

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

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5 XOAQUÍN MOREIRA<sup>1</sup>, LUIS ABDALA-ROBERTS<sup>2</sup>, JOHNATTAN HERNÁNDEZ-  
6 CUMPLIDO<sup>3</sup>, MAXIMILIEN A. C. CUNY<sup>3</sup>, GAÉTAN GLAUSER<sup>4</sup> AND BETTY  
7 BENREY<sup>3\*</sup>

8

9 <sup>1</sup>Misión Biológica de Galicia (MBG-CSIC), Apdo. 28, 36080 Pontevedra, Galicia,  
10 Spain

11 <sup>2</sup>Departamento de Ecología Tropical, Campus de Ciencias Biológicas y Agropecuarias,  
12 Universidad Autónoma de Yucatán, Apartado Postal 4-116, Itzimmá. 97000.  
13 Mérida, Yucatán, México

14 <sup>3</sup>Institute of Biology, Laboratory of Evolutive Entomology, University of Neuchâtel,  
15 Rue Emile-Argand 11, 2000 Neuchâtel, Switzerland

16 <sup>4</sup>Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Rue Emile  
17 Argand 11, 2000 Neuchâtel, Switzerland

18

19 \*Corresponding author:

20 Email: betty.benrey@unine.ch

21 Phone Number: +41 327183132 Fax Number: +41 327183001

22

23

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25

26 **ABSTRACT**

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

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

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

47 *Conclusions:* Overall, this study highlights novel aspects of the specificity of plant  
48 induced responses to multi-species herbivore damage, and uniquely associates such  
49 effects to plant lifetime fitness.

50

51 **Keywords:** *Diabrotica balteata*; *phenolic compounds*; *seed germination*; *seed weight*;  
52 *Spodoptera eridania*; *tolerance*

53

## 54 **INTRODUCTION**

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

65 It is increasingly recognized that plant induced responses to herbivory largely  
66 depend on herbivore identity (Agrawal, 2000; Van Zandt and Agrawal, 2004; Rasmann  
67 and Turlings, 2008; Bingham and Agrawal, 2010; Moreira et al., 2013). Recent  
68 investigations have shown that plants are able to recognize biotic stimuli (e.g.  
69 oviposition secretions and saliva) produced by different herbivores, integrate the  
70 perceived information, and react accordingly in a highly specific manner (Mithöfer and  
71 Boland, 2008). The specificity of plant induced responses to herbivore damage depends  
72 on the type and amount of damage, as well as herbivore diet breadth and feeding guild  
73 (e.g. Rasmann and Turlings, 2008; Clavijo McCormick et al., 2012; Xiao et al., 2012;

74 Carmona and Fornoni, 2013; Moreira et al., 2013). For instance, Rasmann and Turlings  
75 (2008) found that the emission of volatile compounds in maize roots drastically varied  
76 depending on the diet breadth of the root herbivore. Similarly, Moreira et al. (2013)  
77 observed highly specific changes in carbon-based defenses for two pine species after  
78 damage by a phloem-feeder and a folivore. Despite such evidence for the specificity of  
79 induced defenses, much less is known about the specificity of induction of traits  
80 associated with tolerance against herbivory (but see Manzaneda et al., 2010; Carmona  
81 and Fornoni, 2013; Utsumi et al., 2013; Carrillo et al., 2014). One exception is a study  
82 by Utsumi et al. (2013) who reported that insect herbivore community composition  
83 determined the degree of herbivore-induced regrowth intensity of willow trees.  
84 Similarly, Carrillo et al. (2014) demonstrated specificity of tolerance to different  
85 generalist herbivores for native but not for invasive populations of the Chinese tallow  
86 tree.

87 Simultaneous attack by multiple herbivore species often elicits different induced  
88 plant responses than would otherwise be triggered by a single-species attack (Agrawal,  
89 2000; Kessler and Halitschke, 2007; Rasmann and Turlings, 2007; Rodriguez-Saona et  
90 al., 2010; Utsumi et al., 2013). Such variation in responses has been attributed to  
91 synergistic or antagonistic effects from feeding by multiple species, leading to effects  
92 that cannot be predicted based upon the individual effects of each herbivore species. In  
93 addition, the order of arrival and type of damage produced by different herbivore  
94 species can also play an important role in determining the impacts of multiple  
95 herbivores feeding on the same host plant. Although a number of studies have  
96 demonstrated that damage by early herbivores triggers a wide range of plant induced  
97 responses that negatively affect the performance of subsequent herbivores (Rodríguez-

98 Saona et al., 2005; Viswanathan et al., 2007; Poelman et al., 2008; Erb et al., 2011;  
99 McArt et al., 2013; Wang et al., 2014), relatively few studies have addressed how the  
100 chronology of herbivore attack influences induced defense, growth and reproduction in  
101 plants (but see Poelman et al., 2008; Wang et al., 2014). In one of the few available  
102 studies, Wang et al. (2014) observed that the chronological order of aboveground and  
103 belowground herbivory in an herbaceous plant differentially induced the production of  
104 iridoid glycosides in stem and roots.

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

122 biologically realistic scenario where multiple herbivore species co-exist on the same  
123 host plant.

124

## 125 **MATERIAL AND METHODS**

126 *Study system* — *Phaseolus lunatus* (lima bean) is an annual legume distributed along  
127 the Pacific coast from Mexico to South America (Freytag and Debouck, 2002; Heil,  
128 2004; Delgado-Salinas et al., 2006). At our field site, 15 km northwest of Puerto  
129 Escondido, Oaxaca, Mexico (15°55'27.4"N, 97°09'03.0"W), *P. lunatus* germinates  
130 between June and July and flowers at the beginning of October. Seeds are produced  
131 during November and December and disperse in January and February (Freytag and  
132 Debouck, 2002). Leaves are divided into three oval-shaped leaflets that are arranged  
133 alternately on the stem (Freytag and Debouck, 2002).

134 At our field site, *P. lunatus* is attacked by a diverse community of insect  
135 herbivores, including two common leaf-chewers: *Spodoptera eridania* (Stoll)  
136 (Lepidoptera: Noctuidae), a polyphagous moth native to the American tropics whose  
137 larvae feed on the lower surface of leaves, especially at night (Capinera, 2001), and  
138 *Diabrotica balteata* LeConte (Coleoptera: Chrysomelidae), a polyphagous beetle  
139 distributed from North America to Central America whose adults severely defoliate leaf  
140 tips of juvenile and adult plants (Teng et al., 1984). Although at the middle of the  
141 growing season these herbivore species are frequently found feeding simultaneously on  
142 the same *P. lunatus* plants, they typically vary in their order of arrival at the start of the  
143 growing season, with plants being exposed to damage by one species for several days  
144 before the other herbivore arrives (X. Moreira, personal observation).

