Report

Current Biology

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

Highlights

- The pest *Drosophila suzukii* prefers to lay eggs on ripening fruit
- Closely related *Drosophila* species prefer to lay eggs on rotten fruit
- Female flies use chemosensation and mechanosensation to choose an oviposition site
- Orco-dependent detection of ripe fruit odors elicits oviposition in *D. suzukii*

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In Brief

Karageorgi et al. show that the invasive pest *Drosophila suzukii* has evolved a preference to lay its eggs on ripening fruit. The authors dissect the sensory bases of this preference, pointing to a multi-step evolutionary scenario involving the tuning of different sensory modalities.

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

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SUMMARY

The rise of a pest species represents a unique opportunity to address how species evolve new behaviors and adapt to novel ecological niches [1]. We address this question by studying the egg-laying behavior of *Drosophila suzukii*, an invasive agricultural pest species that has spread from Southeast Asia to Europe and North America in the last decade [2]. While most closely related *Drosophila* species lay their eggs on decaying plant substrates, *D. suzukii* oviposits on ripening fruit, thereby causing substantial economic losses to the fruit industry [3–8]. *D. suzukii* has evolved an enlarged, serrated ovipositor that presumably plays a key role by enabling females to pierce the skin of ripe fruit [9]. Here, we explore how *D. suzukii* selects oviposition sites, and how this behavior differs from that of closely related species. We have combined behavioral experiments in multiple species with neurogenetics and mutant analysis in *D. suzukii* to show that this species has evolved a specific preference for oviposition on ripe fruit. Our results also establish that changes in mechanosensation, olfaction, and presumably gustation have contributed to this ecological shift. Our observations support a model in which the emergence of *D. suzukii* as an agricultural pest is the consequence of the progressive modification of several sensory systems, which collectively underlie a radical change in oviposition behavior.

RESULTS AND DISCUSSION

D. suzukii Females Have Evolved a Preference to Lay Eggs in Ripe Rather Than Rotten Strawberries

We analyzed the oviposition behavior of *D. suzukii* and some close relatives using strawberries (genus *Fragaria*), a main target of *D. suzukii*, at different stages of maturation [10]. We first compared ripe, pristine strawberries (hereafter referred to as "ripe fruit"), purchased from a local grocery store, to strawberries from a similar batch left to decay for 4 days (hereafter referred to as "rotten fruit"; see Supplemental Experimental Procedures). We then assessed the egg-laying behavior of *D. suzukii* and five closely related species [11] on ripe and rotten fruit using a two-choice oviposition assay (Figure 1A). We counted the number of eggs laid in each fruit after 19 hr and calculated an oviposition substrate preference index (PI). Using this two-choice assay, we observed a robust oviposition preference for most species and striking differences between *D. suzukii* and the other species (Figures 1B and S1A). *D. suzukii* females laid almost all of their eggs on ripe fruit, whereas *D. ananassae*, *D. melanogaster*, *D. eugracilis*, and *D. takahashii* demonstrated the opposite behavior, targeting rotten fruit almost exclusively. Remarkably, *D. biarmipes* showed an intermediate behavior with no marked preference for either substrate. To test whether the fruit skin is a deterrent barrier resulting in these different behaviors, we repeated the choice assay with ripe fruits sliced in half, to expose their flesh, and rotten fruits. We found that *D. biarmipes* laid approximately equal numbers of eggs on both substrates, while *D. melanogaster* maintained a strong preference for the rotten fruit (Figure S1B). These results establish that the preference for laying eggs on rotten fruit is ancestral to this group of species, and that a preference for oviposition on ripe fruit has evolved in the lineage leading to *D. suzukii*.

To further determine the preferred range of fruit ripening stages [12] targeted by *D. suzukii* for oviposition, we offered *D. suzukii* females the choice between "green," "early/late blushing," and "ripe" fruit (Figures S1C and S1D). Although flies managed to lay a few eggs in the green fruit, the vast majority of eggs were laid in fruit of later maturation stages (Figure 1C). We concluded that *D. suzukii* has access to strawberries at the onset of their maturation but strongly prefers blushing and ripe fruit stages. These results are consistent with previous observations that used different strawberry cultivars and other species of berry [10, 13, 14]. Together, they establish that *D. suzukii*, compared to other closely related species, has shifted its

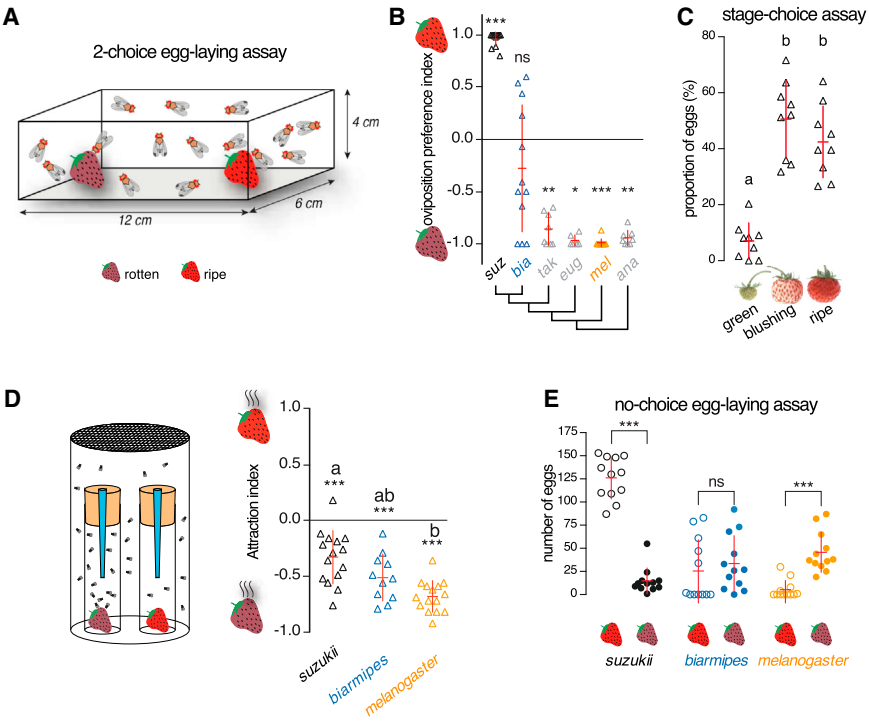

Figure 1. *D. suzukii* Females Have an Evolutionarily Novel Preference for Laying Eggs in the Early Stages of Strawberry Maturation

(A) Two-choice oviposition assay on whole fruits, where ten mated *Drosophila* females (mixed with five males) lay their eggs on two different substrates (ripe versus rotten strawberries). Eggs were counted at the end of each assay.

