Isomers of green leaf volatiles in Nicotiana attenuata and their role in plant-insect interactions
Allmann, S.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Isomerization of green leaf volatiles alters the behavioral responses of female *Manduca* moths

Silke Allmann$^{1,2,\#}$ and Anna Späthe$^{3,\#}$, Sonja Bisch-Knaden$^3$, Mario Kallenbach$^2$, Robert C. Schuurink$^1$, Andreas Reinecke$^3$, Silke Sachse$^3$, Ian T. Baldwin$^2$ and Bill Hansson$^3$

**Footnotes**
1 Department of Plant Physiology, Swammerdam Institute for Life Sciences, Science Park 904, 1098 XH, Amsterdam, NL
2 Department of Molecular Ecology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, GER
3 Department of Evolutionary Neuroethology, Max Planck Institute for Chemical Ecology, Hans-Knöll-Str. 8, D-07745 Jena, GER
\# These authors contributed equally to the paper.
ABSTRACT

Moms always want the best for their children and as such female moths try to find the best plant for their offspring – nutrient rich, poor in toxins and preferably not occupied by other herbivores. Plant volatiles play an important role for female moths in selecting appropriate host plants as these contain valuable information on the plant identity and the presence of other herbivores. Therefore the ability to perceive, discriminate and classify volatile plant compounds is crucial for a moth’s oviposition choice. Green leaf volatiles (GLVs) are rapidly released upon cell disruption and can provide such specific information. When plants are consumed by the larvae of tobacco hawkmoth *Manduca sexta* their oral secretions (OS) cause a rapid conversion of the GLV \((Z)-3\)-hexenal into \((E)-2\)-hexenal leading to a dramatic shift in the \((Z)/(E)\)-ratio of these aldehydes, their alcohols and esters compared to mechanically damaged plants.

*Datura wrightii* is often selected as host by ovipositing hawkmoths. Here we show, through laboratory and field experiments, that the OS-depending shift in *Datura wrightii’s* \((Z)/(E)\)-ratio could be induced during the light and the dark period. However, under artificial night conditions also wounding alone resulted in this shift. Female *Manduca* moths are able to detect and discriminate certain GLVs: by means of functional calcium imaging we found four glomeruli in the antennal lobe of *M. sexta* females being activated upon stimulation with unsaturated C6-aldehydes, alcohols or acetates. Interestingly, two of these showed high specificity for hexenyl acetate isomers i.e. \((Z)-3\)- or \((E)-2\)-isomers. Furthermore, stimulation with different ratios of both acetate isomers resulted in distinct activation patterns. Field experiments demonstrated that *Manduca* moths preferred to oviposit on \((Z)\)-perfumed *Datura wrightii* over \((E)\)-perfumed plants and they discriminated between \((Z)\)- and \((E)\)-GLVs, different \((Z)/(E)\)-ratios and between the two hexenyl acetates. These results show that \((E)\)-GLVs and/or specific \((Z)/(E)\)-ratios are cues hawkmoths can use when selecting host plants for their offspring.

Image description front page

**Upper left panel:** Volatile trapping set-up on a *Datura wrightii* plant in the field. Image courtesy of M. Kallenbach.

**Upper right panel:** Feeding late instar caterpillar of *M. sexta* on a *N. attenuata* leaf. Image courtesy of A.P. van Doorn.

**Lower left panel:** Overlay image of an antennal lobe of a female *Manduca sexta* moth. Image courtesy of A. Späthe.

**Lower right panel:** Ovipositing *Manduca sexta* moth on a *N. attenuata* leaf. Image courtesy of D. Kessler.
INTRODUCTION

Insects rely on olfaction in most aspects of life: volatile signals guide them to food sources, mating partners and oviposition hosts. Especially for herbivorous insects, plant volatiles are important to locate and identify appropriate host plants for their offspring. Upon herbivory, plants respond with an increased release and de-novo synthesis of several volatile compounds from their vegetative tissue (Mumm and Dicke, 2010). The main groups of volatiles released from damaged tissue are green leaf volatiles (GLVs), derived from the fatty acid/lipoxygenase pathway, mono- and sesquiterpenes, from the isoprenoid pathways and aromatic metabolites, such as methyl salicylate, indole and benzenoids from the shikimate/tryptophan pathway (Maffei, 2010). These so-called herbivore induced plant volatiles (HIPV) can provide significant information to the surrounding environment as composition and abundance relies on several biotic and abiotic factors: the quantitative and qualitative amounts of HIPV can differ depending on plant species or cultivar (Gouinguene et al., 2001; Schuman et al., 2009), plant age, season (Hare, 2010), but also depending on the attacking herbivore (De Moraes et al., 1998) or even the developmental stage of the herbivore (Takabayashi et al., 1995). HIPV have been assigned various defense-related functions and they mediate amongst others indirect defenses by attracting predators and parasitoids of the herbivores to the feeding site (Sabelis and Van De Baan, 1983; Mumm and Dicke, 2010). As this ‘cry for help’ (Dicke et al., 1990) might lead to a higher abundance of natural enemies near the herbivore-attacked site, female adult insects should in principal avoid ovipositing at or near herbivore damaged spots. Several studies have shown that females use HIPV to choose appropriate host plants. Evidence exists for both, attractive and repellent effects of a whole volatile bouquet or even single compounds (for reviews see Dicke and van Loon, 2000; Dicke and Baldwin, 2009).

Due to the variable nature of plant volatile signals the ability to perceive, discriminate and classify volatile compounds is crucial for insects to generate appropriate behavioral responses. In insects and more specifically in *M. sexta*, olfactory sensory neurons (OSNs) located on the insect antennae detect odorant molecules, e.g. GLVs (Kalinová et al., 2001; Shields and Hildebrand, 2001; Fraser et al., 2003) and convey this information via electrical potentials to the brain (Fig. 4.1). OSNs expressing the same receptor, and thus responding to the same set of odorants, converge onto the same glomerulus in the antennal lobe (AL; Fig. 4.1 B), the first olfactory processing center (Vosshall, 2000). Glomeruli are spherical, anatomical discrete structures with high synaptic density constituting the functional units of the AL. Odor processing in the AL is maintained by local interneurons (LN), which relay...
information between the glomeruli. Projection neurons (PNs) convey olfactory information to higher brain centers like the mushroom bodies and the lateral horn (for review see Homberg et al., 1989; Hildebrand and Shepherd, 1997; Hansson and Anton, 2000; de Bruyne and Baker, 2008). Spatio-temporal patterns of neuronal activity representing sensory input to the AL can be visualized by optical imaging methods (Joerges et al., 1997; Sachse and Galizia, 2002; Hansson et al., 2003; Skiri et al., 2004; Carlsson et al., 2005; Silbering and Galizia, 2007). This technique allows identifying compound and blend specific activity patterns in the first processing center of the insect brain.

**Figure 4.1. Olfactory system in Manduca sexta moths.** (A) Schematic view of *M. sexta* brain. Sensilla (S) on the antennae house olfactory sensory neurons (OSNs) which send their axons via the antennal nerve (AN) to the first olfactory neuropil, the antennal lobe (AL). After first processing, odor information is conveyed to higher brain centers like the mushroom bodies (MB) and the lateral horn (LH). (B) Olfactory sensory neurons (OSNs, colored lines) with different receptor types detect odorant molecules and convey this odor information to the antennal lobe. OSNs expressing the same receptor converge onto the same glomerulus (glom) in the antennal lobe. Glomeruli are spherical, anatomical discrete structures with high synaptic density constituting the functional units of the AL. They are interconnected by local interneurons (LNs, black lines) which relay information between glomeruli. Projection neurons (PNs, grey lines) convey information to higher brain centers like the mushroom bodies (MB) and the lateral horn (LH). Figure is adapted from Vosshall and Stocker (2007).
The crepuscular hawkmoths *Manduca sexta* and *Manduca quinquemaculata* (Lepidoptera/Sphingidae) are native to Southwestern USA. They use a wide range of plant species for nectar feeding, and for several of these plant species they represent an important pollinator (Alarcón et al., 2008). Interestingly, *Manduca* females almost exclusively oviposit on solanaceous plants (Madden and Chamberlain, 1945; Yamamoto and Fraenkel, 1960; Mechaber and Hildebrand, 2000). In the great basin desert in Utah *Manduca* is often associated with the jimsonweed *Datura wrightii* and the wild tobacco *Nicotiana attenuata* forming an ambivalent relationship as nectar-feeding pollinator on the one hand and as folivorous insect after oviposition on the other (Mechaber et al., 2002; Mira and Bernays, 2002). Both, *N. attenuata* as well as *D. wrightii* respond to *Manduca* herbivore attack by emitting HIPVs (Gaquerel et al., 2009; Hare and Sun, 2011), and these volatiles are thought to influence the host choice of ovipositing *Manduca* females (De Moraes et al., 2001; Kessler and Baldwin, 2001). Reduced oviposition of *Manduca* moths in response to feeding damage has been shown in a few cases: In field experiments undamaged control plants of *N. attenuata* received more eggs of the tomato hornworm *Manduca quinquemaculata* than plants that were already damaged by conspecific caterpillars (Kessler and Baldwin, 2001). Similar results have been shown in the laboratory where female *Manduca sexta* moths preferred to lay eggs on intact tomato plants compared to caterpillar damaged plants (Reisenman et al., 2009). Additionally, *spr2* mutants of tomato plants, which release lower amounts of unsaturated green leaf volatiles (GLVs) and some terpenoids, were preferred over wild type plants by ovipositing *Manduca* moths (Sánchez-Hernández et al., 2006).

