The olfactory coreceptor IR8a governs larval feces-mediated competition avoidance in a hawkmoth

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Finding a suitable oviposition site is a challenging task for a gravid female moth. At the same time, it is of paramount importance considering the limited capability of most caterpillars to relocate to alternative host plants. The hawkmoth, *Manduca sexta* (Sphingidae), oviposits on solanaceous plants. Larvae hatching on a plant that is already attacked by conspecific caterpillars can face food competition, as well as an increased exposure to predators and induced plant defenses. Here, we show that feces from conspecific caterpillars are sufficient to deter a female *M. sexta* from ovipositing on a plant and that this deterrence is based on the feces-emitted carboxylic acids 3-methylpentanoic acid and hexanoic acid. Using a combination of genome editing (CRISPR-Cas9), electrophysiological recordings, calcium imaging, and behavioral analyses, we demonstrate that ionotropic receptor 8a (IR8a) is essential for acid-mediated feces avoidance in ovipositing hawkmoths.

Manduca sexta | Ir8a | CRISPR-Cas9 | insect olfaction | insect–plant interactions

For insects, finding appropriate sites for oviposition is a challenging task, and the decision of a gravid female will have clear consequences for the fitness of its progeny. Due to fragility and limited mobility, the offspring face many threats: limited food availability, intra- and interspecific competition (1), predation (2), and attack by parasitoids (3). Therefore, gravid females must carefully examine the environment prior to selecting the oviposition site. For this, they utilize visual (4, 5), gustatory (6, 7), mechanosensory (8), and olfactory (9, 10) cues. Among these modalities, olfaction plays a pivotal role in an insect's life, as it provides information not only about oviposition sites but also about other biologically relevant resources such as food and mating partners (11).

Insects rely on a sophisticated olfactory system to detect volatile chemicals in the environment. Several protein families are involved, with odorant receptors (ORs) and ionotropic receptors (IRs), 2 types of ligand-gated ion channels, being the key detecting elements (12–14). On the surface of the antenna, the main olfactory organ, numerous hair-like structures (sensilla) contain olfactory sensory neurons (OSNs), which represent the basic units of sensory reception. Sensilla involved in olfaction occur in 3 morphological types: basiconic, trichoid, and coeloconic. In the vinegar fly, *Drosophila melanogaster* (12, 15), as well as in other investigated insect species (16–18), ORs are expressed in the dendritic membrane of OSNs housed in basiconic and trichoid sensilla, whereas IRs are expressed by OSNs housed in coeloconic sensilla. ORs are extremely divergent and different insect species express from 10 *OR* genes in head lice (19) to more than 300 in ants (20). The OR type expressed in an OSN dictates the odorant specificity of the neuron (21). ORs are coexpressed together with the conserved odorant receptor-coreceptor (Orco), which is essential for dendritic localization of ORs and OR-dependent odorant detection (13, 22). IRs usually are less divergent (23), and at least 2 IR coreceptors, ionotropic receptor 8a (IR8a) and IR25a, form ligand-gated ion channels with other odorant-tuned IRs (12, 24). The different receptor types, however, differ not only in their local expression but also in their response profiles. While most ORs are broadly tuned to alcohols, aldehydes, aromatics, esters, or terpenes (21), IRs primarily respond to a restricted subset of odors, including mainly acids and amines (25). At least in *Drosophila* and *Aedes aegypti*, IR8a is required for acid detection (26, 27). IR25a, on the other hand, seems to be coexpressed with IRs responding to amines (28) and is also involved in the detection of temperature (29), humidity (30), and salt (31).

The tobacco hawkmoth *Manduca sexta* (Lepidoptera: Sphingidae) is an established model for insect olfaction (16) and odor-guided behavior (32). The recent identification of 73 *OR* genes and 21 olfactory *IR* genes and their expression patterns in male and female moths (16) and the establishment of the CRISPR-Cas 9 technique in *M. sexta* (33) have made the species an even more powerful model for olfactory neuroethology. The larvae of these moths feed on various plants of the family Solanaceae, including coyote tobacco (*Nicotiana attenuata*) and jimson weed (*Datura wrightii*) (Fig. 1*A*). It was reported that a single *M. sexta* caterpillar consumes 1 to 10 tobacco plants before pupation (1), resulting in complete defoliation of the plants and accumulation of feces under the plant. Therefore, it is crucial for *M. sexta* females to find a suitable host plant that is not already occupied by a conspecific larva.

