their number, weight and size of seeds produced, however, those seeds were more resistant to the attack of seed predators.

Conclusions

The results from this study provide strong evidence of plant-mediated effects triggered by JA induction on insects from different herbivore guilds and trophic levels. While some of the underlying mechanisms for these interactions remain to be elucidated, our results clearly reveal negative effects of JA induction on the Mexican bean weevil and potentially on its larval parasitoid. From the point of view of the plant our study demonstrates that mimicking herbivore attack early in the season can have a positive effect in terms of leaf production, however the negative effects on seed production and seed quality are more significant. This pattern was maintained as well in the next generation. We suggest that Lima bean plants are in a bind, on one hand their fitness could be increased by producing larger and more vigorous seeds with a better germination success, but at the same time these seeds will be more susceptible to the attack by seed predators attracted to this higher seed quality. Future studies should examine the relative advantage of seed size in lima bean plants in environments with high and low density of and seed predators.

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Table 1. Summary of results of the mixed model analyzing the effects of JA induction treatment (fixed factor) on: the number of leaves, pods and seeds of two generations of Lima bean plants. For the second plant generation, seed germination rate and number of flowers were also. The effects of population and population × treatment were considered as random factors.

First generation	**d.f.**			**Number of leaves**	**Number of pods**	**Number of seeds**	**Size**	**Weight**
Treatment	2			989.5***	2.55 †	3.72	31.84***	42.47***
Population	3			191.27***	26.4***	350.5***		
Treatment*Population	6			115.28***	15.81***	12.28***		
Second generation	**d.f.**	**Germination**	**Number of flowers**	**Number of leaves**	**Number of pods**	**Number of seeds**	**Size**	**Weight**
Treatment	2	4.86**	2.05	27.31***	0.51	26.95***	70.25***	73.69***

†* *P*= 0.08, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

Table2. Summary of results of the mixed model analyzing the effects of JA induction treatment and year (fixed factors) on the number of eggs laid, the emergence rate, the developmental time and the sexual proportion in the performance experiment on the Mexican bean beetle. The effects of cup (to account for repeated measures taken on the same experimental unit), population and population × treatment were considered as random factors. F-values, degrees of freedom and associated significance levels (*P*) are shown.

Source	d.f.	Number of eggs	Weight	Development time	Sex ratio
Treatment	2	11.76****	2.64 †	0.97	4.01*
Population	3	1.08	2.14	1.47	0.75
Treatment*Population	6	2.52	1.17	1.47	0.219
Sex	1		2.89	0.09	

† *P*=0.08, **P* < 0.05, ***P* < 0.01, *****P* < 0.0001.

FIGURE LEGENDS

Figure 1. Interaction between bean population (Ca, SMC, Km and Y) and induction treatment (JA application on leaves, JA application on pods, Control) on: (a) the number of leaves per plant, (b) number of pods per plant, and (c) the number of seeds per plant in wild *Phaseolus lunatus*. Points are means ± s.e.m. Different line colors correspond to the different bean populations.

Figure 2. Effect of the induction treatments on the weight (miligrams) and on the size of seeds (millimeters) produced by plants of the two field experiments, the two figures on the left show the weight (a) and the size (b) of the seeds produced by plants from the first generation (mothers) and figures on the right show the weight (c) and size (d) of the seeds of the second generation (progeny) of bean plants. Bars are means ± s.e.m. Different letters indicate significant differences between treatments.

Figure 3. Effect of the induction treatments on bruchid abundance and parasitoid emergence rate in the field for both experiments. The two graphs on the left correspond to a) number of weevils per plant and b) the parasitoids per plant (proportion) that infested seeds inthe first year experiment, while graphs on the right present again i a) the number of weevils per plant and in b) the parasitoids per plant (proportion) found in that infested seeds from the second plant generation. Bars are means ± s.e.m. Different letters indicate significant differences between treatments.

Figure 4. Effect of the induction treatments on *Zabrotes subfasciatus* performance in the laboratory. Herbivore performance was measured as a) number of eggs laid per seed, (b) weight, (c) developmental time and (d) sex ratio of the emerged weevils.

Bars are means ± s.e.m. Different letters indicate significant differences between treatments.

Figure 5. Plant traits of the second plant generation in the field in a) seed germination rate, (b) number of flowers, (c) number of pods and (d) number of seeds produced by plant in all treatments. Bars are means ± s.e.m. Different letters indicate significant differences between treatments.

Figure 6. Effect of plant JA-induction on the production of the glycoside cyanogenic in the seeds: in a) we show linamarin (mg/g of dry weight) and in b) we show Lotaustralin, both in 24 samples obtained from seeds of *Phaseoulus lunatus* plants. Bars are means ± s.e.m. Different letters indicate significant differences between treatments.

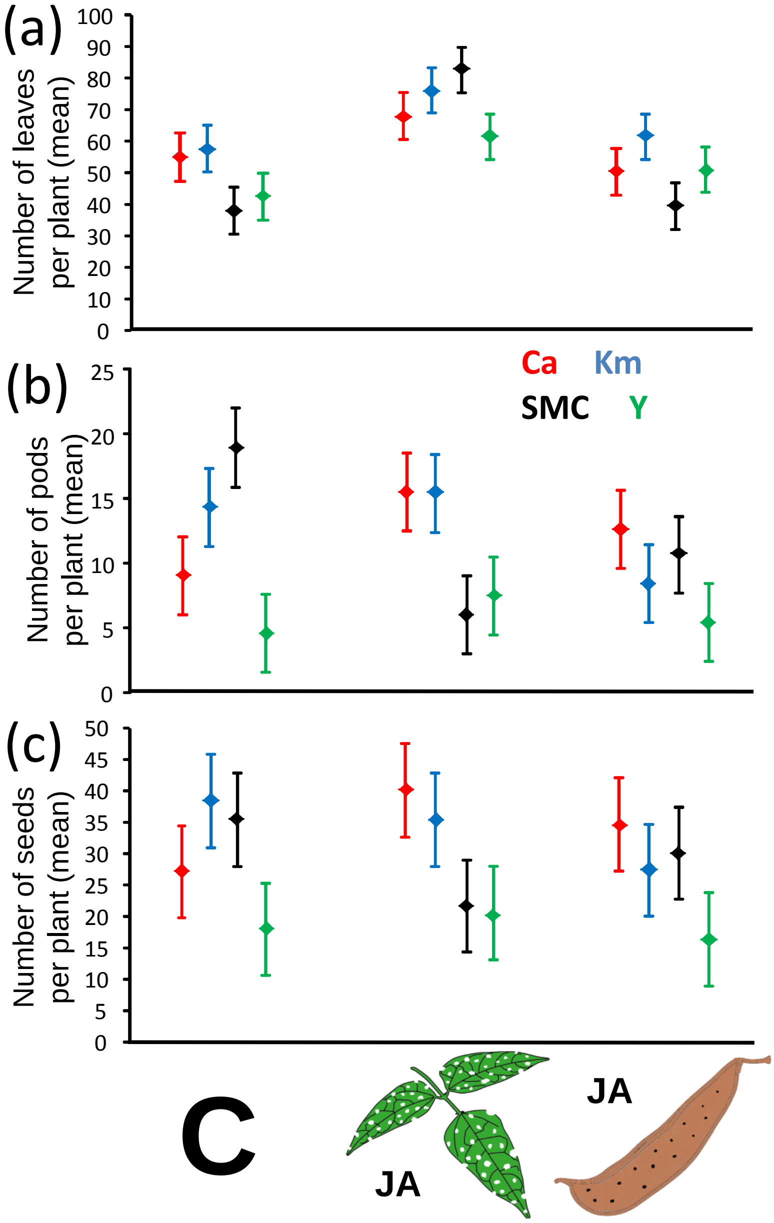


Figure 1. Hernández-Cumplido et al.

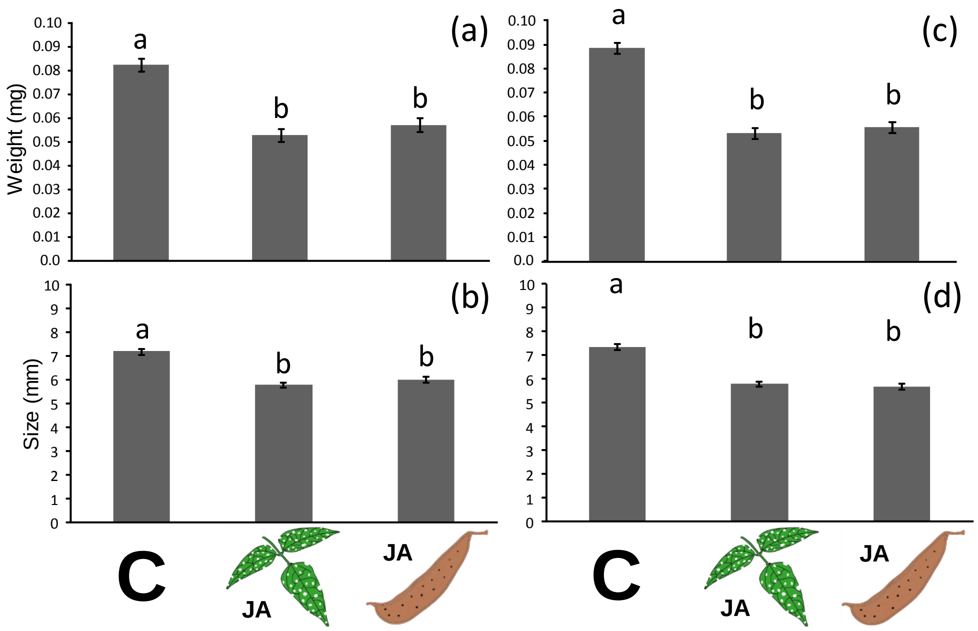
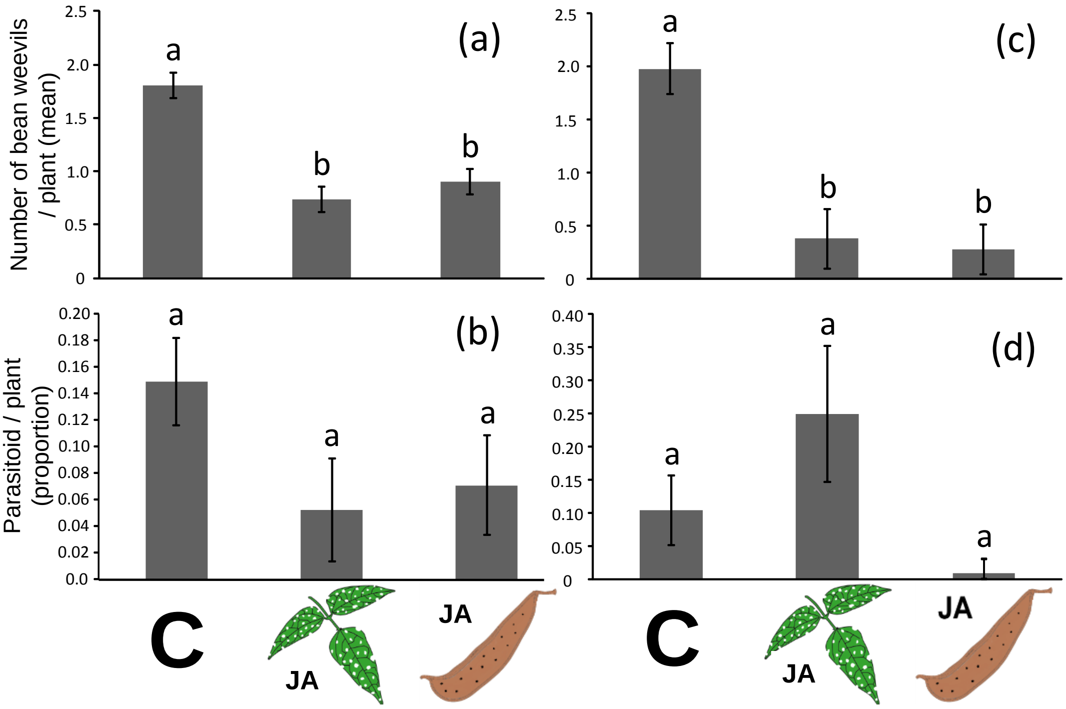


Figure 2 Hernández-Cumplido et al.



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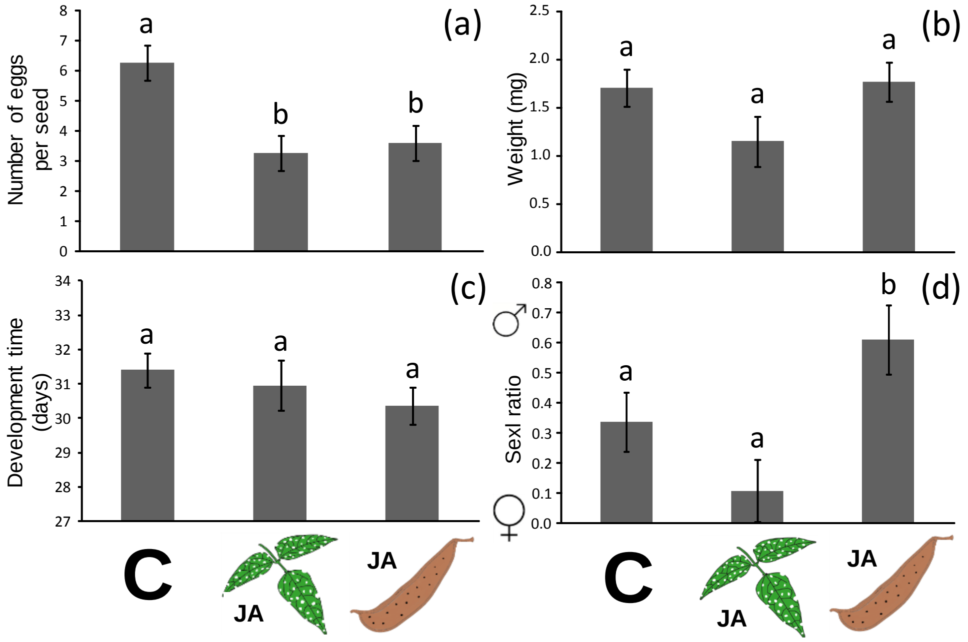

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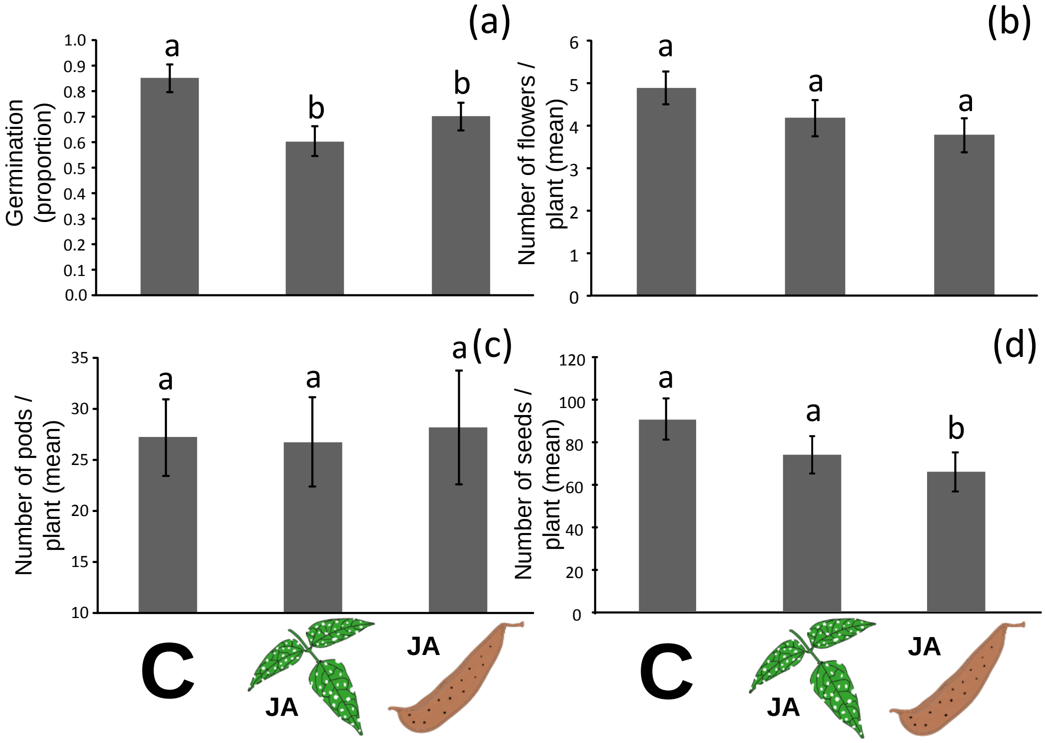

Figure 5. Hernández-Cumplido et al.

