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Top-down and bottom-up plant-mediated interactions on wild Lima bean, *Phaseolus lunatus*

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“Top-down and bottom-up plant-mediated interactions on wild Lima bean, *phaseolus lunatus*”

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CONTENTS

Summary

Plants are attacked by a wide array of insect herbivores, which are in turn attacked by natural enemies. Plants represent the base of these tritrophic interactions, and their traits affect higher trophic levels in many different ways. These effects have been referred to as bottom-up effects. Alternatively, higher trophic levels such as herbivores and their natural enemies can also affect lower trophic levels, this is known as top-down effects. In this thesis, I investigated the complex interactions among different trophic levels comprising several insects from different guilds that feed on various plants organs. I examined the extent to which the actors of such tritrophic interactions can influence each other, as well as other insects in the community.

Lima bean (*Phaseolus lunatus*) is used as a model system in chemical ecology. This plant has been domesticated for the consumption of its seed, leading to many chemical and physical changes in this organ. Such changes can alter the interactions with the insects that attack these seeds. In the first chapter of my thesis, I investigated the effect of Lima bean domestication on one of its main storage pest, the seed beetle *Zabrotes subfasciatus*. Particularly, I focused on the increase of seed size, one of the major effects of domestication, and its impact on the intraspecific competition of this pest insect. I found that as a result of the domestication process, Lima bean seeds were more suitable for the seed beetle. The initial number of eggs on a seed and the weight of female beetles that emerged were negatively correlated, but only for wild seeds. Unravelling the changes in plant insect interactions caused by plant domestication may contribute to the development of pest control strategies.

Seeds of wild lima bean plants are not suitable for human consumption, mainly because they contain toxic defensive compounds in high concentration: cyanogenic glycosides (CNGs). In the second chapter, I examined these secondary compounds that have been mainly studied in the leaves of wild lima bean, and have been shown to be toxic to generalist insect herbivores as well as some mammals. However, a previous study tested the effect of these compounds present in the seeds on seed beetles (*Z. subfasciatus*), and found no correlation between the performance of this insect and the seed CNG concentration. I investigated the potential role of CNG compounds in the seed of wild Lima bean as seedling resistance to herbivore as well as nitrogen storage compounds for plant growth. I found no correlation between seed CNG content and germination rate and seedling growth, but I showed that seeds with high CNG content produced seedling with a higher CNG content.

Chapter three consists on a study on tritrophic interactions. The effect of parasitoids on the plant (top-down) is generally investigated for the potential reduction in herbivory damage they can cause. However, it is increasingly recognized that parasitoids can have other effects on plant, through physiological changes in their host. These types of top-down effect of parasitoids on plant are virtually unexplored. Here, I explored the effect of parasitoid on plant growth. In a field experiment, wild lima bean plants were exposed to three treatments. In the first one, no herbivores were allowed to feed on the plant. In a second one, plants were attacked by an herbivorous caterpillar, *Spodoptera latifascia*, which is naturally present in the same areas as our study plant. Third, some plants were also attacked by herbivores, but in presence of an ectoparasitoid (*Euplectrus platyhypenae*), present in the same area. I found that the herbivores triggered an overcompensation of the plant growth, which was attenuated by parasitoid presence. In addition, parasitoids could reduce the delay in seed production induced by herbivory. These interesting results are among the first to show that parasitoids can influence plant growth through its host.

In the previous chapters, I focused on the separate effects of bottom-up and top-down forces on tritrophic interactions. Recent findings suggest herbivores present on the same plant at different times can influence each other through changes induced in the plant. Yet, this notion remains understudied. In the fourth chapter, I investigated top-down forces in a tritrophic interaction taking place at the end of the season, when wild lima bean plants are producing seeds. One of the main seed predators of seeds of wild Lima bean plants is the bruchid beetle *Z. subfasciatus*, which is in turn parasitized by the braconid ectoparasitoid *Stenocorse bruchivora*. I observed that seeds that had been previously damaged by seed beetles were still able to germinate. Thus, I designed a series of experiments to test the effect of bruchid beetles and their parasitoids on the germination and performance of the seedlings. I found a negative effect of bruchid beetles the germinated seedling performance, which was strongly attenuated by parasitoids. Furthermore, I also tested the bottom-up consequences of seed infestation on subsequent herbivorous insect feeding on plants germinated from seeds that endured different intensity and type of insect damage. I showed that plants produced by seeds with high levels of insect damage are less defended and leaf herbivores perform better on them than plants produced by seeds with low damage or no damage.

In the last chapter of my thesis, I focused on a tritrophic interaction at the seed level, in order to investigate the interactive effects of beetle larval density inside the seed and the presence of parasitoids on bruchid development. Herbivore density has already been shown to influence parasitoid density, without changing the interaction between herbivores and parasitoids. However, the extent to which herbivore density influences the per capita effects of parasitoids on their host remains understudied. In addition, direct effects of parasitoids on their host (parasitism) are well studied, but there are only a few studies that investigate indirect non-consumptive effects of parasitoids on their host (e.g. induction of host defensive behavior by parasitoid sole presence). To study these interactions, we used a tritrophic interaction taking place at the wild lima bean seed level, between a seed beetle (*Z. subfasciatus*) and its parasitoids (*S. bruchivora*). This system is ideal because females of *Z. subfasciatus* glue their eggs on the seed coat and larval development is confined inside a small seed. This allows the manipulation of beetle larval densities in the seeds by removing eggs from the seed coat. The increase in beetle density had a negative effect on beetle performance, certainly due to intraspecific competition. This cascaded up to parasitoids which parasitism rate was significantly reduced. Parasitoids altered beetle development time, which can be explained by parasitoid non-consumptive effects on their host.

Together, the chapters of the current thesis add to the growing body of knowledge on how plant-mediated interactions on different trophic levels and insect guilds affect the ecology and evolution of each of its participants and ultimately the insect community associated with Lima bean plants. Future studies should aim at shedding more light on the mechanisms that underlie herbivore-mediated parasitoids effects on plants, a topic that is particularly understudied.

Résumé

Les plantes sont attaquées par de nombreux herbivores, qui sont à leur tour attaqués par leurs ennemis naturels (parasitoïdes). Les plantes constituent la base des interactions trophiques, et leurs traits affectent les niveaux trophiques supérieurs de plusieurs manières. Ces effets sont nommés « bottom - up effects ». Dans un autre scénario, les niveaux trophiques supérieurs, tels que les herbivores et leurs ennemis naturels peuvent aussi affecter les niveaux trophiques inférieurs (« top - down effects »). Durant ma thèse, j'ai cherché à mieux comprendre les interactions complexes entre niveaux trophiques comprenant plusieurs insectes appartenant à des groupes distincts et se nourrissant de différents organes de plantes. J'ai examiné dans quelle mesure les acteurs de ces interactions trophiques à trois niveaux peuvent s'influencer entre eux, ou influencer d'autres insectes de cette même communauté.

Le haricot de Lima (*Phaseolus lunatus*) est utilisé comme une plante modèle en écologie chimique. Cette plante a été domestiquée pour la consommation de ses graines, ce qui a mené à de nombreux changements physiques et chimiques de cet organe. De tels changements peuvent altérer les interactions avec les insectes qui attaquent ces graines. Dans le premier chapitre de ma thèse, je me suis intéressé aux effets de la domestication du haricot de Lima sur un des principaux ravageurs de graines stockées, la bruche *Zabrotes subfasciatus*. En particulier, je me suis concentré sur l'augmentation de la taille des graines, une des conséquences majeures de la domestication, et sur l'impact de ce changement sur la compétition intra spécifique des bruches. J'ai trouvé qu'en raison du procédé de domestication, les graines domestiquées conviennent mieux aux bruches. Le nombre d'œufs initialement pondus sur les graines était négativement corrélé avec le poids des bruches femelles sortis des graines, mais uniquement pour les graines sauvages. Mieux comprendre les changements de la domestication des plantes et leurs effets sur les interactions avec les insectes pourrait contribuer au développement de nouvelles stratégies de contrôle des ravageurs.

Les graines de haricot de Lima sauvages ne sont pas comestibles pour la consommation humaine, principalement parce qu'elles contiennent des composés toxiques de défense en grande concentration: les glycosides cyanogènes (CNGs). Dans le second chapitre de ma thèse, j'ai étudié ces composés secondaires qui ont principalement fait l'objet de recherche au niveau des feuilles, où il a été démontré qu'ils sont toxiques pour plusieurs herbivores généralistes. Cependant, une étude a testé les effets de ces composés chimiques présents dans les graines sur les bruches *Z. subfasciatus*, et n'a trouvé aucun effet négatif. J'ai donc cherché à comprendre le rôle de ces composés chimiques dans les graines, afin de déterminer s'ils pouvaient servir à la croissance de la plante, ou bien à la défense de la plante qui vient de germer. Je n'ai trouvé aucune corrélation entre la croissance de la jeune pousse et la concentration en CNGs dans les graines. En revanche, j'ai pu démontrer que les jeunes pousses issues d'une graine contenant plus de CNGs contenaient également plus de CNGs.

Le troisième chapitre de ma thèse porte sur l'étude d'une interaction tritrophique. L'effet des parasitoïdes sur la plante (top - down) est généralement étudié pour la réduction potentielle de dégâts fait à la plante. Cependant, il est de plus en plus reconnu que les parasitoïdes peuvent avoir d'autres effets sur les plantes, au travers des changements physiologiques provoqués dans leurs hôtes. Ces types d’interactions « top - down » sont presque inexplorées. Dans ce chapitre, j’ai étudié, lors d’un travail de terrain, les effets des parasitoïdes sur la croissance des plantes de haricot. J’ai trouvé que la réponse de croissance de la plante contre les herbivores était atténuée par la présence de parasitoïdes. De plus, les parasitoïdes ont réduit le délai de production des graines induit par les herbivores. Ces résultats sont parmi les premiers à montrer que les parasitoïdes peuvent influencer la croissance d’une plante à travers leur hôte.

Lors des chapitres précédents, j’ai étudié différents effets « bottom - up » et « top - down » agissant dans les interactions tritrophiques. De récentes études suggèrent que différents herbivores interagissant avec une même plante, même de façon non concomitante, peuvent s’influencer au travers des changements induits dans cette plante. Cependant, cette notion reste peu étudiée. Dans le quatrième chapitre de ma thèse, je me suis intéressé aux forces de type « top - down » présentes dans les interactions tritrophiques qui ont lieu à la fin de la saison, lorsque les haricots sauvages produisent leurs graines. Les bruches qui attaquent et se développent dans ces graines peuvent être attaqués par des parasitoïdes. J’ai observé que les graines endommagées sont toujours capables de germer. J’ai donc étudié l’effet des bruches et de leurs parasitoïdes sur les plantes issues de graines endommagées. J’ai trouvé un effet négatif des bruches sur les pousses de haricot et que celui-ci est grandement atténué par la présence de parasitoïdes. De plus, j’ai démontré que ces effets négatifs sur les plantes issues des graines endommagées pouvaient affecter les insectes herbivores se nourrissant de ces plantes, en raison de changements dans les composés chimiques.

Dans le dernier chapitre, je me suis concentré sur les interactions tritrophiques au niveau des graines de haricots sauvages, dans le but d’étudier l’impact de la densité de larves de bruches dans la graine et de la présence de parasitoïdes. L’effet de la densité des hôtes sur les parasitoïdes a déjà été étudiée dans le contexte de la réponse fonctionnelle, mais peu d’attention a été portée sur l’effet per capita de la densité des hôtes sur les parasitoïdes. De plus, les effets indirects des parasitoïdes sur leurs hôtes ne menant pas à la mort de ces derniers (effets « non - consumptive ») ont été très peu étudiés. L’augmentation du nombre de bruches par graine a eu un effet négatif sur leur poids, probablement par compétition, ainsi que sur leur taux de parasitisme. De plus, les parasitoïdes ont influencé la vitesse de développement des bruches, ce qui peut être expliqué par des effets « non - consumptive ».

Globalement, les différents chapitres de ma thèse participent à l’amélioration de la compréhension des interactions médiées par les plantes à différents niveaux trophiques et avec différentes guildes d’insectes influençant l’évolution et l’écologie des acteurs de cette communauté et des autres insectes associés aux plantes de haricot de Lima. De futures études devraient s’intéresser aux mécanismes cachés des effets des parasitoïdes sur les plantes qui sont médiés par leurs hôtes, un sujet particulièrement peu étudié.

General introduction

Plant bottom-up effects on herbivores

Plants are primary producers of organic matter, and are the first trophic level of most food webs. They are attacked by a wide array of herbivores, which constitute the main second trophic level that affect plants and are affected by them, creating bottom-up and top-down forces in community dynamics (Ehrlich and Raven 1964, Hunter and Price 1992).

Against herbivory, plants have evolved two main types of defensive strategies: tolerance and resistance (Fig. 1). Tolerance response allows the plant to regrow and/or reproduce after herbivore damage, through changes in primary metabolism (Schwachtje and Baldwin 2008). In this case, the herbivore feeding behavior is not reduced and the herbivore is not harmed; the plants only cope better with it (Strauss and Agrawal 1999, Stowe et al. 2000). This strategy is complementary to plant direct resistance which is usually poorly effective against well adapted insect herbivores (Bustos-Segura et al. 2014, Lucas-Barbosa et al. 2016).

Plant resistance strategies affect herbivores by reducing their preference and/or performance (Karban et al. 1997). This type of strategy against herbivores can be divided in two groups: direct and indirect resistance (Fig. 1).

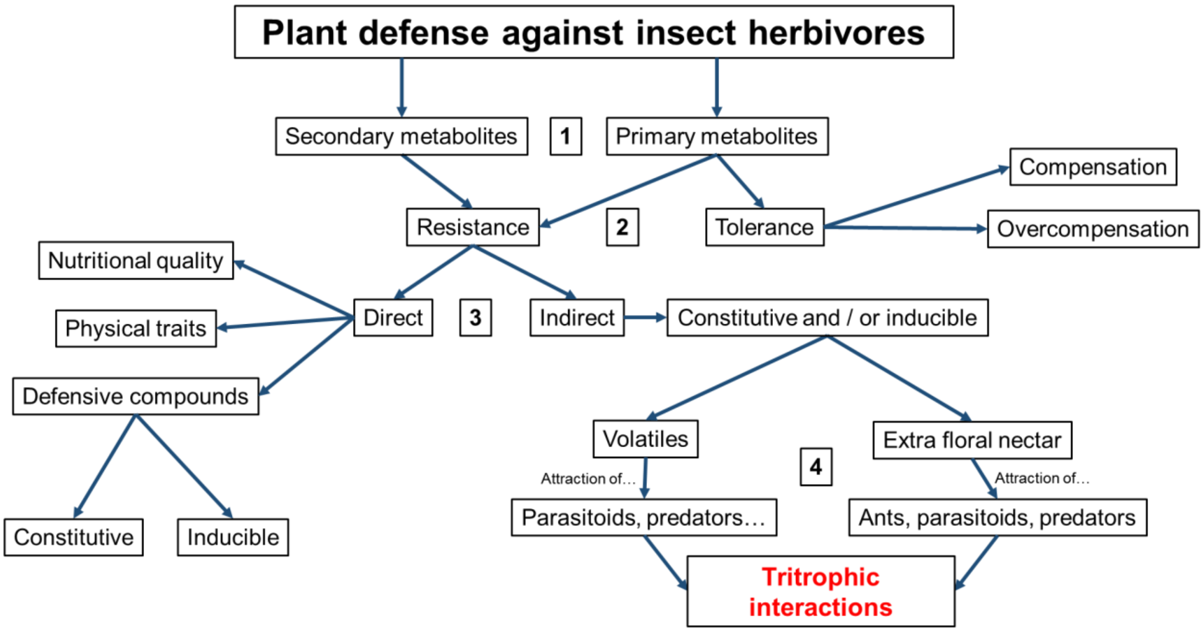

Figure 1. Broad arsenal of defensive strategies/traits which plants use in order to defend themselves against herbivores. 1) Plant defense strategies involve two different types of chemical compounds: primary metabolites, which are essential for plant development, and secondary metabolites, which are used in secondary processes, not essential for plant development. 2) The two main plant defensive strategies against herbivores: tolerance and resistance. 3) Resistance can be divided in two groups: direct resistance, which directly affects negatively the herbivore that feeds on the plant and indirect resistance, which involves other organisms. 4) Different strategies of the plant to attract a third organism in order to defend itself against herbivores, creating interactions among three different trophic levels.

Direct resistance, commonly referred to in the literature as “direct plant defense”, allows plants to act directly against the herbivores in order to reduce their feeding and damage. This strategy can be physical, such as the presence of trichromes on leaves, or leaf toughness (Agrawal and Fishbein 2006, Clissold et al. 2009).

Direct resistance can also involve plant secondary metabolites. These compounds, contrary to primary metabolites, are not essential for the survival of the plant and play an important role in plant interactions with biotic and abiotic environments (Bourgaud et al. 2001, Gols 2014). They can be constitutive, induced or both (Agrawal 2000, Heil 2002). Direct resistance is known to be mainly effective against generalist herbivores that are usually less adapted to plant defensive secondary metabolites than specialist herbivores (Winde and Wittstock 2011). For instance, *Phaseolus lunatus* (lima bean) contains cyanogenic glycosides (CNGs) compounds in their vacuoles. When an herbivore chews on a leaf, breaking the cells, those compounds are activated and there is a release of toxic gaseous hydrogen cyanide (cyanogenesis, Lieberei et al. 1985, Frehner and conn 1987). This well studied direct defense mechanism has been shown to effectively protect the plant against leaf herbivores in nature (Ballhorn et al. 2009). In addition, Ballhorn et al. (2006) also showed that the enzymatic activity of the B-glucosidase increases in response to herbivore attack, highlighting that cyanogenesis is both constitutive and inducible. CNGs are also present in high concentrations in lima bean seeds (Shlichta et al. 2014, 2018). However, these compounds have been shown to be inefficient against one of the most important pest of wild lima beans seeds, the granivorous beetle *Z. subfasciatus*. Therefore, their role in the lima bean seeds remains unknown.

In plant indirect resistance, plants indirectly defend themselves through the help of other organisms (natural enemies), predators or parasitoids (Price et al. 1980, Heil 2004, Hernández-Cumplido et al. 2010). As it is the case for tolerance, plant indirect resistance is more effective than direct resistance against well adapted insect herbivores, as it attracts adapted natural enemies of herbivores through the production of specific herbivore-induced volatiles (Dicke and Baldwin 2010, Turlings et al. 1990, Xiao et al. 2012, Lucas-Barbosa et al. 2016).

Similarly to direct defense, indirect defense can be induced by herbivory (Turlings et al. 1995, Dicke 1999, Kessler and Baldwin 2001). For instance, Dicke (1988) experimentally showed that predatory mites, via the olfaction of volatile chemicals, could distinguish between *Vicia faba* plants attacked or not by spider mites (see Dicke and Sabelis 1988 for a review). Further, the same type of interaction has been shown by Turlings et al. (1990) between plants and parasitoids. They showed that female parasitoids *Cotesia marginiventris* are attracted to terpenoids, plant volatiles mainly released after caterpillars feeding.

From an evolutionary point of view, it has been hypothesized that volatile organic compounds were produced by plants in the first place as a direct defense against herbivores, and which would have been used after by parasitoids and predators to find their hosts (Turlings et al. 1995, Veyrat et al. 2016).

Beside volatile compounds, extrafloral nectar can also attract ants and natural enemies of herbivores (Hernández-Cumplido et al. 2010, Heil 2015). Kost and Heil (2008) performed a field experiment in which they artificially increased the amount of extrafloral nectar of wild lima bean plants (*Phaseolus lunatus*) and found that plants with more extrafloral nectar significantly attracted more insect predators and parasitoids (mainly ants, but also wasps and flies) than plants with less extrafloral nectar, and they also suffered overall less damage. Similarly, Rasmann et al. (2005) found that the same type of plant indirect defense strategy takes place below ground. They showed that entomopathogenic nematodes are attracted by sesquiterpene compounds produced by some maize roots attacked by larvae of *Diabrotica virgifera*.

The few examples of plant indirect resistance that I described above demonstrate different ways in which plants defend themselves by attracting a third actor. In the literature there are many more examples of this and they will be mentioned when appropriate in the following chapters.

Herbivore-mediated plant bottom-up effects on parasitoids

The importance of the third trophic level as an attempt to understand the functioning of ecosystems was first discussed in a seminal paper by Price and collaborators (Price et al. 1980). Because tritrophic interactions are an essential component in many areas of research such as plant defense, plant domestication, behavioral ecology and biological control, they have been and are still very well studied (Kaplan et al. 2016).

One of the main underlying traits believed to be responsible for plant effects on parasitoids are the amount of plant defensive secondary metabolites (Turlings and Benrey 1998, Ode 2006, Gols and Harvey 2009, Harvey et al. 2011). These compounds are known to have negative effects on herbivores feeding on the plant and in turn, affect the third trophic level, such as predators (Kagata et al. 2005) or parasitoids (Turlings and Benrey 1998, Ode 2006). This is particularly the case for koinobiont endoparasitoids, that is, parasitoids that complete their larval development inside their host and that let the host feeding during their whole development, which are particularly exposed to chemical defensive compounds eaten by their host (Gols 2014).

Furthermore, plant secondary metabolites can reduce the growth of herbivores, which may compromise the growth of the parasitoid developing inside its host (Benrey and Denno 1997, Harvey 2005). Thaler (1999) demonstrated that an application of jasmonate on plants led to a decrease in parasitoid performance. In addition, a longer development time of the host may also increase the period of time during which herbivores are exposed to predators and parasitoids (Benrey and Denno 1997). Plant defense may also negatively impact the immune system of the host by affecting the ability of the host to encapsulate parasitoid eggs or larvae (Benrey and Denno 1997, Brodeur and Vet 1995, Bukovinszky et al. 2012).

As previously discussed, several studies have shown that plants can indirectly influence parasitoid through the negative impact of direct resistance on their herbivore host (Turlings and Benrey 1998, Ode 2006, Gols 2014). Another way in which plant can indirectly influence parasitoid through their host is by their other defensive strategy (i.e. tolerance). Very few studies have tested the indirect effect of plant tolerance response on herbivores natural enemies. Lucas-Barbosa et al. (2016) showed that plant tolerance response is not sufficient for the plant to compensate for damage when the herbivore pressure is too high (particularly when herbivores are well adapted against plant direct resistance) and that the presence of parasitoids and predators allow the plant to fully compensate for herbivory damage, in term of seed production. They conclude that indirect defense through natural enemies and plant tolerance response are complementary. Yet, it is still unknown whether parasitoids can be affected by plant tolerance response through the effect on their herbivore host (Kaplan et al. 2016). Contrary to plant direct resistance strategies that negatively affect parasitoids through negative effect on herbivore, we can expect plant tolerance strategy to positively impact parasitoids, as it provides more food for herbivorous insects without negatively affecting their performance (Kaplan et al. 2016).

Herbivore-mediated parasitoid top-down effects on plants

Top-down forces, whereby a trophic level influences a lower one, have been generally less studied in tritrophic interactions than bottom-up forces. Particularly, the impact of parasitoids on plants through qualitative modifications in herbivory via herbivore host physiology and salivary secretions (Korpita et al. 2014, Pennacchio et al. 2014), is still poorly understood (Kaplan et al. 2016).

One of the first studies that explored this phenomenon was the one by Fatouros et al. (2005). They showed that Brussels sprouts plants emit less volatiles when they are damaged by parasitized herbivores compared to unparasitized, and that parasitoids were able to discriminate between parasitized and unparasitized herbivore induced volatiles. Similarly, hyperparasitoids are also able to discriminate between parasitized and unparasitized herbivore induced volatiles, which help them to find their hosts (Poelman et al. 2012, Zhu et al. 2015). These studies showed that the volatile production of the plant can be modified by parasitoids, through modification in oral secretions of their herbivore hosts. Furthermore, Poelman et al. (2011a,b) showed that plant phenotypes, herbivore colonization, herbivore and parasitoid performance can be affected by other parasitoid species developing in herbivores feeding on the same plant. Kaplan et al. (2016) review studies that show how parasitoids indirectly affect plants through changes in the parasitized herbivore physiology. They argue that parasitoids have been considered as bodyguards and that studies have mainly focused on their effect on herbivory rates, ignoring the qualitative effect of parasitoids on the plant through modifications in their host physiology. They conclude that tri-trophic interactions may be far more complicated than previously thought. In one of the few available studies, Ode et al. (2016) demonstrate that *Copidosoma floridanum*, polyembryonic parasitoids, can induce a higher level of non-volatile defensive chemical compounds in the wild cabbage, *Brassica oleracea*, through increasing feeding by its herbivorous host, *Trichoplusia ni*. In a recent study, Cusumano et al. (2018) showed that symbiotic viruses injected into parasitoid hosts are mainly responsible of the changes induced in the host plant. The extent to which parasitoids can alter other plant's defensive strategy, such as tolerance, remains unknown (but see Lucas-Barbosa et al. 2016, Cuny et al., 2018). In addition, it is still not known whether the reduction in plant resistance induced by parasitized larvae has a positive effect on the parasitoid larvae developing inside its herbivore host (Cusumano et al. 2018, Tan et al. 2018).

Finally, parasitoids can also affect herbivores indirectly, by their sole presence (i.e. non-consumptive effects), through induction of host defensive behavior (Fill et al. 2012). Griffin and Thaler (2006) went a step further by demonstrating that predators could indirectly affect plant resistance solely by the effect of their presence on herbivores. However, until now, parasitoid non-consumptive effects on their host, and the indirect effect on plant resistance are not well studied.

Parasitoids top-down effects on plant fitness

As previously discussed, several studies succeeded in showing that predators and parasitoids are attracted by volatiles produced by plants under attack. However, only few studies, so far, have shown that this attractiveness of bodyguards may result in a fitness advantage for the plant, especially in natural conditions and in biological systems in which plants and insects have coevolved together (Gomez and Zamora 1994, Van der Meijden and Klinkhamer 2000, Van Loon et al. 2000, Hoballah and Turlings 2001). In one of the latest study available, Gols et al. (2015) showed in an outdoor garden experiment that the fitness of *Sinapsis arvensis* (Brassicaceae) was significantly increased when *Pieris brassicae* larvae were parasitized by a solitary parasitoid, *Cotesia glomerata*. Previously and the first study that demonstrated this, Gomez and Zamora (1994) experimentally excluded parasitoids by covering the seeds of plants attacked by seed beetle, showing that parasitoid benefit plant fitness.

The impact of a parasitoid on the consumption of its host plant can be highly dependent on the life history strategies of the parasitoid species. Parasitoids have been classified according two types of development strategies (reviewed in Harvey 2005): first, idiobionts develop either in a host stage that does not grow such as eggs or pupae, or they inject venom that paralyzes their host. In this case, the amount of resource available for the parasitoid larvae is fixed, because the host does not grow anymore, and host size when parasitized is usually correlated with parasitoid offspring size (Harvey 2005). Secondly, koinobiont parasitoids attack active hosts which continue feeding and growing during parasitoid development. In this case, the relationship between host quality and parasitoid fitness is more complicated than for idiobionts (Harvey 2005). Solitary and gregarious koinobiont parasitoids both regulate their host growth according to the nutritional requirements of their progeny (review in Harvey and Malcicka 2016).

For solitary koinobionts, if the host final size is close to the adult parasitoid size, then there is generally only a small effect on the host growth (Harvey et al. 1994). However, the host can potentially grow bigger than the adult parasitoid, which can be negative for the parasitoids progeny which may be forced to overeat to leave the host (Harvey 2005). Consequently, the host development will be stopped before it reaches its final size, generally resulting in parasitized larvae being smaller than unparasitized ones (Beckage and Riddiford 1982, Harvey et al. 1999).

Gregarious koinobiont parasitoids can also regulate their host growth, but they need a quantity of resource increasing with the number their progeny (Espírito-Santo et al. 2004). This leads to an increase of the parasitized larvae growth according to the parasitoid progeny number and can eventually result in parasitized larvae growing larger than non-parasitized (Harvey 2000, Harvey and Malcicka 2016). In this case, parasitized larvae can feed more (Coleman et al. 1999; Van der Meijden and Klinkhamer 2000) or for longer periods (Beckage and Riddiford 1982), than unparasitized ones, in which case plants will sustain greater damaged (Smallegange et al. 2008) and increase induced defensive responses (Ode et al. 2016). Smallegange et al. (2008) tested this hypothesis by showing that plants attacked by caterpillars parasitized by only one brood of the gregarious parasitoid *Cotesia glomerata* did not suffer a reduction in their seed production compared to control plants without herbivores, although there was a plant fitness reduction when it was attacked with a higher parasitoid brood size. Xi et al. (2015) went a step further proposing that we should consider the ratio between the size of the herbivore host, and the parasitoid load. In their experiment, the parasitoid fly and its host have similar sizes, and hence, only one single parasitoid larva represents a relatively heavy load, which would explain why they increase the feeding behavior of their host.

Study system

Lima bean, Phaseolus lunatus

Wild lima bean, *Phaseolus lunatus* is an annual plant in the Fabaceae family, widespread throughout the south and southeast of Mexico. Natural populations of *Phaseolus lunatus* can often be found near roads and can reach up to three meters in height or width (personal observation). Lima bean seeds germinate in July, plants flower in October and seeds are produced around December and dispersed until February (Freytag and Debouck 2002). Seed dormancy can be induced by environmental conditions such as humidity and temperature (Degreef et al. 2002).

Wild Lima bean has been used as a model system in chemical ecology (Heil 2004, Ballhorn et al. 2005, Shlichta et al. 2014 and others). Interestingly, *P. lunatus* is the only *Phaseolus* species that contains cyanogenic glycosides (CNG): these well studied chemical compounds are known to have a toxic effect on leaf herbivores (Ballhorn et al. 2005, Ballhorn et al. 2009). However, their role as defensive compounds in the seeds is less clear (Shlichta et al. 2014). This plant also shows tolerance response to artificial and herbivore damage (Blue et al. 2015, Moreira et al. 2015a, Hernández-Cumplido et al. 2016b). All these defense strategies make it an ideal model species for the study of plant direct resistance and tolerance against herbivorous insects. In addition, wild lima beans are known to produce volatiles that are involved in plant-plant communication (Moreira et al. 2016), repellency of herbivores (Heil 2004) and in the attraction of herbivore natural enemies (Dicke 1999, Kost and Heil 2008). *P. lunatus* also produces extrafloral nectar, which is used by pollinators but also by ants which act as plant bodyguards (Kost and Heil 2008, Hernández-Cumplido et al. 2010, 2016a). This plant is involved in complex multitrophic interactions including different guilds of insects feeding on the same plants but on different structures and at different times of the season (Hernández - Cumplido 2016b).

Wild lima bean plants are the ancestors of an economically important crop which has been artificially selected for the human consumption of its seeds. The domestication process has altered several bean traits such as the amount of defensive compounds, pod and seed size (Sotelo et al. 1995, Shlichta et al. 2018). Comparing domesticated versus wild lima bean plants is an ideal method of testing how alteration in plant traits can have bottom-up effects on higher trophic levels (Benrey et al. 1998, Campan and Benrey 2004, Chen et al. 2017). Domesticated plants have generally been found to be more suitable for insect pests, mainly because of a reduction in their defensive chemical compounds that was artificially selected for human consumption (Rosenthal and Dirzo 1997, Benrey et al. 1998, Rodriguez-Saona et al. 2011). However, this is not a consistent pattern, and more research is needed in order to fully understand the consequences of domestication on plant-insect interactions (Turcotte et al. 2014, Chen et al. 2015b, Gaillard et al. 2018). For instance, in a recent study, we found that domestication of wild lima bean reduced the amount of CNGs in the seeds, but not in the leaves, leading to asymmetric insect performance according to the plant structure they feed on (Shlichta et al. 2018). Additionally to defensive compounds, the increase in seed size could have influenced the insect that feed on it. This may be particularly the case for granivorous beetles such as *Z. subfasciatus*, which develop inside the seed until the adult stage, and is therefore highly dependent on the amount of resources present in the seed, and thus highly susceptible to intraspecific competition.

Studying domestication is all the more important knowing that cultivated lima bean is attacked by a wide array of herbivore pests, and that more knowledge can lead to biocontrol solutions (Aebi et al. 2008, Zaugg et al. 2013, Chen et al. 2015a,b).

Early season herbivores and their parasitoids associated with wild lima bean

In our field site near Puerto Escondido (Mexico), leaves of wild lima bean plants are attacked by larvae of *Spodoptera latifascia* (Lepidoptera: Noctuidae). These herbivores are in turn attacked by two generalist parasitoids, *Chelonus insularis* (Hymenoptera: Braconidae) and *Euplectrus platyhypenae* (Hymenoptera:Eulophidae). The first is an egg-larval parasitoid that can parasitize several hosts in a patch of eggs (Jourdie et al. 2010*)*. The second one, *E. platyhypenae*, is a gregarious ectoparasitoid that lays its eggs on the dorsum of third and fourth instars larvae (Fig. 2, Coudron et al. 1990). This parasitoid species has been mostly studied for the venom that the adult wasps injects to stop the host's molting process (Coudron et al. 1990, Nakamatsu and Tanaka 2003a,b) and allows parasitoid larvae to complete their development until pupation, just after they kill their host (Nakamatsu and Tanaka

2004). The relative abundance of these two parasitoid species varies between years (Cuny, personal observation) and the reasons for this temporal variation are not yet understood.

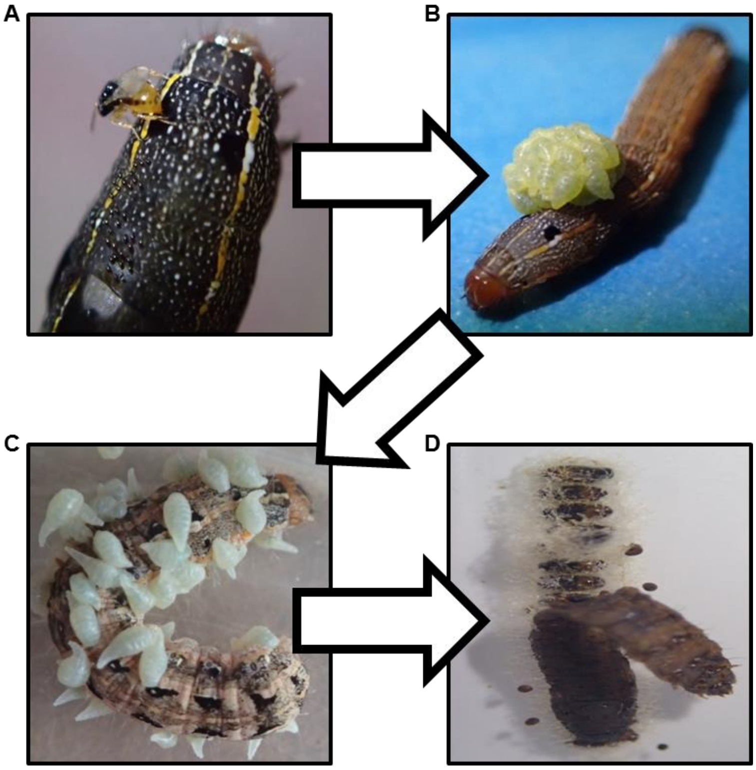

Figure 2. Life cycle of *Euplectrus platyhypenae* on its host, *Spodoptera latifascia*. A) Adult female *E. platyhypenae* laying eggs on a fourth instar larva of *Spodoptera latifascia*. B) Clutch of parasitoid larvae on dorsum of their host. C) After injecting saliva that immobilize and slowly kills their host, parasitoid larvae spread and group under the caterpillar body to pupate. D) Dried body of host caterpillar serves as protection for parasitoid pupae.

Late season herbivores and their parasitoids

Wild lima bean dry seeds are attacked by several species of bruchid beetles such as *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae) (Shlichta et al. 2014, Moreira et al. 2015b), one of the main pests of wild and domesticated bean seeds in Mexico. Females often lay more than one egg per seed leading to scramble competition among the developing beetle larvae (Cuny, personal observation, Teixeira and Zucoloto 2012). During their larval development, inside the seeds, bruchid beetles can be attacked by ectoparasitoids (e.g. *Stenocorse bruchivora,* Hymenoptera: Braconidae) that lay their eggs on the larval host, halting its development (Fig. 3, Campan and Benrey 2004, Aebi et al. 2008, Bustos-Segura, in prep.). Upon completion of their development, adult parasitoids exit the seed by their own hole, cut with their mandibles. Because of the limited amount of resources available and the incapacity of beetle larvae to leave the seed, a strong negative effect of beetle competition is expected. This negative effect of competition could also impact parasitoids. In the literature, apart from studying parasitoid functional responses (Price, 1988), very few studies have looked at the impact of herbivore competition on the performance of parasitoids (Kraaijeveld and Godfray 1997). If the reserves of the seed have not been entirely depleted after beetle and parasitoid development, damage seeds may still be able to germinate (Cuny, personal observation). Overall, virtually no studies have looked at the impact of parasitoids that attack seed eaters whose larvae feed internally on the seed germination, performance of the seedling and subsequent herbivores (but see Nakai et al. 2011).

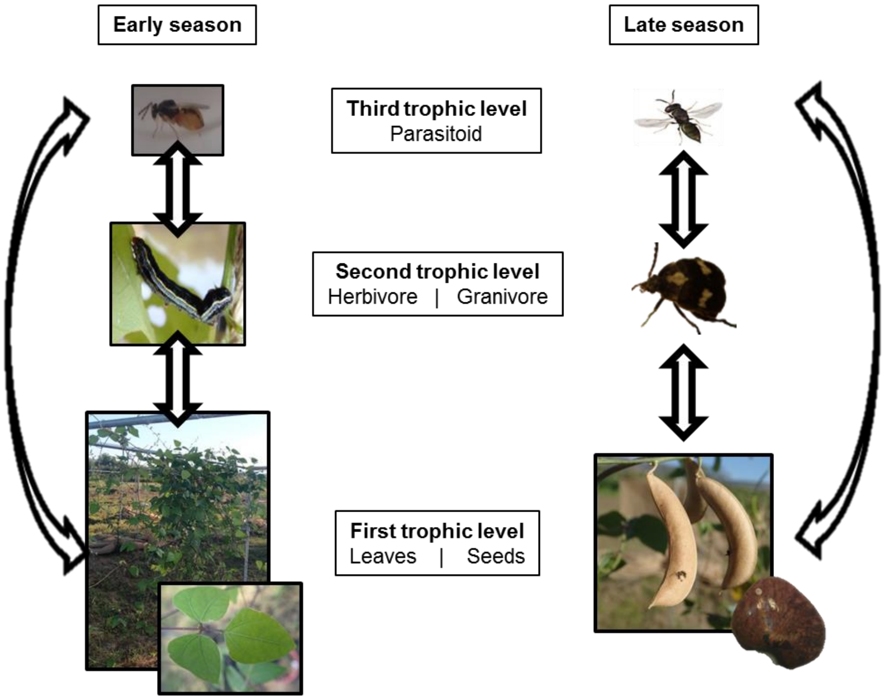

Figure 3. Interactions among three trophic levels during the early and the late season in Mexico. From the bottom to the top for the early season tritrophic system: wild *Phaseolus lunatus*, *Spodoptera latifascia* and *Euplectrus platyhypenae*. From the bottom to the top for the late season tritrophic system: pods and seeds of wild *P. lunatus*, *Zabrotes subfasciatus* and *Stenocorse bruchivora*. Arrows represent positive or negative interactions among the three trophic levels.

Indirect interactions among different trophic levels mediated by wild lima bean plants

Different herbivores feeding on the same lima bean plant at different times in the season can indirectly influence each other through inducible chemical changes in the plant triggered by their feeding (Agrawal and Heil 2012, Poelman et al. 2010). Furthermore, a parasitoid developing inside its host can influence these interactions (Poelman et al. 2011a,b). In the Brassica system, these changes have been found to influence subsequent herbivores feeding or laying eggs on the same plant (Poelman et al. 2008, Poelman et al. 2011a) as well as their parasitoids (Poelman et al. 2011b). Furthermore, recent studies found that this is the virus injected by parasitoids in their host that may be responsible for most of their host changes and not the parasitoid larva itself (Cusumano et al. 2018, Tan et al. 2018). A study from our group (Hernández -Cumplido et al. 2016b) showed that herbivores feeding on wild lima bean leaves in the early season can impact parasitoids of bruchid beetles feeding on the same plant's seeds. So far, very few studies have highlighted this type of interaction which may be vastly underappreciated (but see MacArt et al. 2013).

Conclusion

In conclusion, the study of plant-insect interactions and the factors that affect them are a vast topic of research that include several bottom-up and top-down forces, and despite a growing interest and significant contributions in the past four decades, some questions remain unanswered. For instance, plant variation, as a bottom-up force affecting higher trophic levels, has been well documented but some topics require more empirical studies, such as plant domestication or plant tolerance effect on insects. Top-down forces on tritrophic interaction have been generally less studied, and new types of interactions have been recently unraveled. The effects of parasitoids on plant defense (particularly on plant tolerance) as well as non-consumptive effects on their host have received very little attention. Finally, some recent studies show that top-down and bottom-up forces could affect each other on the same plant, at different time of the season, but this topic remains poorly understood. By addressing all the above mentioned research areas, my thesis built towards a better understanding of top-down and bottom-up forces in plant-insect interactions.

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Chapter 1

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ORIGINAL RESEARCH
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The Large Seed Size of Domesticated Lima Beans Mitigates Intraspecific Competition among Seed Beetle Larvae

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The domestication of beans has selected for larger seeds in cultivated plants compared to their wild relatives. This has not only resulted in an enhanced resource for humans, but also for the insects that feed on these seeds. Seed beetles that attack wild and cultivated seeds often lay several eggs on a single seed. We hypothesized that the larger seed size of domesticated beans will mitigate the competition among the larvae that hatch from these eggs, with important implications for their growth and survival. To test this we examined how seed size of wild and cultivated *Phaseolus lunatus* (lima bean) affect the performance of the Mexican bean weevil *Zabrotes subfasciatus*, an important pest of beans in Mexico. A negative correlation was found between the initial number of eggs on a seed and the weight of female beetles that emerged, but only for the much smaller wild seeds. Similarly, beetle survival was found to be negatively correlated with competition intensity only on wild seeds. Our results imply that by selecting for larger seeds, domestication of *P. lunatus* has reduced the intensity of intraspecific larval competition of *Z. subfasciatus*.

