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Specificity of induced defenses, growth, and reproduction in lima bean (*Phaseolus lunatus*) in response to multispecies herbivory[1]

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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 such induced defenses in plants are herbivore-specific, but less is known about the specificity of compensatory responses. Damage by multiple herbivores may also lead to synergistic effects on induction and plant fitness that differ from those caused by a single herbivore species. Although largely unstudied, the order of arrival and damage by different herbivore species might also play an important role in 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, seed mass, 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 their 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 mass and germination.

CONCLUSIONS: Overall, this study highlights novel aspects of the specificity of plant responses induced by damage from multiple species of herbivores and uniquely associates such effects with plant lifetime fitness.

KEY WORDS *Diabrotica balteata*; Fabaceae; herbivory; lima bean; *Phaseolus lunatus*; phenolic compounds; seed germination; seed mass; *Spodoptera eridania*; tolerance

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, after 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 the nature of induced plant responses largely depends on the identity of the herbivore (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

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of the plant response induced by herbivore damage also 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 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 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 to test 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 induced defenses, growth, and reproduction of the plants. We measured leaf phenolic concentration, plant growth (number of leaves and stems), and reproduction (number of seeds, seed mass, and proportion of germinated seeds) throughout an entire growing season, and because *P. lunatus* is an annual species, measurements of seed output and germination can provide direct estimates of lifetime fitness. Specifically, we addressed the following questions: (1) Are induced plant defenses and effects on growth and reproduction herbivore-specific? (2) Do combined effects of both herbivores differ from individual herbivore species effects? And (3) is specificity of induced responses contingent upon the order of arrival of these herbivore species? By addressing these questions, our work builds toward a better understanding of the specificity of plant responses induced by herbivory under a biologically realistic scenario where multiple herbivore species coexist on the same host plant.

MATERIALS AND METHODS

Study system*—Phaseolus lunatus* (lima bean) is an annual legume distributed in the wild 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 the leaves at the tips of juvenile and adult plants (Teng et al., 1984). Although in 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 of native soil and peat moss. After emergence, we kept all plants in nylon mesh field cages (2 × 2 × 2 m) for 4 wk to prevent undesired herbivory. When plants were 4 wk 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 described). Within each cage, we applied one of the following herbivory treatments to each plant: (1) control (untreated, "herbivore-free" plants), (2) *S. eridania* alone (10 third-instar larvae added), (3) *D. balteata* alone (five adults added), (4) *S. eridania* plus *D. balteata* (10 third-instar larvae of *S. eridania* added and 2 d later five adults of *D. balteata* added), and (5) *D. balteata* plus *S. eridania* (five adults of *D. balteata* added, and 2 d later, 10 third-instar larvae of *S. eridania* added). In both sequential herbivore treatments, the first herbivore continued feeding after the second herbivore was added. In total, there were 50 plants corresponding to 10 cages and five plants per cage (i.e., one plant per herbivory treatment), and plants in treatments 2–5 (above) were exposed to herbivores for 4 d. Within each cage, we individually covered each plant with a nylon mesh to prevent herbivore escape or interference among treatments. Two days after adding the second herbivore for treatments 4 and 5, we removed all

the herbivores and nylon meshes and scored leaf damage for the whole plant in situ using a five-level scale: 0 = undamaged leaves, 1 = <25% damaged leaves, 2 = 25–50% damaged leaves, 3 = >50–75% damaged leaves, and 4 = >75% damaged leaves (i.e., 0–4 score). Throughout the experiment, we watered all plants twice a week.

***Effects of herbivory on induced defenses*—**Immediately after herbivore removal, we randomly collected four young, fully expanded leaves half-way down 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). The phenolics were profiled using ultra-high-pressure liquid chromatography coupled with quadrupole-time-of-flight–mass spectrometry (UHPLC-QTOF-MS) and an Acquity UPLCTM system coupled with a Synapt G2 QTOF-MS (Waters, Milford, Connecticut, USA). Compounds were separated at a flow rate of 400 $\mu L \cdot min^{-1}$ on a reverse-phase Acquity BEH C18 column (50 × 2.1 mm column, particle size 1.7 µm; Waters) 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 for 6 min, 30–100% B for 2 min, 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 capillary voltage at −2.5 kV, cone voltage −25 V, source temperature 120°C, desolvation gas temperature 350°C, desolvation gas flow 800 $L \cdot h^{-1}$. We identified individual phenolic compounds (10 flavonoids and two coumaric acid derivatives; see online Appendix S1, S2, S3) using the MSE mode, which consists of alternate scans at low (4eV) and high (10–30 eV ramp) collision energies. We used argon as the collision gas at a flow of 2.2 $mL \cdot min^{-1}$. The instrument was internally calibrated with an infusion of a solution of 400 $ng \cdot mL^{-1}$ leucine-enkephalin (in 50:50 acetonitrile–water) at a flow rate of 15 $\mu L \cdot min^{-1}$ through the Lock Spray probe. Whenever ion abundance exceeded the linearity domain of the QTOF-MS, we used UV traces obtained from the integrated photodiode array detector of the UPLCTM. We quantified the concentration of phenolics as rutin equivalents using a calibration curve based on a rutin standard at 0.1, 0.5, 2, 10, and 50 $\mu g \cdot mL^{-1}$.

