SPECIFICITY OF INDUCED DEFENSES, GROWTH, AND REPRODUCTION
IN LIMA BEAN (Phaseolus lunatus, Fabaceae) IN RESPONSE TO MULTI-
SPECIES HERBIVORY

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/premise of the study: Following herbivore attack, plants can either reduce damage by inducing defenses or mitigate herbivory effects through compensatory growth and reproduction. It is increasingly recognized that plant induced defenses are herbivore-specific, but less is known about the specificity of compensatory responses. Additionally, damage by multiple herbivores may lead to synergistic effects on induction and plant fitness that differ from those arising from effects of single-species herbivore damage. Relatedly, although largely unstudied, the order of arrival and damage by different herbivore species might also play an important role in determining the impacts of herbivory on plants.

Methods: We investigated the specificity of defense induction (phenolics) and effects on growth (number of stems and leaves), and reproduction (number of seeds, weight and germination rate) from feeding by two generalist leaf-chewing herbivores (Spodoptera eridania and Diabrotica balteata) on Phaseolus lunatus plants, and evaluated whether simultaneous attack by both herbivores and order of arrival influenced such dynamics.

Key results: Herbivory increased levels of leaf phenolics, but such effects were not herbivore-specific. In contrast, herbivory enhanced seed germination in an herbivore-specific manner. For all variables measured, the combined effects of both herbivore species did not differ from their individual effects. Finally, the order of herbivore arrival did not influence defense induction, plant growth, or seed number but did influence seed weight and germination.

Conclusions: Overall, this study highlights novel aspects of the specificity of plant induced responses to multi-species herbivore damage, and uniquely associates such effects to plant lifetime fitness.
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**Keywords:** Diabrotica balteata; phenolic compounds; seed germination; seed weight; Spodoptera eridania; tolerance

**INTRODUCTION**

Plants have evolved multiple strategies associated with the induction of mechanisms or traits in response to herbivore attack (Fineblum and Rausher, 1995; Strauss and Agrawal, 1999; Núñez-Farfán et al., 2007). On the one hand, following herbivore attack plants can increase the production of chemical compounds or physical traits that drastically reduce herbivore damage (Núñez-Farfán et al., 2007; Agrawal, 2011). On the other hand, following herbivore damage, plants can mitigate the negative effects of herbivory by mechanisms of induced tolerance such as compensatory growth and reproduction, increased photosynthetic rates, and changes in nutrient allocation and uptake (Strauss and Agrawal, 1999; Stowe et al., 2000; Núñez-Farfán et al., 2007; Fornoni, 2011; Moreira et al., 2012; Carmona and Fornoni, 2013).

It is increasingly recognized that plant induced responses to herbivory largely depend on herbivore identity (Agrawal, 2000; Van Zandt and Agrawal, 2004; Rasmann and Turlings, 2008; Bingham and Agrawal, 2010; Moreira et al., 2013). Recent investigations have shown that plants are able to recognize biotic stimuli (e.g. oviposition secretions and saliva) produced by different herbivores, integrate the perceived information, and react accordingly in a highly specific manner (Mithöfer and Boland, 2008). The specificity of plant induced responses to herbivore damage depends on the type and amount of damage, as well as herbivore diet breadth and feeding guild (e.g. Rasmann and Turlings, 2008; Clavijo McCormick et al., 2012; Xiao et al., 2012;
Carmona and Fornoni, 2013; Moreira et al., 2013). For instance, Rasmann and Turlings (2008) found that the emission of volatile compounds in maize roots drastically varied depending on the diet breadth of the root herbivore. Similarly, Moreira et al. (2013) observed highly specific changes in carbon-based defenses for two pine species after damage by a phloem-feeder and a folivore. Despite such evidence for the specificity of induced defenses, much less is known about the specificity of induction of traits associated with tolerance against herbivory (but see Manzaneda et al., 2010; Carmona and Fornoni, 2013; Utsumi et al., 2013; Carrillo et al., 2014). One exception is a study by Utsumi et al. (2013) who reported that insect herbivore community composition determined the degree of herbivore-induced regrowth intensity of willow trees. Similarly, Carrillo et al. (2014) demonstrated specificity of tolerance to different generalist herbivores for native but not for invasive populations of the Chinese tallow tree.

Simultaneous attack by multiple herbivore species often elicits different induced plant responses than would otherwise be triggered by a single-species attack (Agrawal, 2000; Kessler and Halitschke, 2007; Rasmann and Turlings, 2007; Rodriguez-Saona et al., 2010; Utsumi et al., 2013). Such variation in responses has been attributed to synergistic or antagonistic effects from feeding by multiple species, leading to effects that cannot be predicted based upon the individual effects of each herbivore species. In addition, the order of arrival and type of damage produced by different herbivore species can also play an important role in determining the impacts of multiple herbivores feeding on the same host plant. Although a number of studies have demonstrated that damage by early herbivores triggers a wide range of plant induced responses that negatively affect the performance of subsequent herbivores (Rodríguez-
Saona et al., 2005; Viswanathan et al., 2007; Poelman et al., 2008; Erb et al., 2011; McArt et al., 2013; Wang et al., 2014), relatively few studies have addressed how the chronology of herbivore attack influences induced defense, growth and reproduction in plants (but see Poelman et al., 2008; Wang et al., 2014). In one of the few available studies, Wang et al. (2014) observed that the chronological order of aboveground and belowground herbivory in an herbaceous plant differentially induced the production of iridoid glycosides in stem and roots.

