A chemical defense deters cannibalism in migratory locusts

Bill Hansson (hansson@ice.mpg.de)
Max Planck Institute for Chemical Ecology
hetan Chang
Sina Cassau
2Department of Animal Physiology, Institute of Biology/Zoology, Martin Luther University Halle-Wittenberg, Hoher Weg 8
Juergen Krieger
2Department of Animal Physiology, Institute of Biology/Zoology, Martin Luther University Halle-Wittenberg, Hoher Weg 8
Xiaojiao Guo
Markus Knaden
Max Planck Institute for Chemical Ecology https://orcid.org/0000-0002-6710-1071
Le Kang
Hebei University https://orcid.org/0000-0003-4262-2329

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A chemical defense deters cannibalism in migratory locusts

Hetan Chang[1], Sina Cassau[2], Jürgen Krieger[2], Xiaojiao Guo[3], Markus Knaden[1], Kang Le[3#] & Bill S. Hansson[1#*]

[1]Department of Evolutionary Ecology, Max Planck Institute for Chemical Ecology, Hans Knöll Strasse 8, D-07745 Jena, Germany
[2]Department of Animal Physiology, Institute of Biology/Zoology, Martin Luther University Halle-Wittenberg, Hoher Weg 8, D-06099 Halle (Saale)
[3]The State Key Laboratory of Integrated Management of Pest Insects and Rodents, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, P.R.China
[#]These authors contributed equally
*Corresponding author hansson@ice.mpg.de

Abstract

Many animals engage in cannibalism to supplement their diets. In swarms of migratory locusts, cannibalism is prevalent and thought to be an important factor for swarm dynamics. We show that in dense swarms, locusts defend themselves by producing an anti-cannibalistic pheromone; phenylacetonitrile (PAN). Both the degree of cannibalism and the production of PAN are density dependent and co-vary. We identify the olfactory receptor responsible for the detection of PAN, and by using genome editing we show that by making this receptor non-functional we abolish the negative response to PAN. We also knock out the gene underlying PAN production and show that locusts lacking PAN lose its protection and are more frequently exposed to intra-specific predation. In conclusion, we reveal an anti-cannibalistic feature built on a specifically produced odor. The system is very likely of great importance in locust swarm dynamics and our results might therefore provide opportunities in locust management.

Cannibalism, defined as intraspecific predation, is a behavioral trait found in a wide variety of animals[1,2]. By consuming conspecifics, extra nutrition and energy can be gained[3]. These events clearly exert a strong selection pressure for the evolution of protective mechanisms that reduce the risk to be cannibalized[4]. However, so far little is known regarding such mechanisms. Here we demonstrate that gregarious nymphs of the migratory locust *Locusta migratoria* produce a specific compound, phenylacetonitrile, to repel conspecifics, thereby preventing intraspecific predation. The cannibalism displays density-dependent changes, with increasing rates following increasing population densities. These dynamics are paralleled by an increase in the production of phenylacetonitrile. We proceed to identify the olfactory receptor specific to phenylacetonitrile. Knockout of this receptor, using CRISPR/Cas9-based genome editing, close to extinguish electrophysiological responses from olfactory sensory neurons in the antennae and totally abolish the effect of phenylacetonitrile in suppressing cannibalism. To further investigate these interactions, we use the same technology to knock out a phenylacetonitrile biosynthesis gene and find that inability to produce the compound increase the vulnerability of gregarious locusts to intraspecific predation significantly. Supplementation with synthetic phenylacetonitrile restore the resistance of mutant locusts against cannibalism fully. Our results thus reveal a novel, anti-

cannibalistic strategy built on purpose-produced olfactory cues, a so-far overlooked strategy that could play an important role in the swarm dynamics of locusts.

Cannibalism is defined as the killing and consumption of all or parts of a conspecific. Although it can sometimes function to eliminate competitors[5,6] or be the price of mating[7], in most cases it is a means of supplementing nutrition[2,3]. When practiced frequently, cannibalism has important ecological consequences for population dynamics and stability, interspecific trophic interactions as well as for pathogen transmission and epidemiology[8-10]. For predatory animals, cannibalism is a straightforward extension of diet and many predators include conspecifics among their prey[11]. A broader range of taxa, including insects, engage in egg cannibalism, facilitated by their small relative size, immobility and defencelessness[12], or in predation on much smaller juvenile conspecifics[8]. For prey of cannibalism, strong selection pressures favor the development of anti-cannibalistic strategies to deter potential predators of their own kind[4]. Among insects, anti-cannibalistic strategies, although having evolved independently in species across taxa, mostly are reported for egg protection[13-15]. Beyond egg protection, prey animals can deploy direct defense against conspecifics. However, such anti-cannibalistic strategies remain largely unknown.

Among species of locusts, cannibalism is a common characteristic, especially when they are deficient in protein. Like several other locust behavioral traits, cannibalism shows phase polyphenism in response to changes in local population density. At low densities, locusts live as solitary individuals avoiding physical contact. As the local density increases beyond a critical value, behavioral repulsion declines and the locusts begin to be mutually attracted, thereby increasing the likelihood of encountering conspecifics and engaging in cannibalistic interactions[16,17]. Cannibalizing vulnerable conspecifics offers the dual benefits to individuals in a group of surviving longer and travelling farther than a solitary individual without the opportunity to cannibalize[16,18]. Furthermore, cannibalistic interactions have been hypothesized as one of the driving forces behind collective mass movement. The threat of cannibalistic attacks, especially from behind, was shown to be an important factor underlying swarm behavior and movement in migratory bands of nymphs[17]. Subsequent models of individual escape and pursuit behavior associated with swarming in cannibalistic insect groups showed that selective repulsion and attraction interactions can lead to the onset of collective motion[19]. The selective repulsion driven by cannibalistic interactions suggests that gregarious locusts may release warning signals to deter conspecific predation. Our recent study on the migratory locust *Locusta migratoria* identified hundreds of volatile compounds emanating from all life stages of the locusts[20]. Several of these compounds were shown to be behaviorally repulsive, but their biological functions remain largely unknown. Here, we use *L. migratoria* as a model system, revealing the importance of one specific olfactory cue involved in protecting individuals against cannibalism at biological levels from detection to behavior.