(B) Oviposition preference (two-choice assay as depicted in A) of six closely related species of the *D. melanogaster* species group. Whereas most species show a strong preference to lay their eggs on rotten fruit, *D. suzukii* females have radically shifted their preference to lay eggs on ripe fruit. Interestingly, *D. biarmipes*, a close relative of *D. suzukii*, displays a mild but not statistically significant preference for rotten fruit. All p values were calculated via Wilcoxon matched-pairs signed-rank test. p values in this and all subsequent figures: ***$p \leq 0.001$; **$p \leq 0.01$; *$p \leq 0.05$; ns, $p > 0.05$, i.e., non-significant difference. In this and all subsequent figures, the red bars indicate the mean ± SD unless otherwise noted. Replicates in preference experiments are represented by triangles (n = 6 to 12 replicates per species).

(C) When given a choice between three stages of the same strawberry cultivar (*Fontaine* strawberries; see Supplemental Experimental Procedures), *D. suzukii* selects predominantly the ripening (blushing) or mature (ripe) fruits. Significant differences are denoted by letters (Friedman test; $X^2(2) = 14$, $p < 0.001$, followed by Conover's test; $p \leq 0.01$). n = 9 replicates per species.

(D) Olfactory attraction assay (trap; design of the assay is shown on the left). All three species (*D. suzukii*, *D. biarmipes*, and *D. melanogaster*) are more attracted to rotten strawberry odors, but *D. suzukii* relatively less than *D. melanogaster*. p values were calculated via one-sample t test for each species; significant differences between species are denoted by letters (ANOVA followed by Tukey's test for multiple comparison; $p < 0.001$). n = 11 or 15 replicates per species.

(E) A no-choice oviposition assay, similar to the two-choice assay in (A), but in which ten females and five males were presented with one type of fruit (ripe or rotten), reveals oviposition on ripe or rotten strawberries for *D. suzukii*, *D. biarmipes*, and *D. melanogaster*. This shows that *D. suzukii* can lay eggs on rotten fruits, although not much, and conversely that *D. melanogaster* can lay eggs on ripe fruits, although not much. *D. biarmipes* appears to lay eggs indifferently on both substrates. All p values were calculated via Mann-Whitney test. In this and all subsequent figures, the number of eggs laid per replicate is presented with open (ripe fruit) or filled (rotten fruit or other) circles. n = 12 replicates per species.

See also Figure S1.

oviposition target from rotten to earlier stages of fruit maturation. We explored this behavioral shift further by focusing on three species: the genetic model *D. melanogaster*; *D. biarmipes*, a close relative of *D. suzukii* not known as a ripe fruit pest; and *D. suzukii* itself.

The Preference of *D. suzukii* for Ripe Fruit Is Specific to Oviposition

We sought to determine whether the preference of *D. suzukii* for ripe fruit is specific to oviposition or a facet of a general ecological shift, as has been observed for other drosophilids [15]. We first found, using an olfactory trap assay, that all three species are more attracted to the odor of rotten fruit, although *D. suzukii* shows a weaker preference (Figure 1D). Hence, the preference for ripe fruit that *D. suzukii* displays in the context of oviposition seems specific to this behavior. Its attraction to rotten fruit may instead relate to feeding. To examine this possibility, we compared feeding behaviors of all three species with an assay similar to that depicted in Figure 1A, but using either ripe or rotten fruit alone (no-choice assay). While *D. suzukii* fed more on ripe strawberries than *D. melanogaster* (as indicated by the red color of the abdomen), both species show a similarly strong appetite for rotten fruit (Figure S1E). We conclude that *D. suzukii* is attracted to rotten fruit mostly for feeding and targets ripe fruit mostly for oviposition, in agreement with published data [16].

We next evaluated the intrinsic capacity of each fruit stage to elicit oviposition using a no-choice oviposition assay. We observed that *D. suzukii* laid more eggs on ripe strawberries alone than on rotten strawberries alone; by contrast, *D. melanogaster* laid more eggs when exposed to rotten fruit, while *D. biarmipes* laid similar numbers of eggs on both fruit stages (Figure 1E). These results suggest that the relative preferences observed in the two-choice assay result directly from the absolute capacity of each substrate to elicit oviposition for each species. We went on to dissect the properties of the fruit that females select as their preferred egg-laying substrates.

D. suzukii Tolerates Stiffer Substrates for Oviposition

The enlarged ovipositor of *D. suzukii* presumably endows the females with the capacity to more easily pierce the stiff skin of a ripe fruit [9]. We wondered whether flies exploit the stiffness of the fruit skin, which decreases with maturation [17], to assess oviposition substrate quality. We exposed females to substrates that differ only in stiffness, in the form of Petri dish halves filled

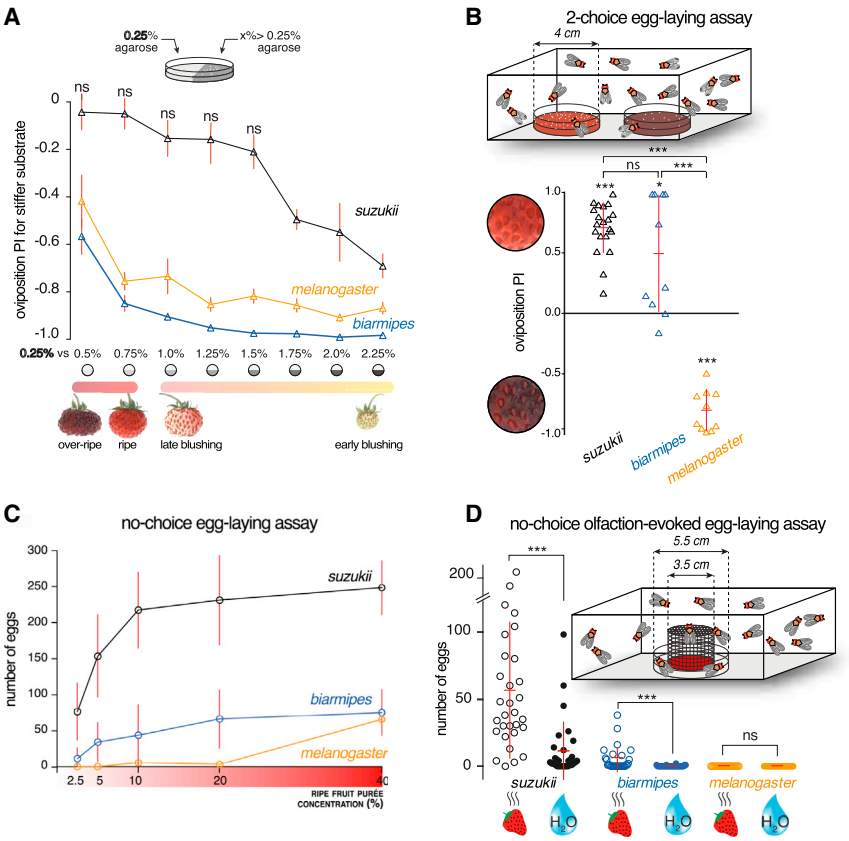

Figure 2. Changes in Mechanosensory and Chemosensory Systems Underlie the Evolution of Oviposition Site Preference

(A) Two-choice oviposition assay for substrate stiffness (schematic depicted above the graph). For each point (n = 8 replicates), a group of females had the choice to lay eggs on 0.25% agarose or an increasingly stiffer substrate from 0.5% to 2.25% agarose in steps of 0.25 percentage point. All substrates were equally supplemented with glucose. The result is expressed as a preference index for the stiffer substrate (red bars indicates here mean ± SEM rather than mean ± SD, for the sake of figure clarity). Whereas *D. melanogaster* and *D. biarmipes* showed a consistent strong preference for the softer substrate —even when the alternative was only marginally stiffer (0.25% versus 0.5%)—*D. suzukii* was relatively indifferent to the substrate stiffness until the concentration difference was higher than 1.5 percentage points. The turning point in the preference of each species matched the stiffness of the fruit stage in which they preferred to lay eggs in choice assays (Figures 1B, 1C, and S1A). All p values were calculated via Wilcoxon tests for each point with comparison to a theoretical value of 0 (no preference). $p \leq 0.01$ for all samples, except where "ns" is indicated.