Effects of herbivory on plant growth and reproduction*—Growth*—Immediately after removing herbivores, we counted the leaves and stems each week for 4 wk until plants started producing pods.

Reproduction—At the end of the growing season (12 wk after the end of herbivory treatments) and once plants started wilting, we collected all mature bean pods per plant on a daily basis until plants dried (about 15 wk after applying herbivory treatments). We then counted the number of seeds. In addition, we weighed five randomly chosen seeds per plant to the nearest 0.00001 g. Finally, we sowed groups of three randomly chosen seeds per plant in plastic cups to evaluate seed germination. We recorded the number of emerged seedlings per cup for 2 wk and estimated the proportion of germinated seeds. In all cases, we selected seeds from a similar phenological stage.

***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 ran sets of models that only included and compared data for control plants, plants attacked by *D. balteata* alone, and *S. eridania* alone (except for leaf damage for which 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 ran sets of models including only plants from the single-species and combined-species (sequential) treatments and conducted a preplanned contrast where we compared the mean of the single-species herbivore treatments to the mean of the combined-species (sequential) herbivore treatments. This test is a conservative test for 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 used 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 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 these 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 that 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 were nonnormal after transformation. PROC MIXED in SAS 9.2 (SAS Institute, Cary, North Carolina, USA) 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 ± SE 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 the plants attacked first by *D. balteata* then 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 concentration did not differ between the plants from the two treatments with a single species, indicating that the magnitude of the induced defense was not herbivore-specific (Fig. 2A). The same pattern was observed for 8 of 12 phenolic compounds analyzed separately (Appendix S1, see Supplemental Data with the online version of this article). On the other hand, we found that the mean 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 online Supplemental Data), indicating that the combined herbivore effects on induced defenses were not greater than the 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 results of the two sequential herbivory treatments did not differ (Fig. 2C), indicating that the order of herbivore arrival did not influence the amount of induced defenses. A similar pattern was observed for five of 12 phenolic compounds analyzed individually (online Appendix S3).

Effects of herbivory on plant growth and reproduction*—Growth*—We found that the number of stems and leaves was not significantly different between plants from the single-species herbivore treatments and control plants or when comparing the single-species herbivore treatments to each other (Fig. 3A, B), i.e., herbivory did not influence stem and leaf production, and such a lack of effect was consistent between herbivore species (i.e., no herbivore-species specificity). Likewise, the number of stems and leaves was not significantly different between the combined herbivore treatments and the single-species treatments (Fig. 3C, D), i.e., combined herbivore effects on plant growth were not greater than individual species effects. In addition, the number of stems and leaves was not significantly different between plants of each sequential herbivory treatment relative to control plants or between plants in the two sequential herbivory treatments (Fig. 3E, F), indicating that there were no effects of herbivore arrival order on plant growth.

Overall, results from previous measurements of growth traits (i.e., 2 and 3 wk after application of the herbivory treatments) were qualitatively similar to those observed at the end of measurements (i.e., 4 wk after application of the herbivory treatments) (data not shown).

Reproduction—The number of seeds was not significantly different between either of single-species herbivore treatments and controls or between the single-species herbivore treatments (Fig. 4A). In addition, although seed mass 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 mean number of seeds, seed mass, and proportion of germinated seeds were not significantly different between plants in the single-species and either of the two-species herbivore treatments (Fig. 4D–F), indicating

