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Spodoptera frugiperda Caterpillars Suppress Herbivore-Induced Volatile Emissions in Maize

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Abstract

The vast spectrum of inducible plant defenses can have direct negative effects on herbivores, or indirect effects, for instance in the form of herbivore-induced plant volatiles (HIPVs) that attract natural enemies. Various arthropods have evolved ways to suppress plant defenses. To test whether this is the case for caterpillar-induced HIPVs, we compared the volatile induction by *Spodoptera frugiperda* (Lepidoptera: Noctuidae), which is particularly well adapted to feed on maize (*Zea mays*), with the induction by three more generalist noctuid larvae. We tested the hypothesis that *S. frugiperda* suppresses HIPV emissions in maize, and thereby reduces attractiveness to natural enemies. HIPV emissions triggered by *S. frugiperda* when feeding on maize were indeed found to be significantly weaker than by *Spodoptera littoralis*, *Spodoptera exigua*, and *Helicoverpa armigera*. The suppression seems specific for maize, as we found no evidence for this when *S. frugiperda* caterpillars fed on cotton (*Gossypium herbaceum*). Artificially damaged maize plants treated with larval regurgitant revealed that HIPV suppression may be related to factors in the caterpillars' oral secretions. We also found evidence that differential physical damage that the caterpillars inflict on maize leaves may play a role. The suppressed induction of HIPVs had no apparent consequences for the attraction of a common parasitoid of *S. frugiperda*, *Cotesia marginiventris* (Hymenoptera: Braconidae). Nevertheless, the ability to manipulate the defenses of its main host plant may have contributed to the success of *S. frugiperda* as a major pest of maize, especially in Africa and Asia, which it has recently invaded.

Keywords Herbivore-induced plant volatiles · Tritrophic interactions · Maize · Cotton · *Spodoptera exigua* · *Spodoptera frugiperda* · *Spodoptera littoralis* · *Cotesia marginiventris* · Parasitoids

Introduction

Numerous studies have revealed that plants are equipped with a broad spectrum of defense mechanisms to protect themselves against herbivorous arthropods. Plants can use direct defenses, such as the production of toxic compounds, either constitutively or induced by insect herbivore attack (Howe and Jander, 2008; Karban and Baldwin, 1997; Wu

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and Baldwin, 2010). In addition, it has been proposed that plants protect themselves indirectly by attracting natural enemies of their herbivores with herbivore-induced plant volatiles (HIPVs) (Dicke et al. 2002; Turlings and Wäckers 2004). The function of HIPVs remains topic of discussion (De Lange et al. 2018; Dicke and Baldwin, 2010; Hare, 2011; Heil 2014; Poelman 2015; Turlings and Erb, 2018), but various studies have shown that they are highly attractive to predators and parasitoids of the herbivores (e.g. De Moraes et al. 1998; Dicke and Sabelis, 1988; Kessler and Baldwin 2001; Thaler 1999; Turlings et al. 1990).

Typically, plants detect elicitors in the oral secretions of arthropods, also known as herbivore-associated molecular patterns, which then triggers the release of volatiles (Acevedo et al. 2015; Erb and Reymond, 2019; Felton and Tumlinson 2008; Schmelz 2015). For example, volicitin present in the regurgitant of *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) larvae induces the emission of HIPVs in maize (*Zea mays* L. ssp. *mays*) (Alborn et al. 1997; Turlings et al. 2000). This and other fatty acid conjugates are also potent elicitors of defense responses in native tobacco (*Nicotiana attenuata*), including the release of volatiles (Halitschke et al. 2003). Similarly, inceptin, isolated from the oral secretions of *Spodoptera frugiperda* Smith, is a potent elicitor of HIPVs in legumes (Carroll et al. 2008; Schmelz et al. 2006). Caeliferins (Alborn et al. 2007) and β-glycosidase (Mattiacci et al. 1995) are further examples of insect-derived elicitors.

Not only do arthropods induce plant defenses, they may also produce repressing compounds to suppress or re-direct inducible plant defenses (Alba et al. 2012b; Pieterse and Dicke, 2007; Walling, 2000). In analogy with plant pathogenic microbes, these repressing compounds are commonly referred to as "effectors" (Boller and He, 2009; Dangl and Jones, 2001; Hogenhout and Bos, 2011). Musser et al. (2002) found that the enzyme glucose oxidase, obtained from oral secretions of the lepidopteran larva *Helicoverpa zea* (Lepidoptera: Noctuidae), is a powerful repressor of toxic nicotine, a direct defense compound of tobacco (*Nicotiana tabacum*), but in tomato (*Solanum lycopersicum*) this enzyme induces defenses (Tian et al. 2012). ATP hydrolyzing enzymes in *H. zea* saliva can suppress direct defenses in tomato (Wu et al. 2012). The regurgitant of Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae), suppresses the expression of wound-inducible genes in tomato (Lawrence et al. 2007). Interestingly, orally secreted bacteria are held responsible for this effect, and bacterial flagellin was identified as a key effector protein (Chung et al. 2013). Indeed, microbial endosymbionts or endosymbiont-like pathogens may manipulate plant defenses to benefit their arthropod hosts (Barr et al. 2010; Casteel et al. 2012; Su et al. 2015). In other cases, the compounds responsible for defense repression remain unknown (e.g. Consales et al. 2011).

If plants actively recruit the natural enemies of their enemies, it can be expected that specialized herbivores have adapted to circumvent and even suppress such indirect plant defenses, similarly to the suppression of direct defenses (Alba et al. 2012a). Indeed, oral secretions of *H. zea* have been found to suppress the emission of HIPVs in tobacco (Delphia et al. 2006). Furthermore, *S. exigua* oral secretions can decrease transcript levels of regulatory genes involved in volatile terpenoid biosynthesis in barrel clover (*Medicago truncatula*) (Bede et al. 2006). A study by Sarmento et al. (2011) showed that feeding by the spider mite *Tetranychus evansi* suppressed the release of HIPVs from its host plant tomato, although two species of predatory mites (*Phytoseiulus longipes* and *Phytoseiulus macropilis*) were still attracted to the herbivore-infested plants (Sarmento et al. 2011). Therefore, the ecological relevance of manipulation of indirect defenses by herbivores has remained uncertain.

In this study, we addressed the possibility that larvae of the moth *S. frugiperda* are capable of suppressing indirect defenses in maize and thereby reduce the plant's attractiveness to their natural enemies. Although *S. frugiperda* is a polyphagous species, it has a strong preference for grasses (Luginbill 1928; Pitre et al. 1983; Sparks 1979), and there are indications that it is adapted to cope with direct defenses specific to grasses, such as silica accumulation (Acevedo 2016). The species tolerates and detoxifies benzoxazinoids, the main direct defense compounds in maize and other grasses (Glauser et al. 2011; Wouters et al. 2014). This further confirms that it is a relative specialist on maize, and, as such, it may also be able to suppress its volatile emissions. In the first study to reveal the potency of caterpillar regurgitants to induce volatile emissions (Turlings et al. 1993), the regurgitant of *S. frugiperda* was indeed one of the least active. Recently, further evidence for the suppressing powers of *S. frugiperda* oral secretions were obtained by Acevedo et al. (2017a, 2018, 2019). In the current study we investigated how this may affect HIPVs and their attractiveness to parasitoids. We compared the volatile blends emitted by maize plants upon feeding by *S. frugiperda* larvae with the blends induced by three generalist lepidopteran larvae, *Spodoptera littoralis* Boisduval, *S. exigua* and *Helicoverpa armigera* Hübner, all of which readily feed on maize in agricultural settings (Luginbill 1928; Hill 1975; Kranz et al. 1977; Sparks 1979; Hill 1987; Fitt 1989).

As *S. frugiperda* and *S. exigua* co-occur in Mexico (Blanco et al. 2014), the country of origin of maize (Matsuoka et al. 2002), we looked at differences in damage patterns and volatile emissions between these species in more detail. Also, we compared the volatile blends induced by *S. frugiperda* and *S. exigua* when feeding on cotton (*Gossypium herbaceum* L.), a plant on which *S. frugiperda* can readily feed (Barros et al. 2010; Luginbill 1928; Sparks 1979), but to which it is not specifically adapted. *S. exigua* also readily feeds on cotton (Greenberg et al. 2001). In additional experiments, we compared HIPVs after the application of regurgitant to damaged leaves, using the regurgitant of three different *Spodoptera* species, to test for a

possible suppressive effect of *S. frugiperda* regurgitant. In a six-arm olfactometer, we also assessed the attractiveness of plant volatiles induced by *S. frugiperda* and *S. exigua* to the solitary koinobiont endoparasitoid *Cotesia marginiventris* Cresson (Hymenoptera: Braconidae), a very common parasitoid of *S. frugiperda* (Hoballah et al. 2004).

Overall, the results imply that *S. frugiperda* is capable of suppressing induced HIPV emissions in maize, but not in cotton. Although suppression of HIPVs did not result in a reduced attractiveness of maize plants to one of the insect's main, probably well adapted, parasitoids, it is likely to reduce the plant's defenses and in part explain the success of *S. frugiperda* as an important pest of maize.