Cannibalism is frequent among crowded gregarious nymphs

Before setting out to study the olfactory background to anti-cannibalism, we established specific traits of cannibalism in *L. migratoria*. We observed that cannibalism is a prevalent characteristic of gregarious locusts at all life stages, even if the insects are supplied with sufficient plant-based food sources. Locusts are hemimetabolous insects with five juvenile instars before reaching

adulthood. The highest rates of cannibalism occurred among nymphs at the fourth-to-fifth instar (Supplementary Fig. 1), when the locusts in general are more aggressive towards conspecifics. We therefore chose this stage to investigate density-dependent variations in the rate of cannibalism. We found that cannibalism among 4-5 instar nymphs displayed density-dependent changes (Fig.1b). At a density of 5 and 25 individuals/9.4l, the behavior was reminiscent of solitary locusts, with little physical contacts observed. When the density was increased beyond 50/9.4l, increasing rates of cannibalism, levelling out at a density of 250 nymphs/9.4l, were noted. Nymphs of the migratory locust thus display a clear, density-dependent rate of cannibalism. Our results are consistent with earlier observations in the desert locust *Schistocerca gregaria*[16]. Based on these initial results, a clear selection pressure for anti-cannibalistic agents and behavior could be expected and we set out in search of potential olfactory cues.

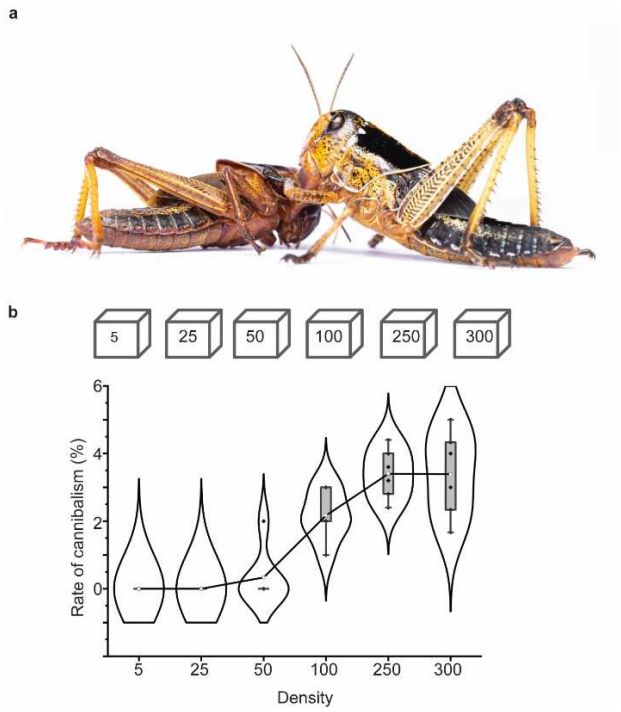

Figure 1 Cannibalism in *L. migratoria*. a, A fifth-instar migratory locust consuming the body of a conspecific. **b**, The rate of cannibalism displayed by gregarious fifth-instar nymphs at different population densities. Data are plotted as violin-box plots (mean: white dot, interquartile range: grey box, black line: upper and lower adjacent value. n = 6 biological replicates for each assay.

To establish candidate odors possibly protecting against cannibalism, we conducted a chemical analysis of all volatile compounds from the body of solitary and gregarious migratory locusts (Fig. 2a). Among these we identified 17 compounds only emitted by the gregarious phase, where cannibalism occurs. Two of the compounds identified, 4-vinylanisole (4VA) and phenylacetonitrile (PAN), have been reported earlier[21], where 4VA was identified as an intraspecific aggregation pheromone[22], while PAN has been shown to be a repellent against bird predators and also a male anti-aphrodisiac[23,24]. The function of PAN as a repellent raised our

interest in this compound and we asked if it might indeed be an intraspecific behavioral antagonist, preventing cannibalism.

