An Orco dependent pathway suppresses egg-laying attraction to ripe fruit volatile compounds in *Drosophila melanogaster* but not in *D. suzukii*

Sebastien Lebreton[1], K. P. Siju[2], Matthieu Cavey[1], Caroline Minervino[1], Maïlys Delcroix[3], Xavier Fernandez[3], Thomas Michel[3], Ilona C. Grunwald Kadow[2,4], Benjamin Prud'homme[1*]

[1] Aix-Marseille Université, CNRS, IBDM, Institut de Biologie du Développement de Marseille, Campus de Luminy Case 907, 13288 Marseille Cedex 9, France

[2] Technical University of Munich, School of Life Sciences, Liesel-Beckmann-Str. 4, 85354 Freising, Germany

[3] Université Côte d'Azur, CNRS, Institut de Chimie de Nice, UMR 7272, 06000 Nice, France

[4] Institute of Physiology, Faculty of Medicine, University of Bonn, Germany, Nussallee 11, 53115 Bonn, Germany

[*] Corresponding author: benjamin.prudhomme@univ-amu.fr

Abstract

Background

Most Drosophila species lay their eggs on a wide range of overripe or rotting fruits. By contrast, *D. suzukii* has evolved a strong preference for undamaged ripe fruits, a shift that has made it one of the most important pests of small fruits worldwide. Growing evidence suggests that this host preference shift is linked to modifications in the fly's olfactory system. Unlike *D. melanogaster, D. suzukii* is attracted by ripe strawberry volatile compounds for oviposition.

Results

In this study, we investigated the mechanisms underlying this behavioral divergence. We identified two strawberry volatiles, hexanoic acid and methyl butyrate, that, together recapitulate the species-specific oviposition preferences. *D. suzukii* is attracted by this two-component blend, whereas in *D. melanogaster* attraction to this blend is suppressed by an OR-dependent mechanism. *In vivo* calcium imaging of the antennal lobes revealed that the perception of these compounds is largely conserved between species, with only subtle differences. This suggests that the divergence in the olfactory perception of this two-component blend arises downstream of sensory neuron activity, within higher levels of olfactory processing.

Conclusions

Taken together, our results suggest that *D. melanogaster* has evolved an as-yet unidentified mechanism for integrating olfactory signals that suppresses attraction to unsuitable oviposition substrates when multiple key volatile are detected simultaneously.

Keywords: evolution, behavior, egg-laying preference, Drosophila, olfaction, antennal lobes, odorant receptors, fruit volatile compounds

Background

Insects must locate suitable hosts to lay their eggs, and most species rely heavily on their olfaction to do this. They detect ecologically relevant volatile compounds that signal appropriate oviposition sites, leading to attraction and egg deposition. However, even closely related species may evolve distinct ecological requirements, resulting in differences in odor perception and oviposition behavior [1].

The evolution of oviposition behavior has been extensively studied in Drosophilids. Some species, such as *D. melanogaster* or *D. simulans*, are generalists that oviposit on a wide variety of fruits, whereas others, like *D. sechellia* or *D. erecta*, specialize on specific hosts [2]. However, most Drosophila species lay their eggs on decaying fruits, where yeasts provide essential proteins for larval development and strongly influences oviposition preference.

D. suzukii represent a striking exception. While it also uses decaying fruit and yeast as resources, it has expanded its host range to include ripe, undamaged fruits, with a strong preference for the latter. This host shift has made *D. suzukii* a major global pest of small fruits and berries worldwide [3–5]. A key adaptation underlying this expansion is its elongated, serrated ovipositor, which enables females to pierce intact fruit skins – a feat impossible for most other Drosophila species [6, 7].

In nearly all documented cases of ecological specialization, behavioral shifts are paralleled by changes in the olfactory system, altering how flies perceive and respond to fruit volatiles [8–11].

The fly olfactory system has been extensively studied, and the response profiles of most the receptors have been described in *D. melanogaster*. Volatile compounds are detected by odorant receptors expressed in olfactory sensory neurons (OSNs) housed in sensilla on the antennae and maxillary palps [12]. Two receptor families mediate insect olfaction: olfactory receptors (ORs)[13] and ionotropic receptors (IRs) [14]. Both require co-receptors for function. ORs depends on the unversal co-receptor Orco [15, 16], whereas IRs use multiple co-receptors, including Ir8a, Ir25a and Ir76b [17].

OSNs project onto the antennal lobes, the first olfactory processing center in nhe brain [18]. The antennal lobes are divided into functional subunits called glomeruli, each receiving input from

OSNs expressing the same receptor. Within the antennal lobes, OSNs connect to second-order projection neurons (PNs), which relay information to higher brain centers [19]. Local interneurons (LNs) interconnect glomeruli, refining olfactory input and shaping odor perception between OSNs and PNs [20].

The evolution of oviposition behavior in Drosophila has frequently been linked to changes in receptor function or OSN sensitivity. For example, in the specialist *D. sechellia*, the tuning of several receptors such as Or22a, Ir75a and Ir75b has shifted towards the detection of specific volatile compounds emitted by Morinda fruit, contributing to the species' exclusive host preference [9, 11, 21–23]. In *D. erecta*, a specialist on Pandanus fruit, the sensitivity of Or22a has also evolved to detect host-specific compounds [9]. In *D. suzukii*, OSNs expressing Or22a specifically detect a leaf volatile that is attractive to this species and may facilitate the search for habitats containing ripe fruit [8, 24]. Furthermore, the coding of several key environmental cues in the antennal lobes has diverged between *D. suzukii* and *D. melanogaster* [25].

Although changes in odorant receptor tuning play a central role in the evolution of olfactory-driven behavior, downstream modifications in the olfactory pathway may also contribute. For instance, differences in the wiring of PNs receiving input from Or22a-expressing OSNs have been reported between *D. melanogaster* and *D. simulans* compared to *D. sechellia* [11].

In this study, we focused on comparing *D. suzukii* and *D. melanogaster* to investigate the opposite behavioral responses to ripe strawberry odors. We identified a blend of two key compounds that is sufficient to recapitulate this divergence, and determined the receptor families involved. Finally, we examined how these compounds are represented in the antennal lobes of the two species to assess whether differences at the OSN level could account for the observed behavioral divergence.

Methods

Fly stocks and husbandry

All flies were maintained on home-made Nutrifly food (http://flystocks.bio.indiana.edu/Fly_Work/media-recipes/germanfood.htm) and reared at 21°C. A stripe of Whatman paper was added in the rearing tube to facilitate pupation. For *D. suzukii*, we used the genomic line WT3 as the wild type strain. For *D. melanogaster*, Oregon R and Canton S lines were used as wild type lines. The w^{1118} line was used as a control for mutant and transgenic lines in a w^- background. Isofemale lines of *D. biarmipes*, *D. eugracilis*, *D. pseudoobscura, D. willistoni* were obtained from the National Drosophila species stock center at Cornell, *D. simulans* and *D. teissieri* were obtained from Virginie Orgogozo. To study the role of Orco-mediated olfaction, the *D. melanogaster* $Orco^1$ and *D. suzukii* $Orco^3$ mutant lines were used [26]. In addition, a *D. melanogaster* Orco-Gal4 line was crossed with a uas-Kir2.1line to inactivate Orco-positive OSNs. To study the role of IRs, Ir8a and Ir25a mutants were used. Ir25a mutants were obtained by crossing two lines carrying a different mutation of the Ir25a gene ($Ir25a^1$ and $Ir25a^2$).
For calcium imaging, Orco-Gal4 lines (*D. suzukii* [26]) and (*D. melanogaster* [15]) were crossed to UAS-GCaMP lines (*D. suzukii* [27]) and (*D. melanogaster* [28]).

