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Odorant detection in a locust exhibits unusually low redundancy

Highlights

- The migratory locust emits sex-, phase-, and developmental-stage-related odorants
- The response to odorants depends on sex, phase, and stage of the behaving animal
- To detect these odorants, locusts use narrowly tuned odorant receptors

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In brief

Locusts differ from other insects because they occur in a solitary and a gregarious phase, of which the latter forms the famous devastating swarms. Here, Chang et al. show that these animals exhibit narrowly tuned odorant receptors, suggesting that, compared with other insects, the locust olfactory coding strategy exhibits an unusually low redundancy.

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Odorant detection in a locust exhibits unusually low redundancy

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SUMMARY

Olfactory coding, from insects to humans, is canonically considered to involve considerable across-fiber coding already at the peripheral level, thereby allowing recognition of vast numbers of odor compounds. We show that the migratory locust has evolved an alternative strategy built on highly specific odorant receptors feeding into a complex primary processing center in the brain. By collecting odors from food and different life stages of the locust, we identified 205 ecologically relevant odorants, which we used to deorphanize 48 locust olfactory receptors via ectopic expression in *Drosophila*. Contrary to the often broadly tuned olfactory receptors of other insects, almost all locust receptors were found to be narrowly tuned to one or very few ligands. Knocking out a single receptor using CRISPR abolished physiological and behavioral responses to the corresponding ligand. We conclude that the locust olfactory system, with most olfactory receptors being narrowly tuned, differs from the so-far described olfactory systems.

INTRODUCTION

The migratory *Locusta migratoria* is considered one of the world's most harmful insect pests, whose swarms can devastate vast areas of crop and pasture.[1,2] This insect appears in two developmental phases—solitary and gregarious—that differ in morphological, physiological, and behavioral traits, with only the gregarious phase forming swarms that threaten agricultural production.[2,3] Numerous behavioral and ecological laboratory and field studies have tried to decipher the phase shift from solitary to gregarious animals and, by doing so, targeted the formation of locust swarms and their biological control.[4–6] Phase shift seems to be governed by population density, which, in turn, is affected by external factors such as weather and food abundance.[7,8] In addition, phase shift is accompanied by up- and down-regulation of phase-specific genes[3] and seems to be triggered by intraspecific communication via visual, tactile, and olfactory cues.[9–11] A well-investigated olfactory cue that turns locusts into the gregarious phase is the so-called locustol (2-methoxy-5-ethylphenol). This compound can be found in locust feces, and long-term exposure to it triggers the development of gregarious-specific behavioral and morphological traits in solitary locusts.[12] Furthermore, several aggregation pheromones, such as veratrole, guaiacol, and phenol, which seem to be involved in the formation of swarms, have been described.[13] Another well-investigated compound, phenylacetonitrile (PAN), has been suggested to both deter cannibalism[14] and repel predators.[15] However, when revisiting the potential function of 35 compounds as aggregation pheromones,[15] previously identified in extracts either from locusts or their feces,[16,17] only 4-vinylanisole turned out to carry this function for all locust developmental stages and both phases.

Olfactory cues, however, are not only involved in phase shift and aggregation but, similar to that in other insects, probably govern food- and reproduction-related behavior as well. During the process of maturation, volatile compounds emitted by male locusts have been shown to promote and synchronize the sexual development of both sexes, whereas compounds produced by young nymphs can retard the maturation of adults.[18–20] Furthermore, volatile cues of solitary females have been reported to attract solitary males,[21] whereas volatiles in the so-called egg pods attract ovipositing females, resulting in a clustered distribution of eggs in the field.[22] Finally, as migratory locusts mainly feed on gramineous plants,[23,24] volatiles might help them identify suitable host plants.

To detect and process all this olfactory information, locusts use an olfactory system that differs considerably from that of other insects. Although the peripheral detection of odors via olfactory sensory neurons (OSNs) housed in basiconic trichoid and coeloconic sensilla resembles that of other insects,[25,26] the first olfactory processing center, i.e., the antennal lobe, is very different.[27,28] As in most animals, including humans, the

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insect antennal lobe contains spherical neuropil subunits, so-called glomeruli, where each glomerulus is usually targeted by OSNs expressing the same odorant receptor (OR) or olfactory ionotropic receptor (IR). From there, the number of glomeruli corresponds well with the number of ORs and IRs present in an insect. Hence, the identity of an odor is typically coded by the combinatorial activation of those glomeruli, whose receptors interact with the odorant. Food odorants often interact with many receptors, resulting in several activated glomeruli, whereas a few odors of specific ecological relevance become detected only by highly specific receptors and hence activate only single glomeruli.[29] In locusts, however, where 142 potential OR genes and 32 IRs have been annotated,[30] the antennal lobe contains more than 1,000 microglomeruli.[27,28] This discrepancy between the number of olfactory receptors and glomeruli results from individual OSNs branching into several glomeruli,[27,31] a trait not observed in other insects. By allowing a much more diverse interaction between OSNs and projection neurons (PNs), the coding capacity of the locust could potentially be increased. However, the functional significance of such a system evolving from a glomerular architecture with unbranched OSNs and with most PNs targeting single glomeruli,[27,28] into a system with thousands of microglomeruli innervated by highly branched OSNs and PNs is still unclear.

Here, we deorphanized 48 fully sequenced ORs (*LmigOR*s) using the *D. melanogaster* empty neuron system. In recordings from single sensilla (SSR), we functionally characterized these receptors using 205 odorants identified in the headspace of 4^{th}–5^{th} instar larval stages (from now on called nymphs), unmated and mated adults (both from the solitary and the gregarious phase), and from host- and non-host plants and thus of potential significance for *L. migratoria* (*Lmig*). Surprisingly, contrary to other insects, where most receptors are broadly tuned,[32–37] almost all investigated locust receptors were narrowly tuned to a single or very few ligands, and most of these best ligands activated only a single receptor. For several developmental stages and both sexes of gregarious and solitary locusts, we then tested the behavioral valence of 22 of such best ligands. We found that more than half of these compounds evoked significantly attractive or aversive behavior. More interestingly, the behavior evoked by an odorant strongly depended on the tested animal's developmental stage, sex, and/or phase. Finally, we used CRISPR-Cas9 to knock out *LmigOR5*, a receptor narrowly tuned to the aversive odorant geranyl acetone, a common locust- and plant-produced odorant. Animals lacking a functional *LmigOR5* lost their antennal sensitivity to geranyl acetone and did not avoid it anymore.

From our results, we conclude that the unorthodox architecture of the locust antennal lobe is paralleled by an almost non-redundant receptor-ligand interaction, an architecture probably underlying a so-far undescribed type of olfactory information processing at these primary levels.

RESULTS

Odorants from ecologically relevant sources

Using solid-phase microextraction-coupled gas chromatography-mass spectrometry (SPME-GC-MS), we identified volatile organic compounds from body and feces of all developmental stages and both sexes of both solitary and gregarious locusts, as well as from two host and two non-host plants, resulting in 32 samples (Figures 1A and 1B). By comparing retention times and MS spectra with those of synthetic standards, we were able to identify 185 compounds (of which 165 could be confirmed by their MS spectra) of potential ecological relevance (Figure 1C). The compounds identified included the majority of those earlier reported from *Lmig* emanations, e.g., PAN, 4-vinylanisole, guaiacol, benzaldehyde, phenol, and 2,3-butanediol.[16]

In order to identify potential additional compounds of lower volatility, such as cuticular hydrocarbons from locust body tissues, we used thermal desorption unit coupled with GC/MS (TDU-GC-MS). We were able to identify 20 additional compounds (of which 19 could be confirmed by their MS spectra) from four body parts (hind leg, wing, abdomen, and tergum) of the different developmental stages and both sexes of both solitary and gregarious animals (Figure 2). Taken together, we identified 205 compounds of potential ecological relevance (Data S2A–S2D).

When comparing the odorants emitted by virgin males and females of both phases, we could confirm an older study on stage-, sex-, and phase-specific odor emissions.[16] Interestingly, we found additional undescribed odorants that were specific either for the sex (L-α-terpineol, emitted by gregarious and solitary males) or the mating state (2,4,6-trimethylpyridine, emitted by mated but not virgin gregarious females; E-2-octen-1-ol, emitted by virgin but not mated solitary females) (for a list of the abundance of odorants in different samples, see Data S2).