The main goal of this study was to investigate the specificity in magnitude and direction of induced plant defense, growth and reproductive responses to feeding by multiple herbivore species. To achieve these goals we carried out a field experiment where we tested the individual and combined effects of two generalist insect leaf-chewers (Spodoptera eridania and Diabrotica balteata) on wild lima bean Phaseolus lunatus L (Fabaceae) plants. For the combined-species treatment, we also tested whether the order of arrival of each herbivore influenced plant induced defenses, growth and reproduction. We measured leaf phenolic concentration, plant growth (number of leaves and stems), and reproduction (number of seeds, weight and proportion of germinated seeds) throughout an entire growing season, and because P. lunatus is an annual species, measurements of seed output and germination provided direct estimates of lifetime fitness. Specifically, we addressed the following questions: (i) Are plant induced defenses and effects on growth and reproduction herbivore-specific? (ii) Do combined effects of both herbivores differ from individual herbivore species effects? And (iii) is specificity of induced responses contingent upon the order of arrival of these herbivore species? By addressing these questions, our work builds towards a better understanding of the specificity of plant induced responses to herbivory under a
biologically realistic scenario where multiple herbivore species co-exist on the same host plant.

MATERIAL AND METHODS

Study system — *Phaseolus lunatus* (lima bean) is an annual legume distributed along the Pacific coast from Mexico to South America (Freytag and Debouck, 2002; Heil, 2004; Delgado-Salinas et al., 2006). At our field site, 15 km northwest of Puerto Escondido, Oaxaca, Mexico (15°55’27.4”N, 97°09’03.0”W), *P. lunatus* germinates between June and July and flowers at the beginning of October. Seeds are produced during November and December and disperse in January and February (Freytag and Debouck, 2002). Leaves are divided into three oval-shaped leaflets that are arranged alternately on the stem (Freytag and Debouck, 2002).

At our field site, *P. lunatus* is attacked by a diverse community of insect herbivores, including two common leaf-chewers: *Spodoptera eridania* (Stoll) (Lepidoptera: Noctuidae), a polyphagous moth native to the American tropics whose larvae feed on the lower surface of leaves, especially at night (Capinera, 2001), and *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae), a polyphagous beetle distributed from North America to Central America whose adults severely defoliate leaf tips of juvenile and adult plants (Teng et al., 1984). Although at the middle of the growing season these herbivore species are frequently found feeding simultaneously on the same *P. lunatus* plants, they typically vary in their order of arrival at the start of the growing season, with plants being exposed to damage by one species for several days before the other herbivore arrives (X. Moreira, personal observation).
Experimental set-up — In early October 2014, we collected seeds from wild plants of *P. lunatus* growing in a population along the Pacific coast of Mexico (Coyuca de Benítez, Guerrero, Mexico; 17°00'40.5"N 100°06'10.2"W; Shlichta et al., 2014). We individually sowed seeds in 5 L pots with a mixture composed of native soil and peat moss. After emergence, we kept all plants in nylon mesh field cages (Bioquip, Outdoor Cage 6’ × 6’ × 6’, 20 × 20 Mesh Lumite) for four weeks to prevent undesired herbivory. When plants were four-weeks old, we counted the number of leaves per plant (“number of initial leaves” hereafter), formed groups of five randomly selected plants, and each group of potted plants was placed in a nylon mesh cage in the field (same cages as above). Within each cage, we applied one of the following herbivory treatments to each plant: (1) control (untreated, “herbivore-free” plants), (2) *S. eridania* alone (we added 10 third-instar larvae), (3) *D. balteata* alone (we added five adults), (4) *S. eridania* plus *D. balteata* (we added 10 third-instar larvae of *S. eridania* and two days later we added five adults of *D. balteata*), and (5) *D. balteata* plus *S. eridania* (we added five adults of *D. balteata* and two days later we added 10 third-instar larvae of *S. eridania*). In both of the sequential herbivore treatment, the first herbivore continued feeding after adding the second herbivore. In total, there were 50 plants corresponding to 10 cages and five plants per cage (i.e. one plant per herbivory treatment) and plants of treatments 2-5 (above) were exposed to herbivores for four days. Within each cage, we individually covered each plant with a nylon mesh to avoid herbivore escape or interference among treatments. Two days after adding the second herbivore for treatments 4-5, we removed all the herbivores and the nylon meshes and scored leaf damage for the whole plant in situ using a five-level scale: 0 = undamaged leaves, 1 = less than 25% damaged leaves, 2 = between 25-50% damaged leaves, 3 = 50-75% damaged leaves, and 4 = more than
75% damaged leaves (i.e. 0–4 score). Throughout the experiment, we watered all the plants twice a week.