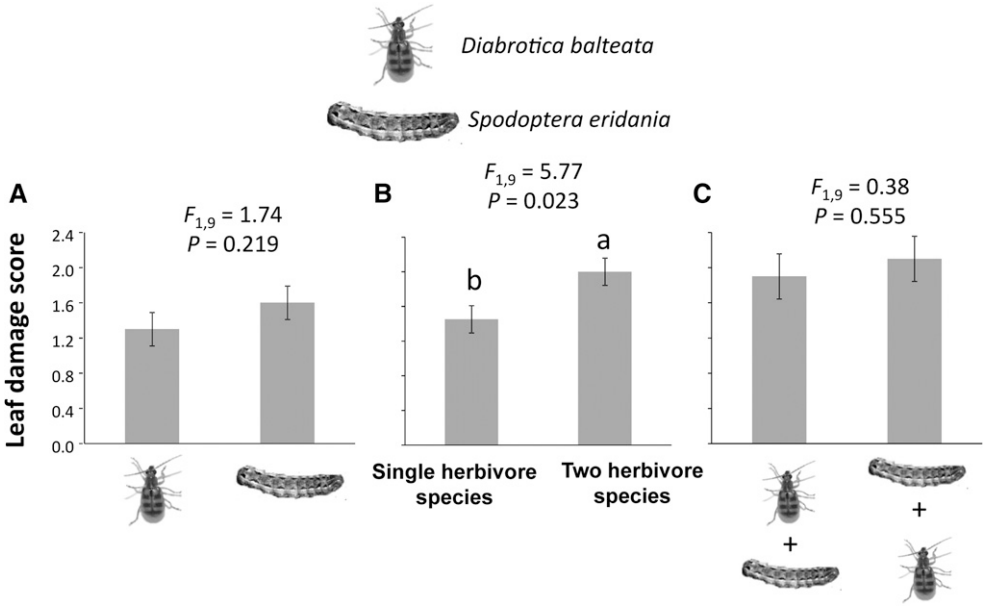

FIGURE 1 Leaf damage scores on *Phaseolus lunatus* after insect herbivory. (A) Specificity of individual effects of each herbivore treatment (plants attacked by *D. balteata* alone and by *S. eridania* alone). (B) Combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments). (C) Effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*). Bars are least square means ± SEM (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.

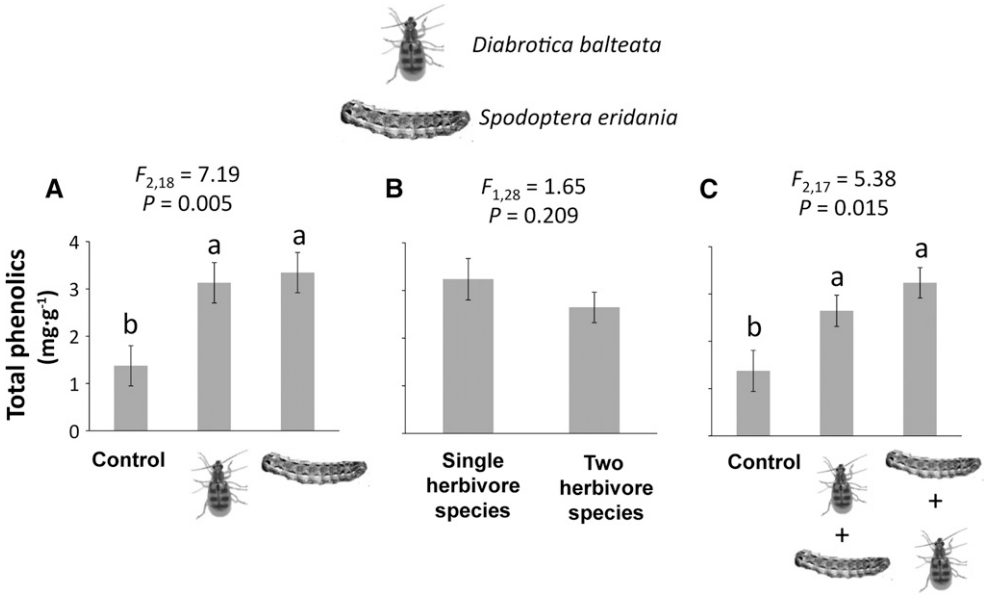

FIGURE 2 Total phenolics concentration in leaves of *Phaseolus lunatus* 4 d after insect herbivory. (A) Specificity of individual effects of each herbivore treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone). (B) Combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments). (C) Effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*). Bars are least square means ± SEM ($N = 10$). *F*-values, degrees of freedom and associated significance levels (*P*) are shown. Different letters indicate significant ($P < 0.05$) differences between herbivory treatments.

that the 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 or between those in the sequential herbivory treatments (Fig. 4G). However, we found that seed mass 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 attacked first by *D. balteata* differed relative to control plants (Fig. 4H, I), whereas plants attacked first by *S. eridania* did not differ from controls, indicating that the order of herbivore arrival determined the effects of herbivory on these seed traits (Fig. 4H, I).

