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Plant Species- and Status-specific Odorant Blends Guide Oviposition Choice in the Moth *Manduca sexta*

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Abstract

The reproductive success of herbivorous insects largely depends on the mother's oviposition preference. In nocturnal insects, olfaction is arguably the most important sensory modality mediating mate finding, foraging, and host location. In most habitats, gravid females select among a number of plants of varying suitability, yet assessment of the neuroethological mechanisms underlying odor-guided choice between host plants is rare. Using a series of behavioral, electrophysiological, and chromatographic analyses in the Hawk moth, *Manduca sexta*, we show that gravid females perform a hierarchical choice among host plants of different species and qualities using olfactory cues. Both relevant plant species and qualities can be distinguished by volatile profiles collected from the headspace of these plants, and olfactory sensilla on female antennae detect more than half of the about 120 analytically detected volatiles in host plant headspace samples. Although olfactory sensory neurons present in antennal sensilla are mainly broadly tuned to multiple host compounds, some sensilla exhibit species and condition-specific responses. In fact, species and quality can be distinguished by the physiologically active components alone. Our findings thus suggest that distinguishing characteristics of both host species and quality are already represented at the sensory periphery.

Key words: *GC-SSR*, host plant choice, *Manduca sexta*, moth, olfaction

Introduction

For animals that do not directly supply food and care to the next generation, the choice of brood site becomes vital for reproductive success. Early life stages are often less mobile (e.g., eggs or larvae), and female substrate choice becomes instrumental in placing eggs in a nourishing and protective environment. Most insects fall into this category, in which an egg-depositing female is often dead when the offspring emerge. For insects accepting several plant species, different criteria define a suitable larval host, for example, palatability, nutritional quality, and shelter from enemies (Courtney et al. 1989; Thompson and Pellmyr 1991; Mayhew 1997). Additionally, ovipositing females encounter ongoing herbivory and predation risks augmented by the plant's induced defense system further shaping host preference (Murphy 2004; Singer et al. 2004). As most moths are nocturnal and/or crepuscular, they have developed a high dependence on olfactory input. This consequently necessitates an olfactory system designed to detect odor cues indicating host plant identity and quality. A number of studies (Hansson et al. 1999; Kalinova et al. 2001; Shields and Hildebrand 2001; Røstelien et al. 2005) have characterized the specificity and sensitivity of antennal olfactory sensory neurons (OSNs) in detecting plant-related odor information and delivering it to early processing centers of the brain. Responses to individual host species or specific volatile compounds have also been investigated (Matsumoto and Hildebrand 1981; Eisthen 2002; Mechaber et al. 2002; Fraser et al. 2003; Reisenman et al. 2004). Direct comparisons of choice among host plants and olfactory reception are, however, rare, and even more so when considering ecologically relevant interactions.

Manduca sexta is a large sphingid moth primarily occurring in arid areas of North and South America. It has been studied as a model system both for insect olfaction (Matsumoto and Hildebrand 1981; Shields and Hildebrand 2001; Eisthen 2002; Reisenman et al. 2004) and for host and food search (Yamamoto and Fraenkel 1960; Mechaber et al. 2002; Fraser et al. 2003; Riffell et al. 2009; Reisenman et al. 2010). These moths choose within a range of host plants, with 3 highly distinctive species playing important roles: *Datura wrightii* (jimsonweed) (Yamamoto and Fraenkel 1960), *Proboscidea parviflora* (devil's claw) (Mechaber and Hildebrand 2000; Mira and Bernays 2002), and *Nicotiana attenuata* (wild tobacco) (Yamamoto and Fraenkel 1960). In the Great Basin of southwestern Utah, United States of America, the impact of *Manduca* larvae in the local ecosystem has been studied in detail (Baldwin et al. 2001). Classic experiments with *N. attenuata* have shown how manipulations of plant volatile emissions can have profound effects on recruitment of the herbivore and its predators (Kessler and Baldwin 2001). Congeneric *M. quinquemaculata* prefer to oviposit on undamaged *N. attenuata* plants compared with plants damaged by feeding *M. sexta* larvae (Baldwin et al. 2001; Kessler and Baldwin 2001). Damaged *N. attenuata* plants in turn produce blends of induced volatiles that are specific for the feeding herbivore, and are used as signals by predators (Halitschke et al. 2001; Kessler and Baldwin 2001). This emission of induced volatiles in concert with direct defenses via secondary metabolites also leads to decreasing larval performance and survival (Kessler and Baldwin 2004). Feeding-induced blends of volatiles from *D. wrightii*, in contrast, do not consistently differ between herbivore species and are rather a general indicator of herbivory (Hare and Sun 2011).

To assess the role of plant odor cues in these condition- and species-dependent oviposition choices, we performed behavioral, chemical, and linked chemical-electrophysiological investigations of the herbivore *M. sexta*. The main questions were as follows: 1) Do females exhibit a choice for oviposition when provided with a selection of host plant species and qualities? 2) Is this choice based on olfactory input? 3) Which plant chemicals potentially constitute the semiochemicals emitted by the plant? and 4) Which of these are detected by OSNs on the female moth antenna? Together our results show that host plant choice exhibited by female *M. sexta* moths is reflected in species- and quality-specific volatile emissions detected by a combinatorial population of both selective and nonselective antennal OSNs.

Materials and methods

Insect rearing

Adult *M. sexta* were maintained at ambient conditions and provided with *N. attenuata* plants for oviposition. Eggs and larvae were kept at 27 °C and 70% humidity on artificial diet (Grosse-Wilde et al. 2011). Wandering fifth instar larvae were separated individually and pupae kept within climate chambers until sexing 1 week before emergence. Individual females were mated during the third night after emergence and used in behavioral experiments the fourth or fifth night. Mating success was confirmed by allowing deposited eggs to develop into vital larvae. All rearing facilities for larvae, pupae, and adults were devoid of any plant material. Adults had unrestricted access to sugar solution in artificial flowers.

Plant breeding

All plants were grown in a greenhouse (23–25 °C, 50–70% relative humidity, 16 h light, Philips Sun-T Agro 400 W Na vapor bulbs, 350–500 µmol/m^2/s photosynthetic photon flux at plant level) until reaching a height of 30–40 cm and were used before flowering.

Datura wrightii seeds were purchased from B & T World Seeds and subsequently harvested from plants bred in the greenhouse. Plants were grown in 2-L pots and used in experiments 40–45 days after sowing.

Nicotiana attenuata seeds were obtained from an isogenic line that had undergone 30–31 generations of inbreeding and was originally derived from an accession collected from a burn in southwestern Utah. After germination (Krügel et al. 2002) seedlings were planted at day 10 in seed flats, transferred to 1-L pots at day 20, and grown under greenhouse conditions until use between 38–42 days after sowing.

Proboscidea parviflora seeds were initially collected from wild plants in southwestern Utah and subsequently harvested from plants grown in the greenhouse. *Brassica oleracea* var. *Rosella* seeds were purchased from Erfurter Samen und Pflanzenzucht GmbH.