Behavioral assay

Description of the assay

A two-choice egg-laying assay was used to assess the oviposition preferences of flies for different volatile compounds. In this assay, ten females (7-10 days old) were placed in a chamber (12 cm x 6 cm x 4 cm) with a choice between two egg-laying plates. Each plate consisted of two parts, as previously described [26]: the inner compartment contained the odor source (or a control) and was covered with a 3D-printed mesh to prevent direct contact of the odor source, while the outer compartment contained an egg-laying substrate made of 5% fructose and 1% agar diluted in water (0.5% agar was used for experiments inolving species other than *D. melanogaster* and *D. suzukii*,). Assays were conducted at 23°C and 60-70% humidity under dark conditions. After 24h, flies were removed from the chambers and eggs were counted. An Oviposition Index was calculated as the number of eggs laid on the stimulus plate minus the number of eggs laid on the control plate,

divided by the total number of eggs laid. Therefore, a positive value indicates a preference for a stimulus while a negative value indicates an avoidance of this stimulus.
A no-choice egg-laying assay was used to assess the stimulatory effect of the odor source on the egg-laying activity of the flies. The experiment was performed as described above except that only one egg-laying plate was provided. The number of eggs was counted after 24h and compared between conditions.

Preparation of the odor source

Frozen organic unsweetened strawberry purée (Sicoly®) was used as the natural strawberry odor source. The strawberry purée was thawed overnight at 4°C and brought to room temperature prior to the experiment. Approximately 3 mL of strawberry purée was added to the inner compartment of the stimulus plate, while an equal volume of water was added to the control plate.
For experiments with synthetic compounds, 100 µL of pure compound was mixed into 100 mL of water containing 1% of agar (final concentration [10^{-3}] (v/v); modified after Lin [29]). The synthetic compound was added once the agar solution had cooled down to 40-50°C. The mixture was homogenized for 1 min, and 5 mL was quickly poured into the inner compartment of each stimulus plate quickly before solidification. Control plates received 5 mL of plain agar (1%) in the inner compartment of the plate. For lower concentrations [10^{-4}] or [10^{-5}], 100 µL of a 10% or 1% dilution of the compound, respectively, was added to 100 mL of plain agar. Following an initial drop in releasing rate just after the agar was poured, the synthetic compounds were gradually released from the egg-laying plates with only a slight, consistent decrease over time (Figure S2D; see "Chemical analysis" for details).

Chemical analysis

Review literature of strawberry volatile compounds

Since *D. melanogaster* and *D. suzukii* are generalist species, their behavioral response to strawberry volatile compounds are expected to be largely independent of specific commercial varieties. To identify the major volatile compounds typically released by strawberries, a literature review was

conducted covering 15 different varieties reported across four studies [30–33]. These studies employed various extraction methods, including Solid-Liquid Extraction and headspace solid phase micro-extraction (HS-SPME). Only data corresponding to ripe or fully red fruits were included in our analysis. When multiple treatments were compared in a given study, only untreated (control) fruits were considered. For each compound reported, the quantity was converted to a percentage of the total volatile profile. Only compounds accounting for at least 0.1% of the total volatiles are reported here.

Chemical analysis of compounds released from egg-laying plates

To assess the release profile of synthetic compounds from the egg-laying substrate, 1% agar was first dissolved in water and heated under stirring until a homogeneous solution was obtained. Once the temperature reached 40 °C, methyl butyrate and hexanoic acid (each at 10% in water) were added. The mixture was stirred vigorously for 1 minute using a metal spatula, and 5 mL of the blend was poured into a 20 mL headspace vial. Vials were sealed immediately prior to gas chromatography–mass spectrometry (GC–MS) analysis.

Volatile compound extraction was performed using static headspace (SHS) sampling. Vials were incubated at 30 °C for 30 minutes, after which 500 µL of the headspace was injected in splitless mode using a 2 mL SHS syringe. Four SHS replicates were performed.

GC separation was carried out on an apolar HP-1 column (Agilent; 100% dimethylpolysiloxane, 50 m × 200 µm × 0.33 µm i.d.), with helium as the carrier gas at a flow rate of 1 mL/min. The temperature program was as follows: from 40 °C to 55 °C at 1 °C/min, then to 85 °C at 3 °C/min, followed by a ramp to 270 °C at 25 °C/min, and a final hold at 270 °C for 10 minutes.

For improved quantification, GC–MS was performed in single ion monitoring (SIM) mode. Methyl butyrate was monitored using m/z 74 and 87 (from 6 to 15 minutes), while hexanoic acid was tracked using m/z 60 and 73 (from 15 minutes onward). Peak areas for each compound were measured over a 26-hour period. These areas are proportional to compound concentrations in the headspace and provide an initial estimate of the release kinetics of the odorants from the agar substrate.

In vivo calcium imaging

Female flies (4–8 days old) of the appropriate genotypes were used for in vivo calcium imaging. Flies were prepared for imaging following a previously described protocol [34]. Imaging was performed using a Leica DM6000 FS upright wide-field fluorescence microscope equipped with a 40×/0.80 NA water-immersion objective and a Leica DFC9000GT fluorescence camera. Time series images were acquired at approximately 25 frames per second in 4×4 binning mode using the Leica LAS X acquisition software.

Using a CS-55 (Syntech, Germany) odor delivery system, throughout the experiments, a continuous charcoal filtered air of 1000 ml/min main airflow was delivered to the fly antenna through an 8 mm Teflon tube positioned 1 cm away from the fly. The odorants, hexanoic acid (10^{-3}) and methyl butyrate (10^{-4}) were diluted in water and the blend was prepared by mixing diluted hexanoic acid and methyl butyrate at 10:1 ratio respectively. Stimulus was delivered to the fly by redirecting 300 ml/min main flow as stimulus flow for 1 sec through a head space vial containing 5 ml of diluted odorant which later rejoined the main flow.

Data analysis

Data were processed using a modified version of an image analysis pipeline described previously[34, 35] and the program codes are available on github https://github.com/sophie63/FlyLFM. Briefly, acquired time series image was first opened in FIJI as image sequence and rigid body transformed using StackReg plugin, thereafter the file was converted to Nifti file format. From this voxel time series file dF/F values were extracted by subtracting and normalizing by a moving average over 20 sec and further noise reduction was achieved by Kalman filtering. Resulting file was used to extract functional regions using principal component analysis (PCA) and independent component analysis (ICA). Well delineated functional components were manually sorted and matched to different antennal lobe glomeruli using previously published AL map references [36, 37]. The relative position of glomeruli within the antennal lobes of *D. melanogaster* and *D. suzukii* are overall well conserved between the two species [36]. The corresponding peak dF/F values from the glomeruli were extracted and further processed for statistical analysis.

Statistical analysis

Oviposition indices were compared to a theoretical value of zero (no preference) using a Wilcoxon's signed rank test. Comparisons between two groups were performed using the non-parametric Mann–Whitney U test. When comparing more than two groups, a Kruskal–Wallis test followed by Dunn's multiple comparison test was applied.

The stimulatory effect of the different stimuli on the egg-laying rate of *D. suzukii* females was compared using a Generalized Linear Model with a negative binomial distribution followed by a multiple comparison test (ghlt, multcomp package) with an fdr adjustment method.

For calcium imaging recordings, to avoid differences due to basal activities and /or calcium sensor activities between species, the activity elicited by water (solvent) was subtracted to the activity obtained for the different stimuli in each glomerulus for each fly. Because the data followed a Gaussian distribution (as confirmed by the Kolmogorov-Smirnov Normality test), responses to a given stimulus were then compared between species in each glomerulus using a t-test. Statistical analyses were computed with R (R 2.1.1, R Development Core Team, Free Software Foundation Boston, MA, USA). Graphics were made either with GraphPad Prism6 or R. Figures were assembled with Adobe Illustrator CS6.

Results

Orco-mediated olfaction blocks egg-laying attraction to strawberry volatile compounds in *D. melanogaster*

To identify and compare species-specific olfactory-driven egg-laying preferences, we used a behavioral assay in which flies were given a choice between two fructose-containing agar substrates: one with a ripe strawberry puree in the center and one with water. Each odor source was covered with a mesh that allowed the diffusion of volatile compounds but prevented the flies from touching, and therefore tasting, the source (Figure 1A).

