Current Biology

CellPress

Report

Cross-generation pheromonal communication drives *Drosophila* oviposition site choice

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https://doi.org/10.1016/j.cub.2023.03.090

SUMMARY

In a heterogeneous and changing environment, oviposition site selection strongly affects the survival and fitness of the offspring.[1,2] Similarly, competition between larvae affects their prospects.[3] However, little is known about the involvement of pheromones in regulating these processes.[4–8] Here, we show that mated females of *Drosophila melanogaster* prefer to lay eggs on substrates containing extracts of conspecific larvae. After analyzing these extracts chemically, we test each compound in an oviposition assay and find that mated females display a dose-dependent preference to lay eggs on substrates spiked with (Z)-9-octadecenoic acid ethyl ester (OE). This egg-laying preference relies on gustatory receptor Gr32a and tarsal sensory neurons expressing this receptor. The concentration of OE also regulates larval place choice in a dose-dependent manner. Physiologically, OE activates female tarsal Gr32a^{+} neurons. In conclusion, our results reveal a cross-generation communication strategy essential for oviposition site selection and regulation of larval density.

RESULTS

Drosophila larval extracts stimulate oviposition in mated females

The presence of conspecific larvae influences the choice of egg-laying site in insects.[9–14] This strategy is thought to accelerate the female's choice of a more nutritious and optimal egg-laying patch. To quantify whether *Drosophila* larvae influence the oviposition preference of mated females via chemical communication, we examined the behavioral effect of larval extracts on oviposition preference using a two-choice oviposition assay (Figures 1A, 1B, and S1A). Although no oviposition preference was observed between hexane and pure agarose (Figure 1C), *CS* females steadily preferred to lay eggs on agarose plates spiked with larval hexane extracts over pure hexane (Figure 1D). Furthermore, hexane washes from internal surfaces of sample vials in which larvae had crawled, stimulated oviposition preference as well, demonstrating that the chemical cues are released and not dependent on larval hemolymph, which also was present in larval extracts (Figures 1E and S1A; see also early report[3]). Moreover, the presence of adults during larval growth had no effect on the oviposition preference elicited by larval extracts (Figure 1F), showing that adult cuticular hydrocarbons (CHCs) have no effect on the egg-laying choice. Finally, we quantified the effect of the duration of larval extraction on site choice and found that an extraction period of 3 h was sufficient to induce oviposition preference (Figure 1G). Taken together, these results indicate that larval extracts contain chemical compounds that consistently elicit oviposition preference in mated females.

Identification of chemical compounds in larval extracts

Larvae deposit multiple chemical cues on the substrate. Two fatty acids have been identified as larval aggregation pheromones.[3] To know which larval compounds underlie the oviposition preference observed here, we analyzed the chemical composition of the larval extract using GC-MS. Multiple compounds were identified in 3- and 24-h larval extracts (Figure 2A). These compounds were also identified in an earlier study,[3] but with four exceptions. First, (Z)-5-tetradecenoic acid (Z5C14OOH) and (Z)-7-tetradecenoic acid (Z7C14OOH) (component 2 and 3 in the present study) were indistinguishable in our analytical setup. Second, we identified compound 9 as ethyl oleate instead of methyl oleate.[3] Third, two heavy alkanes (2-methylpentacosane [2Me-C26] and 2-methylheptacosane [2Me-C28]) and one fatty acid ester (linoleic acid ethyl ester; in short, LE) were identified as additional components in the larval extract (Figure 2B). Fourth, multiple adult pheromones (e.g., Z-9-tricosene [9T], Z-7-pentacosene [7P], and tricosane) were

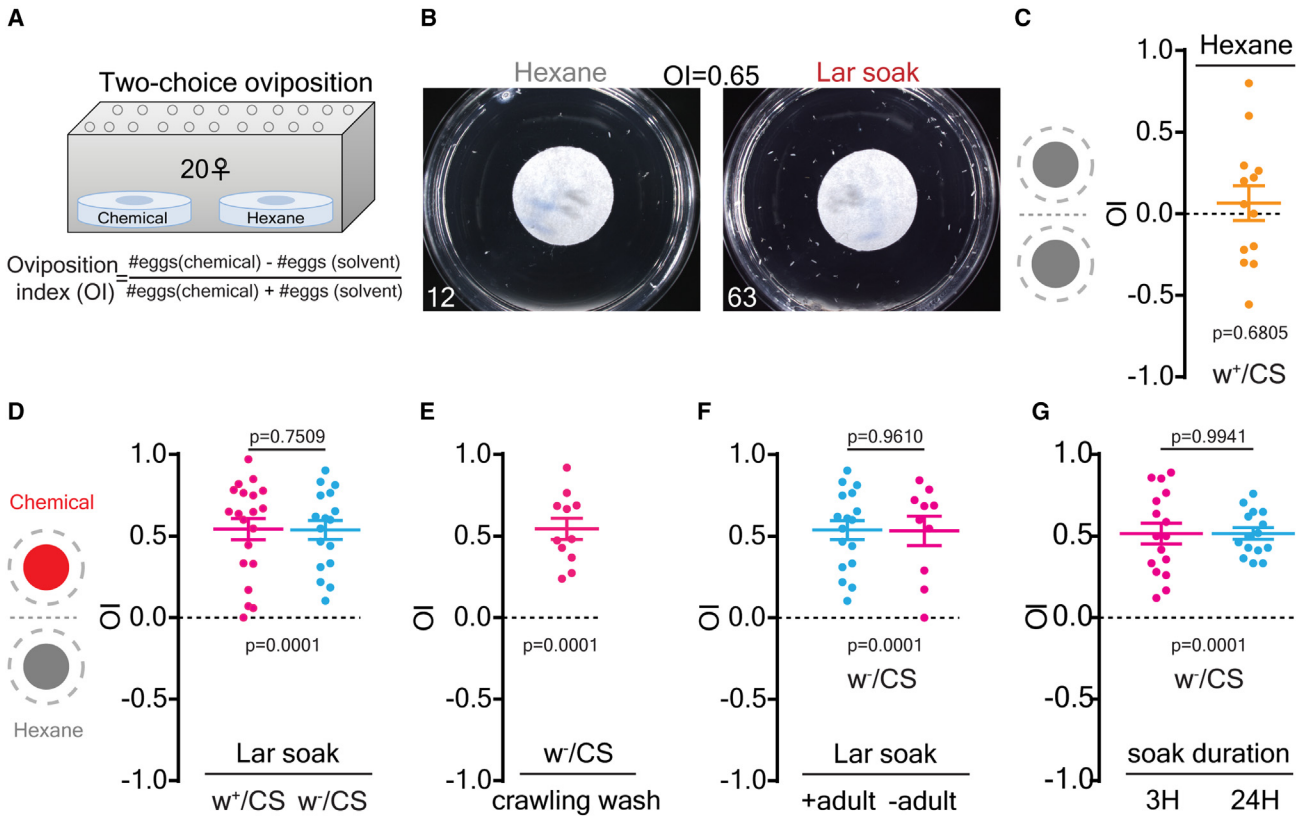


Figure 1. Gravid females prefer to lay eggs on larval extracts

(A) Illustration of the two-choice oviposition preference assay.