Oviposition preference assays

About 1 h before onset of the scotophase, individual females were transferred into screened flight cages in a greenhouse (Figure 1). Females were tested individually. Sucrose solution was provided away from the experimental plants. Females moved freely and oviposited during 1 entire night. Individual egg numbers were assessed the following morning and compared applying a Friedman test and Dunn's Multiple Comparison test for multiple choice assays, and by a Wilcoxon signed-rank test for pairwise comparisons. All plants and females were used only once.

Choice of host species—oviposition on plants

Eight plants of the host species *D. wrightii, N. attenuata* (Solanaceae), and *P. parviflora* (Martinyaceae) and the non-host *B. oleracea* (Brussels sprout) were arranged in 2 patches per species for a total of 32 plants per flight cage. Patches were randomly distributed within the cages (Figure 1A). In total, 26 females were tested individually as described in

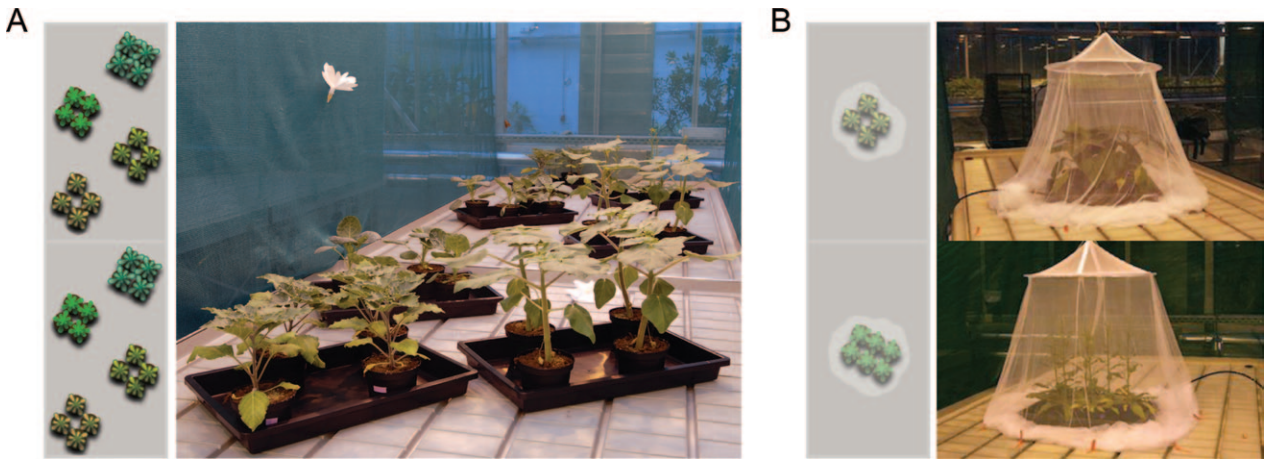

Figure 1 Oviposition preference assay. Individual *Manduca sexta* females were tested in a screened flight cage (7 × 1.8 × 2.3 m) in the greenhouse (23–25 °C, 50–70% relative humidity). Females were allowed to move freely and oviposit during the entire night. Deposited eggs were counted the following morning. (A) 8 plants of the host species *Datura wrightii, Nicotiana attenuata* (both Solanaceae), and *Proboscidea parviflora* (Martinyaceae), and the nonhost *Brassica oleracea* (Brussels sprout; Brassicaceae) were arranged in 2 patches per species for a total of 32 plants per flight cage. Patches were randomly distributed within the cages at distances of 1–1.5 m. (B) 4 *D. wrightii* and 6 *N. attenuata* plants, respectively, were placed under 2 circular gauze tents (top diameter 60 cm, base diameter 1.2 m) with an outlet of charcoal filtered air (30 L/min) at the center pushing volatile compounds out of the tent. Any contact between gauze and plants was avoided.

Table 1 Plant damage treatments

Treatment	Control	Plant signal	Larval signal
Larval feeding	Intact plants	Feeding damage–induced volatiles	Body and feces odors
Temporal larval damage	Intact plants	Feeding damage–induced volatiles	—
Mechanical wounding and spit	Intact plants	Volatiles induced by elicitor present in the regurgitant (Halitschke et al. 2001)	—
Mechanical wounding and chemical induction	Intact plants	Methyl jasmonate-induced volatiles (Halitschke et al. 2000)	—
Caged larvae and feces on intact plants	Intact plants and empty cages	Volatiles from intact plant	Body and feces odors

Oviposition preference assays above. Six females, which did not oviposit, were excluded from statistical analysis.

Choice of host species—contact excluded

Four *D. wrightii* and 6 *N. attenuata* plants, respectively, were placed under 2 circular gauze tents (Figure 1B) with an outlet of charcoal filtered air (30 L/min) at the center pushing volatile compounds out of the tent. Plant numbers were chosen to compensate for silhouette appearance. Any contact between gauze and plants was avoided. Twenty-seven gravid females were used. Sixteen females did not oviposit and were thus considered nonresponders.

Choice of host plant—effect of damage

The effect of feeding damage on female oviposition preference was investigated in *N. attenuata* and *D. wrightii*. Four patches with 3 feeding-damaged and 4 patches with 3 undamaged plants each (see Larval feeding) were placed into the flight cage as described in Oviposition on plants. A total of 29 and 27 individual females were allowed to choose between undamaged and larval feeding–damaged *N. attenuata* or *D. wrightii* plants, respectively. To increase robustness of within-plant species comparison, especially with respect to ratios, females ovipositing less than 10 eggs were excluded from statistical analysis in these experiments (*N. attenuata*, $N = 5$; *D. wrightii*, $N = 5$). Differential oviposition in response to feeding damage was only found for *N. attenuata*, see Results. Oral secretions (OS) may elicit herbivore species-specific volatile emissions in host plants (Halitschke et al. 2000). We therefore applied different treatments (see Table 1) to *N. attenuata* plants to furthermore elucidate whether volatiles nonspecifically emitted by the plant after mechanical damage and chemical defense induction, volatiles specifically emitted by the plant after larval feeding, volatiles emanating from the caterpillar or its feces, or volatiles from both plant and feeding caterpillars contribute to the behavioral preference for undamaged compared with feeding-damaged plants observed in *N. attenuata*. Experimental plants were treated as follows:

Larval feeding.

Three second- to third-stage *M. sexta* larvae were allowed to feed on *N. attenuata* and *D. wrightii* plants starting 48 h before and during the experiment. Control plants were left undamaged.

Temporal larval damage.

Manduca sexta larvae were allowed to feed on test plants starting 36 h before the experiments. Larvae were individually encaged at the undersides of leaves to prevent the accumulation of feces on the plant surface. Cages consisted of 40-mm outer diameter rubber foam rings containing small magnets and covered with wire mesh. The upper side of the leaf was covered with transparent plastic film of the same diameter fixed to the cage by a ferromagnetic steel ring. Control plants carried empty cages. Larvae and cages were removed before start of the experiment.

Mechanical wounding and spit.