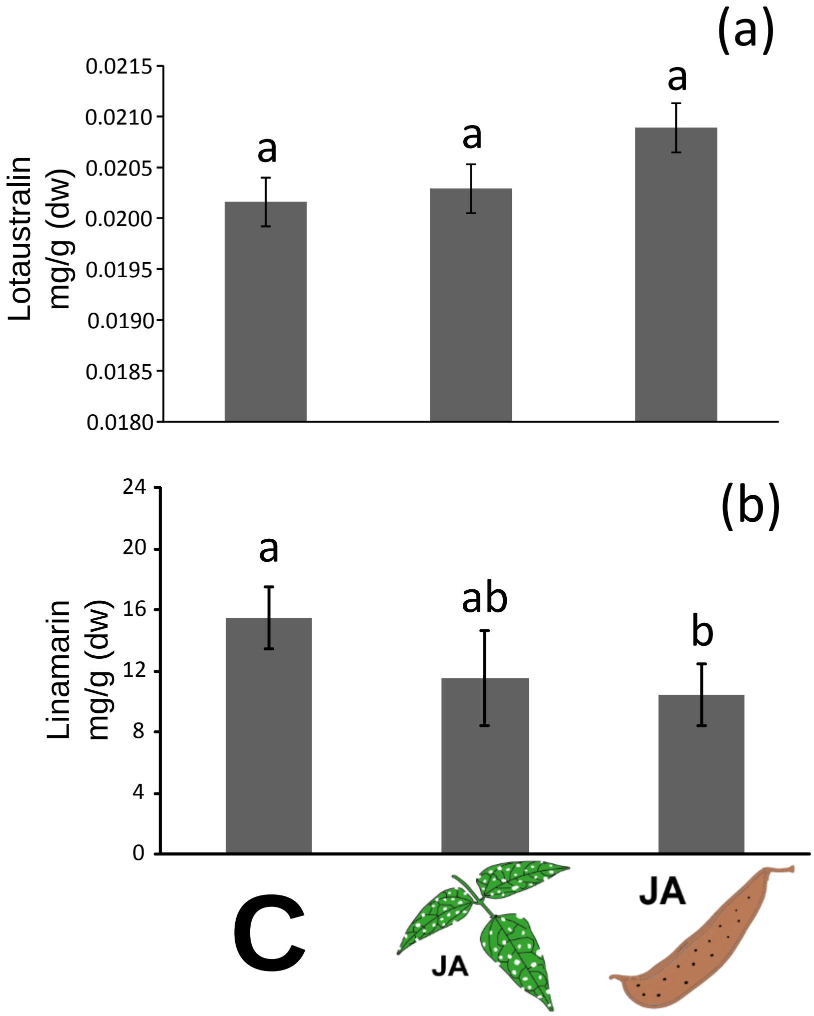

Figure 6. Hernández-Cumplido et al.

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Two:

Effects of early-season herbivory on the abundance and performance of late-season herbivores and their parasitoids

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Number of figures: 5

ABSTRACT

Early herbivory-induced responses in plants can trigger an induced resistance against insect herbivores that attack plants later in the season. These indirect plant-mediated interactions between insects have been well-documented for herbivores that feed on the same plant tissue. Nevertheless, at the moment is still not clear what are the effects of early herbivory by different feeding guilds or how herbivory in separated plant structures can impact the performance of herbivores that later feed on plants. Using field and laboratory experiments, we examined how early season herbivory in wild plants of Lima bean (*Phaseolus lunatus*) by a leaf chewing herbivore *Cerotoma ruficornis,* and the bean pod weevil, *Apion godmani,* affects the abundance and performance of a later seed herbivore *Zabrotes subfaciatus* and of its parasitoid, *Stenocorse bruchivora*. We also explored the consequences of early herbivory-induced defenses for plant fitness. We hypothesized that early-season induction will affect plant fitness and alter the suitability of seeds for late season herbivores, and as a consequence, lower parasitism rates. Plants subjected to early-season herbivores produced fewer flowers, pods and seeds than control plants. In addition, these plants suffered lower infestation by seed beetles and also lower parasitism rates in the field. In performance experiments, fewer beetles and fewer parasitoids emerged from such seeds. The higher abundance and better performance of seed beetles and their parasitoids on seeds from undamaged plants are partly explained by results on chemical analyses conducted on the seeds. Seeds from control plants were larger, heavier and with a higher total protein content than seeds from plants subjected to leaf and pod herbivory, thus, providing a better resource for seed beetles and in turn, for the natural enemies of these beetles. Overall we found that early season herbivory had a negative effect not only on the herbivores that arrive later to the plant but also on the parasitoids that attack these herbivores. We suggest that early-season herbivory alters plant chemistry and these changes are carried on to the reproductive structures. Such cascading effects will have profound effects on the entire community arthropod community associated with wild Lima bean plants.

Keywords: Early-season herbivory*, Host plant-mediated interactions, Induced plant defenses,* Phaseolus lunatus*, Sequential herbivory,* seed beetle*,* parasitoid

INTRODUCTION

Plants are exposed to multiple herbivores with different phenologies and strategies to damage the plants. As a consequence, there are priority effects where the attack of early-season herbivores can drastically influence the abundance and performance of late-season herbivores (Viswanathan et al. 2007, Poelman et al. 2008, Erb et al. 2011, McArt et al. 2013, Wang et al. 2014). Herbivore damage on a plant triggers a diverse array of induced defense responses, such as quantitative and/or qualitative changes in chemical compounds, that might negatively affect the performance of subsequent herbivores (Poelman et al. 2008, McArt et al. 2013, Wang et al. 2014). In particular, an initial herbivore attack may trigger defensive mechanisms that persist and remain in the plant over the season, but can also "prime" plants to respond faster and more strongly during a second herbivore attack (Heil and Kost 2006, Frost et al. 2008, Heil and Ton 2008). As an illustrative example of this phenomenon, Poelman et al. (2008) observed that early-season herbivory by the specialist defoliator *Pieris rapae* on *Brassica oleracea* resulted in an increased expression of genes related to defense biosynthesis and this in turn reduced the performance of three late-season defoliators, two specialists (*P. rapae* and *Plutella xylostella*) and one generalist (*Mamestra brassicae*). Similarly, a recent study conducted by McArt et al. (2013) showed that leaf herbivory by an exotic beetle (*Popilia japonica*), induced an accumulation of the phytohormone jasmonic acid and complex phenolics (ellagitannins and flavonoids) in reproductive tissues of the common evening primrose *Oenethera biennis*, decreasing in turn seed consumption by three late-season lepidopteran species. However, not all studies have supported this phenomenon as a couple of studies have reported that prior stress may alternatively accentuate the response to a secondary herbivore (e.g. (Ton et al. 2007, Erb et al. 2009).

More recently ecologists have been interested not only on how early-season herbivory might affect late-season herbivores but also on the whole arthropod community that interacts with the plants (Shiojiri et al. 2002, Denno and Kaplan 2007, Kessler and Halitschke 2007, Poelman et al. 2010, Poelman et al. 2011). In particular, changes in plant traits after early- season herbivory may modify either herbivore susceptibility or herbivore resistance to their natural enemies (i.e. predators and parasitoids) (Mooney et al. 2012, Singer et al. 2012). For example, the slow-growth/high mortality hypothesis states that herbivore development on a poor quality host plant will be relatively slow, exposing the herbivore to heightened mortality risk from their enemies (Moran and Hamilton 1980, Benrey and Denno 1997). Alternatively, specialist herbivores feeding on plants with high levels of chemical defences might sequester these chemicals which can serve as a defense against their natural enemies, thus increasing herbivore resistance (Moran and Thompson 2001, Singer et al. 2014). In one of the few available examples of induced plant defenses on different trophic levels, Rodríguez-Saona et al. (2005) reported that early damage by the defoliator *Spodoptera exigua* on tomato plants increased the activity of leaf proteinase inhibitors and consequently reduced the performance (reduced number of eggs laid, larvae growth and survival) of subsequent *S. exigua* caterpillars as well as the fitness correlates (reduced pupal mass) of its parasitoid *Cotesia marginiventris*.

In this study, by using a combination of field and laboratory experiments, we examined how early-season herbivory by the leaf-chewing herbivore *Cerotoma ruficornis* (Coleoptera: Chrysomelidae) and the green-pod feeder *Apion godmani*

(Coleoptera: Curculionidae) on wild plants of Lima bean (*Phaseolus lunatus,* Fabaceae) affected the abundance and performance of the late-season seed herbivore *Zabrotes subfaciatus* (Coleoptera: Bruchidae) and its larval parasitoid *Stenocorse bruchivora* (Hymenoptera: Braconidae). Moreover, we further examined whether changes in plant reproductive (number of flowers, pods and seeds and seed weight, size and emergence), defensive (cyanogenic glycoside compounds), and nutritional (total proteins) traits after early-season herbivory serve as underlying mechanisms to explain the observed patterns on the abundance and performance of the late-season herbivore and its parasitoid. By addressing these objectives, our work builds towards a more complete understanding of how changes in plant traits after early-season herbivory might determine late-season herbivore attack and cascade-up to influence higher trophic levels.

MATERIAL AND METHODS

Natural history

Phaseolus lunatus, commonly known as Lima bean, occurs in natural populations along the pacific coast, from Mexico to South America. This species germinates between June and July and starts to produce flowers at the beginning of October. Seeds are produced during November and December and are dispersed in January.

In our study area (15 km North-West of Puerto Escondido, Oaxaca, Mexico, 15°55'33.3"N 97°09'03.0"W), Lima bean is attacked mainly by two early-season specialist herbivores, the leaf-chewing *C. ruficornis* and the green-pod feeder *A. godmani* (Hernández-Cumplido, personal observation). *Cerotoma ruficornis* feeds on the lower surface of the leaves from July to November. *Apion godmani* oviposits in the newly formed pods and new emerged adults feed on the green seeds in the pods from October to November. Therefore, both herbivores can coexist in the same plant during two months. Finally, when the pods are dry and start to open, the Mexican bean weevil *Z. subfasciatus* (also specialist in bean species) enters the dry pods and glues its eggs on the seed coat of mature seeds. Upon hatching of the eggs, the newly emerged larvae enter the seed and feed within it until they complete their life cycle and emerge as adults (Benrey et al. 1998). Second instar larvae of *Z. subfasciatus* are parasitized by the ectoparasitoid *S. bruchivora* (Campan and Benrey 2004).

Lima bean plants contain large quantities of cyanogenic glycoside compounds in their leaves that act as direct defenses (i.e. deterrents) against leaf herbivores (e.g. Ballhorn et al. 2008a, Ballhorn et al. 2008b, Ballhorn et al. 2009).

Field experiment: Effects of early-season herbivory on plant traits and on the abundance of Z. subfasciatus (late-season herbivore) and its parasitoid S. bruchivora

We carried out two field experiments in 2012 (from October 2011 to February 2012; “2012 experiment” hereafter) and 2013 (from August 2012 to February 2013; “2013 experiment” hereafter) at 15 km North-West of Puerto Escondido (Oaxaca, Mexico, 15°55'33.3"N 97°09'03.0"W). Both experiments were conducted with seeds obtained from a single plant sampled next to our field plantation. Plants were sown in 5 L pots. Three weeks after sowing, bean plants were covered with nylon field tents to initiate herbivory treatments (2012 experiment: 12’x12’x6’ Dual Identity Screenhouse and Canopy, Gigatent, Wayne NJ, USA; 2013 experiment: Bioquip, Outdoor Cage 6 x 6 x 6', 20 X 20 Mesh Lumite). We used 11 tents with 10 plants per tent in 2012 (N = 110 plants), and 24 tents with 6 plants per tent in 2013 (N = 144 plants). In 2012, treatments were arranged in the following manner: three tents infested with the leaf herbivore *C. ruficornis*, three tents infested with the green-pod feeder *A. godmani*, three tents infested with both herbivore species, and finally, two tents used as a control without herbivores. Each tent contained 10 *P. lunatus* plants separated 11.5 mts. from each other. In 2013, we had six tents infested with *C. ruficornis*, six tents with *A. godmani*, six tents infested with both species, and six tents as a control without herbivores. In this case, each cage contained six *P. lunatus* plants. For the *C. ruficornis* treatment, when plants were three week-old (±10 fully developed leaves) we introduced 10 adults per week inside the tents during four weeks (i.e. 40 individuals in each tent at the end of the experiment). For the *A. godmani* treatment, when plants were eight weeks-old (±20 full developed leaves) and started to form green pods we introduced 20 adults per week inside the tents during four weeks (i.e 80 individuals at the end of the experiment). For the treatment that included both herbivores, when plants were three week-old we introduced 10 individuals of *C. ruficornis* per week during four weeks and when the plants were 8-week-old we added 20 individuals of *A. godmani* per week during four weeks (i.e. 40 *C. ruficornis* and 80 *A. godmani* at the end of the experiment). We conducted censuses of leaves and flowers produced per plant every week for all the plants during 11 weeks in 2012 experiment (from November 16, 2011 to January 25, 2012), and during 17 weeks in 2013 experiment (from September 24, 2012 to January 14, 2013). Flowers started to appear during the second week of November and flowers blossom during one full month.