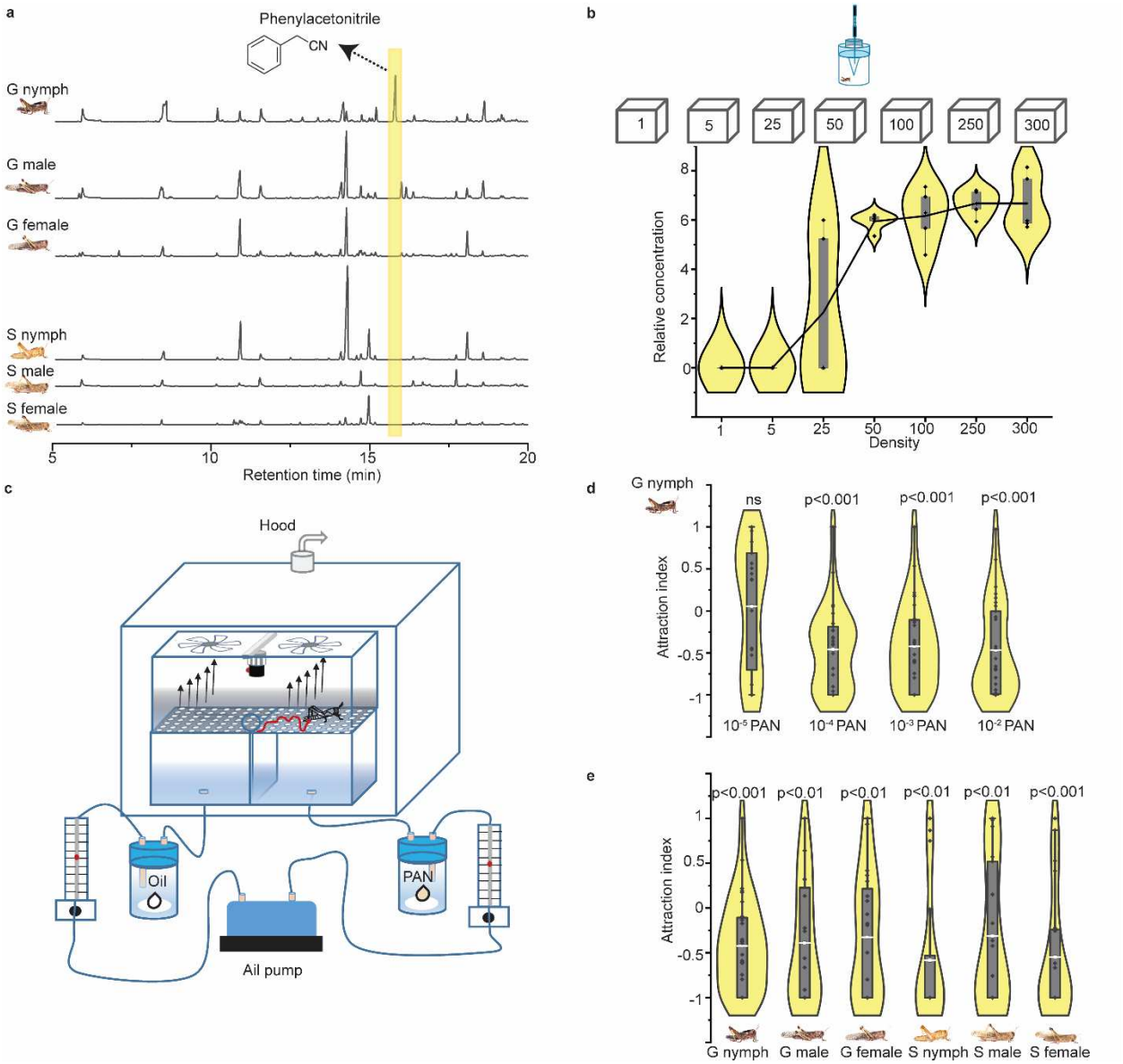

Figure 2 Gregarious nymphs release PAN to repel others. a, Gas chromatograms of locust body volatiles. PAN indicated by yellow bar. **b**, PAN emitted by gregarious nymphs at different population densities, n = 5 replicates for each population density. **c**, Schematic drawing of dual-choice olfactometer experiments. For details, please see Methods. **d**, Attraction index of gregarious nymphs to different dosages of synthetic PAN, n = 20-30 replicates for each concentration. **e,** Behavioral responses of locusts across phase, sex and development stage to 10^{-3} PAN, n = 17-30 replicates for each type. The attraction index in d and e was calculated as (O-C)/(O+C), where O is the total retention time of the locust on the odor side, C the total retention time on the control side. Data in **b**, **d** and **e** are plotted as violin-box plots (mean: white dot and line, interquartile range: grey box, black line: upper and lower adjacent value). P values in **d** and **e** were determined by two tailed unpaired t-test.

Consistent with earlier studies[21], we found that PAN is the dominant volatile component emitted from the body of gregarious nymphs. We also confirmed the presence of PAN in the odor of mated, gregarious males. Moreover, we found that PAN emission by gregarious nymphs displays density-dependent changes that parallel the earlier observations of cannibalism rates (Fig. 2b). Nymphs thus begin to release PAN when the population density exceeds 25/9.4l, whereafter release rates increase gradually with density. This correlation between cannibalism and PAN release rates strongly indicated that PAN could play an important role in nymph interactions at high densities.

Locusts exhibit aversion to phenylacetonitrile

Next, we subjected gregarious fourth-to-fifth instar nymphs to dual-choice olfactometer tests to determine the behavioral valence of PAN (Fig. 2c). We found that the gregarious nymphs displayed significant repulsion towards PAN at concentrations ranging from 10^{-4} to 10^{-2} dilution. The nymphs significantly preferred to move and remain in the zone suffused with vapor of paraffin oil as compared to the zone smelling of PAN. At a lower concentration of 10^{-5}, the negative effect of PAN was absent (Fig. 2d). We proceeded to evaluate the behavioral performance across developmental stages, phases and sexes and found that PAN evoked aversion also among adults of both sexes and both phases (Fig. 2e). We thus conclude that PAN is an aversive compound to migratory locusts regardless of age, sex and phase.

Phenylacetonitrile is mainly detected by one olfactory receptor necessary for aversive behavior

We now turned to the olfactory system of *L. migratoria* in search of the olfactory receptors (ORs) involved in the detection of PAN. Initially, we cloned 50 ORs and functionally expressed them in the at1 empty neuron system of *D. melanogaster*, where the endogenous receptor OR67d is lacking. To establish the molecular specificity of the locust ORs we performed single-sensillum recording (SSR) measurements in the transgene fly lines, while stimulating with PAN. These recordings revealed that the fly line bearing the *L. migratoria* OR70a (LmOR70a) gene displayed the by far highest response to PAN among all the fly lines tested (Fig. 3a). The PAN response of LmOR70a was almost fourfold and fivefold higher than the second and third best LmOR20 and LmOR75, respectively (Fig. 3b). We then asked whether LmOR70a was exclusively tuned to PAN and screened 207 locust volatiles reported previously from our laboratory[20] (tested at 10^{-1} dilution) in continued SSR experiments (S1 table). We identified two additional compounds, structurally similar to PAN (benzaldehyde and cinnamaldehyde), which activated neurons expressing LmOR70a, even though at higher concentrations (Fig. 3c). This result prompted us to return to behavioral experiments, where we tested the two secondary activators of LmOR70a. Here we found that none of these odors elicited neither repulsion nor preference (Fig. 3e), suggesting that these two compounds might be detected also by other ORs modulating the input of LmOR70a-expressing neurons to the locust brain and thereby changing the behavioral response. After testing 50 ORs and more than 200 relevant odors, we thus established LmOR70a as a highly sensitive and specific detector of PAN.

