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Supplemental Information

Evolution of Multiple Sensory Systems Drives Novel Egg-Laying Behavior in the Fruit Pest *Drosophila suzukii*

Marianthi Karageorgi, Lasse B. Bräcker, Sébastien Lebreton, Caroline Minervino, Matthieu Cavey, K.P. Siju, Ilona C. Grunwald Kadow, Nicolas Gompel, and Benjamin Prud'homme

Figure S1

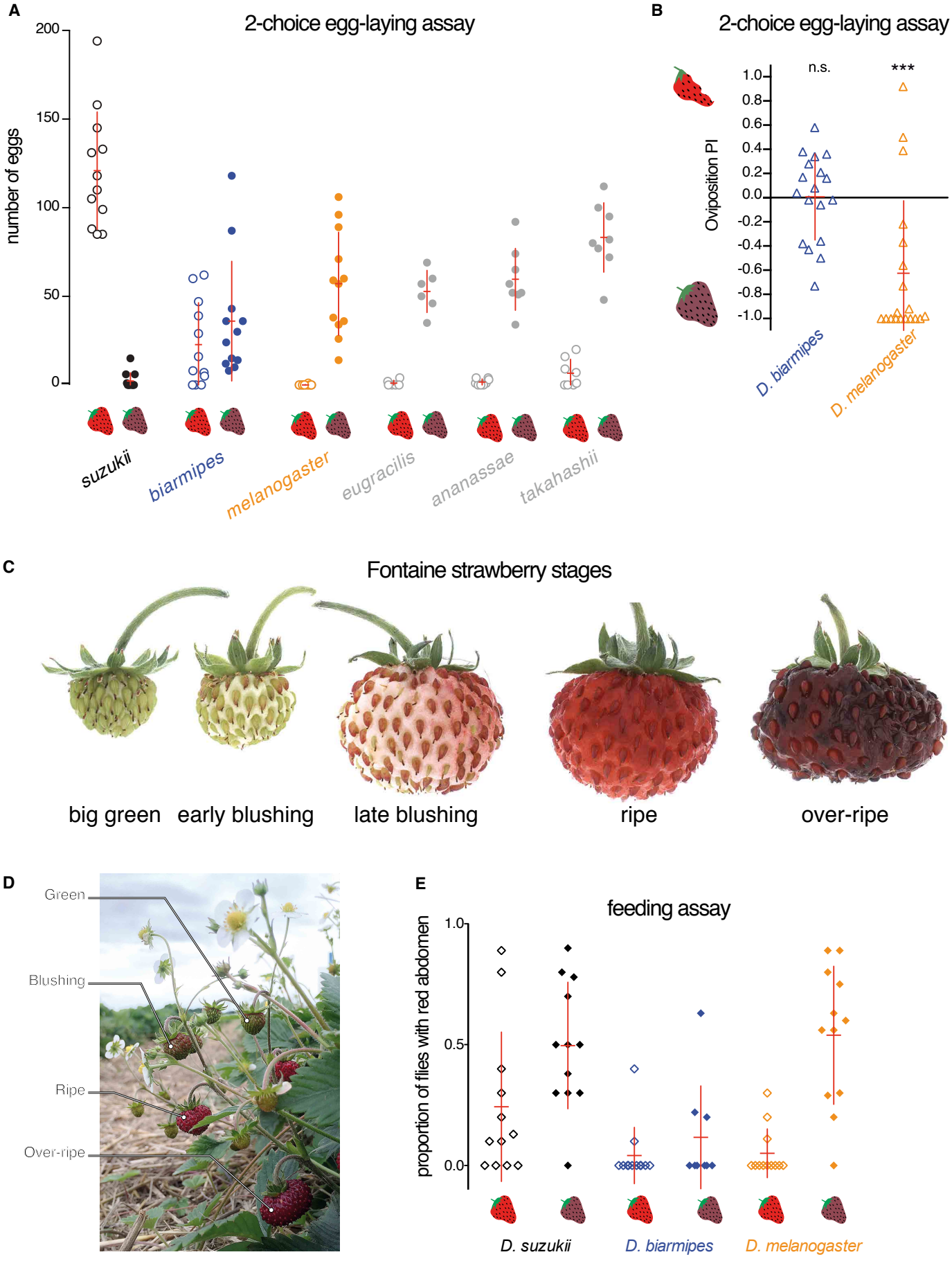

A
2-choice egg-laying assay
number of eggs
200
150
100
50
0
suzukii
biarmipes
melanogaster
eugracilis
ananassae
takahashii
B
2-choice egg-laying assay
Oviposition PI
n.s.
***
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
D. biarmipes
D. melanogaster
C
Fontaine strawberry stages
big green
early blushing
late blushing
ripe
over-ripe
D
Green
Blushing
Ripe
Over-ripe
E
feeding assay
proportion of flies with red abdomen
1.0
0.5
0.0
D. suzukii
D. biarmipes
D. melanogaster

