Sequence of arrival determines plant-mediated interactions between herbivores

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Summary

1. Induced changes in plant quality can mediate indirect interactions between herbivores. Although the sequence of attack by has been shown to influence plant responses, little is known about how this affects the herbivores themselves.
2. We therefore investigated how induction by the leaf-herbivore *Spodoptera frugiperda* influences resistance of teosinte (*Zea mays mexicana)* and cultivated maize *(Zea mays mays)* against root-feeding larvae of *Diabrotica virgifera*. The importance of the sequence of arrival was tested in the field and laboratory.
3. *S. frugiperda* infestation had a significant negative effect on colonization by *D. virgifera* larvae in the field and weight gain in the laboratory, but only when *S. frugiperda* arrived on the plant before the root herbivore. When *S. frugiperda* arrived after the root herbivore had established, no negative effects on larval performance were detected. Yet, adult emergence of *D. virgifera* was reduced even when the root feeder had established first, indicating that the negative effects were not entirely absent in this treatment.
4. The extent of defoliation of the plants was not a decisive factor for the negative effects on root herbivore development, as both minor and major leaf damage resulted in an increase in root resistance and the extent of biomass removal was not correlated with root-herbivore growth. We propose that leaf-herbivore induced increases in feeding-deterrent and/or toxic secondary metabolites may account for the sequence-specific reduction in root-herbivore performance.

5. ***Synthesis:*** Our results demonstrate that the sequence of arrival can be an important determinant of plant-mediated interactions between insect herbivores in both wild and cultivated plants. Arriving early on a plant may be an important strategy of insects to avoid competition with other herbivores. To fully understand plant-mediated interactions between insect herbivores, the sequence of arrival should be taken into account.

Key-words: Above-BG interactions, *Diabrotica virgifera,* induced resistance, plant-mediated effects, plant quality, *Spodoptera frugiperda,* systemic signalling*, Zea mays*, teosinte.

Introduction

The metabolism of plants is remarkably adaptable to environmental stress: Upon attack by insects and pathogens, dedicated signal transduction cascades are activated that help plants to withstand and tolerate the ensuing threats (Howe and Jander, 2008, Dangl and Jones, 2001; Rasmann *et al.*, this issue). Such changes do not only happen locally, but involve non-attacked tissues as well (Schwachtje and Baldwin, 2008, Orians, 2005, Erb *et al.*, 2009c, Heil and Ton, 2008). Systemic effects following herbivory can have fitness consequences for temporally or spatially separated organisms (van Loon *et al.*, 1998, Erb *et al.*, 2009a, Sticher *et al.*, 1997, Poelman *et al.*, 2008a, Viswanathan *et al.*, 2005). Interestingly, it is becoming more and more evident that changes in plant quality may even be more important than direct interference or biomass removal in shaping competitive interactions between herbivores and future attacker communities (Kaplan and Denno, 2007, van Zandt and Agrawal, 2004, Poelman *et al.*, 2010). Some of the most dramatic examples in this context come from studies investigating plant-mediated interactions between root- and leaf- feeding herbivores (Erb *et al.*, 2008): Belowground (BG) herbivores have been shown to profoundly change leaf physiology, thereby affecting aboveground (AG) attackers, and even higher trophic levels (Steinger and Müller-Schärer, 1992, van Dam *et al.*, 2005, Soler *et al.*, 2005, Rasmann and Turlings, 2007) and *vice versa*, AG herbivores can change root physiology and resistance (Moran and Whitham, 1990, Masters, 1995, Soler *et al.*, 2007, Kaplan *et al.*, 2008).

In recent years, it has been hypothesized that plant-quality mediated interactions between herbivores may not only depend on the combination of attackers, but also on their sequence of arrival or timing (Blossey and Hunt-Joshi, 2003). Evidence for this concept comes

for example from a gene-expression study in *Nicotiana attenuata*, where it was found that the order of attack of a sap-feeder and a chewing herbivore is an important determinant explaining the ensuing transcriptional response (Voelckel and Baldwin, 2004). In *Solanum dulcamara*, changes in polyphenol oxidase and peroxidase activity following tortoise and flea beetle attack were determined by the first attacker, but not significantly modified after sequential feeding by either species (Viswanathan *et al.*, 2007). Yet, despite the increasing evidence for the sequential dependence of changes in plant-quality following attack, we are not aware of any study that has tested the effect of an herbivore arriving before or after a second feeder on the performance of the latter. Such experiments are especially difficult to conduct in the AG parts of plants, as simultaneously occurring herbivores may interact directly with each other compared to their sequential presence, thereby confounding direct and plant-mediated effects. As root- and leaf-herbivores are spatially separated and do not have any physical contact during their development, they represent an ideal model to study the effects of the sequence of arrival.

We tested the effect of the sequence of arrival on the impact of leaf-herbivory on root herbivore resistance using leaf-feeding larvae of the specialist noctuid moth *Spodoptera frugiperda* (J.E. Smith) and root feeding larvae of the specialist beetle *Diabrotica virgifera virgifera* (LeConte). These species co-occur in maize (*Zea mays* L.) agroecosystems in North America and natural ecoysystems in Mexico. *D. virgifera* passes the winter and/or dry periods as eggs in the soil, from where the larvae hatch, locate their hosts and start feeding. Larvae can cross distances up to 1m to find or switch host plants (Short and Luedtke, 1970, Suttle *et al.*, 1967). *S. frugiperda* on the other hand overwinters as pupa in tropical regions and the southern

US (Foster and Cherry, 1987), from where adults disperse and oviposit on growing plants. In the main maize growing regions of North America, *S. frugiperda* therefore establishes later on the host than *D. virgifera* (O'Day, 1998). In Mexico, where teosinte (the wild ancestor of maize) and *D. virgifera* are believed to have evolved together (Branson and Krysan, 1981), it can be expected that plants may be attacked first by either herbivore, depending on which species is faster in colonizing its host at the beginning of the growing season. Furthermore, as *D. virgifera* displays an enormous phenotypic plasticity in its diapause behavior (Branson, 1976), late emerging or second generation *D. virgifera* larvae may encounter plants that have already been attacked by both *D. virgifera* and *S. frugiperda*.

A combination of field and laboratory experiments was used to gain insight into the leaf-herbivore induced changes in root resistance and the importance of sequential colonization. In the field, we simulated a natural situation whereby early emerging *D. virgifera* larvae arrived on the plant first, followed by *S. frugiperda* in the leaves and a subsequent second wave of root herbivores. In the laboratory, we explicitly tested if the sequence of arrival influences leaf-herbivore induced changes by adding and removing *S. frugiperda* larvae either before or after the onset of *D. virgifera* feeding. In the laboratory, we not only tested cultivated maize (*Zea mays mays*), but also its wild ancestor teosinte (*Zea mays mexicana*). The complementary assays presented here provide clear evidence for the importance of the sequence of arrival of different insect herbivores for plant-mediated interactions between them.