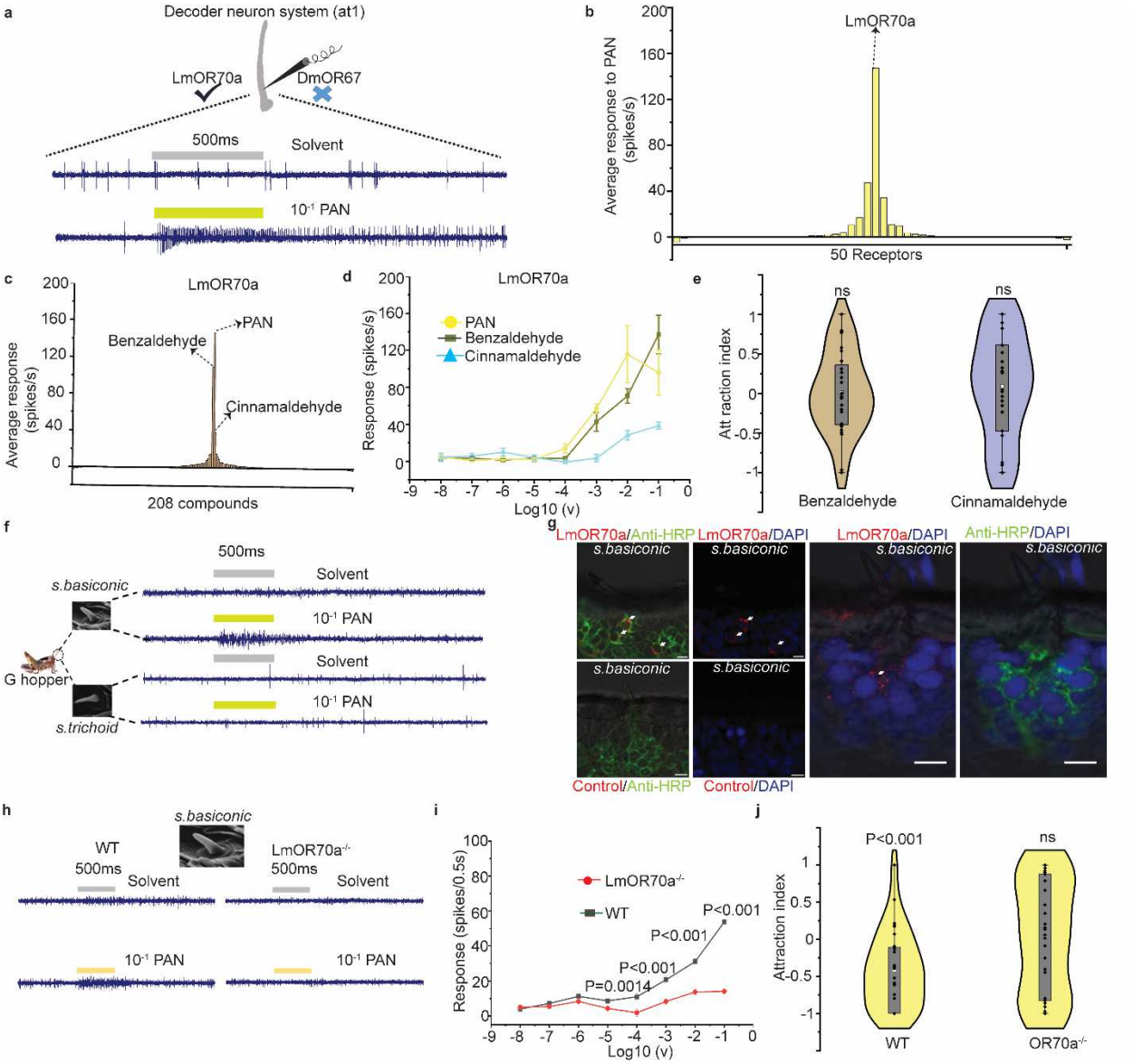

Figure 3 LmOR 70 detects PAN and mediates aversiveness in locusts. a, Representative SSR traces of LmOR70a-expressing *Drosophila* neurons to 10^{-1} PAN. **b**, Response to PAN from *Drosophila* decoder neurons expressing 50 different LmORs**.** n = 5-6 recordings for each receptor**. c,** Response of *Drosophila* decoder neurons expressing LmOR70a to 208 odorants, n = 6 recordings for each odorant. **d,** Dose response curves of *Drosophila* decoder neurons expressing LmOR70a to the three most active odor molecules, n = 6 recordings for each compound. **e,** Attraction index of gregarious nymphs to benzaldehyde and cinnamaldehyde, n = 27-28 biological replicates for each odor. **f,** Representative spike traces from SSR recordings from locust basiconic and trichoid sensilla in response to 10^{-1} PAN**. g,** *In situ* hybridization revealing that LmOR70a-expressing olfactory sensory neurons (OSNs) are housed in basiconic sensilla, white arrows in all images indicate LmOR70a positive cells, white scale bar represent 10 µm**. h,** SSR responses recorded from OSNs present in basiconic sensilla in wild-type and LmOr70a$^{-/-}$ locusts, n = 35 (wild-type) and 27 (LmOr70a$^{-/-}$) sensilla, respectively. **i,** SSR dose response curves in wildtype and LmOR70a$^{-/-}$ locusts. **j.** Attraction index to PAN in wild-type and LmOr70a$^{-/-}$ locusts. n = 29-30 replicates for each assay. Data in **e** and **i** are plotted as violin-box plots (mean: white dot, interquartile range: grey box, black line: upper and lower adjacent value). The attraction index in **e** and **j** was calculated

as (O-C)/(O+C), where O is the total retention time of the locust on the odor side, C the total retention time on the control side. P values in **e**, **h** and **j** were determined by two tailed unpaired t-test.

Olfactory sensory neurons bearing different types of ORs are located in olfactory hairs, sensilla, on the locust antenna. To determine in which type of sensilla the neurons detecting PAN were located, we carried out SSR experiments from two types of locust sensilla (basiconic and trichoid), while stimulating with PAN. These are the sensillum types housing olfactory sensory neurons expressing the olfactory co-receptor *Orco* known to be present in all OR-expressing neurons[25,26]. We found a strong response from neurons present in basiconic sensilla, while no response was observed from neurons in trichoid sensilla (Fig. 3f). We then asked whether it was LmOR70a-expressing neurons that were housed in basiconic sensilla and contributed to the response to PAN. First, we performed RNA *in situ* hybridization to determine the distribution of neurons expressing LmOR70a in the locust nymph antenna and found that LmOR70a-expressing neurons were indeed housed exclusively in basiconic sensilla (Fig. 3g). Next, we generated a loss-of-function allele of the LmOR70a gene using CRISPR-Cas9 genome editing. An sgRNA, targeting the first exon of the LmOR70a gene, introduced an 8-bp deletion, resulting in a truncated LmOR70a protein (Supplementary Fig. 3a). When we then recorded the SSR response of neurons housed in basiconic sensilla to PAN, we found that in the LmOR70a$^{-/-}$ line the response was close to abolished in comparison with that of wild-type nymphs (Fig. 3h,i). Moreover, we tested gregarious nymphs of the LmOR70a$^{-/-}$ line in the dual-choice olfactometer for their behavioral response to PAN and found that the homozygous mutant nymphs had completely lost their aversion to PAN as compared to wild-type insects (Fig. 3j). In sum, we conclude that olfactory sensory neurons expressing LmOR70a are present in basiconic sensilla and are responsible for the negative behavioral response to PAN in migratory locusts. This pathway is thus necessary for the aversion to occur.