Significance

Finding a suitable oviposition site is a challenging task for a gravid female moth. At the same time, it is of paramount importance considering the limited capability of most caterpillars to relocate to alternative host plants. The hawkmoth, *Manduca sexta*, oviposits on solanaceous plants. Larvae hatching on a plant that is already attacked by conspecific caterpillars face food competition. Here, we show that feces from conspecific caterpillars are sufficient to deter a female *M. sexta* from ovipositing on a plant. Furthermore, we not only identify the responsible compound in the feces but also localize the population of sensory neurons that governs the female's avoidance. Hence, our work increases the understanding of how animals cope with a competitive environment.

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Fig. 1. *M. sexta* oviposition on *N. attenuata* and *D. wrightii* is affected by larval feces. (*A*) *M. sexta* host plants coyote tobacco (*N. attenuata*, *Left*) and jimson weed (*D. wrightii*, *Right*). (*B*) Schematic drawing of the wind tunnel assay. (*C*) Oviposition index of mated females toward feces of *M. sexta* caterpillars reared on *N. attenuata* and *D. wrightii*. Oviposition index = (number of eggs on plant with feces − number of eggs on plant without feces)/total egg number. (*D*) GC/MS profile of headspace of feces from a *M. sexta* caterpillar reared on *N. attenuata*. IS, internal standard. (*E*) GC/MS profile of headspace of feces from a *M. sexta* caterpillar reared on *D. wrightii*. (*F*, *Left*) Oviposition index of gravid females to carboxylic acids (at a dilution of 10^{-2}) emitted from *M. sexta* caterpillar feces. (*F*, *Right*) Oviposition index of gravid females to various doses of 3-methylpentanoic acid and hexanoic acid. Deviation of the index against 0 was tested with the Wilcoxon signed-rank test (n = 17 to 20). Orange boxes depict $P < 0.05$. Boxplots depict median and upper and lower quartiles. Whiskers depict quartiles ± 1.5× the IQR. All data were included in the statistical analysis.

Volatiles emitted from larval feces have been shown to act as kairomones and attract parasitoids and predators (2, 3, 34, 35). Therefore, the smell of larval feces not only indicates the occupancy of the host plant, and the resulting potential for intraspecific competition, but also an increased susceptibility to parasitization and predation. Hence, female moths should avoid sites that are already occupied by conspecific larvae and could do so by, for example, detecting chemical cues emanating from larval feces. In several insect species, female oviposition has been found to be deterred by conspecific larval feces (36). Thus, larval feces alone are sufficient to signal potential competition to the female. However, the molecular and cellular mechanisms by which female insects avoid feces remain unknown.

Here, we investigate whether the oviposition of *M. sexta* is deterred by feces from its larvae. We first show that *M. sexta* females, like other insects, display oviposition aversion toward conspecific caterpillar feces stemming from different host plants. Next, we identify specific carboxylic acids emitted from the feces as key compounds that confer oviposition aversion. By performing electrophysiological recordings, calcium imaging, and behavioral analyses with mutant moths that lack either *Orco* or one of the IR coreceptors, *Ir8a* or *Ir25a*, we demonstrate that IR8a is essential for acid-mediated feces avoidance during oviposition.

Results and Discussion

Feces of Caterpillars Fed on *N. attenuata* Repel Oviposition. To test whether gravid females of *M. sexta* avoid ovipositing in the presence of feces, we tested their behavior in a 2-choice assay in a wind tunnel. The moths were allowed to oviposit for 3 min either on an undamaged *N. attenuata* plant that was equipped with 10 g of larval feces (from caterpillars that had fed on other *N. attenuata* plants) or on an undamaged control plant (Fig. 1*B*).

In these experiments, the moths laid, on average, 14.3 ± 1.7 eggs (mean ± SEM) during the 3-min test on both plants. The allocation of eggs depended on the presence of feces, with the moths laying significantly fewer eggs on the plant with caterpillar feces in comparison to the control plant (Fig. 1*C*). A similar preference was observed when moths were given a choice between a plant with feces and a control plant without feces in a steady-air tent (*SI Appendix*, Fig. S1), confirming that feces avoidance is consistent in different behavioral paradigms. We conclude that even in the absence of plant damage, caterpillar feces induce oviposition avoidance in *M. sexta*. Former studies suggested that ovipositing *M. sexta* females mainly use plant- and larva-derived odors to avoid competition (37, 38). In our study, where the amount of feces was higher (but still ecologically reasonable, as we used feces that were produced by a single larva during 1 night), feces alone were sufficient to induce oviposition avoidance. Females tested in our experiments were raised on an artificial diet and had no prior experience with the plants or the feces. We therefore conclude that the feces-induced oviposition avoidance is innate.

