

Host Plant Species Differentiation in a Polyphagous Moth: Olfaction is Enough

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Abstract Polyphagous herbivorous insects need to discriminate suitable from unsuitable host plants in complex plant communities. While studies on the olfactory system of monophagous herbivores have revealed close adaptations to their host plant's characteristic volatiles, such adaptive fine-tuning is not possible when a large diversity of plants is suitable. Instead, the available literature on polyphagous herbivore preferences suggests a higher level of plasticity, and a bias towards previously experienced plant species. It is therefore necessary to take into account the diversity of plant odors that polyphagous herbivores encounter in the wild in order to unravel the olfactory basis of their host plant choice behaviour. In this study we show that a polyphagous moth, *Spodoptera littoralis*, has the sensory ability to distinguish five host plant species using olfaction alone, this being a prerequisite to the ability to make a choice. We have used gas chromatography mass spectrometry (GC-MS) and gas chromatography electroantennographic detection (GC-EAD) in order to describe host plant odor profiles as perceived by *S. littoralis*. We find that each plant emits specific combinations and proportions of GC-EAD active volatiles, leading to statistically distinct profiles. In addition, at least four of these plants show

GC-EAD active compound proportions that are conserved across individual plants, a characteristic that enables insects to act upon previous olfactory experiences during host plant choice. By identifying the volatiles involved in olfactory differentiation of alternative host plants by *Spodoptera littoralis*, we set the groundwork for deeper investigations of how olfactory perceptions translate into behaviour in polyphagous herbivores.

Keywords GC-EAD active volatile profile · Plant volatiles · Diet breadth · Host-plant perception · Non-induced vegetative odor · Olfactory differentiation · *Spodoptera littoralis*

Introduction

Phytophagous insects rely on plant vegetative tissues as a food source to support larval development. Choosing a suitable host for feeding and/or egg laying is crucial for their fitness. However, they live in very complex environments, comprising a large number of different plant species that vary in their nutritional value and defensive chemistry (Mithöfer and Boland 2012), as well as in their sensory appearance. Phytophagous insects have evolved a diversity of strategies to cope with the complexity of their environment. Diet breadth, in particular, ranges from very narrow to very wide, which has important consequences in terms of adaptation to the defence mechanisms of the host(s) and for the exploitation of host-recognition cues. Host plant recognition and host plant choice is mediated to a large extent by olfaction (Bruce et al. 2005), and insects with narrow versus wide diet breadth may show different kinds of adaptations in their olfactory system and in their host-plant searching behaviour.

At one end of the diet breadth spectrum, mono and oligophagous insects accept as hosts, can feed and successfully

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complete their larval development on only one, or on a few closely related plant species. This strategy allows fine-tuned adaptation to the defence mechanisms of the host(s), such as observed in *Asclepias*-associated monarch butterflies, which have evolved an ability to sequester and tolerate cardenolides (Petschenka and Agrawal 2015). Specific adaptations are also observed in the host search behaviour and the olfactory system. For example, volatile isothiocyanates, the specific defence chemicals of Brassicaceae, are attractive to many Brassicaceae specialists that have developed physiological adaptations to tolerate them (Bruce 2014). This fine-tuning of behaviour is achieved by an adaptation of the olfactory circuitry, optimising the perception and neural processing of host-specific volatiles. The monophagous *Drosophila sechellia*, which is strictly associated with toxic *Morindia* fruits, has more olfactory sensilla tuned to *Morindia*-characteristic volatiles on its antennae than the polyphagous *D. melanogaster* (Stensmyr et al. 2003). In *D. sechellia*'s antennal lobe, the region of the brain where primary processing of olfactory information takes place, the corresponding glomeruli are enlarged (Dekker et al. 2006).

At the opposite end of the diet breadth spectrum, polyphagous herbivores accept as hosts, can feed and successfully develop on a wide variety of plant species. This strategy has been proposed, for multivoltine species, as an adaptation to environments where the composition of plant communities varies over space and time in an unpredictable way (Wiklund and Friberg 2009). Despite its advantages, this strategy comes at a cost: when a large number of plant species are suitable, a relationship between sensory cues and suitability would be either non-existent or too complex for insect neural analytical capacity (Bernays 2001; Carrasco et al. 2015). Indeed, it has long been noted that polyphagous females frequently make maladaptive oviposition site choices, and lay their eggs on plant species that are not always optimal for the development of their offspring. A meta-analysis (Gripenberg et al. 2010) showed a weaker correlation between female oviposition preference and larval performance in polyphagous than in oligophagous herbivores. Despite this difficulty with adaptive fine-tuning to host plant recognition cues, several polyphagous species have been shown to make adaptive host plant choices through behavioural plasticity; their host plant preferences are modulated by previous experiences (Anderson and Anton 2014).

In the context of polyphagy, where preferences are plastic and potentially maladaptive, assigning an ecological or adaptive role to any observed preference for olfactory stimulus *x* versus *y* becomes challenging. However, a prerequisite to the ability to make a choice, innate or experienced, is the ability to perceive alternative host plant species as different based on their odors. This ability depends on two parameters: the perceptual abilities of insects, and the presence of species-identity information in plant vegetative odors. The neural organisation

of the insect olfactory system has been studied in depth (Galizia and Rössler 2010). These studies support the idea that the insect brain encodes olfactory information in a combinatorial way. In other words, each volatile or combination of volatiles produces a specific neural representation (Andersson et al. 2015). At the peripheral level, each volatile binds to a unique set of odorant receptor protein types, and therefore stimulates a unique set of olfactory sensory neuron (OSN) types on the antennae. In turn, each olfactory sensory neuron type projects to a specific glomerulus in the antennal lobe (the brain region where primary processing of olfactory information takes place), so that each volatile or blend of volatiles activates a specific combination of glomeruli. This structure of the neural signal allows the detection and differentiation of a wide array of commonly encountered plant volatiles (Bruce et al. 2005), and of plants emitting different combinations or proportions of these volatiles.