Methods and Materials

Plants. Maize seeds (*Z. mays* ssp. *mays*, variety Delprim) were sown in plastic pots (4 cm diameter, 10 cm high) with fertilized commercial soil (Ricoter Aussaaterde, Aarberg, Switzerland). All plants were kept in a climate chamber (27 ± 2 °C; 60% relative humidity; 16 h light/8 h dark; 50.000 lm/m^2). At the beginning of each experiment, the maize plants were 9–12 days old, had a cotyledon, three fully developed leaves and a fourth one emerging from the whorl. Cotton seeds (*G. herbaceum*) were sown in the same plastic pots and were kept under similar conditions as the maize plants. After three weeks, the cotton plants were transplanted to larger pots. At the beginning of the experiments, the cotton plants were 6–8 weeks old, and had 5 fully developed leaves.

Insects *S. littoralis* eggs were provided by Syngenta (Stein, Switzerland). *S. frugiperda* eggs were provided by Bayer CropScience (Monheim, Germany) or were obtained from an in-house colony (Maag et al. 2014). *S. exigua* eggs were provided by Bayer CropScience or from Entomos (Grossdietwil, Switzerland). *H. armigera* eggs were provided by Bayer CropScience. All insect eggs were incubated at room temperature and larvae were reared on artificial diet until they had reached the second instar. Regurgitant was collected as described by Turlings et al. (1993). *C. marginiventris* wasps were reared as described by Turlings et al. (2004). Initial experiments were performed with all four caterpillar species, while additional experiments focused on the three *Spodoptera* species, or only on *S. frugiperda* and *S. exigua* specifically, the two most representative and co-occurring species. *H. armigera* was not included in further studies because its larvae did not feed well in most of our bioassays, causing notable discrepancies in sample sizes between treatments, which affects the reliability of statistical methods.

Detached Leaf Feeding Assays For an initial, quick assessment of the feeding habits of the four caterpillar species, we performed detached leaf feeding assays, similar to Rostás and Turlings (2008). A single second-instar larva of each species ($n = 8$) was weighed and placed in an individual box (2 × 2 cm) with a small piece of maize leaf. After 20 h of overnight feeding, the leaves were scanned into Adobe Photoshop CS2 version 9.0.2. Consumed leaf area was measured using NIH ImageJ software (http://rsb.info.nih.gov/ij/) as described previously (De Lange et al. 2018). Samples when the larvae did not feed were excluded from the analyses (1 sample for *H. armigera*).

Measuring Feeding Patterns For further comparisons and to allow from more replication we worked only with *S. frugiperda* and *S. exigua*. For a more biologically relevant assessment of the feeding habits of these species, we performed clip-cage assays on whole plants as described by Erb et al. (2011b). A single second-instar larva of either *S. exigua* or *S. frugiperda* ($n = 12$) was weighed and placed in a small clip-cage (surface 0.8 cm^2) on the youngest full-grown maize leaf. Larvae were allowed to feed for 6 h and were subsequently weighed again. Larval weight gain was calculated as the final minus the initial weight, and consumed leaf area was determined as described above. When visually characterizing the damaged leaf area, two types of damage could be distinguished: "windowpane" feeding, where the epidermis and mesophyll tissue of only one side of the leaf are ingested, and chewing holes (Erb et al. 2011b; Gouinguené et al. 2003). Consumed leaf area was attributed to each type of damage.

To determine whether differences in feeding patterns and/or differences in mouth parts explain the observed differences in consumed leaf area between *S. exigua* and *S. frugiperda*, we visually inspected feeding damage as well as larval mouth parts by means of scanning electron microscopy (SEM). Leaf material damaged by both species was fixed in a mix of 2% paraformaldehyde and 2.5% glutaraldehyde in a buffer of 0.1 M sodium cacodylate (pH 7.4). After washing the samples three times in the buffer, they were postfixed in a solution of 1% OsO_4 in buffer for 1 h, and then washed in the buffer three more times. Larvae of both species were fixed in 70% ethanol. Samples were dehydrated in a graded acetone series, critical-point-dried in CO_2, mounted on stubs, and coated with a thin gold layer by a sputter coater (SCD 005; Bal-Tec, Balzers, Liechtenstein). They were examined at 10 kV using a Philips XL-30 scanning electron microscope (FEI/Philips Electron Optics, Hillsboro, OR, USA) as described by Roelfstra et al. (2010) and Kessler et al. (2013).

Comparing the Induction of Volatile Emissions by Different Noctuid Caterpillars To assess whether feeding by four different caterpillar species induces different HIPV emissions, we conducted a series of volatile collection experiments. Maize

plants ($n = 12$) were placed in a volatile collection setup under experimental conditions as described previously (De Lange et al. 2016; Ton et al. 2007; Turlings et al. 2004). Infestation by *S. frugiperda*, *S. littoralis*, *S. exigua*, and *H. armigera* was achieved by releasing 4–6, 20–22, 15–16, and 35–37 larvae into the leaf whorl, respectively. The numbers of larvae were chosen to balance the amounts of damage that the larvae inflict (see Results section). After 12–14 h of feeding, volatiles were collected as described below. The larvae remained on the plants during the volatile collections. Control plants received no larvae. Trials in which one species of larvae fed obviously less than the others were excluded from analysis (8 trials for *H. armigera* and 4 trials for *S. frugiperda*). In several cases, the (*Z*)-3-hexenal peak coeluted with the bacterial volatile 2,3-butanediol (D'Alessandro et al. 2014). Therefore, this compound was not included in the total volatile emission data.

We conducted an additional volatile collection experiment with only *S. frugiperda* and *S. exigua*, two of the most common *Spodoptera* species on maize in the Americas (Blanco et al. 2014; Hernandez-Trejo et al. 2019; O'Day and Steffey 1998; Ortega 1987). This time we used equal numbers of caterpillars for both species. The ten second instar larvae per species were chosen such that the *S. frugiperda* larvae were somewhat smaller, but did equal amounts of damage during the 27 h feeding period. Larvae were weighed and damage was assessed as described above for the detached leaf feeding assays. Three-hour volatile collections started when the larvae had fed for 6 h and were repeated when the larvae had fed for 24 h ($n = 6$).

In a third volatile collection experiment, we compared the induction by *S. frugiperda* and *S. exigua* caterpillars on maize plants and cotton plants. Whereas *S. frugiperda* has been shown to tolerate and detoxify direct defense compounds specific to maize (Glauser et al. 2011; Wouters et al. 2014), there are no indications that it is specifically adapted to feed on cotton. Plants were infested with 4, 8, or 16 larvae of each species into the leaf whorl (maize, $n =$ 11–12 for each number of larvae) or onto fully developed leaves (cotton, $n = 6$–7 for each number of larvae). Larvae were left to feed for 16 h on maize plants, or for 48 h on cotton plants. The reason for this difference in timing is that in the case of maize the inducible volatiles are emitted within hours after the caterpillars start feeding (Turlings et al. 1998), whereas for cotton it takes at least a day (Loughrin et al. 1994). Control plants received no larvae. After volatile collections, performed as described below, leaves were detached and scanned and consumed area was measured for each leaf as described above.

Regurgitant Treatments To test if the larval oral secretions of the different noctuids play a role in the observed differences in HIPVs, we also conducted experiments with mechanically damaged plants that were treated with different caterpillar regurgitants (De Lange et al. 2016; Erb et al. 2009; Gouinguené et al. 2003; Ton et al. 2007). Maize plants ($n =$ 12–14) were individually placed in the glass volatile collection vessels after two leaves of each maize plant were damaged and treated with regurgitant of *H. armigera*, *S. frugiperda*. *S. littoralis*, or *S. exigua*, or wounding only. Wounding was inflicted by punching 26 small holes in two leaves at two different locations with a punching device, to damage a total surface of ~4 cm^2 (4 x ~1 cm^2). An amount of 10 μl pure regurgitant of each species was applied on the damaged surface. Wounding and regurgitant treatments took place 12–14 h before the start of volatile collections and were repeated ~1 h before the start of volatile collections. Collections were performed as described below.

A similar experiment was conducted where we only treated specific leaves (damaged plus regurgitant). This was done to test if differential preferences for leaves among the different species could explain the differences in HIPVs. This was also prompted by a recent paper that showed differences in defensive compounds among leaves of different ages in maize plants with three fully developed leaves (Köhler et al. 2015). Again, after damage and regurgitant treatment, maize plants ($n = 4$) were placed in the volatile collection vessels. Either the 2nd, 3rd, or 4th leaf of each maize plant was treated with regurgitant of *S. frugiperda*, *S. littoralis*, or *S. exigua*, or wounding only. In this case, wounding was inflicted with forceps, to damage a surface of ~2 cm^2 (Erb et al. 2015). An amount of 10 μl pure regurgitant of each species was applied on the damaged surface. Volatile collections started 2 h after treatment and were repeated 8 h after treatment.