Immediately after the plants produced pods or were in the drying process (which normally occurs during the first week of January), we removed the tents to allow the Mexican bean weevil *Z. Subfasciatus* and its parasitoid *S. bruchivora* to colonize freely the experimental plants. Pods were collected as soon as they matured and presented an were ready to shed seeds. Seeds from individual pods were stored in plastic containers in an incubator (Percival incubator model: 1-36VL) under controlled conditions (12:8 light:dark, 70-80% RH, 30°C) until the emergence of *Z. Subfasciatus* and its parasitoid *S. bruchivora*. We recorded the number of *Z. Subfasciatus* and *S. bruchivora* adults that emerged from each plant. Plants were monitored until the end of the season (mid-February).

In parallel, we used uninfested mature seeds (randomly chosen) from our experimental plants to quantify seed weight, size, and germination as well as the content of cyanogenic glycosides (10 plants per treatment and year). To evaluate seed germination, we sowed groups of four randomly chosen seeds of each herbivore treatment in plastic cups (20 cups per treatment, N = 80 cups). After five days, we recorded the number of emerged plants in each cup and quantified the proportion of germination. For the extraction and quantification of cyanogenic glycosides (defensive compounds), we grounded up randomly chosen seeds of each plant with liquid nitrogen and used 0.020 grams of ground bean powder from each sample. Then we added 1 mL of cold methanol (70%), and we placed the samples in a heating block at ~90° C for 3 min. After this process we placed the samples on ice, added 10 glass bleads and put them in an ultrasound shaker (Branson 2210) for 4 minutes at maximum speed. When shaking was finished, we centrifuged the samples at 8000 rpm for 3 minutes (centrifuge Vaudax-Eppendorf 5424), and finally we used the supernatant for the HPLC analysis (see more details of extraction in (Shlichta et al. 2014). Markers of interest were identified on the basis of their high resolution mass spectra. Linamarin and lotaustralin reference standards were injected under identical conditions to ascertain the identity of those compounds. Also we choose a set of 75 uninfested seeds per treatment per year and we recorded their size (Vernier Toolzone 150 mm (6") Electronic Digital Vernier Caliper) and weight (balance Mettler AE163). Finally, with another set of uninfested randomly chosen seeds (N = 18 seeds per treatment), we quantified total protein content following the Bradford assay. For the extraction we ground up the seeds with liquid nitrogen and used 0.020 grams of ground bean powder of each sample and add 44 µL of protein extraction buffer per sample (Tris-HCl, pH 6.8, 20% SDS (w/v), DTT, Glycerol 87% and MiliQ water), then we vortex the samples per one minute and we left them incubated for one hour at room temperature. After this procedure, we incubated the samples in a heating block ~100° C X 2 min, then we spin the samples in a centrifuge 30 minutes at 13,000 rpm. Finally we proceed to add 799 µl ddH2O + 1 µl of protein (sample) + 200 µl Bradford. We shake the samples in a vortex and they were incubated for 5 minutes. After this time we measured and recorded the absorbance at 595 nm. From the first year we develop the method for 6 samples per treatment and for the second year we ran 12 per treatment (protocol adapted from: http://www.oregin.info/information/sops.php).

Because our data did not meet the assumptions of normality, plant traits (number of flowers, number of pods, number of seeds) and insect abundance (abundance of *Z. subfasciatus* and its parasitoid *S. bruchivora*) were analyzed using a generalized linear mixed model (PROC GLIMMIX with a Poisson distribution in SAS, SAS 9.2 System, SAS, Cary, NC) (Littell et al. 2006). Herbivory treatment and year were considered as fixed factors. The effect of the tent was considered as a random factor to account for repeated measures taken on the same experimental unit. To account for differences in the number of seeds sampled per plant (which would likely influence herbivore recruitment), we included the number of seeds per plant as a covariate in the herbivore abundance model. Concentration of cyanogenic glycoside compounds and proteins in the seeds and seed size, weight and emergence were analyzed using a general linear model (PROC GLM in SAS) (Littell et al. 2006), with the main effects of treatment as fixed factor.

Laboratory experiment. Effects of early-season herbivory on the performance of Z. subfasciatus (late-season herbivore) and its parasitoid S. bruchivora

We conducted two independent cafeteria experiments to test the effect of early-season herbivory on the performance of *Z. subfasciatus* and its parasitoid *S. bruchivora*. In both cases, we had four treatments: (1) undamaged seeds from control plants, 2) undamaged seeds from plants damaged by *C. ruficornis*, 3) undamaged seeds from plants damaged by *A*. *godmani*, and 4) undamaged seeds from plants damaged by both herbivores. For these experiments we separately used seeds from 2012 and 2013 field experiments.

Zabrotes subfasciatus experiment*:* to measure the performance of the Mexican bean weevil we placed five seeds in a small plastic cups (28 × 23 × 5mm, Semadeni AG, A4686) (10 replicates per treatment, N = 40 cups), in each cup we released two newly emerged *Z. subfasciatus* individuals (one male and one female) from a laboratory colony. This colony has been maintained for two years on red kidney beans (*P. vulgaris*), and every year we add new field collected individuals to add genetic variability. Cups were randomly arranged in an incubator (conditions: 11 h at 27°C /13h at 25°C, 11L/13D, and ~80% RH) and were examined throughout a five-day period to identify *Z. subfasciatus* eggs in the seeds. We carefully individually transferred infested seeds into Eppendorf tubes (2 mL) until adult *Z. subfasciatus* emergence. After adult emergence we recorded (i) the number of eggs laid on each seed, (ii) developmental time of each individual (measured as the number of days until emergence), (iii) percentage of males (measured as the number of males divided by the total number of emerged adults of *Z. subfasciatus*), and (iv) fresh weight of each individual as a proxy of body size. For a closely related beetle species (*Callosobruchus maculatus*, Coleoptera: Bruchidae), it has been shown that female eggs are allocated to high-quality hosts and male eggs to low-quality hosts so a higher percentage of emerging males is directly related to lower beetle fitness (Spitzen and van Huis 2005).

Parasitoid experiment: We placed a pool of 200 seeds of each herbivory treatment in a 2 dL plastic cup. In each cup we added 40 male and female couples of newly emerged adults of *Z. subfasciatus* and allowed them to mate and oviposit. After 24 h, we selected seeds that had three weevil eggs. We placed groups of five of these seeds in individual plastic cups for a total of eight replicates per treatment (N = 32 cups). Once *Z. subfasciatus* larvae reached the third or fourth instar inside the seed and can be parasitized (ca. 17 days after oviposition), we placed two inexperienced wasps (one male and one female) inside each plastic container (Campan and Benrey 2004). We allowed the females to parasitize for a period of five days. During this time, we provided a drop of honey to enhance oogenesis. Adult parasitoids emerged approximately 17 days later. Immediately after emergence, each parasitoid specimen was deep-frozen at -28 ºC and oven-dried for 48 hrs at 65 ºC to constant weight. We recorded (i) tibia length of each individual (using an ocular micrometer) as a proxy of body size, (ii) percentage of males (measured as the number of males divided by the total number of emerged parasitoids), and (iii) survival (measured as the number of emerged parasitoids divided by [number of emerged *Z. subfasciatus* + number of parasitoids]). As for *Z. subfasciatus*, female parasitoid eggs are allocated to high-quality hosts and male eggs to low-quality hosts (Spitzen and van Huis 2005).

Because our data did not meet the assumptions of normality, *Z. subfasciatus* and parasitoid performance traits were analyzed using a generalized linear mixed model (PROC GLIMMIX with a Poisson distribution in SAS) (Littell et al. 2006). The effects of herbivory treatments and year (2012 and 2013) were considered as fixed factors. The effect of the cup was considered as a random factor to account for repeated measures taken on the same experimental unit.

RESULTS

Field experiment: Effects of early-season herbivory on plant traits and on the abundance of Z. subfasciatus (late-season herbivore) and its parasitoids S. bruchivora

Early-season herbivory treatments significantly affected plant reproduction (Table S1, Fig. 1). Specifically, the number of flowers, pods, and seeds was significantly lower in plants attacked by *A. godmani* and plants attacked by both herbivores (*A. godmani* and *C. ruficornis*) than in uninfested control plants and plants attacked only by *C. ruficornis* (Fig. 1a, 1b, 1c). Moreover, seed weight and size and proportion of germinated seeds were significantly lower in plants of the three herbivory treatments than in uninfested control plants (Fig. 1d, 1e, 1f). No significant differences in seed weight and size and proportion of germinated seeds were observed between treatments with herbivores alone or in combination (Fig. 1d, 1e, 1f).

Early-season herbivory treatments significantly affected the concentration of cyanogenic glycosides and total proteins in the seeds (Table S2, Fig. 2). Specifically, lotaustralin concentration was significantly lower in plants attacked by *C. ruficornis* and both herbivores than in control plants (Fig. 2a). Similarly, linamarin concentration was significantly lower in plants attacked by *C. ruficornis* and both herbivores than in control and *A. godamni* attacked plants, but did not differ between control and the *A. godamni* treatment (Fig. 2b). Finally, total protein concentration was significantly lower in plants of the three herbivory treatments than in uninfested control plants (Fig. 2c). No significant differences in total protein concentration were observed between treatments with herbivores alone or in combination (Fig. 2c).

In the field, early-season herbivory treatments significantly affected the abundance of *Z. subfasciatus* (late-season herbivore) (Table S3, Fig. 3a), but not the abundance of its parasitoid *S. bruchivora* (Table S3, Fig. 3b). The number of *Z. subfasciatus* individuals was significantly lower in plants of the three herbivory treatments than in uninfested control plants (Fig. 3a). No significant differences in *Z. subfasciatus* abundance were observed between treatments with herbivores alone or in combination (Fig. 3a).

Laboratory experiment. Effects of early-season herbivory on the performance of Z. subfasciatus (late-season herbivore) and its parasitoids S. bruchivora

Early-season herbivory treatments significantly affected *Z. subfasciatus* performance (Table S4, Fig. 4). The number of *Z. subfasciatus* eggs glued on the seeds was significantly higher in control plants than in plants of the three herbivory treatments (Fig. 4a). No significant differences in the number of *Z. subfasciatus* eggs on the seeds were observed between treatments with herbivores alone or in combination (Fig. 4a). In contrast, early-season herbivory treatments did not significantly affect *Z. subfasciatus* weight, developmental time and proportion of males (Fig. 4b, 4c, 4d).

The performance of the parasitoid *S. bruchivora* was also significantly affected by early-season herbivory treatments (Table S5, Fig. 5). Specifically, parasitoid survival was higher in seeds of control plants than in seeds of plants from the three herbivory treatments (Fig. 5a). No significant differences in parasitoid survival were observed between treatments with herbivores alone or in combination (Fig. 5a). Finally, early-season herbivory treatments did not significantly affect parasitoid sex ratio and size (Fig. 5b, 5c).

DISCUSSION

Our results reveal that early-season herbivory in Lima bean plants negatively affected the abundance (lower number of individuals in the field) and performance (reduced oviposition in the laboratory) of the late-season seed herbivore *Z. subfasciatus*. These results are supported by a number of studies that have shown that early-season herbivory produces changes in plant nutrition, allelochemistry, morphology, and phenology which can subsequently affect the performance of late-season herbivores (Havill and Raffa 2000, Rodríguez-Saona et al. 2005, Denno and Kaplan 2007, Poelman et al. 2010, McArt et al. 2013, Stam et al. 2014). For example, Erb et al. (2011) observed that induction by the leaf herbivore *Spodoptera frugiperda* increased resistance of wild and cultivated maize plants against the subsequent attack of the root-feeding larvae of the Western corn rootworm, *Diabrotica virgifera.* In our system, the negative effects of early herbivory on late-season herbivores seem to be driven by negative effects of early-season herbivory on plant reproductive and nutritional traits. In particular, early-season herbivory drastically reduced the production of flowers, pods and seeds, seed size, weight, emergence and the concentration of total seed proteins and all these traits appear to be of vital importance for the performance of the late-season seed herbivores.

A vast number of studies have demonstrated that variation in the oviposition preference and performance of seed herbivores is strongly affected by seed size and quality (e.g. (Kawecki 1995, Fox 1997, Campan and Benrey 2004, Takakura 2004, Ballhorn et al. 2009, Zaugg et al. 2013, Shlichta et al. 2014). Herbivory has been shown to drastically reduce seed size and weight (reviewed by Crawley 1989) and this in turn can negatively affect the performance of seed herbivores. In our particular system (i.e. *Phaseolus* species), Campan and Benrey (2004) reported that the females of *Z. subfasciatus* oviposit fewer eggs on wild smaller seeds of *Phaseolus vulgaris* than on cultivated larger seeds. Similarly, (Zaugg et al. 2013) found that the performance of the bean bruchid *Acanthoselides obctetus* was positively affected by seed size of different bean populations and varieties. On the other hand, some previous studies have documented that herbivory reduces the levels of proteins in plant tissues (e.g. (Mitra et al. 2008, Rodriguez-Saona et al. 2010) which are considered as important nutrients driving the growth of herbivores (e.g. (Le Gall and Behmer 2014). Further studies should also investigate if changes on individual proteins (not only in the total quantity as we demonstrated here) in the seeds of Lima bean after herbivory damage can drive subsequent seed herbivory by *Z. subfasciatus*.

Our results also showed that differences in the two main cyanogenic glycoside compounds in bean seeds (linamarin and lotoaustralin) between herbivory treatments did not explain the observed patterns in the field. In particular, we observed that seeds from unattacked control plants (with a high number of seed herbivores) had higher concentrations of cyanogenic glycoside compounds compared with plants subjected to herbivory treatments (with a low number of seed herbivores). One plausible explanation for these unexpected results might be that specialist insect herbivores such as *Z. subfasciatus* are able to tolerate and even benefit from the consumption of highly defended plant tissues, through detoxification (Bernays 1998, Mason et al. 2014, Shlichta et al. 2014). In addition to this, some previous studies have suggested that, contrary to the leaves, seeds do not contain enough water to release β-glucosidase, an enzyme that triggers the activity of the cyanogenic compounds and transform them into toxic cyanide (Selmar et al. 1988). Overall, our current study, as well as previous findings (Shlichta et al. 2014), demonstrates that cyanogenic glycoside compounds in the seeds are not closely related to bean resistance against seed herbivores.