Starting 36 h before the experiment, leaves were wounded once on each side of the mid-vein using a pattern wheel. OS from third to fourth instar *M. sexta* larvae (Halitschke et al. 2001) reared on *N. attenuata* plants (5 µL, 1:10 dilution in aqua dest) were pipetted onto the fresh punctures. The treatment was applied 3 times a day to a set of 3 leaves per day. Control plants were left undamaged.

Mechanical wounding and chemical induction.

Following the method described by Halitschke et al. (2000), 40 µL of Lanolin (Sigma Aldrich) containing 300 µg methyl jasmonate (Sigma Aldrich) were applied to the lower part of the elongating stem starting 36 h before the experiment. Leaves were mechanically damaged 4 times per day, see Mechanical wounding and spit above. Control plants were treated with pure lanolin paste and left undamaged.

Caged larvae and feces on intact plants.

Three third instar *M. sexta* larvae previously fed ad libitum on *N. attenuata* plants were placed into Nylon mesh cages (10 × 45-mm internal diameter [ID]; mesh size 1.2 mm; Exo Terra). Cages were fixed on wooden sticks next to undamaged plants 3–4 h before the experiment. Empty cages were placed next to control plants.

Volatile collection and analyses

Volatile collections were performed on whole plants for 1) semiquantitative analysis of the relative composition of odor bouquets emitted by different host plant species and conditions and 2) extracts at high concentrations to characterize the responsive range of OSNs when challenged with natural volatile stimuli. *Nicotiana attenuata* and *D. wrightii* plants were either intact or treated as stated in the Larval feeding section.

1) Headspace collections were performed in 25-L silanized glass cylinders. Teflon discs with a central opening (80-mm ID) separated plant shoots from soil and roots. Charcoal filtered air was introduced at the top (1.2 L/min). Odor-laden air was pulled (1 L/min) through outlets 5 cm above the Teflon disks connected to sorptive filters with 25 mg each of Carbotrap C, B, and X (Sigma Aldrich). Odor collections began 20 min after onset of the scotophase and ran for 6 h. The adsorbents were eluted with 1 mL dichloromethane (DCM) containing 1 µg bromodecane as internal standard (IS). Eluates were concentrated under a gentle stream of N_2 to 30 µL and stored at −80 °C. 2) For physiological studies, flow rates were adjusted to 2.2 (in) and 2 L/min (out), respectively, and volatiles were trapped during 12 h in the dark on 25 mg SuperQ (80/100 mesh, Supelco) or 25 mg activated charcoal as adsorbent. The adsorbent was eluted with 400 µL DCM, the eluate concentrated to 30 µL, and stored at −80 °C. Headspace extracts for gas chromatograph-coupled single sensillum recording (GC-SSR) contained compounds from the lower nanogram to the lower microgram range.

Analytical procedures

All volatile analyses were carried out on 7890A gas chromatographs (GC) (Agilent Technologies) operated in splitless mode, the injection port kept at 230 °C, and 1 µL of sample injected. For compound identification and semiquantification, both nonpolar and polar columns (HP-5 MS ui and Innowax; 30 m, 0.25-mm ID, 0.25-µm film thickness; J&W Scientific) operated under constant He flow (1.1 mL/min) were used with total ion chromatograms recorded by an Agilent 5975C mass spectrometer (MS). The GC oven was kept at 40 °C for 5 min, ramped at 5 °C/min to 260 °C or 280 °C for Innowax or HP-5 columns, respectively. The MS transfer line was maintained at 280 °C and the MS operated in electron impact mode (70 eV, ion source: 230 °C, quadrupole: 150 °C, mass scan range: 33–350 amu). Compounds were identified by comparing mass spectra and Kovats retention time indices to authentic reference compounds or tentatively to those published by the National Institute of Standards and Technologies. For semiquantification the GC-MS system was calibrated with the IS (33, 10, 5, 1, and 0.5 ng; N = 3 replicates). Emission rates were subsequently calculated based on comparison of peak areas of individual compounds and the IS and headspace sampling times. Volatile samples were compared by random forest analysis (Breiman 2001) performed in "R" (randomForest: Breiman and Cutler's random forests for classification and regression' package, version 4.5-34 for R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org). Random forest analysis is similar to a principal component analysis but better suited for nonparametric data sets that include many more variables than samples (Breiman 2001). However, regardless of how well the 2 methods are suited for our analysis, random forest analysis and principal component analysis yielded comparable results (Figure 4 and Supplementary Figure 1). For each analysis, n_{tree} = 100 000 bootstrap samples were drawn with n_{try} set as sqrt(number of compounds). We