3-Methylpentanoic Acid and Hexanoic Acid Govern Oviposition Avoidance to Larval Feces. To identify the active compound responsible for feces avoidance, we raised *M. sexta* caterpillars on *N. attenuata* plants and afterward collected the headspace of the resulting feces using a solid-phase microextraction (SPME) fiber. Gas chromatography/mass spectrometry (GC/MS) analysis revealed similar results as in a previous study (2), with 3-methylpentanoic acid being the most abundant compound, followed by 2 other branched aliphatic acids: 3-methylbutanoic acid and 4-methylpentanoic acid (Fig. 1*D*).

To investigate the impact of these compounds on *M. sexta* oviposition, we pipetted 10 μL of one of the compounds (diluted 10^{-2} in mineral oil) onto a filter paper and attached this filter paper 2 cm upwind of a detached *N. attenuata* leaf before presenting this leaf to a mated female in the wind tunnel. When compared with a control leaf, where the attached filter paper just contained the solvent, only 3-methylpentanoic acid elicited significant avoidance (Fig. 1 *F*, *Left*). To address the behavioral sensitivity of *M. sexta* toward 3-methylpentanoic acid, we further performed the wind tunnel test with lower amounts of the compound and identified the behavioral threshold to be between 10^{-4} and 10^{-3} dilutions (i.e., between 9.3 μg and 93 μg) (Fig. 1 *F*, *Right*), which corresponds well to the reported amount of 30 μg of acids in 1 g of *M. sexta* feces (2). In addition, we have collected volatiles from the feces of *Spodoptera littoralis* larvae (i.e., another common *Nicotiana* herbivore) that were raised on *N. attenuata*. We found the same acids as in the feces from *M. sexta* larvae (*SI Appendix*, Fig. S2), indicating that *M. sexta* is able to detect and avoid feces not only from conspecific but also from allospecific potential competitors. We conclude that 3-methylpentanoic acid is the major compound governing feces avoidance of ovipositing females in the context of *N. attenuata*.

Having shown that feces from caterpillars reduce the attraction of *N. attenuata* plants to ovipositing females, we asked whether this also holds true for the relationship between *M. sexta* and its other main host plant, *D. wrightii*. We now let female moths choose to oviposit either on a *D. wrightii* leaf that was equipped with feces (from caterpillars raised on *D. wrightii*) or on a control leaf without feces. Again, females preferred to oviposit on the control leaf (Fig. 1*C*), suggesting that also at the host plant, *D. wrightii*, feces induce oviposition avoidance in *M. sexta* females.

To identify the active compounds responsible for feces avoidance in *D. wrightii*, we raised *M. sexta* caterpillars on *D. wrightii* plants and then collected and analyzed the volatiles as before. The chemical profile of the feces was dominated by hexanoic acid this time, and accompanied by 2 other minor compounds, heptanoic acid and pentanoic acid (Fig. 1*E*). Again, when an ovipositing female had to choose between a *D. wrightii* leaf that was equipped with one of the 3 acids and a control leaf, only leaves with hexanoic acid (10 μL at a 10^{-2} dilution) were avoided (Fig. 1 *F*, *Left*). This avoidance could still be observed even when we reduced the amount of hexanoic acid 10-fold (Fig. 1 *F*, *Right*). We conclude that hexanoic acid is the major compound governing feces avoidance of ovipositing females in the context of *D. wrightii*. When performing choice experiments in the wind tunnel with additional aliphatic acids and the 2 host plants, we found that only 6-carbon aliphatic acids elicited avoidance (*SI Appendix*, Fig. S3).

