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Arthur De Fouchier, Xiao Sun, Gabriela Caballero-Vidal, Solène Travaillard, Emmanuelle Jacquin-Joly, Nicolas Montagné

HAL Id: hal-01992162

https://hal.sorbonne-universite.fr/hal-01992162v1

Submitted on 24 Jan 2019

Behavioral effect of plant volatiles binding to *Spodoptera littoralis* larval odorant receptors

1 **Arthur de Fouchier[#], Xiao Sun[‡], Gabriela Caballero-Vidal, Solène Travaillard[¶], Emmanuelle**
2 **Jacquin-Joly & Nicolas Montagné[*]**
3
4 Inra, Sorbonne Université, CNRS, IRD, UPEC, Université Paris Diderot, Institute of Ecology and
5 Environmental Sciences of Paris, Paris and Versailles, France
6
7 [#] present address: Laboratoire d'Ethologie Expérimentale et Comparée (LEEC), Université Paris 13,
8 Sorbonne Paris Cité, Villetaneuse, France
9 [‡] present address: Biotic interaction and biosecurity Lab, School of Life Sciences, Henan University,
10 Kaifeng, Henan, China
11 [¶] present address: Aix-Marseille Université, CNRS, Institut de Biologie du Développement de
12 Marseille, Marseille, France
13
14 *** Correspondence:**
15 Nicolas Montagné
16 nicolas.montagne@sorbonne-universite.fr
17
18

19 **Keywords: insect, olfaction, olfactory receptor, volatile organic compound, crop pest,**
20 **caterpillar, Lepidoptera, Noctuidae**
21

22 **Word count: 3732**

23 **Number of figures and tables: 5**

24

25 **Abstract**

26 Phytophagous insects use volatile organic compounds emitted by plants to orient towards their hosts.
27 In lepidopteran pests, crop damages are caused by larval stages – the caterpillars – that feed
28 extensively on leaves or other plant tissues. However, larval host plant choice has been poorly
29 studied, and it is generally admitted that caterpillars feed on the plant where the female laid the eggs.
30 The mobility of caterpillars has been generally overlooked even though several studies showed that
31 they can orient towards odors and change host plant. Recently, a large number of odorant receptors
32 (ORs) tuned to plant volatiles have been characterized in the model pest moth *Spodoptera littoralis*
33 (Noctuidae). In the present work, we identified 9 of these deorphanized ORs as expressed in *S.*
34 *littoralis* caterpillars. In order to understand whether these ORs are involved in host searching, we
35 tested the behavioral significance of their ligands using a larval two-choice assay. This OR-guided
36 approach led to the identification of 9 plant volatiles, namely 1-hexanol, benzyl alcohol,
37 acetophenone, benzaldehyde, (Z)3-hexenol, (E)2-hexenol, indole, DMNT and (Z)3-hexenyl acetate,
38 which are active on *S. littoralis* caterpillar behavior, increasing our knowledge on larval olfactory
39 abilities. To further explore the link between OR activation and behavioral output induced by plant

40 volatiles we used a modeling approach, thereby allowing identification of some ORs whose
41 activation is related to caterpillar attraction. These ORs may be promising targets for future plant
42 protection strategies.
43

1   Introduction

45 Holometabolous insects are characterized by two mobile developmental stages with drastically
46 different morphologies and physiologies. The larval stage constitutes a period of active feeding and
47 growth, while the adult stage is a period devoted to reproduction and dispersal. Larvae and adults
48 thus have different life styles, are not in competition for the same resources, and develop independent
49 adaptations in response to different selective pressures. This distinction between adults and larvae is
50 particularly striking in Lepidoptera. While larvae (or caterpillars) are actively feeding on their host
51 plant, the adults generally live only a few days and feed on the nectar of flowers (Powell, 2009).
52 Almost all plant species are damaged by caterpillars, many of which are pests of both crops and
53 stored products (Stehr, 2009).
54 Host plant choice is a crucial task for phytophagous insects, and it is highly dependent on the sense
55 of smell. The detection of plant-emitted volatile organic compounds (VOC) has been the subject of
56 intense research, notably in crop pest insects (Bruce et al., 2015; Bruce and Pickett, 2011). In a
57 number of lepidopteran pests, VOCs have been identified as attractants towards host plants, as
58 repellents towards non-host or damaged plants or as oviposition stimulants (Borrero-Echeverry et al.,
59 2015; Saveer et al., 2012). However, despite the impact of caterpillars on crop production, most
60 studies focused on the adults and little is known about larval olfaction. A well-admitted theory,
61 referred as "mother knows best", assumes a strong selective pressure for females to lay their eggs on
62 the plant where the larvae will have the highest performance (Carrasco et al., 2015; Jaenike, 1978).
63 However, in some species it has been demonstrated that the caterpillars can leave the plant on which
64 they hatched to select another host plant (Gamberale-Stille et al., 2014; Soler et al., 2012).
65 Consistently, caterpillars exhibit attraction or repulsion behaviors towards VOCs of ecological
66 significance (Becher and Guerin, 2009; Carroll et al., 2006, 2008; Carroll and Berenbaum, 2002;
67 Castrejon et al., 2006; Di et al., 2017; Huang and Mack, 2002; Mooney et al., 2009; Piesik et al.,
68 2009; Poivet et al., 2012; Singh and Mullick, 2002; Zhu et al., 2016) and are even able to perform
69 associative learning (Blackiston et al., 2008; Salloum et al., 2011). This indicates that olfaction may
70 play a more prominent role than initially expected in host plant choice of caterpillars, which could lay
71 foundation for the development of novel pesticide-free strategies for fighting against those insects.
72 The peripheral olfactory system of caterpillars is generally composed of three olfactory sensilla
73 located on the antennae, and four to five olfactory sensilla located on the maxillary palps (Grimes and
74 Neunzig, 1986; Laue, 2000; Poivet et al., 2012; Roessingh et al., 2007; Vogt et al., 2002; Zielonka et
75 al., 2016). These sensilla house the olfactory sensory neurons that express transmembrane odorant
76 receptor (OR) proteins, which bind odorants and allow signal transduction (Leal, 2013). The
77 repertoires of ORs expressed in caterpillar tissues have been identified only in a few species, such as
78 the silkworm *Bombyx mori* (Tanaka et al., 2009), the cotton bollworm *Helicoverpa armigera* (Di et
79 al., 2017) and the cotton leafworm *Spodoptera littoralis* (Poivet et al., 2013). In this latter species, 15
80 ORs (further referred as SlitORs) tuned to plant VOCs have been recently deorphanized (de Fouchier
81 et al., 2017), i.e. their ligands have been identified (Supplementary Figure S1). These VOCs are
82 mainly short-chain alcohols, aldehydes or esters (also referred as green leaf volatiles, abundantly
83 released from damaged leaves), aromatics and terpenes (most of them being ubiquitous odorants,
84 present in high amounts in floral bouquets). However, the effect of these SlitOR ligands on the
85 behavior of *S. littoralis* larvae remains largely unknown. Among them, only 1-hexanol (a green leaf