It is generally accepted that different plant species have distinct average odor profiles (defined as identity and proportions of volatiles emitted). However, for plant odors to convey species-identity information, the intraspecific variation in odor composition has to be sufficiently low compared to interspecific differences. Whether this is generally true in the case of non-induced vegetative odor profiles is difficult to evaluate from the extant literature for two reasons. First, direct statistical comparisons of the non-induced, vegetative odor profiles emitted by different plant species are rare in the literature. Second, odor profiles are generally reported in a way that does not indicate if the variation is purely quantitative (compound emitted by all individuals at varying rates) or has a qualitative component (compound emitted by only some of the individuals). In this study, we aim to evaluate the ability of a polyphagous herbivore to differentiate alternative host plant species, while taking into account both its perceptual abilities (the subset of plant volatiles it can perceive) and the intraspecific variability in host plant odor profiles.

The larvae of the highly polyphagous Egyptian cotton leaf worm, *Spodoptera littoralis*, are known to feed and successfully develop on plants belonging to more than 40 different families (Brown and Dewhurst 1975). Host plant choice behaviour and its plasticity have been extensively studied in this species, using five model host plants representative of Egyptian agroecosystems (cabbage, cotton, clover, cowpea and maize). Plant-naïve adults (individuals reared on artificial diet), show a well-defined innate preference hierarchy among those five plant species. However, experienced adults prefer the plant species on which they have been fed as larvae (Thöming et al. 2013). This plasticity of host plant preference has been found to function in all model host plants, except cabbage, which is the least innately preferred species and is still avoided by individuals that have successfully completed their larval development on it. Female and male choices were found to be mostly consistent with each other, despite the fact

that they were tested in different setups, giving them access to a different range of sensory cues. While females had access to olfactory and contact cues (oviposition choice assay), males only had access to olfactory cues (wind tunnel choice assay). This suggests that olfaction plays a major role in host plant selection (Thöming et al. 2013; Zakir et al. 2013a).

In contrast to the ecological aspects, studies on the olfactory basis of host plant perception and attraction in *S. littoralis* have, to date, mostly focused on a single model host plant: cotton (Borrero-Echeverry et al. 2015; Hatano et al. 2015; Kromann et al. 2015; Saveer et al. 2012). Indeed, GC-EAD active plant volatiles have only been described for cotton (Borrero-Echeverry et al. 2015) plus two non-hosts with a negative impact on *S. littoralis* reproductive behaviour: a toxic plant and a gymnosperm (Binyameen et al. 2013). Olfactory physiology studies suggest that the principle of combinatorial coding of olfactory information described above holds for Lepidoptera in general (De Fouchier et al. 2014). In *S. littoralis* in particular, it is supported by the documented patterns of OSN and antennal lobe activation by plant volatiles (Binyameen et al. 2014; Sadek et al. 2002). However, how this olfactory system enables *S. littoralis* adults to differentiate alternative host plants, and therefore to make a choice among them, has so far never been evaluated.

Here, we provide the first study of olfactory differentiation of alternative host plant species by *S. littoralis*. We compare the GC-EAD active volatile profiles of five host plant species that *S. littoralis* adults are able to distinguish (Thöming et al. 2013), and show that olfaction alone can explain their ability to differentiate them. We discuss our results in the light of already documented differences in behaviour.

Material and Methods

Plants We selected five plant species that have been used previously as model host plants for *Spodoptera littoralis* in host plant choice experiments. We chose our plant material in order to fit that used by Thöming et al. (2013). The selected plants are cabbage (*Brassica oleracea* subsp. *capitata* v. Stonehead, Brassicaceae), cotton (*Gossypium hirsutum* v. Delta Pineland 90, Malvaceae), Egyptian clover (*Trifolium alexandrinum* v. Winner, Fabaceae), cowpea (*Vigna unguiculata* subsp. *unguiculata* v. Dokki 331, Fabaceae) and maize (*Zea mays* v. Tasty Sweet F1, Poaceae). Plants were grown in a greenhouse, in 1.5 l pots filled with commercial soil (80% peat, 20% ceramic clay, supplemented with slow release fertiliser beads, Hasselfors, Örebro, Sweden). Temperature and humidity were set at 22 ± 2 °C and 75% (range 65–100). Natural light was supplemented with sodium lamps of intensity $1 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ between 4 am and 8 pm (Southern Swedish summer day-night cycle). Plants were cultivated until they reached a reasonable biomass for volatile

collection (three to six weeks from germination depending on species).

Odor Collection Protocol Each plant was enclosed in a commercial polyamide roasting bag (Toppits®, Cofresco Frischhalteprodukte, GmbH, Minden, Germany) inside which an aeration column was inserted. Columns consisted of a piece of Teflon tubing (inner diameter 3.0 mm, outer diameter 4 mm) filled with 50 mg of Porapak Q (mesh size 50–80, Waters, Milford, MA, USA). Air was sucked out of the bag through the column for 12 h at $150 \text{ ml}\cdot\text{min}^{-1}$. A control sample (empty bag) was also collected during each odor collection session. After collection, each column was eluted with 500 μl of pentane (*puriss p.a.* Sigma-Aldrich, Saint Louis, MO, USA). In total, we collected odors from 12 individual plants per species and nine control samples.

Coupled Gas Chromatography - Mass Spectrometry (GC-MS) Analysis Samples were injected (2 μl), using an autoinjector (G4567A) on a coupled gas chromatograph mass spectrometer: 7890B GC and 5977A MS detector (all Agilent Technologies, Santa Clara, CA, USA). The injector temperature was 225 °C. The GC was equipped with a 60 m \times 0.25 mm fused silica column coated with DB-Wax (polyethylene glycol, $df = 0.25 \mu\text{m}$, Agilent Technologies). The temperature was programmed from 30 °C (3 min hold) at $8 \text{ }^\circ\text{C}\cdot\text{min}^{-1}$ to 225 °C (10 min hold). Helium was used as the mobile phase, at $35 \text{ cm}\cdot\text{s}^{-1}$, and the electron impact (EI mode) mass spectra were obtained at 70 eV, scanning m/z 29–400, at 3.8 scans per second.

