Olfactory-directed behavior in *Drosophila*

Dissertation

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INTRODUCTION

The sense of smell is probably the oldest sensory modality in the animal kingdom (Strausfeld and Hildebrand, 1999). Olfaction is important for survival and reproduction. Feeding, mating and avoidance of natural enemies depend on a functioning sense of smell. The importance of olfaction to life and health was recognized by the award of the 2004 Nobel Prize in Physiology or Medicine to Linda Buck and Richard Axel for their discovery of olfactory receptor genes which strongly contributed to the understanding of how neurons can deal with the uncountable number of different volatiles (Buck and Axel, 1991).

Drosophila melanogaster was chosen as a genetic animal model at the beginning of 20th Century by Thomas Hunt Morgan (Sturtevant, 1965). Since then it has been a very successful animal model for biological research. This is because *D. melanogaster* has only 4 pairs of chromosomes and its genome, which encodes approximately 14.000 genes is fully sequenced (Adams et al., 2000). Moreover, it is cheap and easy to rear under laboratory conditions. As the understanding of the *Drosophila* genome has yielded in a well-equipped genetic toolkit, that e.g. allows knock-in or knock-out specific genes of interest, nowadays *D. melanogaster* has become a model system for research topics as different as cell development, physiology, and behavior.

Flies of the genus *Drosophila* usually feed on microorganisms, including yeast and bacteria. These microorganisms are often associated with decaying plant materials such as fruits, flowers, tree saps, barks, leaves, or fleshy fungi (Carson, 1971), and are known for producing a wide range of secondary metabolites including volatile organic compounds (reviewed in Davis *et al*., 2013). Thus, *Drosophila* species are immersed in a plethora of odors, which form an olfactory odor space through which the animal has to navigate. This means that the olfactory system must be able to quickly extract relevant information from an enormously complex external environment to elicit the appropriate olfactory odor-guided behavior.

Drosophila olfactory organs

The olfactory system of adult *Drosophila* consists of two peripheral olfactory organs, which are located on the third antennal segment (also known as funiculus) and on the maxillary palps (Figure 1A). The surfaces of both antennae and maxillary palps are covered with hair-like structures, called sensilla.

The surface of antennae is covered with five types of sensilla, large and small basiconic, coeloconic, intermediate, and trichoid sensilla (Dweck et al., 2013; Shanbhag et al., 1999). These sensilla house one to four olfactory sensory neurons (OSNs). Large basiconic sensilla cluster at the medial-proximal side of the antenna, while trichoid and intermediate sensilla are situated at the lateral-distal edge. Small basiconic and coeloconic sensilla are interspersed in the middle-distal region of the antenna. These five sensillum types serve distinct chemosensory functions such as localization and evaluation of food (Ai et al., 2010; Dweck et al., 2015a (Chapter III)), oviposition sites (Dweck et al., 2013, Chapter II) and potential mates (Kurtovic et al., 2007; Grosjean et al., 2011; Dweck et al., 2015b (Chapter IV)) as well as avoidance of natural enemies (Ebrahim et al., 2015, Chapter I) and harmful microbes (Stensmyr et al., 2012).

The maxillary palps contain only basiconic sensilla, which in turn subdivide into three different subtypes, each housing two OSNs. Most of the palp sensilla are located on the distal half of the dorsal and on the lateral edges. A few sensilla are also found on the ventral surface of the palp (Singh and Nayak, 1985). A function connected to taste enhancement has been suggested for maxillary palps in flies (Shiraiwa et al., 2008). However, taste enhancement would be a very general function for six OSNs expressing seven different odorant receptors (Ors). So far it was not known, whether the OSNs of the maxillary palps are dedicated to detect specific ecologically relevant chemical compounds, and if so, what the ecological importance of these compounds is. The last chapter, hence, deals with the identification of compounds that are specifically detected by the maxillary palps (Chapter V).

Contrary to the adult olfactory system *Drosophila* larva smell with two bilaterally symmetric dorsal organs (DO) (Figure 1B). The DO is composed of the central dome and six peripheral sensilla. The dome, whose wall is perforated by thousands of pore tubules through which odors can pass, is innervated by 21 OSNs (Stocker et al., 1994; Gerber and Stocker 2007). This small number of larval OSNs contrasts with the around 1300 OSNs that are present in the adult (Stocker 2001).

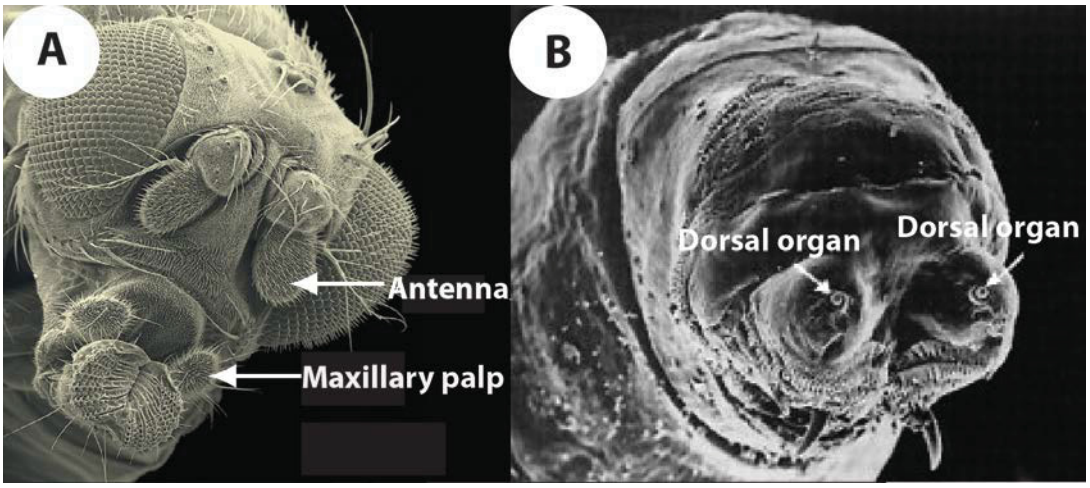

Figure 1. The *Drosophila* olfactory organs. **(A)** Scanning electron micrograph of an adult *Drosophila* head indicating the antenna and the maxillary palp. **(B)** Scanning electron micrograph of a larval *Drosophila* head indicating the dorsal organ. Figure 1A is adapted from Laissue and Vosshall (2008) and Figure 1B is made by Bala Iyengar.

Drosophila olfactory receptors

Drosophila odor receptors (Ors) that were first identified by three independent groups in 1999 (Clyne et al., 1999; Gao and Chess, 1999; Vosshall et al., 1999) contain 60 genes, which are predicted to encode 62 seven-transmembrane-domain proteins via alternative splicing (Robertson et al., 2003). Adult *Drosophila* express 39 Or genes in two antennal trichoid, two antennal intermediate, ten antennal basiconic, and three palp basiconic sensillum types (Vosshall et al., 2000), whereas *Drosophila* larvae express 23 Or genes in the 21 OSNs of the dorsal organs (Fishilevich et al., 2005; Kreher et al., 2005). Thirteen of these 25 Or genes are larval specific and twelve are expressed in both adult and larva (Couto et al., 2005).

Most OSNs of intermediate, basiconic and trichoid sensilla as well as the larval dorsal organ express a single member of the Or family (Couto et al., 2005). These neurons also express a coreceptor, Orco, that heterodimerizes with the Or and is essential for the targeting of the complex to the dendritic membrane (Larsson et al., 2004).

In addition to odor receptors, a genomic analysis identified 66 ionotropic receptor (Ir) genes of three transmembrane domains and a pore loop in *D. melanogaster* (Benton et al., 2009; Croset et al., 2010). Approximately 17 of these genes are expressed in the antenna, mostly in OSNs in the coeloconic sensilla (Benton et al., 2009; Silbering et al., 2011). The four remaining antennal Irs (Ir21a, Ir40a, Ir64a, and Ir93a) are found in arista and sacculus neurons (Benton et al 2009; Ai et al., 2010).

Ir-expressing neurons have narrower tuning breadth than the vast of the majoriety of Or-expressing neurons and are narrowly tuned to select acids and amines, while Ors are tuned to diverse esters, alcohols, ketones and aldehydes (Hallem and Carlson, 2006; Silblerinng et al., 2011)

Olfactory information processing in the *Drosophila* brain

Once binding to the olfactory receptors, the chemical signals of the external world convert to the language of the brain which is the electrical signals. This electrical information is transmitted along the axons of the OSNs and travels down to the primary olfactory processing center, the antennal lobe (AL) (Stocker 1994) (Figure 2). The AL is composed of spheroid structures, called glomeruli (Vosshall et al., 2000). Each glomerulus in the AL receives input from a single OSN population expressing the same Or (Vosshall et al., 2000; Bhalerao et al., 2003; Wilson and Mainen, 2006; Maresh et al., 2008) (Figure 2). The glomeruli of the AL are

also innervated by local interneurons (LNs), which are thought to sharpen the input into the AL via inhibitory interactions (Stocker et al., 1990; Wilson and Laurent, 2005; Seki et al., 2010). The axonal branches of the OSNs within the AL synapse with dendrites of the corresponding class of projection neurons (PNs) (Figure 2). PNs assemble and transmit olfactory information from the glomeruli to the higher brain centers, primarily to the mushroom body (MB) and the lateral horn (LH) (Wong et al., 2002).

As in the adult, the axons of larval OSNs project to the larval AL, in which OSNs synapse with LNs and PNs (Python and Stocker 2002) (Figure 2). The larval AL consists of 21 glomeruli (Fishilevich et al., 2005; Kreher et al., 2005). Each glomerulus is the target of a single OSNs population expressing the same Or, which synapse in most cases with a single PN. Each PN projects through bifurcating axons on the one hand to a stereotyped region of the lateral horn (LH) and on the other hand to one or two of the ~ 35 glomeruli in the mushroom body (MB) calyx (Ramaekers et al., 2005).

Odor-guided behavior

One of the main questions in olfaction is how the neural olfactory components translate into appropriate behavioral responses e.g. attraction and aversion to different ecologically relevant stimuli that are important for finding food sources, mates, oviposition sites as well as for avoiding natural enemies and other dangers.

Flies exhibit innate responses to many odors. Whether an odor is innately attractive or aversive seems to be coded in the LH (Strutz et al., 2014). Innate valence of an odor dictates a primitive reaction of an animal without any previous experience (Gong 2012) and is assumed to be hardwired. This means that upon confronted with a specific stimulus, the animals respond promptly and in a stereotypic and predictable manner (Tinbergen 1951). Innate behavior thus offers an excellent opportunity to explore how complex behaviors are organized in the nervous system and how they are programmed during development. An important first step toward this goal is to understand the neural pathways that mediate a complex innate behavior, from sensory input to behavioral output.

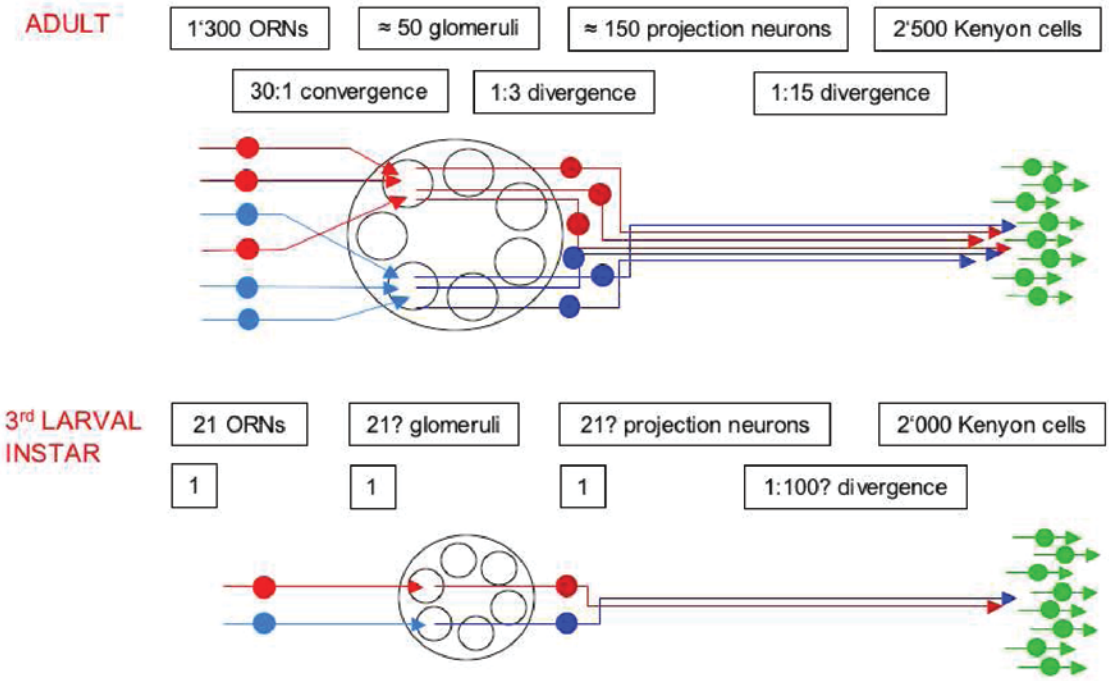

Figure 2. Schematics of the adult and larval olfactory system of *Drosophila* (Ramaekers et al., 2005)

Objectives of this thesis

The main goals of the present thesis are to dissect behavioral responses of *Drosophila* adult and larvae to compounds of highly biological significance and to decipher olfactory pathways underlying displayed behaviors.

Towards this end, I showed that drosophilid flies and their larvae avoid the odors of one genus of parasitoids. This avoidance is mediated by highly specific OSNs, which are necessary and sufficient to govern the parasitoid avoidance behavior (Manuscript I).

I also participated in demonstrating that flies prefer *Citrus* fruits as oviposition substrate. Flies detect terpenes characteristic of these fruits via a single class of OSNs expressing the olfactory receptor Or19a. These neurons are necessary and sufficient for selective oviposition (Manuscript II).

Furthermore, I contributed a study showing that flies are able to detect the presence of hydroxycinnamic acids, i.e. potent dietary antioxidants abundant in fruits, via olfactory cues. Flies are equipped with OSNs expressing Or71a in adult and Or94b in larvae that detect volatile ethylphenols, which are exclusively derived from these acids. These neurons are necessary and sufficient for proxy detection of dietary antioxidants (Manuscript III).

Moreover, I contributed to the identification of three novel fly-produced compounds and dissect the neural mechanisms underlying their detection. All three compounds are detected by the OSNs expressing Or88a that mediate attraction behavior in both sexes. One of these compounds, methyl laurate, is in addition detected by the fruitless-positive OSNs expressing Or47b. This compound promotes optimal male copulation behavior. Both Or47b- and Or88a-based systems and their ligands are conserved in a number of drosophilid species (Manuscript IV).

Finally I participated in examining the contribution of the maxillary palps and their corresponding ligands to odor-guided behavior in the fly (Manuscript V).

OVERVIEW OF MANUSCRIPTS

This thesis is based on the following manuscripts:

Manuscript I

***Drosophila* avoids parasitoids by sensing their semiochemicals via a dedicated olfactory circuit**

Shimaa A. M. Ebrahim*, Hany K.M. Dweck*, Johannes Stökl, John E. Hofferberth, Federica Trona, Kerstin Weniger, Jürgen Rybak, Yoichi Seki, Marcus C. Stensmyr, Silke Sachse, Bill S. Hansson[+] and Markus Knaden[+]

* These authors contributed equally to the work.

[+]These authors share last authorship.

PLoS Biol, 2015, 13(12): e1002318. doi:10.1371/journal. Pbio.1002318

Here, we show that several drosophilid flies and their larvae avoid the odors of one genus of parasitoids. This avoidance is mediated by highly specific OSNs, which in *D. melanogaster* larva express only the olfactory receptor Or49a and respond to the wasps' sex pheromone iridomyrmecin, while in adult express additionally Or85f and respond to the wasps' semiochemicals iridomyrmecin, actinidine and nepetalactol. This information is transferred via projection neurons to a specific part in the lateral horn known to be involved in mediating avoidance. In both *D. melanogaster* adult and larva these neuron are necessary and sufficient to govern odor-driven parasitoid avoidance behavior.

Built on an idea conceived by all authors.

Designed experiments: behavioral experiments BSH, MK, HKMD, SAME (70%)

Performed experiments: Behavioral experiments (100%)

Data analyses: Behavioral experiments MK, SAME (80%).

Wrote manuscript: BSH, MK, HKMD, SAME (30%)

Manuscript II

Olfactory preference for egg laying on citrus substrates in *Drosophila*

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Sophie Kromann, Deni Bown, Ylva Hillbur, Silke Sachse, Bill S. Hansson, and Marcus C. Stensmyr

Current Biology, 2013, 23:1-9

Here, we demonstrate that flies prefer *Citrus* fruits as oviposition substrate. Flies detect terpenes characteristic of these fruits via a single class of OSNs expressing the olfactory receptor Or19a. These neurons are necessary and sufficient for selective oviposition. The *Citrus* preference 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.

Built on an idea conceived by all authors.

Designed experiments: Behavioral experiments MCS, SAME (70%)

Performed experiments: Behavior experiments (100%)

Data analyses: Behavioral experiments MCS, SAME (80%).

Wrote manuscript: MCS, BSH, HKMD, SAME (10%)

Manuscript III

Olfactory proxy detection of dietary antioxidants in *Drosophila*

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Abu Farhan, Bill S. Hansson, and Marcus C. Stensmyr

Current Biology, 2015, 25:455-466

We show that flies are able to detect the presence of hydroxycinnamic acids – potent dietary antioxidant abundant in fruits – via olfactory cues. Flies are equipped with OSNs expressing Or71a in adult and Or94b in larvae that detect volatile ethylphenols, which are exclusively derived from these acids. These neurons are necessary and sufficient for proxy detection of dietary antioxidants. For flies, dietary antioxidants are presumably important to counteract acute oxidative stress induced by consumption or by infection by entomopathogenic microorganisms. The ethylphenol pathway described here adds another layer to the fly's defensive arsenal against toxic microbes.

Built on an idea conceived by all authors.
Designed experiments: Behavioral experiments AF, MCS, SAME (60%)
Performed experiments: Behavioral experiments AF, SAME (70%)
Data analyses: Behavioral experiments AF, MCS, SAME (60%).
Wrote manuscript: MCS, BSH, HKMD, SAME (10%)

Manuscript IV

Pheromones mediating copulation and attraction in *Drosophila*

Hany K. M. Dweck, Shimaa A.M. Ebrahim, Michael Thoma, Ahmed A. M. Mohamed, Ian W. Keesey, Federica Trona, Sofia avista- Llanos, Ales Svatos, Silke Sachse, Markus Knaden and Bill S. Hansson

Proceedings of the National Academy of Sciences USA, 2015, 112: E2829-E2835. doi:10.1073/pnas.1504527112

In this study we demonstrate the presence of three novel fly-produced compounds and dissect the neural mechanisms underlying their detection. All three compounds are detected by the OSNs expressing Or88a that mediate attraction behavior in both sexes. One of these compounds, methyl laurate, is in addition detected by the fruitless-positive OSNs expressing Or47b. This compound promotes optimal male copulation behavior. Both Or47b- and Or88a-based systems and their ligands are conserved in a number of drosophilid species.

Built on an idea conceived by all authors.
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Performed experiments: Behavioral experiments HKMD, MT, SAME (60%)
Data analyses: Behavioral experiments HKMD, MT, MK, SAME (50%)
Wrote manuscript: BSH, MK, HKMD, SAME (10%)

Manuscript V

Olfactory Channels Associated with the Drosophila Maxillary Palp Mediate Short- and Long-range attraction

Hany K.M. Dweck*, Shimaa A. M. Ebrahim*, Mohamed A. Khallaf, Christopher Koenig, Farhan Abu, Regina Stieber, Jerrit Weißflog, Ales Svatos, Ewald Grosse-Wilde, Markus Knaden, Bill S. Hansson

* These authors contributed equally to the work.

Manuscript in preparation

Here, we systematically screened the olfactory sensory neurons (MP-OSNs) housed in the three sensillar types of the maxillary palp of *D. melanogaster* using a large number of natural odor extracts in order to identify novel ligands for each MP-OSN. We found that each MP-OSN is either the sole or the primary detector for a specific chemical compound, and that these neurons detect these compounds with remarkably high sensitivity. We next dissected the contribution of MP-OSNs to behaviors evoked by these compounds and found that maxillary palp contains independent and important olfactory channels that mediate short- and long-range attraction. Our results could provide some insight into why do flies have two olfactory organs.

Built on an idea conceived by all authors.

Designed experiments: Behavioral experiments MAK, SAME (70%)

Performed experiments: Behavioral experiments MAK, SAME (70%)

Data analyses: Behavioral experiments MAK, SAME, HKMD, SAME (60%)

Wrote manuscript: BSH, MK, HKMD, SAME (30%)

MANUSCRIPT I

Drosophila avoids parasitoids by sensing their semiochemicals via a dedicated olfactory circuit

Shimaa A. M. Ebrahim*, Hany K.M. Dweck*, Johannes Stökl, John E. Hofferberth, Federica Trona, Kerstin Weniger, Jürgen Rybak, Yoichi Seki, Marcus C. Stensmyr, Silke Sachse, Bill S. Hansson[+] and Markus Knaden[+]

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PLOS | BIOLOGY

RESEARCH ARTICLE

Drosophila Avoids Parasitoids by Sensing Their Semiochemicals via a Dedicated Olfactory Circuit

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Competing Interests: The authors have declared that no competing interests exist.

Abstract

Detecting danger is one of the foremost tasks for a neural system. Larval parasitoids constitute clear danger to *Drosophila*, as up to 80% of fly larvae become parasitized in nature. We show that *Drosophila melanogaster* larvae and adults avoid sites smelling of the main parasitoid enemies, *Leptopilina* wasps. This avoidance is mediated via a highly specific olfactory sensory neuron (OSN) type. While the larval OSN expresses the olfactory receptor Or49a and is tuned to the *Leptopilina* odor iridomyrmecin, the adult expresses both Or49a and Or85f and in addition detects the wasp odors actinidine and nepetalactol. The information is transferred via projection neurons to a specific part of the lateral horn known to be involved in mediating avoidance. *Drosophila* has thus developed a dedicated circuit to detect a life-threatening enemy based on the smell of its semiochemicals. Such an enemy-detecting olfactory circuit has earlier only been characterized in mice and nematodes.

Author Summary

Detecting danger is a fundamental task for an animal. Larval parasitoids constitute clear danger to the vinegar fly *D. melanogaster* as up to 80% of fly larvae become parasitized in nature. We show that *Drosophila* larvae crawl away from places that smell like the main parasitoid enemies, *Leptopilina* wasps. Furthermore, *Drosophila* adult females avoid laying eggs at those places. This avoidance is mediated via a highly specific olfactory sensory neuron type that is tuned to detect three odors of the parasitoid, including the waps' sex pheromone iridomyrmecin. We identify the neuron type, the receptors, and the odor ligands that mediate this behavior and also show that this neuronal system is both necessary and sufficient to govern the parasitoid avoidance behavior. We also find evidence that this odor-based *Leptopilina* wasp avoidance is conserved across several Drosophila species.

Abbreviations: AL, antennal lobe; FID, flame ionization detector; GC-SSR, gas chromatography-single sensillum recording; LH, lateral horn; MB, mushroom body; OR, odorant receptor; OSN, olfactory sensory neuron; PN, projection neuron; SEM, standard error of the mean; TAAR, trace amine-associated receptor; TIC, total ion current.

Introduction

The olfactory system is tuned to detect cues important to survival and reproduction. One extremely important function is to detect danger [1]. For humans, the odor of smoke is a good example of such an important olfactory warning signal to which we are highly sensitive. Only in two cases, however, have olfactory circuits specifically detecting predator or pathogen odor been characterized regarding the involved olfactory receptor and the danger-derived ligand; the cat urine detection in mice [2] and the pathogen detection in the nematode *Caenorhabditis elegans* [3]. For most insects, olfaction is the primary sense. They use it to find and judge food and oviposition sites [4,5], mates [6,7], or competitors [8], but so far no circuitry has been shown to be involved in detecting life-threatening enemies.

A major cause of death in larvae of the vinegar fly *D. melanogaster* is to be injected with eggs from a parasitoid wasp. The eggs develop into parasitoid larvae, which consume the fly larva from the inside. In some wild subpopulations, up to 80% of the fly larvae are parasitized by different parasitoid wasp species, with *Leptopilina boulardi* and *L. heterotoma* being the most common ones [9,10]. There is thus a very good reason for fly larvae to avoid being parasitized and for female flies to avoid laying eggs where parasitoids are present. With this background, we investigated the reaction of larval and adult vinegar flies to the smell of parasitoids. Both larvae and ovipositing flies showed a clear avoidance behavior to otherwise attractive food and oviposition substrates after these had been perfumed with a *Leptopilina* parasitoid bouquet. We could also demonstrate that in adult flies, avoidance was mediated by the ab10B neuron coexpressing the so-far orphan receptors Or49a and Or85f. This in turn allowed us to identify (-)-iridomyrmecin—a defensive allomone and sex pheromone component of *Leptopilina* [11,12]—as the sole ligand for Or49a and two other parasitoid odorants ((*R*)-actinidine and several stereoisomers of nepetalactol) as ligands for Or85f. As the corresponding neuron in fly larvae only expresses Or49a but not Or85f, larval detection of parasitoids was found to be governed only by iridomyrmecin. When we activated or inactivated the neurons artificially, we could show that they are necessary and sufficient to govern parasitoid avoidance behavior.

Recent investigations revealed that the fly has also developed several other survival strategies to escape parasitoid pressure. Female flies prefer ethanol-rich oviposition sites after they have visually recognized parasitoids. As *D. melanogaster* larvae have higher ethanol tolerance than their parasitoids, the flies self-medicate their offspring [13]. Furthermore, seeing parasitoids leads to sharp decline in flies' oviposition [14]. Finally, upon wasp attack, fly larvae respond with a specific rolling behavior that occasionally flips the attacker to the back [15] and is mediated by a multimodal circuit that includes mechanosensory as well as nociceptive pathways [16]. All these evolutionary adaptations show how important it is for the fly to escape its deadly enemies, the parasitoids.

Results and Discussion

Flies Detect and Avoid Parasitoid Odor

We started our experiments with the main targets of the parasitoid and tested whether *Drosophila* larvae are repelled by the odor of parasitoid wasps (*L. boulardi*). Larvae were strongly repelled by the body wash of parasitoids (Fig 1A, for the behavioral data all figures are based on, see S1 Data). *Orco* mutant larvae, however, lacking functional odorant receptors (ORs), were not repelled (Fig 1A, grey shaded area), indicating that the avoidance behavior is elicited by volatile cues detected by ORs. We next examined the behavior in adult flies. In T-maze and trap assays, we did not observe any avoidance of parasitoid odor (Fig 1B). However, female flies strongly avoided the body wash odor when choosing an oviposition site (Fig 1B). Again,

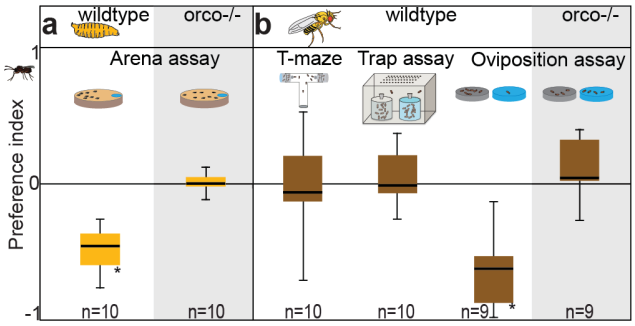

Fig 1. Larvae and ovipositing flies are repelled by parasitoid odor. (**A**) Larval choice assay and preference indices when larvae were exposed to the wash of *L. boulardi*. (**B**) Different choice assays (T-maze, Trap assay, Oviposition assay) for adult flies and resulting preference indices when exposed to the wash of *L. boulardi*. PI = (number of larvae, flies, or eggs in odor side – number in control side) / total number. Bar plots indicate minimum and maximum values (whiskers), the upper and lower quartiles (boxes) and the median values (bold black line). Deviation of the indices against zero was tested with Wilcoxon rank sum test.

doi:10.1371/journal.pbio.1002318.g001

Orco mutant females lacked this avoidance (Fig 1B, grey shaded area). We conclude that the smell of *L. boulardi* is repellent to *D. melanogaster* larvae and ovipositing flies, and that this repellency is mediated by ORs. Obviously, the decision to oviposit seems to be governed by a circuit that is not directly involved in mediating attraction in adult flies—a finding that is in accordance with previous studies showing that both acetic acid [17] and limonene [18] are mediating oviposition but do not attract flies. As the parasitoids do not attack adult flies but only larvae, a general avoidance behavior of adult flies—apart from oviposition avoidance—does not seem to be adaptive.

We next identified the olfactory sensory neuron(s) (OSN) involved in detecting the parasitoid smell. *Drosophila* larvae express 23 different ORs, of which 13 are also expressed in the adult fly [19]. The identification of individual OSN responses in larvae is almost impossible, as all OSNs are colocalized in a single morphological structure, the dorsal organ. However, recording from identified adult OSNs is possible [20–24]. We therefore used a set of diagnostic odors (S1 Fig) to identify adult OSN types and afterwards performed combined gas chromatography-single sensillum recording (GC-SSR) experiments with the headspace of *L. boulardi*. We tested all 48 physiologically different OSN types present on the fly antennae and palps. Although the amount of active odor within the headspace was too low to be visible in the GC trace, a repeatable and strong response was elicited from the ab10B OSN at a specific GC retention time. No other OSN type responded to the extract (Fig 2A). To identify the active compound, we collected a larger quantity of odors by washing wasps in dichloromethane. When repeating the GC-SSR experiments with this wash, the ab10B OSN became activated at the same retention time as found with the headspace (Fig 2B), but now with a visible GC peak. In addition, the same OSN responded to two additional compounds.