2

86 volatile) has been shown to be attractive at high dose toward 2nd and 3rd-instar larvae (Rharrabe et al.,
87 2014).
88 In the present work, we first re-examined the expression pattern of the 15 deorphanized SlitORs in
89 adult and larvae olfactory organs, and identified 9 as expressed at the larval stage. We then used a
90 simple bioassay to carry out a systematic behavioral analysis of 14 VOCs previously identified as
91 ligands of these 9 SlitORs. Using this OR-guided approach, we found 1-hexanol, benzyl alcohol,
92 acetophenone, benzaldehyde, (Z)3-hexenol, (E)2-hexenol, indole, DMNT and (Z)3-hexenyl acetate
93 as active on the behavior of *S. littoralis* caterpillars, increasing our knowledge on larval olfactory
94 abilities. Building on the results of these behavioral assays and on our previous knowledge of SlitOR
95 response spectra (de Fouchier et al., 2017), we used a modeling approach in order to identify possible
96 correlations between the activation of SlitORs and the behavioral response of caterpillars. By doing
97 so, we highlighted ORs whose activation may be critical for larval attraction towards plant volatiles.
98

99 **2   Material and methods**

100 **2.1   Insects and chemicals**

101 *S. littoralis* larvae were reared on a semi-artificial diet (Poitout and Bues, 1974) at 22°C, 60 %
102 relative humidity and under a 16 h light: 8 h dark cycle. The panel of odorants tested was composed
103 of 14 synthetic molecules (Supplementary Table S1) previously shown to be active on SlitORs
104 expressed at the larval stage (de Fouchier et al., 2017). Odorants were diluted in paraffin oil (Sigma-
105 Aldrich, St Louis, MO, USA), except indole that was diluted in hexane (Carlo-Erba Reagents, Val de
106 Reuil, France). The odorants were used at concentrations of 100, 10, 1, 0.1 or 0.01 µg/µl.
107

108 **2.2   RNA isolation and reverse-transcription PCR**

109 Fifty *S. littoralis* male and female adult antennae and 50 pairs of 4th-instar larvae antennae and
110 maxillary palps were dissected and immediately placed in TRIzol™ Reagent (Thermo Fisher
111 Scientific, Waltham, MA, USA) for total RNA extraction. After isolation using phenol-chloroform,
112 RNA was purified using the RNeasy Micro Kit (Qiagen, Venlo, Netherlands), including a DNase I
113 treatment. RNA purity and quantity were measured on a NanoDrop™ ND-2000 spectrophotometer
114 (Thermo Fisher Scientific). cDNA synthesis was performed using 1 µg of total RNA as template,
115 with the iScript Reverse Transcription Supermix (BioRad, Hercules, CA, USA). PCRs were
116 performed using the LightCycler® 480 SYBR Green I Master mix (Roche, Basel, Switzerland) under
117 the following conditions: 95°C for 5 min, followed by 40 cycles of denaturation (95°C for 10 s),
118 hybridation (58-62°C – depending on primer pairs – for 15 s) and elongation (72°C for 15 s). Primer
119 pairs were designed from SlitOR nucleotide sequences using Primer3Plus
120 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi). All primer sequences, annealing
121 temperatures and expected product sizes are listed in Supplementary Table S2. Orco, the obligatory
122 OR co-receptor (Leal, 2013; Malpel et al., 2008), was used as control for the four tissues. For each
123 amplification, negative controls consisted of amplifications run on DNase-treated RNAs and water
124 templates. The amplification products were loaded on 1.5 % agarose gels and visualized using
125 GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). Tissue dissections, RNA
126 extractions and RT-PCR experiments were repeated three times at different periods, to serve as
127 biological replicates.
128

129 **2.3   Behavioral experiments**

130   Two-choice behavioral assays were performed using *S. littoralis* 3rd and 4th-instar larvae, starved for
131   16 to 22 hours prior to experiments. The behavioral assay consisted in placing 10 caterpillars in the
132   center of a Petri dish. Filter papers were placed at two opposite sides of the dish. One was loaded
133   with 10 µl of an odorant solution and the other with 10 µl of the corresponding solvent. Each odorant
134   concentration was tested 10 to 15 times. For each experiment, 10 Petri dishes (containing 10 different
135   odorants) and one control dish with solvent on both sides were recorded during 15 minutes. In each
136   dish, two zones were defined around the filter papers, an "odorant" zone and a "solvent" zone (the
137   layout of the zones are visible in Figure 1). The number of caterpillars in each zone was counted 2.5,
138   5, 10 and 15 minutes after the beginning of the experiment.
139

140   **2.4   Data analysis and modeling**

141   For each time point, a preference index (PI) was calculated using the following formula:

142   $$PI = (N_{odorant} - N_{solvent})/(N_{total})$$

143   $N_{odorant}$ being the number of larvae in the odorant zone, $N_{solvent}$ being the number of larvae in the
144   solvent zone and N_{total} being the total number of larvae in the assay. As this PI varies between -1 and
145   1, a positive value means that the odorant is attractive and a negative value indicates repellency. To
146   test for the statistical significance of the observed PI, we compared the value to a theoretical value of
147   0 with a Wilcoxon two sided unpaired test using R (Package stats version 3.3.2).
148   In order to compare observed PIs with responses of the SlitORs (in spikes.s^{-1}) when expressed in the
149   *Drosophila* empty neuron system (de Fouchier et al., 2017), we performed multiple linear regressions
150   using the "step" and "lm" function of R (Package stats version 3.3.2). To obtain the most efficient
151   equation, we performed stepwise linear regressions relating PI with all possible interactions between
152   the larval SlitOR responses (SlitOR7, 14, 19, 24, 25, 27, 28, 29 and 31). As odorant stimulus
153   quantities used in electrophysiology experiments cannot be directly related to quantities used in the
154   present behavior experiments, we built models for different electrophysiology-behavior odorant
155   quantity relationships (1:1, 1:1/10, 1:1/100 and 1:1/1000). We selected the equation with the highest
156   R² and refined it performing another stepwise multiple linear regression. This model relates the PI
157   with all the interactions between the factors with an impact significantly different from zero (Pr(>t) *p*
158   ≤ 0.05) in the previously selected model. To further simplify the model, we performed a last multiple
159   linear regression relating PI with only additive interactions of the previously used variables.
160   We also built some models to further test the importance of the different SlitORs in predicting larval
161   PI. One using all possible interactions between the responses of SlitOR14, 19, 28, 29 and 31, and
162   four other models using linear regressions of the PI explained by the response from only SlitOR7, 24,
163   25 or 27.
164