145

146 **Experimental set-up** — In early October 2014, we collected seeds from wild plants of  
147 *P. lunatus* growing in a population along the Pacific coast of Mexico (Coyuca de  
148 Benítez, Guerrero, Mexico; 17°00'40.5"N 100°06'10.2"W; Shlichta et al., 2014). We  
149 individually sowed seeds in 5 L pots with a mixture composed of native soil and peat  
150 moss. After emergence, we kept all plants in nylon mesh field cages (Bioquip, Outdoor  
151 Cage 6' × 6' × 6', 20 × 20 Mesh Lumite) for four weeks to prevent undesired herbivory.  
152 When plants were four-weeks old, we counted the number of leaves per plant (“number  
153 of initial leaves” hereafter), formed groups of five randomly selected plants, and each  
154 group of potted plants was placed in a nylon mesh cage in the field (same cages as  
155 above). Within each cage, we applied one of the following herbivory treatments to each  
156 plant: (1) control (untreated, “herbivore-free” plants), (2) *S. eridania* alone (we added  
157 10 third-instar larvae), (3) *D. balteata* alone (we added five adults), (4) *S. eridania* plus  
158 *D. balteata* (we added 10 third-instar larvae of *S. eridania* and two days later we added  
159 five adults of *D. balteata*), and (5) *D. balteata* plus *S. eridania* (we added five adults of  
160 *D. balteata* and two days later we added 10 third-instar larvae of *S. eridania*). In both of  
161 the sequential herbivore treatment, the first herbivore continued feeding after adding the  
162 second herbivore. In total, there were 50 plants corresponding to 10 cages and five  
163 plants per cage (i.e. one plant per herbivory treatment) and plants of treatments 2-5  
164 (above) were exposed to herbivores for four days. Within each cage, we individually  
165 covered each plant with a nylon mesh to avoid herbivore escape or interference among  
166 treatments. Two days after adding the second herbivore for treatments 4-5, we removed  
167 all the herbivores and the nylon meshes and scored leaf damage for the whole plant *in*  
168 *situ* using a five-level scale: 0 = undamaged leaves, 1 = less than 25% damaged leaves,  
169 2 = between 25-50% damaged leaves, 3 = 50-75% damaged leaves, and 4 = more than

170 75% damaged leaves (i.e. 0–4 score). Throughout the experiment, we watered all the  
171 plants twice a week.

172

173 ***Effects of herbivory on induced defenses*** — Immediately after herbivore removal, we  
174 randomly collected four young, fully expanded leaves located half-way along the stem  
175 of each plant to measure the concentration of phenolic compounds. Phenolic  
176 compounds are widely recognized as herbivore deterrents across many plant taxa  
177 (Salminen and Karonen, 2011; Mithöfer and Boland, 2012; Moreira et al., 2014) and  
178 have been demonstrated to confer resistance against leaf herbivores in *P. lunatus*  
179 (Ballhorn, 2011; Ballhorn et al., 2011). We extracted phenolic compounds using 10 mg  
180 of dry plant tissue with 500  $\mu\text{L}$  of 100% methanol in an ultrasonic bath for 15 min,  
181 followed by centrifugation and subsequent dilution of 300  $\mu\text{L}$  of the methanolic extract  
182 with 100  $\mu\text{L}$  water (Moreira et al., 2014). We performed phenolic profiling by Ultra-  
183 High-Pressure Liquid Chromatography coupled with Quadrupole-Time-Of-Flight Mass  
184 Spectrometry (UHPLC-QTOF-MS). We used an Acquity UPLC™ system coupled  
185 with a Synapt G2 QTOF-MS (Waters, Milford, CT, USA). We achieved the separation  
186 of compounds at a flow rate of 400  $\mu\text{L min}^{-1}$  on a reverse-phase Acquity BEH C18  
187 column (50x2.1 mm column, particle size 1.7  $\mu\text{m}$ , Waters) thermostated at 45°C.  
188 Solvents were A= water + 0.05% vol. formic acid; B = acetonitrile + 0.05% vol. formic  
189 acid. The gradient program was as follows: 5-30% B in 6 min, 30-100% B in 2 min,  
190 holding at 100% B for 2 min followed by re-equilibration at 5% B for 2 min. The  
191 injection volume was 2  $\mu\text{l}$ . Mass over charge (m/z) data from the QTOF-MS were  
192 obtained in negative ion mode over an m/z range of 85-1200 Da with the following  
193 parameters: capillary voltage at -2.5 kV, cone voltage -25 V, source temperature 120 °C,

194 desolvation gas temperature 350 °C, desolvation gas flow 800 L hr<sup>-1</sup>. We identified  
195 individual phenolic compounds (10 flavonoids and two coumaric acid derivatives; see  
196 Tables SM1, SM2, SM3) using the MSE mode which consists in alternate scans at low  
197 (4eV) and high (10-30 eV ramp) collision energies. We used argon as collision gas at a  
198 flow of 2.2 mL min<sup>-1</sup>. We obtained internal calibration of the instrument by infusing a  
199 solution of leucine-enkephaline at 400 ng mL<sup>-1</sup> at a flow rate of 15 µL min<sup>-1</sup> through the  
200 Lock Spray<sup>TM</sup> probe. Whenever ion abundance exceeded the linearity domain of the  
201 QTOF-MS, we used UV traces obtained from the integrated photodiode array detector  
202 of the UPLC<sup>TM</sup>. We quantified the concentration of phenolics as rutin equivalents  
203 using a calibration curve made of a rutin standard at 0.1, 0.5, 2, 10 and 50 µg mL<sup>-1</sup>.

204

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

208 *Reproduction* — At the end of the growing season (12 weeks after applying  
209 herbivory treatments), and once plants started wilting, we collected all mature bean pods  
210 present per plant on a daily basis until plants dried (about 15 weeks after applying  
211 herbivory treatments). We shelled the pods and counted the number of seeds. In  
212 addition, we weighed five randomly chosen seeds per plant to the nearest 0.00001 g.  
213 Finally, we sowed groups of three randomly chosen seeds per plant in plastic cups to  
214 evaluate seed germination. We recorded the number of emerged seedlings per cup  
215 throughout a two-week period and estimated the proportion of germinated seeds. In all  
216 cases, we selected seeds from a similar phenological stage.