Effects of herbivory on induced defenses — Immediately after herbivore removal, we randomly collected four young, fully expanded leaves located half-way along the stem of each plant to measure the concentration of phenolic compounds. Phenolic compounds are widely recognized as herbivore deterrents across many plant taxa (Salminen and Karonen, 2011; Mithöfer and Boland, 2012; Moreira et al., 2014) and have been demonstrated to confer resistance against leaf herbivores in P. lunatus (Ballhorn, 2011; Ballhorn et al., 2011). We extracted phenolic compounds using 10 mg of dry plant tissue with 500 µL of 100% methanol in an ultrasonic bath for 15 min, followed by centrifugation and subsequent dilution of 300 µL of the methanolic extract with 100 µL water (Moreira et al., 2014). We performed phenolic profiling by Ultra-High-Pressure Liquid Chromatography coupled with Quadrupole-Time-Of-Flight Mass Spectrometry (UHPLC-QTOF-MS). We used an Acquity UPLCTM system coupled with a Synapt G2 QTOF-MS (Waters, Milford, CT, USA). We achieved the separation of compounds at a flow rate of 400 µL min⁻¹ on a reverse-phase Acquity BEH C18 column (50x2.1 mm column, particle size 1.7 µm, Waters) thermostated at 45°C. Solvents were A= water + 0.05% vol. formic acid; B = acetonitrile + 0.05% vol. formic acid. The gradient program was as follows: 5-30% B in 6 min, 30-100% B in 2 min, holding at 100% B for 2 min followed by re-equilibration at 5% B for 2 min. The injection volume was 2 µl. Mass over charge (m/z) data from the QTOF-MS were obtained in negative ion mode over an m/z range of 85-1200 Da with the following parameters: capillary voltage at -2.5 kV, cone voltage -25 V, source temperature 120 °C,
desolvation gas temperature 350 ºC, desolvation gas flow 800 L hr⁻¹. We identified 194 individual phenolic compounds (10 flavonoids and two coumaric acid derivatives; see 195 Tables SM1, SM2, SM3) using the MSE mode which consists in alternate scans at low 196 (4eV) and high (10-30 eV ramp) collision energies. We used argon as collision gas at a 197 flow of 2.2 mL min⁻¹. We obtained internal calibration of the instrument by infusing a 198 solution of leucine-enkephaline at 400 ng mL⁻¹ at a flow rate of 15 µL min⁻¹ through the 199 Lock SprayTM probe. Whenever ion abundance exceeded the linearity domain of the 200 QTOF-MS, we used UV traces obtained from the integrated photodiode array detector 201 of the UPLCTM. We quantified the concentration of phenolics as rutin equivalents 202 using a calibration curve made of a rutin standard at 0.1, 0.5, 2, 10 and 50 µg mL⁻¹.

**Effects of herbivory on plant growth and reproduction** — *Growth* — Immediately after 205 removing herbivores, for each plant we performed weekly counts of leaf number and 206 stem number throughout a four week-period until plants started producing pods.

*Reproduction* — At the end of the growing season (12 weeks after applying 208 herbivory treatments), and once plants started wilting, we collected all mature bean pods 209 present per plant on a daily basis until plants dried (about 15 weeks after applying 210 herbivory treatments). We shelled the pods and counted the number of seeds. In 211 addition, we weighed five randomly chosen seeds per plant to the nearest 0.00001 g. 212 Finally, we sowed groups of three randomly chosen seeds per plant in plastic cups to 213 evaluate seed germination. We recorded the number of emerged seedlings per cup 214 throughout a two-week period and estimated the proportion of germinated seeds. In all 215 cases, we selected seeds from a similar phenological stage.
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Statistical analyses — We analyzed the individual and combined effects of herbivores on leaf damage, defenses, growth, and reproductive traits using linear mixed models. For growth and reproductive traits, we analyzed cumulative values across sampling dates. For each variable we ran three independent sets of models based on different subsets of the data. First, to evaluate the specificity of individual effects of each herbivore on damage, defenses, growth and reproduction, we performed sets of models that only included and compared control plants, plants attacked by *D. balteata* alone, and *S. eridania* alone (except for leaf damage where we did not include comparisons with the control group). Significant effects of one but not the other herbivore species with respect to the control or significant effects of both herbivore treatments relative to the control but with herbivore treatments differing themselves demonstrate specificity of plant responses. Second, to test for the combined effects of both herbivores, we performed sets of models including only plants from the single-species and combined-species (sequential) treatments and conducted a pre-planned contrast where we compared the mean of the single-species herbivore treatments to the mean of the combined-species (sequential) herbivore treatments. This represents a conservative test of the combined effects of herbivores, since one of the herbivores in the sequential treatments was exposed to plants for half the time relative to the other, i.e. plants were not exposed simultaneously to both herbivores from the start of the experiment. A significant difference between the means of these treatment groups demonstrates the existence of combined effects of these herbivores over and above the individual effects of each herbivore. Third, to evaluate the effect of herbivore arrival order, we performed models that only included and compared control plants, plants attacked by *S. eridania* plus *D. balteata*, and plants attacked by *D. balteata* plus *S. eridania*. Significant effects
of only one of the these herbivore treatments with respect to the control or significant
effects of both herbivore treatments relative to the control but with herbivore treatments
differing themselves would demonstrate an effect of order of arrival on plant defense
induction, growth, or reproduction. We used Tukey tests for pairwise comparisons
among treatment level means for the first and third set of models, as this method
corrects for Type I error inflation due to multiple comparisons. For all of the above
models, herbivory treatment (with a particular combination of treatment levels for each
set of models) was treated as a fixed effect and we included cage as a random effect to
account for non-independence among plants sampled within the same cage. In addition,
to account for differences in initial plant size, we included the number of leaves at the
start of the experiment as a covariate in the models for number of leaves. In addition, to
account for the differences in the amount of damage which could influence plant
responses associated with tolerance to herbivory (i.e. growth, reproduction), as well as
confound effects of amount of damage relative to herbivore species identity (i.e. if one
herbivore species consistently inflicted more damage than the other one), we included
leaf damage score as a covariate in the models for growth and reproductive traits (Hakes
and Cronin, 2011).

Residuals were normally distributed for most variables measured except leaf
damage score which was log-transformed to achieve normality of residuals. In addition,
the proportion of germinated seeds was analyzed using a generalized linear mixed
model with a binomial distribution (logit link) (Littell et al., 2006), as data was non-
normal after transformation. PROC MIXED in SAS 9.2 (SAS Institute, Cary, NC) was
used to run the general linear models (normal distribution), whereas the generalized
linear model was run with PROC GLIMMIX (Littell et al., 2006). In all cases, we provide model least square means ± S. E. as descriptive statistics.