165   **3   Results**

166   **3.1   Expression of SlitORs at the larval stage**

167   The expression pattern of 15 previously deorphanized SlitORs in male and female adult antennae,
168   larval antennae and larval maxillary palps (4th-instar larvae) was re-investigated using RT-PCR. As
169   found previously, all SlitORs were expressed in male and female antennae. Among them, 9 SlitORs
170   were also expressed in larval tissues (Figure 2). Five ORs were expressed in larval antennae
171   (SlitOR14, 19, 24, 28 and 31), and 4 ORs were expressed in both larval antennae and maxillary palps
172   (SlitOR7, 25, 27, 29). Altogether, these 9 ORs were previously found to detect 20 plant VOCs
173   (Supplementary Figure S1) among a panel of 50 molecules from different chemical classes, when

174   expressed in the *Drosophila* empty neuron system (de Fouchier et al., 2017). We then selected a
175   panel of 14 of these odorants, chosen based on the distinct OR activation patterns they elicit, in order
176   to test their effect on larval behavior.
177

178   **3.2   Behavior of *S. littoralis* caterpillars toward SlitOR ligands**

179   We assessed the valence of plant VOCs for *S. littoralis* caterpillars by describing their repartition in a
180   two-choice bioassay (Figure 1) using a preference index (PI) over a period of 15 minutes. Figure 2
181   reports the PIs measured at 2.5 minutes for the different VOCs at different doses. PIs measured for
182   other time points are presented in Supplementary Figure S2. For 2-phenyl acetaldehyde, 1-indanone,
183   (E)-ocimene and eugenol, we observed no significant attraction (PI>0) or repulsion (PI<0), at any
184   dose and any time. Benzyl alcohol, acetophenone, benzaldehyde, indole, 1-hexanol, (Z)3-hexenol and
185   (E)2-hexenol were attractive at least at one dose, with the highest PI measured at 2.5 minutes (Figure
186   3). 1-hexanol displayed the strongest attraction, with a mean PI of 0.50 at 100 µg, and 0.44 at 10 µg.
187   Benzyl alcohol was attractive over the wider range of doses, from 100 down to 1 µg per filter paper.
188   Benzaldehyde elicited attraction at 100 and 10 µg, and acetophenone only at 100 µg. Indole was
189   attractive at 10 and 0.1 µg only and (E)2-hexenol was attractive only at 1 µg. For most of these
190   VOCs, the PI tended to decrease over time (Supplementary Figure S2), which suggests that sensory
191   adaptation occurred. The only stimulus that remained attractive over time was acetophenone, when
192   presented at the highest dose (100 µg). (Z)3-hexenyl acetate differed from the previous VOCs as
193   doses of 100 and 10 µg were found to be attractive after 5 min of experiment, and not after 2.5 min
194   (Supplementary Figure S2).
195   At 2.5 minutes, benzaldehyde (at 0.1 µg) was the only VOC found to be repulsive (Figure 3). (Z)3-
196   hexenyl acetate (1 µg) was repulsive after 5 min, and (E)2-hexenal and DMNT also induced a
197   negative PI (for 0.1 and 100 µg, respectively) at 15 min of observation (Supplementary Figure S2).
198

199   **3.3   Modeling of the relationship between SlitOR activation and behavioral activity induced**
200   **by their ligands**

201   We next aimed to identify which of the SlitORs could be linked to attraction or repulsion towards
202   plant VOCs. To assess the correlation between the valence of odorants and their activation pattern of
203   ORs, we built models relating caterpillar PIs measured here with larval SlitOR responses to the same
204   odorants (previously characterized in de Fouchier et al., 2017). We used stepwise multiple linear
205   regressions, taking into account all possible interactions between the variables. The equations of the
206   first models built are available in Supplementary File S1. The multiple linear regression giving the
207   highest adjusted R^2 (0.6861) was the one using a 1:1 relationship between quantities used in behavior
208   and electrophysiology experiments (Table 1).
209   To identify the SlitORs whose activation is the most critical to the valence of plant odorants for
210   caterpillars, we refined the equation of the 1:1 model. For this, we performed stepwise multiple linear
211   regressions taking into account all possible interactions between the factors with an effect
212   significantly different from zero in the 1:1 model (Pr(>t) $p \leq 0.05$). This model was able to describe
213   the variation of PIs from the responses of 5 SlitORs (SlitOR7, 14, 24, 25 and 27; F-Test, $p \leq 0.001$,
214    $R^2 = 0.6366$, Table 1, Figure 4A and Supplementary Figure S3). The equation of the refined model is
215   given in Supplementary File S1. The intercept value of this model was not different from 0 (Pr(>t) p
216   ≥ 0.05), which predicts that an absence of SlitOR activation would result in an absence of behavioral
217   output. In this refined model, activation of SlitOR24 was predicted to have a positive effect by itself
218   on PIs (Pr(>t) $p \leq 0.05$), whereas activations of SlitOR7, 25 and 27 were predicted to have an effect

219   on PIs only through OR co-activation. SlitOR14 associated coefficients were not different from 0
220   (Pr(>t) $p \geq 0.05$).
221   As the refined model had a complicated equation (20 terms), we then built a simpler model to predict
222   the behavior using only additive interactions. The equation of this minimal model is:
223

$$PI = a + b \times SlitOR7 + c \times SlitOR24 + d \times SlitOR25 + e \times SlitOR27$$

224
225   with *SlitORx* as the ORx responses to the considered odorant in spikes.s^{-1} and *a-e* as coefficients .
226   The values of these coefficients (available in Supplementary File S1) were all different from 0 (Pr(>t)
227    $p \leq 0.05$), except for the intercept. The R² value for this model was 0.6115 (Table 1, Figure 4B and
228   Supplementary Figure S3), which is comparable to the performances of the refined 1:1 model.
229   SlitOR24 had the highest coefficient (2.6070×10^{-3}, $p \leq 0.001$), which further supports a link between
230   this receptor and neuronal circuits driving attraction in *S. littoralis* larvae. It is interesting to note that
231   the coefficient associated with SlitOR7 was negative (-5.0528×10^{-3}, $p \leq 0.05$). This predicts that
232   activation of SlitOR7 has a negative effect of the PI of *S. littoralis* caterpillars.
233   To further confirm the importance of those four SlitORs for models performance in predicting the
234   observed PI, we try to build a model using all interactions between all the SlitORs except SlitOR7,
235   24, 25 and 27. The stepwise multiple linear regressions method was unable to produce a model from
236   these variables, thus highlighting the importance of these receptors for the response of caterpillars to
237   the VOCs tested. We also built models using the responses from only SlitOR7, 24, 25 or 27. The R²
238   values for these models were respectively: 0.15, 0.48, 0.19 and 0.04. The values of the coefficients of
239   the intercept and of the SlitOR response were different from 0 (Pr(>t) $p \leq 0.05$), except for the
240   intercept of the model based on SlitOR24. These observations support that SlitOR24 is the most
241   important receptor to predict the PI observed for the plant volatiles we tested.
242