In this assay, wild-type *D. suzukii* are consistently attracted to lay eggs around the ripe strawberry odor source (Figure 1B, C). The attraction to strawberry volatiles was actually not restricted to *D.*

suzukii, but was widespread among the different species tested (Figure 1B). In addition to *D. suzukii*, *D. biarmipes*, *D. eugracilis*, *D. teissieri* and *D. pseudoobscura* were attracted while *D. willistoni* showed a modest, non-significant preference. In contrast, *D. simulans* and *D. melanogaster* were not attracted to lay eggs by strawberry volatiles. In fact, *D. simulans* was neutral whereas the two strains of *D. melanogaster* tested (Canton S and Oregon R) were significantly repelled (Figure 1C). Remarkably, the behavioral response of *D. melanogaster* to strawberry volatiles was more variable than that of *D. suzukii*, ranging from a significant avoidance (Figure 1B, C) to indifference (Figure S1A).

We then focused on *D. suzukii* and *D. melanogaster*, which show divergent olfactory-driven oviposition preferences, and asked which family of odorant receptors is involved in this difference. To test the role of ORs in the divergence of the response to strawberry volatiles in *D. suzukii* and *D. melanogaster*, we analyzed the egg-laying preference of *Orco* mutant flies. In *D. suzukii*, Orco was not necessary to induce an attraction to strawberry volatiles, suggesting the involvement of IRs in this attraction (Figure 1C, S1B). In contrast, *D. melanogaster Orco* mutants were attracted to strawberry volatiles to lay eggs (Figure 1C). These results show that some ORs repress the attraction to ripe fruit odors in the context of oviposition in *D. melanogaster* but not in *D. suzukii*.

Two key compounds, hexanoic acid and methyl butyrate, are involved in the divergent behavioral response to strawberry volatile compounds

Ripe strawberries release dozens of volatile compounds (Figure S2A) [30–33]. We first sought to identify the key volatile compounds involved in the behavioral divergence between *D. suzukii* and *D. melanogaster*. We therefore replaced strawberry puree with 23 individual synthetic compounds present in strawberry and/or other host fruits in our olfactory-guided egg-laying preference assay (Figure 2A). While most compounds had no significant effect on the egg-laying behavior of *D. suzukii*, four of them (1-hexanol, linalool, butyl acetate, and nerolidol) were significantly repellent (Figure 2A). On the other hand, hexanoic acid and methyl butyrate, two chemicals present in the strawberry headspace (Figure S2A), were the only compounds for which we observed an attraction in *D. suzukii* (Figure 2A).

Hexanoic acid and methyl butyrate elicited similar behavioral responses in *D. suzukii* and *D. melanogaster*, at the different concentrations tested (Figure 2B,C). However, when presented together, the attraction was lost in *D. melanogaster* but not in *D. suzukii* (Figure 2D). Similar to the strawberry odor, attraction to this two-component blend was not restricted to *D. suzukii* (Figure 2E). Indeed, *D. biarmipes*, *D. eugracilis*, *D. simulans* and *D. willistoni* were significantly attracted (Figure 2E). Four species, including *D. suzukii* and *D. melanogaster*, showed a similar behavioral response to the synthetic blend and the strawberry odor (Figure 1B, 2E). Only the most evolutionarily distant species, *D. pseudoobscura* and *D. willistoni*, showed a clearly divergent behavioral response to the two stimuli (Figure 2E). The loss of preference for the blend of hexanoic acid and methyl butyrate in *D. melanogaster* is unlikely to be due to a chemical reaction when the two compounds are mixed, as both are released from the substrate at a relatively stable rate over the course of the experiment (Figure S2B). As for the strawberry odor, the response to the synthetic blend showed some variability in *D. melanogaster*, being either neutral (Figure 2D, 3C, 3D) or repulsive (Figure 3E, S3), but not attractive as in *D. suzukii*. Furthermore, our two-component blend not only attracts *D. suzukii* to lay eggs, but it also stimulates oviposition (Figure S2C).

IRs are likely to be involved in the attraction to the blend of hexanoic acid and methyl butyrate, whereas ORs suppress this attraction in *D. melanogaster*

We next wanted to determine which family of olfactory receptors was involved in the behavioral response to hexanoic acid and methyl butyrate. We first examined the responses to individual compounds and found that IRs are most likely involved in the attraction to hexanoic acid in both *D. melanogaster* and *D. suzukii*, as Orco mutants remain attracted to this compound (Figure 3A). In contrast, Orco mutants in both species are no longer attracted to methyl butyrate (Figure 3B). In this experiment, however, *D. melanogaster* control heterozygous flies were also not attracted to this compound, unlike wild-type flies.

We next examined the response to the blend of hexanoic acid and methyl butyrate. As with the strawberry odor, *D. melanogaster* Orco mutants were attracted, whereas the attraction of *D. suzukii* was unaffected (Figure 3C). The effect of Orco-mediated olfaction in *D. melanogaster* was confirmed when Orco-positive OSNs were inhibited (Figure S3). In contrast to Orco, the behavioral

response to the blend was unchanged when the main IR co-receptors Ir8a and Ir25a were missing (Figure 3D).

Antennal lobe activity in response to a blend of hexanoic acid and methyl butyrate is overall conserved between *D. melanogaster* and *D. suzukii*

Since our results showed that an Orco-dependent pathway suppresses attraction to a blend of hexanoic acid and methyl butyrate in *D. melanogaster* but not in *D. suzukii*, we then investigated how the fly brain perceives these two compounds in the two species. We used a UAS-GCaMP7s line in *D. suzukii* [27] to monitor and compare with *D. melanogaster* the calcium activity of OSNs at the level of the antennal lobes. A broadly expressed Orco-Gal4 [26] driver was used to visualize the activity of OR-expressing OSNs. We stimulated each fly with the individual compounds and the blend.

The relative position of the glomeruli in the antennal lobes of the two species is fairly well conserved between *D. melanogaster* and *D. suzukii*, which enabled us to identify the responding glomeruli [36]. The same six glomeruli (DM1, DM2, DM3, DM5, DM6 and VA2) responded to either methyl butyrate, hexanoic acid or the blend in *D. suzukii* and *D. melanogaster* (Figure 4). Only a few small but significant quantitative differences were observed between the two species in response to the two-component blend. Indeed, in *D. melanogaster*, the neuronal activity was higher in the DM3 and VA2 glomeruli (Figure 4B, C). A stronger response in the DM3 glomerulus (together with the DM5) was also observed in *D. melanogaster* when exposed to methyl butyrate alone. No quantitative difference was observed in the response to hexanoic acid alone, which elicited only a moderate response in each glomerulus (Figure 4B, C).

Discussion

Behavioral divergence between closely related Drosophila species has often been linked to changes

in the perception of semiochemical compounds [1]. In this study, we identified a blend of two strawberry volatiles, hexanoic acid and methyl butyrate, that elicits opposite behaviors in *D. suzukii* and *D. melanogaster*. Remarkably, this blend mimics the behaviors evoked by the natural odor profile of ripe strawberry.

In Drosophila, *D. suzukii* exhibits a uniquely derived oviposition strategy – preferring ripe fruit, while most other species typically lay eggs on decaying substrates. We therefore hypothesized that olfactory-driven attraction to ripe fruit for oviposition might have evolved exclusively in *D. suzukii*. However, this hypothesis was challenged by unexpected observations. Several other Drosophila species were similarly attracted to ripe strawberry odors, despite being unable to penetrate ripe fruit. Conversely, avoidance or indifference to these odors appears more characteristic of *D. melanogaster* and its close relative *D. simulans*. Among the species tested, only the more distantly related *D. willistoni* showed a lack of significant attraction. This pattern suggests that while egg-laying attraction to ripe strawberry odors is widespread across Drosophila, this trait has been selectively lost in a few lineages.