DISCUSSION

***Overview*—**Our study revealed important and novel aspects of the specificity of plant responses induced after 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; *S. eridiana* had a positive effect, whereas *D. balteata* had no influence. Second, except for leaf damage, for which 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. These results suggest, 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 growth and/or reproduction are reduced further. Third, we found that the order of herbivore arrival did not affect the amount of induced defenses or plant growth but did influence seed mass and germination, two important determinants of lifetime fitness in *P. lunatus*. These results suggest that the chronology of plant–herbivore interactions is an important aspect to consider in predicting the impact of multispecies 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 because the single-species treatments caused 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 induced plant 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., 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

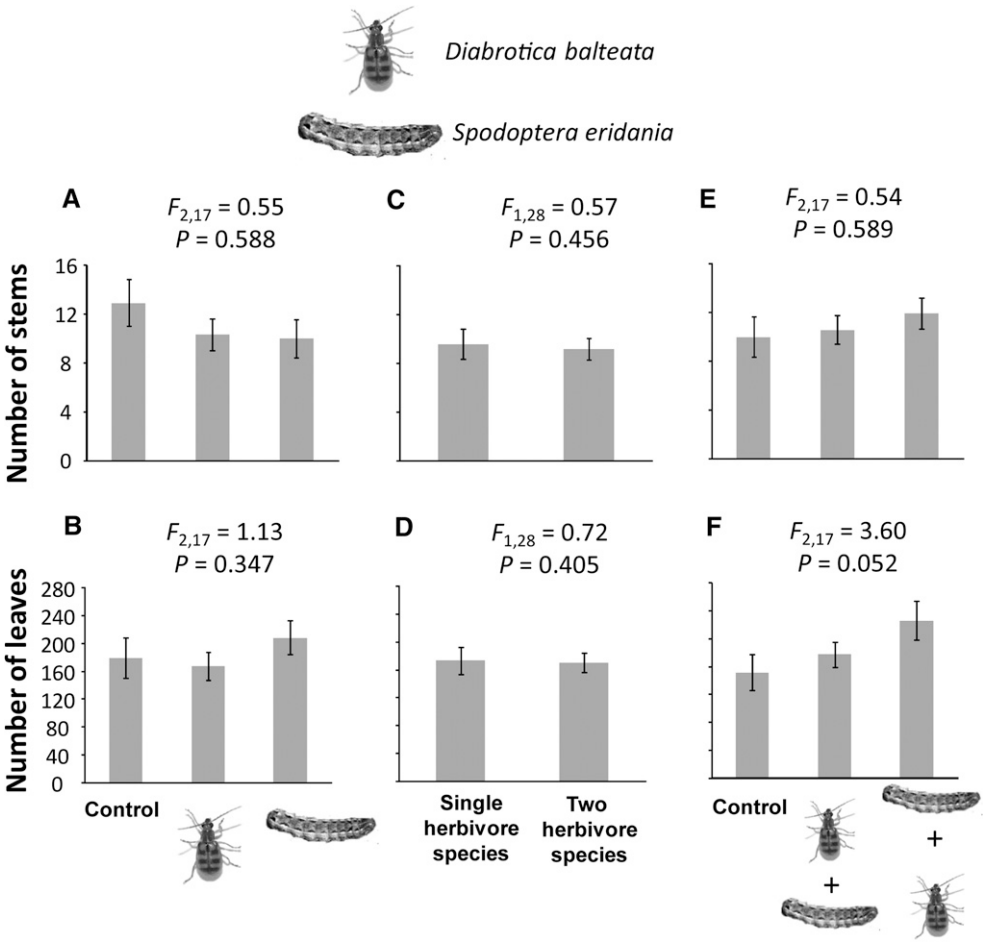

FIGURE 3 Number of stems and leaves on plants of *Phaseolus lunatus* 4 wk after insect herbivory. (A, B) Specificity of individual effects of each herbivore treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone). (C, D) Combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments). (E, F) Effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs. plants attacked by *D. balteata* plus *S. eridania*). The number of initial leaves was used as a covariate in the models for number of leaves but had a nonsignificant effect. Bars are least square means ± SEM (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.