4   Discussion

244   Building upon the previous identification of ligands for a large number of *S. littoralis* ORs, we aimed
245   at identifying behaviorally active odorants for caterpillars, which are pests feeding on a wide range of
246   plants, notably economically important ones (Cabello, 1989; Proffit et al., 2015; Salama et al., 1971;
247   Thöming et al., 2013; von Mérey et al., 2013). Nine *S. littoralis* ORs were confirmed to be expressed
248   in larval chemosensory organs, namely the antennae and the maxillary palps. Our "OR-guided"
249   strategy, by which we tested molecules active on these larval SlitORs, appeared as a good strategy as
250   we could identify plant VOCs being behaviorally active when presented alone, most of them being
251   attractive to caterpillars. Following that work, it will be of interest to test the effect of blends of these
252   VOCs. It has been shown in *H. armigera* that a mixture of the best ligands of four ORs was the most
253   attractive stimulus for first-instar larvae (Di et al., 2017), and one would expect that the same holds
254   true for *S. littoralis*.
255   Our study complements a former study (Rharrabe et al., 2014) that investigated 11 odorants
256   commonly emitted by plants, identifying only a small part of them as behaviorally active. In this
257   previous work, eugenol was found to be repellent and 1-hexanol attractive. Here, attraction towards
258   1-hexanol could be reproduced in our assay but eugenol was inactive. This discrepancy could be
259   explained by the fact that odorants and controls were presented together with food pellets in the
260   aforementioned study while we used only filter papers as odor source. Hence, it is likely that
261   repellent VOCs for *S. littoralis* caterpillars may be identified only when given the choice between
262   food sources (or food odors) with or without the VOC.
263   Another interesting difference between these two types of behavioral assays is that the presence of
264   food will make the larvae stay on the food source once they have made a choice. In our experiments,

265 larvae resumed foraging after their initial choice, which enabled to observe a decrease of the PI in
266 most cases, likely due to sensory adaptation. Another possible explanation for this PI decrease would
267 be that the volume of the Petri dish has been rapidly saturated with the odor, leading to a loss of the
268 odor gradient necessary for larval orientation.
269 A similar OR-guided approach was recently used on another species of pest caterpillars, *H. armigera*,
270 and led to the identification of several OR ligands that were active on the behavior of first-instar
271 larvae (Di et al., 2017). Even if *S. littoralis* and *H. armigera* both belong to the same family
272 (Noctuidae) and are both highly polyphagous herbivores, their larval OR repertoires seem to differ
273 drastically. Indeed, the orthologues of only 3 of the 9 larval SlitORs were also found to be expressed
274 in *H. armigera* larvae (Di et al., 2017). The same holds true when comparing with the more distantly
275 related species *B. mori* (Tanaka et al., 2009). Accordingly, a limited number of odorants identified as
276 active on *S. littoralis* larvae are also active on other species, and vice versa.
277 The most attractive VOC (*i.e.* with the highest PI) was 1-hexanol, an ubiquitous plant volatile
278 (Knudsen et al., 2006), which has been observed to be attractive for caterpillars of the Tortricidae
279 *Lobesia botrana* (Becher and Guerin, 2009). Among other attractive compounds for *S. littoralis*
280 larvae, (Z)3-hexenol was also observed to be attractive to *L. botrana* and *H. armigera* (Di et al.,
281 2017), but not to *B. mori* (Tanaka et al., 2009). (Z)3-hexenyl acetate is a volatile released by plants
282 that suffered attacks from insects and it has been reported to serve as a chemical message between
283 plants (Frost et al., 2008; Helms et al., 2014). It has been observed to be attractive for the larvae of *S.*
284 *littoralis* (this study), *H. armigera*, *L. botrana*, and *B. mori*. This suggests that (Z)3-hexenyl acetate is
285 an important cue for a large spectrum of lepidopteran species. However, at a lower dose (1µg), it is
286 also the most repulsive VOC for *S. littoralis* caterpillars. Further experiments specially designed for
287 the identification of repellents would be necessary to confirm this repulsive effect, but *S. littoralis*
288 might use (Z)3-hexenyl acetate to detect and avoid damaged plants. Indeed, it has been demonstrated
289 previously that *S. littoralis* larvae are able to discriminate between different leaves of a host plant and
290 show a preference for young leaves, this preference being modified by herbivore damage (Anderson
291 and Agrell, 2005). (Z)3-hexenyl acetate is detected via the activation of several ORs (de Fouchier et
292 al., 2017). Their differential activation pattern relative to the dose may encode the concentration, as
293 previously hypothesized for pheromone receptors detecting the same pheromone component in adults
294 (de Fouchier et al., 2015).
295 From the comparison of behavior results with our previous results on SlitOR deorphanization (de
296 Fouchier et al., 2017), we built models that can predict PI values for odorants based on their OR
297 activation pattern. Results of this modelling approach suggest that larval attraction depends on the
298 activation of a particular subset of ORs (i.e. circuit-based) rather than on the summed response of the
299 entire OR repertoire. This will be possible to confirm this hypothesis only when the complete larval
300 OR repertoire will be characterized. In *D. melanogaster*, similar linear regression-based approaches
301 allowed to predict larval behavior from the responses of only 5 ORs (Kreher et al., 2008). Still in *D.*
302 *melanogaster*, a strong link has been identified between larval attraction and activation of two larval
303 ORs, DmelOR42a and DmelOR42b (Asahina et al., 2009; Grewal et al., 2014; Kreher et al., 2008).
304 Here, models supported that SlitOR24, 25 and 27 are involved in pro-attraction neuronal circuits,
305 while SlitOR7 activation would antagonize attraction. Activation of the first three receptors,
306 especially SlitOR24, seems to be sufficient to trigger attraction of *S. littoralis* toward different
307 concentrations of odorants. This will need further experimental validation, notably by identifying
308 new ligands for these receptors and testing their behavioral effect, but it could be a promising way to
309 identify new compounds that could impact the behavior of this important crop pest.
310

311 **Acknowledgments**

319   **Conflict of interest statement**

320   Authors declare that the submitted work was carried out in the absence of any personal, professional
321   or financial relationships that could potentially be construed as a conflict of interest.
322

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456   maize leaf volatiles affect attraction and feeding behavior of *Spodoptera littoralis* caterpillars.
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464   **Tables**

465   **Table 1. SlitOR/behavior multiple linear regression model statistics.**
466   Statistics associated with the models of *S. littoralis* caterpillars PIs. The Shapiro Test column
467   indicates the *p*-value of a normality test for the distribution of the model residuals. ***: $p \leq 0.001$,
468   **: $p \leq 0.01$, *: $p \leq 0.05$, NS: $p > 0.05$.
469