Keywords: plant-insect interactions, bean weevil, seed pest, intraspecific competition, *Phaseolus lunatus*, seed size, domestication syndrome

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INTRODUCTION

Increasing evidence shows that plant domestication has altered the strength and nature of their interactions with other organisms (Chen et al., 2015a; Rowen and Kaplan, 2016; Whitehead et al., 2017). Cultivated plants differ from their wild ancestors in a suite of phenotypic traits, collectively known as the domestication syndrome. These include traits related to the ease of cultivation and harvest, as well as morphological and chemical traits that ensure higher yields and enhanced nutritional value. Selection for these traits has commonly resulted in larger tissue mass or organ size, higher nutrient content and decreases in physical defenses and toxic chemical compounds (Meyer et al., 2012). These changes in cultivated plants have been shown to affect the food choices and performance of insects that attack them (Chen et al., 2015a,b, and references therein). This is particularly evident when crops occur in the native range of their wild relatives (Chen et al., this issue), as insects adapted to wild plants are suddenly faced with a more abundant and often more nutritious and less toxic resource.

Phaseolus lunatus (Lima bean), one of the five domesticated species of the genus *Phaseolus* is of Andean and Mesoamerican origin. Lima beans were domesticated at least twice, one domestication event occurred in the Andean mountains of Ecuador and Northern Peru and a second event in central-western Mexico (Motta-Aldana et al., 2010). Beans went through further domestication events and adapted to a wide variety of climatic regimes and ecological conditions (Martínez-Castillo et al., 2008; Motta-Aldana et al., 2010; Serrano-Serrano et al., 2012; Chacón-Sánchez and Martínez-Castillo, 2017).

Changes resulting from domestication of the genus *Phaseolus* mainly involve an increase in pod and seed size, decreased shattering, reduction in levels of toxins, such as lectins, lectin-like proteins, and cyanogenic compounds (only in *P. lunatus*), and an overall increase in proteins and minerals (Delgado-Salinas, 1988; Smartt, 1988; Sotelo et al., 1995). Throughout their distribution range in Mesoamerica, cultivated and wild bean plants coexist in sympatry (Gepts, 1988; Piñero and Eguiarte, 1988; Martínez-Castillo et al., 2014; Silva et al., 2017), allowing for a frequent exchange of insects and pathogens between wild and cultivated forms (Leroi et al., 1990; Lindig-Cisneros et al., 1997; Alvarez et al., 2007; Zaugg et al., 2013). It is well documented that herbivorous insects that achieve pest status usually continue to exist in natural habitats alongside managed ones (Mitchell et al., 2016). Once cultivated beans are harvested and seeds are transported to storage places, they continue to be in close proximity to wild plants and are exposed to the insects that attack them (Alvarez et al., 2005, 2007). Furthermore, human-mediated migration as a result of farmers exchanging or selling seeds in local or regional markets may increase the spread of insects that originate from wild populations (Alvarez et al., 2007). This constant exchange of insects between wild and cultivated populations has important implications for pest pressures in agriculture. This is particularly true for bruchinae beetles that infest cultivated fields in Mexico, for which it has been shown that geographic distance between cultivated and wild populations greatly explains the patterns of infestation rates (Alvarez et al., 2005, 2007). Moreover, if cultivated plants offer a more reliable and nutritious resource than their wild counterparts this can explain why seed beetles thrive in cultivated seeds.

Numerous studies have shown that seed size greatly influences the oviposition decisions of adult seed beetles, and that size can often be used as a good indicator of seed quality for the developing larvae (Janzen, 1977; Fox and Czesak, 2000; Guedes et al., 2010; Chen et al., 2015b; Oliveira et al., 2015). Indeed, for seeds in the genus *Phaseolus*, seed size has been found to be the best predictor of oviposition choices (Moreira et al., 2015; Hernandez-Cumplido et al., 2016). Thus, we would predict that, faced with a choice, adult females would preferentially oviposit on cultivated seeds rather than on much smaller wild seeds. We further predict that inside the cultivated seeds the larvae will be exposed to lower levels of conspecific competition, which may be an important reason for the oviposition preference.

We tested this hypothesis with the Mexican bean weevil *Zabrotes subfasciatus*, and wild and cultivated seeds of Lima bean, *P. lunatus*. Our specific goal was to test the effects of increased seed size in cultivated varieties on the interaction with the seed beetle. In controlled laboratory experiments using seeds from three cultivated varieties and three wild populations of Lima bean, we investigated the oviposition patterns of adult females and the subsequent performance of their progeny resulting from of seed-size mediated competition among beetle larvae.

MATERIALS AND METHODS

Seeds

For the experiments we used seeds from three cultivated varieties and three wild populations of *P. lunatus* (**Figure 1**). Wild seeds were collected in locations along the Pacific coast of Mexico were Lima bean grows naturally. They are located at: Hidalgo near San Jose Manialtepec ("HGO"; 15.575564, −97.151350), Experimental Campus of the Universidad del Mar ("UMAR"; 15.923366, −97.151892), and near Largartero ("INK"; 15.725127, −96.656343) (as described in Shlichta et al., 2014). We collected seeds from 10 plants per site (only six for HGO).

The following domesticated seed varieties were obtained from W. Atlee Burpee & Co (Warminster, PA, USA): Jackson Wonder, Fordhook 242 Bush Bean and Burpee's Best Pole Bean (we named them "JACK", "FORD," and "BURP," respectively). The choice of these varieties was made based on previous studies with several commercially available cultivated varieties, in which we found that beetles develop well and do not appear to discriminate with respect to their different genetic pool (Shlichta et al. unpublished data). Thus, because we wanted to have extreme variation in seed size in order to test our hypothesis, the choice was made based on this variation and not on their domestication history. These seeds represent a mixture of two and perhaps three genetic pools; "JACK" is of Mesoamerican origin and "FORD" of Andean origin (Nienhuis et al., 1995; Ernest and Kee, 2008), we do not have information regarding the genetic pool "BURP." Although there is variation in seed size and color among these three cultivated varieties, variation in size is greater between wild and cultivated seeds (Supplementary Figure 1).

Insects

The Mexican bean weevil *Z. subfasciatus*, native to Mesoamerica, attacks seeds of several wild and cultivated species in the genus *Phaseolus* throughout Mexico, Central and South America (Credland and Dendy, 1992; Benrey et al., 1998; Romero and Johnson, 2000), It is considered one of the most important pests in bean cultivation and storage (Birch et al., 1985; Leroi et al., 1990), not only in the Americas but also in tropical regions of Asia and Africa (Davies, 1972). Females glue their eggs on the seed coat and upon emergence, first instar larvae bore into the seed, where they feed, develop, pupate and then emerge as adults (Benrey et al., 1998).

This beetle is particularly suited to test our hypothesis because females do not avoid seeds with previously laid eggs and may lay many eggs on a single seed, even when seed availability is not limited. Indeed, a single seed has been observed to present up to 63 eggs lay by multiple females (Teixeira and Zucoloto, 2012), even though larval survival under these conditions is highly unlikely (Cuny, personal observation). Once larvae enter the seed, they are confined to it for their entire development until adulthood. If several larvae are inside the seed, they can experience high levels of competition for both space and food resource.

FIGURE 1 | Seeds of *Phaseolus lunatus* from domesticated varieties: on the top from left to right with the corresponding mean sizes in mm (± SE); Fordhook 242 (10.87 ± 0.47), Burpee's Best (11.18 ± 0.92) and Jackson Wonder (9.18 ± 0.46). On the bottom line seeds from wild populations, from left to right; HGO (4.59 ± 0.2), Umar (4.43 ± 0.1), and Ink (4.32 ± 0.14).

Zabrotes subfasciatus has been reared in our lab for several years on cultivated seeds of *Phaseolus vulgaris* (Vivien Paille red Kidney, obtained from MultiFood, 3238 Gals, Switzerland; see Campan and Benrey, 2006 for details on the rearing). To control for inbreeding effects, every year new field-collected individuals from Mexico are added to the colony and allow to mix for several generations before being used in experiments. All the insects described in this experiment were <4 days old.

Experimental Protocol

Five seeds of one of the varieties or populations were placed in a plastic Petri dish (28 × 23 × 5 mm, Semadeni AG, A4686). Ten Petri dishes were set up for each variety or population (60 in total). One male and one female beetle were introduced into each dish for 5 days, after which the number of eggs laid on each seed was counted and the seeds were individually stored in falcon tubes at 28°C. Beetles complete their development on average in 25 days. Dishes were checked daily and we recorded: larval survival (number of adults that emerged divided by the initial number of eggs laid on the seed), adult sex (determined from elytra patterns and size; Oliveira et al., 2015) and weight (to the nearest 0.01 mg with an analytical balance Mettler AE163, Switzerland). In parallel, in order to confirm the size difference between wild and cultivated lima bean seeds, 20 uninfested seeds (20 seeds per cultivated variety and per wild population) were weighed and measured using a binocular magnifier with an ocular scale.

Finally, we conducted an experiment to evaluate the effect of seed size on female oviposition independent of other factors linked to bean domestication. Seeds from each cultivated variety and wild population were selected and divided in two groups; small and large (chosen from the available natural variation within each seed type). Two seeds of different size from the same variety or population were placed in a Petri dish (as described in the previous experiment), and one male and one female beetle were introduced. Three days later, we counted the number of eggs laid on each seed. Based on previous studies, we know that a 3-day period is sufficient for beetles to make an oviposition choice and at the same time assures that not to many eggs are laid on a single seed (Campan and Benrey, 2006).

Statistical Analysis

Data were analyzed using SAS (SAS Institute, 2002)[1]. SAS Institute Inc., statistical package. Assumptions of normality and homoscedasticity were tested before each test. Linear mixed models (PROC MIXED) or generalized linear mixed models (PROC GLIMMIX), followed by a *post-hoc* analysis (Tukey) were used to compare data on seed size, weight, the number of eggs laid on the seeds, adult sex ratio and survival. Correlations have been tested using Pearson or Spearman correlations tests (PROC CORR). Seeds and Petri dishes were included as random factors in the models and seed domestication status, as well as seed varieties and population nested in domestication status were included as fixed factors (to account for natural variation among the three cultivated varieties and the three wild populations). Seeds with only one egg were not included in the analysis of beetle survival. Females being generally heavier than males, their weight was analyzed separately. For the experiment performed to test the relationship between seed size and number of eggs within each cultivated variety or wild population, seeds with no eggs were excluded from the analysis.

RESULTS

Measurements of seed size and weight confirmed that cultivated seeds are significantly (∼60%) larger and heavier than wild seeds [**Figure 2**, $N = 40$, $F_{(1.74)} = 172.8$, $p < 0.001$, and $N = 40$, $F_{(1.74)} = 281$, $p < 0.001$ for size and weight, respectively]. The fixed factor of population and variety nested within seed domestication status was significant for seed size and weight [$F_{(1.74)} = 6.63$, $p = 0.002$, and $F_{(1.74)} = 59.9$, $p < 0.001$, respectively].

Female beetles laid significantly more eggs (2-fold) on seeds from cultivated varieties than on wild seeds [**Figure 3**, $\mathrm{N}_{\mathrm{wild}} = 41$, $\mathrm{N}_{\mathrm{cultivated}} = 51$, $F_{(1.233)} = 13.32$; $p < 0.001$]. We also found a significant effect of population and variety nested within seed domestication status on the number of eggs laid per seed [$F_{(4.236)} = 28.86$, $p < 0.001$]. Finally, within each variety and population, the relationship between seed size and number of eggs laid was not significant (Supplementary Figure 2). This suggests that the variation in seed size within wild or cultivated seeds is not large enough to influence ovipositing females.

Larval survival (expressed as the percentage of adults that emerged per seed) was negatively correlated with the number of eggs laid on wild seeds (**Figure 4**, $N = 51$, $r = -0.32$, $p = 0.023$), but no significant correlation was found for survival on cultivated seeds ($N = 43$, $r = -0.21$, $p = 0.17$). Similarly, female weight was negatively correlated with the competition intensity (expressed as the number of eggs per the seed) when they developed in wild seeds (**Figure 5B**, $N = 39$, $r = -0.48$, $p = 0.002$), but not in cultivated seeds (**Figure 5A**, $N = 27$, $r = -0.19$, $p = 0.34$). However, male weight was only marginally significant correlated

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[1] Statistical Analysis System (2002).

with number of eggs on in wild seeds (**Figure 5B**, $N = 37$, $r = -0.32$, $p = 0.056$) and this correlation was also not significant in cultivated seeds (**Figure 5A**, $N = 34$, $r = -0.018$, $p = 0.9$). Finally, we did not find a difference in the sex ratio of beetles that emerged from domesticated or wild seeds [$F_{(1.79)} = 0.03$, $p = 0.868$; Supplementary Figure 3], nor a significant effect among cultivated varieties or wild populations [$F_{(4.79)} = 0.74$, $p = 0.57$].

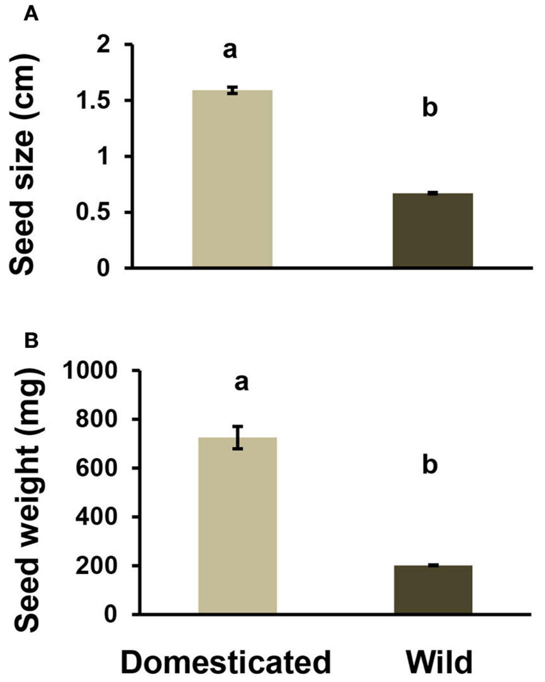

FIGURE 2 | (A) Mean size of domesticated and wild bean seeds. [$N = 40$, $F_{(1.74)} = 172.8$, $p < 0.001$]. **(B)** Mean weight of domesticated and wild bean seeds. [$N = 40$, $F_{(1.74)} = 281$, $p < 0.001$]. Different letters indicate a significant difference. Bars are means ± SE.

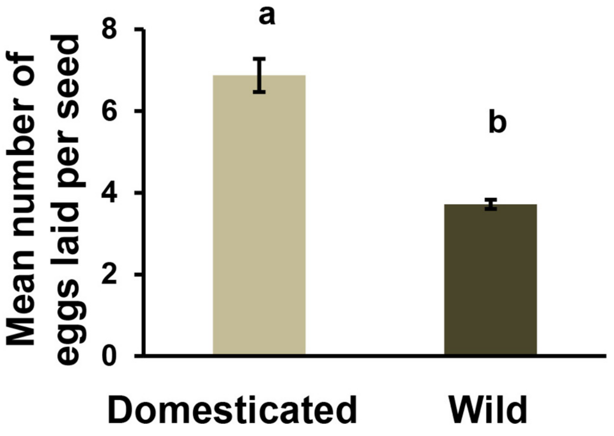

FIGURE 3 | Mean number of eggs laid per domesticated and wild seed in a no-choice experiment. [$N = 121$, $F_{(1.233)} = 13.32$; $p < 0.001$]. Different letters indicate a significant difference. Bars are means ± SE.

DISCUSSION

For pulse crops, larger seed size is one of the major agronomic traits that were selected for during domestication (Evans, 1993; Fuller, 2007). Larger seeds not only result in larger yields (Kluyver et al., 2017), but have also been associated with increases in germination success and seedling competitive ability and survival (Westoby et al., 2002). However, increases in seed size also have been repeatedly shown to be correlated with an increase in the likelihood of herbivore attack (reviewed in Chen et al., 2015b). Here, we found again support for this hypothesis; female beetles laid more eggs on the larger cultivated seeds of Lima bean than on the smaller wild seeds. Further, our results support the hypothesis that larger seeds offer a better resource for the Mexican bean weevil and as a consequence mitigate the intensity and negative effects of larval competition. In addition to and despite the higher number of eggs laid on cultivated seeds, more and larger adults emerged from these seeds.

Earlier studies with *Phaseolus* beans and various species of Bruchinae beetles, support our findings that seed size largely explains the observed patterns of oviposition and larval performance (Paukku and Kotiaho, 2008; Moreira et al., 2015; Oliveira et al., 2015; Hernandez-Cumplido et al., 2016). In a study aimed at examining the role of cyanogenic glycosides of Lima bean seeds on beetle performance, Shlichta et al. (unpublished data) conducted an experiment similar to the one described here but allowing only one larva of *Z. subfasciatus* to develop in each seed. They found that in the absence of larval competition within the seed, whether seeds were wild or cultivated did not affect the survival and average weight of the emerging adults. In another study with wild Lima bean seeds, Hernandez-Cumplido et al. (2016) found that under field and laboratory conditions, beetles laid more eggs on larger seeds. Also, using seeds from different wild bean populations, Moreira et al. (2015) found that two Bruchinae species, *Acanthoscelides obtectus* and *Z. subfasciatus*, laid more eggs and had higher survival on the larger seeds of *P. coccineus* than on the smaller seeds of *P. vulgaris*.

For seed beetles, seed size can be a reliable indicator of seed quality (Fox and Czesak, 2000; Cope and Fox, 2003). For example, Cope and Fox (2003) found that when females of the seed beetle, *Callosobruchus maculatus* were presented with seeds of varying sizes, they distributed their eggs in a manner that maximized resource availability for all offspring. *C. maculatus* rejects seeds that already carry eggs (Messina and Renwick, 1985). For these insects, the presence of previously laid eggs can therefore also serve as a good indicator of the quality of the seed, as it reflects the level of competition that their offspring will face inside the seed. For *Z. subfasciatus* this appears not to be always the case (Campan and Benrey, 2006). Although females prefer to oviposit on uninfested seeds, if they do not have a choice, they will oviposit on seeds that already have eggs (Teixeira and Zucoloto, 2012, M. Cuny, personal observation). Even if the probability of larvae surviving under high egg densities is very low. For females of this species, it seems advantageous to rely on cues such as seed size that will help minimize larval competition and maximize lifetime fitness. Limited amounts of resource inside the seed for the developing larvae will not only affect the intensity of

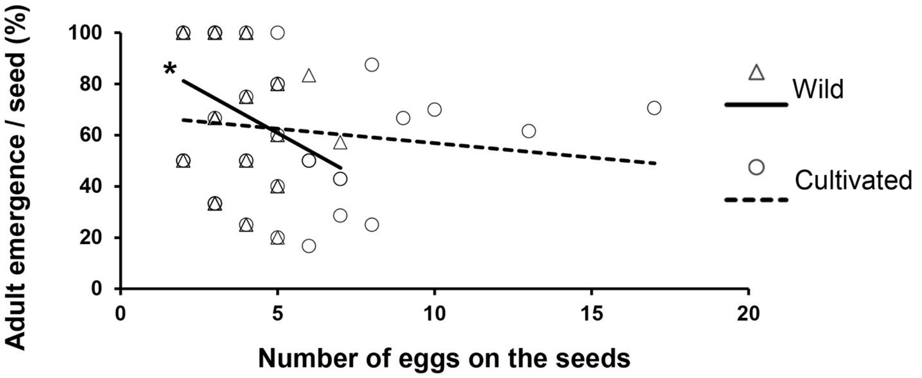

FIGURE 4 | Percentage of adults that emerged from each seed calculated as, number of adults that emerged from the seed/number of eggs laid on the seed * 100. Dashed line indicates linear regression for beetles that developed in cultivated seeds ($N = 43$, Spearman $r = -0.21$, $P = 0.17$) and solid line shows linear regression for beetles that developed in wild seeds ($N = 51$, Spearman $r = -0.32$, $P = 0.023$).

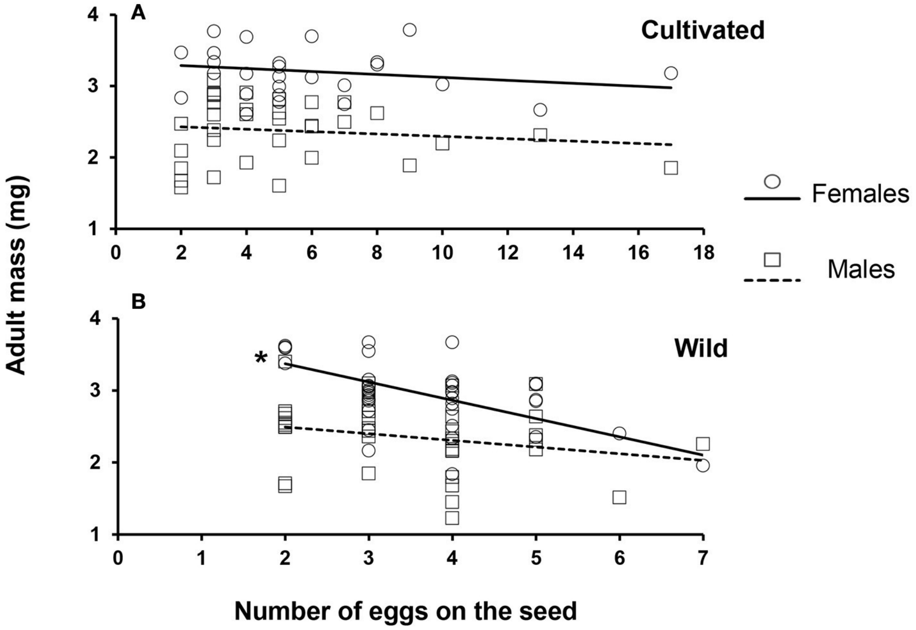

FIGURE 5 | Correlation between the weight of adult females (circles) and males (squares) that emerged from **(A)** domesticated and **(B)** wild seeds carrying different egg densities (as a proxy of the intensity of larval competition inside the seed). Solid lines indicate linear regression for female beetles and dashed lines show linear regression for males. A significant correlation was found for females emerging from wild seeds ($N = 39$, Spearman $r = -0.48$, $P = 0.002$; non on cultivated seeds $P = 0.34$) whereas, for the males no significant correlation was found ($P = 0.056$ wild seeds; $P = 0.9$ cultivated seeds). *Indicates significant difference $p < 0.05$.

competition and subsequent survival, but also the size of the emerging adults, with important consequences for their fitness. Female fitness is dependent on their fecundity, which is directly dependent on body size (Dendy and Credland, 1991; Colegrave, 1993; Callejas, 1996), while male size although not so directly linked to reproductive success, can affect mating success (Savalli and Fox, 1998). Earlier studies with *C. maculatus* found that seed size and the initial number of eggs on the seed influenced the weight of emerging adults (Credland et al., 1986; Giga and Smith, 1991; Colegrave, 1995). For *Z. subfasciatus*, we found that seed size mostly affects female but not male size and only on the smaller wild seeds. This result can be explained by the overall smaller size of males (on average 30% smaller and lighter than females), implying that they may be less limited by the availability of resources for development and thus not as affected by larval competition inside the seeds.

It is important to note that the cultivated and wild seeds used in this study do not only differ in their size, but also in other traits that are part of the domestication syndrome of *Phaseolus* beans resulting from adaptations to cultivation, harvesting practices and human preferences. These other changes in bean traits can all have an influence on beetle oviposition decisions and larval performance. Wild seeds are harder, have a thicker testa and an inconspicuous dark brown color, whereas cultivated seeds have been selected for faster germination, hence are softer and have a thinner seed coat permeable to water and there is a vast color variation among varieties. Physical features of the seeds are known to affect beetle oviposition behavior and the ability of larvae to burrow into the seed (Chavan et al., 1997; Plaza, 2001; Boeke et al., 2004). Similarly, nutritional and defense chemical compounds present in the testa and inside the seed are known to interfere with the development and affect the survival of seed beetles (Goossens et al., 2000; Moraes et al., 2000; Silva et al., 2004), and their concentrations can differ between wild and cultivated accessions (Sotelo et al., 1995; Zaugg et al., 2013). Particularly, for *Z. subfasciatus*, earlier studies have documented differences in its performance when reared on cultivated or wild beans (Schoonhoven et al., 1983; Benrey et al., 1998; Campan and Benrey, 2006), as well as differential performance of beetles on wild seed populations that vary in their protein or phenolic content (Moreira et al., 2015; Hernandez-Cumplido et al., 2016). These differences in physical and chemical traits between wild and cultivated seeds will undoubtedly influence the oviposition decisions and performance of the Mexican bean weevil. Yet, our results unequivocally demonstrate that the difference in seed size between cultivated and wild seeds plays a major role in the oviposition and performance differences. Although we cannot completely disentangle seed size from other factors associated with the domestication status of the seeds, one key finding of this study is that the larger seed size of cultivated beans, independent of their genetic pool of origin, mitigates the potential negative effects of larval intraspecific competition, a process that in nature controls the size of populations (Begon et al., 2009). This additional consequence of bean domestication implies that the presence of bean fields in areas where wild beans occur naturally provides new ecological opportunities for associated insects. The expansion to a new and more profitable resource favors individuals that exploit these novel resources that provide conditions of relaxed competition (Van Valen, 1965). Yet caution should be taken to extrapolate our results to natural situations. The transferability of these results to the field would require additional measurements on variation in seed size and insect oviposition in natural conditions.

Nonetheless, these findings have important evolutionary and applied implications. Divergent selective factors that act on the plants and insects associated with wild and cultivated bean populations can lead to specialization and in extreme cases genetic differentiation and host race formation (Alvarez et al., 2007; Laurin-Lemay et al., 2013; Kenyon et al., 2015). There is further evidence for our bruchid-bean system that shows that bean domestication has selected for different behaviors in host use, not only in seed beetles, but also in the natural enemies of these beetles (Benrey et al., 1998; Campan and Benrey, 2004; Aebi et al., 2008). Yet, strong human-mediated dispersion of cultivated beans and these associated organisms will most likely result in continuous genetic mixing and will prevent selection for divergent behaviors that could lead to genetic differentiation of insects specializing on wild or cultivated seeds (Alvarez et al., 2007; Laurin-Lemay et al., 2013).

Finally, it is important to emphasize that studies in regions where cultivated plants coexist with their wild relatives allow us to understand the interplay between natural and human-mediated selection and how they interact to shape the present-day associations between plants and insects in agricultural and natural systems (Chen et al. this issue). For our study system this is also important from an applied perspective, as beans are a major staple food in many countries of Mesoamerica as well as in other regions of the world (FAO, 2013). The development of strategies that will allow us control pests in this important crop might be facilitated by unraveling the changes in interactions among insects and plants that resulted from plant domestication.

AUTHOR CONTRIBUTIONS

The three authors conceived and designed the experiment and participated to the writing of the paper; MC performed the experiments and analyzed the data.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2017.00145/full#supplementary-material

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

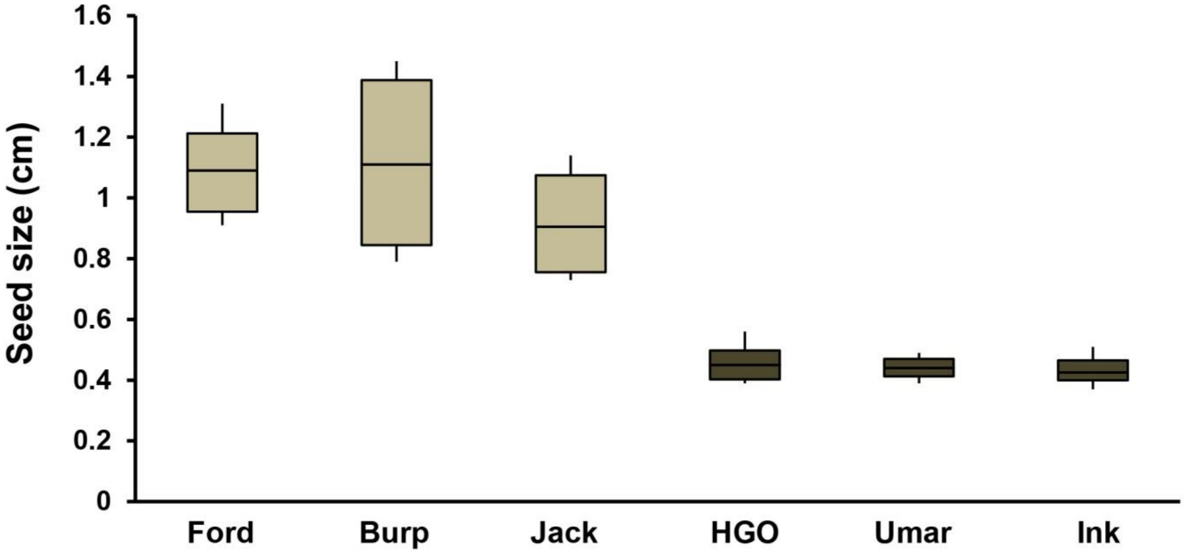

Figure 1 Mean seed size of domesticated and wild beans

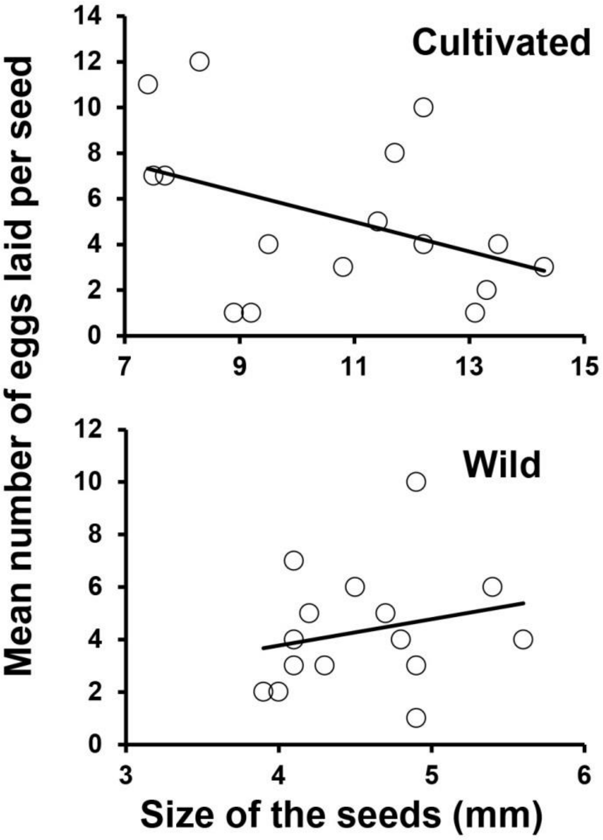

Figure 2 Correlations between the mean number of eggs laid per seed and the size of the seeds for cultivated (upper graph) and wild (lower graph) seeds. No significant correlation were found.

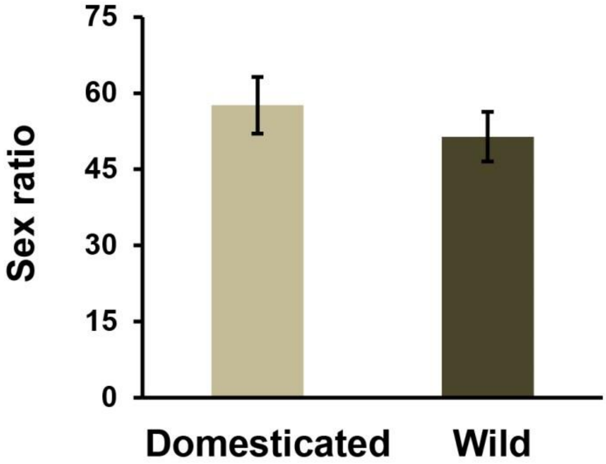

Figure 3 Mean sex ratio of emerged beetles from domesticated and wild seeds. No significant difference were found.

Chapter 2

Role of cyanogenic glycosides in the seeds of wild Lima bean, *Phaseolus lunatus*: Defense, nutrition or both?

Maximilien A.C. Cuny, Diana La Forgia, Gaylord A. Desurmont, Gaetan Glauser and Betty Benrey

Abstract

Wild lima bean plants contain cyanogenic glycosides (CNGs) that are known to defend the plant against leaf herbivores. However, seed feeders appear to be unaffected despite the high levels of CNGs in the seeds. We investigated a possible role of CNGs in seeds as nitrogen storage compounds that influence plant growth, as well as seedling resistance to herbivores. Using seeds from four different wild lima bean natural populations that are known to vary in CNG content we tested two non-mutually exclusive hypotheses: 1) seeds with higher CNG levels germinate better and produce plants that exhibit better growth, and 2) seeds with higher CNG levels produce seedlings that are more resistant against generalist herbivores. Seed CNG content did not correlate with germination rates, nor with seedling growth. However, CNG concentrations increased significantly soon after germination and seeds with the highest CNG concentrations produced seedlings with a higher CNG content in cotyledons and secondary leaves. Moreover, the growth rate of the generalist herbivore *Spodoptera littoralis* was lower on cotyledons with high CNG content. We conclude that CNGs in Lima bean seeds do not play a role in plant growth, but they may contribute seedling defense.

To our best knowledge, this is the first study that examines the potential dual role of cyanogenic glycosides in defense and early plant development in wild lima bean.

Keywords: Secondary metabolites. Nitrogen storage. Herbivory. Cyanogenesis. Seed germination.

Introduction

Throughout their long co-evolutionary history with herbivores, plants have evolved direct chemical defenses in the form of secondary metabolites that can have repellent, anti-nutritional or toxic effects on their attackers. However, the limit between primary and secondary metabolism is not always clear-cut, and increasing evidence suggests that secondary metabolites can, in some cases, be involved in signaling and regulation of primary metabolic pathways (Maag et al. 2015), can work as pigments and thereby influence plant attraction to pollinators or predators (i.e. carotenoids and flavonoids) (Harborne and Williams 2000; Tanaka et al. 2008), or as is the case for phenolic compounds, they can mitigate damage by UV radiation (Dai and Mumper 2010). Cyanogenic glycosides (CNGs) are a group of secondary metabolites for which other functions have been proposed (Forslund and Jonsson 1997; Møller et al. 2010).

CNGs are natural compounds widely distributed in the plant kingdom (Vetter 2000). They are stored in vacuoles, and upon damage of plant tissue they come in contact with ß-glucosidases that hydrolyze them, causing the release of toxic hydrogen cyanide (HCN) (Zagrobelny et al. 2004). This process is referred to as cyanogenesis (Vetter 2000; Gleadow and Woodrow 2002) and is known to be toxic for most living organisms (Cork 1996; Zagrobelny et al. 2004; Jørgensen et al. 2005). Yet, the presence of CNGs is not always harmful for insect herbivores. Contrary to mammals and chewing insects, which cause tissue disruption and trigger cyanogenesis (Frehner and Conn 1987; Poulton 1988, Ballhorn et al. 2009), insects with sucking mouthparts cause minimal tissue disruption and may avoid the defensive reaction (Pentzold et al. 2015), and some specialist chewing insects can cope with CNG toxicity by detoxifying, tolerating, or sequestering CNGs for their own defense (Scriber 1978; Brattsten et al. 1983; Engler-Chaouat and Gilbert 2007; Zagrobelny et al. 2007).

The impact of CNGs also depends on their levels within plant tissues, which can be highly variable, not only among plant populations or cultivars (Ballhorn et al. 2008b; Shlichta et al. 2014, 2018), but also within populations and among different plant structures (Godschalx et al. 2016; Shlichta et al. 2018). Finally, the effectiveness of CNGs as a defense may be dependent on the cyanogenic capacity, which refers to the amount of hydrogen cyanide released resulting from the activation of β-glucosidases (Ballhorn et al. 2005).

Lima bean (*Phaseolus lunatus* L., Fabaceae) is the only species within the entire *Phaseolus* genus that contains CNGs (Vetter 2000). This species has been used as a model in studies on direct and indirect defense (Heil 2004; Ballhorn et al. 2008a, 2009; Yi et al. 2009; Shlichta et al. 2014, 2018; Hernandez-Cumplido et al. 2016). High concentrations of CNGs in the leaves and other vegetative tissues have been shown to negatively affect several generalist leaf herbivores (Ballhorn et al. 2005; Ballhorn and Lieberei 2006; Godschalx et al. 2016; Shlichta et al. 2018). However, seed feeders appear to be unaffected, despite the considerable higher concentrations of these compounds in seeds as compared to leaves (Shlichta et al. 2014, 2018). A possible reason for this is the lack of moisture in the seeds, which might be needed for the degradation of CNGs by the β-glucosidases, thus preventing the release of toxic HCN (Shlichta et al. 2014).

Therefore, it remains unclear to what extent *P. lunatus* benefits from having high levels of CNGs in its seeds. Especially, because generalist seed beetles account for approximately 95% of pre-dispersal seed mortality (Bustos et al. unpublished data). One hypothesis is that CNGs get translocated from the seeds to seedlings (Clegg 1979), providing the latter with protection against leaf-feeding herbivores. Alternatively, CNGs in the seeds could serve as nitrogen storage for plant growth. However, support for this hypothesis is still scarce (Schilcher and Wilkens-Sauter 1986; Selmar et al. 1990; Forslund and

Jonsson 1997; Stensjo and Jonsson 1997), and for Lima bean this putative role of CNGs in plant growth and development has not yet been investigated.

In the current study we investigated the role of CNGs content in the seeds of wild Lima bean on plant defense and as nitrogen storage compounds, influencing plant growth. Using seeds collected from four different natural populations that are known for their variable CNGs contents (Shlichta et al. 2014), we examined the relationship between seed CNGs content and seed germination rate, seedling performance, and plant resistance against a generalist herbivore. We tested two non-mutually exclusive hypotheses: 1) seeds with high CNG levels produce seedlings with high concentrations of CNGs and thus higher resistance to herbivores, and 2) that seeds with high CNG levels have better germination and produce plants that exhibit better growth than seeds with low CNG contents.

Materials and methods

Seeds were obtained from four wild populations collected the previous year in the area of Puerto Escondido in the State of Oaxaca, Mexico. Sampling sites were located from 597 km north to 50 km south Puerto Escondido (see Shlichta et al. 2014 for details). These populations had been previously characterized for the CNGs content in their seeds (Shlichta et al. 2014) and we had an a priori idea on CNG content in the seeds. Nonetheless, to establish a baseline on CNGs content for the subsequent experiments, a sample from each population was analyzed. One sample consisted of 7 seeds and 9-10 samples were analyzed per population. Samples were then analyzed for their content of linamarin and lotaustralin, the two predominant CNGs present in leaves and seeds (Shlichta et al. 2014) and were divided into two groups: high and low CNGs content.

Preparation of plant material

Individual seedling structures

Seeds were selected randomly from two plants of each population, planted in small plastic pots (11cm of height and 4 cm of diameter) and placed in a phytotron (28/24°C, 60-80% R.H. and a light of 550 µmol). As seeds germinated and plants developed, we collected the different plant structures: cotyledons, primary leaves, trifolia of first and third secondary leaves (Figure 1). Only one structure was collected from each individual plant. After removal, samples were directly drowned in liquid nitrogen and stored in a freezer at -80°C until CNG extraction. Between 9 to 16 samples were used for each plant structure of the 4 populations.

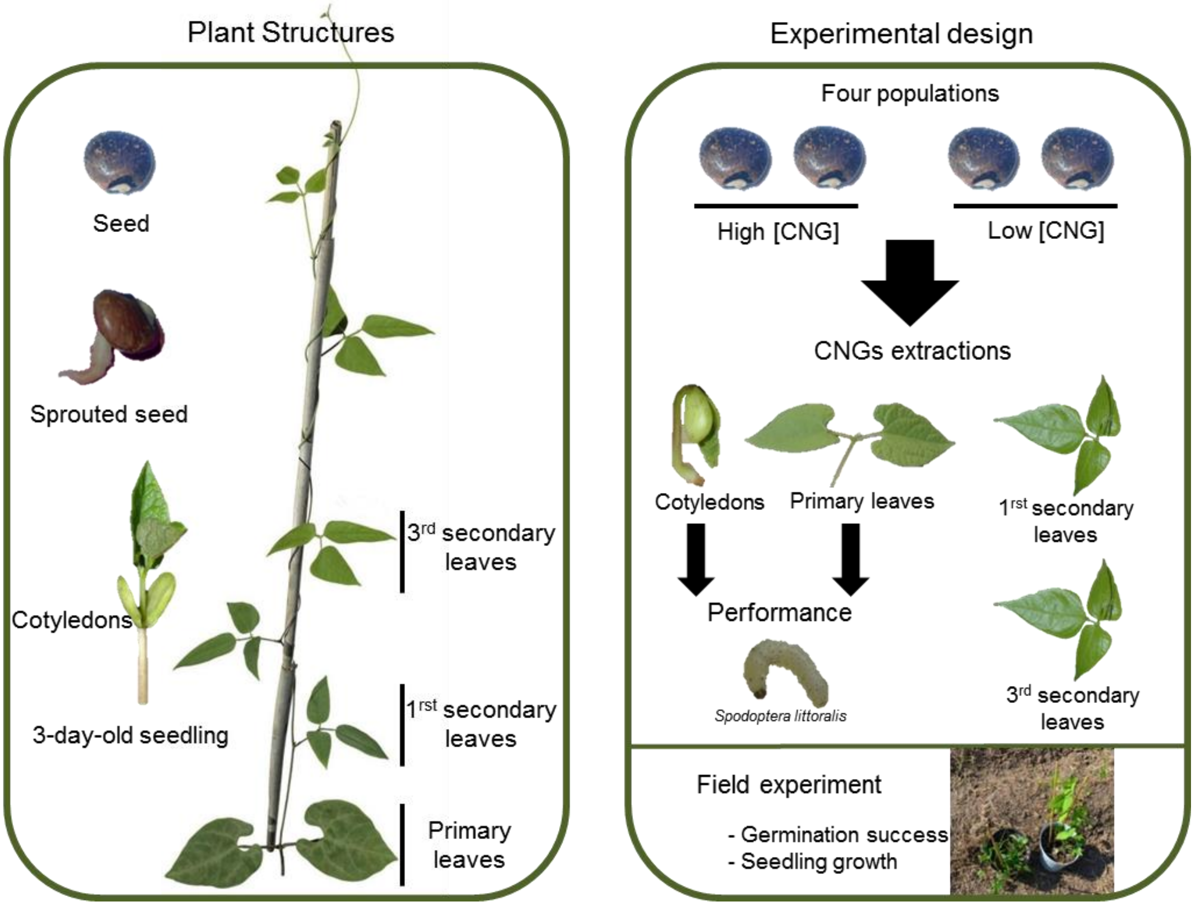

Figure 1 Schematic illustration of the developmental plant stages and tissues of lima bean, *Phaseolus lunatus* used during the study **a**, and of the experimental protocol followed for cyanogenic glycosides (CNGs) extractions, herbivore performance assays and field experiment **b**. Extractions and experiments were conducted with seeds with low and high CNG content

Seedlings at different developmental stages

A group of 30 plants was used to determine the content of CNGs in young plants throughout seedling development. Here, we did not remove individual plant structures, but rather we analyzed the content of cyanogenic glycosides present in the whole young plant at different stages of development. This procedure allowed us to determine if these compounds are transferred from the seeds to seedlings and/or synthetized *de novo*. Because of a lack of seeds, only one population (INK) was used to test this.