Using mass spectrometry, we identified the three active peaks as a non-identified isomer of nepetalactol, (*R*)-actinidine, and (-)-iridomyrmecin (S2 Fig) and confirmed the identification by repeating GC-SSR experiments using synthetic standards (Fig 2B). Furthermore, dose-response experiments revealed that the ab10B neuron exhibited the highest sensitivity to iridomyrmecin (Fig 2C).

Nepetalactol is one of the major compounds of the volatile oil of catnip [25] and has been shown to be an insect repellent [26]. As an insect allomone, however, so far it has only been described for aphids [27]. Both actinidine and iridomyrmecin have been shown to be released by parasitoids. Actinidine acts as a defense compound [28], while iridomyrmecin, which was

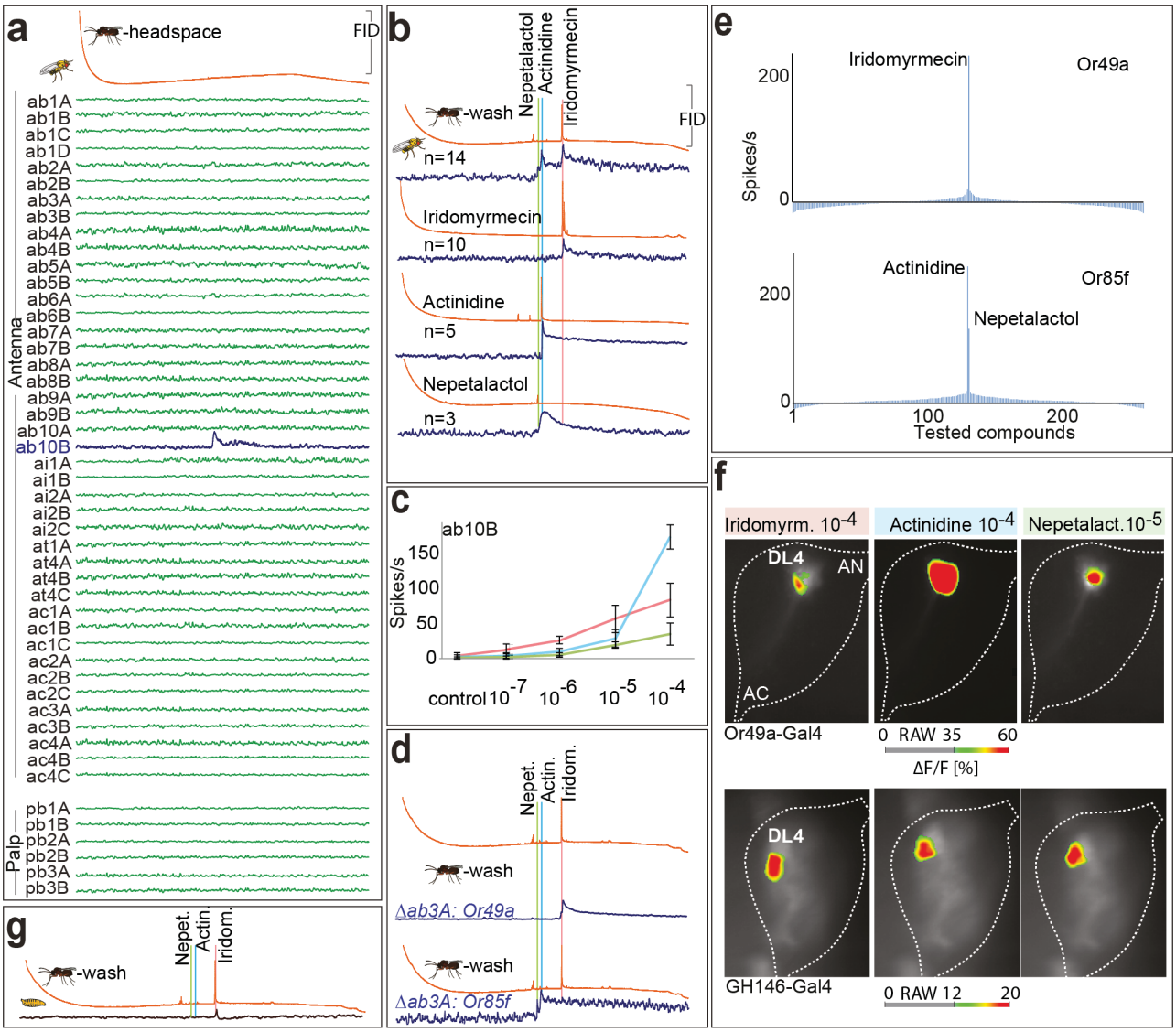

Fig 2. The ab10B neuron detects parasitoid odors. (**A**) Example spike traces of GC-coupled SSR with all *D. melanogaster* OSN types and the headspace of *L. boulardi* (note that the amount of odors within headspace is too low to be detected and analyzed by GC, but is still detected by ab10B). FID, flame ionization detector. (**B**) GC-coupled SSR with the ab10B neuron and the wash of *L. boulardi* (1st panel), as well as the identified active compounds (2nd–4th panel). (**C**) SSR dose-response curves of the ab10B neuron tested with active compounds. (**D**) GC-coupled SSR with mutant ab3A neuron ectopically expressing either Or49a or Or85f. Blue, green, and red lines indicate active compounds. (**E**) Tuning breadths of Or49a and Or85f. 232 odorants are displayed along the *x*-axis according to strengths of responses they elicit from each receptor. Odorants eliciting strongest responses are placed near the center of distribution. Negative values indicate inhibitory responses. For a list of compounds, see S4 Fig; for raw data see S1 Data. (**F**) Identification of glomeruli activated by parasitoid odors (-)-iridomyrmecin, (*R*)-actinidine, and nepetalactol (a mixture of 1S4aR7R7aS, 1R4aS7S7aS-nepetalactol and their enantiomers). 1st to 3rd columns, false color-coded images showing odorant-induced calcium-dependent fluorescence changes in OSNs expressing Or49a or PNs labeled by GH-146-Gal4 at the antennal lobe (AL) level. Flies express UAS-GCaMP3.0 under control of either Or49a-Gal4, or the GH146-Gal4 driver line. (**G**) GC-coupled extracellular recordings from larval dorsal organ and wash of *L. boulardi*. (for more GC-SSR traces of wildtype ab10B neurons and mutant ab3A neurons expressing Or49a or Or85f see S3 Fig)

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first described as a defensive compound of the Argentine ant, *Linepithema humilis* [29] fulfills many functions in the parasitoid wasps of the genus *Leptopilina* [11]. They use iridomyrmecin to defend themselves against predators, to avoid competition among females, and as a major component of the female sex pheromone [11,12]. As a consequence of its ubiquitary use in the

chemical communication of *Leptopilina*, the wasps constantly release at least small amounts of iridomyrmecin, which makes it an ideal key substance to reveal the presence of these parasitoids to their host *Drosophila*.

As the ab10B OSN responds to these iridoids, and as it is one of the cases where two ORs (Or49a and Or85f) are coexpressed within the same OSN type in the adult fly [23], we next explored the role of the individual receptors in parasitoid odor detection. We selectively expressed either Or49a or Or85f ectopically in a *Drosophila* mutant ab3A neuron (i.e., a neuron lacking its own receptor Or22a [30]) and again performed GC-SSR experiments with the body wash of *L. boulardi*. OSNs expressing Or49a responded to iridomyrmecin exclusively (Fig 2D). Of the 16 possible stereoisomers of iridomyrmecin, only 3 (with (-)-iridomyrmecin being the most abundant) have been described to occur in the genus *Leptopilina* [11,12]. When testing those, we found that OSNs expressing Or49a responded only to (-)-iridomyrmecin, but not to (+)-iridomyrmecin or (+)- or (-)-isoiridomyrmecin (S5 Fig).

When we tested OSNs misexpressing Or85f, they became activated by (*R*)-actinidine and nepetalactol (Fig 2D). Further investigation revealed that the ab10B neuron detected both enantiomers of actinidine (although only (*R*)-actinidine is present in *Leptopilina* wasps). Because of chromatographic limitations, we were unable to determine the absolute configuration of the nepetalactol stereoisomer produced by the wasps. However, all synthetically available stereoisomers (i.e., 1S4aR7R7aS-nepetalactol, 1R4aS7S7aS-nepetalactol and their enantiomers) activated OSNs expressing Or85f (S6 Fig). Thus, both ORs expressed in the ab10B OSN are involved in the detection of the parasitoid volatile blend although sensitive to different components thereof. To further test the specificity of the ab10B OSN, we screened neurons ectopically expressing one of both receptors with a set of 232 compounds including odorants from a wide range of different chemical classes (Fig 2E, S4 Fig). From these results and from another study, where Or49a was tested against almost 500 odorants [31], we conclude that the ab10B neuron is highly specific to the *Leptopilina* odorants (-)-iridomyrmecin (Or49a), and (*R*)-actinidine and nepetalactol (Or85f). The spectrum of detection by the ab10B OSN has thus been widened, not by lessening the specificity of a single receptor but by adding a second highly specific one.

We next measured odor-induced activity patterns in the *D. melanogaster* adult antennal lobe (AL) (Fig 2F). We used the Gal4-UAS system to express the Ca^{2+}-sensitive reporter GCaMP3.0 [32] under control of the Or49a promoter. Some of the former studies [23,33–36] did not observe expression of Or49a. However, we expressed GCaMP3.0 under control of *Or49a-Gal4* and validated the functionality of our *Or49a-Gal4* on the adult antenna using confocal microscopy (S7 Fig). All three parasitoid compounds elicited a strong activation. When we used the GH146 enhancer trap line, all three compounds elicited a clear and exclusive activation at the output level of the DL4 glomerulus. Hence, the calcium imaging results confirmed the conclusion from the SSR experiments that the ab10B neurons expressing Or49a and Or85f become activated by the parasitoid odorants (*R*)-actinidine, several enantiomers of nepetalactol, and (-)-iridomyrmecin. They also showed that these signals transfer via dedicated projection neurons to (PNs) higher brain centers. Recent findings suggest that laterally situated glomeruli of the AL mainly become activated by aversive stimuli [37]. Our finding that the DL4 glomerulus becomes activated by odorants governing parasitoid avoidance further supports this segregated representation of positive and negative innate olfactory valence within the medial and lateral AL, respectively.

Interestingly, in contrast to adult flies, larvae do not express Or85f but only Or49a [19]. As expected, when performing GC-coupled extracellular recordings from the dorsal organ with the body wash of parasitoids, we did not observe any responses to the Or85f ligands actinidine and nepetalactol, but observed a strong response to the Or49a ligand iridomyrmecin (Fig 2G).

Contrary to adult females, larvae do not only lose part of their offspring but become killed by the parasitoid. Hence, the larval olfactory system should even be better suited to detect parasitoid odorants. It therefore remains unclear why *Drosophila* larvae do not express Or85f, and by that are restricted to detect iridomyrmecin only.

Parasitoid Odor Activates Avoidance-Specific Brain Region

We next investigated how the information transfers to the next levels of olfactory processing, the mushroom body (MB) calyx and the lateral horn (LH). When characterizing the innervation patterns of DL4 PNs we found that the projection pattern of these neurons in higher brain centers was stereotypic among individual flies as shown for other PNs in previous studies [38,39]. We next compared how this innervation pattern relates to that of DA2-PNs conveying information from another highly repellent olfactory pathway mediating odor information regarding the presence of detrimental microbes [40]. Interestingly, the comparison revealed that the projection pattern of both PN types overlapped strongly within a posteriomedial domain in the LH and in the base MB calyx (Fig 3). The overlapping pattern of two PN populations that both convey information on odorants of strongly negative hedonic valence is in accordance with recent findings that olfactory stimuli with negative and positive valence activate separate domains in the LH [41–43].

Importance of ab10B Neurons for Parasitoid Avoidance

We next tested whether the presence of one of the ab10B ligands was sufficient to inhibit oviposition. Flies exhibited a strong tendency to avoid the Or85f ligand (*R*)-actinidine (we applied 1 μg, i.e., ca 50 wasp equivalents) and oviposited significantly less when confronted with 3 wasp equivalents (i.e., 1 μg) of the Or49A ligand (-)-iridomyrmecin (S8 Fig). Furthermore, larvae only expressing Or49a consequently became significantly repelled by the Or49a ligand (-)-iridomyrmecin but did not avoid the Or85f ligand (*R*)-actinidine (S8 Fig).

Do ovipositing flies lacking a functional ab10B OSN still avoid the parasitoid smell? To silence the ab10B neurons specifically, we expressed the temperature-sensitive mutant dynamin Shibire^{ts} [44] under the control of the Or49a promoter. At the restrictive temperature (30°C), flies carrying this construct did not display any oviposition avoidance toward the parasitoid wash (Fig 4A), while they showed a strong aversion to the wash at the permissive temperature (23°C). Parental lines and wildtype flies avoided the parasitoid odor at the restrictive temperature. As Or49a and Or85f are coexpressed in the ab10B neuron, expressing Shibire^{ts} under the control of the Or49a promotor should erase the functional significance of both receptors. However, we also expressed Shibire^{ts} under the control of the Or85f receptor, which again yielded in reduced olfactory avoidance, when flies were tested at the restrictive temperature (Fig 4A).

Larvae that expressed Shibire^{ts} under the Or49a promoter again exhibited no avoidance at the restrictive temperature, while the permissive temperature resulted in avoidance not different from wild type and parental lines (Fig 4B). As the larvae at the restrictive temperature still targeted the attractant ethyl butyrate, the loss of behavior at restrictive temperature seems to be ligand- and Or49a-specific. We thus conclude that activation of OSNs expressing Or49a in larvae and Or49a and Or85f in adult flies is necessary for behavioral avoidance of parasitoid odors.

To test whether the activation of ab10B neurons was sufficient for avoidance of parasitoid smell, we expressed the photo-activated cation-selective channel channelrhodopsin-2 (*ChR2*) in the neurons by using either the *Or49a-Gal4* or the *Or85f-Gal4* driver. We then tested how blue light (470 nm, i.e., the wave length activating the expressed cation channel) affected adult

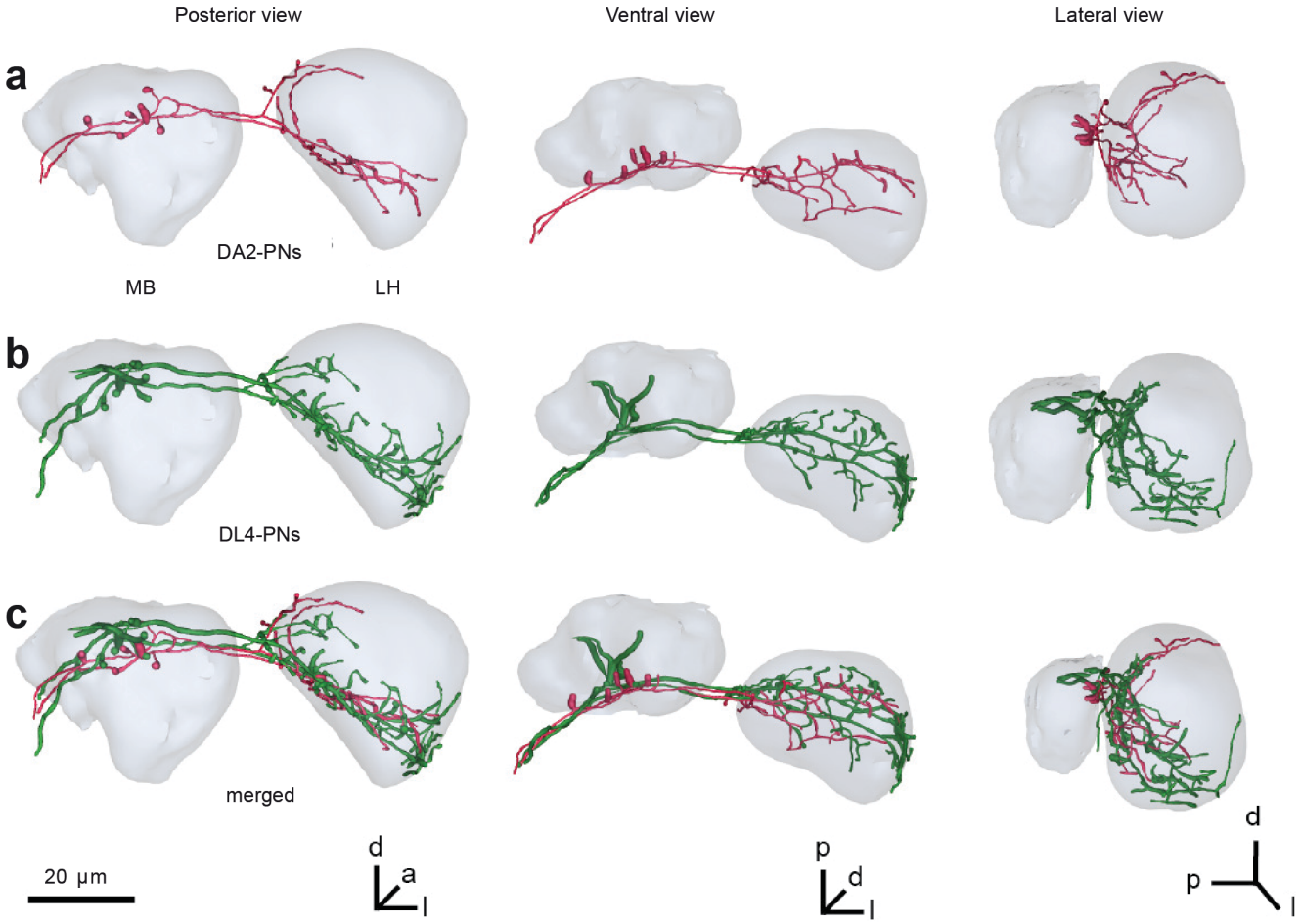

Fig 3. Innervation patterns of DL4 and DA2 PNs in MB and LH. (**A**) Reconstruction of two DA2 PNs. (**B**) Reconstruction of two DL4 PNs. (**C**) comparison of DA2 and DL4 domains after registration of datasets into a common reference space. DA2 and DL4 PNs overlap in the base of the MB and ventroposterior LH. a: anterior, d: dorsal, l: lateral, p: posterior v: ventral.

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oviposition and larval crawling behavior. Contrary to wild-type flies and parental lines, *CHR2*-females avoided illuminated oviposition sources (Fig 4C). The same held true for *CHR2*-larvae that became significantly more repelled by blue light than control lines (Fig 4D).

We conclude that activation of the ab10B neuron and its larval equivalent is necessary and sufficient to elicit avoidance of parasitoid smell in female *D. melanogaster* adults and larvae, respectively.

ab10B Response Is Selective for *Leptopilina* Odor

D. melanogaster becomes parasitized not only by *Leptopilina* wasps but also by other wasp genera. We asked whether the ab10B neuron also responded to odorants from predators or other parasitoid species (Fig 5A). Therefore, we performed GC-SSR experiments with ab10B neurons using body washes of four potential *Drosophila* predators and of four additional parasitoid wasp species (*L. heterotoma*, *Asobara tabida*, *A. japonica*, and *Trichopria* spec.). While the neurons again responded to iridomyrmecin, actinidine, and nepetalactol present in *L. heterotoma*, we could not identify any further ab10B ligand—neither in the odor of the insect predators nor

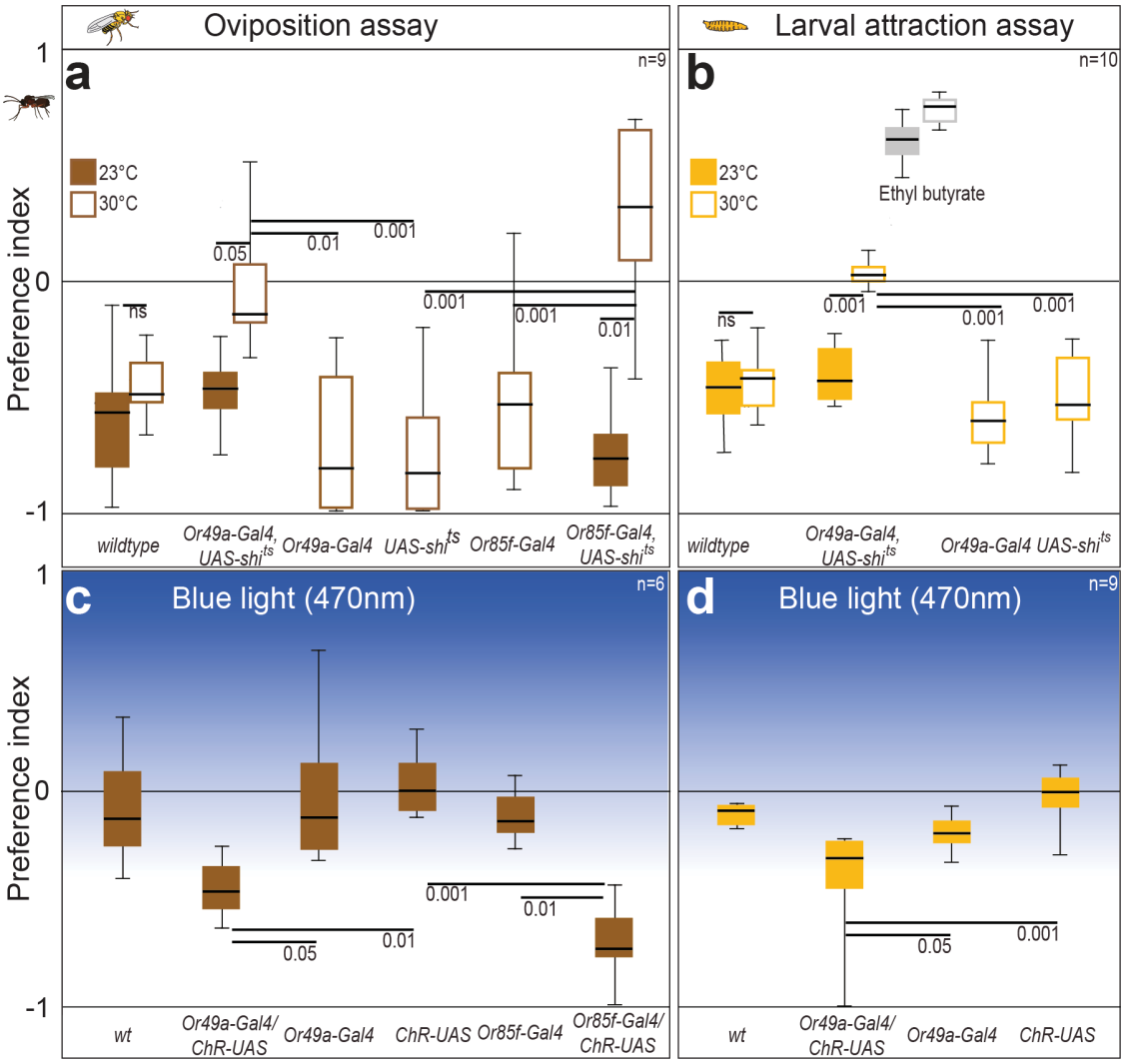

Fig 4. The ab10B neuron is necessary and sufficient to govern oviposition avoidance and larval avoidance behavior in *D. melanogaster*. (**A**) Preference indices of ovipositing wildtype flies, flies expressing *Shibire*^{ts} in ab10B neuron, and corresponding parental lines at restrictive (30°C) and permissive (23°C) temperature when tested with wash of *L. boulardi*. (**B**) Preference indices of the same fly lines when tested in the larval assay. Attraction to ethyl butyrate (grey bars) depict that loss of odor-guided behavior in larvae expressing *Shibire*^{ts} in ab10B neuron is odorant specific. (**C**) Light preference of ovipositing wildtype flies, flies expressing channelrhodopsin in ab10B neuron, and corresponding parental lines. (**D**) Light preferences of the same fly lines when tested in the larval assay. (**A–D**) Bar plots indicate minimum and maximum values (whiskers), the upper and lower quartiles (boxes), and the median values (bold black line). Groups were compared by the Kruskal Wallis test with a Dunn's multiple comparison for selected pairs. For calculation of preference indices, see Fig 1.

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in that of the other parasitoid species. Accordingly, *D. melanogaster* avoided the odor of *L. boulardi* and *L. heterotoma* but neither ovipositing adult flies nor larvae displayed any odor-based avoidance behavior (Fig 5B) towards the predators or the parasitoids belonging to other genera. We conclude that the ab10B-dependent avoidance behavior is restricted to one of the most harmful parasitoid genera [9,10,45], the iridoid-producing *Leptopilina*.

Avoidance of *Leptopilina* Odor Is Not Restricted to *D. melanogaster*

Finally, we asked whether odor-based detection and avoidance of *Leptopilina* wasps is specific for *D. melanogaster*. Parasitoids of the genus *Leptopilina* do not only target *D. melanogaster*

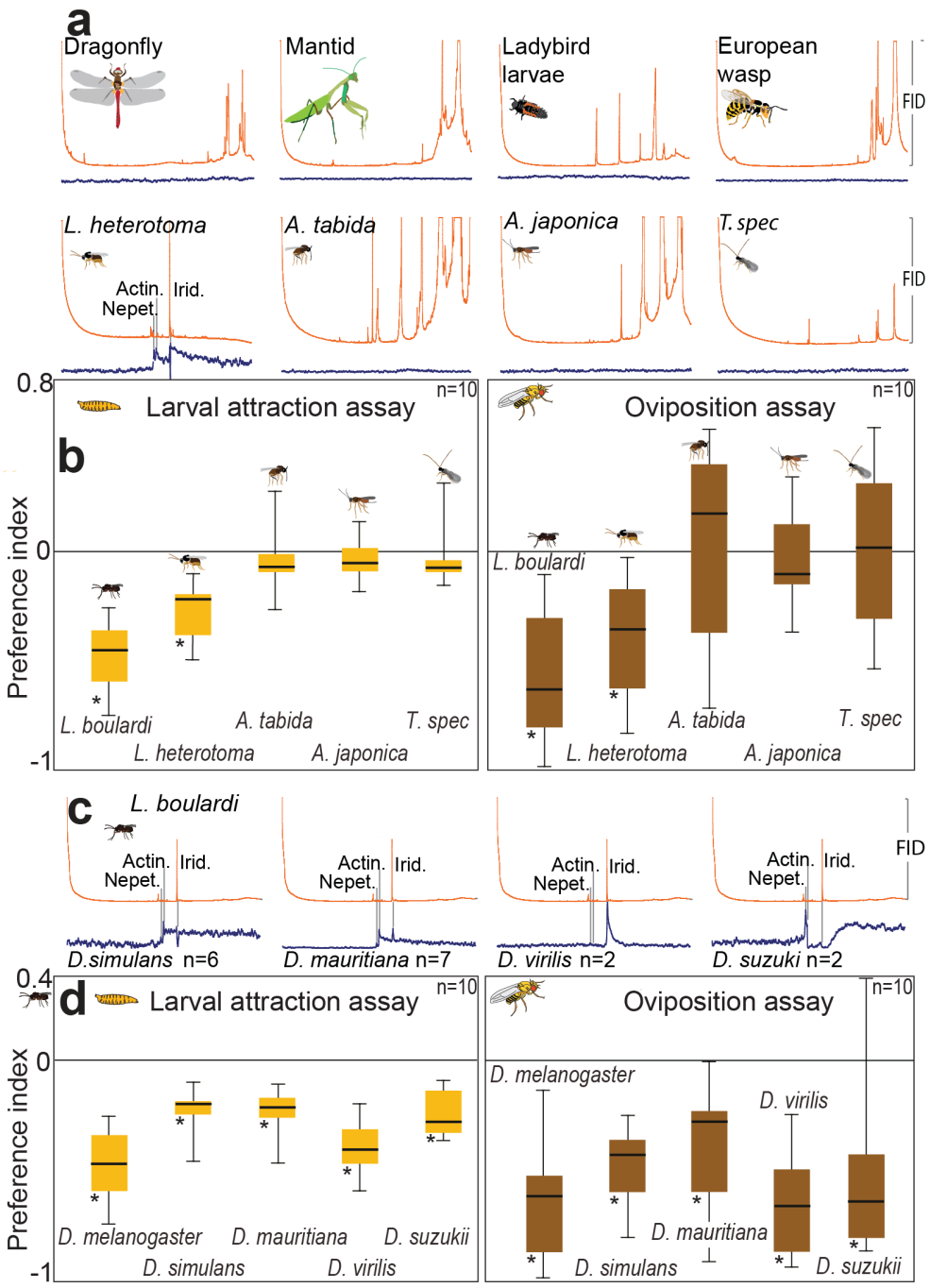

Fig 5. The ab10B neuron detects *Leptopilina* odors only, and detection and avoidance of Leptopilina odor are conserved within many drosophilid flies. (**A**) Example spike traces of GC-coupled SSR recordings of *D. melanogaster* ab10B neurons with washes of four potential predators (top row) and four parasitoid wasp species (bottom row). (**B**) Preference indices of *D. melanogaster* larvae and ovipositing females when tested with washes of five parasitoid species. Note that only washes of *L. boulardi* and *L. heterotoma* contain ligands of the ab10B neuron and provoked avoidance behavior in *D. melanogaster*. (**C**) Example spike traces of GC-coupled SSR recordings of ab10B neurons of 5 different *Drosophila* species with washes of *L. boulardi*. (**D**) Preference indices of larvae and ovipositing females of 5 *Drosophila* species tested with wash of *L. boulardi*. (**A–B**) Deviations of indices against zero were tested with Wilcoxon rank sum test. Asterisks, $p < 0.05$; error bars depict standard deviation.