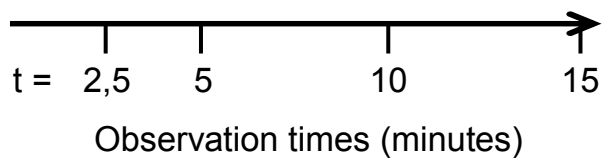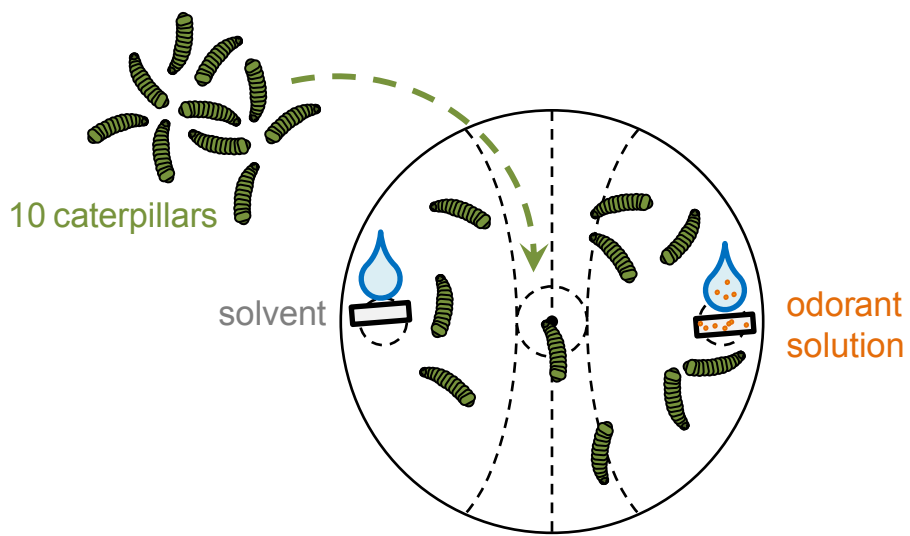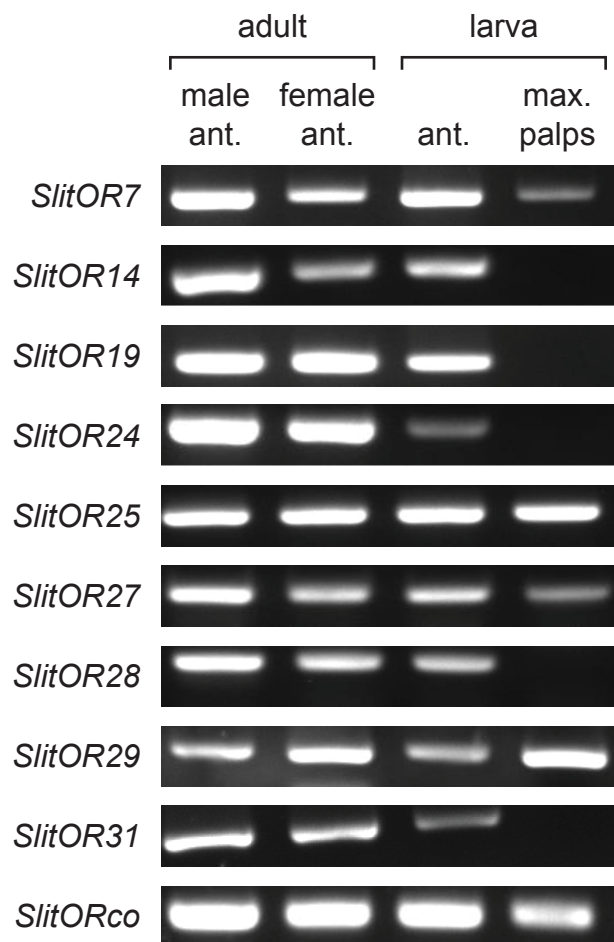| Model	Ajusted R²	Residual standard error	F-test	Shapiro Test
Model 1:1	0.6861	0.09647	***	***
Model 1:1/10	0.6225	0.1048	***	NS
Model 1:1/100	0.5795	0.1106	***	*
Model 1:1/1000	0.3061	0.142	***	NS
Refined 1:1 model	0.6366	0.1038	***	**
Minimal 1:1 model	0.6115	0.1073	***	NS