Material and Methods

Field plants and insects

For the field experiments, maize seeds (var. Delprim) were sown in 16 plots (3.05 m × 3.05 m). Plots were arranged in a 2 x 8 rectangular pattern. All plants were sown on the 1st of June 2009. Because of low initial germination, most plots did not reach the envisaged density of 64 plants per plot. Therefore, new seeds were sown or seedlings were transplanted two weeks later to fill the gaps. To insure that western corn rootworm larvae would not move between plots, a 3.05 m buffer containing no vegetation was maintained between each plot within rows and four rows of commercial buffer maize were planted between the two blocks of eight plots. Four additional rows of buffer maize were also planted at both sides of the study site to minimize wind damage to the screen tents. Eight plots suffered from flooding (2 times for approx. 48 h) during the early stage of the experiment. A block factor (flooding) was added to the statistical model to account for this potential source of variability (see below). All the plots were infested with *D. virgifera* eggs (600 WCR actual eggs every 30.5 cm of maize row) on the 18th of June. A diapausing strain was used for this infestation. Viability of these eggs averaged 83%, so viable egg numbers were close to 500 per 30.5 cm of maize row. On the 3rd of July, when the plants had reached a height of approx. 50 cm and had developed 6 leaves, screen tents (3.35 m × 3.96 m Insta-Clip, The Coleman Company, Inc., Wichita, KS) were placed over the plots to reduce the natural colonization of herbivores. The tents were dug into the soil to a depth of 15 cm to help secure the tents from wind damage. On the 10th of July, half of the plots were infested with 20 neonate *S. frugiperda* larvae/plant using a bazooka/corn grit applicator system (Wiseman et al. 1980). Control plants received the same volume of corn grit without

141 larvae. Because of the high mortality of the neonates after the first application, another 20 *S.*
142 *frugiperda* larvae were added one week later using the same method. Forty *S. frugiperda* larvae
143 per plant are well within the natural range of infestation, as egg batches typically consist of 100
144 or more individuals. On the 22nd of July, when the *D. virgifera* larvae were in the second larval
145 stadium, 4-6 plants with clear caterpillar damage were selected and harvested from each plot.
146 On the 24th of July, when the first *D. virgifera* infestation began to reach the pupal stage and
147 the first maize plants were tasseling, another 500 WCR eggs were added to 8 plants per plot,
148 and the plants were marked for later recovery. These plants had previously been attacked by
149 early emerging *D. virgifera* larvae, followed by either *S. frugiperda* ("infested") or no leaf-
150 herbivory ("controls"). A non-diapausing strain was used for the second infestation. This strain
151 is similar in many aspects to the diapausing *D. virgifera*, but develops somewhat faster on the
152 plants. This enabled a second, successful establishment of the root herbivore larvae on the
153 plants before they were too old (Hibbard *et al.*, 2008). We also hypothesized that in a natural
154 situation in Mexico, late arriving *D. virgifera* larvae would likely be second-generation
155 individuals that did not enter diapause. Two groups of plants were used for this second
156 application: One half that had already reached the tasseling stage and another half that were
157 still in the whorl stage due to late sowing or replanting. On the 7th of August, when the larvae of
158 the first infestation had pupated and the second *D. virgifera* infestation had reached the second
159 instar, the infested plants were harvested. To gain insight into the number of *D. virgifera* larvae
160 that were able to successfully develop to adult beetles, the remaining plants (around 50/plot)
161 were left in the tents until the end of the adult emergence period of the first infestation of *D.*

virgifera. The field experiment was terminated on the 20th of September, when a heavy storm destroyed the tents.

Recovery of D. virgifera *larvae, root damage rating and adult emergence*

Plant root systems (4-8 per plot, see above) were harvested from the field by digging the roots out together with the surrounding soil. The root balls were then transferred to commercial onion bags and suspended in a greenhouse as described by Hibbard *et al.* (2004). Under each bag, a plastic pan filled with water was installed. The high temperature in the greenhouse (40-50° C) dried the soil balls and prompted the *D. virgifera* larvae to move down and fall into the water below. Larvae were counted and recovered twice a day over a period of 10 days and preserved in ethanol. Roots were then washed and rated for damage using the 0 to 3 node-injury scale (Oleson *et al.*, 2005). Starting on the 7th of August, emergence of adult *D. virgifera* beetles in the tents was monitored every week until the 16th of September. The emerging insects were collected, sexed and preserved in ethanol.

Laboratory plants and insects

To confirm the results obtained in the field in a better controlled environment, we carried out additional experiments in the laboratory. Cultivated maize and teosinte plants were grown in bottom-pierced, aluminium-wrapped plastic pots (diameter, 4cm; depth, 11cm) in a phytotron (23±2°C, 60% r.h., 16:8 hr L/D, and 50,000 lm/m^2). Before planting, the seeds were rinsed with water to remove any storage residuals. They were then sown in sand (lower 8 cm) and topped with commercial potting soil (upper 3 cm, Ricoter Aussaaterde, Aarberg,

Switzerland). Cultivated maize plants (*Zea mays mays*, var. Delprim) had two fully expanded primary leaves and were 9-10 days old. Teosinte seeds (*Zea mays mexicana*) had been collected from two wild populations near Texcoco (Mexico) in 1998. As the teosinte plants grew slower than the cultivated hybrid Delprim, they were left in the phytotron for 20 days, until they had 2-3 fully developed leaves. All plants were watered with 10ml of tap water every day. Experiments were carried out under light benches in a climatized laboratory (25±2°C, 40±10% r.h., 16:8 hr L/D, and 8000 lm/m^2). *S. frugiperda* eggs were obtained from an in-house colony reared on artificial diet. *D. virgifera* eggs (non-diapausing strain) were obtained from the USDA-ARS-NCARL Brookings (US) and kept on freshly germinated maize seedlings until use.

D. virgifera performance experiments

Laboratory experiments were carried out to specifically test whether physiological changes in the plants are important for the differential effects of sequence of arrival for the impact of *S. frugiperda* on *D. virgifera*. One experiment was performed using cultivated maize, and a second one with teosinte. The following procedure was used for both trials: Before the beginning of the experiments, the pots of 10 day old plants were covered at the bottom with aluminium foil to prevent root herbivores from escaping through the two drainage holes in the bottom of each pot, and transparent 1,5l PET bottles with their bottoms removed (30cm height, conal shape, top-diameter: 8cm) were placed upside down over the AG part of the plants to confine leaf-herbivores. The PET tubes were held in place with parafilm. The plants were then divided into three groups (n=12-15). All groups were infested with 4 pre-weighed early second instar *D. virgifera* larvae by putting them on the soil with a fine brush. One set of plants had

been infested with 12 L2 *S. frugiperda* larvae 48h prior to root herbivore infestation, while the second set was infested with the leaf herbivore 48h after *D. virgifera* had started feeding. In both cases, the *S. frugiperda* larvae were removed from the plants after 48h of feeding. The third group did not receive any leaf-herbivore treatment. We had intended to add an additional leaf-herbivore treatment to the teosinte experiment, but a lack of suitable *S. frugiperda* larvae prevented this and we therefore had a teosinte control group that consisted of a total of 24 independent replicates. After five days of feeding, the *D. virgifera* larvae were recovered from the soil and weighed to determine their weight increase. Leaves of the different plants were harvested and their fresh weight (FW) was determined.

Data analysis

For the field experiment, the parameters recorded were averaged for the different plots, resulting in eight independent replicate values per treatment. Two-way Analyses of Variance (ANOVAs) were carried out on the number of recovered root herbivore larvae and emerging adults with the factors treatment and environment. The environment was either "flooded" (8 plots) or "non-flooded" (8 plots) depending on the soil-water condition within the field tents, and the two treatments were "control" (8 plots) and "*S. frugiperda* infested" (8 plots). Interaction terms were included in the models. To assess the effect of big and small plants, plant size was included as a nested factor in a general linear model (GLM). Larval growth and leaf fresh-weight in the lab-experiment were assessed using one-way ANOVAs. In all cases, normality and homogeneity of variance was assessed using the Kolmogorov-Smirnov and Levene's test respectively. Because the number of emerged *D. virgifera* adults in the field

228 experiment did not conform to normality and the variance was unequal for this dataset, the
229 analysis was carried out on rank-transformed data. *D. virgifera* weight gain on maize and
230 teosinte were analyzed on $\log_{10}+2$ transformed data to ensure normality of distribution.
231 Significant effects were subjected to pair-wise comparisons using Holm-Sidak post hoc tests.
232 Association between variables was tested using Pearson Product Moment Correlations and
233 Sum-of-Squares linear regression. Statistical analyses were performed with SigmaStat v3.5 and
234 MiniTab v15.
235