RESULTS

Patterns of leaf damage — There was no difference between herbivore species in the amount of damage (Fig. 1a). However, we found that leaf damage was significantly greater for plants exposed to both herbivores relative to plants exposed to a single species (single-species mean vs. two-species mean; Fig. 1b). The order of herbivore species arrival did not influence the amount of leaf damage (Fig. 1c), as leaf damage scores were not significantly different between plants attacked first by S. eridania and subsequently by D. balteata and plants attacked first by D. balteata and subsequently by S. eridania (Fig. 1c).

Effects of herbivory on plant defenses — The concentration of total phenolics in leaves was significantly higher in plants from both single-species herbivore treatments relative to control plants, but the single-species herbivore treatments did not differ themselves which indicates that the magnitude of induced defenses was not herbivore-specific (Fig. 2a). The same pattern was observed for 8 out of 12 phenolic compounds based upon analyses conducted separately for each compound (Appendix S1, see Supplemental Data with the online version of this article). On the other hand, we found that the mean of total concentration of phenolics for the combined herbivore treatment was not significantly different relative to the mean of the single-species treatments (Fig. 2b; similar pattern for individual compound-based analyses, Appendix S2, see Supplemental Data with the online version of this article), indicating that combined
herbivore effects on induced defenses were not greater than individual species effects. In addition, our test of sequential effects indicated that the mean concentration of total phenolics in leaves was significantly greater for both sequential herbivory treatments relative to controls, but the sequential herbivory treatments did not differ themselves (Fig. 2c), indicating that the order of herbivore arrival did not influence the amount of induced defenses. A similar pattern was observed for five out of 12 phenolic compounds based on individual compound-based analyses (Appendix S3, see Supplemental Data with the online version of this article).

Effects of herbivory on plant growth and reproduction — Growth — We found that the number of stems and leaves were not significantly different between plants from the single-species herbivore treatments and control plants, and that the single-species herbivore treatments did not differ themselves (Fig. 3a, 3b), i.e. herbivory did not influence stem and leaf production and such lack of effect was consistent between herbivore species (i.e. no herbivore species specificity). Likewise, the number of stems and leaves were not significantly different between the combined herbivore treatments and the single-species treatments (Fig. 3c, 3d), i.e. combined herbivore effects on plant growth were not greater than individual species effects. In addition, the number of stems and leaves were not significantly different between plants of each sequential herbivory treatment relative to control plants and the sequential herbivory treatments did not differ themselves (Fig. 3e, 3f), indicating that there were no effects of herbivore arrival order on plant growth.

Overall, results from previous measurements of growth traits (i.e. two and three weeks after application of the herbivory treatments) were qualitatively similar to those
observed at the end of measurements (i.e. four weeks after application of the herbivory
treatments) (data not shown).

**Reproduction** — The number of seeds and seed weight were not significantly
different between either of single-species herbivore treatments and controls, and the
single-species herbivore treatments did not differ themselves (Fig. 4a). In addition,
although seed weight was significantly lower for plants from the single-species *D.
balteata* treatment relative to the single-species *S. eridania* treatment, neither one of
these treatment groups differed from controls (Fig. 4b). In contrast, we found that the
proportion of germinated seeds was significantly greater for plants damaged by *S.
eridania* relative to control plants (Fig. 4c), whereas plants damaged by *D. balteata* did
not differ from controls, indicating that herbivore effects on seed germination were
species-specific (Fig. 4c).

The number of seeds, seed weight and proportion of germinated seeds were not
significantly different between the mean of the single-species relative to the mean of the
two-species herbivore treatments (Fig. 4d, 4e, 4f), indicating that combined herbivore
effects did not differ relative to individual species effects.

Finally, the number of seeds was not significantly different between either
sequential herbivory treatment relative to controls, and the sequential herbivory
treatments did not differ themselves (Fig. 4g). However, we found that seed weight and
the proportion of germinated seeds were significantly different between sequential
herbivory treatments. Mean values in both cases were greater for plants attacked first by
*D. balteata* and subsequently by *S. eridania* than for plants attacked first by *S. eridiana*
and subsequently by *D. baleata*. Plants of the former treatment differed relative to
control plants (Fig. 4h, 4i), whereas plants attacked first by *S. eridania* and then by *D.
balteata did not differ from controls, indicating that the order of herbivore arrival determined the effects of herbivory on these seed traits (Fig. 4h, 4i).

DISCUSSION

Overview — Our study revealed important and novel aspects of the specificity of plant induced responses to multi-species herbivore damage, and uniquely associates such dynamics to plant lifetime fitness. First, we found that the individual effects of leaf herbivory by S. eridania and D. balteata produced different types of induced responses in P. lunatus depending on the response variable measured. Such effects included increased production of total phenolics in leaves as well as enhanced seed germination. In the first case, the magnitude of defense induction was the same for both herbivore species. However, for seed germination herbivore effects were species-specific as S. eridiana had a positive effect whereas D. balteata had no influence on this seed trait. Second, except for leaf damage where combined herbivore effects were greater than individual species effects, we found that the combined effects of both herbivore species on defenses, growth and reproduction did not differ from the individual herbivore species effects. This suggests, on the one hand, that the amount of damage inflicted is not proportionally related to the magnitude of induction of chemical defenses by P. lunatus (i.e. combined effects on leaf damage but not on defenses), and on the other that this plant is able to compensate for cumulative effects of multiple herbivores and not to exhibit further reductions in growth and/or reproduction. Third, we found that the order of herbivore arrival did not affect the amount of induced defenses or plant growth but did influence seed weight and seed germination, two important determinants of lifetime fitness in P. lunatus. This suggests that the chronology of plant-herbivore interactions is
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an important aspect to consider in predicting the impact of multi-species herbivory on plant reproduction.