From an evolutionary perspective, being drawn to ripe fruit may offer a competitive advantage [38, 39], enabling early arrival at oviposition sites and readiness for egg-laying as soon as fruit reaches optimal ripeness. In contrast, *D. melanogaster* is ecologically specialized for later stages of fruit decay: it relies heavily on yeast colonizing decomposing fruit [40] and exhibits high ethanol tolerance [41, 42], traits that facilitate survival in fermenting environments. In this context, arriving later and avoiding ripe fruit odors may be adaptive *D. melanogaster* [39] aligning its reproductive behavior with its ecological niche.

The two-component blend of hexanoic acid and methyl butyrate elicited behavioral responses in several species that mirrored their reaction to complex strawberry volatiles, whereas other species responded differently. This suggests that different Drosophila species may rely on distinct volatiles constituents to detect the same fruit source. Specifically, hexanoic acid and methyl butyrate appear to serve as key olfactory cues for *D. suzukii*, *D. biarmipes*, *D. eugracilis* or *D. melanogaster*, guiding them to ripe strawberries. In contrast, species such as *D. pseudoobscura* and *D. willistoni* are likely to use alternative compounds.

Notably, behavioral responses varied substantially across all tested species. For instance, for *D.*

melanogaster exhibited outcomes ranging from strong avoidance to apparent indifference toward the same stimulus. This variability occurred within the same genetic strain, ruling out differences in genetic background as a cause. Moreover, the effect was consistent across both natural (strawberry purée) and synthetic odor blends, making differences in odor source quality an unlikely explanation. Interestingly, these contrasting behavioral responses were tended to occur at different times of the year, suggesting that seasonal or subtle environmental factors may influence the outcome. However, the precise cause of this variability remains unresolved.

Strawberry volatiles, as well as the two-component synthetic blend, not only attract *D. suzukii* for oviposition but also stimulate egg-laying itself, highlighting the key role of hexanoic acid and methyl butyrate in guiding this species towards ripe strawberry. Although the same compounds mediate both attraction and oviposition stimulation, these behaviors are likely triggered through different receptor pathways. In a previous study, we showed that the stimulatory effect of strawberry volatiles on egg-laying rate depends on the co-receptor Orco, and thus on OR family receptors [26]. In contrast, here we show that attraction to oviposition sites is independent of Orco, suggesting the involvement of IR family receptors.

Oviposition is a multistep behavioral sequence that integrate multiple sensory cues through different receptor pathways. Females must first be attracted from a distance, then locate a suitable substrate at close range, probe this substrate, and finally deposit an egg. Such stage-specific sensory processing has been described for acetic acid in *D. melanogaster*, it attracts females from afar, stimulates egg-laying, yet simultaneously induces contact avoidance [43, 44]. These divergent effects rely on the activation of distinct sensory pathways [45, 46]. In the case of the response of *D. suzukii* to hexanoic acid and methyl butyrate, further work will be required to identify the specific IRs and ORs that mediate attraction and oviposition stimulation.

Our results also reveal that a latent IR-mediated attraction persists in *D. melanogaster*, but is overridden by an as-yet-unknown mechanism involving odorant receptors of the OR family. This OR-mediated pathway may act either directly – by inducing repulsion – or indirectly –by inhibiting IR-mediated attraction within the brain. We occasionally observed avoidance of ripe strawberry odors in *D. melanogaster*, suggesting a direct repulsive mechanism at play. The variability in behavioral responses may reflect the relative activation strength of the two pathways: IR-mediated attraction and OR-mediated repulsion. However, some findings challenge the direct repulsion

hypothesis. For example, IR mutants – lacking IR-mediated attraction – did not exhibit strong repulsion, as would be expected if the OR pathway acted solely through a direct mechanism. This supports the possibility of an indirect effect, where ORs suppress attraction rather than directly inducing aversion. Alternatively, both mechanisms might coexist: a direct repulsion triggered only under specific conditions, and an indirect inhibition of the IR pathway.

Several neurophysiological mechanisms could account for the specific loss of attraction observed in *D. melanogaster* but not in *D. suzukii*. One possibility is that the OR responsible for the loss of attraction is strongly activated in *D. melanogaster* but only weakly, if at all, in *D. suzukii*. Alternatively, olfactory perception at the level of the OSN may be conserved across species, but the downstream neuronal circuitry integrating the signal could have diverged. For instance, activation of the OR pathway in *D. melanogaster* might recruit LNs in the antennal lobes that suppress the attractive input conveyed by the IRs. Such LNs could be absent or wired differently in *D. suzukii*. Under this scenario, species-specific differences in brain activity would emerge primarily at the level of the PNs. Finally, the same OR could activate distinct higher-order pathways in the two species, driving attraction in *D. suzukii* but aversion in *D. melanogaster*. Although our results are consistent with these possibilities, they do not allow us to pinpoint the precise mechanism. Further experiments will be required to disentangle these alternatives.

Our calcium imaging results revealed only subtle differences between *D. melanogaster* and *D. suzukii*. Using previously published antennal lobes maps for both species [36], we found that the same six glomeruli responded to the blend of hexanoic acid and methyl butyrate, effectively ruling out the existence of a *D. melanogaster*-specific pathway. Each of these glomeruli also responded to the single compounds. Overall, responses to methyl butyrate were stronger than those to hexanoic acid, consistence with the tendency of acidic compounds to activate IRs [21, 47].

As expected, hexanoic acid evoked activity in the DM2 glomerulus, which is known to be driven by Or22a [48]. However, we also detected small but consistent responses in five additional glomeruli. Among these, only DM3 and VA2 showed quantitatively stronger responses to the blend in *D. melanogaster* compared with in *D. suzukii*. Yet, a similar pattern was observed for methyl butyrate alone in DM3 (and DM5), suggesting these differences are unlikely to underlie the behavioral divergence, since methyl butyrate is equally attractive to both species. VA2 was the only glomerulus to exhibit a blend-specific difference, but in *D. melanogaster* its response to the

blend closely resembled that to methyl butyrate alone..

With the exception of some quantitative differences – or divergences in the processing of specific odorants such as the leaf volatile ß-cyclocitral, which activates the DM2 glomerulus (Or22a) in *D. suzukii* but not in *D. melanogaster* [8, 24, 25, 36] - the antennal lobe responses of these two species appear broadly conserved [36]. It is therefore not surprising that only subtle differences were detected at the OSN level. While we cannot exclude the possibility that small quantitative changes influence behavior, our findings suggest that the mechanisms underlying the divergence in oviposition behavior are more likely to arise downstream of the OSN activity. This interpretation is consistent with the hypothesis of an indirect, OR-mediated pathway inhibitory pathway that suppresses attraction, I which case species-specific differences would only be detectable at the level of the PNs.

Finally, our behavioral data suggest that the OR pathway responsible for repulsion may not be consistently activated. It is therefore possible that this specific pathway was inactive during our calcium imaging experiments and thus remained undetected.

Conclusions

Taken together, our results reveal that a blend of methyl butyrate and hexanoic acid, two prominent strawberry volatiles, attracts *D. suzukii* to lay their eggs, but not *D. melanogaster*. We propose a model in which this blend activates IRs that drive attraction to suitable oviposition sites. In *D. melanogaster*, however, this attraction is overridden by an Orco-dependent pathway through an unknown mechanism. Although we detected subtle changes in the response of OSNs in a few glomeruli, the complexity of oviposition behavior suggest that additional likely occurred between drosophila species. These may include modifications in neuronal connections within the antennal lobes or in higher brain centers that fine-tune host preference. Moreover, differences in the perception of other strawberry volatiles with opposing valence likely contribute to the divergence in oviposition site preference among Drosophila species when confronted with the complex bouquet of fruit odors.