217

218 **Statistical analyses** — We analyzed the individual and combined effects of herbivores  
219 on leaf damage, defenses, growth, and reproductive traits using linear mixed models.  
220 For growth and reproductive traits, we analyzed cumulative values across sampling  
221 dates. For each variable we ran three independent sets of models based on different  
222 subsets of the data. First, to evaluate the specificity of individual effects of each  
223 herbivore on damage, defenses, growth and reproduction, we performed sets of models  
224 that only included and compared control plants, plants attacked by *D. balteata* alone,  
225 and *S. eridania* alone (except for leaf damage where we did not include comparisons  
226 with the control group). Significant effects of one but not the other herbivore species  
227 with respect to the control or significant effects of both herbivore treatments relative to  
228 the control but with herbivore treatments differing themselves demonstrate specificity  
229 of plant responses. Second, to test for the combined effects of both herbivores, we  
230 performed sets of models including only plants from the single-species and combined-  
231 species (sequential) treatments and conducted a pre-planned contrast where we  
232 compared the mean of the single-species herbivore treatments to the mean of the  
233 combined-species (sequential) herbivore treatments. This represents a conservative test  
234 of the combined effects of herbivores, since one of the herbivores in the sequential  
235 treatments was exposed to plants for half the time relative to the other, i.e. plants were  
236 not exposed simultaneously to both herbivores from the start of the experiment. A  
237 significant difference between the means of these treatment groups demonstrates the  
238 existence of combined effects of these herbivores over and above the individual effects  
239 of each herbivore. Third, to evaluate the effect of herbivore arrival order, we performed  
240 models that only included and compared control plants, plants attacked by *S. eridania*  
241 plus *D. balteata*, and plants attacked by *D. balteata* plus *S. eridania*. Significant effects

242 of only one of the these herbivore treatments with respect to the control or significant  
243 effects of both herbivore treatments relative to the control but with herbivore treatments  
244 differing themselves would demonstrate an effect of order of arrival on plant defense  
245 induction, growth, or reproduction. We used Tukey tests for pairwise comparisons  
246 among treatment level means for the first and third set of models, as this method  
247 corrects for Type I error inflation due to multiple comparisons. For all of the above  
248 models, herbivory treatment (with a particular combination of treatment levels for each  
249 set of models) was treated as a fixed effect and we included cage as a random effect to  
250 account for non-independence among plants sampled within the same cage. In addition,  
251 to account for differences in initial plant size, we included the number of leaves at the  
252 start of the experiment as a covariate in the models for number of leaves. In addition, to  
253 account for the differences in the amount of damage which could influence plant  
254 responses associated with tolerance to herbivory (i.e. growth, reproduction), as well as  
255 confound effects of amount of damage relative to herbivore species identity (i.e. if one  
256 herbivore species consistently inflicted more damage than the other one), we included  
257 leaf damage score as a covariate in the models for growth and reproductive traits (Hakes  
258 and Cronin, 2011).

259         Residuals were normally distributed for most variables measured except leaf  
260 damage score which was log-transformed to achieve normality of residuals. In addition,  
261 the proportion of germinated seeds was analyzed using a generalized linear mixed  
262 model with a binomial distribution (logit link) (Littell et al., 2006), as data was non-  
263 normal after transformation. PROC MIXED in SAS 9.2 (SAS Institute, Cary, NC) was  
264 used to run the general linear models (normal distribution), whereas the generalized

265 linear model was run with PROC GLIMMIX (Littell et al., 2006). In all cases, we  
266 provide model least square means  $\pm$  S. E. as descriptive statistics.

267

## 268 **RESULTS**

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

277

278 ***Effects of herbivory on plant defenses*** — The concentration of total phenolics in leaves  
279 was significantly higher in plants from both single-species herbivore treatments relative  
280 to control plants, but the single-species herbivore treatments did not differ themselves  
281 which indicates that the magnitude of induced defenses was not herbivore-specific (Fig.  
282 2a). The same pattern was observed for 8 out of 12 phenolic compounds based upon  
283 analyses conducted separately for each compound (Appendix S1, see Supplemental  
284 Data with the online version of this article). On the other hand, we found that the mean  
285 of total concentration of phenolics for the combined herbivore treatment was not  
286 significantly different relative to the mean of the single-species treatments (Fig. 2b;  
287 similar pattern for individual compound-based analyses, Appendix S2, see  
288 Supplemental Data with the online version of this article), indicating that combined

289 herbivore effects on induced defenses were not greater than individual species effects.  
290 In addition, our test of sequential effects indicated that the mean concentration of total  
291 phenolics in leaves was significantly greater for both sequential herbivory treatments  
292 relative to controls, but the sequential herbivory treatments did not differ themselves  
293 (Fig. 2c), indicating that the order of herbivore arrival did not influence the amount of  
294 induced defenses. A similar pattern was observed for five out of 12 phenolic  
295 compounds based on individual compound-based analyses (Appendix S3, see  
296 Supplemental Data with the online version of this article).

297

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

311 Overall, results from previous measurements of growth traits (i.e. two and three  
312 weeks after application of the herbivory treatments) were qualitatively similar to those

313 observed at the end of measurements (i.e. four weeks after application of the herbivory  
314 treatments) (data not shown).

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

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

329         Finally, the number of seeds was not significantly different between either  
330 sequential herbivory treatment relative to controls, and the sequential herbivory  
331 treatments did not differ themselves (Fig. 4g). However, we found that seed weight and  
332 the proportion of germinated seeds were significantly different between sequential  
333 herbivory treatments. Mean values in both cases were greater for plants attacked first by  
334 *D. balteata* and subsequently by *S. eridania* than for plants attacked first by *S. eridiana*  
335 and subsequently by *D. baleata*. Plants of the former treatment differed relative to  
336 control plants (Fig. 4h, 4i), whereas plants attacked first by *S. eridania* and then by *D.*

337 *balteata* did not differ from controls, indicating that the order of herbivore arrival  
338 determined the effects of herbivory on these seed traits (Fig. 4h, 4i).

339

## 340 **DISCUSSION**

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

361 an important aspect to consider in predicting the impact of multi-species herbivory on  
362 plant reproduction.