Results

Recovery of D. virgifera *larvae*

The tents prevented natural infestation of the two major leaf-pests of corn, *Ostrinia nublilalis* and *S. frugiperda*, as no infestation of the control plots by these species was observed. Individual cattail (*Simyra spp.*) and yellow wollybear (*Spilomena virginica*) caterpillars on the other hand were occasionally encountered on the leaves of control plants. Control plants showing clear damage by these herbivores were not used for root-herbivore recovery. From the first infestation of *D. virgifera*, a total of 216 larvae were recovered from the roots. There was no natural infestation by *D. virgifera* in this particular field. The number of recovered root-herbivore larvae from the first infestation was not affected by the presence of *S. frugiperda* (ANOVA: $p=0.536$). Root masses from plots that had suffered from elevated soil moisture carried significantly lower numbers of larvae than the roots from plots with normal water status (ANOVA: $p<0.001$; Holm-Sidak post-hoc test: $p=0.001$: Fig. 1a). From the second infestation, a total of 129 larvae were retrieved. The first infestation larvae had reached the pupal stage by the time the second generation was sampled. It is therefore unlikely that individuals from this group ended up in the collection pans and indeed, no third instar larvae or pupae were recovered. The environmental block factor (high moisture levels early on) did not show a significant effect on this infestation of *D. virgifera* (ANOVA: $p=0.607$). On the other hand, the presence of *S. frugiperda* significantly reduced the number of surviving root herbivore larvae of the second infestation (ANOVA: $p=0.027$; Holm-Sidak post-hoc test: $p=0.0275$; Fig. 1b). In the plots that were not infested with *S. frugiperda*, an average of 1.5 larvae/plant was retrieved,

257 whereas in the presence of leaf-herbivores, larval recovery was reduced by 79% to 0.3
258 larvae/plant.
259
260 *Influence of plant growth stage and AG damage*
261 It was observed that the smaller plants suffered significantly more from *S. frugiperda*
262 feeding damage than the plants that were already tasseling: In mid-season (during the period
263 when the root herbivores were recovered) the small plants (growth stage V8) were largely
264 defoliated with only the midrib of the youngest leaves remaining, while the bigger plants
265 (growth stage VT, tasseling) showed only traces of herbivory and minimal notable loss of
266 biomass. Only later in the season (at the beginning of the adult-emergence period) did the VT
267 plants also suffer from major defoliation. This difference was most probably due to the fact that
268 tasseling plants had tougher leaves (Williams *et al.*, 1998) and no whorl tissue that serves as an
269 important protective structure for *S. frugiperda*. To test whether this difference in defoliation
270 had an effect on *D. virgifera* resistance, we added plant size (big vs. small) as an additional
271 parameter into the model. The nested ANOVA (with plant size as a nested parameter) showed
272 no significant effect of elevated soil moisture (ANOVA: $p=0.555$) or plant size ($p=0.668$), but the
273 effect of *S. frugiperda* was highly significant for the second infestation (ANOVA: $p=0.008$; Fig.
274 1c).
275
276 *Root damage rating*
277 The clear difference in the numbers of larvae recovered from the differentially shoot-
278 infested plants was not reflected in the observed root damage. One explanation for this is that

overall, the level of *D. virgifera* infestation was relatively low (Hibbard *et al.* 2010), and damage scores were between 0-1 for most root systems, which corresponds to less than one node of pruning. Damage to the first batch of rated plants (attacked by the first infestation of *D. virgifera*) was not affected by *S. frugiperda* feeding (ANOVA: $p=0.815$), but was reduced in plants growing in soil with high early humidity levels (ANOVA: $p=0.022$; Fig. 2a). The second set of plants (sequentially attacked by both infestations of *D. virgifera*) showed the same pattern, with no significant effect of *S. frugiperda* (ANOVA: $p=0.505$) and a negative effect of flooding (ANOVA: $p=0.012$; Fig. 2b).

D. virgifera *adult emergence*

In total, 338 adult *D. virgifera* beetles were collected from the field tents over 6 weeks. The beetles were from the first infestation only, as the larvae of the second infestation did not have enough time to reach the adult stage before the termination of the experiment. The number of adults was affected by the elevated soil moisture factor (ANOVA: $p=0.042$), as well as by *S. frugiperda* feeding ($p<0.001$): Significantly fewer adults emerged from the plots that had experienced flooding, and the same was true for plots in which *S. frugiperda* had fed on the leaves (Figs. 2c and d). When tested separately, the negative effect of *S. frugiperda* feeding was significant for both male (ANOVA: $p<0.001$) and female (ANOVA: $p=0.002$) emergence (data not shown).

D. virgifera *weight gain*

Similarly to the field experiment, larval development of *D. virgifera* was negatively affected by *S. frugiperda* feeding in the laboratory. In both cultivated maize and the wild ancestor teosinte, *D. virgifera* larvae on plants that had previously been infested by *S. frugiperda* gained less weight over 5 days compared to larvae on plants that were free of *S. frugiperda* (Figs. 3a and 4a). Interestingly, *D. virgifera* larvae that had established on the roots before *S. frugiperda* showed similar weight gain as larvae on uninfested maize plants (Fig. 3a) and were affected only slightly on teosinte (Fig. 4a). Leaf-biomass was reduced significantly (~50%) by *S. frugiperda* feeding on the relatively small maize plants used in the laboratory assay (ANOVA: $p<0.001$). The teosinte plants also suffered from a significant reduction of leaf fresh weight (ANOVA: $p<0.001$), although this was less pronounced. Leaf biomass was reduced more for the plants that had been infested first with *S. frugiperda* compared to the ones where *S. frugiperda* attacked the plants after *D. virgifera* (Holm-Sidak post-hoc test: $p<0.05$; Figs. 3b and 4b). As it is known that leaf-to-root effects can directly depend on the extent of defoliation (Kaplan *et al.*, 2008), we tested if there was a relationship between leaf-biomass removal and *D. virgifera* weight gain. In accordance with our observations in the field, no significant correlation was found between these two factors, neither in maize ($R^2=0.032$; Fig. 3c) nor teosinte ($R^2=0.003$; Fig. 4c).

Discussion

To the best of our knowledge, the presented study shows for the first time that the sequence of arrival is an important factor shaping plant-mediated interactions between herbivores. In the field experiment, the number of *D. virgifera* larvae recovered from the roots was not changed by *S. frugiperda* feeding on the leaves if *D. virgifera* established on the plants first (Fig. 1a). However, the root-feeding larvae that arrived after *S. frugiperda* were negatively affected by leaf herbivory (Fig. 1b). The same effect was observed in the laboratory, where larval growth was only impaired when the leaf-feeder had attacked the plant first (Figs. 3a and 4a). In nature, root herbivores may therefore escape this negative effect by arriving early on the plant. Interestingly, early studies on AG-BG interactions reported enhanced herbivore growth rates rather than induced resistance (Masters *et al.*, 1993). This has been attributed to an increase in primary metabolite concentrations in the systemic tissues (van Dam and Heil, this issue; Kaplan et al., 2008). While phloem feeding aphids and plant parasitic nematodes may indeed benefit from such changes, our study adds to the growing evidence the chewing herbivores are suffering from induced defenses after primary attack (van Dam and Heil, this issue). We are currently investigating if the increase in resistance reported in this study is indeed due to an increase in defensive metabolite concentrations in the roots, or if changes in primary metabolism are involved as well (see below).

The laboratory experiments allow a comparison between cultivated and wild maize plants to herbivory. The general pattern regarding the sequence-specificity of leaf-herbivore induced root resistance was similar for teosinte and maize (Figs. 3 and 4), suggesting that the physiological responses have not been altered during the cultivation process. Yet, some small

differences between the two systems were observed. First, teosinte suffered less leaf-herbivory by *S. frugiperda* in terms of biomass loss than cultivated maize (Figs. 3b and 4b). It remains to be determined if the wild plant is naturally more resistant to leaf-herbivory than the cultivar, or if the slightly advanced developmental state of the teosinte plants compared to maize (Figs. 3b and 4b) was responsible for this difference. Second, the effect on root herbivore growth was less pronounced in teosinte than in maize (Figs. 3a and 4a). This may be due to the fact that the plants were less induced by the leaf herbivores. Moreover, the somewhat higher standard deviations indicate higher genetic variability in the field-collected teosinte compared to the genetically uniform background of the cultivar. Future experiments could aim at comparing leaf-herbivore induced root resistance in a variety of wild teosinte populations to get insight into possible evolutionary drivers behind the phenomenon.