Phenylacetonitrile suppresses cannibalism

When observing locust behavior, we found that cannibalism could be categorized into two forms, where one entails the consumption of an already dead body, while the other comprises more active predation on juvenile conspecifics, while still alive. Given this fact, we first wanted to know whether the presence of PAN influences cannibalistic feeding on a dead nymph body. We chose the corpses of fifth-instar gregarious nymphs as potential food sources and noted that these corpses completely stopped releasing PAN 24 h after death (Supplementary Fig. 2). We then compared the consumption of natural corpses, only scented with paraffin oil, with those perfumed with PAN in a group of 20 starved, healthy, fifth-instar gregarious nymphs. The results showed that the tested insects refused to approach and feed on PAN-scented corpses, while happily feasting on the oil-treated ones (Fig. 4b). The feeding nymphs consumed 5 times lower amount weight-wise of the PAN perfumed corpses in comparison with the oil-treated ones. Next, we repeated these experiments but replaced the wild-type feeding nymphs with LmOR70a$^{-/-}$ insects. The LmOR70a$^{-/-}$ nymphs did not show any biting or feeding preference between the corpses loaded with oil or PAN (Fig. 4b). We thus conclude that PAN deters locust nymphs from feeding on dead bodies of conspecifics and that LmOR70a-expressing sensory neurons are responsible for this distinction.

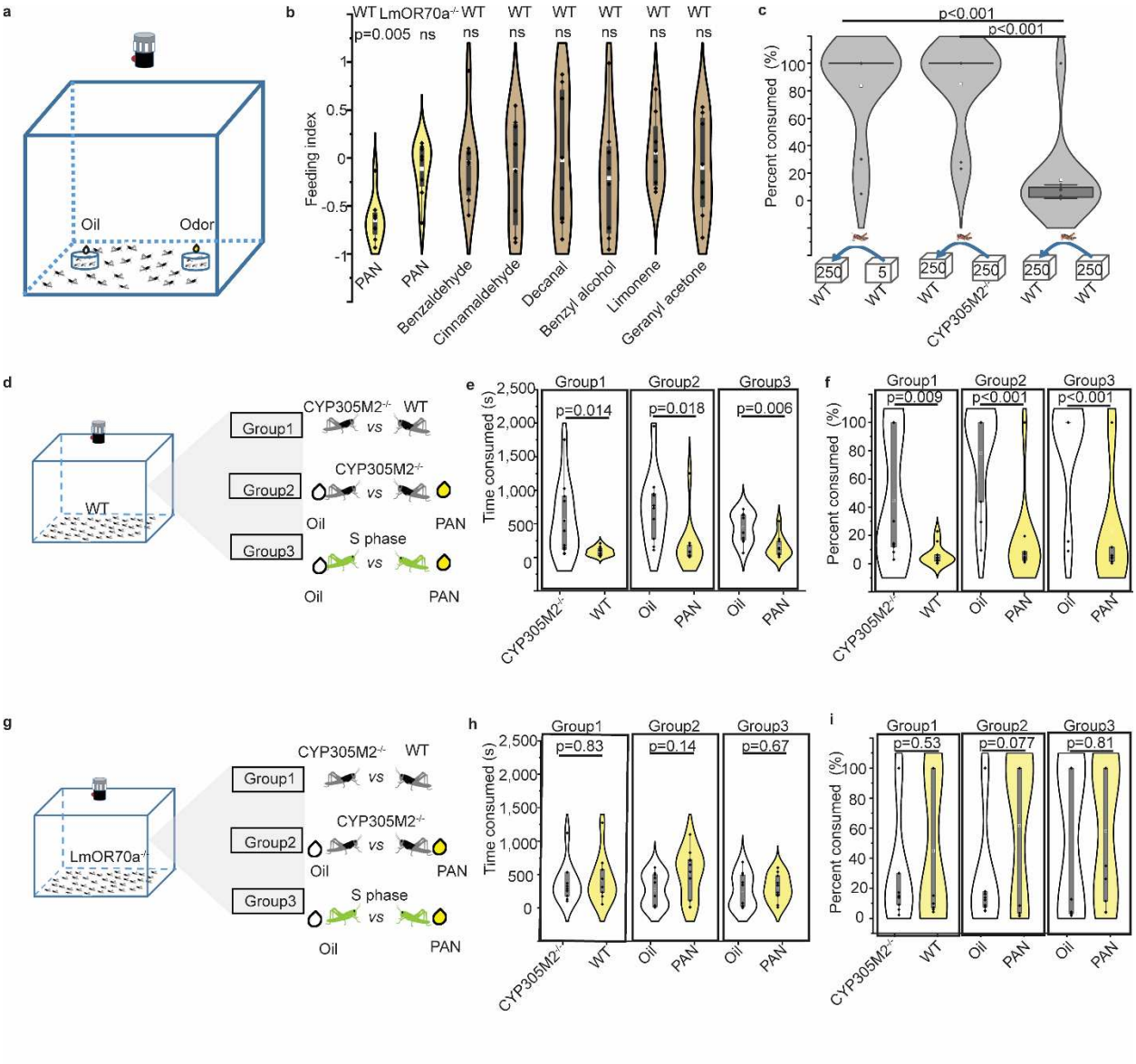

Figure 4 PAN deters locust cannibalism. a, Schematic of the dual-choice predation experiments using nymph corpses. **b,** Choice of starved nymphs between corpses with or without odor, n = 8 biological replicates for each assay. The feeding index was calculated as (O-C)/(O+C), where O is the consumed amount of corpses perfumed with odor, C is the consumed amount of corpses perfumed with paraffin oil as control, P values were determined by two-tailed unpaired t-test. **c,** The rate of cannibalism on individual nymphs transferred from low to high density, from high to high density, and on CYP305M2$^{-/-}$ mutants transferred from high to high density n = 10 biological replicates for each assay. **(d, g),** Schematic of dual-choice cannibalism experiments on three pairs of alive, gregarious CYP305M2$^{-/-}$ or solitary nymphs with or without PAN introduced to either 50 WT **(d)** or 50 LmOR70a$^{-/-}$ **(g)** starved nymphs. **(e,f),** Quantification of predation time on (**e**) and percent consumed (**f**) of prey nymphs exhibited by WT nymphs. **(h, i),** Quantification of predation time on (**h**) and percent consumed (**i**) of prey nymphs exhibited by LmOR70a$^{-/-}$ nymphs. N = 10-11 replicates for each assay in **e, f, h and i,** P values were determined by two-tailed paired t-test. All data were plotted as violin-box plots (mean: white dot, interquartile range: grey box, black line: upper and lower adjacent value).