Volatile Collections Volatiles were collected as described previously (De Lange et al. 2016; Ton et al. 2007; Turlings et al. 2004) using trapping filters containing 25 mg of 80–100 mesh Super Q adsorbent (Alltech Associates, Inc., Deerfield, IL, USA). For the supplementary collections with smaller *S. frugiperda* and larger *S. exigua* larvae and regurgitant bioassays comparing induction of different leaves we used filters with 25 mg of 80–100 mesh HayeSep Q adsorbent (Ohio Valley Specialty Co., Marietta, OH, USA). Volatile collections lasted 3 h. Before use, trapping filters were rinsed with 3 ml of dichloromethane; after each collection, they were eluted with 150 μl (Super Q filters) or 100 μl (HayeSep Q filters) of dichloromethane (Suprasolv, GC-grade; Merck, Dietikon, Switzerland). The samples were stored at −80 °C before analysis.

Analysis of the Volatiles Two internal standards (*n*-octane and nonyl acetate, each 200 ng in 10 μl dichloromethane; Sigma-Aldrich, Buchs, Switzerland) were added to each sample. Volatiles were analyzed with an Agilent 6850 gas chromatograph equipped with a flame ionization detector (GC-FID). A 3-μl aliquot of each sample was injected in pulsed splitless mode onto an apolar capillary column (HP-1 ms, 30 m,

0.25 mm ID, 0.25 μm film thickness; Agilent J&W Scientific, Santa Clara, CA, USA). Helium at constant pressure (18.71 psi) was used as carrier gas. After injection, the temperature was maintained at 40 °C for 3 min, then increased to 100 °C at 8 °C/min and subsequently to 200 °C at 5 °C/min, followed by a post-run of 3 min at 250 °C. The detected volatiles were normalized based on a comparison of their peak areas with those of the internal standards, and identified by comparison of retention times with those from previous analyses (D'Alessandro and Turlings 2005).

To confirm the identities of the different peaks, at least one odor sample per larval species was analyzed using a gas chromatograph (Agilent 6890 Series GC System G1530A) coupled to a mass spectrometer (GC-MS; Agilent 5973 Network Mass Selective Detector; transfer line 230 °C, source 230 °C, ionization potential 70 eV). An aliquot of 2 μl was injected in the pulsed splitless mode onto the same type of column as described above. Helium at constant flow (0.9 ml/min) was used as carrier gas. After injection, the column temperature was maintained at 40 °C for 3 min, and then increased to 100 °C at 8 °C/min and subsequently to 220 °C at 5 °C/min followed by a post-run of 3 min at 250 °C. The detected volatiles were identified by comparison of their mass spectra with those of the NIST05 library, by comparison of their spectra and retention times with those of authentic standards, and by comparison of their retention times with those from previous analyses (Loughrin et al. 1994; D'Alessandro and Turlings 2005; Ngumbi et al. 2009). Volatiles that met only one of these criteria were labelled as tentatively identified.

Six-arm Olfactometer Bioassays To assess a possible effect of the observed differences in HIPV emissions for the attraction of natural enemies, we measured the attractiveness of maize plants induced by *S. exigua* and *S. frugiperda* to one of their principal natural enemies, the parasitoid *C. marginiventris*. Maize plants ($n = 14$) were placed in glass vessels. Infestation by *S. frugiperda* and *S. exigua* caterpillars was achieved by releasing 4 and 16 larvae into the leaf whorl, respectively, which were left to feed overnight. The numbers of larvae were chosen to balance the amounts of damage that the larvae inflict (see Results section). Control plants received no larvae. Bioassays were performed as described previously (De Lange et al. 2016; Turlings et al. 2004). On randomized positions in every other arm, either a *S. frugiperda*-induced, a *S. exigua*-induced, or a control (non-induced) plant was placed. We used mated naïve two- to four-day-old female *C. marginiventris* wasps ($n = 288$ wasps with 14 exchanges of odor sources). They were released into the olfactometer in groups of 6 and per day 1–6 groups of wasps were tested. The wasps were given 30 min to make a choice and were thereafter removed in order to release a new group.

We performed a similar experiment with cotton plants, to which *S. frugiperda* are not specifically adapted. Bioassays with cotton plants ($n = 6$) were performed as described above, with a few modifications. Infestation by *S. frugiperda*, and *S. exigua* caterpillars was achieved by releasing 16 larvae of each species onto fully developed leaves, 48 h before the start of the bioassays. Control plants received no larvae. We used two- to four-day-old naïve mated female *C. marginiventris* wasps ($n = 216$ wasps with 6 exchanges of odor sources).

Statistical Analysis For data on larval weight, damage, and volatile emissions, differences between two treatments were analyzed using Student's t test. Differences between more than two treatments were analyzed using one-way analysis of variance (one-way ANOVA) when data were normally distributed, and Kruskal-Wallis test when data were not normally distributed. All significant effects were subjected to pairwise comparisons using Tukey or Dunn's post hoc tests. When necessary, percentage data were arcsine-square root-transformed, and volatile emission data were log-transformed, to improve normality and homogeneity of variance (non-transformed values are reported). Concerning plant volatiles, we analyzed total volatile emissions (i.e., the sum of normalized peak areas for all individual compounds), as well as emissions of individual compounds. For the latter, only herbivore-induced plants were included in the statistical analyses. Correlations between damage and volatile emissions were analyzed using linear regression, and one-way analysis of covariance (one-way ANCOVA) was conducted to determine differences in the slopes and/or intercepts of the linear regression lines. To compare feeding damage on different maize leaves, and volatile emissions when different maize leaves were damaged, we used two-way ANOVA with treatment and leaf number as factors. Wasp choice data were analyzed using a generalized linear model (GLM) fitted by maximum quasi-likelihood estimation according to Turlings et al. (2004). All analyses were performed with SigmaPlot version 13.0 (Systat Software, San Jose, CA, USA) and the software package R version 3.5.0 (R Core Team 2018).

Results

The Four Caterpillar Species Differ in Leaf Consumption Rate To compare feeding damage on maize by the four different herbivore species, we assessed the extent of damage after 20 h of feeding on a detached leaf by single second-instar larvae of each species. All larvae had a similar starting weight (*H. armigera*: 1.69 ± 0.005; *S. littoralis*: 1.68 ± 0.005; *S. exigua*: 1.69 ± 0.002; *S. frugiperda*: 1.68 ± 0.004; weight (mg) ± SE; *Kruskal-Wallis test,* $H = 0.93$, $df = 3$, $P = 0.82$). However, a *S. frugiperda* larva consumed significantly more leaf area than did a single larva of *S. littoralis*, *S. exigua* and *H. armigera* (*one-way ANOVA*, $F_{(3,27)} = 15.56$, $P < 0.001$; Fig. 1). Since wounding quantitatively influences HIPV emissions

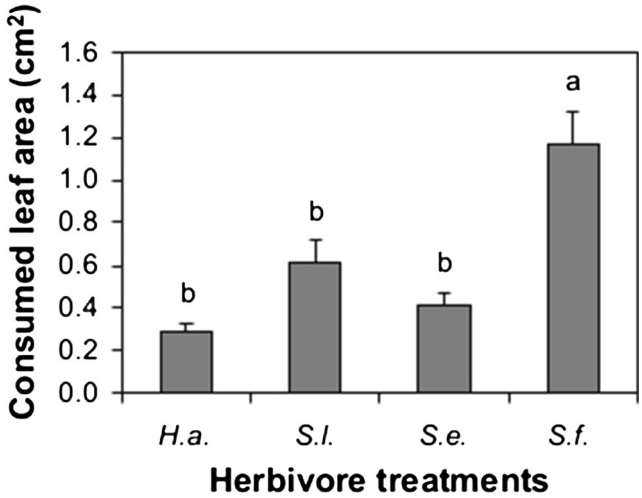

Fig. 1 Herbivory of different lepidopteran larvae on detached maize leaves. Values represent average amounts of leaf area consumption (± SE) ($n = 7$–8). Species: *Helicoverpa armigera* (*H.a.*), *Spodoptera littoralis* (*S.l.*), *Spodoptera exigua* (*S.e.*), and *Spodoptera frugiperda* (*S.f.*). Different letters indicate significant differences (*one-way ANOVA*, $P < 0.05$)

(Gouinguené et al. 2003; Turlings et al. 2004), it was necessary to correct for the observed differences in leaf damage. For this reason, we conducted further experiments with 20–22 *S. littoralis*, 15–17 *S. exigua*, 35–37 *H. armigera*, and 4–6 *S. frugiperda* larvae.