Interestingly, *D. virgifera* infestation has been shown to increase leaf-resistance against *Spodoptera littoralis* in the laboratory (Erb *et al.*, 2009a) and against lepidopteran herbivores in the field (M. Erb, in press). This phenomenon may partially explain why the removal of leaf-biomass was reduced in the laboratory when *S. frugiperda* had to feed on *D. virgifera* infested maize or teosinte plants (Figs. 3b and 4b). Although root herbivore-induced leaf resistance (RISR) is unlikely to be adaptive for the plant (M. Erb, in press), it may help the root herbivore to protect itself against negative effects of AG herbivores. RISR may have contributed to the reduction of negative shoot-to-root effects in the laboratory, but the field experiment was not confounded by this factor because in all treatments, *S. frugiperda* fed on plants that had been infested in the roots before, regardless of the arrival of the second generation. Yet, for the field experiment, it would theoretically be possible that the feeding by the first infestation changed

362 the physiology of the roots differentially depending on the presence of the leaf-herbivore,
363 which then could have influenced the performance of the second infestation. Alternatively,
364 differences in the behavior of the diapausing and non-diapausing strains may have contributed
365 to the observed results (Prischmann *et al.*, 2008). However, the laboratory experiments
366 demonstrate that leaf-herbivore induced root resistance functions independently of such
367 effects, as only one root herbivore generation was present per plant, and the same *D. virgifera*
368 strain was used for all treatments. Taken together, due to their complementary nature, the
369 field and laboratory experiments conclusively show that the sequence of arrival is important for
370 the outcome of plant-mediated insect-plant-insect interactions.

371 AG attack by *S. frugiperda* profoundly influences the physiology and host suitability of
372 maize roots for root-feeding insects. It is unlikely that the lack of assimilate supply from the
373 leaves is responsible for this phenomenon, as i) both heavily defoliated and less-damaged
374 plants supported lower numbers of *D. virgifera* larvae (Fig. 1c), and ii) there was no correlation
375 between the available leaf-biomass and root herbivore growth (Figs. 3c and 4c). On the
376 contrary, leaf-defoliation by grasshoppers has been shown to increase root assimilate flows in
377 maize (Holland *et al.*, 1996). Another possible explanation for the observed reduction in root
378 herbivore performance could be that leaf-herbivory leads to a short-term reduction of root-
379 growth (Hummel *et al.*, 2009) and a long term-decrease of root-biomass (Bardgett *et al.*, 1998).
380 During the course of the field experiment, however, both larval densities and adult emergence
381 numbers were low (Figs. 1 and 2) and the root systems showed only little damage (Fig. 2),
382 implying that root biomass was not a limiting factor. Equally, ample root-biomass was available

383 in the laboratory assays at the end of the experiment. Therefore, the differences in *D. virgifera*
384 performance likely stemmed from changes in secondary metabolism.
385 It has been proposed that highly resistant maize lines produce the defensive protein MIR1-CP in
386 the roots upon leaf-attack by *S. frugiperda* (Lopez *et al.*, 2007). Plants synthesize a variety of
387 secondary metabolites BG to support leaf-defences (Erb *et al.*, 2009c) that may also negatively
388 affect *D. virgifera*. Further research will have to be conducted to characterize the alterations in
389 root physiology that increase BG resistance. It will be interesting to see if these defences are
390 induced differentially in the roots depending on the sequence of arrival. Another focus should
391 be on possible shoot-root signals mediating the interaction. It has been proposed that
392 phytohormone cross-talk may be responsible for a series of plant-mediated interactions
393 between herbivores: The plant's salicylic acid (SA) response for example down regulates
394 jasmonic acid (JA) dependent defense genes (Spoel *et al.*, 2007), which may explain the
395 interference of whiteflies with induced resistance (Zarate *et al.*, 2007) and bacterial
396 colonization belowground (Yang *et al.*, this issue). However, our hormonal profiles suggest
397 that none of the classical stress-response signals (JA, SA and abscisic acid) change in
398 concentration in the roots upon herbivory by *Spodoptera littoralis* (Erb *et al.*, 2009a). This
399 indicates that hormonal crosstalk is not responsible for the reported interaction, and that a
400 hitherto unknown insect-induced compound mediates the increase in systemic resistance BG,
401 which is not surprising, given the complexity of plant hormonal networks (Erb and Glauser,
402 2010).

403 It has also been suggested that early arriving herbivores may "canalize the plant
404 response", making it less reactive to subsequent changes (Viswanathan *et al.*, 2007).

405 Conversely, other studies show that a prior stress may "accentuate" the response to a
406 secondary attacker (Erb *et al.*, 2009b, Ton *et al.*, 2007). In our field experiment, canalization is
407 an unlikely scenario, as the late arriving *D. virgifera* larvae would have benefited equally from
408 the fact that the early arriving root-feeders would have blocked the leaf-herbivore induced
409 changes. For the same reason, an accentuated response is an equally unlikely, as all the
410 "second generation" *D. virgifera* larvae arrived on plants that had previously been induced in
411 the roots by the early arrivers. This raises the question about the nature of the sequence
412 dependent factor. We hypothesize that an increase in feeding-deterrent and/or repellent
413 secondary metabolites is responsible for the observed effects. Such compounds would interfere
414 with the host-location and host-acceptance behavior of herbivores that arrive on the plant, but
415 not necessarily with the feeding behavior of larvae that have already colonized and burrowed
416 into the roots. In the laboratory set-up, the fact that the *D. virgifera* larvae did grow less over 5
417 days on plants that had been pre-infested in the leaves may therefore have been the
418 consequence of the fact that they did not accept the roots as hosts and therefore did not
419 readily initiate feeding. *D. virgifera,* as a highly specialized herbivore*,* has been shown to be
420 very responsive to specific root metabolites (Spencer *et al.*, 2009, Bernklau and Bjostad, 2008),
421 and future experiments will aim at characterizing the behavior and feeding pattern of root
422 herbivores in the presence of leaf-attackers.

423 In conclusion, we demonstrate that the sequence of arrival of different insect herbivore
424 species on a plant can be an important determinant shaping the outcome of plant-mediated
425 interactions between them. Further studies involving other systems will be needed to evaluate
426 if this is a general pattern in plant-insect interactions. Our results suggest that in order to

427 understand the interplay between herbivores sharing a host plant, their sequence of arrival has
428 to be addressed. Experimentally imposed insect-treatments in particular may lead to erroneous
429 interpretations if they do not take into account the natural order of insect-succession during
430 the growing season.
431
432