Despite the increasing evidence of early-season herbivory on subsequent herbivore attacks (Viswanathan et al. 2007, Poelman et al. 2008, Erb et al. 2011, McArt et al. 2013, Wang et al. 2014), there is an emerging question which has been largely ignored in the previous literature. Could the negative effect of early-season herbivory on subsequent herbivores be extended to the natural enemies of these herbivores? Along this line, our study demonstrated that early-season herbivory on leaves and green pods not only negatively affected the performance of late-season seed herbivores, but also the performance (increased developmental time and decreased survival) of their natural enemies (parasitoids). Our results do not support the predictions of the Slow Growth-High Mortality Hypothesis that states that herbivore development on a poor quality host plant will be relatively slow, exposing the herbivore to heightened mortality risk from natural enemies (Moran and Hamilton 1980, Benrey and Denno 1997, Mooney et al. 2012). If this was the case, we would have found more parasitism on seeds from herbivore-damaged plants in which bruchid development was slower. On the other hand, the findings from the parasitoid performance experiment in the laboratory indicate that a poor quality seeds for the bruchid beetles, also results in a poor quality host for the developing parasitoids. Evidence for these patterns has been documented for this and other host-parasitoid systems (see Turlings and Benrey 1998 and Chen et al. 2015 for a review). Supporting our results, (Havill and Raffa 2000) found that poplar plants (*Populus nigra*) that were damaged by larvae of the gypsy moth *Lymantria dispar* negatively affected, not only growth, weight and survival of conspecifics insects that arrived later, but also reduced the developmental and reproductive success of the parasitoid *Glyptapanteles flavicoxis* (Marsh). Similarly, Rodríguez-Saona et al. (2005) reported that early damage provoked by the aphid *Macrosiphum euphorbiae* in tomato plants reduced the subsequent preference of the leaf herbivore *Spodoptera exigua* as well as the performance of its parasitoid *Cotesia marginiventris*.

Conclusions

Early-season herbivory on Lima bean plants had a profound impact on seed predators and their parasitoids. It appears that the most important plant trait leading to these results is the lower quality of the seeds produced by herbivore-damaged plants. Future studies on plant-insect interactions should take into account that early season herbivory can cause chemical changes that affect the plant reproductive structures and their subsequent susceptibility to insect attack. Such cascading effects imply that herbivore-induced defenses may have profound effects on a multitude of herbivores and their natural enemies throughout the season.

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FIGURE LEGENDS

Figure 1. Effects of early herbivory treatments (untreated control, herbivory by the defoliator *Cerotoma ruficornis*, herbivory by the green pod feeder *Apion godmani* and herbivory by both insects together) on the (a) number of flowers, (b) number of pods, (c) number of seeds, (d) seed weight, (e) seed size and (f) proportion of germinated seeds of *Phaseolus lunatus* plants in two consecutive years. All the plant traits were calculated as the average value per plant. Bars are least square means ± s.e.m. (N = 56 for control and N = 66 for the rest of the herbivory treatments). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant ($P < 0.05$) differences between treatments.

Figure 2. Effects of early herbivory treatments (untreated control, herbivory by the defoliator *Cerotoma ruficornis*, herbivory by the green pod feeder *Apion godmani* and herbivory by both insects together) on the concentration (mg g^{-1} d.w.) of (a) linamarin (b) lotoaustralin (two cyanogenic glycoside compounds) and (c) total proteins of *Phaseolus lunatus* plants in two consecutive years. All the plant chemical traits were calculated as the average value per plant. Bars are least square means ± s.e.m. (N = 27 for cyanogenic glycosides and N = 18 for total proteins). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant ($P < 0.05$) differences between treatments.

Figure 3. Effects of early herbivory treatments (untreated control, herbivory by the defoliator *Cerotoma ruficornis*, herbivory by the green pod feeder *Apion godmani* and herbivory by both insects together) on the abundance of (a) the seed herbivore

Zabrotes subfasciatus and (b) its parasitoid *Stenocorse bruchivora* on seeds of *Phaseolus lunatus* plants in two consecutive years. Seed herbivore and parasitoid abundance were calculated as the average value per plant. Bars are least square means ± s.e.m. (N = 56 for control and N = 66 for the rest of the treatments). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant ($P < 0.05$) differences between treatments.

Figure 4. Effects of early herbivory treatments (untreated control, herbivory by the defoliator *Cerotoma ruficornis*, herbivory by the green pod feeder *Apion godmani* and herbivory by both insects together) on (a) number of eggs laid, (b) fresh weight (in mg), (c) developmental time (days until adult emergence) and (d) percentage of males (number of males divided by total individuals) of seed herbivore *Zabrotes subfasciatus* in a laboratory experiment. For this experiment we used seeds produced by *Phaseolus lunatus* plants of our two consecutive year field experiments. All the seed herbivore traits were calculated as the average value per plant. Bars are least square means ± s.e.m. (N = 30). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant ($P < 0.05$) differences between treatments.

Figure 5. Effects of early herbivory treatments (untreated control, herbivory by the defoliator *Cerotoma ruficornis*, herbivory by the green pod feeder *Apion godmani* and herbivory by both insects together) on (a) survival (percentage of emerged parasitoids per number of glued seed predator eggs on the seed coat), (b) percentage of parasitoid males (number of males divided by total individuals), and (c) tibia length (ocular units) of *Stenocorse bruchivora* parasitoid in a laboratory

experiment. For this experiment we used seeds produced by *Phaseolus lunatus* plants of our two consecutive year field experiments. All the seed predator traits were calculated as the average value per plant. Bars are least square means ± s.e.m. (N = 28). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant ($P < 0.05$) differences between treatments.