(B) Two-choice oviposition assay for substrates of different chemical composition. Ten *Drosophila* females (and five males) had the choice between two substrates of equal stiffness (1% agar) but different chemical composition. One egg-laying plate contained 35% w/v ripe strawberry purée, while the other plate contained 35% w/v rotten strawberry purée. The results recapitulate the preference of *D. melanogaster* and *D. suzukii* observed in a similar two-choice assay using whole fruits (Figure 1A), while *D. biarmipes* shows in this assay a mild and variable preference for ripe fruit purée. p values were calculated via Wilcoxon tests with comparison to a theoretical value of 0 (no preference) for each species, and via Kruskal-Wallis test followed by Dunn's test ($p < 0.001$) for interspecies comparisons. n = 10–19 replicates per species.

(C) Oviposition in *D. suzukii* increases in response to increasing concentrations of ripe strawberry purée. *D. melanogaster* hardly responds to this substrate, while *D. biarmipes* shows a moderate response. $logEC_{50}$ of *D. suzukii* (5.7%) and *D. biarmipes* (8.6%) are significantly different (measured via a non-linear regression analysis of dose response, $p < 0.001$); the $logEC_{50}$ of *D. melanogaster* (23.9%) is not statistically comparable to the other species due to a poor fit of the data. n = 16 replicates per species. Each replicate includes ten females.

(D) Oviposition response elicited by the odor of ripe fruit or water in *D. suzukii*, *D. biarmipes*, and *D. melanogaster*. In this no-choice egg-laying assay, females were placed in a chamber similar to the one depicted in Figure 1A and were offered an agar plate for egg laying. A source of odor placed at the center of the plate was covered by a mesh and therefore could not be directly contacted by the flies. *D. suzukii* laid abundantly on the agar when exposed to the odor of ripe strawberry purée but laid significantly less when exposed to water. By contrast, strawberry odors hardly elicited egg laying in *D. biarmipes* and did not elicit egg laying at all in *D. melanogaster*. p values were calculated via Mann-Whitney test. n = 28–30 replicates per condition (15 females and 0 males per replicate).

See also Figures S1 and S2.

with agarose at different concentrations and equally sweetened with glucose to stimulate ovipositioning (Figure 2A, top). We then measured and correlated the stiffness of agarose at different concentrations to that of strawberries at different stages (Figure S2A). Exposed to a choice between agarose at 0.25% (stiffness of a rotten fruit) and any stiffer substrate, up to 2.25% agarose (earlier fruit maturation stages), *D. melanogaster* and *D. biarmipes* always strongly preferred softer substrates. By contrast, *D. suzukii* displayed hardly any preference for softer substrates until the stiffer substrate reached 1.75% agarose (Figure 2A). We concluded that all three species exploit substrate stiffness for oviposition site selection, but in different ranges. The relaxation of the stiffness threshold observed in *D. suzukii* implies functional changes in the mechanosensory system of this species.

Chemical Cues Drive Species-Specific Oviposition Substrate Preferences

Chemical composition also changes with fruit maturation [12, 18]. To test its influence on oviposition site selection, we used substrates of fixed stiffness (1% agarose) containing either ripe or rotten strawberry purée (see Supplemental Experimental Procedures). We then tested oviposition preference in a two-choice assay (Figure 2B, top) in darkness (to circumvent slight color differences). The chemical stimuli were sufficient to recapitulate the egg-laying preference of *D. melanogaster* and *D. suzukii* on whole fruits (compare Figures 1B and 2B), while *D. biarmipes* showed an intermediate behavior (Figure 2B). We concluded that the chemical composition of the substrate is a primary determinant guiding oviposition site selection for *D. suzukii* and *D. melanogaster*. For *D. biarmipes*, the contrasting results

obtained with whole fruits (Figure 1B) or agar-based substrates (Figure 2B) suggest that this species has evolved a mild preference for the chemicals of ripe fruit that is balanced with a strong preference for soft substrates. The previous results suggest that chemical cues from ripe fruit elicit variable oviposition responses in different species. To compare the quantitative response to these cues, we exposed *D. suzukii*, *D. biarmipes*, and *D. melanogaster* females to a dilution series of ripe strawberry purée plates. We observed that the egg laying increased with the concentration of ripe fruit purée (Figure 2C). Although the dose response was shared by all species, *D. suzukii* responded much more, and at lower concentrations of ripe fruit purée, than *D. melanogaster* or, to some degree, *D. biarmipes*. These results show that the oviposition site preference of *D. suzukii* for ripe fruit is mediated at least in part by chemical cues. They also suggest that the chemosensory system involved in oviposition has changed in *D. suzukii* compared to *D. biarmipes* and *D. melanogaster*.

Strawberry Odors Are Sufficient to Evoke Oviposition in *D. suzukii*

We then set out to determine how *D. suzukii* females perceive the chemical cues that elicit their oviposition on ripening fruit. We first tested the sufficiency of olfaction to respond to these cues and elicit oviposition. Specifically, we asked whether the odor of ripe strawberries alone could evoke egg laying in *D. suzukii*, *D. biarmipes*, and *D. melanogaster*. We placed flies in a chamber containing agar plates with, at the center, a cup filled with ripe strawberry purée or water; the cup was covered with a metallic mesh allowing the flies to smell but not to contact its content (Figure 2D). The odor of ripe strawberries alone elicited oviposition by *D. suzukii* on plain agar, and to a lesser extent by *D. biarmipes* as well (Figure 2D). By contrast, it did not elicit *D. melanogaster* to lay eggs at all (Figure 2D). To eliminate the possibility that *D. melanogaster* did not lay eggs simply because females disliked plain agar, we created conditions for an oviposition baseline by supplementing the agar with 5% fructose. We then surveyed oviposition enhancement from this baseline upon exposure to fruit odor and found that the odor of ripe fruit enhanced oviposition in *D. suzukii*, but not in *D. melanogaster* (Figure S2B). Finally, we demonstrated that the oviposition enhancement in *D. suzukii* is not the indirect result of a stronger attraction to the odor source. First, replacing strawberry odors by acetoin, a potent attractant of *D. melanogaster* [19] and *D. suzukii* (Figure S2C), did not enhance *D. suzukii* oviposition (Figure S2B). Second, *D. suzukii* and *D. melanogaster* were equally attracted to ripe strawberry volatiles (Figure S2D). Together, these results reveal that ripe strawberry odors are sufficient to elicit oviposition in *D. suzukii*.