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575
576

Fig. 1

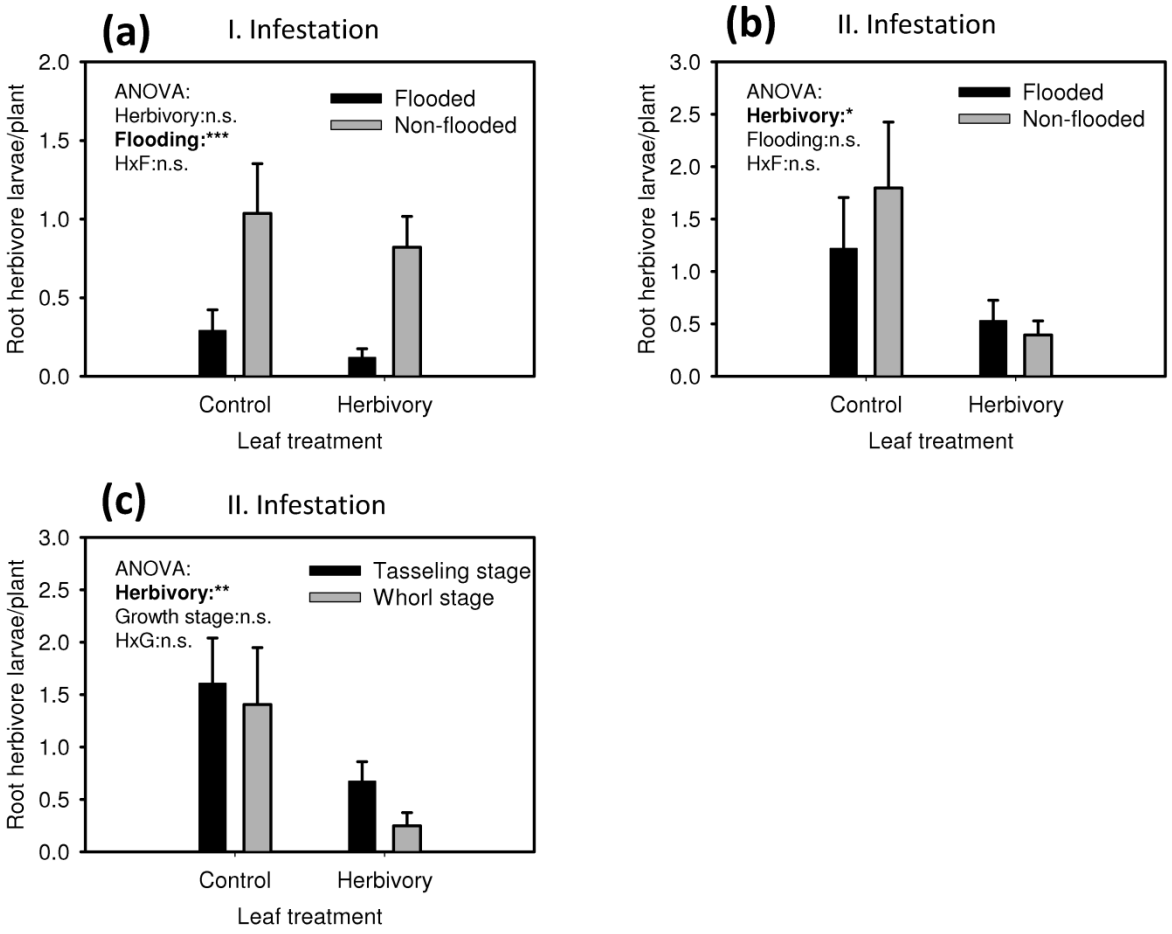

Fig. 1: Influence of leaf herbivory by *S. frugiperda* on recovery rates of root feeding *D. virgifera* larvae. **(a)**: Average number (+SE) of first infestation *D. virgifera* larvae/ plant are shown. *D. virgifera* larvae established on the plants before onset of *S. frugiperda* herbivory. **(b):** Average number (+SE) of second infestation *D. virgifera* larvae/ plant. *D. virgifera* larvae established on the plants after onset of *S. frugiperda* herbivory. Numbers recovered from control plants (left) and *S. frugiperda* infested plants (right) are shown. Plots that suffered from flooding (black bars) are separated from undisturbed plots (grey bars). Results from two-way ANOVAs are included. Effects of Herbivory (*S. frugiperda* and control), flooding (flooded and non-flooded), and their interaction (HxF) are depicted. **(c):** Average number (+SE) of second infestation *D. virgifera* larvae/ plant. Numbers recovered from control plants (left) and *S. frugiperda* infested plants (right) are shown. Tasseling maize plants (black bars) are separated from plants in the late whorl stage (grey bars). Effects of Herbivory (*S. frugiperda* and control), growth stage (whorl and tasseling stage), and their interaction (HxG) are depicted. Stars denote significant factor effects (*p<0.05; **p<0.01; ***p<0.001). N=8.

Fig. 2

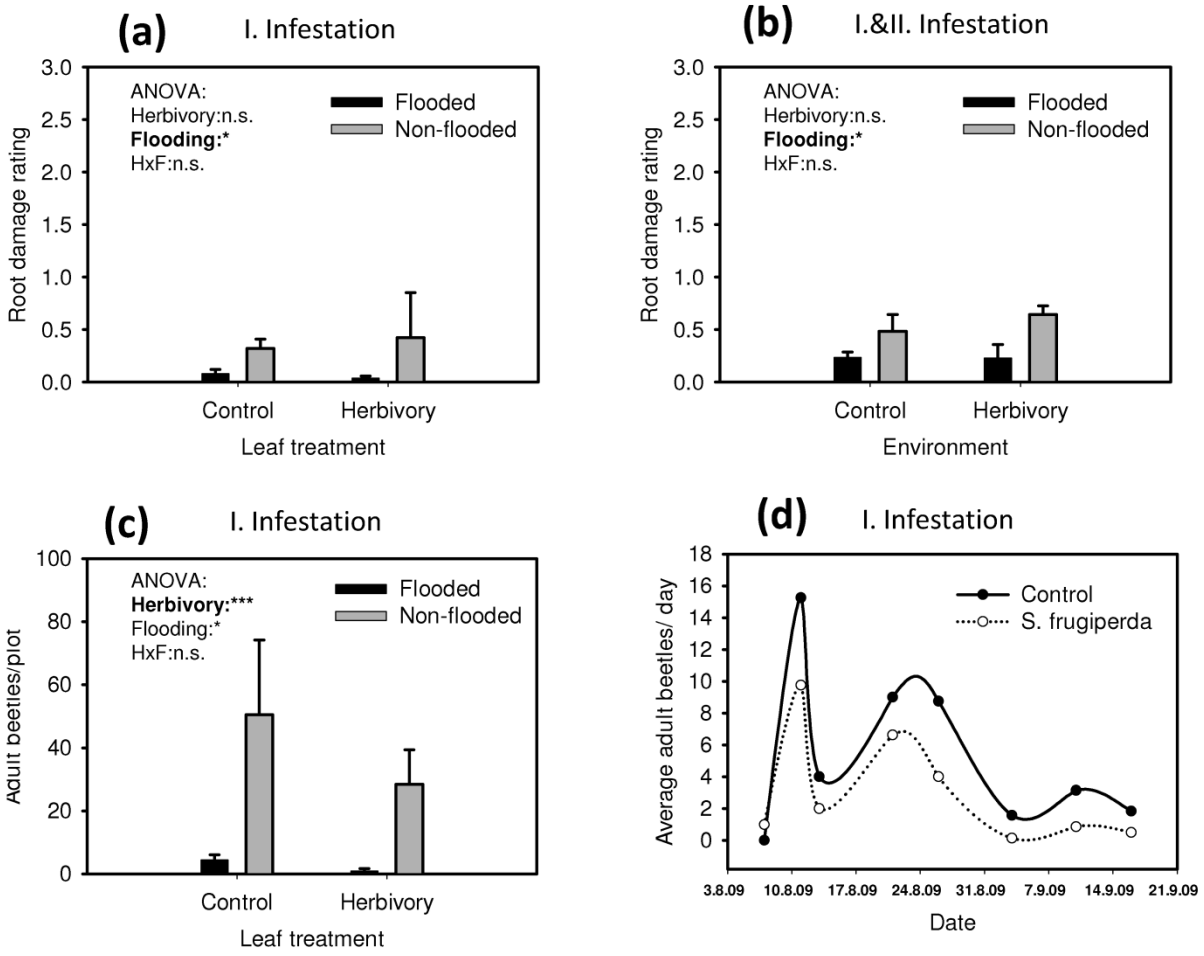

Fig. 2: Effect of leaf herbivory by *S. frugiperda* on *D. virgifera* root damage and adult emergence. **(a)**: Average root rating (+SE) of plants after infestation with the first infestation of *D. virgifera* larvae. **(b):** Average root rating (+SE) of plants after infestation with the first and the second infestation of *D. virgifera* larvae. **(c):** Average number (+SE) of emerging *D. virgifera* adults per plot. Numbers recovered from control plants (left) and *S. frugiperda* infested plants (right) are shown. Plots that suffered from flooding (black bars) are separated from undisturbed plots (grey bars). Results from two-way ANOVAs are included. Effects of Herbivory (*S. frugiperda* and control), flooding (flooded and non-flooded), and their interaction (HxF) are depicted. Stars denote significant factor effects (*p<0.05; **p<0.01; ***p<0.001). **(d):** Time course of emerging adult beetles over the collection period. Average adult beetles per day from control plants (closed circles) and *S. frugiperda* infested plants (open circles) are shown. N=8.