Figure S2

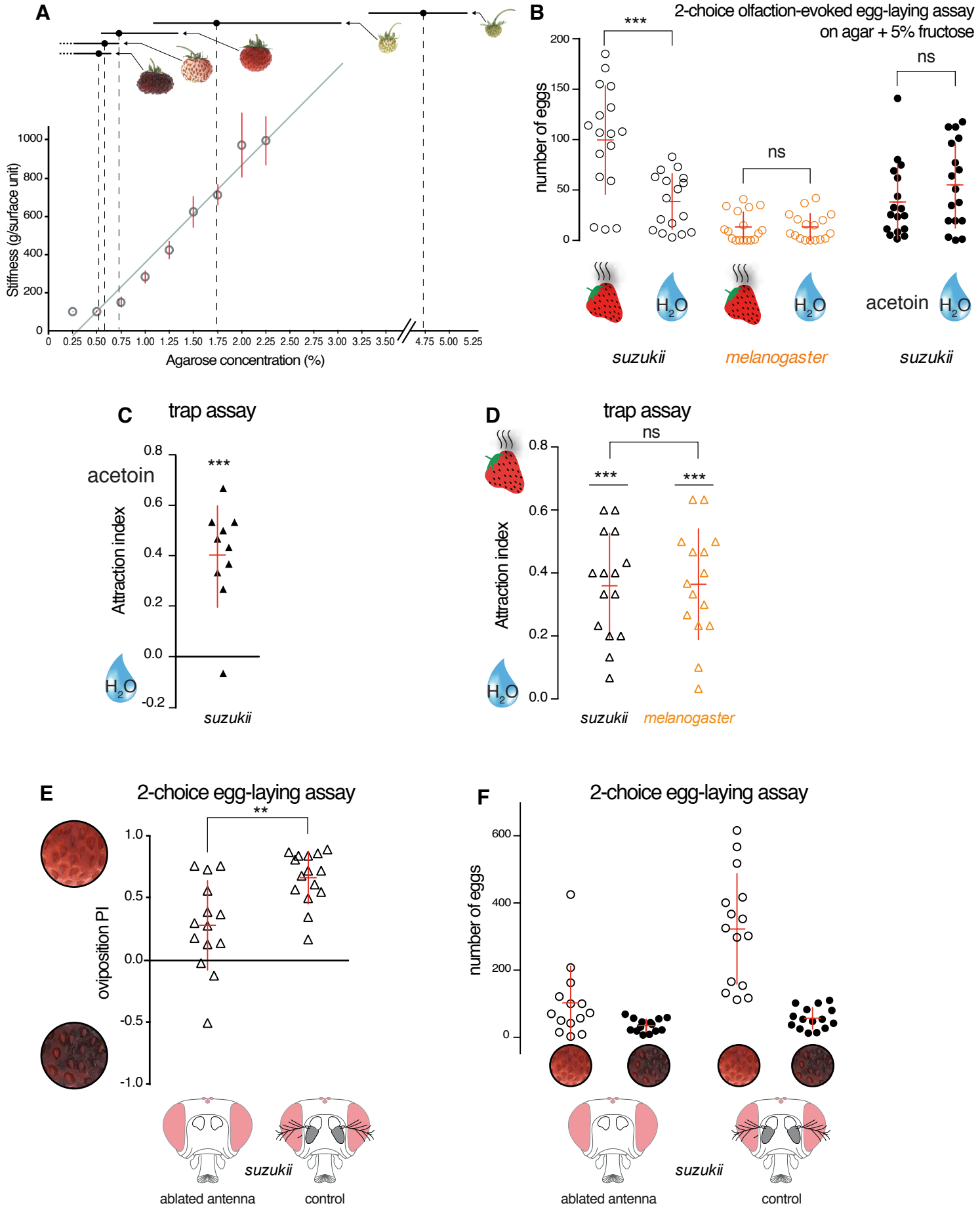
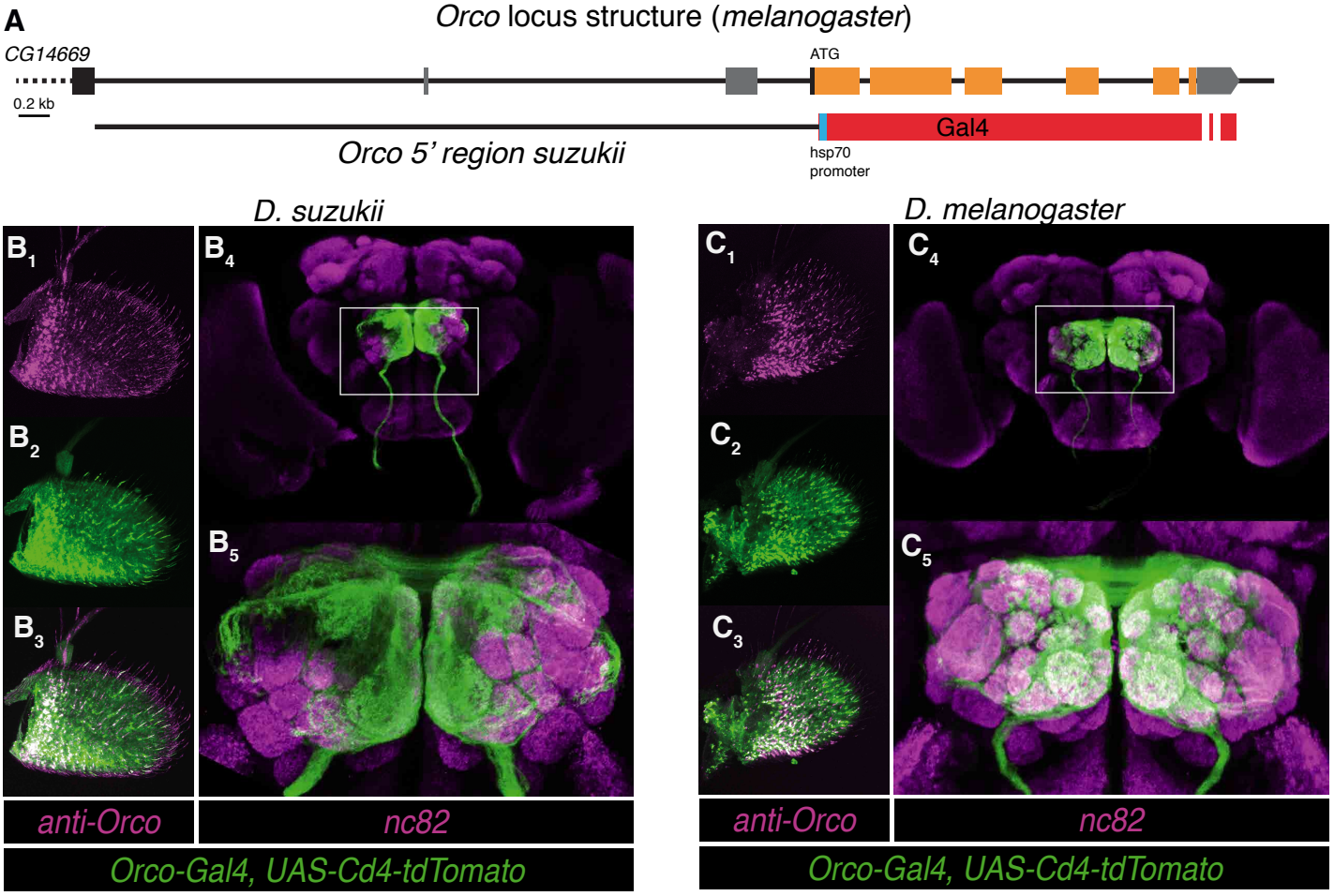
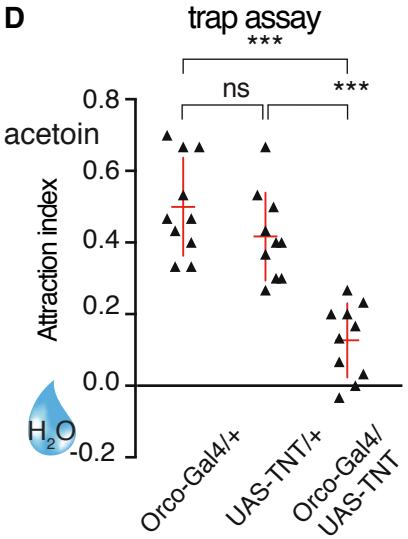
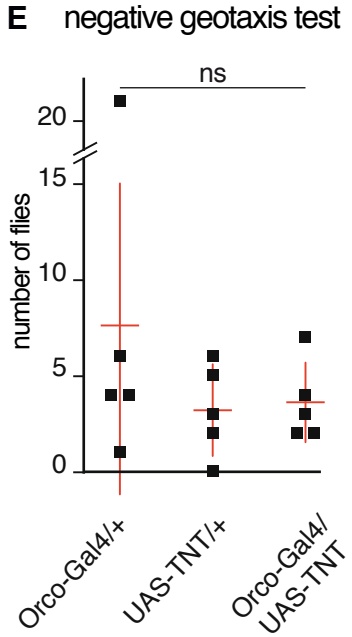