OR-Mediated Olfaction Elicits Oviposition in *D. suzukii*

To measure the contribution of olfaction to the selection of an oviposition site, we first ablated the antennae (the main olfactory organs) of female *D. suzukii*. In a two-choice assay with fruit purée plates, the ablated flies displayed a reduced preference for ripe fruit compared to the non-ablated control flies (Figure S2E), mostly due to a reduction of egg laying on ripe fruit substrate (Figure S2F). We concluded that olfaction from antennae is partially necessary for selecting between ripe and rotten fruits in *D. suzukii*. This also indicates that the maxillary palps (the other olfactory organs) or the perception of chemosensory cues by direct contact can partly compensate for the absence of antennae. We further analyzed the role of olfaction in egg laying in *D. suzukii* by impairing olfaction genetically. We focused on the odorant receptor (OR) system, one of the two olfactory receptor families expressed in antennae chemosensory neurons [20, 21]. We targeted the obligate co-receptor *Orco*, or the neurons that express it, to interfere with OR-mediated olfaction [19, 22].

We first generated a *D. suzukii Orco*-Gal4 line [19] to target *Orco*-positive sensory neurons (Figure S3A) and a UAS-CD4-tdTomato line [23] to visualize the projections of *Orco*-Gal4-positive cells. In *D. suzukii*, the *Orco*-Gal4 and UAS-CD4-tdTomato combination labels neurons projecting to the antennal lobe (Figures S3B_4 and B_5), consistent with what has been described in *D. melanogaster* (Figures S3C_4 and C_5). These neurons also express endogenous Orco, detected by an antibody in the antenna (Figures S3B_1–B_3 and C_1–C_3), showing that the *Orco*-Gal4 construct targets Orco-positive cells.

To block synaptic transmission and impair *Orco* neuron-mediated olfaction, we created a *D. suzukii* UAS-TNT transgenic line [24] and crossed it to our *Orco*-Gal4 line. In a trap assay with acetoin, an odor perceived by *Orco*-expressing neurons [19], we observed a severe reduction of attraction to acetoin (Figure S3D) in *Orco*-Gal4, UAS-TNT flies but normal locomotion (Figure S3E), confirming that these constructs impair OR-mediated olfaction. We then subjected *Orco*-Gal4, UAS-TNT *D. suzukii* females to the olfaction-evoked oviposition assay (Figure 2D) with ripe strawberry odors. We found that olfaction-evoked oviposition was almost abolished compared to the control genotypes (Figure 3A), showing that *Orco*-positive neurons are involved in the oviposition elicited by ripe fruit odors in *D. suzukii*.

We also generated *D. suzukii Orco* mutants using the CRISPR-Cas9 system [25]. We obtained three alleles, named *Dsuz\Orco*^{1}, *Dsuz\Orco*^{2}, and *Dsuz\Orco*^{3} (Figure S4A). These mutants are protein null (Figure S4B). While wild-type *D. suzukii* antennae respond to ripe strawberry odor and to the control odor acetoin in electroantennograms (EAGs), mutant antennae did not respond to either smell (Figure S4E). Consistent with this data, the attraction of *Dsuz\Orco* mutants to acetoin (Figure S4C) or ripe strawberry odors (Figure 3B) was severely impaired, although their locomotion was unaffected (Figure S4D), revealing that they are loss-of-function alleles.

We then exposed *Dsuz\Orco* mutants to the olfaction-evoked oviposition assay (as in Figure 2D). The mutant females were not stimulated, or were significantly less stimulated, to lay eggs in response to ripe strawberry odors compared with the control genotypes (Figure 3C), similar to what we observed upon silencing the Orco-expressing neurons (Figure 3A). Altogether, these results reveal that the OR subsystem is essential in *D. suzukii* for the perception of ripe fruit volatiles and the oviposition elicited by these odors.

Finally, we tested the oviposition preference of the *Dsuz\Orco* mutants in a two-choice assay with whole ripe and rotten strawberries (Figure 1A). We found no difference in oviposition preference between the *Dsuz\Orco* mutants and the wild-type controls (Figure 3D), revealing that OR-mediated olfaction becomes redundant for egg-laying site selection when other

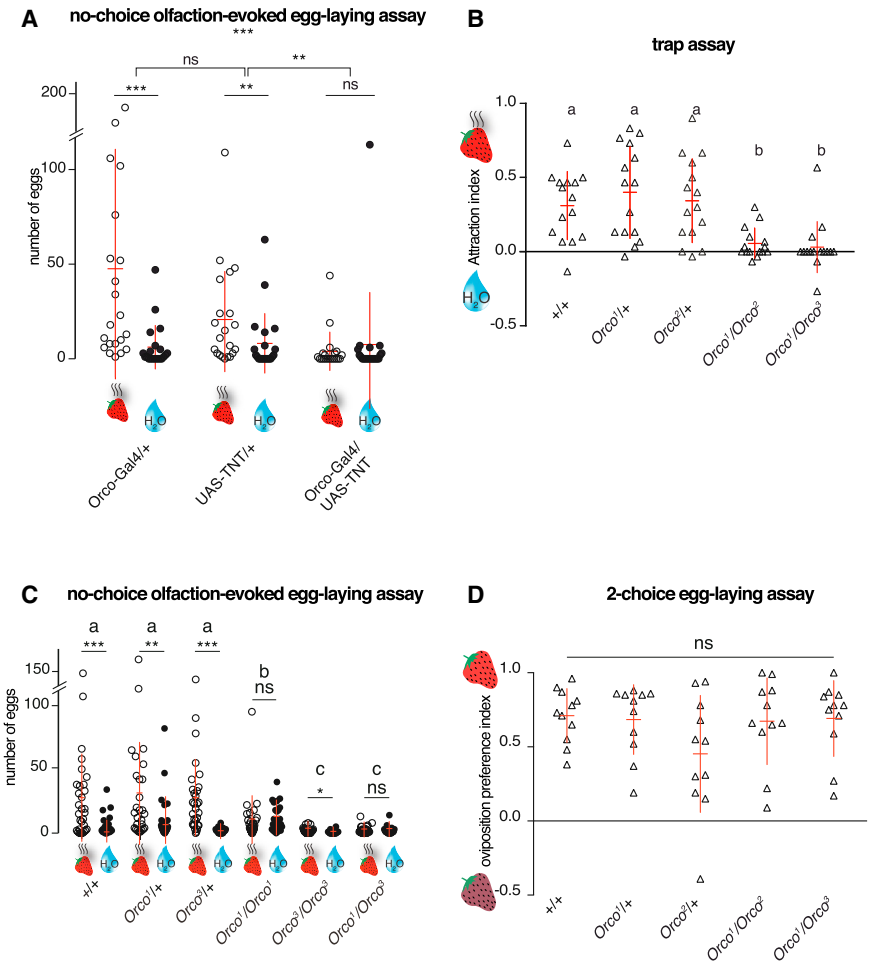

Figure 3. *Orco* and *Orco*-Expressing Neurons Mediate Oviposition Enhancement in Response to the Odor of Ripe Strawberries in *D. suzukii*

(A) In a no-choice olfaction-evoked egg-laying assay (as depicted in Figure 2D), the enhanced egg laying elicited by the odor of ripe fruits in *D. suzukii* was abolished when neurotransmission was specifically blocked in *Orco*-positive neurons. n = 21 replicates per condition (15 females and 0 males per replicate). p values were calculated via Mann-Whitney test for each genotype, using a negative binomial generalized linear model followed by a general linear hypothesis test for multiple comparisons with a false discovery rate (FDR) correction method. n = 21 replicates per condition (15 females and 0 males per replicate).