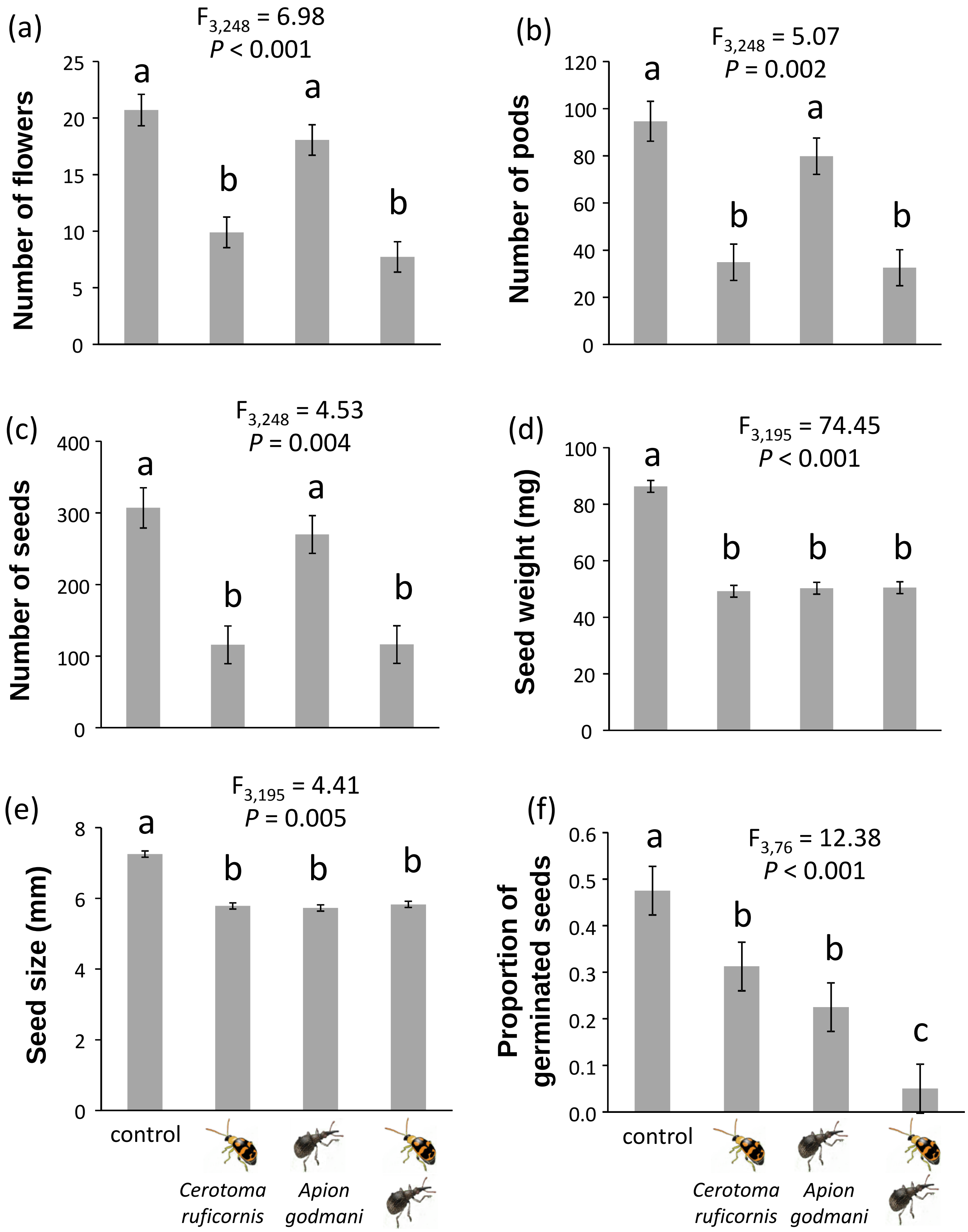

Figure 1. Hernández-Cumplido et al

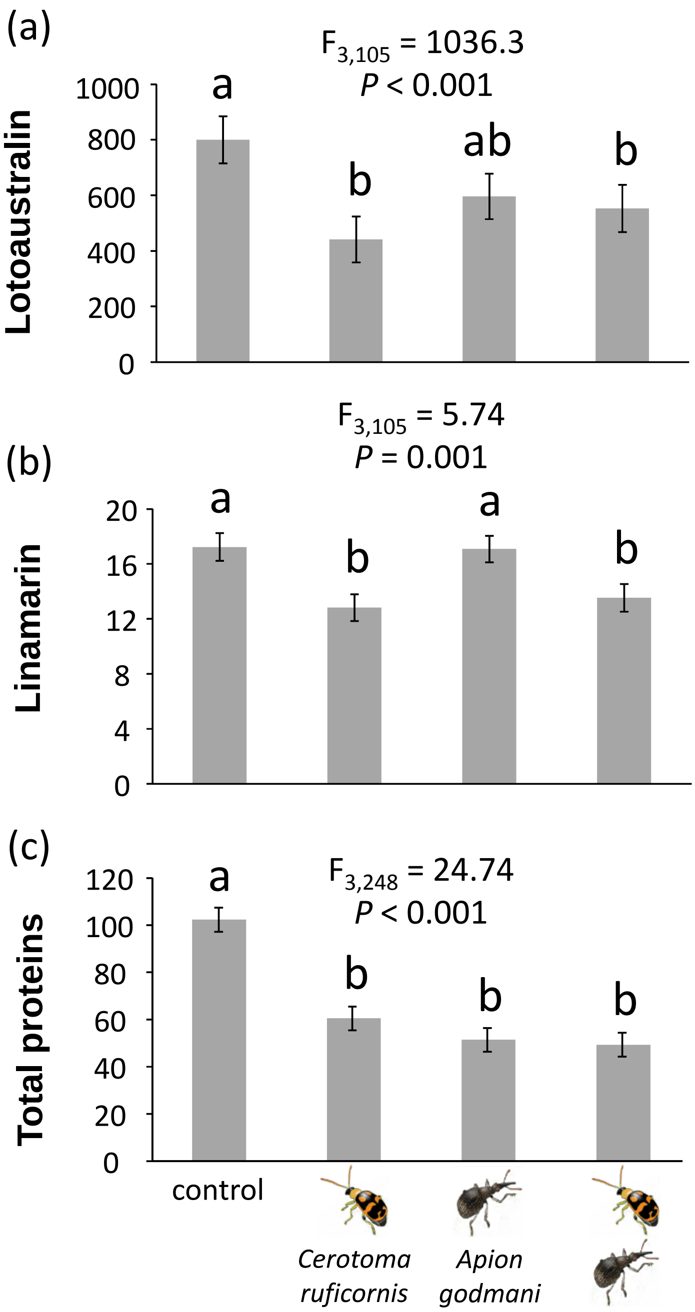

Figure 2. Hernández-Cumplido et al

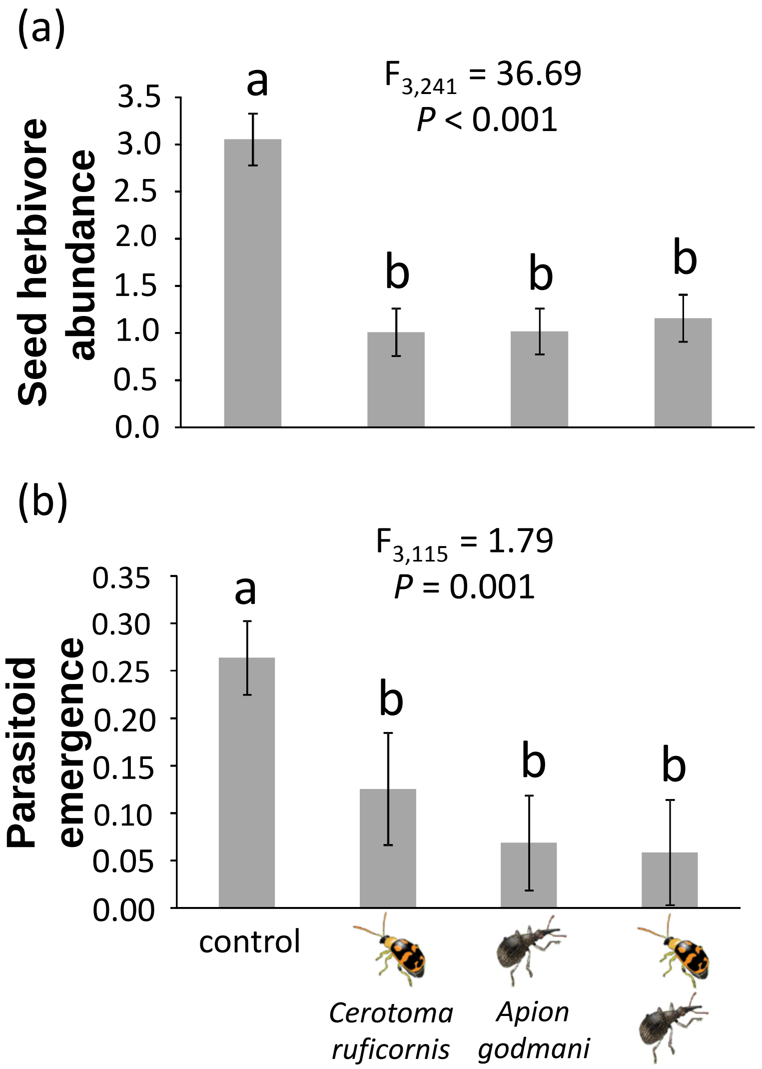

Figure 3. Hernández-Cumplido et al

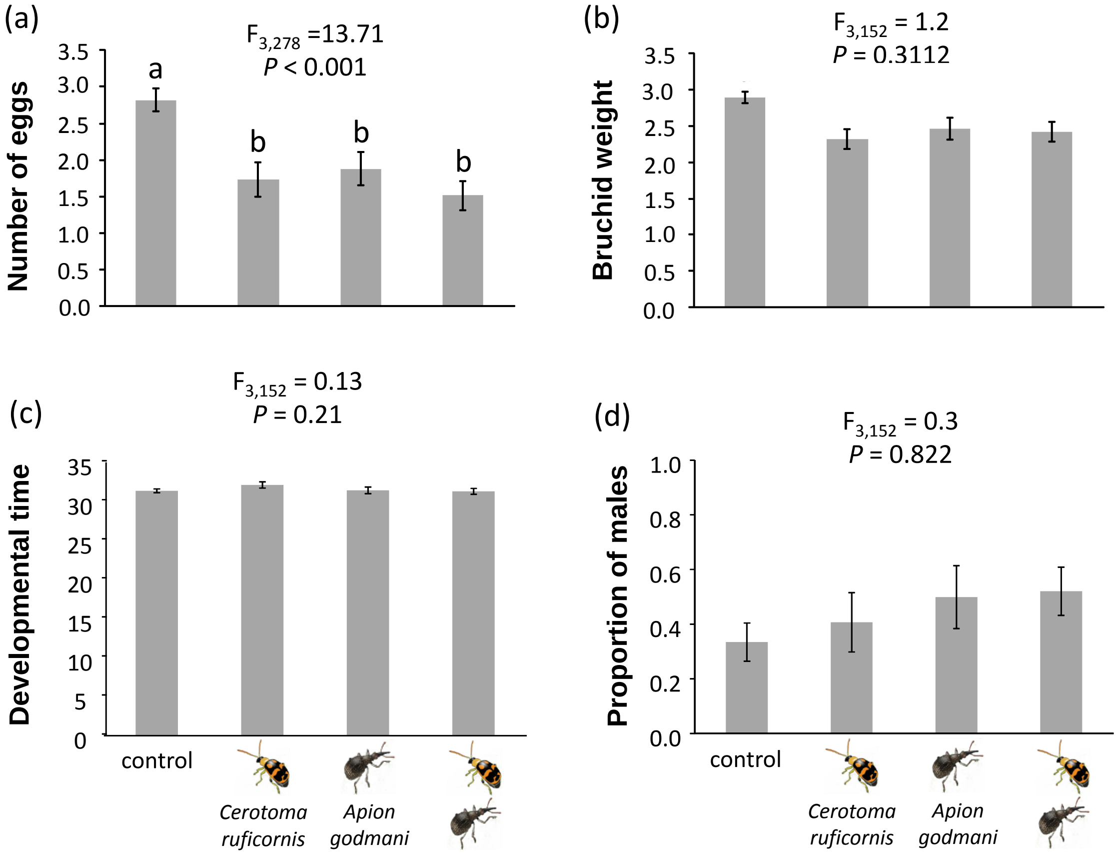

Figure 4. Hernández-Cumplido et al.

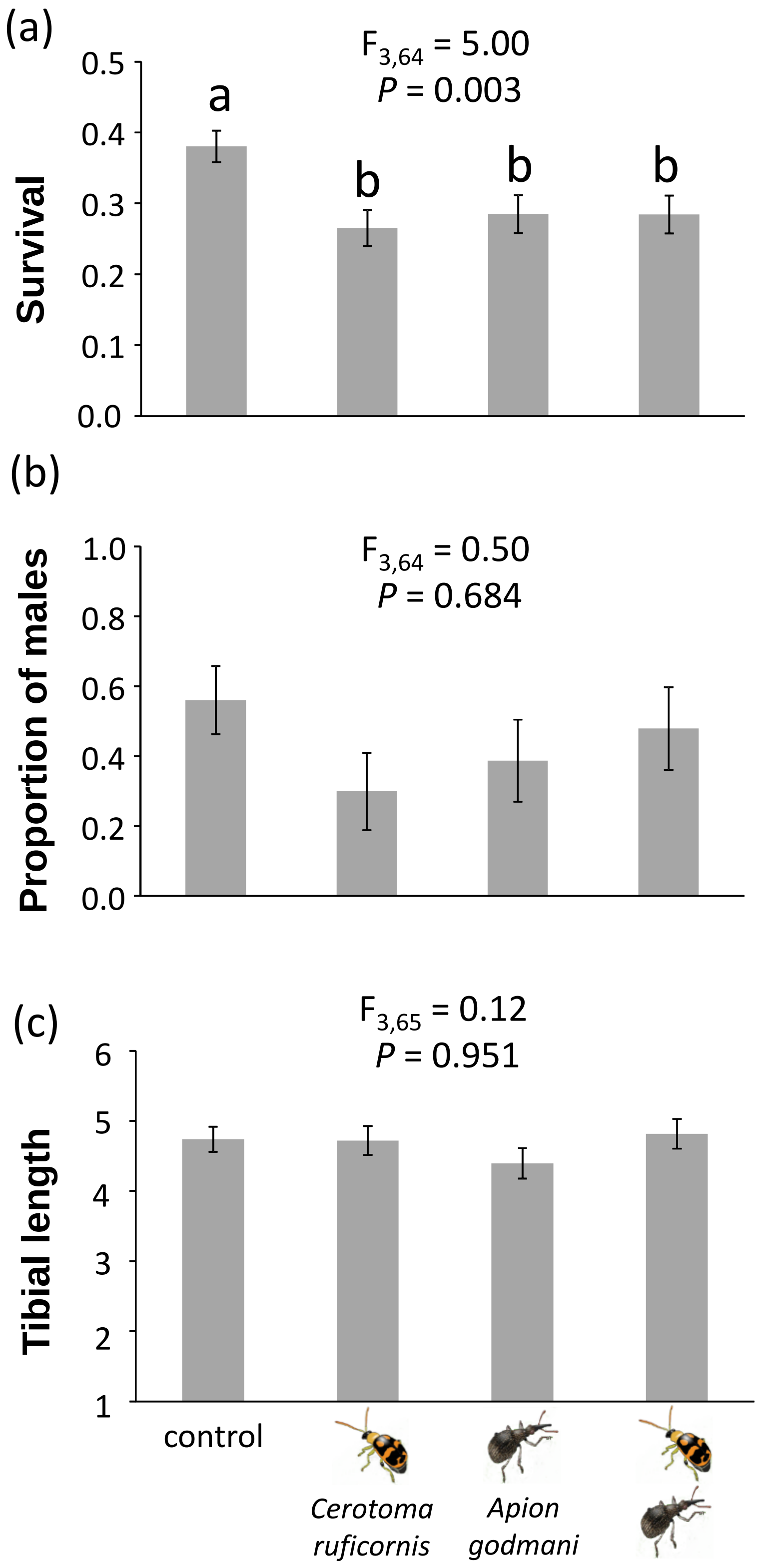

Figure 5. Hernández-Cumplido et al.

Table S1. Summary of results from the mixed model analysis on the effects of the herbivory treatment and year (fixed factors) on the number of flowers, pods and seeds in Lima bean plants. The effect of the tent was considered as a random factor to account for repeated measures taken on the same experimental unit. F-values, degrees of freedom and associated significance levels (*P*) are shown. Significant P values (*P* < 0.05) are typed in bold.

		Flowers		Pods		Seeds	
	df	F	*P*	F	*P*	F	*P*
Treatment	3, 248	6.98	**<0.001**	5.07	**0.002**	4.53	**0.004**
Year	1, 245	276.96	**<0.001**	14.00	**<0.001**	12.62	**<0.001**

Table S2. Summary of results from the linear model analysis on the effects of the herbivory treatment and year on seed weight and size, and the proportion of germinated seeds. F-values, degrees of freedom (within brackets) and associated significance levels (*P*) are shown. Significant P values (P < 0.05) are typed in bold.

	Seed weight		Seed size		Proportion of germinated seeds	
	F	*P*	F	*P*	$F_{3,76}$	*P*
Treatment	$74.45^{(3,195)}$	**<0.001**	$4.41^{(3,195)}$	**0.005**	$12.38^{(3,76)}$	**<0.001**
Year	$4.48^{(1,195)}$	**0.036**	$0.03^{(1,195)}$	0.856		

Table S3. Summary of results from the linear model analysis on the effects of herbivory treatment and year on the concentration of cyanogenic compounds (lotoaustralin and linamarin) and total protein content. F-values and degrees of freedom (within brackets) and associated significance levels (*P*) are shown.

	Lotoaustralin		Linamarin		Proteins	
	F	*P*	F	*P*	F	*P*
Treatment	$3.30^{(3,105)}$	**0.023**	$5.74^{(3,105)}$	**0.001**	$24.74^{(3,67)}$	**<0.001**
Year	$1.21^{(1,105)}$	0.273	$0.82^{(1,105)}$	0.367	$37.11^{(1,67)}$	**<0.001**

Table S4. Summary of results from the mixed model analysis on the effects of herbivory treatment and year on the number of the seed beetle *Z. subfasciatus,* and its parasitoid *Stenocorse bruchivora* in the field. For the abundance of the seed beetles we used the number of seeds as a covariate to control for density-dependency. The effect of the tent was considered as a random factor to account for repeated measures taken on the same experimental unit. F-values and degrees of freedom are shown.

	Seed herbivore		Parasitoids	
	F	*P*	F	*P*
Treatment	$36.69^{(3,241)}$	**<0.001**	$1.79^{(3,115)}$	**0.001**
Year	$3.10^{(1,241)}$	0.079	$1.42^{(1,115)}$	0.234
Seeds	$10.65^{(1,241)}$	**0.001**		

Table S5. Summary of results from the linear mixed model analysis on the effects of herbivory treatment and year on the performance of the seed beetle *Z. subfasciatus*. The effect of cup was considered as a random factor to account for repeated measures taken on the same experimental unit. F-values and degrees of freedom (within brackets) and associated significance levels are shown.

	Eggs		Weight		Developmental time		Proportion of males	
	F	*P*	F	*P*	F	*P*	F	*P*
Treatment	$13.71^{(3,278)}$	**<0.001**	$1.23^{(3,140)}$	0.302	$0.13^{(3,140)}$	0.942	$0.30^{(3,52)}$	0.826
Year	$1.61^{(1,278)}$	0.205	$0.01^{(3,140)}$	0.926	$0.41^{(3,140)}$	0.525	$0.03^{(3,52)}$	0.858

Table S6. Summary of results from the linear mixed model analysis on the effects of herbivory treatment on several parasitoid (*Stenocorse bruchivora*) performance traits. The effect of cup was considered as a random factor to account for repeated measures taken on the same experimental unit. F-values, degrees of freedom and associated significance levels (*P*) are shown. Significant P values (*P* < 0.05) are typed in bold.

		Survival		Proportion of males		Tibial length	
	df	F	*P*	F	*P*	F	*P*
Treatment	3, 65	5.00	**0.003**	0.50	0.684	0.12	0.951

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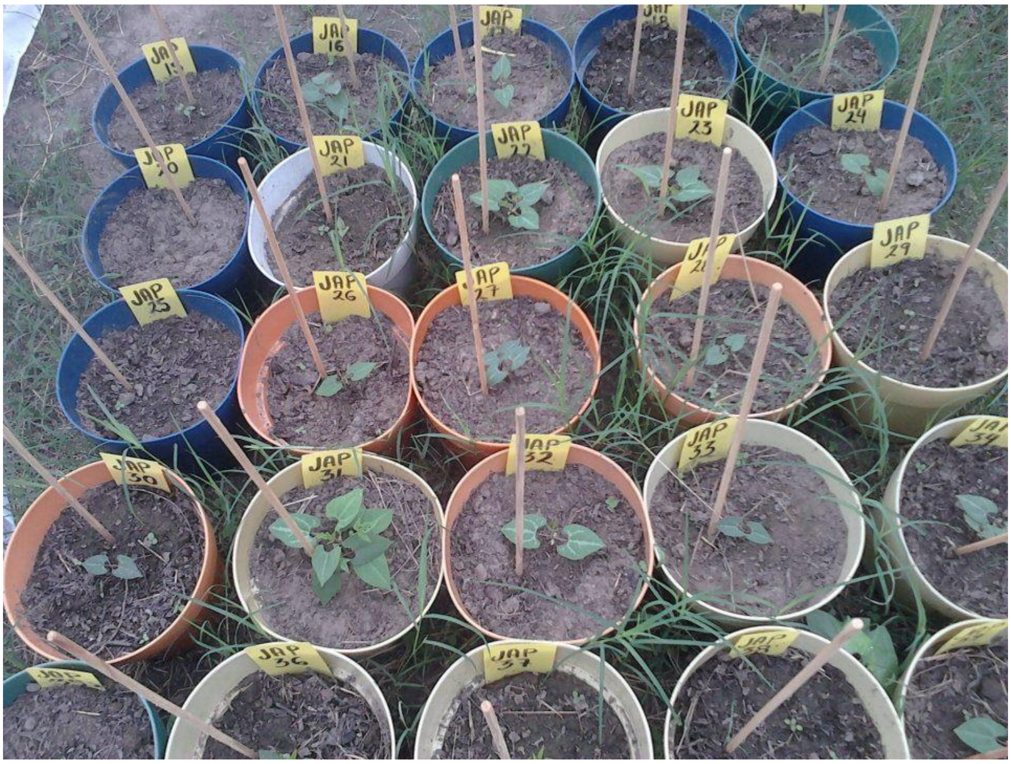
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Attraction of flower visitors to plants that express indirect defence can minimize ecological costs of ant–pollinator conflicts

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Thousands of plant species throughout tropical and temperate zones secrete extrafloral nectar (EFN) to attract ants, whose presence leads to an indirect defence against herbivores (Heil and McKey 2003, Rico-Gray and Oliveira 2007, Heil 2008). Although termed 'extrafloral' because it is not involved in pollination, EFN can in fact be also secreted within inflorescences (Bentley 1977). Because ants tend to defend reliable food sources against all types of putative competitors, it has been assumed that the presence of extrafloral nectaries close to flowers might lead to a competition among ants and pollinators or even to direct defensive actions of ants against pollinators, which would reduce the access to pollinators to flowers and, thereby, pollination efficiency. Such interactions would cause significant 'ecological costs' of an indirect, ant-mediated defence (Heil 2002).

Research into different systems has indeed demonstrated that the presence of ants can reduce pollinator visits to flowers (Ness 2006 and references therein). Flowers of several species can, therefore, release specific odours that act as repellents against ants (Ghazoul 2001) or solve the ant–pollinator conflict by providing rewards in temporal or spatial patterns that allow a separation of ant activity from pollinator activity (Chamberlain & Holland 2008, Galen 2005, Holland *et al*. 2010, Martins 2009, Nicklen&Wagner2006, Raine *et al*.2002, Wagner & Kay 2002). Ecological costs resulting from defender– pollinator conflicts appear a generally accepted paradigm in the context of indirect defence; earlier studies have, however, usually not considered a potential positive effect of the ant-attracting resource, the EFN, on the pollinators.