GLVs constitute a large group of herbivore-induced plant volatiles (HIPVs) with a C6-backbone. While emitted only in trace amounts from healthy, undamaged plant tissue, they are emitted instantly after cell disruption (Turlings et al., 1995; D'Auria et al., 2007). The increase in emission is transient but can be sustained over days by repetitive wounding by feeding herbivores (Turlings et al., 1995). GLVs are generated from C18-fatty acids through the activities of two enzymes, lipoxygenase (LOX) and hydroperoxide lyase (HPL, Allmann et al., 2010). One of the most abundant GLVs, (Z)-3-hexenal, originates from the cleavage of α-linolenic acid through the activity of HPL and it partly isomerizes to (E)-2-hexenal. Both alkenals can be further metabolized by the activity of an alcohol dehydrogenase (ADH) and alcohol acyltransferase (AAT; D'Auria et al., 2007) to the corresponding alcohols and their esters (Matsui, 2006).

GLVs have been assigned various plant defense-associated functions. They may directly inhibit phytopathogens (Hamiltonkemp et al., 1992; Nakamura and Hatanaka, 2002;
Prost et al., 2005). In addition, GLVs also appeared to repel several herbivore species (De Moraes et al., 2001; Kessler and Baldwin, 2001; Vancanneyt et al., 2001; Zhang and Schlyter, 2004) although some Lepidopteran larvae use them as feeding stimuli (Halitschke et al., 2004; Meldau et al., 2009) and other insects, like cockchafers, as sexual kairomones (Ruther et al., 2002; Ruther, 2004; Reinecke et al., 2005). Thirdly, GLVs are associated with the priming of plant defenses in adjacent branches and neighbouring plants (Bate and Rothstein, 1998; Engelberth et al., 2004; Kost and Heil, 2006). Finally, GLVs can establish indirect plant defenses by attracting foraging predators and host-seeking parasitoids to the plant and its attacker (Kessler and Baldwin, 2001; Shiojiri et al., 2006; Halitschke et al., 2008) reminiscent of the role of other HIPVs.

We recently showed that mechanically damaged leaves of *N. attenuata* released large amounts of (Z)-GLVs and low amounts of (E)-GLVs. However, when the plant was attacked by *M. sexta* caterpillars or when puncture wounds of plant leaves were treated with *M. sexta*’s oral secretions (OS), the amount of (E)-GLVs released increased while the amount of (Z)-GLVs decreased leading to a distinct change in the (Z)/(E)-ratio of GLV emissions. This herbivore-induced change in the (Z)/(E)-ratio attracted the generalist hemipteran predator *Geocoris* spp., and the approaching predator decreased the herbivore load on the plant by feeding on caterpillar eggs (Allmann and Baldwin, 2010).

In the present study we addressed the questions if (i) this OS-induced change in GLV emission also occurs in the related species *Datura wrightii* at dusk and night, the active periods of *Manduca* moths, and if so, whether this difference (ii) elicits specific activity patterns in the antennal lobe and (iii) prompts gravid *Manduca* females to avoid ovipositing on plants with shifted (Z)/(E) ratios in nature.

**RESULTS**

*Application of M. sexta OS to leaf wounds triggers pronounced changes in the GLV profile of Datura wrightii*

To investigate whether application of *M. sexta*’s OS onto wounded leaves of *Datura wrightii* plants causes a similar shift in the (Z)/(E)-ratio as seen for *N. attenuata* (Allmann and Baldwin, 2010) we compared the emissions of mechanically wounded *D. wrightii* plants that were treated with either water as a control (w+w) or with *M. sexta*’s OS (w+OS) in growth chamber experiments. During the day the (Z)/(E)-ratios clearly differed between damaged leaves that did or did not come into contact with *M. sexta*’s OS; the application of OS onto the
wounds caused a significant decrease in the (Z)/(E)-ratio of the main GLVs released from Datura plants (hexenals, 17-fold; hexenols, 5-fold and hexenyl acetates, 5-fold decrease in the (Z)/(E)-ratio compared to w+w treated plants (Figs. 4.2A and 4.3A)).

Since Manduca moths are crepuscular and nocturnal insects and thus not active during the day but during sunset and night (Theobald et al., 2010) we repeated the experiment under low light and no-light conditions to mimic sunset and night (see Fig. S4.1 for growth chamber light conditions). Although the (Z)/(E)-ratio of the aldehydes differed significantly between treatments at all measured times, the difference in the ratios decreased gradually with decreasing light intensities, from 17-fold during the day to 16-fold at sunset to 4-fold in complete darkness. This equalization between the treatments was due to a drop in the ratio of w+w treated plant, which was mainly caused by an increase in the (E)-2-hexenal emissions (Fig. 4.3 and Table S4.1A). Similarly, treatment-dependent differences in the (Z)/(E)-ratios for the alcohol and the hexenyl acetate decreased from 5- to 4-fold under lower light conditions and vanished completely during the night (Fig. 4.2A).

To test whether w+w and w+OS treated plants release GLVs in distinguishable ratios during the day and especially during the night under natural conditions we trapped volatiles from a native D. wrightii population in the Utah desert during the field season of 2011. We performed the experiments on two different days using 8 plants for each sampling. Three equally sized leaves of each plant were selected and randomly assigned to one of the treatments (control, w+w or w+OS). For each plant we estimated the total plant damage and counted the number of flowers. The variation in GLV emissions among plants was generally high. However, flower numbers and total plant damage did not account for this variation (data not shown). Similar to previous experiments (Allmann and Baldwin, 2010) we were unable to detect (Z)-3-hexenal in any of the samples.

On the first experimental day, we collected volatiles during both, day and night. During the day the application of OS to the wounds caused a significant 2.5-fold increase in the (E)-2-hexenal emissions when compared to w+w treated leaves (Fig. 4.2B and Table S4.2A). Average (Z)/(E)-ratios of the alcohol and hexenyl acetate decreased from 8 to 7 and from 13 to 9, respectively (Fig. 4.2B). However, due to high variation between plants these changes were not significant.

During the first night-experiment (1st night), plants of both treatments released very high but similar amounts of (E)-2-hexenal, and the calculated (Z)/(E)-ratios of the alcohols and hexenyl acetates were very low and did not differ between treatments, resembling the results of the night trapping in the growth chamber (Figs. 4.2B, 4.3B).
Figure 4.2. Diurnal changes in the emitted (Z)/(E) ratios of GLVs in Datura wrightii plants. 

A) Growth chamber experiment: A single not yet fully developed leaf of each Datura wrightii plant was mechanically wounded and treated with water (W) or M. sexta OS (OS) during three different light conditions to mimic day, sunset and night. B) Field experiment: Three single leaves per plant were chosen and randomly assigned to a treatment (control, w+w (W) or w+OS (OS)). Values of the control leaf were subtracted from the values of treated leaves. As (Z)-3-hexenal was not detectable in any of the field samples (E)-2-hexenal values are displayed in ng*cm^{-2}*2h^{-1}. For visual simplifications (Z)/(E)-ratios <1 are represented as their negative reciprocal. Values above ‘1’ (red dotted line) thus represent treatment-groups that produced more of the (Z)-isomer and values below ‘1’ represent treatment-groups that produced more of the (E)-isomer. Asterisks indicate significant differences between treatments (A: Mann-Whitney U test, p ≤ 0.01 (**), p ≤ 0.05 (*); n=5), (B: Wilcoxon signed-rank test, p < 0.05 (*); n=8). ADH, alcohol dehydrogenase; AAT, alcohol acyl-transferase. Day and 1st night: 3rd and 3rd to 4th of June; 2nd night: 14th to 15th of June 2010. Box outlines mark the 25% and 75% percentiles.

Approximately two weeks later we repeated the night-sampling experiment during a very calm and bright night (2nd night). This time OS-treated plants released significantly higher amounts of (E)-2-hexenal (2-fold increase compared to w+w treated plants) and the (Z)/(E)-ratios of the hexenols and hexenyl acetates were significantly lower compared to mechanically wounded plants that were treated with water only (Fig. 4.2B). Interestingly, all plants independent of the treatment had much lower total GLV emissions than during the first night-sampling (Fig. 4.3 and Tables S4.2B, C).
Figure 4.3. Total amounts of GLVs released from *Datura wrightii* plants at different times of the day in laboratory and field experiments.