To test the unique anti-cannibalistic role of PAN, we tested another four locust volatiles previously reported as aversive to gregarious nymphs[20] for their effects in preventing feeding on conspecific corpses. None of these compounds reduced feeding on dead nymph bodies, suggesting that PAN might be the only compound that specifically suppresses locust cannibalism (Fig. 4b). Neither did the two compounds that activated LmOR70a-expressing neurons to a lower extent than PAN, reduce feeding on dead nymph bodies, pointing again at additional ORs involved in the detection of these odors and modulating their significance.

Next, we wanted to know whether the emission of PAN by alive gregarious locust nymphs protects them from conspecific predation. The perfect test would be to have an alive gregarious locust devoid of PAN but otherwise intact in every way. Here, we could benefit from earlier investigations showing that one member of the cytochrome P450 gene family named CYP305M2 is crucial for PAN biosynthesis in *L. migratoria*[23]. Built on these insights, we generated a loss-of-function allele of CYP305M2 using CRISPR-Cas9 genome editing. An sgRNA targeting the second exon of the CYP305M2 gene introduced a 26-bp deletion, resulting in a truncated CYP305M2 protein (Supplementary Fig. 3b). As shown earlier, PAN biosynthesis is largely determined by population density. Therefore, in order to eliminate density effects, we raised CYP305M2$^{-/-}$ locusts and WT locusts under identical conditions. Chemical analysis of the emissions of CYP305M2$^{-/-}$ and WT locusts showed that the mutant locusts were totally unable to produce PAN (Supplementary Fig. 2), while the release levels of other major compounds, body color as well as mobility characteristics remained unchanged as compared to WT insect. Having access to non-PAN-producing nymphs, we now performed a series of dual-choice predation experiments by introducing a pair of nymphs, one with and one without PAN, into an observation chamber containing 50 starved gregarious nymphs (Fig. 4d). First, we examined the predation choice between CYP305M2$^{-/-}$ and WT nymphs and found that CYP305M2$^{-/-}$ nymphs were frequently attacked and preferentially consumed over WT nymphs. Both the time bitten and the percentage of CYP305M2$^{-/-}$ nymphs consumed were five- and sevenfold higher than that of WT insects, respectively (Fig 4e, f). Second, we used CYP305M2$^{-/-}$ locusts scented with PAN or paraffin oil to test predation choice. CYP305M2$^{-/-}$ locusts were each perfumed with 300 ng PAN, an amount similar to the biosynthesis level in WT nymphs[20]. In predation experiments as described above, oil-treated CYP305M2$^{-/-}$ locusts were in most cases completely consumed by starved conspecifics, whereas PAN-treated CYP305M2$^{-/-}$ nymphs were seldomly injured or consumed (Fig 4e, f).

To further validate these results, we replaced the CYP305M2$^{-/-}$ nymphs with WT solitary ones, as solitary nymphs are also unable to produce PAN. We found that starved nymphs also in this comparison refused to attack and feed on PAN-treated individuals, while happily feeding on the non-scented, solitary locusts (Fig. 4e, f). Taking advantage of our earlier results, showing that nymphs from low-density cages produced no PAN, while those bred in high density produced high levels, we performed one final test. Nymphs transferred from low density to high were directly and completely cannibalized, while those transferred from high to high were left untouched (Fig. 4c). To determine whether LmOR70a was required also for these PAN-dependent interactions, we used LmOR70a$^{-/-}$ locusts as starved predators and repeated the choice experiments including both CYP305M2$^{-/-}$ and solitary locusts (Fig 4g). We found that LmOR70a$^{-/-}$ locusts displayed no

predation preference for prey individuals with or without PAN (Fig. 4h, i). These results together confirm that PAN produced by gregarious nymphs deters intraspecific predation and that the detection of PAN by LmOR70a-expressing neurons governs the suppression of cannibalism among gregarious locust nymphs.

Locusts are infamous for their habit of forming giant swarms, devouring everything in their way. Such enormous densities of individuals raise questions regarding which interactions occur within the swarm. In our present study we reveal an interesting impact of the compound phenylacetonitrile (PAN). The production of PAN in different locust species was reported decades ago, and several hypotheses have been postulated regarding its function. In the desert locust, *Schistocerca gregaria*, it has been proposed as both an aggregation pheromone and as a male courtship inhibitor[24,27,28]. In another exhaustive study, Wei et al described that in *L. migratoria*, PAN acts as an olfactory aposematic signal in defense against general predators, as e.g., birds[23]. However, under crowded conditions, locusts not only place themselves at high risk of exposure to predators, but even more so face the risk of being eaten by conspecifics. Cannibalism has even been hypothesized as being one of the driving forces behind swarm formation[17]. Here, we report for the first time that PAN also has a clear anti-cannibalistic function in swarms of locust nymphs. Actually, taking all results under consideration, we suggest that this might be the original function of the compound, as a more widespread and less density-dependent occurrence might be expected in a purely anti-predatory compound. In fact, only gregarious nymphs of the migratory locust release high amounts of PAN, whereas solitary individuals do not. This dichotomy further emphasizes that the production of PAN comes with specific costs and has a specific function that seems to be restricted to crowded conditions.

Conclusions and outlook

Phenylacetonitrile plays an important role in protecting migratory locust nymphs against cannibalism. Our results strongly imply that the olfactory receptor LmOR70a is crucial for the anti-cannibalistic effect of PAN to occur. When the receptor is inactivated, the distinction between nymphs smelling of PAN, and those that do not, is abolished, as is the major electrophysiological response. The small remaining response can very likely be attributed to PAN interacting with other LmORs of different behavioral significance. We can thus maintain that LmOR70a is necessary for the protective effect of PAN to occur. Still, it remains to be shown if it is sufficient, a level where the non-model status of the locust has not allowed us to reach. Nevertheless, our study reveals how amenable the locust is for genetic manipulations built on CRISPR-cas9 technology.

Future investigations should be aimed at elucidating more behaviorally active odors and their neural substrate, but also to attempt CRISPR-cas9 based insertions of, for instance, genetically encoded, calcium sensitive markers. This would allow us to take the next step in the chain of coding and start to unravel the unorthodox neural architecture of the locust primary olfactory center, the antennal lobe, and its more than 1000 olfactory glomeruli.

Methods

Insects

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 single cylindrical boxes (10.5 cm high × 8 cm 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 and solitary locusts.