363

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

382 Most studies conducted thus far on the specificity of plant induced responses to  
383 herbivory have focused on chemical defenses (e.g. Agrawal, 2000; Van Zandt and  
384 Agrawal, 2004; Rasmann and Turlings, 2008; Bingham and Agrawal, 2010; Erb et al.,

385 2012; Moreira et al., 2013), whereas comparatively fewer studies have addressed the  
386 specificity of other types of growth- or reproduction-related induced responses (e.g. in  
387 responses or traits associated to growth and reproduction; but see Gavloski and Lamb,  
388 2000; Carmona and Fornoni, 2013; Utsumi et al., 2013; Carrillo et al., 2014). Moreover,  
389 even fewer studies have documented the consequences of such specificity for plant  
390 lifetime fitness. In this study, we contribute to filling both gaps in knowledge by  
391 demonstrating the presence of herbivore species-specific induced effects on plant  
392 reproductive (seed) traits associated directly to fitness (measured as seed production and  
393 viability) in this annual plant species. Further work is needed in *P. lunatus*, as well as in  
394 other systems, to compare effects of herbivore species with contrasting traits (e.g. diet  
395 breadth, feeding guild) and measure effects on a broad range of inducible plant traits  
396 (e.g. cyanogenic compounds, nutritional traits, belowground responses, volatiles). In  
397 doing so, we will be able to better describe the full range of herbivore-species specific  
398 plant induced responses, how herbivore traits mediate such dynamics, and in doing so  
399 derive more general and predictable patterns.

400

401 ***Combined effects of herbivores species on P. lunatus*** — For all variables measured, we  
402 found that the combined effects of both herbivore species did not differ relative to their  
403 individual effects. Such lack of combined or cumulative herbivore species effects on  
404 plant defenses, growth, and reproduction occurred despite that leaf damage was  
405 significantly greater for plants exposed to both herbivores relative to plants exposed to a  
406 single species. Such findings contrast with a large body of literature showing that  
407 different herbivore species can exert combined effects on plant induced resistance traits  
408 (e.g. Agrawal, 2000; Kessler and Halitschke, 2007; Huang et al., 2014; Jing et al.,

409 2015). Our findings however agree with work by Rodríguez-Saona et al. (2005) who  
410 also observed that tomato plants simultaneously damaged by aphids and caterpillars  
411 exhibited similar levels of defense induction as plants singly damaged by caterpillars.  
412 The authors of such study argued that the mechanism likely responsible for such finding  
413 was a conflict between defense responses associated with different metabolic pathways  
414 induced by chewers and sap feeders. However, in our study both herbivore species were  
415 chewers and this mechanisms cannot be invoked. Instead, one plausible explanation for  
416 the lack of combined effects of herbivores on the induction of defenses in *P. lunatus*  
417 could be that simultaneous effects of multiple herbivore species feeding on the same  
418 tissue might attenuate plant induced responses through physiological trade-offs (i.e.  
419 physiological limits; Felton et al., 1999). Alternatively, based upon predictions by the  
420 Optimal Defense Theory, it is possible that because the induction of induced responses  
421 in plants is costly (Stamp, 2003), no additional fitness benefits are obtained from further  
422 induction under a particular threshold level of induced responses (Agrawal et al., 2010),  
423 regardless of the number of herbivore species attacking the plant.

424         Likewise, we did not find evidence of combined herbivore species effects on  
425 plant growth or reproduction, indicating that *P. lunatus* plants were able to fully  
426 compensate for cumulative effects of multiple herbivore species. A number of studies  
427 have shown that a plant's ability to mitigate the negative effects of herbivory on fitness  
428 appears to be closely related to the amount of leaf tissue consumed, where low damage  
429 triggers compensation by elevated photosynthetic rates and heavier damage does not  
430 (Mauricio et al., 1993; Koptur et al., 1996; Blue et al., 2015). For example, previous  
431 studies have documented that plants suffering moderate herbivore damage are able to  
432 compensate for the negative impact on plant growth and reproduction through

433 modifications of plant metabolism (i.e. compensatory growth and reproduction  
434 mechanisms; Edenius et al., 1993; Strauss and Agrawal, 1999; Järemo and Palmqvist,  
435 2001; Puettmann and Saunders, 2001; Barton, 2008; Blue et al., 2015). In particular,  
436 Blue et al. (2015) reported that severe herbivore damage in *P. lunatus* (66% leaf area  
437 removed) significantly decreased the number of fruits and seed mass whereas a more  
438 moderate amount of damage (33% leaf area removed) did not. In our study, the amount  
439 of damage inflicted by both species combined was 40% greater than that caused, on  
440 average, by each species individually, and the mean leaf damage score for the combined  
441 species treatment was 2.0 ( $\pm$  0.2) which is equivalent to  $\leq$  50% of leaf tissue consumed.  
442 Therefore, the amount of damage inflicted in the combined herbivore species treatment  
443 could have straddled a threshold where the amount of herbivory was not high enough to  
444 produce concomitant effects on defense induction or negatively influence plant growth  
445 or reproduction.

446

447 *Effects of chronology of herbivore species damage on P. lunatus* — The order of  
448 arrival of different herbivore species to a host plant is considered an important  
449 determinant of plant-mediated interactions between herbivores (Ohgushi, 2005).  
450 However, relatively few studies have addressed whether the chronology of attack by  
451 different herbivore species influences plant induced defense responses, growth, or  
452 reproduction (but see Poelman et al., 2008; Wang et al., 2014). Our results indicated  
453 that the order of herbivore arrival did not affect the magnitude of induced defenses,  
454 growth or seed number, but did influence seed weight and seed germination.  
455 Interestingly, both seed traits exhibited higher values for plants attacked first by *D.*  
456 *balteata* and subsequently by *S. eridania* relative to plants attacked in the inverse order.

457 Such effects were not associated with the amount of herbivory as leaf damage did not  
458 differ between these two treatments and was accounted for, and were therefore mediated  
459 by other aspects of feeding by these herbivores. It is possible that feeding by *D. balteata*  
460 “primed” *P. lunatus* plants which in turn responded more strongly to subsequent attack  
461 by *S. eridiana* (Heil and Kost, 2006; Frost et al., 2008; Heil and Ton, 2008), resulting in  
462 increased seed size and enhanced germination. In contrast, priming by *S. eridiana*  
463 feeding could have been weaker or non-existent, resulting in no effect on seed traits  
464 from the inverse order of attack. However, this argument invokes the presence of  
465 herbivore species-specific priming which has not been shown yet for *P. lunatus* (Heil  
466 and Silva Bueno, 2007), although the potential of damage-specific responses still exists  
467 (see Bricchi et al., 2010). Furthermore, it does not explain the individual effects of each  
468 herbivore species on seed traits. The single-species *S. eridiana* treatment drove an  
469 increase in seed germination whereas the *S. eridiana* plus *D. balteata* treatment did not.  
470 Similarly, the single-species *D. balteata* treatment did not influence seed germination,  
471 but the *D. balteata* plus *S. eridiana* treatment did. This suggests the presence of some  
472 non-additive dynamic (interactive herbivore effects) associated with the chronology of  
473 damage which does not arise when each herbivore feeds independently. Further work is  
474 necessary to understand the mechanism behind this pattern and its specificity.