To prevent any soil particles from contaminating the samples, seeds were germinated in wet cotton (in a phytotron, 28/24°C, 60-80% R.H. and a light of 550 µmol). For the analyses we used seeds, sprouted seeds (less than 24hours) and full seedlings (3-4 days old) (Figure 1). One individual plant was used for each developmental stage, ten plants per stage. Samples were submerged in liquid nitrogen and stored in a freezer at -80 °C until extraction.

Quantification of Cyanogenic Glycosides

Linamarin and lotaustralin were quantified from seeds (one sample consisted of seven seeds), cotyledons (one sample consisted of two cotyledons from 2 plants), primary leaves, first and third secondary leaves, 1-day-old whole sprouted seed and 4-day-old whole seedling. We used a method adapted from two previous studies on CNGs (Franks et al. 2005; Rojas and Morales-Ramos 2010) and fully described in Shlichta et al. (2014). Briefly, samples were taken out of the freezer and immediately submerged in liquid nitrogen and milled with a mortar to obtain a thin powder. Each sample (approximately 20 mg of powder), was then weighed in a microbalance to the nearest 0.1 mg (Mettler Toledo XP6, Columbus, Ohio, USA), put in an Eppendorf tube with 1ml of 70% methanol and immediately placed on a heating block at ~90 °C for 10 min. The tubes were removed from the heating block, cooled for 5 min and placed in a Branson 2210 ultrasonic shaker for 10 min. Then they were centrifuged at 12000 rpm for 10 min and supernatants were collected. Finally, as CNG concentration is higher in the seeds, we diluted seeds and cotyledons samples at 1:25 with 70 % methanol.

The analysis of CNGs was performed using an Acquity UHPLC (Ultra-high-performance liquid chromatography) system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, USA) controlled by Masslynx 4.1 (see Shlichta et al. 2014 for details).

CNGs expressed in fresh and dry weight

CNG concentrations can be measured in fresh or dry weight. From an ecological point of view, concentrations expressed in fresh weight seem to reflect more accurately the amount of CNGs that herbivores face in nature. However, in fresh weight, the amount of water, which can greatly vary according to the plant tissues, is not taken into account. Consequently, plant tissues with higher water content (e.g. leaves) tend to have a lower concentration of CNGs due to a dilution effect, while tissues with low water content (e.g. seeds) display higher concentrations. Thus, in order to follow the variation in CNG content from seed to the early seedling stages, the water content in the different seedling structures has to be considered.

Seeds of the four plant populations were planted and the different seedling structures were collected (5 samples per structure for each population) following the same protocol as in the previous experiment. Samples were weighed to the nearest 0.1mg and oven-dried at 40-60 °C for 48 hours, after which, they were weighed again and the % of water content in each sample was calculated (Supplementary material 1).

Herbivory experiment

This experiment was done to test our first hypothesis on the role of CNGs as defense compounds against a generalist herbivore. We tested the performance of *Spodoptera littoralis* larvae on cotyledons and primary leaves of plants from the four populations. Egg clutches of *S. littoralis* were provided by

Syngenta (Stein, Switzerland) and reared to second instar on artificial diet ("beet armyworm diet", produced by BioServ, U.S.A). Plants used in this experiment were grown under the same conditions as previously described.

To determine larval performance on cotyledons, each second instar of *S. littoralis* was offered one whole cotyledon from young (two-day-old) lima bean seedlings (only one plant was used per larva) of the four populations. This experiment could not be done directly on the plant because cotyledons are very fragile. Consequently, they were set up in petri dishes on wet cotton. Larvae were randomly chosen from a batch of eggs recently hatched and assigned cotyledons from the 4 plant populations (29-35 replicates for low and high-CNG populations). The relative growth rate (larval weight gain divided by the initial larval weight), was calculated by individually weighing larvae before placing them on the cotyledons and 24 hours later.

We followed a similar procedure to test larval performance on primary leaves. We used 1-week-old plants, as soon as the surfaces of primary leaves were large enough, single second instars of *S. littoralis* were placed directly on one of the two primary leaves of the seedling and confined with a plastic clip cage covered with mesh (diameter 1.5 cm, Köhler et al. 2014; 9-17 replicates for low and high CNG-populations). Caterpillar growth rate was also measured by weighing larvae at the start of the experiment and 24 hours later. After this period larvae had eaten a very small amount of leaf, thus they were placed on the second primary leaf of the same plant for an additional 24-hour period.

Field experiment: germination and seedling growth

A field experiment was conducted to test the second hypothesis on the role of CNGs as nutrient storage compounds involved in germination and early seedling development. This experiment was performed in January of 2015 on our field station 15km northwest of Puerto Escondido, Mexico (15°55′27.4″N, 97°09′03.0″W). Seeds from the same four populations with high and low CNGs content (28-30 replicates per population), were sown in individual pots (4 cm, 11 cm high). Pots were placed in a randomized common garden experiment towards the end of the field season (mid-January) when most leaf herbivores are no longer present in the field (Cuny et al. 2018). We recorded the time to germination (number of days between sowing and germination), the germination success and the time to appearance of the first trifolium (number of days between germination and production of the first trifolium).

Statistical analyses

The first CNG extraction from the seeds of the four populations allowed us to separate them in two categories: populations with high or low CNG concentration in the seeds. Based on these results, for all the analyses in which populations are separated in two groups, population nested in CNG group (high and low) were included as fixed factors (to account for natural variation among the two high-CNG and the two low-CNG populations). For the analyses comparing the CNG concentration between different plant structures (in fresh and dry weight), the four populations were used as random factors in the models.

We used linear mixed models (PROC MIXED) to analyze the CNGs concentration (fresh weight) of the different tissues of the seedlings according to the initial CNGs concentration in the seed as well as the performance of *Spodoptera* larvae on cotyledons and primary leaves. When populations were tested separately (not grouped according to their concentration in CNG in the seed), Anovas were used to compare CNG concentrations among them.

The concentration of the different plant tissues expressed in dry weight was analyzed using a generalized linear mixed model (PROC GLIMMIX) following a gamma distribution.

The concentrations of total CNGs (dry weight, INK population) of different early stages of the whole seedling as well as the germination speed of the four populations separated were analyzed using a generalized linear model (PROC GENMOD) following a gamma and a Poisson distribution, respectively.

The same type of model was used for seed germination success (binomial distribution), speed and seedling growth (Poisson distribution).

Correlation tests between linamarin and lotaustralin were done using Pearson correlation tests for the primary leaves, first and third secondary leaves and Spearman for the seeds and cotyledons (PROC CORR).

All statistical analyses were performed with SAS 9.2 software (Littell et al. 2006, SAS Institute, Cary, North Carolina).

Results

Concentrations of linamarin and lotaustralin were highly positively correlated for all plant structures (Supplementary material 2, $r > 0.7$). Linamarin was on average 20 times more concentrated than lotaustralin. Thus, we only report results for linamarin.

CNGs in seedling structures from seeds with high and low content

Fresh weight Based on the results from CNGs content in the seeds, the four plant populations were separated into two groups: 2 high-CNGs populations (INK: 15.7mg/g FW and KM: 11.2 mg/g FW) and two low-CNGs populations (ITC: 7.7 mg/g FW and HHI: 8.7mg/g FW) (Figure 1). On average, seeds in the “high CNG” group, had on average 30% - 50% more CNG content than seeds in the “low CNG” group (Figure 2a, $F_{1,34} = 6.57$, p=0.01).

Overall, high-CNG seeds produced seedlings with higher CNG content, but not in all plant structures. Plants from high-CNG seeds had about 35% more CNGs in their cotyledons (Figure 2b, $F_{1,33} = 11.36$, p=0.002), 55% more in first secondary leaves (Figure 2d, $F_{1,33} = 4.59$, p=0.04) and 50% more in third secondary leaves (Figure 2e, $F_{3,38} = 11.85$, p=0.001) compared with seedlings from the low-CNG treatment group. However, no significant difference was found for the concentration in primary leaves (Figure 2c, $F_{1,52} = 0.35$, p=0.56).

The effect of population nested in the two CNG groups was only significant in the case of first secondary leaves (p=0.002). For all the other plant tissues, this variable was not significant, meaning that the individual variations between populations had no significant impact on the differences in CNG concentration that were observed. CNG concentrations of the first secondary leaves were thus analyzed with populations separated. One population, KM was responsible for driving most of the observed differences (Supplementary material 3, $F_{3,33} = 5.83$, p=0.003). Finally, we compared the different seedling structures according to their concentration of linamarin in both high ($F_{3,84} = 9.94$, $p < 0.001$) and low-CNG ($F_{3,78} = 23.69$, $p < 0.001$) seed groups and found that linamarin content was higher in cotyledons followed by primary leaves (although not significant) and then significantly lower in first and third secondary leaves (Figure 3).

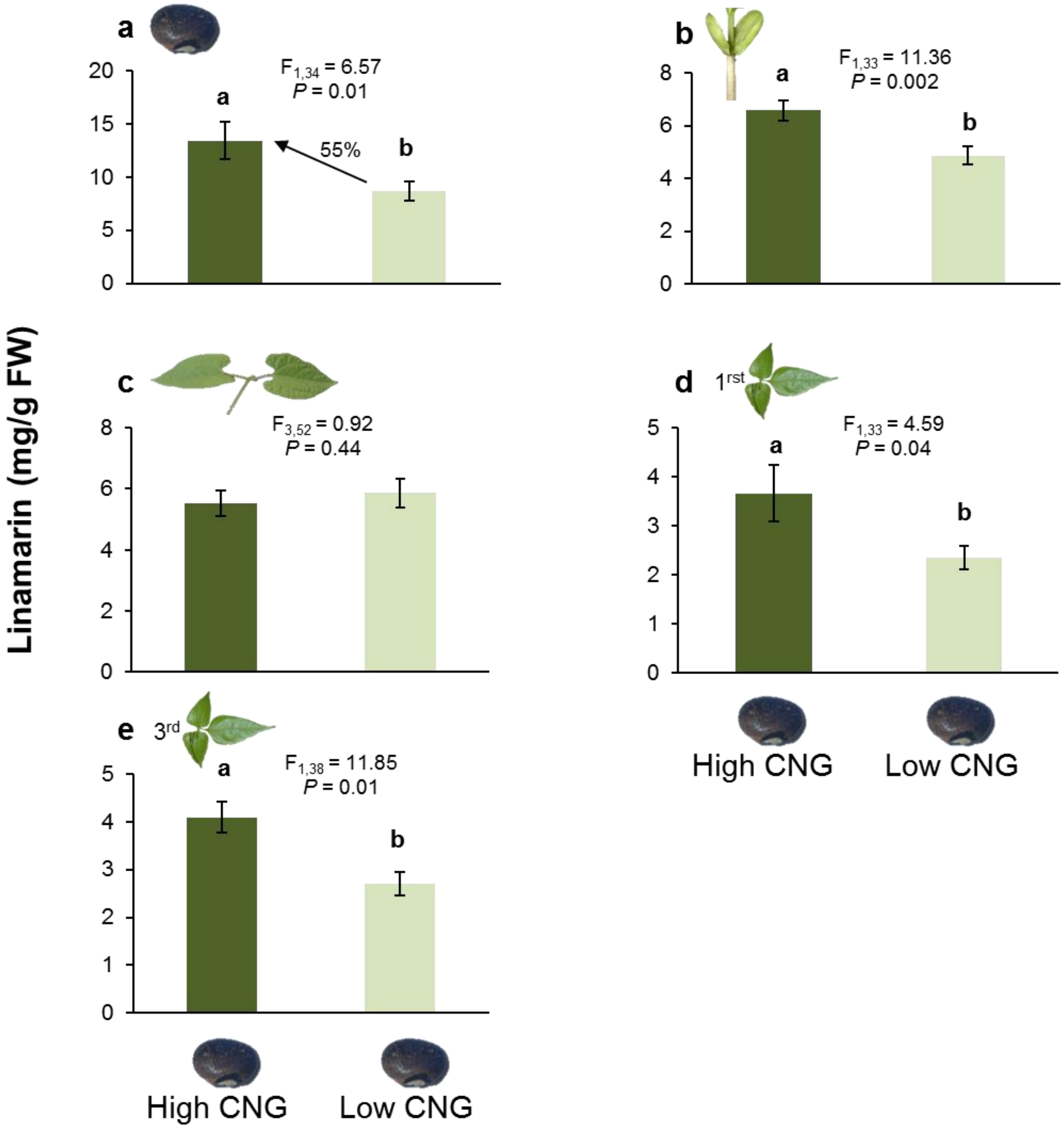

Figure 2 Concentrations of linamarin expressed in fresh weight in individual plant structures of lima bean from seeds with high and low CNG content: **a** seeds, **b** cotyledons, **c** primary leaves, **d** first trifolium and **e** third trifolium. F-values, degree of freedom and p-values (P) are shown. Bars (means ±SE) with different letters are significantly different from each other

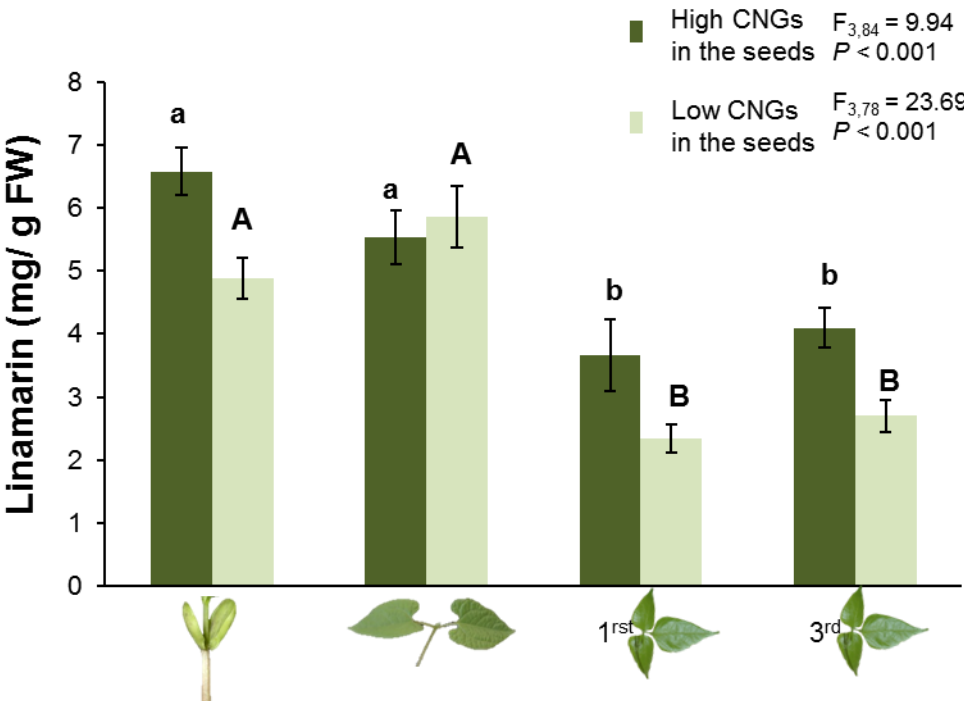

Figure 3 Concentrations of linamarin (**expressed in fresh weight**), in different plant structures throughout seedling development. We compared concentrations in cotyledons, primary leaves, first trifolium and third trifolium, produced by seeds with low or high CNG content. Separate analyses were done for plants from the low and high-CNG seed groups. F-values, degree of freedom and p-values (P) are shown. Bars (means ±SE) with different letters indicate significant differences between plant developmental stages within low or high-CNG seed group

Dry weight The CNG concentrations in the different plant tissues (seed, cotyledon, primary and secondary leaves) expressed in dry weight (in order to remove possible bias from the dilution effect due to the amount of water in the different tissues) were significantly different from each other, for both high (Figure 4, $F_{4,101}$ = 32.93, $p < 0.001$) and low-CNG seeds (Figure 4, $F_{4,97}$ = 45.98, $p < 0.001$). CNG concentration was higher in primary leaves, intermediate in cotyledons and secondary leaves and lower in seeds. These results suggest that there is an increase in CNG concentration relative to dry plant mass when the plant starts to produce leaves.

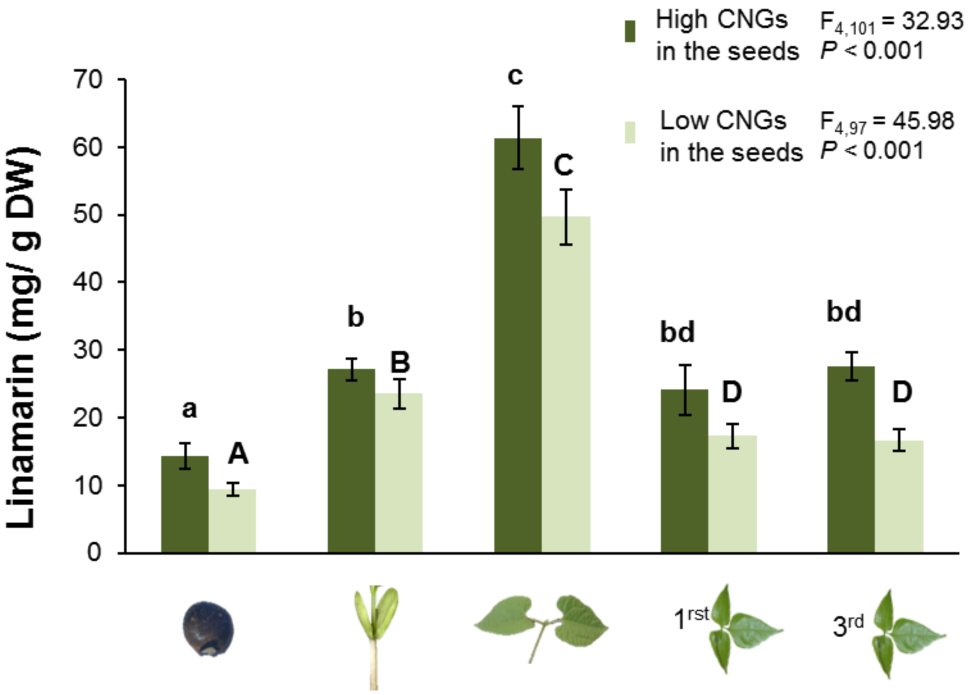

Figure 4 Concentrations of linamarin (**expressed in dry weight**), in different plant structures throughout seedling development. We compared concentrations in cotyledons, primary leaves, first trifolium and third trifolium, produced by seeds with low or high CNG content. Separate analyses were done for plants from the low and high-CNG seed groups. F-values, degree of freedom and p-values (P) are shown. Bars (means ±SE) with different letters indicate significant differences between plant developmental stages within low or high-CNG seed group

CNG concentration (in dry weight) throughout seedling development

This experiment was conducted not only to confirm the results from the previous experiment on whole seedling structures, but also to estimate the increase in CNG levels during plant development. Here we only used one population, INK. Twenty-four hours after germination, the sprouted seed already had two times more CNG compounds (in dry weight) than dry seeds. In addition, 3-4 days later, the total amount of CNG compounds in the entire small seedling (cotyledons and seed coat, small roots, stem, small primary leaves) had tripled compared to the sprouted seed (Figure 5, $\chi^2_{2,27} = 66.41$, $p < 0.001$).

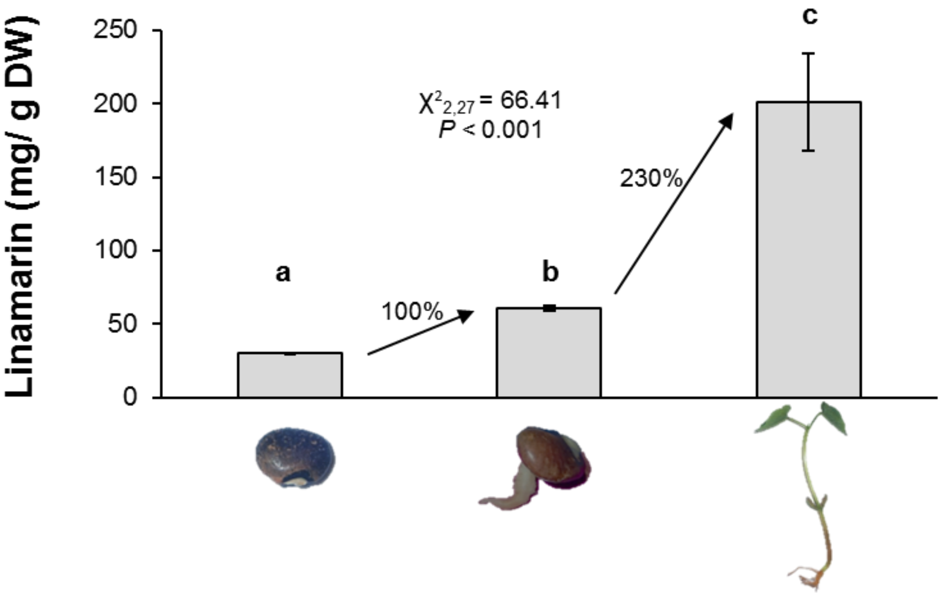

Figure 5 Linamarin concentrations expressed in dry weight in seeds, sprouted seeds and 3-4-day-old seedlings. X^2-value, degree of freedom and p-value (P) are shown. Seeds from only one population, INK were used for this experiment. Bars (means ±SE) with different letters are significantly different from each other. Linamarin content increased 100% from seeds to sprouted seeds, and 230% from sprouted seeds to young seedlings

CNGs effect on the performance of *Spodoptera littoralis*

Cotyledons The relative growth rate of 2nd instar was 37% better when fed with cotyledons with a lower CNG concentration, from low-CNG seeds (Figure 6a, $F_{1,60} = 4.58$, p=0.037). Individual populations had no significant effect (population nested in CNG groups, p=0.784).

Primary leaves No significant difference was observed in the relative growth rate of *S. littoralis* larvae fed with primary leaves from low or high-CNG seeds (Figure 6b, $F_{1,60} = 0.54$, p=0.47). Again, individual populations had no significant effect (population nested in CNG groups, p=0.568).

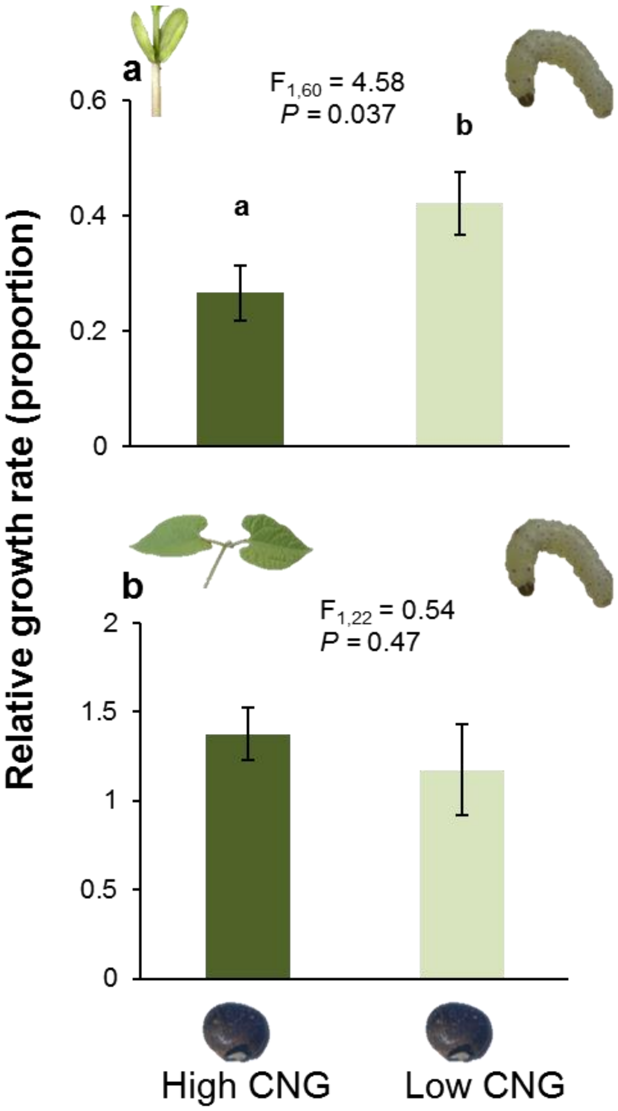

Figure 6 Relative growth rate of *Spodoptera littoralis* feeding on cotyledons **a**, and primary leaves **b**, produced by seeds with high and low CNG content. F-values, degree of freedom and p-values (P) are shown. Bars (means ±SE) with different letters are significantly different from each other. Relative growth rate was calculated for each individual larva as the mass difference between initial and final mass after 24 hours of feeding

CNGs effect on seed germination and seedling growth in the field

Germination success No difference was observed between the germination success of seeds with low or high CNG (Figure 7a, $F_{1,114}$ = 1.84, p = 0.18), nor a significant effect for population.

Germination speed Seedlings from seeds with high CNGs germinated significantly faster than plants from seeds with low CNGs ($F_{1,93}$ = 6.84, p=0.01). However, the fixed factor "population" was also significant (p= 0.005), suggesting that one population could be driving this result. Indeed, when analyzing the four populations separately, we found that the observed difference was driven by the ITC population from low CNG seeds (Supplementary material 4, $F_{3,93}$ = 6.35, p < 0.001). Thus, it appears that the speed of germination is affected more by seed traits associated with the population of origin than by CNG content.

Seedling growth No significant differences were found in time of appearance of the first trifolium in seedlings from seeds with high or low CNG concentration (Figure 7b, $F_{1,76}$ = 1.6, p = 0.21).

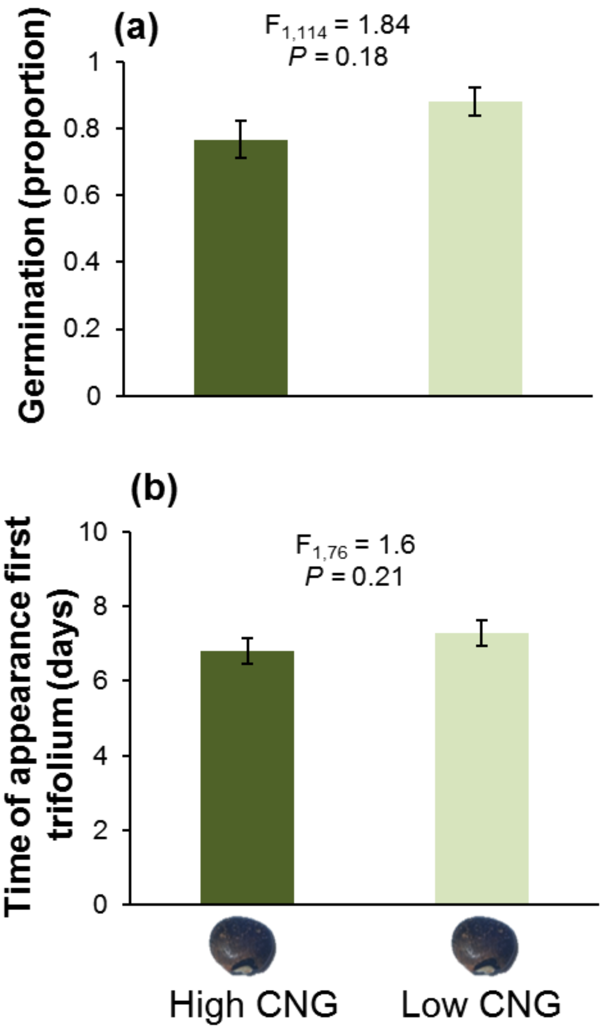

Figure 7 Germination rate of seeds with low and high CNG content **a**, and time of appearance of the first trifolium for plants from seeds with low and high CNG content **b**. F-values, degree of freedom and p-values (P) are shown. Bars are means ±SE

Discussion

Overview

Seeds of *P. lunatus* with higher CNG concentration produced seedlings with a higher CNG content in cotyledons and secondary leaves, but not in primary leaves, than low-CNG seeds. Moreover, cotyledons with high CNG content were better protected against the generalist herbivore *S. littoralis* than low-CNG cotyledons. However, when comparing the CNG concentration between different seedling tissues (measured in dry weight in order to standardize for differences in water content), we found that regardless of the initial concentration in the seed, primary leaves had a higher concentration of these compounds. Further, CNG concentrations increased significantly soon after germination.

Taken together, these results show that initial CNG content in the seeds partially explains the content in subsequent plant structures, where *de novo* production occurs in very high quantities compared to the initial seed level.

Finally, we did not find differences in seed germination and early seedling growth in the field according to the initial CNGs content in the seeds. Therefore, CNGs in seeds do not seem to act as nitrogen storage compounds used for plant growth.

Variation in CNG content among seedling structures according to seed content

High-CNG seeds produced cotyledons that had higher CNG content and that were better defended against herbivore damage than cotyledons from low-CNG seeds. For leaves, this pattern was not so clear. When expressed as fresh weight, which reflects the level of CNGs that the herbivore will face upon plant damage, CNG content in primary leaves was not different between leaves from high and low-CNG seeds. However, for secondary leaves (1st and 3rd trifolium) the difference in CNG content according to the seed content became obvious again. In a study on 6-week old lima bean plants, Ballhorn et al. (2008b) found higher emissions of volatile organic compounds in primary than secondary leaves. They suggested that primary leaves rely more on these and other indirect defenses (i.e. extrafloral nectar), that attract the natural enemies of their herbivores. This may explain why we do not find differences in CNG content (expressed as fresh weight), in primary leaves from the two CNG-seed groups (Figure 2c) and why these differences are detectable again in secondary leaves. Thus, as proposed by Ballhorn et al. (2008a), there appears to be a trade-off between direct and indirect defenses that varies throughout plant ontogeny. Ontogenic changes in plant defense have been widely reported for several plant species (Boege and Marquis 2005; Goodger et al. 2007; Barton and Koricheva 2010; Desurmont et al. 2015). A variable combination of multiple defense mechanisms (direct and indirect) throughout ontogeny could provide lima bean plants with the best strategies to cope with different types of herbivores and other antagonists.

The higher content of CNGs in cotyledons may be the result of two non-mutually exclusive processes. First, CNG compounds from the seeds may be directly transferred to the sprouted seed and then cotyledons. This is supported by cotyledons from high-CNG seeds having higher CNG content than those from low-CNG seeds (Figure 2b). Alternatively, or additionally, a higher CNG content in the seed could provide more of the precursors needed for the production of new CNGs in later plant structures. The proportionally higher de novo content of CNGs in secondary leaves from the high-CNG seed group compared to the low-CNG group seems to support this idea. Further, the comparison among the different seedling structures showed that the younger tissues, cotyledons and primary leaves from both high and low-CNG seeds had overall a higher content of linamarin (Figure 3). This is not an unexpected result. Godschalx et al. (2016) measured the cyanogenic potential (the total amount of CNG compounds) of different plant structures from wild lima bean plants from Costa Rica mainly, flower buds, flowers, seed pods and leaves and found that young leaves had the highest potential.

CNG content and seedling development

To better understand the variation in CNG concentrations during early plant ontogeny, we converted concentrations from fresh to dry weight. Concomitant with the levels of CNGs in the seeds, the total amount of CNG compounds following germination began to increase in the young seedling. CNG content was doubled after 24 hours (from seed to cotyledon), and then tripled after 3-4 days (from cotyledon to primary leaves). These results indicate that most of the CNG compounds present in the young seedling are produced *de novo*, starting as soon as the seed germinates.

In their study, Ballhorn et al. (2008a) showed that primary leaves had a lower CNG concentration than secondary leaves. The difference between their results and ours may be due to the age of the plant examined. They used older lima bean plants, six-weeks-old, compared to one-week-old plants in our study. It may be that primary leaves are first defended by CNGs which then get transferred to other plant tissues, possibly switching the defense strategy towards the production of volatile organic compounds (Ballhorn et al. 2008b). Yet, in a different study, and in agreement with our findings, this same group reported that plants characterized by high cyanogenic potential (HCNp) in their young leaves showed consistently higher HCNp in later developmental stages than plants with low HCNp in young leaves (Ballhorn et al. 2005).

Other studies with this and other plant species have also suggested a transfer of CNG compounds from younger to older plant tissues (Selmar et al. 1987; Frehner et al. 1990). For instance, Selmar et al. (1987) showed that in seeds of the rubber tree *Hevea brasiliensis*, 90% of the CNGs are stored in the endosperm as linamarin and lotaustralin. Upon seed germination, linamarin the dominating CNG is transformed into linustatin which is transferred to the seedling. Similarly, in a study with wild Lima bean plants in Costa Rica, Clegg et al. (1979) found that linamarin content remained constant throughout plant development but, observed a drop in the total CNG content in the plant when the cotyledons were removed. They suggested that the plant transfers the linamarin intact from the seeds to the growing seedling. These findings run counter to our study, as we detected a dramatic increase in linamarin levels during early plant development. We do not have an explanation for this discrepancy.

Ontogenic changes in CNG content have also been reported for other plant species (Schappert and Shore 2000; Dicenta et al. 2002; Ochoa-López et al. 2015). For example, in the tropical shrub *Turnera velutina* (Passifloraceae), hydrogen cyanide is present only in cotyledons and early seedling stages, while in later plant stages the plant relies on other type of direct and indirect defenses such as, trichomes and extrafloral nectar (Ochoa-López et al. 2015, 2018)

For lima bean, contrasting results from the different studies on differential CNG content across plant tissues could also be the result of natural variation among plant populations. Particularly taking into consideration that studies have been conducted with populations from different geographical regions (i.e. from Costa Rica and Mexico), likely subjected to different abiotic and biotic conditions and selective pressures.

Defense or nutrition?

For lima bean, the great majority of studies concerning CNGs focus on their role in defense, while their role as nitrogen storage compounds that might be used during germination and seedling establishment is less understood (Seigler 1998; Møller 2010). Our results indicate that the function of CNG compounds in the seeds is rather defensive than nutritional.

These findings point to a defensive role of CNGs and add to those by numerous studies that have documented how this and other generalist herbivores are affected by the CNG concentrations in their host plants (Jones 1988; Schwarz et al. 1996; Ferreira et al. 1997; Gleadow and Woodrow 2002; Pentzold et al. 2013), including in lima bean, field and lab studies have shown that CNGs effectively protect plants against generalist herbivores (Ballhorn et al. 2007, 2008b, 2009; Rojas and Morales-Ramos 2010; Shlichta et al. 2018).

Conversely, we did not find a significant relationship between the content of CNGs in the seeds (high or low) and the success of germination and early seedling development in the field. As cyanogenic glycosides are nitrogen-based compounds, it has been suggested that in addition to their defensive role, they may also provide a storage place for nitrogen and sugar needed for plant growth (Clegg et al. 1979; Lieberei et al. 1985; Selmar et al. 1988; Forslund and Jonsson 1997; Møller et al. 2010). Evidence for this idea comes mostly from lab studies that examine the biosynthetic pathway of CNGs during plant development. These studies have identified the intermediate products and the enzymes responsible for their catalyzation, and examine how and if these products are translocated and supply nitrogen to latter plant tissues (Forslund and Johnsson 1997; Sanchez-Perez et al. 2008, 2009; Kongsawadworakul et al. 2009; Jørgensen et al. 2011; Kadow et al. 2012).

We did not follow the biosynthetic pathway of CNGs from seed to seedling, therefore we cannot exclude the possibility that some of the intermediate and end products formed during the metabolic pathway serve as nitrogen sources for latter tissues. Yet, by using seeds with high and low CNG content we were able to test the ecological value of CNG variation in the seeds on germination and early seedling development.

Conclusion and future directions

To our best knowledge, this is the first study that examines the potential dual role of cyanogenic glycosides in defense and early plant development in wild lima bean. We found support for our first hypothesis by showing that seeds with more CNG produced seedlings that contain more CNG in cotyledons, which had a significant negative impact on the performance of a generalist herbivore. In addition, we found a significant increase in CNG compounds as soon as the seed germinates. Conversely, we did not find support for our second hypothesis, that a higher CNG content in the seeds would result in higher germination and better seedling growth.

To further understand the varying defense strategies and ecological functions of CNG in this species, we need detailed studies that contemplate different selective pressures by herbivores and other antagonists (i.e. pathogens, fungi, small vertebrates) across lima bean populations coupled with plant ontogeny.

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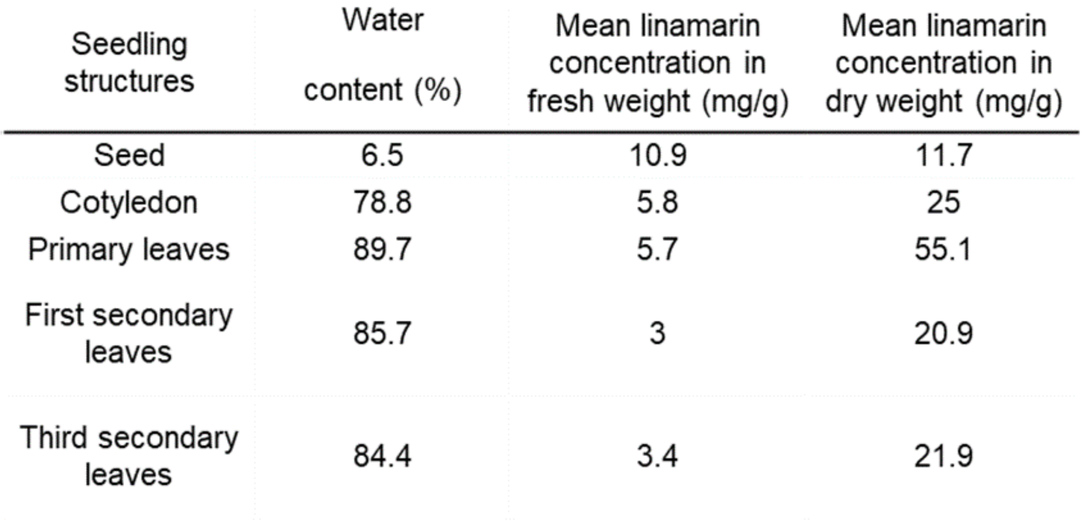
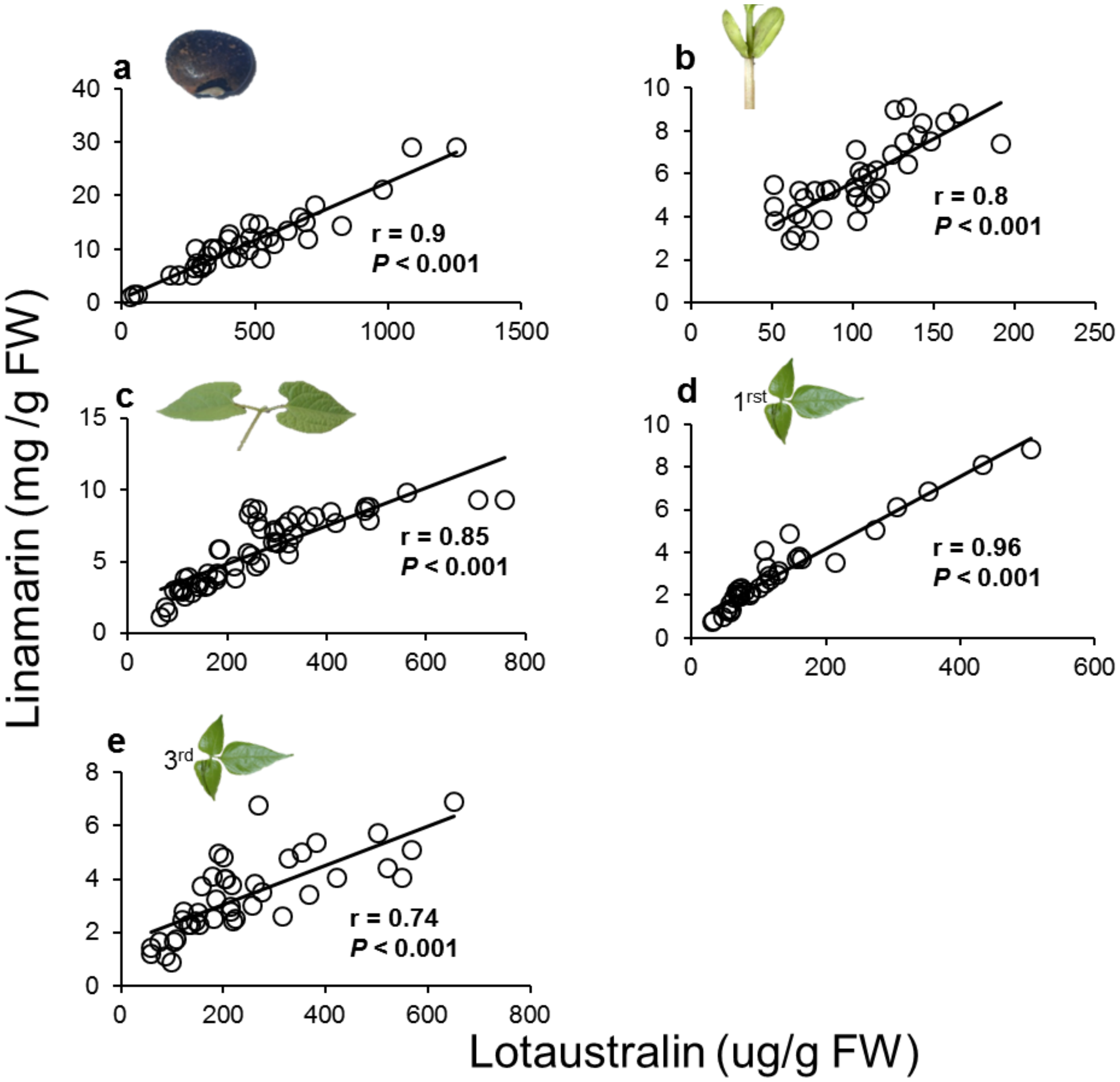
Supplementary material

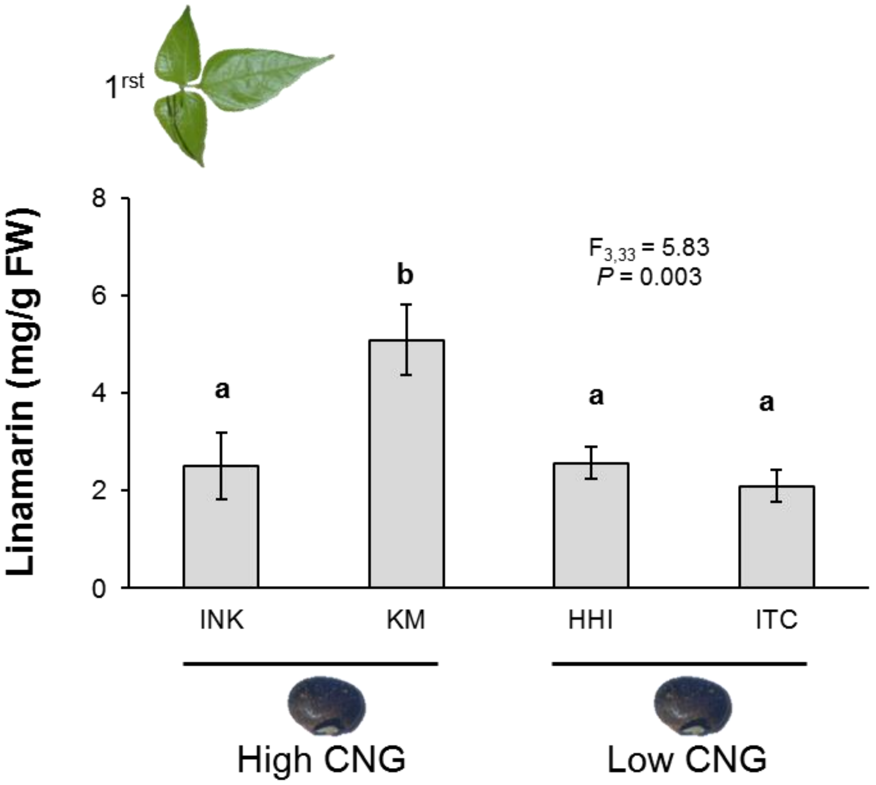
Supplementary material 1. Humidity percentage and linamarin concentration in fresh and dry weight of different plant structures of wild lima bean.