(B) Examples of egg-laying plates. Lar soak: 24-h larval soaking hexane extract.

(C) Oviposition preference of *CS* females for hexane and pure agarose. One-sample t test between zero, n = 13.

(D) Oviposition preference of *CS* females for Lar soak. Two-tailed unpaired Student's t test and one-sample t test between zero, n = 17–20.

(E) Oviposition preference of *CS* females for crawling wash. One-sample t test between zero, n = 11.

(F) Oviposition preference of *CS* females for Lar soak, with and without adult residues. Two-tailed unpaired Student's t test and one-sample t test between zero, n = 10–17.

(G) Oviposition preference of *CS* females for 3- or 24-h Lar soak. Two-tailed unpaired Student's t test and one-sample t test between zero, n = 15–16.

Each solid dot represents one independent trial. Data are represented as mean ± SEM.

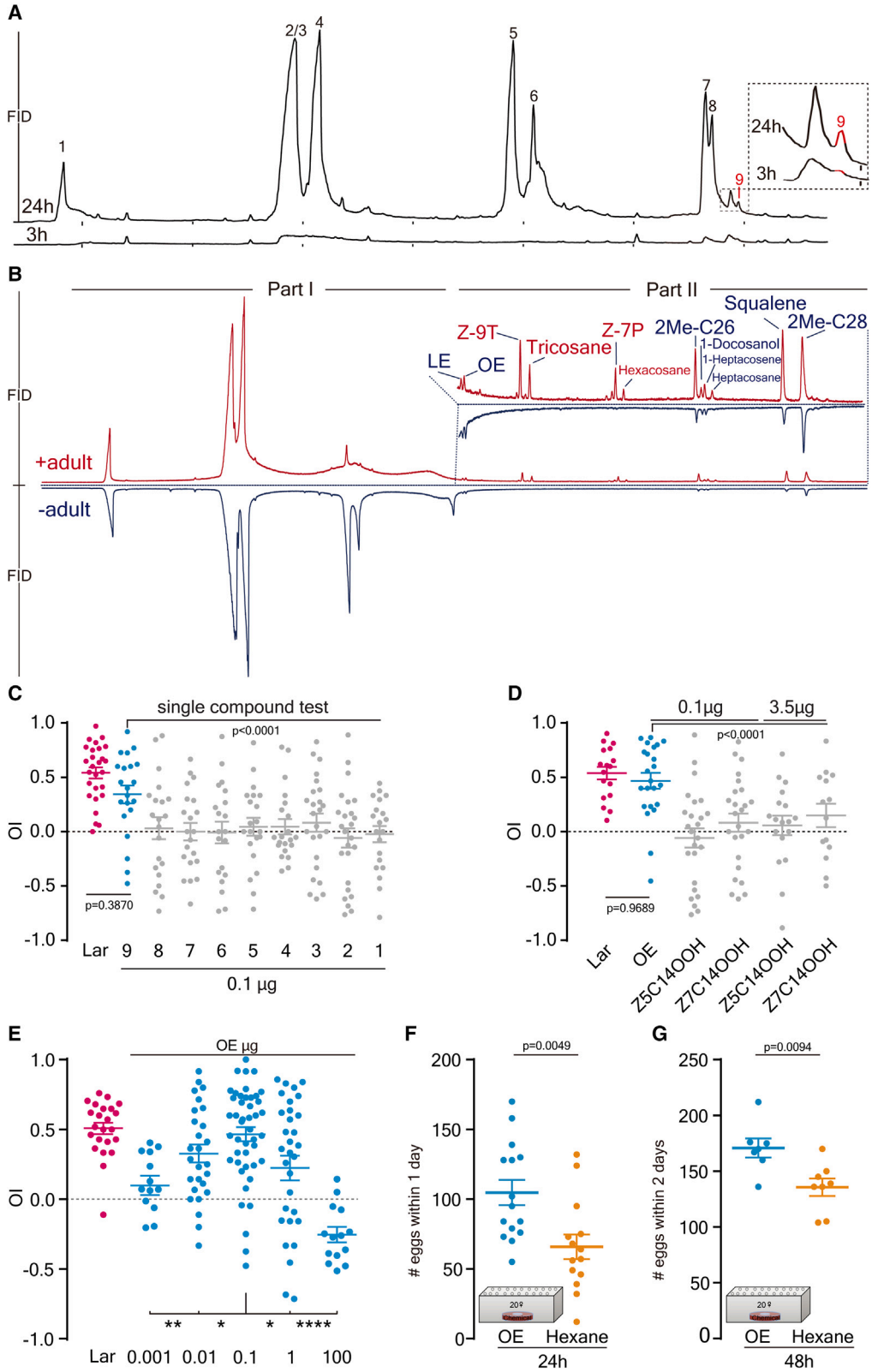
See also Figure S1.

identified in our larval extract. All these adult pheromonal compounds, however, disappeared when adults were removed after egg laying to leave larvae developing free of adults (Figure 2B).

Ethyl oleate acts as an oviposition cue

Next, we measured the oviposition preference for the identified single compounds in the larval extracts. Among the tested synthetic compounds, only (Z)-9-octadecenoic acid ethyl ester (ethyl oleate; in short, OE), at natural concentration of 0.1 μg/plate in the larval extract, elicited oviposition preference comparable to that of the larval extracts (Figure 2C). None of the other single compounds elicited significant oviposition preference at natural concentrations (Figure S1B).