475

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

481 array of plant induced responses to fully understand the mechanisms and general  
482 patterns of specificity of plant induced responses to multi-species herbivory.

483

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492

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688 **FIGURE LEGENDS**

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

698

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

709

710 **Figure 3.** (a, b) Test of the specificity of individual effects of each herbivore treatment  
711 (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone) on the

712 number of stems and leaves. (c, d) Test for the combined effects of both herbivores  
713 (mean of plants from the single-species herbivore treatments vs. mean of plants from  
714 the combined herbivore treatments) on the number of stems and leaves. (e, f) Test for  
715 effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs.  
716 plants attacked by *D. balteata* plus *S. eridania*) on the number of stems and leaves. The  
717 number of initial leaves was used as a covariate in the models for number of leaves but  
718 was non-significant effect. The number of stems and leaves were measured four weeks  
719 after herbivory induction. Bars are least square means  $\pm$  s.e.m. (N = 10). F-values,  
720 degrees of freedom and associated significance levels (*P*) are shown. Different letters  
721 indicate significant ( $P < 0.05$ ) differences between herbivory treatments.

722

723 **Figure 4.** (a, b, c) Test of the specificity of individual effects of each herbivore  
724 treatment (control plants, plants attacked by *D. balteata* alone and by *S. eridania* alone)  
725 on the number of seeds, seed weight and proportion of germinated seeds. (d, e, f) Test  
726 for the combined effects of both herbivores (mean of plants from the single-species  
727 herbivore treatments vs. mean of plants from the combined herbivore treatments) on the  
728 number of seeds, seed weight and proportion of germinated seeds. (g, h, i) Test for  
729 effect of herbivore arrival order (plants attacked by *S. eridania* plus *D. balteata* vs.  
730 plants attacked by *D. balteata* plus *S. eridania*) on the number of seeds, seed weight and  
731 proportion of germinated seeds. All variables were measured 12-15 weeks after  
732 herbivory induction. Bars are least square means  $\pm$  s.e.m. (N = 10). F-values, degrees of  
733 freedom and associated significance levels (*P*) are shown. Different letters indicate  
734 significant ( $P < 0.05$ ) differences between herbivory treatments.

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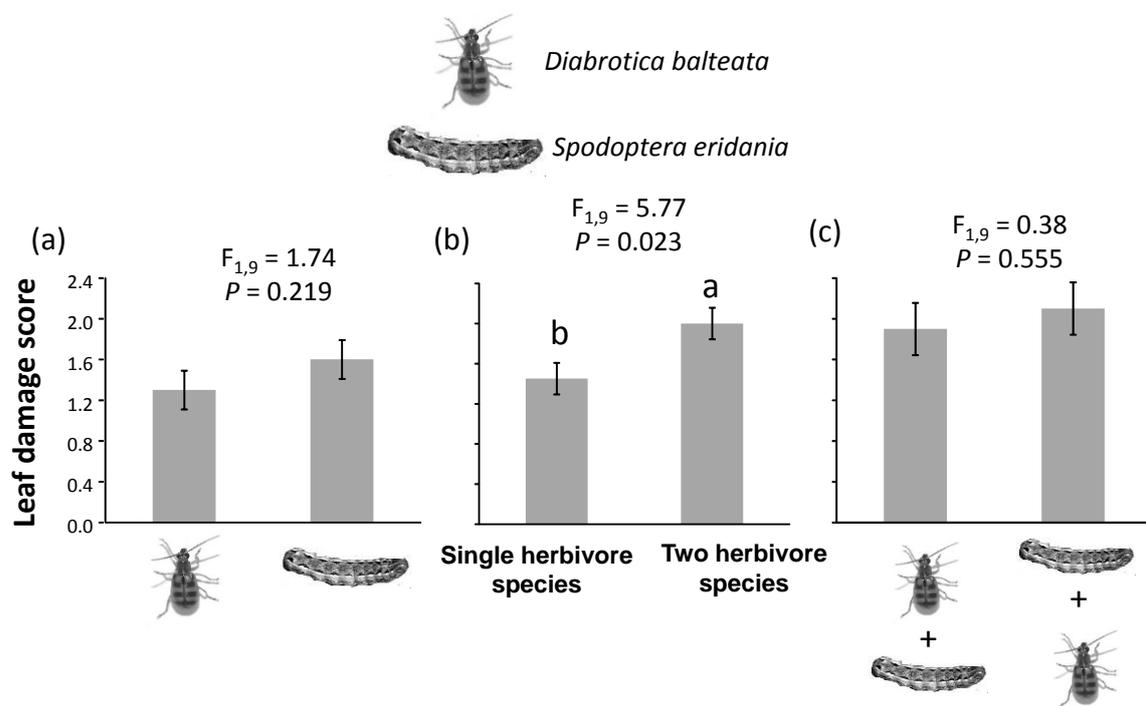
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745 **Figure 1.** Moreira et al.

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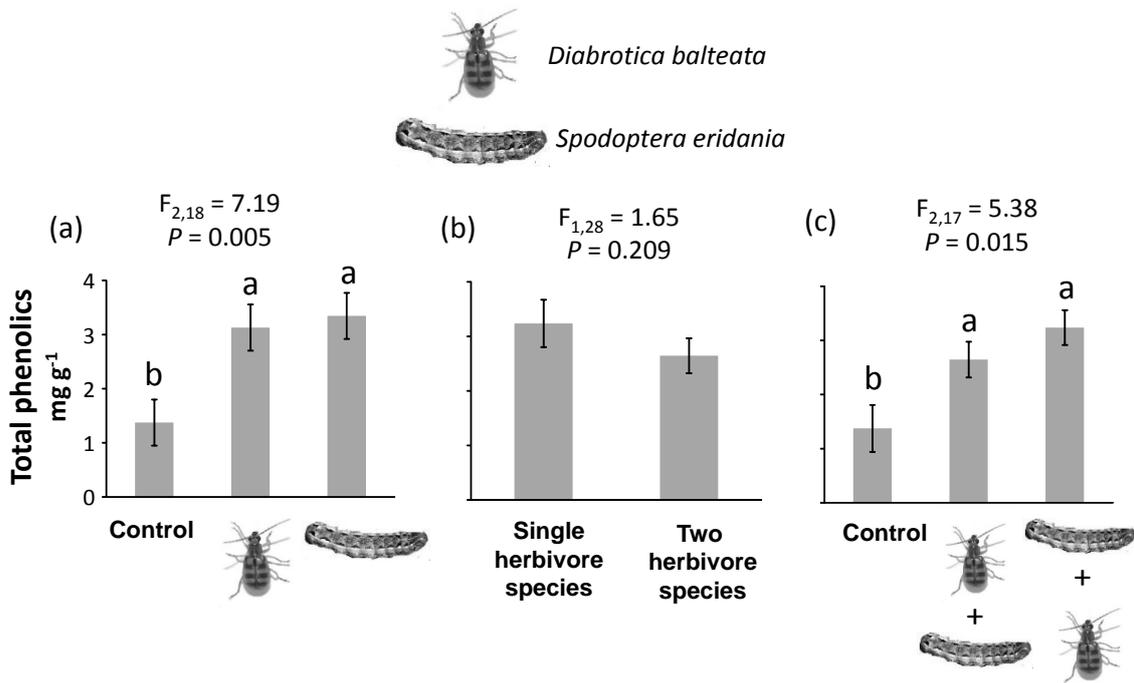
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758 **Figure 2.** Moreira et al.