Chemical analysis

Volatile compounds were collected by solid phase microextraction (SPME) at 30 °C. To compare volatile profiles of gregarious and solitary locusts in nymph (fifth instar) and adult stages, a small number of locusts (six locusts for nymphs or two individuals for adults) were confined into a 100 ml glass bottle. A SPME fiber (PDMS/DVB 65 μm) was then introduced into the bottle through the cap and a PEAK guide piece and collected odor for 10 h. To determine the level of PAN emissions by fifth-instar nymphs at densities of 1–300 individuals, gregarious nymphs were cultured in cages (25 cm × 25 cm × 15 cm), where after one individual was taken from each of these cages and placed into a 20 ml glass bottle for SPME fiber odor collection during 2 h. To determine the level of PAN emissions by WT and CYP305M2$^{-/-}$ animals, we raised WT and CYP305M2$^{-/-}$ nymphs under the same population densities. After reaching the fifth-instar stage, we placed single individuals into a 20 ml glass bottle and used the SPME fiber to collect odors for 2 h. To determine the level of PAN emissions from dead and alive nymphs, we placed five alive or dead nymphs into a 100 ml glass bottle and used the SPME fiber to collect odor for 10 h. 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}, whereafter 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. All of the synthetic odorants that were tested and confirmed were purchased from Sigma–Aldrich.

Single-sensillum recordings (SSR)

To test the function of individual locust olfactory receptors in the *Drosophila* empty neuron system, we performed SSR recordings from fly T1 sensilla according to standard procedures[29,30]. Briefly, adult flies were immobilized in pipette tips, and the third antennal segment was placed in a stable position onto a glass coverslip. Sensillum types were localized under a microscope (BX51WI; Olympus) at ×100 magnification and identified by diagnostic odors listed in table S1. The extracellular signals originating from the OSNs were measured by inserting a tungsten 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) connected to a computer (Syntech). Action potentials were extracted using AutoSpike software, version 3.7 (Syntech). Synthetic compounds were diluted in dichloromethane, hexane or paraffin oil (Sigma-Aldrich, Steinheim, Germany). Before each experiment, 10 μl of 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 600 ml/min through a 8 mm inner diameter stainless steel tube ending 1 cm 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, analyzed in Excel, and illustrated using Adobe Illustrator CS (Adobe systems, San Jose, CA). In addition, we conducted SSRs from locust basiconic and trichoid sensilla with functional ligands identified in the *Drosophila* empty neuron recording. Each locust was confined in a plastic tube 1 ml in diameter and its antennae were fixed with dental wax. The recording and analysis process underwent the same treatment as in the fly.

Cloning of *Locusta migratoria* OR70a

To clone the full-length coding sequence of LmOR70a, we collected 50 antennae from fifth-instar nymphs and 7-day-old adults in both g and s phase. Total RNA of each sample was extracted using an RNA isolation kit (QIAGEN) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was generated from 5 μg of total RNA using oligo dT-primed cDNA synthesis with Superscript III Reverse Transcriptase (Invitrogen) for the generation of templates for subsequent PCR reactions. PCR was performed with Myfi Polymerases (Bioline) (primers listed in table S2). PCR amplification products were separated on a 1.0% agarose gel, 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 LmOR70a and compared to verify polymorphisms as such rather than PCR errors. The sequence of LmOR70a is shown in S1

Transgenic expression of LmORs in *D. melanogaster* at1 neurons

The entire coding region of LmOR70a was sub-cloned into the empty neuron vector PUASt.attb (a gift from J. Bischof) using a combination of restriction enzymes kpnI/XbaI (New England Biolabs). Homozygous UAS-LmOR70a lines (with transgene insertions into chromosome II) were generated at Bestgene (https://www.thebestgene.com). An Or67d-GAL4 stock (provided by B. J. Dickson)[31] was individually crossed to the transgenic UAS-LmOR70a flies, and homozygous lines expressing LmOR70a in the decoder at1 neuron of *D. melanogaster* were established. The UAS-OR70a transgenic line was confirmed by sequencing of genomic DNA prepared from the final stocks.

Behavioral assays

Cannibalism behavior experiments among nymphs of different instars were conducted as shown in figure S1. 20 gregarious nymphs (1^{st}-5^{th} instar) were placed in small boxes (7 cm × 4 cm × 10 cm) in a well-ventilated room. After 48 h, the number of lost nymphs in each box was established. Cannibalism behavior experiments at different densities were conducted as shown in figure 1B. Gregarious nymphs, starved for 24 h, were placed in cages (25 cm × 25 cm × 15 cm) with 5 to 300

fifth-instar nymphs per cage in a well-ventilated room. After 18 h, the number of lost nymphs in each cage were counted.

Dual-choice olfactometer experiments were conducted as shown in Figure 2C. We used a vertical airflow olfactometer, similar to the architecture described in a previous study[23]. Two plastic containers (28 cm × 28 cm × 18 cm) with 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 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 10-15 individuals, the positions of odor and control were reversed to prevent position effects. 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 locust 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).

Dual-choice predation experiments were also conducted in the vertical airflow olfactometer. To test predation choice on locust corpses, we first selected fifth-instar gregarious nymphs to serve as dead prey. The nymphs were frozen for 3 h at -20°C and then placed in a ventilate hood for 24 h until all traces of PAN had vanished. Using these corpses, we offered a series of paired meals, each of which comprised five corpses perfumed with 10^{-4} test odor or with the control, paraffin oil, to 20 starved gregarious fifth-instar nymphs deprived of food for 24 h before testing. Before the assays, each corpse was weighed to determine initial biomass. We then recorded the feeding choice between the pair of meals for 30 min. After the assay, we weighed the remaining biomass and calculated the gross consumed amount of test and control.

To test predation choice on living locusts, we offered a series of paired, living, gregarious fourth-instar nymphs perfumed with PAN or paraffin oil to 50 gregarious fifth-instar nymphs that had been deprived of food for 24 h before testing. Before the assays, each pair of prey nymphs were labeled with two different color tapes and weighed to determine initial biomass. We then recorded cannibalistic behavior for 1 h. After the assay, we weighed the remainder of each prey nymph. To eliminate the influence of color on predation choice, we reversed the color tapes identifying control and test prey after each assay. To quantify behavior, we calculated the biting time (the total time of each prey chewed on by starved conspecifics) and percent consumed (the percentage of the total biomass of each prey consumed). To further investigate the influence of PAN production on cannibalism, we performed the same test as described above, but compared WT prey nymphs with CYP305M2$^{-/-}$ (non-PAN-producing) locusts. In a final comparison, we assessed whether

gregarious locusts preferentially selected and preyed on nymphs bred in low-density cages (no PAN) as compared to those coming from high-density conditions (high PAN). We thus transferred single fourth-instar nymphs either from a density of 5 nymphs per cage or of 250 nymphs per cage, into a 250 fifth-instar nymph cage, and then observed cannibalistic behavior for 1 h. For both the experiments we measured the weight of the introduced nymphs after the observation time had passed.