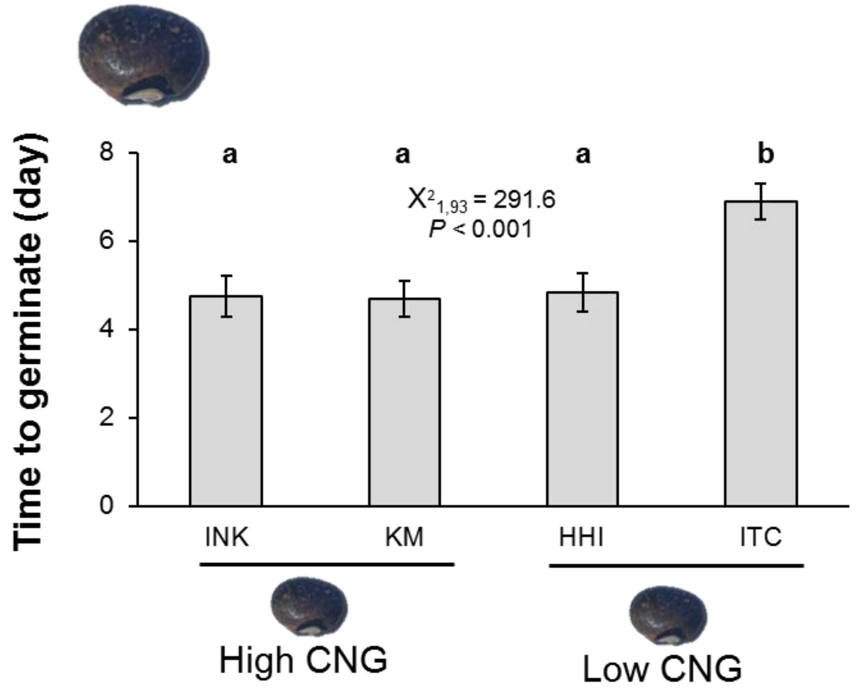
Seedling structures	Water content (%)	Mean linamarin concentration in fresh weight (mg/g)	Mean linamarin concentration in dry weight (mg/g)
Seed	6.5	10.9	11.7
Cotyledon	78.8	5.8	25
Primary leaves	89.7	5.7	55.1
First secondary leaves	85.7	3	20.9
Third secondary leaves	84.4	3.4	21.9



Supplementary material 2. Correlations between concentrations in linamarin and lotaustralin of five plant structures: (a) seed, (b) cotyledons, (c) primary leaves, (d) first trifolium and (e) third trifolium. Pearson (c,d,e) or Spearman (a,b) correlation coefficients and p-values are shown.



Supplementary material 3. CNG concentrations of the first secondary leaves of the four seed populations. F-values, degree of freedom and p-values (P) are shown. Bars (means ±SE) with different letters are significantly different from each other.



Supplementary material 4. Germination time of seeds from the four seed populations. X^2-value, degree of freedom and p-value (P) are shown. Bars (means ±SE) with different letters are significantly different from each other.

Chapter 3

Oecologia
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SPECIAL TOPIC: FROM PLANTS TO HERBIVORES

Changes in plant growth and seed production in wild lima bean in response to herbivory are attenuated by parasitoids

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Abstract

Lima bean plants (*Phaseolus lunatus*) exhibit compensatory growth responses to herbivory. Among the various factors that have been identified to affect plant compensatory growth are the extent and type of tissue damage, the herbivore's feeding mode and the time of damage. Another factor that can greatly impact plant responses to herbivory, but has been largely ignored in previous studies, is the action of parasitoids. In most cases, parasitoids halt or slow down the development of herbivorous hosts, which, can result in decreased leaf damage, thereby affecting plant responses and ultimately plant fitness. Here, we investigated the effects of two koinobiont parasitoids on the amount of leaf damage inflicted by the Southern armyworm *Spodoptera latifascia* to wild lima bean, and the consequences of this for plant growth and seed production in the field. We specifically tested the hypothesis that the action of parasitoids will reduce plant damage and that this reduction will alter plant growth responses and seed production. Indeed, we found that in the presence of parasitoids plants suffered less damage than plants with only herbivores. As a consequence, compensatory growth was reduced and more and heavier seeds were produced earlier in the season, compared to plants exposed to only herbivores.

Keywords Compensation · Plant response · Parasitoid-mediated · Beans · Seed output

Introduction

Plants are attacked by a variety of herbivores. The consequences of these attacks for plant fitness will depend on the intensity and timing of damage (Crawley 1989; Boege and Marquis 2005), and on the plant's defensive and physiological responses (Karban and Myers 1989; Karban and Baldwin 1997), which can act alone or in conjunction with the ecological environment (Strauss and Agrawal 1999). Among the different strategies to cope with tissue loss due to herbivore damage, resistance and tolerance are particularly well studied (Strauss and Agrawal 1999; Núñez-Farfán et al. 2007). Resistance is the ability of a plant to deter and minimize herbivore damage, whereas tolerance is a plastic response that allows a plant to endure or recover from damage through physiological mechanisms or compensatory growth (Agrawal 2000; Stowe et al. 2000). Tolerance response to herbivory has a genetic basis (Mauricio et al. 1997; Kessler and Baldwin 2002; Fornoni 2011), but is also affected by abiotic (Wise and Abrahamson 2005, 2007) and biotic factors such as competition with other plants and the action of herbivores (Edenius et al. 1993; Puettmann and Saunders 2001). For instance, a growing number of studies reveal that as a response to herbivore damage, plants compensate by increasing their photosynthetic capacity, reallocate photoassimilates to different tissues and eventually may produce more leaves, more branches, and flower early (reviewed in Núñez-Farfán et al. 2007). However, the extent to which these responses affect plant reproductive success is still a topic of debate (Dietrich et al. 2005; Heil 2010; Tito et al. 2016).

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Among the various factors that have been identified to affect the type and quantity of compensatory growth are the extent (Martínková et al. 2008) and type of damage (Huhta et al. 2009), the herbivore's feeding mode (Rosenheim et al. 1997; Kotanen and Rosenthal 2000; Tiffin 2000), and the timing and duration of damage (Boege et al. 2007). Another factor that can greatly impact plant responses to herbivory, but so far has received little attention, is the action of the natural enemies of herbivores (Kaplan et al. 2016).

Herbivores are frequently attacked by parasitoids and it is generally accepted that parasitoids can reduce the negative impact of herbivores on plants (Hoballah and Turlings 2001; Poelman et al. 2011; Gols et al. 2015).

Some parasitoids known as idiobionts will kill or paralyze the host immediately upon parasitization, halting host development and stopping it from further feeding (Mackauer and Sequeira 1993; Godfray 1994; Harvey 2005). Conversely, the hosts of koinobiont parasitoids continue to feed after oviposition (Hoballah and Turlings 2001; reviewed in Harvey 2005). Yet the nature and outcome of the interaction between hosts and koinobiont parasitoids is complex and will largely depend on whether the latter are solitary or gregarious, and on the manner in which they affect the growth and development of their host (Smilowitz and Iwantsch 1973; Harvey et al. 1994; Harvey 2005). In some cases parasitized hosts will feed at a slower rate and grow smaller than non-parasitized ones (Hoballah and Turlings 2001). In other cases parasitized herbivores can eat more (Coleman et al. 1999; Van der Meijden and Klinkhamer 2000) or for longer periods (Thorpe 1933; Beckage and Riddiford 1982), than unparasitized ones, in which case plants will sustain greater damaged and this may increase induced defensive responses (Ode et al. 2016). Yet, in virtually all known cases, parasitoids, idiobionts or koinobionts reduce the amount of damage that their hosts inflict to plants (van Loon et al. 2000).

Thus, it could be imagined that by altering the nature and amount of herbivore damage, parasitoids can affect plant growth and ultimately plant reproduction. As yet, to our knowledge, no study has examined the extent to which parasitoids can affect plant growth and compensatory responses. The implications of such effects are of great significance for the presumed positive effects of parasitoids on plant fitness, as to date, field evidence supporting this notion is still very scarce (Gomez and Zamora 1994; Hoballah and Turlings 2001; Gols et al. 2015).

Here, we investigated the effects of two koinobiont parasitoids, one solitary and one gregarious, on the amount of leaf damage inflicted by the lateral lined or velvet armyworm *Spodoptera latifascia* on wild lima bean (*Phaseolus lunatus*) and the consequences for plant growth and seed production. In two field experiments, each with a different parasitoid species, we compared leaf damage among control plants (without herbivory), plants exposed to *Spodoptera* larvae, and plants exposed to both larvae and parasitoids. We counted the number of trifolia per plant as a proxy of plant growth, number and time of appearance of flowers and pods, and measured several seed traits (number, mass and germination success). With these experiments, we tested the hypothesis that the presence of parasitoids will reduce plant damage and that this reduction will result in altered plant growth responses and seed production in the field.

Materials and methods

Study system

Wild lima bean (*Phaseolus lunatus*) plants occur naturally throughout Meso- and South America (Freytag and Debouck 2002). Plant phenology is synchronized with the regional weather. In our study site (see below), the first inflorescences appear in October–November and the seeds are produced at the end of December and early January (Heil 2004; Hernández-Cumplido et al. 2010). At this field site, lima bean plants are attacked by several herbivore species (Ballhorn et al. 2009; Moreira et al. 2015; Hernandez-Cumplido et al. 2016a, b). One of these herbivores is the polyphagous noctuid moth *Spodoptera latifascia.* Its larvae can cause significant leaf damage; one single larva can eat up to 60% of the leaf surface of an older plant or even entirely defoliate a young plant (Cuny, personal observation). Adult moths lay their eggs in batches on the upper surface on the leaves and upon hatching first instar larvae disperse and feed individually on all parts of the leaf (Pogue 2002; Cuny personal observation). Lima bean plants exhibit compensatory growth in response to varying degrees of herbivory (Moreira et al. 2015; Hernandez-Cumplido et al. 2016b). If and how these responses are influenced or altered by the action of the herbivores' natural enemies is not known.

At the field site, larvae of *S. latifascia* are frequently attacked by two generalist parasitoids, *Chelonus insularis* (Hymenoptera: Braconidae) and *Euplectrus platyhypenae* (Hymenoptera: Eulophidae). The former is a solitary egg-larval parasitoid that parasitizes hosts at the egg or first instar stage. One female can parasitize several eggs from the same batch (Ables and Vinson 1981; Jourdie et al. 2010). Parasitized larvae continue to develop until late third instar when parasitoid larvae emerge to pupate and the host dies. *E. platyhypenae* is a gregarious ectoparasitoid that lays its eggs on the dorsum of third and fourth instar larvae of *S. latifascia* and other Lepidoptera (Capinera 2001; Coudron et al. 1990). The relative abundance of the two parasitoid species varies greatly between years (Cuny, personal observation) and the reasons for these fluctuations in abundance are not yet understood.

Plants

Plants used for our experiments originated from seeds collected the previous year in a natural population of wild lima bean plants close to our field site (15°55′14.3″N 97°07′35.1″W and 17°00′40.4″N 100°06′09.9″W) for the first and second experiment, respectively; for more information on these populations see Shlichta et al. (2014). The experiments took place in January 2014 (experiment 1) and from October 2014 to March 2015 (experiment 2) at the Experimental Campus of The Universidad del Mar, located 15 km northwest of the city of Puerto Escondido (Oaxaca, Mexico, 15°55′27.9″N 97°09′04.3″W).

Insects

Colonies of *S. latifascia* and the parasitoids *C. insularis* (first experiment) and *E. platyhypenae* (second experiment) were established early in the season with field-collected insects from wild lima bean plants found in the surroundings of the experimental campus of the Universidad del Mar. Larvae of *S. latifascia* were reared under natural light and temperature conditions on artificial diet ("beet armyworm diet", BioServ, Flemington, NJ, USA). Both parasitoids were reared on larvae of *Spodoptera frugiperda* collected in maize fields close to the experimental field and fed with the same artificial diet. We chose to use this species for the rearing, because of its much higher abundance in the nearby maize fields compared to *S. latifascia*. Previous experiments showed that the two parasitoid species develop successfully on both *Spodoptera* species (Cuny et al. unpublished data). Caterpillars were reared in plastic containers (13 × 15 × 5 cm) with fabric mesh for aeration and adult parasitoids were kept in mating cages (Bugdorm insect rearing cages, 30 × 30 × 30 cm).

Experimental set-up

We conducted two field experiments to determine if parasitoids affected the amount of damage inflicted by *S. latifascia* and the consequences for plant growth. In both experiments lima bean plants were subjected to three herbivory treatments: (1) control (without insects), (2) unparasitized larvae of *Spodoptera latifascia* and (3) either *S. latifascia* larvae parasitized by *C. insularis* (first experiment) or *S. latifascia* larvae in the presence of female *E. platyhypenae* (second experiment). We recorded the amount of leaf damage (first and second experiment), and plant growth and seed production (second experiment).

Experiment 1: herbivory and parasitism by *Chelonus insularis*

The first experiment served to obtain preliminary information on the potential of parasitoids to reduce herbivory on lima bean plants. For this we compared the amount of damage inflicted by *S. latifascia* larvae that were either unparasitized or parasitized by *C. insularis*. Seeds were individually sown in 5-L pots filled with native soil and distributed among 18 field cages (two plants per cage, Bioquip Outdoor Cage 6 × 6 × 6′, 20 × 20 Mesh Lumite). Each pot was placed on a tray filled with water to prevent the experimental larvae from moving between plants and predatory ants from climbing on the plants. To obtain parasitized larvae, three batches of *S. latifascia* eggs were placed in a parasitoid cage with 10 *C. insularis* wasps (males and females) during 48 h. Batches were removed after females were observed parasitizing. Bioassays conducted before the experiment showed that using this method we obtained around 90% parasitism. Parasitized egg batches then were kept in separate containers until larvae emerged from the eggs, at which point they were transferred to artificial diet until the start of the experiment. Although rearing early instar larvae on artificial diet could potentially affect later physiological and behavioral responses, we chose to do this because of the significant mortality of earlier instars when reared on plant material. As larvae were randomly assigned to the different treatments, we can assume that any early effects on their development due to the artificial diet would be the same among the three treatments. Prior to the experiment, to habituate larvae to the switch from artificial diet to plant material, all larvae (unparasitized and parasitized) were fed with *P. lunatus* leaves for 10 h, followed by a 3-h starvation period. We randomly assigned three different treatments to cages (two plants per cage, one treatment per cage and 7–8 cages per treatment): (1) control plants (without *S. latifascia* larvae), (2) plants with twenty unparasitized early-third instars of *S. latifascia* and (3) plants with twenty early-third instar parasitized larvae of *S. latifascia* randomly selected from the containers of "parasitized egg batches". Preliminary experiments revealed that using 20 larvae per plant we could obtain enough damage to quantify potential differences among treatments and at the same time, minimize larval crowding that could prompt dispersion to adjacent plants. Every day, trays were refilled with water and plants were checked for unwanted insects that could have entered the cages. Based on levels of damage found in natural plant populations, we estimated leaf damage on the whole plant using the following scale: (1) no damage, (2) less than 25% of leaf surface eaten, (3) between 25 and 50% of leaf surface eaten and (4) more than 50% of leaf surface eaten. This experiment was repeated twice. To avoid any bias during sampling, plants were coded such that treatments applied to the different plants were not known during the estimation of damage.

Experiment 2: herbivory and parasitism by *Euplectrus platyhypenae*

Based on the results found in the first field experiment, we improved the experimental design and conducted a second experiment the following year. This experiment differed from the first one in that (1) the parasitoid used was *Euplectrus platyhypenae*, because of its much higher abundance in the field during that season, and (2) for the third treatment, instead of using parasitized larvae, we released female parasitoids inside cages with healthy third instar larvae of *S. latifascia*. This was done because trial experiments showed that even with the greatest care, manipulation of parasitized larvae resulted in very high mortality. For this experiment, plants were followed for the whole season until seed production.

Forty-eight plants were grown in 5-L pots and distributed in 24 field cages (Bioquip, Outdoor Cage 6 × 6 × 6′, 20 × 20 Mesh Lumite). Twenty-five days later, we followed the same procedure as in the previous experiment and we randomly assigned the three different treatments to cages with two plants per cage, one treatment per cage and eight cages per treatment. The treatments were: (1) control (plants without *S. latifascia* larvae), (2) herbivore alone (15 third instar larvae of *S. latifascia* per plant), and (3) herbivore + parasitoid (15 third instar larvae of *S. latifascia* per plant, plus five mated *Euplectrus platyhypenae* females per cage) (Fig. 1). Prior to the experiment, to habituate larvae to the switch from artificial diet to plant material, larvae were fed with *P. lunatus* leaves for 10 h, followed by a 3-h starvation period. Insects were left in the cages for 9 days. For each plant, we took a picture of two randomly selected trifolia (between the 5th and the 8th trifolium), and used it to calculate the mean damaged area per trifolium with Adobe Photoshop (Xiao et al. 2005). Two months later, when most of the leaf herbivores were no longer present in the field, cages were removed to reduce humidity and to allow the plants to dry and mature their pods. Plants were watered twice per week, checked every other day and undesirable insects (beetles, grasshoppers) were removed. For each plant, we recorded the following traits: number of trifolia (three leaflets), flowering time, number of tendrils with at least one flower, time of appearance and number of green pods and total number of seeds produced after 4 and 8 weeks. A sample of ten seeds per plant was weighed with a micro-balance (Mettler Toledo XP6, Columbus, Ohio, USA) to the nearest 0.01 mg. The following field season the germination ability of these seeds was measured by sowing them in 5-L pots at the same experimental field site. We used 14–16 plants per treatment, ten seeds per plant (two pots with five seeds per pot).

Statistical analyses

Variables that met model assumptions, number of trifolia, time to flowering and to pod production, number of flowers and green pods, and seed mass, were analyzed with a mixed linear model (PROC MIXED). Conversely, variables that did not meet assumptions of normality were analyzed with a generalized linear mixed model (PROC GLIMMIX): following a gamma distribution for leaf damage in the first experiment, a Poisson distribution for leaf damage in the second experiment, the total number of seeds and for seeds produced early in the season. Finally, a binomial distribution was used for seed germination success (Littell et al. 2006; Moreira et al. 2015; Abdala-Roberts et al. 2016). Pearson correlations (PROC CORR) were used to test for the

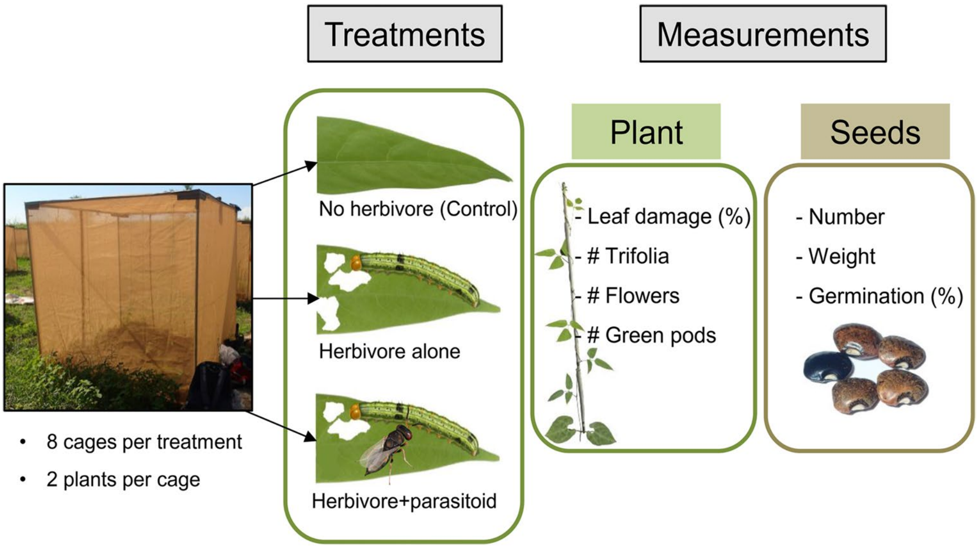

Fig. 1 Schematic illustration of the experimental protocol for the second experiment. The treatments were: (1) control (plants without *Spodoptera latifascia* larvae), (2) herbivore alone (fifteen third instar larvae of *S. latifascia* per plant), and (3) herbivore + parasitoid (fifteen third instar larvae of *S. latifascia* per plant, plus five mated *Euplectrus platyhypenae* females per cage)

potential correlation between the trifolia damage area and the number of trifolia in the second experiment.

For leaf damage in the first experiment, cages as well as the two blocks (the experiment was repeated twice) were considered as random factors (to account for repeated measures taken on the same experimental unit), and the herbivory treatments as fixed factors. For all the variables measured in the second experiment, cages (or pots in the case of the germination test) were considered as random factors, and herbivory treatments as fixed factors.

During the second experiment, one control plant suffered severe damage from an herbivore accidentally entering the cage and was removed from the analysis, as well as two plants from the parasitoid treatment that were destroyed by the wind during seed collection. All statistical analyses were performed with Statistical Analysis System (SAS Institute, Cary, North Carolina, USA), using Kolmogorov–Smirnov (PROC UNIVARIATE) to test model assumptions. For each analysis, we provide means ± SE, with different letters that indicate a significant difference between herbivory treatments.

Results

Experiment 1: herbivory and parasitism by *Chelonus insularis*

Lima bean plants with *S. latifascia* larvae parasitized by *C. insularis* suffered 35% less damage than plants attacked by non-parasitized larvae (Fig. 2a, $F_{2,49} = 127.38$, $P < 0.001$). Despite our efforts to maintain control plants without insect damage, some herbivores found their way inside the cages inflicting some damage (< 0.5 cm^2 per trifolium), but far less than the introduced *S. latifascia* larvae (> 1.5 cm^2 per trifolium). For herbivore-treated plants, unwanted herbivory could not be discerned from the one inflicted by the focal herbivores, but we can assume that it was similar among the three treatments.

Experiment 2: herbivory and parasitism by *E. platyhypenae*

Leaf damage

Plants from the two herbivore treatments (herbivore alone and herbivore + parasitoid) suffered significantly more damage than control plants (Fig. 2b, $F_{2,23} = 3.75$, $P = 0.039$). Although not significant, results show a trend on the presence of parasitoids and a reduction of the amount of damage, with plants from the herbivore + parasitoid treatment suffering almost 30% less damage than plants with only herbivores.

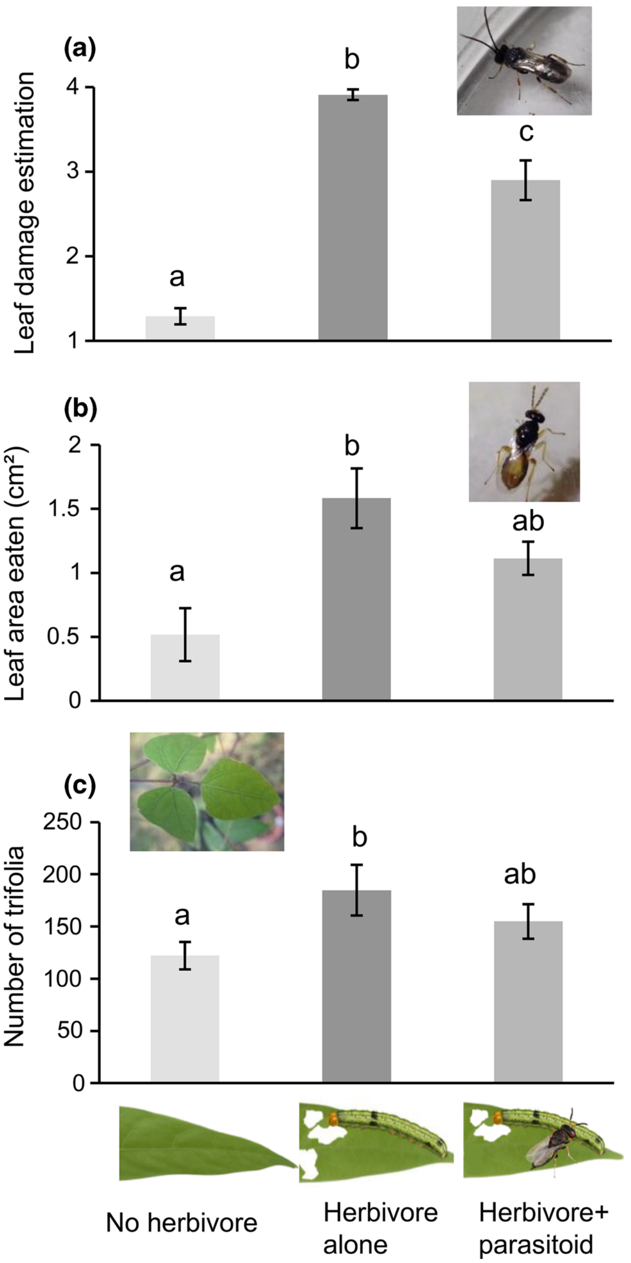

Fig. 2 **a** Estimation of total plant damage by larvae of *Spodoptera latifascia* during the first experiment, measured on a scale from 1 (no damage) to 4 (more than 50%). For this experiment, *Chelonus insularis* was used for the treatment herbivore + parasitoids. Generalized linear mixed model, $P < 0.001$, control: $n = 24$; herbivore alone: $n = 22$; herbivore + parasitoid: $n = 10$. **b** Damaged mean area per plant by larvae of *Spodoptera latifascia* measured in cm^2. For the herbivore + parasitoid treatment, larvae were in presence of females of the parasitoid *Euplectrus platyhypenae*. We used a generalized linear mixed model. $P = 0.039$, control: $n = 15$; herbivore alone: $n = 16$; herbivore + parasitoid: $n = 16$. **c** Mean number of trifolia per plant (three leaflets) produced at the end of the season. Mixed linear model, $P = 0.022$, control: $n = 15$; herbivore alone: $n = 16$; herbivore + parasitoid: $n = 16$. Error bars show standard error of the mean, and different letters indicate statistically significant differences ($P < 0.05$)

Plant growth, time to flowering and pod production

Control plants (without herbivores) produced on average a lower number of trifolia throughout the season, followed by plants from the herbivore + parasitoid treatment, and the highest number of trifolia was produced by plants from the herbivore alone treatment (Fig. 2c, $F_{2,23} = 4.55$, $P = 0.022$). When compared to control plants, plants from the treatment with only herbivores overcompensated as a result of herbivore damage (producing more trifolia than control plants), while plants with both herbivores and parasitoids produced the same number of new trifolia as the control plants.

The first flowers were produced in December, 8 weeks after sowing and the first green pods appeared after 9 weeks. Plants continued to produce flowers and green pods until the end of January. There were not significant differences in the time of flowering ($F_{2,23} = 2.83$, $P = 0.079$) and pod production ($F_{2,23} = 0.2$, $P = 0.81$) among plants from the three treatments. Likewise, no difference was found in total number of flowers ($F_{2,23} = 0.38$, $P = 0.69$) and pods ($F_{2,21} = 0.71$, $P = 0.5$) produced by plants from the different treatments (Online Resource 1a, b).

Seed output and seed traits

Although no differences were found in the total number of seeds produced by plants from the three herbivory treatments at the end of the season (Fig. 3a, $F_{2,22} = 0.35$, $P = 0.71$), highly significant differences were found in the mean number of seeds per plant produced early in the season (during the four first weeks of seed production) (Fig. 3b, $F_{2,22} = 6.14$, $P = 0.008$). During this period, plants with only *S. latifascia* larvae produced significantly fewer seeds than control plants and plants with herbivores and parasitoids (almost 80 and 40% fewer seeds, respectively).

Seeds produced by plants from the control treatment were on average significantly heavier than seeds from plants with only herbivores and plants with both herbivores and parasitoids (Fig. 4a, $F_{2,445} = 5.44$, $P = 0.005$), and no significant differences were found between the two latter treatments. No significant differences were found in germination success of seeds produced by plants from the three treatments (Fig. 4b, $F_{2,92} = 0.23$, $P = 0.79$). Because we detected great variation in the time of seed production among individual plants in all treatments, we performed an additional analysis to examine the relationship between time of seed production and seed mass within each treatment. Within each treatment, plants that produced seeds earlier, produced on average larger seeds than plants that produced seeds late in the season (control plants: $F_{1,6} = 44.84$, $P < 0.001$; herbivore alone: $F_{1,8} = 13.14$, $P = 0.006$ and herbivore + parasitoids: $F_{1,6} = 7.21$, $P = 0.036$, Online Resource 2).

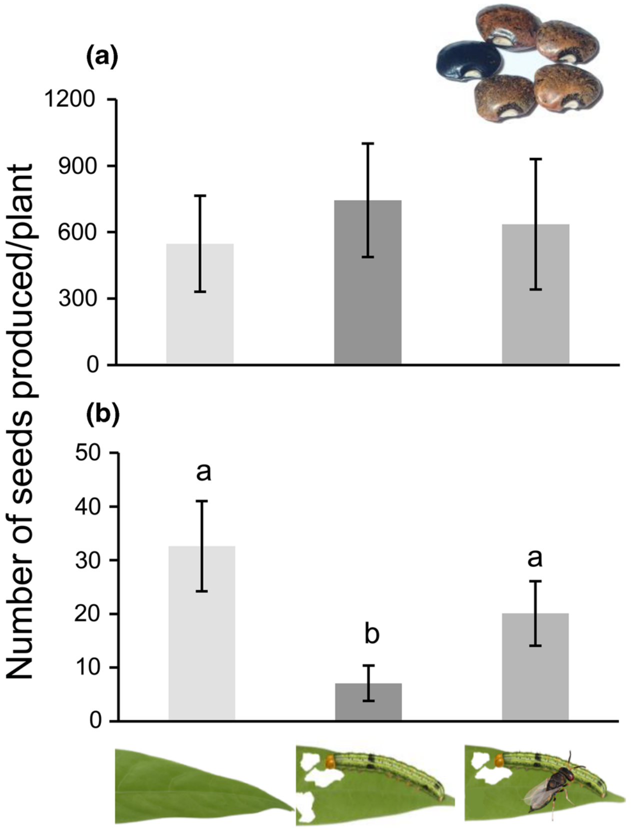

Fig. 3 Mean number of seeds produced per plant **a** for the whole season ($P = 0.71$), and **b** during the first 4 weeks of seed production ($P = 0.008$). Generalized linear mixed models were used for the two variables, and their sample size was the same: control: $n = 15$; herbivore alone: $n = 16$; herbivore + parasitoid: $n = 14$. Error bars indicate standard error of the mean. Different letters indicate statistically significant differences ($P < 0.05$)

Discussion

The overall results from this study reveal that parasitoids can influence plant responses to herbivore damage in different ways. Independent of their life history strategy, solitary (*C. insularis*) or gregarious (*E. platyhypenae*), the two parasitoid species help to reduced leaf damage caused by *Spodoptera latifascia*, and as a result plant compensatory growth was attenuated. Plants exposed to herbivores only overcompensated for the loss of leaf tissue (more trifolia produced compared to control plants), whereas plants in the presence of parasitoids (second experiment) compensated for tissue damage (no difference in number of trifolia compared to control plants). In addition, we found that when plants were in the presence of parasitoids, more and heavier seeds were produced earlier in the season compared to plants that were attacked by caterpillars alone.

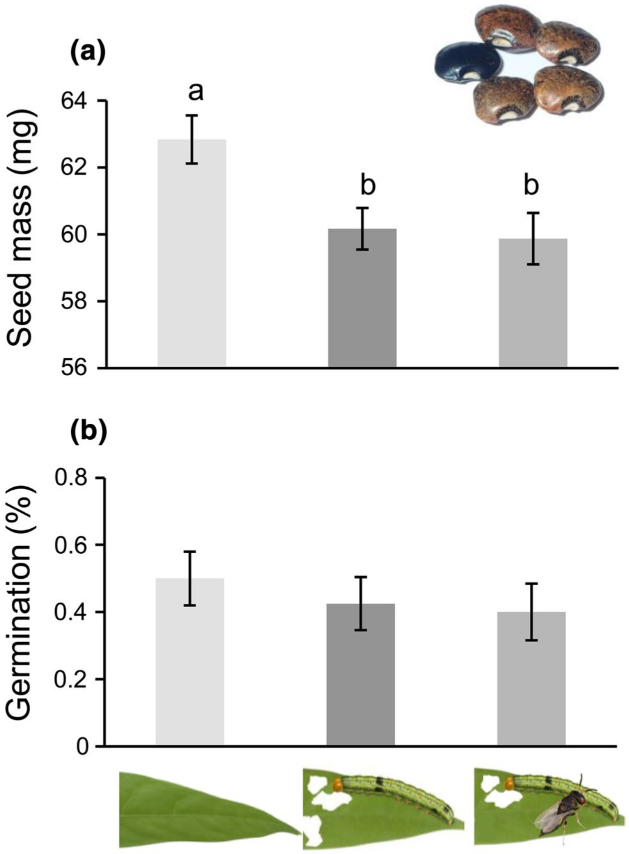

Fig. 4 **a** Mean mass, and **b** mean germination success of seeds produced by plants exposed to the three herbivory treatments. A linear mixed model was used for seed mass: $P = 0.005$, control: $n = 149$; herbivore alone: $n = 160$; herbivore + parasitoid: $n = 140$. For germination, a generalized linear mixed model was used: $P = 0.79$, control: $n = 40$; herbivore alone: $n = 40$; herbivore + parasitoid: $n = 35$. Error bars indicate standard error of the mean. Different letters indicate statistically significant differences ($P < 0.05$)

Parasitoid effects on herbivory

Koinobiont parasitoids, allow their hosts to continue to feed on the plant before they die (Askew and Shaw 1986; Harvey 2005) and, therefore, may not always have positive effects on plants. This is especially true for some gregarious parasitoids that sometimes even cause their host to feed more (Coleman et al. 1999; Van der Meijden and Klinkhamer 2000; Xi et al. 2015). Indeed, we found that reduction on herbivore damage was greater in the presence of the solitary *C. insularis* than of the gregarious *E. platyhypenae* (Fig. 2a). Similarly, Gols et al. (2015) found that plants of *Sinapis arvensis* suffered less damage when larvae were parasitized by either the gregarious *Cotesia glomerata* or the solitary parasitoid *Hyposoter ebeninus*.

Herbivore and parasitoid effects on plant compensatory growth

Herbivory by *Spodoptera latifascia* caused the lima bean plants to produce more trifolia, an overcompensation response that is commonly found in many plants (Strauss and Agrawal 1999). Compensatory growth in lima bean plants in response to natural herbivore damage (Moreira et al. 2015; Hernandez-Cumplido et al. 2016b) and mechanical damage (Blue et al. 2015) was known, but this is the first time that we observed overcompensation. These findings confirm that this response may be context-dependent such that it could vary depending on the amount of damage (Mauricio et al. 1993; Koptur et al. 1996) and the feeding mode of the herbivore (Manzaneda et al. 2010; Utsumi et al. 2013; Moreira et al. 2015).

Our results support this idea coined by Lucas-Barbosa et al. (2016) that plant tolerance responses following herbivory and mortality due to parasitoids are complementary factors that may benefit plants when faced with specialist herbivores. We show that the two are interlinked. One way in which parasitoids may influence the induced regrowth response is simply by reducing the amount of tissue damage. However, we did not find a clear correlation between the amount of plant damage and plant regrowth (Online Resource 3). It may be that this relationship is not linear and that at some level of damage plants can no longer compensate. For example, Blue et al. (2015) found that while a moderate amount of mechanical damage (33% of leaf area removed) inflicted on lima bean plants resulted in compensation, larger amounts of damage (66% of leaf area removed) significantly decrease fruit number and seed mass. Similarly, compensatory growth after artificial damage in the herbaceous biennial *Gentianella campestris* has been found to be the highest for plants that suffered intermediate levels of damage (Juenger et al. 2012).

Alternatively, parasitized larvae may induce a different response in plants than unparasitized larvae, both in terms of direct (Ode et al. 2016) and indirect defenses (Fatouros et al. 2005; Poelman et al. 2012). The oral secretions of the caterpillars may be affected by parasitization (Poelman et al. 2012). That factors in oral secretions are important for plant growth responses was shown by Korpita et al. (2014), who found that tomato plants that were mechanically damaged and then treated with regurgitate of *Manduca sexta* had more regrowth than plants damaged and treated with water. It is, therefore, well possible that the patterns of herbivore-induced plant regrowth that we observed are the result of the combined effect of loss of leaf tissue, and differential physiological reactions to attacks by unparasitized or parasitized larvae. Studies are underway to examine this hypothesis.

Herbivore and parasitoid effects on plant reproduction and seed traits

Even though control plants suffered considerably less damage than plants from the two herbivory treatments, at the end of the season the herbivore-treated plants had fully caught up and the total number of seeds that they produced was the same as for control plants. These findings agree with a large body of literature showing that moderate amount of damage allow plants to fully recover (Mauricio et al. 1993; Koptur et al. 1996; Blue et al. 2015; Moreira et al. 2015). Yet, early in the season control plants and plants from the herbivore + parasitoid treatments produced more seeds than plants from the herbivore alone treatment (Fig. 3b). We were surprised by these results as in other studies with this same system, but a different herbivore (adult beetles), we found that plants exposed to either herbivores or mechanical damage flowered and produced seeds earlier than control plants (Hernandez-Cumplido et al. 2016a, b). Variables such as the type, time and frequency of damage, but also considerable environmental variation among years, may all have contributed to the differential plant responses. For example, the relevant field season of 2014 experienced an "El Niño" event, with more frequent rains and colder mean temperatures during the winter months (corresponding to our field season) (CONAGUA 2014).

The observed differences in time to reproduction and seed size can have important ecological and evolutionary consequences for plant populations and plant–insect interactions (Elzinga et al. 2007; Brody et al. 2007). Plants that flower relatively early or late typically receive less damage from flower and seed herbivores than plants that flower at the peak flowering period (Johnson et al. 2015). This could be very important for lima bean, as its seeds are frequently consumed by larvae of bruchid beetles (Alvarez et al. 2006; Aebi et al. 2008, Shlichta et al. 2014). These beetles start appearing in the field as pods mature in early January, and their densities build up as the season progresses reaching a peak towards the end of January (Hernandez-Cumplido et al. 2016a, b; Bustos et al. unpublished data). Any shift in the timing of seed production may have important consequences for the exposure to seed predators.

We found that seeds from control plants were in general heavier than seeds produced by plants exposed to the two herbivory treatments (with and without parasitoids) (Fig. 4a). This appeared to be mainly due to the fact that, within each treatment, seeds produced early in the season (first 4 weeks) were heavier than seeds produced later in the season (last 4 weeks) (Online Resource 2). We could speculate that by producing more seeds earlier in the season, plants exposed to herbivores + parasitoids produced on average heavier seeds than plants with caterpillars alone. Larger seeds may have a selective advantage during adverse conditions (Leishman et al. 2000), can better tolerate predispersal seed predation (Mack 1998), and can improve seedling vigor and competitive ability (Moles and Westoby 2004). Nevertheless, larger seed size may not always be advantageous. In lab and field studies, we have consistently found that seed beetles lay more eggs on larger seeds, in which they perform better (Campan and Benrey 2006; Zaugg et al. 2013; Hernandez-Cumplido et al. 2016b).

Overall impact of parasitoids on plant fitness

One of the unexpected but most interesting results from this study was that, in the absence of parasitoids, herbivores trigger an overcompensation response (i.e. more trifolia were produced by herbivore-damaged than by control plants). This runs against the general assumption that the action parasitoids may benefit plants. Indeed, only a handful of studies suggest that parasitoids may positively affect plant fitness (Gomez and Zamora 1994; Van Loon et al. 2000; Hoballah and Turlings 2001; Smallegange et al. 2008; Gols et al. 2015), and only three of these studies were conducted under (semi-)field conditions. Gomez and Zamora (1994) found that by excluding parasitoids from fruits of *Hormathophylla spinosa*, Brassicaceae, the incidence of damage by seed weevils was increased and seed production significantly decreased. Hoballah and Turlings (2001) found that maize plants produced more seeds when attacked by parasitized caterpillars than plants attacked by unparasitized caterpillars. Finally, in a recent study, Gols et al. (2015) showed in an outdoor garden experiment that the fitness of *Sinapsis arvensis* (Brassicaceae) was significantly increased when *Pieris brassicae* larvae were parasitized by two parasitoid species. Our study adds to this scarce field evidence on the potential beneficial effects of parasitoids for plant fitness, but also shows that parasitoids may indirectly affect plant compensatory growth in responses to herbivory.

Conclusions and future directions

The beneficial effects of parasitoids on plant performance in natural and agricultural systems have been widely accepted, but the mechanisms underlying these effects, particularly under natural conditions, remain largely underexplored. Our results provide further insight into how the presence of parasitoids can alter the outcome of plant–herbivore interactions. We conclusively showed that the combined effects of the plant's ability to tolerate and compensate for herbivore damage, and the parasitoid-mediated reduction in leaf damage, mitigated the negative effects of herbivory, which ultimately resulted in more and heavier seeds produced earlier in the season. It should be noted that in this study we only looked at one herbivore species and one parasitoid species during one particular time of the season. To fully understand the impact of parasitoids on herbivore-mediated plant responses, it would be necessary to manipulate herbivore pressure and parasitoid presence throughout the entire growing season.