**Herbivore species-specific effects on P. lunatus** — Our results showed that individual damage by each herbivore increased the concentration of leaf chemical defenses (phenolic compounds) in *P. lunatus*. Similarly, previous work with *P. lunatus* has also shown that leaf damage by a generalist herbivore drove an increase in the concentration of cyanogenic glycoside compounds in leaves (Ballhorn et al., 2010). Nonetheless, we found that herbivore effects on *P. lunatus* defense induction were not species-specific. These findings run counter to a study by Bingham and Agrawal (2010) who found that the induction of latex exudation on leaves of *Asclepias syriaca* was greater after feeding by larvae of the monarch butterfly *Danaus plexippus* than after feeding by larvae of the milkweed tussock moth *Euchaetes egle*. We did, however, observe evidence of herbivore species-specific effects on other plant traits. Specifically, the proportion of germinated seeds, an important proxy of plant fitness as it involves seed viability and offspring, was greater for plants attacked by *S. eridania* relative to control plants whereas plants attacked by *D. balteata* did not differ from controls. This effect was not contingent upon the amount of leaf damage as the single-species treatments exhibited similar levels of damage and leaf damage was accounted for, indicating that other features of herbivore feeding (rather than the amount of damage) were responsible for this effect.

Most studies conducted thus far on the specificity of plant induced responses to herbivory have focused on chemical defenses (e.g. Agrawal, 2000; Van Zandt and Agrawal, 2004; Rasmann and Turlings, 2008; Bingham and Agrawal, 2010; Erb et al.,
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2012; Moreira et al., 2013), whereas comparatively fewer studies have addressed the
specificity of other types of growth- or reproduction-related induced responses (e.g. in
responses or traits associated to growth and reproduction; but see Gavloski and Lamb,
2000; Carmona and Fornoni, 2013; Utsumi et al., 2013; Carrillo et al., 2014). Moreover,
even fewer studies have documented the consequences of such specificity for plant
lifetime fitness. In this study, we contribute to filling both gaps in knowledge by
demonstrating the presence of herbivore species-specific induced effects on plant
reproductive (seed) traits associated directly to fitness (measured as seed production and
viability) in this annual plant species. Further work is needed in P. lunatus, as well as in
other systems, to compare effects of herbivore species with contrasting traits (e.g. diet
breadth, feeding guild) and measure effects on a broad range of inducible plant traits
(e.g. cyanogenic compounds, nutritional traits, belowground responses, volatiles). In
doing so, we will be able to better describe the full range of herbivore-species specific
plant induced responses, how herbivore traits mediate such dynamics, and in doing so
derive more general and predictable patterns.

Combined effects of herbivores species on P. lunatus — For all variables measured, we
found that the combined effects of both herbivore species did not differ relative to their
individual effects. Such lack of combined or cumulative herbivore species effects on
plant defenses, growth, and reproduction occurred despite that leaf damage was
significantly greater for plants exposed to both herbivores relative to plants exposed to a
single species. Such findings contrast with a large body of literature showing that
different herbivore species can exert combined effects on plant induced resistance traits
(e.g. Agrawal, 2000; Kessler and Halitschke, 2007; Huang et al., 2014; Jing et al.,
Our findings however agree with work by Rodríguez-Saona et al. (2005) who also observed that tomato plants simultaneously damaged by aphids and caterpillars exhibited similar levels of defense induction as plants singly damaged by caterpillars. The authors of such study argued that the mechanism likely responsible for such finding was a conflict between defense responses associated with different metabolic pathways induced by chewers and sap feeders. However, in our study both herbivore species were chewers and this mechanisms cannot be invoked. Instead, one plausible explanation for the lack of combined effects of herbivores on the induction of defenses in *P. lunatus* could be that simultaneous effects of multiple herbivore species feeding on the same tissue might attenuate plant induced responses through physiological trade-offs (i.e. physiological limits; Felton et al., 1999). Alternatively, based upon predictions by the Optimal Defense Theory, it is possible that because the induction of induced responses in plants is costly (Stamp, 2003), no additional fitness benefits are obtained from further induction under a particular threshold level of induced responses (Agrawal et al., 2010), regardless of the number of herbivore species attacking the plant.

Likewise, we did not find evidence of combined herbivore species effects on plant growth or reproduction, indicating that *P. lunatus* plants were able to fully compensate for cumulative effects of multiple herbivore species. A number of studies have shown that a plant’s ability to mitigate the negative effects of herbivory on fitness appears to be closely related to the amount of leaf tissue consumed, where low damage triggers compensation by elevated photosynthetic rates and heavier damage does not (Mauricio et al., 1993; Koptur et al., 1996; Blue et al., 2015). For example, previous studies have documented that plants suffering moderate herbivore damage are able to compensate for the negative impact on plant growth and reproduction through
modifications of plant metabolism (i.e. compensatory growth and reproduction mechanisms; Edenius et al., 1993; Strauss and Agrawal, 1999; Järemo and Palmqvist, 2001; Puettmann and Saunders, 2001; Barton, 2008; Blue et al., 2015). In particular, Blue et al. (2015) reported that severe herbivore damage in *P. lunatus* (66% leaf area removed) significantly decreased the number of fruits and seed mass whereas a more moderate amount of damage (33% leaf area removed) did not. In our study, the amount of damage inflicted by both species combined was 40% greater than that caused, on average, by each species individually, and the mean leaf damage score for the combined species treatment was 2.0 (± 0.2) which is equivalent to ≤ 50% of leaf tissue consumed. Therefore, the amount of damage inflicted in the combined herbivore species treatment could have straddled a threshold where the amount of herbivory was not high enough to produce concomitant effects on defense induction or negatively influence plant growth or reproduction.