Compounds were identified by comparison with mass spectra (MS) and Kováts retention indices (RI) from our custom library (records made on our own GC-MS devices), supplemented with commercially available MS libraries (NIST, Wiley) and published RI libraries (El-Sayed 2014; Jennings and Shibamoto 1980). A compound was considered as reasonably identified and assigned a precise chemical name only if the MS library match was above 90% and the difference in RI with the library was no more than 10 units. Some of the compound identities were confirmed by comparison with a synthetic standard. This was done for all compounds suspected to be GC-EAD active, with specific procedures described in the dedicated material and methods section. Compounds were quantified on the basis of their total ion abundance, and relative to the total ion abundance shown by 10 ng of heptyl acetate, injected separately as an external standard.

Insects Egyptian cotton leaf worm (*Spodoptera littoralis*) females were obtained from a laboratory culture established from wild individuals collected near Alexandria, Egypt, in 2008 and regularly refreshed with new wild individuals. The entire rearing cycle takes place in climate chambers at

25 ± 2 °C, humidity $70 \pm 2\%$ and 16 h day: 8 h night cycle. Larvae were reared on an artificial diet based on potato (Hinks and Byers 1976). Males and females were separated at the pupal stage based on their sexual dimorphism.

Coupled Gas Chromatography - Electroantennographic Detection (GC-EAD) Analysis All odor samples collected were pooled per plant species and concentrated to approximately 1 h-equivalent per microliter. Each of these concentrates was used to perform combined GC - electroantennographic detection (GC-EAD, 3 to 5 replicates per concentrate), using an EAG setup (IDAC-2; Syntech, Kirchzarten, Germany) with an Agilent 7890A GC. We also recorded GC-EAD responses from synthetic standards in order to confirm the identity of the volatiles responsible for the responses recorded from the concentrates (5 replicates per compound). The amounts injected were set so as to stimulate the antenna with either 1 h of emission equivalent of plant odour concentrate or 10 ng of synthetic standard.

GC columns and the temperature programmes were the same as for the GC-MS analysis. Hydrogen was used as the mobile phase at an average linear flow of $45 \text{ cm}\cdot\text{s}^{-1}$. At the GC effluent, 4 psi of nitrogen was added and split 1:1 in a Gerstel 3D/2 low dead volume four way cross (Gerstel, Mülheim, Germany) between the flame ionization detector and the EAD. The GC effluent capillary for the EAD passed through a Gerstel ODP-3 transfer line, that tracked GC oven temperature, into a glass tube (length 10 cm, inner diameter 6.7 mm), where it was mixed with charcoal filtered, humidified air ($1.5 \text{ l}\cdot\text{min}^{-1}$, which makes $0.7 \text{ m}\cdot\text{s}^{-1}$).

One to five-day-old, virgin females were gently grasped by the wings and held under a binocular microscope. One antenna was excised at the base and immediately inserted into a glass electrode filled with Beadle-Ephrussi Ringer solution. This electrode was mounted on a $10\times$ preamplifier probe (Syntech) connected to the IDAC box. Then, the tip of the antenna was inserted into the second glass electrode, immediately after having cut the distal segment. The antenna was positioned as close as possible to the outlet of the glass tube.

Since the mobile phases differ between GC-MS and GC-EAD (helium and hydrogen, respectively), retention indices were not always the same for a given compound on both setups. Indeed, discrepancies of up to 15 RI units were commonly observed between the two setups for any given compound (see Table S3 in Online Resource 1). For this reason, we applied a more stringent procedure than usual when it came to confirming the identity of putatively GC-EAD active compounds. We co-injected all concentrates with a blend of n-alkanes from octane to eicosane on both our GC-MS and our GC-EAD setups in order to calculate precise RI for the GC-EAD responses observed as well as the compounds suspected to be responsible for them. We did the same for all the synthetic standards we tested.

We marked a compound as GC-EAD active if 1) The compound was part of the volatile profile of at least some plant species (compound listed in Table 1, identity confirmed as described above, RI discrepancy with the standard no more than five units), 2) a GC-EAD response was frequently or consistently detected at the correct RI from at least some of the plant concentrates (RI discrepancy with the standard no more than one unit), and 3) The synthetic standard elicited a consistent GC-EAD response (in at least four out of five recordings).

Computation of Plant Volatile Profiles The volatile profile of each individual plant sampled was determined from the GC-MS data (identity of emitted compounds, emission rate expressed as $\text{ng}\cdot\text{h}^{-1}$, and % of each compound in the blend). We defined two kinds of volatile profiles, based on two subsets of the GC-MS peaks detected, in order to describe both the overall volatile emission of each plant, and its odor as perceived by *S. littoralis*.

Overall volatile profiles of each individual plant were computed from all the compounds listed in Table 1. These include all detected volatiles, except those that were possible contaminants (we excluded compounds present in at least one third of the control samples and/or whose MS library match clearly indicated a non-natural compound). Table S1 (in Online Resource 1) presents an extended version of Table 1, including the occurrence of each retained compound in control samples. For those compounds that were occasionally found in the controls, the plant released amounts presented in Table 1 are after subtracting the amounts found in the corresponding controls. Compounds that we have considered as contaminants but that can be produced by plants and/or are GC-EAD active are listed in Table S2 (Online Resource 1).

GC-EAD active odor profiles are meant to reflect the way each individual sampled plant would be perceived by *S. littoralis*' olfactory system. We computed them from the same data as for the overall volatile profiles, but retained only the compounds that we had marked as GC-EAD active (compounds in bold in Table 1).

Statistical Analyses: Differences in Volatile Profiles between the five Host Plant Species We used a set of multivariate statistical methods implemented in the R package Vegan in order to compare the volatile profiles, expressed as % composition, of the five host plant species (R Core Team 2014; Oksanen et al. 2014). PERMANOVA and NMDS analyses (see below), contrary to the more classical MANOVA and PCA, allow the use of the Bray-Curtis distance metric, which is recommended for datasets that contain many zero values. The procedures described below were applied separately to overall volatile profiles and to GC-EAD active odor profiles. The aim was to characterise overall volatile differences as well as odor differences as perceived by adult female *S. littoralis*.

