**Supplementary Information**

# Odor-regulated oviposition behavior in an ecological specialist

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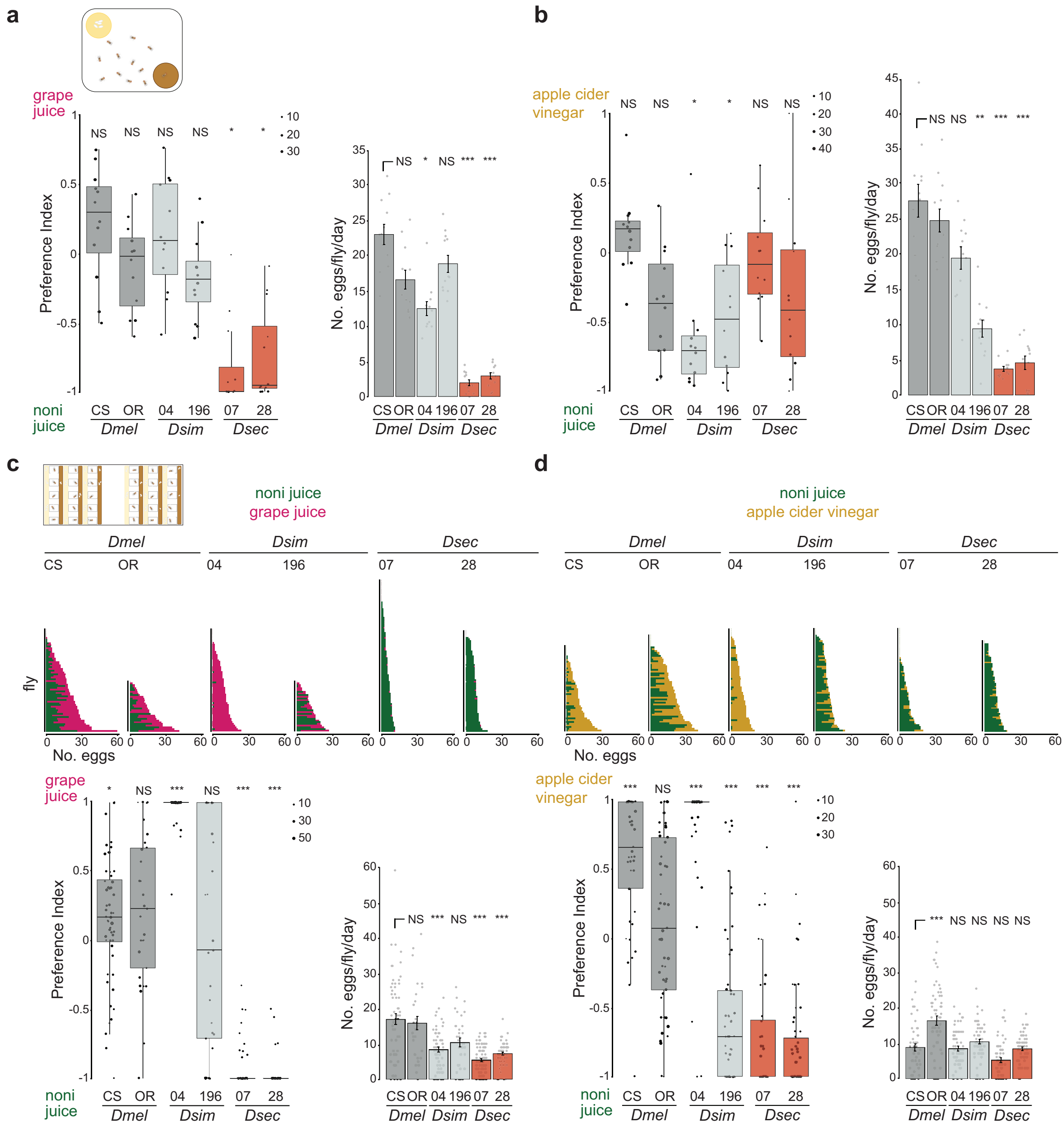