Because larval social pheromones (Z5C14OOH and Z7C14OOH, component 2 and 3 in this study) occupied larger flame ionization detector (FID) peak areas than OE in the GC-MS measurements (Figure 2A), we tested high doses of 3.5 μg/plate, which is equivalent to natural concentrations. Increased dosages of these compounds elicited only low, if any, oviposition preference (Figures 2D and S1C). We also tested synthetic compounds of the common residuals 2Me-C26, 2Me-C28, and LE. None of these compounds triggered oviposition comparable to OE (Figure S1D). In general, *Drosophila* mating and oviposition occur on the same site where sexual pheromones are present. We thus assayed egg-laying responses to most of these adult pheromones. Specifically, we tested 9T, 7T, 7P, 7, 11-HD, 7, 11-ND, and ML, each at a concentration of 0.1 μg/plate. We found no oviposition preference for any of the tested adult pheromones (Figure S1E). Lastly, because mutation of the *desat1* gene (*desat1*$^{1573\text{-}1}$) greatly decreases the production of unsaturated hydrocarbons on the cuticle of flies by disrupting oenocytes,[15,16] we anticipated that the production of OE could be impaired because of the lack of unsaturated (Z)-9-octadecenoic acid. Extracts from *desat1*$^{1573\text{-}1}$ mutant larvae did not elicit any preference for egg laying by *CS* females, however, larval extracts from its genetic rescue line *desat1*$^{1573\text{-}N2}$ did. Supplementation of OE into larval extracts of *desat1*$^{1573\text{-}1}$ mutant also restored the attractivity (Figures S1F–S1H).



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We next hypothesized that the quantity of OE reflects the density of the larval population. To test this hypothesis, we investigated the oviposition preference to doses of OE, ranging from 0.001 to 100 μg/plate. The concentration of 0.1 μg/plate induced a robust response that was indistinguishable from that of the larval extract, whereas lower (0.001 μg/plate) and higher (100 μg/plate) concentrations produced decreased oviposition preferences indistinguishable from zero (Figures 2E and S2A). Also, larval extracts prepared from different numbers of larvae (30, 100, and 400 larvae) contained different OE concentrations (0.0074, 0.02, and 0.1 μg per 50 μL larval extracts) and triggered different oviposition preferences (Figure S2B). Furthermore, different soaking durations yielded different OE concentrations (Figures S2C and S2D). From these results, we conclude that gravid females may use OE concentration to evaluate larval density to make an informed decision regarding where to lay eggs.

In nature, food chemicals interact with pheromones to direct oviposition behavior.[6,7,17,18] We thus tested the oviposition preference to OE when combined with the food cue of grape juice. Females consistently preferred to oviposit on substrates spiked with larval extracts or OE, regardless of the presence of grape juice (Figures S2E and S2F). In addition, females laid more eggs on OE-supplemented plates (0.1 μg/plate) compared with solvent-treated plates after both 24 and 48 h in a single-choice oviposition chamber (Figures 2F and 2G). Together, we conclude that only OE among the identified compounds in the larval extracts acts as an oviposition stimulant.

Gustatory signaling is required for larvae-induced oviposition preference

To understand the chemosensory pathways of OE-induced egg-laying behavior, we started by examining the role of olfaction. First, we used an olfactory trap assay (Figure S3A) to evaluate whether mated females could smell larval extract. Consequently, neither *CS* nor *Orco*^{2}[2,19] females were preferentially attracted to traps containing either larval extracts (Figure S3A) or OE (Figure S3B). Second, we tested whether olfaction was involved in the oviposition preference. Indeed, *Orco*^{2} females showed normal oviposition preference for larval extracts (Figure S3C). These results imply that Orco-dependent olfactory pathways are not required for the female preference for larval extracts or OE. In addition, we demonstrated that OE has no effect on female locomotion or position preference before egg laying (Figures S2G and S2H). Next, we turned our attention to gustation. Peripheral taste inputs are blocked by mutating *poxn*, a transcription factor controlling taste bristle development.[20,21] This null allele (*poxn*^{70}) transforms gustatory bristles on the labellum and legs into mechanosensory bristles, while this phenotypic trait can be partially or fully rescued in two alleles: *Full-1* and *SuperA158* (Figures S3D and S3E). We found that *poxn*^{70} females showed no oviposition preference for larval extracts or OE (Figures 3A and 3B), while in the two rescue lines, *Full-1* and *A158*, the response was fully restored (Figures 3A and 3B). Taken together, gustation is required for the oviposition preference for larval extracts and OE, and this preference might rely on leg tarsal gustatory bristles.

Gr32a mediates the oviposition preference for OE

Gustatory coding of taste substances relies on multiple taste receptor families. To identify the specific molecules required for sensing larval extracts, we examined a repertoire of chemosensory receptor mutants.[5,22–25] Among the tested mutants, *Ir25a*^{1}, *ΔGr32a*, *Δppk23*, and *ΔGr5a/64a*^{2} showed decreased oviposition preference for larval extracts (Figure 3C). As Ir25a is involved in acid-induced egg-laying preference[6] and is widely expressed all over the fly body,[26] we did not include this receptor for further analysis. Subsequent experiments revealed that only *ΔGr32a* females lost oviposition preference specifically for OE (Figures 3D and 3E). It is worth noting that *ΔGr32a* females laid comparable numbers of eggs to heterozygous control females (Figures S3F–S3H). Furthermore, the *ΔGr32a* mutant can be rescued through insertion of the transgene (*ΔGr32a*$^{\#1/2}$).[23] The oviposition preference for larval extracts and OE in *ΔGr32a* females was fully restored to normal levels in *ΔGr32a*$^{\#1/2}$ females (Figures 3F and 3G). Taken together, these results strongly indicate that Gr32a is required to mediate the oviposition preference for OE.

Gr32a modulates larval chemotaxis response to OE

We also wondered whether larval social behavior is regulated by OE. To this end, we measured larval chemotaxis in a two-choice arena over a time course of 5 min (Figure 3H). The positive control (100 mM sucrose) attracted more larvae (Figure 3H, lower left), whereas larvae avoided 1 μg OE (Figure 3H, lower right). Additional assays indicated that larvae were strongly attracted to 0.1 μg OE but repelled by 1 and 10 μg OE (Figure 3I). The taste receptor Gr32a is expressed in the larval terminal organ.[27] Similar to the adult female, *ΔGr32a* mutant larvae attenuated their response intensity at all OE doses, while this mutant

Figure 2. Ethyl oleate (OE) is an egg-laying stimulant in larval extracts

(A) Gas chromatography traces from 3- and 24-h Lar soak. Inset indicates amplified traces revealing component 9. Compound numbers: (1) dodecanoic acid, (2/3) (Z)-5-tetradecenoic acid or (Z)-7-tetradecenoic acid, (4) tetradecanoic acid, (5) (Z)-9-hexadecenoic acid, (6) hexadecanoic acid, (7) (Z, Z)-9,12-octadecadienoic acid, (8) (Z)-9-octadecenoic acid, and (9) Z-9-Octadecenoic acid ethyl ester (ethyl oleate, OE).

(B) Gas chromatography traces from 24-h Lar soak, with and without adult residues. LE; ethyl linoleate; 2Me-C26, 2-methylpentacosane; 2Me-C28, 2-methylheptacosane; 9T, 9-tricosene; 7P, 7-pentacosene.