Most *Locusta* ORs are narrowly tuned

To identify *LmigOR*s involved in the detection of the compounds identified, we amplified the coding regions of 48 OR genes from antennal cDNA of adult locusts, covering main clades and subclades of annotated OR genes (Figure 3A). The *LmigOR* genes were ectopically expressed in the *Drosophila melanogaster* empty neuron system (Figure 3B). Previous *in situ* hybridization studies in the desert locust *Schistocerca gregaria* revealed that many OR genes are co-expressed with the sensory neuron membrane protein 1 (*SNMP1*).[38] We, therefore, expressed the receptors of interest in the *D. melanogaster* at1 empty neuron that is known to express *SNMP1*[39,40] (for a comparison of tuning characteristics of locust receptors when being expressed in either the empty at1 or the empty ab3 system, see Figure S1A). From 48 cloned *LmigOR*s, 42 turned out to be functional in the *Drosophila* at1 neuron, conferring a characteristic, regular, spontaneous firing rate (Figure 3C). The remaining five receptors were non-functional because the at1 neurons displayed an abnormal spontaneous firing rate with bursts of action potentials, a phenotype reminiscent of that observed in mutant at1 neurons lacking their native receptor.[3,9] Next, we systematically examined the odorant detection spectrum of the 42 functional *LmigOR*s by using a set of 205 locust- or food-derived compounds. At 10^{-1} dilution (i.e., 1 μL of a given compound dissolved in 10 μL solvent), all but one (*LmigOR2*) of the functional receptors exhibited significant responses (we used a cutoff of more than 15 spikes/s net increase) to at least one ligand. Of the 42 responding ORs, only one (*LmigOR20*) exhibited an extremely broad response pattern (Figure 3C, for detailed response patterns of

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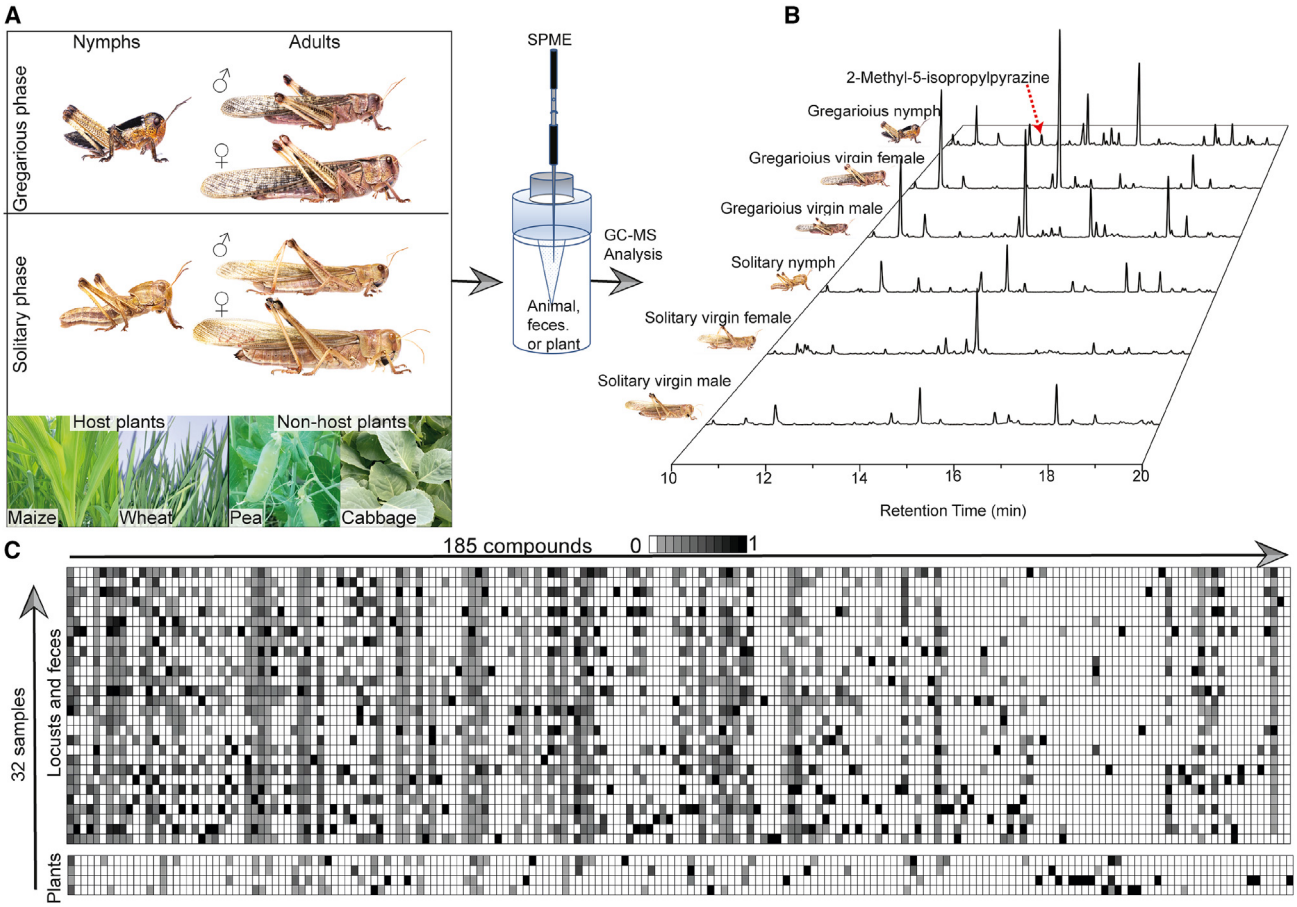

Figure 1. Odorants of potential ecological relevance

(A) Example sources of which headspace samples were analyzed (i.e., 32 headspaces of both phases of nymphs, of immature, virgin, and mated females and males, and the corresponding feces of all of them, as well as 2 host- and 2 non-host plants).

(B) Gas chromatograms revealing 2-methyl-5-isopropylpyrazine as an odorant specific for 4–5 instar nymphs.

(C) Heatmap presentation of 185 volatile compounds identified in the headspace of different sources normalized for each compound (n= 4–8 per source). Locust photos, Benjamin Fabian (see also Data S2A and S2B).

42 receptors to 205 odorants, see Table S1), i.e., responded to more than 100 out of the tested 205 odors of different chemical classes. In addition, the response kinetics of this receptor depended on the tested odorant (Figure S1B). However, surprisingly, 41 *LmigOR*s (98%) turned out to be narrowly tuned to a single or very few compounds (Figure 3D; Table S1).

The tuning width of an olfactory receptor can be calculated as its lifetime sparseness (LTS)[41–43] with values ranging from 0 to 1, with 0 signifying widely tuned, generalist receptors, whereas sparsely tuned, specialized receptors result in an LTS close to 1. With a median LTS of 0.95, the locust system exhibits a significantly more specialized set of receptors than described for other insect species investigated so far (*D. melanogaster*, 0.75[44]; *Anopheles gambiae*, 0.78,[37] Figure 3D). When we repeated the analysis with only those 18 odorants that were used in all three studies (Data S3A), the locust receptors still turned out to be more narrowly tuned (Figure S1C). One should mention here that the deorphanized fly and mosquito receptors were expressed in the *Drosophila* Or22a neuron instead of the *Drosophila* Or67d neuron used in our study. However, several studies have shown that the type of empty neuron used does not strongly affect the tuning width of an investigated receptor.[45,46] Furthermore, one should mention that by using the *Drosophila* empty neuron system, the locust ORs of interest become co-expressed with the *Drosophila* olfactory receptor coreceptor (ORCO) instead of the corresponding locust coreceptor. However, to our knowledge, no studies exist that show that the tuning characteristics of an investigated receptor depend on the identity of the co-expressed ORCO. To exclude the possibility that the observed high tuning rates of the locust receptors are an artifact of the expression system, we, in addition, expressed several of the locust receptors either in the *Drosophila* Or22a neuron or in frog oocytes. Irrespective of the expression system, the tested receptors exhibited extremely narrow tuning properties (Figures S1A and S1F). Although we performed our experiments only with odors that we identified either from body tissues, feces, or food, the 205 tested odorants included 46 compounds that were also used for the deorphanization of fly and mosquito receptors. When restricting our comparison of LTS values to responses to those 46 odorants, the