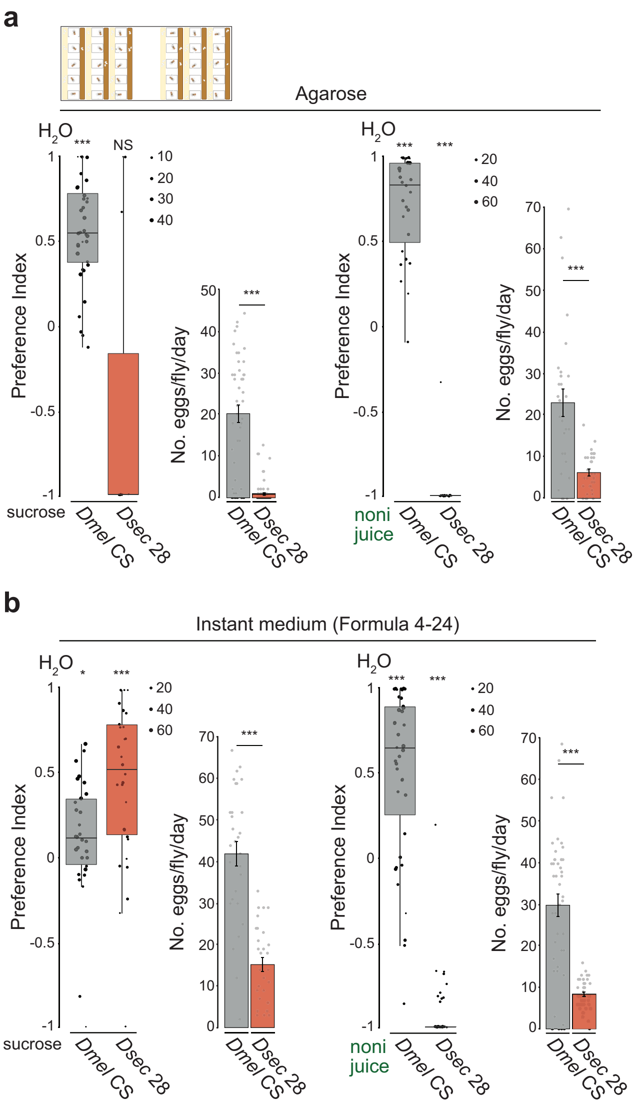

**Supplementary Figure 1. Oviposition preference assays using instant medium-based substrates.**

**a** Group oviposition preference assays for noni juice versus grape juice in instant medium using the same strains as in Fig. 1c. Left: box plots of oviposition preference index. Statistically-significant differences from 0 (no preference) are indicated (as in Fig. 1c); *N* = 12 (representing 4 assays of 40-80 flies, each scored on 3 successive days with fresh oviposition plates each day). Right: bar plots of egg-laying rate. Mean values ± standard error of the mean (SEM) are shown in these and all other bar plots. Statistically-significant differences from the *D. melanogaster* Canton-S (CS) strain are indicated (as in Fig. 1c).

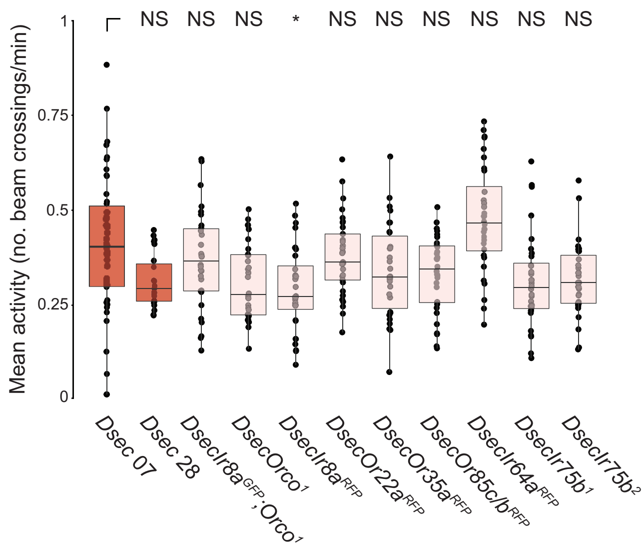
**b** Group oviposition preference assay, as in **a**, for noni juice versus apple cider vinegar; *N* = 12 (representing 4 assays of 40-80 flies, each scored on 3 successive days with fresh oviposition plates each day).

**c** Single-fly oviposition preference assay for noni juice versus grape juice in instant medium for the same strains as in **a**. Top: total number of eggs laid in each substrate by each female. Bottom left: oviposition preference index. Bottom right: egg-laying rate.

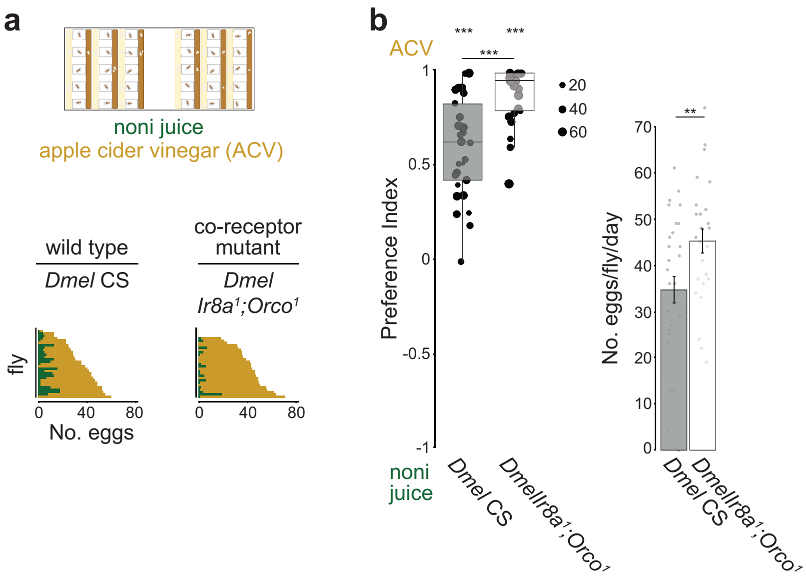
**d** Single-fly oviposition preference assays, as in **c**, for noni juice versus apple cider vinegar; *N* = 43-60 flies across 2 technical replicates.