Fig. 3

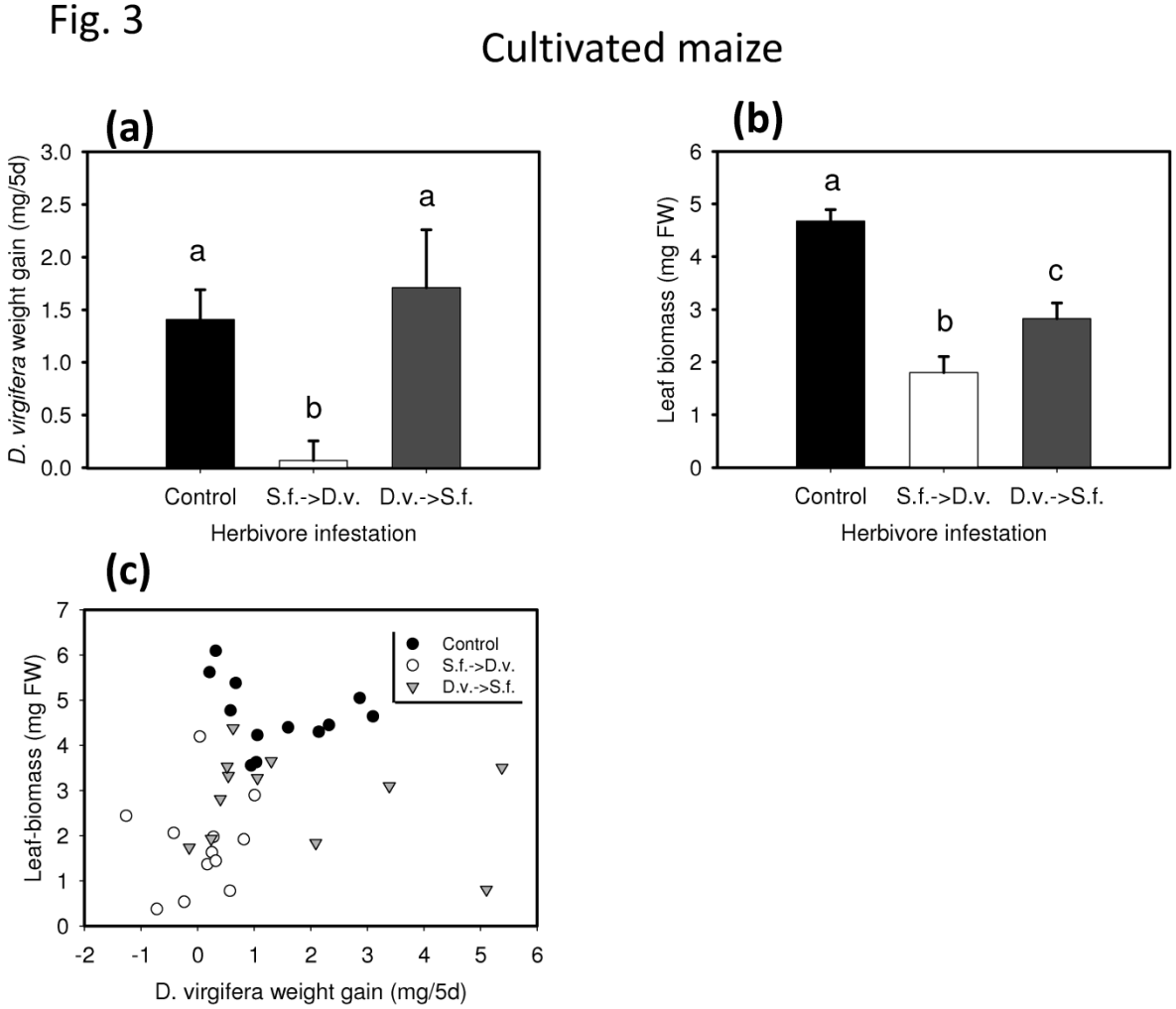

Fig. 3: Influence of leaf herbivory by *S. frugiperda* on *D. virgifera* growth on cultivated maize. **(a):** Average weight gain (+SE) of *D. virgifera* larvae feeding on leaf-herbivore free plants (control, black bars), previously *S. frugiperda* infested plants (before onset of root herbivory, S.f.->D.v., open bars) and late *S. frugiperda* infested plants (after onset of root herbivory, D.v.->S.f., grey bars) are shown. **(b):** Average leaf-biomass of *D. virgifera* and *S. frugiperda* infested plants. Different letters indicate significant differences between treatments ($p<0.05$). **(c):** Correlation between leaf-biomass and *D. virgifera* weight gain on leaf herbivore free plants (filled circles), previously *S. frugiperda* infested plants (empty circles.) and simultaneously *S. frugiperda* infested plants (gray triangles). N=12-15.