A
Stiffness (g/surface unit)
Agarose concentration (%)
B
2-choice olfaction-evoked egg-laying assay
on agar + 5% fructose
number of eggs
***
ns
ns
H2O
acetoin
suzukii
melanogaster
suzukii
C
trap assay
acetoin
Attraction index
***
H2O
suzukii
D
trap assay
ns
***
***
Attraction index
H2O
suzukii
melanogaster
E
2-choice egg-laying assay
**
oviposition PI
suzukii
ablated antenna
control
F
2-choice egg-laying assay
number of eggs
suzukii
ablated antenna
control

Figure S3


A
Orco locus structure (melanogaster)
CG14669
ATG
0.2 kb
Gal4
Orco 5' region suzukii
hsp70
promoter
D. suzukii
D. melanogaster
B1
B2
B3
B4
B5
C1
C2
C3
C4
C5
anti-Orco
nc82
Orco-Gal4, UAS-Cd4-tdTomato
anti-Orco
nc82
Orco-Gal4, UAS-Cd4-tdTomato


D
trap assay
***
ns
***
acetoin
Attraction index
0.8
0.6
0.4
0.2
0.0
-0.2
H2O
Orco-Gal4/+
UAS-TNT/+
Orco-Gal4/
UAS-TNT


E
negative geotaxis test
ns
number of flies
20
15
10
5
0
Orco-Gal4/+
UAS-TNT/+
Orco-Gal4/
UAS-TNT

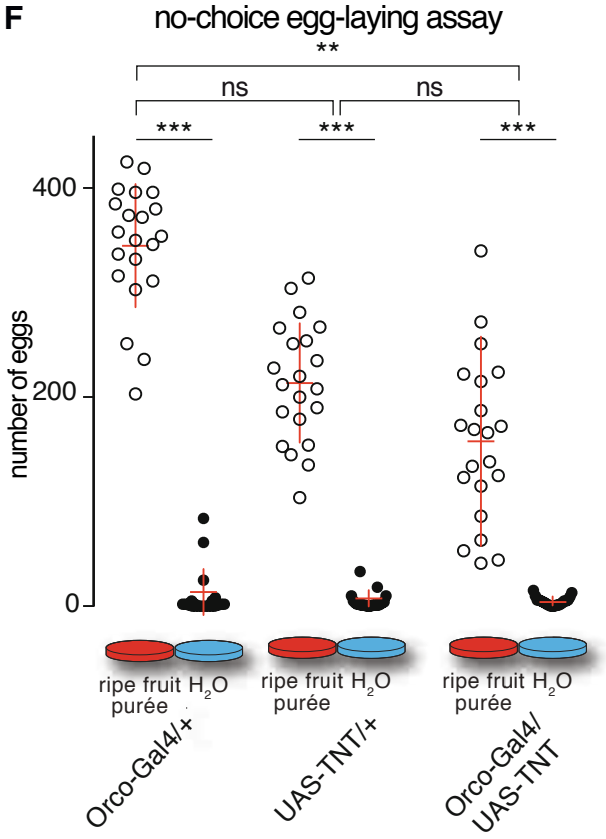
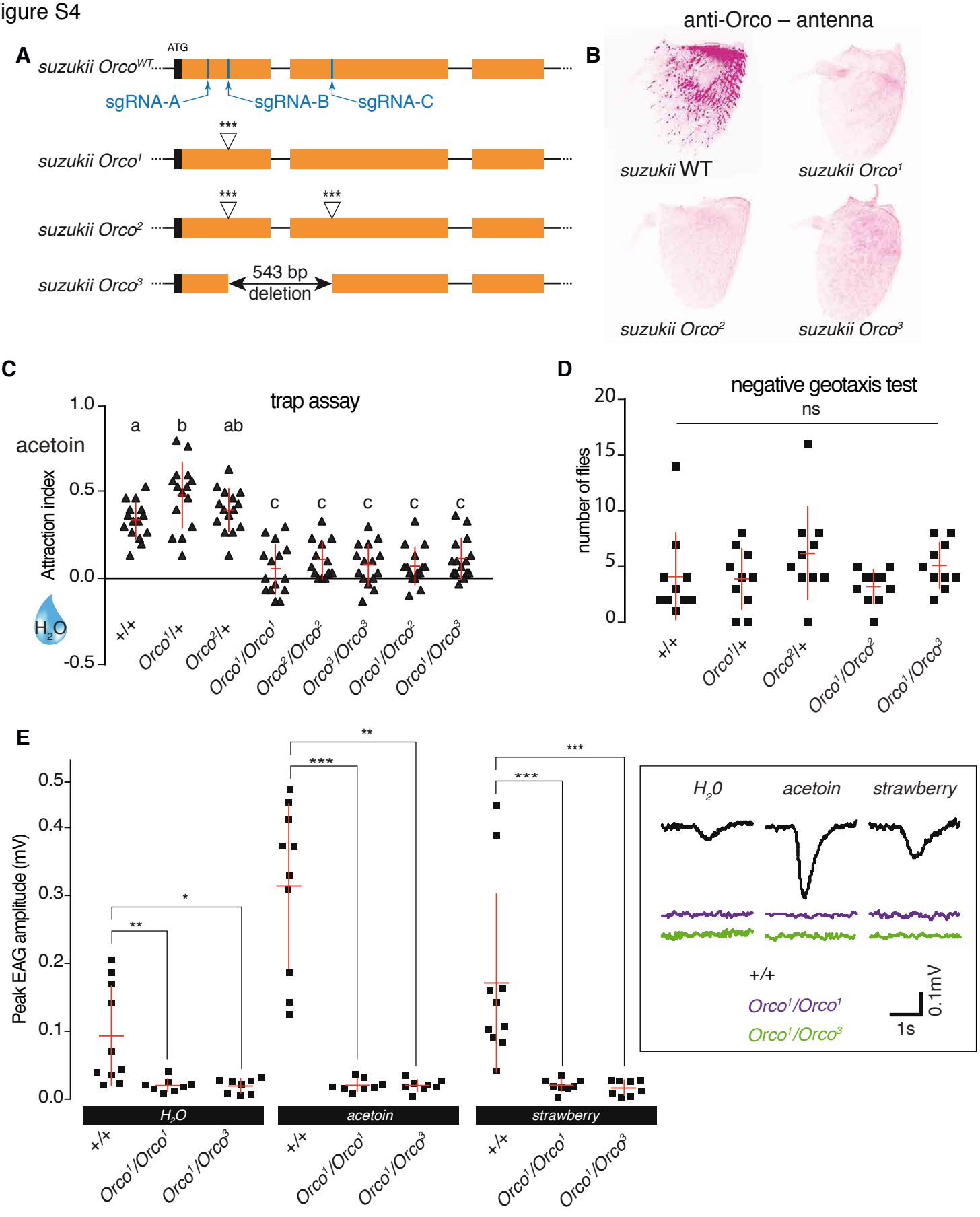