**Supplementary Figure 2. Establishment of single-fly oviposition assays.**
**a** Single-fly oviposition preference assays testing $H_2O$ versus either 150 mM sucrose or noni juice in agarose. For the box-plots of oviposition preference index, statistically-significant differences from 0 (no preference) are indicated: *** $P < 0.001$; * $P < 0.05$; NS (not significant) $P > 0.05$ (Wilcoxon test with Bonferroni correction for multiple comparisons); $N$ = 30-45 flies across 1-2 technical replicates. Preference of *D. melanogaster* for the plain substrate resembles previous observations[1]. Bar plots of egg-laying rate indicate mean values ± SEM. Statistical comparisons between genotypes are shown: *** $P < 0.001$ (two-sample t-test).
**b** Single-fly oviposition preference assays, as in **a**, using instant medium instead of agarose; $N$ = 30-45 flies across 1-2 technical replicates. The higher egg-laying rate of both species on instant medium compared to agarose substrates might reflect differences in chemical composition, texture and/or moistness. Most experiments were performed using simpler agarose substrates, to minimize interference with responses to complex stimuli (i.e., juices). However, instant medium substrates were used in most single-odor experiments, as egg-laying rate was very low on agarose substrates with single chemical stimuli, especially for *D. sechellia* strains.



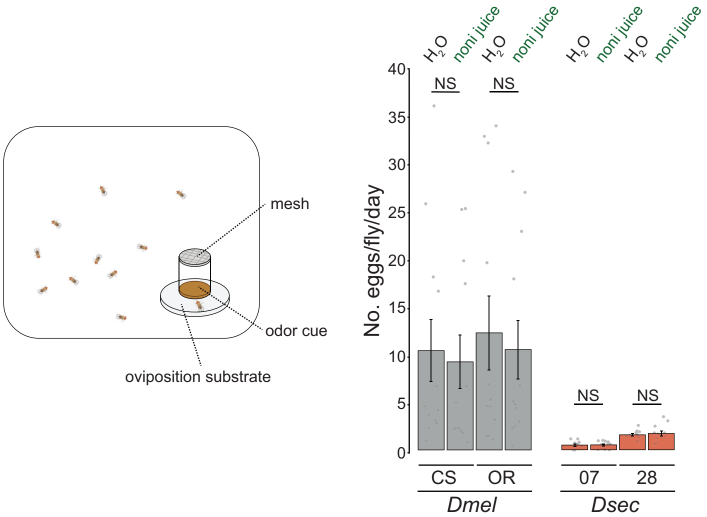

**Supplementary Figure 3. Locomotor activity of olfactory receptor mutants.** Mean activity of the indicated *D. sechellia* strains represented by the number of beam crossings per minute per fly recorded over 3 complete 12 h light: 12 h dark cycles. Statistically-significant differences from *Dsec* 07 (the genetic background strain for all mutants) are indicated: * $p < 0.05$; NS $P > 0.05$ (Wilcoxon test with Bonferroni correction for multiple comparisons); $N$ = 23-46 flies per genotype across at least 2 technical replicates.