(C) Oviposition preference for individual larval compounds. Compounds are marked with the same number as in (A), except for compound 2: (Z)-5-tetradecenoic acid, and component 3: (Z)-7-tetradecenoic acid. The concentration used for each compound was 0.1 μg/plate. One-way ANOVA with Dunnett's test, n = 21–26.

(D) Oviposition preference for larval aggregation pheromones. Two doses of 0.1 and 3.5 μg/plate were used. One-way ANOVA with Dunnett's test, n = 14–26.

(E) Oviposition preference of CS females for different doses of OE. One-way ANOVA with Dunnett's test, n = 13–45.

(F and G) Single-choice oviposition assay for OE at 24 h (F) and 48 h (G). 15% grape juice was supplemented in the agarose. Two-tailed unpaired Student's t test, n = 7–15.

Each solid dot represents one independent trial. *p < 0.05, **p < 0.01, ****p < 0.0001. Data are represented as mean ± SEM.

See also Figures S1 and S2 and Table S1.

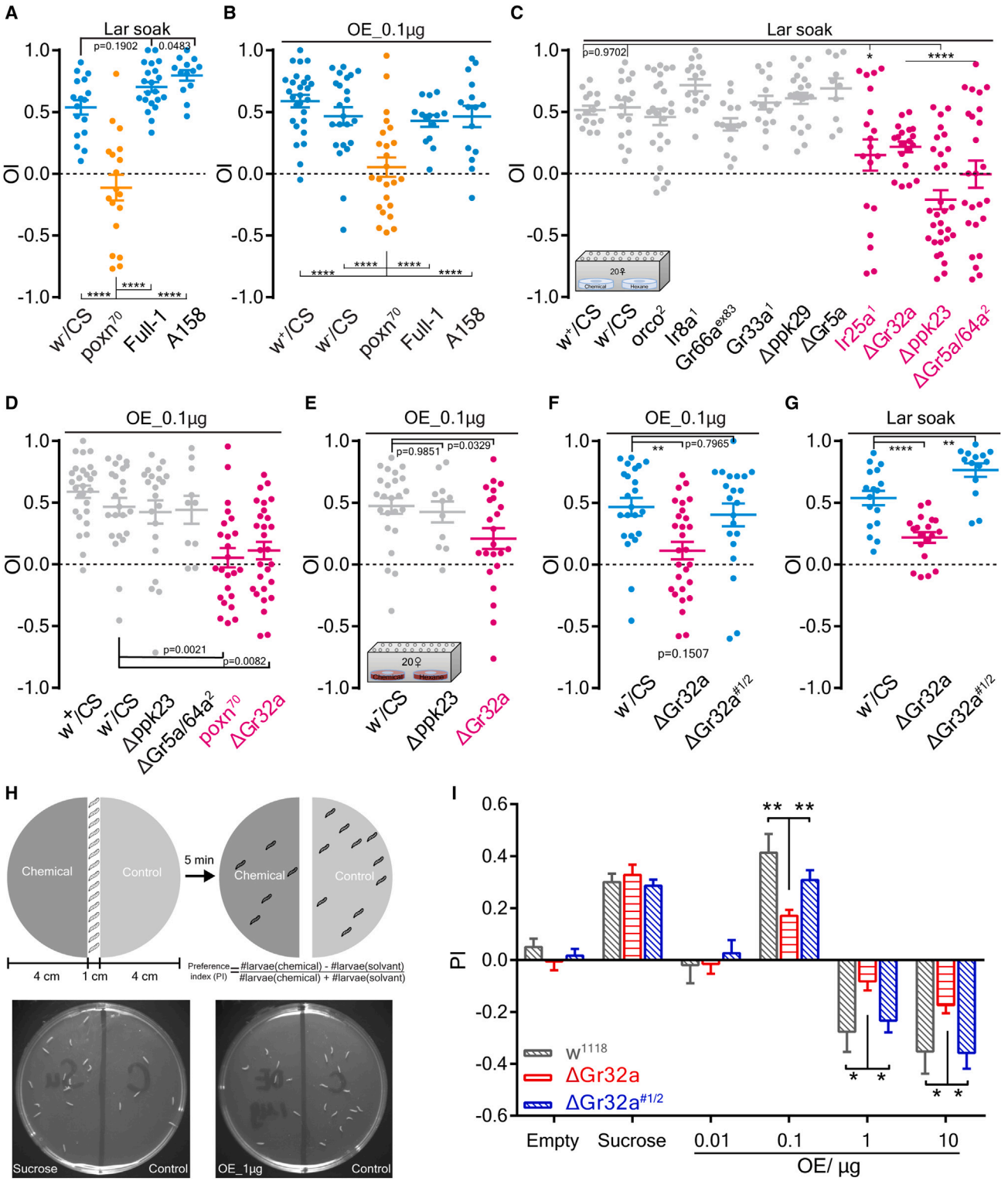

Figure 3. Gustatory receptor Gr32a is required for oviposition preference to OE

(A and B) Oviposition preference of taste bristle mutants for Lar soak (A) and OE (B). One-way ANOVA with Dunnett's test, n = 13–23.

(C) Oviposition preference of indicated genotypes for Lar soak. Pink color indicates mutants with significantly reduced oviposition index (OI) compared with *CS* females. One-way ANOVA with Dunnett's test, n = 16–30.

(D and E) Oviposition preference of *ΔGr32a* females for OE. Pure agarose plates (D) and 15% juice plates (E) are used separately. One-way ANOVA with Dunnett's test, n = 11–28.

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phenotype was restored in $\Delta Gr32a^{\#1/2}$ larvae (Figure 3I). Gr32a thus also plays a role in regulating larval density to potentially optimize the utilization of food patches.

Gr32a$^+$ tarsal neurons are required for physiological and behavioral responses to OE

To directly measure the response of Gr32a-expressing tarsal neurons to OE, we performed *ex vivo* calcium imaging as earlier reported.[28] Expression of the calcium indicator GCaMP6s was driven by Gr32a-LexA[29] and neuronal activity in the last two tarsal segments (T4 and T5) were monitored when stimulated with different taste stimuli. The Gr32a-LexA line marks the same population of Gr32a$^+$ tarsal neurons as indicated by the Gr32a-Gal4 line.[29] First, a bitter compound (1 mM denatonium benzoate) evoked intensely elevated cytoplasmic calcium levels, significantly higher than those of ethanol and sucrose (Figures 4A and 4B). Second, the Gr32a$^+$ neurons also showed dose-dependent responses to OE (Figures 4C and 4D), where 0.1 μg/μL elicited the highest fluorescence change. The solvent ethanol elicited a weak response in itself, while sucrose (diluted in ethanol) stimulation did not increase the response further. In addition, we knocked down expression of Gr32a using RNAi and found the significant suppression of tarsal Gr32a$^+$ neuronal activity to denatonium benzoate and OE (Figure 4E). We thus demonstrate that tarsal Gr32a-positive neurons are physiologically responsive to OE.

