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Article

Olfactory Preference for Egg Laying on *Citrus* Substrates in *Drosophila*

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Summary

Background: Egg-laying animals, such as insects, ensure the survival of their offspring by depositing their eggs in favorable environments. To identify suitable oviposition sites, insects, such as the vinegar fly *Drosophila melanogaster*, assess a complex range of features. The fly selectively lays eggs in fermenting fruit. However, the precise cues and conditions that trigger oviposition remain unclear, including whether flies are also selective for the fruit substrate itself.

Results: Here, we demonstrate that flies prefer *Citrus* fruits as oviposition substrate. Flies detect terpenes characteristic of these fruits via a single class of olfactory sensory neurons, expressing odorant receptor *Or19a*. These neurons are necessary and sufficient for selective oviposition. In addition, we find that the *Citrus* preference is an ancestral trait, presumably representing an adaptation toward fruits found within the native African habitat. Moreover, we show that endoparasitoid wasps that parasitize fly larvae are strongly repelled by the smell of *Citrus*, as well as by valencene, the primary ligand of Or19a. Finally, larvae kept in substrates enriched with valencene suffer a reduced risk of parasitism.

Conclusions: Our results demonstrate that a single dedicated olfactory pathway determines oviposition fruit substrate choice. Moreover, our work suggests that the fly's fruit preference—reflected in the functional properties of the identified neuron population—stem from a need to escape parasitism from endoparasitoid wasps.

Introduction

For egg-laying animals, such as insects, the capacity to discriminate and choose appropriate sites for oviposition is of profound importance to the fitness of the future generation. The limited mobility of (most) insect larvae also means that the female parent must be able to make an informed decision about any potential oviposition site's future prospects as a suitable home for the larvae. Gravid females accordingly make use of multiple sensory modalities when evaluating the suitability of potential oviposition sites. For example, oviposition site selection in mosquitoes depends upon evaluation of a complex range of chemical and physical factors of their aquatic niches, ranging from, e.g., optical density, pool reflectance, salinity, chemical cues from conspecifics, and the presence of anuran tadpoles to the composition of the surrounding vegetation [1, 2].

The vinegar fly *Drosophila melanogaster*, which utilizes fermenting fruit as breeding substrate, likewise assesses a wide range of factors prior to choosing its oviposition site. Flies are selective, e.g., for (or against) color [3], ethanol and sugar content [4–6], temperature [7], fermentation volatiles [8, 9], endoparasitoid wasps [10, 11], substrate texture [12], and microbial composition [13]. Of the sensory cues involved, olfactory input plays a crucial role. The smell of acetic acid alone acts as a strong oviposition stimulant [14], whereas the smell of geosmin, an indicator of harmful microbes, prevents egg laying [13]. The microbial composition of the potential oviposition substrate is clearly a critical factor; however, whether flies also display partiality with respect to the substrate itself on which the microbes grow, i.e., the fruit, remains unclear. Do flies have an oviposition preference for certain fruits, and are there fruit-produced volatiles that, similar to acetic acid, act as oviposition stimulants?

We here investigated oviposition preference toward fruit in *D. melanogaster*. We find that flies indeed have an innate olfactory preference for certain fruits, preferring *Citrus* spp. and fruits with similar characteristics. We also find that this preference is mediated via a single class of olfactory sensory neurons, dedicated to the detection of terpenes typical of flavedo (i.e., the colored rind found in *Citrus*). The *Citrus* partiality likely reflects an ancestral preference toward specific fruits found in the native African habitat. Finally, we demonstrate that the *Citrus* preference has likely been driven by needs to avoid parasitization from endoparasitoid wasps.

Results and Discussion

Flies Prefer *Citrus* Fruits for Oviposition

We first assessed the egg-laying preference of *Drosophila melanogaster* toward different fruits using a multiple-choice oviposition assay in which flies had unrestricted access to presented fruits (six at a time). Importantly, we screened only ripe, undamaged fruits, to exclude yeast that might influence the flies' choice. In three iterative trials, wild-type (WT) flies consistently chose sweet oranges as oviposition substrate over the 15 other fruits tested (Figure 1A). Flies (n = 30 per trial, 10 trials per treatment, each lasting 24 hr) deposited on average 103.0 ± 51.1 (SD) eggs on the oranges, compared to between 0 and 30.9 ± 20.4 on the other fruits. Flies clearly showed little liking for lemon, not unexpected given the acidity of this fruit. However, the effect of orange could be recapitulated by grapefruit (data not shown), suggesting that except for the most acidic taxa, given a choice, flies will prefer to oviposit on *Citrus* spp. Accordingly, we conclude that flies do not indiscriminately oviposit on any fruit but display a preference for certain fruits, in our screen represented by *Citrus* spp. Since the tested flies had no prior experience with fruit, we further conclude that this preference is innate.

The Oviposition Preference for *Citrus* spp. Is Dependent on Limonene

Flies, like many other insects, rely on their sense of smell to locate objects of importance [15]. Hence, we next sought to