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Author contribution statement BB originally formulated the idea, MACC, JG, JHC and BB designed the experiments, MACC, JG and JHC conducted fieldwork, MACC analyzed the data, MACC and BB wrote the manuscript.

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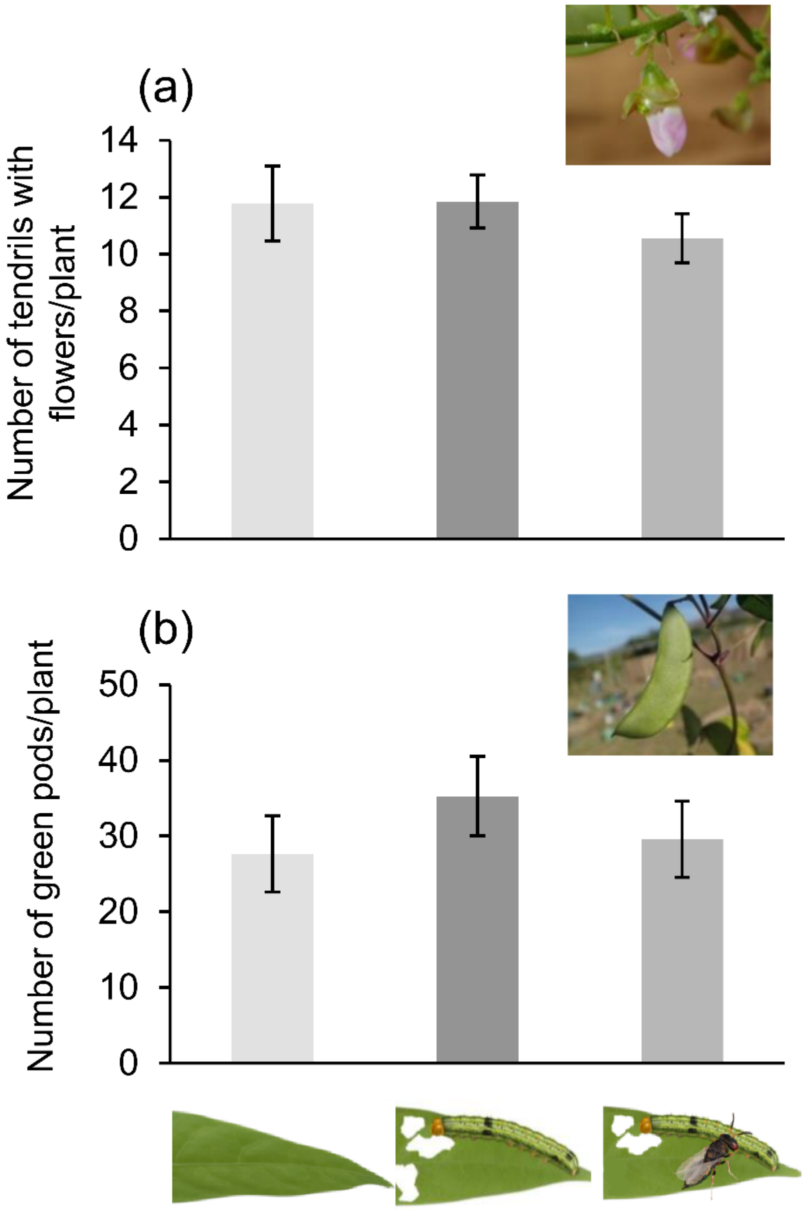
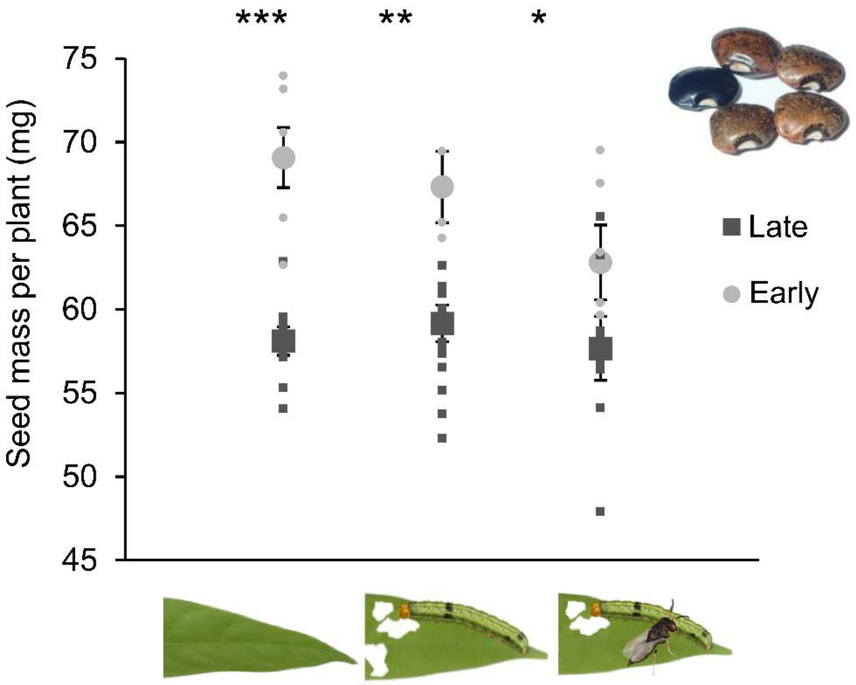
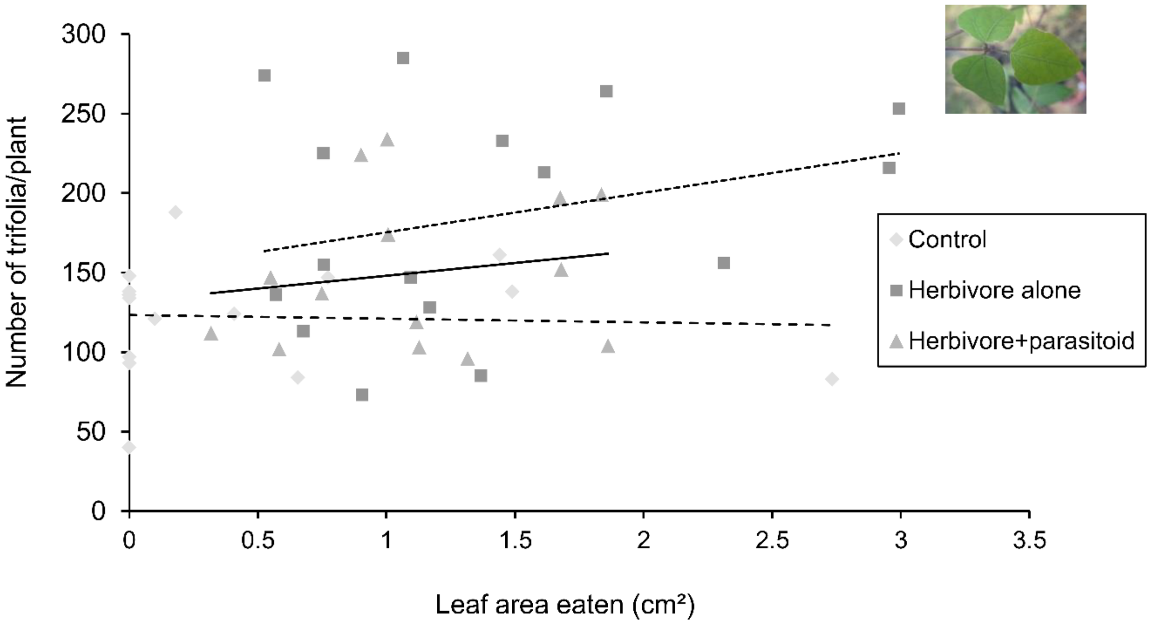
Supplementary material



Online Resource 1 (a) Mean number of tendrils with at least one flower per plant. Linear mixed model, P = 0.69, control: n = 15; herbivore alone: n = 16; herbivore+parasitoid: n = 16. (b) Mean number of green pods produced per plant. Error bars indicate standard error of the mean. Linear mixed model, P = 0.5, control: n = 14; herbivore alone: n = 16; herbivore+parasitoid: n = 14



Online Resource 2 Mean seed mass of seeds produced by plants during the season. Grey circles represent seeds produced during the first 4 weeks of seed production and black squares represent seeds produced during the last 4 weeks of seed production. Linear mixed models were used to compare the seed mass of early and late seeds within each treatment. Control: $P < 0.001$, early: $n = 6$, late: $n = 9$; Herbivore alone: $P = 0.006$, early: $n = 4$, late: $n = 12$; Herbivore+parasitoid: $P = 0.036$, early $n = 5$, late: $n = 9$. Asteriks indicate significant differences within each treatment; ***<0.001, **<0.01 and *<0.05



Online Resource 3 Relationship between the mean number of trifolia per plant and leaf damage (area eaten in cm2) for plants from the three treatments. Pearson correlation tests were used for the three treatments: control: $r = -0.05$, $P = 0.86$, $n = 15$, herbivore alone: $r = 0.29$, $P = 0.28$, $n = 16$, herbivore+parasitoid: $r = 0.18$, $P = 0.57$, $n = 14$

Chapter 4

Top-Down cascading effects of seed predators and their parasitoids on plant responses and early season herbivores

M.A.C. Cuny, D. La Forgia, G. A. Desurmont, G. Glauser and B. Benrey

Abstract

For natural plant populations, pre-dispersal seed predation can have devastating effects on subsequent seedling recruitment and plant fitness. Damaged seeds may still be able to germinate and produce healthy seedlings as long as their reserves are not entirely depleted. In this context, the action of natural enemies such as parasitic wasps that kill seed predators may reduce the impact of seed predation on plant fitness. Here we evaluated the impact of bruchid seed predators on the germination success of wild Lima bean seeds and the subsequent performance and later interactions with other insects of the germinated plants. Moreover, we studied how parasitism of bruchid larvae inside the seeds by parasitoids affected these measures of plant performance. We found enhance germination of bruchid-infested seeds, but reduced chemical resistance of the resulting plants against a generalist herbivore *Spodoptera latifascia*. These bruchid feeding effects were significantly mitigated when larvae in the seeds were parasitized.

This first evidence for season-long beneficial effects of parasitoids of seed-feeding insects on plant performance implies that seeds too may have evolved traits that help to recruit parasitoids, for instance via herbivore-induced volatiles.

Key words: tritrophic interactions, pre-dispersal seed predation, *Phaseolus lunatus*, plant growth, germination, larval performance, cyanogenic glycosides, phenolics

Introduction

Seed-feeding insects have been shown to be key players in the regulation of plant populations (Leimu and Lehtilä 2006, Maron and Crone 2006). Although the effects of pre-dispersal seed predation are in general considered to be negative on plant fitness (Mack 1998, Tomaz et al. 2007, Fox et al. 2010, Tuller et al. 2015, Yirgu and Tsega 2015), few studies have also reported positive effects of seed predators on plant populations (Vallejo-Marin et al. 2006, Fox et al. 2012). For example, Takakura (2002) showed that seeds of the legume *Gleditsia japonica* could only germinate if attacked by the specialist seed beetle *Bruchidius dorsalis*. The authors argue that this is probably the by-product of an evolutionary arms race between the two species. In another study, Vallejo-Marin et al. (2006) artificially damaged seeds from 11 woody tropical species and found that depending on the seed species, damage could have negative or positive effects on germination dynamics. The extent of these potential positive effects will depend on the intensity of damage to the endosperm and on the amount of tissue consumed that serves as the resource for the developing seedling.

Because parasitoids halt host development and ultimately kill their hosts, it can be expected that parasitism on seed beetles would result in reduced seed damage, thereby, increasing seed germination success and seedling performance (Nakai et al. 2011). For instance, Savidan (2002) found that the generalist parasitoid *Anisopteromalus calandrae* significantly reduced the seed weight loss caused by infestations of the seed-feeding insect *Sitophilus zeamais* in maize, although the germination success was not affected. In another example, Martins (2013) found that parasitoids of *bruchidius* sp. increase seed germination success of A*cacia drepanolobium* by interrupting the development of bruchid beetle larvae. Hence, the presence of parasitoids can alleviate the negative effects of seed infestations by reducing seed damage. Yet, the fate of the plant may not be the same once the seed has been damaged.

This beneficial effect of parasitoids on plants has been well studied for parasitoids of leaf-feeding herbivores and it is thought to be one of the reasons why herbivore-damaged leaves emit specific blends of volatiles that attract parasitoids (Gols et al. 2015, Cuny et al. 2018). In this context, previous studies have examined how damage by early-season herbivores can trigger plant responses that affect subsequent herbivores and their parasitoids (Poelman et al. 2008, Poelman et al. 2011, Hernandez-Cumplido et al. 2016), and the consequences of these effects for plant fitness (McArt et al. 2013, Pashalidou et al. 2015, Hernandez-Cumplido et al. 2016)

However, there are no comparable studies that examine how a decrease in damage by seed predators due to the action of parasitoids can affect subsequent plant performance and the herbivores that feed on the resultant plants.

In this study we evaluated the impact of bruchid seed predators on the germination success of wild Lima bean seeds (*Phaseolus lunatus*) and on the subsequent performance and later interactions with other insects of the germinated plants. Moreover, we studied how parasitism of bruchid larvae inside the seeds by parasitoids affected these measures of plant performance.

We tested the hypotheses that seeds that surmount seed predation and are able to germinate, will have fewer resources to allocate to plant growth and defense. As a result, we expect that plants produced by damaged seeds will be less performant and less resistant to herbivores than plants produced by undamaged seeds, and the nature of this outcome may be dependent on the degree of seed damage. In addition, we expect that by halting host development and eventually killing the seed beetle larvae, parasitoids will mitigate the negative effects of seed predation.

This study examines for the first time the potential season-long beneficial effects of parasitoids of seed-feeding insects on plant performance.

Materials and methods

Biological System

Wild lima bean *Phaseolus lunatus* occurs naturally along the Pacific coast of Mexico (Heil 2004, Hernandez-Cumplido et al. 2016). Seeds germinate and produce seedlings during June and July and plants flower at the beginning of October. Seed dispersal is mainly guaranteed by explosive opening of dry pods and seed dormancy is induced by environmental conditions such as temperature and humidity (Degreef et al. 2002). Seeds of *P. lunatus* are attacked by several bruchinae species such as *Zabrotes subfasciatus*, one of the main granivorous pests worldwide (Leroi et al. 1990, Benrey et al. 1998, Shlichta et al. 2014). Adult females lay their eggs on the seed coat and larvae bore into the seed upon hatching where they complete their development (Benrey et al. 1998).

Females can lay several eggs on a single seed and even lay eggs on seeds containing eggs previously laid by other females. The amount and intensity of seed damage will depend on the number of bruchid larvae developing within the seed, and the extent of tissue damage. If the endosperm is not completely damaged or left intact, infested seeds can still germinate (Cuny personal observation). Newly-developed adults bore a whole with their mandibles to exit the seed. Beetle larvae are attacked by hymenopterous ectoparasitoids that lay their eggs on the larval host halting its development (Campan and Benrey 2004). Upon completion of their development, adult parasitoids exit the seed making their own and smaller exit holes.

The velvet armyworm, *Spodoptera latifascia*, is a generalist herbivore naturally present in Mexico, which readily feeds on the leaves of wild lima bean (Cuny et al. 2018). Insects were collected in the field in Mexico (near our field site in Puerto Escondido, Oaxaca) and reared on artificial diet (“beet armyworm diet”, BioServ, Flemington, NJ, USA) at the University of Neuchatel.

Figure 1: Example of seeds used in the lab experiments. A: uninfested seed, B: seed with one beetle exit hole, C: Seed with two beetle exit holes and D: Seed with one beetle and one parasitoid exit holes. Exit holes are designated with the circles.

First laboratory experiment: effects of pre-dispersal seed damage by beetles and their parasitoids on germination and seedling growth.

We did not have enough seeds from only one population to conduct this experiment, so we used four different Lima bean populations, collected in 2013 (YEL: 16°15'03.8"N 97°48'10.0"W, AZUL: 15°53'30.3"N 97°07'04.0"W, ITC: 17°00'40.4"N 100°06'09.9"W and MAR: 16°35'43.9"N 98°46'06.1"W). These populations are located in the state of Oaxaca, near Puerto Escondido and have been used in previous studies (Shlichta et al. 2014, Hernandez-Cumplido et al. 2016). Seeds were taken to the lab and kept in plastic containers until the emergence of granivorous beetles and parasitoids. In cases where several insects develop in the same seed, each beetle or parasitoid (if the beetle was parasitized) leaves its own emergence hole. The holes are smaller for parasitoids, therefore it allows us to easily discern on the identity and number of insects that emerged from each seed (Fig. 1). Before initiating the germination experiment, a sample of seeds with different combinations of insect infestation levels were measured using an electronic digital Vernier caliper (Vogel, Germany) and weighed to the nearest 0.01mg with an analytical balance (Mettler AE163, Switzerland). This was done to determine whether beetles and parasitoids preferentially attacked seeds of a given size, which could bias the interpretation of the results from the experiment (Supplementary material, Fig.1). In addition, the highly significant positive correlation between seed size and weight (Supplementary material, Fig.2) allowed us to use seed weight as a proxy of the quantity of seed eaten in the different infestation treatments.

The germination experiment was performed in order to investigate the effect of pre-dispersal seed insects (beetles and parasitoids) on seed germination as well as germinated seedling performance. First, we selected seeds with different combinations of exit holes: control (no hole), one beetle hole, one parasitoid hole, two beetle holes, one beetle and one parasitoid hole, three beetle holes, two beetle and one parasitoid holes, one beetle and two parasitoid holes. Seeds were then weighed to the nearest 0.1mg in order to quantify the amount of seed mass that remained after seed damage by granivorous beetles. Later, seeds were individually sowed in pots (11cm of height and 4 cm of diameter) with soil (Einheitserde company, profi substrat, Germany) and placed in a room with controlled light, temperature and humidity conditions (LD 16-8, 24°C, 35% R.H). Plants were checked daily and watered every other day. For each plant, we recorded the time to germination and germination success, the time to production of the fourth trifolium, and aboveground fresh biomass (shoot separated from the root and weighed to the nearest 0.1mg soon after the plants produced their fourth trifolium). Finally, we collected the seeds that did not germinate and determined if they were healthy or decomposed. In total, we used 218 seeds (the number of replicates per treatment varied from 10 to 43 depending on seed availability).

We did not record the species identity of the beetles and parasitoids that had emerged from the seeds used in these experiments. However, from our studies in previous years, we are confident that granivorous insects were all bruchinae beetles, mainly *Zabrotes subfasciatus* and parasitic wasps were mainly *Stenocorse bruchivora*, a specialist on *Z. subfasciatus* (personal observation, Campan and Benrey 2004, Hernandez-Cumplido et al. 2016).

Second laboratory experiment: effects of pre-dispersal seed damage by beetles and their parasitoids on plant chemistry and on the subsequent performance of the leaf herbivore Spodoptera latifascia.

We conducted a second experiment to determine the effect of seed damage on the resistance of germinated plants against leaf chewing insect. We collected enough seeds from one population in January 2017 to perform this experiment (AZUL: 15°53'30.3"N 97°07'04.0"W). Seeds were kept in plastic containers until insect emergence and they were then selected for different combination of seed insect holes. The following combinations of seed insect infestation were selected, based on their availability: control (no exit holes), one bruchid hole, one parasitoid hole, two beetle holes, one beetle and one parasitoid hole, three beetle holes, one beetle and two parasitoid holes, three parasitoid holes.

To increase germination success, we slightly scratched the seed coat of all control seeds by removing about 0.5mm² of seed coat with a fine point forceps, before sowing all the seeds under the same conditions as in the previous experiment. The experiment started as soon as all the plants had grown at least one trifolium (composed of three leaflets). Second instar larvae (*S. latifascia*) were weighed to the nearest 0.01mg and placed on one leaflet (of the second or third trifolium, depending on plant growth) using plastic clip cages (3cm diameter, covered with mesh nylon screen (Shlichta et al. 2018)) to prevent them from leaving the leaf. Larvae were left on the plants for 48 hours, after which they were weighed again, in order to calculate their relative growth rate ((ln(weight2)-ln(weight1))/days). Immediately after removing the larvae, two undamaged leaflets of the same trifolium were collected and drown in liquid nitrogen. They were then stored at -80°C until chemical analyses.

For each sample we quantified the concentrations of the two main cyanogenic glycosides present in lima bean (linamarin and lotaustralin) and phenolics (total flavonoid compounds) using the same protocol as used in previous studies (Shlichta et al. 2014, Moreira et al. 2015a, Cuny et al. 2018). Samples were first ground using cold mortars and pestles. Approximately 0.02 mg of thin powder was put in screw cap tubes, and 1ml of 70% methanol was added. Samples were heated at 90° for 10 min and placed in an ultrasonic shaker (Branson 2210) for 15 min. Then the tubes were centrifuged at 14000 rpm for 10 min and the supernatant was transferred to a new tube. Finally, each sample was separated in two for each chemical compound to extract (CNGs and phenolics). CNG samples were diluted at 1:5 for the leaves and 1:50 for the seeds. No dilution was done for phenolic samples, which are less concentrated than CNGs. An Acquity ultra-high pressure liquid chromatography (UPLC) system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, MA, USA) was used to measure chemical defensive compounds (CNGs and phenolics).

Field experiment: effects of pre-dispersal seed damage by seed beetles and their parasitoids on seed germination, plant growth and reproduction and on leaf herbivory.

We conducted experiment to determine the fate of plants produced by damaged seeds in a natural field setting. This experiment was performed in an open field site located 15km northwest of Puerto Escondido (Oaxaca, Mexico, 15°55'27.9"N 97°09'04.3"W). Due to seed availability, we used seeds from two wild Lima bean populations in this experiment (AZUL: 15°53'30.3"N 97°07'04.0"W and INK: 15°43'28.1"N 96°39'11.3"W). Treatments consisted of undamaged seeds and seeds with one beetle exit hole. The unavailability of seeds with different degrees of bruchid and parasitoid infestation did not allow us to replicate all treatments used in the laboratory experiment.

Three seeds were sown in individual pots (biodegradable pots, 8*8 cm) for a total of 19 pots per treatment, and were kept in a field cage to protect plants from unwanted herbivory. After recording seed germination, only one plant was allowed to grow in each pot. Plants were watered every other day. Two weeks later, plants were transplanted into the soil, in a common garden seeting outside the cages, where they were exposed to natural populations of herbivores. Plant size (total number of trifolia) and leaf damage (percentage of damaged leaves, calculated as the number of damaged leaves divided by the total number of leaves and multiplied by 100) were recorded one month after the transfer of the plants to the common garden. Later in the season, we individually recorded the date of the production of the first flower when seed pods were dry and ready to pop seed were collected and counted.

Statistical analyses
Data were analyzed using the SAS statistical package (ver. 9.4, SAS Institute, Cary NC, Littell et al. 2006). When residuals were normally distributed, we used linear mixed models (PROC MIXED) while generalized linear mixed models (PROC GLIMMIX) were used whenever residuals were not normally distributed (Bolker et al. 2009). Tukey tests for pairwise comparisons were used after significant results. The different combinations of seed infestation were treated as a fixed effect. In the models analyzing data with seeds from different lima bean populations, "population" was treated as a random factor. For data of the second lab experiment on herbivore relative growth, quantity of plant consumed and plant defensive compounds, the trifolium was used as a random factor.

Laboratory experiments Linear mixed models were used for seed size and weight, plant production time of the fourth trifolium, herbivore relative growth rate and phenolic compounds, and generalized linear models were used for proportion of germinated seeds, germination speed, biomass and CNG compounds. For the proportion of germinated seeds, we used a binomial distribution. A poisson distribution was used for germination speed (discrete data), whereas gamma distributions were used for plant shoot biomass and CNGs concentration (continuous data) (Zuur et al. 2009). Finally, a logistic regression was used to investigate the association between seed weight and proportion of germination in infested seeds (excluding undamaged seeds), using seed weight as the independent variable and seed germination (0 = seed did not germinate; 1 = seed did germinate) as dependent variable (JMP12 statistical package, $\alpha = 0.05$).

Field experiment We ran linear mixed models testing for the effect of seed infestation on germination speed, leaf herbivory (percentage of damaged leaves) and flowering time. In contrast, the proportion of germinated seeds, the total number of trifolia and the total number of seeds were analyzed using generalized linear models. We used poisson distributions for plant size (number of trifolia) and total amount of seeds produced (discrete data). A binomial distribution was used for the proportion of germinated seeds.

Results

Seed weight - laboratory
We found significant differences in seed weight according to the infestation status of the seeds ($F_{7,207} = 14.47$, $P < 0.001$). Uninfested seeds and seeds attacked by three beetle larvae were the heaviest and lightest, respectively, compared to all other treatments (fig. 2A). Each additional beetle larvae inside the bean had an effect on seed weight. Seeds with three beetle larvae were lighter than seeds with two, which were in turn lighter than seeds with one larva. The presence of parasitoids did not have any

significant effect on seed weight when only one beetle developed in it. However, when two or three beetles attacked the seeds, the presence of one parasitoid was enough to significantly reduce seed mass loss. Indeed, when three beetle larvae developed inside the seed, seeds with parasitoids were on average 20% heavier than seeds without parasitoids.

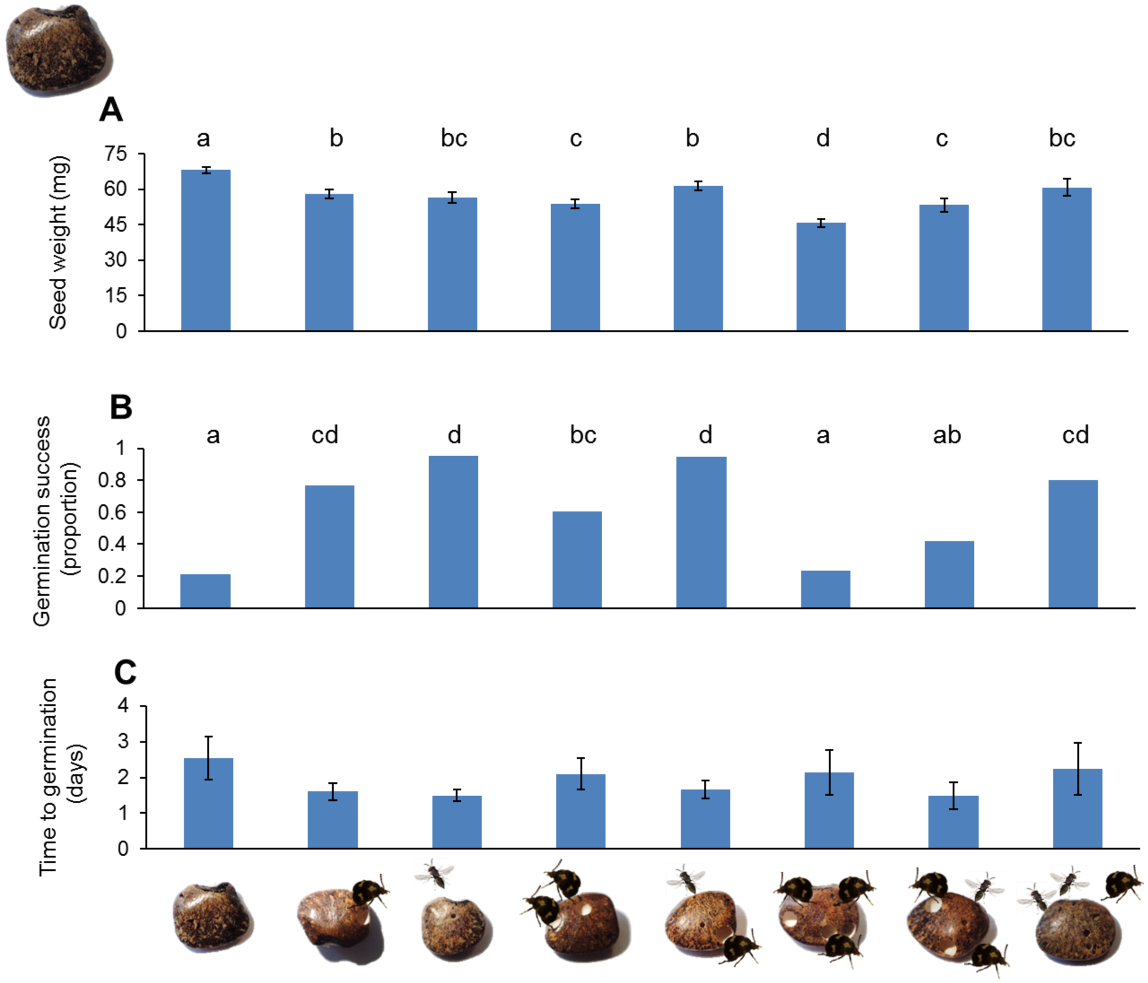


Figure 2: Laboratory experiment. A: Mean weight (+ SEM) of non-infested seeds and seeds infested with different combinations of insects (bruchid beetles and parasitoids). Insect symbols correspond to the number of adults that emerged from the seeds. Number of replicates: n= 43,43,21,33,19,30,19,10, respectively to the X axis treatments. B: Proportion of *P. lunatus* seeds that successfully germinated according to their infestation treatments. Number of replicates: n= 42,43,21,33,19,30,19,10, respectively to the X axis treatments. C: Mean time (+ SEM), measured in days, between seed sowing and germination. Number of replicates: n= 9,33,20,20,18,7,8,8, respectively to the X axis treatments. Different letters indicate statistically significant differences (P < 0.05).

Germination success and speed - laboratory

Overall, germination success (proportion of germinated seeds) significantly increased with seed infestation ($F_{7,206} = 7.41$, $P < 0.001$) (Fig. 2B). Seeds infested by one and two beetles had a germination success 3.6 and 2.8 times respectively, higher than undamaged seeds. However, seeds attacked by three healthy beetles, or by two healthy and one parasitized beetle did not germinate better than uninfested seeds. Parasitoid presence had only a marginal positive effect ($P = 0.086$) on germination for seeds attacked by one beetle. Yet, parasitoids significantly increased germination success when one out of two beetles was parasitized, as well as when two out of three beetles were parasitized (germination success 1.5 and 3.5 times higher, respectively), compared with seeds infested by two and three healthy beetles.

No differences were found in the time to seed germination among the different infestation treatments, despite a trend for a faster germination for seeds with a low infestation density ($F_{7.112} = 1.05$, $P = 0.4$) (fig. 2C). Finally, among the 94 seeds that did not germinate (61 infested and 33 uninfested) the recovery rate of uninfested seeds at the end of the experiment was 100%. However, we could not recover any of the infested seeds. These seeds were undoubtedly dead and decomposed in the soil. Consequently, we assume that the mortality of non-germinated infested seeds was 100%.

Effect of seed consumption on germination rate - laboratory

The probability that a seed did not germinate was significantly reduced when the weight of infested seeds was increased ($\chi^2 = 32.0$, $R^2 = 0.15$, $P<0.001$, Fig. 3). This result shows a direct relationship between the amount of seed damage and the probability of germination.

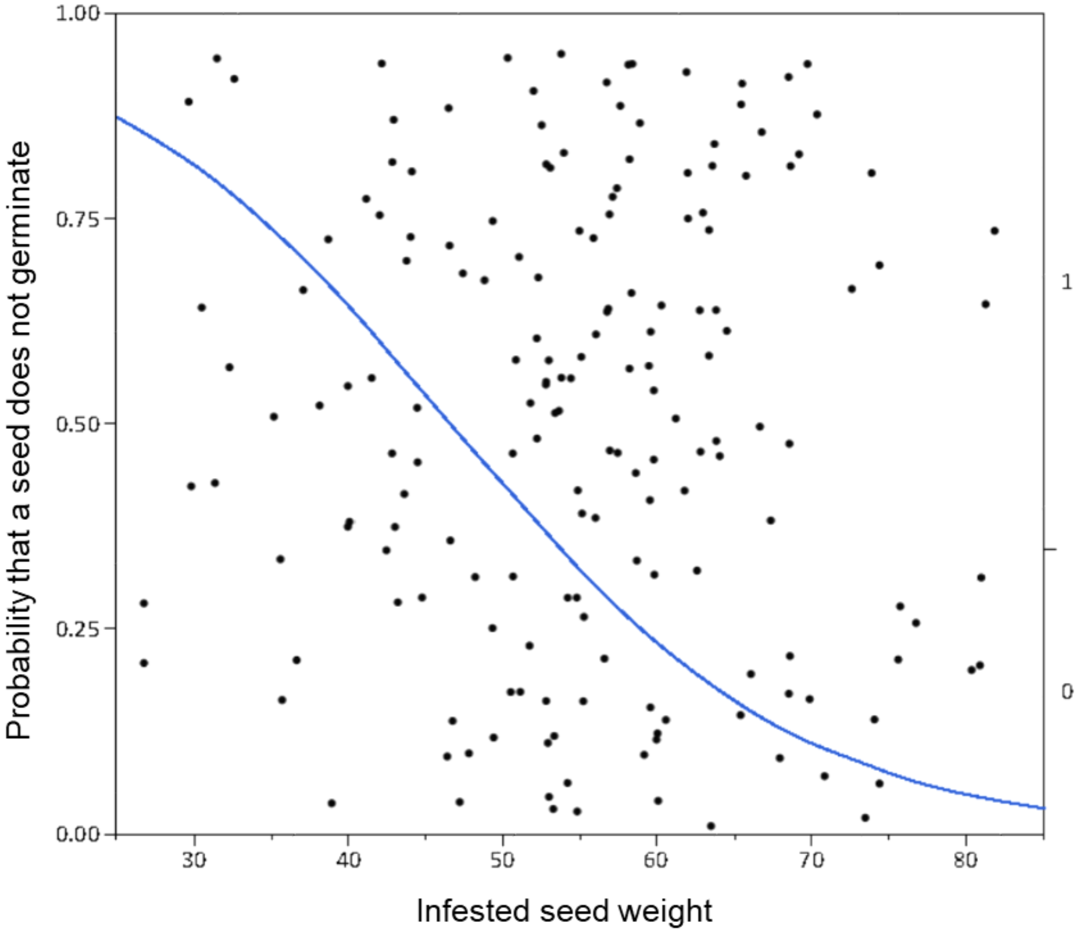


Figure 3: Laboratory experiment. Logistic regression between seed weight and their probability to not germinate (right Y-axis: 0 = no germination, 1 = germination) only for seeds infested by at least one insect.

Plant growth – time of appearance of the fourth trifolium – laboratory

Plants from uninfested seeds grew significantly faster than plants from the other treatments ($F_{7,81}$ = 7.11, $P < 0.001$) (Fig. 4A). Specifically, the length of time between seed germination and the appearance of the fourth trifolium for plants from seeds infested by one or three beetle larvae was slower (17% to 40% respectively) than for plants from uninfested seeds. Interestingly, plants from seeds infested by one parasitized beetle grew as fast as plants from uninfested seeds. When three beetles developed in the seeds, the presence of two parasitoids significantly increased the rate at which plants grew (15% faster).

Seedling mass – laboratory

Shoot fresh mass at the time of production of the fourth trifolium was significantly higher for plants from uninfested seeds compared to most of the other treatments ($F_{7,81}$ = 7.87, $P < 0.001$) (Fig. 4B). For example, shoots of plants from seeds attacked by one or three healthy beetles weighed 30% and 50% respectively, less than plants from uninfested seeds.

As it was the case for seedling development, shoot mass of plants from seeds with one parasitized beetle larva was not significantly different compared to plants from uninfested seeds. Parasitoids significantly increased the shoot mass of plants from seeds infested by two beetles (24% heavier). Concerning treatments with three beetle larvae, parasitoids did not significantly affect the plant’s shoot mass.

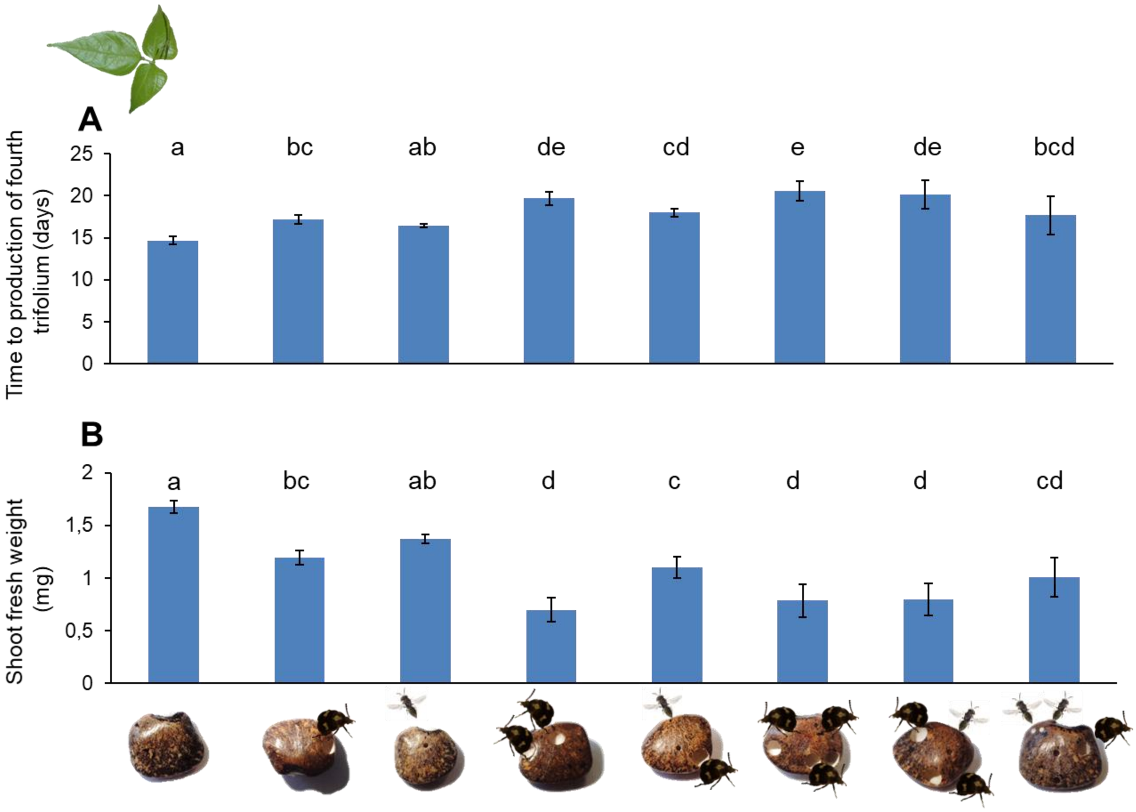


Figure 4: Laboratory experiment. A: Average development time of seedlings, individually measured from seed germination until the production of the fourth trifolium (mean ± SEM). Number of replicates: n= 9,27,19,7,13,5,6,6, respectively to the X axis treatments. B: Fresh shoot weight (mean ± SEM) of plants grown from seeds with different insect infestation treatments. The measurements were done just after plants produced their fourth trifolium. Number of replicates: n= 9,27,19,7,13,5,6,6, respectively to the X axis treatments. Different letters indicate statistically significant differences ($P < 0.05$).

Herbivore performance – laboratory

The infestation of Lima bean seeds had a significant positive impact on the subsequent performance of larvae of *S. latifascia* ($F_{7,128} = 2.33$, $P = 0.0283$). When lima bean seeds were infested by three healthy beetles, the relative growth rate of second instar larvae was significantly improved by 25%, compared to uninfested seeds. In contrast, intermediate levels of beetle infestation and parasitoid presence had similar effects on larval performance (Fig. 5). No significant effect was found on the quantity of leaf eaten by the larvae ($F_{7,128} = 2.05$, $P = 0.053$).

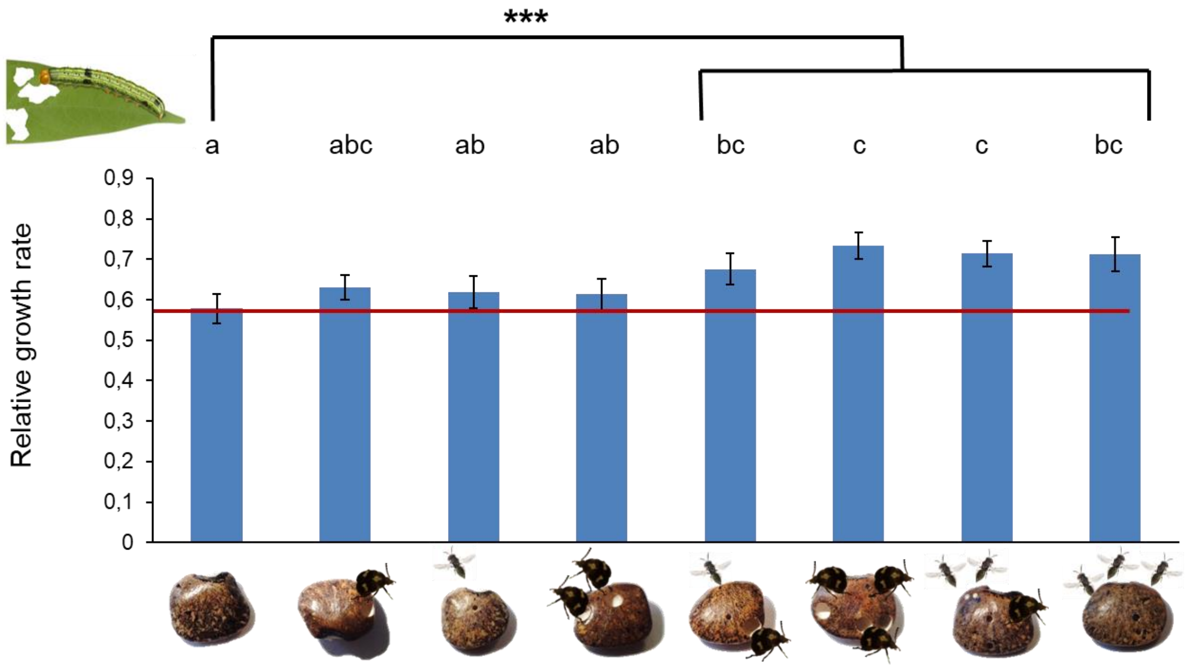


Figure 5: Laboratory experiment: mean relative growth rate (± SEM) of *Spodoptera latifascia* larvae fed on wild lima bean leaves germinated from undamaged (control) or damaged seeds with parasitized or unparasitized beetles. The red line shows the mean relative growth rate for control treatment (plant germinated from undamaged seeds). *S. latifascia* that fed on control plants grew significantly less than larvae fed on the 4 last treatments. Number of replicates: n=22,21,21,19,20,11,13,10, respectively to the X axis treatments. Different letters as well as asterisks indicate statistically significant differences ($P < 0.05$).

