1   **The olfactory co-receptor IR8a governs larval-frass mediated competition avoidance in a**

2   **hawkmoth**

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12   **Abstract**

13   Finding a suitable oviposition site is a challenging task for a gravid female moth. At the same time, it is of

14   paramount importance considering the limited capability of most caterpillars to relocate to alternative host

15   plants. The hawkmoth, *Manduca sexta* (Sphingidae), oviposits on solanaceous plants. Larvae hatching on

16   a plant that is already attacked by conspecific caterpillars can face food competition, as well as an increased

17   exposure to predators and induced plant defenses. Here, we show that frass from conspecific caterpillars is

18   sufficient to deter a female *M. sexta* from ovipositing on a plant and that this deterrence is based on the

19   frass-emitted carboxylic acids 3-methylpentanoic acid and hexanoic acid. Using a combination of genome

20   editing (CRISPR/Cas9), electrophysiological recordings, calcium imaging and behavioral analyses we

21   demonstrate that the ionotropic co-receptor IR8a is essential for acid-mediated frass avoidance in

22   ovipositing hawkmoths.

23

24   **Introduction**

25   For insects, finding appropriate sites for oviposition is a challenging task and the decision of a gravid female

26   will have clear consequences for the fitness of its progeny. Due to fragility and limited mobility, the

27   offspring faces many threats: limited food availability, intra- and interspecific competition, predation and

28   attack by parasitoids. Therefore, gravid females must carefully examine the environment prior to selecting

29   the oviposition site. For this, they utilize visual[1, 2], gustatory[3, 4], mechanosensory[5], as well as olfactory[6, 7]

30   cues. Among these modalities, olfaction plays a pivotal role in an insect's life, as it provides information

31   not only about oviposition sites but also about other biologically relevant resources such as food and mating

32   partners[8].

33   Insects rely on a sophisticated olfactory system to detect volatile chemicals in the environment. Several

34   protein families are involved, with odorant receptors (ORs) and ionotropic receptors (IRs), two types of

35   ligand-gated ion channels, being the key detecting elements[9-11]. On the surface of the antenna, the main

36   olfactory organ, numerous hair-like structures (sensilla) contain olfactory sensory neurons (OSNs), which

37   represent the basic units of sensory perception. Sensilla involved in olfaction occur in three morphological

38   types: basiconic, trichoid, and coeloconic. In the vinegar fly, *Drosophila melanogaster*[9, 12], as well as in

39   other investigated insect species[13-15], ORs are expressed in the dendritic membrane of OSNs housed in

40   basiconic and trichoid sensilla, whereas IRs are expressed by OSNs housed in coeloconic sensilla. ORs are

41   extremely divergent and different insect species express from ten *OR* genes in head lice[16] to more than 300

42   in ants[17]. The OR type expressed in an OSN dictates the odorant specificity of the neuron[18]. ORs are co-

43   expressed together with the conserved odorant receptor-co-receptor (Orco), which is essential for dendritic

44   localization of ORs and OR-dependent odorant detection[10, 19]. IRs usually are less divergent[20] and at least

45   two IR co-receptors, IR8a and IR25a, form ligand-gated ion channels with other odorant-tuned IRs[9, 21]. The

46   different receptor types, however, do not only differ regarding their local expression but also in their

47   response profiles. While most ORs are broadly tuned to alcohols, aldehydes, aromatics, esters, or terpenes[18],

48   IRs primarily respond to a restricted subset of odors including mainly acids and amines[22]. At least in

49   *Drosophila* and *Aedes aegypti*, IR8a is required for acid detection[23, 24]. IR25a, on the other hand, seems to

50   be co-expressed with IRs responding to amines[25] and is also involved in the detection of temperature[26],

51   humidity[27] and salt[28].

52   The tobacco hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae) is an established model for insect

53   olfaction[13] and odor-guided behavior[29]. The recent identification of 73 *OR* genes and 21 olfactory *IR* genes

54   and their expression patterns in male and female moths[13] and the establishment of the Crispr/Cas 9

55   technique in *M. sexta*[30] has made the species to an even more powerful model for olfactory neuroethology.

56   The larvae of these moths feed on various plants of the family Solanaceae, including coyote tobacco

57   (*Nicotiana attenuata*) and jimson weed (*Datura wrightii*). It was reported that a single *M. sexta* caterpillar

58   consumes 1-10 tobacco plants until pupation[31], resulting in complete defoliation of the plants and

59   accumulation of frass under the plant (Fig. 1 a). Therefore, it is crucial for female *M. sexta* to find a suitable

60   host plant that is not already occupied by a conspecific larva.

61   Volatiles emitted from larval frass have been shown to act as kairomones and attract parasitoids and

62   predators[32-35]. The smell of larval frass, therefore, not only indicates the occupancy of the host plant, and

63   the resulting potential for intra-specific competition, but also an increased susceptibility to parasitization

64   and predation. Hence, female moths should avoid sites that are already occupied by conspecific larvae and

65   could do so by e.g. detecting chemical cues emanating from larval frass. In several insect species, female

66   oviposition has been found to be deterred by conspecific larval frass[36]. Thus, larval frass alone is sufficient

67   to signal potential competition to the female. However, the molecular and cellular mechanisms by which

68   female insects avoid frass remain unknown.

69   Here we investigate whether the oviposition of *M. sexta* is deterred by frass from its larvae. We first show

70   that *M. sexta* females, like other insects, display oviposition aversion toward conspecific caterpillar frass

71   stemming from different host plants. Next, we identify specific carboxylic acids emitted from the frass as

72   key compounds that confer oviposition aversion. By performing electrophysiological recordings, calcium

73   imaging and behavioral analyses with mutant moths that either lack *Orco*, or one of the IR co-receptors,

74   *Ir8a* or *Ir25a*, we demonstrate that IR8a is essential for acid-mediated frass avoidance during oviposition.

75   **Results and discussion**

76   **Frass of caterpillars fed on *N. attenuata* repels oviposition.** To test whether gravid females of *M. sexta*

77   avoid ovipositing in the presence of frass, we tested their behavior in a two-choice assay in a wind tunnel.

78   The moths were allowed to oviposit for 3 min either on an undamaged *N. attenuata* plant that was equipped

79   with 10 g of larval frass (from caterpillars which had fed on other *N. attenuata* plants) or on an undamaged

80   control plant (Fig. 1 b). In these experiments, the moths laid on average 14.3 ± 1.7 eggs (mean $\pm$ SEM)

81   during the 3 min test on both plant. The allocation of eggs depended on the presence of frass with the moths

82   laying significantly less eggs on the plant with caterpillar frass as compared to the control plant (Fig. 1 c).

83   A similar preference was observed when moths were given a choice between a plant with frass and a control

84   plant without frass in a steady-air tent (Supplementary Fig. 1), confirming that frass avoidance is consistent

85   in different behavioral paradigms. We conclude that even in the absence of plant damage caterpillar frass

86   induces oviposition avoidance in *M. sexta*. Former studies suggested that ovipositing *M. sexta* females

87   mainly use plant- and larva-derived odors to avoid competition[37, 38]. In our study, where the amount of frass

88   was higher (but still ecologically reasonable, as we used frass that was produced by a single larvae during

89   one night), frass alone was sufficient to induce oviposition avoidance. Females tested in our experiments

90   were raised on artificial diet and had no prior experience with the plants or the frass. We therefore conclude

91   that the frass-induced oviposition avoidance is innate.

92   **3-methylpentanoic acid and hexanoic acid govern oviposition avoidance to larval frass.** To identify

93   the active compound responsible for frass avoidance, we raised *M. sexta* caterpillars on *N. attenuata* plants

94   and afterwards collected the headspace of the resulting frass using a solid phase micro extraction (SPME)

95   fiber. Gas chromatography–mass spectrometry (GC-MS) analysis revealed similar results as in a previous

96   study[33], with 3-methylpentanoic acid being the most abundant compound followed by two other branched

97   aliphatic acids, 3-methylbutanoic acid and 4-methylpentanoic acid (Fig. 1 d).