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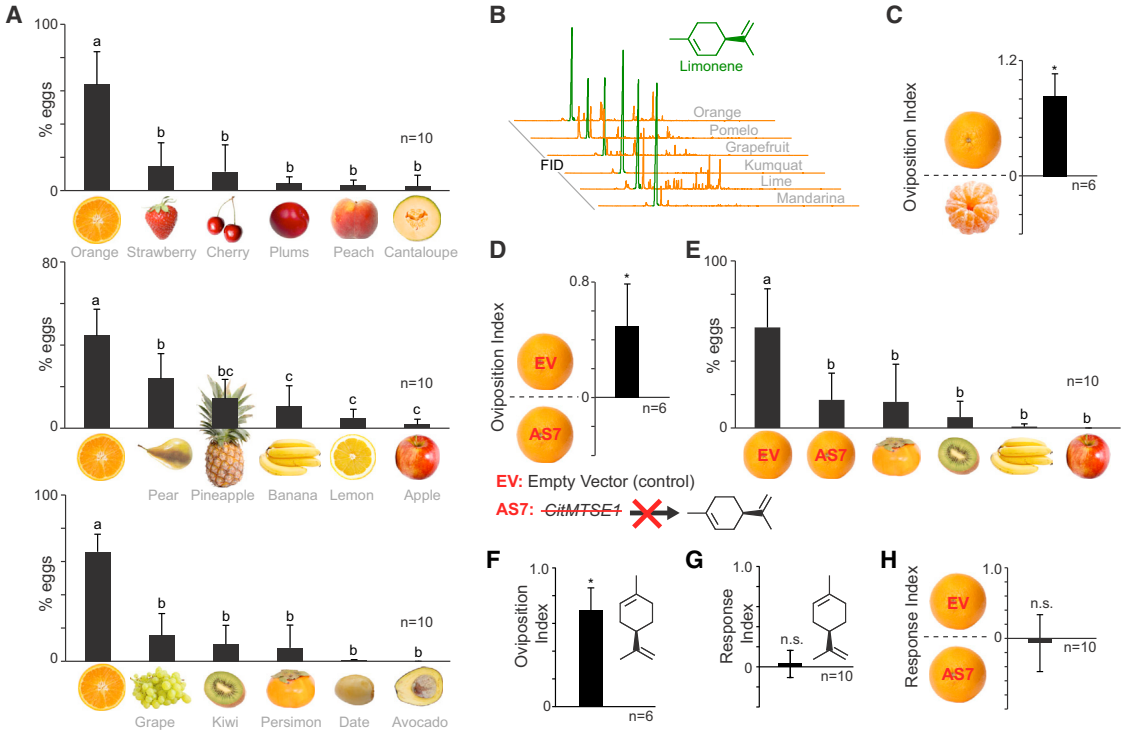

Figure 1. Flies Prefer *Citrus* as Oviposition Substrate

(A) Percentage of eggs deposited on fruits presented in six-way choice oviposition experiments. Error bars represent SEM. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(B) Flame ionization (FID) traces from headspace collections of various *Citrus* varieties. Limonene is the major volatile constituent.

(C) Oviposition index (OI) from a binary choice between intact and peeled oranges. OI = 1 denotes all eggs deposited on intact oranges; OI = −1 denotes all eggs deposited on peeled oranges. Deviation of the OI against zero (no choice) was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(D) OI from a binary choice between oranges transfected with empty vector (EV) and oranges with antisense downregulation of a limonene synthase gene (*CitMTSE1*) (AS7). Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(E) Percentage of eggs deposited on fruits in a six-way choice oviposition experiment. Abbreviations are as per (D). Error bars represent SEM. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(F) OI to limonene (10^{-2} dilution). Deviation of the OI against zero was tested by Student's t test ($p < 0.05$).

(G) Response index (RI) to limonene (10^{-2} dilution). Error bars represent SEM. Deviation of the RI against zero was tested by Student's t test ($p < 0.05$).

(H) RI from a binary choice between the orange lines described in (D). Error bars represent SEM. Deviation of the RI against zero was tested by Student's t test ($p < 0.05$).

identify olfactory cues mediating the fruit partiality. In terms of volatile chemistry, *Citrus* fruits are characterized by a high content of terpenes, in particular limonene. This volatile occurs in extraordinary amounts in most *Citrus* varieties [16] (Figure 1B), where it accumulates in the flavedo. The flavedo further contains a plethora of other terpenes in high amounts [16]. In a binary choice oviposition assay [13], flies clearly preferred intact oranges over peeled oranges (Figure 1C), implying that chemicals present in the flavedo are important. To determine the role of limonene, we tested in our binary oviposition assay a transgenic line (AS7) of sweet oranges with reduced limonene content due to antisense downregulation of a key gene involved in limonene synthesis (*CitMTSE1*) [17] against a control line with normal limonene content. Flies strongly preferred the control line (Figure 1D). Likewise, in a multifruit comparison, flies did not choose the AS7 line as egg-laying substrate over other fruits: flies laid as many eggs on the AS7 line as they did on apple, persimmon, kiwi, or banana (Figure 1E). We accordingly conclude that the presence of limonene is necessary for the increased rate of oviposition seen toward *Citrus* fruits.

Is limonene sufficient to induce oviposition? In a binary olfactory choice oviposition assay [13], flies strongly preferred to oviposit on food plates spiked with synthetic limonene (Figure 1F). This result could however also be explained by flies having an innate attraction to limonene, thus spending more time on the baited plate and hence laying more eggs. In other words, limonene could be acting as an oviposition attractant rather than an oviposition stimulant [18]. To exclude this possibility, we examined the behavioral valence of limonene using a modified olfactory trap assay [9, 19]. Limonene was neutral, with flies displaying neither attraction nor repulsion (Figure 1G). Moreover, flies exposed to the odor of the AS7 and empty vector (EV) lines in the olfactory trap assay likewise showed no preference for either genotype (Figure 1H). We hence conclude that volatile limonene by itself is a genuine oviposition stimulant, in a fashion similar to acetic acid [14].

Limonene Is Detected by OSNs Housed in an Antennal Intermediate Sensillum Type

We next sought to identify the olfactory sensory neurons (OSNs) that detect limonene, via a system-wide single-sensillum recording (SSR) screen from all OSN classes found on the third antennal segment and maxillary palps, while stimulating OSNs with limonene. Only antennal intermediate sensillum type 2A (ai2A) neurons [20] responded strongly to

limonene (Figures 2A and 2B). Apart from ai2A, we additionally noted a weaker response to limonene from antennal basiconic sensilla type 9A (ab9A) (Figure 2A). To verify that limonene is detected primarily via the ai2A neurons, we examined the response threshold toward limonene for these two OSNs. Indeed, the limonene detection threshold of ai2A was at least three orders of magnitude lower than that of ab9A (Figure 2C). Thus, we conclude that at ecologically relevant concentrations, the presence of limonene is mediated solely via a pathway receiving input from ai2A OSNs.