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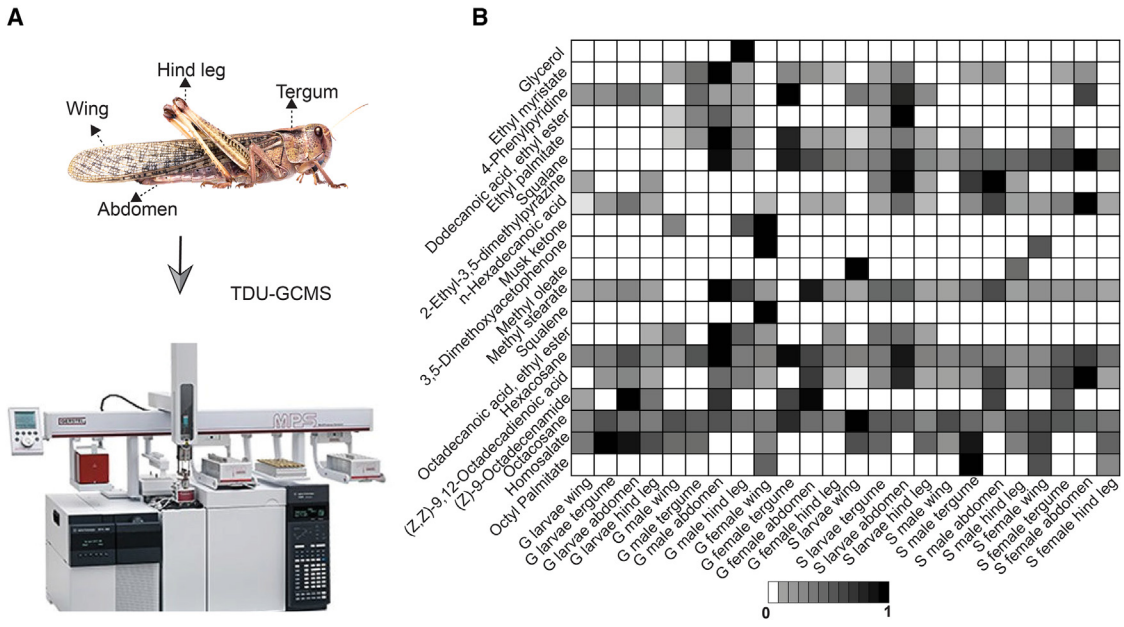

Figure 2. Cuticular hydrocarbons of potential ecological relevance

(A) Example sources (highlighted body parts of different stages, phases, and sexes of locusts) that were analyzed in the thermal-desorption-coupled GC-MS. (B) Heatmap presentation of the 20 compounds identified in the different samples (N = 4–8 per source). Locust photo, Benjamin Fabian (see also Data S2C and S2D).

tuning of locust receptors remained significantly narrower (Figure S1C). Furthermore, this panel of ecologically relevant odors covered basically all functional groups tested in the other two studies (Data S3A). One could also argue that, based on the locust origin of many of the tested odorants, our odor panel could have been skewed toward potential pheromones, for which the detection by narrowly tuned receptors would not have been surprising. Anyhow, when comparing the LTS values of the locust ORs gained either from a screen with body odors or from a screen with plant odors, we did not find any significant difference (Figure S1D). We therefore conclude that the observed narrow tuning of most locust receptors seems to be reliable.

In parallel to the highly specific *LmigOR*s, each best ligand activates only a single or very few receptors (Figure 4), resulting in a significantly higher lifetime sparseness of ligands in the locust system compared with the fly and mosquito systems (for a statistical analysis, see Figure S1E). This means that the locust olfactory system offers fewer opportunities for across-fiber coding at the first neural level. Therefore, it is less redundant than in other insect species, where many receptors are broadly tuned to several chemically diverse ligands and where single ligands are detected by many receptors.[36,44,47]

Behavioral screening of identified ligands

Narrowly tuned receptors are often suggested to respond to odorants of high biological relevance.[29] Without aiming for a detailed behavioral characterization of each identified ligand, we screened 22 of the identified best ligands for their general valence in a vertical two-way olfactometer (Figure 5A). In this assay, a freely moving animal can decide between two rectangular areas (28 cm × 28 cm), of which one area contains the test odor, whereas the control area does not (Figure 5B). By measuring the time spent in both areas, we calculated an attraction index ([time at odor − time at control]/total time) that could range from −1 (strongly aversive) to +1 (strongly attractive). We performed this experiment with 4^{th}–5^{th}-stage nymphs and virgin females and males of both phases.

Many of the tested odorants exhibited either attraction or repulsion to different subgroups of locusts (Figure 5C, for trajectory examples, see Figure S3). Geranyl acetone, an odorant emitted by all tested developmental stages and phases, as well as by non-host plants (Data S2A and S2B), and detected by *LmigOR5*, repelled all gregarious stages but was neutral to the solitary animals (for the statistical impact of stages and phases on the valence of the different odorants, see Data S3B). We also found 2-methyl-5-isopropylpyrizine (an odorant characteristically emitted in high concentrations in gregarious nymphs and their feces) (Data S2A and S2B) to be specifically attractive to the nymphs of both phases but neutral to all tested adults. However, the ecological relevance of these odorants and their attractiveness to specific subgroups of locusts remain elusive. Interestingly, although some of the compounds elicited strong responses in one or several subgroups of animals, none of them was as attractive to all animals as could have been expected from, for example, an odor such as linalool or linalool oxide, which are strongly emitted by host plants.

Typical pheromone receptors of other insects have been shown to be as narrowly tuned as locust ORs.[39,48] Therefore, we hypothesized that some deorphanized *LmigOR*s would be involved in pheromone detection. Consistent with this hypothesis, some receptors were narrowly tuned toward compounds that could potentially fulfill a pheromonal role based on their presence in a specific sex, phase, or developmental stage.

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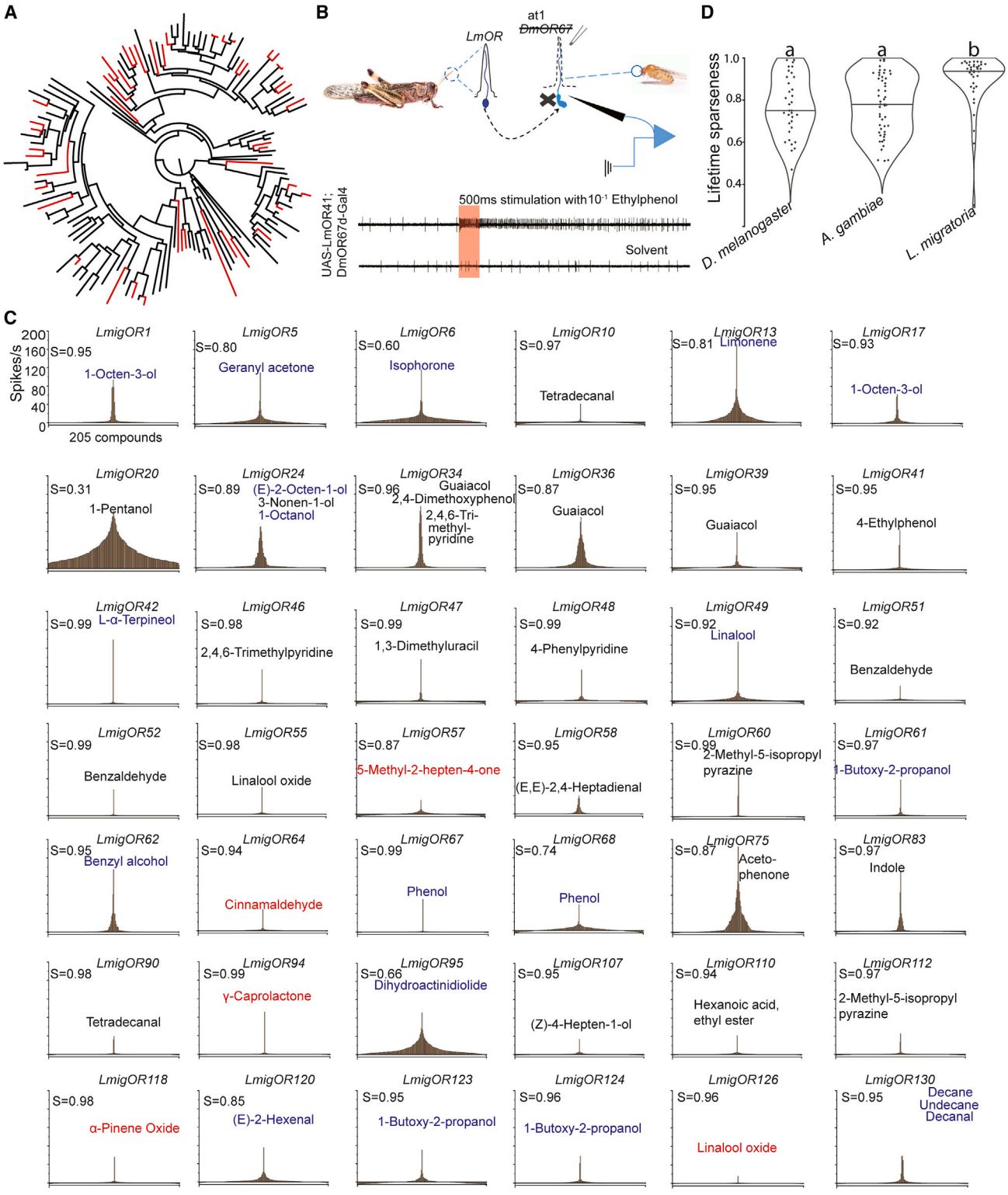

Figure 3. Response profiles of locust odorant receptors

(A) Maximum likelihood tree based on amino acid sequences of 139 annotated odorant receptors genes of *L. migratoria*. Red, genes of receptors deorphanized in this study (for sequences of deorphanized receptors, see Data S1).