S. frugiperda Induces the Release of Lower Amounts of Volatiles than S. exigua, S. littoralis, and H. armigera All lepidopteran larvae induced a significant amount of volatiles compared to control, non-attacked maize plants, but *S. frugiperda* larvae induced considerably lower amounts of HIPVs than larvae of the other three species (*one-way ANOVA*, $F_{(4,43)} = 93.05$, $P < 0.001$; Fig. 2). Statistical tests

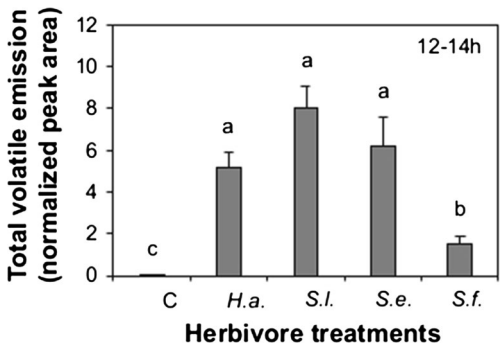

Fig. 2 Volatile emissions of maize plants infested with different lepidopteran larvae. Values represent average total amounts of volatiles (± SE), i.e. the sum of normalized peak areas for all individual compounds ($n = 4$–12). Treatments: Control (C), feeding by *Helicoverpa armigera* (*H.a.*), *Spodoptera littoralis* (*S.l.*), *Spodoptera exigua* (*S.e.*), or *Spodoptera frugiperda* (*S.f.*). Volatiles were collected after 12-14 h of feeding. Because of coelution with another compound, (*Z*)-3-hexenal was not included in the total volatile emission data. Different letters indicate significant differences (*one-way ANOVA*, $P < 0.05$)

for emissions of individual compounds were performed on data for herbivore-induced plants only (not for control plants). *S. frugiperda* feeding triggered lower emissions of the green leafy volatiles (GLVs) (*Z*)-3-hexenyl acetate and (*E*)-2-hexenyl acetate than feeding by *S. littoralis* and *S. exigua*. Most monoterpenes, sesquiterpenes, and esters were also emitted in lower quantities in response to feeding by *S. frugiperda* than in response to feeding by *S. littoralis* and *S. exigua* (Table 1).

An additional volatile collection experiment with only *S. frugiperda* and *S. exigua*, in which we used equal numbers of caterpillars (10 per plant), yielded very similar results. The *S. frugiperda* larvae were smaller at the beginning of the experiment (*S. exigua*: 2.52 ± 0.080; *S. frugiperda*: 1.53 ± 0.048; weight (mg) ± SE; *t-test*, $t = 9.07$, $df = 5$, $P < 0.001$), but since they showed a higher feeding rate, the two species inflicted equal amounts of damage (*S. exigua*: 398.1 ± 59.9; *S. frugiperda*: 336.5 ± 28.4; damage (mm^2) ± SE; *t-test*, $t = 1.26$, $df = 5$, $P = 0.26$). After 6 h, both larvae induced a significant amount of volatiles compared to control, non-attacked maize plants, but there were no significant differences in total volatile emissions between the two species (*one-way ANOVA*, $F_{(2,15)} = 67.93$, $P < 0.001$; Fig. 3a). After 24 h, total volatile emissions were lower for *S. frugiperda*-damaged plants than for *S. exigua*-damaged plants (*one-way ANOVA*, $F_{(2,15)} = 223.32$, $P < 0.001$; Fig. 3b). Again, statistical tests for emissions of individual compounds were performed on data for herbivore-induced plants only (not for control plants). These results show that after 6 h, several GLVs as well as (*Z*)-β-ocimene, (3*E*)-4,8-dimethyl-1,3,7-nonatriene, and geranyl acetate were released in lower quantities by *S. frugiperda*-damaged plants than by *S. exigua*-damaged plants. After 24 h, most of the inducible compounds were released in lower quantities by *S. frugiperda*-damaged plants, but not the GLVs (Table 2). These discrepant differences in GLV emissions for the two time points could be due to the initial size differences between the larvae, with the smaller *S. frugiperda* causing less physical damage at the beginning of the experiment, resulting in lesser amounts of GLVs being released.

S. frugiperda Induces Lower Amounts of HIPVs than S. exigua in Maize but not in Cotton To examine the relationship between herbivory and HIPV emissions in further detail, we correlated inflicted damage on maize plants with HIPV emissions upon feeding by *S. frugiperda* and *S. exigua*. Plant HIPV emissions increased steadily with increasing amounts of consumed leaf area for both *S. exigua* (*linear regression*, $R^2 = 0.48$, $F_{(1,33)} = 30.10$, $P < 0.001$) and *S. frugiperda* (*linear regression*, $R^2 = 0.41$, $F_{(1,34)} = 23.47$, $P < 0.001$). However, the slopes of the regression lines were significantly different (*one-way ANCOVA*, $F_{(1,67)} =$

Table 1 Individual volatiles emitted by herbivore-induced maize plants

No.	Compound	Class	C	*H.a.*	*S.l.*	*S.e.*	*S.f.*	P
1	(*Z*)-3-hexenal*	GLV	10.1 ± 3.6	189.9 ± 88.2	193.1 ± 33.8	243.5 ± 50.2	115.7 ± 28.3	NA
2	(*E*)-2-hexenal	GLV	0 ± 0	40.3 ± 13.6ab	162.3 ± 50.5a	158.1 ± 51.2ab	28.8 ± 12.6b	**0.02**
3	(*Z*)-3-hexen-1-ol	GLV	0 ± 0	1.2 ± 1.2	8.4 ± 3.3	5.6 ± 2.0	0 ± 0	***0.04***
4	β-myrcene	Monoterpenes	4.9 ± 1.3	22.6 ± 1.7ab	28.9 ± 2.4a	22.6 ± 2.7ab	11.3 ± 1.7b	**<0.001**
5	(*Z*)-3-hexenyl acetate	GLV	0 ± 0	289.9 ± 113.6a	197.5 ± 34.9a	200.7 ± 42.6a	44.4 ± 7.1b	**0.009**
6	(*E*)-2-hexenyl acetate[N]	GLV	0 ± 0	10.1 ± 4.8ab	34.7 ± 12.3a	24.8 ± 7.7a	1.9 ± 1.3b	**0.01**
7	(*Z*)-β-ocimene	Monoterpenes	0 ± 0	7.5 ± 3.1ab	14.7 ± 2.4a	13.7 ± 2.4a	2.2 ± 1.5b	**0.004**
8	linalool	Monoterpenes	53.4 ± 9.1	322.3 ± 35.2ab	580.7 ± 45.4a	568.3 ± 88.5a	166.8 ± 33.8b	**<0.001**
9	(3*E*)-4,8-dimethyl-1,3,7-nonatriene	Homoterpenes	0 ± 0	386.2 ± 57.5ab	505.5 ± 72.5a	402.6 ± 78.1a	102.3 ± 36.1b	**0.003**
10	phenylmethyl acetate[N]	Esters	0 ± 0	17.8 ± 2.0ab	32.1 ± 5.8a	37.1 ± 8.2a	5.4 ± 2.8b	**0.003**
11	2-phenylethyl acetate	Esters	0 ± 0	25.3 ± 6.8ab	56.9 ± 8.6a	54.5 ± 12.5a	14.5 ± 5.5b	**0.02**
12	indole	Aromatics	1.8 ± 1.2	677.9 ± 245.1ab	938.4 ± 130.2a	723.2 ± 172.7ab	234.2 ± 60.1b	**0.02**
13	methyl anthranilate	Aromatics	0 ± 0	53.4 ± 36.6	45.0 ± 10.9	41.1 ± 13.0	16.8 ± 8.5	0.36
14	geranyl acetate	Monoterpenes	0 ± 0	169.9 ± 26.4ab	307.9 ± 45.4a	242.2 ± 50.0a	58.3 ± 21.3b	**0.001**
15	(*E*)-β-caryophyllene	Sesquiterpenes	4.3 ± 4.3	110.5 ± 14.8ab	366.1 ± 63.2a	215.1 ± 58.6ab	74.3 ± 28.9b	**0.002**
16	(*E*)-α-bergamotene	Sesquiterpenes	0 ± 0	888.3 ± 99.7ab	1405.3 ± 210.0a	1067.4 ± 259.9a	217.2 ± 69.5b	**0.002**
17	(*E*)-β-farnesene	Sesquiterpenes	0 ± 0	1962.6 ± 234.8ab	3043.6 ± 476.2a	2232.9 ± 555.1ab	460.8 ± 147.6b	**0.002**
18	β-sesquiphellandrene[N]	Sesquiterpenes	0 ± 0	113.9 ± 12.3ab	190.5 ± 34.4a	137.7 ± 36.4a	25.0 ± 9.5b	**0.002**
19	(*E*)-nerolidol	Sesquiterpenes	0 ± 0	65.1 ± 6.1a	67.4 ± 14.3a	46.6 ± 10.2ab	11.3 ± 7.4b	**0.007**
20	(3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	Homoterpenes	0 ± 0	21.6 ± 5.6	33.2 ± 7.7	32.7 ± 7.8	9.3 ± 4.7	0.12

Values represent means ± SE in normalized peak area. Treatments: Control (C), feeding by *Helicoverpa armigera* (*H.a.*), *Spodoptera littoralis* (*S.l.*), *Spodoptera exigua* (*S.e.*) or *Spodoptera frugiperda* (*S.f.*) larvae. [N] = tentative identification. * = Because of coelution with another compound, this compound was not included in statistical analyses. $N = 4–12$. NA = not applicable. GLV = green leaf volatiles. Numbers in bold and different letters indicate significant differences (*one-way ANOVA* or *Kruskal-Wallis test*, $P < 0.05$). Numbers in italics indicate the post hoc test did not reveal significant differences. Only herbivore-induced plants were included in statistical analyses

