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*

**Authors**
Marianthi Karageorgi, Lasse B. Bräcker, Sébastien Lebreton, ..., Ilona C. Grunwald Kadow, Nicolas Gompel, Benjamin Prud’homme

**Correspondence**
gompel@biologie.uni-muenchen.de (N.G.),
benjamin.prudhomme@univ-amu.fr (B.P.)

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

Mariantiki Karageorgi,1 Lasse B. Bräcker,2,4 Sébastien Lebreton,1,4 Caroline Minervino,1 Matthieu Cavey,1 K.P. Siju,3,5 Ilona C. Grunwald Kadow,3,6 Nicolas Gompel,2,* and Benjamin Prud’homme1,6,*

1 Aix-Marseille Université, CNRS, IBDM, Institut de Biologie du Développement de Marseille, Campus de Luminy Case 907, 13288 Marseille Cedex 9, France
2 Ludwig-Maximilians Universität München, Fakultät für Biologie, Biozentrum, Grosshaderner Straße 2, 82152 Planegg-Martinsried, Germany
3 Max Planck Institute of Neurobiology, Am Klopferspitz 18, 82152 Planegg-Martinsried, Germany
4 These authors contributed equally to this work
5 Present address: School of Life Sciences Weihenstephan, Technische Universität München, Liesel-Beckmann-Strasse 4, 85354 Freising, Germany
6 Lead Contact
*Correspondence: gompel@biologie.uni-muenchen.de (N.G.), benjamin.prudhomme@univ-amu.fr (B.P.)

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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...
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
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. logEC50 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 logEC50 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 attraction to the odor source. First, replacing strawberry odors by acetoin, a potent attractant of 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 S3B4 and B6), consistent with what has been described in D. melanogaster (Figures S3C4 and C6). These neurons also express endogenous Orco, detected by an antibody in the antenna (Figures S3B3 and C3), 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 DszuOrco1, DszuOrco2, and DszuOrco3 (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 DszuOrco 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 DszuOrco 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 DszuOrco mutants in a two-choice assay with whole ripe and rotten strawberries (Figure 1A). We found no difference in oviposition preference between the DszuOrco mutants and the wild-type controls (Figure 3D), revealing that OR-mediated olfaction becomes redundant for egg-laying site selection when other
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. biarmipes has progressively increased, as in D. melanogaster, 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. In this stepwise scenario, the evolution of the ovipositor of D. suzukii was certainly a key acquisition, but it was secondary
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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