**Supplementary Figure 4. Near-anosmic *D. melanogaster* do not display defects in oviposition behavior.**

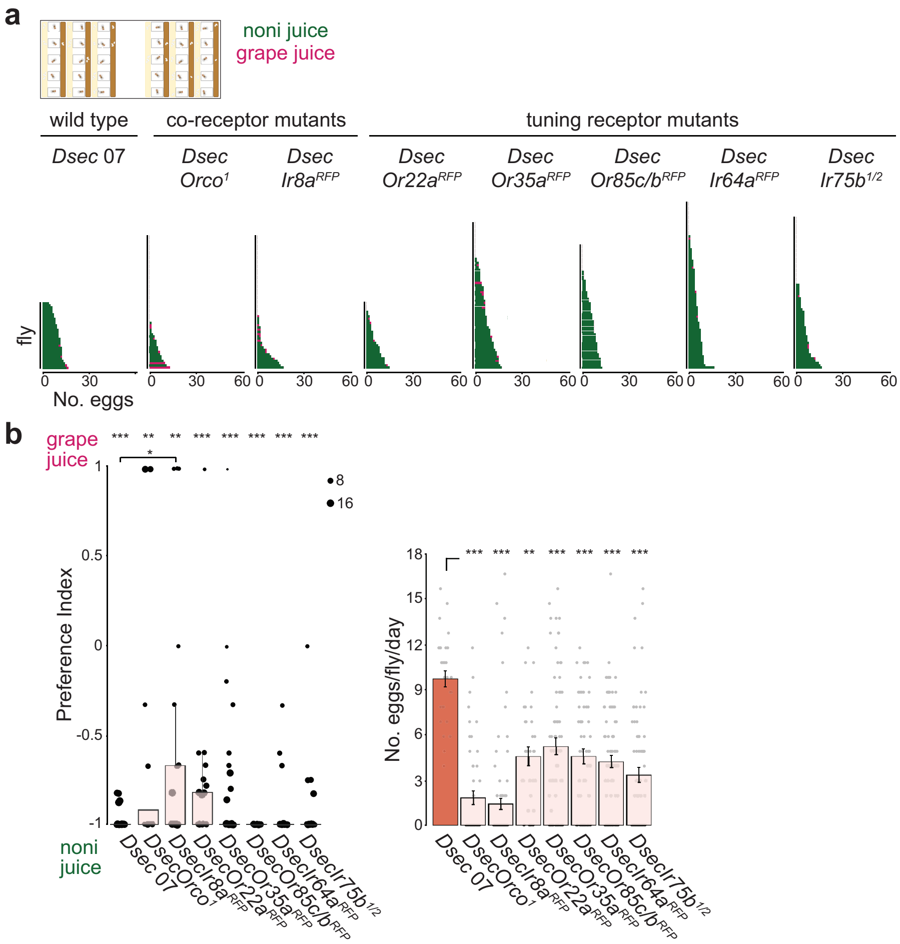
**a** Single-fly oviposition preference assays for apple cider vinegar versus noni juice in agarose for the wild-type *D. melanogaster* Canton-S (CS) and near-anosmic *DmelIr8a$^1$;Orco$^1$* mutants. The plots show the number of eggs laid per fly; *N* = 26-29 flies across 1 technical replicate.

**b** Left: oviposition preference index for the assays shown in **a**. Statistically-significant differences from 0 (no preference) are indicated: *** $P < 0.001$ (Wilcoxon test with Bonferroni correction for multiple comparisons). The two genotypes are statistically-significantly different: $P = 6.334\times10^{-5}$ (Wilcoxon test with Bonferroni correction). Right: egg-laying rate. The two strains are statistically-significantly different: ** $P < 0.01$ (Kruskal-Wallis rank sum test with Nemenyi post-hoc test). Note, however, that as the *Ir8a$^1$;Orco$^1$* mutant is not in the CS genetic background, it is unclear if the slight increase in preference for apple cider vinegar and in egg-laying rate in the mutant strain reflects a function for these olfactory co-receptors or an effect of genetic background.