We next sought to determine which other compounds the ai2A OSNs might respond to. We tested in our SSR assay 450 synthetic chemicals—a set that contained multiple representatives from all biologically relevant chemical classes (Figure 2D; see also Figure S1 available online). Out of the 450 screened substances, only 5% yielded a response of >50 spikes/s, and only seven compounds produced a firing rate of >100 spikes/s. These seven compounds were all terpenes, as well as sharing other structural features with limonene (Figure 2D). The strongest response was not recorded from limonene but from valencene, another characteristic *Citrus* volatile [21]. To determine the most efficient ligands for ai2A, we subsequently examined dose-response relationships for 28 compounds, a subset that included the most efficient ligands from the initial screen and a range of other terpenes (Figure 2E). The dose-response trials revealed that the most efficient activator for this OSN population was indeed valencene, followed by β-caryophyllene, β-caryophyllene oxide, and limonene oxide, with the latter three showing similar efficiency at activating ai2A (Figure 2E). These three substances, although commonly occurring in nature, are nevertheless typically also found in *Citrus* headspace, in particular limonene oxide [16].

Do the additional ai2A ligands elicit a behavioral response similar to limonene? To address this question, we tested four of the ligands in the oviposition as well as in the olfactory trap assay. Indeed, all of these compounds triggered oviposition (Figure 2F), but no apparent chemotaxis (Figure 2G), and thus similarly act as oviposition stimulants. Moreover, we would also expect that ai2A OSNs are activated by the smell of genuine *Citrus* fruits. Thus, we next used gas chromatography (GC)-linked SSR to stimulate ai2A OSNs with headspace from a range of *Citrus*. As expected, all seven *Citrus* varieties screened strongly activated the ai2A neurons (Figure 2H). We thus conclude that the ai2A OSNs are configured specifically for the detection of terpenes, particularly those associated with *Citrus*.

ai2A Neurons Express Or19a and Target the DC1 Glomerulus

To identify the odorant receptor (OR) underlying the response property of the ai2A neurons, we visualized the activity of antennal lobe (AL) glomeruli using in vivo calcium imaging and delineated the identity of the corresponding OR by virtue of the published map of OR expression in the fly AL [22, 23] (Figure 3A). Stimulation with limonene, valencene, and β-caryophyllene primarily activated a region in the AL corresponding to the DC1 glomerulus (Figures 3B and 3C). In line with the SSR data, we also noted weaker responses to limonene from the D glomerulus (Figure 3C), which is the target of OSNs expressing *Or69a* and housed in the ab9 sensillum [22]. DC1 receives input from OSNs expressing *Or19a* and *Or19b* [22, 23], of which the former has previously been found to bind limonene [24]. Indeed, misexpression of Or19a in Δab3A OSNs [25] endows these neurons with a response profile inseparable from that of ai2A OSNs when stimulated with synthetic volatiles (Figures 3D and 3E), as well as with *Citrus* headspace via GC (Figure 3F). The function of *Or19b*, if any, remains to be elucidated. We accordingly conclude that the terpene responsiveness of the ai2A OSNs is due to *Or19a*.

ai2A OSNs Are Necessary and Sufficient for the Oviposition Preference toward *Citrus*

Are the ai2A neurons necessary for the observed behavior? We next used the temperature-sensitive mutant dynamin *Shibire^{ts}* [26] expressed from the *Or19a* promoter to shut down synaptic transmission in ai2A OSNs. First, we examined the oviposition behavior toward limonene, valencene, and β-caryophyllene. At the restrictive temperature (32°C), flies carrying this construct displayed no oviposition preference toward these compounds (Figure 3G), unlike flies with the same genotype tested at a permissive temperature (25°C) and control lines. Strikingly, thermogenetic silencing of the ai2A neurons also completely abolished the preference for *Citrus* fruit at the restrictive temperature in a binary oviposition choice test with oranges versus plums (Figure 3H). As expected, silencing of the ab9A OSNs, via expression of *Shibire^{ts}* from the *Or69a* promoter, had no effect on the oviposition behavior toward valencene (Figure S1A), or any effect in the oranges-versus-plums oviposition test (Figure S1B).

We next wondered whether activation of this OSN population is sufficient to induce oviposition. We subsequently expressed the temperature-sensitive cation channel *dTRPA1* in the ai2A OSNs, which allowed us to conditionally and specifically activate these neurons at temperatures above 26°C [27]. In a binary choice oviposition assay, flies bearing the *Or19a-Gal4,UAS-dTRPA1* construct preferred to deposit eggs on plates heated to 26°C over plates held at room temperature (20°C), in contrast to parental controls and WT flies, which showed no such preference (Figure 3I). Specific activation of these neurons is hence sufficient to induce oviposition. To further explore the sufficiency of these neurons in guiding oviposition site selection, we again provided flies with the choice to oviposit on either oranges or plums, but now adding valencene—the key ligand for *Or19a*—to the plums. Indeed, adding this volatile alone to the plums abolished the *Citrus* preference (Figure 3J). In summary, we conclude that *Or19a* is both necessary and sufficient for the oviposition preference toward *Citrus*.