Table 1 Average volatile profiles of the 5 host plant species, expressed as mean emission rate of each detected volatile compound (ng·h⁻¹, 12 samples per species)

Compound	RI	Cabbage		Cotton		Clover		Cowpea		Maize	
		mean	O	mean	O	mean	O	mean	O	mean	O
<i>Monoterpenoids</i>		5.09	12	5.10	12	0.2	9	18	12	4.42	12
α-thujene	1021	0.65	10	0	0	0	0	0.53	10	0	0
β-pinene ^a	1105	0.34	11	1.56	11	0.18	9	0.25	10	0.05	3
sabinene	1118	1.90	12	0.09	2	0	0	1.12	12	0.08	1
myrcene^a	1158	0.64	12	1.48	11	0.02	2	14.23	12	3.70	11
β-phellandrene ^a	1206	0.03	2	0.28	8	0	0	0.51	9	0.01	1
eucalyptol^a	1208	1.27	12	0	0	0	0	0.92	11	0	0
(Z)-β-ocimene^a	1228	0	0	0.07	1	0	0	0.18	5	0	0
(E)-β-ocimene^a	1245	0	0	0.05	7	0	0	0.14	11	0.00	1
terpinolene ^a	1282	0.26	8	0.03	3	0	0	0.12	3	0.03	1
trans-furanoid-linalool oxide ^a	1469	0	0	0.70	12	0	0	0	0	0	0
linalool^a	1533	0	0	0.84	9	0	0	0	0	0.55	6
neral ^a	1683	0.03	1	0	0	0	0	0.07	1	0	0
<i>Sesquiterpene hydrocarbons</i>		0.06	2	2.23	10	0	0	0.11	2	40.18	11
sesquiterpene 1	1498	0	0	0	0	0	0	0	0	37.33	10
sesquiterpene 2	1506	0	0	0	0	0	0	0	0	0.58	8
sesquiterpene 3	1591	0.03	1	0	0	0	0	0.04	2	0.24	6
sesquiterpene 4	1609	0	0	0	0	0	0	0	0	0.13	5
trans-β-caryophyllene ^a	1613	0	0	1.44	7	0	0	0	0	0	0
α-humulene ^a	1692	0	0	0.34	7	0	0	0	0	0	0
sesquiterpene 5	1703	0	0	0	0	0	0	0	0	0.40	9
sesquiterpene 6	1737	0	0	0.01	1	0	0	0	0	0.09	6
δ-cadinene	1769	0	0	0.44	6	0	0	0	0	0.40	10
sesquiterpene 7	1776	0	0	0	0	0	0	0	0	0.74	11
sesquiterpene 8	1807	0	0	0	0	0	0	0	0	0.12	5
sesquiterpene 9	2169	0	0	0	0	0	0	0	0	0.15	4
<i>Other terpenoids</i>		1.99	12	87.24	12	0	0	2.08	11	1.24	4
unidentified terpenoid	1221	0	0	0.78	6	0	0	0	0	0	0
(E)-DMNT^{a,b}	1301	0	0	27.73	12	0	0	1.99	11	1.13	4
β-isophorone	1411	0.49	12	0	0	0	0	0	0	0	0
α-isophorone^a	1606	0.10	12	0	0	0	0	0	0	0	0
TMTT stereoisomer ^a	1775	0	0	0.18	3	0	0	0	0	0	0
(E,E)-TMTT ^a	1802	0	0	58.55	12	0	0	0.09	2	0.11	2
diterpene hydrocarbon	2388	1.40	7	0	0	0	0	0	0	0	0
<i>Fatty-acid derivatives</i>		0.57	10	1.57	12	10.38	12	3.70	12	0.85	10
isopentyl acetate ^a	1117	0	0	0.70	11	0	0	0	0	0	0
unidentified ester	1243	0	0	0.19	4	0	0	0	0	0	0
(Z)-3-hexenyl acetate^a	1309	0.32	9	0.72	11	6.51	12	2.07	12	0.43	8
(Z)-3-hexen-1-ol^a	1374	0.07	5	0.10	3	3.46	12	0.95	7	0.40	7
unidentified acetate ester	1378	1.06	9	0	0	0	0	0	0	1.69	12
1-octen-3-ol ^a	1437	0.17	5	0.05	2	0.40	8	0.68	6	0.02	1
<i>Other/unidentified</i>		4.75	12	2.47	12	0.28	10	1.28	8	1.47	12
Unidentified 1	965	0	0	0	0	0	0	0.57	5	0	0
Unidentified 2	1007	0.21	6	0.24	2	0.02	1	0.13	1	0.22	1
5-hepten-2-one	1244	0	0	0	0	0	0	0	0	0.95	9
Unidentified 3	1248	0.40	4	0.53	5	0	0	0	0	0	0

Table 1 (continued)

Compound	RI	Cabbage		Cotton		Clover		Cowpea		Maize	
		mean	O	mean	O	mean	O	mean	O	mean	O
cyclopentanol ^a	1294	1.83	12	0.42	6	0.21	10	0.04	3	0.04	1
2,5-dimethyl pyrazine ^a	1321	0.03	1	0.68	11	0	0	0.00	1	0	0
Unidentified 4	1396	0.80	6	0	0	0.03	1	0	0	0	0
Unidentified 5	1429	1.34	12	0	0	0.02	1	0	0	0	0
Unidentified 6	1767	0	0	0	0	0	0	0.53	1	0.20	1
Unidentified 7	1771	0.14	5	0	0	0	0	0	0	0	0
methyl salicylate^a	1783	0	0	0.60	5	0	0	0	0	0.06	4

Compounds in bold are GC-EAD active. *RI* = Kováts Retention index in GC-MS (DB-Wax column, mobile phase helium). *O* = occurrence (number of samples in which the compound is detected)

^a Identity confirmed by comparison with a reference standard

^b (3*E*)-4,8-Dimethyl-1,3,7-nonatriene

^c (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

First, we tested whether the five host plant species had significantly different volatile profiles using Permutational Multivariate Analysis of Variance (PERMANOVA, based on Bray-Curtis distances calculated from % composition data, 999 permutations; Anderson 2001). Whenever a significant effect of species identity was detected, we tested differences between all possible pairs of species using pairwise PERMANOVAs with *Bonferroni* correction for multiple testing. Differences between species in levels of multivariate dispersion were tested using the PERMDISP procedure and *Tukey* tests for pairwise comparisons (Anderson 2006).