Mean release of major GLVs from *Datura wrightii* plants at different times of the day and at different light intensities. Grey and white bars represent (Z)- and (E)-GLVs, respectively. Single leaves were mechanically damaged and volatiles were trapped for two hours immediately after wounds had been treated with either water (W) or with *M. sexta*’s OS (OS). **A**) GLV emissions of *Datura* plants under controlled light conditions in a growth chamber. Light conditions are explained in Figure S1. Quantities are given in nmol/g fresh mass (FM)/2h; n=5. **B**) GLV emissions of *Datura* plants naturally grown in the field. Quantities are given in pmol/cm²/2h; n=8. For an approximate comparison between (A) and (B): 50 cm² leaf area ≈ 1g FM; aldehydes, red line; alcohols, green line; acetates, blue line;

**(Z)- and (E)-GLVs show different activation patterns in the antennal lobes of Manduca sexta**

Plant volatiles provide important information for female *Manduca* moths to choose appropriate host plants for their offspring (De Moraes et al., 2001; Kessler and Baldwin, 2001; Reisenman et al., 2010). To test whether female *Manduca* moths are physiologically able to discriminate between (Z)- and (E)- GLVs and between different (Z)/(E)-ratios we performed functional calcium imaging of the antennal lobes (AL) of female *Manduca sexta* moths (Hansson et al., 2003).
Odor-evoked calcium changes to the (E)- and (Z)-isomers of hexenal, hexenol and hexenyl acetate led to discrete activity regions most likely corresponding to specific glomeruli in the AL of *M. sexta* females (Fig 4.4 A, B). Aldehyde and alcohol isomers activated one specific region (ROI 2, region of interest 2, green), with stronger responses to the (E)-2-isomers compared to (Z)-3-isomers (Fig 4.4B and Table 4.2). (Z)-3-hexenyl acetate and its (E)-2-isomer activated three different regions in the female AL: a (Z)-3-specific (ROI 3, blue), an (E)-2-specific (ROI 4, pink) and an isomer-unspecific region (ROI 1, grey, Fig 4.4B and Table 4.1). Similarity of AL activation patterns was compared by calculating the correlation coefficients for the (Z)-3 versus (E)-2-isomers ((Z) vs. (E)) of each GLV and as a control the correlation coefficients for repeated measurements of the same isomer (E/E, Z/Z, Fig 4.4C).
For (Z)-3-hexenyl acetate and its (E)-2-isomer (Z/E) correlation coefficients differed significantly from repeated stimulations with the same isomer suggesting that the two odors (Z)-3- and (E)-2-hexenyl acetate activated OSNs expressing different sets of odorant receptor types on the female antennae. Correlation coefficients did not differ for hexenal and hexenol, which leads to the assumption that for M. sexta females changes in the volatile emission of (Z)-3- and (E)-2-GLVs might primarily be detected via hexenyl acetate.

Since it is very unlikely for an insect to encounter pure isomers in nature, we decided to study the AL representation of the acetate isomers in more detail by stimulating with blends of (Z)-3- and (E)-2-hexenyl acetate in different ratios (given as Z/E: 100/0, 80/20, 50/50, 20/80, 0/100) to the female AL. In ROI 3 (blue) calcium signals evoked by (Z)-3-hexenyl acetate-containing mixtures were significantly higher compared to stimulations with pure (E)-2-hexenyl acetate, which in turn did not differ from the mineral oil control (Fig. 4.5B and Table 4.2). Similar responses were observed for the (E)-2-specific ROI 4 (pink): stimulation with pure (Z)-3-hexenyl acetate led to significantly lower calcium responses when compared to pure (E)-2-hexenyl acetate and the 20/80 ratio, but was not different from the mineral oil control (Fig. 4.5B and Table 4.2). As expected, calcium responses of the unspecific ROI 1 (in grey) did not differ between the isomers and isomeric mixtures (Table 4.2 and Fig. 4.5B).

To compare the odor-evoked activation patterns of different (Z)/(E)-ratios we calculated the ratio of ROI activation using the calcium responses evoked in (Z)-3- and (E)-2-specific regions (ROI 3, 4). Stimulations with pure isomers as well as the 20% (Z) / 80% (E) mixture led to different levels of neural activity in the isomer-specific regions resulting in a ratio of ROI activation significantly different from 1, the value which would result from equal

Figure 4.4. Calcium activity patterns of the (Z)- and (E)-isomers in the M. sexta antennal lobe (AL). (A) Morphological view onto the AL of a Manduca sexta female after bath application with the calcium-sensitive dye Calcium-Green-AM. Stimulations with any of the six tested GLVs resulted in the activation of 4 regions in the AL corresponding to single glomeruli (4 ROIs, regions of interest) (B) Representative false color-coded images show calcium responses in the AL after odor stimulation. Images are individually scaled to the strongest activation (given by the max value in each image). Time traces showing activity of ROI 1, 2, 3 and 4 (n=10) in response to odor stimulation (2sec. grey bar). Error bars represent standard error of mean. For hexenal and hexenol stimulations with (E)-2-isomers activated ROI 2 significantly higher than stimulations with (Z)-3-isomers (Wilcoxon signed-rank test: hexenal: p<0.01, hexenol: p<0.05). ΔF = change in fluorescence; F = background fluorescence. (C) Comparison of response pattern similarity for repeated stimulations of one isomer ((Z) vs. (Z) or (E) vs. (E), white boxes) and for both isomers ((E) vs. (Z), grey boxes); sample size is given above the boxes (Mann-Whitney U test: hexenal: p>0.05; hexenol: p>0.05, hexenyl acetate: p<0.001).
activation of ROI 3 and ROI 4 (Fig. 4.5C). Activation patterns differed significantly for the pure isomers, between pure (E)-2-hexenyl acetate and the 50/50 and 80/20 mixture as well as between pure (Z)-3-hexenyl acetate and the 20/80 mixture (Fig. 4.5C). However, no differences could be found between the isomeric mixtures (20/80; 50/50; 80/20).

Besides the isomer-specificity for the two hexenyl acetates, ROI 3 and 4 displayed different response characteristics which are shown in Figure 4.5D. The level of activation of the (Z)-3-hexenyl acetate specific ROI 3 (X-axis) is solely dependent on the presence of the (Z)-3-isomer and does not differ between various amounts in the isomeric mixes (ranging from 50ng in 20/80 to 250ng in 100/0) suggesting that the input gained by ROI 3 about (Z)-3-hexenyl acetate to the AL is restricted to a binary presence/absence information. In contrast, the calcium signal in ROI 4 (Y-axis) increases gradually with increasing percentage of the (E)-2-isomer in the isomeric mixtures. Thus, both ROIs act in concert to convey the information about the perceived ratio of (Z)-3- and (E)-2-hexenyl acetate.

Table 4.1. Average calcium responses (± SEM) in ROI 1-4 to the pure isomers (% ΔF/F).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Maximal change in fluorescence in % ΔF/F</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unspecific ROI 1 (grey)</td>
</tr>
<tr>
<td>(Z)-3-hexenal</td>
<td>0.36 ± 0.09</td>
</tr>
<tr>
<td>(E)-2-hexenal</td>
<td>0.44 ± 0.13</td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>0.44 ± 0.10</td>
</tr>
<tr>
<td>(E)-2-hexenol</td>
<td>0.53 ± 0.12</td>
</tr>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>0.97 ± 0.12</td>
</tr>
<tr>
<td>(E)-2-hexenyl acetate</td>
<td>0.96 ± 0.16</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>0.15 ± 0.04</td>
</tr>
</tbody>
</table>

(Z)- and (E)-GLVs induce divergent behavioral responses in ovipositing Manduca moths in nature

To test whether female Manduca moths use the herbivore-specific shift in the (Z)/(E)-ratio to choose appropriate host plants for their offspring we performed oviposition assays in the field during the field season of 2010. We selected two native populations of D. wrightii plants located close to the Lytle Preserve research station (Santa Clara, UT, USA). On each experimental day we tested two mixes that contained either only (Z)- or only (E)- GLVs or both isomers but in different ratios. Since our calcium imaging data suggested that M. sexta
possesses (Z)-3- and (E)-2-hexenyl acetate specific glomeruli we also tested these two compounds separately (see Table S4.3 for composition of each GLV-mixture). Experiments were done in a paired design (Fig. 4.6A) to minimize the volatile ‘noise’ caused by e.g. different numbers of flowers, different grades of damage or different plant ages.

When plants were perfumed with single isomeric mixtures that consisted of either (Z)- or (E)-GLVs (aldehyde, alcohol, hexenyl acetate and hexenyl butyrate, Table S4.3) Manduca moths laid significantly more eggs on the (Z)- than on the (E)-baited side of the plant (median difference of 1 egg per plant, Fig. 4.6B). When we tested two GLV mixes that contained all tested (Z)- and (E)-GLVs in a balanced isomer ratio (1:1) or in a high (Z) versus (E) ratio (9:1) we counted significantly more eggs on those sides of the plants that were scented with the higher (9:1) (Z)/(E)-ratio (median difference of 1 egg per plant, Fig. 4.6B). Finally, when we tested the two different hexenyl acetates we found on average one more egg per plant on those sides that we had scented with the (Z)-isomer (Fig. 4.6B).

**DISCUSSION**

In this study we demonstrate that the (Z)/(E)-ratio of the GLV bouquet emitted from *D. wrightii* plants differs depending on the presence or absence of *M. sexta* oral secretions at wound sites. As OS-specific changes in the (Z)/(E)-ratio were detectable during the night in field experiments, this volatile signal is likely to be encountered by ovipositing *Manduca* females searching for host plants. Optical imaging experiments showed that *M. sexta* females possess different OSN populations detecting (Z)-3- and (E)-2-hexenyl acetate, leading to different activation patterns being evoked by these two isomers in the AL. In field experiments *Manduca* females laid less eggs on plants scented with GLV mixtures with increased percentages of (E)-GLVs.