Concentration of CNGs and phenolic compounds – laboratory

Leaves from seeds previously infested with at least two bruchids had a lower concentration of CNGs and phenolic compounds (about 65% and 45 % lower for CNGs and phenolics, respectively) than leaves from control seeds (linamarin: $F_{7,71} = 2.16$, $P = 0.048$; lotaustralin: $F_{7,68} = 2.48$, $P = 0.025$, phenolics: $F_{7,71} = 3.06$, $P = 0.007$) (Fig. 6). However, no differences were found among treatments with or without parasitized beetles.

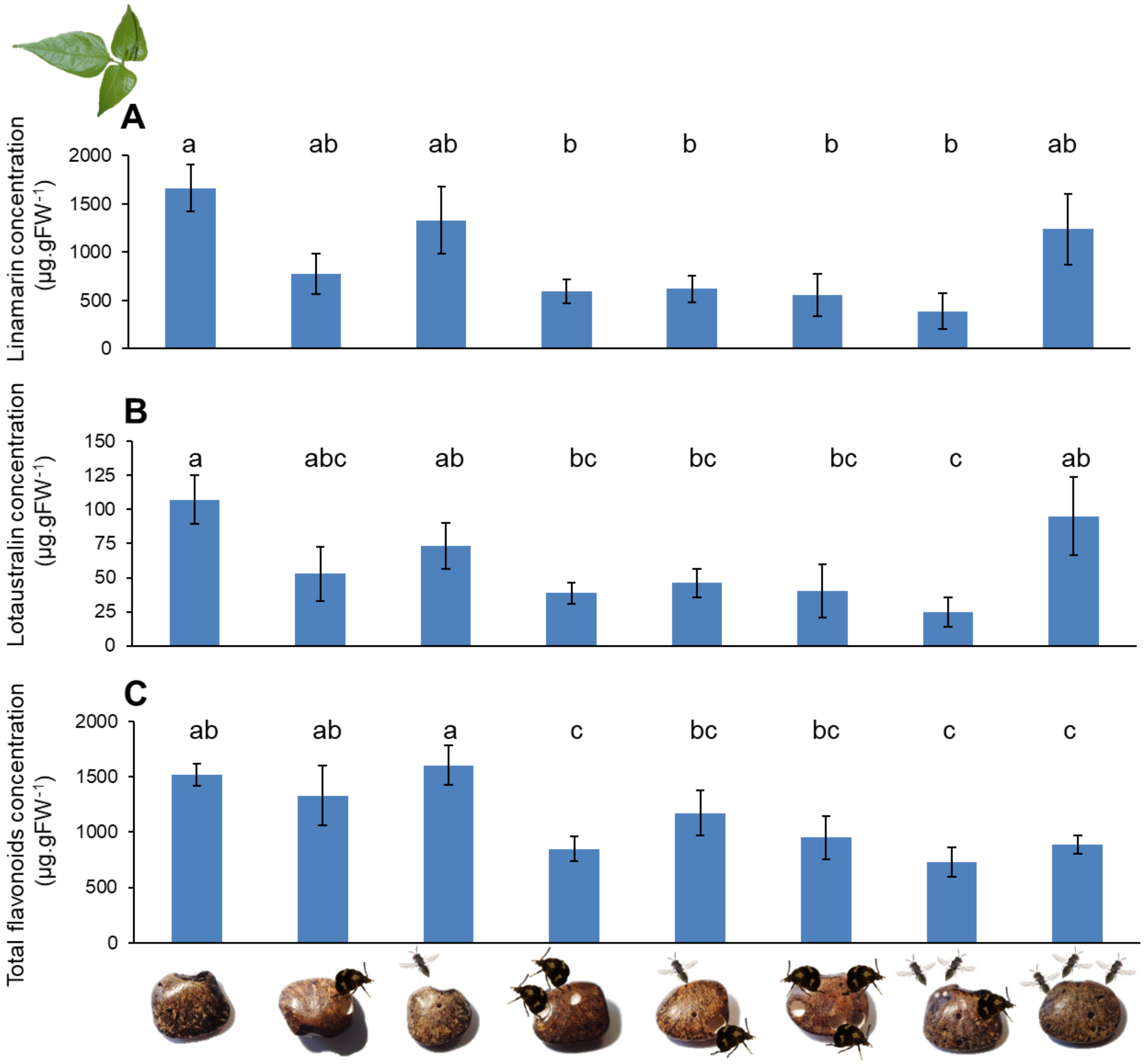


Figure 6: Laboratory experiment: mean (± SEM) concentration in A: linamarin, B: lotaustralin and C: phenolics in trifolia of wild lima bean plants germinated from damaged and undamaged seeds. Number of replicates for linamarin: n=11,10,10,11,10,10,11,8, for Lotaustralin: n= 11,9,10,11,10,9,10,8 and for phenolics: n= 11,10,10,11,10,10,11,8, respectively to the X axis treatments. Different letters indicate statistically significant differences ($P < 0.05$).

Germination success and speed – field

Germination success (proportion of germinated seeds) in the field was not affected by seed damage ($F_{1,141} = 0.03$, $P = 0.86$) (Fig. 7A). However, we found a significant effect of infestation on the germination speed ($F_{1,46} = 12.42$, $P = 0.001$) (Fig. 7B). Damaged seeds germinated 20% faster than uninfested seeds.

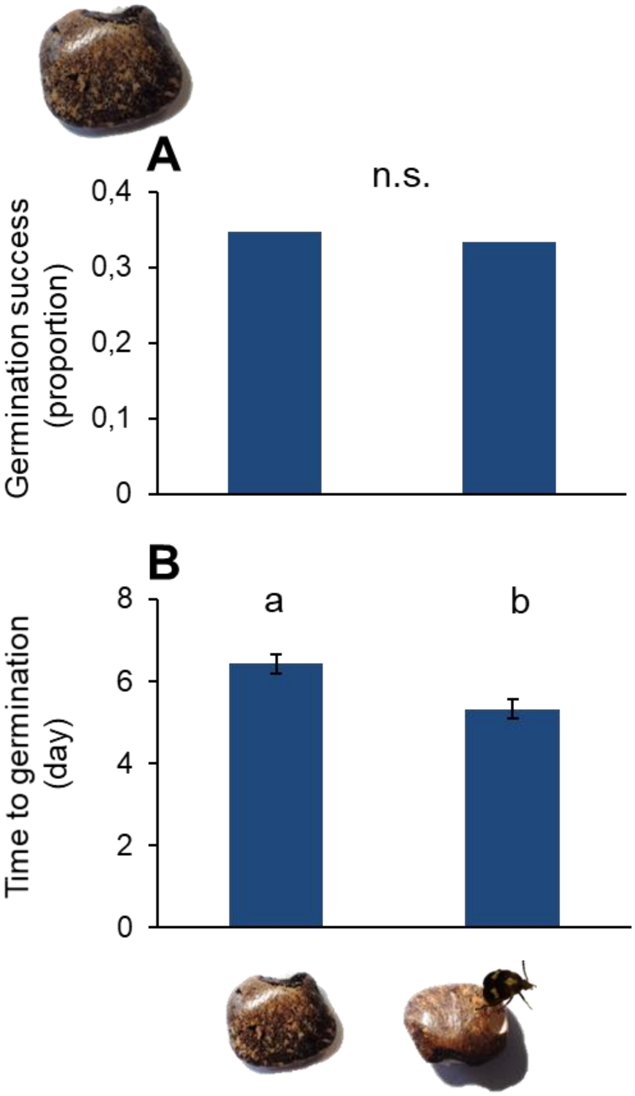


Figure 7: Field experiment. A: Proportion of seeds that successfully germinated. Number of replicates: n = 72 for each treatment. B: Mean (± SEM) time between seed sowing and germination. Number of replicates: n = 25 for uninfested seeds and 24 for damaged seeds. Different letters indicate statistically significant differences ($P < 0.05$).

Number of trifolia – field

Plants from seeds attacked by one bruchid beetle produced on average 25% fewer trifolia than plants from uninfested seeds ($F_{1,36} = 23.06$, $P < 0.001$) (Fig. 8A).

Leaf herbivory – field

The percentage of leaves damaged by leaf-chewer herbivores was doubled for plants produced by damaged seeds compared to plants from control, undamaged seeds (10% vs 22%, respectively) ($F_{1,36} = 16$, $P < 0.001$) (Fig. 8B).

Reproductive phenology – field

Plants from undamaged seeds produced flowers 30% times earlier than plants from damaged seeds ($F_{1,31} = 6.14$, $P = 0.018$) (Fig. 8C).

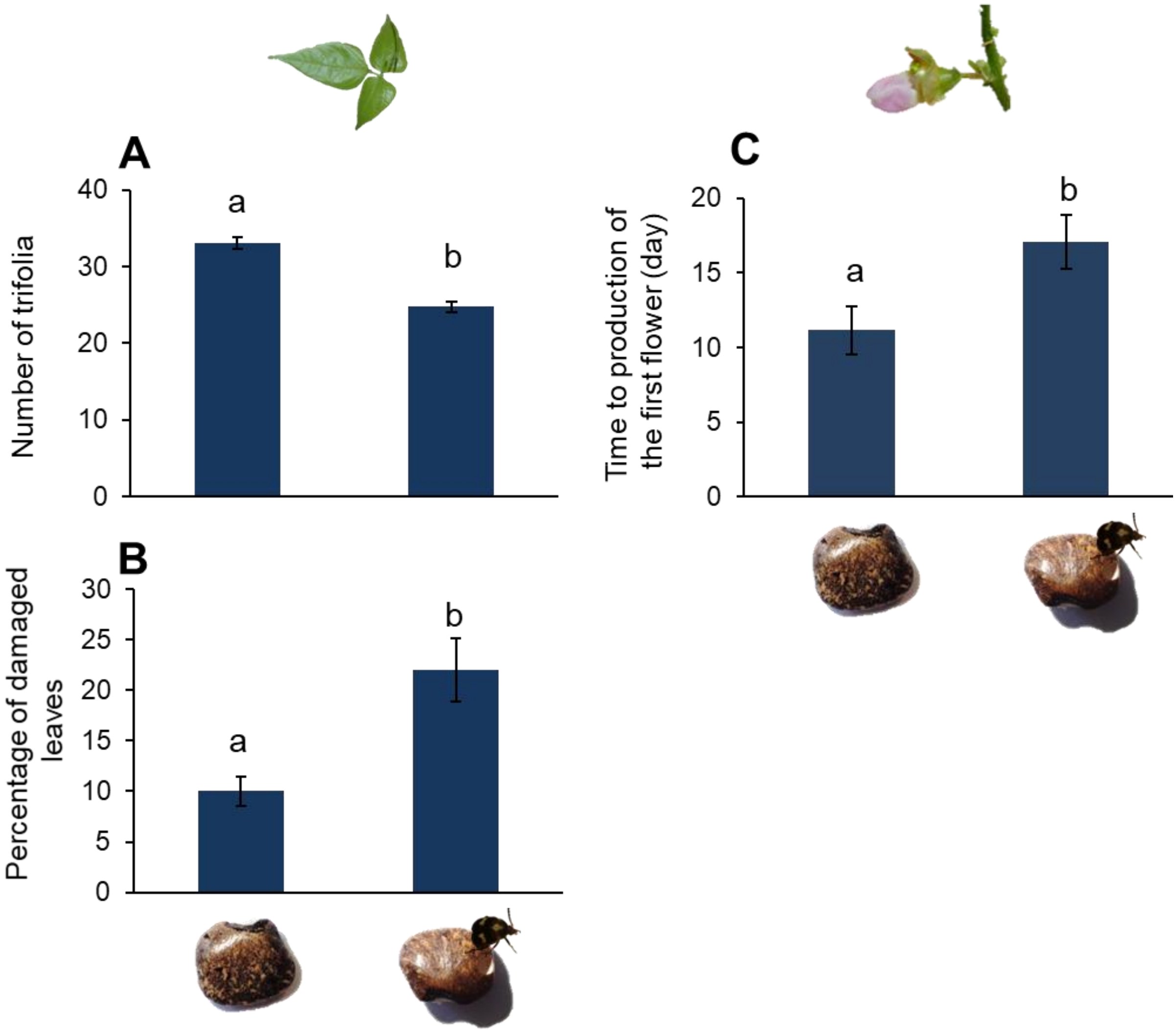


Figure 8: Field experiment. A: Mean (+ SEM) number of trifolia per plant. Number of replicates: n= 21 for plants from uninfested seeds and 19 for plants from damaged seeds. B: Average percentage of damaged leaves per plant (number of damaged leaves / total number of leaves). This measurement was repeated twice during the season, the number of survey was added as fixed factor in the model, and the individual plants as a random factor. Number of replicates per survey: n = 21 for plants from uninfested seeds and 19 for plants from damaged seeds. C: Average number of days (+ SEM) per plant for the production of their first flower. Day counting started with the first plant producing its first flower. Number of replicates: n = 18 for plants from uninfested seeds and 16 for plants from damaged seeds. Different letters indicate statistically significant differences ($P < 0.05$).

Number of seeds – field

The total number of seeds produced per plant at the end of the season was not significantly different between plants germinated from control or damaged seeds, but a slight trend can be observed as plants from undamaged seeds produced on average 10% more seeds ($F_{1,31} = 0.03$, $P = 0.86$) (Supplementary material, Fig. 3).

Discussion

Overview

Our study revealed strong positive and negative effects of pre-dispersal seed predation and parasitoid attack on seed germination and plant performance in wild lima bean. Low levels of seed infestation enhanced germination, but this positive effect did not continue during plant growth. In contrast, high levels of seed infestation by bruchid beetles resulted in lower germination success and poor plant performance. In the lab, germination success of infested seeds was directly correlated with seed weight, indicating that the impact of herbivory on seed germination is indeed threshold-dependent. Most importantly, the presence of parasitoids had a clear positive impact on seed survival and seedling performance: parasitoids overall reduced seed weight loss due to granivory, and mitigated the negative effects of bruchid beetles on plant performance. In other words, we showed that parasitoids positively affect lima bean plants at two levels: the fitness of the mother plant (germination rate of infested seeds improved) and the performance of the seedling (faster development and increased shoot biomass). In addition, seed damage also altered the chemical resistance of seedlings. Plants produced by damaged seeds had lower content of CNGs and phenolic compounds which most likely lead to the higher growth rate of the leaf herbivore on these plants.

In the field, we confirmed that seed damage improved germination but negatively impacted plant growth. In addition, we showed that granivory by bruchid beetles affected leaf herbivores feeding on the plant that germinated from damaged seeds, which have, to our knowledge, never been shown before. The field study allowed us to go further than the laboratory experiment, showing that the negative impact of bruchid beetles on seedling performance also impacted plant reproductive traits such as flower production, although plants were able to fully compensate for seed production, probably due to the relatively optimal growing conditions (water ad libitum and no plant competition).

Bottom-up effects of pre-dispersal seed predation on germination and plant traits

Seed weight loss due to granivory was directly proportional to the number of beetles infesting the seed as previously reported by Fox et al. (2012). In addition, the weight of infested seeds and germination success were directly correlated. Yet, germination success of uninfested seeds was significantly lower than seeds attacked by one or two beetles, and did not differ from seeds attacked by three beetles, despite the lower weight of infested seeds due to granivory, when tested in laboratory.

These results indicate that 1) the presence of holes in the seed coat facilitates seed germination and compensate for the negative impact of seed weight loss (Degreef et al. 2002, Takakura 2002, Baskin and Baskin 2004) and 2) damage by more than two beetles greatly reduces the viability of seeds. This suggests that germination success is directly linked to the amount and type of tissue (embryo and cotyledon) eaten by granivorous beetle larvae. From our data, we have no way of knowing the relative amount eaten of these two tissues, although it can be expected that higher amount of damage increases the chances of damage to the embryo, which makes a seed unviable. Similarly, other studies found that seed germination can be increased by bruchid predation, but reduced with more beetles attacking the seed (Fox et al. 2012, Nakai et al. 2011).

We did not find any effect of beetle damage on the speed of germination in laboratory. However, when tested in the field, we found that infested seeds germinate faster than uninfested, but we did not find any difference in the germination success.

The discrepancy in these results may be due to different conditions between laboratory and field (soil, light, temperature, humidity…). However, the trend is still the same because beetle infestation at low levels acted positively on seed germination by accelerating it, which has been shown to be an important trait for plants (Verdú and Traveset 2005).

This mechanism can be explained by a facilitated turgescence of infested seeds, in which water can easily enter and break dormancy. To confirm this hypothesis, we placed 40 lima bean seeds in wet cotton after slightly scratching half of them, and found a significant three-fold increase in seed germination rate in the scratched group (Supplementary. material. Fig. 4).

Degreef et al. (2002) showed that water can only enter in dormant *P. lunatus* seeds through the hilum, which opens only under certain environmental conditions (high temperature and humidity). In lima bean and legumes in general, the impermeability of the seed is very important for the induction of seed dormancy. In their study they manually clipped the seed coat in *P. lunatus* and compared their germination success with healthy seeds. Clipped seeds germinated about 25% more than non-clipped ones, showing that the introduction of water into the seed is one of the main factors triggering germination (Degreef et al. 2002). Takakura (2002) found a similar result by mechanically damaging seeds of the legume *Gleditsia japonica*. Seed germination is a good proxy of plant fitness, because it directly measures the number of viable offspring (Moreira et al. 2015a). In some systems, pre-dispersal seed predation is sometimes necessary for germination to occur (Fox et al. 2012, Takakura 2002). However, germination represents only one of a suite of plant traits that are likely to influence plant fitness.

In our laboratory experiment, we found that both plant development and shoot biomass were negatively affected by beetle infestation. These negative effects were significantly higher for seeds infested by two or three beetles compared to seeds infested by one beetle, which confirms the density dependent effect of beetles in seeds. Plant growth speed can be an important trait as it can give a competitive advantage and counteract the negative impact of herbivores. For instance, Allcock and Hik (2004) found that plants with faster growth mitigated the negative effects of herbivores and that many seedlings die due to overgrowth by surrounding vegetation. Shoot biomass is also a very important plan trait, often used as a proxy for plant fitness, with the assumption that larger plants will produce more or larger seeds (Vega et al. 2000). In the field experiment, we found similar and strong results. Indeed, plants from infested seeds had 25% fewer trifolia than plants from undamaged seeds, even if only one beetle developed in those seeds. Thus, despite facilitating seed germination, the ultimate effect of beetle granivory for Lima bean plants may be detrimental. Most of the few other studies that have assessed seedling performance following seed predation by granivorous insects also found negative effects (Mack 1998, Mateus et al. 2011, Fox et al. 2012, Nikolova 2016). Our study not only adds to this knowledge but also reveals that the negative effects of seed predation on plant fate depend on a threshold set by a combination of the intensity of seed damage and the action of parasitoids that attack the seed beetles inside the seeds.

Top-down effects of pre-dispersal seed predation on subsequent plant resistance and herbivore performance

In our second laboratory experiment, we found strong evidence that damage by seed beetles resulted in an increased herbivore performance on plants produced by these seeds. Larvae of *S. latifascia* grew better (relative growth rate increased by 25%) when feeding on a plants whose seeds had been attacked by three healthy bruchid beetles, compare to uninfested seeds. Furthermore, we found a dramatic reduction in CNG (-65%) and phenolic (-45%) compounds in leaves from damaged seeds. This is all the more important as CNGs and phenolics in Lima bean plants have been shown to be important defensive chemical compounds against herbivorous insects (Ballhorn et al. 2009, Moreira et al. 2015a, Shlichta et al. 2018). These results were confirmed by our field experiment, in which we found that plants from damaged seeds suffered twice more damage from generalist chewing herbivores (120% more) than plants from undamaged seeds.

One plausible explanation for the observed pattern may be that bruchid damage reduced the resources available for the seedling, thus affecting plant growth and compromising the biosynthesis of defensive compounds. This type of complex plant-mediated interactions between two insects of different guilds feeding on the same plant but on different plant tissues and at different times has been rarely studied (but see, McArt et al. 2013, Hernandez-Cumplido et al. 2016). Moreover, the few studies that we found focused on how early-season leaf damage affects late-season insects that attack the plant’s reproductive structures (McCall and Karban 2006, McArt et al. 2013). For instance, a recent study on the same system showed that seeds produced by plants that had suffered damage by leaf herbivores were less attacked by bruchid beetles (Hernandez-Cumplido et al. 2016). Here we show that the cascading effects of herbivory can take place in the opposite direction as it has been previously shown, whereby plants produced by damaged seeds were less defended and endured more damage than plants produced by healthy seeds.

Effects of parasitoids on seed traits, plant growth and herbivore performance

The presence of parasitoids in the seeds was overall beneficial for the plant. Seeds with parasitoid exit holes were less damaged, germinated more and faster than seeds with only beetle exit holes. It appears that parasitoids significantly reduced the amount of seed tissue eaten by beetle larvae. However, this was only the case for seeds infested by two or three beetles, with at least one of them parasitized and not for seeds infested by only one beetle. This may be due to non-consumptive effects (Thaler and Griffin 2008) of parasitoids whereby their foraging affects the behavior and development of non-parasitized beetle larvae that ate less when they perceived the wasp walking on the seed (Zaugg et al. 2013, Cuny et al. unpublished data). Our findings agree with work on other systems that showed that the presence of parasitic wasps resulted in reduced consumption of seed tissue by weevils (Savidan 2002, Nakai et al. 2011, Martins 2013). The presence of prasitoids also increased seed germination success for highly damaged seeds. More seeds with parasitoid exit holes germinated (3.5 times) compared to seeds with high levels of beetle infestation (three beetle exit holes).

Moreover, at the end of the experiment, we checked the 94 seeds that did not germinate and recovered 0% of the 61 infested seeds compared to 100% of the 33 uninfested seeds. This suggests that in nature infested seeds that do not immediately germinate are decomposed in the soil in less than three months. Seed beetle damage has been shown to increase the risk of mortality due to pathogen or fungal infection (Chang et al. 2011, Mateus et al. 2011). Hence, by improving infested seeds germination success, parasitoids indirectly affect plant fitness by saving a significant number of seeds. The beneficial effects of parasitoids on the seeds continued after seed germination. Plant performance was about 10% (growth) and 35% (shoot mass) higher for plants produced by beetle-infested seeds attacked by parasitoids than those from seeds infested with only beetles. Our results are in accordance with a study with the pea *Pisum sativum* (Nikolova 2016), in which they found that *P. sativum* seeds with beetle (*Bruchus pisorum*) emergence holes had lower germination, growth and seed production than control undamaged seeds and seeds with a parasitoid emergence hole. Conversely, Mateus et al. (2011) found no difference in the germination rate and seedling growth between seeds (*P. sativum*) damaged by parasitized and unparasitized beetles (*Bruchus pisorum*). It is clear however, the need for more studies that measure the impact of parasitoids of seed beetles on plant fitness.

Finally, contrary to the results found for plants from seeds infested with only beetles, the presence of parasitoids in the seeds did not alter plant chemistry, nor the growth of the generalist caterpillar. Unfortunately, due to the lack of seeds, we were not able to test the effect of parasitoid-infested seeds in the field, yet based on the strong evidence found from the lab experiments we believe that parasitoid-infested seeds will also have improved germination and seedling growth in natural conditions. But this remains to be shown.

Ecological and evolutionary consequences of beetle and parasitoid effects on seed germination and plant performance

The first consequence of beetle infestation in the seed is the break of dormancy followed by the initiation of the germination process. Under certain conditions early and faster germination can be advantageous. For example, in areas or periods where precipitation and humidity are low, seeds that germinate faster can have a competitive advantage over slower-germinating seeds (Hyatt and Evans 1998). Fast germination can also reduce post-dispersal seed predation by both invertebrate and vertebrate predators (Vallejo-Marin et al. 2006). Finally, early and fast germination can reduce the incidence of fungal and pathogen attack (Dalling et al. 2011, García-Guzmán and Heil 2014).

Thus, little or moderate seed infestation as a result of low beetle damage and to the action of parasitoids could even be beneficial for lima bean seeds. On the other hand, an early termination of dormancy could be disadvantageous, specifically, when dormancy allows germination to be delayed until environmental conditions are suitable for plant growth. Indeed, the uninfested seeds that did not germinate during our experiment and were later recovered were still able to germinate in the lab, whereas attacked seeds that did not germinate were most likely rotten in the soil (Mateus et al. 2011).

Thus, our results have important implications for the demography and population dynamics of wild Lima bean plants. The accumulation of healthy seeds in the soil contributes to the seed bank, which ensures the persistence and long-term survival of a plant species in an ecosystem (Alexander and Schrag 2003). A study conducted in natural populations of Lima bean in Costa Rica (Degreef et al. 2002), showed that factors that affect the size of the seed bank for example, unusual weather conditions could have dramatic consequences for future colonization and plant population densities. Therefore, seed predation would very likely affect the persistence of seeds and reduce the seed bank recruitment. In previous studies we have reported relatively high levels of seed infestation which are both spatially and temporally dynamic (Alvarez et al. 2006, Zaugg et al. 2013, Moreira et al. 2015b). In such situations, seed predators and parasitoids could play a significant role in driving bean plant population dynamics.

This study is the first to demonstrate that the effect of beetles feeding on a seed can cascade up to the herbivores that feed on the plant produced by the same seed. Further, when the beetles are attacked by parasitoids, there are top-down effects that improve seed performance.

Finally, although in this study we used seeds from wild bean populations, our findings have significant applied implications. Beans are the most important grain legumes for human consumption in the world (Broughton et al. 2003). In Mexico, where this study was conducted, beans comprise a major food staple providing together with maize, the basic diet for the majority of the population. The outcome of the current study confirms the important role of the third trophic level in protecting beans and other grain crops, and strategies that conserve and enhance the presence of parasitoids in bean cultivation should be strongly encouraged.

Conclusions

Our study is among the first to test the combined effect of different densities of granivorous beetles and parasitoids on seed traits and subsequent plant performance (growth and chemical defense). We have shown that despite the initial positive effects on seed germination, negative effects of seed predation can appear at later stages of plant development. Moreover, our study revealed the effectiveness of parasitoids in reducing the negative impact of granivory and thus positively affect plant fitness. We also showed that seed predation altered plant chemical defense and hence resistance against leaf herbivores. Our findings provide new insights into the complex consequences of multitrophic interactions and on the frequently debated effect of parasitoids on plant fitness.

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Supplementary material

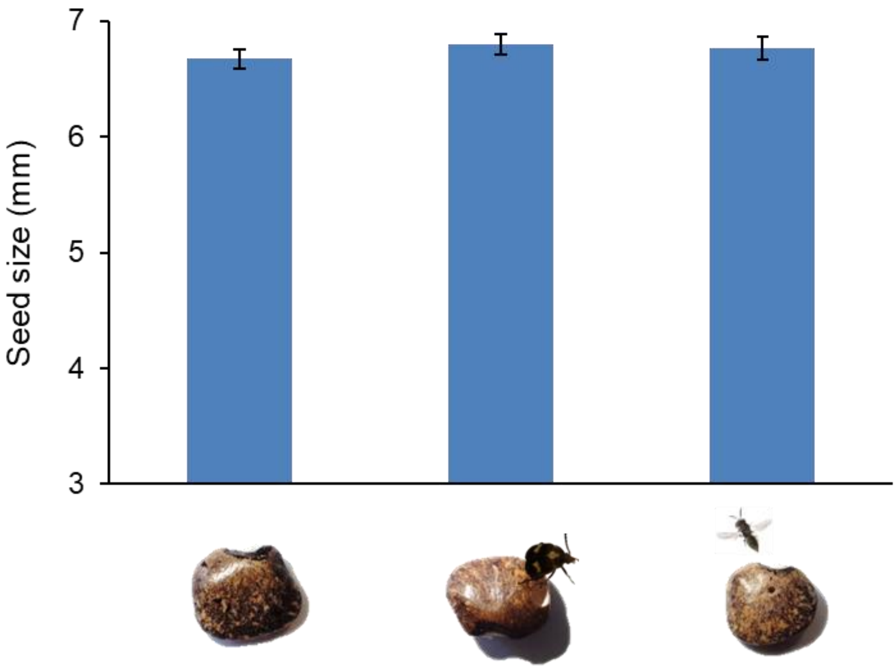

Figure 1: Seed size of field-collected Lima beans (mean ± SEM). X-axis symbols indicate: no insect infestation, seeds infested by healthy beetles and seeds infested by a combination of healthy beetles and at least one parasitized. Number of replicates: n= 62,90,35, respectively to the X axis treatments. No significant difference in the size of the seeds with different infestation combination was found ($F_{2,179}$ = 1.31, p-value=0.27).

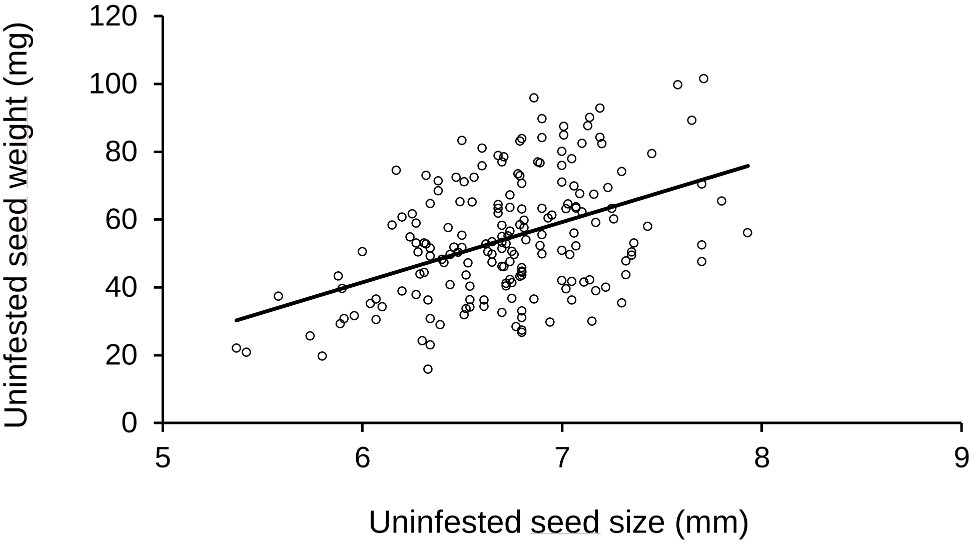

Figure 2: Significant positive correlation between seeds weight and size of infested seeds. r (pearson coefficient) =0.44, p-value < 0.001. Number of replicates: n =175.

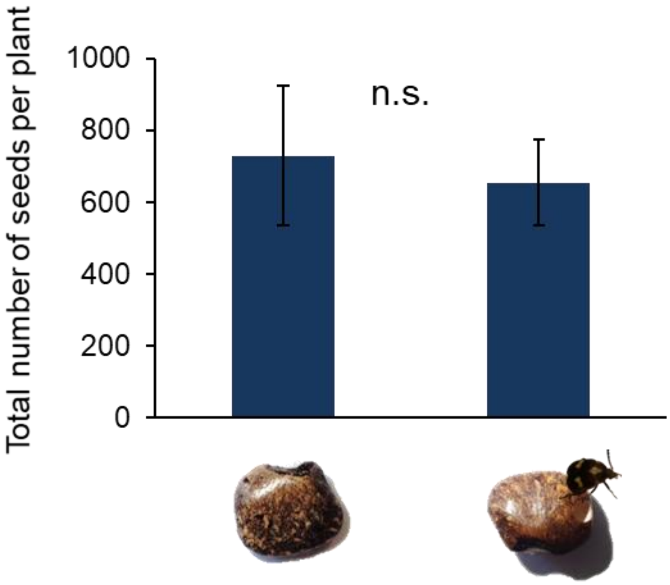

Figure 3: Means (+ SEM) of the total number of seeds produced by plants grown from uninfested and damaged seeds in the field experiment in Mexico. No significant difference was found ($F_{1,31}$ = 0.03, P = 0.86). Number of replicates: n=17 for the two treatments.

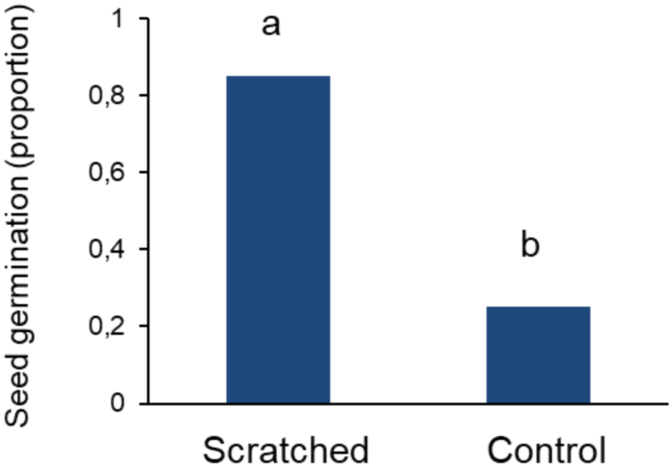

Figure 4: Mean proportion of germination success of wild lima bean seeds (slightly scratched or undamaged) in wet coton ($X^2_{1,38}$=15.55, P<0.001). n=40 seeds. This experiment was performed to confirm that a hole in the seed coat facilitates seed turgescence and germination.

Chapter 5

Host density and parasitoid non-consumptive effects on the performance of a seed beetle and an ectoparasitoid on seeds of wild Lima bean, *Phaseolus lunatus*

M.A.C. Cuny, J. Traine, C. Bustos-Segura, G. Glauser and B. Benrey

Abstract

For a seed beetle, the two most important factors that affect their development and survival are the seed in which they develop and the natural enemies that attack them. Because seed beetles complete their development inside a single seed they are often subject to strong intraspecific competition. Parasitoids will also influence their performance and survival through consumptive and non-consumptive effects.

Here, we used seeds of two natural populations of wild Lima bean, *Phaseolus lunatus*, to examine the impact of larval density of the bruchid beetle *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae), on bruchid and parasitoid performance. In addition, we also investigated consumptive and non-consumptive effects of parasitoids on beetle performance.

Results showed that an increase in larval beetle density inside the seed had a negative effect on bruchid performance. These effects cascaded up to the third trophic level and strongly reduced the parasitism rates. Parasitoid presence dramatically reduced bruchid emergence, influenced their development time and had an overall negative effect on female bruchid weight. The performance of bruchids and parasitoids was different according to the bean population and in an opposite way, which can be explained by seed size.

Key words: tritrophic interaction, seed predation, *Zabrotes subfasciatus*, *Stenocorse bruchivora*, parasitism rate

Introduction

Among the numerous ecological factors that affect the performance and survival of seed beetles, are the seed in which they develop and the natural enemies that attack them. Seed beetles lay their eggs on the seed coat and upon hatching the larvae burrow inside the seed where they complete their development and are confined to this close space (Toquenaga and Fujii 1991). Several chemical traits of the seed are known to affect seed beetle performance, such as nutritional quality and defensive compounds (Nwanze and Horber 1976, Moraes et al. 2000, Campan and Benrey 2006, Moreira et al. 2015).

Seed physical characteristics can also be very important for the performance of seed beetles. Particularly, seed size is directly linked with the total amount of resource available for the developing larvae of seed beetles (Oliveira et al. 2015, Cuny et al. 2017). This is all the more important when several larvae are developing in the same seed, leading to competition for resources (Denno 1995, Kaplan and Denno 2007). This biological system is ideal for the study of competition as bruchids often oviposit more than one egg per seed, often affecting their adult weight, mortality and development time (Oliveira et al. 2015, Cuny et al. 2017).

Also because they cannot escape by leaving the seed or hide, seed beetle larvae are often attacked by parasitoids (Benrey et al. 1998, Campan et al. 2005, Moreira et al. 2015) which results in the death of the beetle larvae after parasitism. Alternatively, parasitoids can indirectly impact their host's performance, through their sole presence, usually by induction of host defensive behaviors (Gross 1993, Lima 1998). These types of predator non-consumptive effects are less studied than direct effects, especially for seed beetles and parasitoids, although they are believed to be very important in shaping ecological interactions (Morrison 1999, Preisser et al. 2005, Thaler and Griffin 2008, Zaugg et al. 2013). For instance, Fill et al. (2012) tested non-consumptive effects of an aphid parasitoid (*Aphidius colemani*) on non-hosts pea aphids (*Acyrthosiphon pisum*). They found that because of an increase in defensive behavior of pea aphids (dropping from the plant), the parasitoids presence significantly reduced their population growth, without parasitism.

Reciprocally, parasitoid's performance can be affected by their host. Host traits responsible for changes in parasitoid performance have received considerable attention in the past decades (Godfray 1994, Harvey 2005, Harvey and Malcika 2016). For instance, parasitoids may benefit from a weaker immune system of their host or a slower development, that could prolong the host's vulnerability (Dushay and Beckage 1993, Benrey and Denno 1997, Turlings and Benrey 1998). Parasitoid performance can also be negatively affected when herbivores sequester toxic chemical compounds from plants (Opitz and Müller 2009). Similarly, changes in herbivore size can affect parasitoid's performance, depending on their larval development strategies (i.e. idiobiont or koinobiont). In the case of idiobiont parasitoids, which usually paralyze their host and prevent them from feeding and growing, the host size is generally positively correlated with parasitoid offspring size (Harvey 2005). Conversely, koinobiont parasitoid hosts continue to feed and grow during parasitoid development. Hence, the relationship between the host size and koinobiont parasitoid offspring is much more complex (Harvey 2005).

The interactive effects of larval competition and the action of parasitoids have seldomly been studied together (but see Kraaijeveld and Godfray 1997, Kraaijeveld et al. 2001). In addition, very little is known on parasitoid non-consumptive effects on their hosts.

In the present study, we used the bruchid *Zabrotes subfasciatus* (Coleoptera: Chrysomelidae), to examine the impact of beetle larval density inside the seed, on bruchid and parasitoid performance. In addition, we also investigated consumptive and non-consumptive effects of parasitoids on beetle performance. To do this, we determined bruchid and parasitoid development, survival (parasitism rate for parasitoids) and the weight and sex ratio of emerging adults in seeds with either one or two beetle larvae, in presence of absence of parasitoids

Material and Methods

Study system

The bruchid beetle *Zabrotes subfasciatus* is one of the most important pests of stored beans worldwide (Benrey et al. 1998). This species is multivoltine and in its native range in southern Mexico and Central America, it attacks wild and cultivated species in the genus Phaseolus, among them, *Phaseolus lunatus,* Lima bean (Zaugg et al. 2013). Females enter the mature pod through a hole in order to glue eggs on the seed coat (Campan and Benrey 2006). Few days later, larvae pierce the seed coat and burrow into the seed, where they will complete their entire development until adult stage (Campan and Benrey 2006). Several eggs can be laid on the same seed, but in the field we mostly find one or two eggs per seed (Cuny, personal observation). Because larvae cannot leave the seed before completing their entire development, they often face strong intraspecific competition (Oliveira et al. 2015, Cuny et al. 2017).

Stenocorse bruchivora is one of the main parasitoids of *Z. subfasciatus* (Campan and Benrey 2004). This solitary ectoparasitoid attacks the third and fourth instar beetle larvae and can lay up 67 eggs throughout their lifespan (Benrey et al. 1998). This is an idiobiont (it paralyzes its host before parasitism, preventing it from feeding and growing), and because its host range is limited to a few species, it is considered a specialist (Hetz and Johnson 1988).

Seed infestation by bruchids

Previous studies with this bean species showed great variation among different populations in the chemical profiles of beans and seeds (Shlichta et al. 2014). To account for this variation, in our experiments we used seeds from two natural populations (BLO: N16 35.732 W98 46.102, YEL: N16 15.071 W97 48.169, see Shlichta et al. 2014). Seeds were collected in 2012 and transported to the lab where they were stored in a cold incubator (13°C). *Z. subfasciatus* beetles were collected in 2015 (N15 55.330 W97 09.132) and reared in our laboratory on *Phaseolus vulgaris* seeds. Parasitoids of S*tenocorse bruchivora* were obtained from the same location by collecting bruchid-infested seeds. Seeds of each population were placed in eight plastic jars (8 x 10 cm), four jars for each population, with 100 seeds per jar.

Experimental set-up

We obtained seeds with eggs of *Z. subfasciatus* by placing fifty mated females in each jar. Twenty-four hours later, seeds were checked for eggs under a binocular microscope. We obtained densities of one or two eggs per seed, that match those that are naturally found in nature. Under lab conditions females sometimes lay higher densities; we removed excess in egg densities by carefully using fine forceps. This process was repeated for another 24 hours with the seeds that did not receive any eggs until we obtained approximately 300 seeds with one or two eggs for each of the two plant populations. These seeds were stored in an incubator (30/24°C, 12/12). Few days after egg-laying, we checked that every egg became white and opaque, meaning that the eggs were hatched and that the bruchid larvae buried into the seed to complete their development (Dendy and Credland 1991). Twenty days after eggs were laid on the seeds, we set-up petri dishes (9 x 1.5 cm) with eight wild Lima bean seeds in each using a factorial design with the number bruchid larvae in the seeds (one or two), parasitoid presence and seed populations (BLO or YEL), leading to a total of eight different treatment combinations. We set-up the same total number of seeds and bruchid larvae per petri dish for each treatment (a total of eight seeds and eight bruchid larvae per petri dish) while modifying the density of the bruchid larvae per seed (one or two per seed). Consequently, in the treatments with only one bruchid larva per seed, all the eight seeds were infested. On the other hand, in the treatments with two bruchid larvae per seed, only half of the eight seeds were infested with two bruchid larvae, and the other four seeds remained uninfested. We used this type of design in order to offer the same number of seeds and hosts to the parasitoids.

In the treatments with parasitoids, one adult female parasitoid was introduced in each petri dish when the bruchid host larvae were approximately 20-day-old. They were left in the petri dish for three days, after which each seed from all the treatments was individually transferred to a 1.5 ml Eppendorf tube until insect emergence. We recorded bruchid development time (number of days from the first day of egg-counting until the day of emergence), parasitoid development time (number of days estimated from the second day of parasitism until adult emergence), ratio of bruchid emergence (total number of eggs on the seeds divided by the number of bruchid emerged), parasitism rate (total number of parasitoids emerged divided by the total number of bruchid host). We also measured bruchid and parasitoids male sex ratio and adult weight (measured in mg with balance Mettler Toledo XP6, Columbus, Ohio, USA).

In parallel, in order to investigate the potential seed traits that vary between the two populations, we measured 50 seeds from each population with an electronic digital caliper (Vogel, Germany), to the nearest 0.01mm.