List of abbreviations

OSN: Olfactory Sensory Neuron

OR: Olfactory Receptor

IR: Ionotropic Receptor

PN: Projection Neuron

LN: Local Interneuron

GC-MS: Gas Chromatography – Mass Spectrometry

SHS: Static Headspace

HS-SPME: Headspace – Solid Phase Micro-extraction

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

The dataset generated during the current study are available in Table S1.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

SL, MC and BP conceived and designed the study. SL performed and analyzed the behavioral experiments. CM and BP generated *D. suzukii* transgenic lines. KPS and ICGK performed and analyzed the calcium imaging recordings. MD, XF and TM performed the chemical analyses. SL, MC and BP wrote the original draft. All authors reviewed and approved the final manuscript.

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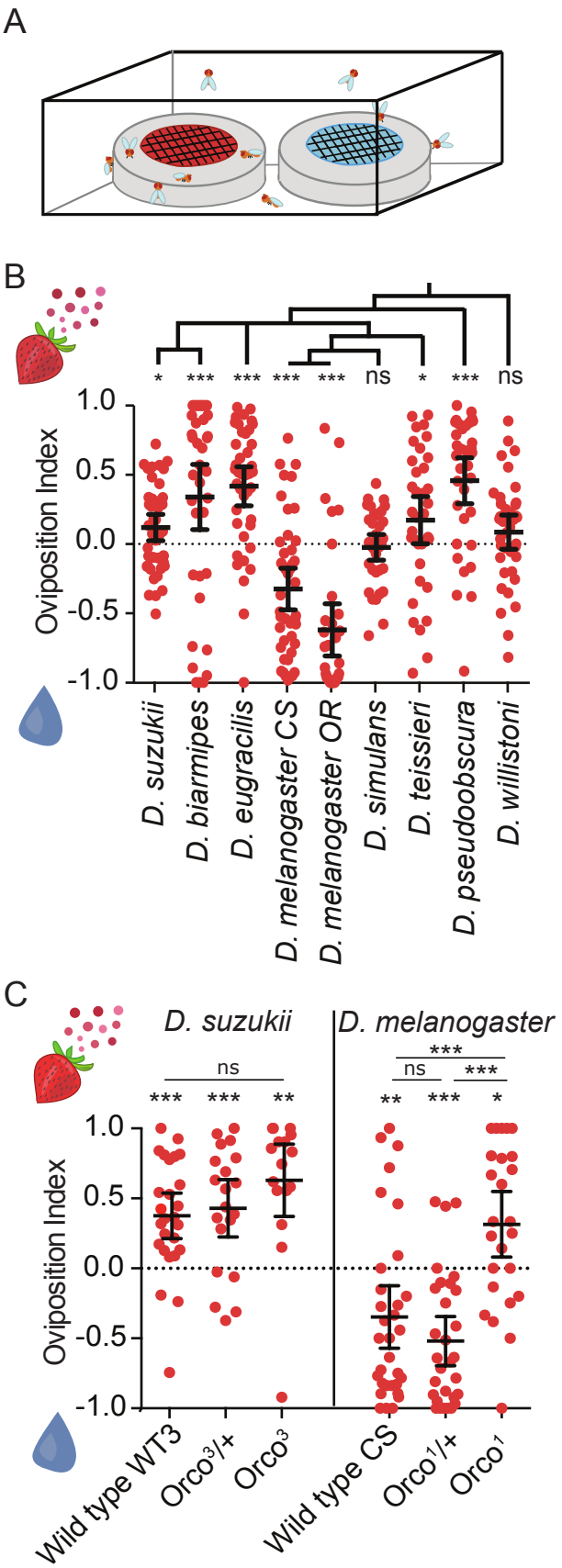
Figure 1. An Orco-dependent pathway suppresses the preference to lay eggs on ripe strawberry odor substrates in *D. melanogaster*. (A) Schematic of the egg-laying assay. Ten females were offered a choice between two substrates (5% fructose) containing either ripe strawberry puree or water at the center. The odor source was covered with a mesh to prevent direct physical contact. (B) Oviposition preference for ripe strawberry volatile compounds in the subgenus Sophophora. Attraction to these volatiles is widespread among species but has been lost in the *D. melanogaster – D. simulans* clade. (C) Role of Orco in mediating egg-laying preference for ripe strawberry odors in *D. suzukii* and *D. melanogaster*. Loss of function of Orco had no effect in *D. suzukii*, whereas *D. melanogaster* exhibited attraction in the absence of the co-receptor. Each point represents a replicate of ten females. Horizontal bars indicate mean values, and error bars show 95% confidence intervals. Asterisks above groups indicate significant attraction (above zero) or avoidance (below zero) of the stimulus (Wilcoxon signed rank test). Asterisks between groups represent significant differences between genotypes (Anova followed by a multiple comparison test with a fdr adjustment method). ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

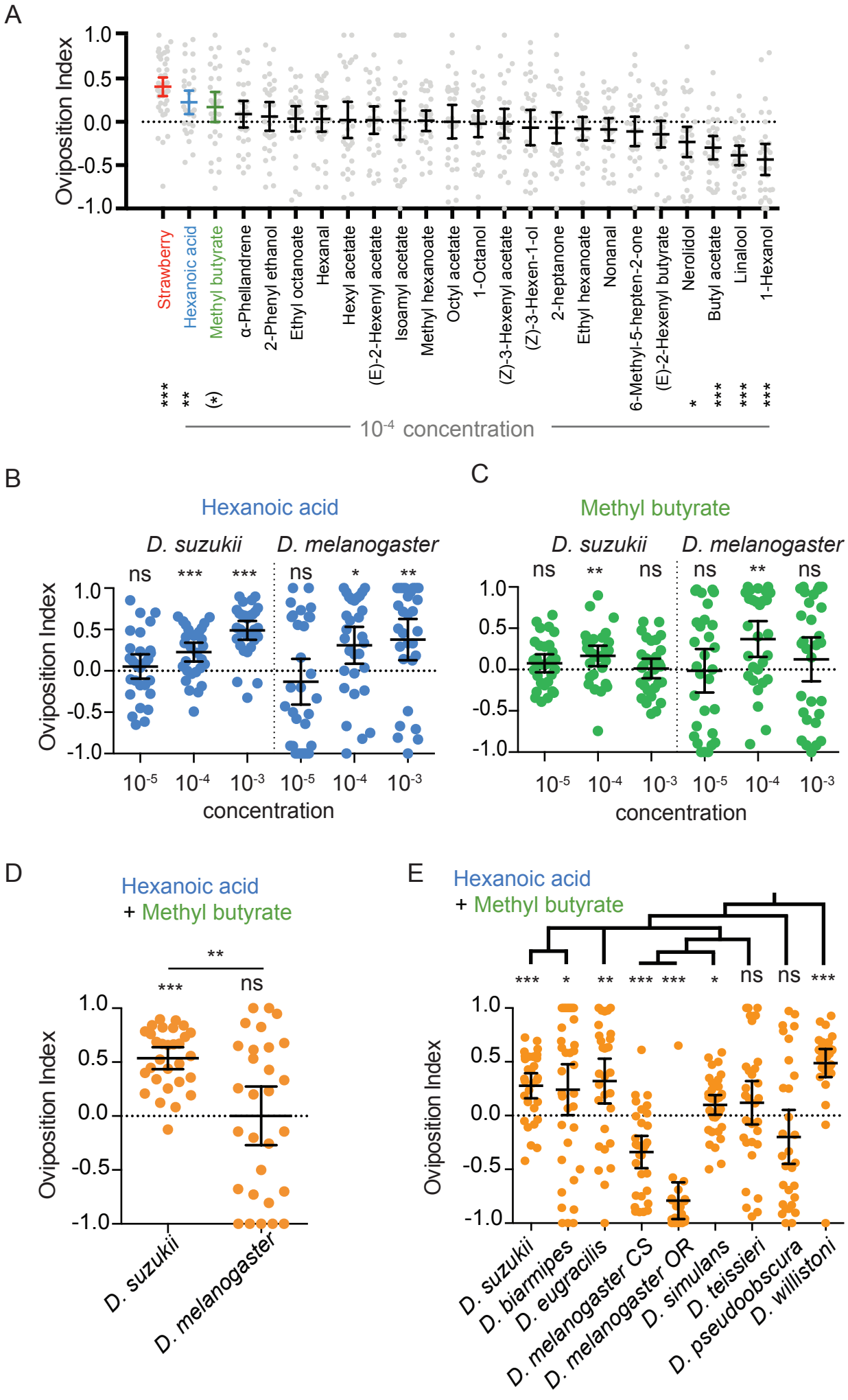
Figure 2. Specific volatile compounds mediate behavioral divergence between *D. suzukii* and *D. melanogaster*. (A) In addition to strawberry puree, twenty-three synthetic compounds identified in strawberry or other host fruits (cherry, raspberry, blueberry, and blackberry) were tested for their behavioral activity in *D. suzukii* (WT3) at a concentration of 10^{-4} (v/v). Hexanoic acid was significantly attractive, and flies showed a trend toward attraction to methyl butyrate ($p = 0.059$). (B and C) Dose-dependent behavioral responses to hexanoic acid (B) and methyl butyrate (C) were assessed in D. suzukii WT3 and D. melanogaster Canton S at three concentrations. (D) A two-component blend of hexanoic acid [10^{-3}] and methyl butyrate [10^{-4}] attracted *D. suzukii* WT3 but not *D. melanogaster* Canton S for egg-laying. (E) Egg-laying behavior of eight Drosophila species to the hexanoic acid + methyl butyrate blend. Each point represents a replicate of ten females. Horizontal bars indicate mean values and error bars shows 95% confidence intervals. Asterisks above groups indicate significant attraction (above zero) or avoidance (below zero) of the stimulus (Wilcoxon signed rank test). Differences between groups were tested using a Mann-Whitney test. ns = not significant, (*) $p = 0.059$, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