F
no-choice egg-laying assay
**
ns
ns
***
***
***
number of eggs
400
200
0
ripe fruit H2O
purée
ripe fruit H2O
purée
ripe fruit H2O
purée
Orco-Gal4/+
UAS-TNT/+
Orco-Gal4/
UAS-TNT

Figure S4


A
ATG
suzukii Orco^WT
sgRNA-A
sgRNA-B
sgRNA-C
suzukii Orco¹
suzukii Orco²
suzukii Orco³
543 bp deletion
B
anti-Orco – antenna
suzukii WT
suzukii Orco¹
suzukii Orco²
suzukii Orco³
C
trap assay
acetoin
Attraction index
1.0
0.5
0.0
-0.5
H2O
a
b
ab
c
+/+
Orco¹/+
Orco²/+
Orco¹/Orco¹
Orco²/Orco²
Orco³/Orco³
Orco¹/Orco²
Orco¹/Orco³
D
negative geotaxis test
ns
number of flies
20
15
10
5
0
E
Peak EAG amplitude (mV)
0.5
0.4
0.3
0.2
0.1
0.0
H2O
acetoin
strawberry
+/+
Orco¹/Orco¹
Orco¹/Orco³
H2O
acetoin
strawberry
0.1mV
1s

Supplemental Figure legends

Figure S1. Egg-laying and feeding preferences of Drosophila species on various strawberry substrates. Relates to Figures 1 and 2.

(A) Absolute egg numbers laid per replicate (10 females and 5 males) on entire intact strawberries in a 2-choice assay and used to calculate the preference indices of Figure 1B.

(B) Oviposition preference of *D. biarmipes* and *D. melanogaster* in a 2-choice assay between a rotten strawberry and one half of a ripe strawberry sliced in two, providing flies with access to the fruit flesh. p-values were calculated *via* a Wilcoxon signed rank tests.

(C) Representative images of Fontaine strawberries at different stages of fruit maturation.

(D) The choice between strawberries at different stages of maturation exists in natural settings, including as shown here, in a strawberry field. The vicinity of the berries at different stages confronts the gravid female with an actual choice, a choice that is much broader and realistic than the 2 options of the assay depicted in Figure 1A.

(E) Comparison of feeding capacity on entire fruit, measured as the proportion of flies with a red ventral abdomen after 19 hours of exposure to either only ripe or only rotten strawberries (same flies as in Figure 1E).

Figure S2. Analysis of sensory stimuli that influence egg laying. Relates to Figure 2.

(A) An empirical scale of egg-laying plate stiffness as a function of agarose concentration. The stiffness of Fontaine strawberries at different stages was measured independently with a penetrometer. The range of stiffness (horizontal black lines) and the mean stiffness (black dots) are plotted onto the agarose scale (vertical dashed line),

thereby establishing a correspondence between fruit stiffness and agarose concentration. Horizontal dashed black line for the ripe and rotten strawberries indicates uncertainty in the stiffness measurement at the very low end of the scale.

(B) Two-choice experiment, similar to the olfactory oviposition assay presented in Figure 2D, but with 5% fructose in the agar to elicit a baseline of *D. melanogaster* oviposition. Addition of ripe strawberry odors boosts oviposition of *D. suzukii*, but not of *D. melanogaster*. In the same assay, replacing strawberry odors with acetoin, a potent Drosophila attractant (see Figure S2C), does not enhance *D. suzukii* oviposition. p-values were calculated *via* Wilcoxon matched-pairs signed rank test. n=17 replicates per condition.

(C) *D. suzukii* is attracted by acetoin odors; p-value was calculated *via* Wilcoxon test.