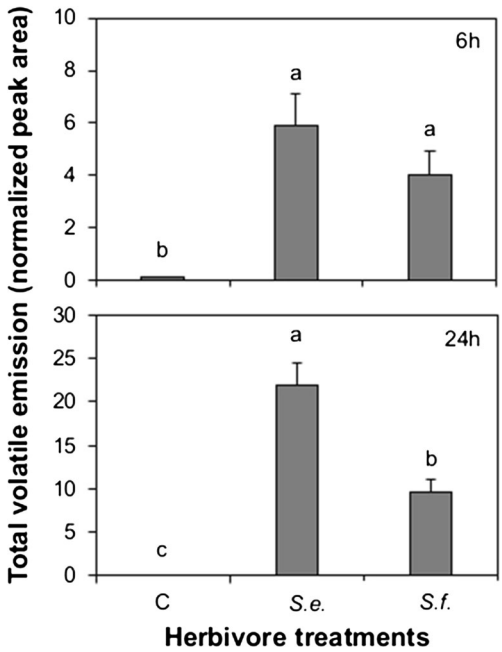

Fig. 3 Volatile emissions of maize plants 6 h (a) and 24 h (b) after infestation with lepidopteran larvae. Values represent the average total amounts of volatiles (± SE), i.e. the sum of normalized peak areas for all individual compounds ($n = 6$). Treatments: feeding by *Spodoptera exigua* (*S.e.*) or *Spodoptera frugiperda* (*S.f.*). At the start of the experiment, *S. frugiperda* larvae were smaller than *S. exigua* larvae, so that the two species inflicted equal amounts of damage. Different letters indicate significant differences (*one-way ANOVA*, $P < 0.05$)

7.80, $P = 0.007$), confirming that *S. frugiperda* induced lower amounts of HIPVs per unit of leaf damage than *S. exigua* (Fig. 4a). We also observed that the different lepidopteran species preferred to feed on different maize leaves (*two-way ANOVA*, treatment: $F_{(1,276)} = 0.01$, $P = 0.91$, leaf: $F_{(3,276)} = 29.01$, $P < 0.001$, interaction: $F_{(3,276)} = 13.93$, $P < 0.001$) (Supplementary Fig. 1). This prompted us to perform an additional experiment, in which we assessed HIPV emissions after treating leaves of different ages (see below).

When performing a similar experiment with cotton plants, on which *S. frugiperda* is not specialized, there was also an increase of HIPV emissions with increased damage for both *S. exigua* (*linear regression*, $R^2 = 0.37$, $F_{(1,16)} = 9.23$, $P = 0.008$) and *S. frugiperda* (*linear regression*, $R^2 = 0.69$, $F_{(1,19)} = 41.35$, $P < 0.001$). For cotton, the slopes of the regression lines did not differ (*one-way ANCOVA*, $F_{(1,35)} = 0.90$, $P = 0.35$), nor did the intercepts (*one-way ANCOVA*, $F_{(1,36)} = 0.16$, $P = 0.69$), implying that *S. exigua* and *S. frugiperda* induced similar amounts of HIPVs per unit of leaf damage (Fig. 4b). These results provide further evidence that *S. frugiperda* is capable of specifically suppressing HIPV emissions in maize.

The Regurgitants of Different Spodoptera Species Trigger Different Amounts of HIPVs Our observation that *S. frugiperda* and *S. exigua* prefer to feed on different maize leaves, prompted us to test if induction of different leaves resulted in the release of different amounts of HIPVs. Therefore, we compared total HIPV emissions after standardized regurgitant treatment of different leaves, using regurgitant from all three *Spodoptera* species. Two hours after treatment, *S. frugiperda* regurgitant resulted in the release of significantly lower total amounts of volatiles than regurgitant of the other species, independent of the leaf that was treated. Overall, *S. exigua* regurgitant induced the highest total quantity of HIPVs, which was significantly higher than in response to wounding only. Treatment with *S. littoralis* regurgitant did not affect HIPV emissions, as it was the same as wounding only, and, interestingly, plants treated with *S. frugiperda* regurgitant released even less HIPVs than the plants with only wounding (*two-way ANOVA*, treatment: $F_{(3,36)} = 45.18$, $P < 0.001$, leaf: $F_{(2,36)} = 0.90$, $P = 0.42$, interaction: $F_{(6,36)} = 0.61$, $P = 0.72$) (Fig. 5a). Eight hours after treatment, the leaves that were treated with *S. frugiperda* regurgitant still released considerably less HIPVs than those treated with the regurgitant of the other two *Spodoptera* species. Again, induction with *S. exigua* regurgitant increased HIPV emissions the most and treatment with *S. littoralis* regurgitant was intermediate, but not different from wounding only (*two-way ANOVA*, treatment: $F_{(3,36)} = 13.11$, $P < 0.001$, leaf: $F_{(2,36)} = 0.78$, $P = 0.47$, interaction: $F_{(6,36)} = 1.55$, $P = 0.19$) (Fig. 5b). Clearly, these results indicate that the three leaves responded similarly, but that caterpillar regurgitant affected the volatile emissions quite differently. Note that control, non-treated plants were not included in this experiment.

We also conducted an experiment in which we punched 26 tiny holes in two of the leaves and treated the leaves with regurgitant of all four different caterpillar species, 12-14 h before HIPV collections. Treatments were repeated ~1 h before HIPV collections. In this case, we only found significant differences in volatile emissions between wounding only and regurgitant of the four species (*one-way ANOVA*, $F_{(4,57)} = 10.57$, $P < 0.001$) (Supplementary Fig. 2). The absence of HIPV suppression may be due to the low amount of inflicted damage, or the time points at which HIPV emissions were measured in this experiment.

No Differences between S. frugiperda and S. exigua Feeding at the Microscale To study the feeding behavior of *S. frugiperda* and *S. exigua* on maize plants in further detail, we observed the mouth parts of both species as well as leaf tissue damaged by both species under the SEM. At microscale, second-instar *S. frugiperda* (Fig. 6a) and *S. exigua* (Fig. 6b) larvae looked strikingly similar. For both species,

Table 2 Individual volatiles emitted by maize plants, 6 h and 24 h after herbivore induction

No.	Compound	Class	6 h				24 h			
			C	*S.e.*	*S.f.*	P	C	*S.e.*	*S.f.*	P
1	(*Z*)-3-hexenal	GLV	0 ± 0.0	180.9 ± 35.9	52.4 ± 20.4	**0.01**	0 ± 0.0	184.9 ± 40.3	204.6 ± 36.7	0.73
2	(*E*)-2-hexenal	GLV	0 ± 0.0	72.0 ± 12.4	33.9 ± 14.0	0.07	0 ± 0.0	166.5 ± 58.3	182.2 ± 40.2	0.83
3	(*Z*)-3-hexen-1-ol	GLV	0 ± 0.0	27.6 ± 4.2	4.7 ± 2.4	**<0.001**	0 ± 0.0	56.8 ± 22.2	20.1 ± 10.3	0.17
4	β-myrcene	Monoterpenes	1.9 ± 0.6	32.5 ± 6.7	13.2 ± 2.7	**0.02**	1.8 ± 1.0	59.7 ± 8.0	30.4 ± 3.5	**0.007**
5	(*Z*)-3-hexenyl acetate	GLV	0 ± 0.0	201.3 ± 40.1	92.8 ± 12.6	**0.03**	0 ± 0.0	557.7 ± 142.1	398.6 ± 87.2	0.36
6	(*E*)-2-hexenyl acetate[N]	GLV	0 ± 0.0	16.7 ± 4.7	8.4 ± 3.3	0.18	0 ± 0.0	111.3 ± 52.4	73.0 ± 24.0	0.52
7	(*Z*)-β-ocimene	Monoterpenes	0 ± 0.0	18.5 ± 5.0	6.1 ± 1.2	**0.04**	0 ± 0.0	17.2 ± 2.1	9.0 ± 1.6	**0.01**
8	linalool	Monoterpenes	80.1 ± 15.0	871.2 ± 151.1	684.6 ± 147.1	0.40	83.1 ± 14.1	1574.8 ± 206.8	1121.6 ± 129.2	0.09
9	(3*E*)-4,8-dimethyl-1,3,7-nonatriene	Homoterpenes	6.9 ± 3.1	556.0 ± 113.0	258.7 ± 59.0	**0.04**	10.7 ± 4.0	1090.1 ± 145.3	465.2 ± 66.9	**0.009**
10	phenylmethyl acetate[N]	Esters	0 ± 0.0	19.0 ± 5.3	6.4 ± 2.4	0.055	0 ± 0.0	55.3 ± 11.4	29.2 ± 8.4	0.09
11	2-phenylethyl acetate	Esters	0 ± 0.0	39.6 ± 10.2	32.3 ± 10.5	0.63	0 ± 0.0	106.0 ± 16.7	73.4 ± 22.3	0.27
12	indole	Aromatics	0 ± 0.0	561.9 ± 126.4	596.6 ± 179.3	0.88	0 ± 0.0	2319.6 ± 297.0	1189.7 ± 205.8	**0.01**
13	methyl anthranilate	Aromatics	0 ± 0.0	13.2 ± 4.4	39.9 ± 19.9	0.09	0 ± 0.0	32.0 ± 2.5	38.9 ± 15.9	0.39
14	geranyl acetate	Monoterpenes	0.4 ± 0.4	252.8 ± 60.9	88.2 ± 30.3	**0.04**	0.9 ± 0.9	514.7 ± 61.2	256.4 ± 34.1	**0.004**
15	(*E*)-β-caryophyllene	Sesquiterpenes	0 ± 0.0	149.7 ± 45.2	119.3 ± 32.7	0.60	10.1 ± 9.2	2648.1 ± 325.3	372.6 ± 74.2	**0.002**
16	(*E*)-α-bergamotene	Sesquiterpenes	2.8 ± 2.8	968.6 ± 218.4	647.1 ± 165.1	0.27	19.3 ± 17.6	3946.8 ± 479.2	1819.4 ± 266.7	**0.003**
17	(*E*)-β-farnesene	Sesquiterpenes	0 ± 0.0	1774.4 ± 397.9	1204.6 ± 293.1	0.28	0 ± 0.0	7414.6 ± 832.8	3082.8 ± 417.9	**<0.001**
18	β-sesquiphellandrene[N]	Sesquiterpenes	0 ± 0.0	96.0 ± 24.5	69.5 ± 23.8	0.46	2.1 ± 2.1	694.7 ± 98.2	260.8 ± 42.6	**0.002**
19	(*E*)-α-farnesene	Sesquiterpenes	0 ± 0.0	8.3 ± 3.0	5.9 ± 4.3	0.49	0 ± 0.0	288.4 ± 68.8	15.3 ± 3.8	**0.002**
20	(3*E*, 7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	Homoterpenes	0 ± 0.0	3.6 ± 1.2	8.3 ± 6.3	0.94	0 ± 0.0	131.5 ± 25.5	67.8 ± 11.2	**0.045**