To further test the involvement of tarsal Gr32a-positive neurons in the sensing of OE, we first surgically ablated females' tarsi and found that this manipulation abolished the egg-laying preference for OE, suggesting the necessity of tarsal segments of legs for OE detection (Figure S4A). Second, we used a calcium-responsive transcription factor NFAT-based neural-tracing-method CaLexA, designed for labeling active neurons in behaving animals.[30] The CaLexA-GFP signal is expressed and observed in physiologically activated neurons.[30] Using this method, we observed a strong GFP signal in tarsal Gr32a$^+$ neurons after stimulation with OE but not with the solvent (Figures S4B and S4C). We also observed that genetic ablation of Gr32a$^+$ neurons via expression of the pro-apoptotic gene head involution defective (*hid*)[31] (Figures S4D and S4E) led to a significant reduction in the egg-lay preference for OE (Figure S4F). Finally, we demonstrated the sufficiency of Gr32a$^+$ neurons in egg-laying potentiation via artificial activation with heat (Figures S4G and S4H). Together, we demonstrate that tarsal Gr32a-positive neurons are required for physiological and behavioral responses to OE.

DISCUSSION

We have identified a novel larval chemical compound, OE, that *D. melanogaster* larvae release into food substrates. Unlike the earlier reported larval social pheromones Z5C14OOH and Z7C14OOH,[3] OE acts as both oviposition stimulant and larval attractant within a certain dose range. Our data indicate that both oviposition preference and larval attraction to OE rely on the gustatory receptor Gr32a, which is expressed in many taste neurons in both adult flies and larvae. However, larval aggregation elicited by Z5C14OOH and Z7C14OOH requires two members of the degenerin/epithelial sodium channels (DEG/ENaCs) family, ppk23 and ppk29. Larvae, similar to adults, thus rely on different gustatory receptor signaling cascades to guide social interactions via sensing external chemical cues.

In contrast to pheromonal marking strategies in adult flies,[9,32] the existence of chemical cues emitted by larvae in an egg-laying patch potentially indicates its suitability as a breeding substrate to ovipositing adults. Such a cross-generation communication strategy might result in substantial benefits by saving energy and efforts to scan and search for appropriate egg-laying sites.[14] However, the benefits from exploiting previously occupied egg-laying patches may be at least partially offset by the fitness costs of increased competition.[33] As a potential countermeasure toward over-crowding, our findings demonstrate that larvae use the same chemical compound, OE, to regulate larval place choice, given that a high dose of OE is aversive while a low dose is attractive. Thus, OE potentially represents a trade-off mechanism between a suitable substrate and competition.

Gr32a plays a role in recognizing bitter compounds involved in male social aggression, sexual discrimination, and sexual isolation between *Drosophila* sibling species.[22,34–37] For instance, in gustatory inputs via the mouth, Gr32a neurons promote male aggression and form synaptic connections with octopaminergic suboesophageal ganglion neurons,[36] whereas male Gr32a$^+$ bristle neurons are required to inhibit male *D. melanogaster* from mating with females of other *Drosophila* species.[22] This suggests that Gr32a sensory neurons on different appendages mediate distinct behaviors by projections into various downstream intermediate neurons in the central nervous system. Thus, it would be interesting to identify the postsynaptic VNC interneurons that relay OE signaling into the newly identified central oviposition descending neurons' oviDNs[38] or some other central neural circuits involved in egg-laying modulation.

Mated females are assumed to evaluate a potential oviposition patch through wide integration of multiple oviposition stimulants, especially regarding food cues and egg-laying pheromones. Although the addition of grape juice did not alter the oviposition preference for larval compounds, the egg-laying intensity was augmented (Figure S2F). This enhanced egg-laying might be potentiated by cross-talk between neural circuitry relaying information about attractive food cues and larval chemical signals. Such a delicate synergistic strategy has been uncovered in *D. melanogaster*, where virgin females display enhanced receptivity in the presence of a mixture of the complex food odor, vinegar, and the male pheromone cVA. This strategy was suggested to be mediated by electrical synapses between excitatory local interneurons and projection neurons in the antennal lobe.[39] The integrated oviposition decision-making model

(F and G) Oviposition preference of indicated phenotypes for 0.1 μg OE (F) and Lar soak (G). One-way ANOVA with Dunnett's test, n = 17–28.
(H) Top: illustration of larval two-choice chemotaxis assay. Bottom: examples of chemotaxis behavior to 100 mM sucrose and 1 μg OE.
(I) Chemotaxis responses of the indicated genotypes to serial doses of OE. One-way ANOVA with Dunnett's test, n = 7–13.
Each solid dot represents one independent trial. *p < 0.05, **p < 0.01, ****p < 0.0001. Data are represented as mean ± SEM.
See also Figures S2 and S3.