**Supplementary Figure 5. Noni odor cues are not sufficient to promote egg-laying behavior.**

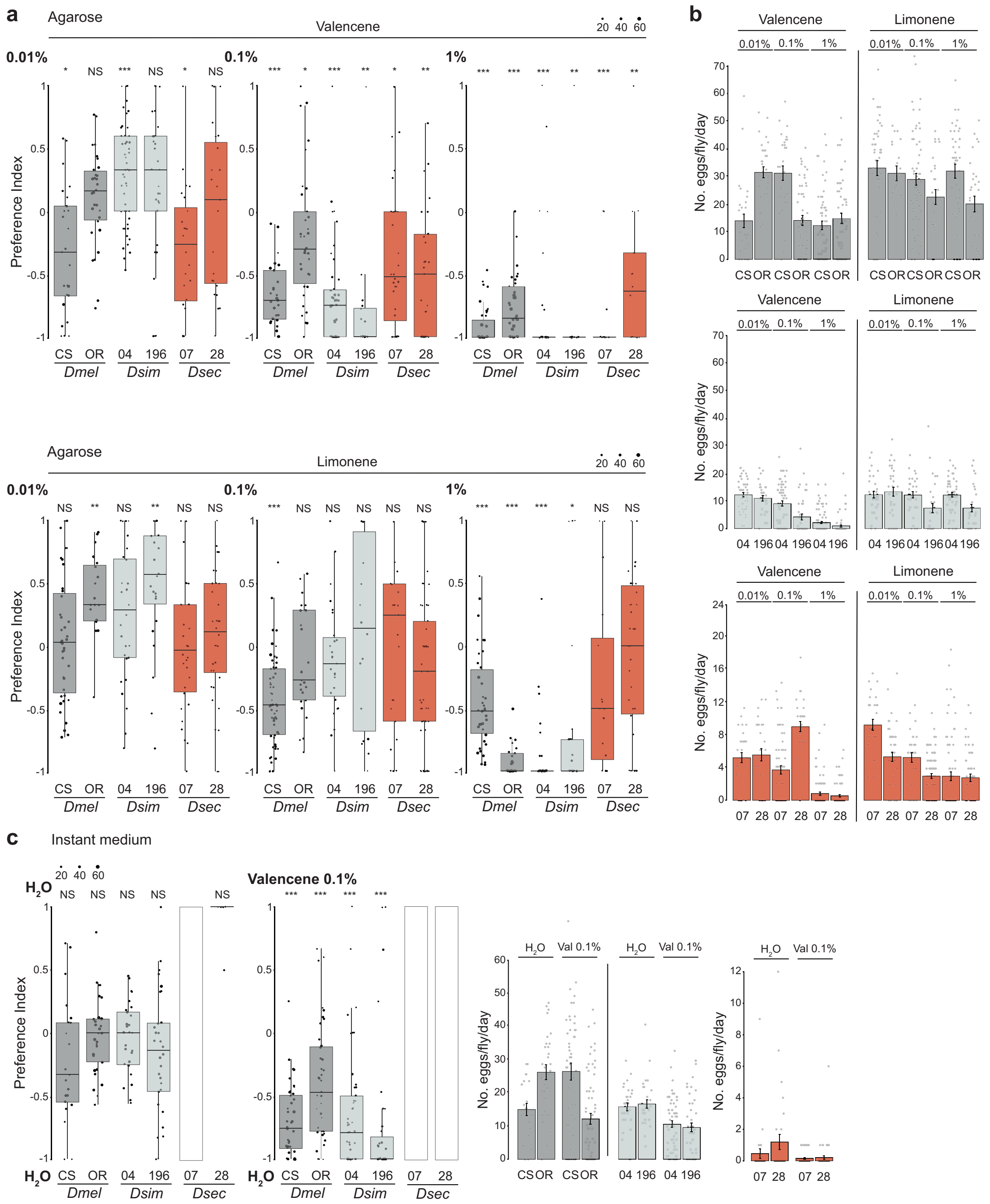
Left: schematic of the one-choice group oviposition assay in the presence of a non-accessible noni juice source or $H_2O$ control. Agarose plates supplemented with 150 mM sucrose were provided as oviposition substrates. Right: egg-laying rate. No statistically-significant differences between the conditions were observed: NS $P > 0.05$ (Kruskal-Wallis rank sum test with Nemenyi post-hoc test); $N = 12$ (representing 4 assays, each scored on 3 successive days with fresh oviposition plates each day).




**Supplementary Figure 6. Olfactory pathways required for *D. sechellia* oviposition.**

**a** Single-fly oviposition preference assays for noni juice versus grape juice in agarose for the indicated genotypes. The plots show the number of eggs laid per fly; *N* = 30-60 flies across 1-2 technical replicates.

**b** Single-fly oviposition assay for the same strains and conditions as in **a**. Left: oviposition preference index. Statistically-significant differences from 0 (no preference) are indicated: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$ (Wilcoxon test with Bonferroni correction for multiple comparisons); *N* = 30-60 flies, 1-2 assays. *Dsec 07* and *DsecIr8a*$^{RFP}$ show a statistical difference: $P = 0.0223$ (Wilcoxon test with Bonferroni correction). Right: egg-laying rate in these assays. *** $P < 0.001$; ** $P < 0.01$ (Kruskal-Wallis rank sum test with Nemenyi post-hoc test).