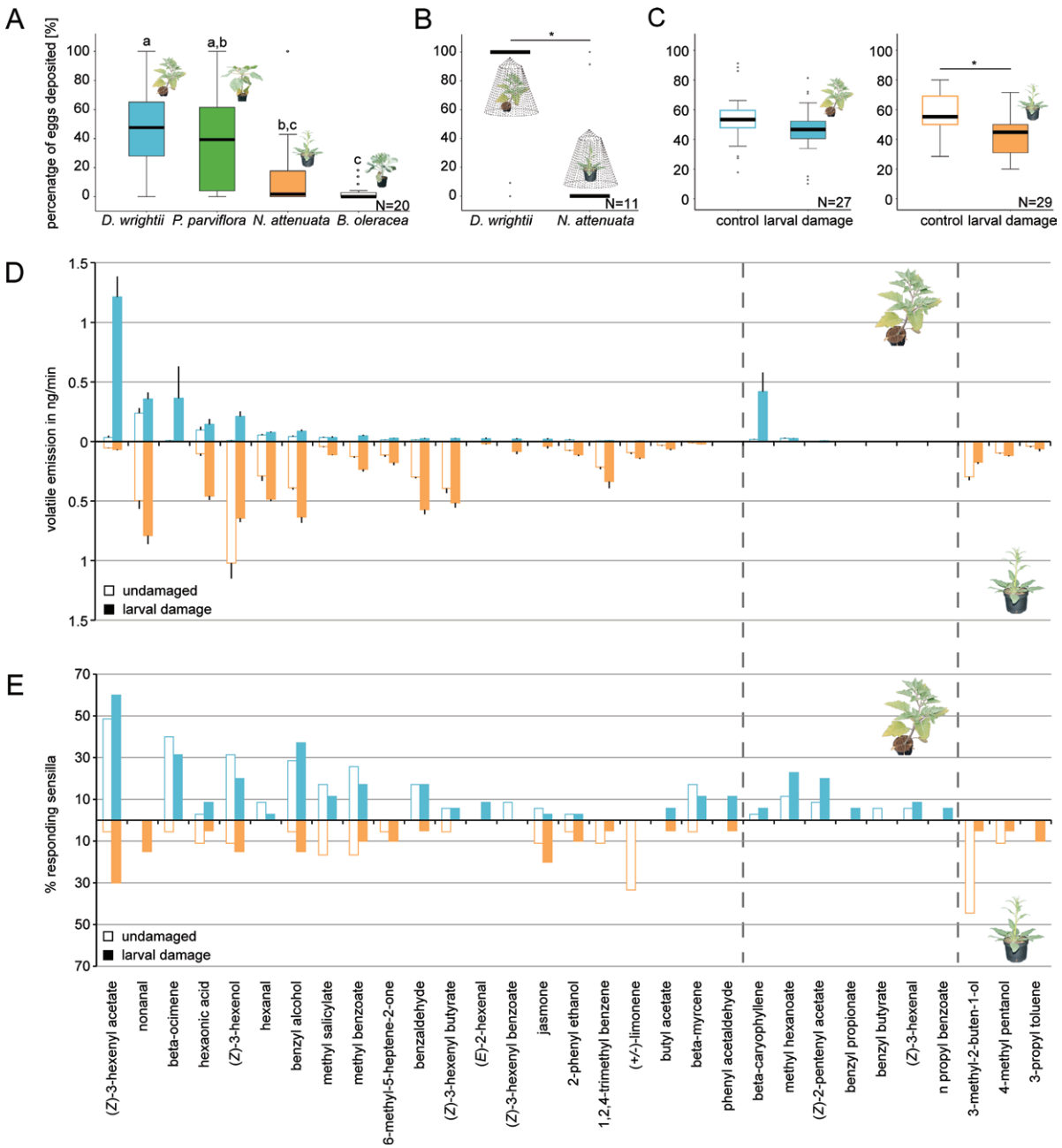

Figure 2 Oviposition preference and olfactory response of *Manduca sexta* females to host plants is species and condition specific. (A–C) Oviposition choice experiments. (A) In a flight cage, individual *M. sexta* females preferred to oviposit on nonflowering *Datura wrightii* and *Proboscidea parviflora* over *Nicotiana attenuata* and/or the nonhost *Brassica oleracea* (letters depict significant differences between species). (B) Individual females preferred to oviposit on gauze tents hiding *D. wrightii* compared with tents hiding *N. attenuata* plants. (C) Females preferred to oviposit on undamaged (open boxes) versus larval-damaged (filled boxes) *N. attenuata* plants (right), but did not prefer any *D. wrightii* plant state (left). (D) Average scotophase volatile emissions (ng/min) of *N. attenuata* (bottom) and *D. wrightii* (top) plants. Only compounds that elicited repeated responses in the GC-SSR experiments are listed. Dashed lines separate species-specific compounds. Error bars denote standard errors ($N = 5$). (E) Percentage of *M. sexta* sensilla responding in GC-SSR experiments using *N. attenuata* (bottom) and *D. wrightii* (top) headspace samples (Supplementary Table 2). Dashed lines separate species and condition-specific (filled vs. open bar) responses.

performed the analysis for all headspace volatiles and for those volatiles found to be active in the SSR experiments. In comparing SSR active with headspace volatiles (Figure 2), we only considered volatiles eliciting responses in at least 2 sensilla that were also detected in the semiquantitative headspace analyses, or those that elicited responses in at least 10% of recorded sensilla.

GC-SSR experiments were conducted on an instrument equipped with an HP-5 MS column (30 m, 0.32-mm ID, 0.25-µm film; J&W Scientific) at constant He flow (2 mL/min).

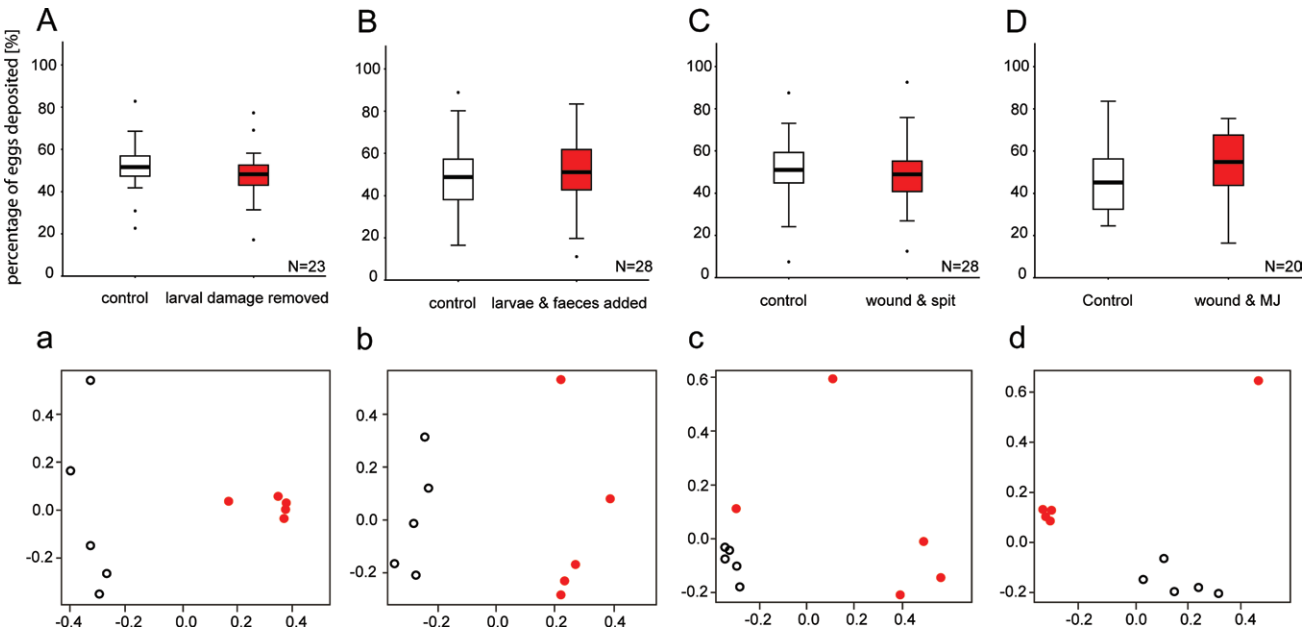

Figure 3 Oviposition preference requires complete volatile information on larval damage. (A–D) The oviposition preference of individual *Manduca sexta* females disappeared when offered different plant treatments (filled boxes) aiming to disentangle the volatile information emitted by the herbivore host complex. Experiments presented either (A) feeding-induced plant volatiles without larvae and feces, (B) larval and fecal odors only, (C) plant volatiles released after mechanical damage and application of *M. sexta* OS, or (D) plant volatiles released after mechanical damage and application of methyl jasmonate. (a–d) Metric multidimensional scaling representation of volatile blends from treated plants (filled circles) versus control plants (open circles) based on SSR-active volatiles (see Table 1). Random forest analysis revealed clear separation of treatment and control samples based on physiologically active compounds.

The oven was programmed at 40 °C for 1 min with a ramp of 20 °C/min to 280 °C held 10 min. The effluent flow was split 40:60 with make-up gas added (He, 30 psi) via a Gerstel 3D/2 connector (Gerstel) and inert restriction columns to synchronously reach a flame ionization detector (350 °C) and antennal preparations via a modified olfactory detection port (ODP3, Gerstel). The transfer line was kept at 280 °C.