Statistical analyses

All statistical analyses were performed with Rstudio. For all tests, “seed identity” (i.e. each individual seeds) nested within petri dish was used as a random factor.

For the analysis of bruchid emergence we used a generalized linear mixed model (function glmer from the *lme4* package) with a binomial distribution. Explanatory variables were bruchid density, parasitoid presence, seed population and all their interactions. We performed the same model for parasitoid emergence, without parasitoid presence as an explanatory variable. Bruchid weight was tested using a linear mixed model (function lmer from the lme4 package) following a normal distribution. Bruchid density per seed, parasitoid presence, seed population and all their interactions were used as explanatory variables. The same model was used for parasitoid weight, without parasitoid presence as a variable. Bruchid and parasitoid development times were analyzed using mixed models with a cox distribution (function coxme from the *coxme* package) where we fitted development time according to density per seed, parasitoid presence (only for models concerning bruchids), seed population and all their interactions. Tukey post-hoc analyses were used for male bruchid weight and female bruchid development time. Seed size from the two wild Lima bean populations was tested using a t-test.

Results

Bruchids

Bruchid emergence was not influenced by their density in the seeds ($DF = 1$, $X^2 = 0.07$, $p = 0.78$). As it was expected, bruchid emergence was 76 % lower when parasitoids were present (Fig. 1A, $DF = 1$, $X^2 = 115.9$, $p < 0.001$). Additionally, 22% more bruchids emerged from the Lima bean population “BLO” compared to “YEL” (Fig. 1B, $DF = 1$, $X^2 = 6.8$, $p = 0.009$).

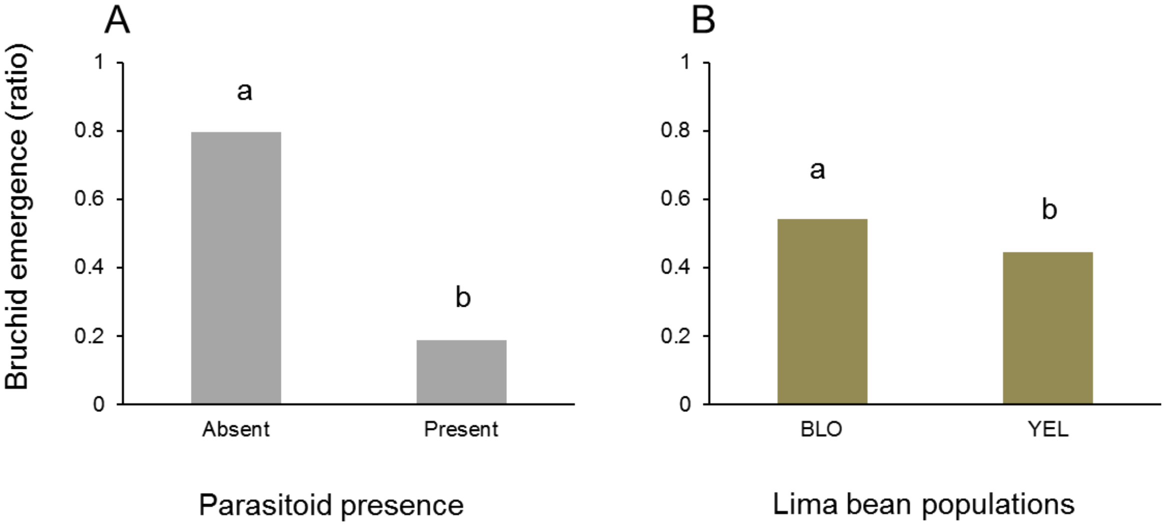

Figure 1: The effect of **A)** parasitoid presence and **B)** seed population on ratio of bruchid emergence. Significant differences are indicated by different letters. Parasitoids absent : n = 416, parasitoids present : n = 416, BLO population : n = 384, YEL population : n = 448.

The development time of female bruchids was not affected by the density of bruchid larvae per seed (DF = 1, X^2 = 1.21, p = 0.23) nor by seed population (DF = 1, X^2 = 0.72, p = 0.47). The interaction between parasitoid presence and population had a significant effect on female bruchid development time (DF = 1, X^2 = 3.27, p = 0.001, figure 2). Parasitoid presence reduced significantly the development time of bruchid beetles, although this was only the case in the Lima bean population “BLO” (Fig. 2, DF = 3, X^2 =15.67, p = 0.003). The reverse pattern was observed in the other population, but it was not statistically significant (p = 0.4). No significant differences were found in the development time of male bruchids (density per seed: DF = 1, X^2 = 1.19, p = 0.23; parasitoid presence: DF = 1, X^2 = 0.75, p = 0.45; seed populations: DF = 1, X^2 = 1.56, p = 0.12).

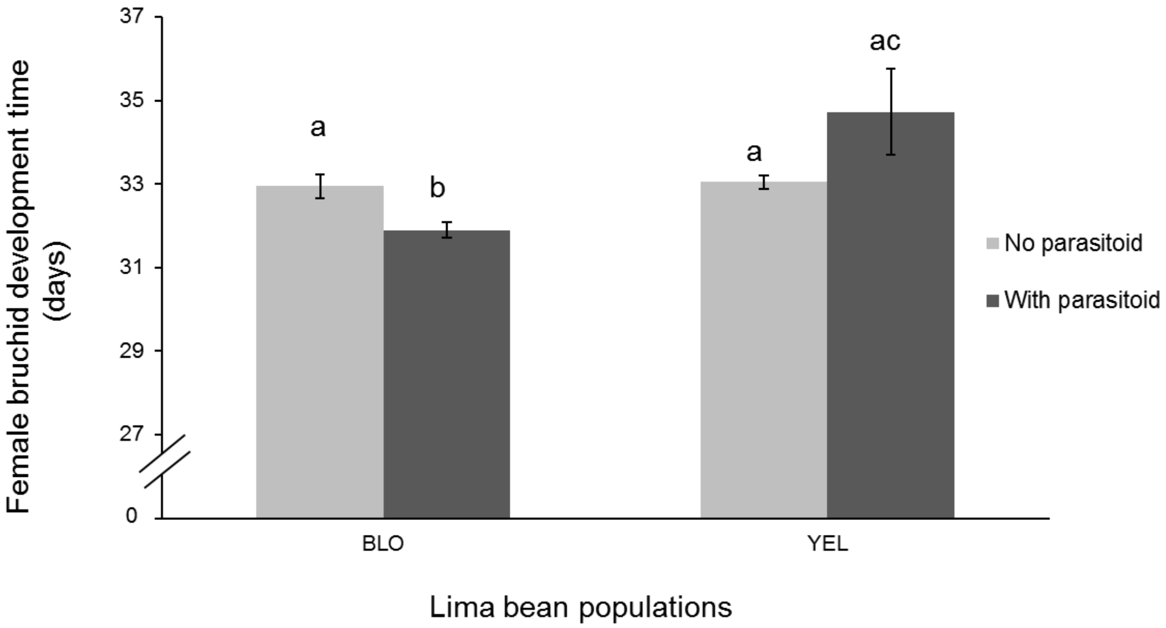

Figure 2: The effect of the presence of parasitoid on the development time of female bruchids developing in different Lima bean seed populations. Bars represent the standard error of the mean. Significant differences are indicated by different letters. The Y axis was cut for clarity purpose. For beetles developing in seeds from BLO population: no parasitoid: n = 71, with parasitoid: n = 18. In seeds from YEL population: no parasitoid: n = 80, with parasitoid: n = 11.

Bruchid females that emerged from seeds with only one egg were 7 % heavier than females from seeds with two eggs (Fig. 3A, DF = 1, F = 14.38, $p < 0.001$). Similarly, bruchid females that emerged from seeds of the BLO population were 4.5% heavier than females that developed in YEL seeds (Fig. 3B, DF = 1, F = 4.74, p = 0.029). Parasitoid presence had a marginal negative effect on the weight of adult female bruchids (Fig. 3C, DF = 1, F = 3.12, p = 0.08) and did not influence the weight of adult male bruchids (DF = 1, F = 0.016, p = 0.9). The interaction between population and density had a significant effect on male bruchid weight (DF = 1, F = 4.36, p = 0.037), but not on females. Consequently, the effects of bruchid density and seed population on male bruchid weight are represented together in figure 4. Males emerging from BLO seeds with a density of two bruchids were significantly bigger from the male bruchids emerging from all YEL seeds (Fig. 4, DF = 3, F = 16.17, p = 0.001).

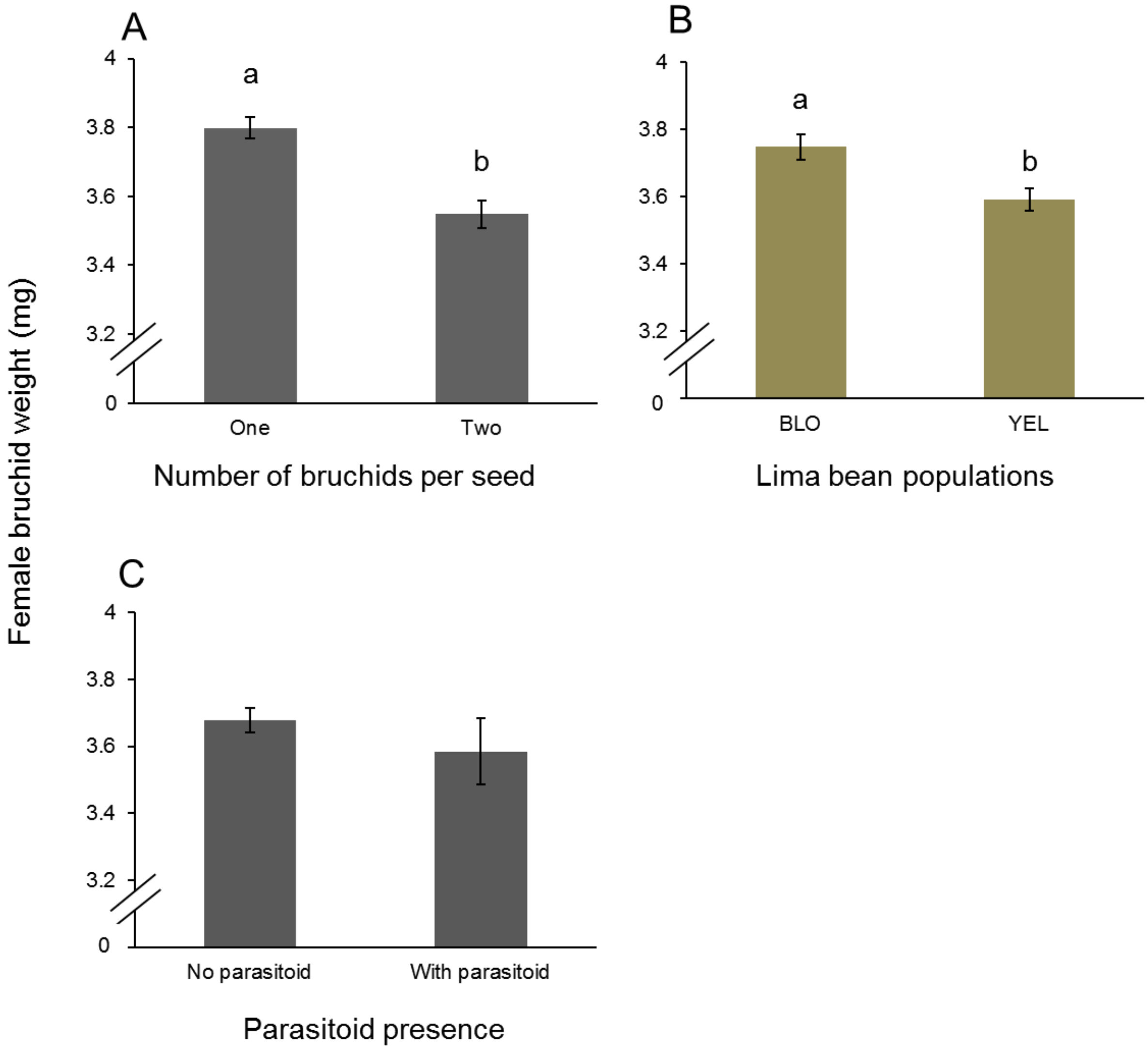

Figure 3: The effect of **A** bruchid density per seed, **B** seed population and **C** parasitoid presence on female bruchid weight. Bars are standard error of the mean. The Y axis was cut for clarity purpose. Significant differences are indicated by different letters. One bruchid per seed: n = 89, two bruchids per seed: n = 95, YEL population: n = 92, BLO population: n = 92.

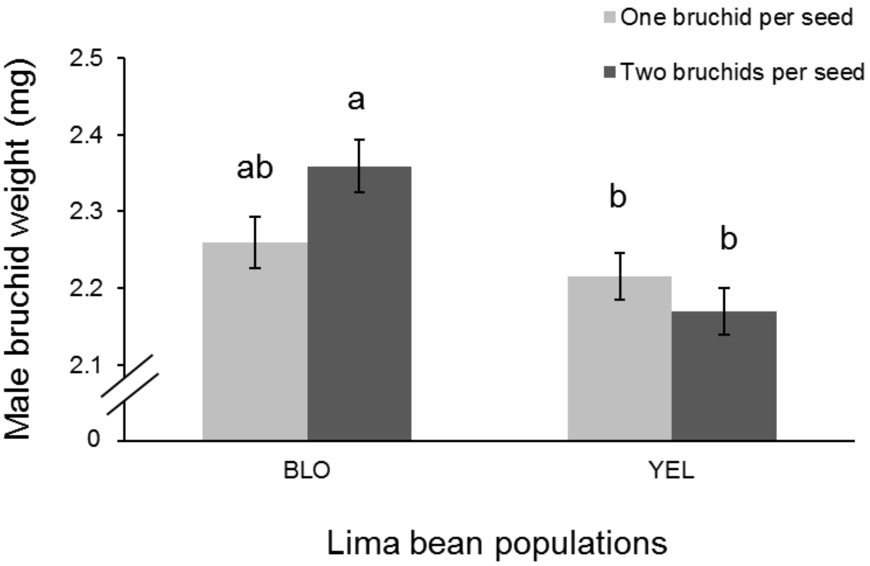

Figure 4: The effect of seed population and density per seed on male bruchid weight. Bars represent the standard error of the mean. Different letters indicate significant differences. The Y axis was cut for clarity purpose. For bruchids developing in seeds from BLO population: one bruchid per seed: n = 53, two bruchids per seed: n = 55. For bruchids developing in seeds from YEL population: one bruchid per seed: n = 57, two bruchids per seed n = 51.

Finally, bruchid sex ratio was not affected by any of the treatments (bruchid density per seed: DF = 1, $X^2 = 0.336$, p = 0.56; parasitoid presence: DF = 1, $X^2 = 2.37$, p = 0.13; Lima bean population: DF = 1, $X^2 = 0.01$, p = 0.98).

Parasitoids

Parasitism rate was 30.8 % higher when parasitoids were offered seeds with only one beetle larva compared to seeds with two larvae (Fig. 5A, DF = 1, $X^2 = 5.66$, p = 0.01) and was 36 % higher when they developed in seeds of the YEL populations compared to BLO seeds (Fig. 5B, DF = 1, $X^2 = 7.41$, p = 0.006). The development time of female parasitoids was not affected by the seed population (DF = 1, $X^2 = 1.42$, p = 0.15), nor by the density of bruchid larvae in the seed (DF = 1, $X^2 = 0.65$, p = 0.51). The same results were found for the development time of male parasitoids (seed population: DF = 1, $X^2 = 0.63$, p = 0.53; density of bruchid per seed: DF = 1, $X^2 = 0.27$, p = 0.79). Bruchid larval density per seed had no significant effect on parasitoid weight, for both males (DF = 1, F = 0.005, p = 0.94) and females (DF = 1, F = 1.98, p = 0.16). Similarly, parasitoid weight was not affected by the population of the seed in which they developed (Males: DF = 1, F = 0.06, p = 0.8; Females: DF = 1, F = 0.51, p = 0.47).

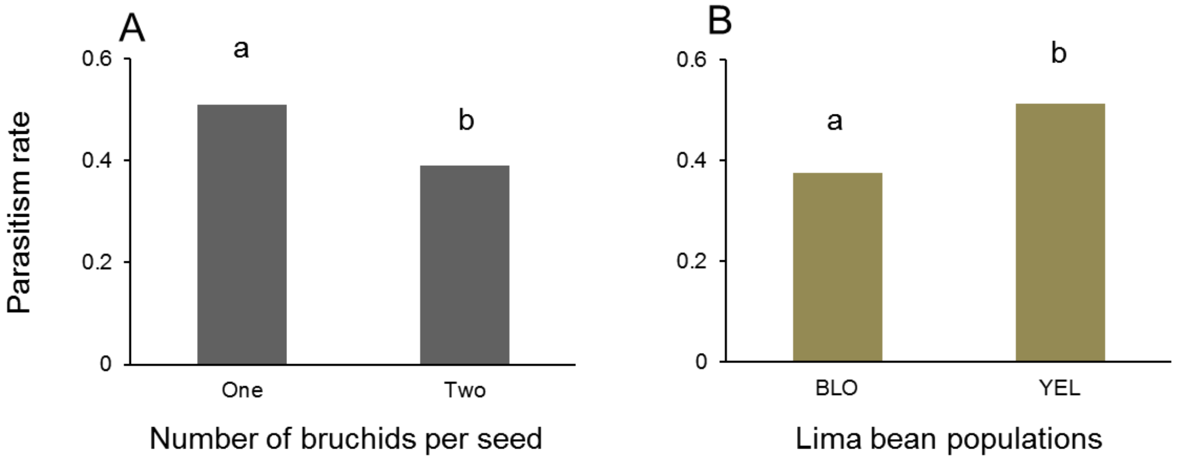

Figure 5: The effect of **A)** bruchid density per seed and **B)** seed population on parasitism rate (total number of parasitoid emerged divided by the total number of available host). Significant differences are indicated by different letters. One bruchid per seed: n = 208, two bruchids per seed: n = 208, BLO: n = 192, YEL: n = 224.

Wild Lima bean seeds originated from “BLO” population were 3% larger than “YEL (Supplementary material, Fig. 1, DF=97.1, t=3.04, p=0.003).

Discussion

Overview

The increase in bruchid density per seed had a negative effect on their performance (weight), most likely due to negative consequences of intra-specific larval competition. These effects cascaded up to the third trophic level and strongly reduced the parasitism rate. Parasitoid presence had both direct and indirect effects on bruchids: parasitoids dramatically reduced bruchid emergence, influenced their development and had a low but negative effect on female bruchid weight. The performance of bruchids and parasitoids was different according to the bean population and in an opposite way, which can be explained by seed size.

Bruchid density

The increase in bruchid larval density per seed had a negative impact on the weight of female bruchids, but not on males. This may be explained by the limited amount of resource present in a seed of wild Lima bean, which may not be sufficient for two bruchid larvae to fully complete their development. Consequently, because males are smaller than females, they probably also need less resources to grow, and were less affected by larval competition. Similarly, in a previous study on this system, Cuny et al. (2017) found that female beetles that emerged the smaller wild seeds, were more negatively affected by larval intraspecific competition inside the seed than females that emerged from the larger cultivated seeds, whereas for males there was no difference. We did not find any effect of bruchid density per seed on their development time and emergence rate. A number of studies have reported that bruchid competition can have negative effects on their development time and emergence, but they used a higher level of competition intensity than we used in this study (Teixeira and Zucoloto 2012, Oliveira et al. 2015, Cuny et al. 2017).

The negative effect of bruchid density on adult weight did not cascade up on parasitoid weight and development time. Surprisingly, although bruchid emergence was not affected by bruchid density per seed, parasitism rate was strongly reduced (about 25% lower) when two bruchids were present in the seed, compared to only one. One hypothesis could be that parasitoids parasitized less when at a higher bruchid density. However, this would mean that more bruchids would emerge from this treatment, which was not the case. Alternately, it may be that an increase in bruchid density increases host mortality after parasitism, killing the parasitoid larvae at the same time.

The effect of host density has been well studied in the past in the context of parasitoid functional responses (Cloutier 1984, Price 1988, Montoya et al. 2000, Sagarra et al. 2000, Farkas and Singer 2013). In these type of studies, the traits that are usually measured are the total number of host available, total number of host parasitized and the number of emerging parasitoids. These studies used different herbivore abundances, which may in turn influence parasitoid abundance (density-mediated effects). However, they did not measure the effect of changes in herbivore density on herbivore and parasitoid traits (trait-mediated effects, Mooney and Singer 2012). In our study, the total number of host available was constant, but their distribution per seed was different (one or two bruchids per seed), which led to a negative impact on bruchid performance, certainly due to larval competition. The effect of host intraspecific competition on parasitoids performance has virtually never been experimentally tested. This is surprising, considering that competition is considered one of the main factors shaping ecological communities (Denno et al. 1995, Kaplan and Denno 2007) it should be expected to influence the quality of host-parasitoid relationships. In the only study we could find, Kraaijeveld and Godfray (1997) reared different lines of *Drosophila melanogaster* in presence or absence of parasitoids, in order select host lines with a better ability to encapsulate parasitoid eggs. Then, they exposed the lines to different levels of intra-specific competition, and showed that *D. melanogaster* lines that were selected for an increase in parasitoid egg encapsulation had a lower survival in conditions of high competition, compared to the lines that were not selected for egg encapsulation. This suggests the existence of a trade-off between host traits involved in resistance against parasitoids (i.e. egg encapsulation) and traits involved in intra-specific competition (i.e. survival) (Kraaijeveld et al. 2001).

Parasitoid presence

Parasitoid presence or absence had two types of effects on bruchid beetles. On one hand they drastically reduced the emergence of bruchids through direct parasitism, which is not surprising. But more interestingly, parasitoid presence had an indirect effect on the development time of non-parasitized female bruchids, and this effect was different in the two bean populations. In one population, female bruchid development time was significantly reduced in the presence of parasitoids, but the opposite pattern was found in the other population. In addition, we found a marginal negative effect of the presence of parasitoids on female bruchid adult weight. In accordance with our results, Zaugg et al. (2013) found that the presence of the parasitoid *Dinarmus basalis* reduced the mean weight of emerged (non-parasitized) beetles (*Acanthoscelides obtectus*) as well as their development time. They hypothesized that bruchid larvae can sense parasitoids searching for them, using vibration cues created by the parasitoid antenna. In response, they would stop feeding in order to limit their own vibrations, as it has been shown in several species of leafminers (Bacher et al. 1997, Meyhöfer et al. 1994, Djemai et al. 2004). However, very little is known about vibrations perceived by bruchid larvae inside the seed. Non-consumptive predator effects have been shown to have important effects on herbivore population dynamics (Lima 1998, Werner and Peacor 2003, Preisser et al. 2005). For instance, Thaler and Griffin (2008) showed that the negative impact of a predatory stinkbug (*Podisus maculiventris*) on an herbivorous insect prey (*Manduca sexta*) was approximately the same when they removed the predator’s rostra, preventing them from killing their prey.

We found that the weight as well as the emergence rate of bruchids was significantly affected by the Lima bean population in which they developed. This is not surprising, as we know that there can be strong variation between different populations of wild Lima bean plants (Shlichta et al. 2014). Shlichta et al. (2014) tested the performance of *Z. subfasciatus* larvae developing in seeds from 12 populations of wild Lima bean plants. They found an effect of population on bruchid performance and also showed that it could not be explained by variations in cyanogenic glycosides, one of the main defensive compounds of this plant. In order to unravel the traits responsible for the observed effect of seed population, we measured the seeds and found that seeds from the “BLO” population, in which bruchids performed better, are significantly larger than seeds from the other population. Hence, seed size may be one trait responsible for the observed results, although we cannot exclude that other non-measured traits played a role, such as other chemical compounds or nutritional quality (Zaugg et al. 2013, Moreira et al. 2015).

Parasitism rates were also significantly affected by the population of Lima bean. Interestingly, the population on which parasitoids performed better was the one on which bruchids performed worse (“YEL”). In a previous study with *S. bruchivora* but in this case on bruchid hosts attacking the common bean *P. vulgaris*, Campan et al. (2005) also found significant effect of plant population on *S. bruchivora* performance which did not cascade up to parasitoids. This is somehow surprising because *S. bruchivora* is an idiobiont and their performance is often positively correlated with the weight of their host (Benrey et al. 1998, Harvey 2005). However, it is also possible that some defensive compounds present in the wild populations of Lima bean are more toxic for the parasitoid larvae than for its host (Turlings and Benrey 1998, Opitz and Müller 2009, Gols 2014). This idea remains to be tested. Finally, other differences in seed traits may also account for the observed results. Moreira et al. (2015) found higher rates of parasitism on bruchid-infested seeds of *P. vulgaris* than on seeds of *P.*

coccineus. In the lab, they showed that bruchid development was slower on *P. vulgaris*, suggesting a wider window of vulnerability for parasitism (Benrey and Denno, 1997). Thus, our findings indicate that in this system, bruchid and parasitoid performance on seeds of wild Lima bean result from the interaction between host-mediated, such as host density, and seed trait-mediated effects. We suggest that future work should investigate the specific mechanisms that drive these interactions.

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General discussion and Conclusions

Interactions between plants, herbivorous insects and their natural enemies are widespread in natural and agricultural systems. In order to have a better understanding of these interactions, I studied different bottom-up and top-down forces involved in wild and cultivated plants of Lima bean *Phaseolus lunatus* (Figure 1).

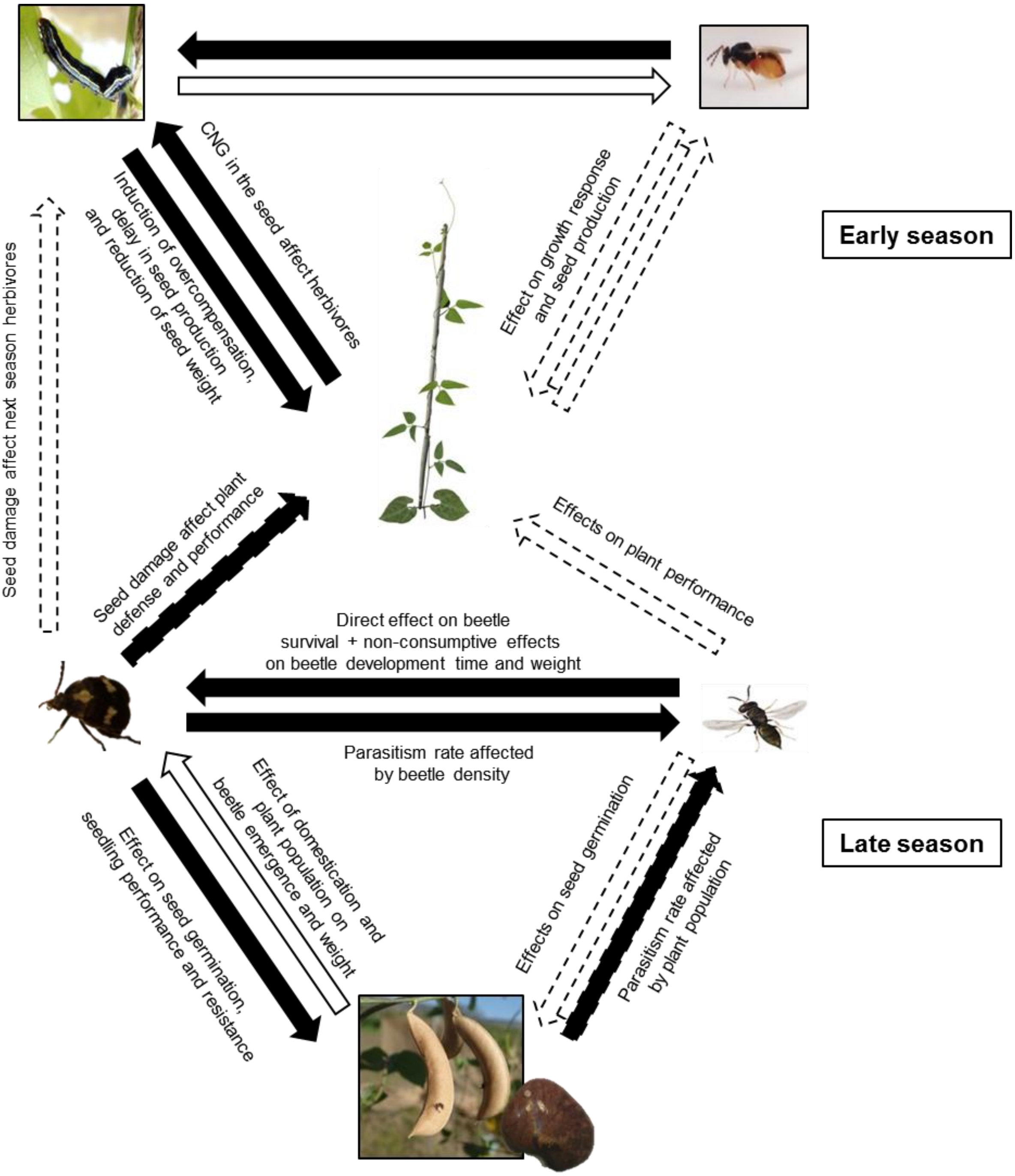

Figure 1. Summary of the interaction outcomes found in my thesis, taking place during the early and late seasons. White arrows represent interactions with positive outcome and black arrows interactions with negative outcome. Indirect interactions are represented by dashed arrows, while plain arrows show direct interactions.

The process of plant domestication, the selection of plant traits that favor human consumption, has often led to a decrease in plant resistance against herbivorous insects (Whitehead et al. 2017). I showed that domestication significantly reduced the concentration of cyanogenic glycosides (CNGs), one of the main chemical defensive compounds present in wild lima bean seeds (Shlichta et al. 2018, Supplementary material) and in many other plants (Vetter 2000, Zagrobelny et al. 2004, Ballhorn et al. 2009). Nevertheless, because these compounds do not appear to have any effect on bruchid beetle (*Z. subfasciatus*) performance (Shlichta et al. 2014), the lower level of these compounds observed in domesticated lima bean seeds is probably not the only factor responsible for the better performance of bruchid beetles in this type of seeds (Shlichta et al. 2018 Supplementary material). Among the other lima bean seed traits that have been altered during domestication, there is a dramatic increase in size, which is often the case for plant structures used for human consumption (Chen et al. 2015). In the first chapter, I showed that this increase in seed size for lima bean (*Phaseolus lunatus*) has significantly reduced the potential negative impact of intraspecific competition among larvae of the bruchid beetle (*Zabrotes subfasciatus*). This is interesting because this result highlights a case of a negative consequence of domestication that is not caused by a direct effect on plant defense. Such result may help our understanding on why so many domesticated species are vulnerable to insect pests.

The fact that cyanogenic glycosides do not seem to be effective against bruchid larvae, one of the main pests of lima bean seeds, raises the following question: why are these compounds present at such high levels in the seeds? In the second chapter of this thesis, I attempted to fill the gap of knowledge regarding the role of CNGs present in the seeds of wild *Phaseolus lunatus* seeds. I found that seeds with a higher amount of CNGs, produced seedling with more CNGs, which allowed the seedling to be better defended against a generalist insect (*Spodoptera littoralis*). The total amount of CNG compounds in the seedling significantly increased during early plant development, suggesting an important production de novo. Hence, I hypothesize that the higher amount of CNG compounds in the seed increase the production of CNG compounds in the seedling. Finally, CNGs concentration present in the seeds did not appear to have an effect on seedling growth and may therefore not be used as nitrogen storage. Alternatively, CNGs in the seeds may also serve as defense against other non-insect organism, such as birds or small mammals.

In lima bean leaves, CNGs are also present and have been shown to be efficient against generalist herbivores (Ballhorn et al. 2009). Other strategies against leaf herbivores include tolerance (Blue et al. 2015, Moreira et al. 2015) and attraction of parasitoids (Kost and Heil 2008). Lucas-Barbosa et al. (2016) showed that the former two strategies are complementary. However, virtually no study has investigated whether the action of parasitoids can affect plant tolerance responses. In the third chapter, I tested the hypothesis that by influencing the feeding behavior and likely physiology of their herbivorous host, parasitoids can affect the growth of wild lima bean plants. I found that when attacked by herbivores in the absence of parasitoids, plants exerted an overcompensation response.

This however was no longer the case when parasitoids were present, probably due to a reduction in herbivore damage. In addition, parasitoid presence also increased the number and weight of seeds produced by the plants. This study opens a new area of research in the already vast and complex area of tritrophic interactions. Furthermore, the impact of parasitoids on plant growth may be an important factor to consider for future biological control programs.

Results from this study also suggest that leaf herbivores delayed seed production in wild lima bean plants, and that the presence of parasitoids could attenuate this delay. Another study from our group (Hernandez-Cumplido et al. 2016a) found that lima bean plants induced by jasmonic acid produced flowers earlier than control plants. This result tends to run counter to our study, as the phenology of flower production has been shown to strongly influence plant seed production (Herrera et al. 1992, Elzinga et al. 2007). Consequently, the influence of leaf herbivory on the time of production of plant reproductive structures appears to be context-dependent (Elzinga et al. 2007).

But the main question that remains to be answered is the following: is there an advantage for the plant to produce their seeds earlier than other plants? From the point of view of plant-plant competition, one could argue that seeds that fall into the ground faster may also germinate faster and gain a net competitive advantage over other plants (Hyatt and Evans 1998). However, lima bean is an annual plant which seeds are added to a seedbank that can stay in the ground for many years and germinate mainly during the rainy season, when ideal conditions of temperature and humidity are met (Degreef et al. 2002). Therefore, producing seeds some days earlier than other plants does not seem to provide a real advantage in the context of plant competition. However, this idea remains to be tested.

An alternative hypothesis is that seeds that are produced earlier in the season may suffer less attack from bruchid beetles, which are synchronized with lima bean seasonal seed production (Hernandez-Cumplido et al. 2016b). This hypothesis is supported by the meta-analysis from Elzinga et al. (2007), in which they found that pre-dispersal seed predators tend to be less present at the beginning and at the end of seed production period. Consequently, plants that produce seeds earlier than other individuals in the population, may suffer less damage from pre-dispersal seed predators.

As mentioned before, different interactions can take place on the same lima bean plant. These occur on different plant organs and at different times during the season. In chapter 4, I asked whether interactions at the seed stage could influence seedling growth. I found that moderate seed damage by beetles increases seed germination. Yet, if damage is high, plants growth can be negatively affected. Parasitoids mitigate the damage caused by the seed beetles and their presence increases plant fitness. In addition, I showed that wild lima bean seed damage caused by bruchid beetles had a negative impact on the defense response of germinated seedlings, which are more likely to be attacked by generalist herbivores. In this context, a previous study from our lab (Hernandez-Cumplido et al. 2016b), showed that wild lima bean plants damaged by leaf herbivores produced seeds that were less attacked by bruchid beetles. Consequently, damage at the level of the leaves leads to a reduction in damage at the seed level, while damage at the level of the seed leads to an increase in damage at the leaf level. Combined, these two studies suggest that two opposite selective forces are present at the leaf and seed levels, that may result in a trade-off for the plant for defense in leaves and seeds. From an evolutionary point of view, I can expect the level of defensive compounds in the seeds and leaves to stabilize toward an ideal amount that would minimize the negative impact of seed damage on the germinated seedling and maximize the positive impact of leaf damage on seed predation.

In Chapters 3 and 4, I found strong evidence that parasitoid presence can benefit wild lima bean plants. I demonstrated that parasitoids reduced plant damage inflicted by herbivores on the leaves (Chapter 3) and seeds (Chapter 4), suggesting that the two parasitoids I studied have potential use in biological control of these insect pest. Many studies have shown that parasitoids can be used as biological control agents in order to reduce the population of insect pests and their negative impact on agriculture (Greathead and Greathead 1992, Cross et al. 1999). This has been investigated in greenhouses (Van Lenteren et al. 1996), as well as open fields (Settle et al. 1996, Dahlsten et al. 1998). It is interesting to notice that from the point of view of evolutionary ecology, only few studies have demonstrated that parasitoids efficiently provide a benefit that results in increased plant fitness under field conditions (Gomez and Zamora 1994, Van der Meijden and Klinkhamer 2000, Van Loon et al. 2000, Hoballah and Turlings 2001). This is particularly the case for koinobiont parasitoids and tritrophic systems in which the actors have a joined evolutionary history (Gols et al. 2015).

One of the main hypothesis that dominates the tritrophic (plants, herbivores and parasitoids) literature, is that plants release volatiles when they are under attack to attract parasitoids and defend themselves against herbivores (Kessler and Baldwin 2001, Heil 2004, Poelman et al. 2009), although this hypothesis is still debated (Dicke and Baldwin 2009). Parasitoids use plant cues, such as volatiles, to detect the plants on which their host is feeding (Turlings et al. 1990, Vet and Dicke 1992, Turlings and Wackers 2004, Turlings and Erbs 2018). Plant volatiles are believed to be efficient at long distance. At short distance, other cues related to the hosts themselves may be involved (Geervliet et al. 1994, Goubert et al. 2013). Concerning tritrophic systems in which parasitoid's host complete their whole development in a seed, parasitoids use several cues at short distance to discriminate between infested and non-infested seeds, such as their host kairomones (Onodera et al. 1992) and feces (Steidle and Schöller 1997). Zaugg et al. (2013) hypothesized that vibrations are also used by parasitoids to find their host in the seeds. This behavior has already been shown for parasitoids of leaf miners (Meyhöfer et al. 1994). Concerning long-distance cues used by parasitoids of seed predators, it appears that the grain itself is more attractive than the vegetative parts of the plant (Cortesero et al. 1993). More studies in this topic could lead to new biological control methods against seed insect pests.

In the last chapter I found that an increase in the density of seed beetle (*Z. subfasciatus*) larvae developing inside the seeds influenced the weight of emerging adults, probably due to the negative impact of intraspecific competition. This effect cascaded up to the parasitoids (*S. bruchivora*), and parasitism rate was negatively affected. These results reveal that host density may have an effect on the quality of its interaction with parasitoids and should therefore be taken into account in future studies.

Moreover, I showed in the first chapter of this thesis that the negative impact of intraspecific competition was attenuated when beetles were developing in domesticated seeds. Hence, *S. bruchivora* performance may be improved when developing in domesticated seeds, in which their host beetle suffered less negative effect from competition. Most of the studies conducted so far focused on density-mediated interactions between herbivores and parasitoids, that is, an increase in host density leads to an increase in parasitoid density (Mooney and Singer 2012). Surprisingly, very few studies have examined the effect of herbivore density on the qualitative interaction (per capita effect) between herbivores and parasitoids (but see Kraaijeveld and Godfray 1997). In addition, parasitoids exerted non-consumptive effects by significantly altering the development time of seed beetles, but this effect was dependent on the seed population in which they developed. Indirect effects of parasitoid presence are not well studied, yet they are believed to have an important impact on herbivores (Preisser et al. 2005, Fill et al. 2012).

The first part of my thesis consisted in revisiting bottom-up effects in plant-insect interactions, adding a different angle (plant domestication) and the unknown role of CNGs in Lima bean seeds. I could thus confirm that plants can have strong bottom-up effects in this system. My thesis also focused on top-down effects of parasitoids on their hosts and on the host's plants, for which there is very little information, particularly concerning their effect on plants through parasitized herbivores. I showed that parasitoids could influence plant performance, through leaf and seed herbivores. Therefore, my findings contribute to the awareness that parasitoids can have significant effects on plants, and suggest that these effects may be widespread in nature.

The aim of this thesis was to further our understanding of plant-mediated insect interactions. However, new results led to new questions which would benefit from additional research. In chapter 5, I showed that parasitoids were very effective at parasitizing bruchid beetles. However, I also found that parasitoids were negatively affected by beetle competition, even at low host density, which often happens in nature (Cuny, personal observation). Interestingly, I showed in the first chapter that the increase in seed size caused by domestication improved the performance of bruchid beetles by mitigating the negative effect of competition. Consequently, domestication may have a positive effect on parasitoids, and could make *Stenocorse bruchivora* a good candidate for biological control against bruchid beetle pest. In chapter 3, I found that the presence of parasitoids altered plant growth responses to herbivory. However, the mechanisms underlying this result are still unknown. It may be due to a reduction in herbivore damage with the presence of parasitoids. Alternatively, because parasitism induces many changes in the host's physiology, changes in parasitized herbivore saliva may have affected plant growth. Therefore, future research should aim at understanding the extent to which host saliva modification due to parasitism affects plant tolerance responses. In chapter 4, I showed that bruchid beetles and their parasitoids could have a dramatic effect on seedling performance, as well as on leaf herbivores. However, I did not investigate the consequences on the belowground system of the plant and I predict that the plant roots would also be affected by seed damage, which could in turn affect root herbivores. Current work in our lab is examining this question.

Taken together, the results from this thesis show that even from the well-studied bottom-up perspective, there are still unanswered questions concerning the mechanisms by which plants affect higher trophic levels. Moreover, I showed that top-down forces, which are vastly underappreciated in tritrophic interactions, can have significant effects on lower trophic levels and should therefore receive more attention.

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Supplementary material

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Contrasting consequences of plant domestication for the chemical defenses of leaves and seeds in lima bean plants

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Abstract

Plant domestication is assumed to result in reduced levels of defensive compounds in crops, because this makes the plants more suitable for consumption by humans and livestock. We argue that this should mainly be reflected in the concentrations of defense compounds in the plant parts that are used for consumption and not necessarily for other parts of crop plants. We tested this hypothesis for domesticated lima bean (*Phaseolus lunatus*), by comparing its chemical defenses against a leaf herbivore, the beet armyworm *(Spodoptera exigua),* and a seed predator, the beetle *Zabrotes subfasciatus*. For seeds and leaves we determined the concentrations of cyanogenic glycosides (CNGs) in cultivated varieties and wild populations and evaluated the preference and performance of the herbivores when exposed to leaves and seeds from wild and cultivated plants. Concentrations of CNGs were significantly different between wild and cultivated plants. In the leaves the concentration of CNGs in the cultivated varieties were more than double that of the wild leaves. In contrast, seeds from cultivated plants had up to 20 times lower CNG concentration compared to seeds from the wild populations. Insect preference and performance do not parallel the chemical data. Larvae of *S. exigua* preferred wild leaves but had higher survival on cultivated leaves. The beetles, however, strongly preferred seeds from cultivated plants and females developed more quickly on these seeds. We conclude that domestication of *P. lunatus* has altered the concentration of CNGs in both the seeds and the leaves in opposite directions. This results in differential effects on the herbivores that attack these two plant structures. The contrasting effect of domestication on different plant tissues can be explained by the fact that bean plants have been specifically selected for human consumption of the seeds. Tissue-specific effects of plant domestication on plant defenses can be expected for other crops as well.