470

471   **Figure legends**

472   **Figure 1. Schematic of the behavior assay design.** Ten 3rd and 4th-instar caterpillars were put in the
473   center of a Petri dish after being starved for 16 to 22 hours. On one side of the dish, a filter paper
474   with 10 µl of an odorant solution was placed. Another filter paper with 10 µl of solvent was put at the
475   opposite side of the dish. The numbers of caterpillars in the different zones were recorded at 2.5, 5,
476   10 and 15 minutes. The preference index, ranging for 1 (attraction) to -1 (repulsion), was calculated
477   for each observation time.
478
479   **Figure 2. Tissue-specific expression of larval *S. littoralis* ORs identified by RT-PCR.** Each RT-
480   PCR was repeated three times on three separate RNA extractions. Only SlitORs found to be
481   expressed in larval antennae or maxillary palps in the 3 replicates are shown.
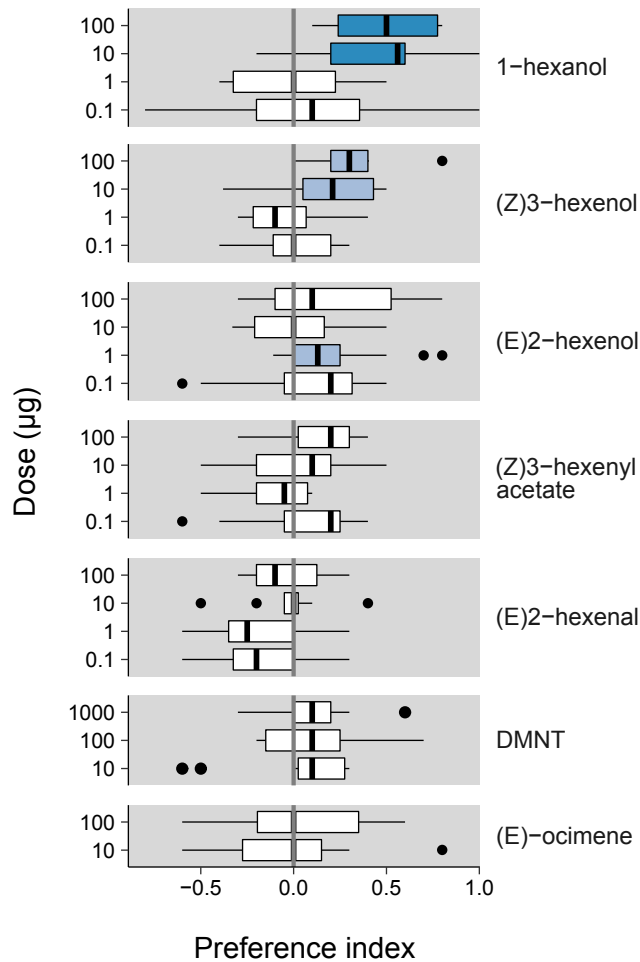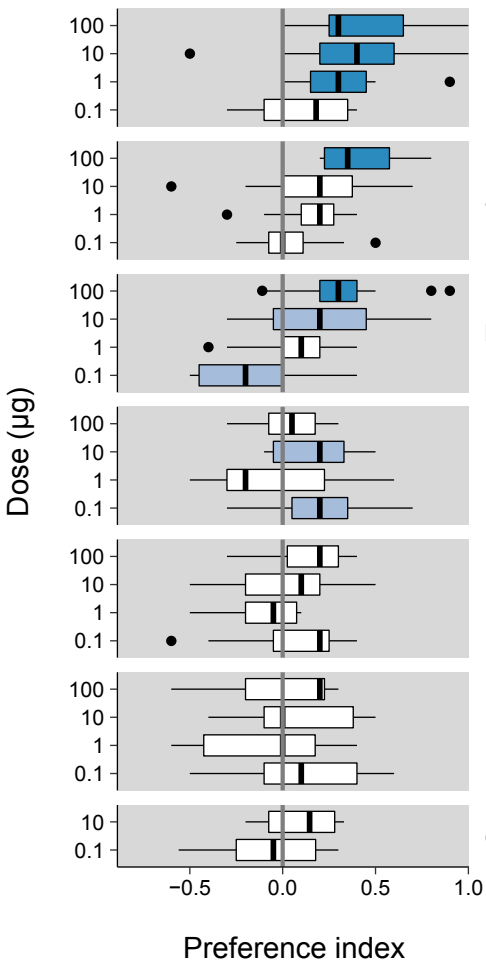482
483   **Figure 3. *S. littoralis* larval preference index (PI) measured 2.5 minutes after exposure to**
484   **different odorant stimuli.** Box plots show the median PI and the 25th and 75th percentiles ($n = 8$-15).
485   Outliers are indicated with black dots. *p*-values are indicated using a color code (Wilcoxon test).
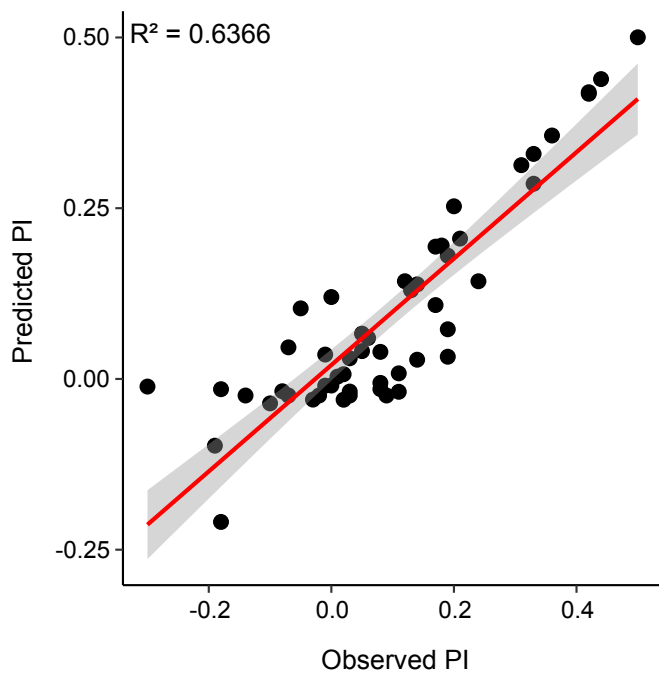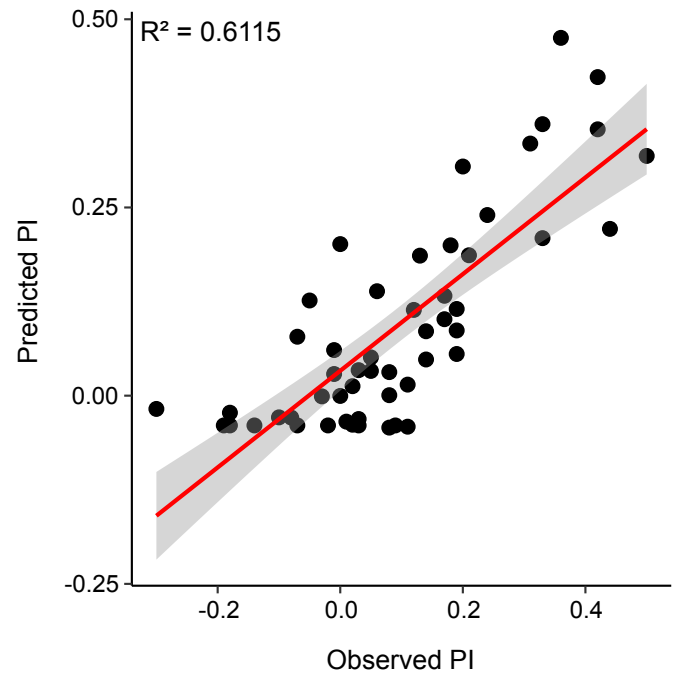486
487   **Figure 4.** Predicted preference index (PI) plotted as a function of the observed PI for the refined (**A**)
488   and minimal models (**B**). Red lines depict the linear trend while the overlaying gray band is the SE
489   for the fit.

10 caterpillars

solvent

odorant
solution

t = 2,5   5   10   15

Observation times (minutes)

	adult		larva	
	male ant.	female ant.	ant.	max. palps
SlitOR7				
SlitOR14				
SlitOR19				
SlitOR24				
SlitOR25				
SlitOR27				
SlitOR28				
SlitOR29				
SlitOR31				
SlitORco				

p-value   <0.01   <0.05   NS

Dose (µg)

benzyl alcohol

acetophenone

benzaldehyde

indole

1−indanone

2−phenyl acetaldehyde

eugenol

1−hexanol

(Z)3−hexenol

(E)2−hexenol

(Z)3−hexenyl acetate

(E)2−hexenal

DMNT

(E)−ocimene

Preference index

A Refined 1:1 Model

R² = 0.6366

B Minimal 1:1 Model

R² = 0.6115

Supplementary Material

Behavioral effect of plant volatiles binding to *Spodoptera littoralis* larval odorant receptors

Arthur de Fouchier[#], Xiao Sun[‡], Gabriela Caballero-Vidal, Solène Travaillard[¶], Emmanuelle Jacquin-Joly & Nicolas Montagné[*]

Inra, Sorbonne Université, CNRS, IRD, UPEC, Université Paris Diderot, Institute of Ecology and Environmental Sciences of Paris, Paris and Versailles, France

[#] present address: Entomology Department, Max-Planck Institute for Chemical Ecology, Jena, Germany
[‡] present address: School of Life Sciences, Henan University, Kaifeng, Henan, China
[¶] present address: Aix-Marseille Université, CNRS, Institut de Biologie du Développement de Marseille, Marseille, France

* Correspondence:
Nicolas Montagné
nicolas.montagne@sorbonne-universite.fr

Supplementary Table S1. Synthetic volatile organic compounds used in behavioral assays.