***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 the significantly greater leaf damage on 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 induced resistance traits in plants (e.g., Agrawal, 2000; Kessler and Halitschke, 2007; Huang et al., 2014; Jing et al., 2015). 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 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, so their hypothesized mechanism 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 the induced responses through physiological tradeoffs (i.e., physiological limits; Felton et al., 1999). Alternatively, based upon predictions of the optimal defense theory, the induction of these responses in plants may be costly (Stamp, 2003), so no additional fitness benefits are obtained beyond a particular threshold level of the responses (Agrawal et al., 2010), regardless of the number of herbivore species attacking the plant.

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 these 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 responses induced in plants, how herbivore traits mediate such dynamics, and in doing so, derive more general and predictable patterns.

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; 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

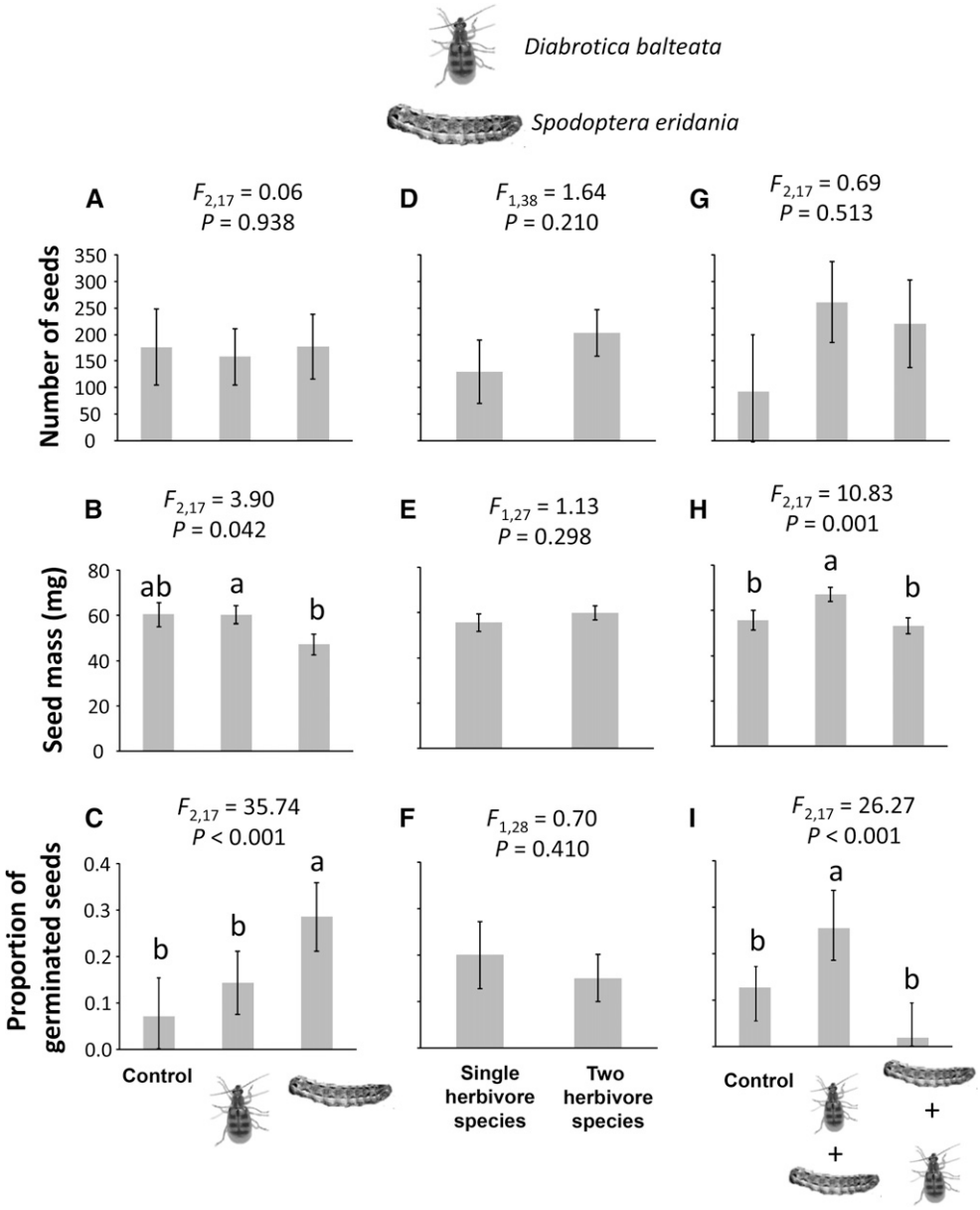

FIGURE 4 Seed number, mass, and proportion of germinated seeds of *Phaseolus lunatus* 12–15 wk after insect herbivory. (A–C) Specificity of individual effects of each herbivore treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone). (D–F) Test for the combined effects of both herbivores (mean of plants from the single-species herbivore treatments vs. mean of plants from the combined herbivore treatments). (G–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*). Bars are least square means ± SEM (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.