(B) Schematics of the heterologous expression system using an empty *Drosophila* at1 neuron with example traces from an at1 neuron expressing *LmigOR41* stimulated with ethylphenol (upper trace) or solvent (red bar, stimulus duration).

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LmigOR46, for example, responded narrowly to 2,4,6 trimethylpyridine, a compound emitted by gregarious females only after mating and therefore potentially involved in post-copulatory mate guarding, as described for several drosophilid flies.[49,50] Furthermore, *LmigOR24* was tuned to E-2-octen-1-ol (emitted by virgin but not mated solitary females) and *LmigOR42* to L-α-terpineol, a male-specific odor in both the gregarious and the solitary phases (Data S2A and S2B).

However, none of the behavioral results pointed at a pheromonal function of any of the tested odorants. The aforementioned 2,4,6-trimethyl pyridine, for example, an odor emitted mainly by mated gregarious females (Figure 1) but in lower amounts also by other subgroups and their feces (Data S2A and S2B) and detected by OSNs expressing *LmigOR46* (Figure 3), was significantly attractive to all tested gregarious stages while eliciting no response in any solitary stages (Figure 5C). If this odor would, as discussed before, be involved in post-copulatory mate guarding, we would rather have expected a repellent effect on virgin males. Therefore, although it still might be a pheromone compound, its ecological significance remains elusive. Furthermore, E-2-octen-1-ol (emitted by virgin but not mated solitary females) or L-α-terpineol (emitted only by males of both phases) (Data S2A and S2B) did not elicit a specific response in the opposite sex (Figure 5C), suggesting that these odorants either do not play a major role in sexual behavior or do so only in the context of other cues.

Another characteristic trait of sex pheromone receptors is that their expression is often upregulated or even specific for the receiving sex.[51] We, therefore, analyzed the expression patterns of ORs in different developmental stages and both sexes of both the solitary and the gregarious phases to investigate whether any of the aforementioned *LmigOR*s that were tuned to sex- or mating-state-specific odorants would be upregulated in the potential recipient of this information. To do so, we examined the mRNA expression levels of olfactory receptors *using* NanoString nCounter, a technology primarily developed for gene expression analysis[52] (for a detailed list of expression patterns of ORs and IRs in gregarious and solitary nymphs, virgin females, and virgin males, see Data S4B).

When we analyzed those receptors that detect any of the before-mentioned sex- or stage-specific compounds, we found that the expression level of *LmigOR46* detecting 2,4,6 trimethylpyridine was seven times higher in gregarious males than in females (Data S4B). This could potentially suggest a role of this receptor and its corresponding ligand in mating behavior. At the same time, the expression levels of receptors detecting E-2-octen-1-ol (*LmigOR24*) and L-α-terpineol (*LmigOR42*) were not differentially expressed in a given developmental stage or sex (Data S4B), which does not support any specific involvement of these receptors in pheromone interactions. However, behavioral responses to a given pheromone do not necessarily rely on sex-, stage-, or phase-specific expression of pheromone receptors but can also be due to processing differences at higher brain centers.[53] Therefore, our analysis does not rule out the existence of pheromones in *Lmig*.

Drosophila larvae[54] and adults[55] exhibit behavioral attraction to a given food odorant, even when lacking an olfactory receptor detecting this odorant. The reason behind these results seems to be the redundancy of the *Drosophila* system, where olfactory coding is based on the participation of many receptors that detect partly overlapping sets of odorants, so-called across-fiber coding.[36] Therefore, receptors with overlapping tuning profiles can compensate for the absence of a single receptor. Having shown that in the locust, most *LmigOR*s are narrowly tuned (Figure 3C) and that many odorants seem to be detected by single or few ORs, we next aimed to probe the presence or lack of redundancy in locust odorant detection. Because geranyl acetone provoked strong repellency in gregarious locusts (Figure 5), we asked whether knocking out *LmigOR5*, a receptor specifically tuned to geranyl acetone, would impair the detection and behavioral responses to this odorant. To address this question, we generated an *LmigOR5* mutant line using CRISPR-Cas9 genome editing (Figure 6A). The obtained *LmigOR5*$^{-/-}$ mutant line contained a 149-bp deletion with a 59-bp deletion in the non-coding 5′ end, which was adjacent to exon1, and a 90-bp deletion in exon1. As the remaining part of the gene has a start codon 18 bp downstream of the deletion, the deletion results either in the lack of the full protein or at least in a protein lacking 36 amino acids. When recording either from the whole antenna (electroantennogram [EAG] recordings) or from basiconic sensilla (single sensillum recordings [SSRs]), mutant locusts detected odorants not related to *LmigOR5* (isophorone and 2,4,6-trimethylpyridine) such as wild-type animals (Figures 6B and 6C). They also exhibited strong attraction toward 2,4,6 trimethylpyridine, indicating the lack of any relevant phenotypical off-target effects of the CRISPR-Cas9 manipulations (Figure 6D; in addition, we searched the potential off-target binding sites by using bioinformatics tools; no off-target binding sites were found at other gene regions, showing a high specificity of this single guide RNA [sgRNA]). However, the electrophysiological response to geranyl acetone, both at the level of the antenna and the sensillum (Figures 6B and 6C), as well as the wild-type typical avoidance of this odor (Figure 6D), was fully abolished in the mutant animals, suggesting the necessity of *LmigOR5* for the detection of geranyl acetone and the corresponding behavioral response. No across-fiber coding patterns did thus ameliorate the effects of the missing receptor.

DISCUSSION

We conclude that, because most of the 42 deorphanized *LmigOR*s, which represent the genetic diversity of all 142 annotated

(C) Tuning curves of 42 receptors when stimulated with 205 compounds (tuning curves depict average responses of n = 6–8 sensillum recordings per receptor). Compounds are displayed along the x axis according to the strength of the responses they elicit from each receptor. Compounds eliciting the strongest responses are placed near the center, with weaker responses toward the edges of the distribution. The most active identified ligands are depicted (for tuning properties, when receptors are expressed in other expression systems, see Figure S1; for quantitative response values, see Table S1).
(D) Lifetime sparseness of receptors of the fly *D. melanogaster* (n = 35 receptors), the mosquito *Anopheles gambiae* (n = 50 receptors), and the locust *L. migratoria* (n = 42 receptors) (letters above violin plots signify differences between groups, $p < 0.01$, Kruskal-Wallis test with Dunn's multiple comparison test). Locust photo, Benjamin Fabian. Red compound names, compounds found in plants; black compound names, compounds found in locusts; blue compound names, compounds found in both plants and locusts (see also Figure S1 and Table S1).