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but also many other *Drosophila* species [46,47]. All of the 12 *Drosophila* species, whose genomes have been published, express Or49a and Or85f. However, the orthologs of Or49a (Or85f) only show 57.1% (63.5%) amino acid similarity with a dN/dS value of 0.23 (0.19), respectively. We therefore asked whether detection and avoidance can also be observed in *D. simulans*, *D. mauritania*, *D. virilis*, and *D. suzukii*. As all four species either originate from Africa or Asia, i.e., continents where wasps of the genus *Leptopilina* also occur, they should all have a long evolutionary history with at least one *Leptopilina* species. In GC-SSR experiments with each of the *Drosophila* species, we always found an OSN that responded to the headspace of *L. boulardi* (Fig 5C). We cannot exclude that the OSN responding to wasp odors in those flies expressed another so-far unidentified receptor. However, in all species, the colocalized neurons exhibited similar responses like the *D. melanogaster* ab10A neuron (S9 Fig, for raw data see S1 Data). Therefore—also in the other species—it was most probably the ab10 sensillum that detected the wasp odor, which supports the general involvement of Or49a and Or85f in parasitoid detection of the genus *Drosophila*. Furthermore, larvae and ovipositing adults of all tested species avoided the parasitoid headspace (Fig 5D and 5E). We conclude that iridoid-based *Leptopilina* avoidance is a conserved feature of several *Drosophila* species. Whether or not the detection of the *Leptopilina* odors is mediated in all species by Or49a and Or85f remains open. However, the comparatively low conservation of these receptors is not necessarily a counterargument. It has been shown before that receptors exhibiting low sequence conservation in different *Drosophila* species still can be tuned to the same ligands [48]. Another functionally conserved receptor, Or56a, on the other hand is also one of the molecularly most conserved receptors among the drosophilids. Evolution obviously finds different ways to preserve function among olfactory receptors.

Conclusion

We show that drosophilid flies and their larvae avoid the odor of one genus of parasitoids. The question, why *Drosophila* exhibits a dedicated circuit to the odors of *Leptopilina* wasps while the odors of the other tested parasitoid wasps did not evoke any olfactory avoidance behavior at all, remains open. Although *Leptopilina* seems in some habitats to be the most abundant parasitoid, parasitation rates by other genera can become high [9,10,45]. However, *Drosophila* does not only exhibit olfactory parasitoid avoidance but also avoidance based on visual cues [13,14] as well as on mechanosensory and nociceptive cues [15]. It, therefore, might be that the nonolfactory cues become especially important in the avoidance of those parasitoids that do not carry any of the ab10B ligands. The animals tested in our experiments had never experienced parasitoids before. Hence, the observed avoidance behavior is governed by innate rather than learned negative hedonic valence. Dedicated pathways involved in detection and processing of innate valence are usually restricted to odorants of outstanding ecological valence [49] and are not restricted to insects [50]. Zebrafish detect the death-associated odor cadaverine based on the specific and highly sensitive receptor TAAR13c [51]. Mice detect predators like cats, snakes, and rats [52] as well as conspecifics [53] based on olfactory cues. It has been shown that the trace amine-associated receptor 4 (TAAR4) mediates innate repellence to cat urine [2], while TAAR5 detects a compound in mouse urine and mediates attraction towards conspecifics [53]. Recent findings in *Drosophila* revealed several dedicated olfactory pathways that either mediate avoidance [40,54], mating [55], or oviposition [18]. Enemy avoidance based on learned cues have been shown for *C. elegans*. After first contact with the pathogen *Serratia marcescens*, the nematode learns to avoid the bacterium based on a specific bacterium-derived peptide [3]. We show that the fly's ab10B neuron specifically mediates innate aversion towards the parasitoid-derived odorants (-)-iridomyrmecin, (*R*)-actinidine and different

enantiomers of nepetalactol. To our knowledge, this presents the first case in which an animal not only smells and avoids its enemy but does this based on the enemy's semiochemicals, including a sex pheromone. The evolutionary strategy to use the odor-based sex communication system of an enemy to avoid it should be highly adaptive. The possibility for evolutionary countermeasures from the parasitoid side should be limited as it is difficult to resign the sex communication system.

Methods

Experimental Procedures

***Drosophila* stocks.** All experiments with wild type (WT) *D. melanogaster* were carried out with the Canton-S strain. Species other than *D. melanogaster* were obtained from the *Drosophila* species 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 the w^{118}; Δ*halo/cyo; UAS-Or49a*, w^{118}, and Δ*halo/cyo; UAS-Or85f*, which were a kind gift from Dr. J.R. Carlson (Yale University, USA), and w[*]; P{w [+ mC] = UAS − ChR2.S}3, which was a kind gift from Dr. Andrè Fiala (Georg August University, Germany).

Rearing of wasps. We used *D. melanogaster* as the host to rear *L. boulardi*. To rear a cohort of *L. boulardi*, 20–30 *Drosophila* flies of mixed age and sex were put into a jar containing an approximately 2 cm thick layer of standard corn based rearing medium. After 48 h, the flies were removed and 5 to 10 mated females of *L. boulardi* were put in the jar. Wasps emerge approximately 28 d after oviposition and were kept at 25°C, 60% humidity and a 16:8 h light: dark cycle. For experiments, we either used the headspace of 20 parasitoid wasps or the body wash of 100 parasitoid wasps (solved in 1,000 μl of dichloromethane).

T-maze assays. T-maze experiments were carried out as described in [40]. In brief, 30 4–5 d-old female flies were introduced into the bottom part of a t-shaped tube (length of each arm, 4 cm; diameter, 1 cm) and over 40 min were allowed to enter (but not to leave) via pipette tips (tip opening, 2 mm) eppendorff caps attached to the two upper arms of the t-shaped tube. The lids of the Eppendorff caps contained 0.5 ml agar (1%). In addition, each Eppendorff cap contained a piece of filter paper that was loaded either with 50 μl of dichloromethane containing the equivalent of the wash of 3 wasps or with solvent only. We let the solvent evaporate for 5–10 min before the filter papers were added to the caps. The attraction index (AI) was calculated as AI = (O − C) / 30, where O is the number of flies entered the odorant containing trap and C is the number of flies entered the solvent containing trap.

Trap assays. Trap assay experiments were performed as described in [37]. In brief, 50 4–5 d old female flies were introduced in to a small box (length, 10 cm; width, 8 cm; height, 10 cm) that contained two smaller containers (height, 4.5 cm; diameter, 3 cm). For 24 hr, flies could enter (but not leave) these containers through a pipette tip (tip opening, 2 mm). Containers were equipped with the lid of an eppendorff cap that was loaded either with 50 μl of dichloromethane containing the equivalent of the wash of 5 wasps or with solvent only. The attraction index (AI) was calculated as AI = (O − C) / 50, where O is the number of flies entered the odorant containing trap, and C is the number of flies that entered the solvent-containing trap.

Oviposition assays. Oviposition assay experiments were carried out in a cage (50 x 50 x 50 cm) that was equipped with two petri dishes (diameter, 9 cm) containing agar (1%), of which one was loaded with 50 μl of dichloromethane containing the equivalent of the wash of 5 wasps or with solvent only. Thirty 4–5 d-old female flies were placed in each cage. Experiments were carried out in a climate chamber (25°C, 70% humidity, 12 h light:12 h dark cycle). The number of eggs was counted after 24 hr. 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.

Larval two-choice assays. The larval olfactory choice assay was performed as described in [56]. Briefly, 50 second or third instar larvae were placed in the center of a Petri dish, filled with 1% agrose. The Petri dish contained two discs of filter paper (diameter, 0.5 cm) placed at opposite positions at the periphery of the dish. Filter papers were loaded either with 20 μl of dichloromethane containing the equivalent of the wash of 2 wasps or with 20 μl of dichloromethane only. Larvae were allowed to crawl for 5 min before their position on the Petri dish was determined. Attraction index was calculated as ((O − C) / T), with O being the number of larvae on the side of the dish loaded with wasp odor, C being the number of larvae on the solvent side, and T being the total number of larvae.

Shibire experiments. The experiments with larvae or flies expressing shibire^{ts} were performed as described above except that the temperature was set as either 23°C (permissive temperature) or at 30°C (restrictive temperature).

Channelrhodopsin experiments. For channelrhodopsin-2 (ChR2) experiments, larvae or adult flies were raised in darkness on food inoculated with 200 μl of 150 mM all-trans retinal (Sigma, Germany). Oviposition and larval experiments were performed in a petri dish, filled with 1% agarose, which contained a single LED-emitting blue light (480 nm wavelength) on one side, while the other side was not illuminated. In the larval experiment, 50 larvae were allowed to crawl for 5 min before their position on the Petri dish was determined. Attraction index was calculated as ((O − C) / T), with O being the number of larvae on the illuminated side of the dish, C being the number of larvae on the nonilluminated side, and T being the total number of larvae. The oviposition experiment with flies expressing channelrhodopsin-2 was slightly modified from the oviposition assay mentioned above. A single Petri dish (illuminated on one side by a blue LED) was located in a small container (length, 10 cm; width, 10 cm; height, 10 cm) that prohibited the desiccation of the illuminated (and hence slightly heated) agar. After 24 hr, eggs on the illuminated and the nonilluminated side were counted. Attraction index was calculated as ((O − C) / T), with O being the number of eggs on the illuminated side of the dish, C being the number of eggs on the nonilluminated side, and T being the total number of eggs.

SSR/GC-SSR

Adult flies were immobilized in pipette tips, and the third antennal segment or the palps were placed in a stable position onto a glass coverslip. Sensilla were localized under a binocular at 1,000x magnification and the extracellular signals originating from the OSNs were measured by inserting a tungsten wire electrode in the base of a sensillum. The reference electrode was inserted into the eye. Signals were amplified (10x; Syntech Universal AC/DC Probe, www.syntech.nl), sampled (10,667 samples/s), and filtered (100–3,000 Hz with 50–60 Hz suppression) via USB-IDAC connection to a computer (Syntech). Action potentials were extracted using Syntech Auto Spike 32 software. We used a set of diagnostic odors (S1 Fig, i.e., odors that have been shown to activate neurons expressing a specific receptor [20]) to identify adult OSN types. As in *D. melanogaster*, the colocalization of neurons expressing different receptors in a single sensillum is conserved; we were able to identify even those neurons with orphan receptors by checking out the response patterns of colocalized neurons. Neuron activities were recorded for 10 s, starting 2 s before a stimulation period of 0.5 s. Responses from individual neurons were calculated as the increase (or decrease) in the action potential frequency (spikes/s) relative to the prestimulus frequency. For GC stimulation, 1 μl of the odor sample (1 μl of actinidine and nepetalactol solutions correspond to ca 50 wasp equivalents, 1 μl of iridomyrmecin solution corresponds to 3 wasp equivalents, 1 μl of wasp wash corresponds to 0.1 wasp equivalent) was injected onto a DB5 column (Agilent Technologies, http://www.agilent.com),

fitted in an Agilent 6890 GC, equipped with a four-arm effluent splitter (Gerstel, www.gerstel.com), and operated as previously described (Stökl et al., 2010) except for the temperature increase, which was set at 15°C min^{-1}. GC-separated components were introduced into a humidified airstream (200 ml min^{-1}) directed toward a mounted fly. Signals from OSNs and FID were recorded simultaneously.

Chemical Analysis

Solvent extract of 100 wasps were analysed by gas chromatography coupled with mass spectrometry (GC-MS). Samples were analysed on a Shimadzu GC2010 gas-chromatograph (GC) connected to a QP2010 plus mass-spectrometer (MS; Shimadzu, Germany) and an Agilent 7890GC coupled with an 5975c MS (Agilent Technologies, Germany). The Shimadzu GC-MS was equipped with either a nonpolar capillary column (BPX-5, 30 m length, 0.25 mm inner diameter, 0.25 μm film thickness; SGE Analytical Science, UK), or a BetaDex 225 cyclodextrin column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness; Sigma-Aldrich, Germany) or a GammDex 120 cyclodextrin column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness; Sigma-Aldrich, Germany). When using the BPX5 column, Helium was used as a carrier gas with a constant linear velocity of 50 cm/s^{-1} and the temperature program of the GC oven started at 80°C and was raised by 5°C/min^{-1} to 280°C. For both cyclodextrin columns, the carrier gas flow was reduced the 35 cm/s^{-1} and the oven temperature started at 50°C and was raised by 2°C/min^{-1} to 220°C. The MS was run in electron impact (EI) mode at 70 eV and set to a scan range from 35 to 600 mz^{-1}. All samples were injected splitless. The Agilent GC-MS was equipped with a Cyclosil-B cyclodextrin column (30 m length, 0.25 mm inner diameter, 0.25 μm film thickness; Agilent Technologies, Germany). Helium was used as a carrier gas with a constant linear velocity of 36 cm/s^{-1}. The initial temperature of the GC oven of 40°C was held for 2 min and afterwards raised by 2°C/min^{-1} to 170°C and then with 70°C to 250°C. The MS was run in electron impact (EI) mode at 70 eV and set to a scan range from 33 to 350 mz^{-1}. All samples were injected splitless. Compounds were identified by comparing the mass spectrum and retention time with that of synthetic reference compounds (S2 Fig).

Synthetic Compounds

Stereoisomers of Iridomyrmecin were synthesized as described in [12] and [57]. Actinidine and Nepetalactol were synthesized as described by [58].

Optical Imaging

Flies were prepared for optical imaging as previously described by [59]. Briefly, flies were anesthetized on ice; the head capsule was opened by incising the cuticle between the antennae and the eyes. With the brain immersed in Ringer's saline (130 mM NaCl, 5 mM KCl, 2 mM MgCl2, 2 mM CaCl2, 36 mM sucrose, 5 mM Hepes, [pH 7.3]), the ALs were exposed by removing muscle tissue, glands, and the trachea. We used a Till Photonics imaging system with an upright Olympus microscope (BX51WI) equipped with a 20x Olympus objective (XLUM Plan FL 20x/0.95W). A Polychrome V provided light excitation (475 nm) and a filter set ensured passage of only relevant wavelengths (excitation: SP500, dicroic: DCLP490, emission: LP515). The emitted light was captured by a CCD camera (Sensicam QE, PCO AG) with a symmetrical binning of 2 (0.625 x 0.625 μm/pixel). For each measurement, a series of 40 frames was taken (4 Hz). Odors were applied for 2 sec. Pure compounds were diluted in mineral oil (Carl Roth GmbH + Co. KG); 6 μl of the diluted odors were pipetted onto a small piece of filter paper (~1 cm^2, Whatman), placed inside a glass Pasteur pipette. Filter papers were prepared ca. 30 min before every experimental session. For odor application, a stimulus controller (CS-55, Syntech)

provided a continuous air flow (l/min) in which odor injection was applied via two disposable Pasteur pipettes. For odor stimulation, the air stream switched from a blind Pasteur pipette to the stimulus pipette. Calcium imaging recordings were processed using custom-written software in IDL (ITT Visual Information Solutions) as described in detail in [59]. An in vivo 3-D atlas of the *Drosophila* AL [60] served to link the calcium signals to identified glomeruli.

Registration of uPNs into Reference Space in Central Brain

Neurons were intracellulary stained with biocytin or Lucifer yellow and histologically processed as described previously [40,61]. For brain neuropil background staining, the synaptic antibody nc82 [62] was used. High-resolution scans were done with a Zeiss LSM 510 confocal microscope using a 63 x water immersion objective. Image stacks were imported to Amira 5.6, and neuronal arborizations and neuropil were manually segmented. For neuron reconstruction, the Skeleton plugin [63] of Amira was used. For registration of datasets, a label template of the central brain (MB and LH) was chosen out of $n = 58$ preparations following the method of [64]. The warping of segmented labels of the MB and LH onto the template was done in a two-step process: an affine transformation with 12 degree of freedom (12 DOF) followed by an elastic registration using modules of Amira. The calculated transformation matrix was applied to the neuron reconstruction and thus transformed to the template reference space.

Supporting Information

S1 Data. Raw data the figures of this manuscript are based on.
(XLSX)

S1 Fig. Diagnostic set of odors used to identify OSNs during SSRs. OSNs that are expected to exhibit strong responses to a specific odor are given in brackets.
(TIF)

S2 Fig. Iridoid compounds produced by *L. boulardi* and *L. heterotoma*. Total ion current (TIC) chromatograms on a nonpolar (BPX5) GC column of an extract of females of (**A**) *L. boulardi* and (**B**) *L. heterotoma*. (**C**) Molecular structure of the iridoid compounds found in *L. boulardi* and *L. heterotoma*. Numbers correspond to the peaks in (A) and (B). (**D–G**) Identification of (*R*)-actinidine: (**D**) TIC chromatograms on a cyclodextrin (CycloSil B) GC column of synthetic (*S*)- and (*R*)-actinidine, and extracts of *L. boulardi* and *L. heterotoma*. (**E**) Mass spectrum of synthetic (*R*)-actinidine and the indicated peak in *L. boulardi* (**F**) and *L. heterotoma* (**G**). The peaks found in the extracts of *L. boulardi* and *L. heterotoma* show the same retention time and mass spectrum as (*R*)-actinidine.

Identification of nepetalactol (**H–M**): (**H**) TIC chromatograms on a cyclodextrin (CycloSil B) GC column of racemic samples of synthetic 1R4aS7S7aS- and 1S4aR7R7aS-nepetalactol. The dashed lines indicate the peaks of nepetalactol. (**I**) and (**J**) mass spectra of the first peak in 1R4aS7S7aS- and 1S4aR7R7aS-nepetalactol, respectively. (**K**) TIC chromatograms of extracts of *L. boulardi* and *L. heterotoma* and the mass spectrum of the indicated peak in *L. boulardi* (**L**) and *L. heterotoma* (**M**). The peaks in the extracts of *L. boulardi* and *L. heterotoma* show the same retention time and mass spectrum as nepetalactol. The four stereoisomers of nepetalactol available as authentic standards could not be separated on any of the three cyclodextrin column tested. Therefore, the absolute configuration of the nepetalactol produced by the wasps remains unknown.

The color of the mass spectra corresponds to the color of the chromatograms in the same row.
(TIF)

S3 Fig. Electrophysiological recordings with *Drosophila* OSNs and wasp odours. (A–D) SSR responses of wildtype ab10B neurons tested with the headspace **(A)** or wash **(B)** of *L. boulardi*, or synthetic (-)-iridomyrmecin **(C),** (*R*)-actinidine **(D),** or nepetelactol (a mixture of 1S4aR7R7aS-Nepetalactol, 1R4aS7S7aS-Nepetalactol and their enantiomers) **(E)**. **(F)** Dorsal-organ recordings of wildtype larvae tested with the bodywash of *L. boulardi*. **(G–H)** SSR responses of mutant ab3A neuron-expressing Or49a **(G)** or Or85f **(H)** tested with bodywash of *L. boulardi*.
(TIF)

S4 Fig. Odor panel used to screen Or49a and Or85f in the empty neuron system, color-coded by functional group (red, alcohols; blue, esters; gray, acids; brown, ketones; pink, aldehydes; light green, nitrogen-containing compounds; purple, terpenes; dark green, alkanes; black, other compounds).
(TIF)

S5 Fig. GC-SSR responses of a neuron misexpressing Or49a to different isomers of iridomyrmecin. Top line named with the compound depicts the flame ionization detector (FID) signal of the GC.
(TIF)

S6 Fig. GC-SSR responses of a neuron misexpressing Or85f to different isomers of actinidine and nepetalactol. Top line named with the compound depicts the FID signal of the GC.
(TIF)

S7 Fig. Localization of OSNs expressing Or49a on the antenna of a female *D. melanogaster*. OSNs are visualized by expressing GCaMP3.0 under control of *Gal4-Or49a* driver line.
(TIF)

S8 Fig. Behavioral avoidance of synthetic compounds. Larval choice assay and oviposition assay and resulting preference indices when exposed to the synthetic (-)-iridomyrmecin and (*R*)-actinidine. Deviation of the indices against zero was tested with Wilcoxon rank sum test. Asterisks, $p < 0.05$; error bars depict standard deviation. PI = (number of larvae, flies, or eggs in odor side − number in control side) / total number.
(TIF)

S9 Fig. Response profiles of neurons paired with iridomyrmecin-, actinidine-, and nepetalactol -responsive neurons shown in Fig 4C (*n* = 3). Error bars represent standard error of the mean (SEM).
(TIF)

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Author Contributions

Conceived and designed the experiments: SAME HKMD JS MCS BSH MK. Performed the experiments: SAME HKMD FT KW. Analyzed the data: SAME HKMD JS FT SS JR YS MK KW. Contributed reagents/materials/analysis tools: JEH. Wrote the paper: BSH MK HKMD. No.

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MANUSCRIPT II

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

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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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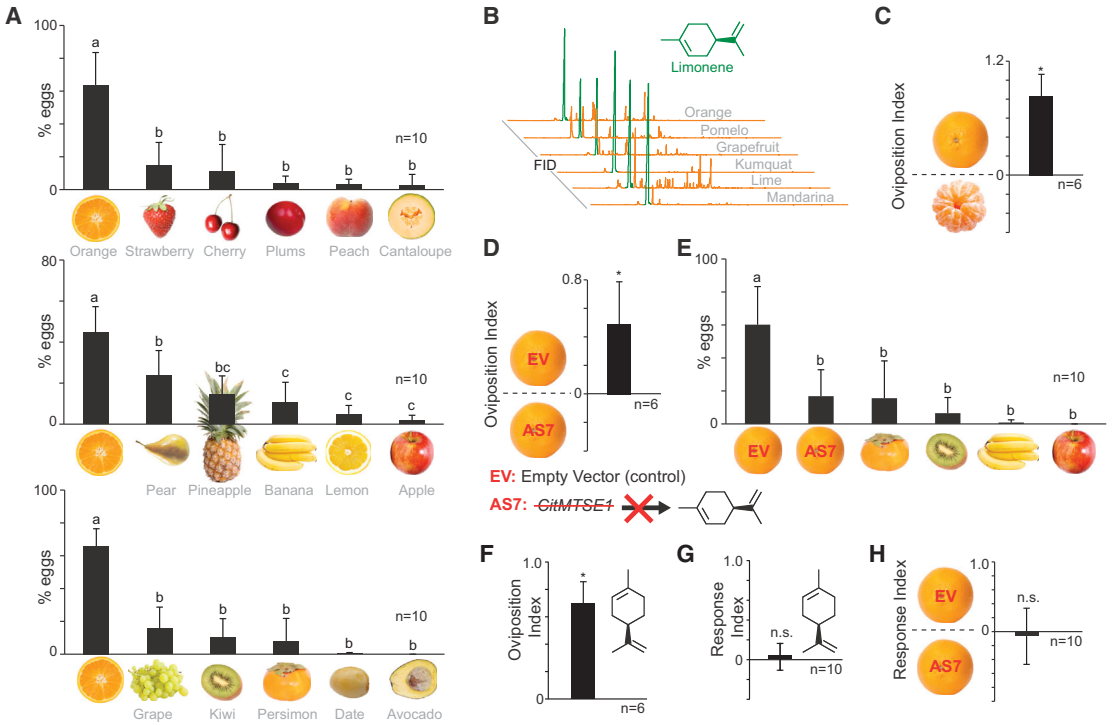

Figure 1. Flies Prefer *Citrus* as Oviposition Substrate

(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

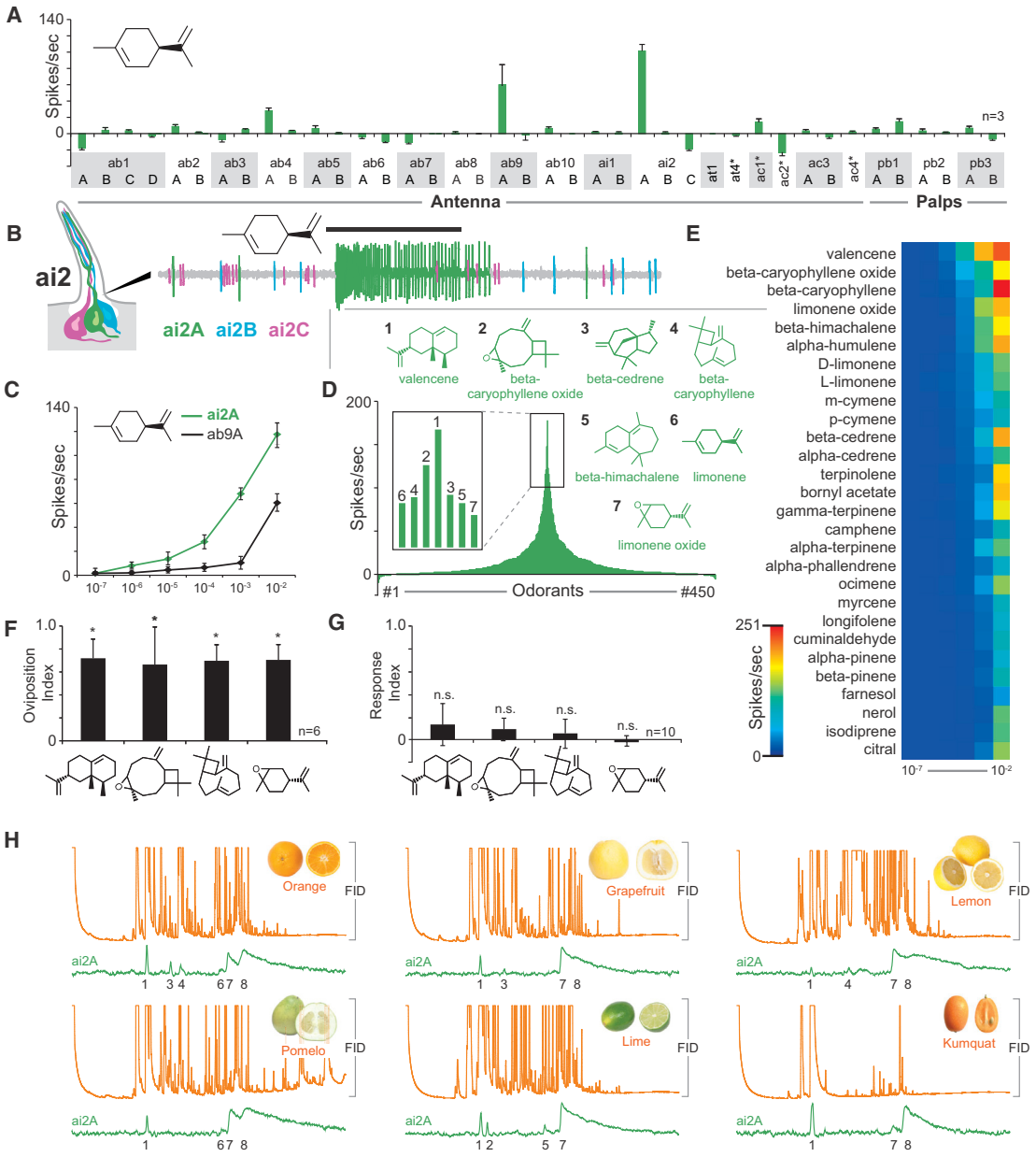

Figure 2. *Citrus* Odorants Are Detected by the ai2A Neurons

(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.