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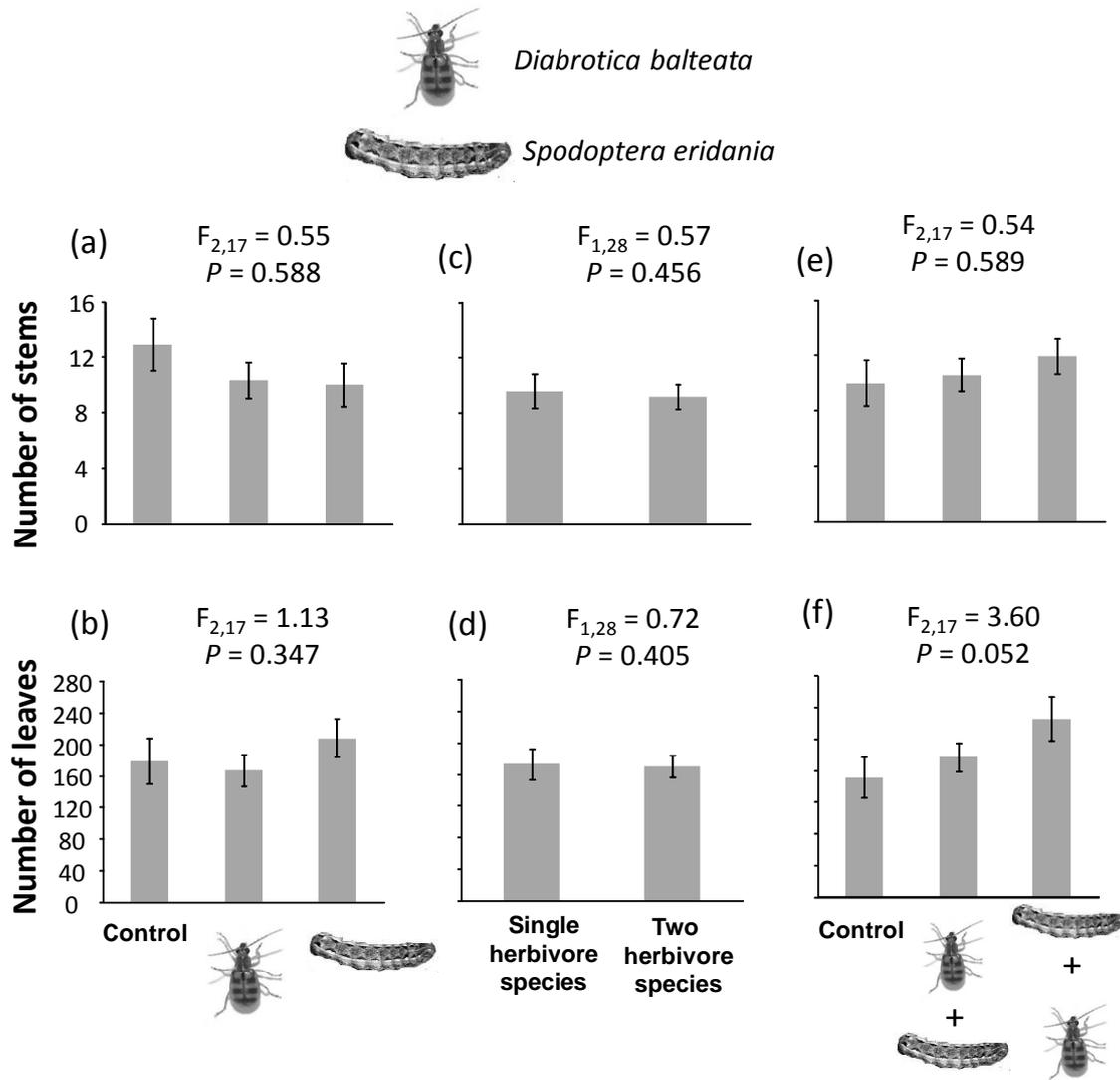
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771 **Figure 3.** Moreira et al.