Fig. 4

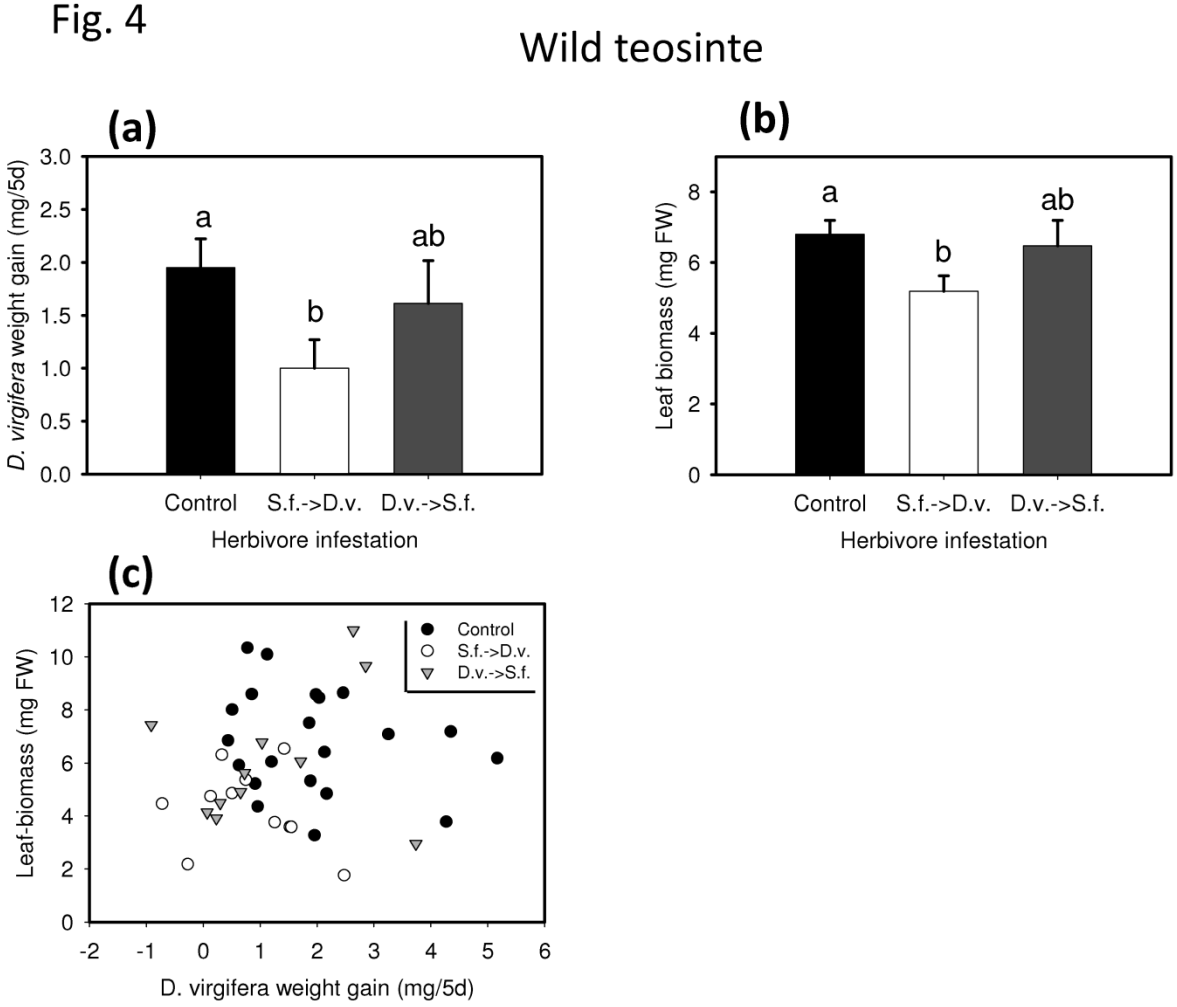

Fig. 4: Influence of leaf herbivory by *S. frugiperda* on *D. virgifera* growth on teosinte. **(a):** Average weight gain (+SE) of *D. virgifera* larvae feeding on leaf-herbivore free plants (control, black bars), previously *S. frugiperda* infested plants (before onset of root herbivory, S.f.->D.v., open bars) and late *S. frugiperda* infested plants (after onset of root herbivory, D.v.->S.f., grey bars) are shown. **(b):** Average leaf-biomass of *D. virgifera* and *S. frugiperda* infested plants. Different letters indicate significant differences between treatments ($p<0.05$). **(c):** Correlation between leaf-biomass and *D. virgifera* weight gain on leaf herbivore free plants (filled circles), previously *S. frugiperda* infested plants (empty circles.) and simultaneously *S. frugiperda* infested plants (gray triangles). N=12.

Fig. 1

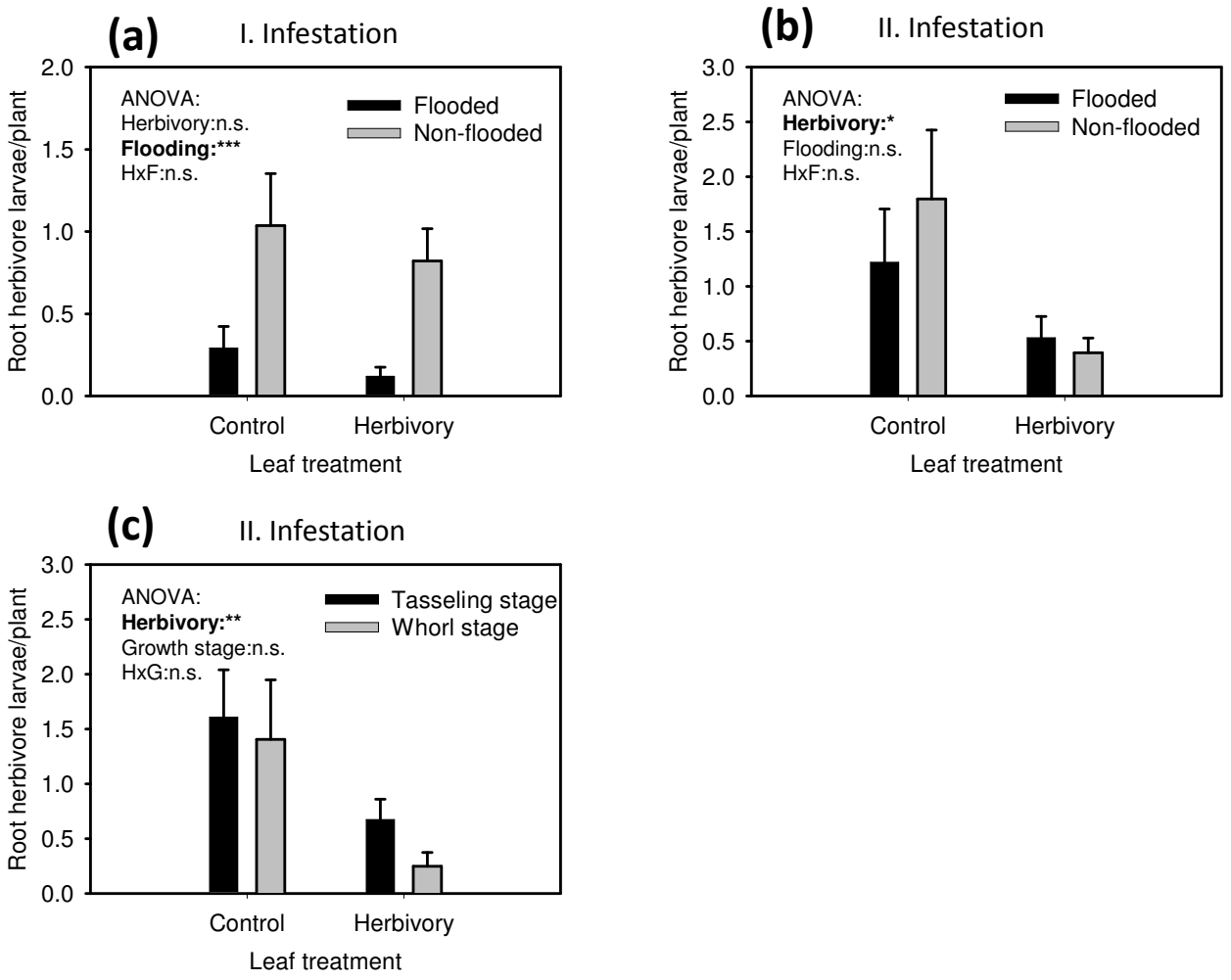
(a)
I. Infestation
ANOVA:
Herbivory:n.s.
Flooding:***
HxF:n.s.
Flooded
Non-flooded
Root herbivore larvae/plant
2.0
1.5
1.0
0.5
0.0
Control
Herbivory
Leaf treatment
(b)
II. Infestation
ANOVA:
Herbivory:*
Flooding:n.s.
HxF:n.s.
Flooded
Non-flooded
Root herbivore larvae/plant
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Herbivory
Leaf treatment
(c)
II. Infestation
ANOVA:
Herbivory:**
Growth stage:n.s.
HxG:n.s.
Tasseling stage
Whorl stage
Root herbivore larvae/plant
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Herbivory
Leaf treatment