Keywords: Cyanogenic glycosides; Domestication; Seed predator; Herbivore

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Introduction

Comparative studies of species interactions using plant systems in which cultivated and wild varieties coexist, provide an opportunity to examine how alterations in plant traits

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affect herbivores and their natural enemies in the same environment. Recently there has been an increasing interest in using wild and cultivated systems to study species interactions (Turcotte, Turley, & Johnson 2014; Chen, Gols, & Benrey 2015; Chen, Gols, Stratton, Brevik, & Benrey 2015; Milla, Osborne, Turcotte, & Violle 2015; Rowen & Kaplan 2016; Whitehead, Turcotte, & Poveda 2017). Artificial selection has directly or indirectly altered the defensive chemistry, architecture, and/or nutritional content of cultivated plants in a relatively short time span. Contrasting these cultivars with their wild counterparts can shed light on the way in which specific changes on plant traits can mediate species interactions.

Studies comparing cultivated and wild species have, in general, found that cultivated plants offer a better resource for herbivores (Rosenthal & Dirzo 1997; Benrey, Callejas, Rios, Oyama, & Denno 1998; Gols et al. 2008; Rodriguez-Saona et al. 2011). As a result of selective breeding, domesticated plants and their wild counterparts often differ substantially in their physical defense traits, allelochemistry, and nutrient content (Gouinguené, Degen, & Turlings 2001; Chen & Welter 2007; Wang et al. 2009; Delgado-Baquerizo, Reich, García-Palacios, & Milla 2016; Rowen & Kaplan 2016). In many cases the defensive chemistry of cultivars has been drastically reduced (Gols et al. 2008; Chacón-Fuentes et al. 2015; Whitehead et al. 2017). The pattern of reduced resistance to herbivory in domesticated crops has not been consistent across cultivated plants and their herbivores (Chen, Gols, Stratton et al. 2015; Gaillard, Glauser, Robert, & Turlings 2018; Turcotte et al. 2014), although there is evidence that changes in secondary metabolites (most often a reduction) happen to more than half of crop plants (Meyer, DuVal, & Jensen 2012). An extensive analysis of plant resistance across 29 domestication events using two generalist herbivores, the beet armyworm (*Spodoptera exigua*), and the green peach aphid (*Myzus persicae*) found that domestication significantly reduced plant resistance to attack by armyworm but did not have an effect on plant resistance to aphids (Turcotte et al. 2014). In addition, they found that different plant traits were associated with insect resistance to these two species of insects. Further, the pattern of resistance associated with domestication varies across location, history of domestication, and the form or life stage of the plant examined (Meyer et al. 2012; Chen, Shapiro, Benrey, & Cibrián-Jaramillo 2017; Whitehead et al. 2017).

We propose that the effects of domestication may be tissue-specific and dependent on which part of the plant has been bred for consumption. A recent review by Chen, Gols & Benrey (2015) makes the same argument, but points out that as yet the evidence for this notion is scarce. Here we test this hypothesis of tissue-specific domestication effects on plant defenses by comparing chemical defenses and insect performance for leaves and seeds in wild and domesticated lima bean.

Phaseolus lunatus (lima bean), was domesticated at least twice, once in the Andean mountains of Ecuador and Northern Peru, and a second time in central-western Mexico (Motta-Aldana, Serrano-Serrano, Hernández-Torres, Castillo-Villamizar, & Debouck 2010). Once domesticated it spread throughout the Americas, and now is widely distributed in the tropics. Domestication of beans in the genus *Phaseolus* has dramatically altered morphological, chemical and nutritional traits. For cultivated beans these changes mainly involve an increase in seed and pod size, decrease in shattering, an overall decrease in toxins, and an increase in proteins and minerals (Salinas 1988; Sotelo, Sousa, & Sanchez 1995; Beebe, Gonzalez, & Rengifo 2000; Cuny, Shlichta, & Benrey 2017).

Lima bean is unique in that it is the only reported species out of approximately 52 species in the genus *Phaseolus* that contains cyanogenic glycosides (CNGs) in both the leaves and the seeds (Vetter 2000). These chemical compounds are known to be highly toxic to most living organisms (Shragg, Albertson, & Fisher 1982; Yamane et al. 2010). CNGs are toxic for some herbivores (Gleadow & Woodrow 2002; Ballhorn, Lieberei, & Ganzhorn 2005), but they act as a feeding stimulant for others (Brattsten, Samuelian, Long, Kincaid, & Evans 1983). Generalist herbivores, such as *Schistocerca gregaria* and *Spodoptera littoralis*, avoid cyanogenic glycosides (Schwarz, Wray, & Proksch 1996), but other polyphagous species, such as *Spodoptera eridania*, prefer plants with higher CNGs (Brattsten et al. 1983). Furthermore, CNGs are known to influence oviposition preferences of the Mexican leaf-bean beetle (Ballhorn & Lieberei 2006) and affect the leaf beetle's behavior and development (Ballhorn, Heil, Pietrowski, & Lieberei 2007).

In plants, CNGs are stored in the vacuoles (Vetter 2000). When plant tissue is damaged by herbivore attack, CNGs get into contact with ß-glucosidases that hydrolyze the CNGs, this causes a release of toxic hydrogen cyanide (HCN) (Zagrobelny et al. 2004). The seeds of cultivated varieties of *Phaseolus lunatus* have been shown to release less hydrogen cyanide (HCN) than their wild counterparts (Lucas & Sotelo 1984). The effect of varying CNG concentrations on the leaf consumption and feeding behavior has been assessed for leaf-feeding herbivores (Ballhorn, Kautz, Lion, & Heil 2008; Ballhorn, Kautz, & Lieberei 2010) and the performance of a seed predator on wild *P. lunatus* seeds has also been evaluated (Shlichta, Glauser, & Benrey 2014). However, little is known about the consequences of domestication of *P. lunatus* on the preference and performance of the leaf and seed herbivores. Most studies on the effects of plant domestication on susceptibility to herbivores have focused on insects that attack the same plant structure (i.e., leaves). Leaf herbivores and seed predators have different foraging strategies and feeding behaviors; therefore, there may also be differences in the effect that plant chemicals have on their behavior and performance.

In this study we examine the predicted effects of lima bean domestication on its interaction with a leaf herbivore and a seed predator. We test the hypothesis that changes in insect resistance due to breeding, are not uniform in all plant tissues, but are more pronounced in tissues consumed by humans. This is tested with lima beans and two different herbivores that specialize on different parts of the plants (leaves and seeds). Using plants and seeds from three cultivated varieties and three wild populations of *P. lunatus*, our specific objectives were to: (1) analyze and characterize both cultivated varieties and wild accessions for their concentration of CNGs in leaves and seeds, (2) determine whether both herbivore species prefer the cultivated varieties, and (3) determine whether both species of herbivores have higher performance on cultivated varieties.

Materials and methods

Study system

Seeds for domesticated varieties of *P. lunatus* were obtained from W. Atlee Burpee & Co. (Warminster, PA, USA). The varieties used were: Fordhook 242 Bush Bean (FORD), Burpee's Best Pole Bean (BURP), and Jackson Wonder (JACK). These seeds belong to the two most important genetic groups of lima bean; "JACK" is of Mesoamerican origin and "FORD" of Andean origin (Nienhuis, Tivang, Skroch, & dos Santos 1995; Ernest & Kee 2008). We could not find information regarding the genetic origin of the BURP variety.

Wild seeds were collected from populations in Southeast Mexico where wild lima bean occurs naturally. From December to February 2011–2012 seeds were collected in three sites located along the coast from 26 km north to 50 km south of Puerto Escondido in the state of Oaxaca. The sites were, Hidalgo near San Jose Manialtepec (HGO; 15.575564, −97.151350), Universidad del Mar Experimental Campus (UMAR; 15.923366, −97.151892), and near Largartero (INK; 15.725127, −96.656343) (see Shlichta et al. (2014) for more details on the populations). Seed samples were taken from 10 plants per site, with the exception of HGO where only 6 plants were available. An average of 250–500 seeds were obtained from each plant. Pods were shelled and seeds were separated for chemical analyses and beetle experiments. Plants for the experiments with the leaf herbivore were grown from the seeds collected (wild) or purchased (cultivated) at the same location/time as the seeds that were used for the beetle experiments.

For all caterpillar experiments, *Spodoptera exigua* (Lepidoptera: Noctuidae) caterpillars were used. *Spodoptera exigua* eggs were supplied by Entomos AG (Grossdietwil, Switzerland). Caterpillars were reared on artificial diet described by Turlings, Davison, and TamÒ (2004) until they were transferred to leaves (3–5 days-old) for preference or performance trials.

The bean weevil, *Zabrotes subfasciatus* (Coleopetera: Bruchidae) attacks mature seeds of wild and cultivated species in the genus *Phaseolus* and it is considered one of the most important pests in bean cultivation and storage (Leroi, Bonet, Pichard, & Biemont 1990; Benrey et al. 1998). Females glue their eggs on the surface of the bean and the larvae hatch and burrow into the seed where they feed until their development is complete and emerge as adults (Benrey et al. 1998; Teixeira, Barchuk, Medeiros, & Zucoloto 2009). The main host plants of *Z. subfasciatus* are seeds of *Phaseolus* species. *Zabrotes subfasciatus* is ubiquitous on *P. lunatus* in Oaxaca, Mexico, and readily feeds and develops on *P. lunatus* in the laboratory (Shlichta et al. 2014). We used our laboratory colony of *Z. subfasciatus* to examine the preference and performance of bruchid beetles on seeds from the different varieties and populations. This colony was established with a mixture of beetles collected from the Mexico City area and Puerto Escondido (UMAR). The beetles were reared on *Phaseolus vulgaris* beans to eliminate any effects or selection for CNGs resistance (Shlichta et al. 2014).

Cyanogenic glycoside quantification

Leaves

Plants were grown in the same soil and under controlled temperature, light and humidity conditions (LD 16:8 at 28 °C) in the same phytotron at University of Neuchatel. Leaves were removed for extraction when plants were 30 days old. To prepare leaf samples for quantification of CNGs, 1 primary and 2 secondary leaves from 5 different plants from each cultivated variety or wild population were removed with a razor blade and immediately submerged in liquid nitrogen. Each leaf sample was individually ground with a clean mortar and pestle to obtain a fine powder. This process was repeated for 5 samples for each cultivated variety and wild population. Approximately 50 mg of prepared leaf 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.

Seeds

The extraction method for the seed samples was very similar to the leaf extraction, except 20 mg of prepared bean powder per sample was used for the seed CNG extraction to account for the additional water in the leaf tissue.

In addition to quantifying the concentration of CNGs, we also quantified the loss of CNG concentration for the seeds and leaves in order to indirectly assess the β-glucosidase activity responsible for the hydrolysis of cyanogenic glycosides into hydrogen cyanide (HCN capacity or HCN-c) (see Appendix A in Supplementary material). This technique has been used in a previous study to indirectly assess HCN-c or the release of HCN when the beetles are feeding on seeds (Shlichta et al. 2014). CNGs were analyzed using an Acquity UPLC system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, USA) controlled by Masslynx 4.1 (CNG analysis method detailed in Shlichta et al. (2014). We focused on two CNGs, linamarin and lotaustralin, because they are the most abundant in the plant (Shlichta et al. 2014). To extract these CNGs from the leaf samples, we used a method adapted from two previous studies on CNGs (Franks, Hayasaka, Choimes, & Van Heeswijck 2005; Rojas & Morales-Ramos 2010) and described in detail in Shlichta et al. (2014).

Caterpillar preference (choice experiment)

Caterpillar preference was tested using leaf disc assays. This method is commonly used to test the preference of herbivores for host plants (Hägele & Rahier 2001; Kazemi-Dinan, Thomaschky, Stein, Krämer, & Müller 2014). Leaf discs measuring 1.5 cm in diameter were cut out with the open end of a 15 ml Falcon^{TM} tube that was sharpened with a file. Discs were cut from leaves that were approximately the same age/at the same area on the plant and were kept moist throughout the experiment on moist filter paper that was misted every 4 h. Caterpillars were given a choice between leaf discs cut from cultivated varieties and wild populations. The choice test was done in a 9 × 2 cm petri dish. Each dish contained four leaf disks, one disk from two different cultivated varieties and one disk from two wild populations. All possible combinations (9 total) were tested for the three cultivated varieties and three wild populations. Each dish received a 2nd instar caterpillar and placed under LD 16:8 for 24 h at room temperature (approximately 23 °C). In order to compare all the 6 varieties in all possible combinations, the total initial sample size was 760 leaf discs/190 caterpillars (some caterpillars were removed from the final analysis if they did not consume any of the discs). There were three trials (3 blocks); trial 1 with 256 disks/64 caterpillars, trial 2 with 252 disks/63 caterpillars, and trial 3 with 252 disks/63 caterpillars. If the caterpillar died during the experiment, the sample was not used for analysis. Leaf discs were scanned and analyzed in Adobe Photoshop® CS4 for percent of leaf disk eaten per dish. In order to verify CNG concentration in the leaf disks, a sample of disks were cut in parallel and tested for change in linamarin and lotaustralin concentration over 24 h. Results indicate that leaf disks maintain on average up to 80% of their original CNG concentration for at least 6 h with a few exceptions (Shlichta et al., unpublished data).

Caterpillar performance

To measure caterpillar performance, larvae were randomly assigned to a plant variety/population. One-month-old plants, which had been grown in 5 kg pots in a phytotron (28 °C, LD 12:12), were transported to the laboratory. Two 2nd instar (3-day-old) or two 3rd instar (5-day-old) caterpillars of similar size were weighed and placed on each plant in a 3 cm diameter clip cage that provided the caterpillars with ample room to move and feed but prevented them from leaving the leaf. A clip cage consisted of a small plastic cylinder covered on one side with a thin mesh nylon screen. Caterpillars were gently placed on the leaf and enclosed with the clip cage. A cardboard circle of the same size as the plastic cylinder covered with cotton wool was placed on the other side of the leaf to prevent injury. Finally, a single metal hair clip was used to secure the plastic dish on the top and the cotton-cardboard piece on the leaf. This method has been used in other studies to keep caterpillars on a leaf for a defined time period (Rostás & Wölfling 2009). Six plants were used for each variety/population. Our approximate sample size for each trial was 12 caterpillars per variety. Three trials were run in 2013. Plants were placed under LD 16:8 and caterpillars were allowed to feed freely for 3 days at room temperature (approximately 23 °C). After 3 days, the caterpillars were removed and weighed again. Dead caterpillars were noted and the remaining living caterpillars were used to calculate survival. Mean relative growth rate (mass difference divided by initial mass) was calculated for each of the living caterpillars.

Bruchid beetle preference (choice experiment)

Beetle preference for a cultivated variety or wild population of *P. lunatus* seeds was also tested in two-choice experiments. Choice experiments which allow seed beetles to choose to oviposit on a selection of seeds are commonly used in other studies with Bruchinae beetles (Cope & Fox 2003; Paukku & Kotiaho 2008). The wild beans were carefully checked for holes or any sign of previous infestation. A male and female bruchid were aspirated from our rearing colony and placed in a cup with the beans. The choice test was done in small plastic 28 × 23 × 5 mm (Semadeni AG, A4686) cups with lids. Each cup contained 2 cultivated beans and 2 wild beans. There were 9 possible combinations of the 3 wild and 3 cultivated varieties. Each combination was replicated 30 times. The choice test was repeated 2 times in 2013. Beetles were less than four days old at the start of the experimental trial and individual beetles were only used once. Each cup with a mating pair was placed on a tray and put in a rearing chamber under controlled conditions (LD 11:13 at 25 °C). Beetles were allowed to lay eggs undisturbed for 3 days. On the third day the number of eggs per bean per cup was noted. If either of the beetles died during these 3 days, the sample was not used for analysis.

Bruchid beetle performance

We used the same experimental set-up as for the choice tests except that each cup contained 5 beans from the same variety or population. In brief, a male and female were aspirated from the beetle colony and placed in a cup with the beans. We prepared approximately 10 cups for each population for a total of 50 beans and 10 mating pairs per population for each experimental trial. We repeated the entire experiment twice in 2013. After three days each bean was examined for eggs, if beans had more than one egg, we haphazardly removed eggs until only one egg remained per bean. Each bean was placed into a 15 ml Falcon^{TM} tube. Only one egg

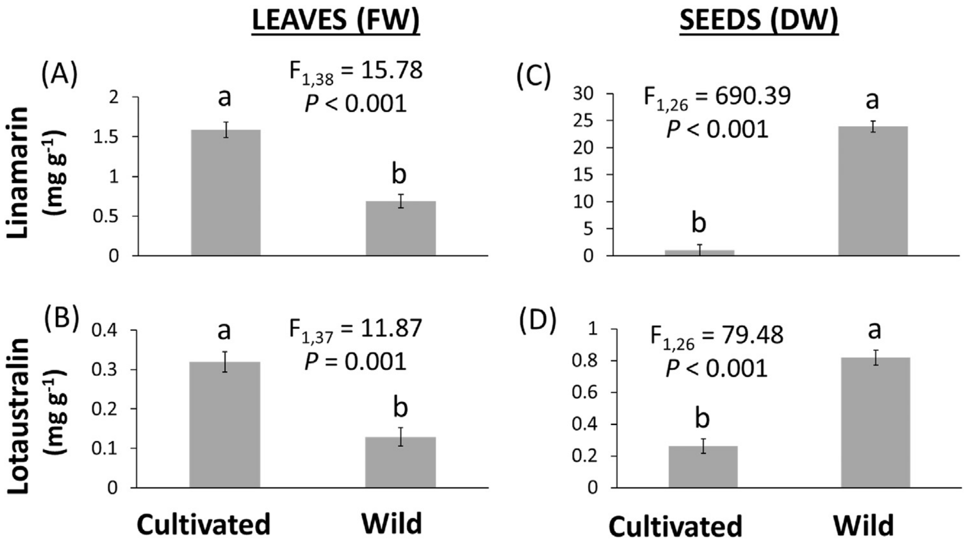

Fig. 1. Concentration of cyanogenic glycosides (CNGs) in cultivated varieties and wild populations of lima bean, *Phaseolus lunatus*. Shown are means and ±SE of concentrations of CNGs in mg/g for linamarin (top) and lotaustralin (bottom), quantified from (A) leaves in fresh weight (FW), and from (B) seeds in dry weight (DW). Letters indicate significant pairwise differences.

was allowed to remain on each bean. If additional eggs were laid on a single bean they were destroyed with a pin to prevent competition between beetle larvae in the same bean. Several small holes were made in the cap of each Falcon^{TM} tube containing a bean with a single egg to allow for air exchange. Tubes were returned to the rearing chamber and checked daily for 7 days until the adult beetles emerged. Date of emergence and sex were noted and the adult beetle was weighed.

Statistical analysis

Data from the chemical analyses were processed with Masslynx 4.1. Linamarin and lotaustralin were quantified by measuring peak areas from the corresponding LC–MS extracted ion chromatograms. The concentration (μg/mL) was calculated using the calibration curve of each standard and further converted into μg or mg/g dry weight (DW) or fresh weight (FW) based on the initial amount of leaf or dry bean powder. The effects of plant domestication (wild vs. cultivated) on plant defences in the seeds and leaves, the performance of *Spodoptera exigua* and bruchid preference were analysed using linear models (PROC GLM in SAS 9.2, SAS Institute, Cary, NC) (Littell, Milliken, Stroup, Wolfinger, & Schabenberger 2007). The effects of plant domestication on bruchid (weight and developmental time) fitness correlates were analysed using linear mixed models (PROC MIXED in SAS) (Littell et al. 2007).

Whenever residuals were not normally distributed, we first used a log-transformation of the data. If normality assumptions were still not met after data transformation, generalized linear mixed model (PROC GLIMMIX in SAS 9.2) were used. This was the case for testing the effects of plant domestication on the percentage of leaf damage by 3-day-old and 5-day-old larvae and on the beetle survival (following a binomial and a gamma distribution, respectively) (Littell et al. 2007).

All the models included plant domestication status (cultivated or wild) and plant population/varieties nested within plants domestication status (to account for natural variation among the three cultivated varieties and the three wild populations) as fixed factors.

Blocks (repetition of the whole experiment at a different time) were included as random factors. When petri dishes were used, their effect was also treated as a random factor to account for repeated measures taken on the same experimental unit.

Because in this species females are larger than males (Campan & Benrey 2006), we separately analysed the weight, and developmental time for males and females. In all cases, we provide model least square means ± S. E. as descriptive statistics.

Results

Cyanogenic glycoside quantification in leaves

The cyanogenic concentration in the leaves of *P. lunatus* was significantly different between cultivated and wild plants (Fig. 1A and B). Significant differences were found among cultivated varieties and wild populations for both linamarin (1A, PROC GLM, $F_{1,38} = 15.78$, $P < 0.001$) and lotaustralin (Fig. 1B, PROC GLM, $F_{1,37} = 11.87$, $P = 0.001$). Leaves from cultivated varieties had more than double the concentration of both linamarin and lotaustralin than leaves from wild populations (Fig. 1A and B), yet these concentrations were highly variable (see Appendix A in Supplementary material).

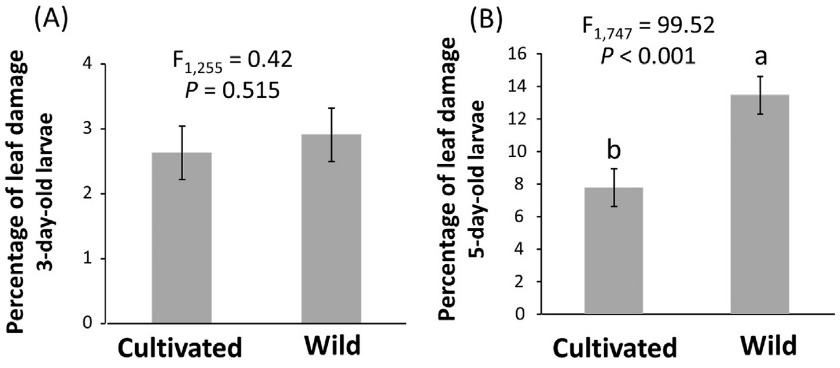

Fig. 2. Percentage of leaf area eaten by *Spodoptera exigua* larvae on cultivated varieties and wild populations of lima bean *Phaseolus lunatus*: (A) leaf area eaten by 3-day-old larvae, and (B) leaf area eaten by 5-day-old larvae. Bars (means ±SE) with different letters are significantly different from each other.

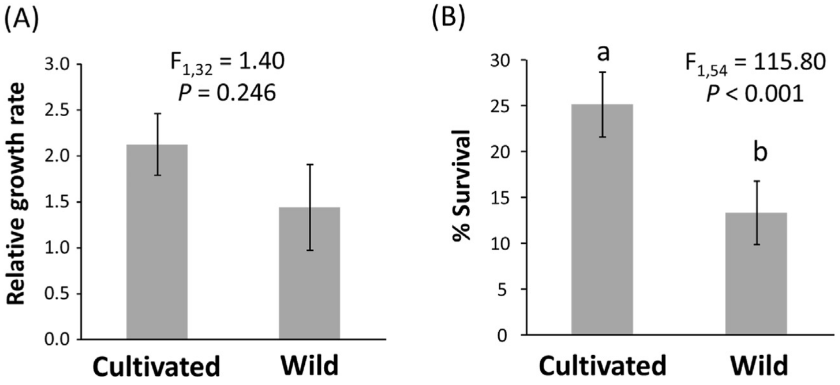

Fig. 3. Performance of *Spodoptera exigua* larvae on leaves of cultivated varieties and wild populations of lima bean *Phaseolus lunatus*. Shown are means and ±SE for (A) relative growth rate, and (B) percent survival. Bars with different letters are significantly different from each other. Relative growth rate was calculated for each individual larva as the mass difference between initial and final mass after 3 days of feeding.

Caterpillar preference

There was no significant difference in the percentage of leaf damage for 3-day-old *S. exigua* caterpillars feeding on cultivated versus wild leaves and damage ranged from 0 to 48% (Fig. 2A, PROC GLIMMIX, $F_{1,255} = 0.42$, $P = 0.515$). In contrast, leaves from wild plants suffered almost a two-fold increase in herbivory by 5-day-old *S. exigua* caterpillars compared to leaves from cultivated plants (Fig. 2B, PROC GLIMMIX, $F_{1,747} = 99.52$, $P < 0.001$).

Caterpillar performance

No significant difference in the relative growth rate of *S. exigua* caterpillars was found on cultivated varieties compared to wild populations (see Fig. 3A, PROC GLM, $F_{1,32} = 1.40$, $P = 0.246$). Caterpillar survival was two times higher on cultivated than on plants from wild populations (see Fig. 3B, PROC GLM, $F_{1,54} = 115.80$, $P < 0.001$).

Cyanogenic glycoside quantification in seed

CNG concentrations for linamarin ($F_{1,26} = 690.39$, $P < 0.001$) and lotaustralin ($F_{1,26} = 79.48$, $P < 0.001$) in the seeds from wild populations were significantly higher than in seeds from the cultivated varieties (see Fig. 1C and D PROC GLM). β-glucosidase activity was shown only to be present when the enzyme was added to the seed. This was the same for all varieties and cultivars (see Appendix A in Supplementary material, ANOVA on ranks, linamarin: $P < 0.0001$; lotaustralin; $P < 0.0001$, Tukey's HSD; $P < 0.05$).

Bruchid beetle preference

When female beetles had the choice between cultivated and wild seeds, they showed a preference for cultivated beans. Beetles laid significantly more eggs on beans from all of the cultivated varieties compared to the wild populations (PROC GLM, $F_{1,615} = 330.56$, $P < 0.001$, Fig. 4).

Bruchid beetle performance

There was no significant difference between the adult mass of female and male *Z. subfasciatus* that fed on seeds from cultivated varieties or wild populations (see Fig. 5A and C, PROC MIXED, $F_{1,64} = 3.03$, $P = 0.086$ and $F_{1,54} = 2.72$, $P = 0.105$, respectively). We found a significant difference in the development time of females ($F_{1,64} = 5.25$, $P = 0.025$, on

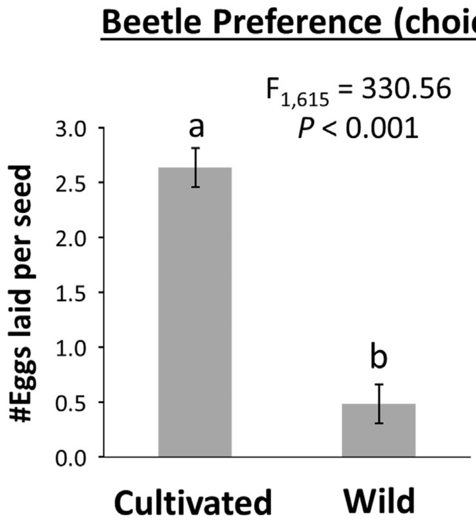

Fig. 4. Number of eggs laid by *Zabrotes subfasciatus* beetles on wild and cultivated seeds of lima bean *Phaseolus lunatus*. Bars (means ± SE) with different letters are significantly different from each other.

average 2–3 days difference) but not the males ($F_{1,54} = 1.05$, $P = 0.309$) in cultivated versus wild seeds (see Fig. 5B and D, PROC MIXED).

Discussion

The objective of our study was to examine the extent to which domestication of lima bean has altered its chemical defenses in leaves and seeds and how this affects insect herbivores that attack these different plant structures. As generally expected, we found that domestication of *P. lunatus* has altered the concentration of CNGs, but as we predicted, it has differentially affected leaves and seeds. Leaves from cultivated varieties had significantly higher CNGs than leaves from the wild populations. Conversely, seeds from the cultivated plants were found to have dramatically lower concentrations. This result can be explained by the fact that cultivated lima bean plants have been specifically selected for human consumption of the seed and, among other traits, lower concentrations of toxic and deterrent compounds must be a result, at least in part, of this selection process. It is evident

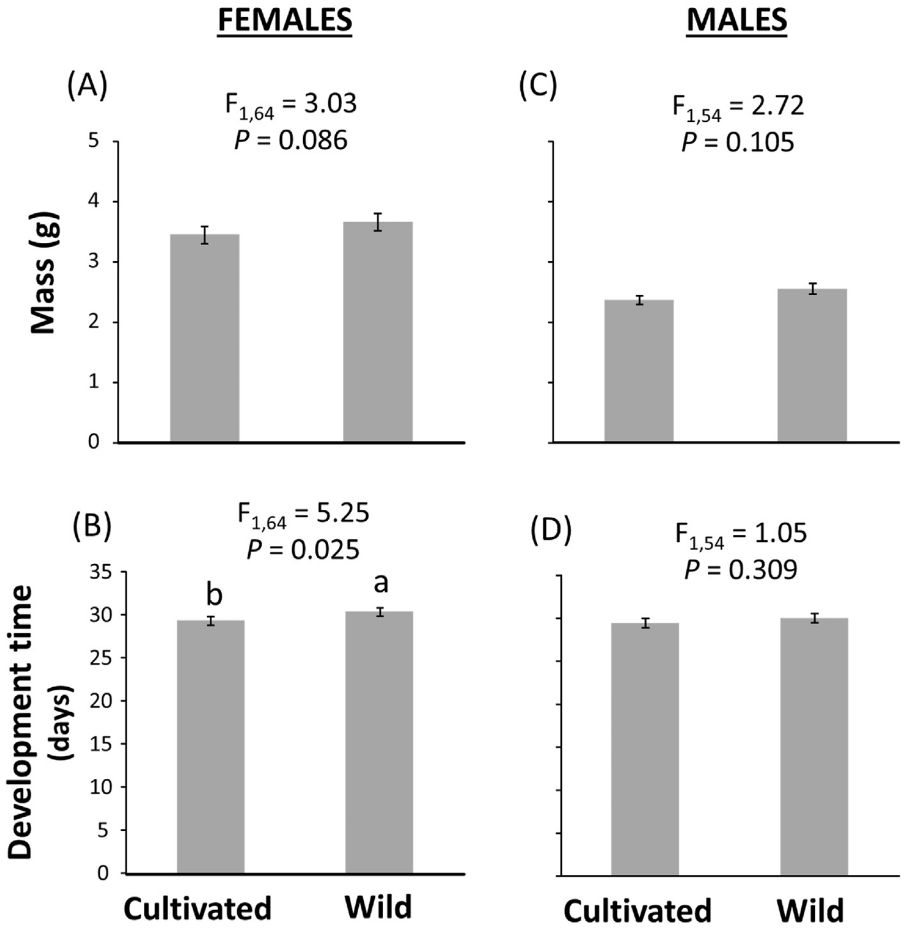

Fig. 5. Performance of *Zabrotes subfasciatus* beetles reared on wild and cultivated seeds of lima bean *Phaseolus lunatus*: (A) body mass of adult females, and (C) males, and (B) development time of females, and (D) males. Bars (means ± SE) with different letters are significantly different from each other.

from our study that selection against secondary compounds in one plant structure is not necessarily correlated with the reduction of the toxic compound in all structures of the plant. While selection for bean consumption may adequately explain our findings, plant allocation of defenses to the most valuable tissues could also play a role. In a recent study, Godschalx, Stady, Watzig, and Ballhorn (2016) showed that among the reproductive organs of lima bean plants, young pods had higher cyanogenic potential than mature pods, and the cyanogenic potential of young leaves was higher than that of old leaves. Our results are also in accordance with these findings. We found that independent of the domestication status (wild or cultivated) seeds are overall better protected than leaves (Fig. 1).

The results for the leaves of wild and cultivated lima bean plants also support those reported by Ballhorn et al. (2010). Their study included 4 cultivated varieties and one wild type. Similar to our findings, they found high variation in the concentration of CNGs in the leaves of their cultivated varieties. However, conversely to our results, the one wild variety used in their study had a higher concentration of CNGs compared to 2 of the cultivated varieties. In addition, they found that lower concentrations of CNGs in the leaves were correlated with the preference of their generalist herbivore, *Schistocerca gregaria*. In our study, three-day-old larvae of *S. exigua* did not appear to prefer the cultivated varieties (Fig. 2A). On the other hand, consumption by five-day old larvae showed a strong preference for leaves from wild plants (Fig. 2B). Since leaves from wild populations clearly have lower CNGs, caterpillars appear to be targeting leaves that are lower in CNGs, at least to some degree, when given a choice. Yet, CNG concentrations may not be perfectly indicative of toxicity, as the liberation of the toxic gaseous hydrogen cyanide (HCN) is also dependent on beta-glucosidase activity (Ballhorn et al. 2010). In fact, HCN might be used by this caterpillar as a cue to avoid toxic leaves, and HCN release is not necessarily correlated with CNG concentration. Alternatively, it is likely that other plant traits operate in conjunction with CNGs and may play a role in caterpillar preference. For example, in a study conducted in Madagascar, Ballhorn, Kautz, and Rakotoarivelo (2009) found that lemurs preferred to feed on the more toxic younger leaves of bamboo plants that contained more proteins than on the less toxic older leaves. Additionally, trade-offs may exist between CNGs and other chemical defenses and between chemical and mechanical defenses (Ballhorn et al. 2008; Ballhorn, Godschalx, & Kautz 2013). For example, a trade-off between concentrations of CNGs in leaves and volatile emissions has been reported for lima bean (Ballhorn et al. 2013) and a trade-off between CNGs and tolerance to drought in white clover (Kooyers, Gage, Al-Lozi, & Olsen 2014).

The results for the seed beetles also support our predictions. Beetles consistently laid more eggs and performed better on seeds of cultivated varieties with lower concentration of CNGs. But again, it is important to note that concentration of CNGs is just one of the suite of plant traits that has been changed during bean domestication and we can therefore not conclude that it is the sole factor that accounts for the observed oviposition preference. Indeed, a recent study with wild and cultivated seeds of lima bean showed that the larger seed size of cultivated seeds can attenuate the intensity of larval competition inside the seed and this results in increased beetle survival (Cuny et al. 2017). In our study, it is unlikely that seed size was a major driver of beetle performance as we controlled for the number of larvae inside the beans. Although we cannot disentangle the contribution of each of the traits that are part of the domestication syndrome in beans, it is clear that cultivated seeds are more suitable for the beetles and this is not entirely the case for cultivated leaves. Other external characteristics of the seed, such as shape, hardness and contact chemicals of the seed coat can influence oviposition choice in other seed beetles (Szentesi & Jermy 1995; Thiéry, Jarry, & Pouzat 1994). The specific effects of CNGs on the beetle's oviposition decisions, as well as their presence in the seed coat remain to be examined.

In addition to domestication affecting the preference of herbivores, it is predicted that herbivores should perform better on cultivated varieties. However, we found no significant difference in relative growth rate of *S. exigua* on leaves of cultivated varieties compared to leaves from wild populations. *Spodoptera* is a generalist and bean plants are not among its most common host plants, therefore they are not adapted to process CNG, even in small concentrations. When given a choice, caterpillars will feed on the wild plants with the lower CNGs even though the long-term effect is a reduction in survival and a possible side effect is that they consume more leaf material to obtain the necessary nutrition to reach an adequate mass to molt into the next instar or pupate. Regardless of CNG concentration, this generalist caterpillar's survival was higher when fed on the leaves from the cultivated plants. This is not surprising because Turcotte et al. (2014) found, also for *S. exigua*, that domestication of several plant species was associated with higher larval survival compared to the wild relatives. Furthermore, CNG concentration does not appear to be a determining factor for short-term caterpillar growth but appears to have an effect on the overall survival. This result was counter to our expectations. Thus, *S. exigua* caterpillars choose to feed on a plant with a lower CNG concentration, but their survival was higher when they fed on leaves from cultivated plants with higher concentrations. This result indicates that other traits of leaves from cultivated plants are important in long-term survival and potentially outweigh the cost of the secondary chemistry. Nutritional components such as the mineral content among cultivars (Beebe, Toro Ch, Gonza, Chaco, & Debouck 1997; Gouveia, Freitas, de Brito, Slaski, & de Carvalho 2014) may play a significant role in increasing the survival of this generalist caterpillar.

Similarly, in cultivated beans selection for reduced toxicity appears to be associated with increases in nutrient content (De La Vega & Sotelo 1986). Thus, it is no surprise that beetles developed more quickly in the cultivated seeds since increased nutrient content in plants is generally correlated with an increase in herbivore performance (Benrey et al. 1998; Chen, Gols, Stratton et al. 2015). Our previous studies on *Zabrotes subfasciatus* show high variation of CNGs in the wild populations of *P. lunatus* along the coast of Oaxaca, Mexico, but beetles do not significantly vary in their performance (development time or adult mass) on beans from these different populations (Shlichta et al. 2014). This implies that beetles are able to withstand high concentrations of CNGs when necessary and therefore their preference for cultivated varieties may not be based on CNG concentrations. The mechanism behind their tolerance to high concentrations of CNGs is still unknown.

Finally, caution should be taken about generalizing our results, as we only used three cultivated varieties and three wild accessions. Significant variation has been found in the concentration of CNGs among wild accessions and cultivated varieties of lima bean both in leaves (Ballhorn et al. 2008) and seeds (Shlichta et al. 2014) Yet, in this and other studies we have consistently found that differences in CNGs and other plant traits is greater between wild and cultivated plants than variation within each domestication status (Cuny et al. 2017; Zaugg, Benrey, & Bacher 2013) (Appendix A in Supplementary material).

In conclusion, this study presents support for the hypothesis that the reduction in chemical defenses in domesticated plants pertains primarily to plant parts that have been selected for consumption. In cultivated lima bean we found far lower concentration of CNGs in seeds in comparison with the concentrations found in seeds of wild lima bean. Such differences were not observed for the leaves, for which the CNG concentrations were higher. These findings are not unique for lima bean plants. Dicenta et al. (2002), found that in sweet almonds, concentrations of cyanogenic compounds are much higher in leaves and roots than in the edible seeds. These results imply that the common assumption that crops have reduced defenses needs fine-tuning. Similar studies with other crops are needed to further substantiate our premise that plant breeding has predominantly caused such reductions in the edible parts of the crops. Moreover, future studies should also examine the relationship between plant domestication status and resistance against herbivores in non-edible crops such as ornamentals and crops used in fiber production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.baae.2018.05.012.

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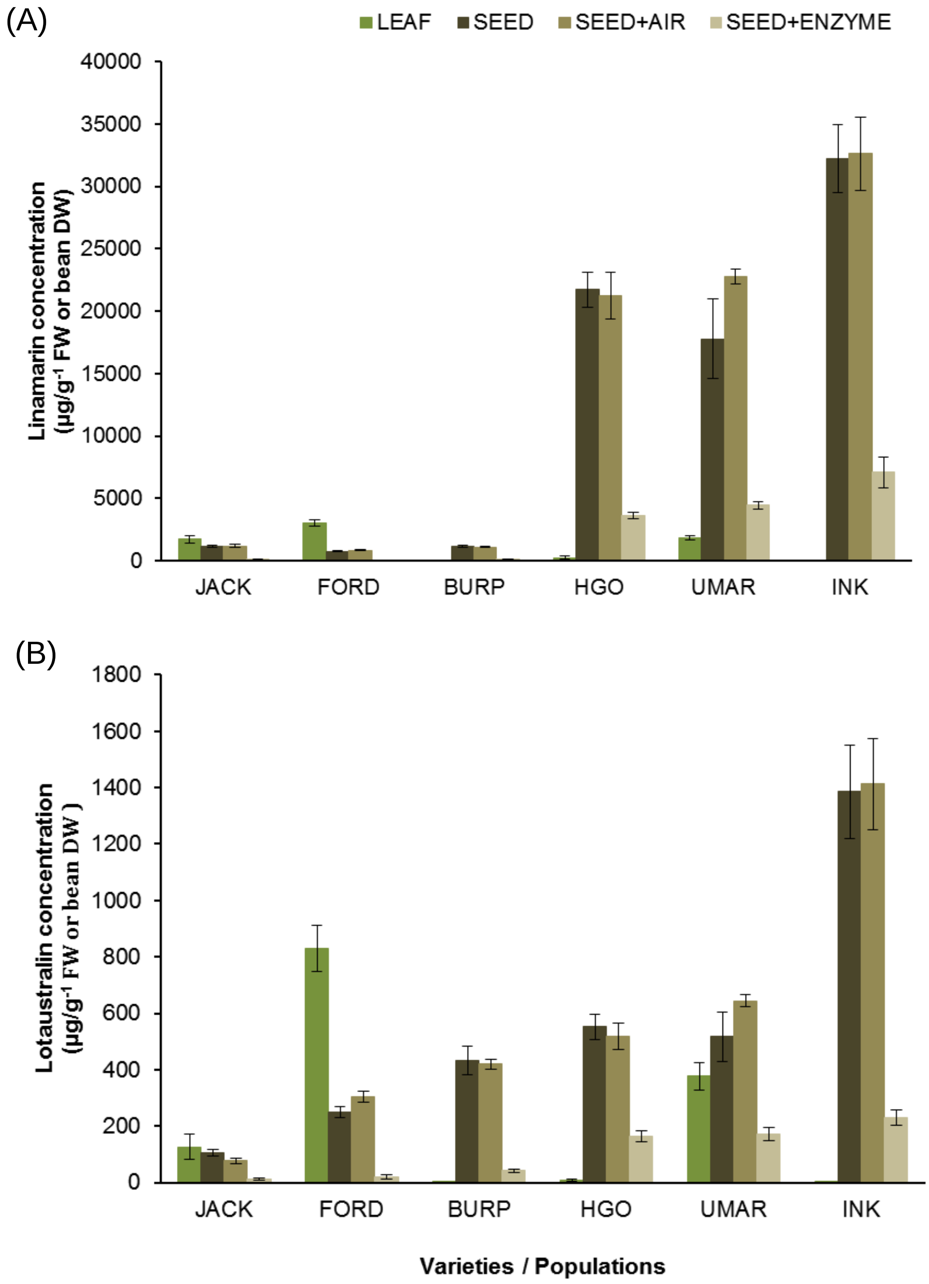
Supplementary material



Appendix A. Analysis of β-glucosidase activity in beans compared to leaf control. Graph shows the mean concentration of the cyanogenic glycoside compound (A) linamarin, and (B) lotaustralin, quantified from bean dry weight (DW) and leaf fresh weight (FW). Concentration in µg/g. Analysis was performed on six samples from leaves or beans from plants from each cultivated variety and wild population. Values shown are means±SE. Samples in which β-glucosidase enzyme extract was added to ground bean were found to be signifcantly different from seed samples and the seed samples exposed to air for all populations and cultivars (ANOVA on ranks, linamarin: $P<0.0001$; lotaustralin; $P<0.0001$). Significant differences calculated for beans by a post-hoc test (Tukey's HSD; $P<0.05$) after one-way ANOVA on rank

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