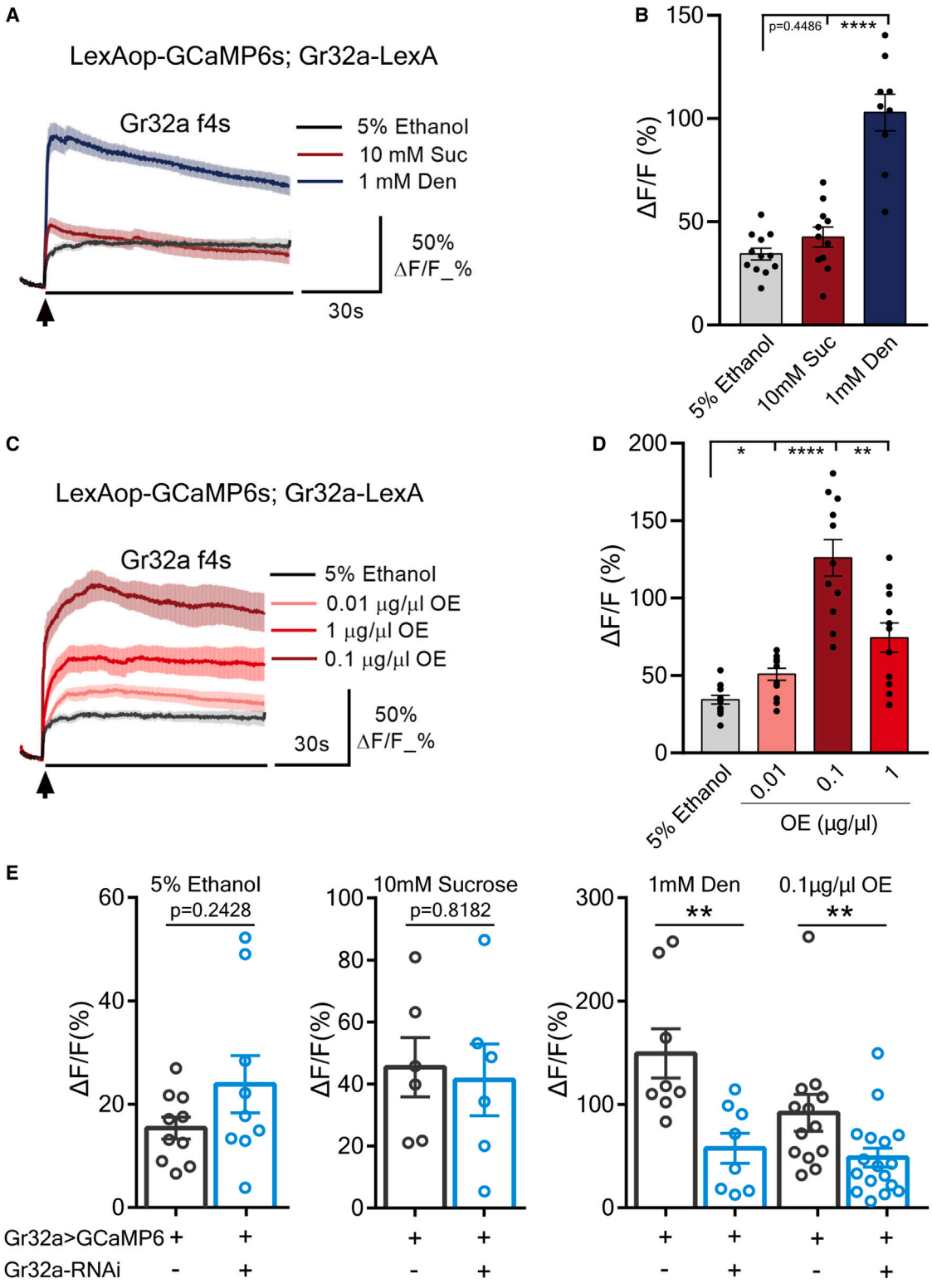

Figure 4. OE elicits physiological response in tarsal GR32a$^+$ neurons

(A) Calcium imaging traces of Gr32a$^+$ tarsal neurons with serial stimulations by the solvent ethanol, 10 mM sucrose, and 1 mM denatonium. Genotype is indicated. Arrowhead indicates the onset of stimulus application.

(B) Response statistics of Gr32a$^+$ tarsal neurons after stimulation with control chemicals. One-way ANOVA with Dunnett's test, n = 9–12 for each stimulation.

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established here may help to further elucidate which central circuits combine and encode the multiple stimulus inputs that determine egg-laying fitness.

STAR★METHODS

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

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS
  - Drosophila stocks
- METHOD DETAILS
  - Preparation of larval extracts
  - Chemical analysis
  - Egg-laying site choice assay
  - Olfactory trap assay
  - Larval two-choice chemotaxis assay
  - Immunohistochemistry and confocal imaging
  - Fly locomotion assay
  - Calcium imaging of tarsal neurons
- QUANTIFICATION AND STATISTICAL ANALYSIS

ACKNOWLEDGMENTS

We thank Dr. Yan Chen at Yunnan University for providing excellent experimental assistance in calcium imaging and Dr. Wei Zhang at Tsinghua University for supplying numerous fly stocks. We also thank Dr. Hubert Amrein, Dr. Werner Boll, Dr. Kristin Scott, Dr. Anupama Dahanukar, and the BDSC for sharing fly stocks. We would like to acknowledge Dr. Veit Grabe, Regina Stieber, and Kerstin Weniger for technical support. We thank Dr. Xiao Xu for assistance with chemical analysis during the revision. This work was supported by the Max Planck Society (Liwei Zhang, E.G.-W., B.S.H., and H.K.M.D.), the grant for innovation project from Shandong Academy of Agricultural Sciences (Long Zhang, CXGC2021B12), and the China Scholarship Council (Liwei Zhang).

AUTHOR CONTRIBUTIONS

Liwei Zhang designed and performed most experiments. H.S. performed experiments in Figures 3H and 3I. E.G.-W., Long Zhang, B.S.H., and H.K.M.D. supervised the project. All authors wrote the manuscript together. All authors discussed and commented on the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: July 15, 2021
Revised: January 9, 2023
Accepted: March 31, 2023
Published: April 24, 2023