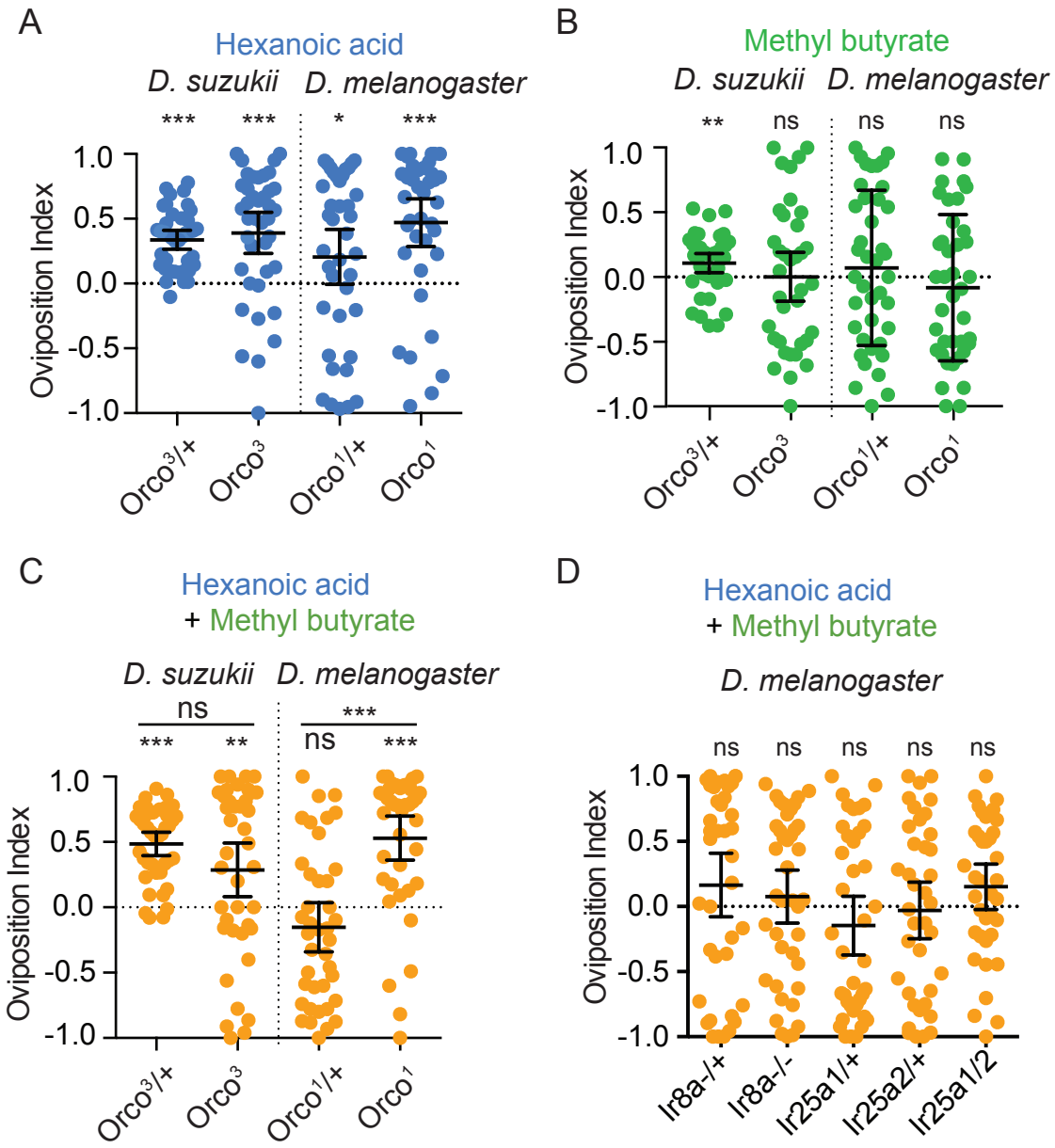
Figure 3. Distinct receptor families mediate egg-laying response to methyl butyrate and hexanoic acid. (A) Responses of Orco mutant to hexanoic acid [10^{-3}] in *D. suzukii* and *D. melanogaster*. (B) Responses of Orco mutant to methyl butyrate alone [10^{-4}] in *D. suzukii* and *D. melanogaster*. (C) Egg-laying behavior of *D. suzukii* Orco mutants in response to the synthetic two-component blend remain unchanged, whereas *D. melanogaster* Orco mutants were attracted to the blend. (D) Responses od IR co-receptor mutants (Ir8a and Ir25a) in *D. melanogaster* to the two-component blend. Asterisks above groups indicate significant attraction (above zero) or avoidance (below zero) of the stimulus (Wilcoxon signed rank test). Differences between groups were tested using a Kruskal-Wallis test, followed by a Dunn's multiple comparison test. ns = not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