Several biotic and abiotic factors can have an influence on a plant’s volatile profile (Hare, 2011) and when studying a single stress factor, in our case simulated herbivore attack mimicked by the application of *M. sexta*’s OS onto leaf wounds, it is often necessary to perform experiments under controlled laboratory conditions. Under such controlled conditions we could show that the (Z)/(E)-ratio of GLVs released from wounded *D. wrightii* plants was clearly decreased by application of *M. sexta*’s OS onto leaf wounds. However, we also saw a positive correlation between (Z)/(E)-ratios in wounded plants and light intensities; lower light intensities resulted in lower (Z)/(E)-ratio of w+w treated *Datura* leaves (Fig. 4.2). This light-dependent decrease in the (Z)/(E)-ratio mainly resulted from a proportionally higher increase in the (E)- than in the (Z)-GLVs under low-light conditions (Fig. 4.3, Table S4.1A). It has
been shown that darkening can cause a temporary burst of GLVs in plants (Graus et al., 2004; Brilli et al., 2011). Furthermore, in *N. attenuata* the lipoxygenase NaLOX2, which specifically provides oxygenated fatty acids for the GLV-pathway, has its highest transcript levels during the night (Allmann et al., 2010), and, while this might explain the overall increase in GLVs with decreasing light intensities, it does not explain the specific increase in (E)-GLVs in w+w treated plants (Fig. 4.3). (3Z):(2E)-enal isomerase activity has been found in crude extracts of some plants (alfalfa and soybean Takamura and Gardner, 1996; Noordermeer et al., 1999), but not in *N. attenuata*, and it needs to be tested whether *Datura* plants possess such an enzyme with a nocturnal peak activity.

During the day the total amounts of GLVs emitted did not differ between treatments (Fig. 4.3). However, under simulated sunset and night conditions OS-treated *Datura* plants emitted overall less GLVs compared to w+w treated plants. It has been shown that *M. sexta* OS is able to repress the emission of certain compounds of *N. attenuata*’s volatile bouquet strongly. Suppressed metabolites included several GLVs, and this OS-induced repression could largely be attributed to the action of fatty acid-amino acid conjugates (FACs; Gaquerel et al., 2009). FACs are elicitor compounds present in the OS of *M. sexta* caterpillars (Halitschke et al., 2001) and other (lepidopteran) species (Yoshinaga et al., 2007) and they generally induce plant defenses (Bonaventure et al., 2011). *Manduca*’s OS also contain glucose oxidase (GOX; Diezel et al 2009), an enzyme that has an inhibitory effect on the plant’s direct and indirect defense responses (Musser et al., 2005; Bede et al., 2006; Delphia et al., 2006; Diezel et al., 2009). Although FACs and/or GOX could account for the observed OS-induced GLV suppression, it remains an open question why these OS-induced differences in the total GLV emissions become obvious only under low light and only under controlled laboratory conditions.

Most research on HIPVs has been done in laboratory studies under controlled conditions (Hunter, 2002; Kigathi et al., 2009). While these studies provide useful information about the influence of a single stress factor on plant-plant, plant-herbivore or tritrophic interactions they often fail to include the entity of all biotic and abiotic stresses, which influence volatile production under natural conditions (Kigathi et al., 2009). To account for this, but also to determine whether w+w and w+OS treated *Datura* plants do or do not differ in their (Z)/(E) ratios during the night, as concluded from the laboratory studies, we trapped volatiles in the field from native populations of *D. wrightii* plants. These field-grown plants released highly variable amounts of GLVs during day and night (Table S4.2), subsequently leading to a high variation in (Z)/(E)-ratios within one treatment group (Fig.
This high variation in GLV emissions between plants was likely due to several factors, including differences in the degree and history of herbivore damage (Shiojiri et al., 2010; Hare and Sun, 2011), plant age (Hare, 2010) and/or the water supply (Gouinguene and Turlings, 2002).

Figure 4.5. Female antennal lobe (AL) shows isomer specific calcium responses to (Z)-3- and (E)-2-hexenyl acetate

(A) Representative false color-coded images show calcium responses in the AL after odor stimulation. Images are individually scaled to the strongest activation (given by the max value in each image). Time traces show activity of ROI 1, 3 and 4 (n=10) in response to odor stimulation (grey bar). Error bars represent standard error of mean. (B) Change in fluorescence in ROI 1, 3 and 4 to the pure isomers and isomeric mixtures, normalized to the highest activation in every animal. Filled boxes mark responses significantly different from mineral oil (MO) control, different letters denote significantly different calcium responses (Kruskal-Wallis and Dunn’s multiple comparison test). (C) Both isomer-specific regions ROI 3 and ROI 4 are shown as ratio of ROI activation (ROI 3 / ROI 4) at stimulations with 250 ng. Asterisks indicate significant differences from 1, the ratio at which activation is equal for ROI 3 and 4 (Wilcoxon signed-rank test, 100/0: 3.6 ± 0.7, 0/100: -2.8 ± 0.3, p<0.01, 20/80: -1.45 ± 0.2, p<0.05). Isomers and isomeric mixtures were tested with Kruskal-Wallis and Dunn’s multiple comparison test, different letters denote significantly different ratios. (D) Calcium signals of ROI 3(X-axis) and ROI 4 (Y-axis) (% norm ΔF/F, separated by axes) in response to odor stimulation (colored boxes) and the solvent mineral oil (grey box). Points denote the median values, box outlines mark the 25% and 75% percentiles.
Night-GLV emissions were measured at two different dates, and while \((Z)/(E)\)-ratios were the same in w+w and w+OS treated plants during the first experimental night, shortly after new moon, they significantly differed during full moon, the second experimental night. While only little is known about lunar rhythms in plants (Vogt et al., 2002) and the scientific acceptance of such rhythms is rather low, lunar rhythms are well established for a number of insect species, especially for marine species that experience tidal fluctuations (Kaiser et al., 2011) and it needs to be tested whether these rhythms also have an influence on GLV-emissions in plants. Additionally, although quantitative differences in light intensities between the two experimental nights were not detectable in measurements they were visible to the human eye. It is known that for several volatile compounds the quantitative but also the qualitative emission pattern exhibits diurnal photoperiodicity (Loughrin et al., 1994; Turlings et al., 1995). Acyclic terpenes like \(\beta\)-farnesene and \(\beta\)-ocimene were emitted in a diurnal fashion while GLVs and few terpenes did not show such a clear diurnal pattern (Loughrin et al., 1994). Diurnal-rhythm-dependent emission was also observed in \textit{N. tabacum} after feeding by larvae of \textit{Heliothis virescens}, \textit{M. sexta} and \textit{Helicoverpa zea} as these plants released higher amounts of \((E)\)-2-hexenal during the night and emitted other GLVs exclusively in the dark period (De Moraes et al., 2001). Experiments on lima beans revealed that leaves damaged during the scotophase responded with an almost immediate nocturnal emission of \((Z)\)-3-hexenyl acetate, while the main emission of \(\beta\)-ocimene was delayed and peaked during the photophase (Arimura et al., 2008). These studies affirm that light plays an important regulatory role in volatile emissions, but it is not clear whether moonlight could serve as a sufficient light signal to regulate volatile emissions.

**Table 4.2.** Average change in fluorescence (± SEM) in hexenyl acetate-activated ROIs for the pure isomers and isomeric mixtures, normalized to the highest activation in every animal (% norm \(\Delta F/F\)).

<table>
<thead>
<tr>
<th>((Z)/(E)) %</th>
<th>Unspecific ROI 1 (grey)</th>
<th>((Z))-specific ROI 3 (blue)</th>
<th>((E))-specific ROI 4 (pink)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100/0</td>
<td>43.1 ± 10.3</td>
<td>59.8 ± 7.1</td>
<td>19.5 ± 6.7</td>
</tr>
<tr>
<td>80/20</td>
<td>48.9 ± 7.8</td>
<td>63.1 ± 7.8</td>
<td>51.8 ± 10.4</td>
</tr>
<tr>
<td>50/50</td>
<td>44.7 ± 8.4</td>
<td>63.2 ± 7.7</td>
<td>58.7 ± 6.6</td>
</tr>
<tr>
<td>20/80</td>
<td>49.5 ± 8.4</td>
<td>58.8 ± 6.0</td>
<td>80.8 ± 7.5</td>
</tr>
<tr>
<td>0/100</td>
<td>44.5 ± 9.1</td>
<td>28.6 ± 3.9</td>
<td>72.7 ± 7.1</td>
</tr>
<tr>
<td>Mineral oil</td>
<td>-1.5 ± 2.5</td>
<td>-2.3 ± 2.6</td>
<td>2.6 ± 2.6</td>
</tr>
</tbody>
</table>
In any case, GLVs seem to play an important role in volatile ‘communication’ as almost every green plant releases them upon various stress conditions. Furthermore, GLVs are released instantly from plant tissue upon damage (Turlings et al., 1995; D’Auria et al., 2007), independent of the time of the day, while terpenoids are released with a delay (Kant et al., 2004) and several terpenoids not at all during the night as they are linked to photosynthesis (Arimura et al., 2008). This makes GLVs an important cue for ovipositing Manduca moths as they are active during sunset and night (Theobald et al., 2010) and thus need to rely on signals that are released by the plant during the scotophase.