CRISPR-Cas9–based genome editing

The establishment of LmOR70a and CYP305M2 mutant locusts by CRISPR–Cas9 was performed as previously described[22,26]. 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 guide RNA were mixed to final concentrations of 400 and 150 ng μl^{-1}, respectively (27.2 nl), and were injected in the embryos using a 2010 nanoliter injector (World Precision Instruments) with a glass micropipette tip under an anatomical lens. Then, the embryos were placed in a 30 °C incubator for approximately 12 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 wheat food. To observe that mutants had been generated successfully, we collected part 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). We then used a 2 μl template to amplify the targeted fragments and sequenced these. To further investigate the exact mutation models, we performed TA cloning and Sanger sequencing for PCR amplification of all mutated locust individuals to confirm mutation type. Selected lines of locusts were subsequently bred to generate a homozygous mutant line. Mutations in G0 locusts were evaluated by using PCR-based genotyping. G0 mutants were crossed with the wild-type locusts to obtain G1 heterozygotes. G1 heterozygotes with one DNA strand containing the same mutation type were in-crossed to obtain homozygote G2 locusts. Finally, LmOR70a and CYP305M2 mutant lines with 8-bp deletions and 26-bp deletions, respectively, were successfully established.

Generation of riboprobes for *in situ* hybridization

The digoxigenin-labelled antisense and sense riboprobes for LmOR70a were synthesized using the pCR II Vector (Thermo Fisher Scientific, Waltham, Massachusetts, USA) containing the corresponding sequence and the T7/Sp6 RNA transcription system (Roche Diagnostics, Mannheim, Germany) as recommended by the manufacturer.

In situ hybridization experiments

With few modifications, *in situ* hybridization experiments were performed as described in detail previously[32]. For tissue preparation, antennae of 5 instar larval *L. migratoria* were surgically removed and embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Alphen aan den Rijn, The Netherlands). Longitudinal sections (12 μm) through the antennae were prepared with a Cryostar NX50 cryostat (Thermo Fisher Scientific, Waltham, Massachusetts, USA) at - 20 °C. Sections were thaw-mounted on Super Frost Plus slides (Menzel, Braunschweig, Germany) and immediately used for *in situ* hybridization experiments. Briefly, sections were transferred into a fixation solution (4% paraformaldehyde in 0.1 M NaHCO3, pH 9.5) for 22 min at 4 °C. Next, sections were washed in PBS (0.85% NaCl, 1.4 mM KH2PO4, 8 mM Na2HPO4, pH 7.4) for 5

min, incubated in 0.2 M HCl for 10 min and washed twice in PBS for 2 min each. Then, sections were incubated for 10 min in acetylation solution (0.25% acetic anhydride freshly added in 0.1 M triethanolamine), washed three times in PBS for 5 min each and incubated in pre-hybridization solution [5xSSC (0.75 M NaCl, 0.075 M sodium citrate, pH 7.0) and 50% formamide] for 10 min. Subsequently, each slide was covered with 130 µl hybridization solution [50% formamide, 25% H2O, 25% Microarray Hybridization Solution Version 2.0 (GE Healthcare, Freiburg, Germany)] containing either the labeled antisense or sense RNA probe. After placing a coverslip on top, slides were incubated in a humid box (50% formamide) at 60 °C overnight. The slides were then washed two times for 30 min each in 0.1x SSC at 60 °C. This was followed by a blocking step in 1% blocking reagent (Roche Diagnostics, Mannheim, Germany) in Tris-buffered Saline (TBS; 100 mM Tris, 150 mM NaCl, pH 7.5) supplemented with 0.3% Triton X-100 for 30 min at RT. Afterwards, 130 µl of anti-digoxigenin alkaline phosphatase-conjugated antibody (Roche Diagnostics) 1:500 diluted in 1% blocking reagent in TBS, 0.3% Triton X-100 was added on each slide. A coverslip was placed on top, and slides were incubated for 1 h at 37 °C. The sections were washed three times for 5 min in TBS, 0.05% Tween-20 and then transferred into 150 mM Tris-HCl solution (pH 8.3) for 5 min. For visualization of the digoxigenin-labelled probes the Vector red alkaline phosphatase substrate kit (Vector Laboratories, Burlingame, CA, USA) was used according to the manufacturer's instructions. Briefly, 50 µL of each Vector red reagent (1, 2, and 3) were diluted in 5 ml of 150 mM Tris-HCl (pH 8.3) to create the substrate solution, which was then applied to each section for 50 min at RT. The sections were washed three times for 5 min in TBS with 0.05% Tween-20. Counterstaining was performed with goat-anti-HRP Alex Fluor 647-conjugated antibody (1:200) (Jackson ImmunoResearch, Ely, Great Britain) and DAPI (1:500) diluted in TBS for 1 h at RT. Finally, the sections were rinsed with H_2O for 5 min and mounted in Mowiol. Sections were analyzed with a confocal LSM 780 laser scanning microscope (Carl Zeiss Microscopy, Jena, Germany). Confocal image Z-stacks were acquired from antennae in the blue, red and green fluorescence channels along with the transmitted light channel. In the figures shown, combinations of the different fluorescence channels were overlayed either with one another or with the transmitted light channel.

Statistics and figure preparation

All statistics were analyzed using SPSS 18.0 and preliminary figures were constructed using Software Origin 2018 (https://www.originlab.com/). Figures were drawn using Adobe Illustrator CS5.

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Author Contributions

H.T.C, M.K., K.L. and B.S.H conceptualized and designed the study. All authors contributed to experimental design, analysis and interpretation of results. H.T.C prepared all figures and collected experimental data. S.C and J.K performed in situ hybridization experiments. H.T.C., M.K and B.S.H wrote the manuscript. All authors commented on the final version of the manuscript

Competing Interests

The authors declare no competing interests.

Materials & Correspondence

Correspondence and material requests should be directed to Prof Dr Bill S Hansson (hansson@ice.mpg.de)

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