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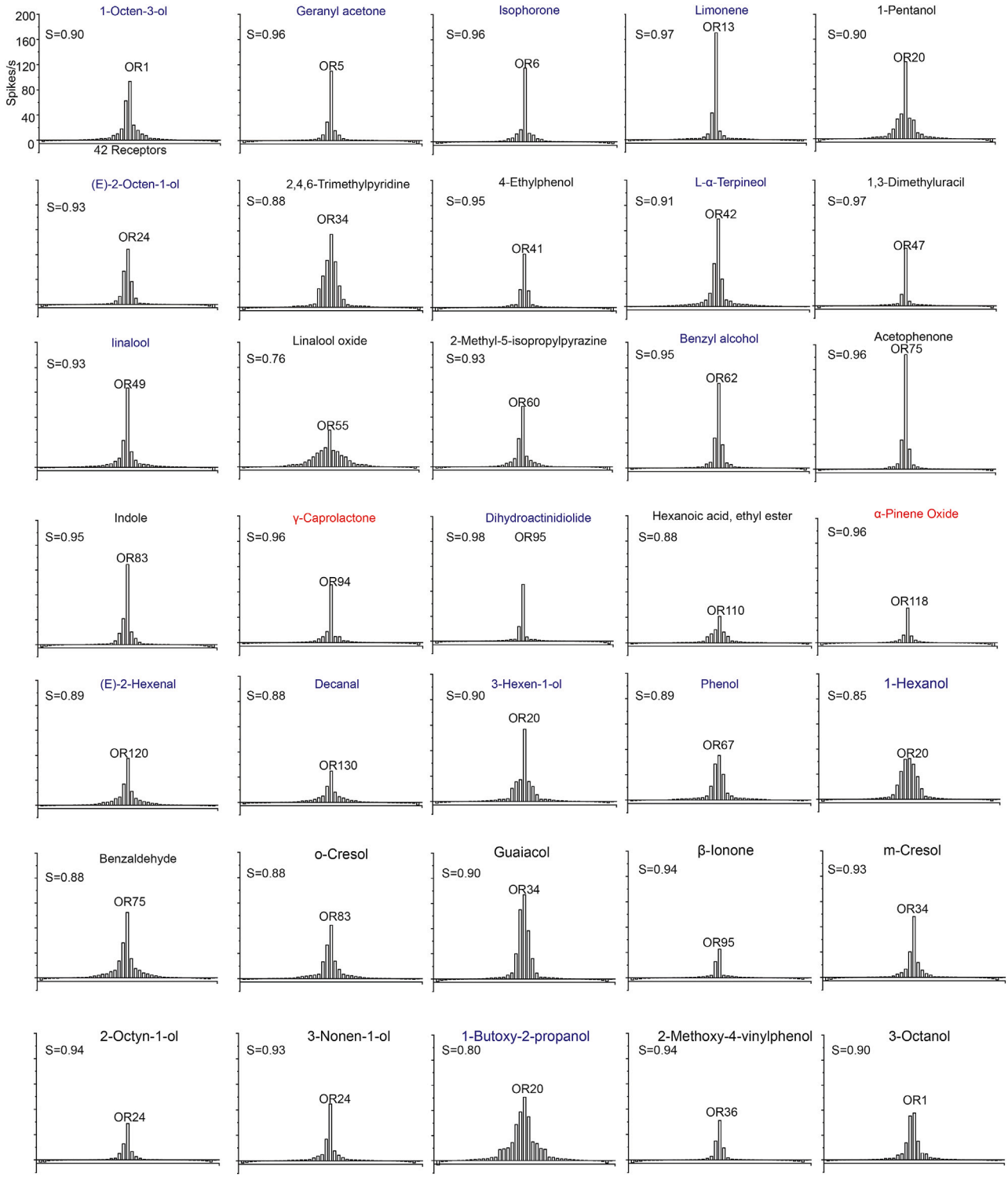

Figure 4. Tuning curves for odorants

The responses of the 42 locust *OR*s are ordered along the x axis according to the magnitude of the response they generate for a given compound. Strongest (weakest) responding receptors placed near the center (edges) of the distribution, and weakest responding receptors are placed near the edges. Strongest responding receptors are depicted S, lifetime sparseness value (for formular of calculation see STAR Methods). Red compound names, compounds found in plants; black compound names, compounds found in locusts; blue compound names, compounds found in both plants and locusts (see also Figure S2 and Data S3A).

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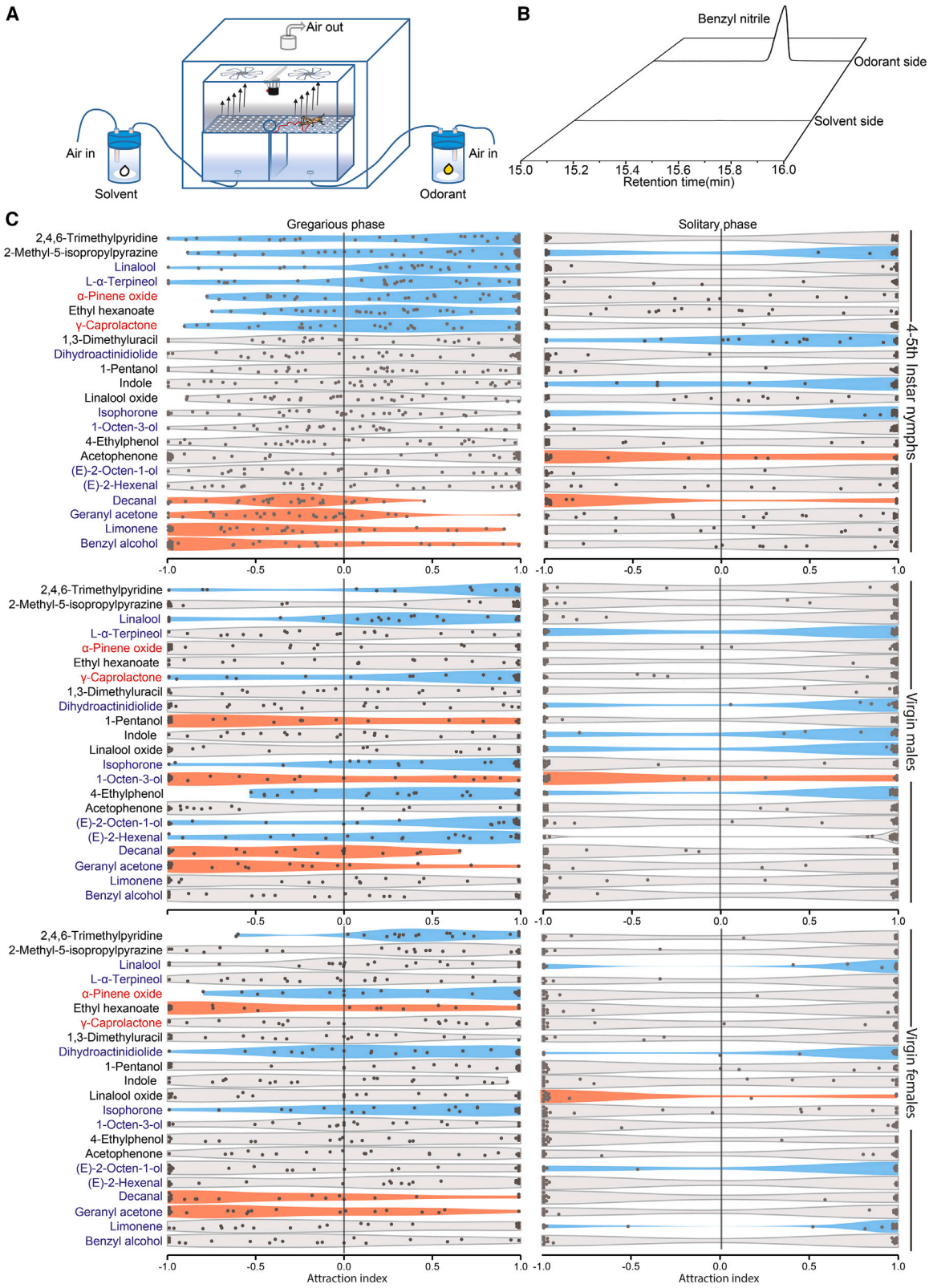

Figure 5. Behavioral responses of nymphs and virgin adults of both sexes to the identified best ligands

(A) Vertical two-choice olfactometer, in which an animal can move within a solvent control side and an odorant-enriched test side. Test duration of individual animals, 10 min. For details, see STAR Methods section.

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OR genes well (Figure 3A), were narrowly tuned to very few compounds, the olfactory sensory system of *Lmig* exhibits a very low redundancy and, by that, might differ dramatically from other so-far-described olfactory systems. Up to now, it was suggested that insects, as well as vertebrates, can identify a virtually infinite number of odors by using a combinatorial code, in which one OR recognizes multiple odorants and one odorant is recognized by multiple ORs, so that different odorants are recognized by different combinations of ORs.[56] Such a strategy demands numerous broadly tuned ORs, and only one out of 42 deorphanized *LmigOR*s (*LmigOR20* responding to >100 out of 205 odorants) fulfilled this demand, *Lmig* obviously has evolved an alternative way to code odor information. Because the response kinetics of *LmigOR20* differ depending on the different tested odorants, temporal information might increase the discriminatory power of the locust olfactory system (Figure S1B). For example, information about odor identity might not only be coded by the combination of responding OSNs but also by the temporal characteristics of their individual responses.[57,58]

Although the final strategy of olfactory coding in locusts remains elusive, it might very well relate to the enormously complex structure of the locust antennal lobe,[27,28] where highly complicated connections between neurons might allow the equivalent resolution to be gleaned as in a system built on first-order across-fiber coding. In fact, in other species of locusts, PNs are generally shown to respond to multiple odors, employing a combinatorial code.[59,60] Future investigations will reveal whether locusts can discriminate between different odorants, as well as insects having access to a more redundant system already at the periphery at their disposal.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.11.017.