The use of volatile blends for host location by insects depends heavily on the ability to perceive and process olfactory signals. The insect’s olfactory system is highly sophisticated and enables detection of odors at very low concentrations (Bruce and Pickett, 2011). However, it is a smelly world out there and insects thus need to filter host odors against a high background noise. Plant volatiles are detected by OSNs and these can be tuned to very specific (Bruce and Pickett, 2011) or to ubiquitous host plant compounds (Hansson et al., 1999; Bruce et al., 2005). We found that stimulations with hexenal- and hexenol-isomers led to activation of one distinct region in the AL (Fig. 4.4). However, calcium signals evoked in this region by (E)-2-isomers were significantly stronger compared to (Z)-3-isomers. This difference in activation intensity may arise due to different binding affinities of the isomers to the receptor expressed by the OSNs (Hallem et al., 2004; Hooper et al., 2009) or due to differences in the abundance of isomer-specific OSNs, which project onto the same ROI in the AL (Couto et al., 2005; Fishilevich and Vosshall, 2005). In the latter case, AL processing could result in different signals for both isomers in higher brain centers like mushroom bodies or lateral horn and thus, contribute to different behavioral responses.

Of all tested compounds, only the (Z)-3- and (E)-2-isomers of hexenyl acetate activated two different discrete regions in the AL of M. sexta females (Figs. 4.4 and 4.5) suggesting different isomer-specific OSN types on the insect antenna. Given the limitation in receptor numbers to about 70 in female M. sexta (Grosse-Wilde et al., 2011) the investment in isomer-specific receptors and glomeruli to detect and process rather ubiquitous volatiles like GLVs underlines the information content transferred by the respective compounds and their ratios. Specific responses towards different types of green leaf volatiles have been reported both at the physiological (Hansson et al., 1999; Røstelien et al., 2005) and the behavioral level (Reinecke et al., 2005). Recently, Reisenmann et al (2010) reported that Manduca moths were able to distinguish between two enantiomers; while (+) linalool was attractive to ovipositing M. sexta females, (-) linalool had the adverse effect. Interestingly, Manduca did not
discriminate between these two enantiomers when foraging, which suggests that isomers can have differential effects on feeding and oviposition through different neural pathways: one that is sexually isomorphic and non-enantioselective, and another that is female-specific and enantioselective (Reisenman et al., 2010). In this context it would be interesting to test whether the isomer-specificity for hexenyl acetate of the female AL can be found also in males.

A timescale of experiment

B difference in egg numbers per plant

1) (Z)- minus (E)- isomers
2) 9:1 minus 1:1 ratio
3) (Z)- minus (E)- hexenyl acetates

isomers: p=0.022 (n=30)
ratios: p=0.001 (n=16)
acetates: p=0.001 (n=44)
For hexenyl acetate the AL activation pattern for stimulations with mixtures of both isomers were purely additive suggesting no mixture interaction at the OSN and AL input level, which is consistent with other studies (Galizia et al., 1999; Deisig et al., 2006; Carlsson et al., 2007; Silbering and Galizia, 2007). When comparing the ratio of ROI activation, we did not find any difference between the isomeric mixtures (Fig. 4.5C). This result is not surprising when taking into account the different response characteristics of ROI 3 and 4. Calcium activity of ROI 4 in response to mixes with increasing percentages of the (E)-2-isomer resembled a dose-dependent response whereas the activation of ROI 3 to the same mixes resembled more an ‘on-off’ mechanism and was thus solely dependent on the presence of the (Z)-3-isomer, leading to a constant bias towards (Z)-3-hexenyl acetate (Fig. 4.5B, D). Correspondingly, AL mixture processing and projection neuron output has been described as a highly combinatorial but predominantly non-linear scheme not necessarily reflecting ratiometric coding (Kuebler, 2011).

The different response characteristics of ROI 3 and 4 might mirror the relevance of the odors for *M. sexta* females. (Z)-3-hexenyl acetate is a rather ubiquitously occurring plant volatile, which is released in high amounts after plant damage irrespective of its origin (Arimura et al., 2008; Mumm and Dicke, 2010). Electrophysiological experiments revealed a high grade of representation of this compound in the female *M. sexta* olfactory system: 60% of the tested sensilla on female antennae (personal communication, Späthe) as well as 21 out of 34 cells in the female AL (Kuebler et al., 2011) responded to this compound. (E)-2-hexenyl acetate, in contrast, is rather rarely reported in insect-plant interactions besides its emergence after larval feeding of *M. sexta* on *N. attenuata* (Allmann and Baldwin, 2010) as an indication of actual larval damage. Thus, the presence of each isomer can contain specific information but at different levels of resolution. In the case of (Z)-3-hexenyl acetate, the detected signal

**Figure 4.6. Manduca moths laid more eggs on the (Z)- than on the (E)-baited side of the plant.**

A) The effect of different GLV-mixes on the ovipositing behavior of female *Manduca* moths was tested during the field season 2010 on native *Datura wrightii* plants. On each experimental day two different mixes were tested in a paired design. Mixes used on different experimental days are plotted above the timeline. The composition of each mixture is described in Table S3. B) Difference in egg numbers oviposited per plant. Higher oviposition rates were observed for the (Z)-baited side of the *Datura* plant, while the (E)-baited side had less oviposited eggs. The composition of all mixtures is described in Table S3. Treatment pairs with no oviposited eggs were excluded prior to the statistical analysis (Wilcoxon signed-rank test). Mean (±SEM) number of eggs oviposited per plant: (Z)-3-hexenyl acetate 1.5 ± 0.4, (E)-2-hexenyl acetate 0.89 ±0.2; (Z)-isomers 1.0 ± 0.2, (E)-isomers 0.45 ±0.1; “9:1”-ratio 1.75 ± 0.2; “1:1”-ratio 0.56 ± 0.2
might also be relevant in long-range host location and host choice. Information about \((E)-2\)-hexenyl acetate, gained by ROI 4 in a dose-dependent fashion should be most valuable at short distance to the plant, possibly to locate the best spot for oviposition depending on the actual amounts emitted by different plant sites.

Experiments in which insects have been exposed to single volatile compounds or to volatile mixtures have revealed that volatile blends evoke a stronger behavioral response than single compounds (Bruce et al., 2005; Bruce and Pickett, 2011; van Wijk et al., 2011). In a recent study Riffell et al. (2009) showed that foraging Manduca moths responded only to flower-specific volatile mixtures with a host-directed flight behavior and not when random mixes or single compounds were given as odor source. Furthermore, numerous studies suggest that the ratio of plant volatiles is an important component of the olfactory signal (Visser and Avé, 1978; Bruce et al., 2005; Bruce et al., 2009; Cha et al., 2011). In the case of the oriental fruit moth Grapholita molesta the ratio of a minor compound to the remaining components of a plant-derived synthetic mix determined the behavioral acceptance of this mix, which could be linked to the response of two glomeruli in the female AL (Piñero et al., 2008; Najar-Rodriguez et al., 2010).

We found that ovipositing Manduca moths distinguished between different \((Z)/(E)\)-ratios and that they used these volatile cues to choose appropriate oviposition sites. However, independent of whether the mixes tested were rather complex in their composition (9:1 vs. 1:1 ratios), or less complex (only \((Z)\)-GLVs vs. only \((E)\)-GLVs), or consisted only of a single compound ((Z)-3-hexenyl acetate vs. \((E)-2\)-hexenyl acetate) ovipositing Manduca moths continuously made a choice and always preferred those sides of the plant that smelled more like \((Z)\)-GLVs, or less like \((E)\)-GLVs (Fig. 4.6B). From our results we cannot conclude whether a complex GLV bouquet in different ratios provided more reliable information for the moths than single compounds, but our results do show that adding one single component to the background volatile bouquet of native D. wrightii plants altered the choice of ovipositing Manduca moths.

CONCLUSION

Nature is not only colorful but also very fragrant as almost every green plant releases volatiles in a highly variable fashion. This makes it a challenge for host searching insects to simultaneously extract useful information while flying through the odor plumes of multiple sources. Our results show that the antennal lobe, the first odor processing center of the insect
brain, has the capacity to resolve the composition of GLV blends as emitted by highly relevant host plants. Correspondingly, gravid females make an informed choice. They prefer oviposition sites where predation and competition risks are reduced for their offspring, as indicated by the plant volatile bouquet. Future work will reveal whether increasing amounts of (E)-GLVs or rather changes in the (Z)/(E)-ratio at the background of other host odors provide the crucial information for female Manduca moths. Little is known about nocturnal volatile emissions from vegetative tissues and, thus, more work is needed to understand their regulatory mechanisms.