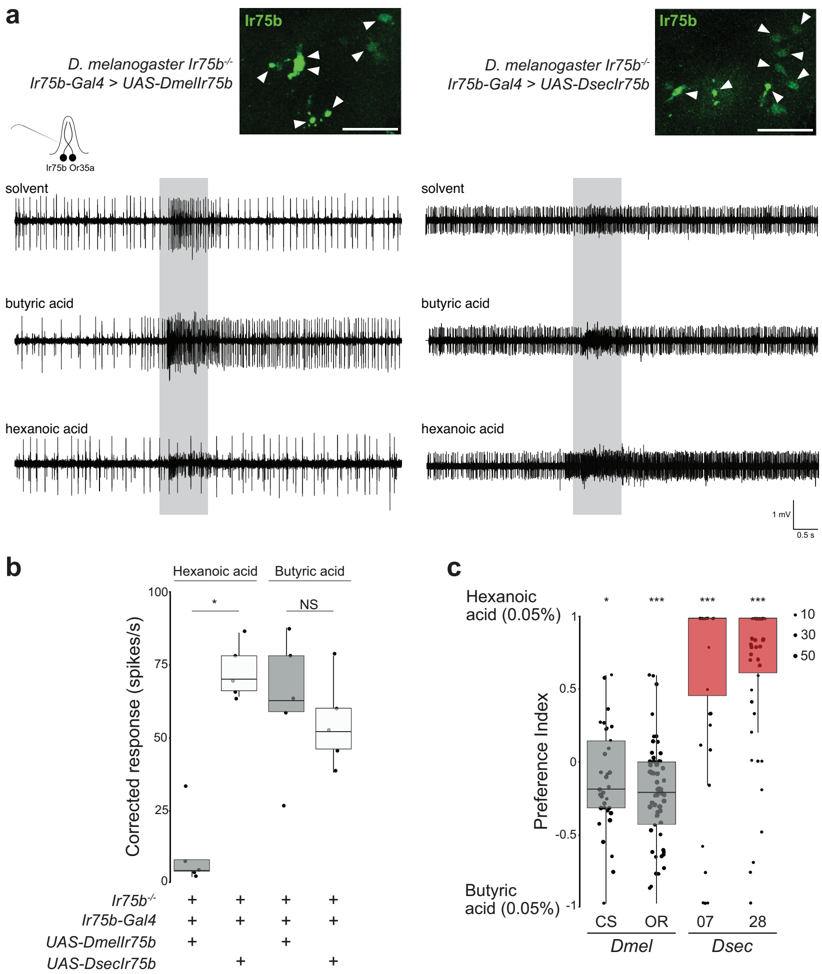



**Supplementary Figure 7. Influence of valencene and limonene on oviposition behavior.**

**a** Single-fly oviposition assays for valencene and limonene at the indicated concentrations. Odors were diluted in apple cider vinegar (for *D. melanogaster* and *D. simulans*) or noni juice (for *D. sechellia*) and agarose. For further information about fly strains and statistical analyses, see the legend to Fig. 1c. Statistically-significant differences from 0 (no preference) are indicated: *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$; NS $P > 0.05$ (Wilcoxon test with Bonferroni correction for multiple comparisons); $N$ = 30-90 flies across 1-3 technical replicates.

**b** Bar plots of egg-laying rates for flies in the assays in **a**.

**c** Single-fly oviposition assays of the same strains as in **a**, with an instant medium substrate. Left: oviposition preference index in control ($H_2O$ versus $H_2O$) or experimental (0.1% valencene versus $H_2O$) conditions. Statistically-significant differences from 0 (no preference) are indicated: *** $P < 0.001$; NS $P > 0.05$ (Wilcoxon test with Bonferroni correction for multiple comparisons); $N$ = 30-60 flies across 1-2 technical replicates. Right: egg-laying rate in these assays (Val = valencene). For some strains (indicated with a white rectangle), the low number of flies laying eggs prevented calculation of a preference index.