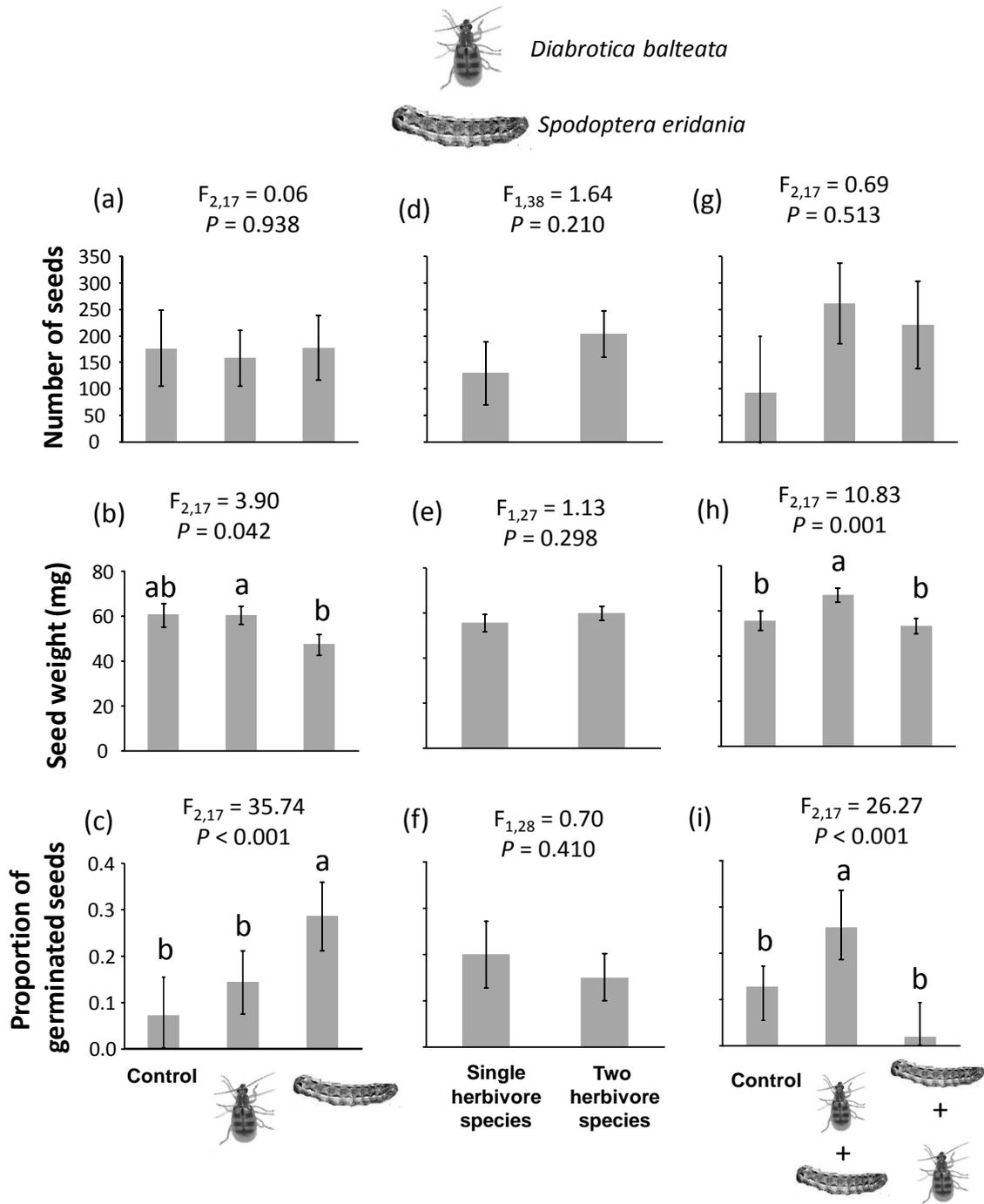
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778 **Figure 4.** Moreira et al

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781 **Appendix S1.** Test for the specificity of individual effects of each herbivore treatment (control plants, plants attacked by *Diabrotica balteata*  
 782 alone, and *Spodoptera eridania* alone) on the concentration of individual phenolics in the leaves (10 flavonoids and two coumaric acid derivatives).  
 783 Least-square means  $\pm$  SE are shown. PROC MIXED in SAS 9.2 was used to run the general linear models. F-values, degrees of freedom (within  
 784 brackets) and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between treatments.  
 785 Significant *P*-values are shown in bold. We used Tukey tests for pairwise comparisons among treatment level means.  
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COMPOUND	Control	<i>D. balteata</i>	<i>S. eridania</i>	F	<i>P</i>
Rutin	33.0 $\pm$ 20.5 <b>a</b>	100.8 $\pm$ 27.1 <b>a</b>	76.2 $\pm$ 19.1 <b>a</b>	2.27 <sup>(2,8)</sup>	0.166
Quercetin hexoside rhamnoside	62.4 $\pm$ 67.4 <b>a</b>	141.0 $\pm$ 63.1 <b>a</b>	179.9 $\pm$ 59.5 <b>a</b>	0.93 <sup>(2,12)</sup>	0.420
Kaempferol triglycoside	78.8 $\pm$ 45.3 <b>b</b>	182.6 $\pm$ 45.3 <b>a</b>	270.8 $\pm$ 45.3 <b>a</b>	5.08 <sup>(2,18)</sup>	<b>0.018</b>
Isorhamnetin triglycoside	171.7 $\pm$ 60.2 <b>b</b>	361.0 $\pm$ 60.2 <b>a</b>	456.3 $\pm$ 60.2 <b>a</b>	6.23 <sup>(2,18)</sup>	<b>0.009</b>
Kaempferol hexoside rhamnoside	146.7 $\pm$ 85.1 <b>b</b>	456.4 $\pm$ 85.1 <b>a</b>	352.6 $\pm$ 85.1 <b>a</b>	4.49 <sup>(2,18)</sup>	<b>0.026</b>
Isorhamnetin hexoside rhamnoside	598.3 $\pm$ 214.7 <b>b</b>	1204.1 $\pm$ 214.7 <b>a</b>	1348.4 $\pm$ 214.7 <b>a</b>	3.54 <sup>(2,18)</sup>	<b>0.048</b>
Methylkaempferol hexoside	17.2 $\pm$ 6.2 <b>b</b>	35.3 $\pm$ 6.2 <b>a</b>	40.5 $\pm$ 6.2 <b>a</b>	4.19 <sup>(2,13)</sup>	<b>0.039</b>
Dimethylkaempferol hexoside	21.8 $\pm$ 7.6 <b>a</b>	40.6 $\pm$ 7.6 <b>a</b>	33.0 $\pm$ 7.6 <b>a</b>	1.69 <sup>(2,12)</sup>	0.226
Methoxyflavone hexoside	92.1 $\pm$ 32.7 <b>b</b>	234.5 $\pm$ 36.5 <b>a</b>	224.9 $\pm$ 34.5 <b>a</b>	5.57 <sup>(2,15)</sup>	<b>0.016</b>
Methylkaempferol or isomer	16.8 $\pm$ 5.1 <b>a</b>	23.5 $\pm$ 4.9 <b>a</b>	19.7 $\pm$ 5.1 <b>a</b>	0.93 <sup>(2,10)</sup>	0.426
Coumaric acid derivative	147.3 $\pm$ 85.5 <b>b</b>	458.7 $\pm$ 85.5 <b>a</b>	354.3 $\pm$ 85.5 <b>a</b>	4.50 <sup>(2,18)</sup>	<b>0.026</b>
Coumaric acid derivative2	25.5 $\pm$ 8.8 <b>b</b>	47.8 $\pm$ 8.8 <b>a</b>	67.7 $\pm$ 8.8 <b>a</b>	5.51 <sup>(2,18)</sup>	<b>0.014</b>

787 **Appendix S2.** Test for the combined effects of both herbivores (plants from single-species herbivore treatments, and plants from the two  
 788 combined herbivore treatments) on the concentration of individual phenolics in the leaves (10 flavonoids and two coumaric acid derivatives).  
 789 Least-square means  $\pm$  SE are shown. PROC MIXED in SAS 9.2 was used to run the general linear models. F-values, degrees of freedom (within  
 790 brackets) and associated significance levels ( $P$ ) are shown. Different letters indicate significant ( $P < 0.05$ ) differences between treatments. We  
 791 used Tukey tests for pairwise comparisons among treatment level means.