MATERIAL AND METHODS

Plant Material and Growing Conditions

Datura wrightii seeds were initially purchased from B & T World Seeds, Paguignan, France and subsequently harvested from plants propagated in the greenhouse. Plants were grown in 2 L pots in the greenhouse (23 to 25°C, 50-70 % humidity, 16 h light supplemented by Philips Sun-T Agro 400 W Na-vapor bulbs, 350 - 500 µmol m² s⁻¹ photosynthetic photon flux at plant level) and used for experiments 35 - 40 days after sowing.

For field experiments we used native populations of D. wrightii plants which were located close to the Lytle Preserve research station (Santa Clara, UT, USA).

Plant Treatments

For all treatments in the growth chamber and in the field plants were wounded with a fabric pattern wheel to punch three rows of holes on each side of the midrib. Wounded leaves were immediately treated with 20 µL of deionized water (w+w) or with 1:3 (v/v) diluted Manduca sexta oral secretions (w+OS) which were pipetted directly onto the wounded leaf and gently dispersed across the surface. The OS was collected from 3rd to 5th instar caterpillars which were fed on D. wrightii plants, and OS was stored at -20°C until usage. Prior to use OS was centrifuged to remove any particles.

Volatile Collection

Volatile collections were performed in a growth chamber (temperature 23-25°C, humidity 50-60%) on shelves equipped with diode arrays of white (ca 420 – 690 nm), red (630 – 690 nm) and UV (380 – 420 nm). Light intensity and composition of the diode types were changed within 24 h to simulate sunrise, daylight, sunset and night conditions accordingly (Details of the program are described in Fig S1).
D. wrightii plants were placed in the chamber two days prior the experiment to acclimatize. On the experimental day one single leaf per plant was enclosed immediately after treatment between two 50 mL food-quality plastic containers (Huhtamaki, Bad Bertricher, Germany; www.polarcups.de) secured with miniature claw-style hair clips. Ambient air was pulled through the collection chamber and a glass tube (ARS, Inc., Gainsville, FL, USA; www.ars-fla.com) packed with glass wool and 20 mg of Super Q (Alltech, Düsseldorf, Germany; www.alltech.com). Airflow was created by a vacuum pump (model DAAV114-GB, Gast Mfg, Benton Harbour, MI, USA; www.gastmfg.com) as described by Halitschke et al. (2000). For each time point and each treatment we trapped volatiles from five replicate plants. Directly after volatile sampling we determined the fresh mass (FM) of each trapped leaf for further calculations.

In the field, we selected 8 plants for each measurement in a 10 m radius. For each plant we estimated the total leaf damage and we counted the number of flowers. To account for differences in volatile emissions caused by different degrees of leaf damage we selected 3 equally sized leaves of each plant and randomly assigned each leaf to one of the treatments (control, w+w or w+OS). Since trappings were done in the field we did not measure the fresh mass of the trapped leaves but photographed each leaf and calculated the leaf area. We subsequently subtracted the amounts of volatiles emitted from untreated control leaves from those emitted from treated leaves of the same plant. We used a Li-COR Li-250A light meter with a Li-190SA quantum sensor (http://www.licor.com) to measure the photosynthetic active radiation during the different trapping periods. The first two trappings were done on the 3rd and 4th of June, closely after new moon. During the day volatiles were sampled from 1:30 to 3:30 pm at an average light intensity of 1450 µmol s⁻¹ m⁻². During the night volatiles were sampled from 0 to 2 am; light intensity was below the detection limit. The second trapping was done in the night from the 14th to the 15th of June. Although it was a very calm and bright night (full moon) the average light intensity was below the detection limit. On the experimental day we enclosed single leaves directly after elicitation in polystyrene chambers fitted with holes at both ends. Air was pulled through the chamber and subsequently through a single-use charcoal trap (Orbo M32, Sigma-Aldrich, Germany) as described in Kessler and Baldwin (2001). Charcoal traps were equipped with MnO₂-coated copper gauze as ozone scrubbers (OBE Corporation, Fredericksburg, TX, USA) to prevent oxidation of volatiles.

In all experiments volatiles were trapped for 2 h immediately after elicitation.
Both, charcoal and SuperQ traps were eluted with 250 μL dichloromethane (DCM) into a GC vial after spiking each SuperQ trap with 320 ng and each charcoal trap with 240 ng tetralin (Sigma-Aldrich, Germany; www.sigmaaldrich.com) as an internal standard.

**Volatile Analysis and Quantification**

Samples were analyzed on an Agilent 7890A instrument (Agilent Technologies, CA, USA) with the injection port kept at 230°C, operated in split-less mode and connected to an Agilent 5975C mass spectrometer. One μL of each sample was injected on a polar column (Innowax; 30 m, 0.25 mm ID, 0.25 μm film thickness; J&W Scientific, Folsom CA, USA) operated under a constant He flow of 1.1 mL/min. The MS was operated in electron impact mode (70 eV) with the ion source at 230°C and the quadrupole at 150°C. The GC oven was programmed to hold 40°C for 5 min, to increase the temperature at 5°C/min to 130°C, then increasing temperature at 30°C/min to a maximum of 240°C. The maximum temperature was held for 15 min. The transfer line to the MS was kept at 260°C. To increase detector sensitivity the MS was operated in selected ion monitoring (SIM) mode (hexenals: m/z 55, 69, 83; hexenols: m/z 55, 57, 67, 82; hexenyl acetates: m/z 67, 71, 82; tetralin: m/z 104, 132).

GLVs were identified using standard solutions of (Z)-3-hexenal, (E)-2-hexenal, (Z)-3-hexenol, (E)-2-hexenol, (Z)-3-hexenyl acetate, (E)-2-hexenyl acetate, (Sigma-Aldrich, Germany; www.sigmaaldrich.com) and quantifications were done with calibration curves for each compound (33 ng, 10 ng, 5 ng, 1 ng, 0.5 ng, 0.1 ng; n = 3 replicates) using single ion traces (hexenal m/z 83, hexenol and hexenyl acetate m/z 82). Emission rates were calculated based on fresh mass or surface area of the sampled leaves.

(Z)/(E)-ratios were calculated for each sample dividing the amount of the (Z)-GLV by the amount of its corresponding (E)-isomer. For visual simplifications (Z)/(E)-ratios <1 were depicted as their negative reciprocal.

**Insect rearing**

*Manduca sexta* (Lepidoptera, Sphingidae) females were reared as described in (Grosse-Wilde et al., 2011). Pupae were kept separately in paper bags at 25°C and 70% relative humidity under a 16 h:8 h light/dark cycle until beginning of physiological experiments. Naïve adult females were tested 2-4 days post emergence.
CHAPTER 4

Odorants tested with optical imaging

Odors were chosen based on the results of a previous study (Allmann and Baldwin, 2010) and on volatile collections of D. wrightii plants done for this study (Fig 1). We tested GLVs that showed a change in the ratio of (Z)-3- and (E)-2-isomers in the volatile emission of N. attenuata and D. wrightii plants caused by the presence of oral secretion of M. sexta larvae in plant wounds: hexenal, hexenol and hexenyl acetate (Sigma Aldrich, Germany; www.sigmaaldrich.com). Odors were diluted in mineral oil (Sigma Aldrich, Germany; www.sigmaaldrich.com) and used in doses of 25 ng, 250 ng and 2500 ng for the comparison of pure isomers. Hexenyl acetate was additionally tested as percentage mixtures of its (Z)-3- and (E)-2 isomers ranging from 0/100, 20/80, 50/50, 80/20 to 100/0 percent (v/v) in doses of 250 ng and 1250 ng.

Preparation and staining of adult females of Manduca sexta

Moths were restrained in 15 ml Falcon tubes with the head exposed and fixed with dental wax (Surgident, Heraeus Kulzer, Dormagen, Germany). The head capsule was opened and all tissues covering the antennal lobes were carefully removed. The brain was stained with Calcium Green-2 AM (30 μmol; Invitrogen, Darmstadt, Germany, http://www.invitrogen.com) containing physiological saline solution with 6% Pluronic F-127, (Invitrogen, Darmstadt, Germany; http://www.invitrogen.com) for 90 min at 4°C. After staining the brain was rinsed several times with Ringer’s solution to remove remaining dye.

Optical imaging of the antennal lobes

For imaging we used a Till Photonics imaging system (Martinsried, Germany) equipped with a CCD camera (PCO Imaging, Sensicam) connected to an upright microscope (Olympus BX51WI). Monochromatic excitation light was given at 475 nm (500 nm SP; Xenon arc lamp, Polychrome V) and fluorescence was detected with a LP515 emission filter and transmitted by a DCLP490 dicroic filter. The set-up was controlled by the software Tillvision 4.0 (Till Photonics). Images were taken with a water immersion objective (Olympus, 10x/0.30). Four-fold symmetrical binning resulted in image sizes of 344 x 260 pixels with one pixel corresponding to an area of 4 μm x 4 μm (10 x magnifications).

Odorant stimulation

Six μL of the odorant mixtures were pipetted freshly prior to every experiment on a filter paper (Whatman, http://www.whatman.com/) in glass pipettes using doses of 25 ng,
250 ng, 1250 ng and 2500 ng, respectively. The same volume of mineral oil served as a control stimulus. The glass pipette was inserted into a glass tube which delivered a constant flow of clean humidified air (1 L/min) along one antenna. Alteration of the airflow during stimulation was prevented by a second glass pipette adding a continuous clean airstream (0.1 L/min) which could be switched to the odor-containing pipette (Syntech Stimulus Controller CS-55).