Second, whenever the PERMANOVA analyses indicated a significant effect of species identity on plant volatile profiles, we visualised those differences using non-metric multidimensional scaling (NMDS, based on Bray-Curtis distances calculated from % composition data, three synthetic variables/axes). NMDS belongs to the family of ordination methods (as for example Principal Component Analysis). It aims to summarise a multivariate dataset into a small number of synthetic variables/axes that retain as much of the original variance as possible, and can easily be visualised as a scatterplot. The NMDS algorithm first calculates distances between all possible pairs of samples, then, given the desired number of synthetic variables/axes, computes a locus for each sample such that the final distances on the ordination plot are as close as possible to the original distances. On the resulting plot, each point represents one volatile sample. Samples located close to each other have similar volatile profiles, while samples located far from each other have contrasted volatile profiles. The discrepancy between distances on the ordination plot and the original distances calculated from raw data is measured by the stress value, which varies from 0 (perfect correspondence) to 1 (no correspondence). In our case, three synthetic axes were enough to achieve a stress lower than 0.15, the highest value considered as acceptable (Proffitt and Johnson 2009).

Statistical Analysis: Characterisation of Species Identity Cues in the GC-EAD Active Odor Profiles For the GC-EAD active odor profiles, we aimed to further characterise the differences we had detected among plant species. Which features could serve as a cue for the olfactory differentiation of host plant species? Are those features conserved enough among individual plants for previously experienced plant species to be recognisable if encountered again? We tested if each plant species displayed conserved ratios among some of the GC-EAD active compounds it emitted, using *Kendall's* tests for correlation on absolute emission rates ($\text{ng}\cdot\text{h}^{-1}$) of all possible pairs of GC-EAD active compounds. Multiple testing was accounted for by applying *Benjamini-Hochberg's* procedure for the control of false discovery rate. When significant correlations between two given compounds were found in more than one species, we tested if the ratio differed significantly between species using ANCOVA models explaining the emission rate ($\text{ng}\cdot\text{h}^{-1}$) of one compound by emission rate of the other, species identity and the interaction between both factors.

Results

Overall Volatile Profiles: Composition of the Volatile Emissions of the five Plant Species A total of 48 compounds were detected in the headspace of the five sampled plant species, 23 of which could be identified (Table 1, extended version available as Table S1 in Online Resource 1). The amounts of volatiles emitted by these non-induced plants are very small. For instance, the highest observed value was $59 \pm 46 \text{ ng}\cdot\text{h}^{-1}$ for the most abundant compound of cotton, (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene ((E,E)-TMTT). The detection limit for the analyses was around $10 \text{ fg}\cdot\text{h}^{-1}$. Cabbage odor was dominated by monoterpenes

(mostly sabinene, 15% of the blend, and eucalyptol, also known as 1,8-cineole, 10%) as well as cyclopentanol (15%). Cotton emitted mostly the irregular terpenes (*E,E*-TMTT and (*E*)-4,8-dimethyl-1,3,7-nonatriene (*E*)-DMNT) (58% and 26% of the blend, respectively). Clover odor was dominated by the fatty-acid derivatives (*Z*)-3-hexenyl-acetate and (*Z*)-3-hexen-1-ol (58 and 33% of the blend, respectively). Cowpea emitted mostly monoterpenes, the major compound was myrcene (54% of the blend). Finally, maize mostly emitted sesquiterpene hydrocarbons (68% of the blend) and was the only species where at least some of these were consistently detected (found in at least ten out of twelve samples). The most abundant compounds emitted by maize were sesquiterpene 1, myrcene and an unidentified acetate ester. Several species also emitted species-specific volatiles. The irregular terpenoids α - and β -isophorone as well as one still unidentified compound (“unidentified 5”) were virtually only found in cabbage. Cotton was the only species to produce 2,5-dimethyl pyrazine, isopentyl acetate and linalool oxide. Two unidentified sesquiterpene hydrocarbons were only detected in maize (“sesquiterpene 1” and “sesquiterpene 7”).

In accordance with the above description, average overall volatile profiles significantly differ between species, both globally (PERMANOVA on % composition data, $P = 0.001$) and in pairwise comparisons (pairwise PERMANOVAs, all *Bonferroni*-corrected- $P = 0.01$). This is illustrated by the NMDS ordination presented as Fig. 1a, where all five host plant species are spatially segregated.

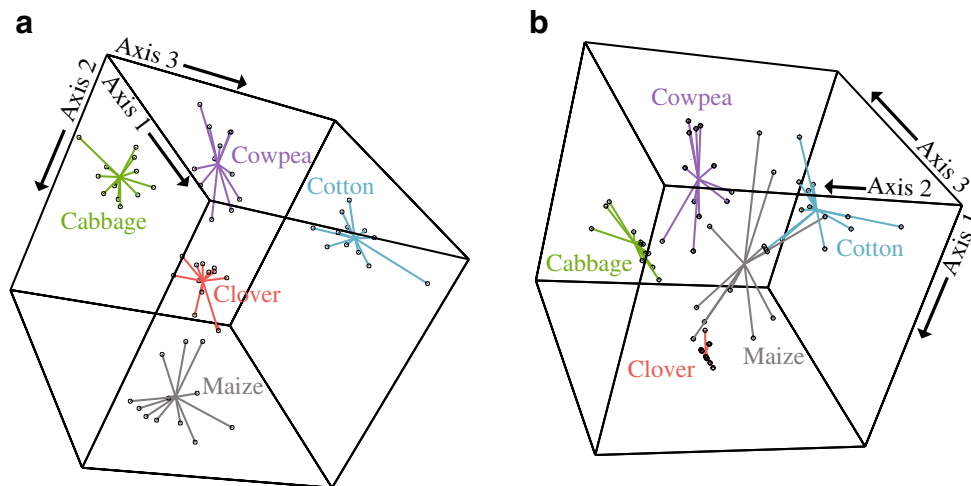
Odor Profiles: Olfactory Perception of the five Host Plant Species by *S. littoralis* Females We detected a total of 15 electroantennographic responses across the five plant species, ten of which could be assigned a putative identity (see Table S3 in Online Resource 1). These ten GC-EAD active compounds are highlighted in bold in Table 1. They are among the most commonly reported volatiles from green plant odors, with the exception of the cabbage-specific α -

isophorone. We only found two mentions of α -isophorone in the plant volatile literature, both from varieties of *Brassica oleracea* (Fernandes et al. 2009; Weldegergis et al. 2015).