In the present study we used Lima bean (*Phaseolus lunatus* L.) to study the effect of defence induction within the inflorescences on the interaction between ants and flower visitors. The study was conducted in the coastal area of the state of Oaxaca, Mexico. The climate in the study area is characterized by one main rainy season from June to October, which follows a bimodal distribution peaking in July and September. The site selected was located 15 km north-west of Puerto Escondido (15◦55.596N, 97◦9.118W). Here, Lima bean grows naturally along dirt roads that lead to extensively used pastures or plantations. All experiments were performed on this native population in December 2009 during the transition from wet to dry season. Lima bean is a suitable model plant to investigate our question because it exhibits an inducible EFN secretion both on leaves and in the inflorescences, thus allowing an easy manipulation of the rates of EFN secretion (Heil 2004). We selected plants with inflorescences that presented more than 50% of fully open flowers. To induce EFN secretion, an aqueous solution of jasmonic acid (1mMl−1 of JA, the EFN-inducing wound hormone) was applied to the youngest parts of each shoot (comprising the seven youngest fully unfolded leaves and inflorescences). JA and a ring of ant-excluding resin (Tangletrap The Tanglefoot Corporation, Grand Rapids, MI, USA) around the base of the shoot were applied in a complete two-factorial design: (1) ants present, no JA (control); (2) ants present, JA application; (3) ant exclusion, no JA; and (4) ant exclusion, JA application. We used plants at three sites in the field and realized three censuses (at 10h00, 12h00 and 14h00) with four replicates (four different shoots) for each treatment. During every census all flower visitors and ants entering the inflorescences were counted: for the purpose of this study we considered all Hymenopterans and Dipterans (bees, wasps and flies) that entered flowers and stayed formorethan5s as ‘flower visitors’. Ants were considered when they completely entered the inflorescence. Visits per day were averaged for every plant and were evaluated with a two-factorial nested ANOVA for the effect of JA and ant presence (with ‘site’ as the nested factor because due to the clonal growth of lima bean we cannot exclude genetic identity among some of the shoots that were investigated at the same site) on numbers of flower visitors. Tukey-Kramer tests were applied as a post hoc test. Independent plants were used to confirm the EFN-inducing effect of JA within inflorescences. We used 10 plants at each site: on five of them we applied jasmonic acid, while the other five plants were used as controls: both plant groups were protected against EFN consumers with mesh bags. Nectar concentration was measured 4 h later as described by Heil (2004).

Our results confirmed earlier observations (Heil 2004) that EFN secretion within the inflorescences responds positively to JA treatment (Control: mean ± SE = 2.96 ± 0.53 mg, JA: 11.3 ± 2.89 mg, *t*(1,9) = 2.84, P = 0.009) and that an increased EFN secretion leads to increased numbers of ant visits to the inflorescences. In the censuses of insect visits, our treatments of ant exclusion and JA application significantly affected the number of ants entering the inflorescences and of flower visitors. Approximately 2.6 ± 0.27 flower visitors were counted in JA-treated inflorescences to which ants had access whereas 7.4 ± 0.61 flower visitors attended JA treated inflorescences from which ants had been excluded.

Without JA application, *c*. 1.8±0.63 flower visitors were counted on inflorescences to which ants had access and 2.5 ± 0.29 when ants were excluded (*F*3,35 = 40.1, P < 0.001, according to two-factorial nested ANOVA) (Figure 1). The JA-mediated induction of EFN secretion had, thus, a significant effect on the number of visits to the flowers, which became particularly obvious under ant exclusion conditions (7.4 visits to induced plants vs. 2.5 visits to controls; *F*3,35 = 13.1, P < 0.001). This part of our results clearly supports the general assumption of an ant–pollinator conflict (Ghazoul 2001, Holland *et al*. 2010). Most interestingly, however, the negative effect of the presence of ants and the positive effect of the JA treatment nearly cancelled each other out, because no significant difference could be found between the number of flower visitors on the inflorescences of control shoots (no ant exclusion and no JA treatment) and the visits to the induced shoots in the presence of ants ($F_{3,35} = 0.1$, P = 0.090, Figure 1). Although some studies have found evidence for temporal differences in the activity patterns of ants and pollinators (Holland *et al*. 2010, Martins 2009, Raine *et al*. 2002) we found that ants and flower visitors can co-occur at the same time on the inflorescences of Lima bean.

We conclude that a JA-treatment of lima bean with the resulting increase of EFN secretion within the inflorescences can increase the intensity of indirect, ant-mediated defence without decreasing the frequency of visits to flowers, as compared with control plants. What is the causal mechanism that underlies this phenomenon? Flower visitors have occasionally been observed to feed on Lima bean EFN and significantly more Hymenopteraand Diptera were observed on plants that had been experimentally supplied with an EFN mimic (Kost & Heil 2005, 2008). Thus, the increased EFN flow within the inflorescences might have been the main attractant for the flower visitors in our study. Most recently, however, a positive effect of JA on flower nectar secretion has been observed for rape (*Brassica napus*) although it remains to be studied whether JA also induces floral nectar secretion in other species (Radhika *et al*. 2010). Finally, JA might have affected flower odours and thereby increased their attractive effect. Future studies will have to investigate why flower visitors were attracted to JA-treated inflorescences of Lima bean. Independently of the underlying physiological mechanism, however, future studies on ant–pollinator conflicts will have to consider the potential positive effect on flower visitors of a resistance induction within the inflorescences and its consequence for pollination efficiency.

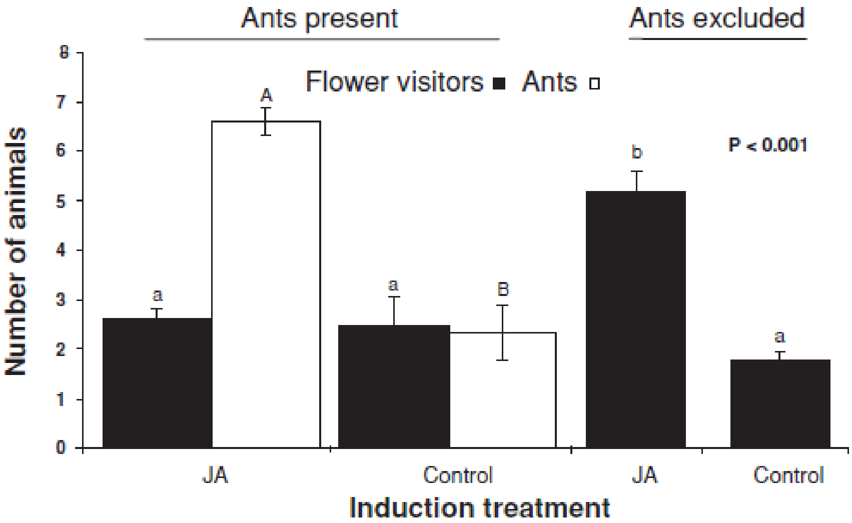

Figure 1. Conflict among ants and flower visitors after induction of extrafloral nectar secretion. Numbers of flower visitors and ants are presented for the four treatments (JA+ants+, JA−ants+, JA+ants− and JA−ants−). Bars represent means ± SE, different letters indicate significant differences ($P < 0.001$ according to Tukey–Kramer test, lowercase letters represent flower visitors, uppercase letters represent ants).

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Four:

Induction in floral and extrafloral nectar production in Lima bean (*Phaseolus lunatus*): Effects on the interaction between ants and pollinators and its consequences for plant fitness

Running title: Ant-pollinator interaction and plant fitness

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ABSTRACT

Thousands of plant species throughout tropical and temperate zones secrete extrafloral nectar (EFN) to attract ants, whose presence provides an indirect defense against herbivores. These structures are located close to flowers and may modify competition between ants and pollinators. Here, we used Lima bean (*Phaseolus lunatus* L.) to study the interaction between ants and flower visitors and its consequences on 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). Then we measured floral and extrafloral nectar production, the number of patrolling ants and flower visitors as well as some 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 pollinator attraction. Finally, ant presence did not result in a decrease in the number of pollinator visits, but our results suggest the existence of an ant-pollinator conflict. In particular, JA-induced plants without ants produced a higher amount of flowers and seeds compared with the JA-treated plants with ants, suggesting that ants could negatively affect pollination efficiency.

Keywords: *Indirect defenses, jasmonic acid,* Phaseolus lunatus, *seeds, ant-pollinator conflict*

INTRODUCTION

Plant defenses can be broadly classified as direct and indirect (Karban and Baldwin 1997). Plant 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 and 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, Turlings et al. 1995, Heil 2008, Xiao et al. 2012). Such top-down control of herbivores by natural enemies can be transmitted indirectly to plants through so called “trophic cascades” and drive an increase in plant biomass (i.e. “the green world hypothesis”) meaning that natural enemies keep herbivore populations in check (Hairston et al. 1960). In particular, extrafloral nectaries (EFN) have been shown to attract predatory ants that defend plants against herbivores and positively affect plant fitness (Heil and Mckey 2003, Rico-Gray and Oliveira 2007, Heil 2008, Chamberlain and Holland 2009, Moreira et al. 2012). Despite ample evidence on 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 and Kay 2002, Ness 2006, Nicklen and Wagner 2006). For instance, ants can discourage pollination by robbing nectar from the flowers, by chasing or attacking pollinators or simply by patrolling the plant (Galen 1999, Nicklen and Wagner 2006, LeVan et al. 2014). Such antagonistic interactions could result in trade-offs between indirect defenses and pollination (Heil 2002, Heil 2008, Hernández-Cumplido et al. 2010, Ona and Lochman 2011).

Several mechanisms have been suggested to counteract this potential trade-off between pollination and indirect defense by ants (Lach 2008, Ona and Lochman 2011). Plants can produce specific odors that act as repellents against ants. For example, Gazhoul (2001) reported that two acacia-ant mutualists were repelled by floral tissue chemicals from their own host plant species but also from other 13 plant genera, showing that ant repellents are widespread among different plant families. Moreover, plants can also create chemical barriers that deter ants from the flowers (Nicklen and Wagner 2006, Ballantyne and Willmer 2012). In this sense, Ballantyne and Willmer (2012) documented that at least one third of 49 plants species in a Costa Rican dry forest were ant-repellent usually via repellent pollen. Finally, plants can solve the ant-pollinator conflict by providing rewards in temporal or spatial patterns that allow a separation between ant and pollinator activity (Raine et al. 2002, Wagner and Kay 2002, Galen 2005, Nicklen and Wagner 2006, Holland et al. 2010). Nicklen and Wagner (2006) observed that pollinators and ants associated with *Acacia constricta* visited plants at different times of the day. The ecological costs 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 and 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 and Kay 2002, Chamberlain and Holland 2008, Holland et al. 2011). Nevertheless, very few studies have considered the role of the EFN on pollinator behavior and even less on the on plant fitness (Ness 2006, Nicklen and 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 predatory ants and pollinators (Hernández-Cumplido et al. 2010). In particular, 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, despite these convincing findings, in this previous paper we did not examine the effect of JA application on floral nectar (i.e. pollinator reward) and the potential consequences of increased extrafloral nectar on plant fitness (i.e. reproduction). Additionally, as we did not manipulate the presence/absence of predatory ants we did not specifically test the effects of ants on pollinator abundance (i.e. ant-pollinator conflicts). Therefore, the goal of this study was to further explore the independent and interactive effects of exogenous application of JA and predatory ants on floral and extrafloral nectar, ant and pollinator abundance, and plant reproductive success. To address these objectives we carried out two field experiments using *P. lunatus* plants and manipulated the presence of ants and nectar production via induction with jasmonic acid. Then we measured floral and extrafloral nectar production, the number of patrolling ants and flower visitors and some plant fitness traits, such as flower production and seed set. Previous studies on this and other plant systems have shown that jasmonic acid induces the production of extrafloral nectar (Heil 2004). Moreover, a repeated induction of EFN flow can significantly benefit plants due to a nearly 10-fold reduction of herbivore damage (Heil et al. 2001). Specifically, in the present study we asked: 1) Do floral and extrafloral nectar secretion and ant attraction increase after JA exogenous application?, 2) How does increased nectar production in both flowers and extrafloral nectaries (EFN), as well as ant presence, affect ant–pollinator interactions along the season?, and finally 3) what are the effects of the interaction between ants and pollinators on plant reproductive success?

MATERIAL AND METHODS

Natural history

Phaseolus lunatus L. (Fabaceae), commonly known as Lima bean, occurs naturally throughout Meso and Southamerica. This study was conducted in the coastal area of the state of Oaxaca, Mexico, 15 km north-west of Puerto Escondido (15°55.596’N, 97°9.118’W). At this site, *P. lunatus* plants in wild populations start to germinate between June-July. The first inflorescences appear in October-November and the seeds are produced at the end of December and early January. Plant phenology is synchronized with the regional weather, which is characterized by one main rainy season from June to October, and follows a bimodal distribution peaking in July and September (Kost and Heil 2005). As many species of the family Fabaceae, Lima bean bear EFN located on their bracts or arranged pairwise at the stipules of the trifoliate leaves as well as the petioles of the individual leaflets (Heil 2004). This plant species exhibits an inducible EFN secretion both on leaves and inflorescences, thus allowing an easy manipulation of the rates of nectar production (Heil 2004).

Experimental design

Experiment 1: Effect of JA-induction treatment 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. In February 2011, seeds of *P. lunatus* were collected from wild populations close to Puerto Escondido (Oaxaca, Mexico). In November 2011, plants were sown and grown in 5L pots in the greenhouse at the experimental campus of the Universidad del Mar (Oaxaca, Mexico). In December 2011 (transition from the rainy to the dry season), twenty potted plants were transplanted to the field. To avoid undesirable herbivory, plants were grown in field cages for the first month until they started to produce flowers. The experiment was initiated when the plants presented more than 50% of fully open flowers (plants with at least 5 flowers completely open). The plants were divided into two groups. In the first group of 10 plants, all leaves were sprayed with a solution of jasmonic acid (1 mM) applied with an atomizer. The other 10 plants were used as controls and sprayed with distilled water. JA-induced plants were treated every seven days during 4 weeks. Both plant groups were covered 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 on the plants (Mooney and Agrawal 2008). Plants were treated at 6h00 AM and the concentration of nectar from EFN and flowers for each plant was measured 4 h later as described by (Heil 2004).

The quantity of nectar (floral and extrafloral) was measured with 5µL micropipettes (graduated by 1µl divisions to make a direct measurement of nectar volume) and we used a temperature-compensated portable refractometer (ATAGO hand refractometer, L. Kübler, Karlsruhe, Germany) to measure the concentration of soluble solids. To remove the nectar we used 5 µl of distilled water applied into all nectaries. This procedure was repeated until we had concentrations of <1%. Values from all collections conducted for the nectaries on one leaf were summed to quantify a leaf 's overall production of solid EFN compounds, in the case of the flowers we followed the same procedure; see (Stephenson 1982) for details concerning this method.