98   To investigate the impact of these compounds on *M. sexta* oviposition, we pipetted 10 µl of one of the

99   compounds (diluted 10^{-2} in mineral oil) on a filter paper and attached this filter paper 2 cm upwind of a

100 detached *N. attenuata* leaf before presenting this leaf to a mated female in the wind tunnel. When compared

101   to a control leaf, where the attached filter paper just contained the solvent, only 3-methylpentanoic acid

102   elicited significant avoidance (Fig. 1 f, left panel). To address the behavioral sensitivity of *M. sexta* toward

103   3-methylpentanoic acid, we further performed wind tunnel test with lower amounts of the compound and

104   identified the behavioral threshold to be between 10^{-4} and 10^{-3} dilutions (i.e. between 9.3 µg and 93 µg)
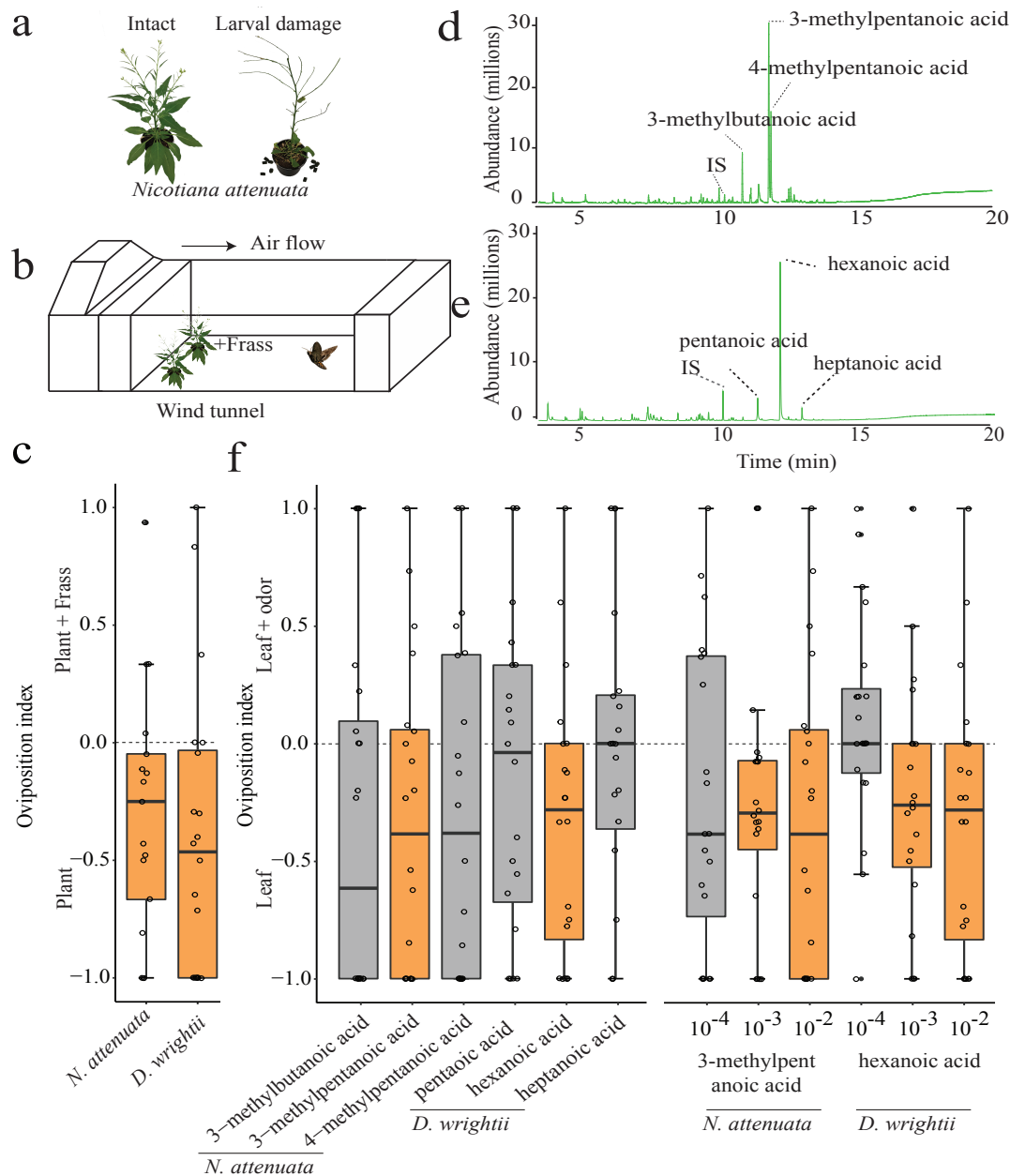
105   (Fig. 1 f, right panel).

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Figure 1 | *M. sexta* oviposition on *N. attenuata* and *D. wrightii* are affected by larval frass.

108   (**a**) *N. attenuata* plant with and without larval damage. (**b**) Schematic drawing of wind tunnel assay.

109   (**c**) Oviposition index of mated females toward frass of *M. sexta* caterpillars reared on *N. attenuata*

110   and *D. wrightii*. Oviposition index = (number of eggs on plant with frass - number of eggs on plant

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111   without frass) / total egg number. (**d**) GC-MS profile of headspace of frass from *M. sexta* caterpillar

112   reared on *N. attenuata*. IS, internal standard. (**e**) GC-MS profile of headspace of frass from *M.*

113   *sexta* caterpillar reared on *D. wrightii*. IS, internal standard. (f) Oviposition index of gravid females

114   to carboxylic acids emitted from *M. sexta* caterpillar frass (left panel). (**f**) Oviposition index of

115   gravid females to various doses of 3-methylpentanoic acid and hexanoic acid (right panel).

116   Deviation of the index against zero was tested with Wilcoxon signed-rank test (n=17-20). * p<0.05.

117   Boxplots depict median and upper and lower quartile; whiskers depict quartiles +/- 1.5× the

118   interquartile range (IQR). Any data points above the superior or below the inferior whisker values

119   are considered as outliers. All data were included in the statistical analysis. Orange boxes depict

120   significant repulsion of frass and/or individual compounds.

121

122   Having shown that frass from caterpillars reduces the attraction of *N. attenuata* plants to ovipositing females,

123   we asked whether this holds true also for the relationship of *M. sexta* and its other main host plant *D.*

124   *wrightii.* We now let female moths choose to oviposit either on a *D. wrightii* leaf that was equipped with

125   frass (from caterpillars raised on *D. wrightii*) or on a control leaf without frass. Again, females preferred to

126   oviposit on the control leaf (Fig. 1 c), suggesting that also at the host plant *D. wrightii*, frass induces

127   oviposition avoidance in *M. sexta* females.

128   To identify the active compounds responsible for frass avoidance in *D. wrightii*, we raised *M. sexta*

129   caterpillars on *D. wrightii* plants and then collected and analyzed the volatiles as before. This time the

130   chemical profile of the frass was dominated by hexanoic acid, and accompanied by two other minor

131   compounds, heptanoic acid and pentanoic acid (Fig. 1 e). When again an ovipositing female had to choose

132   between a *D. wrightii* leaf that was equipped with one of the three acids and a control leaf, only leaves with

133   hexanoic acid (10 µl at 10^{-2} dilution) were avoided (Fig. 1 f, left panel). This avoidance could still be

134   observed even when we reduced the amount of hexanoic acid tenfold (Fig. 1 f, right panel). We conclude

135   that hexanoic acid is the major compound governing frass avoidance of ovipositing females in the context

136   of *D. wrightii.* When performing choice experiments in the wind tunnel with additional aliphatic acids and

137   the two host plants, we found that only six-carbon aliphatic acids elicited avoidance (Supplementary Fig.

138   2).

139   **Both the odorant co-receptor Orco and the ionotropic co-receptor 8a participate in acid sensing.** To

140   determine which olfactory pathway is governing the detection of 3-methylpentanoic acid and hexanoic acid,

141   we performed electroantennography (EAG) measurements on wild type (WT) moths, and on odorant co-

142   receptor heterozygous (*Orco+/-*) and homozygous (*Orco-/-*) moths that were recently generated in our lab

143   using CRISPR/Cas9 genome editing[30]. While WT moths and *Orco+/-* moths exhibited robust EAG

144   responses to the acids, *Orco-/-* moths showed reduced responses (Supplementary Fig. 3). However, clear

145   EAG responses to the acids remained, indicating that the IR pathway is also involved in acid detection.

146   To address whether the remaining response to acids in *Orco-/-* moths were indeed resulting from activation

147   of the IR pathway, we generated two IR mutant lines, *Ir8a-/-* and *Ir25a-/-*, using again CRISPR/Cas9

148   genome editing. The resulting *Ir8a-/-* mutant contained a 339bp deletion (93bp at exon2, 170bp at intron2

149   and 76bp at exon3) while the *Ir25a-/-* mutant contained a 154bp deletion (154bp at exon2) in the genome.

150   As both deletions resulted in frame-shifts and the occurrence of premature stop codons (Supplementary Fig.

151   4A), we expected both mutations to result in non-functional ionotropic co-receptors. We found no

152   difference regarding pupal weight and length in neither *Ir8a-/-* nor *Ir25a-/-* mutants, when compared to the

153   heterozygous controls (Supplementary Fig. 4B). Furthermore, in EAG experiments both mutants exhibited

154   normal responses to the OR-detected pheromone, bombykal (Supplementary Fig. 4C) suggesting the

155   absence of relevant off-target effects.