After restricting the analysis to GC-EAD active volatiles only, the five host plant species still differ significantly in their odour profiles, both globally (PERMANOVA on % composition data, $P = 0.001$) and in pairwise comparisons (pairwise PERMANOVAs, all *Bonferroni*-corrected- $P = 0.01$). This pattern can be visualised on the NMDS ordination presented as Fig. 1b, where all five host plant species are clearly spatially segregated, in a similar way as in the case of overall odor profiles (Fig. 1a). However, maize no longer has a clearly defined profile. Maize samples now plot in between all other species, and are much more dispersed around their average position than the other species, reflecting a significantly higher level of multivariate dispersion (PERMDISP analysis, $P = 1.21 \times 10^{-11}$; *Tukey* test for pairwise comparisons, $P < 10^{-6}$ when comparing maize to any other species, $P > 0.05$ when comparing other species to one another). This pattern is explained by the fact that contrary to the other four species, none of the GC-EAD active volatiles are present in all 12 out of 12 maize samples, making maize GC-EAD active odour profile much more variable among individual plants than in any other species (see Fig. S6 in Online Resource 2). Indeed, none of the sesquiterpenes that dominate maize volatile emissions elicited any GC-EAD response.

The statistical patterns described above reflect the fact that with the exception of maize, the plant species studied here emit specific combinations of GC-EAD active compounds, as illustrated in Fig. 2. Each of these four species is characterised by one or two major GC-EAD active compounds whose identity or combination is different for each of them. Cabbage is characterised by myrcene and eucalyptol (around 50% and 30% of the GC-EAD active odor profile, respectively), cotton by a (*E*)-DMNT-dominated blend (80%), clover by a combination of (*Z*)-3-hexenyl acetate (60%) and (*Z*)-3-hexen-1-ol (40%), and cowpea by a myrcene-dominated blend (70%).

Fig. 1 NMDS ordination on the relative composition (percentage of each compound) of the volatiles emitted by the five host plant species, based on (a) overall volatile profiles (all compounds, stress = 0.092), (b) GC-EAD active odour profiles (stress = 0.08). Each point represents the volatile profile of one individual plant. Individual plants of the same species are connected by lines converging on the species' centroid



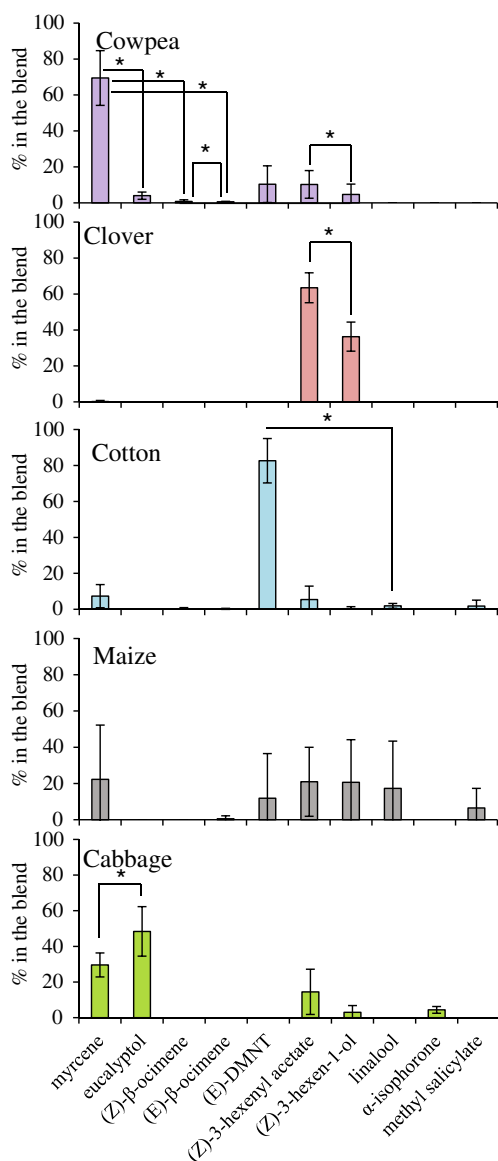


Fig. 2 The olfactory perception of the five host plant species by *Spodoptera littoralis*. The average GC-EAD active odor profile of each plant species, expressed as a percentage of each compound in the GC-EAD active blend. Brackets and asterisks indicate pairs of compounds whose absolute emission rates ($\text{ng}\cdot\text{h}^{-1}$) are significantly positively correlated with one another (pairwise Kendall's correlation tests, false discovery rate < 0.05)

Can GC-EAD Active Compound Ratios Convey Information about Species Identity? All four host plants that have a clearly defined GC-EAD active odour profile show significant positive correlations between the emission rates of either the two main GC-EAD active compounds (in cabbage and clover) or between the main compound and at least one of the minor compounds (cotton: (E)-DMNT with linalool; cowpea: myrcene with eucalyptol, (E)- and (Z)-β-ocimene, summarised in Fig. 2, see Figs. S1 to S5 in Online Resource 2 for details), indicating that some of the compound ratios are conserved across plant individuals and can constitute a

species-specific signature. In all cases, the compounds involved belong to the same biosynthetic class (terpenoids, or fatty-acid derivatives), and the correlation might simply be the result of shared biosynthetic pathway. No significant correlation between the emission rates of any pair of individual compounds was observed in maize.