Citrus Fruits Are Not the Ancestral Host of *D. melanogaster*

Citrus fruits are native to Southeast Asia [28], whereas *D. melanogaster* stems from Africa [29]. How can *D. melanogaster* have evolved a tight association with fruits that it has not coevolved with? One explanation could be that the preference for *Citrus*, and in turn the tuning of *Or19a* toward volatiles of a *Citrus* character, represents an ancestral trait. The *melanogaster* species subgroup comprises an African offshoot of a Southeast Asian radiation. One could envision that the ancestral Asian population from which *D. melanogaster* stems utilized *Citrus*, and that this preference, reflected in the olfactory makeup, was retained when Africa was colonized during late Miocene [30]. Once in Africa, the colonists would have found fruits with chemical (and physical) properties similar to those of *Citrus*. A GC-SSR comparison of 13 species from across the subgenus *Sophophora* (Figures S3A and S3B), with orange headspace as stimulus, demonstrated that there are indeed Asian relatives with ai2A

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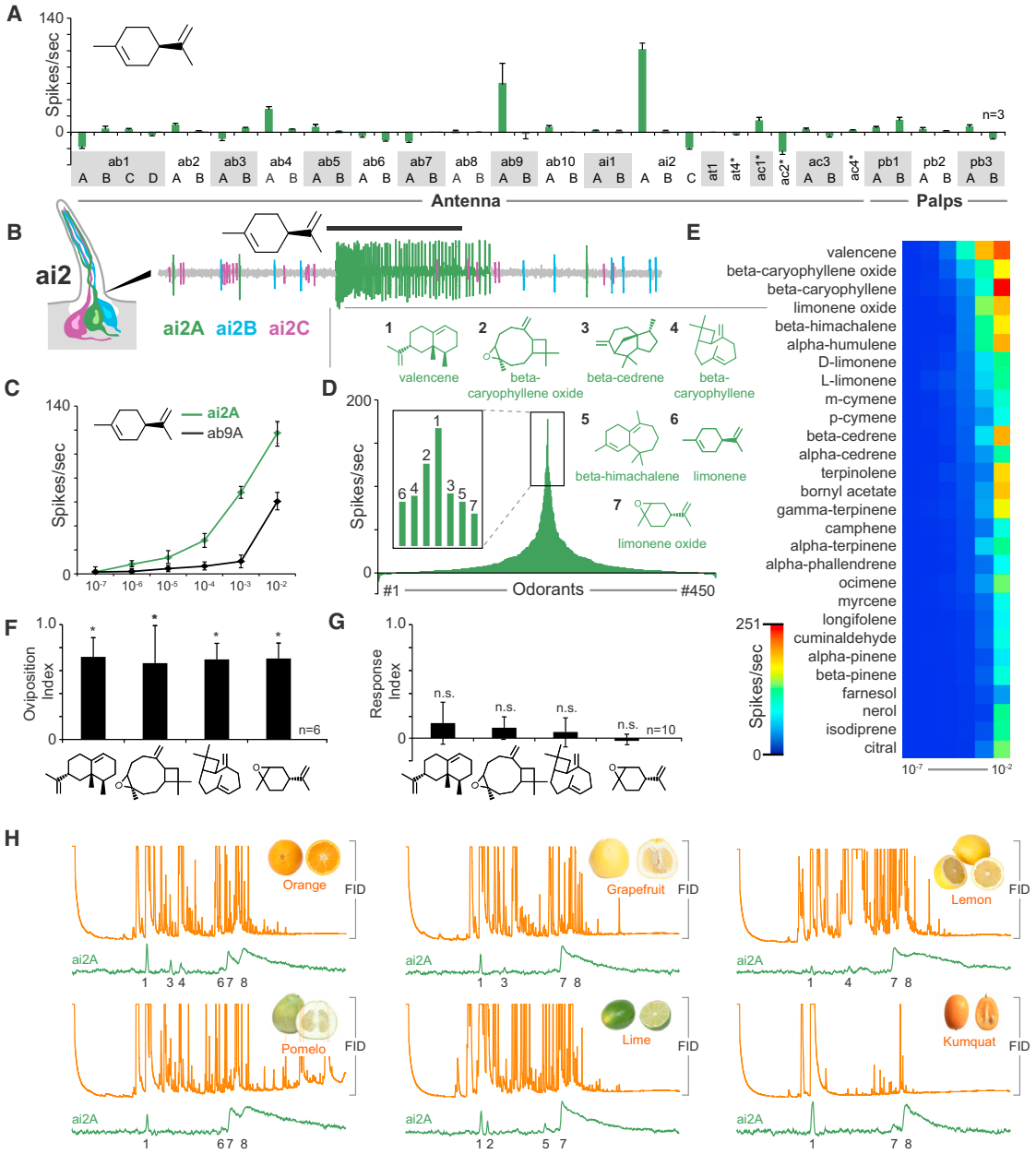

Figure 2. *Citrus* Odorants Are Detected by the ai2A Neurons

(A) Single-sensillum recording (SSR) measurements from all olfactory sensilla, with limonene (10^{-3} dilution) as a stimulus. ab, antennal basiconic sensilla (s.); ac, antennal coeloconic s.; at, antennal trichoid s.; ai, antennal intermediate s.; pb, palp basiconic s. Asterisks denote that activity from individual OSNs was not separated. Error bars represent SEM.

(B) Representative SSR traces from an ai2 sensillum. The larger-amplitude spiking neuron, i.e. ai2A, responds to limonene (10^{-3} dilution). The duration of stimulus delivery (0.5 s) is marked by the black bar.

(C) Dose-response curve from ai2A neurons toward limonene. Error bars represent SEM.

(D) Tuning curve for the ai2A neuron type based on a screen of 450 synthetic substances (10^{-2} dilution). Error bars represent SEM.

(E) Heatmap based on dose-response profiles of ai2A neurons toward 28 compounds.