156   However, when performing EAG experiments with *Ir8a-/-* and *Ir25a-/-* moths, only *Ir8a-/-* moths exhibited

157   significantly reduced response to both behaviorally active acids when compared to WT moths, while the

158   acid responses in *Ir25a-/-* moths remained unaffected (Fig. 2a).

159   **IR8a pathway is essential for detecting and avoiding acids from caterpillar frass.** We next asked which

160   sensillum type is involved in the detection of the acids in caterpillar frass. According to the well-studied

161   *Drosophila* [9, 18, 39, 40], IR-expressing OSNs are mainly housed in coeloconic sensilla. Furthermore, in *M.*

162   *sexta,* previous single-sensillum recordings (SSRs) from trichoid and basiconic sensilla showed little to no

163   responses to acids[41, 42]. We therefore hypothesized that coeloconic sensilla of *M. sexta* house IR-expressing

164   OSNs that are involved in acid detection. Contrary to the antenna of female *D. melanogaster*, which

165   contains only 54 coeloconic sensilla[40], the antenna of female *M. sexta* carries about 3600[41]. This makes

166   the identification and recording from identified individual coeloconic sensilla almost impossible. We,

167   therefore, recorded from 28 coeloconic sensilla from the middle part of the antenna, which should cover a

168   wide range of functional types, and stimulated them with a set of 52 odorants from different chemical

169   classes (Supplementary Fig. 5). Consistent with previous studies in *D. melanogaster*[40] and *Bombyx mori*[43],

170   OSNs housed in coeloconic sensilla in wild type *M. sexta* were mainly activated by acids and amines. The

171   two behaviorally active acids activated mainly OSNs in non-overlapping groups of coeloconic sensilla. The

172   number of coeloconic sensilla responding to hexanoic acid was about two times higher than those

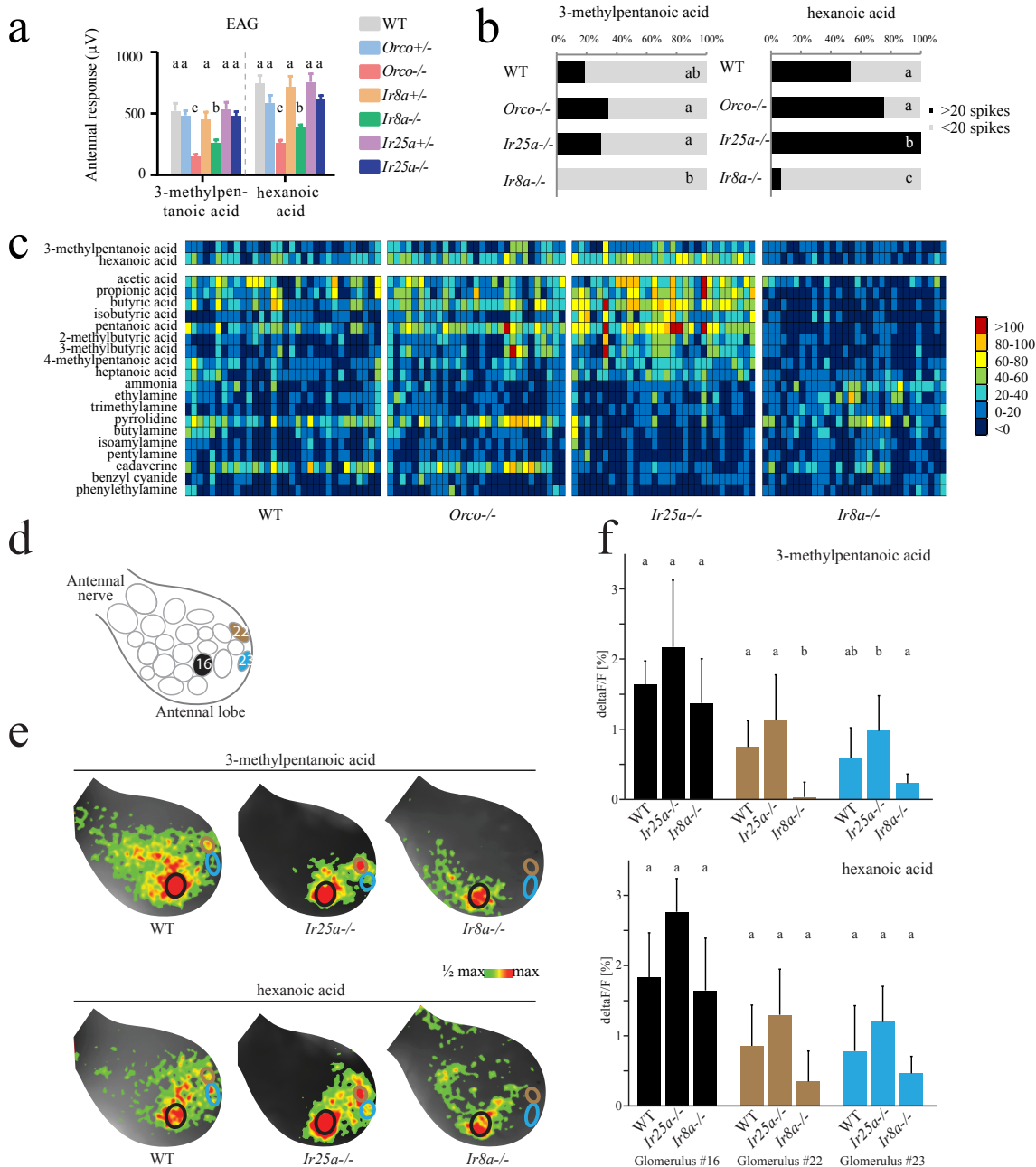173   responding to 3-methylpentanoic acid and the intensity of responses to hexanoic acid was stronger.

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Figure 2 | Detection and processing of frass-emitted (**a**) Electroantennogram responses (EAG, in µV ± SEM, the response to solvent was subtracted) of *M. sexta* antennae isolated from wild type (WT), *Orco-/-* (Orco mutant), *Orco+/-* (Orco heterozygous), *Ir8a-/-* (Ir8a mutant), *Ir8a +/-* (Ir8a heterozygous), *Ir25a-/-* (Ir25a mutant), *Ir25a+/-* (Ir25a heterozygous). EAG responses to 3-methylpentanoic acid and hexanoic acid. (**b**) Percentage of coeloconic sensilla responding to the two behaviorally active acids in different genotypes (**c**) Heat map representation of SSR responses of coeloconic sensilla from different moth genotypes. (**d**) Schematic of 23 putative

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183   olfactory glomeruli at the dorsal surface of the right antennal lobe; the schematic was created for

184   each individual moth based on the activation patterns of 19 diagnostic odorants[45]; numbers

185   identify glomeruli that were most strongly activated by the tested acids (#16), or that showed acid-

186   specific activation (#22, #23). (**e**) Examples of calcium imaging recordings in wildtype, *IR25a-/-*,

187   and *IR8a-/-* female moths after stimulation with the two behaviorally active acids. The increase of

188   fluorescence is color coded (see scale) and superimposed onto the view of the antennal lobe;

189   circles indicate positions of glomeruli #16 (black outline), #22 (brown), #23 (blue). (**f**) Bars show

190   the mean response of a glomerulus (after subtraction of the solvent response) to an odorant; error

191   bars indicate standard deviation; bars with the same letter are not significantly different from each

192   other (ANOVA, n=4-6 females/genotype).

193

194   We found reduced numbers of coeloconic sensilla responding to 3-methylpentanoic acid and hexanoic acid

195   in *Ir8a-/-* moths, when comparing to the other three genotypes (Fig. 2b and c). Interestingly, increased

196   numbers of coeloconic sensilla exhibited enhanced responses to acids in *Ir25a-/-* moths, whereas the

197   responses to amines were almost abolished. The enhanced responses in *Ir25a-/-* moths toward acids could

198   be due to more energy being available to OSNs responding to acids, as these OSNs no longer have to

199   compete with amine-tuned OSNs in the same sensillum. Such a phenomenon has been reported for

200   gustatory receptors (GRs), where sensory neurons in the same sensillum have been shown to interact,

201   exhibiting competition, inhibition or activation[44]. In conclusion, our results show that IR8a, but neither

202   Orco nor IR25a, is required for acid detection in OSNs of coeloconic sensilla.

203   We conclude that IR8a is involved in the detection of the key compounds governing frass avoidance. We

204   next asked, where in the antennal lobe (i.e. the first olfactory processing center of the moth's brain) this IR-

205   related acid detection becomes processed. A recently published functional analysis of the moths' antennal

206   lobe[45] revealed three glomeruli that strongly responded to acids. In another study[30], activation of two of

207   these glomeruli was not affected by knocking out the OR-coreceptor Orco, supporting that these two

208   glomeruli become innervated by IR-expressing OSNs. When performing calcium imaging experiments with

209   moths that either lacked a functional IR25a or IR8a, the responses to acids in *Ir25a* mutants were unaffected

210   compared to control animals (Fig. 2 e, f). However, when testing *Ir8a* mutants, we observed a slightly

211   reduced response to hexanoic acid and a significantly reduced response to 3-methylpentanoic acid (Fig. 2

212   e, f) in only those two glomeruli that in the former study were independent of Orco. Together with the EAG

213   results, we conclude that both Orco and IR8a, but not IR25a, are involved in acid sensing and that IR8a-

214   expressing OSNs involved in the detection target a subset of glomeruli on the medial surface of the antennal

215   lobe.