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COMPOUND	Single herbivore species	Two herbivore species	F	$P$
Rutin	84.4 $\pm$ 16.9 <b>a</b>	59.1 $\pm$ 16.9 <b>a</b>	1.12 <sup>(1,13)</sup>	0.309
Quercetin hexoside rhamnoside	160.9 $\pm$ 41.1 <b>a</b>	121.5 $\pm$ 41.1 <b>a</b>	0.49 <sup>(1,23)</sup>	0.489
Kaempferol triglycoside	226.7 $\pm$ 32.0 <b>a</b>	173.5 $\pm$ 32.9 <b>a</b>	1.34 <sup>(1,28)</sup>	0.256
Isorhamnetin triglycoside	408.6 $\pm$ 45.4 <b>a</b>	302.9 $\pm$ 46.6 <b>a</b>	2.83 <sup>(1,28)</sup>	0.104
Kaempferol hexoside rhamnoside	404.5 $\pm$ 71.7 <b>a</b>	354.3 $\pm$ 73.1 <b>a</b>	0.34 <sup>(1,28)</sup>	0.564
Isorhamnetin hexoside rhamnoside	1276.3 $\pm$ 157.3 <b>a</b>	979.3 $\pm$ 161.1 <b>a</b>	1.94 <sup>(1,28)</sup>	0.174
Methylkaempferol hexoside	38.4 $\pm$ 7.3 <b>a</b>	37.3 $\pm$ 6.8 <b>a</b>	0.01 <sup>(1,24)</sup>	0.904
Dimethylkaempferol hexoside	36.5 $\pm$ 7.2 <b>a</b>	33.3 $\pm$ 6.6 <b>a</b>	0.11 <sup>(1,24)</sup>	0.744
Methoxyflavone hexoside	232.9 $\pm$ 27.7 <b>a</b>	193.0 $\pm$ 26.4 <b>a</b>	1.33 <sup>(1,25)</sup>	0.259
Methylkaempferol or isomer	21.1 $\pm$ 4.3 <b>a</b>	21.2 $\pm$ 4.1 <b>a</b>	0.00 <sup>(1,21)</sup>	0.990
Coumaric acid derivative	406.5 $\pm$ 72.0 <b>a</b>	356.1 $\pm$ 73.5 <b>a</b>	0.34 <sup>(1,28)</sup>	0.564
Coumaric acid derivative2	54.8 $\pm$ 8.0 <b>a</b>	57.8 $\pm$ 8.1 <b>a</b>	0.14 <sup>(1,28)</sup>	0.713

793 **Appendix S3.** Test for effect of herbivore arrival order (control plants, plants attacked by *S. eridania* plus *D. balteata*, and plants attacked by *D.*  
 794 *balteata* plus *S. eridania*) on the concentration of individual phenolics in the leaves (10 flavonoids and two coumaric acid derivates). Least-  
 795 square means  $\pm$  SE are shown. PROC MIXED in SAS 9.2 was used to run the general linear models. F-values, degrees of freedom (within  
 796 brackets) and associated significance levels (*P*) are shown. Different letters indicate significant (*P* < 0.05) differences between treatments.  
 797 Significant *P*-values are shown in bold. We used Tukey tests for pairwise comparisons among treatment level means.  
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COMPOUND	Control	<i>D. balteata</i> plus <i>S. eridania</i>	<i>S. eridania</i> plus <i>D.</i> <i>balteata</i>	F	<i>P</i>
Rutin	33.0 $\pm$ 17.6 <b>a</b>	56.6 $\pm$ 16.5 <b>a</b>	64.1 $\pm$ 23.3 <b>a</b>	0.72 <sup>(2,8)</sup>	0.514
Quercetin hexoside rhamnoside	63.6 $\pm$ 37.6 <b>a</b>	150.9 $\pm$ 33.2 <b>a</b>	90.1 $\pm$ 35.2 <b>a</b>	1.75 <sup>(2,12)</sup>	0.216
Kaempferol triglycoside	78.8 $\pm$ 27.6 <b>b</b>	176.2 $\pm$ 28.8 <b>a</b>	172.2 $\pm$ 27.6 <b>a</b>	5.75 <sup>(2,17)</sup>	<b>0.012</b>
Isorhamnetin triglycoside	171.7 $\pm$ 46.7 <b>b</b>	316.9 $\pm$ 48.4 <b>a</b>	296.5 $\pm$ 46.7 <b>a</b>	5.27 <sup>(2,17)</sup>	<b>0.016</b>
Kaempferol hexoside rhamnoside	146.7 $\pm$ 71.9 <b>a</b>	341.6 $\pm$ 75.8 <b>a</b>	351.9 $\pm$ 71.9 <b>a</b>	2.54 <sup>(2,17)</sup>	0.108
Isorhamnetin hexoside rhamnoside	598.3 $\pm$ 163.2 <b>b</b>	989.5 $\pm$ 169.0 <b>a</b>	1001.8 $\pm$ 163.2 <b>a</b>	3.98 <sup>(2,17)</sup>	<b>0.038</b>
Methylkaempferol hexoside	16.3 $\pm$ 9.6 <b>a</b>	43.8 $\pm$ 9.6 <b>a</b>	30.9 $\pm$ 9.1 <b>a</b>	2.67 <sup>(2,16)</sup>	0.100
Dimethylkaempferol hexoside	21.8 $\pm$ 10.9 <b>a</b>	37.7 $\pm$ 10.2 <b>a</b>	29.2 $\pm$ 9.7 <b>a</b>	0.65 <sup>(2,15)</sup>	0.537
Methoxyflavone hexoside	92.1 $\pm$ 27.9 <b>b</b>	194.4 $\pm$ 28.8 <b>a</b>	190.9 $\pm$ 27.9 <b>a</b>	9.80 <sup>(2,17)</sup>	<b>0.001</b>
Methylkaempferol or isomer	14.4 $\pm$ 6.4 <b>a</b>	23.7 $\pm$ 6.0 <b>a</b>	19.0 $\pm$ 5.7 <b>a</b>	0.56 <sup>(2,12)</sup>	0.584
Coumaric acid derivative	147.3 $\pm$ 72.3 <b>a</b>	343.3 $\pm$ 76.2 <b>a</b>	353.6 $\pm$ 72.3 <b>a</b>	2.55 <sup>(2,17)</sup>	0.108
Coumaric acid derivative2	25.5 $\pm$ 8.0 <b>b</b>	61.4 $\pm$ 8.4 <b>a</b>	53.1 $\pm$ 8.0 <b>a</b>	5.34 <sup>(2,17)</sup>	<b>0.016</b>

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