We found two pairs of volatiles whose emission rates were significantly positively correlated in more than one species. For the first pair, myrcene and eucalyptol, the slope of the relationship between their respective emission rates significantly differs between cowpea and cabbage (ANCOVA analysis, significant interaction term Table 2A, Fig. 3a), indicating that the two species emit these two compounds in contrasted ratios. In the case of the second pair, (Z)-3-hexenyl acetate and (Z)-3-hexen-1-ol, emission rates are positively correlated in clover and cowpea (significant), as well as in cotton and maize (non-significant tendency, see Figs. S2 and S5 in Online Resource 2). The slope of the relationship does not significantly differ among these four species (ANCOVA analysis, non-significant interaction term, Table 2B), indicating that the ratio between these two closely related compounds is conserved across species (Fig. 3b).

Discussion

Our results clearly show that non-induced vegetative volatiles do convey information on species identity. The five plant species investigated in this study show different average volatile profiles, and an absence of overlap of their envelopes of variation (Fig. 1a). Each of them emits specific combinations and proportions of common volatiles, as well as some species-

Table 2 ANCOVA models for pairs of GC-EAD active volatiles whose emission rates are significantly correlated in more than 1 species (see Fig. 2 and Figs. S 1 to S5 in Online Ressource 2), explaining the absolute emission rate of one compound by the emission rate of the other and the species identity

A. Eucalyptol = Myrcene * Species (levels for species: Cabbage, Cowpea)				
factor	df	F	P	% variance explained
myrcene	1	0.84	0.37	2.3
species	1	7.2	0.01	20
interaction	1	8.0	0.01	22
residuals	20			55
B. (Z)-3-hexen-1-ol = (Z)-3-hexenyl-acetate * Species (levels for species: Cotton, Clover, Cowpea, Maize)				
factor	df	F	P	% variance explained
(Z)-3-hexenyl acetate	1	412	<2.10 ⁻¹⁶	88
species	3	3.6	0.021	2.3
interaction	3	1.4	0.27	0.88
residuals	40			8.6

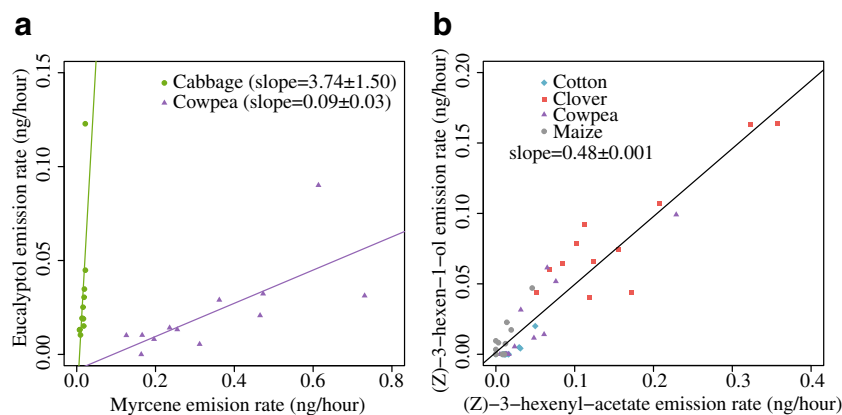


Fig. 3 Regression plots for pairs of GC-EAD active compounds that are significantly positively correlated with one another in more than one host plant species. **a** The slope of the relationship between myrcene and eucalyptol (representing the ratio between both compounds) is significantly different in cabbage and cowpea. Multiple R^2 of the

ANCOVA model = 0.45. **b** The slope of the relationship between (Z)-3-hexenyl acetate and (Z)-3-hexen-1-ol does not vary among host plant species. Multiple R^2 of the ANCOVA model = 0.91. Slopes are estimates from the ANCOVA model

specific volatiles (Table 1). The subset of volatiles we found to be GC-EAD active in *S. littoralis* is sufficient to differentiate alternative host plant species using olfaction alone, since GC-EAD active odor profiles are conserved within plant species and contrasted between species. Within species, we found that at least some of the GC-EAD active compounds were emitted by every single individual and/or in conserved ratios for four out of five species. Similarly, Webster et al. (2010) reported that ratios among volatiles emitted by *Vicia faba* are conserved among individual plants. Between species, the combinations and/or proportions of GC-EAD active compounds emitted differ (Fig. 2), which is illustrated by the spatial segregation of plant species on the NMDS graph (Fig. 1b). Using the same statistical approach, Späthe et al. (2012) also found that the subset of volatiles perceivable by *Manduca sexta* was sufficient for them to differentiate *Nicotiana attenuata* and *Datura wrightii*.

Elements of olfactory profiles that are conserved among host plant individuals seem a necessary condition for the fine-tuned adaptation of a monophagous insect's olfactory system to their host's characteristic odor (Webster et al. 2010), or for a polyphagous insect to be able to act upon previous olfactory experiences in the context of experienced host plant choice. We do find such conserved elements for most of the plant species we have investigated here. The case of maize, however, seems to challenge this view. The results from Thöming et al. (2013) show that *S. littoralis* adults are able to utilize experiences of maize perceptual appearance that they have formed during larval development (individuals grown on maize prefer it as adults), whereas we did not detect any conserved feature in the GC-EAD active odor profile of this plant. It is, however, possible that the picture of olfactory perception of maize by *S. littoralis* we have depicted is incomplete. We have excluded from our analyses a number of compounds that are known to be emitted by some plant species, but that cannot be reliably quantified in our study because they are

abundant in ambient air (Table S2 in Online Resource 1). In addition, previous single sensillum recording (SSR) studies have reported as electrophysiologically active several compounds for which we have detected no GC-EAD response (for example, β -pinene, β -phellandrene or TMTT in SSR by Binyameen et al. 2014). Responses to compounds that excite rare sensilla types might be lost in the noise or filtered out when recording from whole antennae, but might still contribute to odor coding in the antennal lobes.