8

216   Finally, we asked whether any of the three co-receptors governs the behavioral avoidance towards acids in
217   ovipositing *M. sexta*. Unfortunately, the oviposition rates of *Orco*-/- moths were too low to draw any
218   conclusions regarding the involvement of Orco in the oviposition avoidance. One explanation for the
219   conflicting result with our previews study[30] in terms of oviposition in *Orco*-/- moths is that Fandino et al
220   (2019) used whole and large *D. wrightii* plants which probably provided a very strong visual stimulation.
221   Interestingly, however, mutation of *Ir25a* did not affect the oviposition behavior (Fig. 3a), while *Ir8a*-/-
222   moths were no longer repelled by the tested acids (Fig. 3b). We conclude that ovipositing females rely on
223   IR8a for detection of acids from caterpillar frass.
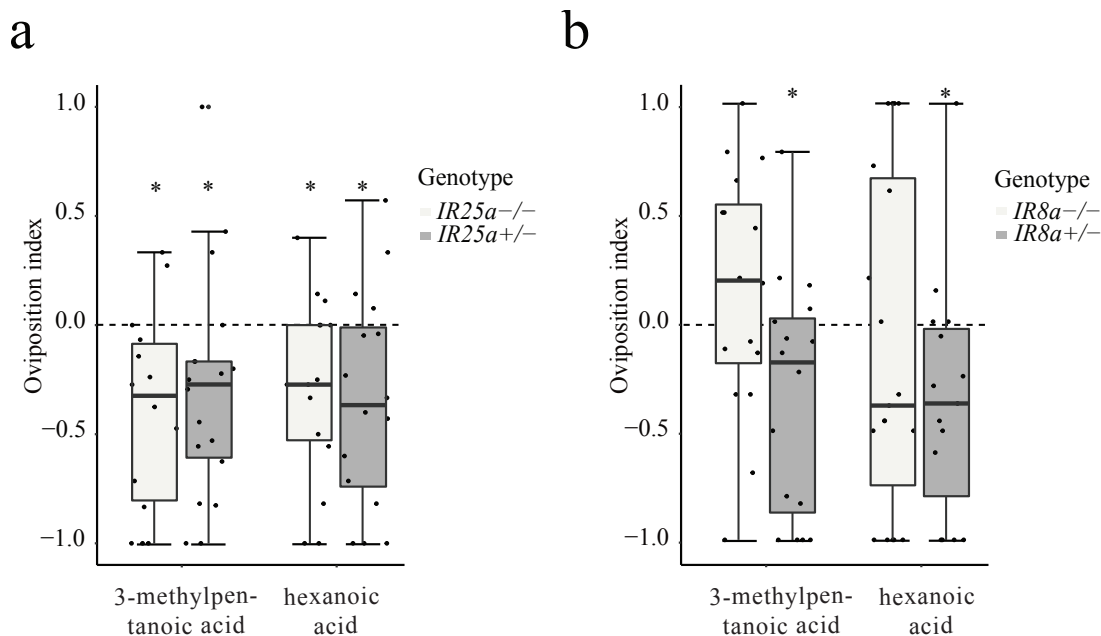

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Figure 3 | IR8a is necessary for acid avoidance of ovipositing *M. sexta* females. Two-choice
assay showing the oviposition indexes of the homozygous and heterozygous (as a control) of
Ir25a (**a**) and *Ir8a* (**b**) mutants for the frass-emitted compounds 3-methylpentanoic acid and
hexanoic aicd (for details on choice assay see Fig. 1 and methods section).

229

230   Several studies have shown that larval frass and its odors deter female oviposition[36]. Frass-emitted acids
231   play a crucial role in oviposition avoidance in moth species like *Ostrinia* species[46] and *Helicoverpa*
232   *armigera*[47]. Moreover, it was shown that female parasitoid wasps, *Cotesia glomerata*, use acids emitted by
233   host larvae as cues to locate their host[48], and *M. sexta* caterpillar frass-emitted acids play a major role in
234   attracting predators like ants[33]. Finally, one of the acids we identified in the caterpillar frass (hexanoic acid)
235   has been shown to induce plant defenses against herbivores[49]. Obviously, carboxylic acids are potent signals
236   for a gravid female to realize that at a given plant the female's offspring might face conspecific competitors,
237   parasitoids and predators, as well as an already induced plant defense. Therefore, our finding that

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238   ovipositing *M. sexta* females, like other moths, avoid emitted acids from larval frass is not unexpected.

239   However, the neural and molecular mechanisms as well as the exact chemistry underlying this behavior

240   remained elusive. In this study, we do not only show that *M. sexta* display oviposition aversion toward

241   caterpillar frass, but also find that only the major volatile compounds (C_6 carboxylic acids) emitted are

242   aversive for gravid females. By testing mutant moths in which we knocked out different olfactory co-

243   receptors, we show that the co-receptors IR8a and Orco, but not IR25a, participate in acid detection. We

244   also find that IR8a is necessary for the acid avoidance behavior of gravid *M. sexta* females, which helps the

245   moth to protect its offspring from conspecific competition.

246   It has been reported that *M. sexta* lay significantly less eggs on plants treated with herbivore-induced

247   volatile organic compounds due to high predation rate on those treated plants[31]. Our finding that *M. sexta*

248   avoids competition by sensing not only plant-emitted, but also frass-emitted compounds adds another layer

249   of regulation to host choice in *M. sexta* to distinguish between healthy and damaged plants.

250

251   **Methods**

252   **Insect rearing and plant material.** All animals were reared at the Max Planck Institute for Chemical

253   Ecology, Jena, Germany, as already described[13]. Briefly, eggs were collected from female *M. sexta* moths,

254   which could freely oviposit on *D. wrightii* plants. Larvae used in the experiments were reared on artificial

255   diet, under 16:8 h light: dark photo period with a relative humidity of 40% at 26℃. Naïve females were

256   mated the second night after emergence and tested during the subsequent night. *M. sexta* frass was collected

257   daily from fourth to fifth instar caterpillars which were raised on either *N. attenuata or D. wrightii*.

258   All plants were grown in a greenhouse as described[50]. Plants used for experiments were not yet flowering.

259   Approximately 7 days before being used, plants were transferred into a climate chamber with the same

260   settings as the moth flight cage (16:8 h light: dark photo period with a relative humidity of 40% at 26℃.).

261   **Chemical analysis.** We identified the volatiles of caterpillars frass using SPME (Solid Phase

262   Microextraction) coupled with GC-MS (Gas chromatography–mass spectrometry). One gram of frass from

263   caterpillars raised on either *D. wrightii* or *N. attenuata* were put into a 500-mL plastic container. A circular

264   filter paper (diameter: 12 mm Whatman, Sigma-Aldrich USA) loaded with 10 µL of diluted bromodecane

265    $(1:10^4$ in hexane) was used as an internal standard. Through a hole in the lid of the container, a SPME fiber

266   (50 µm Divinylbenzene / Carboxen / Polydimethylsiloxane coating; Supelco) was exposed to the container

267   headspace for 30 min at room temperature without agitation, and then introduced into the injector inlet for

268   2 min at 250℃ in split-less mode. The compounds adsorbed on the fiber were then analyzed by GC-MS

269   (Agilent 6890 GC & 5975C MS, Agilent, USA). After fiber insertion, the column temperature was

270   maintained at 40°C for 2 min and then increased to 260°C at 15°C min^{-1}, followed by a final stage of 5 min

271   at 260°C. Compounds were identified by comparing mass spectra against synthetic standards and NIST 2.0

272   library matches. All of the synthetic odorants that were tested and confirmed were purchased from Sigma

273   (www.sigmaaldrich.com) and were of the highest purity available.