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AUTHOR CONTRIBUTIONS

Conceptualization, H.C., B.S.H., and M.K.; chemical analysis, H.C. and K.W.; cloning of receptors, H.C., S. Bucks, Q.C., Y.L., and G.W.; establishment of bioassay, A.P.U.; behavioral experiments, H.C.; tissue collection and RNA extraction, H.C., A.P.U., and S. Brase; gene expression analysis, M.T.T., S.B.-K., and L.C.L.; writing of first draft, H.C.; all authors contributed to the final manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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(B) Solid-phase-micro-extraction followed by GC-analysis confirms odor-free control side and odor-enriched test side of the olfactometer.
(C) Attraction indices of gregarious (left) and solitary (right) nymphs (top), virgin males (middle), and virgin females (bottom) were tested with the 22 identified best ligands. Attraction index = (time in odorant side − time in control side)/(time in odorant side + time in control side), with 1 signifying maximum attraction, and −1 signifying maximum aversion. Blue violins, significant attraction; red violins, significant aversion; Wilcoxon rank-sum test against 0; gregarious nymphs, n = 28–30 (tested individuals per odorant); gregarious males and females, n = 20; solitary nymphs, males, and females, n = 16. Red compound names, compounds found in plants; black compound names, compounds found in locusts; blue compound names, compounds found in both plants and locusts (see also Figure S3 and Data S3B).

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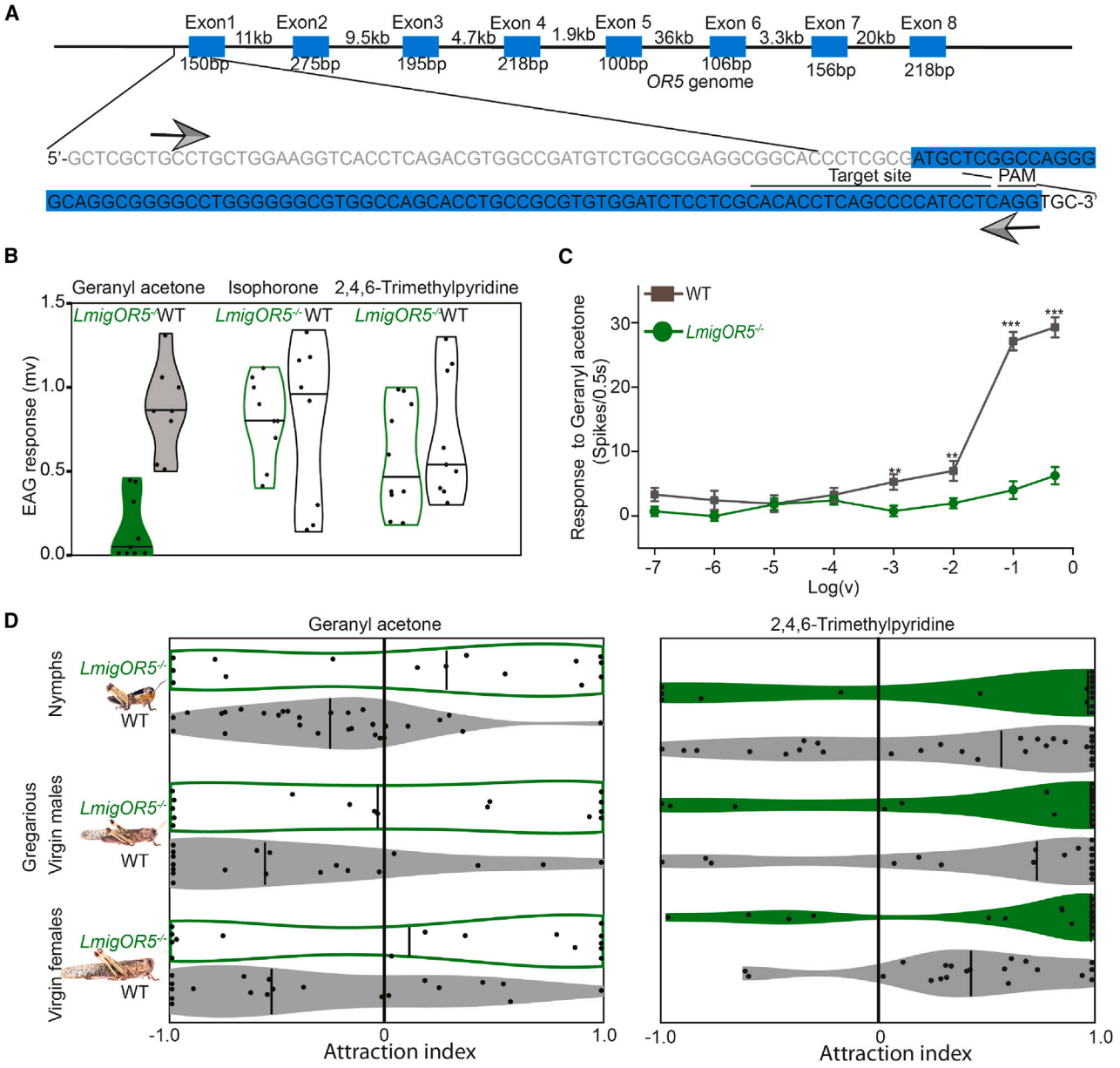

Figure 6. *LmigOR5* is necessary for the detection and aversive responses to geranyl acetone

(A) Schematic of the CRISPR-Cas9 strategy to knock out *LmigOR5*. Exons 1–8, coding regions of the *LmigOR5* gene; nucleotide sequence depicts the CRISPR-Cas9-induced deletion of 149 bp of the non-coding (gray letters) and coding (blue-shaded letters) region; deleted part depicted by arrows; target site and the proto adjacent spacer motive (PAM) are highlighted.

(B) Electroantennogram (EAG) responses of *LmigOR5*-knockout animals and wild-type animals to the *LmigOR5* ligand geranyl acetone and two control odorants (filled boxes differ from each other ($p < 0.01$, n = 8–10 per test; Mann-Whitney U test; black lines, median). For EAG dose-response curves, see Figure S4.

(C) Single sensillum recording (SSR) dose-response curves for geranyl acetone in *LmigOR5*-knockout and wild-type locusts. SSR experiments were performed with responsive (wild type: 90% of all tested sensilla) basiconic sensilla of wild-type animals and unselected (as we did not find any responsive ones in knockout animals) basiconic sensilla from knockout animals (knockout animals, n = 20 recordings; wild-type animals, n = 19 recordings; dots, mean; error bars, standard error; Mann-Whitney U test).

(D) Left, lack of repulsion of geranyl acetone toward *LmigOR5* knockouts (green violins) compared with wild-type animals (gray violins). Right, wild-type-like behavior of *LmigOR5* knockouts toward the attractant 2,4,6-trimethylpyridine, detected by *LmigOR46*. All experiments performed with gregarious animals. Filled violins, significant attraction or repulsion (Wilcoxon rank-sum test, knockout animals, n = 15–16, wild-type animals, n = 20–30, filled violins significantly differ from 0). Locust photos, Benjamin Fabian (see also Figure S4).

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
One Shot™ Chemically Competent *E. coli*	Thermofisher	K450002
Chemicals, peptides, and recombinant proteins		
KpnI restriction enzyme	New England Biolabs	R3142S
XbaI restriction enzyme	New England Biolabs	R0145S
EcoRI restriction enzyme	New England Biolabs	R0101S
XhoI restriction enzyme	New England Biolabs	R0146S
Dimethyl sulfoxide	Sigma-Aldrich	276855
Mineral oil	Sigma-Aldrich	M5904
Paraffin Oil	Sigma-Aldrich	18512
Dichloromethane	Sigma-Aldrich	270997
See Data S3 for chemicals used as odorants	N/A	N/A
Experimental models: Organisms/strains		
D. melanogaster OR67d-GAL4 line	Barry Dickson lab	N/A
Oligonucleotides		
See Table S2 for primer sequences	eurofins genomics	N/A
Recombinant DNA		
pUAST.attb	J. Bischof	N/A
Software and algorithms		
Adobe Illustrator CS5	Adobe Systems Incorporated	N/A
AutoSpike32 (v3.7)	Syntech	N/A
FastTree 2.1.3	N/A	N/A
FigTree version 1.4.2	http://tree.bio.ed.ac.uk/software/figtree/	N/A
GraphPad Instat	https://www.graphpad.com/scientific-software/instat	N/A

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Markus Knaden (mknaden@ice.mpg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- All raw data of this study are provided in the Tables S1 and S2.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

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EXPERIMENTAL MODEL AND SUBJECT DETAILS

Animals and plant

The gregarious and solitary locusts (*Locusta migratoria*) used in the experiments were reared at the Max Planck Institute for Chemical Ecology. In brief, gregarious locusts were reared in cages (30 cm × 30 cm × 30 cm) with 400 to 500 first-instar locusts per cage in a well-ventilated room. Solitary locusts were raised in a single cylindrical box (10.5cm high × 8cm diameter), each box with a separate ventilation system. Both the gregarious and solitary locusts were maintained for at least three generations before the experiments were conducted. All locusts were cultured under the following conditions: a 14 h:10 h light:dark photoperiod, temperature of 30 ± 2 °C, relative humidity of 50 ± 5%, and a diet of fresh, greenhouse-grown wheat seedlings for gregarious locusts and solitary locusts. As all experiments were performed with insects, no approvement of any ethics institution was needed.