Both the Odorant Coreceptor Orco and IR8a Participate in Acid Sensing. To determine which olfactory pathway is governing the detection of 3-methylpentanoic acid and hexanoic acid, we performed electroantennography (EAG) measurements on wild-type (WT) moths and on odorant coreceptor heterozygous ($Orco^{+/-}$) and homozygous ($Orco^{-/-}$) moths that were recently generated in our laboratory using CRISPR-Cas9 genome editing (33). While WT moths and $Orco^{+/-}$ moths exhibited robust EAG responses to the acids, $Orco^{-/-}$ moths showed reduced responses (*SI Appendix*, Fig. S4). However, clear EAG responses to the acids remained, indicating that the IR pathway is also involved in acid detection.

To address whether the remaining response to acids in $Orco^{-/-}$ moths were indeed resulting from activation of the IR pathway, we generated 2 IR mutant lines, $Ir8a^{-/-}$ and $Ir25a^{-/-}$, again using CRISPR-Cas9 genome editing. The resulting $Ir8a^{-/-}$ mutant contained a 339-base pair (bp) deletion (93 bp at exon2, 170 bp at intron2, and 76 bp at exon3), while the $Ir25a^{-/-}$ mutant contained a 154-bp deletion (154 bp at exon2) in the genome. As both deletions resulted in frameshifts and the occurrence of premature stop codons (*SI Appendix*, Fig. S5*A*), we expected both mutations to result in nonfunctional ionotropic coreceptors. We found no difference regarding pupal weight and length in either $Ir8a^{-/-}$ or $Ir25a^{-/-}$ mutants, when compared with the heterozygous controls (*SI Appendix*, Fig. S5*B*). Furthermore, in EAG experiments, both mutants exhibited normal responses to the OR-detected pheromone bombykal (*SI Appendix*, Fig. S5*C*), suggesting the absence of relevant off-target effects.

However, when performing EAG experiments with $Ir8a^{-/-}$ and $Ir25a^{-/-}$ moths, only $Ir8a^{-/-}$ moths exhibited significantly reduced responses to both behaviorally active acids when compared with WT moths, while the acid responses in $Ir25a^{-/-}$ moths remained unaffected (Fig. 2*A*).

IR8a Pathway Is Essential for Detecting and Avoiding Acids from Caterpillar Feces. We next asked which sensillum type is involved in the detection of the acids in caterpillar feces. According to the well-studied *Drosophila* species (12, 21, 39, 40), IR-expressing OSNs are mainly housed in coeloconic sensilla. Furthermore, in *M. sexta*, previous single-sensillum recordings (SSRs) from trichoid and basiconic sensilla showed little to no response to acids (41, 42). We therefore hypothesized that coeloconic sensilla of *M. sexta* house IR-expressing OSNs that are involved in acid detection. In contrast to the antenna of female *D. melanogaster*, which contains only 54 coeloconic sensilla (40), the antenna of female *M. sexta* carries about 3,600 (41). This makes the identification and recording from identified individual coeloconic sensilla almost impossible. We therefore recorded from 28 coeloconic sensilla from the middle part of the antenna, which should cover a wide range of functional types, and stimulated them with a set of 52 odorants from different chemical classes (*SI Appendix*, Fig. S6). Consistent with previous studies in *D. melanogaster* (40) and *Bombyx mori* (43), OSNs housed in coeloconic sensilla in WT *M. sexta* were mainly activated by acids and amines. The 2 behaviorally active acids activated mainly OSNs in nonoverlapping groups of coeloconic sensilla. The number of coeloconic sensilla responding to hexanoic acid was about 2-fold higher than the number responding to 3-methylpentanoic acid, and the intensity of responses to hexanoic acid was stronger.