274   **Behavioral experiments in the wind tunnel.** To investigate the behavioral significance of *M. sexta* frass

275   from caterpillars which had fed on *N. attenuata*, we performed two choice tests in a transparent wind tunnel

276   ($220 \times 90 \times 90$ cm^3) at 25 °C, 70% relative humidity, 0.3 lux illumination, and a wind speed of 40 cm/s.

277   Two non-flowering *N. attenuata* of similar size were placed at the upwind end of the wind tunnel. An empty

278   petri dish (control) or a petri dish loaded with 10 gram of freshly collected frass (treatment) was placed at

279   the base of the plant. A single 5th instar larva produce about 10 gram of frass per day. As described before[45],

280   mated female moths were released at the downwind side of the wind tunnel and during 3 min were allowed

281   to oviposit on both plants. Afterwards, the number of eggs on both plants was counted and the eggs were

282   gently removed after each test. Moths were tested only once and plants were exchanged after two tests. The

283   positions of treatment and control plant within the wind tunnel were swapped after every second moth. The

284   oviposition indexes were calculated as $(T-C)/(T+C)$ where T is the number of eggs on the treatment site

285   and C is the number of eggs on the control site.

286   To test the effect of *M. sexta* frass from caterpillars that had raised on *D. wrightii*, we conducted a similar

287   two choice test in the wind tunnel. Due to the large size of *Datura* plants, we trimmed plants seven days

288   before the experiments in a way that two leaves of similar size remained on opposite directions. An empty

289   petri dish (control) or a petri dish loaded with 10 gram of freshly collected frass (treatment) was placed 10

290   cm beneath the leaves. Again, mated female moths were allowed to oviposit on both control and treatment

291   leaves and the resulting eggs and oviposition indexes were calculated afterwards.

292   To determine the functional significance of different volatiles emitted by the frass, we conducted two-

293   choice tests in the wind tunnel. This time two freshly detached leaves of similar size were presented to the

294   gravid female. Each leaf was attached to the tip of one of two upright acrylic glass poles (40 cm high and

295   placed at the upwind end of the wind tunnel with a distance of 40 cm between them). Beneath each leaf we

296   attached a square filter paper (2×2 cm^2) loaded with 10 µL of diluted odorant ($1:10^2$) or the solvent mineral

297   oil alone. Moths, leaves and filter papers were tested only once. Experiments were conducted both with

298   leaves from *N. attenuata* and *D. wrightii*.

299   **CRISPR/ Cas 9-based genome editing.** To determine which co-receptor is involved in the acid detection

300   and acid-driven oviposition avoidance, we used olfactory receptor co-receptor (Orco) mutant moths[30], and

301   generated Ionotropic receptor 8a (Ir8a) and Ir25a two mutant lines. The *M. sexta* genome v.1.0 (Mansexv1.0)

302   fasta file and the GFF3 file were submitted to the CHOPCHOP (http://chopchop.cbu.uib.no) database for

303   CRISPR/ Cas9 target selection sites. The OGS2.0 gene names, i.e. Msex2.10447-RB, isoform 1 and

304   Msex2.02645-RA, isoform 1, were used to select target site. The sgRNA and Cas9 were synthesized by

305   IDT   (https://eu.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system).   The

306   microinjection and genotyping were carried out according to previously established procedures[30]. After the

307   mutant lines were established, mutations were reconfirmed by Sanger sequencing.

308   **Electrophysiology.** To investigate the antennal responses to frass-emitted carboxylic acids, we

309   performed EAG (Electroantennography) recording. We therefore clipped the antenna of a 3-day-old female

310   moth directly above the scapulum and before the third last flagellum. Antenna preparation, stimuli delivery,

311   data acquisition and analysis were carried out according to previously established procedures[50]. Odorants

312   for EAG analyses were selected based on compounds identified in the headspace of caterpillar frass as well

313   as structurally similar chemicals. 10 μl of diluted odor ($1:10^2$) or solvent alone were pipetted onto a circular

314   filter paper (diameter: 12 mm) and placed into a glass pipette. In addition, we performed single-sensillum

315   recordings from coeloconic sensilla as described before[42]. Coeloconic sensilla were identified by their

316   characteristic morphology. 29-32 Coeloconic sensilla were recorded in each genotype. Responses were

317   quantified by counting all spikes recorded from an individual sensillum due to difficulties in reliably

318   distinguishing spikes from individual neurons[22, 40]. The response was calculated as the difference in spike

319   number observed 0.5 s before and after the stimulus onset. Heatmap was generated in Excel. Calcium

320   imaging experiments were conducted as described previously[45]. CAS number and purities for odorants is

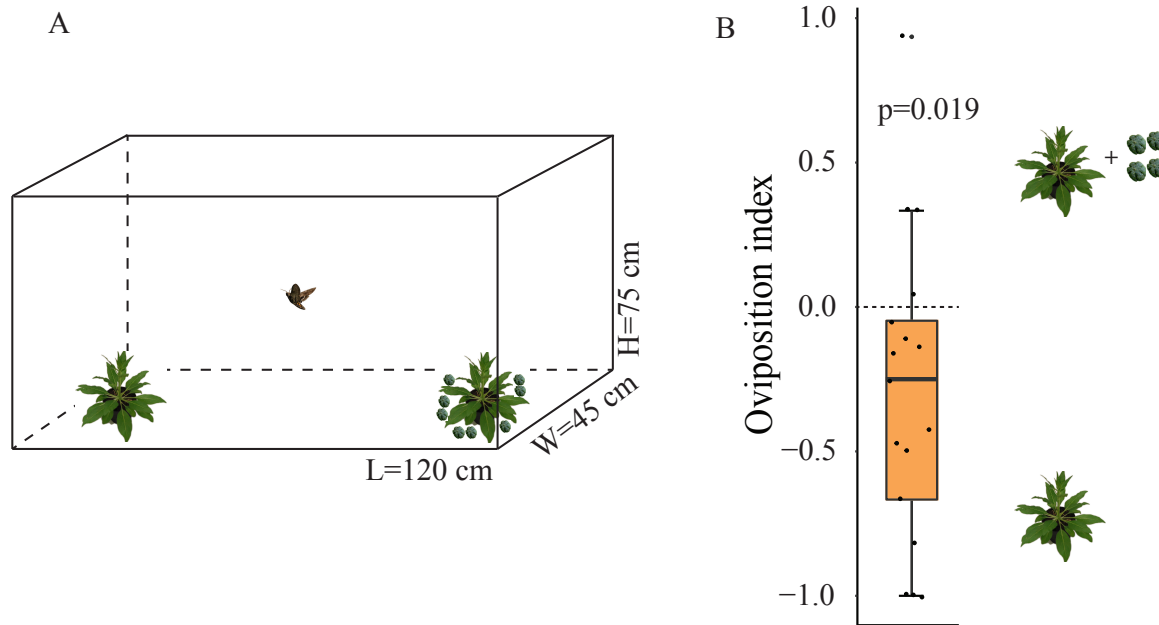321   listed in supplementary table 1.

322   **Statistics and figure preparation.** Sample size of behavioral experiments was determined based on a

323   previous study[45]. Data were analyzed and plotted using RStudio (Version 1.1.414), R (Version 3.4.2; The

324   R Project for Statistical Computing) and GraphPad InStat 3 (https://www.graphpad.com/scientific-

325   software/instat/), while figures were organized and prepared using Adobe Illustrator CS5. The Wilks–

326   Shapiro test was used to determine normality of each data set. Normally distributed data were assessed

327   using t-tests. Not normally distributed data were analyzed using Wilcoxon signed-rank test, with the null

328   hypothesis that the median of sampled values differs from zero. For the boxplots the whiskers were

329   calculated as follows: the upper whisker equals the third quartile plus 1.5× the interquartile range (IQR)

330   and the lower whisker equals the first quartile minus 1.5× the IQR. Any data points above the superior or

331   below the inferior whisker values are considered as outliers. All data were included in the statistical analysis.

332   **Figure 1 | *M. sexta* oviposition on *N. attenuata* and *D. wrightii* are affected by larval frass. (a)** *N.*

333   *attenuata* plant with and without larval damage. (**b**) Schematic drawing of wind tunnel assay. (**c**)

334   Oviposition index of mated females toward frass of *M. sexta* caterpillars reared on *N. attenuata* and *D.*

335   *wrightii*. Oviposition index = (number of eggs on plant with frass - number of eggs on plant without frass)

12

336   / total egg number. (**d**) GC-MS profile of headspace of frass from *M. sexta* caterpillar reared on *N. attenuata*.