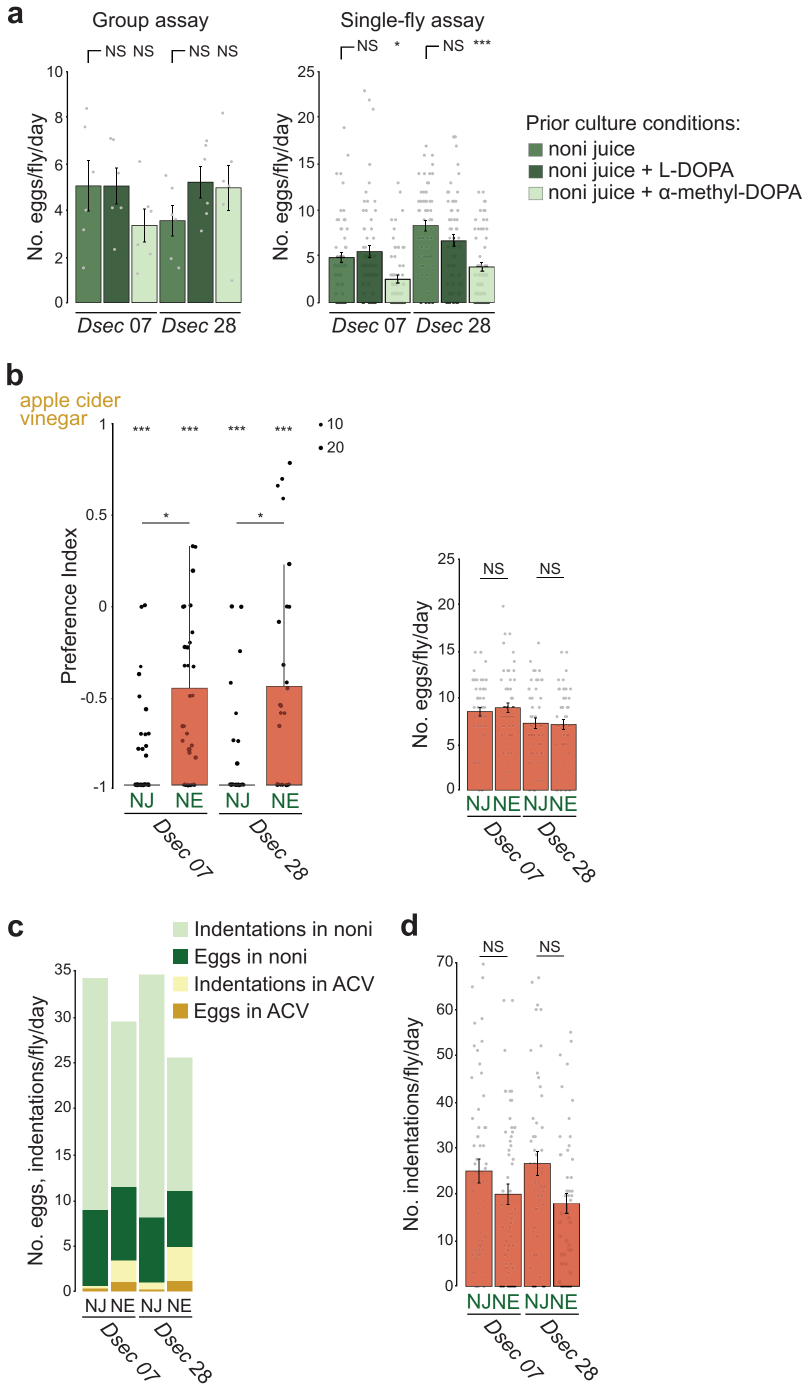



**Supplementary Figure 8. Validation of the expression and species-specific function of heterologously-expressed Ir75b variants.**

**a** Top: immunohistochemistry for Ir75b on whole-mount antennae of flies expressing *D. melanogaster* or *D. sechellia* Ir75b in *D. melanogaster Ir75b* mutant neurons (see Fig. 6d for the full genotypes). Scale bars, 25 μm. Although immunofluorescence signals of soma (arrowheads) were variable between neurons within an antenna – possibly reflecting the heterogeneous strength of the *Ir75b* promoter-Gal4 driver – they were comparable between the two genotypes. 5 and 6 antennae were examined for the *D. melanogaster* and *D. sechellia* Ir75b rescue genotypes, respectively. Bottom: representative traces of extracellular recordings from the same genotypes of neuronal responses in antennal sensilla housing Ir75b neurons (and Or35a neurons) to the indicated odors or solvent ($H_2O$). The grey bars indicate the stimulus period (1 s).

**b** Quantification of odor-evoked electrophysiological responses of the indicated genotypes (see the legend to Fig. 6d for the full genotypes). Each dot represents a measurement from an independent olfactory sensillum. Statistically-significant differences are indicated. * $P < 0.05$; NS $P > 0.05$ (pairwise Wilcoxon test with Bonferroni correction).

**c** Single-fly oviposition preference assays for hexanoic acid versus butyric acid in instant medium for the indicated wild-type strains of *D. melanogaster* and *D. sechellia*. Statistically-significant differences from 0 (no preference) are indicated. *** $P < 0.001$; * $P < 0.05$ (Wilcoxon test with Bonferroni correction for multiple comparisons); $N$ = 34-60 flies across 2 technical replicates.




**Supplementary Figure 9. Impact of modulation of L-DOPA levels, and of fresh noni extract, on oviposition behavior of *D. sechellia*.**

**a** Egg-laying rate of flies that had been grown on control standard noni juice food or that supplemented with L-DOPA or α-methyl-DOPA (see Methods). Left: group oviposition assays, representing 2 assays of either 10 (*D. melanogaster*) or 20 (*D. sechellia*) flies each, scored on 3 successive days with a fresh non-supplemented noni paste oviposition plate provided each day. Right: single-fly oviposition assays using noni paste as oviposition substrate on both sides of the chamber. Statistically-significant differences from flies grown on non-supplemented noni juice food are shown: *** $P < 0.001$; * $P < 0.05$; NS $P > 0.05$ (Kruskal-Wallis rank sum