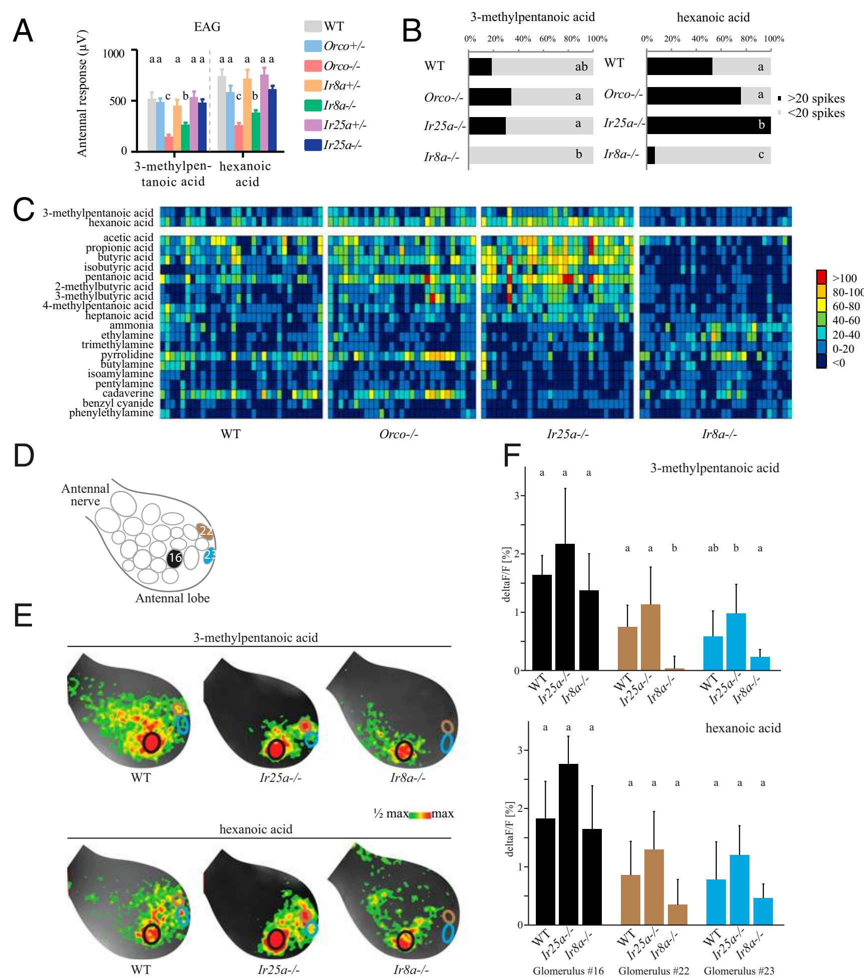

Fig. 2. Detection and processing of feces-emitted odors. (*A*) EAG responses (in microvolts ± SEM; the response to solvent was subtracted) of *M. sexta* antennae isolated from WT, $Orco^{-/-}$ (Orco mutant), $Orco^{+/-}$ (Orco heterozygous), $Ir8a^{-/-}$ (Ir8a mutant), $Ir8a^{+/-}$ (Ir8a heterozygous), $Ir25a^{-/-}$ (Ir25a mutant), and $Ir25a^{+/-}$ (Ir25a heterozygous). EAG responses to 3-methylpentanoic acid and hexanoic acid are shown. Bars with same letter are not significantly different from each other. ANOVA; n = 20 to 25 females per genotype. (*B*) Percentage of coeloconic sensilla responding to the 2 behaviorally active acids in different genotypes. Bars with same letter are not significantly different from each other. Fisher's exact test with Bonferroni–Holms correction for multiple comparisons; n = 29 to 32 sensilla. (*C*) Heat map representation of SSR responses of coeloconic sensilla from different moth genotypes. (*D*) Schematic of 23 putative olfactory glomeruli at the dorsal surface of the right antennal lobe. The schematic was created for each individual moth based on the activation patterns of 19 diagnostic odorants (45), and numbers identify glomeruli that were most strongly activated by the tested acids (16) or that showed acid-specific activation (22 and 23). (*E*) Examples of calcium imaging recordings in WT, $IR25a^{-/-}$, and $IR8a^{-/-}$ female moths after stimulation with the 2 behaviorally active acids. The increase of fluorescence is color-coded (scale) and superimposed onto the view of the antennal lobe; circles indicate positions of glomeruli 16 (black outline), 22 (brown), and 23 (blue). max, maximum. (*F*) Bars show the mean response of a glomerulus (after subtraction of the solvent response) to an odorant. Error bars indicate SD. Bars with the same letter are not significantly different from each other (ANOVA; n = 4 to 6 females per genotype).