337   IS, internal standard. (**e**) GC-MS profile of headspace of frass from *M. sexta* caterpillar reared on *D. wrightii*.

338   IS, internal standard. (f) Oviposition index of gravid females to carboxylic acids emitted from *M. sexta*

339   caterpillar frass (left panel). (**f**) Oviposition index of gravid females to various doses of 3-methylpentanoic

340   acid and hexanoic acid (right panel). Deviation of the index against zero was tested with Wilcoxon signed-

341   rank test (n=17-20). * p<0.05. Boxplots depict median and upper and lower quartile; whiskers depict

342   quartiles +/- 1.5× the interquartile range (IQR). Any data points above the superior or below the inferior

343   whisker values are considered as outliers. All data were included in the statistical analysis.

344   **Figure 2 | Detection and processing of frass-emitted** (**a**) Electroantennogram responses (EAG, in µV ±

345   SEM, the response to solvent was subtracted) of *M. sexta* antennae isolated from wild type (WT), *Orco-/-*

346   (Orco mutant), *Orco+/-* (Orco heterozygous), *Ir8a-/-* (Ir8a mutant), *Ir8a +/-* (Ir8a heterozygous), *Ir25a-/-*

347   (Ir25a mutant), *Ir25a+/-* (Ir25a heterozygous). EAG responses to 3-methylpentanoic acid and hexanoic

348   acid. (**b**) Percentage of coeloconic sensilla responding to the two behaviorally active acids in different

349   genotypes (**c**) Heat map representation of SSR responses of coeloconic sensilla from different moth

350   genotypes. (**d**) Schematic of 23 putative olfactory glomeruli at the dorsal surface of the right antennal lobe;

351   the schematic was created for each individual moth based on the activation patterns of 19 diagnostic

352   odorants[45]; numbers identify glomeruli that were most strongly activated by the tested acids (#16), or that

353   showed acid-specific activation (#22, #23). (**e**) Examples of calcium imaging recordings in wildtype,

354   *IR25a-/-*, and *IR8a-/-* female moths after stimulation with the two behaviorally active acids. The increase

355   of fluorescence is color coded (see scale) and superimposed onto the view of the antennal lobe; circles

356   indicate positions of glomeruli #16 (black outline), #22 (brown), #23 (blue). (**f**) Bars show the mean

357   response of a glomerulus (after subtraction of the solvent response) to an odorant; error bars indicate

358   standard deviation; bars with the same letter are not significantly different from each other (ANOVA, n=4-

359   6 females/genotype).

360   **Figure 3 | IR8a is necessary for acid avoidance of ovipositing *M. sexta* females**. Two-choice assay

361   showing the oviposition indexes of the homozygous and heterozygous (as a control) of *Ir25a* (**a**) and *Ir8a*

362   (**b**) mutants for the frass-emitted compounds 3-methylpentanoic acid and hexanoic aicd (for details on

363   choice assay see Fig. 1 and methods section).

A

B

364

Supplementary Figure 1 | Frass avoidance of ovipositing *M. sexta* females is conserved in different behavioral assays. (A) Schematic drawing of oviposition cage. (B) Oviposition index of WT *M. sexta* given a choice between *N. attenuata* and *N. attenuata* containing caterpillar frass in the oviposition cage. Deviation of the index against zero was tested with Wilcoxon signed-rank test (n=20). * p<0.05. Boxplots depict median and upper and lower quartile; whiskers depict quartiles +/- 1.5× the interquartile range (IQR). Any data points above the superior or below the inferior whisker values are considered as outliers. All data were included in the statistical analysis.

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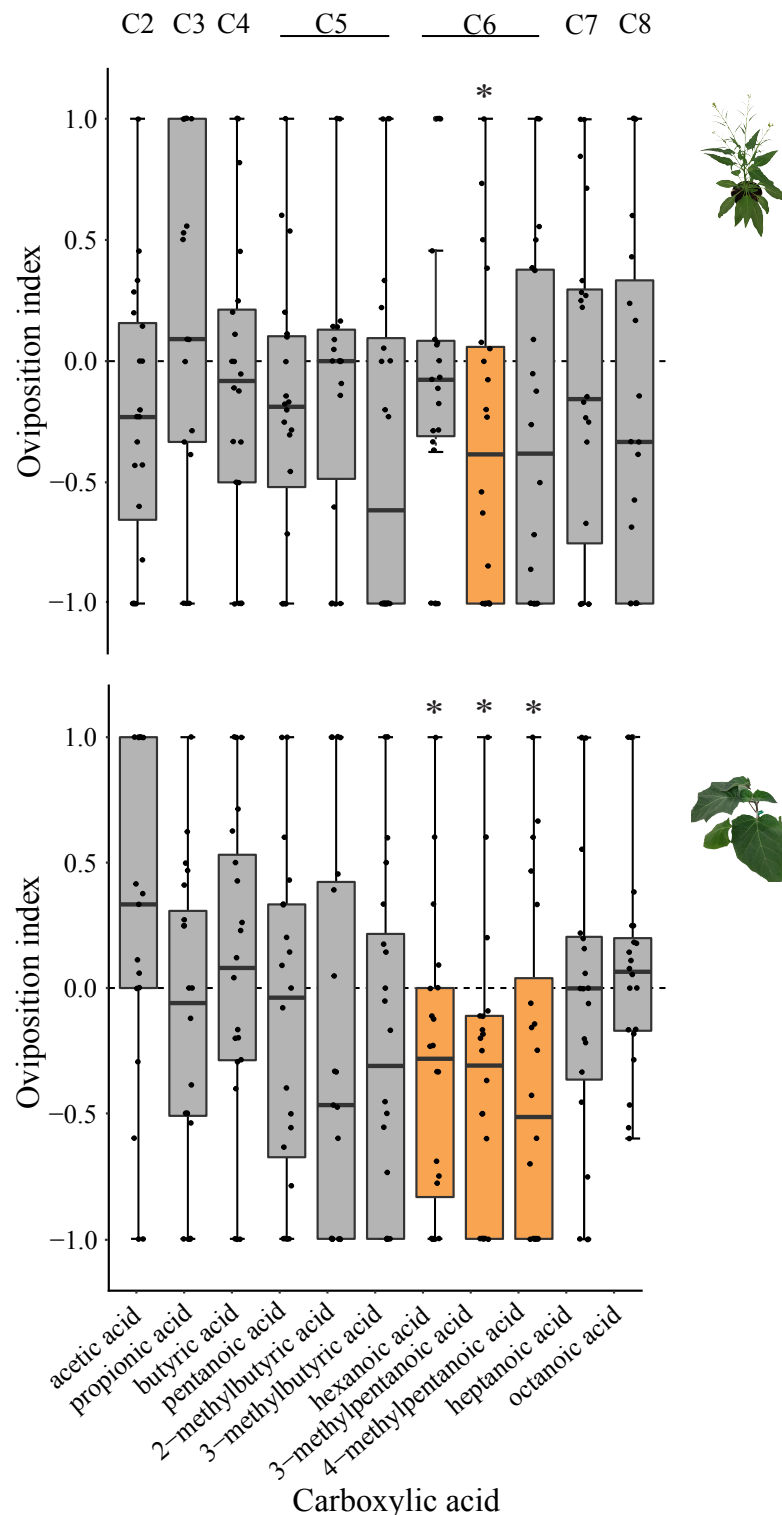
Supplementary Figure 2 | Host-plant-specific effects of different carboxylic acids on *M. sexta*'s oviposition choice. (**A**) Two-choice assay showing the preference of wild-type (WT) females for carboxylic acids over control in the context of detached *N. attenuata* leaf. (**B**) Two-choice assay showing the preference of wild-type (WT) females for carboxylic acids over control

377   in the context of detached *D. wrightii* leaf. Deviation of the index against zero was tested with

378   Wilcoxon signed-rank test (n=20). * p<0.05. Part of these data are already shown in Fig. 1 f.

379   Please also consider that hexanoic acid obviously deters oviposition only in the right context, i.e.

380   when females oviposit on *Datura wrightii*.