Electrophysiological recordings—GC-SSR

Manduca sexta females (3–4 days after eclosion) were fixed dorsally with wax to glass slides in disposable 20 mL Falcon tubes with the head protruding. Electrode positioning was achieved using piezoelectric micromanipulators (PM-10 Märzhäuser). Electrolytically sharpened tungsten electrodes connected to a 10× preamplifier head stage (Syntech) were inserted randomly into the base of trichoid and basiconic olfactory sensilla with a reference electrode placed in the eye. Signals from OSNs were digitized by an IDAC-4 interface (Syntech) and recorded on a PC using Syntech Autospike 32 software.

The antennal preparation was continuously flushed with charcoal filtered and humidified air (0.5 m/s, Syntech CS 55 Stimulus Controller) through glass tubing (10-mm ID) positioned 10 mm from the recording site and connected to the olfactory detection port of the GC. Synthetic reference or test stimuli were introduced 10 cm from the tubing outlet. Sensilla showing spontaneous spiking activity were screened for responses to full host plant headspace extracts loaded onto filter paper strips inserted into disposable Pasteur pipettes. If responses were detected, GC-SSR recordings were initiated.

Action potentials originating from OSNs within a sensillum were extracted as digital spikes from the analog signal using Syntech Auto Spike 32 software. Due to the difficulty in separating action potential (spike) amplitudes from colocated OSNs within a sensillum (ranging from 1 to 3 in basiconic and trichoid sensilla; Shields and Hildebrand 1999), all analyses were based on the total response from all OSNs within each sensillum. Instantaneous spike frequencies for all OSNs within a sensillum were recorded in 1 s bins for the entire GC run time. To be considered a significant excitatory or inhibitory response, the instantaneous spike frequency after solvent elution must be 4 standard deviation (SD) above or below the average of the first 100 s before solvent elution. Responses were then matched with the respective GC peak. If sensilla were still responsive after GC-SSR recording, they were stimulated with reference compounds in hexane tested at 1 µg loadings on filter paper strips (0.5 × 1 cm) in Pasteur pipettes delivered at 0.5 L/min for 0.5 s duration.

Sensillum responses were compared as a population using PASW 18.0 software. Number of sensilla responding per compound type was assessed using chi-square tests. Sensillum response profiles to extracts and synthetic stimuli were clustered using Ward's method with squared Euclidian distance. Species and condition-specific sensilla were

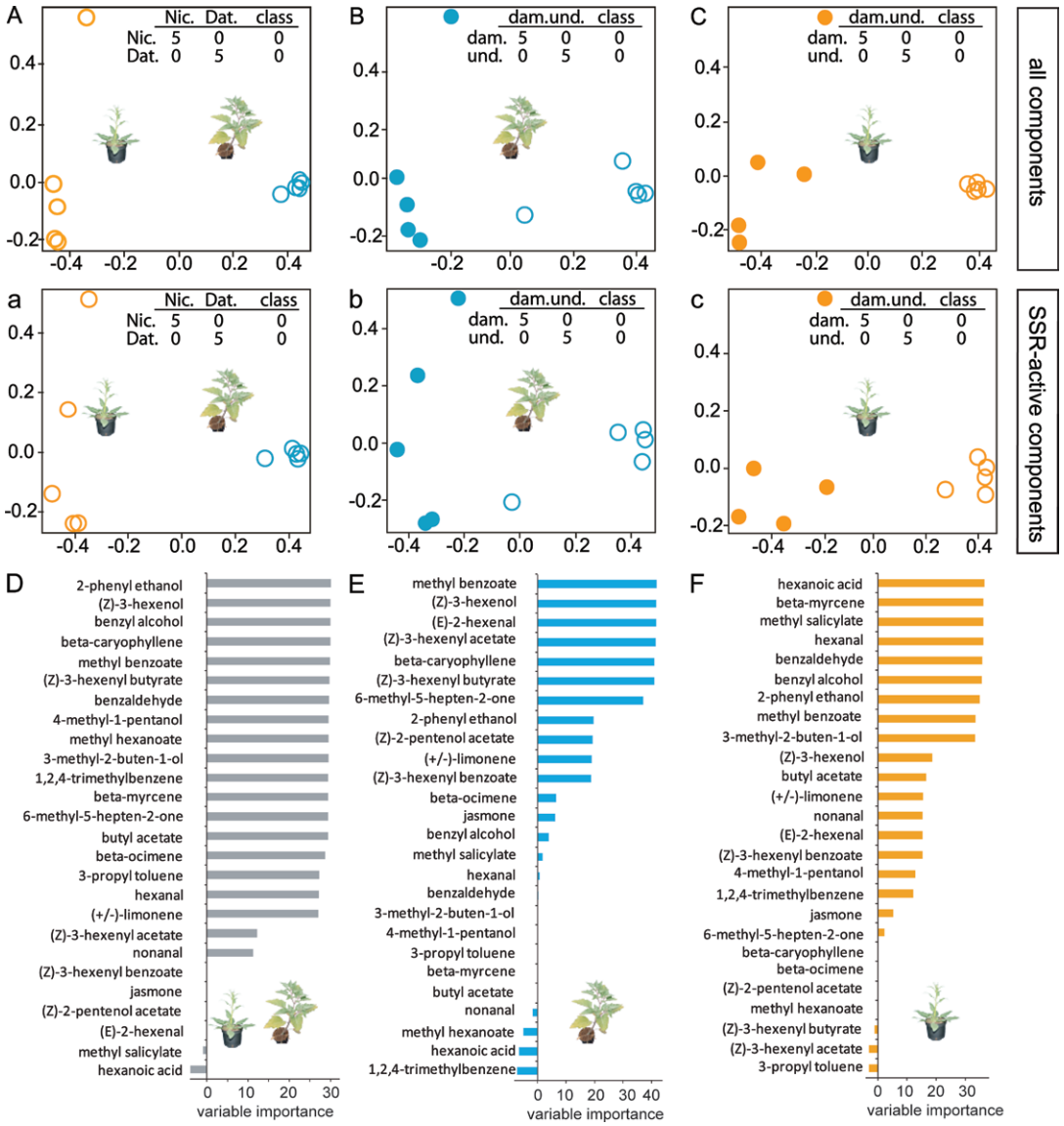

Figure 4 Host plant species and plant state can be separated by both volatile emission and GC-SSR response. (A–C) Metric multidimensional scaling representation of *Datura wrightii* and *Nicotiana attenuata* based on all volatiles found in headspace samples of the 2 host plants. (A) species representation, (B) and (C) comparison of headspace samples of undamaged (open circles) and larval-damaged (full circles) *D. wrightii* and *N. attenuata*, respectively. (a–c) The same type of analysis using SSR active compounds only. During 10 0000 replicated tree constructions samples were always assigned correctly (see inlet confusion matrices; Nic, *N. attenuata*; Dat, *D. wrightii*; dam, damaged; und, undamaged; class error, number of classification errors). (D–F) Contribution of different compounds to the separation of samples in (a–c). High absolute value of variable importance depicts strong contribution.

determined as sensilla responding to at least 1 compound that only elicited responses in the presence of a certain species or condition, but did not respond to compounds specific to other species or conditions.