We found reduced numbers of coeloconic sensilla responding to 3-methylpentanoic acid and hexanoic acid in $Ir8a^{-/-}$ moths, when compared with the other 3 genotypes (Fig. 2 *B* and *C*; representative recording traces are shown in *SI Appendix*, Fig. S7). Interestingly, increased numbers of coeloconic sensilla exhibited enhanced responses to acids in $Ir25a^{-/-}$ moths, whereas the responses to amines were almost abolished. The enhanced responses in $Ir25a^{-/-}$ moths toward acids could be due to more energy being available to OSNs responding to acids, as these OSNs no longer have to compete with amine-tuned OSNs in the same sensillum. Such a phenomenon has been reported for gustatory receptors, where sensory neurons in the same sensillum have been shown to interact, exhibiting competition, inhibition, or activation (44). In conclusion, our results show that IR8a, but neither Orco nor IR25a, is required for acid detection in OSNs of coeloconic sensilla.

We conclude that IR8a is involved in the detection of the key compounds governing feces avoidance. We next asked where in the antennal lobe (i.e., the first olfactory processing center of the moth's brain) this IR-related acid detection becomes processed. A recently published functional analysis of the moth's antennal lobe (45) revealed 3 glomeruli that strongly responded to acids. In another study (33), activation of 2 of these glomeruli was not affected by knocking out Orco, supporting that these 2 glomeruli become innervated by IR-expressing OSNs. When performing calcium imaging experiments with moths that lacked either a functional IR25a or IR8a, the responses to acids in *Ir25a* mutants were unaffected compared with control animals (Fig. 2 *E* and *F*). However, when testing *Ir8a* mutants, we observed a slightly reduced response to hexanoic acid and a significantly reduced response to 3-methylpentanoic acid (Fig. 2 *E* and *F*) in only those 2 glomeruli that were independent of Orco in the former study. Together with the EAG results, we conclude that both Orco and IR8a, but not IR25a, are involved in acid sensing and that IR8a-expressing OSNs involved in the detection target a subset of glomeruli on the medial surface of the antennal lobe.

Next, we asked whether any of the 3 coreceptors governs the behavioral avoidance toward acids in ovipositing *M. sexta*. Unfortunately, the oviposition rates of *Orco*$^{-/-}$ moths were too low to draw any conclusions regarding the involvement of Orco in oviposition avoidance, which is in accordance with the findings of a former study, where knocking out the Orco receptor resulted in significantly reduced oviposition behavior in *M. sexta* (33). Interestingly, however, mutation of *Ir25a* did not affect oviposition behavior (Fig. 3*A*), while *Ir8a*$^{-/-}$ moths were no longer repelled by the tested acids (Fig. 3*B*). We conclude that ovipositing females rely on IR8a for avoidance of acids from caterpillar feces.

Several studies have shown that larval feces and odors deter female oviposition (36). Feces-emitted acids play a crucial role in oviposition avoidance in moth species like *Ostrinia* species (46) and *Helicoverpa armigera* (47). Moreover, it was shown that female parasitoid wasps, *Cotesia glomerata*, use acids emitted by host larvae as cues to locate their host (48), and *M. sexta* caterpillar feces-emitted acids play a major role in attracting predators like ants (2). Finally, one of the acids we identified in the caterpillar feces (hexanoic acid) has been shown to induce plant defenses against herbivores (49). Obviously, carboxylic acids are potent signals for a gravid female to realize that, at a given plant, the female's offspring might face conspecific competitors, parasitoids, and predators, as well as an already induced plant defense. Therefore, our finding that ovipositing *M. sexta* females, like other moths, avoid emitted acids from larval feces is not unexpected. However, the neural and molecular mechanisms, as well as the exact chemistry underlying this behavior, remained elusive. In this study, we only show that *M. sexta* displays oviposition aversion toward caterpillar feces but also find that only the major volatile compounds (C_6 carboxylic acids) emitted are aversive for gravid females. As these acids are present not only in feces of *M. sexta* larvae but also in feces of *S. littoralis* larvae (i.e., another herbivore from *N. attenuata* plants), feces avoidance might provide the ovipositing *M. sexta* female with the opportunity to avoid not only conspecific but also allospecific competition. By testing mutant moths in which we knocked out different olfactory coreceptors, we show that the coreceptors IR8a and Orco, but not IR25a, participate in the detection of larval feces and that at least IR8a is necessary for feces avoidance.

It was reported that *M. sexta* lay significantly fewer eggs on plants that were damaged by herbivores (1). From an evolutionary perspective, the plant might tag caterpillars with a distinctive (acid) odor that provides spatial and temporal information about feeding larvae to predators (2). Our data suggest that the ovipositing females can recognize these odors and avoid them to optimize the survival chance of their offspring, which adds another layer of regulation to host choice in *M. sexta*.