381
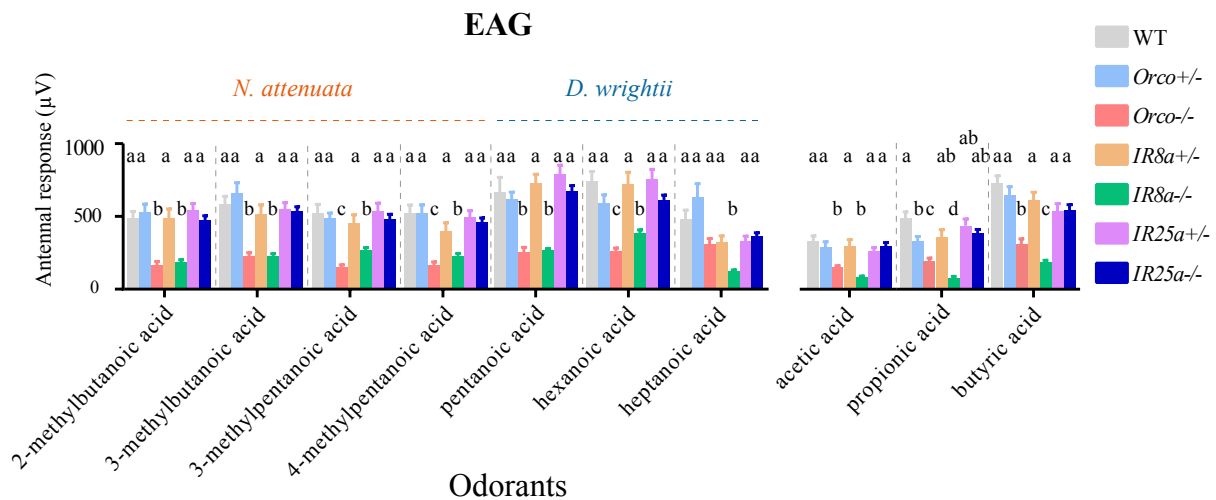
382   **Supplementary Figure 3 | Both O***rco-/-* **and** *Ir8a-/- M. sexta* **exhibit reduced**

383   **electrophysiological responses to carboxylic acids.** Electroantennogram responses (EAG, in

384   mV ± SEM, the response to solvent was subtracted) of *M. sexta* antennae isolated from wild-type

385   (WT), O*rco-/-* (*Orco* mutant), *Orco+/-* (*Orco* heterozygous), *Ir8a-/-* (*Ir8a* mutant), *Ir8a +/-* (*Ir8a*

386   heterozygous), *Ir25a-/-* (*Ir25a* mutant), *Ir25a +/-* (*Ir25a* heterozygous). Responses to 3-

387   methylpentanoic acid and hexanoic acid have been shown in Figure 2. Different letters above

388   each odorant response indicate significant differences (one-way anova; p < 0.05); a is different

389   from b and c, and b is different from c and ab is not different from either a or b.
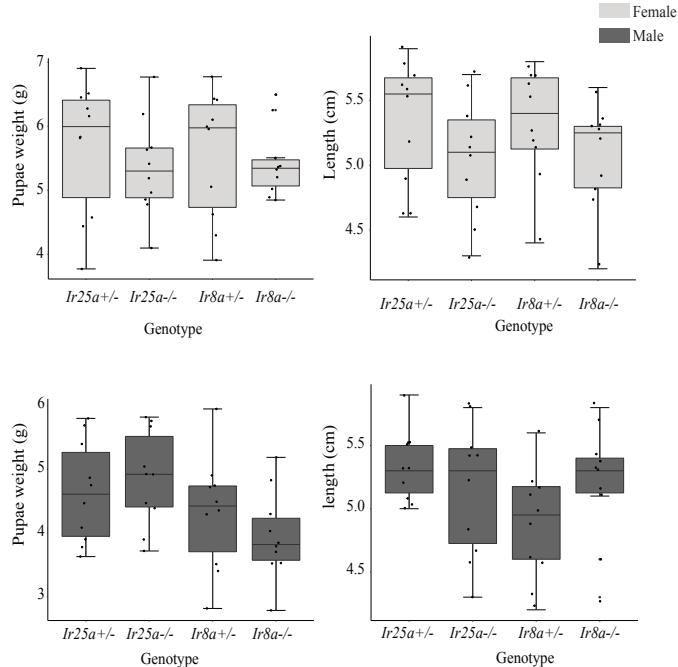
390
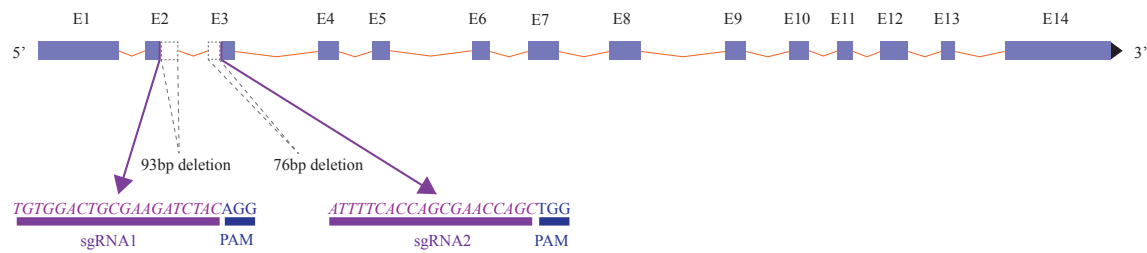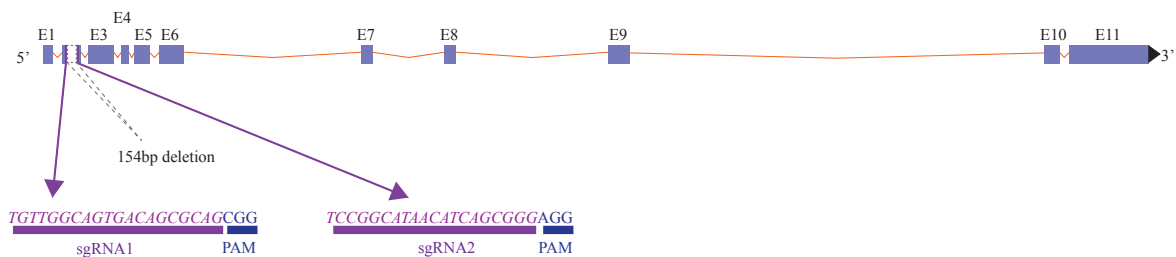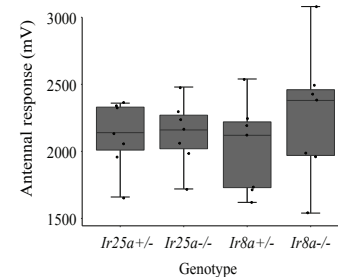
Supplementary Figure 4 | Gene editing technique and potential off-target effects. (**A**) **Schematic of the genes targeted via CRISPR-Cas9.** *Ir8a*-/- mutant contained a 339bp deletion (93bp at exon2, 170bp at intron2 and 76bp at exon3) while the *Ir25a*-/- mutant contained a 154bp

394   deletion (154bp at exon2) in the genome. PAM protospacer adjacent motif. (B**)** Weight (left) and

395   length (right) of female pupae from both *Ir8a* (upper panels) and *Ir25a* (lower panels) mutant and

396   heterozygous lines. There were no statistical differences among corresponding genotypes

397   (Wilcoxon signed-rank test, n=10). (C) EAG response of male adults toward pheromone

398   (bombykal) in all genotypes. There were no statistical differences among genotypes (one-way