(D) *D. suzukii* and *D. melanogaster* are similarly attracted to the odors of ripe strawberries in a trap assay. p-values were calculated with unpaired t test (with comparison to 0 for no preference), and Mann-Whitney test to compare species. n=15 replicates per condition.

(E) 2-choice oviposition assay for ripe *vs.* rotten egg-laying agar plates showing that flies devoid of antennae (ablated) have a reduced preference for ripe substrate compared with non-ablated controls. p-value was calculated with a Wilcoxon-Mann-Whitney test ($p=0.003$). n=14 to 15 replicates (30 females and 15 males per replicate).

(F) Raw data (egg numbers) for the results presented in (E), showing that the reduced preference for ripe substrate results mostly from a reduction of egg laying on ripe substrate.

Figure S3. Generation and validation of *Dsuz\Orco* alleles. Relates to Figure 3.

(A) A schematic map of the *Orco* (*Or83b*) locus from *D. melanogaster* and the orthologous 5' region from *D. suzukii* used to build an *Orco*-Gal4 reporter construct.

(B_1-B_3) *Orco*-Gal4, UAS-CD4-tdTomato and endogenous Orco distribution in *D. suzukii* adult female antennae, revealed by immunochemistry. (B_1) endogenous Orco protein; (B_2) CD4-tdTomato distribution; (B_3) merged.

(B_4-B_5) *Orco*-Gal4, UAS-CD4-tdTomato-positive sensory neurons innervate multiple glomeruli in the antennal lobe of *D. suzukii*.

(C_1-C_3) For comparison to (B_1-B_3), *Orco*-Gal4, UAS-CD4-tdTomato and endogenous Orco distribution in *D. melanogaster* adult female antenna, revealed by immunochemistry. (C_1) Endogenous Orco protein; (C_2) CD4-tdTomato distribution; (C_3) merge.

(C_4-C_5) For comparison to (B_4-B_5), *Orco*-Gal4, UAS-CD4-tdTomato-positive sensory neurons innervate multiple glomeruli in the antennal lobe of *D. melanogaster*. Note the similar Orco expression and similar innervation patterns of *Orco*-Gal4 positive neurons between *D. melanogaster* and *D. suzukii*.

(D) Functional validation of the *Orco*-Gal4 line in *D. suzukii*. In a trap assay, wild type flies (here *Orco*-Gal4/+ and UAS-TNT/+) show a strong attraction to the volatile acetoin. This attraction is severely reduced when neurotransmission is specifically blocked in *Orco*-positive neurons using UAS-TNT. p-values were calculated *via* an ANOVA followed by multiple comparison test with a false discovery rate (FDR) correction. n=10 replicates per condition (15 males and 15 females in each replicate).

(E) Negative geotaxis test on *Orco*-Gal4, UAS-TNT, and Orco-Gal4, UAS-TNT flies to measure and compare locomotor activity. The number of times flies crossed the midline in an empty rearing tube during 5 minutes is similar across genotypes, suggesting that *Orco*-Gal4, UAS-TNT flies do not have altered locomotor activity. p-value were calculated *via* a Kruskal-Wallis test (p=0.65). n=5 replicates per condition (10 males in each replicate).

(F) In an assay similar to Figure 3A, but where the flies can physically contact the ripe strawberry purée, blocking neurotransmission in Orco-positive neurons had almost no effect on oviposition. p-values were calculated *via* Mann-Whitney test for each genotype, and using a negative binomial Generalized Linear Model followed by a general linear hypothesis test for multiple comparisons with a FDR correction method. n=21 replicates per condition, (15 females, 0 male in each replicate).

Figure S4. Generation and validation of *Orco* alleles in *D. suzukii*. Relates to Figure 3.

(A) Schematic representation of loss-of-function *Orco* alleles obtained by CRISPR/Cas9-mediated mutagenesis in *D. suzukii*. All alleles map to the first 2 coding exons and were created using the sgRNAs indicated on the first line (sgRNA-A, -B, and -C). The triangles indicate insertion sites of a 56 bp DNA cassette containing stop codons in all 3 reading frames (asterisks).

(B) In all three alleles, Orco protein is undetectable in whole-mount immunochemistry of antennae compared to wild type flies. The images are negatives of confocal image projections of the third antennal segment of an adult antenna for each genotype.

(C) Functional validation of *D. suzukii Orco* loss-of-function alleles. In a trap assay (see Figure 1D), wild type flies (+/+) as well as heterozygous *Orco* mutants ($Orco^1$/+ and $Orco^2$/+) show a strong attraction to the volatile acetoin. This attraction is lost in homozygotes and trans-heterozygotes ($Orco^1/Orco^1$; $Orco^2/Orco^2$; $Orco^3/Orco^3$; $Orco^1/Orco^2$; $Orco^1/Orco^3$). Significant differences are denoted by letters (ANOVA followed by Tukey's test for multiple comparison; p value < 0.001). n=15 replicates per condition (15 males and 15 females per replicate).

(D) Negative geotaxis test. The number of times flies crossed the midline in an empty rearing tube during 5 minutes is similar across genotypes, suggesting that *Orco* mutants do not have altered locomotor activity and do not have a general defect in climbing. p-values were calculated *via* a Kruskal-Wallis test (p=0.11).

(E) Absolute peak amplitude values measured from electroantennograms recordings using different fly genotypes of *D. suzukii* (wild type and *Dsuz\Orco* mutants) exposed to water, acetoin, or ripe strawberry odor. p-values were calculated *via* a Kruskal-Wallis test, followed by Dunn's tests for comparisons between genotypes for each stimulus. n= 8 or 10 females per condition. Inset shows sample traces for each genotype and stimulus.

Supplemental Experimental Procedures

Fly stocks and husbandry. We raised all stocks on home-made NutriFly (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/germanfood.htm) or standard cornmeal food. For species other than *D. melanogaster*, we added a strip of Whatmann paper into the culture to facilitate pupation. We used Canton S and Oregon R as wild type *D. melanogaster* stocks, *D. biarmipes* genome stock [S1], a *D. biarmipes* isofemale line from Bangalore, India, and different *D. suzukii* stocks (the genomic line WT3 [S2]; an isofemale line from Japan, and another one from France (Alpes Maritimes, "AM")). All *D. suzukii* stocks behave consistently.