(B) The loss of *Orco* function in *D. suzukii* prevents attraction to the odors of ripe strawberries in a trap assay against water. Significant differences are denoted by letters (ANOVA followed by Tukey's test for multiple comparison; $p < 0.001$). n = 10–13 replicates per condition (15 males and 15 females per replicate).

(C) The enhanced egg laying elicited by the odor of ripe fruits in a no-choice olfaction-evoked egg-laying assay was also significantly diminished or abolished in *D. suzukii* when the function of *Orco* is lost. p values were calculated via Mann-Whitney test for each genotype, using a negative binomial generalized linear model followed by a general linear hypothesis test for multiple comparisons with a FDR correction method to compare the genotypes. n = 26–30 replicates per condition (10 females and 0 males per replicate).

(D) In a two-choice assay with whole strawberries, in which the flies could contact and presumably taste the fruits, *D. suzukii Orco* mutants preferred to lay eggs on ripe fruits as much as wild-type females did. n = 11 replicates per condition (15 females and 0 males per replicate) (ANOVA followed by Tukey's test for multiple comparison; $p < 0.001$).

See also Figures S3 and S4.

sensory stimuli are available. Consistent with this, when females in which Orco neuron output is silenced could contact strawberry purée, and presumably taste it, they laid eggs at levels comparable to wild-type flies (Figure S3F). These results suggest that contact chemosensation, in conjunction with olfaction, also contributes to the oviposition behavior of *D. suzukii* on ripe fruit.

A Multi-step Evolutionary Scenario for the Making of a Pest Species

The evolution of *D. suzukii* as a pest species could be regarded as the result of a single key innovation: its enlarged, serrated ovipositor that enables females to pierce the skin of many ripe fruits. We have found that the egg-laying substrate preference of *D. suzukii* has evolved in concert with its morphology and was instrumental in the shift to a new reproductive niche. Our work shows that the divergence in oviposition behavior is associated with the modification of multiple sensory modalities, namely mechanosensation and chemosensation, that determine differences in the egg-laying site choice between *D. melanogaster*, *D. biarmipes*, and *D. suzukii*.

The comparison of *D. suzukii* with multiple closely related species, in particular *D. biarmipes*, suggests a possible scenario for the evolution of *D. suzukii* as a pest species (Figure 4). In this scenario, oviposition in species like *D. melanogaster* is strongly elicited by rotten fruit and is inhibited by stiff substrates. In the lineage leading to *D. suzukii*, the oviposition response to ripe fruit has progressively increased, as in *D. biarmipes*, whose behavior is intermediate between that of *D. melanogaster* and *D. suzukii*. Such species, however, can only exploit ripe or slightly damaged fruit of sufficient softness. Presumably, the small ovipositor in these species prevented the full exploitation of the ripe fruit niche. Only in *D. suzukii*, and its close relative *D. subpulchrella* [9], did chemosensory specialization for ripe fruit cues, broadening of substrate stiffness preference, and evolution of an enlarged ovipositor come together and endow the flies with the capacity to fully use ripe fruit as oviposition substrates. Finally, additional physiological adaptations (e.g., metabolic changes [27]) may have provided *D. suzukii* with the potential to adapt to different environments and invade new geographical areas. In this stepwise scenario, the evolution of the ovipositor of *D. suzukii* was certainly a key acquisition, but it was secondary

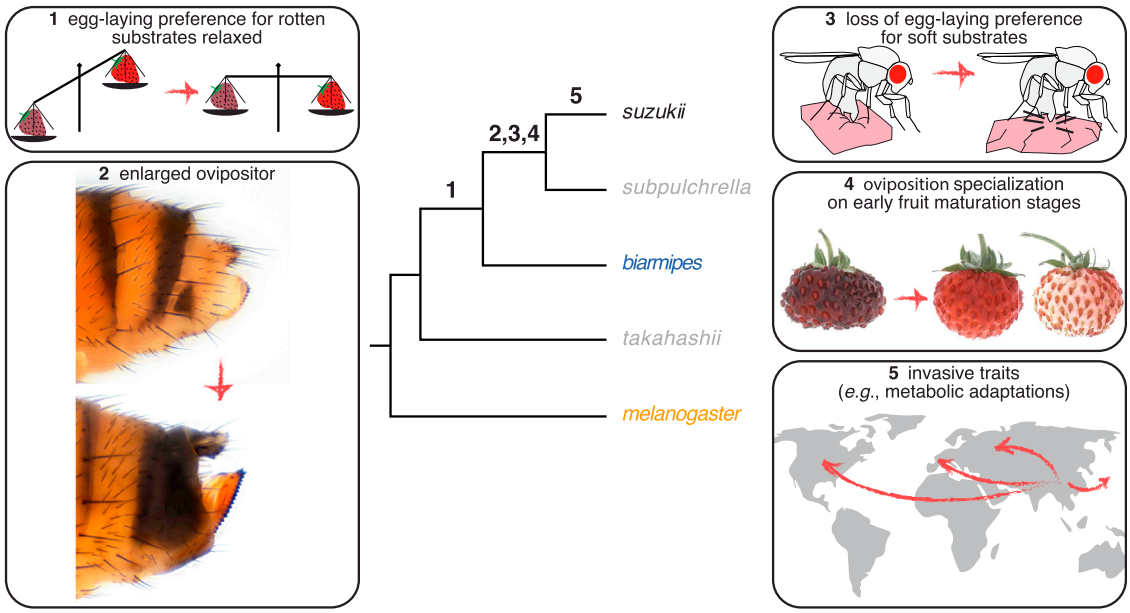

Figure 4. A Possible Evolutionary Scenario for the Shift in *D. suzukii* Oviposition Behavior

The emergence of *D. suzukii* as a pest species is associated with multiple evolutionary changes, either identified or suspected. These comprise (1) a broadening of the range of possible oviposition sites to include earlier fruit maturation stages, (2) the morphological expansion and strengthening of the ovipositor, (3) the tuning of mechanosensory preferences to accept stiffer substrates, (4) the tuning of chemosensory modalities to earlier stages of fruit maturation in the context of egg laying, and (5) physiological and metabolic changes enabling the fly to spread across a broad geographic range. The order of these changes is speculative, but the intermediate state of *D. biarmipes* for some of these traits suggests the genetic potentiation of the clade containing these species to gain such traits [26].

to the behavioral changes that endowed some ancestors with an opportunistic egg-laying behavior toward ripe fruits.