399   ANOVA, n=7).

400
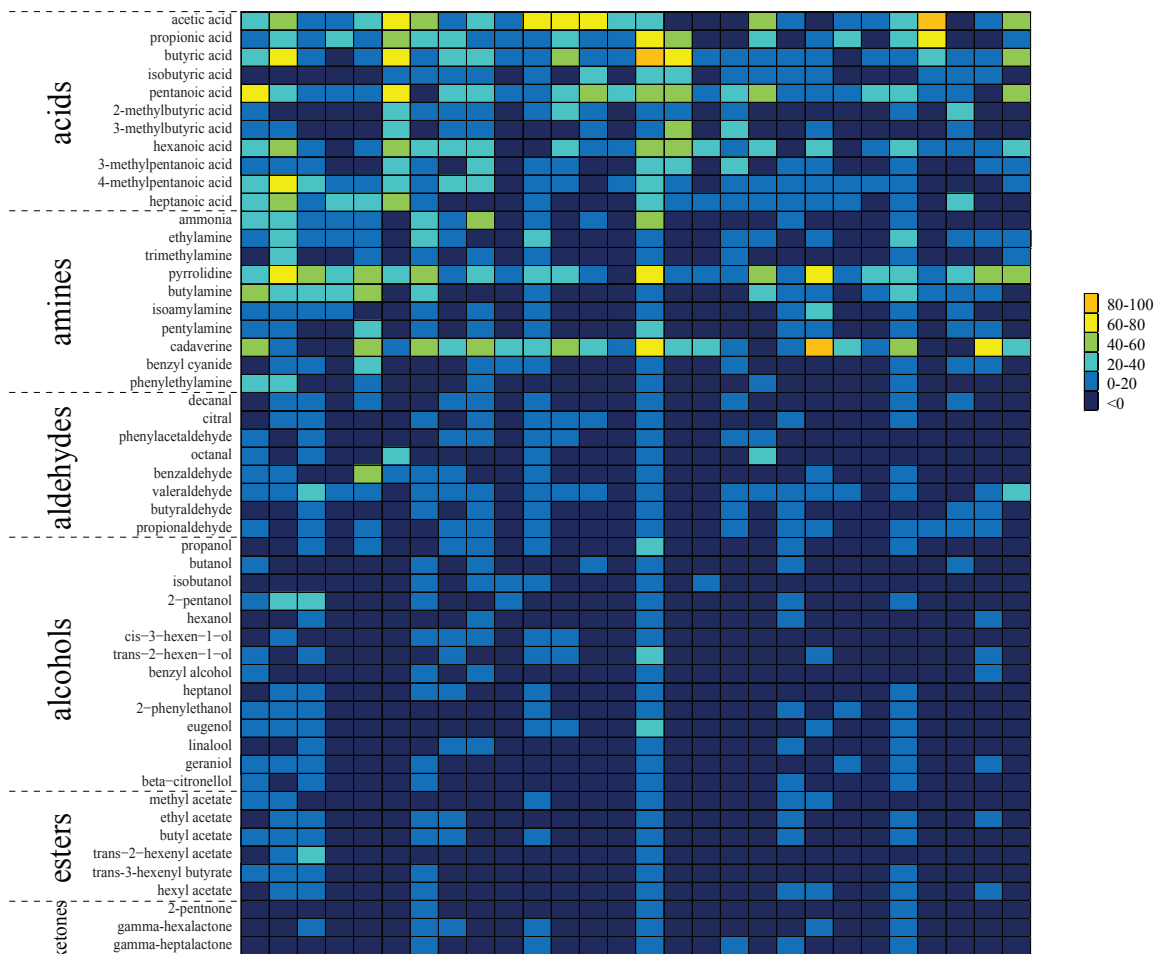
Supplementary Figure 5 | Heatmap based on SSRs of 28 coeloconic sensilla from WT *M.*

sexta* to 52 screened odors. Color-coded numbers depict difference in spikes/0.5s before and

after stimulus onset.

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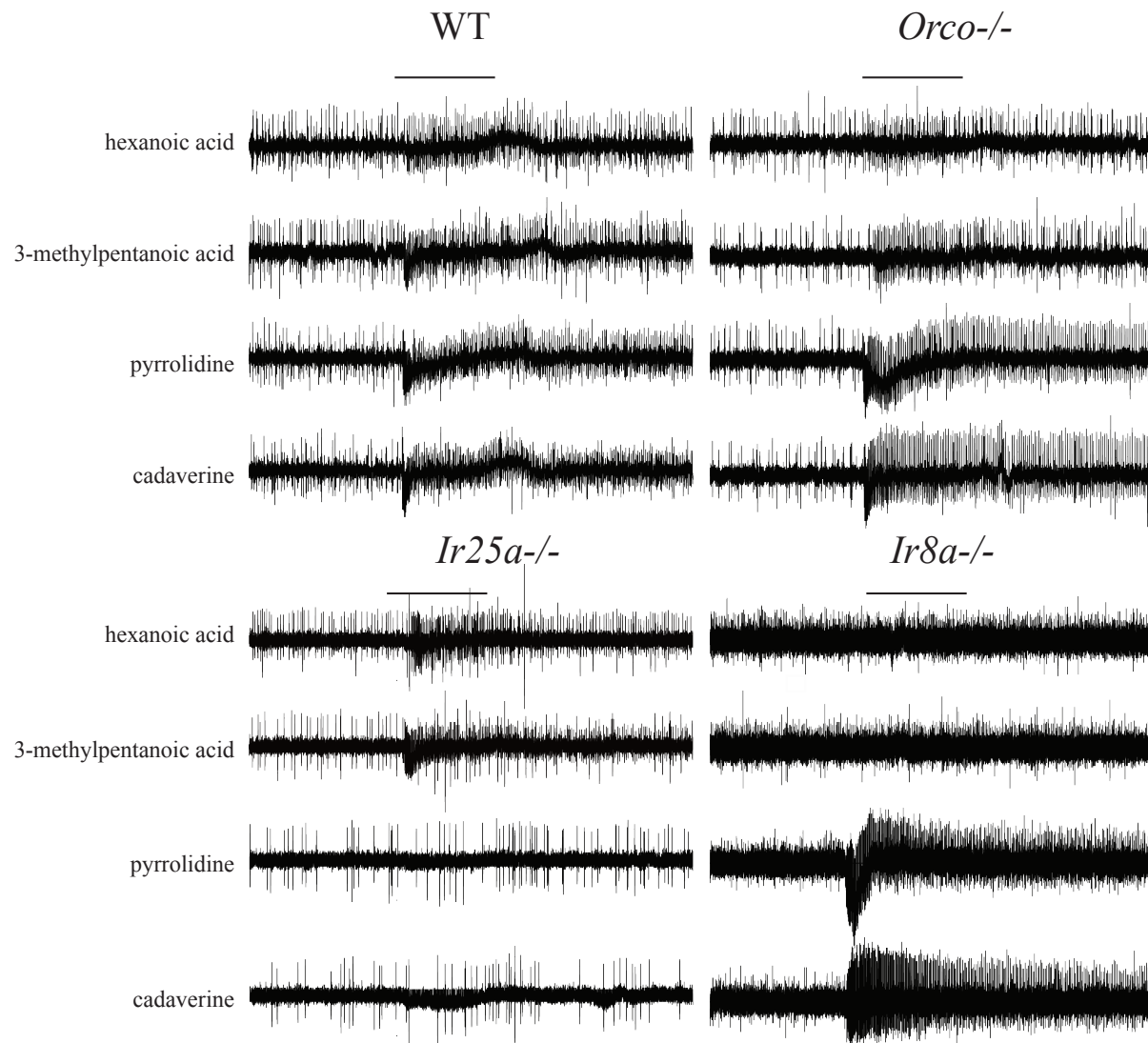
405 **Supplementary Figure 6 | Representative SSR traces of coeloconic sensilla**. Bars above the traces mark

406 0.5 s stimulus time.

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572   **Author contributions**
573   J.Z., M.K. and B.S.H. designed the study; J.Z. performed all SSR and GC-MS experiments. J.Z. and S.W.Y.
574   conducted wind tunnel assays, EAG experiments and measured pupae weight. J.Z., R.F., G.F.O., and
575   E.G.W designed sgRNA and established CRISPR/Cas9 knockouts. S.B.K. performed the calcium imaging
576   experiments. The original manuscript was written by J.Z., subsequently edited by M.K., S.B.K. and B.S.H.,
577   and all coauthors contributed to the final version of this paper.

578

579

580   Table S1. List of 52 tested stimuli.

Odorant name	CAS Number	Odorant name	CAS Number
acetic acid	64-19-7	propanol	71-23-8
propanoic acid	79-09-4	butanol	71-36-3
butyric acid	107-92-6	isobutanol	78-83-1
isobutyric acid	79-31-2	2−pentanol	6032-29-7
pentanoic acid	109-52-4	hexanol	111-27-3
2-methylbutyric acid	116-53-0	cis−3−hexen−1−ol	928-96-1
3-methylbutyric acid	503-74-2	trans−2−hexen−1−ol	928-95-0
hexanoic acid	142-62-1	benzyl alcohol	202-859-9
3-methylpentanoic acid	105-43-1	heptanol	111-70-6
4-methylpentanoic acid	646-07-1	2−phenylethanol	60-12-8
heptanoic acid	111-14-8	eugenol	97-53-0
ammonia	7664-41-7	linalool	78-70-6
ethylamine	75-04-7	geraniol	106-24-1
trimethylamine	75-50-3	beta−citronellol	106-22-9
pyrrolidine	123-75-1	methyl acetate	79-20-9
butylamine	109-73-9	ethyl acetate	141-78-6
isoamylamine	107-85-7	butyl acetate	123-86-4
pentylamine	110-58-7	trans−2−hexenyl acetate	2497-18-9
cadaverine	462-94-2	trans-3-hexenyl butyrate	53398-84-8
benzyl cyanide	140-29-4	hexyl acetate	142-92-7
phenylethylamine	64-04-0	2-pentnone	107-87-9
decanal	112-31-2	gamma-hexalactone	695-06-7
citral	5392-40-5	gamma-heptalactone	105-21-5
phenylacetaldehyde	122-78-1	octanal	124-13-0
butyraldehyde	123-72-8	benzaldehyde	100-52-7
propionaldehyde	123-38-6	valeraldehyde	110-62-3

581