Fig. 2

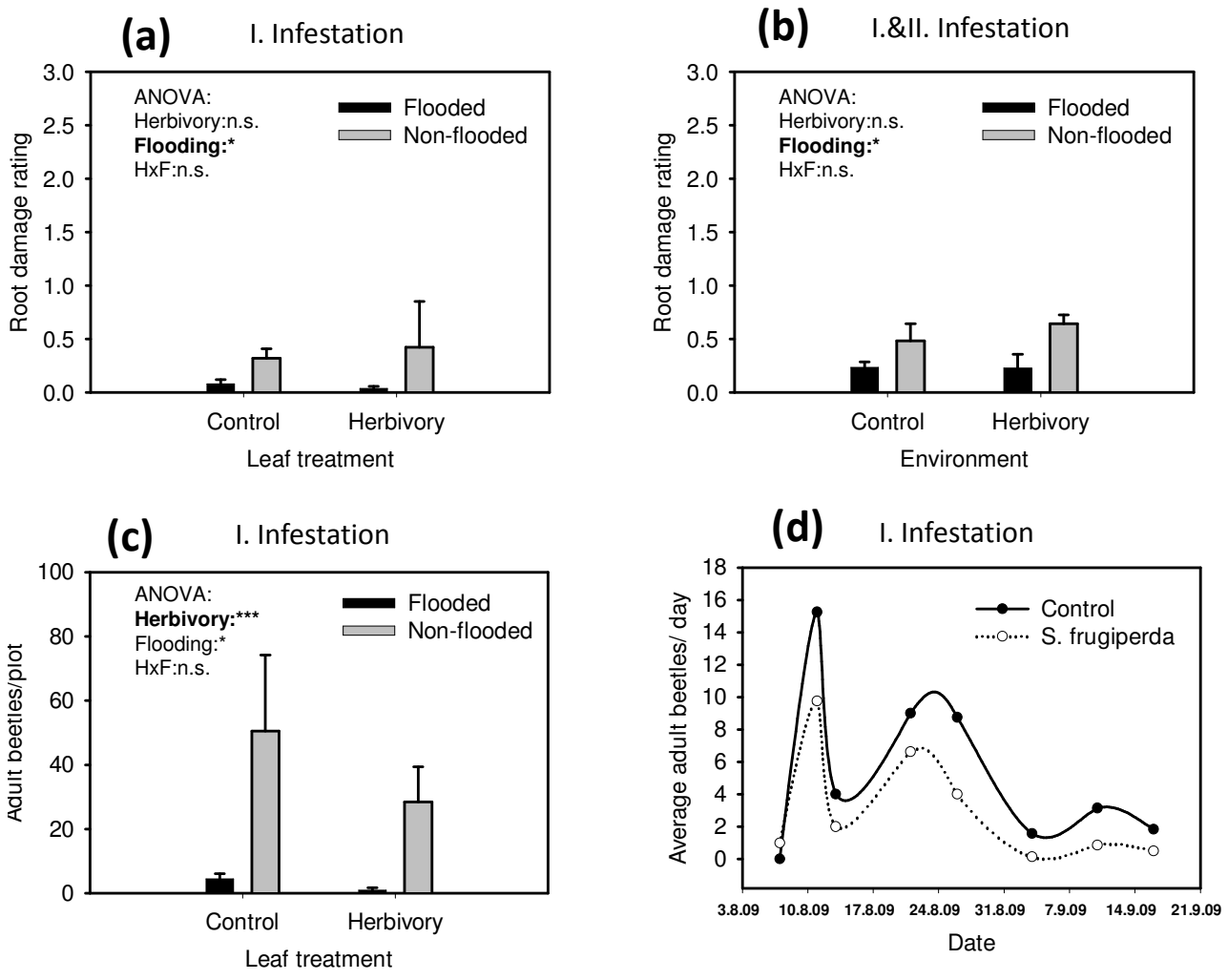
(a)
I. Infestation
ANOVA:
Herbivory:n.s.
Flooding:*
HxF:n.s.
Flooded
Non-flooded
Root damage rating
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Control
Herbivory
Leaf treatment
(b)
I.&II. Infestation
ANOVA:
Herbivory:n.s.
Flooding:*
HxF:n.s.
Flooded
Non-flooded
Root damage rating
Control
Herbivory
Environment
(c)
I. Infestation
ANOVA:
Herbivory:***
Flooding:*
HxF:n.s.
Flooded
Non-flooded
Adult beetles/plot
100
80
60
40
20
0
Control
Herbivory
Leaf treatment
(d)
I. Infestation
Control
S. frugiperda
Average adult beetles/ day
18
16
14
12
10
8
6
4
2
0
3.8.09
10.8.09
17.8.09
24.8.09
31.8.09
7.9.09
14.9.09
21.9.09
Date

Fig. 3

Cultivated maize

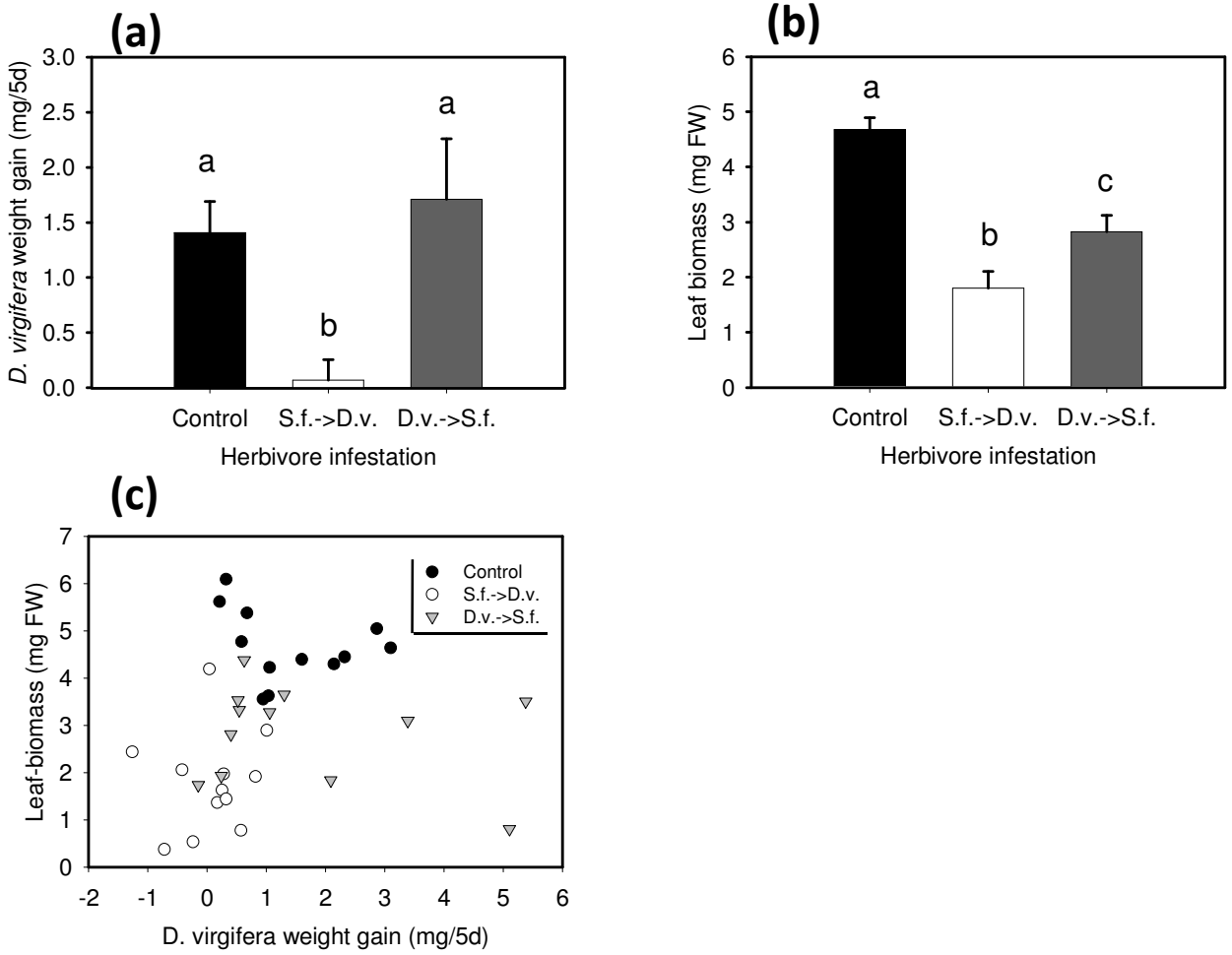

Fig. 4

Wild teosinte