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Silene latifolia temporal patterns of volatile induction and suppression after floral interaction by the nursery pollinator, *Hadena bicruris* (Lepidoptera: Noctuidae)

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After 24-hour *Hadena bicruris* floral interaction on *Silene latifolia* (Caryophyllaceae) with or without oviposition, we examined temporal volatile emission patterns for 3 days from plants with moth interaction and from neighboring plants only exposed to plant volatiles. Several lilac aldehydes and veratrole were progressively reduced after moth exposure without oviposition (by 30 to 40% after 24 hours and by 60 to 90% after 72 hours), but β-myrcene and β-pinene emissions increased by 200 to 300% only when exposure involved oviposition. Exposing *S. latifolia* to *H. bicruris* floral interaction without oviposition yielded no change in volatile organic compound (VOC) emission of neighboring *S. latifolia*; with oviposition, neighboring plants had 80 to 126% increases in emission rates for β-myrcene and β-pinene. Progressive reduction of *S. latifolia* VOC emission rates might help plants to avoid nursery pollinator oviposition. In contrast, with *H. bicruris* oviposition on *S. latifolia* flowers some VOCs (common herbivore-induced plant volatiles i.e. HIPVs) were induced. Whether oviposition occurred on *S. latifolia* strongly influenced neighboring plant VOC emission.

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1. Introduction

Over 1,000 vegetative and floral volatile terpenes, fatty acid derivatives, benzenoids, phenylpropanoids, aromatics, and amino acid derived metabolites are emitted across plant species (Knudsen *et al.* 2006). Volatile organic compound (VOC) induction is associated with a range of biotic and abiotic stress factors (Peñuelas & Llusiŕ 2003, Holopainen & Gershenzon 2010, Boczek *et al.* 2013). Induction of VOCs has been extensively studied in relation to direct and indirect defenses against pathogens and herbivores (Kessler & Baldwin 2001, Wang & Dorn 2003, Mumm *et al.* 2008, Unsicker *et al.* 2009, Holopainen & Gershenzon 2010).

Direct defenses act on the attacking agent, while indirect defenses influence natural enemies to come and act on the attacking agent (Unsicker *et al.* 2009). Induced VOCs prime, or even induce, defenses of uninjured plant organs (Heil & Silva Bueno 2007) or different uninjured (Engelberth *et al.* 2004, Baldwin *et al.* 2006, Piesik *et al.* 2010) or uninfected (Piesik *et al.* 2011, 2013) plants. Priming is not induction, but instead refers to an unattacked organism being prepared to more quickly and strongly mount defense responses to future biotic attack (Engelberth *et al.* 2004).

Defense induction follows herbivore oviposition in some plant systems (Hilker & Meiners 2011). More specifically, plant VOC induction after insect oviposition was inferred by greater parasitoid attraction to plants in the system involving field elm trees (*Ulmus minor* Mill.) and elm leaf beetles (*Xanthogaleruca luteola* Müller (Coleoptera, Chrysomelidae); Meiners & Hilker 1997, 2000, Hilker *et al.* 2002, Colazza *et al.* 2004); one early study in this system showed VOC induction from direct measurement (Wegener *et al.* 2001). Other systems have also reported strong VOC induction after herbivore oviposition onto a plant (Tamiru *et al.* 2011, Piesik *et al.* 2013). Yet, in some plant systems herbivore oviposition only slightly induces (Conti *et al.* 2008), does not change (Mumm *et al.* 2003), or even suppresses VOCs (Bruce *et al.* 2010, Peñaflor *et al.* 2011, Piesik *et al.* 2013).

Floral emitted VOCs are important for pollinator attraction (Raguso 2008, Schiestl 2010). Nocturnal plant species emit strong floral scents (Jürgens *et al.* 2002) since visual cues are less effective at night (Raguso *et al.* 2003). Spatial fragrance patterns within flowers are used by pollinators for orientation on flowers (Vogel 1963, Dötterl & Jürgens 2005, Effmert *et al.* 2005) to guide visitors to floral rewards (Dobson 1994). *Silene latifolia* Poir. ssp. *alba* (Mill.) Greut. and Burdet (white campion; Caryophyllaceae) is a dioecious European native perennial plant that is pollinated at night. Several attributes make this plant an interesting and useful subject for the study of plant-pollinator interactions and VOC emission. First, *S. latifolia* emits a strong nocturnal floral scent that has been characterized (Jürgens *et al.* 2002, Dötterl *et al.* 2005, Muhlemann *et al.* 2006). Second, *S. latifolia* floral scent is responsible for the attraction of flower visitors, mostly nocturnal Lepidoptera species (Brantjes 1976a, b).

A nursery pollinator moth, *Hadena bicruris* Hufnagle 1766 (Noctuidae), is attracted by *S. latifolia* floral scent (Dötterl & Jürgens 2005, Dötterl *et al.* 2006a, b). Nursery pollinators effectively pollinate flowers, but also oviposit onto them for herbivorous larval development (Dufaÿ & Anstett 2003). There are 14 *Hadena* spp. as nursery pollinators of 26 *Silene* spp.; these interactions range from being characterized as antagonistic to mutualistic (Kephart *et al.* 2006). Adult *H. bicruris* are effective pollinators of *S. latifolia*, but female *H. bicruris* also oviposit onto female *S. latifolia* flowers, where herbivorous larvae subsequently consume seeds (Bopp 2003, Bopp & Gottsberger 2004). Lilac aldehydes and veratrole emitted by *S. latifolia* flowers (Dötterl & Jürgens 2005) are attractive to *H. bicruris* adults (Dötterl *et al.* 2006a, b), and guide adults to elicit landing onto flowers (Brantjes 1976a, b). Adult *H. bicruris* can discriminate among different stereoisomers of lilac aldehydes (Dötterl *et al.* 2006b).

On one hand, *S. latifolia* seems to benefit by having flowers effectively pollinated by adult *H. bicruris*. However, *S. latifolia* also suffers a cost as *H. bicruris* larvae feed on developing seeds (Wolfe *et al.* 2002) and *S. latifolia* aborts up to 40% infested fruits (Burkhardt *et al.* 2009). In highly infested fruits where more seeds are more likely to be consumed, plants could reduce re-

source allocation into fruits likely to yield low seed numbers and might also cause the deaths of *H. bicruris* larvae. Many plants have evolved to attract pollinators with floral VOCs, but then lower flower VOC emission once they are pollinated (Raguso 2008). It has been hypothesized that *S. latifolia* reduces floral VOC emission from pollinated flowers partly in order to reduce future *H. bicruris* visits and thereby minimizing oviposition and subsequent larval seed-predation (Dötterl *et al.* 2005, Muhlemann *et al.* 2006). After hand-pollination of two *S. latifolia* flowers, both flowers almost completely wilted and nearly eliminated VOC emission within 24 hours (Fig. 2c in Dötterl *et al.* 2005). Muhlemann *et al.* (2006) tested the effect of hand-pollination on one Swiss population (n=16). They reported that pollinated flowers did not wilt, but had progressively reduced emission rates of lilac aldehydes (LA) A and B, and veratrole (VER), over the following 48 hours (Muhlemann *et al.* 2006). Moreover, habitat fragmentation can affect the levels of herbivory in plant populations if plants and herbivores are differentially affected by fragmentation (Elzinga *et al.* 2005).

With four experiments, we examined *S. latifolia* whole plant VOC response 24 to 72 hours after floral exposure to an actual insect nursery pollinator, *H. bicruris*. In two experiments, we studied *S. latifolia* VOC emission after exposure involved *H. bicruris* floral interaction either with or without oviposition. We did this to test whether *H. bicruris* floral interaction was sufficient to cause progressive decline of whole plant VOC emission, including specific floral VOCs like LA A–D and VER (Dötterl & Jürgens 2005), and VOC induction occurred when *H. bicruris* floral interaction included oviposition. In two additional experiments, we examined *S. latifolia* VOC emission when exposed to VOCs from a conspecific that had received *H. bicruris* floral interaction with or without oviposition. We did this to test whether the exact nature of *H. bicruris* floral interaction (with or without oviposition) influenced the VOC emission of a neighboring plant.

2. Material and methods

2.1. Plant culture

Experiments were performed at the Plant Growth Centre at University of Technology and Life Sciences (Bydgoszcz, Poland) from spring 2010 to 2011. Female *S. latifolia* were planted from seed (National Botanic Garden of Belgium) with two plants per pot (14 cm diameter, 12 cm height) in sterilized soil (Castorama, Bydgoszcz, Poland) in a greenhouse for 10 weeks. Plants were grown with supplemental light at ambient relative humidity, watered four times per week, and received 100 ppm Peters® General Purpose Fertilizer (J.R. Peters Inc., Allentown, Pennsylvania, USA) in aqueous solution twice a week. Then, plants were transferred to a growth chamber and raised in a 16:8 (day:night) photoperiod with 30 $\mu mol\ m^{-2}\ s^{-1}$ light intensity. Daytime temperature was 22 ± 2°C and the overnight temperature was 18 ± 2°C. Plants were kept in the growth chamber until flowers were blooming, so that plants were ready for experimental use. Experimental *S. latifolia* were used at stage 65 of Biologische Bundesanstalt, Bundessortenamt und Chemische Industrie (BBCH). The BBCH is a commonly used plant development scale which represents common plant phenology stages; stage 65 specifically refers to plants having 50% of their flowers open.