We propose that the evolutionary origin of *D. suzukii* as a pest species was therefore made possible by the progressive tuning of multiple sensory systems, which might be mirrored in changes in its sensory receptor genes [28, 29] or the determinants of neuronal connectivity. Our results suggest that these traits may have emerged in a clade predisposed [26] for this behavioral shift.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2017.01.055.

AUTHOR CONTRIBUTIONS

B.P. and N.G. conceived the project. B.P., N.G., M.K., L.B.B., and S.L. designed the experiments. M.K., L.B.B., and S.L. carried out the behavioral experiments and the statistical analyses. C.M. and M.K. built the constructs used in Figure 3 and S3 (Orco-Gal4 [C.M.]; UAS-CD4-tdTomato and UAS-TNT [M.K.]). M.C. characterized the expression of Orco and reporter genes (Figures S3 and 4). B.P., C.M., and M.K. generated all transgenic and CRISPR mutant lines. N.G. documented the strawberry stages. I.C.G.K. and K.P.S. designed and analyzed the electrophysiology experiments (Figure S4E), which K.P.S. carried out. B.P. and N.G. wrote the manuscript with the help of all authors.

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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

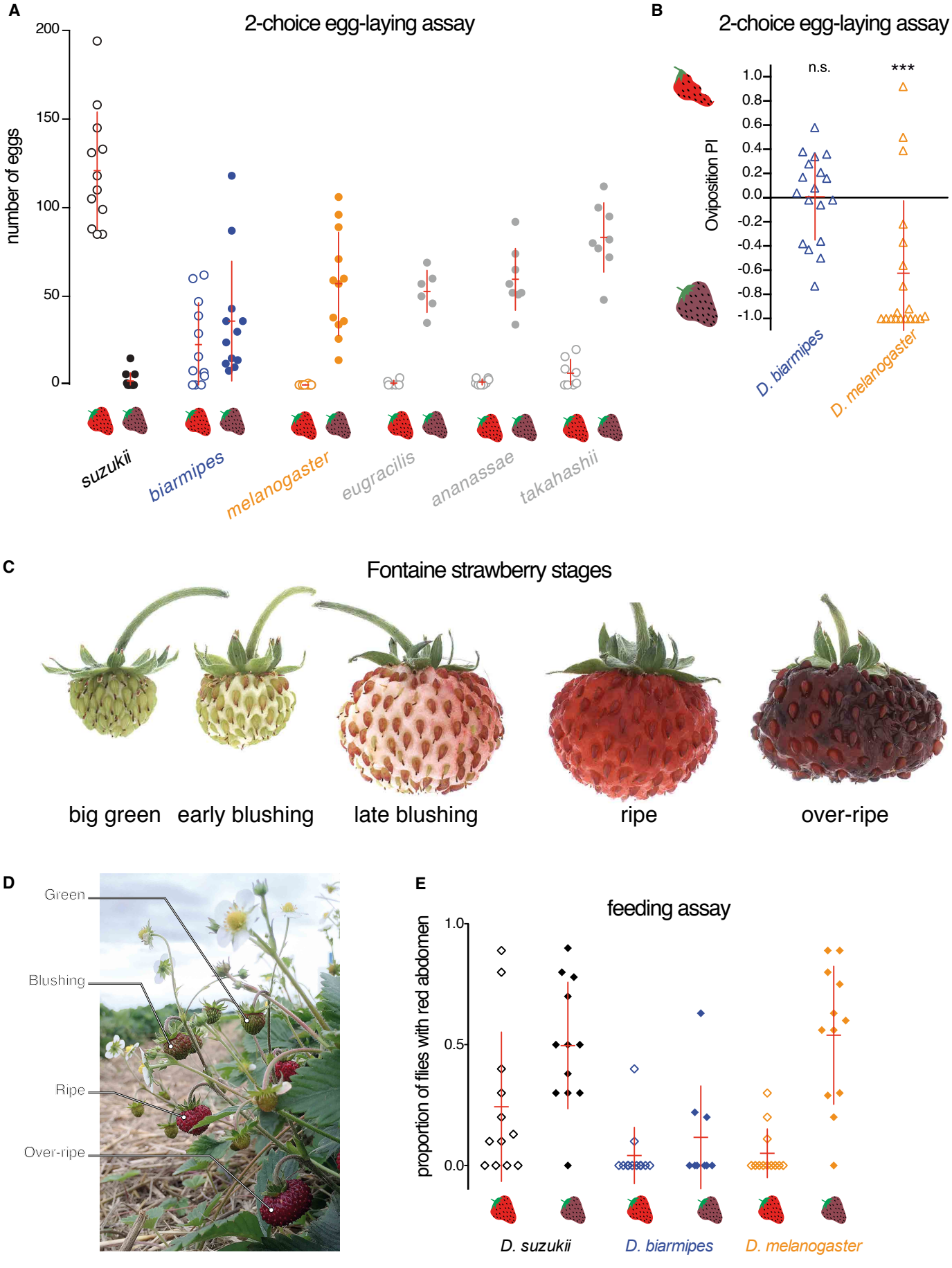

A
2-choice egg-laying assay
number of eggs
200
150
100
50
0
suzukii
biarmipes
melanogaster
eugracilis
ananassae
takahashii
B
2-choice egg-laying assay
Oviposition PI
n.s.
***
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
D. biarmipes
D. melanogaster
C
Fontaine strawberry stages
big green
early blushing
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