S. littoralis adults show a clear innate preference hierarchy among the five host plant species we have studied here (Thöming et al. 2013). In Fig. 2, the five species are ordered according to this hierarchy, with the most preferred plant at the top, and the least preferred one at the bottom. This arrangement does not reveal any obvious correlation between emission rate or proportion of a specific volatile and ranking in the hierarchy. Understanding how olfactory perceptions translate into host plant preferences would therefore require the comparison of the neural representation of alternative host plant odors in the moth's brain. Thöming et al. (2013) report that host plant preference hierarchies are mostly consistent between males and females despite the fact that female choice was tested in a setup that gives them access to a wider range of sensory cues: oviposition assay for females versus wind tunnel for males. This observation suggests that olfactory cues do explain a major part of host plant choice behaviour.

The case of clover constitutes an interesting counterexample (Thöming et al. 2013). Plant-naïve males ranked it second to last (wind tunnel assay, only olfactory cues available), whereas it was first *ex-aequo* with cowpea in the female oviposition preference hierarchy (olfactory and contact cues available). We found the clover GC-EAD active volatile profile to be characterised by the virtually exclusive emission of (Z)-3-hexenyl acetate and (Z)-3-hexen-1-ol (Fig. 2). However, these two compounds are emitted by all plant species with the

same ratio (Fig. 3b). Clover therefore appears to have no species-specific olfactory signature that we can detect, and would probably be inconspicuous to *S. littoralis* olfaction when encountered in a complex plant community. (Z)-3-hexenyl acetate is biosynthesised directly from (Z)-3-hexen-1-ol (Matsui 2006), which could explain the conservation of their ratio across plant species. Further behavioural experiments are required to test whether the low ranking of clover in wind tunnel assays is due to a lack of attraction or to an active avoidance. If the olfactory inconspicuousness of clover is confirmed, its high ranking in the preference of unexperienced females would then be explained by contact cues. Clover's olfactory inconspicuousness would make it an interesting tool to control for visual and contact cues while manipulating olfactory cues in host plant choice assays.

The second plant for which Thöming et al. (2013) evidenced an unusual behaviour is cabbage. They found it to be the least innately preferred host plant species, despite the fact that larvae perform as well on cabbage as on more preferred host plants in laboratory conditions (personal observation). Furthermore, cabbage-reared *S. littoralis* individuals still preferred any alternative host plant to cabbage (Thöming et al. 2013). We find that cabbage is the only plant whose GC-EAD active odor profile comprises a species-specific volatile. This volatile, α -isophorone, is biosynthesised via the degradation of carotenoids (Fernandes et al. 2009), which does not indicate any obvious link with glucosinolates and isothiocyanates, the toxins specific to Brassicaceae. However, another carotenoid degradation product (β -ionone), part of canola's induced response to herbivores, has been found to repel generalist herbivores as well as Brassicaceae specialists (Cáceres et al. 2016). Another specific feature of cabbage odor is the high proportion of eucalyptol in its GC-EAD active volatile profile. In bark beetles eucalyptol strongly reduces pheromone attraction, and inhibits the activation of pheromone-OSN, through the activation of a co-localised OSN (Andersson et al. 2010).

The exception to behavioural plasticity represented by the hard-wired avoidance of cabbage, even by individuals that successfully developed on it, suggests that at least a part of the innate choice behaviour is ecologically and adaptively relevant. The emission of certain volatiles might consistently correlate with low host plant suitability at the scale of a plant community. Such correlations could possibly arise from a given volatile being biosynthesised through the same pathway as a frequently encountered plant toxin. Indeed, many biosynthetic classes of secondary metabolites do comprise both volatiles and toxins, and some toxins do have volatile precursors (for example putrescine and nicotine, Mithöfer and Boland 2012). Alternatively, such a correlation could be a fortuitous consequence of the composition of the local plant community,

in which case outlier plant species may explain occasional maladaptive choices.

In *S. littoralis*, only one compound has so far been confirmed as a behavioural antagonist. (*E*)-DMNT inhibits male attraction towards the sex pheromone (Hatano et al. 2015) and is part of a volatile blend that is responsible for the avoidance of herbivore-induced cotton plants by ovipositing females (Zakir et al. 2013b). The investigation of its neural representation in the antennal lobe evidenced that (*E*)-DMNT inhibits overall antennal lobe activation by the sex pheromone and by an attractive synthetic blend mimicking host plant odor (Hatano et al. 2015). Whether this pattern reflects the neural mechanism responsible for avoidance of unsuitable plants by *S. littoralis* can only be confirmed by replicating the experiment with other behavioural antagonists. As pointed out above, candidates can be found among the volatiles emitted by plants that are known to negatively impact reproductive behaviour, such as described in Binyameen et al. (2013), or possibly cabbage.

Host plant choice behaviour in polyphagous herbivores is likely the result of a complex and plastic neural process, evolved under conflicting selective pressures by a complex and changing plant community. Here we show that olfaction is sufficient for a polyphagous herbivore insect to differentiate alternative host plant species, which is a prerequisite to the exhibition of a choice behaviour. However, we do not find any straightforward link between odor profile and ranking in the innate preference hierarchy. The investigation of neural representation (patterns of antennal lobe activation) of alternative host plant odors appears as the next step in order to crack the code behind innate host plant preferences. However, in polyphagous herbivores, the way in which odor perceptions translate into behaviour also strongly depends on previous experiences (Proffitt et al. 2015; Thöming et al. 2013). The neural mechanisms by which experience modulates the behavioural outcome of a given perception are still poorly understood (Anderson and Anton 2014). Studying the neural representation of behavioural antagonists identified from plants eliciting a hard-wired avoidance (such as cabbage) or by non-host plants (Binyameen et al. 2013) can help shed light on the mechanisms behind avoidance of unsuitable plants. By identifying the volatiles involved, our results set the groundwork for further investigations of how olfactory perceptions translate into behaviour in polyphagous herbivores.

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Compliance with Ethical Standards

Conflicts of Interest The authors declare that they have no conflict of interest.

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