2.2. *Silene latifolia* floral exposure by *H. bicruris*

Each experimental plant was transferred in the morning on the day of its *H. bicruris* exposure treatment to a greenhouse bay, and had roughly 12 hours to adjust to experimental conditions. At night (22:00 h) of the same day, each plant was placed into a cage for its *H. bicruris* exposure treatment. Each cage (60 × 30 × 20 cm) contained two glass sides, two mesh sides, and wire (small holes to prevent insect escape) on the top and bottom of the cage; this allowed for light transmission and gas exchange.

Adult *H. bicruris* were provided from INRA (France) and collected from the field in western part of Poland and near Bydgoszcz, brought back

to the lab, and placed onto non-experimental *S. latifolia* prior to use. It is possible that two moth species were involved in the experiments, because the closely similar *H. capsincola* occurs in Poland (Hacker *et al.* 2002). Also at 22:00 hours, adult *H. bicruris* were placed on their respective *S. latifolia* plants for 24 hours in both experiments. Each moth was used only once and only in one experiment.

In the first experiment, treatment *S. latifolia* plants (*n*=8) received a *H. bicruris* male–female pair that was not "*in copula*" immediately prior to introduction. These experimental plants were expected to receive floral interaction without oviposition. The lack of oviposition was confirmed when no eggs were found from each of the five flowers randomly dissected from each treatment plant one week following *H. bicruris* floral exposure. Caged plants in the first experiment, that were not exposed to *H. bicruris,* served as unexposed controls (*n*=8).

Treatment *S. latifolia* in the second experiment (*n*=8) received a *H. bicruris* male–female pair that was "*in copula*" immediately prior to introduction. These experimental plants were expected to receive eggs during floral interaction. Floral oviposition was confirmed with each treatment plant one week later as more than one (2 to 5) of the five randomly chosen flowers dissected from each plant contained an egg of *H. bicruris*.

Plants that were not exposed to *H. bicruris* served as unexposed controls (*n*=8); this was a different group of plants than used in the first experiment. Since VOCs were collected from four plants simultaneously, five groups of plants (including two blanks) were measured for 3 days (24, 48, and 72 h) in each of these two *H. bicruris* exposure experiments.

2.3. *Silene latifolia* exposure to a conspecific after *H. bicruris* exposure

Healthy (unmanipulated and undamaged) *S. latifolia* plants were placed as neighbors nearby to a conspecific that had just received 24 hours *H. bicruris* exposure with or without oviposition (different individuals than in the previous two experiments). Neighbor plants in these two experiments were maintained separately from *H. bicruris* exposed conspecifics used for neighbor exposure, both before and after plant–plant VOC exposure. The experimental treatments differed by the distance that a *S. latifolia* plant exposed to *H. bicruris* was from a neighbor, and the degree to which exposed plants were blocked with a Nalophan bag (20 cm diameter, 50 cm height; polyethylene teraphtalate; odor and taste-free cooking bags made of a plastic film resistant in the temperature range from –60°C to +220°C; Charles Frčres-Saint Etienne-France). In each experiment, the exposed plant was located one meter away and entirely surrounded by a Nalophan bag; 1 meter or 3 meters away and partially surrounded by a Nalophane bag (bag between the exposed plant and its neighbor, but not surrounding the exposed plant); or 1 meter or 3 meters away and completely open (unbagged).

There were eight *S. latifolia* plants (*n*=8) assigned to each of these five treatments in each plant–plant exposure experiment, so there were 40 neighbors from which VOCs were collected in each experiment. One experiment consisted of neighboring plant exposure to a conspecific plant with *H. bicruris* floral exposure but no oviposition, while the second experiment consisted of neighboring plant exposure to a conspecific plant with *H. bicruris* floral exposure and oviposition. We confirmed that all plants with *H. bicruris* exposure but no expected oviposition lacked eggs one week later (five flowers dissected from each plant). We also confirmed, that all plants with *H. bicruris* exposure and oviposition had an egg on at least one flower (five flowers dissected from each plant). *Silene latifolia* plants exposed to a neighboring plant with *H. bicruris* exposure with or without oviposition was moved from a growth chamber to a greenhouse bay for neighboring plant exposure (different from the bay used for *H. bicruris* exposure) that started at 22:00 hours and lasted for 24 hours. There were 11 groups of plants (and two blanks) measured for 3 days (24, 48, and 72 h) in each of these two experiments.

2.4. Volatile collection system

Volatiles were collected separately and simultaneously from four Nalophan enclosed *S. latifolia*

Table 1. *F*-statistics from one-way repeated measures analysis of variance of total VOC and 14 specific VOCs in four experiments involving *S. latifolia* floral exposure to *H. bicruris* moths.

	a. Floral exposure without oviposition (NoO)			b. Floral exposure with oviposition (O)		
Factor	NoO	Day	NoO×Day	O	Day	O×Day
Degrees of freedom	1, 14	2, 28	2, 28	1, 14	2, 28	2, 28
Total VOC emission	12,771****	464****	343****	6.2*	9.4***	6.4**
Lilac aldehyde B	12,089****	1,236****	1,231****	123****	4.1*	3.4*
Lilac aldehyde A	2,544****	90****	79****	218****	ns	9.0**
Veratrole	1,697****	271****	170****	22***	ns	ns
Lilac aldehyde D	646****	13****	16****	ns	4.4*	ns
Lilac aldehyde C	611****	45****	30****	21***	38****	11***
(E)-β-farnesene	ns	3.6*	ns	63****	32****	17****
(Z)-β-ocimene	ns	6.7**	ns	125****	15****	11***
β-caryophyllene	11**	ns	ns	119****	14****	7.6**
Linalool	49****	3.9*	ns	127****	ns	5.0**
Phenylacetaldehyde	ns	4.0*	ns	7.6*	ns	ns
β-myrcene	5.1*	6.4**	ns	1,281****	ns	ns
4-heptanone	ns	7.3**	ns	29****	ns	ns
β-pinene	18****	ns	ns	2,596****	ns	ns
Linalool oxide	62****	ns	10***	7.7*	ns	ns

	c. Exposure to neighbor without oviposition (NeNoO)			d. Exposure to neighbor with oviposition (NeO)		
Factor	NeNoO	Day	NeNoO×Day	NeO	Day	NeO×Day
Degrees of freedom	4, 35	2, 70	8, 70	4, 35	2, 70	8, 70
Total VOC emission	ns	9.8***	ns	7.7***	19****	ns
Lilac aldehyde B	ns	ns	ns	ns	4.1*	ns
Lilac aldehyde A	ns	32****	ns	3.6*	ns	6.3****
Veratrole	ns	16****	2.3*	4.8**	9.8***	ns
Lilac aldehyde D	ns	3.1*	ns	ns	22****	ns
Lilac aldehyde C	3.4*	19****	4.7****	ns	47****	ns
(E)-β-farnesene	ns	26****	3.8**	9.6****	32****	6.1****
(Z)-β-ocimene	4.5**	49****	2.5*	15****	6.3**	ns
β-caryophyllene	2.7*	15****	3.9**	13****	ns	5.1****
Linalool	5.5**	14****	ns	4.8**	5.8**	4.2***
Phenylacetaldehyde	5.0**	26****	ns	2.7*	5.6**	ns
β-myrcene	ns	ns	ns	241****	ns	ns
4-heptanone	ns	ns	2.5*	ns	19****	ns
β-pinene	6.0**	121****	3.0**	70****	11****	8.4****
Linalool oxide	3.3*	9.3***	3.9**	17****	85****	10****

ns: not significant, *: $p<0.05$, **: $p<0.01$, ***: $p<0.001$, ****: $p<0.0001$

plants. Each plant was entirely surrounded by its bag; the bag was tied at the base of the stem above the pot's soil, and a volatile collector trap was placed on the top of each bag and held in place with a frame. A volatile collector trap (6.35 mm OD, 76 mm long glass tube; Analytical Research Systems, Inc., Gainesville, Florida, USA) containing 30 mg of Super-Q (Alltech Associates, Inc., Deerfield, Illinois, USA) adsorbent was inserted into each of four Tygon tubes (connection between airflow meter and collector trap). Purified, humidified air was delivered at a rate of 1.0 L min^{-1} over the plants, and a vacuum pump sucked 20% less (0.8 L min^{-1}) to avoid collecting odors of outside air via any system gaps. Two blanks (odors collected from empty Nalophan