Values represent means ± SE in normalized peak area. Treatments: Control (C), feeding by *Spodoptera exigua* (*S.e.*) or *Spodoptera frugiperda* (*S.f.*) larvae. [N] = tentative identification. $N = 6$. GLV = green leaf volatiles. At the start of the experiment, *S. frugiperda* larvae were smaller than *S. exigua* larvae, so that the two species inflicted equal amounts of damage. Numbers in bold indicate significant differences (*t-test*, $P < 0.05$). Only herbivore-induced plants were included in statistical analyses

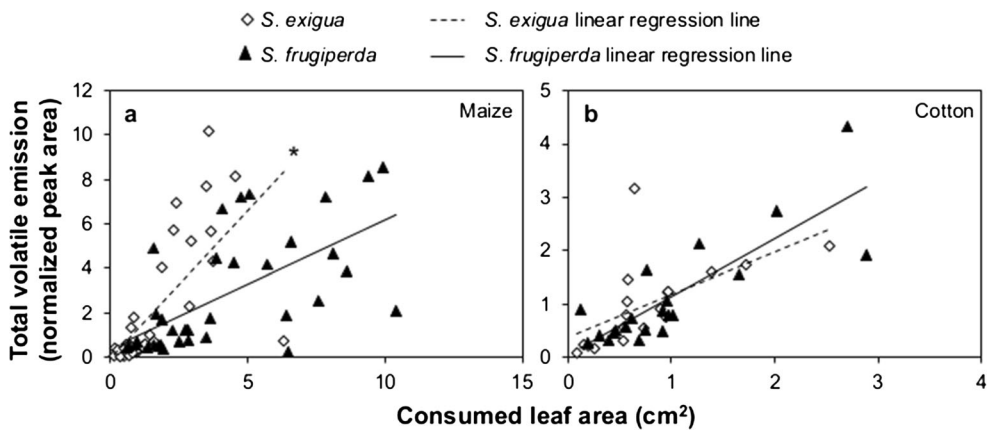

Fig. 4 Correlation between herbivore-inflicted damage and total volatile emissions in maize (a) and cotton (b). Open diamonds represent *Spodoptera exigua* and filled triangles represent *Spodoptera frugiperda*. The dashed line represents the linear regression line for *S. exigua* (maize: $R^2 = 0.48$; cotton: $R^2 = 0.37$) and the solid line represents the linear regression line for *S. frugiperda* (maize: $R^2 = 0.41$; cotton: $R^2 = 0.69$). For maize, $n = 35$–36 and for cotton, $n = 18$–21. For both *S. frugiperda* and *S. exigua*, on both maize and cotton, there was a positive linear relationship between amount of damage and volatile emissions (*linear regression*, $P < 0.005$). An asterisk indicates significant differences between the slopes of the linear regression lines (*one-way ANCOVA*, $P < 0.05$)

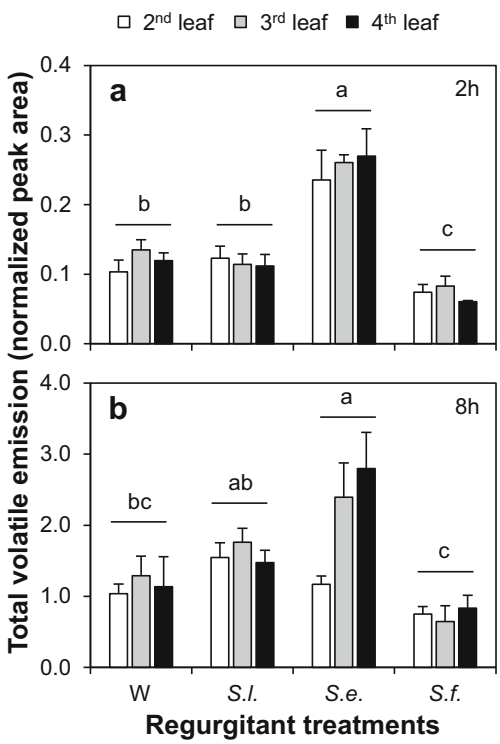

Fig. 5 Volatile emissions of maize plants 2 h (a) and 8 h (b) after different leaves were treated with larval regurgitant. Values represent the average total amounts of volatiles (± SE), i.e. the sum of normalized peak areas for all individual compounds ($n = 4$). Treatments: Wounding only (W), regurgitant application of *Spodoptera littoralis* (*S.l.*), *Spodoptera exigua* (*S.e.*), or *Spodoptera frugiperda* (*S.f.*). Wounding was inflicted with forceps. Different letters indicate significant differences between regurgitant treatments, represented by the line above the bars (*two-way ANOVA*, $P < 0.05$). There were no significant differences between the different leaves (*two-way ANOVA*, $P > 0.05$)

we could observe windowpane feeding, where larvae consume the epidermis and mesophyll from one side of the leaf, while leaving the cuticle and the epidermis of the other side of the leaf intact (Fig. 6c,d).

S. frugiperda Takes Larger Bites than S. exigua To further study the feeding damage, we compared larval growth and leaf area eaten on maize plants by *S. frugiperda* and *S. exigua* in a clip-cage. While all larvae had the same starting weight (*S. exigua*: 0.72 ± 0.037; *S. frugiperda*: 0.72 ± 0.032; weight (mg) ± SE; *t-test*, $t = 0.02$, $df = 22$, $P = 0.98$) after feeding for 6 h, *S. frugiperda* gained significantly more weight than *S. exigua* larvae (*t-test*, $t = 6.46$, $df = 22$, $P < 0.001$; Fig. 7a). Furthermore, *S. frugiperda* consumed significantly more leaf area than *S. exigua* (*t-test*, $t = 5.31$, $df = 22$, $P < 0.001$; Fig. 7b). When distinguishing two types of damage, *S. frugiperda* chewed relatively more holes, and inflicted relatively less windowpane damage than *S. exigua* (*t-test*, $t = 3.33$, $df = 22$, $P = 0.003$) (Fig. 7c–e). These results suggest that *S. frugiperda* may have a stealthier way of feeding, avoiding the activation of plant defenses by reducing the number of damaged cells.

No Difference in Wasp Attractiveness of Maize Plants Damaged by S. frugiperda or S. exigua A possible ecological relevance of HIPV suppression by *S. frugiperda* was studied by comparing attraction of *C. marginiventris* parasitoids to HIPVs induced by similar amounts of leaf damage incurred by *S. exigua* and *S. frugiperda* larvae. The wasps strongly preferred the odor of herbivore-induced maize plants over the odor of non-induced plants (control) and empty arms, but did not show a preference for either *S.*