Every stimulation experiment lasted for 10 sec, with 2 sec of odor stimulation. Inter-stimulus time of at least 1 min was chosen to reduce adaptation effects. Every mix was presented first in the lower concentration. The animal’s response to the higher concentration was only analyzed in absence of a response to the lower one. The sequence of the stimulations was changed from animal to animal. In some females (hexenal n=5, hexenol n=7, hexenyl acetate n=4), the odors were repeatedly measured to test for the reproducibility of the evoked activity patterns within an animal.

**Processing of optical imaging data**

All stimulation experiments were recorded with 4 Hz resulting in a series of 40 consecutive frames which were analyzed with custom written software (IDL, ITT Visual Informations Solutions). Data were corrected for background fluorescence, bleaching of the dye and movement during the measurement to increase the signal-to-noise ratio (Sachse and Galizia, 2002, 2003). Additionally, a spatial median filter with a width of 5 pixels was applied to remove outliers.

Odor responses represented as change in fluorescence ($\Delta F/F$) at spatially distinct activity spots were recorded at the spot center in an area of the size of a small to medium-sized glomerulus (60 µm x 60 µm). Time traces of $\Delta F/F$ were smoothed by averaging three successive frames for each activity spot. The maximum $\Delta F/F$ value after stimulus onset was averaged with the pre- and postmaximum value. For every animal the odor responses were normalized to the maximal response and were taken into account if they reached $\geq 30\%$ of the maximal value in this animal in at least one activity spot.

**Analysis of activity patterns in the moth antennal lobe (AL)**

Due to the lack of a glomerular map in the *M. sexta* AL observed activity regions for the tested odors can’t be directly compared between animals. Thus, for every isomeric pair of hexenal, hexenol and hexenyl acetate observed activation patterns were used to calculate
correlation coefficients providing a relative measurement of similarity. Repeated stimulations with the same isomer and the correlation coefficients thereof were used as control.

To compare activity patterns between the different isomeric mixtures of hexenyl acetate we calculated the difference in activity of both isomer-specific glomeruli resulting from the ratio of activity in the (Z)-specific and the (E)-specific glomerulus. For visual simplifications values below 1 (representing cases in which the (E)-specific glomerulus was more active than the (Z)-specific glomerulus) were displayed as their negative reciprocal and all values were presented on a scale without the range between “-1” and “1” (Fig. 4.5C).

**Oviposition Assay in the Field**

Experiments were done between 26th of May and 1st of July 2010 in southwestern Utah. This area is part of the native habitat of the tobacco and tomato hawkmoths *Manduca sexta* and *M. quinquemaculata*. Eggs of both species were counted for this experiment. We selected between 15 and 17 plants of two native populations of *D. wrightii* plants which were located close to the Lytle Preserve research station (Santa Clara, UT, USA). All plants were carefully inspected and oviposited *Manduca* eggs were removed prior the experiment. On each experimental day two mixes were tested in a paired design: every evening before sunset cotton swabs were dipped into the GLV-scented lanolin pastes and stuck onto two opposing branches of one *Datura* plant. This paired design was chosen to minimize the effect that different numbers of flowers or different grades of leaf damage might have on the oviposition behavior of the moths. On the next day freshly laid *Manduca* eggs were counted in a defined area on the plant, approx 30 cm around the scented cotton swabs and afterwards removed. Treatment sides were switched every day. The GLV-scented lanolin pastes were prepared by warming up lanolin and adding different GLV-mixtures to the liquefied lanolin paste shortly before it solidified again. The GLV mixes used are described in Table S4.3.

**Statistical analysis**

All statistical tests were performed with SPSS 17.0 and XLSTAT 2009 software packages.

**ACKNOWLEDGEMENTS**

We thank Mariana Stanton for her help with the volatile trapping experiments in the field and the Brigham Young University for the use of their Lytle Ranch Preserve field station.
REFERENCES


Bate NJ, Rothstein SJ (1998) C-6-volatiles derived from the lipoxygenase pathway induce a subset of defense-related genes. Plant Journal 16: 561-569


CHAPTER 4


Gaquerel E, Weinhold A, Baldwin IT (2009) Molecular Interactions between the Specialist Herbivore Manduca sexta (Lepidoptera, Sphingidae) and Its Natural Host Nicotiana attenuata. VIII. An Unbiased GCxGC-ToFMS Analysis of the Plant's Elicited Volatile Emissions. Plant Physiology 149: 1408-1423


Hare JD, Sun J (2011) Production of Induced Volatiles by Datura wrightii in Response to Damage by Insects: Effect of Herbivore Species and Time. Journal of Chemical Ecology 37: 751-764

Hare JD (2011) Ecological Role of Volatiles Produced by Plants in Response to Damage by Herbivorous Insects. Annual Review of Entomology 56: 161-180


(Z)/(E)-CONVERSION OF GLVS AS SIGNAL FOR MANDUCA


Madden AH, Chamberlain FS (1945) Biology of the tobacco hornworm in the southern cigar-tobacco district. In, Vol 896, Div. of Truck Crop and Garden Investigations, Bureau of Entomology and Plant Quarantine; Agricultural Research Administration


Piñero JC, Giovanni Galizia C, Dorn S (2008) Synergistic behavioral responses of female oriental fruit moths (Lepidoptera:Tortricidae) to synthetic host plant-derived mixtures are mirrored by odor-evoked calcium activity in their antennal lobes. Journal of Insect Physiology 54: 333-343


Sabelis M, Van De Baan H (1983) Location of distant spider mite colonies by phytoseid predators: Demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. Entomologia Experimentalis et Applicata 33: 303-314


### Supporting Information

#### Table S4.1A. GLV emission of *Datura wrightii* plants in the first 2h after w+w or w+OS treatment with 100% light (day).

<table>
<thead>
<tr>
<th>class</th>
<th>common Name</th>
<th>RT</th>
<th>volatile release in nmol / g fresh mass</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>(Z)-3-hexenal</td>
<td>8.54</td>
<td>6.48 ± 2.98</td>
<td>0.140</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenal</td>
<td>10.49</td>
<td>2.25 ± 1.11</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-3-hexenol</td>
<td>14.98</td>
<td>13.01 ± 5.10</td>
<td>0.696</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>15.57</td>
<td>0.58 ± 0.34</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>hexenylester</td>
<td>(Z)-3-hexenyl acetate</td>
<td>13.28</td>
<td>11.21 ± 3.11</td>
<td>0.536</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>13.75</td>
<td>0.12 ± 0.03</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>17.07</td>
<td>0.17 ± 0.05</td>
<td>0.245</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>17.44</td>
<td>0.04 ± 0.02</td>
<td>0.209</td>
</tr>
</tbody>
</table>

#### Table S4.1B. GLV emission of *Datura wrightii* plants in the first 2h after w+w or w+OS treatment with 20 - 10% light (sunset).

<table>
<thead>
<tr>
<th>class</th>
<th>common Name</th>
<th>RT</th>
<th>volatile release in nmol / g fresh mass</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>(Z)-3-hexenal</td>
<td>8.54</td>
<td>16.50 ± 5.10</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenal</td>
<td>10.49</td>
<td>13.09 ± 7.89</td>
<td>0.708</td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-3-hexenol</td>
<td>14.98</td>
<td>16.22 ± 4.32</td>
<td>0.225</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>15.57</td>
<td>4.52 ± 3.14</td>
<td>0.964</td>
</tr>
<tr>
<td>hexenylester</td>
<td>(Z)-3-hexenyl acetate</td>
<td>13.28</td>
<td>11.37 ± 3.03</td>
<td>0.633</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>13.75</td>
<td>1.25 ± 0.84</td>
<td>0.894</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>17.07</td>
<td>0.23 ± 0.06</td>
<td>0.279</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>17.44</td>
<td>0.08 ± 0.02</td>
<td>0.554</td>
</tr>
</tbody>
</table>

#### Table S4.1C. GLV emission of *Datura wrightii* plants in the first 2h after w+w or w+OS treatment with 0% light (night).

<table>
<thead>
<tr>
<th>class</th>
<th>common Name</th>
<th>RT</th>
<th>volatile release in nmol / g fresh mass</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>(Z)-3-hexenal</td>
<td>8.54</td>
<td>17.48 ± 7.46</td>
<td>0.121</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenal</td>
<td>10.49</td>
<td>24.72 ± 5.31</td>
<td>0.093</td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-3-hexenol</td>
<td>14.98</td>
<td>11.81 ± 3.50</td>
<td>0.463</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>15.57</td>
<td>7.91 ± 1.40</td>
<td>&lt; 0.05</td>
</tr>
<tr>
<td>hexenylester</td>
<td>(Z)-3-hexenyl acetate</td>
<td>13.28</td>
<td>4.41 ± 1.88</td>
<td>0.835</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>13.75</td>
<td>0.65 ± 0.28</td>
<td>0.847</td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>17.07</td>
<td>0.13 ± 0.05</td>
<td>0.904</td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>17.44</td>
<td>0.06 ± 0.01</td>
<td>0.758</td>
</tr>
</tbody>
</table>

Mean (± SEM; n=5) release of GLVs in *Datura Wrightii* plants. A single not yet fully developed leaf of each plant was mechanically wounded and treated with water (w+w) or *M. sexta* OS (w+OS) during the day (A, 100% light), sunset (B, 20-10% light) and night (C, 0% light). Volatiles are listed by chemical classes and with their retention time. Differences between w+w and w+OS emissions were determined with a Student’s t-test and significant differences were displayed in bold (p<0.05).
Table S4.2A. GLV emission of native *Datura wrightii* plants in the field (2011) during the first 2h after w+w or w+OS treatment; Day (1:30-3:30pm).