reproduction by modifying their 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 fruit number 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), 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 the defense responses induced or subsequent growth, and 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 mass and germination. Interestingly, values for these two seed traits were higher in plants attacked first by *D. balteata* then by *S. eridania* relative to plants attacked in the inverse order. Such effects were not associated with the amount of herbivory, because leaf damage did not differ between these two treatments, 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 nonexistent, 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. These results suggest 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 induced plant responses to herbivory under a biologically realistic scenario where multiple herbivore species coexist 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) and measure a diverse array of plant responses to fully understand the mechanisms and general patterns of specificity of plant responses induced by multispecies herbivory.

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LITERATURE CITED

Agrawal, A. A. 2000. Specificity of induced resistance in wild radish: Causes and consequences for two specialist and two generalist caterpillars. *Oikos* 89: 493–500.

Agrawal, A. A. 2011. Current trends in the evolutionary ecology of plant defense. *Functional Ecology* 25: 420–432.

Agrawal, A. A., J. K. Conner, and S. Rasmann. 2010. Tradeoffs and adaptive negative correlations in evolutionary ecology. *In* M. Bell, W. Eanes, D. Futuyma, and J. Levinton [eds.], Evolution after Darwin: The first 150 years, 243–268. Sinauer, Sunderland, Massachusetts, USA.

Ballhorn, D. J. 2011. Constraints of simultaneous resistance to a fungal pathogen and an insect herbivore in lima bean (*Phaseolus lunatus* L.). *Journal of Chemical Ecology* 37: 141–144.

Ballhorn, D. J., S. Kautz, M. Jensen, I. Schmitt, M. Heil, and A. D. Hegeman. 2011. Genetic and environmental interactions determine plant defences against herbivores. *Journal of Ecology* 99: 313–326.

Ballhorn, D. J., S. Kautz, and R. Lieberei. 2010. Comparing responses of generalist and specialist herbivores to various cyanogenic plant features. *Entomologia Experimentalis et Applicata* 134: 245–259.

Barton, K. E. 2008. Phenotypic plasticity in seedling defense strategies: compensatory growth and chemical induction. *Oikos* 117: 917–925.

Bingham, R. A., and A. A. Agrawal. 2010. Specificity and trade-offs in the induced plant defence of common milkweed *Asclepias syriaca* to two lepidopteran herbivores. *Journal of Ecology* 98: 1014–1022.

Blue, E., J. Kay, B. S. Younginger, and D. J. Ballhorn. 2015. Differential effects of type and quantity of leaf damage on growth, reproduction and defence of lima bean (*Phaseolus lunatus* L.). *Plant Biology* 17: 712–719.

Bricchi, I., M. Leitner, M. Foti, A. Mithöfer, W. Boland, and M. E. Maffei. 2010. Robotic mechanical wounding (MecWorm) versus herbivore-induced responses: Early signaling and volatile emission in Lima bean (*Phaseolus lunatus* L.). *Planta* 232: 719–729.

Capinera, J. L. 2001. Handbook of vegetable pests. Academic Press, San Diego, California, USA.

Carmona, D., and J. Fornoni. 2013. Herbivores can select for mixed defensive strategies in plants. *New Phytologist* 197: 576–585.

Carrillo, J., D. McDermott, and E. Siemann. 2014. Loss of specificity: Native but not invasive populations of *Triadica sebifera* vary in tolerance to different herbivores. *Oecologia* 174: 863–871.

Clavijo McCormick, A., S. B. Unsicker, and J. Gershenzon. 2012. The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. *Trends in Plant Science* 17: 303–310.

Delgado-Salinas, A., R. Bibler, and M. Lavin. 2006. Phylogeny of the genus *Phaseolus* (Leguminosae): A recent diversification in an ancient landscape. *Systematic Botany* 31: 779–791.

Edenius, L., K. Danell, and R. Bergstrom. 1993. Impact of herbivory and competition on compensatory growth in woody plants: Winter browsing by moose on Scots pine. *Oikos* 66: 286–292.

Erb, M., S. Meldau, and G. A. Howe. 2012. Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science* 17: 250–259.

Erb, M., C. Robert, B. Hibbard, and T. Turlings. 2011. Sequence of arrival determines plant-mediated interactions between herbivores. *Journal of Ecology* 99: 7–15.