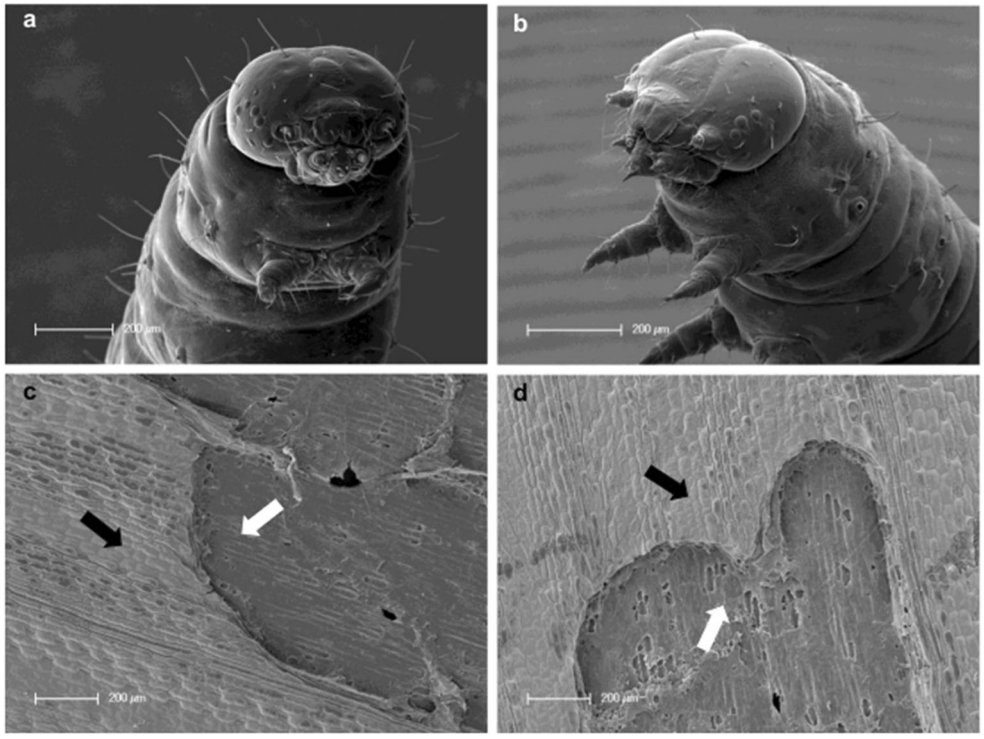

Fig. 6 Scanning electron microscopy (SEM) images of *Spodoptera* larvae and the damage they inflict on maize plants. (a) Second-instar *Spodoptera frugiperda* larva. (b) Second-instar *Spodoptera exigua* larva. (c) Damage inflicted by *S. frugiperda*. (d) Damage inflicted by *S. exigua*. Black arrows indicate undamaged leaf tissue, while white arrows indicate damaged leaf tissue. The larvae inflict so-called windowpane damage, consuming the epidermis and mesophyll from one side of the leaf, while leaving the cuticle and the epidermis of the other side of the leaf intact

exigua- or *S. frugiperda*-attacked plants (*GLM*, $F_{(3,284)} = 22.20$, $P < 0.001$; Fig. 8a). These results imply that the attraction of *C. marginiventris*, a very common parasitoid of *S. frugiperda*, is not affected by *S. frugiperda*'s capacity to suppress maize HIPV emissions.

No Difference in Wasp Attractiveness of Cotton Plants Damaged by S. frugiperda or S. exigua We also compared the attractiveness of cotton HIPVs to *C. marginiventris* parasitoids between plants that were damaged by *S. exigua* or *S. frugiperda* larvae. Again, the wasps preferred the odor of herbi-

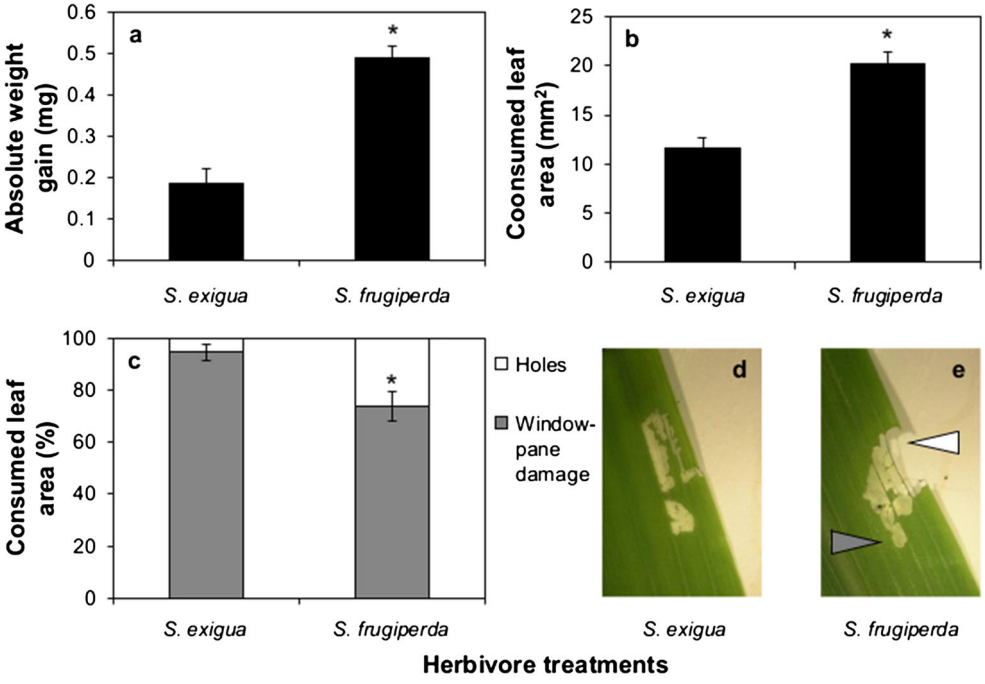

Fig. 7 Weight gain and feeding damage of *Spodoptera frugiperda* and *Spodoptera exigua* larvae on maize plants. (a) Absolute weight gain (± SE) of the larvae after feeding for 6 h in a small clip-cage. (b) Total amount of damage (± SE) inflicted by the larvae. (c) Different types of feeding damage (± SE). For all measurements, $n = 12$. (d) A representative example of feeding damage of *S. exigua*. (e) A representative example of feeding damage of *S. frugiperda*. Two types of feeding damage were distinguished: grey bars and arrows indicate windowpane feeding while white bars and arrows indicate chewing holes. An asterisk indicates significant differences (*t-test*, $P < 0.05$)

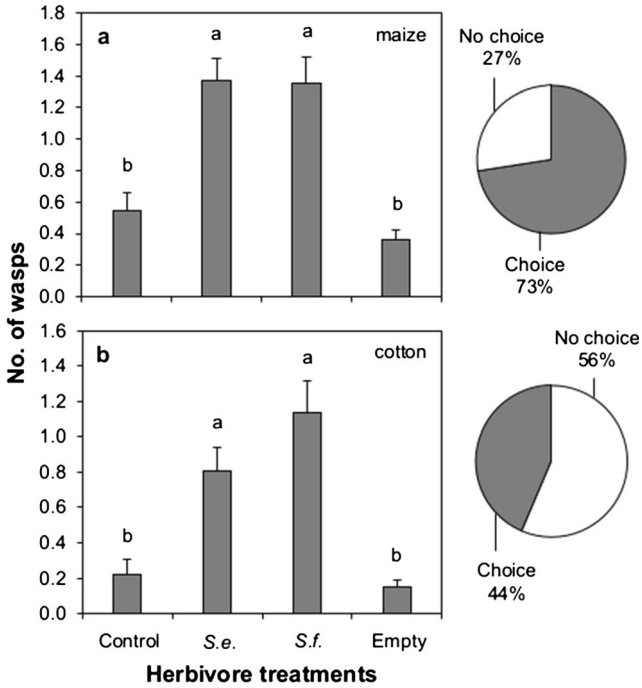

Fig. 8 Responsiveness of naïve female *Cotesia marginiventris* parasitoid wasps to volatiles of *Spodoptera exigua* (*S.e.*)- and *Spodoptera frugiperda* (*S.f.*)-induced maize (a) and cotton (b) plants in a six-arm olfactometer. Values represent the average number of wasps per release of 6 wasps (± SE). Control: non-induced plants. Empty: empty vessels (average value of three vessels). The pie chart indicates the proportion of wasps choosing an arm. For (a), $n = 288$ wasps with 14 exchanges of odor sources. For (b), $n = 216$ wasps with 6 exchanges of odor sources. Different letters indicate significant differences (*GLM*, $P < 0.05$)

vore-induced plants over non-induced plants (control) and empty arms, but showed no significant difference in their choices for *S. exigua*- and *S. frugiperda*-damaged plants (*GLM*, $F_{(3,212)} = 19.93$, $P < 0.001$; Fig. 8b).

Discussion

This study confirms that *S. frugiperda* larvae are capable of specifically suppressing herbivore-induced volatiles in maize. This suppression is associated with lower elicitation activity of the regurgitant and differences in leaf damage patterns. The plant's attractiveness to a common parasitoid wasp does not seem to be affected by this HIPV suppression, however, suggesting that parasitoids can overcome plant defense manipulation by *S. frugiperda*.

The exact mechanism behind the observed suppression remains to be elucidated, but we provide evidence that it involves compounds present in the insect's regurgitant (Fig. 5). Sarmento et al. (2011) found something similar for the spider mite *T. evansi*, which suppresses HIPV emissions in tomato compared to *T. urticae* Koch, yet the predatory mite *P. longipes* did not distinguish between plants induced by either spider mite species. Effector-like proteins in the saliva of both spider mite species were shown to suppress defenses when expressed in *Nicotiana benthamiana* (Villarroel et al. 2016). Putative defense suppression activity has also been reported for the regurgitant of *S. exigua* and *S. frugiperda*, as the regurgitants of both species have been shown to suppress GLV emissions in ground maize tissue (Jones et al. 2019). *S. exigua* regurgitant reportedly decreased transcript levels of terpene-related genes in *M. truncatula* (Bede et al. 2006). It has also been shown that *S. frugiperda* regurgitant contains bacteria that can downregulate the activity of two defensive proteins in tomato (Acevedo et al. 2017a). *S. frugiperda*, *S. exigua*, and *S. littoralis* regurgitant all contain volicitin, which induces HIPV emissions in maize (Alborn et al. 1997; Spiteller et al. 2001; Turlings et al. 2000). It is possible that the levels of volicitin and volicitin-related compounds in the regurgitant of the three species is different, as has been reported for other lepidopteran species (Mori et al. 2003). Volicitin does not induce HIPV release in lima bean (*Phaseolus lunatus*), cotton (*Gossypium hirsutum*), or cowpea (*Vigna unguiculata*) (Schmelz et al. 2009; Spiteller et al. 2001), indicating that the effects of elicitors, and possibly also suppressors, is host plant-specific (Louis et al. 2013). Our results imply that, in addition to elicitors, *S. frugiperda* regurgitant contains effectors that are specifically active in maize. Alternatively, *S. frugiperda* regurgitant may contain lower levels of elicitors than the regurgitant of the other tested lepidopteran species.