test with Nemenyi post-hoc test); *N* = 60 flies across 2 technical replicates.
**b** Single-fly oviposition preference assays for commercial noni juice (NJ) or fresh noni extract (NE) versus $H_2O$ in agarose for the indicated wild-type strains of *D. sechellia*. Left: oviposition preference index. Statistically-significant differences from 0 (no preference) are indicated. *** $P < 0.001$ (Wilcoxon test with Bonferroni correction for multiple comparisons). Statistically-significant differences exist between experimental conditions (NJ or NE) in both *Dsec 07* and *Dsec 28*: * $P < 0.05$ (pairwise Wilcoxon test with Bonferroni correction); *N* = 49-56 flies across 2 technical replicates. Right: egg-laying rate. NS $P > 0.05$ (Kruskal-Wallis rank sum test with Nemenyi post-hoc test).
**c** Rate and distribution of egg-laying and indentation formation of the flies in the assays in **b**.
**d** Rate of indentation formation observed in the experiments in **b**. No statistically-significant differences were observed between conditions: NS $P > 0.05$ (Kruskal-Wallis rank sum test with Nemenyi post-hoc test).

## Supplementary Table 1. *Drosophila* strains.

| Strain | Source | Identifier |
|---|---|---|
| *D. melanogaster* wildtype Canton-S (CS) | | |
| *D. melanogaster* wildtype Oregon-R (OR) | | |
| *D. melanogaster* $ovo^{D1}$ *v24 / Y / C(1)Dx, y f* | Daniel Pauli | |
| *D. melanogaster* $Ir8a^{1}$ | [2] | RRID:BDSC_41744 |
| *D. melanogaster* $Orco^{1}$ | [3] | RRID:BDSC_23130 |
| *D. melanogaster* $Ir75b^{DsRed}$ | [4] | |
| *D. melanogaster Ir75b-Gal4* | [5] | |
| *D. melanogaster UAS-DmelIr75b* | [5] | |
| *D. melanogaster UAS-DsecIr75b* | [5] | |
| *D. simulans* wildtype 04 | *Drosophila* Species Stock Center [DSSC] | 14021-0251.004 |
| *D. simulans* wildtype 196 | DSSC | 14021-0251.196 |
| *D. sechellia* wildtype 07 | DSSC | 14021-0248.07 |
| *D. sechellia* wildtype 28 | DSSC | 14021-0248.28 |
| *D. sechellia* $Orco^{1}$ (strong hypomorph) | [6] | |
| *D. sechellia* $Or22a^{RFP}$ | [6] | |
| *D. sechellia* $Or85c/b^{RFP}$ | [6] | |
| *D. sechellia* $Or35a^{RFP}$ | [6] | |
| *D. sechellia* $Ir8a^{GFP}$ | [6] | |
| *D. sechellia* $Ir8a^{RFP}$ | [6] | |
| *D. sechellia* $Ir64a^{RFP}$ | [6] | |
| *D. sechellia* $Ir75b^{1}$ | [6] | |
| *D. sechellia* $Ir75b^{2}$ | [6] | |

## Supplementary Table 2. Chemicals.

| Chemical | Supplier | CAS |
|---|---|---|
| Agarose | Promega | - |
| Apple cider vinegar | Migros | - |
| BACTO Agar | BD | 214010 |
| Balsamic vinegar | Antica Modena | - |
| Butyric acid | Sigma-Aldrich | 107-92-6 |
| 3,4-dihydroxyphenylalanine (L-DOPA) | Sigma-Aldrich | 53587-29-4 |
| Formula 4-24® instant *Drosophila* medium ("instant medium") | Carolina Biological Supply Company | - |
| Grape juice | Beutelsbacher Bio | - |
| 2-heptanone | Sigma-Aldrich | 110-43-0 |
| Hexanoic acid | Sigma-Aldrich | 142-62-1 |
| Limonene | Sigma-Aldrich | 5989-27-5 |
| $\alpha$-methyl-DOPA | Sigma-Aldrich | 41372-08-1 |
| Methyl hexanoate | Sigma-Aldrich | 106-70-7 |
| Noni juice | Raab Vitalfood Bio | - |
| Octanoic acid | Sigma-Aldrich | 124-07-2 |
| Sucrose | Sigma-Aldrich | 57-50-1 |
| Valencene | Sigma-Aldrich | 4630-07-3 |

**Supplementary References**


1. Gou B, Zhu E, He R, Stern U, Yang CH. High Throughput Assay to Examine Egg-Laying Preferences of Individual *Drosophila melanogaster*. *J Vis Exp.* e53716 (2016).

2. Abuin L, Bargeton B, Ulbrich MH, Isacoff EY, Kellenberger S, Benton R. Functional architecture of olfactory ionotropic glutamate receptors. *Neuron.* **69**, 44-60 (2011).

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6. Auer TO*, et al.* Olfactory receptor and circuit evolution promote host specialization. *Nature.* **579**, 402-408 (2020).