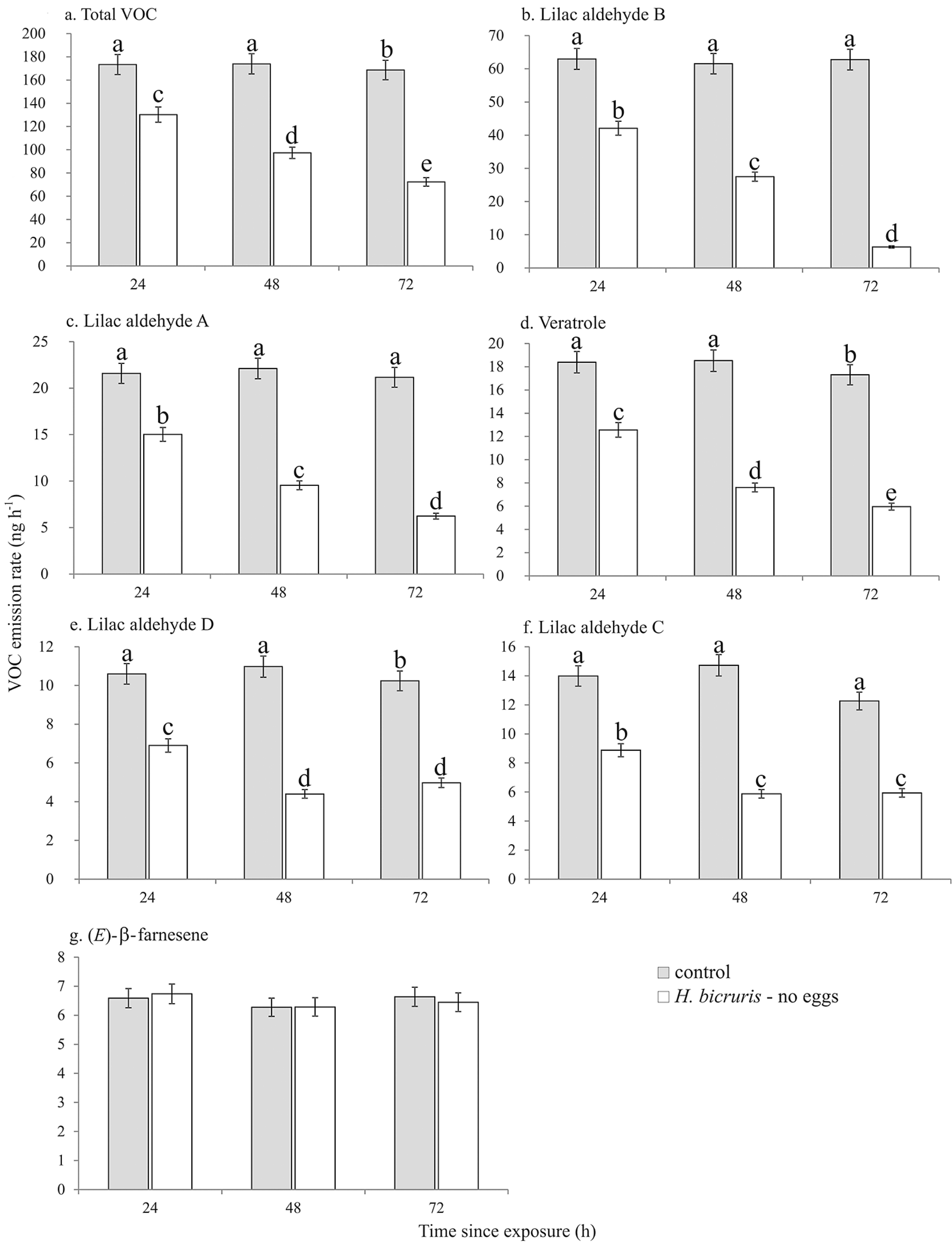
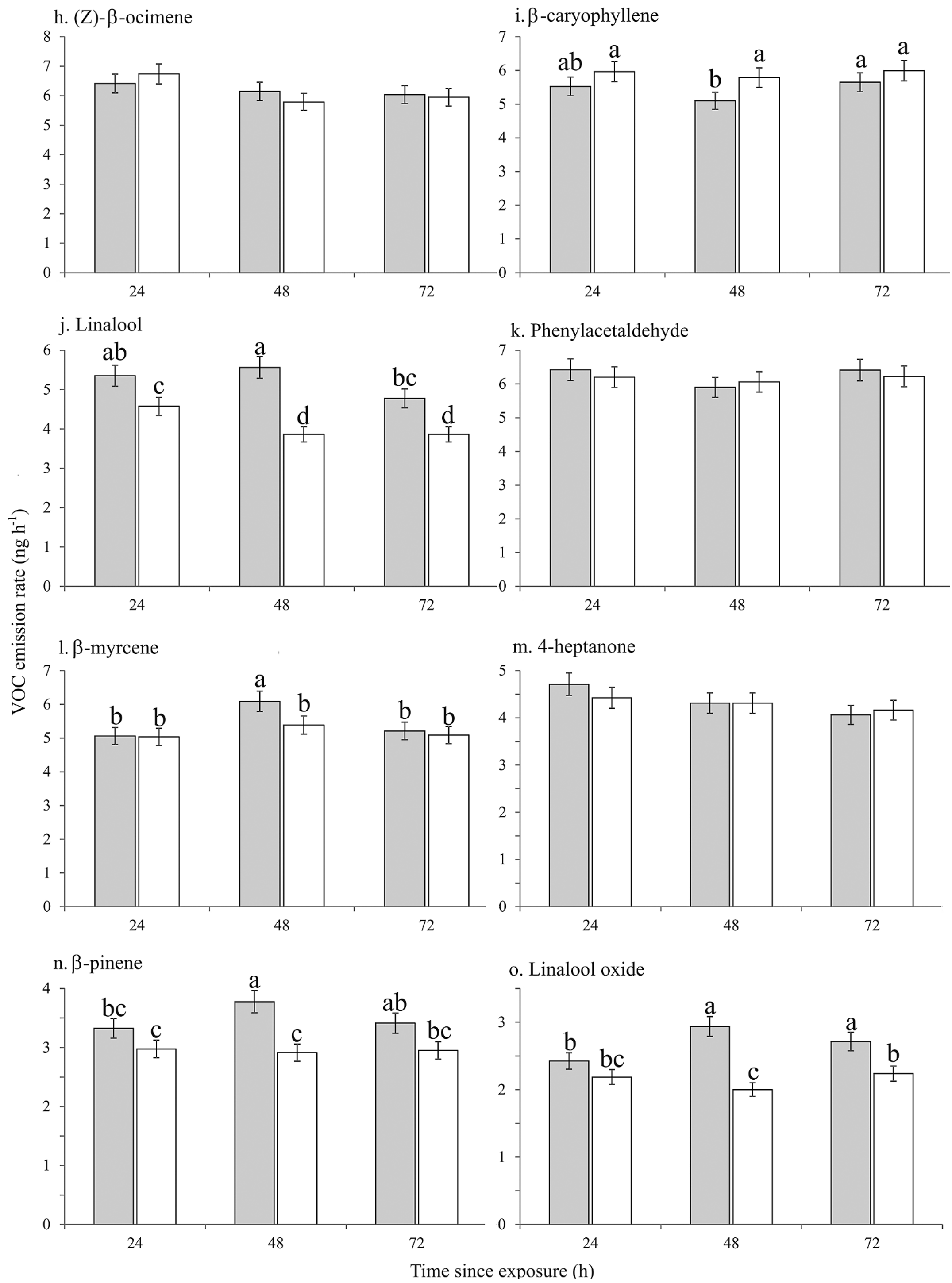


Fig. 1. Effects of *H. bicruris* floral exposure without oviposition (lasting 24 h) on *S. latifolia* VOC emission rates (mean ± 1 SD) at 24, 48, and 72 hours following initial plant exposure to *H. bicruris* or not (control). Treatments with the same letter within each measurement date were not significantly different when tested with LSD *post-hoc* tests. – a. Total VOC. – b–o. Specific VOCs (h–o on next page).



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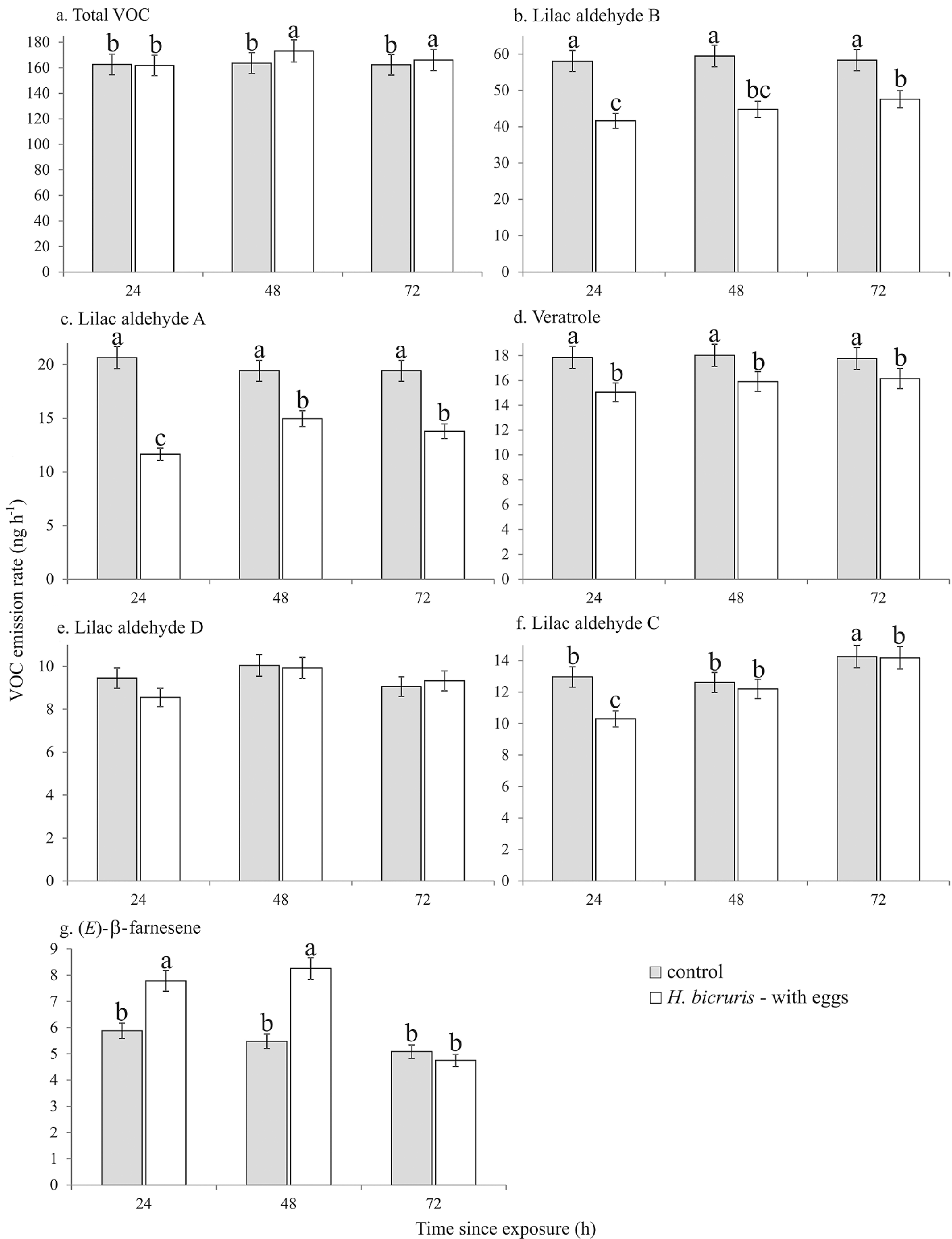