All plant species (maize, wheat, pea and cabbage) used in this study were grown at the Max Planck Institute for Chemical Ecology, Jena, Germany, for several generations. Plants were grown in the greenhouse at 23–25 °C, 50–70% relative humidity and a light:dark cycle of 16:8 h (Philips Son-T Agro 400 W Na vapour bulbs, 350–500 μmol m−2 s−1 photosynthetic photon flux at plant level) until elongation.

Transgenic fly lines were generated following the described method. The complete coding region of each LmigOR was subcloned into an empty neuron vector, pUAST.attb, generously provided by J. Bischof, using two different combinations of restriction enzymes: kpnI/XbaI or EcoRI/XhoI (New England Biolabs). Homozygous UAS-ORX lines, with transgene insertions on chromosome II, were created at Bestgene (https://www.thebestgene.com). Each of the transgenic UAS-Lm-ORX flies was individually crossed with an OR67d-GAL4 stock, kindly supplied by B. J. Dickson, resulting in homozygous lines expressing the Or gene of interest in the decoder at1 neuron of *D. melanogaster*. The authenticity of each UAS-transgenic line was confirmed through genomic DNA sequencing obtained from the final crosses. *Drosophila* stocks were raised on a cornmeal-agar-molasses medium and kept under a 12-hour light/12-hour dark cycle at 25°C with 60% relative humidity. These final stocks were utilized in electrophysiological experiments

METHOD DETAILS

Chemical analysis

The volatile compounds from all these different samples were collected by solid phase microextraction (SPME) for 10 hours at 30 °C. To compare volatile profiles of gregarious and solitary locusts in nymphs (fourth to fifth instar) and adult stages (immature adult refers to 2-day-old post-adult eclosion, mature adult is 10-day-old post-adult eclosion before mating, mated adult was 1-day-old after mating), a small number of locusts (6 individuals for nymphs or 2 individuals for adults) were confined into a 100 ml glass bottle, a SPME fiber (PDMS/DVB 65 μm) was introduced inside of bottle through the cap and a PEAK guide piece which served as barrier to avoid direct contact of the insects with the fiber. Meanwhile, 300mg of fresh feces were collected from each stage of locusts and enclosed in a 1.5 ml Agilent vial with a silicone septum, the fiber was exposed to the headspace by directly penetrating the septum of the cap with the SPME fiber holder. Plant materials were subjected to the similar SPME procedure that was used for body volatile sampling. Approximately 5g of fresh leaves were collected from 4 plant species, including two host plants (*Zea mays* and *Triticum aestivum*) and two non-host plant (*Brassica oleracea* and *Pisum sativum*) and placed individually in 500ml glass vials for subsequent headspace sampling. For each type of odor collection, the SPME volatiles collected from an empty glass vial for 10 hours served as control. After each odor collection, the SPME fiber was retracted and immediately inserted into the inlet of a gas chromatography–mass spectrometry (GC–MS) system equipped with an HP5 column. After fiber insertion, the column temperature was maintained at 40 °C for 3 min and then increased to 150 °C at 5 °C·min^{-1}, thereafter it was increased to 260 °C at 10 °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. Most of the synthetic standards that were tested and confirmed were purchased from Sigma–Aldrich.

Cloning of Locust ORs

To clone the full-length coding sequences of locust ORs, we collected > 50 antennae and palps from 7-day-old post-adult in both G and S phase. RNA of each sample was extracted using an RNA isolation kit (QIAGEN) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized from 5 μg of total RNA pooled in equivalent amounts from all tissue sampled using oligo dT-primed cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen). PCR was performed with MyTaq DNA or Myfi Polymerases (Bioline) and primers designed (Table S2) according to published sequences available at GenBank.[24] PCR amplification products were separated on a 1.0% agarose gel and were cloned into the PCR 2.1 TA cloning vector (Invitrogen) and verified by sequencing. Sequences of at least 4 independent clones were obtained for each OR and compared to verify polymorphisms as such rather than PCR errors. The cloning OR sequences which have been used for functional analysis are listed in supplementary material.

Single-Sensillum Recordings (SSR)

To test the function of individual locust odorant receptors in the *Drosophila* empty neuron system, we performed SSR recordings from fly at1 sensilla according to standard procedures.[49] Briefly, adult flies were immobilized in 200ul pipette tips, and the third antennal segment was placed in a stable position onto a glass coverslip. The at1 sensillum type was identified under a microscope

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(BX51WI; Olympus) at ×100 magnification and tested with odors listed in Table S1. The extracellular signals originating from the OSNs were measured by inserting a tugsten wire electrode into the base of a sensillum and a reference electrode into the eye. Signals were amplified (Syntech Universal AC/DC Probe; Syntech), sampled (96000 samples/s), and filtered (500 to 3000 Hz with 50/60-Hz suppression) via USB-Universal Serial Bus-Intelligent Data Acquisition Controller (IDAC) (Syntech) connected to a computer. Action potentials were extracted using AutoSpike software, version 3.7 (Syntech). Synthetic compounds were diluted in dichloromethane, hexane or mineral oil (Sigma-Aldrich, Steinheim, Germany). Before each experiment, 10 μl of the diluted odor was freshly loaded onto a small piece of filter paper (1 cm^2, Whatman, Dassel, Germany) and placed inside a glass Pasteur pipette. The odorant was delivered by inserting the tip of the pipette into a constant, humidified airstream flowing at 600ml/min through an 8mm inner diameter stainless steel tube ending 1cm from the antenna. Neural activity was recorded for 10 s, starting 3 s before the stimulation period of 0.5 s. Responses from individual OSNs were calculated as the increase (or decrease) in action potential frequency (spikes per second) relative to the pre-stimulus frequency. Traces were processed by sorting spike amplitudes in AutoSpike and analyzed in Excel. In addition, we conducted SSRs from locust basiconic sensilla with functional ligands identified in the *Drosophila* empty neuron recording according to previous description[17]. Briefly, we place each male locust in a plastic tube 1cm in diameter, exposed its head and the antenna were fixed with dental wax. A tungsten wire was inserted into the bottom of the sensilla as recording electrode. The reference electrode (Ag/AgCl wire) was inserted into the locust's eye. The signals were then processed and analyzed and underwent the same treatment as described for SSR methods in the fly.

Electroantennograms (EAGs)

Electroantennograms (EAGs) of OR5 mutant locusts and wildtype animals were performed by cutting off the antenna of male animals at the bases of the flagellum. The cut end was immediately placed into a glass capillary containing locust ringer solution (140 mM NaCl, 5 mM $CaCl_2$, 5 mM KCl, 4 mM $NaHCO_3$, 1 mM $MgCl_2$, 6.3 mM Hepes, 15 mM Sucrose). Both capillaries were then placed on Ag-AgCl wires of which one was connected to a grounding electrode, while the other was connected to a 10x a high-impedance d.c. amplifier. Both capillaries were then placed on Ag-AgCl wires, of which one was connected to a grounding electrode, while the other was connected to a 10x a high-impedance DC amplifier (Syntech; The Netherlands). The signal was then sent to an analog/digital converter (IDAC-4, USB, Syntech) and transferred to a computer. Finally, the data were analyzed and saved by using AutoSpike software, version 3.7 (Syntech). The odorant was delivered by inserting the tip of the pipette into a constant, humidified airstream flowing at 600 ml/min through an 8 mm inner diameter stainless steel tube ending 1cm from the antenna. The response was recorded for 10 s, starting 3 s before the stimulation period of 0.5 s.

Locust behavioral responses and video-tracking system

Dual-choice olfactometer experiments were conducted as shown in Figure 5A. We used a vertical airflow olfactometer, similar to the architecture described in a previous study.[15] Two plastic containers (28 cm × 28 cm × 18 cm) with an open-top, seamlessly connected to a glass chamber (60 cm × 30 cm × 30 cm), constituted the main structure of the behavioral observation chamber. The top of each container was equipped with a plastic plate. These plates had small holes 1 mm in diameter at a 1 cm distance from each other. The bottom of each container was connected to an air purification system consisting of a compressed air cylinder, a charcoal filter and a molecular sieve filter. A flowmeter guaranteed a constant rate of airflow (3l/min) through each plastic container at each side (zone) of the arena. The glass chamber enclosed the area above the two plates and thus formed the behavioral observation area. The top of the chamber was equipped with two fans to provide vertical airflow and a video camera was installed in the gap between the fans. The bioassay setup was placed in an observation room (60 cm × 70 cm × 80 cm) with a ventilation system at the top. White light panels were located in the ceiling to provide uniform lighting. The bioassay provided two choices for locusts tested: a clean, vertical airflow in the control zone and an adjacent vertical airflow filled with the odor tested. For the series of behavioral tests, locusts entered the arena through a small door in the middle of the Plexiglas chamber and were allowed to stay in the olfactometer for 10 min. The diluted odorant was applied to a piece of filter paper (3 cm × 3 cm; Whatman No. 1), and paraffin oil was applied in a similar way to serve as a control. After testing 8–15 individuals, the positions of odor and control were reversed to prevent position deviation. The container was then cleaned with 75% ethanol and ventilated for 30 min to remove any odor residues. By using a HD digital video camera, combined with media recorder software (Media recorder 2.5), we captured the locusts' behavioral activities during 10 min at 30 frames s^{-1} after introduction into the arena. Video recordings were analyzed by manually observing the total time spent on each side (unit: s). Valence of the tested odorants was quantified with an attraction index (AI), calculated as: AI = (O-C)/(O+C), where O is the time of the locust spent in the odorant panel and C the time spent in the control panel.