Experiment 2: Effect of ant and JA-induction treatments on the abundance of ants and pollinators 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 (i.e. pollinators) 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 2 ant treatments × 2 induction treatments × 10 replicates.

Seeds were collected, sown and plants were grown as described in the previous experiment. The experiment was initiated when the inflorescences presented more than 50% of fully open flowers. As in the previous experiment, plants were sprayed with either a solution of jasmonic acid (1 mM) or with distilled water and ants were excluded by applying tanglefoot around the base of the plant. For the treatments with JA, plants were treated twice a week in order to stimulate a permanent induction. Induction was always conducted at 6h00AM to avoid wind-spreading of JA in the experimental plot.

A census was conducted every day, for 21 days (three times per day at: 10h00hrs, 12h00hrs and 14h00hrs), from January 29 to February 14. During this period, for each plant we counted: the number of flowers, number of pollinators and number of patrolling ants (including ants visiting extrafloral nectaries and flowers). We considered all hymenopterans and dipterans as 'flower visitors' when they entered the flowers and stayed more than 5 seconds. Ants were counted only when they were seen patrolling along the plant. At the end of the experiment, mature and dry pods of each plant were collected, shelled and seeds were counted.

Statistical Analyses

Analyses of extrafloral and floral nectar (and their specific compounds) were carried out using a generalized linear model (PROC GLM in SAS System, version 9.2, SAS Institute, Cary, NC) (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) on each specific compound of nectar 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.

As residuals were not normal, analyses of pollinator abundance and the number of flowers were carried out using a generalized linear mixed model (PROC GLIMMIX with a Poisson distribution in SAS) (Littell et al. 2006). The main effects of ants (A), induction treatment (T) and the A × T interaction were treated as fixed factors. The effect of the time (21 days) was treated as a random factor to account for repeated measures taken on the same experimental unit. The effect of census hour (10.00, 12.00, 14.00) was treated as a fixed factor because the model did not converge using this factor as a random factor. Analyses of ant abundance were carried out using a generalized linear mixed model (PROC GLIMMIX in SAS) with the induction treatment and hour of census as fixed factors and time (day of census) as a random factor (Littell et al. 2006).

Finally, analyses of the number of seeds were carried out 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 pollinators and their consequences for plant reproductive success.*

Ant presence (vs. absence) did not significantly affect the abundance of pollinators (Table 1, Fig. 2a). Exogenous application of JA significantly affected the abundance of ants, but not the abundance of pollinators (Table 1). Specifically, we found that the number of ants was 3-fold greater in JA-induced plants than in control plants (Fig. 2b). The interaction between ant and JA treatments did not significantly affect the abundance of pollinators (Table 1).

Ant presence significantly affected the number of flowers (Table 2), which was 2-fold greater in plants with ants than in ant-excluded plants (Fig. 3a). Ant presence did not significantly affect the number of seeds (Table 2). Exogenous application of JA significantly affected the number of flowers and seeds (Table 2). Specifically, the number of flowers and seeds was 1.4-fold 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 six days that followed the application of JA. Flower production per day during the first week was 2.5-fold greater in plants treated with JA than untreated control plants ($F_{1,269} = 60.69$; $P < .0001$; JA-treated plants: 6.85 ± 0.41, Control plants: 2.25 ± 0.41).

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, attraction of ants markedly increased in JA-induced plants. Despite an increase in floral nectar in JA plants, application of this hormone had no significant effects on pollinator attraction. Finally, ant presence did not result in a decrease in the number of pollinator visits, but our results suggest the existence of an ant-pollinator conflict. In particular, JA-induced plants without ants produced a higher amount of flowers and seeds compared with the JA-treated plants with ants, suggesting that ants could negatively affect pollination efficiency.

Our results showing that exogenous JA application increased the amount of floral and extrafloral nectar confirm earlier observations on the same plant system but using tendrils under 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 extrafloral nectar than control plants. In this regard, (Heil 2011) documented that JA-induction markedly increased the amount of floral nectar, suggesting that nectar production in flowers and EFN of Lima bean are subject to a joined 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 predatory ant attraction after JA application should result in significant alterations in the arthropod community and plant fitness. However, in the present study we observed that pollinators were not influenced by the JA induction treatment. Previous research has reported contrasting findings on the role of this hormone on the frequency of pollinator visits (Bruinsma and Dicke 2008, Radhika et al. 2010). For example, (Bruinsma et al. 2008) observed that rates of flower visitation by honeybees and syrphid flies were similar in JA-induced plants of *Brassica nigra* and in untreated controls. In contrast, (Radhika et al. 2010) found that *B. napus* plants induced by JA application showed increased pollinator visits, enhancing thus pollination efficiency. Our results showed that pollinator visits did not decrease in plants with ant presence, and this seems to show an absence of conflict between ants and pollinators. However, when we analyzed plant fitness traits, we observed that JA-treated plants without ants produced much more seeds than JA-treated plants with ants (Fig. 3b), suggesting thus the presence of an ant-pollinator conflict. Supporting our results, previous studies have observed that ants interfere with the 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 ant aggressive behavior, but they patrolled actively the entire plant and even went to flowers (Hernández-Cumplido, personal observation). Another plausible explanation would be that ants could have negatively affected the development and performance of the flowers. In this sense, some species of ants such as *Crematogaster dohrni* can castrate flowers from *Humboldtia brunonis* by damaging them when the style and stamensare still folded (Gaume et al. 2005). Taken together all of this, an emerging question from our study would be if ants benefit plants via a cascade with both antagonistic (i.e. herbivores) and mutualistic (i.e. pollinators) interactions.

Our results showed that exogenous application of JA had a positive effect on plant fitness (i.e. number of flowers and seeds), which indicates no reproductive costs associated with induction by JA. These findings contrast with those from several previous studies which 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 and Baldwin 2001, Cipollini 2007, Cipollini 2010). Fitness costs associated with JA induction have been usually explained by emerging trade-offs between induced defenses and fitness traits (Cipollini 2010, Sampedro et al. 2011). Nevertheless, our results of non-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 3-fold 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 flowers considerably earlier than non-treated plants. This is further supported by findings from 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. unpublished). 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 Lima bean system, as pod and seed beetles 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 bean (i.e. pollinator and ants) and affect plant reproductive traits. In particular, Lima bean plants under JA induction produce more nectar in both structures EFNs and flowers, which suggest a joint hormonal control, attract more ants and produce more seeds than non-induced plants. Additionally, we found strong support to suggest a potential ant-pollinator conflict which may very likely be responsible for the increased plant fitness in JA-induced plants. Future studies should address the mechanisms (e.g. specific nectar compounds that might attract predatory ants) underlying some of the patterns observed in our study.

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Table 1. Summary of mixed models analysing independent and interactive effects of ant (presence vs. absence) and induction (control vs. jasmonic acid) treatments (fixed factors) on the abundance of pollinators and ants. The effect of census hour (10.00, 12.00, 14.00) and day (21 days) were treated as fixed factors. Number of flowers was used as a covariate for pollinator abundance analysis. F-values, degrees of freedom and associated significance levels (*P*) are shown. Significant P values ($P < 0.05$) are typed in bold.

	Pollinators			Ants		
	$Df_{num,den}$	F	*P*	$Df_{num,den}$	$F_{1,2615}$	*P*
Ant (A)	1, 2609	0.86	0.355			
Treatment (T)	1, 2609	0.40	0.527	1, 2595	21.98	**<0.001**
A × T	1, 2609	0.05	0.829			
Day	20, 2609	9.82	**<0.001**	20, 2595	3.93	**<0.001**
Hour	2, 2609	14.09	**<0.001**	2, 2595	0.24	0.783
Flowers	1, 2609	1080.67	**<0.001**			

Table 2. Summary of mixed models analysing independent and interactive effects of ant (presence vs. absence) and induction (control vs. jasmonic acid) treatments (fixed factors) on the number of flowers and seeds. The effect of census hour (10.00, 12.00, 14.00) and day (21 days) were treated as fixed factors for the number of flower analysis. F-values, degrees of freedom and associated significance levels (*P*) are shown. Significant P values ($P < 0.05$) are typed in bold.

	Flowers			Seeds		
	$Df_{num,den}$	F	*P*	$Df_{num,den}$	$F_{1,2615}$	*P*
Ant (A)	1, 2611	56.76	**<0.001**	1, 36	2.58	0.117
Treatment (T)	1, 2611	81.19	**<0.001**	1, 36	5.88	**0.020**
A × T	1, 2611	2.62	0.106	1, 36	2.87	0.099
Day	20, 2611	18.92	**<0.001**			
Hour	2, 2611	111.01	**<0.001**			

FIGURE LEGENDS

Figure 1. Effect of induction treatment (white bars for untreated control and grey bars for jasmonic acid) on the concentration of (a) extrafloral nectar and (b) floral nectar in *Phaseoulus lunatus* plants. Nectar secretion was measured as mg of soluble solids secreted per g dry mass 4 h after treatments. Bars are means ± s.e.m. (N = 10). F-values, degrees of freedom and associated significance levels (*P*) are shown Different letters indicate significant differences between constitutive and induced conditions.

Figure 2. Effect of presence of mutualistic ants (two levels: presence or absence) and induction treatment (white bars for untreated control and grey bars for jasmonic acid) on (a) pollinator abundance in *Phaseoulus lunatus* plants. Effect of induction treatment (white bars for untreated control and black bars for jasmonic acid) on (b) ant abundance. Measurements were carried out during 21 consecutive days. Bars are means ± s.e.m. (N = 10). Asterisks in figure 3a indicate significant differences between constitutive and induced conditions within each ant treatment at $P < 0.01$ (**). n.s. = non-significant. Different letters in figure 3b indicate significant differences between constitutive and induced conditions.

Figure 3. Effect of presence of mutualistic ants (two levels: presence or absence) and induction treatment (white bars for untreated control and grey bars for jasmonic acid) on the number of (a) flowers and (b) seeds in *Phaseoulus lunatus* plants. Measurements of flowers were carried out during 21 consecutive days. Bars are means ± s.e.m. (N = 10). Asterisks indicate significant differences between constitutive and induced conditions within each ant treatment at $P < 0.01$ (**) and $P < 0.001$ (***). n.s. = non-significant.

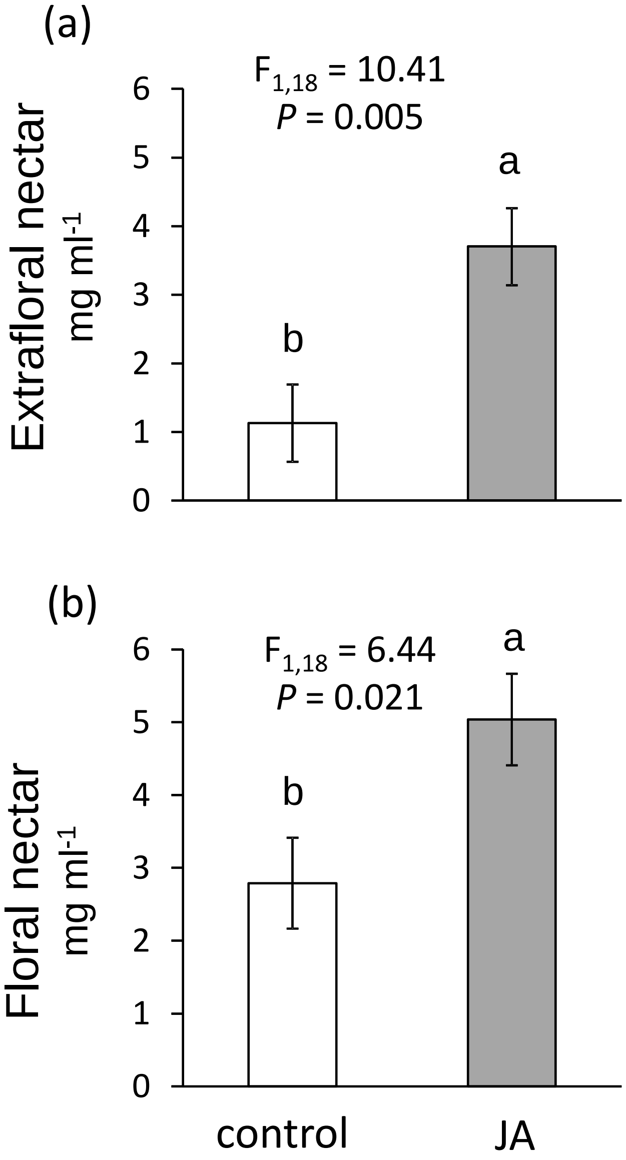

Figure 1. Hernández-Cumplido et al.

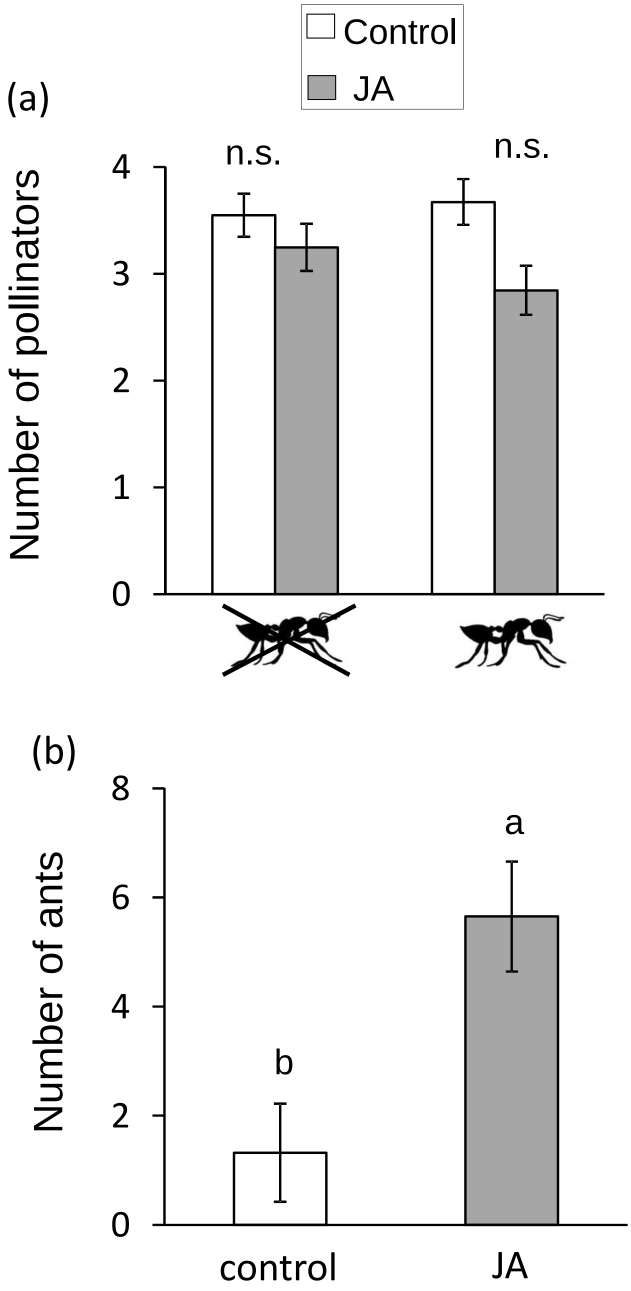

Figure 2. Hernández-Cumplido et al.