(F) Oviposition indices (OI) to valencene, β-caryophyllene, β-caryophyllene oxide, and limonene oxide. Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(G) Response indices (RI) from olfactory trap assay experiments toward the same compounds as in (F). Deviation of the RI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(H) Representative gas chromatography (GC)-linked SSR measurements from ai2A neurons. The orange trace represents the FID, photos depict the screened odor sources, and the green trace depicts the simultaneously recorded neural activity of ai2A neurons. Numbers refer to the identity of active FID peaks (as determined via GC-MS): 1, limonene; 2, γ-terpinene; 3, limonene oxide; 4, unidentified; 5, γ-elemene; 6, β-cubebene; 7, β-caryophyllene; 8, valencene.

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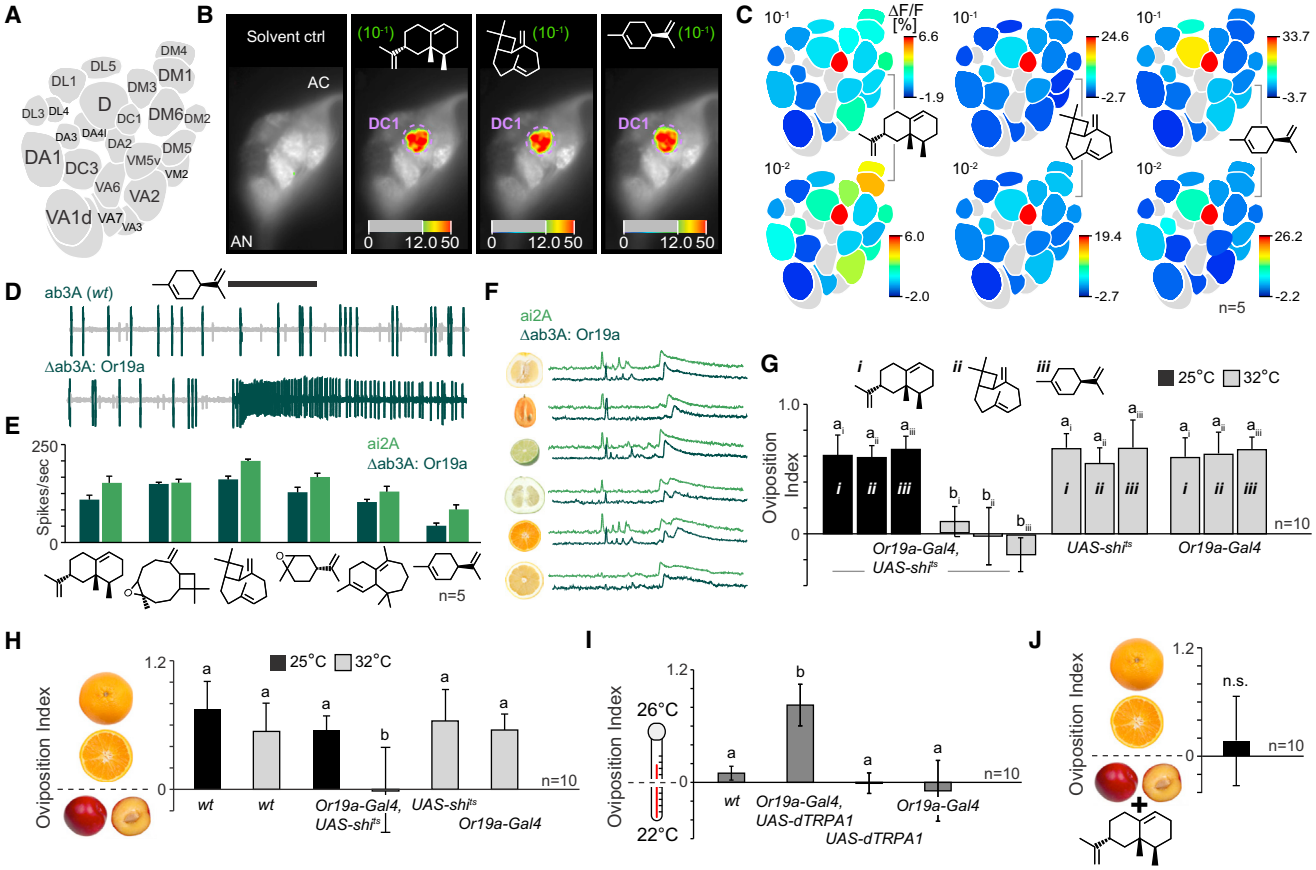

Figure 3. *Or19a* Is Necessary and Sufficient for the *Citrus* Preference

(A) Glomerular atlas of the antennal lobe (AL).

(B) False color-coded images showing solvent-induced and odorant-induced calcium-dependent fluorescence changes in the AL of a fly expressing the activity reporter GCaMP3.0 from the *Orco* promoter. AC, antennal commissure, AN, antennal nerve.

(C) Odor-induced activity plotted on schematic ALs (average % DF/F).

(D) Representative SSR traces from measurements of WT ab3 (above) and *Δab3:Or19a* (*Δhalo;Or22a-GAL4/UAS-Or19a*) (below) stimulated with limonene (10^{-3}). The duration of the stimulus delivery (0.5 s) is marked by the black bar.

(E) Quantified SSR responses toward valencene, β-caryophyllene, β-caryophyllene oxide, limonene oxide, β-himachalene, and limonene from ai2A (green) and Δab3:Or19a OSNs (dark green). Error bars represent SEM.

(F) Representative GC-SSR traces from ai2A and *Δab3:Or19a* OSNs stimulated with a variety of *Citrus* spp. Color coding is as per (E).