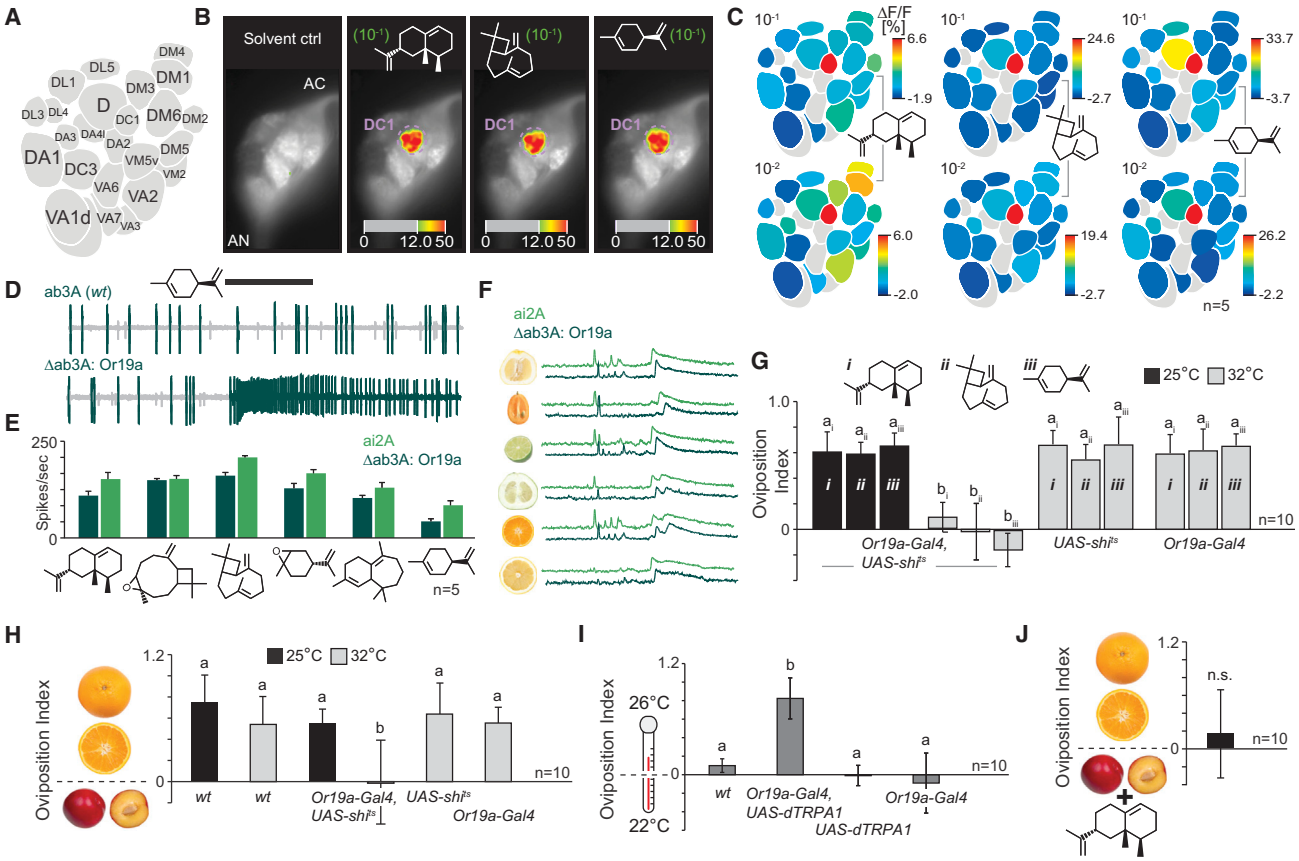

Figure 3. *Or19a* Is Necessary and Sufficient for the *Citrus* Preference

(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

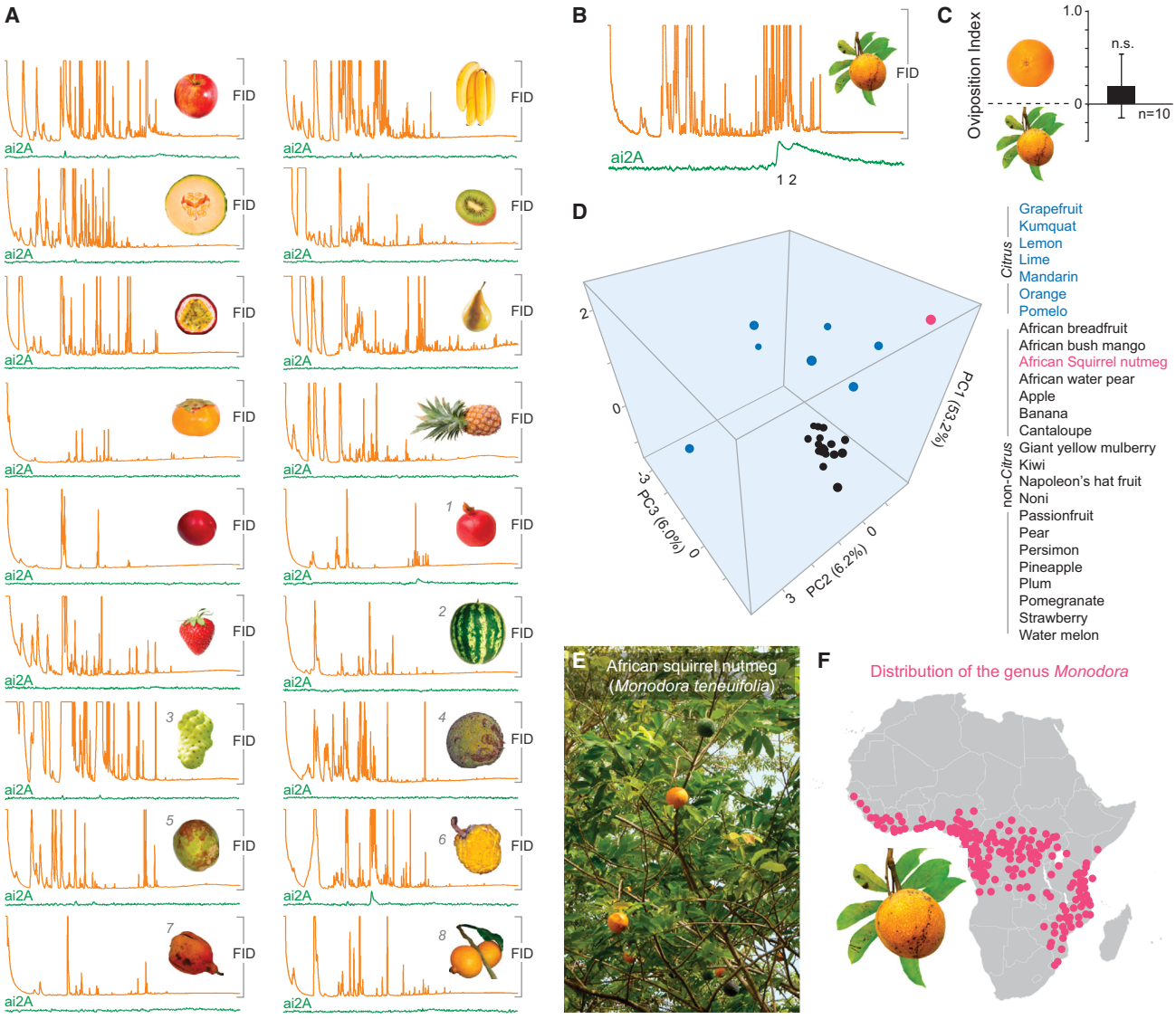

Figure 4. The *Citrus* Preference of *D. melanogaster* Is an Ancestral Trait

(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*.

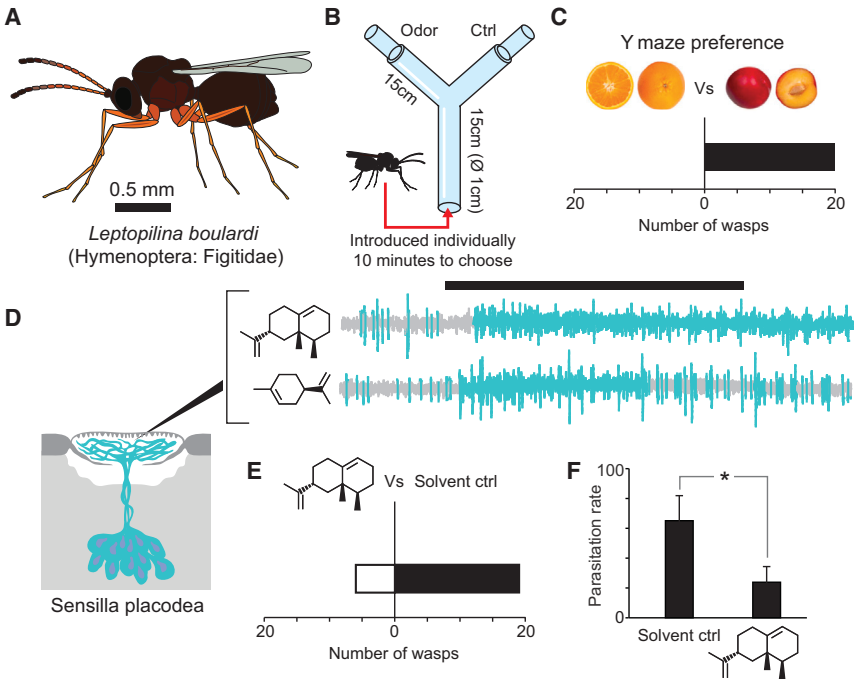

Figure 5. *Citrus* Volatiles Confer Protection against Endoparasitoid Wasps

(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.

Acknowledgments

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Current Biology, Volume *23*

Supplemental Information

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

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Sophie Kromann, Deni Bown, Ylva Hillbur, Silke Sachse, Bill S. Hansson, and Marcus C. Stensmyr

Supplemental Inventory

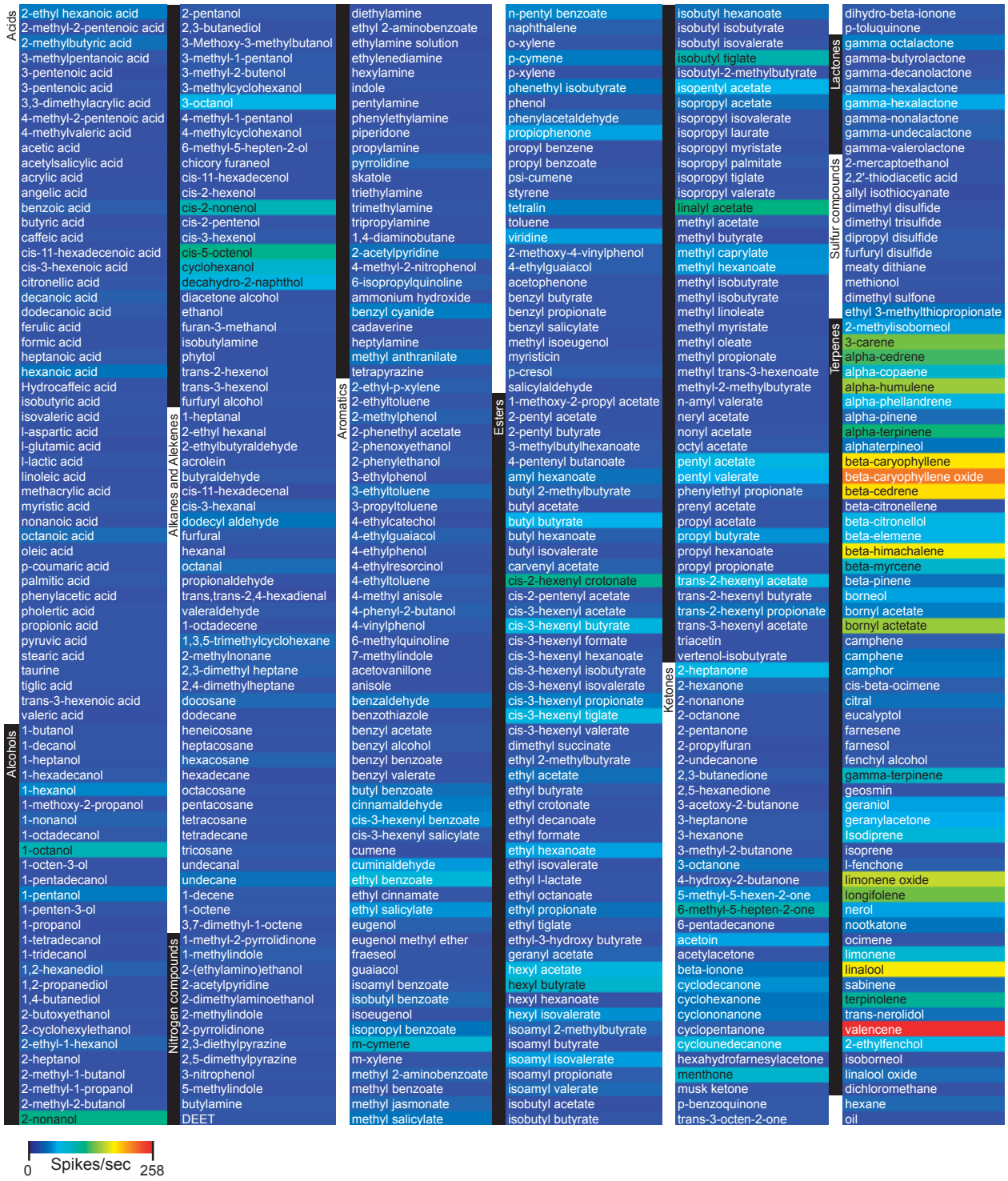


Figure S1 (related to Figure 2).
Heatmap based on responses of ai2A neurons towards 450 screened compounds.

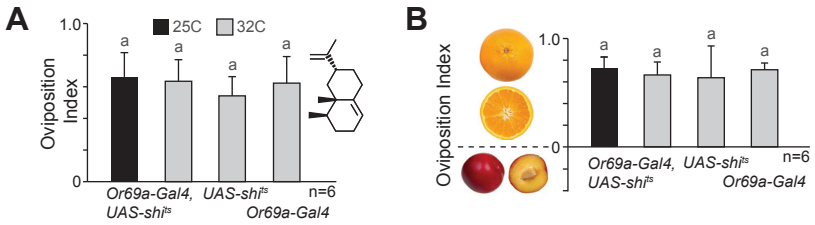


Figure S2 (related to Figure 3).
(A) Oviposition index (OI) to valencene (10^{-2}) of flies expressing *Shibire^{ts}* from the *Or69a* promoter and corresponding parental lines. Significant differences are denoted by letters (analysis of variance [ANOVA] followed by Tukey's test; $p < 0.05$). Error bars represent SEM.
(B) OIs of flies expressing *Shibire^{ts}* from the *Or69a* promoter and corresponding parental lines presented with a choice to oviposit on either oranges or plums. Significant differences are denoted by letters (analysis of variance [ANOVA] followed by Tukey's test; $p < 0.05$). Error bars represent SEM.

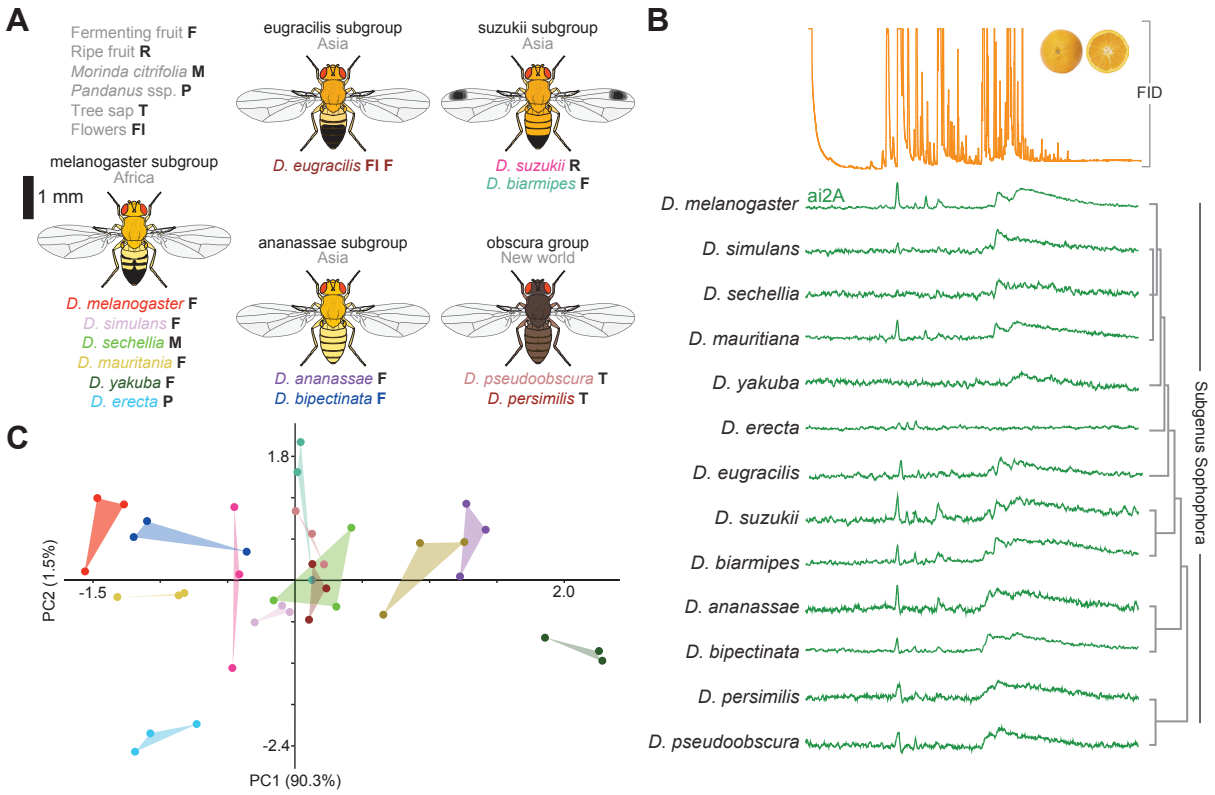


Figure S3 (related to Figure 4).
(A) Examined drosophilid species (subgenus *Sophophora*), sorted according to taxonomic relationship and with breeding substrate indicated.
(B) Representative GC-SSR measurements from 13 species of flies, stimulated with the same orange headspace sample. Phylogenetic relationships of the examined species are given on the right-hand side.
(C) Two-dimensional principal component analysis plot based upon GC-SSR response profiles of 13 species of drosophilids towards orange headspace; exemplified in (B). Color code as in panel (A).

MANUSCRIPT III

Olfactory proxy detection of dietary antioxidants in *Drosophila*

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Abu Farhan, Bill S. Hansson, and Marcus C. Stensmyr

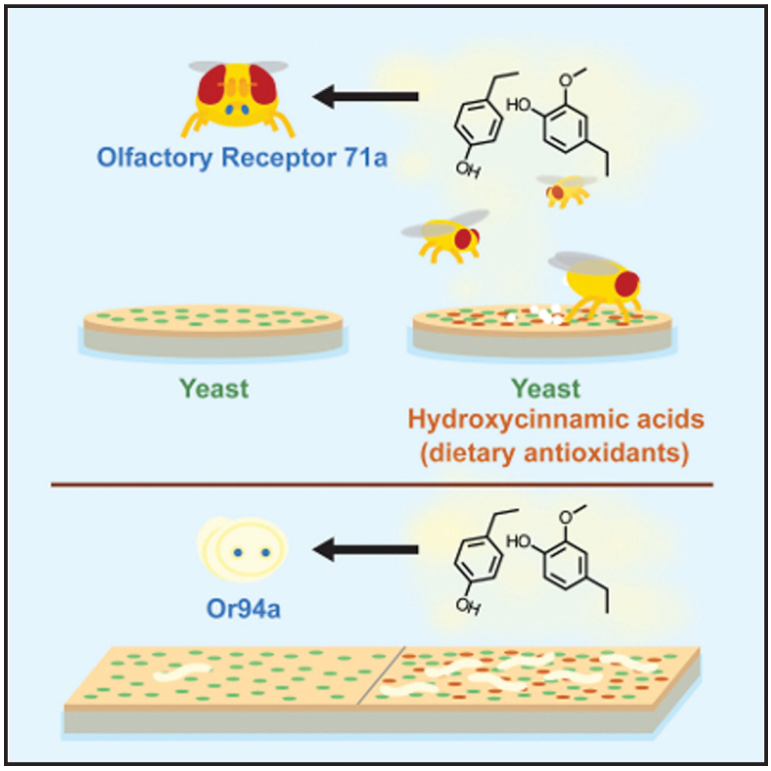
Current Biology, 2015, 25:455-466

Article

Current Biology

Olfactory Proxy Detection of Dietary Antioxidants in *Drosophila*

Graphical Abstract



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In Brief

Dweck et al. demonstrate that the vinegar fly detects the presence of hydroxycinnamic acids (HCAs), which are potent dietary antioxidants abundant in fruit, through olfactory cues. Flies are unable to smell HCAs directly but have dedicated olfactory sensory neurons detecting yeast-produced ethylphenols that are exclusively derived from HCAs.

Highlights

- Flies prefer the smell of yeast grown on media enriched with dietary antioxidants
- Attraction is due to yeast-produced ethylphenols derived from the antioxidants
- The ethylphenols are detected by maxillary palp neurons expressing *Or71a*
- Larvae are also attracted to the same ethylphenols but rely on a different receptor

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Article

Olfactory Proxy Detection of Dietary Antioxidants in *Drosophila*

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Summary

Background: Dietary antioxidants play an important role in preventing oxidative stress. Whether animals in search of food or brood sites are able to judge the antioxidant content, and if so actively seek out resources with enriched antioxidant content, remains unclear.
Results: We show here that the vinegar fly *Drosophila melanogaster* detects the presence of hydroxycinnamic acids (HCAs)—potent dietary antioxidants abundant in fruit—via olfactory cues. Flies are unable to smell HCAs directly but are equipped with dedicated olfactory sensory neurons detecting yeast-produced ethylphenols that are exclusively derived from HCAs. These neurons are housed on the maxillary palps, express the odorant receptor *Or71a*, and are necessary and sufficient for proxy detection of HCAs. Activation of these neurons in adult flies induces positive chemotaxis, oviposition, and increased feeding. We further demonstrate that fly larvae also seek out yeast enriched with HCAs and that larvae use the same ethylphenol cues as the adults but rely for detection upon a larval unique odorant receptor (*Or94b*), which is co-expressed with a receptor (*Or94a*) detecting a general yeast volatile. We also show that the ethylphenols act as reliable cues for the presence of dietary antioxidants, as these volatiles are produced—upon supplementation of HCAs—by a wide range of yeasts known to be consumed by flies.
Conclusions: For flies, dietary antioxidants are presumably important to counteract acute oxidative stress induced by consumption or by infection by entomopathogenic microorganisms. The ethylphenol pathway described here adds another layer to the fly's defensive arsenal against toxic microbes.

Introduction

Dietary antioxidants play a fundamental role in preventing oxidative stress by regulating levels of free radicals and other reactive oxygen species [1]. Dietary antioxidants thus constitute a significant nutritional reward [2]. Indeed, for example, frugivorous birds actively seek out fruit with a high content of antioxidants and, furthermore, are able to judge the fruit's antioxidant content by relying on visual cues alone [3]. Whether feeding partiality toward food enriched with dietary antioxidants, as well as the ability to judge antioxidant content, is widespread remains, however, an open question.

Oxidative stress is of importance not only to long-lived organisms, but also to animals with shorter lifespan, such as insects, in which, apart from aging [4], oxidative stress has also been shown to accrue from, e.g., cold exposure [5] and through ingestion of environmental toxins [6]. Here we examine how the vinegar fly *Drosophila melanogaster* reacts to the presence of two polyphenolic dietary antioxidants, the hydroxycinnamic acids (HCAs) p-coumaric acid and ferrulic acid (Figure 1A). These two HCAs are particularly abundant in fruit [7], the primary breeding substrate of flies [8], and therefore are presumably important antioxidants in wild fly populations. In flies, polyphenol antioxidants have been shown to offer protection against induced oxidative stress [9], and also to prolong lifespan [10].

We demonstrate here that flies are able to detect the presence of HCAs via olfactory cues. Flies are, however, unable to smell HCAs directly, but they are equipped with a dedicated olfactory sensory neuron (OSN) class—localized on the maxillary palps—that detects volatile ethylphenols, which are exclusively derived from HCAs. Larval flies also do the proxy detection of HCAs via the same ethylphenols, albeit with a different, but similarly tuned, larval unique odorant receptor (OR). Our results provide the first indication that animals are able to use olfactory cues to judge content of dietary antioxidants.

Results and Discussion

Flies Are Unable to Smell HCAs Directly

We first sought to confirm that a diet supplanted with HCAs remedies the negative effects of induced oxidative stress. We fed flies with 20 mM paraquat (a pesticide that induces oxidative stress [11]) dissolved either in yeast medium or in HCA-inoculated yeast medium. Flies fed with paraquat dissolved in HCA-inoculated yeast showed a significant enhancement in both survival and locomotor activity compared to flies treated with paraquat dissolved in the yeast medium alone (Figures 1B and 1C). Can flies smell HCAs? We employed three different olfactory assays monitoring chemotaxis, oviposition, and feeding, respectively [12, 13]. In none of these assays did flies show any reaction to p-coumaric acid or ferulic acid (Figure 1D). A lack of behavior does not, however, mean that flies are unable to smell these substances. Hence, we next turned to electrophysiology, more specifically to single-sensillum recordings (SSRs), to investigate whether stimulation with HCAs induce alterations in spike firing rate. Using the two HCAs as a stimulus (10^{-2}), we performed a system-wide screen across all 48 olfactory sensory neuron (OSN) classes present on the flies' antennae and maxillary palps. Neither HCA yielded any activity from any of the contacted OSNs (Figure S1A). We thus conclude that the olfactory system is unable to detect these two chemicals.

Flies Are Attracted to HCA-Derived Yeast Volatiles

Although flies are unable to smell the HCAs directly, they could still be able to detect the presence of these chemicals via proxies. Many yeast species, including those consumed by flies, are known to be able to metabolize HCAs into ethylphenols [14], specifically 4-ethylphenol and 4-ethylguaiacol (Figure 1E). We first sought to verify that fruits utilized by flies contain HCAs. Indeed, high-performance liquid chromatography (HPLC) analysis of banana pulp revealed the presence of

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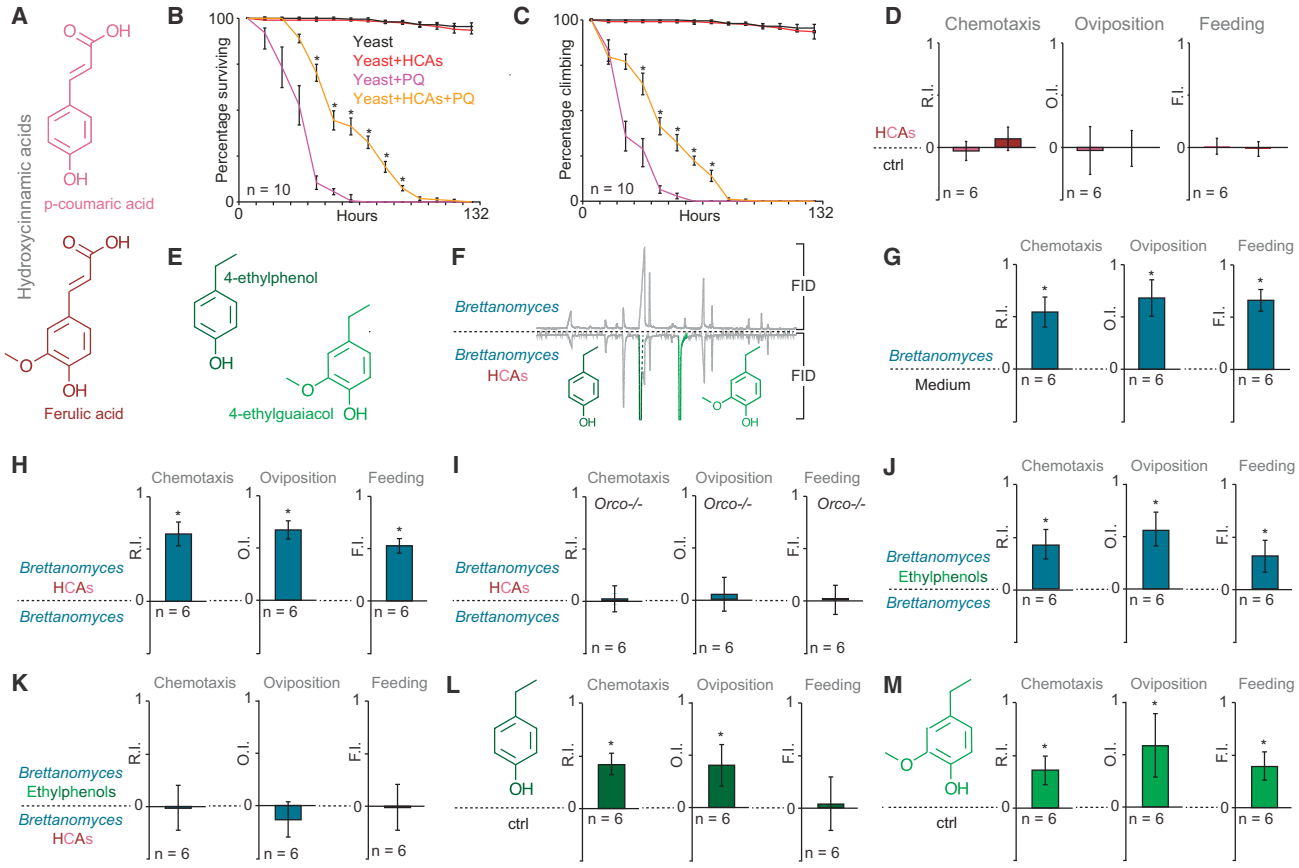

Figure 1. Flies Detect HCAs via Volatile Ethylphenols

(A) Chemical structures of p-coumaric acid and ferulic acid.

(B) Percentage of flies—treated with combinations of yeast, hydroxycinnamic acids (HCA) and paraquat (PQ)—surviving over time. Colors refer to different food treatments. Error bars represent the SD. Significant differences (between yeast + PQ versus yeast + PQ + HCAs) are denoted by asterisks (ANOVA followed by Tukey's test; $p < 0.05$).

(C) Percentage of flies—treated with combinations of yeast, HCAs, and paraquat—climbing over time. Color coding is the same as in (B). Error bars represent the SD. Significant differences (between yeast + PQ versus yeast + PQ + HCAs) are denoted by asterisks (ANOVA followed by Tukey's test; $p < 0.05$).

(D) Chemotaxis, oviposition, and feeding response indices from a binary-choice assay between p-coumaric acid or ferulic acid against solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test.

(E) Chemical structures of 4-ethylphenol and 4-ethylguaiacol.

(F) Flame ionization detection (FID) traces from headspace collections of *Brettanomyces* and HCA-inoculated *Brettanomyces*.

(G) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between *Brettanomyces* and media. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(H) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between *Brettanomyces* and HCA-inoculated *Brettanomyces*. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(I) Chemotaxis, oviposition, and feeding indices of $Orco^{-/-}$ flies from a binary-choice assay between *Brettanomyces* and HCA-inoculated *Brettanomyces*. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(J) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between *Brettanomyces* and *Brettanomyces* spiked with ethylphenols. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(K) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between HCA-inoculated *Brettanomyces* and *Brettanomyces* spiked with ethylphenols. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(L) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between 4-ethylphenol and solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(M) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between 4-ethylguaiacol and solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

See also Figure S1.

both p-coumaric acid and ferulic acid (Figure S1B). We next investigated whether the HCA amounts present in banana were sufficient to induce production of ethylphenols by yeasts. We inoculated banana-based medium with *Brettanomyces bruxellensis*, a yeast species isolated from wild flies [15] and known for its potent ability to convert HCAs into ethylphenols [16–19]. Indeed, in yeasts grown on medium mixed with banana pulp, we identified ethylphenols in the headspace (Figure S1C). Similarly, growth of *Brettanomyces* on medium supplanted with HCAs resulted in the production of ethylphenols, but not when *Brettanomyces* was grown on standard medium (Figure 1F).