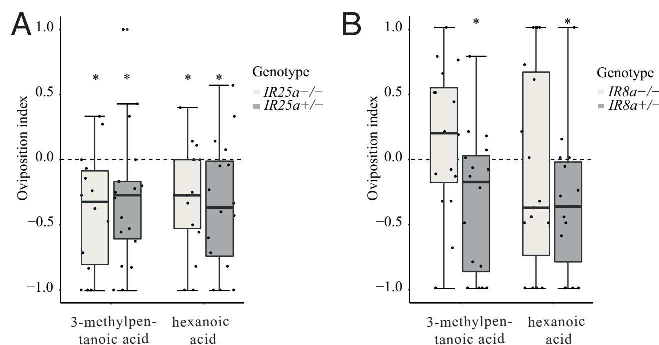

Fig. 3. IR8a is necessary for acid avoidance of ovipositing *M. sexta* females. A 2-choice assay shows the oviposition indexes of the homozygous and heterozygous (as a control) *Ir25a* (*A*) and *Ir8a* (*B*) mutants for the feces-emitted compounds 3-methylpentanoic acid and hexanoic acid (details on choice assay are provided in *Methods* and Fig. 1). Deviation of the index against 0 was tested with the Wilcoxon signed-rank test (n = 14 to 19). $^{*}P < 0.05$.

Methods

Insect Rearing and Plant Material. All animals were reared at the Max Planck Institute for Chemical Ecology, as already described (16). Briefly, eggs were collected from female *M. sexta* moths, which could freely oviposit on *D. wrightii* plants. Larvae used in the experiments were reared on an artificial diet, under a 16:8-h light/dark photoperiod, with a relative humidity of 40% at 26 °C. Naive females were mated the second night after emergence and tested during the subsequent night. *M. sexta* feces were collected daily from fourth- to fifth-instar caterpillars that were raised on either *N. attenuata or D. wrightii*.

All plants were grown in a greenhouse, as described (50). Plants used for experiments were not yet flowering. Approximately 7 d before being used, plants were transferred to a climate chamber with the same settings as the moth flight cage (16:8-h light/dark photoperiod with a relative humidity of 40% at 26 °C).

Chemical Analysis. We identified the volatiles of caterpillars feces using SPME coupled with GC/MS. One gram of feces from caterpillars raised on either *D. wrightii* or *N. attenuata* was put into a 500-mL plastic container. A circular filter paper (12-mm diameter, Whatman; Sigma–Aldrich) loaded with 10 μL of diluted bromodecane (1:10^4 in hexane) was used as an internal standard. Through a hole in the lid of the container, a SPME fiber (50 μm of divinylbenzene/carboxen/polydimethylsiloxane coating; Supelco) was exposed to the container headspace for 30 min at room temperature without agitation, and then introduced into the injector inlet for 2 min at 250 °C in split-less mode. The compounds adsorbed on the fiber were then analyzed by GC/MS (GC: Agilent 6890, Agilent; MS: 5975C MS, Agilent). After fiber insertion, the column temperature was 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 at 260 °C. Compounds were identified by comparing mass spectra against synthetic standards and NIST 2.0 library matches. All of the synthetic odorants that were tested and confirmed were purchased from Sigma–Aldrich (https://www.sigmaaldrich.com/).

Behavioral Experiments in the Wind Tunnel. To investigate the behavioral significance of *M. sexta* feces from caterpillars that had fed on *N. attenuata*, we performed 2 choice tests in a transparent wind tunnel (220 × 90 × 90 cm^3) at 25 °C, 70% relative humidity, 0.3-lux illumination, and a wind speed of 40 cm·s^{-1}. Two nonflowering *N. attenuata* plants of similar size were placed at the upwind end of the wind tunnel. An empty Petri dish (control) or a Petri dish loaded with 10 g of freshly collected feces (treatment) was placed at the base of the plant. A single fifth-instar larva produces about 10 g of feces per day. As described before (45), mated female moths were released at the downwind side of the wind tunnel and were allowed to oviposit on both plants for 3 min. Afterward, the number of eggs on both plants was counted, and the eggs were gently removed after each test. Moths were tested only once, and plants were exchanged after 2 tests. The positions of the treatment and control plants within the wind tunnel were swapped after every second moth. The oviposition indexes were calculated as (T−C)/(T+C), where T is the number of eggs on the treatment site and C is the number of eggs on the control site.