Egg-laying assays. For 2-choice and no-choice assays of Figures 1B, E, 2B-D, 3A, C-D, S1A, B, D and S2B, E-F, flies were placed in custom-made transparent Plexiglas chambers (12x6x4 cm, see Figure 1A). The chambers contained various egg-laying substrates, depending on the assay, and as depicted in Figures 1A, 2B, 2D.

Flies were collected and left to age in food vials for 9-10 days. For each trial, flies were placed into each chamber without anesthesia through a small funnel that fits in the lid of the chamber, and left for 19 or 24 hours. Eggs were counted from each substrate, and when applicable an oviposition preference index was calculated as follows: (#eggs on ripe substrate – #eggs on rotten substrate)/(#eggs on ripe substrate + #eggs on rotten substrate). The specifics of each experiment are given below.

2-choice and no choice on entire fruits (Figures 1B, E, 3D): for each trial (19 hours), two strawberries (one ripe and one rotten, two ripe, two rotten, or one rotten and one half of a ripe strawberry sliced in two) were placed in the chamber as in Figure 1A with 10 female and 5 male flies, except in Figure 3D (15 females, 0 male).

2-choice and no-choice on strawberry purée/agar plates (2-choice, Figures 2B, S2B, E-F; no-choice, Figures 2C-D, 3A, C, S3F): same conditions as above, with 10 or 15 female and 0 or 5 male flies, but egg-laying substrates were two Petri dishes (Ø 40 mm) filled with 35% w/v strawberry purée (ripe or rotten) in 1% agar. The experiment of Figure 2C used plates with increasing concentrations of ripe strawberry purée (2.5 to 40%) and each trial contains 10 females and no males. Strawberry purée was mixed together with molten agar cooled to 45-50°C. Trials last 24 hours and are carried out in the dark. n= 16 replicates for each species.

Antennae ablation (Figure S2E, F): antennae of anesthetized adult females were dissected manually with tweezers 3 days before the oviposition assay. The assay is the 2-choice strawberry purée/agar plate described above (Figure 2B, top), using 30 females per replicate.

Olfaction-evoked oviposition assays (Figures 2D, 3A, C, S2B): same conditions as above, but with 10 or 15 females and no male flies, and a modified egg-laying substrate (see schematics on Figure 2D). A Petri dish (Ø 35 mm) containing 5 g of strawberry purée and covered with a metallic mesh was placed at the center of another Petri dish (Ø 55 mm) filled with 1% plain agar. The flies were exposed to the odor of strawberry purée, but could not touch or taste it. Trials last 24 hours and were carried out in the dark. A similar experiment was conducted in a 2-choice setting (strawberry odors *vs.* water, or acetoin *vs*. water) and with 5% fructose in the agar to elicit a baseline of oviposition in *D. melanogaster* (Figure S2B). Egg-laying assays of Figure 1C were performed in round cages (Plexiglass tubes, 5 cm high, Ø 6 cm, with a metal gauze on top and a Petri dish underneath).

Entire fruits at multiple maturation stages (Figure 1C): 45 5-6 day-old mated *D. suzukii* females (no males) were presented with three Fontaine strawberries produced under controlled condition in a green house (one "big green" 16-17 days post-pollination (dpp), one "blushing" ~20 to 22 dpp, and one "ripe" 24 dpp) and left to lay eggs for 16 hours. Fruit were examined under a stereoscope and eggs were counted at the end of each assay.

Stiff vs. *soft agarose preference* (Figure 2A): Petri dishes (Ø 55 mm) were filled with 0.5 M glucose and increasing concentrations of agarose (0.25-2.25%). The solution with the higher agarose content was poured into the plate and left to harden. One half was removed and replaced by filling the empty half with 0.25% agarose/0.5 M glucose. The stiffness of glucose medium with different agarose concentrations, as well as the stiffness of the strawberry stages displayed at the bottom of the figure, was measured using a penetrometer (model FT301 Matzner, Munich). Mixed sex groups of 5-6 day-old flies were separated into groups of ~80 flies (using a mouth aspirator, no anesthesia applied). Groups were transferred to round cages with a 2-choice agarose plate for 16 hours at 25°C, 50% humidity and 8 h light: 8 h dark cycle. Eggs were counted on each side of the plate at the end of the assay. Oviposition preference for stiffer substrate was calculated as follows: (#eggs on the stiff side – #eggs on the soft side)/(#eggs on the stiff side + #eggs on the soft side).

Feeding assay (Figure S1E). At the end of the egg-laying no-choice assay (Figure 1E), the food intake of females was scored by inspecting their ventral abdomen. Females with reddish abdomens were marked as fed, while females with whitish abdomen were marked as unfed. The proportion of fed females was calculated as number of females with red abdomens over the total number of females.

Trap assays (Figures 1D, 3B, S2C-D, S3D, S4C). We adapted the assay from [S3] and [S4] to compare attraction to odors among genotypes, or preference for ripe *vs.* rotten strawberry odors among species and genotypes. The test chamber was assembled with a plastic cylinder (14 x 8 cm) covered by a ventilated lid and a base made from a plastic Petri dish (diameter 9 cm). The test chamber contained an odor-baited trap and a control trap or a different odor-baited trap. The traps were made of transparent plastic vials (9.5 x 3 cm) and were sealed with a cotton plug perforated with a cut pipet tip (Ø ~4 mm). Flies were placed in each test chamber through a small funnel that fits into a hole in the lid. To compare attraction to acetoin (CAS: 513-86-0, Sigma) and strawberry, 30 5-8 day-old flies (15 males and 15 females; starved beforehand for 24 hours in humidified vials) were used for each trial. The odor-baited trap contained 200 µl of acetoin diluted in water (1:100 of pure odor) on a filter paper, or 400 µl of strawberry purée. The control trap contained an equal volume of water. The experiment lasted for 6 hours. To compare preference for ripe *vs.* rotten strawberry odor, 30 7-8 day-old flies (15 males +15 females; flies not starved) were used for each trial. Each odor-baited trap contained 400 µl of strawberry purée. Each trial lasted 24 hours. The attraction index was calculated as follow: (#flies in odor-baited trap 1– #flies in control or odor-baited trap 2)/(#total number of flies).