<table>
<thead>
<tr>
<th>class</th>
<th>common Name</th>
<th>RT</th>
<th>volatile release in nmol / cm² leaf</th>
<th>control</th>
<th>w + w</th>
<th>w + OS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>(E)-2-hexenal</td>
<td>10.87</td>
<td>0.062 ± 0.006</td>
<td>1.016 ± 0.233</td>
<td>2.433 ± 0.597</td>
<td>&lt;0.05</td>
<td></td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-3-hexenol</td>
<td>15.38</td>
<td>0.137 ± 0.067</td>
<td>1.212 ± 0.280</td>
<td>2.074 ± 0.465</td>
<td>0.063</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>15.97</td>
<td>0.248 ± 0.035</td>
<td>0.368 ± 0.088</td>
<td>0.571 ± 0.148</td>
<td>0.068</td>
<td></td>
</tr>
<tr>
<td>hexenylester</td>
<td>(Z)-3-hexenyl acetate</td>
<td>13.66</td>
<td>0.260 ± 0.083</td>
<td>11.138 ± 1.881</td>
<td>12.277 ± 2.067</td>
<td>0.692</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>14.13</td>
<td>0.010 ± 0.002</td>
<td>0.872 ± 0.396</td>
<td>1.342 ± 0.564</td>
<td>0.053</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>17.44</td>
<td>0.011 ± 0.002</td>
<td>0.217 ± 0.181</td>
<td>0.193 ± 0.142</td>
<td>0.577</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>17.8</td>
<td>0.004 ± 0.001</td>
<td>0.006 ± 0.002</td>
<td>0.006 ± 0.002</td>
<td>0.305</td>
<td></td>
</tr>
</tbody>
</table>

Table S4.2B. GLV emission of native *Datura wrightii* plants in the field during the first 2h after w+w or w+OS treatment; 1st night (0-2am).

<table>
<thead>
<tr>
<th>class</th>
<th>common Name</th>
<th>RT</th>
<th>volatile release in nmol / cm² leaf</th>
<th>control</th>
<th>w + w</th>
<th>w + OS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>(E)-2-hexenal</td>
<td>10.87</td>
<td>0.103 ± 0.013</td>
<td>24.592 ± 7.844</td>
<td>22.493 ± 5.312</td>
<td>0.846</td>
<td></td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-3-hexenol</td>
<td>15.38</td>
<td>0.032 ± 0.006</td>
<td>9.573 ± 2.028</td>
<td>8.202 ± 3.734</td>
<td>0.695</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>15.97</td>
<td>0.296 ± 0.023</td>
<td>4.121 ± 0.955</td>
<td>2.894 ± 0.855</td>
<td>0.263</td>
<td></td>
</tr>
<tr>
<td>hexenylester</td>
<td>(Z)-3-hexenyl acetate</td>
<td>13.66</td>
<td>0.165 ± 0.028</td>
<td>10.684 ± 3.621</td>
<td>11.527 ± 4.291</td>
<td>0.884</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>14.13</td>
<td>0.009 ± 0.001</td>
<td>1.137 ± 0.371</td>
<td>1.151 ± 0.306</td>
<td>0.980</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>17.44</td>
<td>0.006 ± 0.000</td>
<td>0.022 ± 0.008</td>
<td>0.040 ± 0.022</td>
<td>0.314</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>17.8</td>
<td>0.002 ± 0.000</td>
<td>0.006 ± 0.002</td>
<td>0.007 ± 0.003</td>
<td>0.865</td>
<td></td>
</tr>
</tbody>
</table>

Table S4.2C. GLV emission of native *Datura wrightii* plants in the field during the first 2h after w+w or w+OS treatment; 2nd night (0-2am).

<table>
<thead>
<tr>
<th>class</th>
<th>common Name</th>
<th>RT</th>
<th>volatile release in nmol / cm² leaf</th>
<th>control</th>
<th>w + w</th>
<th>w + OS</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>aldehyde</td>
<td>(E)-2-hexenal</td>
<td>10.87</td>
<td>0.057 ± 0.009</td>
<td>4.720 ± 1.878</td>
<td>9.525 ± 4.010</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td>alcohol</td>
<td>(Z)-3-hexenol</td>
<td>15.38</td>
<td>0.036 ± 0.018</td>
<td>3.954 ± 1.221</td>
<td>2.939 ± 0.519</td>
<td>0.433</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenol</td>
<td>15.97</td>
<td>0.185 ± 0.020</td>
<td>0.974 ± 0.431</td>
<td>1.454 ± 0.559</td>
<td>0.399</td>
<td></td>
</tr>
<tr>
<td>hexenylester</td>
<td>(Z)-3-hexenyl acetate</td>
<td>13.66</td>
<td>0.097 ± 0.028</td>
<td>4.787 ± 2.199</td>
<td>9.381 ± 4.713</td>
<td>0.385</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl acetate</td>
<td>14.13</td>
<td>0.010 ± 0.003</td>
<td>0.741 ± 0.505</td>
<td>1.764 ± 0.973</td>
<td>0.325</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Z)-3-hexenyl butyrate</td>
<td>17.44</td>
<td>0.001 ± 0.001</td>
<td>0.024 ± 0.015</td>
<td>0.035 ± 0.012</td>
<td>0.375</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(E)-2-hexenyl butyrate</td>
<td>17.8</td>
<td>0.001 ± 0.001</td>
<td>0.004 ± 0.003</td>
<td>0.006 ± 0.002</td>
<td>0.644</td>
<td></td>
</tr>
</tbody>
</table>

Mean (± SEM; n=5) release of GLVs in *Datura Wrightii* plants in nature. A single not yet fully developed leaf of each plant was mechanically wounded and treated with water (w+w) or *M. sexta* OS (w+OS) during the day (A, 1:30-3:30pm) and during night (B, 1st night, C, 2nd night, 0-2am). Volatiles are listed by chemical classes and with their retention time. Differences between w+w and w+OS emissions were determined with a paired sample T-test and significant differences were displayed in bold (p<0.05).
Table S4.3. GLV-mixtures used for oviposition assay in the field.

<table>
<thead>
<tr>
<th>Compounds (common names)</th>
<th>(Z)/(E)-mix 1:1; = w+OS (µg/µl lanolin)</th>
<th>(Z)/(E)-mix 9:1; = w+w (µg/µl lanolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Z)-3-hexenal (50% in triacetin)</td>
<td>5.0</td>
<td>9.0</td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>5.0</td>
<td>9.0</td>
</tr>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>(Z)-3-hexenyl butyrate</td>
<td>0.05</td>
<td>0.09</td>
</tr>
<tr>
<td>(E)-2-hexenal</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(E)-2-hexenol</td>
<td>5.0</td>
<td>1.0</td>
</tr>
<tr>
<td>(E)-2-hexenyl acetate</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>(E)-2-hexenyl butyrate</td>
<td>0.05</td>
<td>0.01</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds (common names)</th>
<th>Only (Z)-mix (µg/µl lanolin)</th>
<th>Only (E)-mix (µg/µl lanolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Z)-3-hexenal (50% in triacetin)</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(Z)-3-hexenol</td>
<td>10.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>(Z)-3-hexenyl butyrate</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>(E)-2-hexenal</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>(E)-2-hexenol</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>(E)-2-hexenyl acetate</td>
<td>0.0</td>
<td>0.1</td>
</tr>
<tr>
<td>(E)-2-hexenyl butyrate</td>
<td>0.0</td>
<td>0.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compounds (common names)</th>
<th>(Z)-3-hexenyl acetate (µg/µl lanolin)</th>
<th>(E)-2-hexenyl acetate (µg/µl lanolin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Z)-3-hexenyl acetate</td>
<td>5.0</td>
<td>0.0</td>
</tr>
<tr>
<td>(E)-2-hexenyl acetate</td>
<td>0.0</td>
<td>5.0</td>
</tr>
</tbody>
</table>
Figure S4.1. Light conditions during laboratory volatile collection
Light composition and intensity changed within 24 hours to simulate day (1), sunset (2) and night (3) condition. Photosynthetically active radiation (PAR, μmol photons*m⁻²*s⁻¹) was measured for every light composition and ranged from 0.39 ± 0.01 SE at night to 138.37 ± 0.09 SE at full day conditions. For the graph values were logarithmized. Grey areas denote volatile collection events, respective light spectra are shown on the right. For representational reasons time scale starts at 2 am.