Results

Oviposition preference for host species and condition

In the initial experiments with full access to the plants, *M. sexta* females deposited almost 6 times more eggs on *D. wrightii* plants (a total of 29.6 eggs per female ± 33.7 SD; Figure 2A) compared with *N. attenuata* (5.3 ± 7 SD; Figure 2A), whereas *P. parviflora* elicited an intermediate preference (24 ± 29.9 SD; Figure 2A) and the nonhost *B. oleracea* was not preferred (1.2 ± 2.7 SD; Figure 2A). Egg numbers were significantly different for *D. wrightii* and *P. parviflora* compared with *B. oleracea*, and for *D. wrightii* compared with *N. attenuata* (Friedman test, $P < 0.0001$; Dunn's Multiple Comparison test, $P < 0.05$). Among the host plants, *D. wrightii* and *N. attenuata* exhibited the clearest difference in oviposition preference and were thus chosen for further analyses. In subsequent experiments, gravid females also deposited significantly more (Figure 2B)

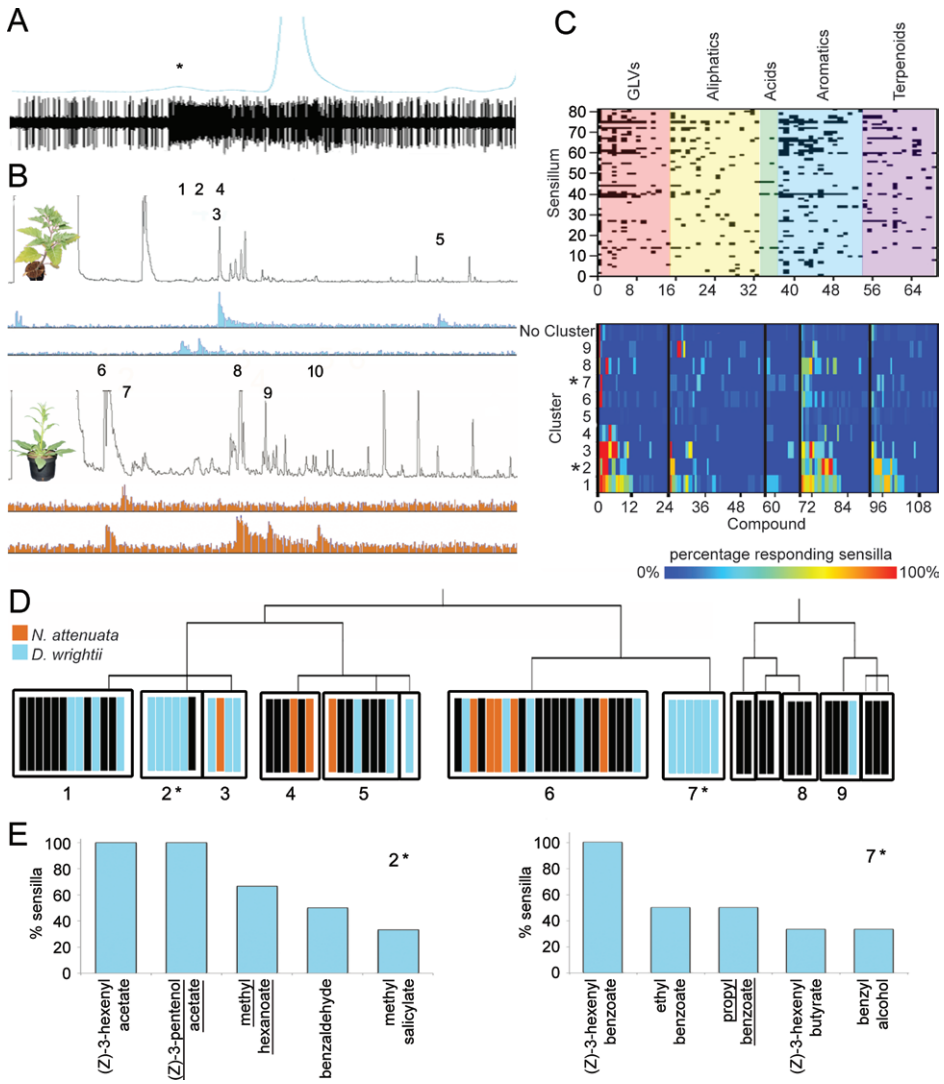

Figure 5 GC-single sensillum responses are both specific and broadly tuned. (A) Responses (t = 50 s) of a single sensillum to volatiles from feeding-damaged *Datura wrightii* headspace; * response to benzaldehyde. (B) Sample traces showing the specificity of response for 4 sensilla to an undamaged *D. wrightii* (top) or *Nicotiana attenuata* (bottom) extract, respectively. The responses (labeled with numbers) correspond to the following compounds: 1) methyl hexanoate, 2) benzaldehyde, 3) (Z)-3-hexenyl acetate, 4) (E)-2-hexenyl acetate, 5) α-farnesene, 6) 3-methyl-2-buten-1-ol, 7) 4-hydroxy-2-pentanone, 8) limonene, 9) methyl benzoate, and 10) methyl salicylate. (C, top) Response matrix for all 81 sensilla tested to plant and synthetic stimuli. The range of responses across the 5 main categories of compounds (GLVs, aliphatics, acids, aromatics, and terpenoids) shows the broad response profiles exhibited by many sensilla. Only compounds that elicited a response in at least 2 sensilla are shown. (C, bottom) Response matrix for the 9 clusters shown in (D). "No cluster" refers to the few sensilla in (C) that established clusters with maximum 2 sensilla. Color scale represents percentage of sensilla within the cluster that elicited a response to a particular compound from 0% to 100%. (D) Cluster analysis (Ward's method; squared Euclidian distance) for all 81 recorded sensilla (bars) shows the presence of species-specific clusters. Sensilla are labelled as *D. wrightii* specific or *N. attenuata* specific if they exhibited responses specific to 1 plant species. Black bars indicate nonspecific sensilla. (E) Response profiles of the 2 *D. wrightii* specific clusters in (D) (cluster 2 and 7). Both response profiles show only compounds common to 2 or more sensilla in the cluster.

eggs on tents housing *D. wrightii* (36 ± 69.2 SD) compared with *N. attenuata* (2.3 ± 6.1 SD, Wilcoxon signed-rank test, $P < 0.035$) precluding physical contact with the plants.

We also examined whether conspecific larval damage affected oviposition choice. We did not find any significant impact of larval damage on the oviposition preference of females on *D. wrightii* (number of eggs laid for undamaged, 97.6 ± 42.9 SD vs. damaged, 77.9 ± 31.42 SD, Wilcoxon signed-rank test, $P = 0.102$, Figure 2C, left). However, females deposited significantly more eggs on undamaged (74.8 ± 44.7 SD) versus larval-damaged *N. attenuata* plants (56.9 ± 30.6 SD, Wilcoxon signed-rank test, $P < 0.018$, Figure 2C, right). This reduction in oviposition was only detected in the presence of both larvae and feeding damage, rather than with either damage-specific plant or larval volatiles alone (see Figure 3A–D).