Negative geotaxis test (Figure S3E, S4D). To verify that the locomotor activity of *Orco*-Gal4, UAS-TNT and *Orco* mutant flies was not affected, we performed a negative geotaxis test [S5]. For each genotype (n= 10 for the *Orco* mutants, n=5 for the *Orco*-Gal4, UAS-TNT) ten males were placed in an empty plastic vial and left 1-2 hours to acclimate. Vials were then briefly tapped three consecutive times to initiate negative

geotaxis. Flies were video-recorded for 5 min. The number of times a fly crossed the midline was then counted.

Strawberries. Most experiments involving strawberries were carried out using different varieties of *Fragaria ×ananassa* and different batches obtained from local grocery stores or producers. Alternatively, frozen strawberry purée, supplemented with 10% sucrose (purchased from SICOLY, ref. PUFR50XB01) was used. Variations in strawberry origin did not affect the fly preferences (ripe *vs.* rotten) and *D. suzukii* showed no oviposition preference for substrates made of agar mixed with frozen/thawed ripe strawberry purée *vs.* agar mixed with freshly prepared ripe strawberry purée (data not shown). Batches of ripe strawberries were split in two. Half were immediately used for an experiment (for instance with another batch that was prepared 4 days earlier), while the other half was left to rot in a plastic box at 25°C and 75% humidity for 4 days. Strawberries at different stages (ripe or rotten) in the same experiment had the same origin (either frozen, or purchased from the same grocery store). Ripe strawberries were carefully examined under a stereoscope and only intact fruits with no bruises or wounds were selected.

Experiments in Figures 1C and S1C were carried out with Fontaine strawberries, a cultivar selected from a cross between *Fragaria vesca* x *Fragaria iinumae* and registered under European Plant Breeders Rights (application no. 2014/1495). Fontaine plants were maintained in our green house and fruits were staged at harvest. For "big green" strawberries, achenes have started to separate, but remain green, as does the receptacle. "Late blushing" strawberries have reached their final size, their receptacle is white with occasional patches of red and their achenes are red. Ripe strawberries have a similar size as "late blushing", their achenes and receptacle are bright red, but they show no wrinkles

or other signs of over-maturation. Fruit stiffness (Figures 2A and S2A) was measured using a fruit penetrometer (model FT301 Matzner, Munich).

Molecular Biology.

Sequences of all the primers indicated in this section are in the list of primers (below).

D. suzukii 5' Orco regulatory regions orthologous to those of *D. melanogaster* [S3] were amplified by PCR on *D. suzukii* WT3 genomic DNA using primers 5'orco.cons F2 & 5'orco.cons R1. The 4.4 kb amplicon was subsequently cloned into a PiggyBac-Gal4 transformation vector [S6, S7].

PiggyBac UAS-TNT and PiggyBac UAS-CD4-tdTomato vectors were built by cloning UAS-effector gene fragments into a PiggyBac transformation vector backbone. These fragments were obtained by PCR on genomic DNA of the respective *D. melanogaster* transgenic lines (BDSC #3569 and #35846) using the following primers: TeTxLc_Fw1 & TeTxLc_Rv1 ; cd4-tdTom-ER1_Fw1 & cd4-tdTom-ER1_Rv1. All PiggyBac constructs were injected in the *D. suzukii* "AM" stock.

Transgenesis and genome editing.

Germline transformation. Fly embryos were injected with PiggyBac constructs as in [S6]. Of note, of the 8 independent transgenic lines we recovered from the injection of the *Piggybac-suzukii 5'_orco-Gal4* construct, only one drives robust expression in the antennae and the brain.

Orco *sgRNA design and synthesis*. We targeted the following GGN18NGG sequences following the protocol of reference [S8]: TTTATGCACAACTTCACGGGCGG (orco-A, plus strand), GGCCATGTTGACCAGGATGAAGG (orco-B, minus strand), CCACCGTGGCCTCGGCCACCGCC (orco-C, minus strand). Oligos were used to

synthesize *in vitro* the three sgRNA with T7 RNA polymerase: sgRNA-orcoA-F, sgRNA-orcoB-F, sgRNA-orcoC-F ; each of which was coupled with the generic oligo sgRNA-R. The three sgRNAs and their corresponding single-stranded oligos (ssODN) were all co-injected, together with Cas9 protein (http://pnabio.com , ref. CP01), at the following concentrations: 50 ng/µl of each sgRNA, 300 mg/µl of protein and 125 ng/µl of each single-stranded DNA oligo (ssODN_orcoA, ssODN_orcoB, ssODN_orcoC).

Adults and their progeny were screened by PCR on genomic DNA extracted from single legs or entire individuals (single flies or pairs of flies) to detect insertions of single-stranded DNA oligos or deletions. Hits were confirmed by Sanger sequencing of the PCR product. Primers used for screening were: orcoA-F & orcoA-R; orcoB-F & orcoB-R; orcoC-F & orcoC-R.

Electroantennography (EAG) For EAG recordings, 4-8 days old female flies of appropriate genotypes were used (n= 8-10 females per genotype). A fly was restrained in a pipette tip with its antenna protruding and mounted on a coverslip. Glass electrodes filled with 0.1 M KCl were used for both recording and reference electrodes. The reference electrode was inserted into the eye and the recording electrode was placed on the third antennal segment. The signals were recorded using a CV-7B headstage and MultiClamp 700B amplifier, and digitized by a Digidata 1440A (Molecular Device). Clampex 10.2 software was used for acquiring the signals and the signals were low pass-filtered at 10 Hz and sampled at 5 kHz. Peak amplitude voltage and traces were extracted using Clampfit 10.2 software offline. Data analysis was carried out using Excel and R for the statistics.