To test the effect of *M. sexta* feces from caterpillars that were raised on *D. wrightii*, we conducted a similar 2-choice test in the wind tunnel. Due to the large size of *Datura* plants, we trimmed plants 7 d before the experiments in such a way that 2 leaves of similar size remained in opposite directions. An empty Petri dish (control) or a Petri dish loaded with 10 g of freshly collected feces (treatment) was placed 10 cm beneath the leaves. Again, mated female moths were allowed to oviposit on both control and treatment leaves, and the resulting eggs and oviposition indexes were calculated afterward.

To determine the functional significance of different volatiles emitted by the feces, we conducted 2-choice tests in the wind tunnel. This time, 2 freshly detached leaves of similar size were presented to the gravid female. Each leaf was attached to the tip of one of 2 upright acrylic glass poles (40 cm high and placed at the upwind end of the wind tunnel with a distance of 40 cm between them). Beneath each leaf, we attached a square filter paper (2 × 2 cm^2) loaded with 10 μL of diluted odorant (1:10^2) or the solvent mineral oil alone. Moths, leaves, and filter papers were tested only once. Experiments were conducted with leaves from both *N. attenuata* and *D. wrightii*.

CRISPR-Cas9–Based Genome Editing. To determine which coreceptor is involved in the acid detection and acid-driven oviposition avoidance, we used olfactory receptor-coreceptor (Orco) mutant moths (33), and generated Ir8a and Ir25a, 2 mutant lines. The *M. sexta* genome v.1.0 (Mansexv1.0) fasta file and the GFF3 file were submitted to the CHOPCHOP (http://chopchop.cbu.uib.no) database for CRISPR-Cas9 target selection sites. The OGS2.0 gene names (i.e., Msex2.10447-RB,

isoform 1; Msex2.02645-RA, isoform 1) were used to select the target site. The single guide RNA (sgRNA) and CRISPR-associated protein 9 (Cas9) were synthesized by Integrated DNA Technologies (https://www.idtdna.com/pages/products/crispr-genome-editing/alt-r-crispr-cas9-system). The microinjection and genotyping were carried out according to previously established procedures (33). After the mutant lines were established, mutations were reconfirmed by Sanger sequencing.

Electrophysiology. To investigate the antennal responses to feces-emitted carboxylic acids, we performed EAG recordings. We therefore clipped the antenna of a 3-d-old female moth directly above the antenna basis and before the third last flagellum. Antenna preparation, stimuli delivery, and data acquisition and analysis were carried out according to previously established procedures (50). Odorants for EAG analyses were selected based on compounds identified in the headspace of caterpillar feces as well as structurally similar chemicals. Ten microliters of diluted odor (1:10^2) or solvent alone was pipetted onto circular filter paper (12-mm diameter) and placed into a glass pipette. In addition, we performed SSRs from coeloconic sensilla as described previously (42). Coeloconic sensilla were identified by their characteristic morphology. A total of 29 to 32 coeloconic sensilla were recorded in each genotype. Responses were quantified by counting all spikes recorded from an individual sensillum due to difficulties in reliably distinguishing spikes from individual neurons (25, 40). The response was calculated as the difference in spike number observed 0.5 s before and after the stimulus onset. A heat map was generated in Excel. Calcium imaging experiments were conducted as described previously (45). Chemical Abstracts Service (CAS) numbers for odorants are listed in *SI Appendix*, Table S1.

Statistics and Figure Preparation. The sample size of behavioral experiments was determined based on a previous study (45). Data were analyzed and plotted using RStudio (version 1.1.414), R (version 3.4.2; The R Project for Statistical Computing), and GraphPad InStat 3 (https://www.graphpad.com/scientific-software/instat/), while figures were organized and prepared using Adobe Illustrator CS5. The Wilks–Shapiro test was used to determine the normality of each dataset. Normally distributed data were assessed using *t* tests. Nonnormally distributed data were analyzed using the Wilcoxon signed-rank test, with the null hypothesis that the median of sampled values differs from 0. For the boxplots, the whiskers were calculated as follows: the upper whisker equals the third quartile plus 1.5× the interquartile range (IQR), and the lower whisker equals the first quartile minus 1.5× the IQR. All data were included in the statistical analysis.

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