Felton, G. W., K. L. Korth, J. L. Bi, S. V. Wesley, D. V. Huhman, M. C. Mathews, J. B. Murphy, et al. 1999. Inverse relationship between systemic resistance of plants to microorganisms and to insect herbivory. *Current Biology* 9: 317–320.

Fineblum, W. L., and M. D. Rausher. 1995. Tradeoff between resistance and tolerance to herbivore damage in a morning glory. *Nature* 377: 517–520.

Fornoni, J. 2011. Ecological and evolutionary implications of plant tolerance to herbivory. *Functional Ecology* 25: 399–407.

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, Texas, USA.

Frost, C. J., M. C. Mescher, J. E. Carlson, and C. M. De Moraes. 2008. Plant defense priming against herbivores: Getting ready for a different battle. *Plant Physiology* 146: 818–824.

Gavloski, J. E., and R. J. Lamb. 2000. Compensation for herbivory in cruciferous plants: Specific responses to three defoliating insects. *Environmental Entomology* 29: 1258–1267.

Hakes, A. S., and J. T. Cronin. 2011. Resistance and tolerance to herbivory in *Solidago altissima* (Asteraceae): Genetic variability, costs, and selection for multiple traits. *American Journal of Botany* 98: 1446–1455.

Heil, M. 2004. Induction of two indirect defences benefits Lima bean (*Phaseolus lunatus*, Fabaceae) in nature. *Journal of Ecology* 92: 527–536.

Heil, M., and C. Kost. 2006. Priming of indirect defences. *Ecology Letters* 9: 813–817.

Heil, M., and J. C. Silva Bueno. 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proceedings of the National Academy of Sciences, USA* 104: 5467–5472.

Heil, M., and J. Ton. 2008. Long-distance signalling in plant defence. *Trends in Plant Science* 13: 264–272.

Huang, W., E. Siemann, L. Xiao, X. Yang, and J. Ding. 2014. Species-specific defence responses facilitate conspecifics and inhibit heterospecifics in above–belowground herbivore interactions. *Nature Communications* 5: 4851.

Järemo, J., and E. Palmqvist. 2001. Plant compensatory growth: A conquering strategy in plant–herbivore interactions? *Evolutionary Ecology* 15: 91–102.

Jing, J., C. Raaijmakers, O. Kostenko, M. Kos, P. P. J. Mulder, and M. Bezemer. 2015. Interactive effects of above- and belowground herbivory and plant competition on plant growth and defence. *Basic and Applied Ecology*.

Kessler, A., and R. Halitschke. 2007. Specificity and complexity: The impact of herbivore-induced plant responses on arthropod community structure. *Current Opinion in Plant Biology* 10: 409–414.

Koptur, S., C. L. Smith, and J. H. Lawton. 1996. Effects of artificial defoliation on reproductive allocation in the common vetch, *Vicia sativa* (Fabaceae: Papilionoideae). *American Journal of Botany* 83: 886–889.

Littell, R. C., G. A. Milliken, W. W. Stroup, R. Wolfinger, and O. Schabenberger. 2006. SAS system for mixed models, 2nd ed. SAS Institute, Cary, North Carolina, USA.

Manzaneda, A. J., K. V. Prasad, and T. Mitchell-Olds. 2010. Variation and fitness costs for tolerance to different types of herbivore damage in *Boechera stricta* genotypes with contrasting glucosinolate structures. *New Phytologist* 188: 464–477.

Mauricio, R., M. D. Bowers, and F. A. Bazzaz. 1993. Pattern of leaf damage affects fitness of the annual plant *Raphanus sativus* (Brassicaceae). *Ecology* 74: 2066–2071.

McArt, S. H., R. Halitschke, J.-P. Salminen, and J. S. Thaler. 2013. Leaf herbivory increases plant fitness via induced resistance to seed predators. *Ecology* 94: 966–975.

Mithöfer, A., and W. Boland. 2008. Recognition of herbivory-associated molecular patterns. *Plant Physiology* 146: 825–831.

Mithöfer, A., and W. Boland. 2012. Plant defence against herbivores: Chemical aspects. *Annual Review of Plant Biology* 63: 431–450.

Moreira, X., L. Lundborg, R. Zas, A. Carrillo-Gavilán, A. K. Borg-Karlson, and L. Sampedro. 2013. Inducibility of chemical defences by two chewing insect herbivores in pine trees is specific to targeted plant tissue, particular herbivore and defensive trait. *Phytochemistry* 94: 113–122.