Fig. 2. Effects of *H. bicruris* oviposition and floral bud injury exposure (lasting 24 h) on *S. latifolia* VOC emission rates (mean ± 1 SD) at 24, 48, and 72 hours following initial plant exposure to *H. bicruris* oviposition or not (control). Treatments with the same letter within each measurement date were not statistically significantly different when tested with LSD *post-hoc* tests. – a. Total VOC. – b–o. Specific VOCs (h–o on next page).

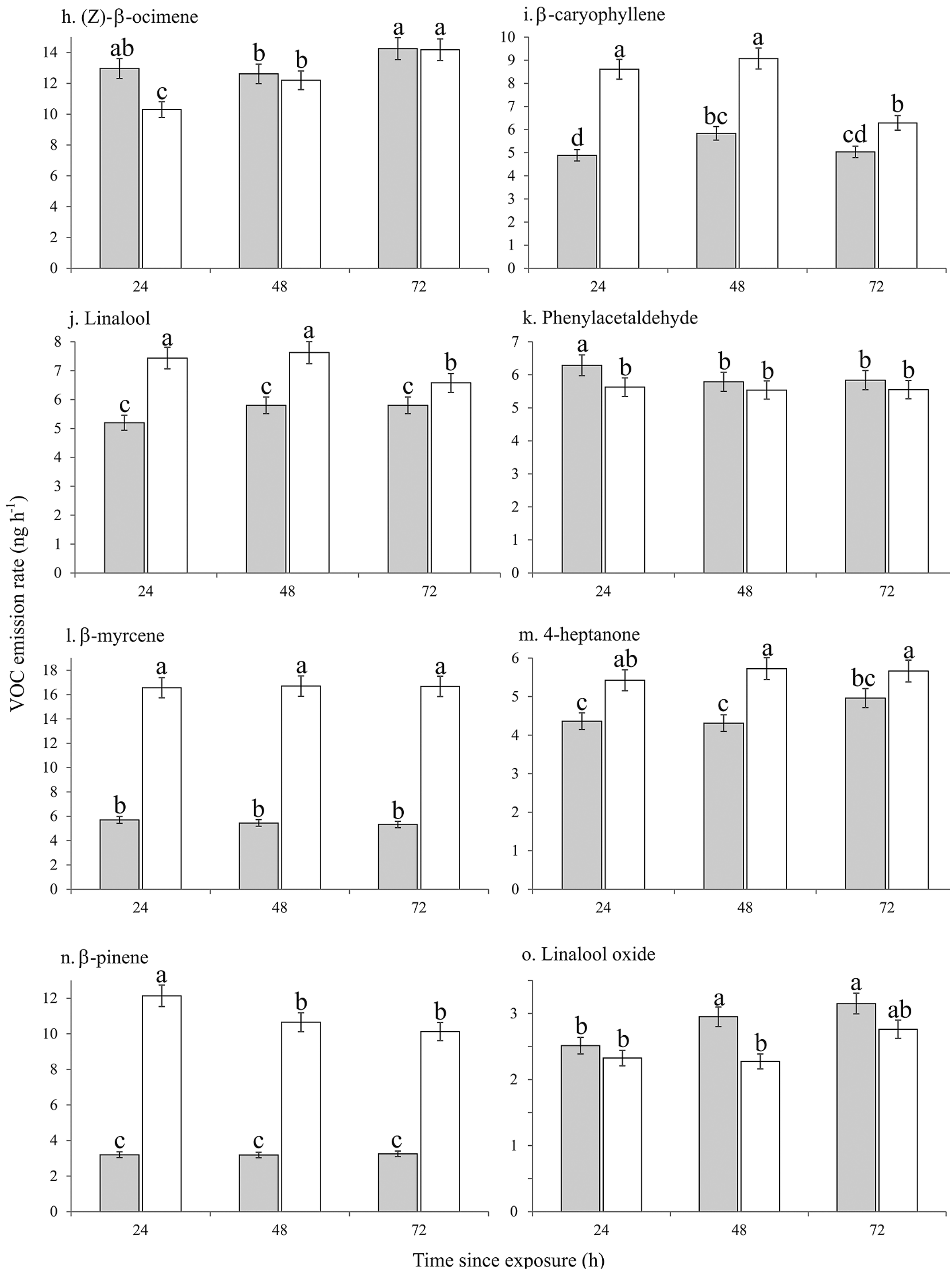


Fig. 2. Continued from previous page.

bags) were also collected in each of the above four experiments to verify that measured VOCs were not background air (non-plant) contaminants. The nocturnal volatile collection sequence lasted for two hours, from 22:00 to 24:00, like in other nocturnal floral emission studies (Jürgens *et al.* 2002, Dötterl *et al.* 2005, Dötterl & Jürgens 2005, Muhlemann *et al.* 2006). This was because this is the time of peak *S. latifolia* VOC emission (Dötterl *et al.* 2005).

2.5. VOC analytical methods

Our VOC analysis was similar to previous studies (Piesik *et al.* 2010, 2011, 2013, Wenda-Piesik *et al.* 2010). Volatiles were eluted from the Super-Q in each volatile collection trap with 225 μL of hexane. Then, seven ng of decane was added to each sample as an internal standard, which allows for the quantification of other VOCs by comparing each chromatographic peak area relative to the peak area from the known quantity of decane (e.g., Muhlemann *et al.* 2006, Piesik *et al.* 2010, 2011, 2013, Wenda-Piesik *et al.* 2010). Individual samples (1 μL) were injected and analyzed by coupled gas chromatography-mass spectrometry (GC/MS). The GC/MS Auto System XL/Turbomass (Perkin Elmer Shelton, CT, USA) fitted with a 30 m Rtx-5MS capillary column (0.25 mm ID, 0.25 μm film thickness; Restek, USA). The temperature program increased from 40°C to 200°C at 5°C min^{-1}. Tentative VOC identification was initially based on matches to compounds from the NIST 1998 database. Then, each VOC ID was verified by matching retention times and mass spectra from authentic standards. Several compounds were purchased from a commercial source (Sigma-Aldrich, MO, USA) with the caveat that the β-ocimene standard contained multiple isomers, while lilac aldehydes A–D and veratrole were obtained from the Institute of Systematic Botany at the University of Zürich. VOC concentrations (ng h^{-1}) were calculated by comparing chromatogram peak area of each VOC relative to the peak area of the internal standard (e.g., Muhlemann *et al.* 2006, Piesik *et al.* 2010, 2011, 2013). Data for a VOC was reported if minimal levels were consistently detected (> 0.1 ng h^{-1} from all individuals within a control or experimental treatment).

2.6. Statistical analysis

Parametric MANOVA was conducted using Proc GLM in SAS v. 9.2 (SAS Institute 2008) since VOC data was continuous and had multivariate normality (J. Bocianowski, personal communication). Separate analyses were performed from data for each measurement day, because small treatment sample sizes relative to number of VOCs measured prevented repeated measures MANOVA. We used MANOVA to examine whether an overall trend of individual VOC emission rate increased or decreased after *H. bicruris* or neighboring plant exposure treatments. Following significant MANOVA for each measurement day in each experiment, one-way repeated measures analyses of variance (ANOVA) were conducted on the variability of emission rates of each VOC separately using PROC MIXED in SAS (SAS Institute, 2008). The normality of data distributions from each VOC was tested using Shapiro-Wilk's normality test (Shapiro and Wilk 1965). All VOC induction experiments were carried out using a completely randomized design involving repeated measures. Day was the repeated measures factor, plant was the subject, *S. latifolia* exposure to *H. bicruris* (or neighboring plant) was the fixed main effect, and the "day*exposure" term was the interaction to examine whether treatment effects weakened or strengthened across measurement days. For all analyses, statistical significance was set at $\alpha = 0.05$. The least significant differences (LSD) post-hoc test was used to distinguish significant treatments for analyses with significant exposure or "day*exposure" terms.