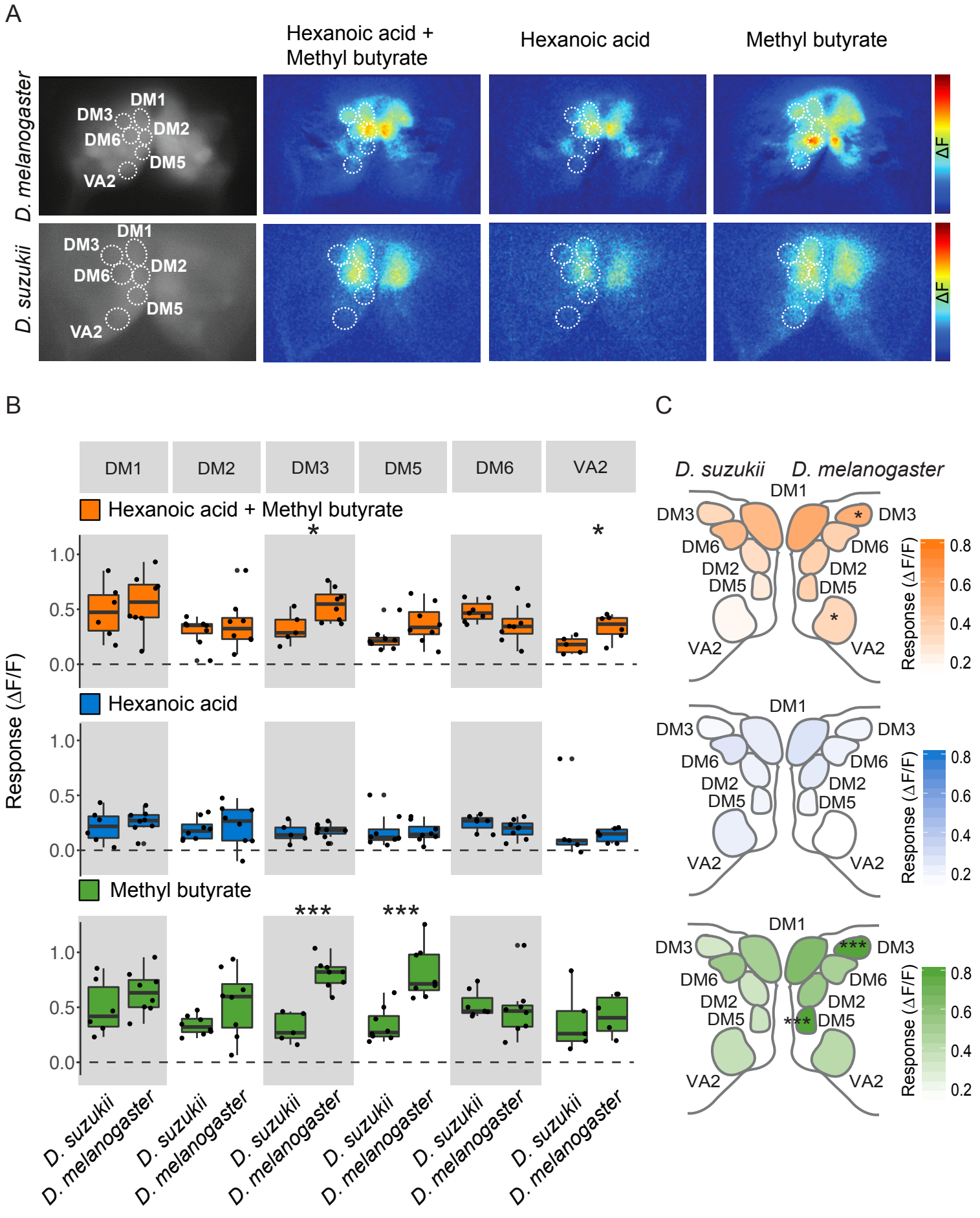
Figure 4. Calcium imaging of antennal lobe responses to hexanoic acid and methyl butyrate in female *D. melanogaster* and *D. suzukii*. Neuronal activity was recorded using the calcium

sensor GCaMP expressed in sensory neurons under the control of an Orco-Gal4 transgene. (A) Representative antennal lobe images showing responses to the three stimuli in *D. melanogaster* and *D. suzukii*. Responsive glomeruli are outlined. (B) Individual responses of the six activated glomeruli to the three stimuli tested in both species. (C) Color-coded average responses (mean) mapped onto a schematic of the antennal lobe for each species. Asterisks indicate statistically significant differences in the response between *D. suzukii* and *D. melanogaster* (t-test). * $p<0.05$, *** $p<0.001$.


A
B
* *** *** *** *** ns * *** ns
1.0
0.5
0.0
-0.5
-1.0
Oviposition Index
D. suzukii
D. biarmipes
D. eugracilis
D. melanogaster CS
D. melanogaster OR
D. simulans
D. teissieri
D. pseudoobscura
D. willistoni
C
D. suzukii
D. melanogaster
ns
*** *** **
*** ns ***
** *** *
1.0
0.5
0.0
-0.5
-1.0
Oviposition Index
Wild type WT3
Orco3/+
Orco3
Wild type CS
Orco1/+
Orco1


A
Oviposition Index
1.0
0.5
0.0
-0.5
-1.0
Strawberry
Hexanoic acid
Methyl butyrate
α-Phellandrene
2-Phenyl ethanol
Ethyl octanoate
Hexanal
Hexyl acetate
(E)-2-Hexenyl acetate
Isoamyl acetate
Methyl hexanoate
Octyl acetate
1-Octanol
(Z)-3-Hexenyl acetate
(Z)-3-Hexen-1-ol
2-heptanone
Ethyl hexanoate
Nonanal
6-Methyl-5-hepten-2-one
(E)-2-Hexenyl butyrate
Nerolidol
Butyl acetate
Linalool
1-Hexanol
*** ** (*) * *** *** ***
10-4 concentration
B
Hexanoic acid
D. suzukii
D. melanogaster
ns *** *** ns * **
Oviposition Index
10-5 10-4 10-3 10-5 10-4 10-3
concentration
C
Methyl butyrate
D. suzukii
D. melanogaster
ns ** ns ns ** ns
Oviposition Index
10-5 10-4 10-3 10-5 10-4 10-3
concentration
D
Hexanoic acid
+ Methyl butyrate
**
*** ns
Oviposition Index
D. suzukii
D. melanogaster
E
Hexanoic acid
+ Methyl butyrate
*** * ** *** *** * ns ns ***
Oviposition Index
D. suzukii
D. biarmipes
D. eugracilis
D. melanogaster CS
D. melanogaster OR
D. simulans
D. teissieri
D. pseudoobscura
D. willistoni


A
Hexanoic acid
D. suzukii
D. melanogaster
***
***
*
***
Oviposition Index
1.0
0.5
0.0
-0.5
-1.0
Orco3/+
Orco3
Orco1/+
Orco1
B
Methyl butyrate
D. suzukii
D. melanogaster
**
ns
ns
ns
Oviposition Index
1.0
0.5
0.0
-0.5
-1.0
Orco3/+
Orco3
Orco1/+
Orco1
C
Hexanoic acid
+ Methyl butyrate
D. suzukii
D. melanogaster
ns
***
***
**
ns
***
Oviposition Index
1.0
0.5
0.0
-0.5
-1.0
Orco3/+
Orco3
Orco1/+
Orco1
D
Hexanoic acid
+ Methyl butyrate
D. melanogaster
ns
ns
ns
ns
ns
Oviposition Index
1.0
0.5
0.0
-0.5
-1.0
Ir8a-/+
Ir8a-/-
Ir25a1/+
Ir25a2/+
Ir25a1/2


A
Hexanoic acid + Methyl butyrate
Hexanoic acid
Methyl butyrate
D. melanogaster
D. suzukii
DM1
DM2
DM3
DM5
DM6
VA2
ΔF
B
C
Response (ΔF/F)
Hexanoic acid + Methyl butyrate
Hexanoic acid
Methyl butyrate
D. suzukii
D. melanogaster