(G) OIs to valencene, β-caryophyllene, and limonene (all at 10^{-1}) of flies expressing *Shibire^{ts}* from the *Or19a* promoter and corresponding parental lines. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(H) OIs of flies expressing *Shibire^{ts}* from the *Or19a* promoter and corresponding parental lines presented with a choice to oviposit on either oranges or plums. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

(I) OIs of flies expressing *dTRPA1* from the *Or19a* promoter, the corresponding parental lines, and WT flies in an oviposition assay with a choice between 22°C and 26°C. Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(J) OIs of flies confronted with a choice between oranges and plums spiked with valencene (10^{-3}). Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

OSNs tuned as in *D. melanogaster* (Figure S3C). The species most similar to *D. melanogaster* is in fact *D. bipectinata*, a widespread species occurring from India to Samoa [31]. Although the ecology of this species is poorly known, given an oviposition choice between oranges and plums, *D. bipectinata* also strongly preferred oranges (oviposition index 0.97 ± 0.05 [average ± SD]; $p = 0.0001$, Student's t test against zero [1.0 = full preference for oranges]). It is hence not inconceivable that the *Citrus* partiality, and tuning of the ai2A OSNs, constitutes an ancestral trait that has remained conserved in the lineage leading to *D. melanogaster*.

Irrespective whether the observed behavior is an ancestral attribute or was acquired independently after the colonization of Africa, there should presumably be fruits with chemical properties similar to those of *Citrus* within the native range of *D. melanogaster*. We subsequently went to the field and obtained headspace collections from a variety of native African noncultivated fruits (n = 6) and examined the GC-SSR activity pattern of ai2A OSNs. We then compared the responses triggered by these fruits to those elicited by a host of other non-*Citrus* (n = 12) and the previously examined *Citrus* (n = 7). With two exceptions, none of the non-*Citrus* varieties elicited any noticeable responses from the ai2A neurons (Figure 4A). Stimulation with giant yellow mulberry (*Myrianthus arboreus*) triggered a single response (unidentified peak), whereas stimulation with headspace from African squirrel nutmeg (*Monodora tenuifolia*) yielded a response pattern akin to that of *Citrus* (Figure 4B). Flies given a binary oviposition choice

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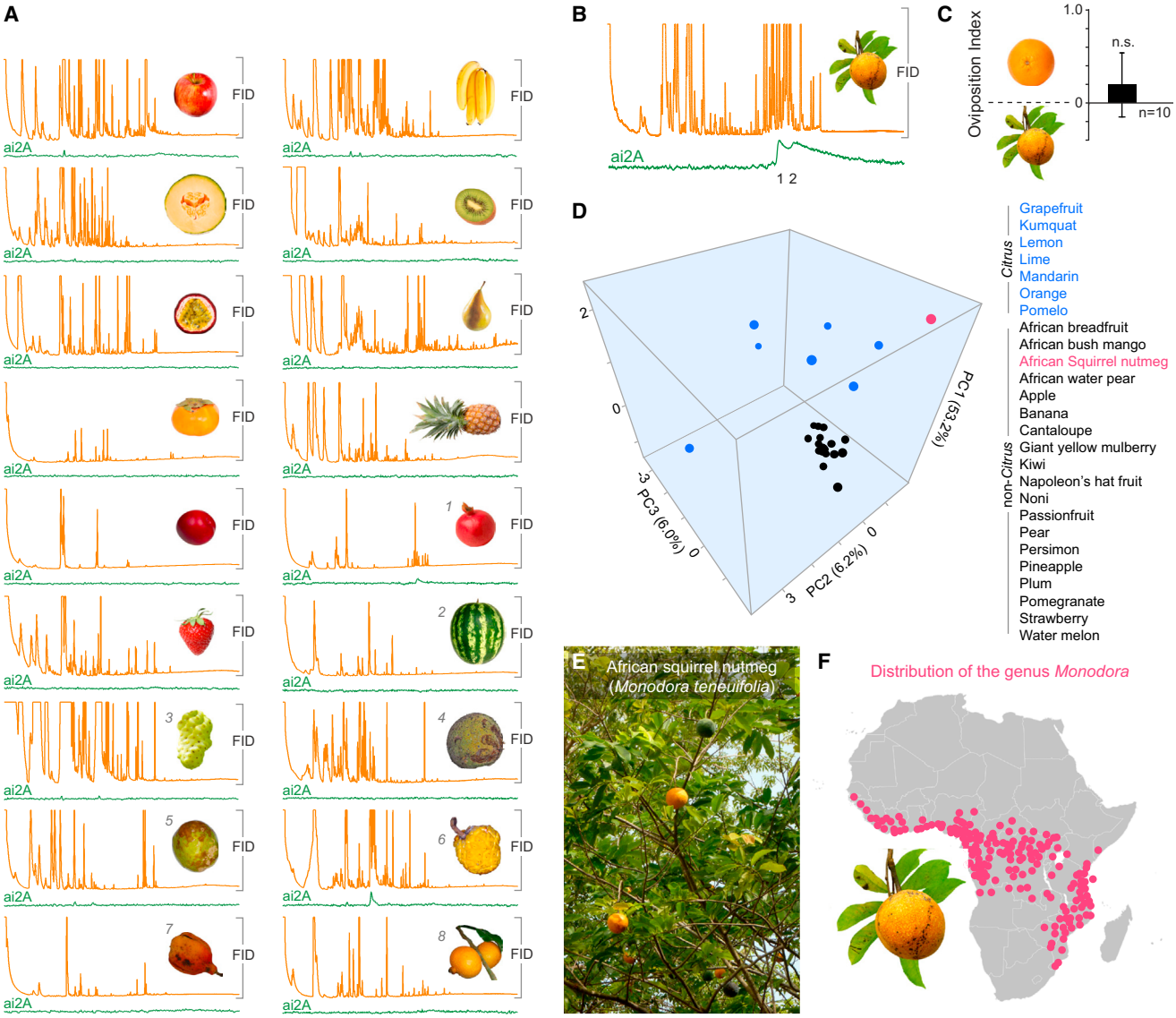

Figure 4. The *Citrus* Preference of *D. melanogaster* Is an Ancestral Trait

(A) Representative GC-SSR traces from *D. melanogaster* stimulated with a range of fruit. Gray numbers indicate (1) pomegranate, (2) watermelon, (3) noni *Morinda citrifolia*, (4) African breadfruit *Treculia africana*, (5) African bush mango *Irvingia wombulu*, (6) African giant mulberry *Myrianthus arboreus*, (7) Akee apple *Blighia sapida*, (8) Napoleon's hat fruit *Napoleona imperialis*.