3. Results

3.1. *Silene latifolia* exposure to *H. bicruris* without oviposition

The exposure of *S. latifolia* to *H. bicruris* floral interaction without oviposition led to progressively lower *S. latifolia* total VOC emission: by 25% at 24 hours, 44% at 48 hours, and 56% by 72 hours (Table 1, Fig. 1a). We found significant exposure terms from MANOVA conducted on data from 14 VOCs (Table 1, Fig. 1a–o) measured at

24 (Wilk's λ = 1.1 × 10–5; $F_{1,14}$ = 6,369; p < 0.001), 48 (Wilk's λ = 1.0 × 10–5; $F_{1,14}$ = 7,730; p < 0.001), and 72 (Wilk's λ = 1.3 × 10–5; $F_{1,14}$ = 5,415; p = 0.011) hours after initial *H. bicruris* introduction. Univariate repeated measures ANOVA for each VOC resulted in the "day*exposure" interaction being significant for lilac aldehydes A–D (LA A–D) and veratrole (VER, Table 1a), where *H. bicruris* exposed *S. latifolia* had progressively lower emission concentrations of these VOCs, with 30 to 40% reductions at 24 hours, but 60 to 90% reductions by 72 hours relative to unexposed plants (Fig. 1b–f). The "exposure" main effect was significant for β-pinene (β PIN), β-myrcene (β MYR), linalool oxide (LINOX, also "day*exposure"), and linalool (LIN, Table 1a), as these four monoterpenes had small degrees of suppression in exposed plants at one or more measurement days (Figs. 1j, l, n, o). The significant exposure term for β-caryophyllene (β CAR, Table 1a) reflected a small degree of induction (Fig. 1i). There was no significant change in the emission of (E)-β-farnesene (E β FAR), (Z)-β-ocimene (Z β OCI), phenylacetaldehyde (PHE), and 4-heptanone (4HEP) due to *H. bicruris* exposure (Figs. 1g, h, k, m).

3.2. *Silene latifolia* exposure to *H. bicruris* with oviposition

Oviposition during *H. bicruris* exposure resulted in slightly increased *S. latifolia* total reported VOC emission (+8%), but only at 48 hours (Fig. 2a). However, there was a significant exposure term from MANOVA conducted from data collected at 24 (Wilk's λ = 1.3 × 10–4; $F_{1,14}$ = 557; p = 0.033), 48 (Wilk's λ = 1.1 × 10–4; $F_{1,14}$ = 633; p = 0.031), and 72 (Wilk's λ = 0.0 × 10–5; $F_{1,14}$ = 20,104; p = 0.006) hours after initial *H. bicruris* introduction. The "day*oviposition" interaction and the "oviposition" main effect were both significant for LA A–C (Fig. 2c), Z β OCI (Fig. 2h), LIN (Fig. 2j), β CAR (Fig. 2i) and E β FAR (Fig. 2g). "Oviposition" was also significant for 4HEP (Fig. 2m), β MYR (Fig. 2l), β PIN (Fig. 2n), VER (Fig. 2d), PHE (Fig. 2k), and LINOX (Table 1b). Plants receiving eggs had consistently larger induction of β MYR (~167%) and β PIN (200 to 300%) from 24 to 72 hours compared to control plants (Fig. 2l, n). Significant but smaller degrees of induction (10 to 80%) were measured from plants with eggs at 24 hours for E β FAR (Fig. 2g), Z β OCI (Fig. 2h), β CAR (Fig. 2i), LIN (Fig. 2j), and 4HEP (Fig. 2m), where the degree of induction decreased or was not significant by 72 hours. No VOC had progressively greater emission level reductions over 72 hours (Fig. 2). Plants with eggs also had significant reductions (10 to 40%) in emission rates compared to control plants; over 72 hours for LA B (Fig. 2b), LA A (Fig. 2c), and VER (Fig. 2d), significant reduction only on one measurement day for LA C (Fig. 2f), PHE (Fig. 2k), and LINOX (Fig. 2o), and no significant change for LA D (Fig 2e).

3.3. *Silene latifolia* exposure to a conspecific with *H. bicruris* exposure (without oviposition)

Exposure of *S. latifolia* to a *H. bicruris* -exposed (no oviposition) neighboring conspecific did not significantly affect total VOC emission rates on any measurement day (Fig. 3a). There was a significant neighbor exposure main effect from MANOVA conducted from data collected at 24 (Wilk's λ = 0.041; $F_{56,87}$ = 1.99; p = 0.002), 48 (Wilk's λ = 0.030; $F_{56,87}$ = 2.22; p = 0.0002), and 72 (Wilk's λ = 0.047; $F_{56,87}$ = 1.88; p = 0.004) hours after initial exposure to a neighboring *S. latifolia* plant. Many VOCs had a significant "neighbor exposure" main effect and/or a "day*exposure" interaction from univariate ANOVA (Table 1c), yet all significant treatment differences were small (10 to 20%, Fig. 3). Plants had significantly lower emission on one or more measurement days of β CAR (Fig. 3i), LIN (Fig. 3j), β PIN (Fig. 3n), and INLOX (Fig. 3o), or higher emission of E β FAR (Fig. 3g), PHE (Fig. 3k), and β MYR (Fig. 3l), when exposed 1 meter from an unbagged neighbor compared to an entirely bagged neighbor. VER (Fig. 3d), LA C (Fig. 3f), Z β OCI (Fig. 3h), 4HEP (Fig. 3m), and LINOX (Fig. 3o) had significant differences on one or more measurement days only between plants exposed 3 meters from an unbagged neighbor compared to an entirely bagged neighbor 1 meter away. Some significant differences for E β FAR (Fig. 3g), Z β OCI (Fig. 3h), and β CAR