Compound	CAS number	Provider	Purity
benzyl alcohol	100-51-6	Aldrich	99
acetophenone	98-86-2	Acros	99
benzaldehyde	100-52-7	Aldrich	99.5
Indole	120-72-9	Aldrich	99
1-indanone	83-33-0	Aldrich	99
2-phenyl acetaldehyde	122-78-1	Aldrich	98
Eugenol	97-53-0	Aldrich	98
1-hexanol	111-27-3	Aldrich	98
(Z)3-hexenol	928-96-1	Aldrich	98
(E)2-hexenol	928-97-2	Aldrich	96
(Z)3-hexenyl acetate	3681-71-8	Aldrich	98
(E)2-hexenal	6728-26-3	Aldrich	98
(E)-ocimene	3779-61-1	Aldrich	65 (E)
(E)-4,8-dimethyl-1,3,7-nonatriene (DMNT)	19945-61-0	Gift from Pr. Wittcko Francke, Hamburg	99

**Supplementary Table S2.** Primers used in RT-PCR experiments.

Odorant receptor	Forward primer sequence	Reverse primer sequence	T_m (°C)	Product size (bp)
SlitOR3	GTATGGGATGCTGGTGAGAGAAG	AGTGGATTGAAGACCTGGATATGC	58	163
SlitOR4	GCGCTTCAAGAACTGACGGCTAT	AACCGCAACAGTACACTGCCAT	60	427
SlitOR7	CCTTCCTATCGATGGCTCTG	CCCAGGTACCACTTGCAGTT	60	115
SlitOR14	CGTCATCACCCACAACCTCAC	CCCAATAGTCACCCAGCCAAAG	58	196
SlitOR17	GTAGCGATCGGTAACACAACAAT	CGAGCTCTCCACTGTTACTTCAT	60	414
SlitOR19	AAACGTGACTCCGTGAGCTT	CCGCCATCAACGTATTTTCT	62	148
SlitOR24	CGCATCCGTTTATCGACTTT	CAAACCAGACCACAAGAGCA	60	116
SlitOR25	AGCTTTCTGTTCCTGGCGTA	ATGATGGTAGACCGCACTCC	62	186
SlitOR27	ACCAAATTGGCGTTTCTGTC	ATGGTACAGTTGGGGGTTGA	60	80
SlitOR28	TGTAACTGGCGAGGGAAATCAC	GCTCTATATGGCTGCGGTTGG	58	133
SlitOR29	CGTCATCACCCACAACCTCAC	CCCAATAGTCACCCAGCCAAAG	58	196
SlitOR31	TTGGGGAAGCAAACTGCCTTCA	GAATCTTGGCTTGCGCATAGAACG	60	379
SlitOR32	TCTGAATAGGGCGAAGTTTGTAA	TGTGTAGGTCTTCACTCGTAGCA	60	944
SlitOR35	TGCGACCTGCCGACTATG	CTCCTCACGAACACGAACC	53	179
SlitOR36	GTCTCCATACTCCTGAGGGTTCT	GCTGCAAAAATGTATTCTCCAAC	60	904