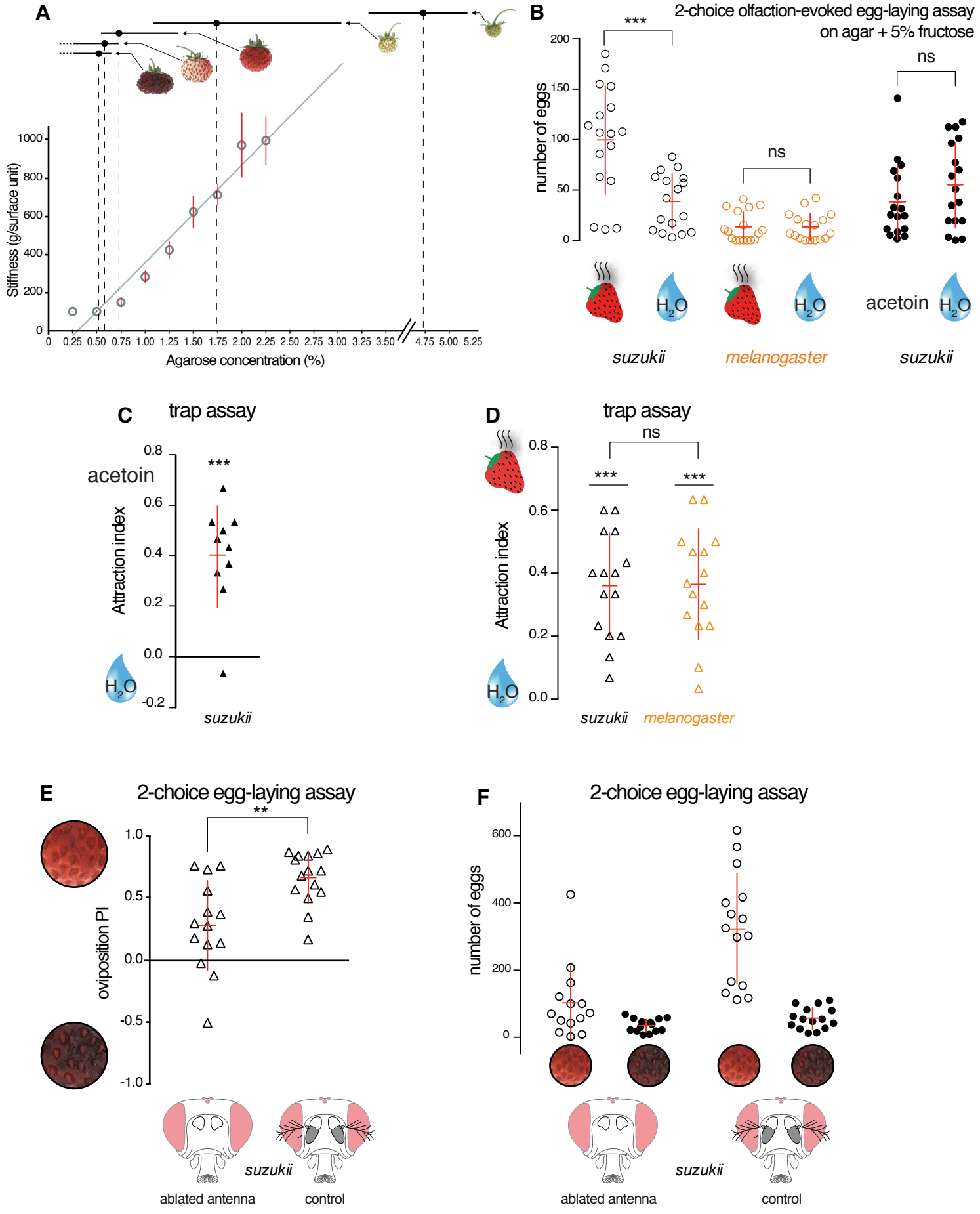
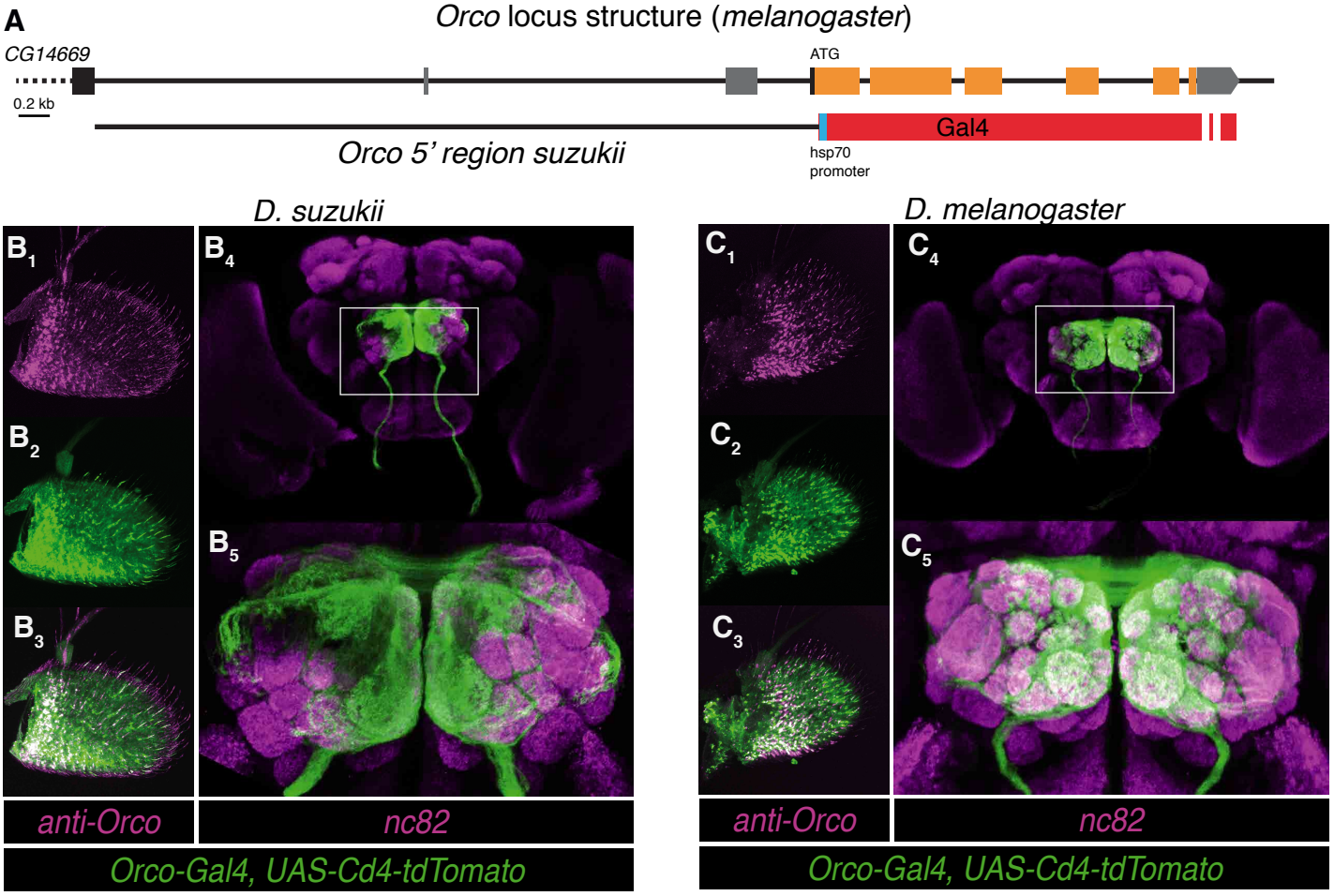
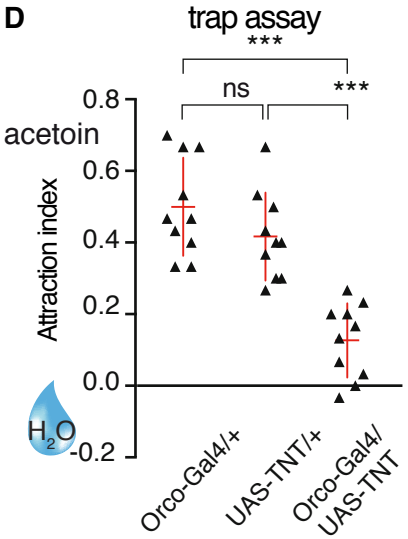
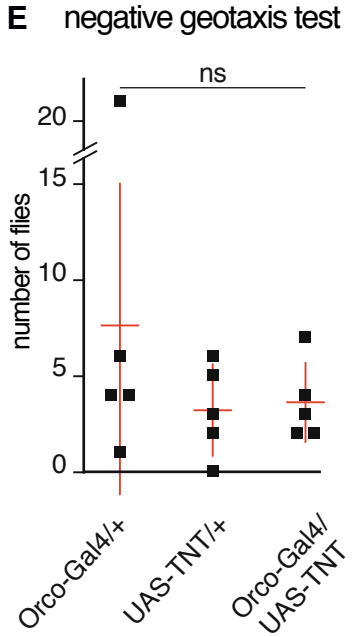