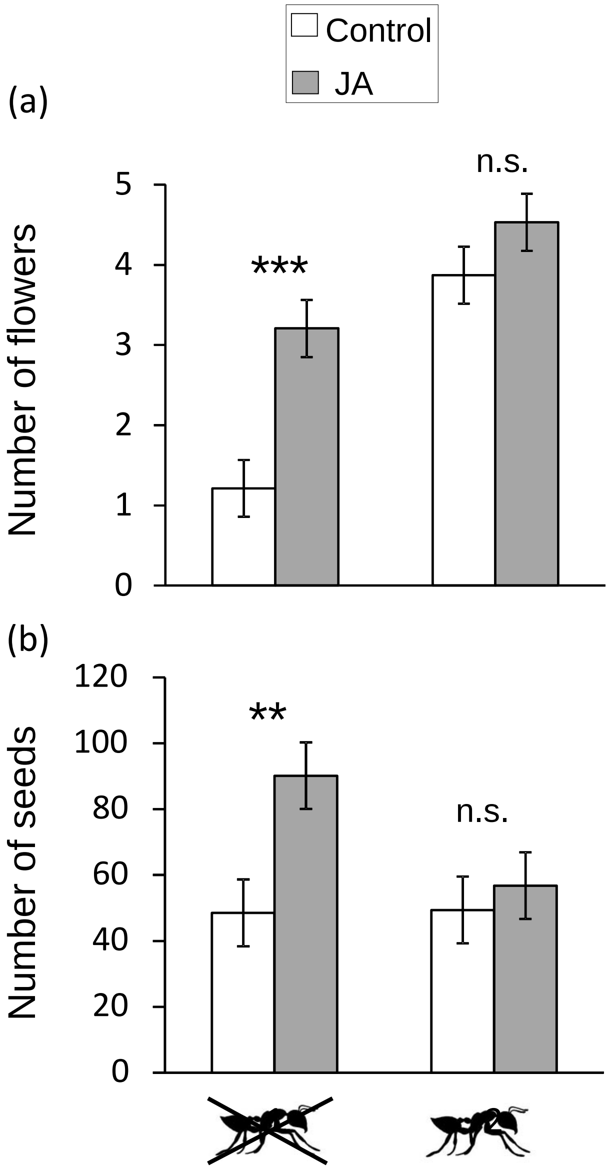

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Figure 3. Hernández-Cumplido et al.

General discussion

During the last few decades, numerous empirical studies and literature reviews have examined the wide array of mechanisms that plants have develop to defend themselves against their attackers (see the reviews by Kessler and Baldwin 2002, Heil 2008, Stam et al. 2014). As examples of direct defenses studies have examined morphological traits such as spines, thorns and wax layers, which may hamper herbivore colonization and movement on the plant (e.g. Fordyce and Agrawal 2002). Others have focused on the production of plant chemicals, such as carbon- and nitrogen-based compounds, may interfere with the physiology of the herbivores and reduce their growth and survival (e.g. Ruhola et al. 2001). Finally, a number of studies have examined indirect plant defenses such as, alternative food resources (e.g. the production of extrafloral nectar) and shelter for the herbivore's natural enemies (Heil 2008).

One of the general conclusions of all these previous studies and reviews has been that plant and insect communities and their dynamics are highly influenced by plant defensive traits. Herbivory triggers physical and chemical changes on the plant that can affect not only the first event of herbivory but also the entire community of insects associated with that particular plant.

The studies that are presented in the current thesis examine the effect of direct and indirect defenses of wild lima bean plants, *Phaseolus lunatus*, on the associated natural herbivores and their natural enemies, as well as on ants and pollinators that interact with this plant species.

***Sequential herbivory*:**

During the last five years, the differential responses of plants against multiple insect herbivores have been studied using an integrative approach (Stam et al. 2014). However, only few studies have explicitly examined how herbivore induced plant responses shape insect communities (Kessler and Halitschke 2007) and much less is known about intraspecific variation in plant responses to multiple herbivores. Insect herbivores can indirectly influence each other even when they are even partially or temporally separated. This can take place via plant mediation through induced changes in plant quality (Denno et al. 1995, Denno and Kaplan 2007). In this thesis, using first as a tool the phyhormone jasmonic acid (JA) and supported by experiments with natural herbivores, we demonstrated that induction with this hormone in two plant structures of Lima bean; leaves and green pods markedly affected plant performance. In addition, the performance of the seed-feeder (Mexican bean weevil) that arrives later in the season in both field and laboratory experiments was shown to be negatively affected. As it has been discussed in chapters one and two of this thesis, these finding are supported by numerous studies that have shown that secondary herbivores are affected negatively by the early damage made by other herbivores (Kaplan and Denno 2007, Poelman et al. 2010). For example, McArt et al (2013) found that leaf herbivory caused by the beetle *Popilia japonica* increases plant fitness in evening primrose (*Oenethera biennis*) via induced resistance to seeds predators. In this case, leaf herbivory induced an accumulation of jasmonic acid and some other complex phenolics in reproductive tissues which negatively affected seed predation by three species of Lepidotera that feed on seeds (reduction of 77% compared with plants that were damaged by the leaf herbivore).

We also found that JA induction on Lima bean plants reduced seed predation by the Mexican bean weevil. Similar results were found in the study in which real herbivores where used. Here again, plants under early-herbivore induction were less damaged at the seed stage than control plants. Moreover, the abundance and performance of the parasitoids that attack the bean weevils were also lower on seeds from plants that were induced early in the season.

The effects of induced-plant chemical changes cannot only affect herbivores that feed on the plant during different times along the season, but they could even last longer and be passed on to the next generation of plants (Agrawal et al. 1999, Rasmann et al. 2012). By exploring the existence of a transgenerational effects of early-defense induction (via JA), we found that induced mother plants not only produce seeds that are more resistant to seed weevils compared to non-induced plants but also this trait was transmitted to their daughters, such that, the effects of early application of JA will persist on the plant's offspring. Overall, seeds from control plants were larger and heavier than seeds produced from JA-induced plants, but surprisingly these seeds contained higher quantities of linamarin, a cyanogenic glycoside that has been implicated in defense against leaf herbivores (Ballhorn et al. 2008). However, these seeds were also more attacked by the Mexican bean weevil, and the same pattern was consistent for the seeds produced by the daughters of these plants. Thus, even though these seeds contained higher concentration of the cyanogenic glycoside, linamarin, they did not appear to be better defended against the Mexican bean weevil.

As for the parasitoids that attack the bean weevil, in the first experiment we found a marginal but non-significant effect of JA-induction on parasitism rates. However, no significant differences on parasitism rates were found for seeds produced by treated and non-treated plants in the offspring generation. The results obtained in the experiment in which we used real herbivores, yielded similar results. Here, we manipulated damage by exposing the plants to different treatments consisting of no herbivores or two different guilds of herbivores. Again, seeds produced by plants that were exposed to either type of herbivore were smaller, lighter and less attacked than the seeds produced by plants on which herbivores were absent. In this study, it was also found that fewer parasitoids emerged from seeds produced by plants exposed to herbivores. It appears that these seeds are of lower quality than seeds produced by herbivore-free plants. Performance experiments in the lab support this idea. Overall, beetle performance was lower on seeds produced by herbivore-attacked plants. Results from other studies have shown similar patterns. For example, Poelman et al. (2010) found that early season leaf herbivory by larvae of the cabbage butterfly, *Pieris rapae* in two varieties of *Brassica* plants resulted in lower subsequent attack by generalist herbivores from different feeding guilds (Poelman et al. 2010). Similarly, Rodríguez-Saona et al. (2005) showed that early herbivory by the aphid *Macrosiphum euphorbiae* in tomato plants reduced the subsequent preference of the leaf herbivore *Spodoptera exigua* as well as the performance of its parasitoid *Cotesia marginiventris*.

Thus, the studies that conform this thesis do not only contribute to the growing knowledge on plant-induced mediated effects on plant-insect interactions, but also provide unequivocal evidence that early-induction both, by exogenous application of JA and by damage produced by real herbivores in the field, has a strong effect on the late-season seed weevils and their parasitoids and these effects are maintained in the following generation of plants.

As it was previously discussed in chapter one, only a handful of studies have examined the consequences of early season herbivory for the plant's offspring. Among these, are the ones of Agrawal et al. (1999) and Rasmann et al (2012). Both of these studies showed that herbivore-induced plant resistance can be passed on to the next generation of plants. In the first case offspring of damage plants had a higher concentration of hidroxylated glucosinolates and in the second case, larvae of *P. rapae* perform better on control plants than on plants under JA induction and this pattern persisted along two plant generations.

Despite the growing evidence on transgenerational effects of plant resistance, no previous study has examined the consequences of these plant maternal effects on insects that feed on different plant structures than the ones where the original damage was inflicted, nor on insects that belong to different trophic guilds or trophic levels. The novelty of the studies presented in this thesis lies in the fact that we explored these transgenerational effects of plant resistance on herbivores from different guilds and extended this question to the natural enemies of these herbivores. Thus, the results from this thesis not only add to the newly growing field that explores transgenerational plant effects on herbivore-mediated interactions, but also adds new levels of complexity to this fascinating question.

It is important to stress that some questions pertaining the changes in the chemistry and quality of bean seeds as a resource for seed beetles and their parasitoids remain unanswered. For example, we still do not know which are the signals and mechanisms that are driving the choice for seeds in these insects. Contrary to our expectations, seeds produced by control plants in all experiments had higher CNGs concentrations than the seeds from induced plants. This may be explained by results from a previous study in our group that examined variation in cyanogenic compounds in natural populations of Lima bean (Shlichta et al. 2014). They found no correlation between the amount of CNGs and the performance of the Mexican bean weevil. Future research should focus on the analyses of content of proteins and other chemical compounds that can confer resistance against seed herbivores and that may play a role in the host selection behavior and subsequent performance of bean weevils and their parasitoids. For example phaseolin has been shown to affect the larval development of *Callosobruchus maculatus* (Moraes et al. 2000). Similar patterns have been found for arcelins that belong to a family of proteins and are known to be toxic to the larvae of certain bruchid species such as *Zabrotes subfasciatus* (Paes et al. 2000). Also in our group, Zaugg et al. (2013) reported the presence of a new arceline-like protein in a related bean species, the common bean *Phaseolus vulgaris* that was associated with resistance against bruchid beetles. We do not know however, if this group of proteins is found in wild Lima bean seeds.

Indirect defenses in Lima bean

Indirect defenses are known to be involved in the expression of traits that increase the effectiveness of predators and parasitoids in locating their prey (Heil 2008). Such is the case of extrafloral nectaries that have been shown to attract natural enemies of herbivores, (i.e. ants) that enhance plant protection against these herbivores. In the second part of the thesis I explore the interaction between ants and pollinators and the extent to which this indirect interaction is mediated by the nectar produced by flowers and extrafloral nectaries in wild plants of Lima bean. We found that plants subjected to JA induction attract more ants. Similarly, an increase in nectar secretion in JA-induced plants resulted in a higher number of flower visitors, but only when ants where excluded. This finding indicates that the presence of ants interferes with pollinator attraction and/or efficiency. A second study (chapter four) examined the consequences of a potential conflict for Lima bean plants between the attraction of ant-defending ants and flower visitors on plant fitness. Here we not only confirm again that an increase in nectar production by JA-induced plants attracts more ants, and although we did not find significant differences in visits of pollinators as in the third chapter, plants that were under induction and without ants produced more flowers and seeds that plants in which ants were present.

These results together suggest that there is indeed a potential conflict for the plant between ant and pollinator attraction, but this conflict is only apparent when plants are under induction and nectar production is increased. Support for these findings are results from other studies that show that the presence of ants on plants can have a negative effect on pollinators, either by ants robbing nectar from the flowers, by chasing or attacking the pollinators or simply because of interference while they patrol the plant (Galen 1999, Nicklen and Wagner 2006, LeVan et al. 2014). Although we did observe any of these behaviors, we can speculate that as nectar secretion increased on JA-induced plants, and more ants were present, it is likely that they affected the time that pollinators spent on the flowers. This idea is supported by the fact that plants from the two treatment groups where nectar secretion was increased via JA-induction, those plants on which ants were excluded, produced more seeds than plants on which ants were present.

General conclusions

Overall, this thesis presents a multidisciplinary approach on the study of interactions among different members of the community related to wild plants of Lima bean (herbivores, parasitoids, pollinators and ants), supported by studies conducted in the field, in the laboratory and complemented by chemical analyses. We found first, that natural herbivores and exogeneous application of JA, trigger an induction response that affects not only the same plant structures but also that is transferred to the reproductive tissues (such as flowers and seeds), and this affects negatively the performance of the herbivores that attack the plant later in the season, also supported by the performance experiment with the Mexican bean weevil.

Secondly, from the plant's perspective, early-induction appears to create a trade-off where seeds produced by non-induced plants are larger and germinate more than seeds from induced plants, but at the same time the former ones present a more attractive resource for seed beetles which results in higher rates of infestation. Third, we showed that in *P. lunatus* there is a potential conflict between attracting defending ants and pollinators. This conflict could only be detected in situations of an increased production of extrafloral and floral nectar by plants induced with JA.

Finally, by integrating the results regarding both direct and indirect defenses in Lima bean we can conclude that the complexity of the plant responses is driven by hormonal control. As soon as the first visitor arrives to the plant it triggers a series of responses that will affect not only the next visitor's choice but also will affect the plant defense in the next generation. Nevertheless, the mechanisms underlying the patterns observed still need to be elucidated. As for the role of CNGs the seeds, it seems that they are not involved in defense against the Mexican bean weevil beetles.

It is possible that they are acting at some other levels of plant defense or development such as plant germination, seedling establishment or seedling defense

In conclusion, together the results of the different studies that comprise this thesis reveal that changes in plant phenotype as a result of the first events of herbivory in the field, can trigger a complex set of plant responses towards subsequent plant enemies and visitors from different guilds; including different leaf herbivores, pod and seed feeders, parasitoids, ants and pollinators.

As it is usually the case with most scientific work, by answering some questions, we open the door to new and exciting questions. For the particular case of this doctoral thesis, some of these unsolved and exciting questions are:

1) What is the mechanism that drives the host selection process and performance of the Mexican bean weevil?

2) What is the role and purpose of the high concentrations of CNGs present in the seeds? and finally,

3) Is there a toxic or repellent effect of the nectar secreted in both structures (floral and extrafloral)?

I believe that the answers to these new open questions would allow a better understanding of the specific mechanisms underlying the patterns observed in this multitrophic system.

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