In addition, we observed the frequency of plant contacts for each of 10 gravid females choosing between damaged

and undamaged *N. attenuata* or *D. wrightii*, respectively. We observed no difference in the ratio of rejection after plant contact versus oviposition behavior (contacts resulting in abdomen curling) on either undamaged or damaged plants of both species (the odds ratio comparing conditions was 0.98 in *N. attenuata* and 1.18 in *D. wrightii* [Fisher's Exact test: *N. attenuata*, $P = 0.933$; *D. wrightii*, $P = 0.383$]). This suggests that physical contact with the plants does not affect choice between plant conditions in this species. Thus olfactory cues must play a primary role in the overall preference of *Manduca* for undamaged *N. attenuata*.

Chemical profile of host species and condition

Both species-specific and condition-specific compounds were detected in headspace samples from undamaged and feeding-damaged *N. attenuata* and *D. wrightii* plants (Figure 2D; Supplementary Table 1). The specificity of compounds was assessed in relation to the tested host species and conditions. Thus, the term "specific" refers to compounds that were emitted by *D. wrightii* but not by *N. attenuata* and vice versa or by undamaged plants only in comparison to larval-damaged plants. Compounds common to both species and/or states differed with respect to their proportion of the total blend (Figure 2D; Supplementary Table 1). Green leaf volatiles (GLVs) and their derivatives, terpenoids, and aromatic and aliphatic compounds all contributed to the characteristics of each blend. Overall, *N. attenuata* had significantly higher volatile emission rates (Figure 2D; total emission rate ± standard error: undamaged 10.5 ± 0.5 ng/min, feeding damaged 18.8 ± 1.4 ng/min) compared with *D. wrightii* (undamaged 1.8 ± 0.3 ng/min, feeding damaged 5.4 ± 1.2 ng/min).

We computed "random forest" classification trees (Breiman 2001; Ranganathan and Borges 2010) for complete volatile profiles of *D. wrightii* and *N. attenuata*. The analysis led to clear separation of *D. wrightii* and *N. attenuata* headspace samples (Figure 4A) as well as distinct clustering of feeding-damaged and undamaged samples in both species (Figure 4B,4C). When comparing headspace samples of differently induced *N. attenuata* plants, the volatile bouquet of all treatments also separated well from the headspace composition of control plants (Figure 3a–d).

Electrophysiological analysis of host species and condition

OSNs in a total of 81 *M. sexta* female olfactory sensilla were tested against headspace volatiles of damaged as well as undamaged *N. attenuata* and *D. wrightii* plants delivered via gas chromatography. The sensilla were also tested against synthetic standards (Supplementary Table 2). OSNs exhibited robust and discrete responses to compounds (Figures 2E and 5A,B). Sensilla thus demonstrated a number of different response profiles (Figure 5B,5C), although individual OSNs within single sensilla could not be unambiguously identified. Sensilla responded to a total of 119 natural headspace and synthetic compounds (Figure 2E; Supplementary Table 2). Overall, a significantly higher percentage of sensilla responded to GLV and aromatic compounds rather than aliphatics, acids, or terpenoids (Supplementary Table 2; Figure 2E; chi-square = 87.6, $n = 81$, $P < 0.001$). In 51% of the tested sensilla, more than 5 compounds elicited a response (Figure 5C, median = 6), whereas only 30% responded to 3 or fewer compounds.

When tested against headspace extracts, a higher percentage of sensilla responded to GLVs and aromatics from *D. wrightii*, whereas more sensilla responded to acids and aliphatics from *N. attenuata* (chi-square = 23.5, $n = 52$, *D. wrightii* and $n = 34$, *N. attenuata*, $P < 0.001$). Reducing the chemical data sets in the "random forest" analysis to the compounds found to be physiologically active in the GC-SSR experiments did not diminish the distinct grouping of headspace samples along physiological states or species (Figure 4a–c). The reduced set of SSR active compounds was thus sufficient to provide information on both species and state of the volatile source. A small number of compounds contributed to all 3 tested classifications of species or state (2-phenyl ethanol, (*Z*)-3-hexenol, benzyl alcohol, methyl benzoate, 6-methyl-5-hepten-2-one, hexanal, and (+/−) limonene; Figure 4D–F).

In addition, a number of responses to compounds present in only 1 of the 2 tested host species were found by GC-SSR (dashed sections of Figure 2D,2E). Sensilla with responses to *D. wrightii*-specific compounds also grouped into at least 2 separate clusters according to response profile (Figure 5D,5E). Conversely, sensilla with condition-specific responses (Figure 2E; compare filled vs. open bars) did not cluster according to response profile. In summary, the OSNs assessed here exhibit both broadly tuned and specific responses that allow discrimination of both host species and quality by volatile profile alone.

Discussion

The natural environment of an herbivorous insect consists of a number of plant species and conditions, which provide more or less suitable oviposition sites. We show that *M. sexta* females make a clear distinction between relevant species, and in some cases, between plants of differing condition, which translates into host quality in the field (Mira and Bernays 2002; Kessler and Baldwin 2004). Both host plant species and quality could be distinguished by volatile profile, and these volatiles were detected by an antennal periphery that exhibits both broadly tuned and specific responses to species and states. The set of sensilla recorded here detected more than half of the volatiles emitted by the 2 main host plants in this study, and the sensory arsenal present on the *Manduca* antenna is equipped to distinguish both host species and state. Building on the wealth of prior knowledge concerning *M. sexta* olfaction, we can now draw a direct

connection between behavioral preference, volatile release, and peripheral detection that has important implications for understanding the evolution of the olfactory system to meet the reproductive needs of a species.

The oviposition behavior of a female moth is a complex chain of decisions based on sensory input and the physiological state of the female (Yamamoto et al. 1969; Ramaswamy 1988). Here, the physiological state was kept as constant as possible, and the sensory input was compared. In our study, female moths made a choice between different species of unattacked potential host plants. *Datura wrightii* was the most accepted, *P. parviflora* received an (insignificantly) intermediate number of eggs, whereas *N. attenuata* was the least preferred. The *B. oleracea* nonhost—as expected—elicited almost no oviposition visits at all. When excluding direct contact with the host plant and providing the moths only with plant-derived olfactory cues, again the moths oviposited preferably on a net surrounding *D. wrightii* versus *N. attenuata*. As oviposition is the ultimate choice of the female, it represents a highly conservative measurement of host acceptance, and suggests the importance of olfactory input in female choice. However, without physical contact to the plants the rate of nonresponding females increased, indicating that contact and/or visual cues also contribute to the full sequence of egg laying behavior (Raguso and Willis 2005).