Do flies react to the HCA induced changes in the yeast's volatile headspace? We first verified that flies reacted to the

smell of *Brettanomyces* yeast, which they did, with flies displaying strong preference for this yeast in the three previously mentioned assays (Figure 1G). Next, we confronted flies with a choice between *Brettanomyces* grown with or without HCAs (henceforth referred as HCA^+ and HCA^-). In all assays, flies clearly preferred HCA^+ yeasts (Figure 1H). To verify that this preference is mediated via olfaction, we repeated this experiment with flies lacking *Orco*, a co-receptor necessary for function in the majority of all OSNs [20]. Indeed, $Orco^{-/-}$ flies did not differentiate between the two treatments in any of the three assays (Figure 1I), demonstrating that OSNs expressing ORs are necessary for this behavior. We next wondered whether the preference for HCAs is mediated via ethylphenols. To address this issue, we provided flies with a binary choice of *Brettanomyces* (grown on standard medium) spiked with either 4-ethylguaiacol and 4-ethylphenol (10^{-4} dilution) or solvent (mineral oil). Flies preferred the *Brettanomyces* with added ethylphenols in all three assays (Figure 1J). Similarly, flies that were given a choice between HCA^+ *Brettanomyces* and yeasts grown on standard medium, but spiked with ethylphenols, showed no preference either way in all assays (Figure 1K). Finally, we examined the behavioral valence of the ethylphenols themselves, and as expected, flies in all three assays showed a strong preference for these yeast metabolites (Figures 1L and 1M). We hence conclude that although flies are unable to smell HCAs directly, they are able to detect volatiles derived from HCAs.

Ethylphenols Activate a Single Class of OSNs on the Maxillary Palps

How do flies detect the ethylphenols? We performed a system-wide SSR screen stimulating with the two ethylphenols (Figure 2A). Strong responses to these two chemicals (at 10^{-4} dilution) were exclusively observed from a single OSN class, namely palp basiconic type 1B (pb1B) (Figure 2A). To determine the specificity of these neurons, we next tested a battery of 154 compounds (screened at a higher dose [10^{-2}] to obtain the upper limit of the receptive range). The chosen stimulus included representatives of all relevant chemical classes but focused on substances of structural similarity to the HCA derived ethylphenols (Figure 2B). Out of the screened chemicals, none produced a stronger response than 4-ethylguaiacol, and only nine of the compounds—all structurally similar to 4-ethylguaiacol—yielded a response of >100 spikes/s (Figures 2C and 2D). We next examined dose-response relationships for the six most efficient agonists using gas chromatography (GC) for controlled stimulus delivery (Figure 2E). As suspected, 4-ethylguaiacol was indeed the most efficient ligand, triggering responses already at 10^{-7} dilution. To determine whether the additional ligands for pb1B also activate other OSN classes, we performed an exhaustive SSR screen, this time stimulating with the seven primary agonists for pb1B (at 10^{-4} dilution) across all 48 OSN classes. With the exception of guaiacol, which also strongly activated antennal basiconic type 6B (ab6B, expressing *Or49b* [21]), none of the other volatiles triggered significant activity from OSN classes other than pb1B (Figure 2F). We hence conclude that at ecologically relevant concentrations, the ethylphenols and structurally similar phenolic compounds exclusively activate the pb1B pathway.

Pb1B Is Necessary and Sufficient for Proxy Detection of HCAs

The presence of HCAs might also lead to other changes in the yeast's volatile profile, which in turn could activate other subpopulations of OSNs. To control for this eventuality, we repeated the system-wide SSR screen, but now employed GC to screen headspace collections from HCA^+ and HCA^- *Brettanomyces*. Stimulation with the former activated 12 OSN classes (Figures 3A and 3C), whereas nine were activated with the latter (Figures 3B and 3C). The additional OSN classes activated by the HCA^+ *Brettanomyces* headspace were pb1B, ab5B, and ab9A. The pb1B neurons were, as expected, triggered by 4-ethylguaiacol and 4-ethylphenol (as identified via GC-linked mass spectroscopy). The large amount of 4-ethylguaiacol in the HCA^+ sample was also sufficient to trigger weak activity from ab9A, whereas the response from ab5B in the HCA^+ sample stemmed from greatly increased levels of phenylethanol compared to the HCA^- treatment.

We next sought to determine which of these three OSN classes are necessary for the proxy detection of HCAs. We used the temperature-sensitive mutant dynamin $Shibire^{ts}$ to shut down synaptic transmission [22] in the OSN classes specifically activated in the HCA^+ sample. At the restrictive temperature (32°C), flies expressing $shibire^{ts}$ from the promoter of the OR expressed in pb1B OSNs—*Or71a* [23]—displayed no preference toward HCA-inoculated yeasts in any of the three employed assays (Figure 3D). The preference of flies with ab9A and ab5A silenced (via $Shibire^{ts}$ expression from the promoters of *Or69a* and *Or47a*, respectively [21]) was, however, not different from that of flies tested at a permissive temperature (25°C) or from parental control lines at restrictive temperature (Figure 3D). We hence conclude that *Or71a* alone is necessary for the substitute detection of HCAs. Is activation of pb1B then sufficient to induce the observed preference? We next drove expression of the temperature-sensitive cation channel *dTRPA1* in the pb1B OSNs, which enabled us to conditionally activate this specific OSN population at temperatures above 26°C [24]. Specific activation of pb1B neurons indeed triggered attraction, egg laying, and feeding (Figure 3E). In short, the *Or71a* pathway is both necessary and sufficient for the detection of the HCA derived yeast volatiles.

Ethylphenols Constitute a Reliable Signal for the Presence of HCAs

In nature, flies are not only confronted with *Brettanomyces*, but also encounter a wide range of yeast species [25]. If the ethylphenols indeed serve as a general signal enabling identification of HCA enriched substrates, we would expect that other yeast growing on HCA-containing sources would also produce these volatiles. To investigate this issue, we examined HCA-induced production of volatile phenols in a range of additional yeast species, namely *Wickerhamomyces anomalus*, *Torulaspora delbrueckii*, *Hanseniaspora uvarum*, *Metschnikowia pulcherrima*, and *Saccharomyces cerevisiae*. All of these yeasts have previously been isolated from the surface or guts of drosophilid flies [26–28]. The conversion of HCAs into volatile phenols involves two steps: first a hydroxycinnamate decarboxylase enzyme converts the HCAs into vinyl derivatives, which are subsequently reduced by a vinyl phenol reductase into the corresponding ethyl derivatives (4-ethylphenol and 4-ethylguaiacol) [14]. The examined yeasts ability to complete these synthesis steps differed (Figure 4A), with none of the yeasts being able to synthesize 4-ethylphenol. Nevertheless, when stimulated with the HCA^+ yeast headspace, the amounts and types of volatile phenols present in were sufficient to activate pb1B OSNs in GC-SSR measurements (Figure 4B). Moreover, flies confronted with the same

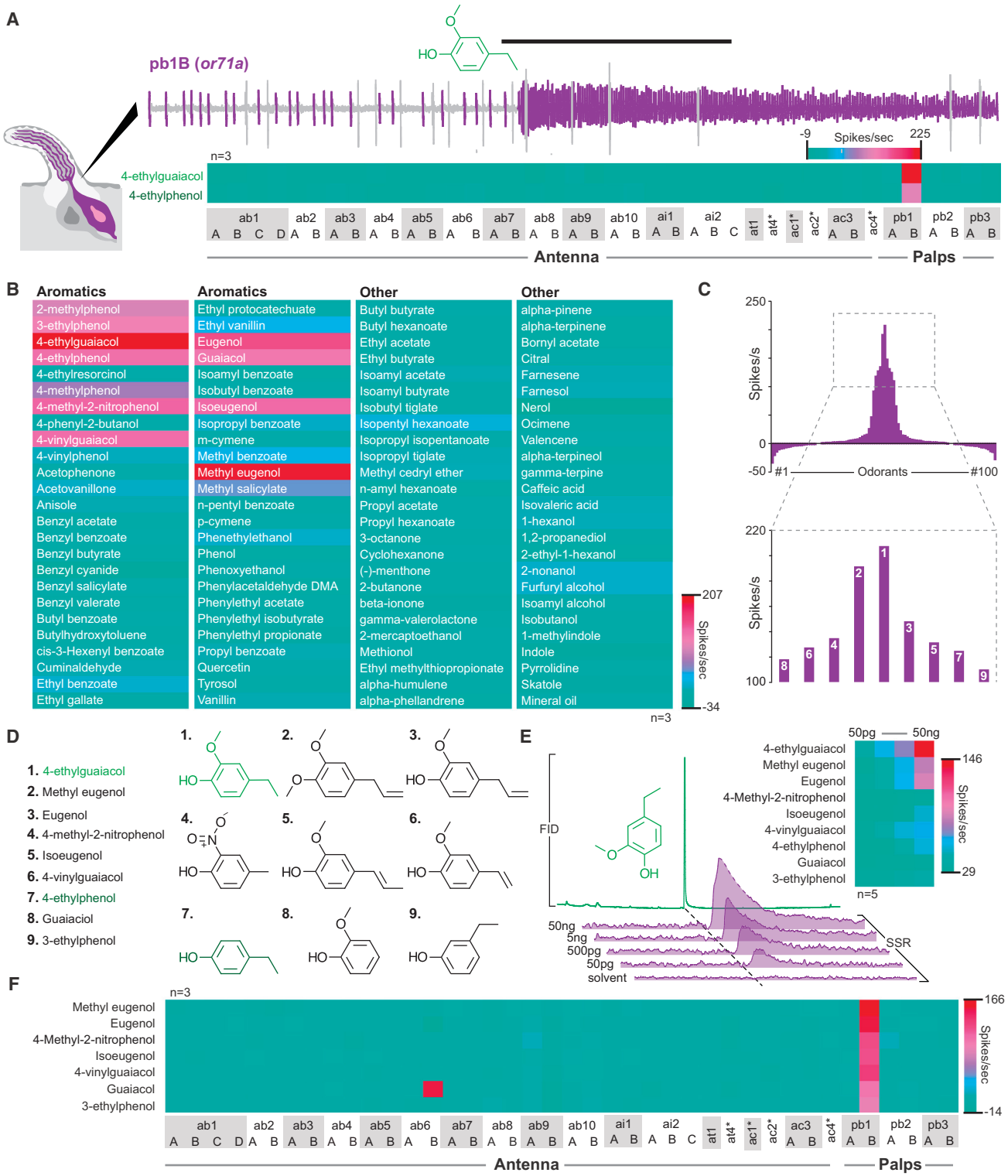

Figure 2. HCA-Derived Ethylphenols Are Detected by pb1B OSNs

(A) Representative SSR trace from a pb1B neuron stimulated with 4-ethylguaiacol (top). The heatmap depicts the average SSR responses from all OSN classes stimulated with 4-ethylguaiacol and 4-ethylphenol (10^{-4} dilution; bottom).

(B) Heatmap depicting average SSR responses from pb1B neurons stimulated with 100 synthetic volatiles (10^{-2} dilution).

(C) Tuning curve for pb1B based on a screen of 100 synthetic compounds (as shown in B).

(D) Chemical structures of the best ligands for pb1B.

(E) Linked GC-SSR response traces from pb1B stimulated with different concentrations of 4-ethylguaiacol (left). The heatmap is based on GC-SSR dose-response profiles of the best ligands for pb1B (right).

(F) Heatmap depicting average SSR responses toward the best ligands of pb1B across all OSN classes.

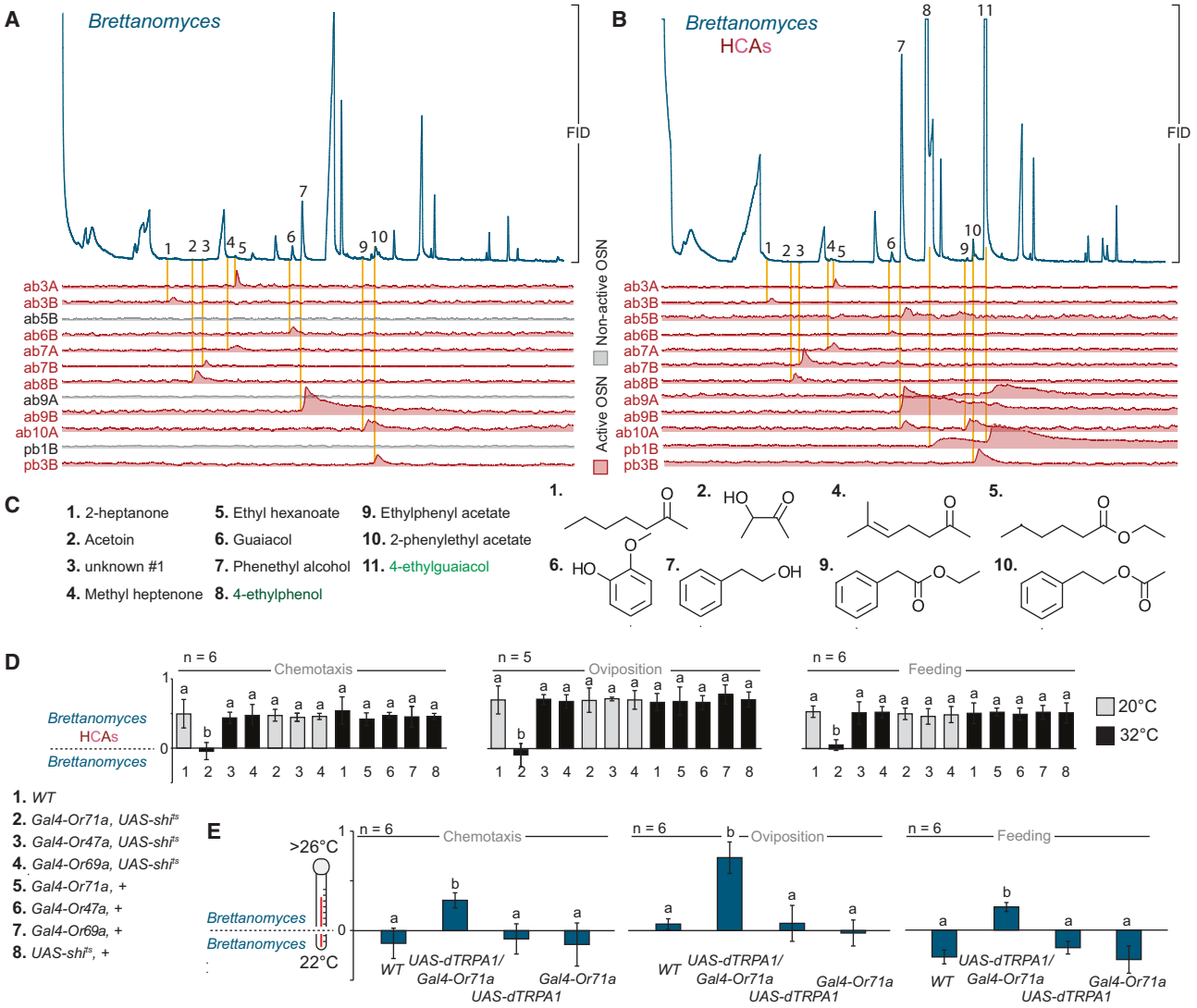

Figure 3. Pb1B Is Necessary and Sufficient for Proxy Detection of HCAs

(A) Representative GC-SSR traces from activated OSN classes stimulated with headspaces of *Brettanomyces* yeast.

(B) Representative GC-SSR traces from activated OSN classes stimulated with the headspaces of HCA-inoculated *Brettanomyces*.

(C) Chemical structures of the active compounds from (A) and (B).

(D) Chemotaxis, oviposition, and feeding indices of flies expressing *Shibire*^{ts} from the promoters of *Or47a*, *Or69a*, and *Or71a*, respectively, the corresponding parental lines, and wild-type (WT) flies confronted with a choice between *Brettanomyces* yeast grown with or without HCAs. Error bars represent the SD. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(E) Chemotaxis, oviposition, and feeding indices of flies expressing *dTRPA1* from the *Or71a* promoter, the corresponding parental lines, and WT flies provided a choice between 22°C and 26°C. Error bars represent the SD. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

binary choice between HCA$^+$ and HCA$^-$ yeasts as before clearly preferred the odor of HCA$^+$ yeasts in all assays (Figure 4C).

It is, however, not inconceivable that HCAs in combination with other yeast might cause other changes in the volatile profile than does the combination of *Brettanomyces* and HCAs. To examine this issue, we again performed a system-wide GC-SSR screen, now stimulating with the headspace from the five above mentioned yeasts. Although the other yeast headspace activated a slightly different subset of OSNs than did *Brettanomyces*, only ab9A and pb1B were additionally recruited by stimulation with the HCA$^+$ yeast headspace compared to HCA$^-$ (Figures 4D and 4E). We hence conclude that ethylphenols serve as a consistent and reliable signal for the presence of HCAs.

Drosophila Larvae Detect Ethylphenols

Being able to detect HCA-enriched patches and favorable food yeasts should be important not only for adult flies, but also for larvae. Although essentially confined to their food, the microhabitat of larvae is not uniform, and thus being able to navigate toward suitable pockets within the fruit home should be an important ability. Although *Or71a* is not expressed in the larval stage [29], it's possible that among the larval unique OR genes, there are receptors that are able to make the same proxy detection of HCAs as adults

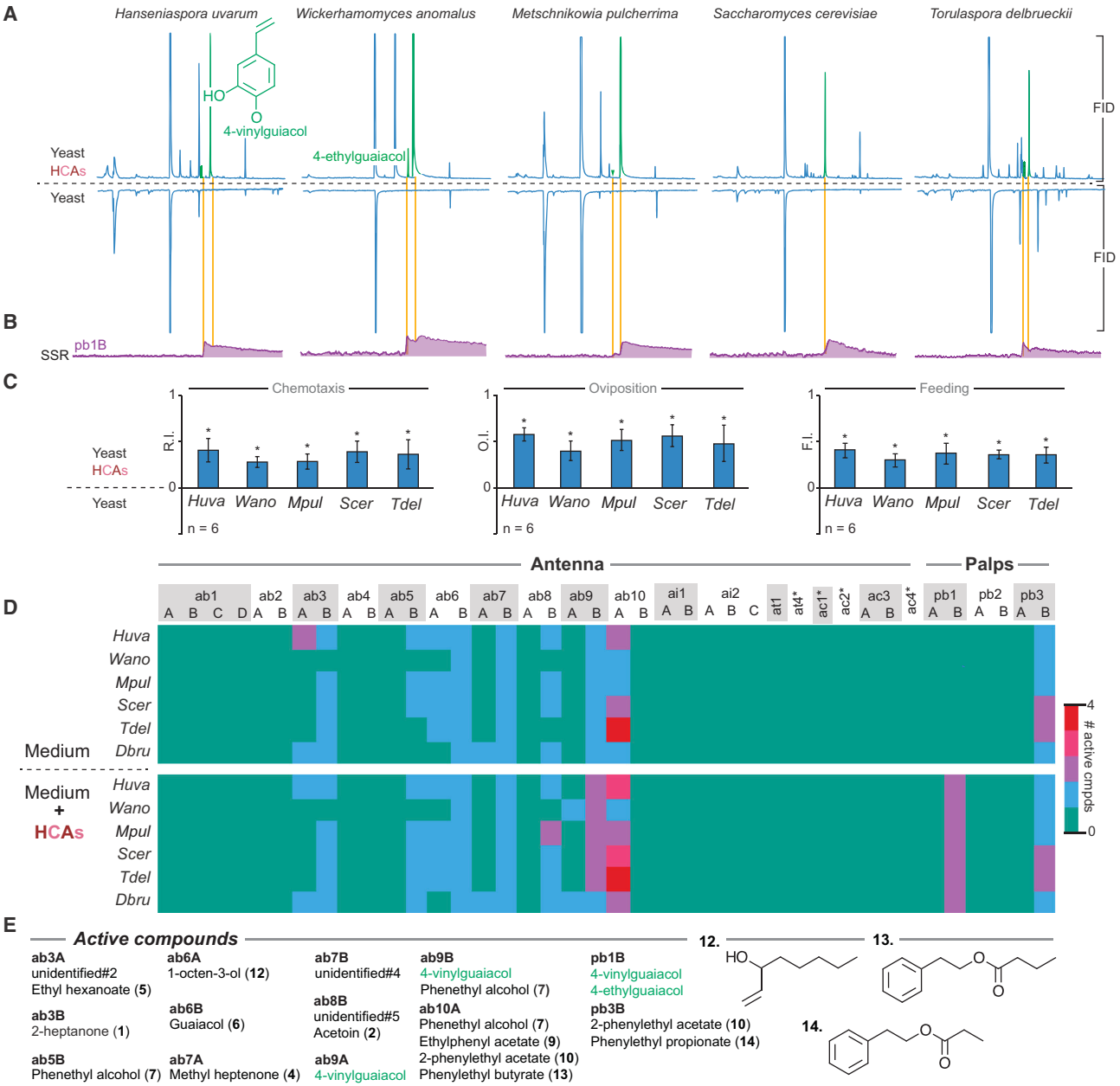

Figure 4. Ethylphenols Constitute a Reliable Signal for the Presence of HCAs

(A) FID traces from the headspace collected from a range of yeasts grown with or without HCAs.

(B) Corresponding SSR traces from pb1B stimulated with HCA-inoculated yeasts.

(C) Chemotaxis, oviposition, and feeding indices from a binary-choice assay between yeast grown with or without HCAs. *Huva*, *Hanseniaspora uvarum*; *Wano*, *Wickerhamomyces anomalus*; *Mpul*, *Metschnikowia pulcherrima*; *Scer*, *Saccharomyces cerevisiae*; *Tdel*, *Torulaspora delbrueckii*. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(D) Heatmap comparison depicting number of active compounds present in headspace of yeasts grown with or without HCAs as determined via a system-wide GC-SSR screen across all OSN classes. Abbreviations are as in (C).

(E) Activated neuron classes and the respective identified ligands. Numbers refer to the chemical structure drawings shown to the left or in Figure 3C.

do, or, alternatively, to detect HCAs directly. We first examined whether larvae respond behaviorally to HCAs. Larvae confronted with HCAs in a binary-choice larval olfactory preference assay (Figure 5A) showed no reaction to the HCAs (Figure 5B). Although displaying no overt behavior in response to the presence of HCAs, larvae could still be able to smell HCAs. To examine whether larvae can smell HCAs, we next performed SSR from the dorsal organ (DO)—the larval nose (Figure 5C) [30, 31]. The DO is innervated by 21 OSNs, and by gently inserting the recording electrode into this structure, we were able to simultaneously record the activity of (presumably) all OSNs residing within the DO. Stimulation with HCAs yielded no activity from any of the discernable neurons in multiple recordings (Figure 5D). We thus conclude that larvae, like adults, are unable to detect the presence of HCAs directly.

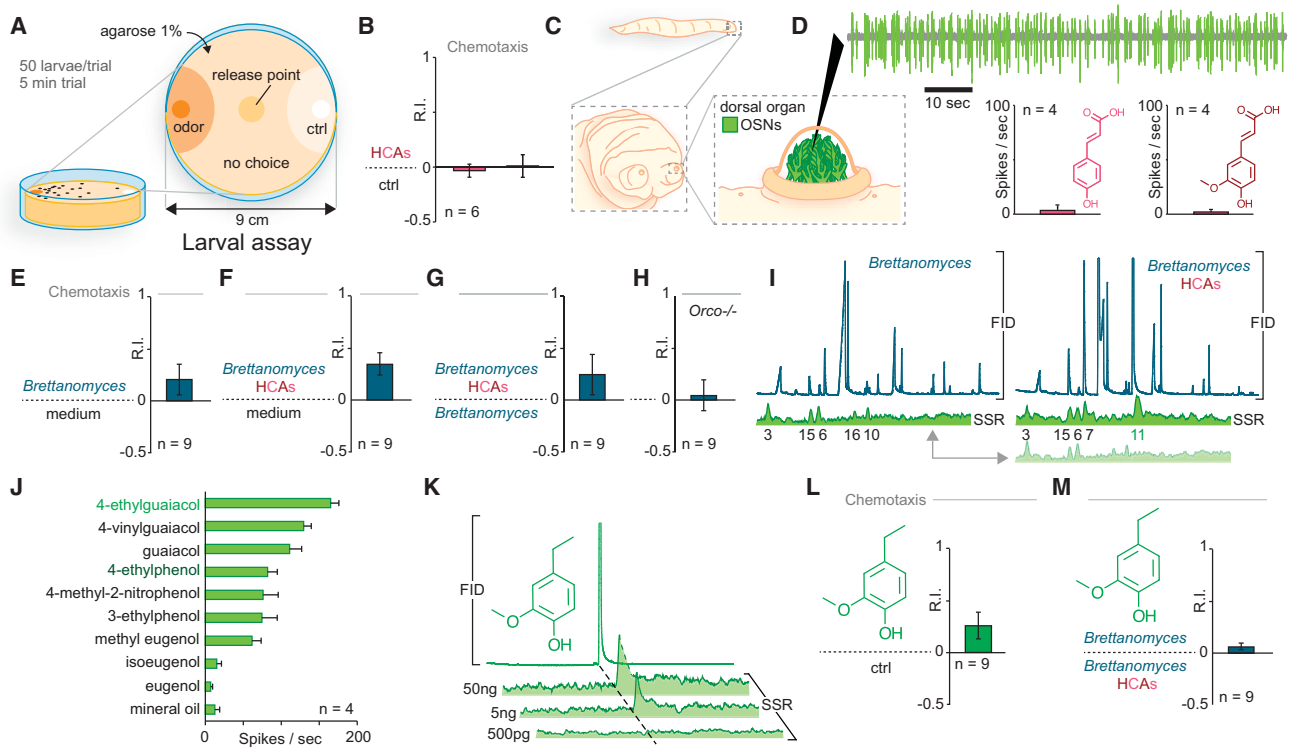

Figure 5. Larvae Also Detect HCAs via Ethylphenols

(A) Schematic drawing of the larval olfactory choice assay.

(B) Larval response indices from a binary-choice assay between either p-coumaric acid or ferulic acid against solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(C) Schematic drawing of the larval nose—the dorsal organ.

(D) Representative SSR trace from the larval dorsal organ (top). The average number of spikes/s was recorded from the larval dorsal organ stimulated with p-coumaric acid (bottom left) and ferulic acid (bottom right). Error bars represent the SD.

(E). Larval response index from a choice between *Brettanomyces* and medium control. Error bars represent the SD. Deviation of the response index against zero was treated with a Student's t test ($p < 0.05$).

(F) Larval response index from a choice between HCA-inoculated *Brettanomyces* and medium control. Error bars represent the SD. Deviation of the response index against zero was treated with a Student's t test ($p < 0.05$).

(G) Larval response index from a choice between *Brettanomyces* grown with or without HCAs. Error bars represent the SD. Deviation of the response index against zero was treated with a Student's t test.

(H) Larval response index of *Orco*$^{-/-}$ larvae from a choice between *Brettanomyces* grown with or without HCAs. Error bars represent the SD. Deviation of the response index against zero was treated with a Student's t test.

(I) GC-SSR measurements from the larval dorsal organ stimulating with *Brettanomyces* grown with or without HCAs. Numbers refer to the chemical structure drawings shown in Figure 3C.

(J) The average number of spikes/s recorded via SSR from the larval dorsal organ stimulated with the best ligands from pb1B. Error bars represent the SD.

(K) GC-SSR response traces from the larval dorsal organ stimulated with different concentrations of 4-ethylguaiacol.