Moreira, X., K. A. Mooney, S. Rasmann, W. K. Petry, A. Carrillo-Gavilán, R. Zas, and L. Sampedro. 2014. Trade-offs between constitutive and induced defences drive geographical and climatic clines in pine chemical defences. *Ecology Letters* 17: 537–546.

Moreira, X., R. Zas, and L. Sampedro. 2012. Genetic variation and phenotypic plasticity of nutrient re-allocation and increased fine root production as putative tolerance mechanisms inducible by methyl-jasmonate in pine trees. *Journal of Ecology* 100: 810–820.

Núñez-Farfán, J., J. Fornoni, and P. L. Valverde. 2007. The evolution of resistance and tolerance to herbivores. *Annual Review of Ecology, Evolution and Systematics* 38: 541–566.

Ohgushi, T. 2005. Indirect interaction webs: Herbivore-induced effects through trait change in plants. *Annual Review of Ecology, Evolution and Systematics* 36: 81–105.

Poelman, E. H., C. Broaekgaarden, J. J. A. Van Loon, and M. Dicke. 2008. Early season herbivore differentially affects plant defense responses to subsequently colonizing herbivores and their abundance in the field. *Molecular Ecology* 17: 3352–3365.

Puettmann, K. J., and M. R. Saunders. 2001. Patterns of growth compensation in eastern white pine (*Pinus strobus* L.): The influence of herbivory intensity and competitive environments. *Oecologia* 129: 376–384.

Rasmann, S., and T. C. J. Turlings. 2007. Simultaneous feeding by aboveground and belowground herbivores affects the plant-mediated attraction of their respective natural enemies. *Ecology Letters* 10: 926–936.

Rasmann, S., and T. C. J. Turlings. 2008. First insights into specificity of belowground tritrophic interactions. *Oikos* 117: 362–369.

Rodríguez-Saona, C., J. A. Chalmers, S. Raj, and J. S. Thaler. 2005. Induced plant responses to multiple damagers: Differential effects on an herbivore and its parasitoid. *Oecologia* 143: 566–577.

Rodríguez-Saona, C. R., R. O. Musser, H. Vogel, S. M. Hum-Musser, and J. S. Thaler. 2010. Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. *Journal of Chemical Ecology* 36: 1043–1057.

Salminen, J.-P., and M. Karonen. 2011. Chemical ecology of tannins and other phenolics: We need a change in approach. *Functional Ecology* 25: 325–338.

Shlichta, J. G., G. Glauser, and B. Benrey. 2014. Variation in cyanogenic glycosides across populations of wild lima beans (*Phaseolus lunatus*) has no apparent effect on bruchid beetle performance. *Journal of Chemical Ecology* 40: 468–475.

Stamp, N. 2003. Out of the quagmire of plant defense hypotheses. *Quarterly Review of Biology* 78: 23–55.

Stowe, K. A., R. J. Marquis, C. G. Hochwender, and E. L. Simms. 2000. The evolutionary ecology of tolerance to consumer damage. *Annual Review of Ecology and Systematics* 31: 565–595.

Strauss, S. Y., and A. A. Agrawal. 1999. The ecology and evolution of plant tolerance to herbivory. *Trends in Ecology & Evolution* 14: 179–185.

Teng, H. J., V. Waddill, F. Slansky, and J. Strayer. 1984. Performance and host preference of adult banded cucumber beetles, *Diabrotica balteata*, when offered several crops. *Journal of Agricultural Entomology* 1: 330–338.

Utsumi, S., Y. Ando, H. Roininen, J.-i. Takahashi, and T. Ohgushi. 2013. Herbivore community promotes trait evolution in a leaf beetle via induced plant response. *Ecology Letters* 16: 362–370.

Van Zandt, P. A., and A. A. Agrawal. 2004. Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. *Oikos* 104: 401–409.

Viswanathan, D. V., O. A. Lifchts, and J. S. Thaler. 2007. Consequences of sequential attack for resistance to herbivores when plants have specific induced responses. *Oikos* 116: 1389–1399.

Wang, M., A. Biere, W. H. Van der Putten, and T. M. Bezemer. 2014. Sequential effects of root and foliar herbivory on aboveground and belowground induced plant defense responses and insect performance. *Oecologia* 175: 187–198.

Xiao, Y., Q. Wang, M. Erb, T. C. J. Turlings, L. Ge, L. Hu, J. Li, et al. 2012. Specific herbivore-induced volatiles defend plants and determine insect community composition in the field. *Ecology Letters* 15: 1130–1139.