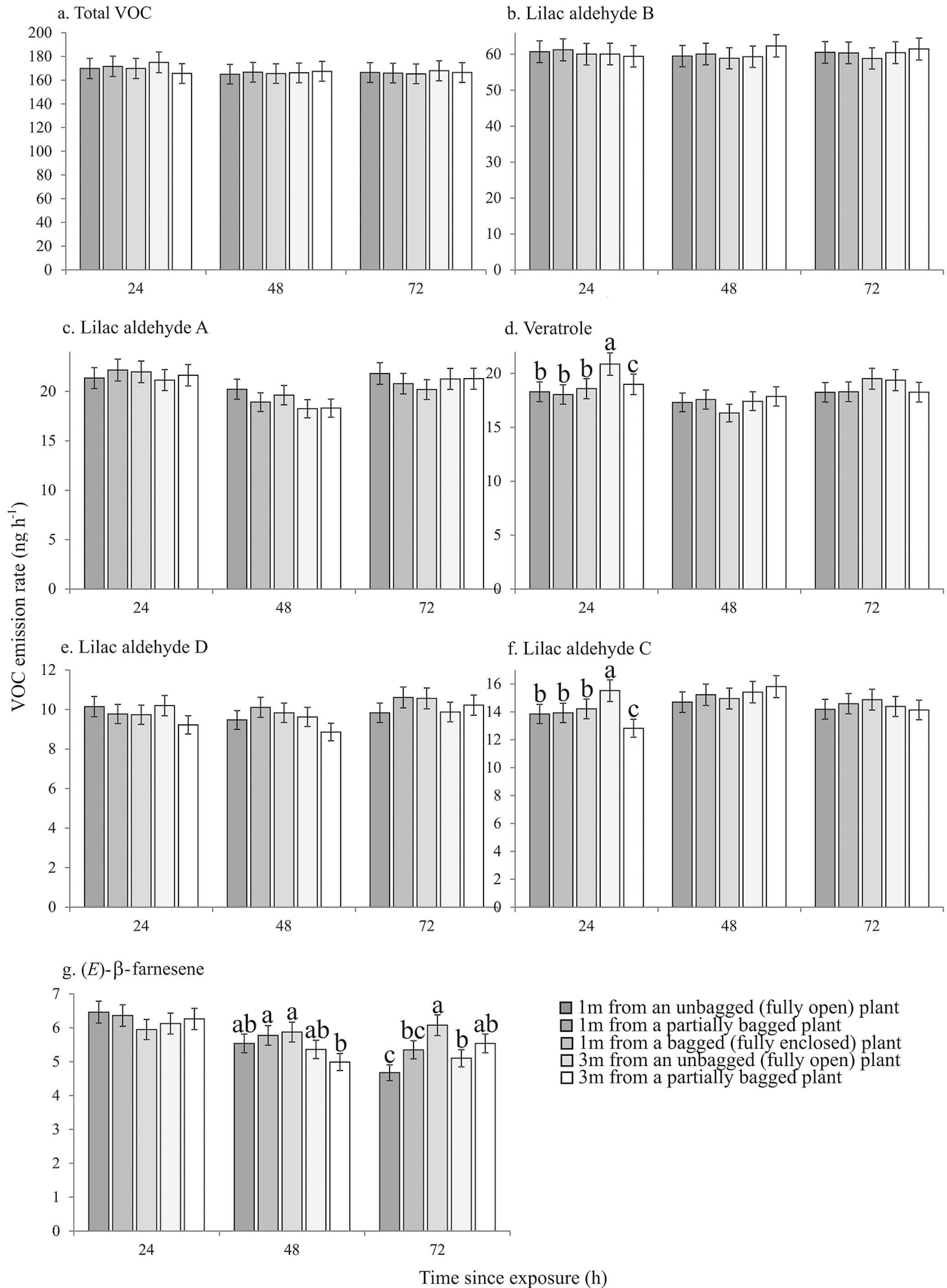
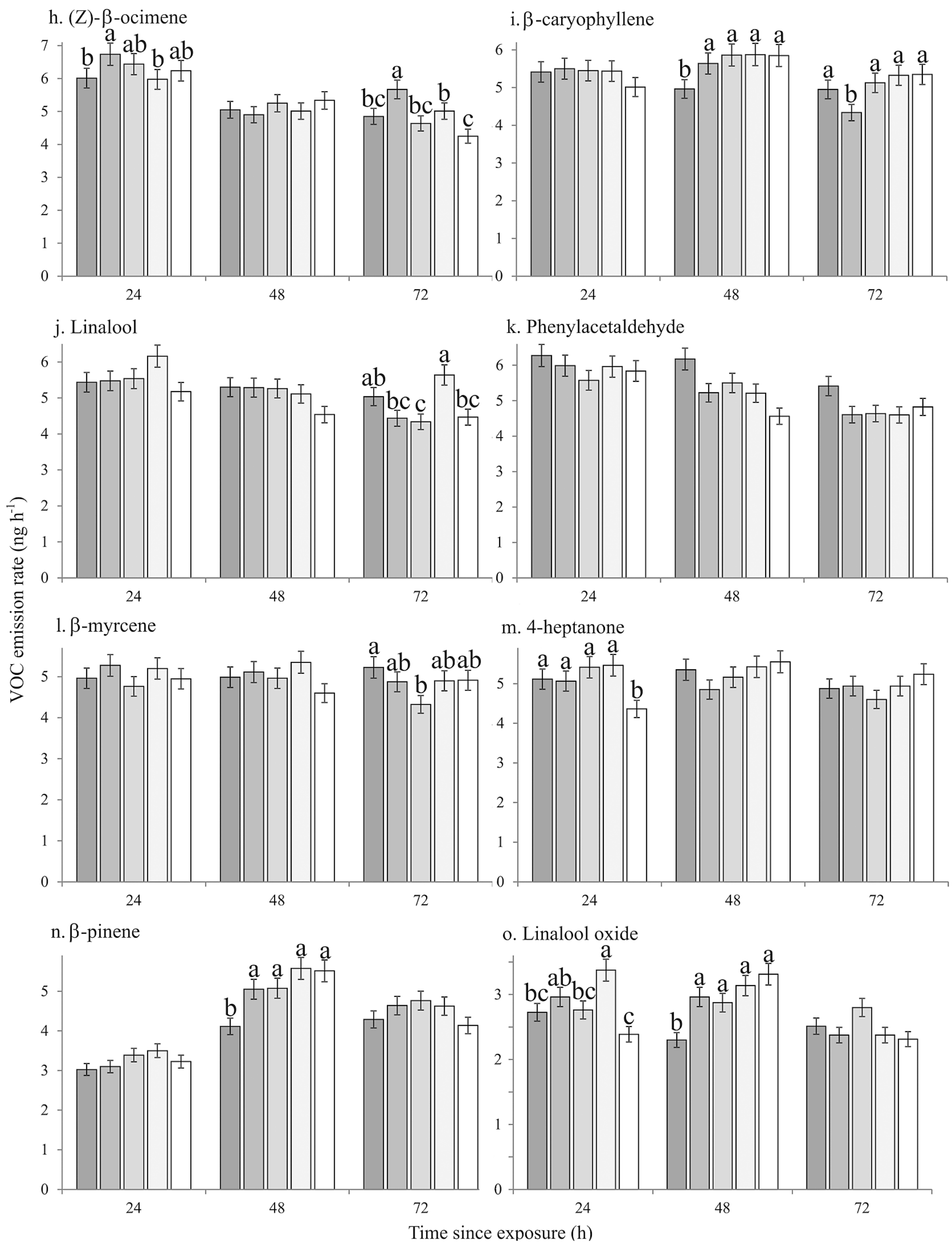


Fig. 3. Effects of *S. latifolia* exposure for 24 hours to a neighboring plant that had just finished receiving *H. bicruris* floral exposure, but no oviposition for 24 hours, on *S. latifolia* VOC emission rates (mean ± 1 SD) at 24, 48, and 72 hours following initial plant exposure to a neighbor. Treatment plants were located 1 meter or 3 metres from plants exposed to *H. bicruris* entirely or partially enclosed by a clear Nalophan bag, or were unbagged. Treatments with the same letter within each measurement date were not significantly different when tested with LSD *post-hoc* tests. – a. Total VOC. – b–o. Specific VOCs (h–o on next page).



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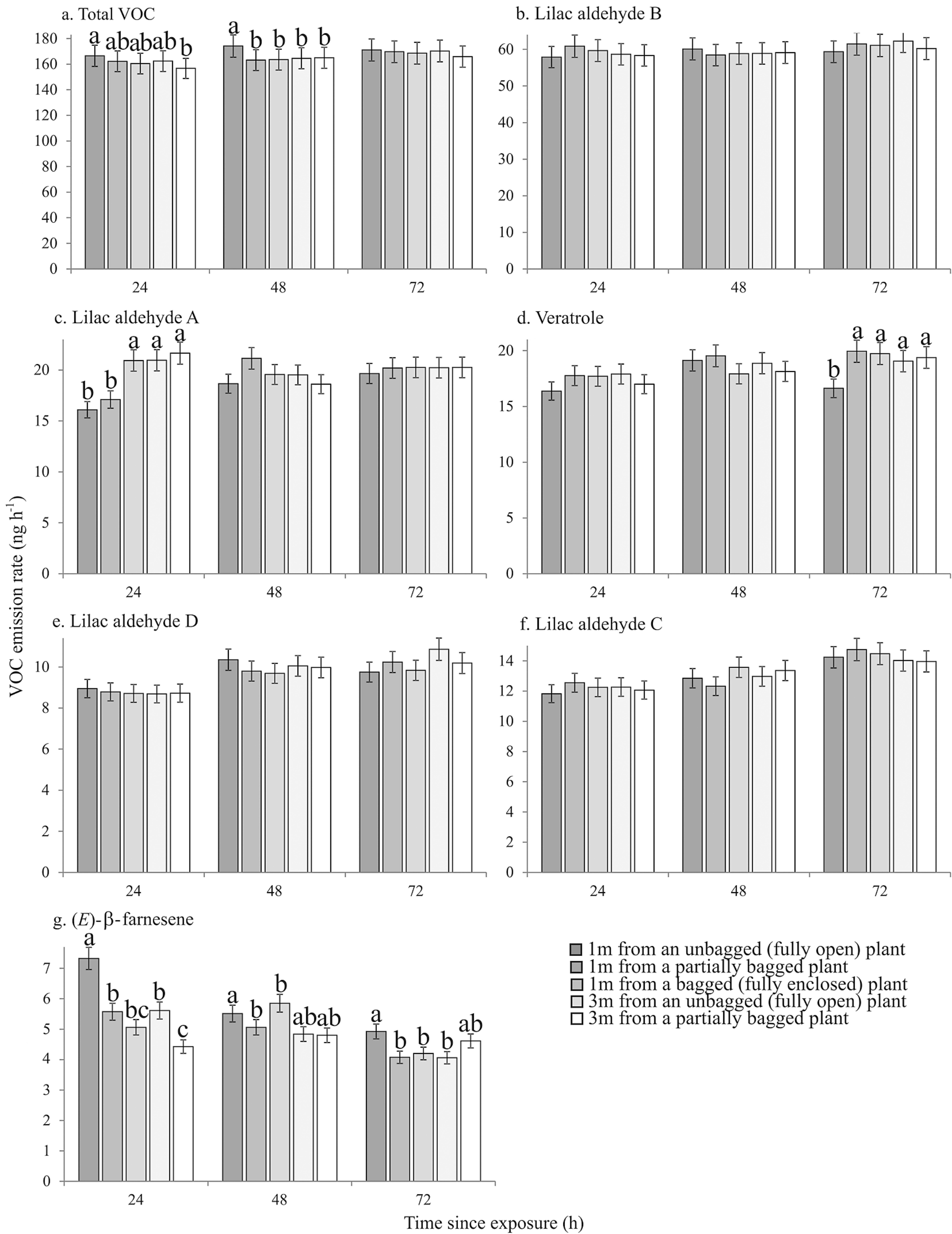
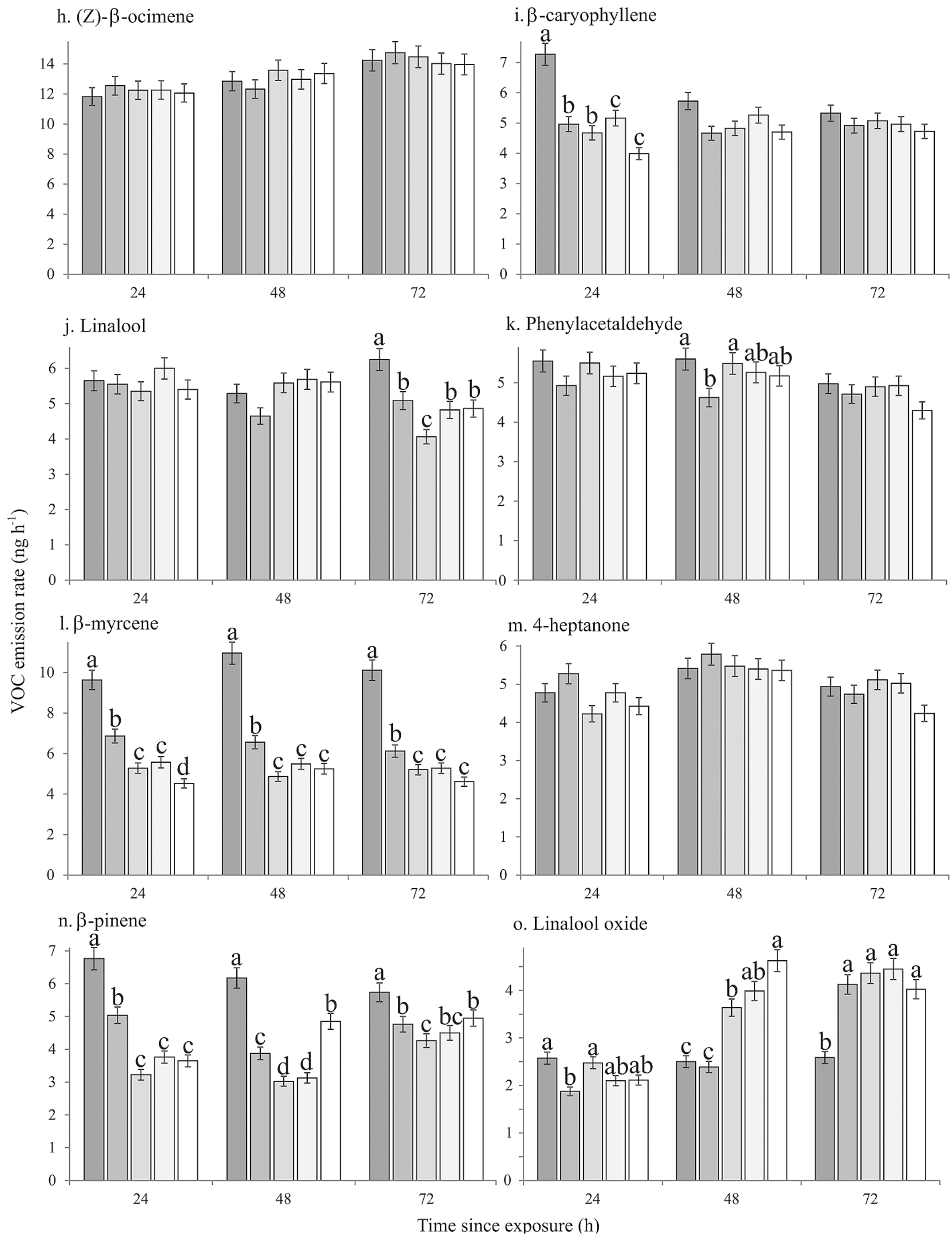