(B) GC-SSR trace from *D. melanogaster* stimulated with headspace of African squirrel nutmeg. Numbers refer to identity of active FID peaks, as determined via GC-MS. 1, β-caryophyllene; 2, unidentified terpene.

(C) Oviposition index from a binary choice between orange and African squirrel nutmeg. Deviation of the OI against zero was tested by Student's t test ($p < 0.05$). Error bars represent SEM.

(D) Three-dimensional principal component analysis plot based on the GC-SSR traces in (A) and (B).

(E) The African squirrel nutmeg in nature (photo by D.B.).

(F) Distribution of the genus *Monodora*. Image adapted from African Plant Database (www.ville-ge.ch/musinfo/bd/cjb/africa/).

test between *Monodora* and oranges showed no significant preference either way (Figure 4C). The similarity could also be seen in a three-dimensional principle component analysis plot based on the response pattern (Figure 4D), where all non-*Citrus*, with the exception of African squirrel nutmeg, cluster together separately from *Citrus*. African squirrel nutmeg also shows an overall likeness to oranges (Figure 4E) that extends to color, shape, and size. Similar to *Citrus*, *Monodora* fruits have a thick epicarp, where presumably the terpenes triggering activity from ai2A neurons accumulate. Are *Monodora* fruits then the ancestral breeding substrate of *D. melanogaster*? Probably not. First of all, members of the genus *Monodora* are restricted to the tropical rainforest zone (Figure 4F). The presumed evolutionary cradle of *D. melanogaster*, however, lies in drier habitats further south, possibly in the Miombo forest zone [32]. Moreover, although the flies readily laid eggs on these fruits, the mesocarp of *Monodora* fruits is quite dry in comparison with fruits typically utilized by *D. melanogaster*, making the suitability of these fruits as larval substrate questionable. Nevertheless, the African squirrel nutmeg serves as proof of principle that there are fruits in Africa with properties similar to those of *Citrus*.

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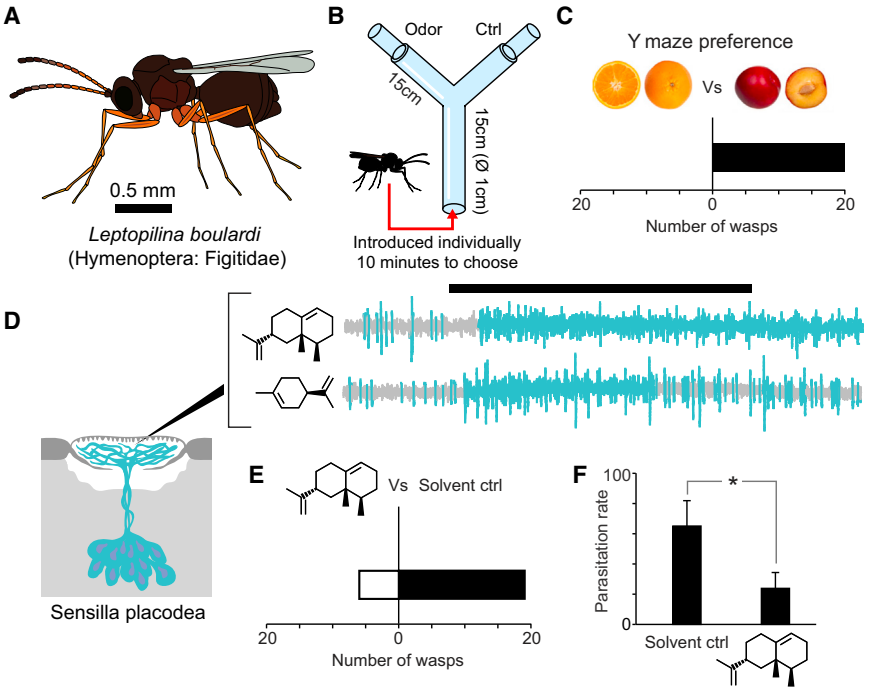

Figure 5. *Citrus* Volatiles Confer Protection against Endoparasitoid Wasps

(A) Schematic drawing of the endoparasitoid wasp *Leptopilina boulardi*, a major larval parasite of *D. melanogaster*.

(B) Schematic drawing of the Y maze olfactory assay used for the wasp behavioral experiments.

(C) Number of wasps choosing oranges versus plums, both infected with fly larvae, in Y maze choice experiments (n = 20).

(D) Representative SSR traces from antennal sensilla placodea of *L. boulardi*, stimulated with valencene and limonene, respectively (at 10^{-2} dilution). As in other Hymenoptera, individual OSNs cannot be discerned. The duration of the stimulus delivery (0.5 s) is marked by the black bar.

(E) Number of wasps moving toward valencene or solvent control in Y maze choice experiments (n = 25). Deviation against even distribution was tested by χ^2 test ($\chi^2 = 6.8$, $p < 0.01$).

(F) Parasitization rate, measured as the number of emerging flies divided by number of eggs laid on plates inoculated with either valencene or solvent control. Asterisk denotes significant difference by Student's t test ($p < 0.05$). Error bars represent SEM.

Identifying the actual ancestral breeding substrate will be a daunting task involving also finding genuinely wild populations of *D. melanogaster*, a feat no one has accomplished so far [29]. The present work, however, provides clear hints as to the characteristics of the ancestral fruit substrate, which should narrow down the search.