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(C) Calcium imaging traces of Gr32a$^+$ tarsal neurons after stimulation with the solvent ethanol and serial dilutions of OE (μg/μL). Genotype is indicated. Arrowhead indicates the onset of stimulus application.
(D) Response statistics of Gr32a$^+$ tarsal neurons to OE doses. One-way ANOVA with Dunnett's test and two-tailed unpaired Student's t test, n = 10–14 for each stimulation.
(E) Ca^{2+} response of tarsal Gr32a$^+$ neurons to 5% ethanol, 10 mM sucrose, 1 mM denatonium, and 0.1 μg/μL OE, before and after Gr32a-RNAi. Genotypes, Gr32a>GCaMP6: UAS-GCaMP6s/+; Gr32a-Gal4/+, and Gr32a>GCaMP6; Gr32a-RNAi: UAS-GCaMP6s/UAS-Gr32a-RNAi; Gr32a-Gal4/+. Two-tailed unpaired Student's t test, n = 6–17 for each column.
Each dot represents one independent trial. * $p < 0.05$, ** $p < 0.01$, and **** $p < 0.0001$.
Data are represented as mean ± SEM.
See also Figure S4.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit polyclonal anti-GFP	Invitrogen	Cat# A-11122; RRID: AB_221569
Goat anti-Rabbit-Alexa 488	Invitrogen	Cat# A-11008; RRID: AB_143165
Chemicals, peptides, and recombinant proteins		
Dodecanoic acid	Tokyo Chemical Industry (TCI)	Cat# L0011-25G
(Z)-5-Tetradecenoic acid	NMR department in Max Planck Institute for Chemical Ecology	N/A
(Z)-7-Tetradecenoic acid	NMR department in Max Planck Institute for Chemical Ecology	N/A
Tetradecanoic acid	Tokyo Chemical Industry (TCI)	Cat# M0476-25G
(Z)-9-Hexadecenoic acid	Tokyo Chemical Industry (TCI)	Cat# H0072-100MG
Hexadecanoic acid	Sigma-Aldrich	Cat# P0500-10G
(Z, Z)-9,12-Octadecadienoic acid	Sigma-Aldrich	Cat# L1376-10MG
(Z)-9-Octadecenoic acid	Sigma-Aldrich	Cat# O1008-1G
(Z)-9-Octadecenoic acid ethyl ester	Sigma-Aldrich	Cat# 55441-250MG
Denatonium Benzoate	Aladdin	Cat# D124654-5g
Experimental models: Organisms/strains		
D.melanogaster. w; Orco^{2}	Bloomington Drosophila Stock Center	Stock# 23130
D.melanogaster. w, Ir8a^{1}	Bloomington Drosophila Stock Center	Stock# 41744
D.melanogaster. w; Gr66a^{ex83}	Bloomington Drosophila Stock Center	Stock# 25027
D.melanogaster. w; Gr33a^{1}	Bloomington Drosophila Stock Center	Stock# 31427
D.melanogaster. w; Ir25a^{1}	Bloomington Drosophila Stock Center	Stock# 41736
D.melanogaster. wCS; poxn^{70}	Dr. Werner Boll (University of Zürich)	Boll and Noll[20]
D.melanogaster. wCS; Full-1	Dr. Werner Boll (University of Zürich)	Boll and Noll[20]
D.melanogaster. wCS; SuperA158	Dr. Werner Boll (University of Zürich)	Boll and Noll[20]
D.melanogaster. w; ΔGr32a (Gr32a^{KO})	Dr. Hubert Amrein (Texas A&M University)	Miyamoto and Amrein[23]
D.melanogaster. w; Gr32a$^{+t5.9\ \#1}$	Dr. Hubert Amrein (Texas A&M University)	Miyamoto and Amrein[23]
D.melanogaster. w; Gr32a$^{+t5.9\ \#2}$	Dr. Hubert Amrein (Texas A&M University)	Miyamoto and Amrein[23]
D.melanogaster. Δppk29	Dr. Kristin Scott (UC, Berkeley)	Thistle et al.[24]
D.melanogaster. Δppk23	Dr. Kristin Scott (UC, Berkeley)	Thistle et al.[24]
D.melanogaster. w; ΔGr5a (Gr5a$^{\Delta EP5}$)	Dr. Anupama Dahanukar (UC, Riverside)	N/A
D.melanogaster. w; ΔGr5a/64a2	Dr. Anupama Dahanukar (UC, Riverside)	N/A
D.melanogaster. w; Gr32a-Gal4	Dr. Yi Rao (Peking University)	Fan et al.[22]
D.melanogaster. wCS; UAS-Stinger-hid	Dr. Yi Rao (Peking University)	Fan et al.[22]
D.melanogaster. wCS; UAS-Stinger	Dr. Yi Rao (Peking University)	Fan et al.[22]
D.melanogaster. w; desat1$^{1573\text{-}N2}$	Dr. Yi Rao (Peking University)	N/A
D.melanogaster. w; desat1$^{1573\text{-}1}$	Dr. Yi Rao (Peking University)	N/A
D.melanogaster. w; UAS-dTrpA1	Dr. Wei Zhang (Tsinghua University)	Liu et al.[29]
D.melanogaster. Canton-S (w^{+}/CS)	Dr. Wei Zhang (Tsinghua University)	Liu et al.[29]
D.melanogaster. w^{1118} (w^{-}/CS)	Dr. Wei Zhang (Tsinghua University)	Liu et al.[29]
D.melanogaster. w; LexAop-GCaMP6s/CyO; Gr32a-LexA/TM6B	Dr. Wei Zhang (Tsinghua University)	Liu et al.[29]
D.melanogaster. w; UAS-GCaMP6s/CyO; Gr32a-Gal4/TM6B	Dr. Wei Zhang (Tsinghua University)	Liu et al.[29]
D.melanogaster. w; UAS-CaLexA (UAS-mLexA-vp16-NFA, LexAOP-CD8-GFP-2A-CA8-GFP/TM6B)	Dr. Yufeng Pan (Southeast University)	N/A
D.melanogaster. yv; UAS-Gr32a-RNAi	TsingHua Fly Center	Stock# TH04853.N

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Liwei Zhang (lwzhang@cau.edu.cn).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All data reported in this paper will be shared by the lead contact upon request. 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.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Drosophila stocks

Flies were kept on standard cornmeal media under 25°C with constant periodic duration (12h:12h). Canton-S (w^+/*CS*) and isogenic w^{1118} (w^-/*CS*) were used as wild type control accordingly in the study. For heat-activation experiments in Figure S4H, all genotypes were raised under 22°C between embryo to first three days of adult stage, and subsequent mating was under 25°C for 3 hours. Females were separated for egg-laying under either 22°C or 31°C. For RNAi experiments in Figure 4E, 7 days-old mated females were used in calcium imaging to allow maximal RNAi efficiency.

METHOD DETAILS

Preparation of larval extracts

Well-cleaned 3rd instar Canton-S larvae were used for chemical extraction. Specifically, larvae were isolated from food using sucrose solution (15%, g/ml), and then washed with water several times to completely remove any visible food debris in a cell culture petri dish (CELLSTAR® cell culture dish, 100/10 MM). To dry the larvae, we transferred these larvae into a filter paper with a fine brush and rolled them until no residual water. 0.7g (roughly 400 larvae) of clean larvae were fully immersed in 1ml hexane in a 2 ml Amber vial (Thermal Fisher) under room temperature for soaking incubation. After the soaking, the upper layer solution was transferred into a new sample vial and stored at -20°C. For preparing larval extracts without adult residues, gravid females were allowed to lay eggs for 4 hours in food vials, and then were removed immediately. All eggs were developed free of adult flies before extracting larval chemicals. The abbreviation "Lar soak" means larval compounds extraction for 24 hours with hexane. For crawling wash, similar number of *CS* larvae were restricted inside 2 ml Amber vial (Thermal Fisher) for one hour free-moving, and subsequently, the larvae were removed and 1ml Hexane was added into the empty vial for half an hour incubation to prepare sufficient crawling wash extraction.