Fig. 4. Effects of *S. latifolia* exposure for 24 hours to a neighboring plant that had *H. bicruris* floral exposure and oviposition/bud injury on *S. latifolia* VOC emission rates (mean ± 1 SD) at 24, 48, and 72 hours following initial plant exposure to a neighbor. Treatment plants were located 1 meter or 3 metres from plants exposed to *H. bicruris* entirely or partially enclosed by a clear Nalophan bag, or were unbagged. Treatments with the same letter within each measurement date were not significantly different when tested with LSD *post-hoc* tests. – a. Total VOC. – b–o. Specific VOCs (h–o on next page).



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(Fig. 3i) were between 1 meter exposure to partially bagged plants compared to unbagged and entirely bagged plants at 1 meter, and/or to plants exposed at 3 meters.

3.4. *Silene latifolia* exposure to a conspecific with *H. bicruris* exposure (with oviposition)

Exposure of *S. latifolia* to a neighboring conspecific with *H. bicruris* oviposition significantly increased total plant VOC emission rates (+8%), but only at 48 hours (Fig. 4a). There was a significant neighbor exposure main effect from MANOVA at 24 (Wilk's $\lambda = 0.001$; $F_{56,87} = 7.24$; $p < 0.0001$), 48 (Wilk's $\lambda = 0.0019$; $F_{56,87} = 6.33$; $p < 0.0001$), and 72 (Wilk's $\lambda = 0.0046$; $F_{56,87} = 4.70$; $p < 0.0001$) hours after initial neighboring plant exposure. There were significant "day*exposure" interactions and "exposure" main effects from univariate ANOVA for LA A (Fig. 4c), β PIN (Fig. 4n), LIN (Fig. 4j), LOX (Fig. 4o), β CAR (Fig. 4i) and E β FAR (Fig. 4g), while only the "exposure" main effect was significant for β MYR (Fig. 4l), VER (Fig. 4d), PHE (Fig. 4k), and Z β OCI (Fig. 4h) (Table 1d). Induction of β MYR (Fig. 4l) from 24 to 72 hours by *S. latifolia* exposed 1 meter from unbagged plants with *H. bicruris* oviposition was 82 to 126% higher compared to entirely enclosed neighboring plants (Fig. 4l). The magnitude of other changes ranged from increases of 50 to 105%, or decreases of 15 to 50% (Fig. 4).

The greatest degree of induction for E β FAR (Fig. 4g), β CAR (Fig. 4i) and, β PIN (Fig. 4n) occurred at 24 hours after plant exposure 1 meter from a neighboring plant with oviposition, with small or no significant induction by 72 hours. Induction of LIN was significant only at 72 hours (Fig. 4j). Plants 1 meter from a neighboring plant with oviposition also had significantly lower LA A only at 24 hours (Fig. 4c), only at 72 hours for VER (Fig. 4d) and LINOX (Fig. 4o), and Z β OCI at 48 to 72 hours (Fig. 1h). There was no significant "exposure" main effect for LA B (Fig. 4b), LA D (Fig. 4e), and LA C (Fig. 4f), and 4HEP (Fig. 4m). The "day" term was significant for most VOCs in each experiment (Table 1a–d) to reflect temporal variation in emission of plant VOCs (Figs. 1–4).

4. Discussion

4.1. *Silene latifolia* floral exposure to *H. bicruris* without or with oviposition

When *S. latifolia* received *H. bicruris* floral exposure without oviposition, there were striking progressive reductions in the emission rates of several lilac aldehydes and veratrole. This was the driver of reductions in whole plant total VOC emission rate, and in the emitted VOC blend. In contrast, when *S. latifolia* received *H. bicruris* floral exposure with oviposition, there were smaller reductions in the emission rates of several lilac aldehydes and veratrole, and a striking induction of some herbivore-induced plant volatiles (HIPVs). Overall, whole plant emission remained largely unchanged because reductions of some VOCs were offset by induction of other VOCs; mainly, *S. latifolia* VOC blend dramatically changed after *H. bicruris* exposure. In some systems, insect oviposition leads to host plant VOC induction (e.g., Wegener *et al.* 2001, Tamiru *et al.* 2011, Piesik *et al.* 2013), which is consistent with our results. Also, insect floral interaction without oviposition, but likely including pollination, often leads to subsequent decreased floral scent emission (Tollsten & Bergström 1989, Tollsten 1993, Schiestl *et al.* 1997, Theis & Raguso 2005, Muhlemann *et al.* 2006, Hossaert-McKey *et al.* 2010).

Following *S. latifolia* floral exposure to *H. bicruris* without oviposition, whole plant emission concentrations of LA A–D and VER progressively decreased over 72 hours, a few terpenes had small reductions, one terpene was induced to a small degree, and no floral wilting was observed. We suspect that floral wilting did not occur after *H. bicruris* floral exposure, because it is not a general *S. latifolia* response to pollination, neither when using hand-pollination (Muhlemann *et al.* 2006) or insect pollination (current study). In fact, the floral wilting observed in Dötterl *et al.* (2005) happened only with the two hand-pollinated flowers tracked, and may not reflect a general *S. latifolia* response to insect pollination. We did not confirm/observe *S. latifolia* female floral pollination by *H. bicruris*, so we can only state that floral interaction occurred without subsequent oviposition. Progres-

sive reduction of several lilac aldehydes and veratrole, which are attractive to *H. bicruris* adults (Dötterl *et al.* 2006a, b), was previously reported after hand-pollination (Muhlemann *et al.* 2006). Our *S. latifolia* VOC results were very striking because this occurred after *H. bicruris* interactions despite a lack of confirmed pollination. In earlier studies, larval *H. bicruris* destroyed an average of 25% of seeds (range of 0 to 100%; Wolfe 2002) and caused up to 40% of early fruit abortion (Burkhardt *et al.* 2009). Thus, *S. latifolia* might benefit from *H. bicruris* pollination, but then take steps to avoid potentially large costs from attracting subsequent *H. bicruris* oviposition and larval herbivory; this can make *S. latifolia–H. bicruris*-interaction antagonistic to *S. latifolia* (Dufaÿ & Anstett 2003, Kephart *et al.* 2006).