(L) Laval response index from a choice between 4-ethylguaiacol and solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

(M) Larval response index from a choice between *Brettanomyces* inoculated with HCAs against *Brettanomyces* spiked with ethylphenols. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

Larvae could still, however, make the same proxy detection of HCAs as adults. We first examined whether larvae respond behaviorally to the odor of *Brettanomyces*—grown with or without HCAs. Both HCA$^+$ and HCA$^-$ *Brettanomyces* triggered positive chemotaxis from the larvae in the olfactory preference assay (Figures 5E and 5F). Larvae confronted with a binary choice between HCA$^+$ and HCA$^-$ cultured *Brettanomyces* clearly preferred the odor of the former (Figure 5G). *Orco*$^{-/-}$ larvae presented with the same choice did not show any preference, verifying that ORs indeed mediate this preference (Figure 5H). Which volatiles do the larvae rely on? We next performed larval GC-SSR measurements, stimulating with HCA$^+$ and HCA$^-$ *Brettanomyces* headspace collections. Compared with HCA$^-$, stimulation with HCA$^+$ samples yielded additional responses toward 4-ethylguaiacol and phenethyl alcohol, the latter again most likely due to the increased amounts in the HCA$^+$ samples (Figure 5I). Larvae also displayed increased spike firing rate in response to stimulation with the other primary ligands for *Or71a*, and, similarly to the situation in the adults, 4-ethylguaiacol elicited the strongest response (Figure 5J). In GC-SSR dose-response trials, larvae were, however, less sensitive to 4-ethylguaiacol than were adults, with discernable responses to 4-ethylguaiacol requiring a 3-fold larger dose in larvae than in adults (Figure 5K). How do larvae react behaviorally to 4-ethylguaiacol? Application of 4-ethylguaiacol in the larval olfactory choice assay resulted in positive chemotaxis (Figure 5L). Moreover, larvae given a choice between HCA$^+$ *Brettanomyces* and HCA$^-$ *Brettanomyces* spiked with

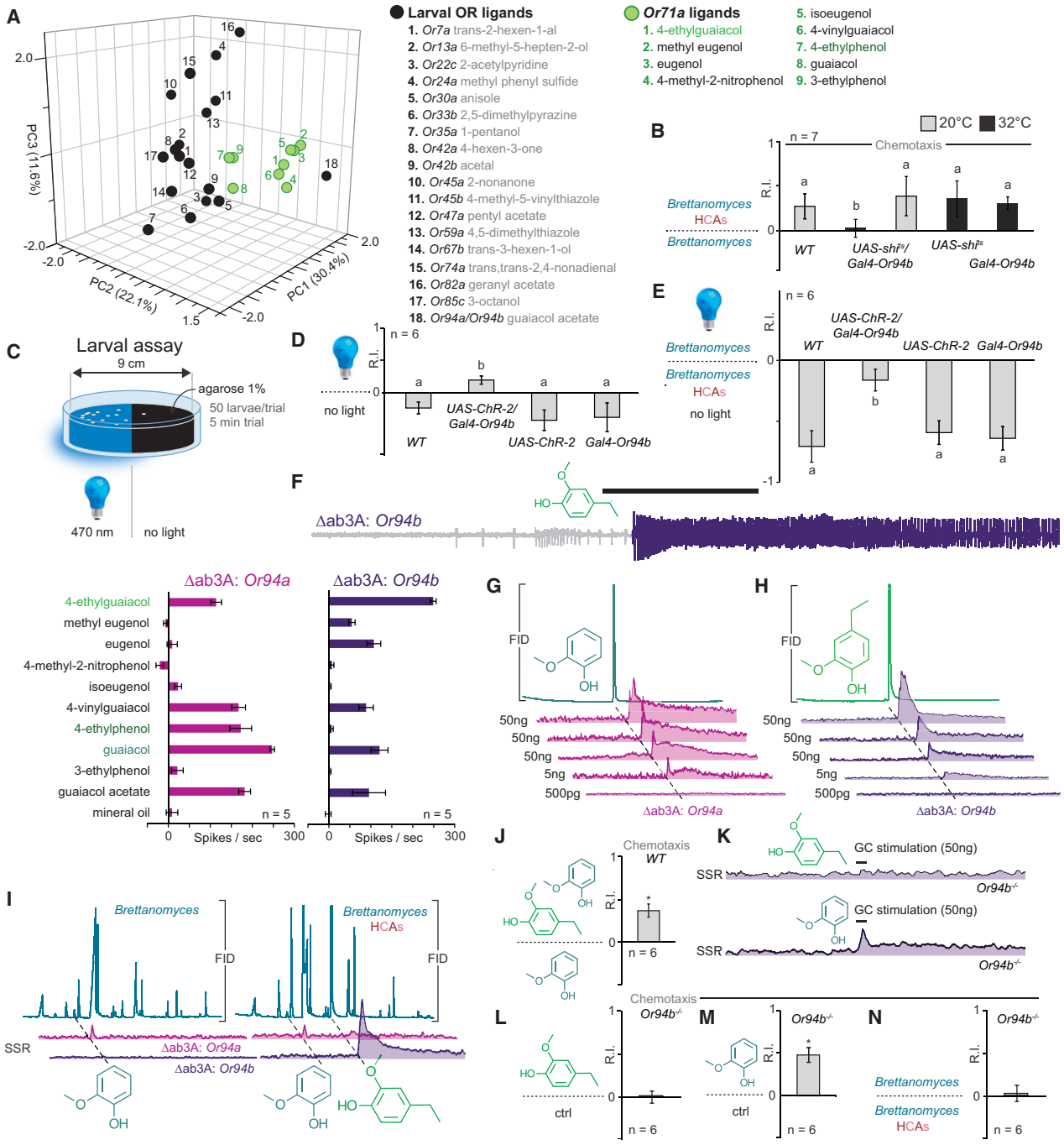

Figure 6. Larvae Detect Ethylphenols via *Or94a*

(A) PCA plot showing the distribution of the best ligands for the larval olfactory system (black dots) [32] and the main ligands for *Or71a* (green dots) in the odor space, defined by 32 physiochemical [33].

(B) Response indices from larvae expressing *Shibire^{ts}* from the *Or94b* promoter, the corresponding parental lines, and WT larvae confronted with a choice between *Brettanomyces* yeasts grown with or without HCAs. Error bars represent the SD. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(C) Schematic drawing of the larval choice assay used for the optogentic experiments.

(D) Response indices from larvae expressing *Channelrhodopsin-2* (*ChR-2*) from the *Or94b* promoter, the corresponding parental lines, and WT larvae confronted with a choice between an illuminated side (blue light) and a dark side. Error bars represent the SD. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p < 0.05$).

(E) Response indices from larvae expressing *ChR-2* from the *Or94b* promoter, the corresponding parental lines, and WT larvae confronted with a choice of HCA$^+$ and HCA$^-$ *Brettanomyces*, the latter illuminated with blue light. Error bars represent the SD. Significant differences are denoted by letters (ANOVA followed by Tukey's test; $p <$

(F) Representative SSR traces from *Δab3:Or94b* (*Δhalo;Or22a-GAL4/UAS-Or94b*) stimulated with 4-ethylguaiacol (top). The duration of the stimulus delivery (0.5 s) is marked by the black bar. Quantified SSR responses from *Δab3:Or94a* and *Δab3:Or94b* neurons toward the primary *Or71a* ligands are shown (bottom).

(legend continued on next page)

4-ethylguaiacol showed no preference either way (Figure 5M), suggesting that the presence of 4-ethylguaiacol in the HCA$^+$ samples indeed confers the attraction. We thus conclude that the larvae perform the same proxy detection of HCAs as adults, relying on the presence of ethylphenols to identify antioxidant-enriched patches.

The Larval OSN Populations Detecting Ethylphenols Also Detect Guaiacol

We next sought to determine which OR(s) in the larva detect the ethylphenols. In a recent study, Carlson and colleagues [32] deorphaned 19 out of the 21 expressed larval ORs using a panel of ~500 chemicals. Although the ethylphenols were not included in the test panel, chemicals of structural proximity were. To identify candidate OR(s) detecting the ethylphenols, we first undertook a chemometric approach [33]. We plotted the ethylphenols in a 32-dimensional odorant space together with the primary larval OR ligands identified by Mathew et al. [32]. A principal component analysis (PCA) plot revealed that the primary *Or71a* ligands clustered closest with the aromatic ligand for *Or94a* and *Or94b*, namely guaiacol acetate (or 2-methoxyphenyl acetate; Figure 6A). Thermogenetic silencing of the OSNs expressing *Or94a* and *Or94b* by expression of *Shibire^{ts}* from the promoter of the latter (the two ORs are co-expressed in the same OSN [34]) indeed abolished preference in a binary-choice test between HCA$^+$ and HCA$^-$ *Brettanomyces* (Figure 6B). Furthermore, optogenetic activation of the *Or94a/Or94b* pathway induced attraction in larvae expressing *Channelrhodopsin-2* (*ChR-2*) from the *Or94b* promoter, with larvae preferring the side illuminated with blue light (470 nm, activating the *ChR-2* molecules; Figures 6C and 6D), in contrast to parental lines and wild-type (WT) larvae, which are all repelled by blue light. Similarly, larvae confronted with a choice of HCA$^+$ and HCA$^-$ *Brettanomyces*—the latter illuminated with blue light—showed no preference either way (Figure 6E).

To verify that *Or94a/Or94b* respond to the ethylphenols, we next utilized the "empty-neuron" system [35] to determine the response properties of these two receptors. Heterologous expression of *Or94a* and *Or94b*, respectively, in ab3A OSNs conferred responsiveness toward the ethylphenols (Figure 6F). Out of the nine primary ligands of *Or71a* (Figures 3C and 3D), *Or94b* responded most strongly to 4-ethylguaiacol. This compound, however, only elicited minor responses from *Or94a*, which instead was strongly activated by guaiacol. Moreover, GC dose-response trials showed that these ligands induced responses already at very low concentrations from the respective ORs (Figures 6G and 6H). Both *Or94a* and *Or94b* were also activated by stimulation with the *Brettanomyces* headspace in GC-SSR experiments (Figure 6I). We note with interest that guaiacol—similar to 4-ethylguaiacol—activates a different receptor than in the adults, although with similar tuning. Guaiacol is a common microbial volatile (produced, e.g., by all the yeasts examined here), and its presence in nature would reliably indicate the occurrence of microbes, to larvae as well as adults.

Given that *Or94a* and *Or94b* are co-expressed in the same neurons, how do larvae distinguish HCA$^-$ from HCA$^+$ *Brettanomyces* when the headspace activates the same neural pathway? A possible explanation could be that the dual activation of *Or94a* and *Or94b* by the HCA$^+$ *Brettanomyces* sample would lead to a stronger signal into the central nervous system, in turn causing the behavioral preference. To test this notion, we next challenged the larvae with a mixture of 4-ethylguaiacol and guaiacol (10^{-4} dilution, total volume 10 μl) against guaiacol (10^{-4} dilution, 10 μl volume), a situation chemically mimicking the HCA$^-$/HCA$^+$ *Brettanomyces* choice. Indeed, larvae displayed a significant preference for the mixture over the single component (Figure 6J). Preference for the mixture remained even when double amounts (i.e., 20 μl) of guaiacol were tested against 10 μl of the mixture (data not shown), a treatment that would presumably compensate for any effects stemming from an increased volatility of the mix. Next, we examined an available *Or94b* null mutant (no expression of *Or94b* was detected in RT-PCR experiments with larval cDNA; data not shown). As expected, *Or94b$^{-/-}$* larvae showed no response to stimulation with 4-ethylguaiacol in SSR experiments (Figure 6K), nor did these larvae show any reaction to 4-ethylguaiacol in behavioral tests (Figure 6L), whereas the response to guaiacol was no different from that of WT larvae (Figure 6M). Furthermore, *Or94b$^{-/-}$* larvae confronted with a choice between HCA$^+$ and HCA$^-$ *Brettanomyces* displayed no preference either way (Figure 6N). In summary, larvae, like adults, identify the presence of HCAs via ethylphenols. Curiously, detection is done via a separate receptor from adults, albeit with similar tuning, which moreover is co-expressed with a receptor detecting a general yeast signal. The larval *Or94a/Or94b* OSNs thus offers coincidence detection of two distinct, but ecologically related, volatiles.

Conclusions

We have here shown that flies are able to recognize substrates enriched with HCAs. Flies—adults as well as larvae—do so by relying on specific volatile ethylphenols (4-ethylphenol and 4-ethylguaiacol), which are exclusively derived from HCAs. In adult flies, the ethylphenols are detected by maxillary palp OSNs that express *Or71a*. This neuron population is both necessary and sufficient for the proxy detection of HCAs. We demonstrate that the ethylphenols are generated by a wide range of yeasts consumed by flies and thus act as a consistent and reliable signal for the presence of HCAs. We further show that larvae perform the same proxy detection of HCAs via the

(G) Linked GC-SSR response traces from *Δab3:Or94a* neurons stimulated with different amounts of guaiacol.
(H) GC-SSR response traces from *Δab3:Or94b* neurons stimulated with different amounts of 4-ethylguaiacol.
(I) GC-SSR response traces from *Δab3:Or94a* and *Δab3:Or94b* neurons stimulated with headspace from Brettanomyces grown with or without HCAs.
(J) Larval response index from a choice between 4-ethylguaiacol and guaiacol against guaiacol. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).
(K) GC-SSR response traces from *Or94b$^{-/-}$* larvae stimulated with 4-ethylguaiacol (top) and guaiacol (bottom).
(L) Response index from *Or94b$^{-/-}$* larvae provided a choice between 4-ethylguaiacol and solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).
(M) Response index from *Or94b$^{-/-}$* larvae provided a choice between guaiacol and solvent control. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).
(N) Response index from *Or94b$^{-/-}$* larvae provided a choice between *Brettanomyces* grown with or without HCAs. Error bars represent the SD. Deviation of the response indices against zero was treated with a Student's t test ($p < 0.05$).

ethylphenols as the adults, but do so via a different OR (*Or94b*) only expressed in the larval stage.

In humans, oxidative stress has been implicated in triggering or enhancing a range of diseases typically associated with aging, inter alia cancer and neurodegenerative disorders [36, 37]. For a short-lived species like the fly, the need to prevent the onset of aging related diseases would appear to be an unlikely reason for having a dedicated proxy detection system for dietary antioxidants. For flies, antioxidants could play an important role in counteracting acute oxidative stress induced by immune defense responses and detoxification processes upon consumption or infection by entomopathogenic microorganisms, which co-occur with beneficial food yeasts in the flies' habitat. The importance played by toxic microbes in the fly's ecology is also illustrated by the remarkably sensitive and selective detection system for geosmin, a volatile indicating the presence of harmful microorganisms [38]. The ethylphenol pathway described here thus adds another layer to the fly's defensive arsenal against toxic microbes.

We propose here that the ecological significance of the pb1B circuit is to alert flies to the presence of dietary antioxidants. Proxy detection of non-volatile nutrients and health-promoting compounds is most likely an important function of the olfactory system. Many volatiles that humans perceive as having a positive impact on flavor are in fact derived from essential nutrients or from other compounds having direct health benefits [39]. These volatiles are accordingly attractive to humans precisely because they reliably signal the presence of their health-promoting precursors. For a generalist species such as the fly, having dedicated OSNs tuned to volatiles indicating the presence of essential nutrients would make sense. Further research will surely reveal more instances of proxy detection of nutrients in the fly's olfactory system, as well as in other organisms.

The pb1B pathway joins a growing number of non-pheromonal OSN classes for which dedicated and non-redundant functions has been assigned. Functionally segregated pathways identified so far include the above-mentioned geosmin circuit fed by *Or56a* [38], CO_2 avoidance mediated via *Gr21a* and *Gr63a* [40–42], aversion toward select acids via *Ir64a* [43], oviposition preference for citrus-like fruits via *Or19a* [13], attraction toward farnesol (exact ecological function unclear) via *Or83c* [44], attraction toward vinegar via *Or42b* and *Or92a* [45], preference for the yeast metabolites phenylacetic acid and phenylacetaldehyde via *Ir84a* [46], and attraction to ammonia and select amines through *Ir92a* [47]. It is our suspicion that precise and non-redundant functions, linked to ecologically relevant behaviors can be assigned to most, if not all, of the flies' (known) 48 classes of OSNs. Thus, in contrast to the widespread notion that individual odorants are predominantly decoded via combinatorial patterns of glomerular activation, the fly's olfactory system appears to mainly extract information from its chemical surrounding via dedicated olfactory pathways. Although functionally segregated, the respective pathways would still function in concert, with behavioral decisions arising based on the relative input—or lack thereof—into combinations of dedicated circuits, each carrying a distinct ecological message.

Experimental Procedures

Fly Stocks

All experiments with WT *D. melanogaster* were carried out with the Canton-S strain. Transgenic lines were obtained from the Bloomington *Drosophila* Stock Center (http://flystocks.bio.indiana.edu), except for *Δhalo; Or22a-GAL4/UAS-Or94a/Or94b*, which were a kind gift from Dr. J.R. Carlson (Yale University).

Yeast Species and Cultivation

The six different yeast species used in this study were obtained from the Leibniz Institute DSMZ (German Collection of Microorganisms and Cell Cultures) under DSM numbers 2768 (*Hanseniaspora uvarum*), 70001 (*Brettanomyces bruxellensis*), 70130 (*Wickerhamomyces anomalus*), 70336 (*Metschnikowia pulcherrima*), 70451 (*Saccharomyces cerevisiae*), and 70497 (*Torulaspora delbrueckii*). Except for *B. brexellensis*, all yeasts were cultivated on broth media, which consisted of 3 g yeast extract, 3 g malt extract, 5 g peptone (from soybeans), 10 g glucose, and 1 l distilled water. The media used for *B. bruxellensis* consisted of 1.67 g nitrogen (from yeast) and 10 g glucose. For preparation of HCA-inoculated yeast samples, 0.1 g each of p-coumaric acid and ferulic acid was added to the media. The cultures were kept at 28°C under continuous shaking for 3 days, except for *B. bruxellensis*, which was kept for 21 days.

Stimuli and Chemical Analysis

All synthetic odorants tested were acquired from commercial sources (Sigma, http://www.sigmaaldrich.com; Bedoukian, http://www.bedoukian.com) and were of the highest purity available. Stimuli preparation and delivery followed Stökl et al. [12]. The headspace collection of volatiles was carried out according to standard procedures. For GC stimulation, 1 μl of the odor sample was injected onto a DB5 column (Agilent Technologies, http://www.agilent.com), fitted in an Agilent 6890 GC, equipped with a four-arm effluent splitter (Gerstel, http://www.gerstel.com), and operated as previously described [12] except for the temperature increase, which was set at 15°C min^{-1}. GC-separated components were introduced into a humidified airstream (200 ml min^{-1}) directed toward the antennae, palps, or dorsal organ of a mounted fly or larva. Signals from OSNs and FID were recorded simultaneously. GC mass spectrometry analysis was performed as previously described [12]. So that the presence of HCAs in banana pulp could be verified, HCAs were extracted from 25 mg of banana pulp in 50% aqueous methanol (methanol:water 50:50, v:v). The methanol extract was then analyzed by HPLC on an Agilent 1200 HPLC system (Agilent Technologies) coupled to an API 3200 tandem mass spectrometer (AB SCIEX, http://www.absciex.com) equipped with a turbospray ion source.

Toxicity Assay

The paraquat toxicity assay was performed on virgin 2- to 3-day-old flies collected overnight and kept on regular food medium. Fifty separated adult flies were then starved in empty vials for 3 hr at 25°C. Then, each treatment was provided to the flies in a capillary tube similar to the feeding assay described in Stensmyr et al. [38]. Red food dye (McCormick red food coloring, http://www.mccormick.com) was added to ensure homogeneity and food intake. The locomotor assay was performed as described in Ortega-Arellano et al. [48]. In brief, treated flies were placed in empty plastic vials. After a 10 min rest period, the flies were tapped to the bottom of the vials, and the number of flies able to climb 5 cm in 6 s was recorded at each interval of time.

Behavioral Assays

T-maze, oviposition, and feeding experiments were carried out as described in Stensmyr et al. [38]. In brief, trap and oviposition assay experiments were carried out in a cage (50 × 50 × 50 cm) with a treatment and a control either traps or oviposition plates. Thirty 4- to 5-day-old flies were placed in each cage. Experiments were carried out in a climate chamber (25°C, 70% humidity, 12-hr light:12-hr dark cycle). The number of flies or eggs was counted after 24 hr. The attraction index was calculated as (O − C) / T, where O is the number of flies in the odorant trap, C is the number of flies in the control trap, and T is the total number of tested flies. 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. Feeding experiments were conducted as outlined in Figure 6G in Stensmyr et al. [38]. A feeding index was calculated as (O − C) / (O + C), where O is the amount of food consumed from the odorous solutions and C is the amount from control sucrose-only solutions. The larval olfactory choice assay is illustrated in Figure 5A. For measurement of olfactory responses, 50 larvae were briefly dried on a filter paper before being placed in the center of a Petri dish filled with 1% agarose. The Petri dish contained 10 μl of odor (or solvent control) dispensed on each of the two 0.5-cm-radius filter discs placed in the two opposite odor zones. After

5 min of larvae placement and covering of the Petri dish, the number of larvae in respective zones was counted and a response index was calculated ((O − C) / T). For channel channelrhodopsin-2 (*ChR2*) experiments—outlined in Figure 6C—the petri dish was divided in two halves, one illuminated with blue light (480 nm wavelength) and the other half dark. Other experimental conditions and the calculation of the response index were done as described above.

SSRs

Larvae used in electrophysiology were taken from the culture during the first 24 hr of the third instar (48–72 hr after egg hatching). Each larva were subsequently placed on its dorsum on a pipette tip and fixed with a silver wire, which served also as reference electrode. The recording electrode was inserted at the rim of the dome of the dorsal organ. Adult flies were immobilized in pipette tips, and the third antennal segment or palps were placed in a stable position onto a glass coverslip. Sensilla were localized at 1,000× magnification, and the extracellular signals originating from the OSNs were measured by insertion of a tungsten wire electrode in the base of a sensillum. The reference electrode was inserted into the eye. Signals were amplified (10×; Syntech Universal AC/DC Probe, http://www.syntech.nl), sampled (10,667.0. samples/s), and filtered (100–3,000 Hz with 50/60 Hz suppression) via a USB-IDAC connection to a computer (Syntech). Action potentials were extracted with Syntech Auto Spike 32 software. Neuron activities were recorded for 10 s, starting 2 s before a stimulation period of 0.5 s. Responses from individual neurons were calculated as the increase (or decrease) in the action potential frequency (spikes/s) relative to the pre-stimulus frequency.

Data Analysis

Chemometric analysis was performed as outlined in Haddad et al. [33] In brief, chemodescriptors for the selected volatiles were calculated using Dragon (http://www.talete.mi.it), from which 32 descriptors were selected and subsequently *Z* score normalized [33], and were used as basis for a PCA performed in PAST (http://folk.uio.no/ohammer/past/). All other statistical procedures were performed with SPSS (http://www.spss.com).

Supplemental Information

Supplemental Information includes one figure and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2014.11.062.

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Current Biology

Supplemental Information

Olfactory Proxy Detection of Dietary Antioxidants in *Drosophila*

Hany K.M. Dweck, Shimaa A.M. Ebrahim, Abu Farhan, Bill S. Hansson, and Marcus C. Stensmyr

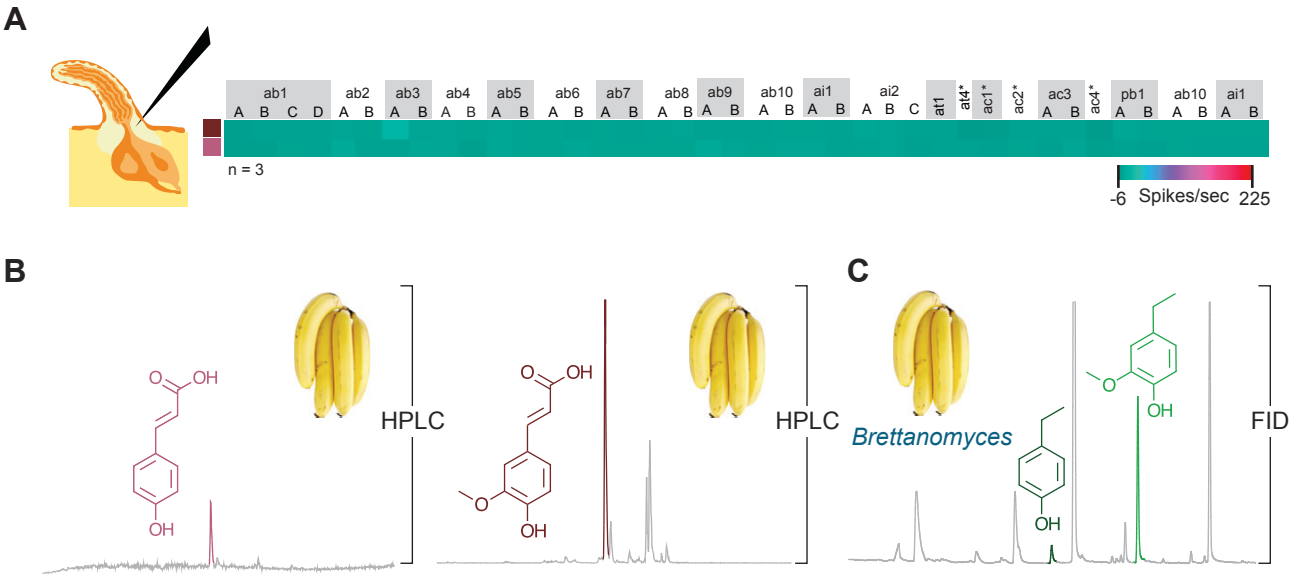

Figure S1 (related to Figure 1).
(A) Heatmap of the average Single Sensillum Recording (SSR) responses from all known OSN classes stimulated with p-coumaric acid and ferulic acid (10-2 dilution). ab, antennal basiconic sensilla (s.); ac, antennal coeloconic s.; at, antennal trichoid s.; ai, antennal intermediate s.; pb, palp basiconic s. Stars denote that activity from individual OSNs was not separated.
(B) High Performance Liquid Chromatography (HPLC) trace of banana pulp.
(C) Flame Ionization Detection (FID) trace from a headspace collection of *Brettanomyces* yeast grown on banana medium.

MANUSCRIPT IV

Pheromones mediating copulation and attraction in *Drosophila*

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Pheromones mediating copulation and attraction in *Drosophila*

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Intraspecific olfactory signals known as pheromones play important roles in insect mating systems. In the model *Drosophila melanogaster*, a key part of the pheromone-detecting system has remained enigmatic through many years of research in terms of both its behavioral significance and its activating ligands. Here we show that Or47b-and Or88a-expressing olfactory sensory neurons (OSNs) detect the fly-produced odorants methyl laurate (ML), methyl myristate, and methyl palmitate. Fruitless (*fru^M*)-positive Or47b-expressing OSNs detect ML exclusively, and Or47b- and Or47b-expressing OSNs are required for optimal male copulation behavior. In addition, activation of Or47b-expressing OSNs in the male is sufficient to provide a competitive mating advantage. We further find that the vigorous male courtship displayed toward oenocyte-less flies is attributed to an oenocyte-independent sustained production of the Or47b ligand, ML. In addition, we reveal that Or88a-expressing OSNs respond to all three compounds, and that these neurons are necessary and sufficient for attraction behavior in both males and females. Beyond the OSN level, information regarding the three fly odorants is transferred from the antennal lobe to higher brain centers in two dedicated neural lines. Finally, we find that both Or47b- and Or88a-based systems and their ligands are remarkably conserved over a number of drosophilid species. Taken together, our results close a significant gap in the understanding of the olfactory background to *Drosophila* mating and attraction behavior; while reproductive isolation barriers between species are created mainly by species-specific signals, the mating enhancing signal in several *Drosophila* species is conserved.

Drosophila | pheromone | mating | olfaction | olfactory circuit

In the vinegar fly *Drosophila melanogaster*, cuticular hydrocarbons (CHCs) act as pheremones and play important roles in courtship and aggregation behaviors. These pheremones include the female-specific aphrodisiacs (*Z*,*Z*)-7,11-heptacosadiene (7,11-HD) and (*Z*,*Z*)-7,11-nonacosadiene (7,11-ND) and the male specific antiaphrodisiacs (*Z*)-7-tricosene (7-T) and 11-cis-vaccenyl acetate (cVA) (1). However, several lines of evidence suggest that other unidentified pheromones likely contribute to courtship and aggregation behaviors. Previous studies have demonstrated that an unidentified volatile sex pheromone produced by female flies stimulates male courtship (2–6). Flies anosmic to cVA exhibit residual attraction to live male flies, suggesting that other attractive cues are produced by flies that are independent of cVA and its neural circuit (7). Furthermore, no specific ligands other than cVA have been identified for the potential pheromone receptors expressed in OSNs of antennal trichoid sensilla (8). Moreover, OSNs expressing olfactory receptors Or47a and Or88a housed in trichoid sensilla respond to unidentified odors in male and female body wash extracts (9).

Although the CHC profile of *D. melanogaster* has been characterized by several analytical techniques (10–14), it is not yet complete (3). In the present study, we used thermal desorption-gas chromatography-mass spectrometry (TD-GC-MS) to determine whether flies harbor so far unidentified CHCs. TD-GC-MS provides a highly sensitive and labor-saving alternative to solvent extraction, and allows analysis of a wider volatility range of components than all previously mentioned techniques. In addition, this method has been applied to confirm the composition of sex pheromones in other insect species (15, 16).

Here we demonstrate the presence of a truly positive fly-produced signal mediating mating and dissect the neural mechanism underlying its detection. With our findings, the understanding of male olfactory-based sexual arousal is becoming more complete, with all *fru*-positive OSNs now with known ligands. We also report the presence of the first fly odorants that exclusively mediate attraction in both sexes via a pathway separated from that involved in sexual and social behaviors. Interestingly, both systems and their ligands are remarkably conserved over a number of drosophilid species.

Results and Discussion

Flies Produce Previously Unidentified Candidate Pheromones. To determine whether *D. melanogaster* harbors so far unidentified CHCs, we used TD-GC-MS to measure CHC profiles of individual flies. Intact flies of different ages were placed in thermal desorption tubes, which were subsequently heated. The cuticular compounds released were trapped by cooling and then transferred to the GC-MS device by rapid heating. Eighty-five cuticular compounds, including alkanes, methyl-alkanes, monoenes, dienes, aldehydes, ketones, esters, and amides, were identified (Fig. S1 and Table S1). Sixty-four were found in both

Significance

Mating interactions in *Drosophila melanogaster* depend on a number of sensory cues targeting different modalities like hearing, taste, and olfaction. From an olfactory perspective, only negative fly-derived signals had been identified, whereas a positive signal mediating mating was missing. Here we demonstrate the presence of such a signal (methyl laurate) and dissect the neural mechanism underlying its detection. We also show that the same odorant together with two additional fly-derived odorants (methyl myristate and methyl palmitate) mediate attraction via a pathway separated from that involved in courtship. Interestingly, the odorants identified are attractive to several closely related species. Thus, we describe two highly important neural circuits involved in mating and attraction that seem to be conserved in *Drosophila*.

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males and females, whereas 11 were female-specific and 10 were male-specific.