Gene expression analysis

Antennal tissues from stage 4 nymphs and virgin adult males and females of both solitary and gregarious phase *L. migratoria* were collected in 2 ml microcentrifuge tubes, immediately immersed in liquid N_2, and stored at -80°C until further processing. Total RNA was extracted from the tissues using RNeasy Mini Kit (Qiagen, Germany) and RNase-free DNase Set (Qiagen, Germany) was used for the DNase digestion step. The concentration and purity of the extracted total RNA were checked on a NanoDrop™ One (Thermo Scientific) and three samples for each experimental group were selected.

We used the nCounter Elements XT gene expression assay (NanoString Technologies, Inc, USA). Probes A and B for the target genes were designed based on sequences published on GenBank. Off-targets were checked by BLAST to Locust genome assembly v2.4.1 predicted coding sequences (http://locustmine.org/index.html). Master stocks for Probe A pool and Probe B pool were

generated by Integrated DNA Technologies (IDT, Inc.). TagSets for 192 targets and buffers were purchased from NanoString Technologies, Inc, USA. The hybridization reaction (15 μl) was set-up using standard protocol (MAN-10086-01, Page 16). Based on initial standardization, 300 ng total RNA was used. Hybridization was done at 67°C for 16 h, after which 20 μl Merck water was added to the sample. 30 μl of each sample was then loaded on nCounter SPRINT Cartridge (NanoString, USA) and processed on nCounter SPRINT Profiler (NanoString, USA).

The raw data from the gene expression assay was processed using nSolver4.0 (NanoString, USA). Quality control for mRNA data was done on each sample using default parameters for nCounter SPRINT Profiler according to NanoString Gene Expression Data Analysis Guidelines (MAN-C0011-04). The parameters were Imaging QC: 75; Binding Density QC: 0.1 – 1.8; Positive Control Linearity QC: 0.95; Positive Control Limit of Detection QC: 2 standard deviations. Background subtraction was done first using the raw counts of the 8 negative control probes (mean +/- 2 standard deviations). After that, two normalization steps were performed, first using the geometric mean counts of the 6 external positive control probes, and second, using the geometric mean counts of two endogenous reference genes (18S rRNA and EF1-alpha) (Data S4A). The geometric mean normalized data are given in Data S4B.

Establishment of the *LmigOR5* mutant line using CRISPR–Cas9

The establishment of *LmigOR5* mutant locusts by CRISPR–Cas9 was performed as previously described.[17] In brief, the embryos of locusts were collected from egg pods, washed with 75% ethanol, and placed on 1% agarose gel. The purified Cas9 protein and single-guided RNA were mixed to final concentrations of 400 and 150 ng μl^{-1}, respectively, and 27.2 nl were injected into the embryos using a nanoliter injector (World Precision Instruments) with a glass micropipette tip under an anatomical lens (for the target site see Figure 6A). Then, the embryos were placed in a 30 °C incubator for approximately 14 days until the locusts hatched. The first-instar nymphs were placed in cages (30 cm × 30 cm × 30 cm) with a 14 h:10 h light:dark cycle and sufficient food. In order to screen for successful mutations, we collected parts of adult legs and lysed them with a 45 μl NAOH buffer (50 mM) at 95 °C in a PCR machine for 30 min and added 5 μl Tris-HCL (pH 8.0, 1 M). Then, we used a 2 μl template to amplify the targeted fragments and sequenced the fragments to identify whether the mutants were generated. The PCR reaction volume contained 5μL 5x MyTaq Reaction Buffer, 2μL (10 μM) F primer, 2ul (10 μM) R primer, 0.5 μL MyTaq HS DNA polymerase, 13.5μL nuclease-free water and 2μL template. The PCR reaction condition was 95°C 1min, 40 cycles of 95°C 15s, 60°C 15s and 72°C 30s, followed by a final 10min extension period of 72°C. In 78 locust individuals, 16 locusts with mutations in exon 1 (mutation efficiency: 20.5%) were identified. To further investigate the exact mutation models, we performed Sanger sequencing of PCR amplicons from all mutated locust individuals. Mutations in G0 locusts were evaluated using PCR-based genotyping (for primer information, see Table S5). G0 mutants were crossed with the wild type to obtain G1 offspring. G1 locusts, whose DNA strands contained a 149-bp deletion, were crossed with each other to establish stable lines. For the expanding mutant population, 149-bp-deleted homozygotes of G2 locusts were further crossed with each to generate a line of OR5 homozygous mutant lines.

Polygenetic tree construction

To construct the *LmigOR* phylogenetic tree, a total of 139 amino acid sequences from *L. migratoria* were aligned with MAFFT. The phylogenetic tree was built with FastTree version 2.1.3 using approximated maximum likelihood method and the resulting tree was visualized using FigTree version 1.4.2.

Receptor expression in oocytes and electrophysiological recordings

The open reading frames (ORFs) encoding of DmOrco, LmigOR49, LmigOR39 and LmigOrco were amplified and cloned into pT7Ts vector. The cRNAs were synthesized using the mMESSAGE Mmachine T7 kit (Ambion, Austin, TX). Electrophysiological recordings were performed according to previously reported protocols.[32,33] Mature healthy oocytes (stage V–VII) (Nasco, Salida, California) were treated with collagenase I (GIBCO, Carlsbad, CA) in washing buffer (96mM NaCl, 2mM KCl, 5mM $MgCl_2$, and 5mM HEPES [pH= 7.6]) for about 1h at room temperature. After being cultured overnight at 18 °C, oocytes were microinjected with 27.6ng ORs cRNA and 27.6ng Orco cRNA. After injection, oocytes were incubated for 4–7 days at 18 °C in 1X Ringer's solution (96mM NaCl, 2mM KCl, 5mM $MgCl_2$, 0.8mM $CaCl_2$, and 5mM HEPES [pH= 7.6]) supplemented with 5% dialysed horse serum, 50mg/ml tetracycline, 100mg/ml streptomycin and 550mg/ml sodium pyruvate. Whole-cell currents were recorded from the injected *Xenopus* oocytes with a two-electrode voltage clamp. Odorant induced currents were recorded with an OC-725C oocyte clamp (Warner Instruments, Hamden, CT) at a holding potential of 280mV. Data acquisition and analyses were carried out with Digidata 1440A and pCLAMP 10.2 software (Axon Instruments Inc., Union City, CA). Tested compounds were dissolved in dimethyl sulfoxide (DMSO) to 1M stock solutions and stored at −20 °C. Before testing, the stock solutions were diluted with 1 X Ringer's buffer (96mM NaCl, 2mM KCl, 5mM $MgCl_2$, 0.8mM $CaCl_2$ and 5mM HEPES [pH= 7.6]).

Calculation of lifetime sparseness values

We compute the lifetime sparseness value of each receptor according to the formula $S=[1/(1-1/N)]*\{1-[(\sum^{N}_{J=1} R_j/N)^2/(\sum^{N}_{J=1} (R_j)^2/N)]\}$, where N=number of odors and R_j is the response of the receptor to odor j [spikes/s]. Any values of R_j <0 were set to zero before computing lifetime sparseness.

Correspondingly, we compute lifetime sparseness value of each odor according to the formula $S=[1/(1-1/n)]*\{1-[(\sum^{n}_{J=1} r_j/n)^2/(\sum^{n}_{J=1} (r_j)^2/n)]\}$, where n=number of receptors and rj is the response intensity of the compound to receptor j [spikes/s]. Any values of r_j <0 were set to zero before computing lifetime sparseness.

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QUANTIFICATION AND STATISTICAL ANALYSIS

All statistics were analyzed usinsg GraphPad Instat (https://www.graphpad.com/scientific-software/instat) and preliminary figures were conducted using PAST (https://past.en.lo4d.com). Figures were then processed with Adobe Illustrator CS5. Details regarding sample sizes, levels of significance and statistical tests used are provided in the figures and their corresponding legends.