The olfactory-based species choice of *M. sexta* females can be placed into both an ecological and evolutionary context. *Datura wrightii* and *N. attenuata* grow side by side in Utah's Great Basin desert (Kessler and Baldwin 2002). They do, however, exhibit dramatically different life histories. *Datura wrightii* is a vigorous herbaceous perennial plant with large leaves and flowers, and of more or less constant yearly availability (Bronstein et al. 2009). It thus provides ample and predictable biomass for consumption by larvae. *Nicotiana attenuata*, on the other hand, is an annual plant with a basal rosette and erect slender stems supporting small flowers. It occurs in extreme numbers after bush fires, but is otherwise dormant in seed banks (Wells 1959). Each *N. attenuata* plant supports only a single larva (McFadden 1968; Kessler and Baldwin 2001), but when these plants occur they constitute a considerable resource. Consequently, when both plants are present, females may exhibit a preference for *D. wrightii*, but must be prepared to accept either species due to availability.

Previous field and chemical analyses with these 2 plant species suggest that *Manduca* exhibits a preference for unattacked *N. attenuata* in the field that could relate to its herbivore-specific induced volatiles (Baldwin et al. 2001; Kessler and Baldwin 2001). In contrast, the volatile profiles of *D. wrightii* do not depend on herbivore species (Hare and Sun 2011). Correspondingly, in our study, female moths exhibited a significant preference for nonattacked *N. attenuata* plants, whereas in *D. wrightii* they did not. This behavior also connects well to the already discussed idea, that females would be well advised to refrain from oviposition on the smaller, already attacked *N. attenuata*, whereas oviposition on the larger, though occupied *Datura* still might prove beneficial. Interestingly, only the combination of an attacked *Nicotiana* plant with the odor of larvae and their waste products provided a choice-eliciting stimulus in our behavioral assays (Kessler and Baldwin 2001). Such a combined stimulus indicates that not only has the plant been attacked but the attackers are still actively feeding.

How has the olfactory system evolved to meet these ecological needs? By recording from 81 individual sensilla responding to volatile emissions of *D. wrightii* and *N. attenuata*, we performed one of the largest GC-SSR studies so far, thus gathering chemical as well as detailed information on peripheral olfactory specificity to host plants. The chemical investigations revealed that both species and condition provide volatile signatures that could be clearly distinguished at the periphery (e.g., Figure 4). The female has an excellent olfactory opportunity to remotely utilize these patterns for her choice, without needing to contact the leaves. Yet, although both *Datura* and *Nicotiana* changed their volatile emissions drastically after attack, this change was only behaviorally relevant in the case of *Nicotiana*. Thus, having the sensory capability to make a choice does not mean that an animal always makes use of it.

The female sensilla investigated detected roughly 60% of all odorants produced by the plants, indicating that the antennal OSNs can detect a large portion of the volatile emissions emanating from these potential hosts. Given that the majority of OSNs were also broadly tuned to a number of the host volatiles, the odor "image" of these plants must in large part be relayed combinatorially to the central nervous system. Nevertheless, we also found that female *Manduca* possessed separate sensillum types with OSNs detecting compounds specific to only one of the tested host species, suggesting that host species can still be segregated at the antennal periphery. The peripheral coding of *D. wrightii* and *N. attenuata* odor emissions thus seems to contain 2 components; 1 ratiometric, with sensilla detecting molecules emitted by both species in different proportions, and 1 detecting species-specific odorants. This creates a sensory system in which both specific and broadly tuned OSNs have shared, but nonoverlapping response profiles: a "selectively nonselective" system (Alicia Anderson, APACE, Honolulu, HI, 2009).

An interesting comparison can be made between olfactory-based oviposition and nectar-feeding preference in *Manduca* as studied by Riffell et al. (2009). These authors elegantly showed that moths orient toward *D. wrightii* flowers, and that the volatile mixture emitted by the flower can be mimicked by a highly reduced blend. Although both antagonistic and mutualistic interactions can take place on the same plant, resulting in a typical trade-off situation (Bronstein et al. 2009), the selection pressures between oviposition and nectar feeding are very different. When a female moth looks for an oviposition site, it is vital that she optimizes her choice. Choosing a poor host for her offspring may dramatically

reduce the genetic output in the next generation. In contrast, the search for a nectar-rich flower is not under immediate selection pressure, and it is possible to repeat the search expending only time and energy. In addition, the search for a nectar source is more or less always a search for a reward, so both the feeding moth and the pollinated flower are interested in a successful outcome. Simple and unambiguous signals, for example, odor constancy (Raguso 2008) will therefore be favored over evolutionary time. Correspondingly, a widely reduced and physiologically active blend of flower volatiles attracts foraging hawk moths (Riffell et al. 2009). Conversely, host search is antagonistic; the moth female wants to locate a host plant, which naturally benefits by escaping the fate of being consumed. A stronger crypsis in plant odor emissions should thus be favored, creating a stronger selection pressure on the moth olfactory system to detect these kairomones. In parallel, however, such pressures might be counteracted by physiological processes directly involved in plant defense against *Manduca* moths and other potential herbivores (Dicke and Baldwin 2010).

Our study indicates that female *M. sexta* make an adapted choice of host plants based on olfactory information, and that the olfactory periphery is equipped to discriminate different species and qualities. Nevertheless, visual and contact cues may also provide additional information to host searching insects (Stenberg and Ericson 2007; Städler and Reifenrath 2009; Kuehnle and Mueller 2011). Lower response rates in our experiment with obstructed visual and contact stimuli indicate that these cues also play a role for the ovipositing *M. sexta* female. Herbivory by a single species is also quite unnatural, and volatile emission from the simultaneous feeding of different guilds cannot be anticipated from herbivore-specific plant responses (Delphia et al. 2007). Further investigations should dissect responses to more "natural" plants, having been attacked by a host of herbivores, bacteria, fungi, and other natural enemies. Another important aspect is the flexibility of the system. To what degree can the female choice of host plant be modified through experience? Attraction to *Agave* flowers is not innate, but has to be learned, unlike *Datura* (Riffell et al. 2008). Similarly, adult experience can shape oviposition behavior in Lepidoptera (Tammaru and Javois 2005; Olsson et al. 2006) as well as other insect taxa (Jaenike 1983; Kaur et al. 2003). Corresponding investigations in ovipositing *Manduca* necessitate field studies to properly simulate natural conditions. A deeper understanding of higher levels of olfactory processing, that is, in the antennal lobe, mushroom body, and lateral horn, is also crucial to further increase our understanding of how the female decodes kairomone signals into attraction or nonattraction.

Conclusions

We show that female *M. sexta* sphingid moths display an odor-dependent oviposition preference between different ecologically relevant host species, and also between herbivore-attacked and -nonattacked plants. Combined chemical and physiological analysis of 2 of these host plants, *D. wrightii* and *N. attenuata*, show that both host plant species and quality can be distinguished by volatile profile, and that these volatiles are detected by an antennal periphery that exhibits both broadly tuned, and specific responses to species and states. Out of more than 100 compounds identified, more than 60% were detected by the OSNs measured in this study. Our findings suggest that the odor image of a host is already represented at the sensory periphery, and that distinct olfactory activation patterns for both host species and quality are conveyed to the brain, allowing the female moth to distinguish both host species and quality. We can now draw a direct connection between behavioral preference, volatile release, and peripheral detection that highlights the important role of olfactory cues in *M. sexta* oviposition choice.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/

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