*Effects of chronology of herbivore species damage on *P. lunatus* — The order of arrival of different herbivore species to a host plant is considered an important determinant of plant-mediated interactions between herbivores (Ohgushi, 2005). However, relatively few studies have addressed whether the chronology of attack by different herbivore species influences plant induced defense responses, growth, or reproduction (but see Poelman et al., 2008; Wang et al., 2014). Our results indicated that the order of herbivore arrival did not affect the magnitude of induced defenses, growth or seed number, but did influence seed weight and seed germination. Interestingly, both seed traits exhibited higher values for plants attacked first by *D. balteata* and subsequently by *S. eridania* relative to plants attacked in the inverse order.*
Such effects were not associated with the amount of herbivory as leaf damage did not differ between these two treatments and was accounted for, and were therefore mediated by other aspects of feeding by these herbivores. It is possible that feeding by *D. balteata* “primed” *P. lunatus* plants which in turn responded more strongly to subsequent attack by *S. eridiana* (Heil and Kost, 2006; Frost et al., 2008; Heil and Ton, 2008), resulting in increased seed size and enhanced germination. In contrast, priming by *S. eridiana* feeding could have been weaker or non-existent, resulting in no effect on seed traits from the inverse order of attack. However, this argument invokes the presence of herbivore species-specific priming which has not been shown yet for *P. lunatus* (Heil and Silva Bueno, 2007), although the potential of damage-specific responses still exists (see Bricchi et al., 2010). Furthermore, it does not explain the individual effects of each herbivore species on seed traits. The single-species *S. eridiana* treatment drove an increase in seed germination whereas the *S. eridiana* plus *D. balteata* treatment did not. Similarly, the single-species *D. balteata* treatment did not influence seed germination, but the *D. balteata* plus *S. eridiana* treatment did. This suggests the presence of some non-additive dynamic (interactive herbivore effects) associated with the chronology of damage which does not arise when each herbivore feeds independently. Further work is necessary to understand the mechanism behind this pattern and its specificity.

**Future directions** — Overall, our work provides insight and an improved understanding of the specificity of plant induced responses to herbivory under a biologically realistic scenario where multiple herbivore species co-exist on the same host plant. We call for further studies that account for herbivore traits (e.g. diet breadth and feeding guild) and plant damage intensity (from low to severe defoliation), as well as measure a diverse
array of plant induced responses to fully understand the mechanisms and general patterns of specificity of plant induced responses to multi-species herbivory.

ACKNOWLEDGEMENTS

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LITERATURE CITED


Moreira et al. Specificity of plant induced responses


FREYTAG, G. F., AND D. G. DEBOUCK. 2002. Taxonomy, distribution and ecology of the genus Phaseolus (Leguminosae-Papilionoideae) in North America, Mexico and Central America. BRIT Press, Fort Worth, TX.


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Moreira et al. Specificity of plant induced responses


FIGURE LEGENDS

Figure 1. (a) Test of the specificity of individual effects of each herbivore treatment (plants attacked by *D. balteata* alone and by *S. eridania* alone) on leaf damage score. (b) Test for the combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments) on leaf damage score. (c) Test for effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*) on leaf damage score. Bars are least square means ± s.e.m. (N = 10). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between herbivory treatments.

Figure 2. (a) Test of the specificity of individual effects of each herbivore treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone) on the concentration of total phenolics in the leaves. (b) Test for the combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments) on the concentration of total phenolics in the leaves. (c) Test for effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*) on the concentration of total phenolics in the leaves. Bars are least square means ± s.e.m. (N = 10). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between herbivory treatments.

Figure 3. (a, b) Test of the specificity of individual effects of each herbivore treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone) on the
number of stems and leaves. (c, d) Test for the combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments) on the number of stems and leaves. (e, f) Test for effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*) on the number of stems and leaves. The number of initial leaves was used as a covariate in the models for number of leaves but was non-significant effect. The number of stems and leaves were measured four weeks after herbivory induction. Bars are least square means ± s.e.m. (N = 10). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between herbivory treatments.

**Figure 4.** (a, b, c) Test of the specificity of individual effects of each herbivore treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone) on the number of seeds, seed weight and proportion of germinated seeds. (d, e, f) Test for the combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments) on the number of seeds, seed weight and proportion of germinated seeds. (g, h, i) Test for effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*) on the number of seeds, seed weight and proportion of germinated seeds. All variables were measured 12-15 weeks after herbivory induction. Bars are least square means ± s.e.m. (N = 10). F-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between herbivory treatments.
Figure 1. Moreira et al.
Figure 2. Moreira et al.
Figure 3. Moreira et al.
Moreira et al. Specificity of plant induced responses