Chemical analysis

Larval chemicals were analyzed using GC-MS (Agilent 6890GC & 5975bMS, Agilent Technologies), which is equipped with a four-arm effluent splitter (Gerstel). The GC was equipped with an HP5-MS column (19091S-413U HP-5MS UI, Agilent Technologies) with helium used as carrier gas (1.1 ml min^{-1} constant flow) and the splitless mode was applied. The inlet temperature was set to 280°C. The temperature of the GC oven was held at 40°C for 3 min and then increased by 20°C min^{-1} to 280°C. The MS transfer-line was held at 280°C, the MS source at 230°C, and the MS quad at 150°C. Mass spectra were taken in EI mode (at 70 eV) in the range from 29 mz^{-1} to 500 mz^{-1}. GC-MS data were processed with the MDS-Chemstation software (Agilent Technologies). Compounds were identified with the NIST 2.0 mass spectra database using the NIST algorithm. Identification was confirmed by comparison with synthetic standards (spectrum and retention time) that were obtained from commercial sources, and *de novo* synthesis with highest available purity. Sources of all standard chemicals used in this study are listed in Table S1.

Egg-laying site choice assay

To maximize the number of eggs in the behavioral assay, all gravid females were prepared from virgin individuals. Briefly, virgin females and naïve males were picked and isolated in food vials over 3 days, and then mixed into one food vial to mate for 3-4 hours. Under CO_2 anesthesia, males were discarded and females were used within 2 days for egg-laying. We designed two-choice and one-choice egg-laying assays supplemented with or without 15% grape juice to test the fly's ability for site choice. Specifically, for the two-choice assay, two small cell culture petri dishes (CELLSTAR® cell culture dish, 35/10 MM) filled with 0.25% agarose solution were dried at RT, and subsequently loaded with 50μl chemicals per plate. After 5 min air-drying, both petri dishes were enclosed into a colorless plastic box containing 20 gravid females for indicated days. Boxes containing $\geq$ 15 eggs were considered for statistical analysis. For the no-choice assay, either solvent or chemicals-loaded dishes were separately tested in a colorless plastic box with 20 gravid females over indicated days. For experiments with grape juice, pure grape juice was diffused into 0.25%

agarose solution with final concentration of 15% (v/v). The whole setup was under 25°C, 70% RH and 12h:12h periodic duration. Oviposition index (OI) was calculated as follows: (#eggs on chemicals treatment – #eggs on solvent control) / (#eggs on chemicals treatment + #eggs on solvent control). The value of OI ranges between +1(complete preference) to -1(complete avoidance). For surgical ablation, tarsal parts of a pair of front legs were surgically removed after courtship and before the egg-laying. For heat activation assays, egg-laying intensity in single-choice OE-less oviposition assay was quantified under restrictive temperature (31°C) and permissive temperature (22°C). The egg-laying plates are consisted of 1% agarose and 100mM sucrose. All tested genotypes were kept at 22°C since fertilized eggs, and the courtship and mating were subjected under 25°C.

Olfactory trap assay

The two-choice trap assay was modified from early report.[40] Briefly, 30 gravid females were subjected to choosing one of two traps without direct contact with chemicals. After 3 days, the number of flies in each trap was counted. Attraction index (AI) was calculated as follows: (#females in chemicals-baited vials – #females in solvent-baited vials) / (#females in chemicals-baited vials + # females in control-baited vials). The resulting index value varies between +1(complete attraction) to -1(complete avoidance).

Larval two-choice chemotaxis assay

Each half of the 2.5% agarose plate (d=90mm) was loaded with either Hexane (control solvent) or a chemical (OE or sucrose) with different dosages. Thirty larvae were tested in each plate and the number of larvae in each half was quantified after 5min. The assay was carried out in the darkness under 25°C. Larval preference index (PI) for tested chemicals was calculated as PI= (# in the chemical side-# in the control side)/30.

Immunohistochemistry and confocal imaging

Females expressing CaLexA-GFP using Gr32a-Gal4 were exposed to either Hexane or 0.1μg OE overnight in food vial. Subsequently, standard immunostaining protocol was applied to visualize the GFP signal on VNC. Primary antibody rabbit anti-GFP (1:500, A11122, Invitrogen) and secondary antibody Alexa 488-goat anti-rabbit (1:200, A11008, Invitrogen) were used to visualize the CaLexA-GFP signal in this study. For imaging Gr32a-Gal4^{+} neurons on leg tarsus in Figures S4B and S4C, intrinsic nls-Stinger signals were captured directly with confocal microscope. Fluorescent imaging was performed on an Olympus FV1000 confocal microscope. Stereotype microscope (Olympus SZX16) equipped with digital camera (Olympus DP74) was used to observe morphology of taste bristles on legs and labellum in Figures S3D and S3E. All images were processed with ImageJ software.

Fly locomotion assay

Female fly locomotion was monitored on a Petri dish filled with 0.25% agarose based on a previous report.[41] In brief, 15 mated or virgin females were immediately transferred into the 2.5% agarose-filled Petri dish, and then their movement was captured for 3 hours by Canon 60D camera. This Petri dish was back-illuminated by white LED arrays. The time-lapse recordings (interval: 10min, each exposure duration: 1s) started after 2min resting from when flies were introduced to the Petri dish; this was considered as time 0. At the onset, most of the flies were awake and moved freely. The fraction of flies on the 0.1μg OE side was calculated out of the total of 15 at each time point.

Calcium imaging of tarsal neurons

Tarsal calcium imaging was carried out as reported before.[28] Briefly, forelegs of 4-7 days-old mated females of indicated genotypes (LexAop-GCaMP6s; Gr32a-LexA) were prepared as follows: after cutting off the femur, the lower part of the tibia and the first three tarsal segments were dipped into silicone oil to prevent leakage and dehydration, and the preparation was placed on double-sided scotch tape stuck on a glass bottom dish. The preparation was fixed by covering it with 1% agarose, so that only the fourth and fifth tarsal segments were exposed, and then was covered with 100μl of water. Tarsal f4s sensilla[42] expressing Gr32a was targeted for calcium imaging. 100μl of test solution (2x of the final concentration of the indicated ligands) was added. Imaging was performed with a Nikon ECLIPSE Ni-U microscope using a 40x water objective, and data acquisition was performed with NIS-Elements software (Nikon). Images were acquired every 200 ms, 10 s before and 90 s after ligand application. Each preparation was tested with 2-4 different compounds/concentrations. Measurements of fluorescence intensity were taken in the cell bodies, while adjacent regions were used as control to determine background auto fluorescence. ΔF was calculated as ΔF = F_cell bodies – F_control (F = Intensity of fluorescence), and ΔF_baseline = Average of 10 frames taken immediately before the application of ligand, so that ΔF/F % = (ΔF-ΔF_baseline)/ ΔF_baseline*100. Maximum ΔF/F % within 90 s after ligand application was used as the representation of ligand response.

QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism 7 was used to graph and statistically analyze data. No sample size estimation was conducted in this study. N represents number of replications/trails. Two-tailed unpaired Student's t-test, one-sample t-test, and one-way ANOVA with Dunnett's test were used for statistical analysis, accordingly. All error bars are SEM. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.