Studies with nursery pollinator systems have shown leaf VOC peaks during floral receptivity and subsequent decline (Dufa *et al.* 2004), post-pollination declines after hand-pollination (Muhlemann *et al.* 2006) or actual insect pollinators (Proffit *et al.* 2008, Chen *et al.* 2009, current study), or from pollen-feeding of an insect pest on a crop (Piesik *et al.* 2013). Post-pollination floral VOC reductions also occur in non-nursery pollinator systems (Tollsten & Bergström 1989, Tollsten 1993, Schiestl *et al.* 1997, Theis & Raguso 2005). Competing hypotheses, but not necessarily with non-overlapping predictions, have tried to explain why post-pollination floral scents decrease (Hossaert-McKey *et al.* 2010). One explanation is that VOC emission decreases are merely due to floral senescence. However, not all flowers immediately wilt or rapidly senesce after pollination. Other possibilities for why flowers reduce post-pollination VOC emission may be to 1) minimize resource and energy allocation when pollinator attraction is no longer needed for a flower (Pichersky *et al.* 1994, but see Grisson-Pigé *et al.* 2001), 2) minimize apparency to floral herbivores (Euler & Baldwin 1996), or 3) make it easier for pollinators to find remaining unpollinated flowers on a plant by reducing attractive VOCs (Schiestl *et al.* 1997) or increasing a repellent VOC (Schiestl & Ayasse 2001). Plants that commonly experience net negative reproductive consequences from non-obligate nursery pollinators might emit attractive floral VOCs to benefit from pollination, but then reduce post-pollination VOCs to minimize subsequent costs due to oviposition and larval herbivory (Dötterl *et al.* 2005, Muhlemann *et al.* 2006, Hossaert-McKey *et al.*2010).

Our results are the first to report that *H. bicruris* floral interaction without oviposition is sufficient to lead to progressive reductions of key VOCs known to be attractive to *H. bicruris*, and is the first published study to examine *S. latifolia* VOC responses after interaction with an actual insect (Dötterl *et al.* 2005, Muhlemann *et al.* 2006). More needs to be understood about how floral VOC suppression after pollination subsequently affects the attraction of *H. bicruris* and other pollinators of *S. latifolia*, as well as other *Hadena* spp. interactions with other *Silene* spp. host plants. This question could be addressed by using VOC bouquets in lab and field experiments, and quantifying geographical and environmental variation in these interactions (Kephart *et al.* 2006, Hossaert-McKey *et al.* 2010).

There were more complicated changes in *S. latifolia* whole-plant VOC emission when *H. bicruris* floral interaction involved oviposition. Two common terpene HIPVs (ßPIN, ßMYR) had a consistently larger induction compared to both unexposed plants and exposed plants without oviposition. Other common terpene HIPVs (ZOCI, LIN, β CAR, and E β FAR) had small degrees of brief inductions. Several lilac aldehydes and VER were suppressed from 24 to 72 hours after oviposition, but the degree of suppression was much smaller than in plants exposed to *H. bicruris* without oviposition, and there was no progressive reduction of any VOCs. A mix of induced and suppressed VOCs quantitatively altered *S. latifolia* VOC blend after egg deposition, with little overall effect on the total VOC emission. Such changes in VOC blend from *S. latifolia* plants with *H. bicruris* oviposition might be of relevance if natural enemies are attracted to eggs or larvae (Unsicker *et al.* 2009), or if certain VOCs deter future herbivores from trying to oviposit onto a plant (Kessler and Baldwin 2001); both possibilities need to be tested.

Increased parasitoid attraction that was related to VOC induction (Wegener *et al.* 2001), has been shown for plants that received oviposition from a herbivore compared to control

plants (Meiners & Hilker 1997, 2000, Hilker *et al.* 2002, Colazza *et al.* 2004). However, later studies have reported more variable results after insect oviposition across other plant-herbivore systems: suppression of a single VOC (Bruce *et al.* 2010) or several VOCs (Peñaflor *et al.* 2011); no change in VOC concentrations (Mumm *et al.* 2003); slight VOC induction (Conti *et al.* 2008); or a large induction of some VOCs but the suppression of others (Piesik *et al.* in review, and current study). Injury caused during oviposition, compounds excreted to hold an egg(s) to a plant, or other factors may be the stimuli to which plants respond to oviposition (Mumm *et al.* 2003). Some plants can rapidly alter VOC emission after receiving oviposition, which may help to prevent future oviposition or attract natural enemies to eggs or future larvae (Kessler & Baldwin 2001, Mumm *et al.* 2003).

4.2. Exposure to neighboring *S. latifolia* with *H. bicruris* floral exposure with or without oviposition

Plants exposed to *H. bicruris* without oviposition mainly reduced VOC emissions. In the second set of two experiments, we demonstrated that *S. latifolia* had very little alteration in VOC emission when exposed to a conspecific neighbor with *H. bicruris* floral exposure but no oviposition. This makes sense as fewer VOCs should reach neighboring plants, and pollinator attraction should still be important to unpollinated *S. latifolia* lacking direct *H. bicruris* exposure.

Neighboring *S. latifolia* had some reduced VOC emission rates and other VOCs induced when exposed to a conspecific that had received *H. bicruris* oviposition in the greenhouse; the effects were stronger when the neighbor was 1 meter vs. 3 meters away. These results were strengthened by having bag treatments, since induced or reduced VOC levels were detected when plants were exposed to an unbagged neighbor, but not when exposed to a completely bagged neighbor. This has been shown previously for plant VOC induction when exposed to another plant with herbivory injury (Piesik *et al.* 2010). We believe, that because emitted VOCs (even induced ones) from a fully bagged plant do not reach a nearby conspecific, they do not influence it. In contrast, VOCs from an unbagged plant can reach a neighbor, and greater amounts of induced VOCs are able to reach and influence neighboring plants. Although weaker and briefer, the quantitative pattern of neighboring plant VOC alteration generally matched terpene responses of those plants that had received eggs.

Our results are in agreement with previous studies, which show, that mainly lilac aldehyde and veratrole emissions of *S. latifolia* are important for pollinator attraction (Dötterl *et al.* 2005, 2006a, b, Muhlemann *et al.* 2006). Our responding *S. latifolia* test plants were unpollinated and lacked eggs, so the plants might not benefit from any VOC emission change that reduces pollinator attraction, seed-predating or otherwise. A future study could examine whether neighboring *S. latifolia* has primed defenses when exposed to a plant with eggs, to quickly respond to future oviposition attempts from seed-predating nursery pollinators like *H. bicruris*.

5. Conclusions

We found that *H. bicruris* interaction with *S. latifolia* flowers altered the subsequent whole-plant emission rates of several floral VOCs and common HIPVs. Floral interaction by *H. bicruris* without oviposition progressively reduced emission rates of several lilac aldehydes and veratrole (pollinator attractants), but had little effect on HIPVs. The reduction in pollinator attractant VOCs after floral pollination is thought to be due to evolutionary pressures to reduce resource allocation for pollinator attraction, and for *S. latifolia* more specifically, to reduce attraction of nursery pollinators like *H. bicruris* (Muhlemann *et al.* 2006). In contrast, *H. bicruris* floral interaction with oviposition induced several common HIPVs from *S. latifolia*, coupled by smaller lilac aldehyde and veratrole emission rate reductions. The HIPV induction suggests a possible direct and/or indirect defensive response to oviposition specifically, rather than insect floral contact. Thus, *S. latifolia* VOC emission was sensitive to floral VOC suppression after general *H. bicruris* floral interaction, while HIPV induction only occurred after oviposition.

A second important finding from our experimental results is, that whether there was *H.*

bicruris oviposition during floral interaction with *S. latifolia* or not, not only influenced VOC emission rates from the plant itself, but also influenced whether VOCs from the focal plant altered VOC responses in neighboring plants. Floral interaction without oviposition had very little influence of neighboring plant VOC emission rates, as lower VOC levels should reach neighboring plants. In contrast, when *H. bicruris* floral interaction included oviposition, there was quantitatively weaker but qualitatively similar HIPV induction in neighboring plants. The plant receiving oviposition has several HIPVs induced, so that higher levels should reach a neighboring plant to stimulate HIPV induction. This explanation is further supported, because HIPV induction did not occur in a neighboring plant when receiving a VOC bouquet from *S. latifolia* plants with *H. bicruris* oviposition fully surrounded by a Nalophan bag. This is probably because the bag blocked induced HIPVs from reaching the neighboring plant. Future studies should explore active vs. passive neighboring *S. latifolia* VOC induction and determine which HIPV blend(s) stimulate neighboring plant HIPV induction. Studies with other pollinators would help illuminate, whether the *S. latifolia* results reported here are specific to *H. bicruris* or more generally to *S. latifolia* insect pollinators. Finally, using an actual insect pollinator, *H. bicruris*, is more realistic when studying plant VOC responses after floral interaction/pollination than after hand-pollination.

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