A recent study showed that protein content in *S. frugiperda* regurgitant differs depending on insect diet (Acevedo et al. 2017b). In fact, two *S. frugiperda* strains occur, a "corn strain" associated with maize and cotton (*Gossypium* spp.), and a "rice strain" associated with rice (*Oryza sativa*). Individuals of both strains displayed differential gene expression when fed on the same diet, indicating alimentary divergence and possible specialization (Roy et al. 2016). Regurgitant of the corn strain suppresses the activity of a defensive protein in Bermuda grass (*Cynodon dactylon*), but not in maize, whereas the regurgitant of the rice strain induces the activity of defensive proteins in both plants. Larvae seem to benefit from plant defense suppression, as lower levels of defensive protein activity were correlated with higher weight gain. Interestingly, the authors propose that changes in larval saliva content could lead to adaptation to novel food sources (Acevedo et al. 2018). Suppressing factors in *S. frugiperda* regurgitant may contribute to its status as a major pest in maize, and its rapid invasion in Africa and Asia, which is currently taking place (Day et al. 2017; Stokstad 2017; Nagoshi et al. 2019).

Our experiments focused on HIPV emissions, and revealed that *S. exigua* regurgitant strongly induces HIPVs, while *S. frugiperda* regurgitant represses the emissions compared to wounding alone (Fig. 5). The relatively low HIPV amounts emitted by maize plants treated with *S. frugiperda* regurgitant is in line with the findings by Turlings et al. (1993). When they

incubated excised maize seedlings in diluted regurgitant of different lepidopteran species, the regurgitant of *S. frugiperda* was one of the least active. Another, more recent, study showed that *S. frugiperda* regurgitant induces the release of HIPVs in maize, but there were significant differences between the two maize varieties that were tested (Block et al. 2018). A possible explanation for the discrepancies between the studies is that different maize varieties were used, and it is known that there is a high level of variability in defense responses in different plant genotypes (Degen et al. 2004; De Lange et al. 2019; Erb et al. 2011a). Schmelz et al. (2009) found that the elicitor volicitin does not induce volatiles in all maize varieties, indicating that the effects of elicitors, and possibly also suppressors, may be genotype specific. The type of wounding and exposure to regurgitant may also make a difference. When we used a different method to wound the plants, and volatiles were collected 12–14 h after treatment (which was repeated 1 h before collections), rather than after 2 and 8 h, the application of *S. frugiperda*, *S. littoralis*, *S. exigua*, and *H. armigera* regurgitant induced very similar amounts of HIPVs in maize plants, and the emissions were significantly higher than for wounding alone (Supplementary Fig. 2). It is, therefore, possible that defense suppression properties of the regurgitant change with time. Alternatively, defense suppression may result from interactions between wound-derived and herbivore-derived molecular patterns, resulting in different outcomes depending on the method used for wounding and application of oral secretions. Future studies on the oral secretions of *S. frugiperda* larvae should determine if possible effectors from their saliva (Musser et al. 2006) or other compounds in their regurgitant are responsible for the suppression of maize HIPVs. Future studies should also include other plant species, to reveal whether *S. frugiperda*'s suppressive ability is truly limited to maize.

Besides differences in herbivore-derived elicitors, it could also be that the observed variations in HIPV quantities are due to distinct feeding behaviors that lead to differences in the type of damage caused by the lepidopteran species. Two experiments showed that *S. frugiperda* reduced emissions of monoterpenes, homoterpenes, sesquiterpenes, aromatics, and esters, compared to *S. exigua* feeding, but there were no consistent reductions in emissions of GLVs (Tables 1, 2), except in the early collection (after 6 h) of the second experiment, when the smaller *S. frugiperda* probably had inflicted less damage than the *S. exigua* larvae. That GLVs can be subject to manipulation by insects was shown by Allman and colleagues, who found isomeric rearrangement of GLVs by caterpillars (Allmann and Baldwin, 2010; Allmann et al. 2013). Moreover, Jones et al. (2019) found that caterpillar regurgitant, including that of *S. frugiperda* and *S. exigua*, can suppress the emission of GLVs in ground maize tissue. These studies suggest that GLVs are particularly important for plant defense and that it is worthwhile to further explore how and why caterpillars have evolved to reduce their emissions (Jones et al. 2019).

The fact that *S. frugiperda*-infested and *S. exigua*-infested maize plants were equally attractive to *C. marginiventris* wasps suggests that, at least in the case of this parasitoid that frequently parasitizes *S. frugiperda*, its larvae do not benefit from their ability to suppress HIPV induction (Fig. 8a). *S. frugiperda*-infested and *S. exigua*-infested cotton plants were also equally attractive to the parasitoid (Fig. 8b). *C. marginiventris* is a generalist that attacks a wide variety of early instar lepidopteran larvae (Bahena-Juárez 2008; Cave 1995) and is a very common natural enemy of *S. frugiperda* (Cortez-Mondaca et al. 2012; De Lange et al. 2014; Hoballah et al. 2004; Jourdie et al. 2008; Molina-Ochoa et al. 2004; Von Mérey et al. 2012), as well as *S. exigua* (Alvarado-Rodriguez 1987; Stewart et al. 2001). Therefore, it is to be expected that the wasp has evolved to readily recognize plant volatiles induced by suitable hosts. Indeed, *C. marginiventris* is attracted to herbivore-induced volatiles of maize, teosintes (i.e., the wild ancestors of maize), cotton (*G. hirsutum*) and cowpea (De Lange et al. 2016; Tamò et al. 2006) and shows strong antennal responses to volatiles from these plants (Gouinguené et al. 2005; Ngumbi et al. 2009). From several laboratory studies we already knew that total quantities of HIPVs are not of key importance for the attraction of *C. marginiventris* (Block et al. 2018; D'Alessandro and Turlings 2005; Fritzsche Hoballah et al. 2002; Sobhy et al. 2012). This is again shown here, and our results also support the notion that minor, as yet unknown compounds in the HIPV blends may be essential for the attraction of *C. marginiventris* (D'Alessandro et al. 2009). *S. frugiperda* and *S. exigua* are attacked by numerous natural enemies in their natural habitat (Cortez-Mondaca et al. 2012; Stewart et al. 2001; Von Mérey et al. 2012), and it can be expected that other parasitoids or predators are affected by changes in the maize HIPV blend. Hence, the full ecological implications for HIPV suppression on interactions with the third trophic level remain to be determined.

We found that *S. frugiperda* and *S. exigua* had distinct preferences for specific leaves to feed on. This finding was corroborated by Köhler et al. (2015). Using maize plants with three up to seven leaves, they found that *S. frugiperda* prefers younger leaves while *S. littoralis* prefers older leaves; the younger leaves were associated with higher levels of direct defense compounds, which *S. frugiperda* can tolerate (Glauser et al. 2011). We found a similar difference in leaf preference (Supplementary Fig. 1), but this apparently does not explain the difference in HIPV emissions. Induction of the different leaves resulted in very similar amounts of volatiles (Fig. 5).

An increasing number of studies have shown that arthropod pests can manipulate plant defenses, from insect eggs with defense-suppressing effects (Bruessow et al. 2010; Peñaflor et al. 2011) to whiteflies (Kempema et al. 2007; Zarate et al.

2007), aphids (Elzinga et al. 2014; Naessens et al. 2015), spider mites (Sarmento et al. 2011; Schimmel et al. 2017), and beetles (Lawrence et al. 2007). Specific feeding patterns (Dussourd 2017), as well as suppressing proteins (Elzinga et al. 2014; Naessens et al. 2015; Villarroel et al. 2016) and bacteria (Chung et al. 2013; Acevedo et al. 2017a) in arthropod oral secretions are responsible for the suppression. A recent study showed that even compounds in *S. frugiperda* frass can suppress defenses in maize (Ray et al. 2016). Hence, defense manipulation appears to be quite common.

In summary, we show here that larvae of *S. frugiperda*, a ferocious pest that is particularly well adapted to feed on maize, is able to repress HIPV emissions in maize. However, the reduced emissions did not change the attractiveness of infested plants to a common and important natural enemy. *S. frugiperda* recently appeared in Africa and Asia, where it is rapidly spreading and causing tremendous crop losses. Sustainable control options are badly needed. Unraveling the mechanisms employed by the pest to manipulate their host plants will provide a better understanding of its adaptations to maize and will set the stage for the development of novel crop protection strategies that could interfere with its ability to overcome and manipulate maize defenses.

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