Supplementary Figure S1. Heat map summarizing the mean responses of the 9 larval SlitORs to 100 µg of plant VOCs when expressed in the *Drosophila* empty neuron system (adapted from de Fouchier et al., 2017). Responses are color-coded according to the scale on the right (values are spikes.s^{-1}).
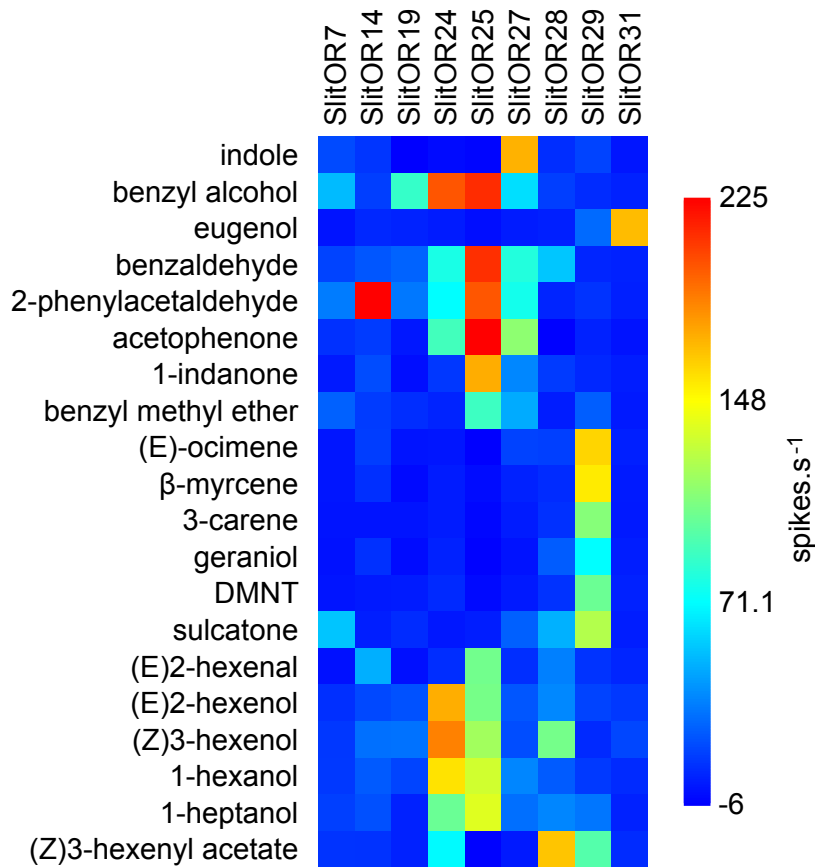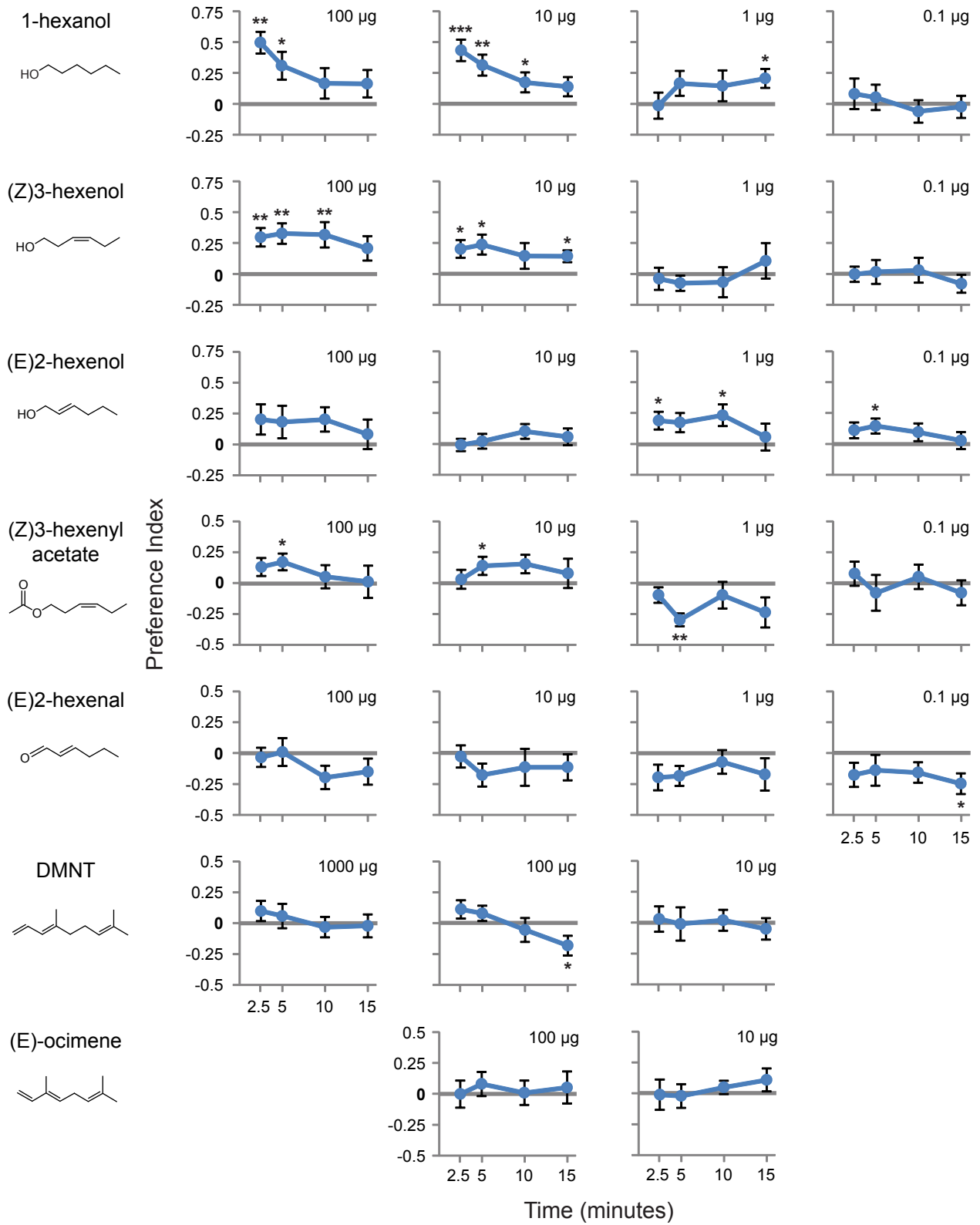
Supplementary Figure S2. *S. littoralis* mean preference index (PI) measured for different doses of plant VOCs after 2.5, 5, 10 and 15 minutes of experiment. Error bars indicate s.e.m. (*n* = 8-15). *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$ (Wilcoxon test).
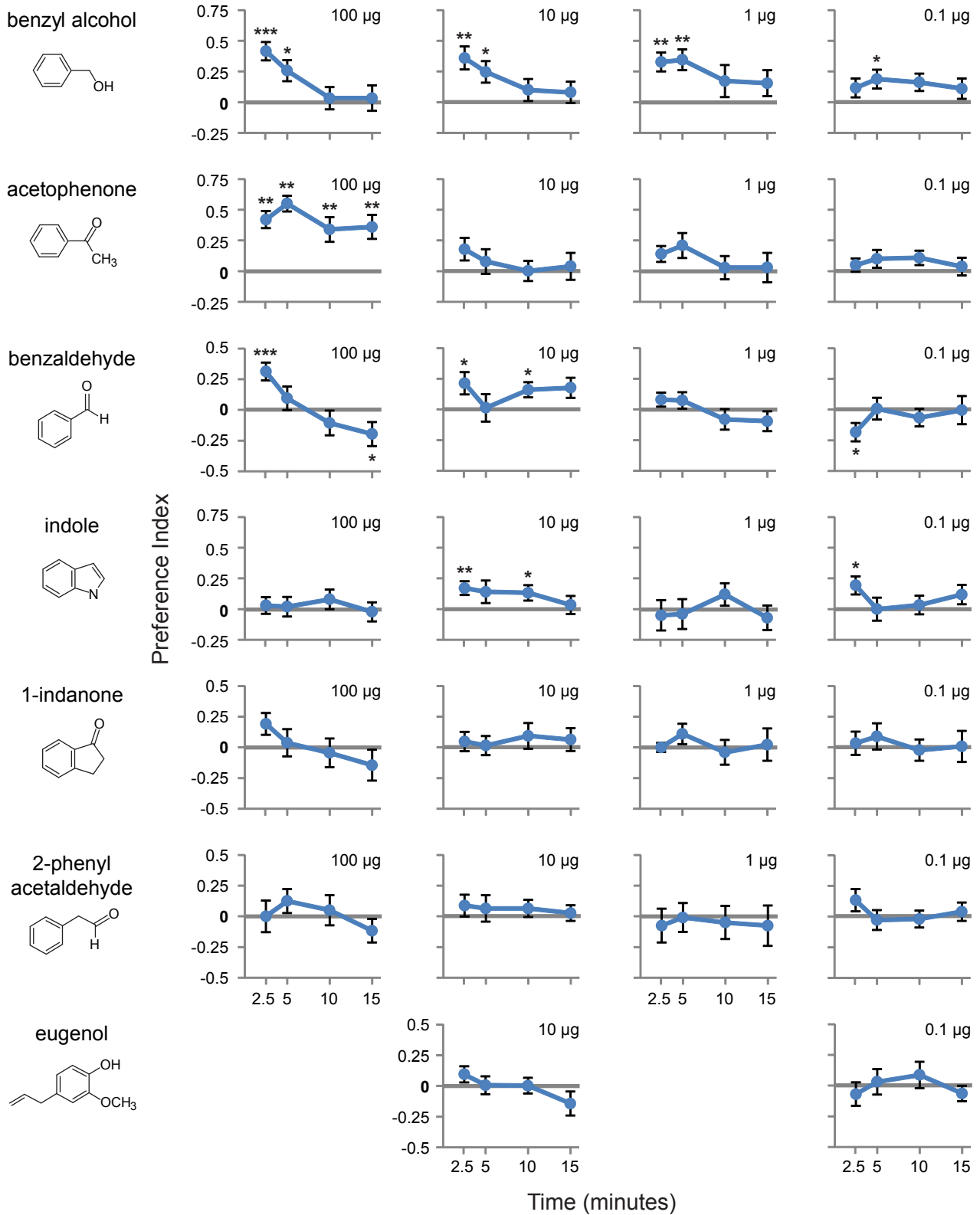
Supplementary Figure S2. continued

Supplementary Figure S3. *S. littoralis* mean preference index (PI) observed (green) or predicted from the refined (orange) or minimal (purple) models for different doses of plant VOCs.