Odor delivery

With a custom-made odor delivery system (Smartec, Martinsried, Germany) a continuous and humidified airstream (1000 ml/min) was delivered to the fly through an 8 mm glass tube positioned 10 mm away from the preparation. For odor stimulation, 1 ml of water, strawberry purée, or acetoin (1:100) were freshly pipetted into glass vials and a 300 ml/min odor pulse was delivered for 1 second through the headspace of the vials into the continuous airstream. In each experiment odors were delivered in a random order.

Antibody staining. Immunochemistry was performed on fly brains and antennae following standard protocols [S9]. Briefly, dissected samples were fixed in 4% formaldehyde, washed in PBT, blocked with 4% goat or calf serum and incubated with primary antibody overnight at 4°C. Samples were then washed, incubated in secondary antibody for 2 hours at room temperature, washed again, and mounted. The following antibodies were used: primaries: rabbit anti-RFP (Rockland), 1:1000 for brains, 1:500 for antennae; mouse nc82 (Hybridoma bank) 1:40; guinea pig anti-Orco ([S10], a gift from R. Benton) 1:800; secondaries (Rockland) used at 1:200 for brains, 1:100 for antennae: donkey anti-rabbit alexa 488; donkey anti-rabbit alexa 647; donkey anti-mouse alexa 647; goat anti-guinea pig alexa 488.

Imaging. Reporter expression and fluorescent antibody stainings were imaged on a LSM 510 confocal microscope (Carl Zeiss AG, Oberkochen, Germany).

Images of strawberry maturation stages, strawberries infested with eggs, or ovipositors were taken under a Leica M420 Makroscop equipped with a Manta CCD G-609 camera (Allied Vision Technologies, Germany), with identical lighting conditions (Aputure AL-H160 Amaran LED plates, placed laterally).

Images were enhanced using Adobe Photoshop in compliance with *Current Biology* guidelines.

Statistical analysis. Statistical analyses were performed with GraphPad Prism6 and various R packages. Briefly, for oviposition choice and odor attraction tests, the preference index of each species was compared to a theoretical value of 0 (no preference) using a t test or Wilcoxon's signed rank test. Within-species comparisons in no-choice assays were made using a Mann-Whitney test. The proportions of eggs laid in 3-choice assays were compared with a Friedman test, followed by a pairwise comparison using Conover's test (package PMCMR). Inter-species and inter-genotypes comparisons of preferences were done with a Mann Whitney test. When there were more than two groups under comparison, attraction and oviposition preference indices were compared between fly lines and species using an ANOVA followed by a multiple comparison test (glht) with an FDR correction method, or using a Kruskal-Wallis test, followed by Dunn's test.

For oviposition no-choice tests of Figures 3 and S3, the number of eggs laid by the different genotypes were compared using a negative binomial Generalized Linear Model (package MASS) followed by a general linear hypothesis test (glht, package multcomp) for multiple comparisons with a false discovery rate (FDR) correction method.

The dose-responses to ripe strawberry purée were compared using a non-linear regression analysis (after a log transformation and normalization of the data). Comparison of logEC50 (the concentration of strawberry purée eliciting 50% of the maximum response) was calculated for *D. suzukii* and *D. biarmipes* (but not for *D. melanogaster* due to a poor fit of the data).

Signals from the EAG were compared between genotypes for each stimulus using an ANOVA followed by a multiple comparison test (glht) with a FDR correction method.

List of primers. Primers used for reporter constructs and CRISPR/Cas9 experiments. Relates to main-text Experimental Procedures.

5'orco.cons F2
GTGAAGTTGTGCATAAGGGCGAATT

5'orco.cons R1
GCTGACAGGGCGACAGATTC

TeTxLc_Fw1
GAATTCGCAATTAAGGAGATAATAG

TeTxLc_Rv1
AAGCTTACCACCCCCAACCTG

cd4-TdTom-ER1_Fw1
ATGAATCCCAAGAGCGAAGTCCTC

cd4-TdTom-ER1_Rv1
TTAGAGGGCAACTTCATTTTCATAGC

orcoA-F
GAAATTAATACGACTCACTATAGGTATGCACAACTTCACGGGGTTTTAGAGC
TAGAAATAGC

sgRNA-orcoB-F
GAAATTAATACGACTCACTATAGGGGCCATGTTGACCAGGATGAGTTTTAGA
GCTAGAAATAGC

sgRNA-orcoC-F
GAAATTAATACGACTCACTATAGGCGGTGGCCGAGGCCACGGGTTTTAGAGC
TAGAAATAGC

sgRNA-R
AAAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATT
TTAACTTGCTATTTCTAGCTCTAAAAC

orcoA-F

CGCAGGATGACGACCTCGATGC

orcoA-R
TCGTGTTGCCCGACAGCTCGTT

orcoB-F
GAAGAAGGTCTACTCCTCGGTGC

orcoB-R
CTGTTAAAAAGTGGGGTAGATTAAAAAGAACACA

orcoC-F
TGAATATATGGAACCAGGTCAACACGCACC

orcoC-R
ATCATCGAGAAGAGCACGTAGTAGATCTGAAAGG

ssODN_orcoA
GGGCCTGGTGGCCGACCTGATGCCCAATATACGGGCGATGAAGTACTCGGGC
CTGTTTATGCACAACTTCACAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTC
TCTCAGTTGGGGGCGTAGAATTCGGGCGGCAGTGCCTTCATGAAGAAGGTCT
ACTCCTCGGTGCACCTGGTGCTCCTGCTGATGCAGTTCGCCTT

ssODN_orcoB
GCGGCAGTGCCTTCATGAAGAAGGTCTACTCATCGGTGCACCTGGTGCTCCTG
CTGATGCAGTTCGCCTTCAAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTCT
CTCAGTTGGGGGCGTAGAATTCTCCTGGTCAACATGGCCCTCAACGCCGAGG
AGGTGAACGAGCTGTCGGGCAACACGATCACCACCCTCTTCT

ssODN_orcoC
CAGACGCGCGCTACCACTCGATCGCCCTGGCCAAGATGAGGAAGCTGTTCTT
CCTGGTGATGCTGACCACCGAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTC
TCTCAGTTGGGGGCGTAGAATTCTGGCCTCGGCCACCGCCTGGACCACGATC
ACCTTCTTCGGGGACAGCGTCAAGATGGTGGTGGACCACGAAA

Supplemental References

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