Citrus Confers Protection against Endoparasitoid Wasps

Why do *D. melanogaster* then prefer fruits with *Citrus*-like characteristics as oviposition substrate? One reason could be that fruits with a thick epicarp offer protection from parasitoids. In the wild, parasitization from endoparasitoid wasps is a major cause of mortality in drosophilid flies, and in *D. melanogaster*, populations with a >80% parasitization rate have been reported [33]. *Citrus*-like fruits may be advantageous for the reason that the thick rind would form a physical barrier against probing wasps. If a hard epicarp constitutes an obstacle in the parasitization process, we could assume that wasps avoid searching out larvae in fruits with these characteristics. To investigate this, we next examined olfactory-guided behavior of *Leptopilina boulardi* (Figure 5A), an endoparasitoid wasp specialized upon *D. melanogaster* [34], in a Y maze assay (Figure 5B). Confronted with a choice of oranges or plums in the Y maze, wasps made the opposite choice as compared to flies, strongly preferring the smell of plums (Figure 5C). The innate preference of the wasps is accordingly contradictory to that of flies. We next wondered whether the evident repulsion caused by oranges is mediated via the same flavedo terpenes that trigger oviposition in flies. We first used SSR to examine whether wasps can smell these compounds. Recordings from sensilla placodea of the wasps, which contain multiple OSNs (>20) [35], revealed increased spike firing from an unknown number of OSNs in response to stimulation with valencene and limonene (Figure 5D). Having confirmed that wasps are equipped with the machinery to detect these compounds, we next examined the behavioral effect in the Y maze assay. The wasps clearly avoided valencene (Figure 5E). We thus conclude that wasps are repelled by the odor of *Citrus* and that the repellency resides in part or wholly with the presence of terpenes. A fly depositing eggs in a substrate containing valencene and similar terpenes should hence run a reduced risk of having its offspring parasitized. To test this notion, we placed second-instar fly larvae (n = 100 for each treatment) on plates with either fly food baited with valencene or solvent control (mineral oil) added. We thereupon exposed the larvae to ten female wasps for 48 hr, after which we transferred the larvae to vials and then waited for either adult parasitoids or flies to emerge. Indeed, larvae maintained on valencene suffered a significantly decreased rate of parasitism as compared to those maintained on plates with solvent only (Figure 5F). In summary, the *Citrus* preference of flies is presumably a consequence of the lowered parasitization risk conferred by this type of breeding substrate.

Conclusion

We demonstrate that flies prefer fruits with *Citrus* characteristics as oviposition substrate. We show that this preference is mediated via a single class of OSNs expressing *Or19a*, which is both necessary and sufficient for this behavior. In addition, we find that the *Citrus* preference is an ancestral trait, presumably representing an adaptation to fruits found within the native African habitat. Moreover, we show that endoparasitoid wasps—parasites upon fly larvae—are strongly repelled by the smell of *Citrus*, as well as by valencene, the primary ligand of *Or19a*. Finally, larvae maintained on substrates enriched with valencene suffer a reduced risk of parasitism.

Choosing where to lay eggs is a complex behavior that relies upon input from multiple sensory modalities. Although the choice requires complex sensory input overall, our findings suggest that a limited number of olfactory pathways are involved in oviposition site selection. As we show, oviposition preference toward the fruit substrate itself is in fact mediated via only a single olfactory channel. Even though flies choose

to preferentially oviposit on *Citrus*, flies are evidently able to utilize a wide variety of fruits [15, 36]. In nature, flies oviposit in fermenting fruit, where other signals additionally come into play, guiding oviposition site selection. In terms of olfactory cues, the presence of acetic acid is clearly an important factor [14] that presumably serves as a fermentation indicator to the flies. The pathway being fed by input to the ai2A neurons accordingly acts in concert with other circuits—olfactory as well as taste, visual, and tactile—in guiding oviposition site choice. Future work will need to decipher the relative roles of each of these stimuli in mediating this complex behavior.

Experimental Procedures

Fly Stocks

All experiments with WT *D. melanogaster* were carried out with the Canton-S strain. Species other than *D. melanogaster* were obtained from the UCSD Drosophila Stock Center (https://stockcenter.ucsd.edu/info/welcome.php). Transgenic lines were obtained from the Bloomington Drosophila Stock Center (http://flystocks.bio.indiana.edu/), except for *Δhalo;Or22a-GAL4/UAS-Or19a*, which was a gift from J.R. Carlson (Yale University). The *Leptopilina boulardi* strain (established from individuals wild caught in southern France) was a kind gift from J. Stökl (Universität Regensburg).

Stimuli and Chemical Analysis

All synthetic odorants tested were acquired from commercial sources (Sigma, www.sigmaaldrich.com, and Bedoukian, www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery followed Stökl et al. [9]. The headspace collection of volatiles was carried out according to standard procedures. The transgenic orange lines were gifts from L. Peña (Centro de Protección Vegetal y Biotecnología, Instituto Valenciano de Investigaciones Agrarias). GC stimulation analysis was performed as described previously [9, 13].

Behavioral Assays

Trap assay experiments were performed as described previously [9], with response index (RI) calculated as (O − C)/T, where O is the number of flies in the baited vial, C is the number of flies in the control vial, and T is the total number of flies used in the trial. The resulting index ranges from −1 (complete avoidance) to 1 (complete attraction). Oviposition experiments were carried out as described in Stensmyr et al. [13]. Oviposition index was calculated as (O − C)/(O + C), where O is the number of eggs on a baited plate and C is the number of eggs on a control plate. Y maze experiments with wasps were performed as outlined in Figure 5B. For the *dTRPA1* experiments, oviposition plates were placed on silicon heat mats (RS Components, http://www.rs-components.com/index.html) connected to PT100 temperature sensors and a Siemens LOGO! control module (www.siemens.com).

Physiology and Morphology

SSR measurements were performed as described previously [13]. Functional imaging of odor-induced glomerular activity was conducted as outlined in Stökl et al. [9].

Supplemental Information

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2013.10.047.

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