Figure 4. Moreira et al
Appendix S1. Test for the specificity of individual effects of each herbivore treatment (control plants, plants attacked by *Diabrotica balteata* alone, and *Spodoptera eridania* alone) on the concentration of individual phenolics in the leaves (10 flavonoids and two coumaric acid derivatives). Least-square means ± SE are shown. PROC MIXED in SAS 9.2 was used to run the general linear models. F-values, degrees of freedom (within brackets) and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between treatments. Significant *P*-values are shown in bold. We used Tukey tests for pairwise comparisons among treatment level means.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>Control</th>
<th><em>D. balteata</em></th>
<th><em>S. eridania</em></th>
<th>F</th>
<th><em>P</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>33.0 ± 20.5</td>
<td>100.8 ± 27.1 a</td>
<td>76.2 ± 19.1 a</td>
<td>2.27(2,8)</td>
<td>0.166</td>
</tr>
<tr>
<td>Quercetin hexoside rhamnoside</td>
<td>62.4 ± 67.4 a</td>
<td>141.0 ± 63.1 a</td>
<td>179.9 ± 59.5 a</td>
<td>0.93(2,12)</td>
<td>0.420</td>
</tr>
<tr>
<td>Kaempferol triglycoside</td>
<td>78.8 ± 45.3 b</td>
<td>182.6 ± 45.3 a</td>
<td>270.8 ± 45.3 a</td>
<td>5.08(2,18)</td>
<td>0.018</td>
</tr>
<tr>
<td>Isorhamnetin triglycoside</td>
<td>171.7 ± 60.2 b</td>
<td>361.0 ± 60.2 a</td>
<td>456.3 ± 60.2 a</td>
<td>6.23(2,18)</td>
<td>0.009</td>
</tr>
<tr>
<td>Kaempferol hexoside rhamnoside</td>
<td>146.7 ± 85.1 b</td>
<td>456.4 ± 85.1 a</td>
<td>352.6 ± 85.1 a</td>
<td>4.49(2,18)</td>
<td>0.026</td>
</tr>
<tr>
<td>Isorhamnetin hexoside rhamnoside</td>
<td>598.3 ± 214.7 b</td>
<td>1204.1 ± 214.7 a</td>
<td>1348.4 ± 214.7 a</td>
<td>3.54(2,18)</td>
<td>0.048</td>
</tr>
<tr>
<td>Methylkampferol hexoside</td>
<td>17.2 ± 6.2 b</td>
<td>35.3 ± 6.2 a</td>
<td>40.5 ± 6.2 a</td>
<td>4.19(2,13)</td>
<td>0.039</td>
</tr>
<tr>
<td>Dimethylkaempferol hexoside</td>
<td>21.8 ± 7.6 a</td>
<td>40.6 ± 7.6 a</td>
<td>33.0 ± 7.6 a</td>
<td>1.69(2,12)</td>
<td>0.226</td>
</tr>
<tr>
<td>Methoxyflavone hexoside</td>
<td>92.1 ± 32.7 b</td>
<td>234.5 ± 36.5 a</td>
<td>224.9 ± 34.5 a</td>
<td>5.57(2,15)</td>
<td>0.016</td>
</tr>
<tr>
<td>Methylkaempferol or isomer</td>
<td>16.8 ± 5.1 a</td>
<td>23.5 ± 4.9 a</td>
<td>19.7 ± 5.1 a</td>
<td>0.93(2,10)</td>
<td>0.426</td>
</tr>
<tr>
<td>Coumaric acid derivative</td>
<td>147.3 ± 85.5 b</td>
<td>458.7 ± 85.5 a</td>
<td>354.3 ± 85.5 a</td>
<td>4.50(2,18)</td>
<td>0.026</td>
</tr>
<tr>
<td>Coumaric acid derivative2</td>
<td>25.5 ± 8.8 b</td>
<td>47.8 ± 8.8 a</td>
<td>67.7 ± 8.8 a</td>
<td>5.51(2,18)</td>
<td>0.014</td>
</tr>
</tbody>
</table>
Appendix S2. Test for the combined effects of both herbivores (plants from single-species herbivore treatments, and plants from the two combined herbivore treatments) on the concentration of individual phenolics in the leaves (10 flavonoids and two coumaric acid derivates). Least-square means ± SE are shown. PROC MIXED in SAS 9.2 was used to run the general linear models. F-values, degrees of freedom (within brackets) and associated significance levels (P) are shown. Different letters indicate significant (P < 0.05) differences between treatments. We used Tukey tests for pairwise comparisons among treatment level means.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>Single herbivore species</th>
<th>Two herbivore species</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>84.4 ± 16.9 a</td>
<td>59.1 ± 16.9 a</td>
<td>1.12(^{(1,13)})</td>
<td>0.309</td>
</tr>
<tr>
<td>Quercetin hexoside rhamnoside</td>
<td>160.9 ± 41.1 a</td>
<td>121.5 ± 41.1 a</td>
<td>0.49(^{(1,23)})</td>
<td>0.489</td>
</tr>
<tr>
<td>Kaempferol triglycoside</td>
<td>226.7 ± 32.0 a</td>
<td>173.5 ± 32.9 a</td>
<td>1.34(^{(1,28)})</td>
<td>0.256</td>
</tr>
<tr>
<td>Isorhamnetin triglycoside</td>
<td>408.6 ± 45.4 a</td>
<td>302.9 ± 46.6 a</td>
<td>2.83(^{(1,28)})</td>
<td>0.104</td>
</tr>
<tr>
<td>Kaempferol hexoside rhamnoside</td>
<td>404.5 ± 71.7 a</td>
<td>354.3 ± 73.1 a</td>
<td>0.34(^{(1,28)})</td>
<td>0.564</td>
</tr>
<tr>
<td>Isorhamnetin hexoside rhamnoside</td>
<td>1276.3 ± 157.3 a</td>
<td>979.3 ± 161.1 a</td>
<td>1.94(^{(1,28)})</td>
<td>0.174</td>
</tr>
<tr>
<td>Methylkampferol hexoside</td>
<td>38.4 ± 7.3 a</td>
<td>37.3 ± 6.8 a</td>
<td>0.01(^{(1,24)})</td>
<td>0.904</td>
</tr>
<tr>
<td>Dimethylkaempferol hexoside</td>
<td>36.5 ± 7.2 a</td>
<td>33.3 ± 6.6 a</td>
<td>0.11(^{(1,24)})</td>
<td>0.744</td>
</tr>
<tr>
<td>Methoxyflavone hexoside</td>
<td>232.9 ± 27.7 a</td>
<td>193.0 ± 26.4 a</td>
<td>1.33(^{(1,25)})</td>
<td>0.259</td>
</tr>
<tr>
<td>Methylkaempferol or isomer</td>
<td>21.1 ± 4.3 a</td>
<td>21.2 ± 4.1 a</td>
<td>0.00(^{(1,21)})</td>
<td>0.990</td>
</tr>
<tr>
<td>Coumaric acid derivative</td>
<td>406.5 ± 72.0 a</td>
<td>356.1 ± 73.5 a</td>
<td>0.34(^{(1,28)})</td>
<td>0.564</td>
</tr>
<tr>
<td>Coumaric acid derivative2</td>
<td>54.8 ± 8.0 a</td>
<td>57.8 ± 8.1 a</td>
<td>0.14(^{(1,28)})</td>
<td>0.713</td>
</tr>
</tbody>
</table>
Appendix S3. Test for effect of herbivore arrival order (control plants, plants attacked by *S. eridania* plus *D. balteata*, and plants attacked by *D. balteata* plus *S. eridania*) on the concentration of individual phenolics in the leaves (10 flavonoids and two coumaric acid derivates). Least-square means ± SE are shown. PROC MIXED in SAS 9.2 was used to run the general linear models. F-values, degrees of freedom (within brackets) and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between treatments. Significant *P*-values are shown in bold. We used Tukey tests for pairwise comparisons among treatment level means.