A
Stiffness (g/surface unit)
Agarose concentration (%)
B
2-choice olfaction-evoked egg-laying assay
on agar + 5% fructose
number of eggs
***
ns
ns
H2O
acetoin
suzukii
melanogaster
suzukii
C
trap assay
acetoin
Attraction index
***
H2O
suzukii
D
trap assay
ns
***
***
Attraction index
H2O
suzukii
melanogaster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

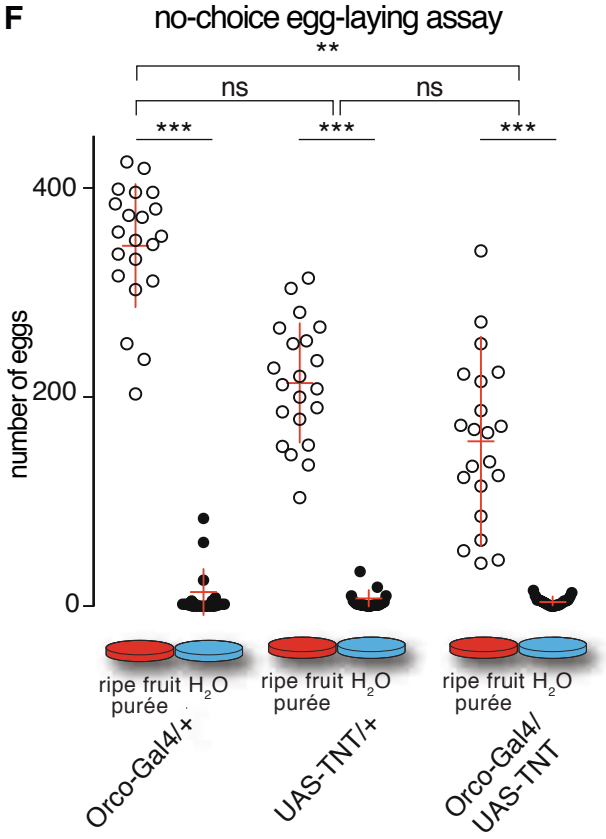
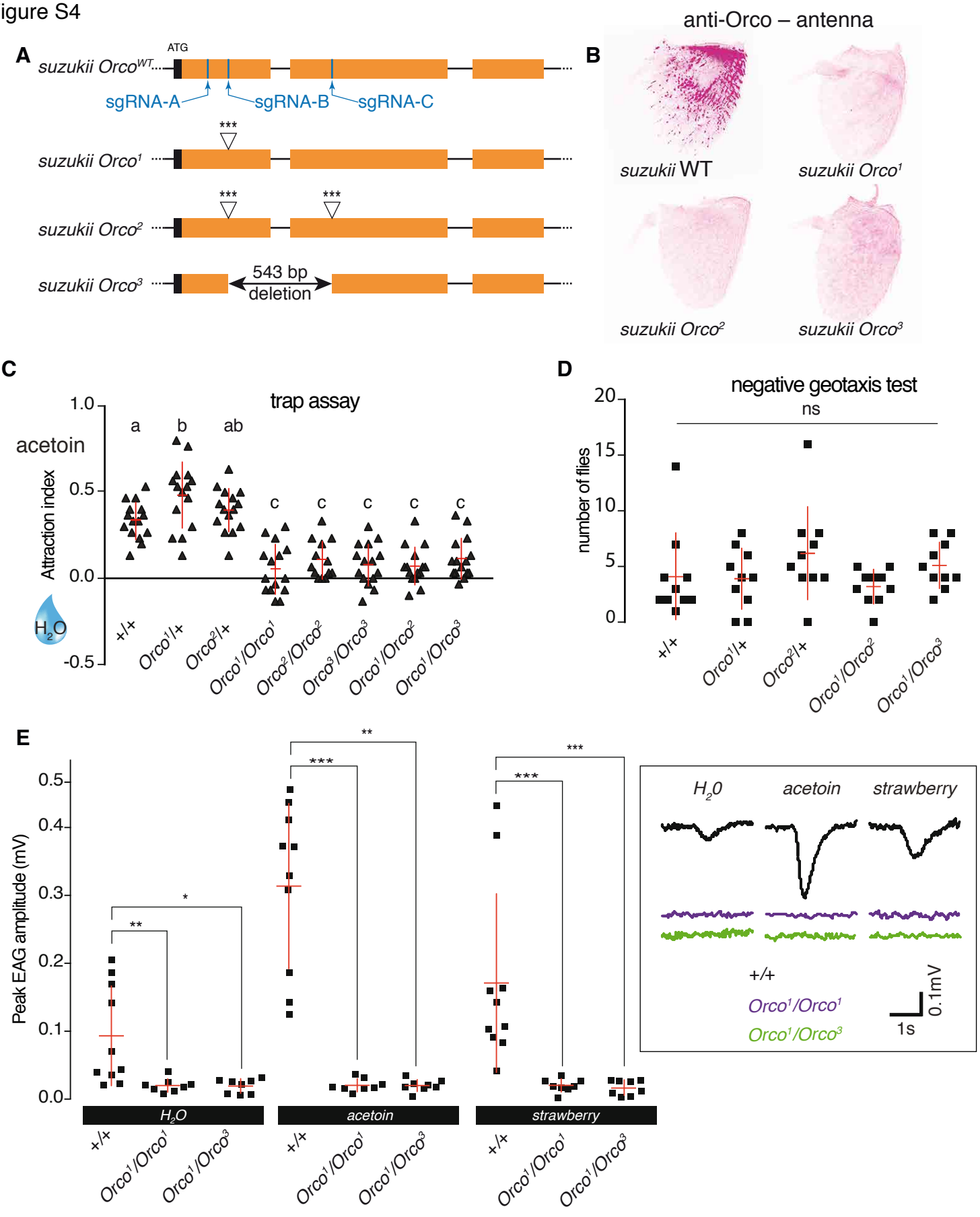

F
no-choice egg-laying assay
**
ns
ns
***
***
***
number of eggs
400
200
0
ripe fruit H2O
purée
ripe fruit H2O
purée
ripe fruit H2O
purée
Orco-Gal4/+
UAS-TNT/+
Orco-Gal4/
UAS-TNT

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

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