OR47b- and OR88a-Expressing OSNs Detect Methyl Laurate, Methyl Myristate, and Methyl Palmitate. To test for olfactory detection of the fly-produced compounds identified in the analytical study, we obtained single-sensillum recording (SSR) measurements from all OSN types housed in trichoid sensilla (at1 and at4) using 42 synthetic compounds as stimuli. These compounds were chosen to represent all chemical classes identified. In addition to cVA, three other fly-produced odorants activated two OSN types, both present in the antennal trichoid sensillum type 4 (at4)

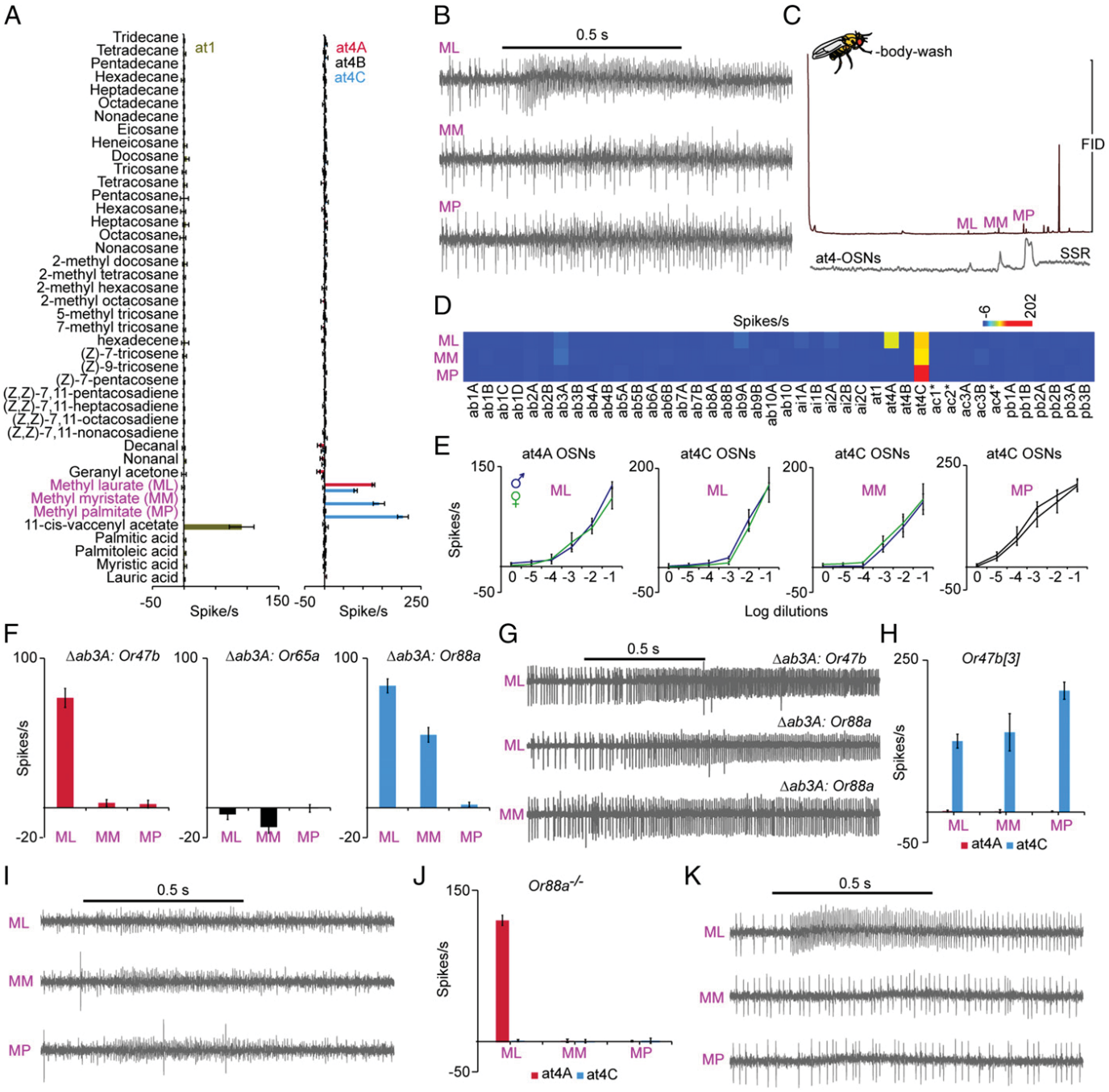

Fig. 1. OR47b- and OR88a-expressing OSNs detect ML, MM, and MP. (*A*) Average SSR responses from all OSNs housed in trichoid sensilla after stimulation with 42 cuticular compounds (10^{-1} dilution) ($n = 5$). (*B*) Representative SSR traces from measurements of WT at4 OSNs stimulated with ML, MM, and MP (10^{-1} dilution). (*C*) Representative GC-SSR measurements from at4 OSNs stimulated with GC-fractionated fly body wash extracts ($n = 4$). (*D*) Heat map of the average SSR responses from all OSN classes stimulated with ML, MM, and MP (10^{-1} dilution) ($n = 3$). Asterisks denote the total activity of an OSN when spike sorting failed. (*E*) Dose–response curves from at4A and at4C OSNs to ML, MM, and MP ($n = 5$). (*F*) Average SSR responses from Δab3A: *Or47b*, Δab3A: *Or65a*, and Δab3A: *Or88a* to ML, MM, and MP (10^{-1} dilution) ($n = 5$). (*G*) Representative SSR traces from Δab3A: Or47b and Δab3A: Or88a stimulated with ML and MM (10^{-1} dilution). (*H*) Average SSR responses from at4A and at4C OSNs of *Or47b[3]* mutant flies stimulated with ML, MM, and MP (10^{-1} dilution) ($n = 5$). (*I*) Representative SSR traces from at4 OSNs of *Or47b[3]* mutant flies stimulated with ML, MM, and MP (10^{-1} dilution). (*J*) Average SSR responses from at4A and at4C OSNs of *Or88a*$^{-/-}$ mutant flies stimulated with ML, MM, and MP (10^{-1} dilution) ($n = 5$). (*K*) Representative SSR traces from at4 OSNs of *Or88a*$^{-/-}$ mutant flies stimulated with ML, MM, and MP (10^{-1} dilution).

(Fig. 1 *A* and *B*). The at4 sensillum in total houses three OSNs (A–C), which have been shown to respond to previously unidentified odors secreted by both male and female flies (9). The at4A OSN responded exclusively to methyl laurate (ML), whereas the at4C OSN responded to ML, methyl myristate (MM), and methyl palmitate (MP) (Fig. 1 *A* and *B*).

Because not all fly odors were tested in our initial screening, we proceeded to obtain linked GC-SSR measurements from at4 OSNs using fly body wash extracts to further test whether the three fly odors were the exclusive ligands for at4A and at4C OSNs. In these experiments, only three flame ionization detector (FID) peaks corresponded to responses from the at4 OSNs (Fig. 1*C*). Using GC-MS and synthetic standards, we found that these three FID peaks are ML, MM, and MP. Thus, we conclude that ML is the sole fly-produced ligand for at4A OSNs, whereas ML, MM, and MP are the ligands for at4C OSNs.

To establish whether these three active compounds activate other OSNs types as well, we proceeded to test them in SSR

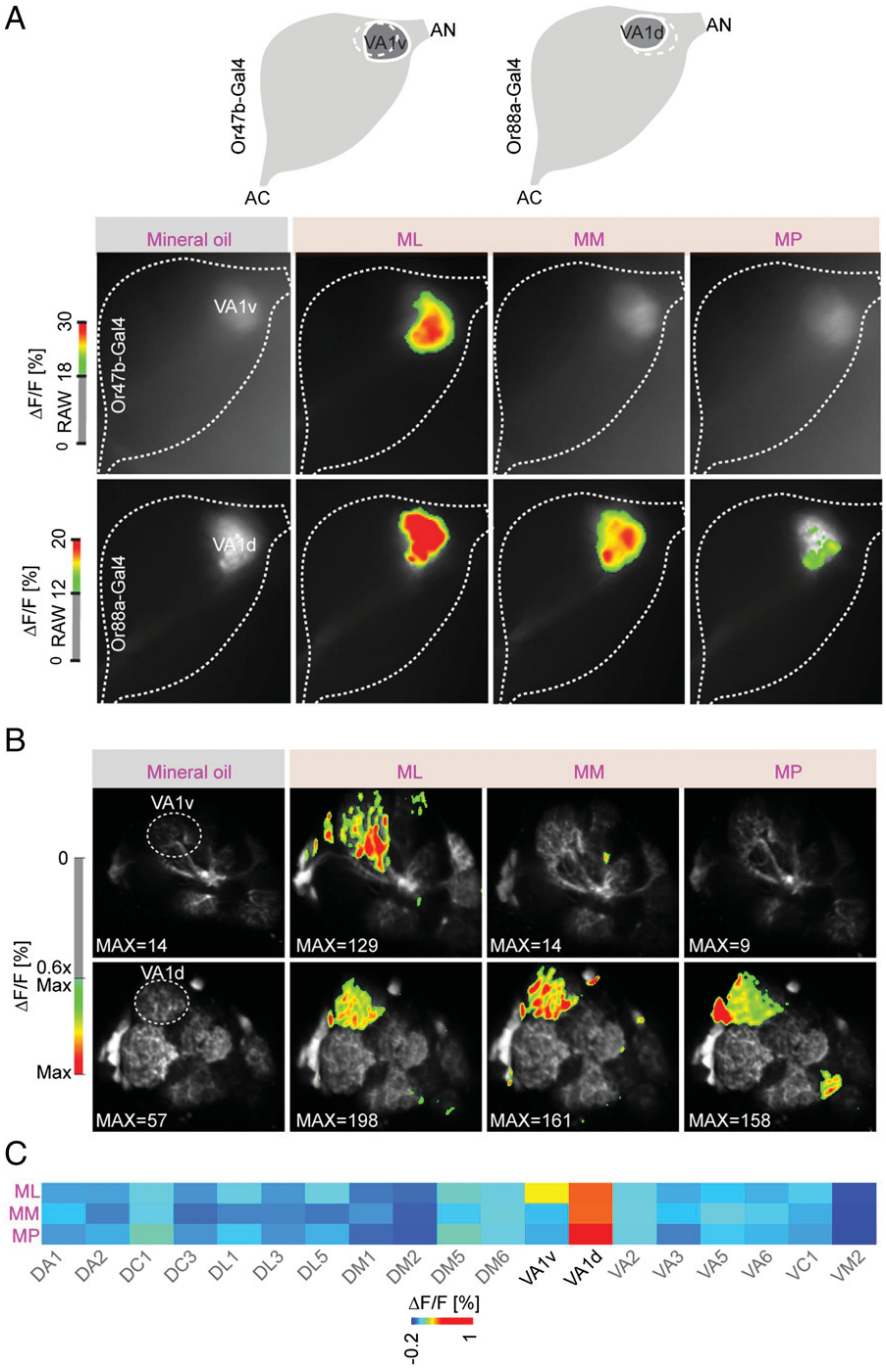

Fig. 2. ML, MM, and MP peripheral signals are transferred via dedicated neural lines from the antennal lobe to higher brain centers. (*A*) False color-coded images showing mineral oil-, ML-, MM-, and MP-induced calcium dependent fluorescence changes in the AL of a representative fly expressing the activity reporter GCaMP3.0 from Or47b and Or88a promotors (10^{-1} dilution) ($n = 5$). (*B*) False color-coded images of a representative fly showing mineral oil-, ML-, MM-, and MP-induced calcium signals in PNs of the AL via GCaMP6s expression under control of the GH146-GAL4 driver (10^{-1} dilution). (*C*) Heat map of the average ML-, MM-, and MP-evoked calcium signals in PNs in the AL as shown in *B* ($n = 3$).

experiments including all OSN types located on the third antennal segment and maxillary palp. None of the compounds elicited a reliable response from any OSN type beyond at4A and at4C (Fig. 1*D*); thus, we conclude that these three active fly odorants activate exclusively at4A and at4C OSNs.

We next examined dose–response relationships of at4A and at4C OSNs for ML, MM, and MP. In contrast to the strong sexual dimorphism in antennal responses to pheromones observed in moths (17, 18), responses of at4A and at4C OSNs to the three fly odorants were quantitatively indistinguishable between the sexes (Fig. 1*E*). However, the at4A OSNs were two orders of magnitude more sensitive to ML compared with the at4C OSNs, whereas at4C OSNs were activated by MP at lower doses than by MM or ML (Fig. 1*E*).

The three neurons of the at4 sensillum express Or47b, Or88a, and the closely related genes Or65a, Or65b, and Or65c (19). To identify the Or expressed in at4A and at4C OSNs, we misexpressed *Or47b*, *Or88a*, and *Or65a* in *Δab3A* OSNs using the *Drosophila* empty neuron system (20). OSNs misexpressing *Or47b* responded exclusively to ML, whereas OSNs misexpressing *Or88a* responded to ML and MM, but not to MP (Fig. 1 *F* and *G*). The latter finding is enigmatic, but the detection of MP may require other crucial factors in the native trichoid environment, such as odorant-binding proteins (7, 21). This relationship remains to be investigated, however. None of the three fly odorants activated OSNs misexpressing *Or65a* (Fig. 1*F*). Furthermore, in an *Or47b* mutant (22), which has two identical independent knockout alleles, *Or47b[2]* and *Or47b[3]* (in all experiments, we used only *Or47b[3]* after backcrossing it to the Canton-S background to minimize genetic background effects), the responses of at4A OSNs to ML were completely abolished, whereas at4C OSNs still responded to the three fly odorants (Fig. 1 *H* and *I*). In contrast, in an *Or88a* mutant, which was generated by imprecise excision (as a gift from L. B. Vosshall) and validated by RT-PCR experiments (Fig. S2), the responses of at4C OSNs to the three fly odorants were abolished, whereas the responses of at4A OSNs to ML remained unaffected (Fig. 1 *J* and *K*). These results suggest that the responsiveness of at4A OSNs to ML is due to the expression of Or47b, whereas the responsiveness of at4C OSNs to ML, MM, and MP is due to the expression of Or88a.

ML, MM, and MP Peripheral Signals Are Transferred from the Antennal Lobe to Higher Brain Centers in Dedicated Lines. We verified that Or47b- and Or88a-expressing OSNs are the peripheral channels for the three fly odorants by expressing the calcium-sensitive protein *GCaMP* (23) under control of the two corresponding *Or* lines (19) (Fig. 2*A*). To further investigate how the input signals were transferred via projection neurons (PNs) to higher processing centers, we expressed *GCaMP* (24) under control of the *GH146* (25) driver line and performed two-photon calcium imaging at the level of PN dendrites in the antennal lobe (AL). As expected, the VA1v glomerulus, which receives input from Or47b (19), was exclusively activated by ML but not by MM or MP, whereas the VA1d glomerulus, which receives input from Or88a (19), was activated by all three fly odorants (Fig. 2 *B* and *C*). Thus, we conclude from the SSR and imaging data that ML, MM, and MP are detected exclusively by Or47b- and Or88a-expressing OSNs, and that this information enters and leaves the AL through these two channels only.

ML Acts as a Stimulatory Pheromone to Promote Male Copulation. Male courtship behavior is controlled by neural circuitry expressing male-specific isoforms of the transcription factor Fruitless (fru^M) (26, 27). Blocking of synaptic transmission of all *fru*-expressing neurons significantly reduces male courtship (27). The Or47b OSN population is one of only three expressing fru^M (26, 27). In addition, the VA1v glomerulus, the target of Or47b neurons in the AL, is larger in males than in females (27). These facts suggest a role for ML, the sole ligand of Or47b-expressing neurons, in mediating male courtship behavior. We investigated this hypothesis in single pair courtship assays. Coating WT females with 100 pg of ML (the equivalent quantity of an individual fly; Fig. S3) significantly increased the number of

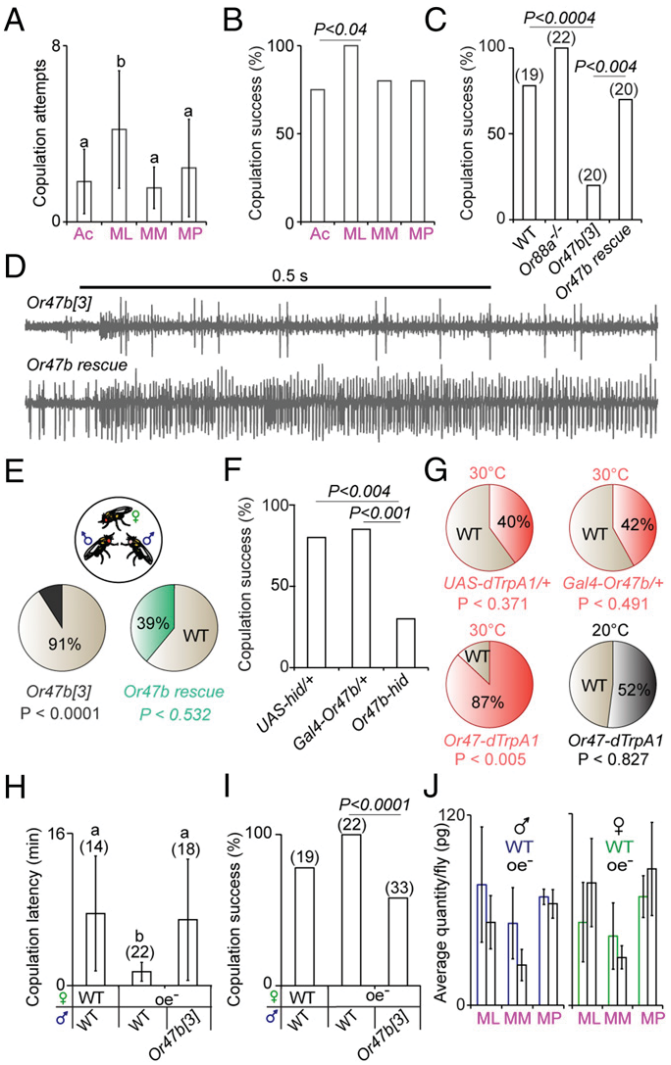

Fig. 3. Or47b promotes male copulation. (*A*) Average number of copulation attempts of WT males courting WT females painted with acetone (Ac), ML, MM, or MP ($n = 20$). Error bars represent SD. Significant differences are denoted by letters ($P < 0.05$, ANOVA followed by Tukey's test). (*B*) Percentage of copulation success of WT males courting WT females painted with acetone, ML, MM, or MP ($n = 20$) (Fisher's exact test). (*C*) Percentage of copulation success of WT, *Or47b[3]*, $Or88a^{-/-}$, and *Or47b rescue* males courting WT females (Fisher's exact test). Sample sizes are given in brackets above bars. (*D*) Representative SSR traces from at4 OSNs of *Or47b[3]* and *Or47b rescue* flies stimulated with ML (10^{-1} dilution). (*E*) WT males competing with either *Or47b[3]* (gray) or *Or47b rescue* (green) males for mating with WT females in competition assays ($n = 25$) (χ^2 test). (*F*) Percentage of copulation success of males expressing *UAS-hid/+*, *Gal4-Or47b/+*, and *UAS-hid* from *Or47b* promoter courting WT females ($n = 20$) (Fisher's exact test). (*G*) Males expressing *dTrpA1* from *Or47b* promoter competing with WT males for mating with WT females in competition assays ($n = 25$) (χ^2 test). (*H*) Copulation latency of WT and *Or47b[3]* males courting either WT or oe^- females. Error bars represent SD. Significant differences are denoted by letters ($P < 0.05$, ANOVA followed by Tukey's test). Sample sizes are given in brackets above bars. (*I*) Percentage of copulation success of WT and *Or47b[3]* males courting either WT or oe^- females (Fisher's exact test). Sample sizes are given in brackets above bars. (*J*) Average quantity of ML, MM, and MP in WT and oe^- flies ($P > 0.05$, independent-samples *t* test; $n = 6$).

copulation attempts and copulation success in WT males (Fig. 3 *A* and *B*). The other sequences of the male courtship ritual remained unaffected (Fig. S4). WT females coated with 100 pg of MM, MP, or acetone elicited no significant change in the courtship behavior of WT males (Fig. 4 *A* and *B* and Fig. S4). Thus, we conclude that only ML, and not MM or MP, acts as a stimulatory pheromone to promote male copulation behavior.

Or47b- and Or47b-Expressing OSNs Are Required for Optimal Male Copulation Behavior. Because ML activates both Or47b- and Or88a-expressing OSNs, we asked whether normal levels of male copulation behavior require only one or both of these receptors. Pairs of either *Or47b[3]* or *Or88a*$^{-/-}$ males with virgin WT females were placed in courtship chambers and the percentage of copulation success was observed after 30 min. When courting WT females, *Or47b[3]* males, but not *Or88a* mutant males, displayed a significant reduction in copulation success compared with control males (Fig. 3*C*). This result is consistent with a previous finding that a reduced size of the VA1v glomerulus, the target of Or47b-expressing neurons, causes courtship deficits (28). To verify that the observed phenotype was due to the loss of *Or47b* function, we rescued this function by introducing *UAS-Or47b* under control of *Or47b-Gal4* into *Or47b[3]*. Restoration of *Or47b* function was accompanied by restoration of normal levels of spontaneous activity and responses to ML in at4A OSNs (Fig. 3*D*). As expected, *Or47b* rescue males, in contrast to *Or47b[3]* males, copulated as much as control males when courting WT females (Fig. 3*C*).

To avoid any variation dependent on female receptivity, we further examined the importance of Or47b for male copulation success in competitive mating assays. In these assays, one WT male with intact Or47b and one male with mutation in *Or47b* were allowed to compete for copulation with the same WT female for 30 min. The genotypes of the competing males were verified by eye color. Indeed, males with mutation in *Or47b* had significantly lower copulation success than WT males when competing for copulation with WT females (Fig. 3*E*). This defect was fully restored to the levels of WT males by rescuing *Or47b* function (Fig. 3*E*). Thus, we conclude that Or47b is required for optimal male copulation behavior.

We proceeded to examine whether Or47b-expressing OSNs are also required for promoting male copulation behavior. We expressed the programmed cell death gene, *head involution defective* (*UAS-hid*) (29), coupled with *UAS-Stinger II* from the *Or47b* promoter to generate flies lacking Or47b neurons. The combination of *StingerGFP* with *hid* allowed us to visualize the absence of GFP-labeled Or47b neurons from males lacking *Or47b* neurons in the fluorescence microscope. Indeed, in single pair courtship assays, males lacking *Or47b* neurons had significantly less copulation with WT females compared with control males (Fig. 3*F*). The percentage of copulation success with WT females was similar in males lacking *Or47b* neurons and males with disrupted *Or47b*. Thus, we conclude that the activity of Or47b neurons is required for optimal male copulation behavior.

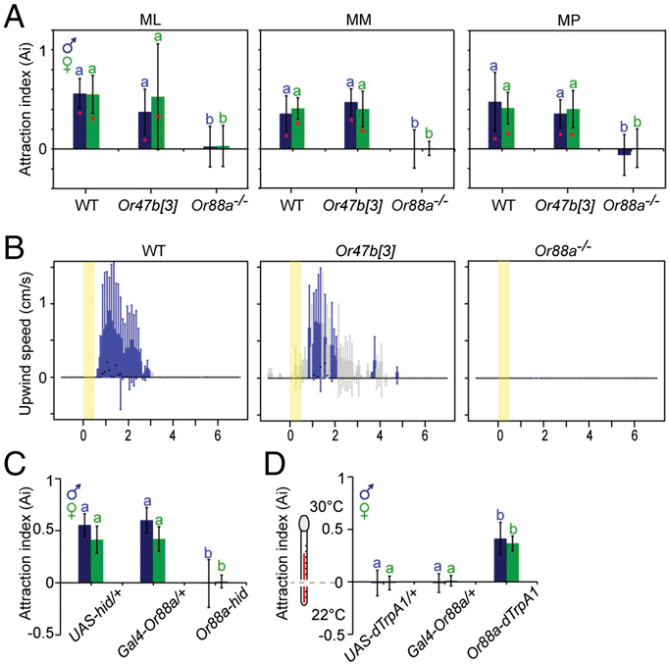

Fig. 4. Or88a is required for the attraction behavior toward ML, MM, and MP. (*A*) Attraction indices of WT, *Or47b[3]*, and *Or88a*$^{-/-}$ in a binary choice assay between ML, MM, or MP against solvent control. Error bars represent SD. Deviation of the response indices against zero was tested with the Student *t* test; significant differences are denoted by asterisks. For comparison between groups, ANOVA followed by Tukey's test was used, and significant differences are denoted by letters ($P < 0.05$). (*B*) Boxplot representation of odor-induced changes in the *FlyWalk* assay in upwind speed. Black lines indicate median values; box, interquartile range; whiskers, 90th and 10th percentiles; blue boxplots, significantly increased upwind speed compared with the upwind speed during the solvent control situation within the corresponding 100-ms time frame ($P < 0.05$, Wilcoxon signed-rank test; $n = 30$ flies); gray boxplots, no significant difference in upwind speed. (*C*) Attraction indices of flies expressing *UAS-hid/+*, *Gal4-Or88a/+*, and *UAS-hid* from *Or88a* promoter in a binary choice assay between ML and solvent control. Error bars represent SD. ANOVA followed by Tukey's test was used for comparisons between groups. Significant differences are denoted by letters ($P < 0.05$). (*D*) Attraction indices of flies expressing *UAS-dTrpA1/+*, *Gal4-Or88a/+*, and *UAS-dTrpA1* from *Or88a* promoter in a binary choice assay between 22 °C and 30 °C. Error bars represent SD. ANOVA followed by Tukey's test was used for comparisons between groups. Significant differences are denoted by letters ($P < 0.05$).

Activation of Or47b-Expressing OSNs Provides a Competitive Mating Advantage. We next tested whether activation of Or47b OSNs is sufficient to provide a competitive mating advantage. We therefore generated males expressing the heat-activatable cation channel, dTrpA1 (*UAS-dTrpA1*), from the *Or47b* promoter to artificially activate Or47b neurons by shifting the temperature to 30 °C. Indeed, males carrying *UAS-dTrpA1* (30) from the *Or47b* promoter exhibited significantly greater copulation success than WT males when competing for copulation with WT females at 30 °C (Fig. 3*G*). This effect was not observed in males of the same genotype at the permissive temperature (20 °C), or in the parental lines at the restrictive temperature (30 °C) (Fig. 3*G*). Thus, activation of Or47b neurons is important for providing a competitive mating advantage.

Vigorous Courtship Toward Oenocyte-Less Flies Is Due to Sustained Production of the Or47b Ligand, ML. In *D. melanogaster*, CHCs are synthesized in specialized cells called oenocytes (31). Genetic manipulation of oenocyte cells (oe^{-}) eliminates CHCs (32), but does not affect the level of cVA, which is synthesized in the ejaculatory bulb (33). A previous study reported that WT males exhibit decreased copulation latency toward oe^{-} females compared with WT females (32). We investigated whether this decreased copulation latency requires Or47b. For this purpose, we paired either WT or *Or47b* mutant males with oe^{-} females in single pair assays and observed copulation latency and copulation success. Compared with WT males, *Or47b* mutant males exhibited increased copulation latency (Fig. 3*H*) and reduced copulation success when courting oe^{-} females (Fig. 3*I*). This result, together with the previously reported idea that mutation in *Or47b* suppresses increased levels of courtship toward oe^{-} males (22), strongly suggest that oe^{-} flies still synthesize the ligand for Or47b. We investigated this hypothesis by analyzing

CHC profiles of oe$^-$ flies. Indeed, we found no significant difference in the average quantity of ML found on oe$^-$ and WT flies, even though all other known nonvolatile pheromones except cVA were completely eliminated from oe$^-$ flies (Fig. 3*J*). These findings provide further support that Or47b and its ligand ML mediate the vigorous courtship observed toward oe$^-$ flies, and that ML is the key stimulatory pheromone necessary for optimal male copulation behavior in *D. melanogaster*.

ML, MM, and MP Elicit Attraction in Males and Females. Aggregation can facilitate mate finding. Drosophilid flies use aggregation pheromones to assemble on breeding substrates, where they feed, mate, and oviposit communally (34, 35). The well-known aggregation pheromone in *D. melanogaster* is cVA, which in addition to its role in social and sexual behaviors elicits aggregation in both males and females (36). Flies anosmic to cVA display residual attraction to live male flies, indicating that other attractive cues are produced by flies that are independent of cVA and its neural circuit (7). Therefore, we investigated whether the three so far unidentified fly odorants mediate a behavior similar to the aggregation function of cVA. None of these three fly odorants elicited any significant upwind long-range flight attraction in wind tunnel assays; however, in the trap assay (37), the three fly odorants elicited short-range attraction in both males and females (Fig. 4*A*). Furthermore, pulses of ML presented in the FlyWalk assay (38, 39) were attractive to both males and females (Fig. 4*B*). Thus, we conclude that ML, MM, and MP mediate short-range attraction in both males and females.

Or88a- and Or88a-Expressing OSNs Are Required for the Attraction Behavior Toward ML, MM, and MP. We next asked whether both receptors, Or47b and Or88a, are necessary for the observed attraction behavior. Although *Or88a* mutant flies were not attracted to any of the three fly odorants in the trap assay, *Or47b* mutant flies were still attracted to all three (Fig. 4*A*). Correspondingly, the ML attraction in the FlyWalk assay disappeared in *Or88a* mutant flies, but not in *Or47b* mutant flies (Fig. 5*B* and Fig. S5). In addition, we verified that the observed phenotype of *Or88a* mutant flies does not reflect a general deficit in attraction behavior by exposing *Or88a* mutant flies in the FlyWalk assay to pulses of ethyl acetate (EtA), a well-known attractant to flies. Indeed, both *Or88a* mutant males and females were attracted to EtA, similar to WT flies (Fig. S5). Consequently, we conclude that ML, MM, and MP activate Or88a to mediate short-range attraction in both sexes.