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>Control</th>
<th><em>D. balteata</em> plus <em>S. eridania</em></th>
<th><em>S. eridania</em> plus <em>D. balteata</em></th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rutin</td>
<td>33.0 ± 17.6 a</td>
<td>56.6 ± 16.5 a</td>
<td>64.1 ± 23.3 a</td>
<td>0.72(2,8)</td>
<td>0.514</td>
</tr>
<tr>
<td>Quercetin hexoside rhamnoside</td>
<td>63.6 ± 37.6 a</td>
<td>150.9 ± 33.2 a</td>
<td>90.1 ± 35.2 a</td>
<td>1.75(2,12)</td>
<td>0.216</td>
</tr>
<tr>
<td>Kaempferol triglycoside</td>
<td>78.8 ± 27.6 b</td>
<td>176.2 ± 28.8 a</td>
<td>172.2 ± 27.6 a</td>
<td>5.75(2,17)</td>
<td><strong>0.012</strong></td>
</tr>
<tr>
<td>Isorhamnetin triglycoside</td>
<td>171.7 ± 46.7 b</td>
<td>316.9 ± 48.4 a</td>
<td>296.5 ± 46.7 a</td>
<td>5.27(2,17)</td>
<td><strong>0.016</strong></td>
</tr>
<tr>
<td>Kaempferol hexoside rhamnoside</td>
<td>146.7 ± 71.9 a</td>
<td>341.6 ± 75.8 a</td>
<td>351.9 ± 71.9 a</td>
<td>2.54(2,17)</td>
<td>0.108</td>
</tr>
<tr>
<td>Isorhamnetin hexoside rhamnoside</td>
<td>598.3 ± 163.2 b</td>
<td>989.5 ± 169.0 a</td>
<td>1001.8 ± 163.2 a</td>
<td>3.98(2,17)</td>
<td><strong>0.038</strong></td>
</tr>
<tr>
<td>Methylkampferol hexoside</td>
<td>16.3 ± 9.6 a</td>
<td>43.8 ± 9.6 a</td>
<td>30.9 ± 9.1 a</td>
<td>2.67(2,16)</td>
<td>0.100</td>
</tr>
<tr>
<td>Dimethylkaempferol hexoside</td>
<td>21.8 ± 10.9 a</td>
<td>37.7 ± 10.2 a</td>
<td>29.2 ± 9.7 a</td>
<td>0.65(2,15)</td>
<td>0.537</td>
</tr>
<tr>
<td>Methoxyflavone hexoside</td>
<td>92.1 ± 27.9 b</td>
<td>194.4 ± 28.8 a</td>
<td>190.9 ± 27.9 a</td>
<td>9.80(2,17)</td>
<td><strong>0.001</strong></td>
</tr>
<tr>
<td>Methylkaempferol or isomer</td>
<td>14.4 ± 6.4 a</td>
<td>23.7 ± 6.0 a</td>
<td>19.0 ± 5.7 a</td>
<td>0.56(2,12)</td>
<td>0.584</td>
</tr>
<tr>
<td>Coumaric acid derivative</td>
<td>147.3 ± 72.3 a</td>
<td>343.3 ± 76.2 a</td>
<td>353.6 ± 72.3 a</td>
<td>2.55(2,17)</td>
<td>0.108</td>
</tr>
<tr>
<td>Coumaric acid derivative2</td>
<td>25.5 ± 8.0 b</td>
<td>61.4 ± 8.4 a</td>
<td>53.1 ± 8.0 a</td>
<td>5.34(2,17)</td>
<td><strong>0.016</strong></td>
</tr>
</tbody>
</table>