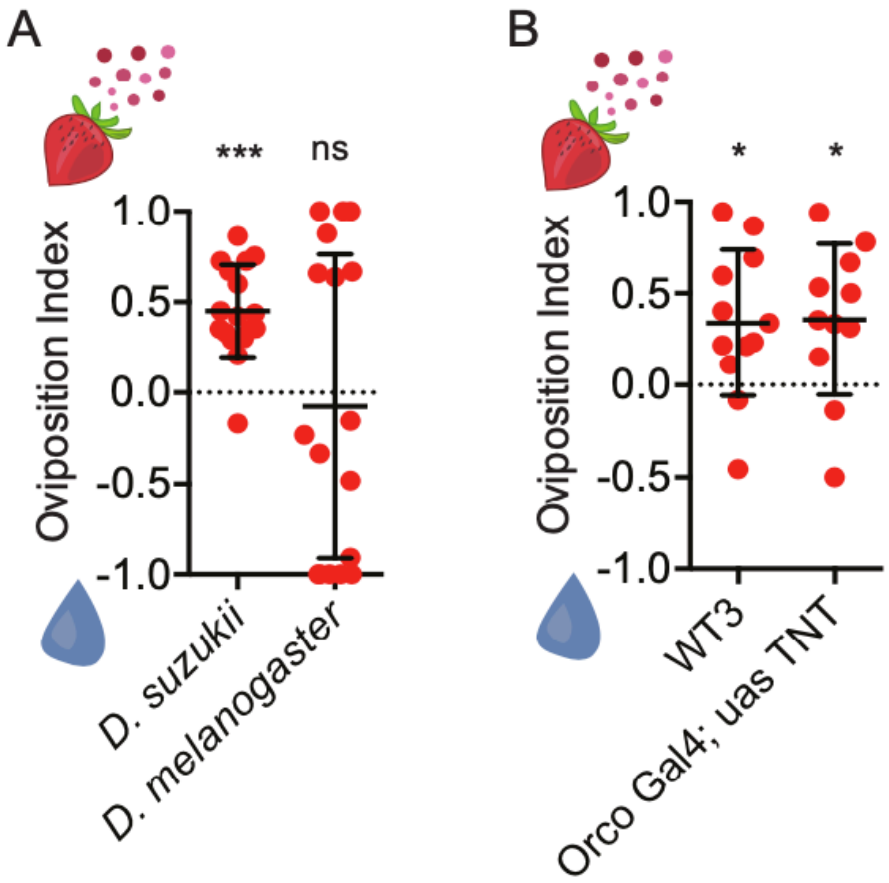


Figure S1 (related to Figure 1). Orco-dependent olfaction suppresses oviposition preference for ripe strawberry odors in *D. melanogaster*. (A) Behavioral assays show that *D. suzukii* (WT3) are attracted to lay eggs on substrates releasing strawberry odors whereas *D. melanogaster* (Canton S) females remain unresponsive (B) Suppressing of the activity of Orco-positive OSNs does not alter the behavioral response of *D. suzukii* females. Asterisks above groups indicate significant attraction (above zero) or avoidance (below zero) of the stimulus (Wilcoxon signed rank test). ns = not significant, * $p<0.05$, *** $p<0.001$.

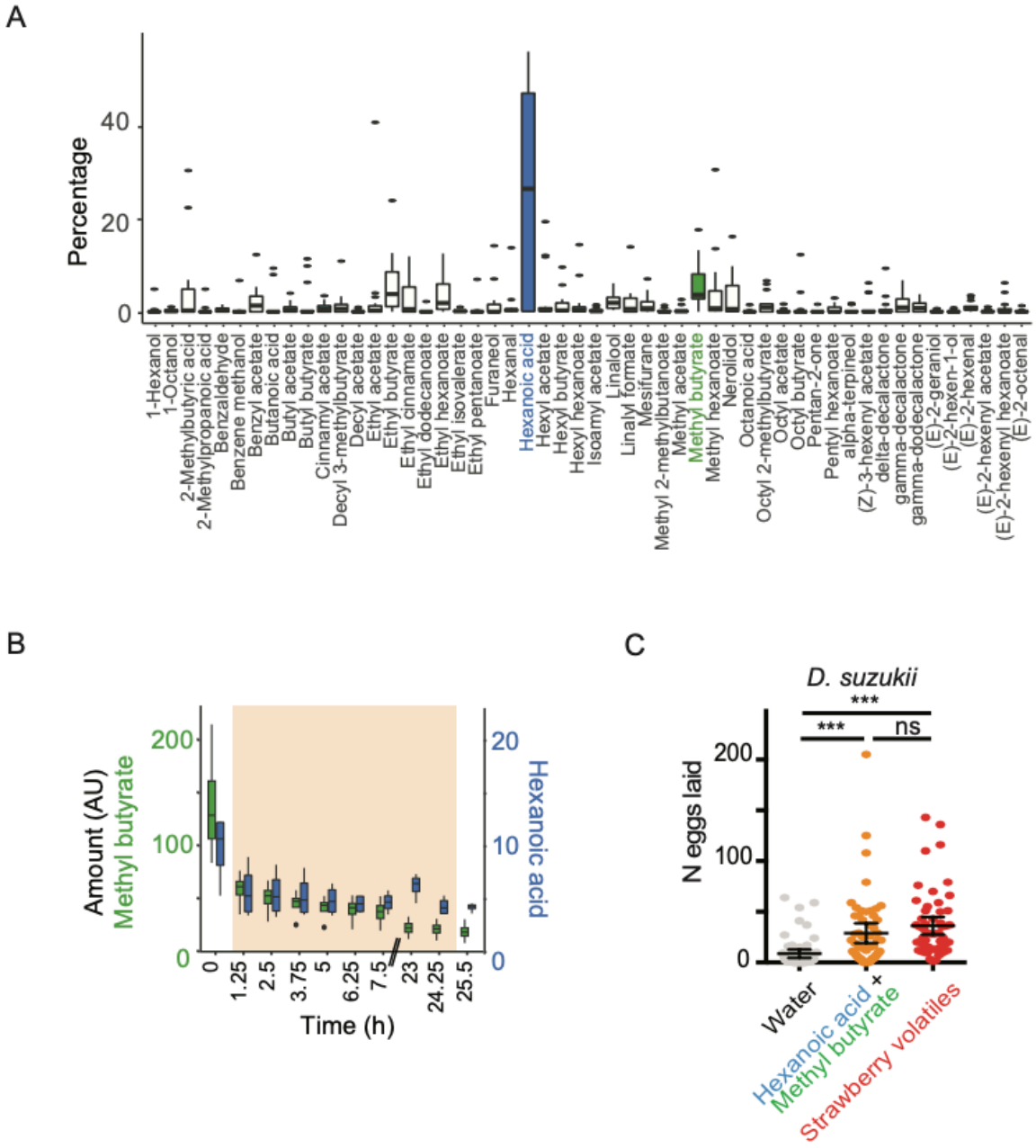


Figure S2 (related to Figure 2). A blend of hexanoic acid and methyl butyrate, two prominent compounds found in strawberries, recapitulates the behavioral response induced by natural strawberry volatiles. (A) Average composition of strawberry volatile compounds compiled from a literature survey of 15 varieties. The two attractive compounds, hexanoic acid and methyl butyrate are highlighted. (B) Levels (in arbitrary units) of hexanoic acid and methyl butyrate extracted from egg-laying plates supplemented with the two-component blend over time. The yellow shading indicates the 24h time period during which fly behavior was observed. (C) A synthetic blend of hexanoic acid [10^{-3}] and methyl butyrate [10^{-}

[4] stimulates oviposition in wild-type *D. suzukii* females to a similar extent as natural strawberry volatiles. Differences between groups were tested using a GLM with a negative binomial distribution followed by a multiple comparison test with an FDR adjustment method. ns = not significant, *** $p<0.001$.

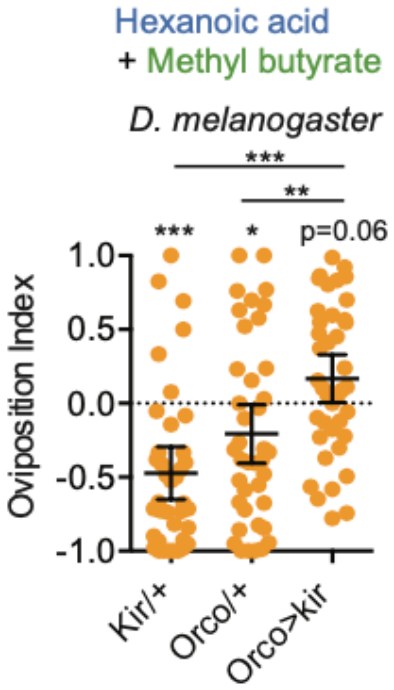


Figure S3 (related to Figure 3). Orco-mediated olfaction modulated attraction to the two-component blend. Inhibition of the activity of Orco-positive OSNs increases attraction to a blend of hexanoic acid [10^{-3}] and methyl butyrate [10^{-4}] in *D. melanogaster.* Asterisks above groups indicate significant attraction (above zero) or avoidance (below zero) of the stimulus (Wilcoxon signed rank test). Differences between groups were tested by a Mann-Whitney test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.