We further investigated whether Or88a-expressing OSNs are required for the observed attraction behavior. We generated flies expressing *UAS-head involution defective* (*UAS-hid*) and *UAS-Stinger II* in *Or88a* neurons to ablate *Or88a* neurons. Attraction toward ML was abolished in flies lacking *Or88a* neurons, but not in the corresponding parental lines (Fig. 4*C*). These experiments suggest that Or88a neurons are necessary for fly attraction behavior induced by Or88a ligands.

We next determined the sufficiency of Or88a OSN activity to induce attraction behavior. For this purpose, we drove the expression of dTrpA1 in Or88a neurons, to conditionally activate this specific OSN population at 30 °C. Consistent with the attraction behavior induced by Or88a ligands, flies carrying *Gal4-Or88a* and *UAS-dTrpA1*, but not the corresponding parental lines, preferred traps heated to 30 °C over traps held at 20 °C (Fig. 4*D*). In short, we conclude that Or88a neurons are necessary and sufficient for the observed attraction toward ML, MM, and MP.

Both Or47b- and Or88a-Based Systems and Their Ligands Are Remarkably Conserved over a Number of Drosophilid Species. In addition to ML, we also found MM and MP present in oe$^-$ flies (Fig. 3*J*). Interestingly, oe$^-$ females are courted by males of four *D. melanogaster* sibling species (4, 32). Based on these results, we hypothesized

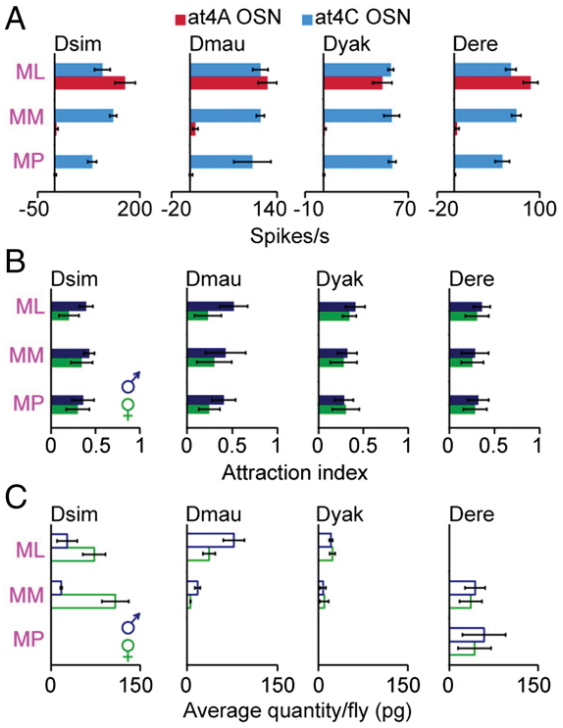

Fig. 5. Both Or47b- and Or88a-based systems and their ligands are remarkably conserved over a number of drosophilid species. (*A*) Average SSR responses of ML, MM, and MP from at4A and at4C OSNs of *D. simulans* (Dsim), *D. mauritiana* (Dmau), *D. yakuba* (Dyak), and *D. erecta* (Dere) (10^{-1} dilution) ($n = 5$). (*B*) Attraction indices from a binary choice assay between ML, MM, or MP and solvent control. Error bars represent SD. Deviation of the response indices against zero was tested with the Student *t* test, and all were found to be significant ($P < 0.05$) ($n = 10$). (*C*) Average quantity of ML, MM, and MP ($n = 3$).

that male copulation and aggregation behaviors are driven by the novel pheromones also in these other species. Notably, we found that the other four species detect all three compounds with the same set of OSNs (Fig. 5*A*) and also show attraction toward these fly odors in trap assays (Fig. 5*B*). Finally, we found ML and MM (but not MP, which seems to be *D. melanogaster*-specific) in the CHC profiles of all four sibling species (Fig. 5*C*). These data suggest that closely related drosophilid species rely on these pheromones to promote male copulation and aggregation behaviors, although the last common ancestor with *D. melanogaster* lies 2–10 million years back through evolutionary time (40).

Conclusions

The mating of *D. melanogaster* is clearly governed by a number of sensory cues targeting different detector systems. Already the complexity of the olfactory signals involved in the interplay between positive and negative cues determining the ultimate outcome of an encounter between the sexes is quite astounding. One factor lacking among the so-far unidentified chemical signals has been a truly positive fly-derived olfactory signal mediating mating. We have demonstrated the presence of such a signal (ML) and dissected the neural mechanism (Or47b) underlying its detection. With our findings, the understanding of male olfactory-based sexual arousal is becoming more complete, with all *fru*-positive OSNs now having known ligands. We also demonstrate the presence of the first fly odorants, MM and MP, which, together with ML, exclusively mediate attraction in both sexes via a pathway (Or88a) separated from that involved in sexual behavior. Interestingly, the compounds identified are attractive

to several closely related species. We conclude that in several *Drosophila* species, the mating enhancing signal is conserved, whereas reproductive isolation barriers between species are created mainly by species-specific signals.

Materials and Methods

TD-GC-MS. Individual flies were placed in standard microvials in thermal desorption tubes and transferred using a GERSTEL MPS 2 XL multipurpose sampler into a GERSTEL thermal desorption unit (www.gerstel.de). After desorption at 200 °C for 5 min with solvent venting, the analytes were trapped in the liner of a GERSTEL CIS 4 Cooled Injection System at −50 °C, using liquid nitrogen for cooling. The components were transferred to the GC column by heating the programmable temperature vaporizer injector at 12 °C/s up to 210 °C and then held for 5 min. The GC-MS device (Agilent GC 7890A fitted with an MS 5975C inert XL MSD unit; www.agilent.com) was equipped with an HP5-MS UI column (19091S-433UI; Agilent Technologies) and operated as follows. The temperature of the gas chromatograph oven was held at 40 °C for 3 min and then increased by 5 grd/min to 200 °C and then by 20 grd/min to 260 °C, with the final temperature held for 15 min. For MS, the transfer line was held at 260 °C, the source was held at 230 °C, and the quad was held at 150 °C. Mass spectra were taken in EI mode (at 70 eV) in the range from 33 *m/z* to 500 *m/z*. The structures of most of the cuticular compounds were confirmed by comparison with reference compounds measured at the same conditions.

Details on *Drosophila* stocks, compound quantification, genetic elimination of female CHCs, perfuming of female flies with cuticular compounds, single sensillum recordings, imaging, and the different behavioral assays are provided in *SI Materials and Methods*.

Or88a Mutant Generation and Genotyping Information. The *Or88a* mutant was generated by Leslie Vosshall in collaboration with Tim Tully in 2001–2003 by imprecise excision of a P-element from the E4365 strain. This line was generated at Cold Spring Harbor Laboratory as part of a large-scale learning and memory mutant screen in the Tully Lab, supported by the John A. Hartford Foundation. The original strain contains a P-element with the *white* eye color marker inserted 728 bp upstream of the *Or88a* ATG translation initiation codon. The P-element insertion site E4365 is indicated by <**X**> in the following sequence:

TAAGTGTTTGCGTAAACTTACCCCCGTTTTGAGCAGTGCACGCCTCGGAC<**X**>-ATATTACGAAATGCACGAGGGGCATCCACTACGCACAAATAATAGCTCAA-TTTCAT

Standard P-element mobilization was carried out, and *white*$^{-/-}$ strains were isolated and genotyped by PCR to detect deletions 3′ of the P-element insertion site. A single imprecise excision line, E4365#181, was isolated and contains a 1,229-bp deletion that stretches from the P-element insertion site downstream to the middle of the first protein-coding exon. In addition to this deletion, there is a 25-bp insertion in the breakpoint region. The breakpoint of the E4365-181 deletion is indicated by <Δ>, and the 25-bp insertion is indicated in lowercase bold type below:

TAAGTGTTTGCGTAAACTTACCCCCGTTTTGAGCAGTGCACGCCTCGGAC<Δ>-**catgatgaaataacaataatagata**<Δ>ATACTCCTGTTGCCCAGCACGAGCAGCTC-CTTGGAGGATGGCTGCCATGCGGTG

This deletion removes the first 168 amino acids of Or88a and is predicted to be a null mutation. The strain is homozygous viable, and the deletion does not affect any other known protein-coding genes in this part of the genome. However, in the time since the mutant was generated and characterized, the *Drosophila* genome consortium has annotated a noncoding RNA (*CR44237*) located on the other strand and contiguous with the *Or88a* gene. This theoretical gene has no known function and has not been characterized.

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Supporting Information

Dweck et al. 10.1073/pnas.1504527112

SI Materials and Methods

***Drosophila* Stocks.** All experiments with WT *D. melanogaster* were carried out with the Canton-S strain. Species other than *D. melanogaster* were obtained from the *Drosophila* Species Stock Center (https://stockcenter.ucsd.edu/info/welcome.php). Transgenic lines were obtained from the Bloomington *Drosophila* Stock Center (flystocks.bio.indiana.edu/), except for the w^{118}*;Δhalo/cyo;UAS-Or47b*, w^{118}*;Δhalo/cyo;UAS-Or65a/TM3*, and w^{118}*;Δhalo/cyo;UAS-Or88a/TM3*, which were a kind gift from J. R. Carlson (Yale University); *+;PromE(800)-Gal4, tubP-Gal80^{ts}*;+, +;*UAS-StingerII*;+, and +;*UAS-StingerII,UAS-hid/CyO*;+, which were a kind gift from J.-C. Billeter, University of Groningen, Groningen, The Netherlands; *Or88a* mutant flies, which were a kind gift from L. B. Vosshall, Rockefeller University, New York; and *pJFRC124-20XUAS-IVS-dTrpA1 (attP18)*, which was a kind gift from G. M. Rubin, Janelia Farm Research Campus, Ashburn, VA.

Compound Quantification. Quantification of the average amounts of ML, MM, and MP from individual flies was done by a standard addition method, comparing total ion current values with 0.58 ng/fly of deuterated methyl laurate, which served as the internal standard.

Genetic Elimination of Female CHCs. Ablation of oenocytes was achieved as described previously (1). Male *+;PromE(800)-GAL4, tub-GAL80^{TS};+* flies crossed to female *+;UAS-StingerII, UAS-hid/CyO;+* at 18 °C. Female pupae were collected at room temperature and kept at 18 °C until emergence. Newly emerged females were kept at 25 °C for 24 h. Subsequently, oenocyte-eliminated females were maintained at 30 °C during the nighttime and at 25 °C during the daytime for another 3 d. On day 5, females were checked for GFP fluorescence and left to recover for at least 24 h before use in experiments.

Perfuming of Female Flies with Cuticular Compounds. The procedure for perfuming female flies was adapted from previous work (1). In brief, for each compound of interest, 10 μL of a 1 ng/μL stock solution was pipetted into a 1.5-mL glass vial. After the solvent had evaporated under a nitrogen gas flow, one female fly was transferred to the vial and subjected to three medium vortex pulses lasting for 30 s, with a 30-s pause between each pulse. The treated female fly was then transferred to a fresh food vial for 1 h to recover. The recovered fly was then used in either a courtship assay or GC to confirm that equivalent amount of WT female cuticular compound (~100 pg) was transferred to individual females.

Chemicals. All chemicals were purchased in high purity from Sigma-Aldrich and Cayman Chemical except for 2-methyl docosane, 2-methyl tetracosane, 2-methyl hexacosane, 2-methyl octacosane, 5-methyl tricosane, and 7-methyl tricosane, which were kind gifts from Jocelyn G. Millar, University of California, Riverside, CA and J. Weißflog, Max Planck Institute for Chemical Ecology, Jena, Germany. The fly body wash extracts were obtained by washing 500 flies in 1 mL of methanol for 24 h. For GC stimulation, 2 μL of the odor sample was injected onto a DB5 column (Agilent Technologies; www.agilent.com), fitted in an Agilent 6890 gas chromatograph equipped with a four-arm effluent splitter (Gerstel; www.gerstel.com), and operated as described previously (2) except for the temperature increase, which was set at 15 °C min^{-1}, and the split mode, which was 1:30 to ensure that a small aliquot of the injected sample went to the FID of the gas chromatograph, with the remainder going to the antennal preparation. GC-separated components were introduced into a humidified airstream (200 mL min^{-1}) directed toward the antennae of a mounted fly. Signals from OSNs and FID were recorded simultaneously.

SSR. The SSR procedure was performed as described previously (3). Adult flies were immobilized in pipette tips, and the third antennal segment or the palps were placed in a stable position onto a glass coverslip. Sensilla were localized under a binocular at 1,000× magnification, and the extracellular signals originating from the OSNs were measured by inserting a tungsten wire electrode into the base of a sensillum. The reference electrode was inserted into the eye. Signals were amplified (10×; Syntech Universal AC/DC Probe; www.syntech.nl), sampled (10,667 samples/s), and filtered (100–3,000 Hz with 50/60-Hz suppression) via a USB-IDAC connection to a computer (Syntech). Action potentials were extracted using Syntech Auto Spike 32 software. Neuron activities were recorded for 10 s, starting 2 s before a stimulation period of 0.5 s. Responses from individual neurons were calculated as the increase (or decrease) in the action potential frequency (spikes/s) relative to the prestimulus frequency.

Optical Imaging. Flies were prepared for optical imaging as described previously (4). Imaging of the two specific Or lines was performed with a Till Photonics imaging system with an upright Olympus microscope (BX51WI) and a 20× Olympus objective (XLUM Plan FL 20×/0.95 W). A Polychrome V provided light excitation (475 nm), which was then filtered (excitation: SP500; dicroic: DCLP490; emission: LP515). The emitted light was captured by a CCD camera (Sensicam QE; PCO) with a symmetrical binning of 2 (0.625 × 0.625 μm/pixel). For each measurement, a series of 40 frames was obtained (1 Hz) with a frequency of 4 Hz. Odors were applied during frames 8–15. Pure compounds were diluted (10^{-1}) in mineral oil (Carl Roth); 6 μL of the diluted odors was pipetted onto a small piece of filter paper (~1 cm^2; Whatman), placed inside a glass Pasteur pipette. Filter papers formulated with solvent alone were used as blanks. Filter papers were prepared ~30 min before each experimental session. For odor application, a stimulus controller (Stimulus Controller CS-55, Syntech) was used, which produced a continuous airstream with a flow of 1 L min^{-1}, monitored by a flowmeter (0.4–5 LPM Air; Cole-Parmer). An acrylic glass tube guided the airflow to the fly's antennae. Within the constant air stream, the applied odor stimuli were also diluted ~1:10. Data were analyzed with custom-written IDL software (ITT Visual Information Solutions). All recordings were manually corrected for movement. To achieve a comparable standard for the calculation of the relative fluorescence changes (ΔF/F), the fluorescence background was subtracted from the averaged values of frames 0–7 in each measurement, so that basal fluorescence was normalized to zero. The false color-coded fluorescent changes in the raw-data images were calculated by subtracting frame 6 from frame 12. A 3D map of the fly AL (5) served to link the active area to individual glomeruli.

All experimental flies contained the calcium-dependent fluorescent sensor *G-CaMP3.0* (6) together with a promoter *Gal4* insertion to direct expression of the calcium sensor to specific neuron populations. Stimulus-evoked fluorescence in these flies arises from the population of labeled neurons that are sensitive to the specific odor. For specific OSNs, two transgenic lines expressing *G-CaMP3* in ORs, *Or47b-GAL4* and *Or88a-GAL4*, were used.

Two-Photon Imaging. Flies were prepared for optical imaging as described previously (4). Imaging was performed with a two-photon

laser scanning microscope (2PCLSM, Zeiss LSM 710 meta NLO) equipped with an infrared Chameleon UltraTM diode-pumped laser (Coherent). Both the 2PCLSM and Chameleon laser were placed on a smart table (UT2; New Corporation). The excitation wavelength for imaging was 925 nm (BP500-550) using a 40× lens (W Plan-Apochromat 40×/1.0 DIC M27). For each measurement, a series of 40 frames was taken with a frequency of 4 Hz. Odors were applied during frames 8–15 (i.e., after 2 s for 2 s). Pure compounds were diluted (10^{-1}) in mineral oil (Carl Roth); 2 mL of the diluted odors was added to glass bottle (50 mL, Duran Group), with two sealed openings for the air inflow and outflow. Odor was applied using a stimulus controller (CS-55; Syntech) with a continuous airstream with a flow of 1.5 L min^{-1}, monitored by a flowmeter (Cole-Parmer). A peek tube guided the airflow to the fly's antennae. At odor onset, the headspace of the odor (0.5 L min^{-1}) was guided to the fly's antennae. Data were analyzed with custom-written IDL software (ITT Visual Information Solutions). All recordings were manually corrected for movement. To achieve a comparable standard for the calculation of relative fluorescence changes (ΔF/F), the fluorescence background was subtracted from the averaged values of frames 0–7 in each measurement, so that basal fluorescence was normalized to zero. A 3D atlas of the fly AL (5) served to link the active area to individual glomeruli.

All experimental flies expressed the calcium-sensitive fluorescent sensor *G-CaMP6.0s* (7) under control of the *GH146-GAL4* (8) driver line to direct expression of the calcium sensor to the majority of PNs.

Single Pair Courtship and Comparative Mating Assays. Male and female pupae were collected individually and in groups, respectively, and then kept for 6–9 d before use in experiments. Courtship assays were performed in the lid of an Eppendorf (1 cm diameter × 0.5 cm depth) covered with a plastic slide. Courtship behaviors were recorded for 30 min and analyzed by a blinded observer. All courtship experiments were performed with 7- to 9-d-old flies under red light (660-nm wavelength) at 25 °C (unless stated otherwise) and 70% humidity, and without food to avoid activating the *fru*-positive IR84a-expressing OSNs with food-derived odors (9).

Courtship latency was defined as the time that the male takes until performing any sequence of the courtship ritual. The courtship index was calculated as the percentage of time that the male spends courting the female during the first 10 min. Wing extension was measured as the duration of unilateral wing vibration of the male in the first 10 min. Copulation latency was measured as the time that the male takes until copulation. A copulation attempt was counted whenever the male bended his abdomen forward to start copulation. Copulation success was calculated as the percentage of males that copulated.

Trap Assays. Trap assay experiments were performed as described previously (10). A treatment and a control traps made from 30-mL transparent plastic vials were placed into 500-mL cups with ventilation holes in the lids. The treatment and control traps contained 10 μL of the test odorant and solvent, respectively. Thirty 4- to 5-d-old starved flies were placed in each test box. Experiments were carried out in a climate chamber (25 °C, 70% humidity, 12:12 light:dark cycle). The number of flies inside and outside the traps was counted after 24 h. Attraction index (RI) was calculated as $(O - C)/T$, where O is the number of flies in the odorant trap, C is the number of flies in the control trap, and T is the total number of tested flies.

Wind Tunnel. The wind tunnel was built as described previously (11), with the airstream in the tunnel (0.3 m/s) produced by a fan and filtered through activated charcoal. The wind tunnel was maintained within a climate chamber set to 25 °C and 70% humidity, with bright white overhead light. Flies aged 2–7 d were released in groups of 10. No differences between the sexes were noted, and thus the data were pooled. A dilution of the odor in solvent was delivered onto a dental cotton wick contained within a plastic container that was suspended within the airstream opposite the point at which the flies were released. Experimental observations lasted 10 min for each group of flies, with data tabulated for each fly that contacted or entered the source of the odor.

FlyWalk Assay. Apart from few technical modifications on the behavioral setup (see below), the FlyWalk experiments were performed and analyzed as described previously (12) with 4- to 6-d-old virgin male and female flies starved for 24 h before the start of the experiments. In short, 15 individual flies were placed in glass tubes (0.8 cm i.d.). The glass tubes were aligned in parallel, and flies were monitored continuously by an overhead camera (HD Pro Webcam C920; Logitech). XY positions were recorded automatically at 20 fps using Flywalk Reloaded v1.0 software (Electricidade Em Pó; flywalk.eempo.net). Experiments were performed under red LED light (peak intensity at λ, 630 nm).

During the experiments, flies were continuously exposed to a humidified airflow of 20 cm/s (70% relative humidity, 20 °C). Flies were repeatedly presented with pulses of various olfactory stimuli at interstimulus intervals of 90 s. Stimuli were added to the continuous airstream and thus travelled through the glass tubes at a constant speed.

In brief, 100 μL of odor dilution was prepared in 200-μL PCR tubes, which were placed into odor vials made of polyetheretherketone. The odor vials were tightly sealed and connected to the stimulus device via ball-stop check valves that allowed only unidirectional airflow through the odor-saturated headspace. Odor stimulation was achieved by switching an airflow otherwise passing through an empty vial (compensatory airflow) to the odor-containing vial. Odor pulses were 500 ms in duration, with an interstimulus interval of 90 s. Tracking data were analyzed using custom-written routines programmed in R (www.r-project.org).

Flies were assigned to individual glass tubes using the Y coordinates and thus could be unambiguously identified throughout the whole experiment. As flies are allowed to distribute freely within their glass tubes, they may encounter the odor pulse at different times. This is compensated for by calculating the time of odor encounter for each individual tracking event based on the X position of the fly, system intrinsic delay, and airspeed. The time of encounter was set to 0, and the speed of movement was interpolated in the interval between 10 s before and 10 s after an encounter at 10 Hz. Because the tracking system does not capture the entire length of the glass tubes, not every fly was tracked for every stimulation cycle, and some entered or left the region of interest during the tracking event; thus, we decided to consider only complete trajectories in the interval between 1 s before and 7 s after odor encounter for further analysis.

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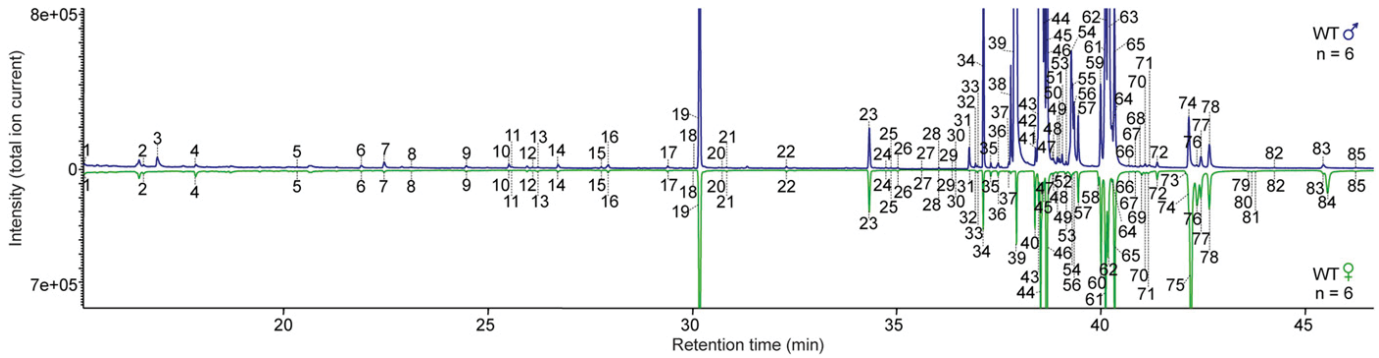

Fig. S1. Representative GC-MS traces of single virgin 4-d-old WT male and female. Peak numbers refer to the compounds listed in Table S1.

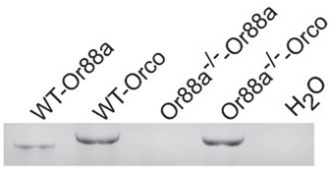

Fig. S2. PCR validation of the Or88a$^{-/-}$ mutant allele.

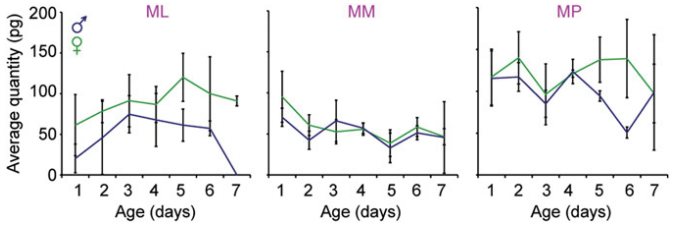

Fig. S3. Age-related variation in the average quantity of ML, MM, and MP (n = 3).

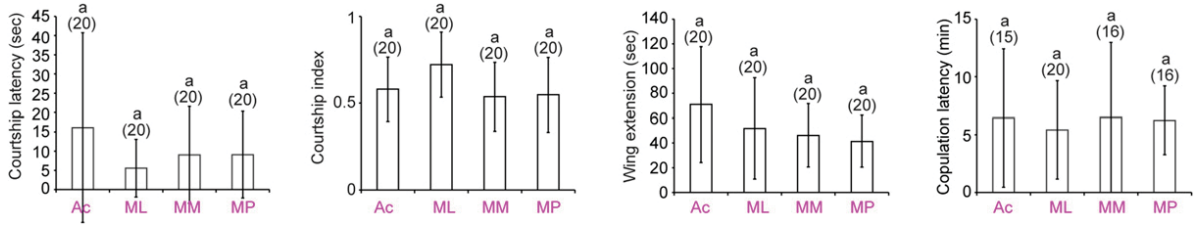

Fig. S4. Courtship parameters of WT males with WT females painted with acetone, ML, MM, or MP. Error bars represent SD. Significant differences are denoted by letters ($P < 0.05$, ANOVA followed by Tukey's test).

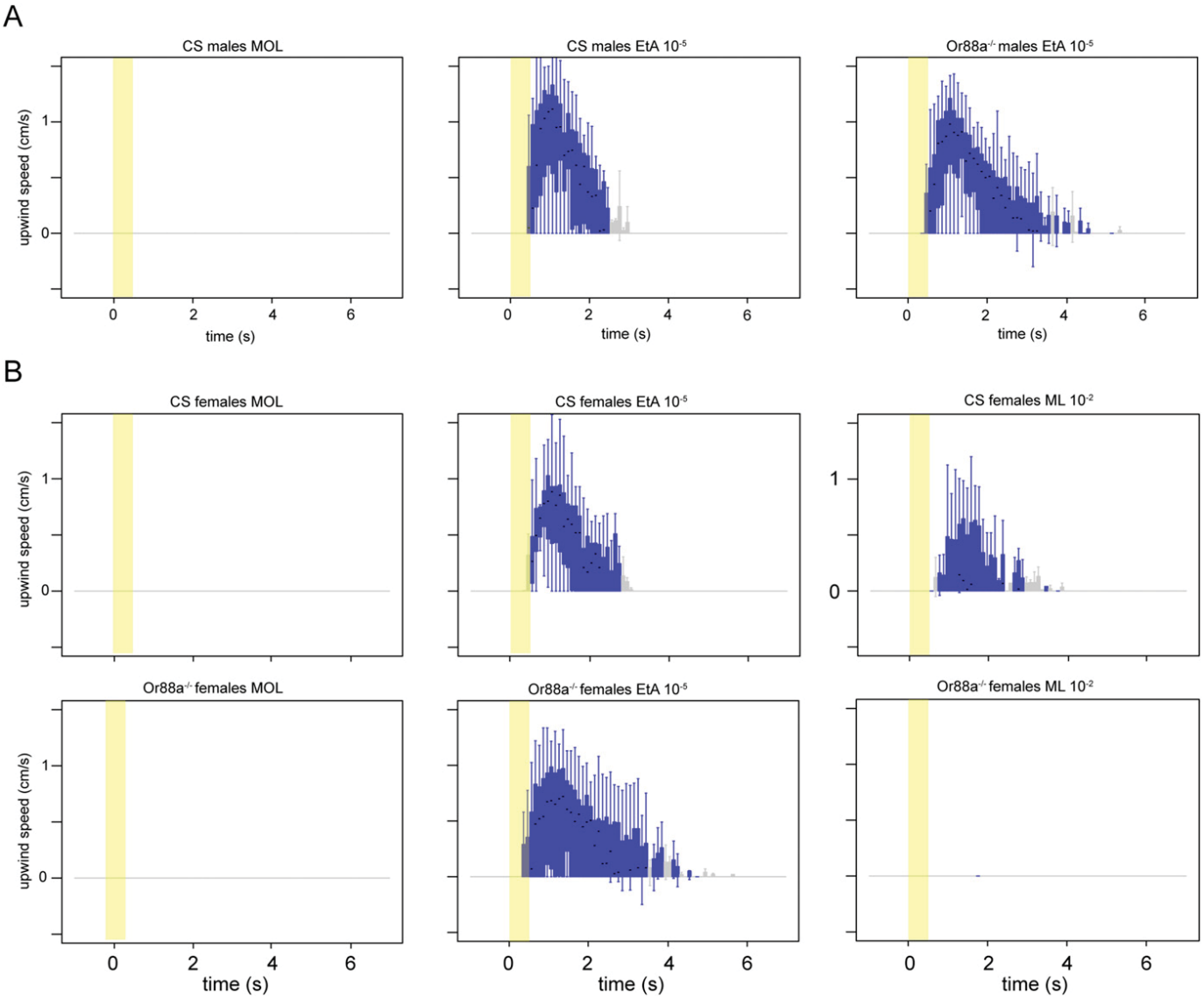

Fig. S5. Quantified behavior from individual flies stimulated with mineral oil (Mol), EtA, and ML in the FlyWalk assay. (*A* and *B*) Boxplot representation of odor-induced changes in upwind speed; black line, median upwind speed; box, interquartile range; whiskers, 90th and 10th percentiles. The blue boxplots depict significantly increased upwind speed compared with the upwind speed during the solvent control situation within the corresponding 100-ms time frame; gray boxplots depict no significant difference in upwind speed.

Table S1. Cuticular compounds on virgin 4-d-old WT flies

Peak no.	Kovats index	Compound name	Male	Female
1	1105	Nonanal*	+	+
2	1163	Unknown	+	+
3	1175	Dehydromevalonic lactone*	+	—
4	1206	Decanal*	+	+
5	1300	Tridecane*	+	+
6	1354	Butyrate ester*	+	+
7	1375	Butyrate ester*	+	+
8	1400	Tetradecane*	+	+
9	1455	Geranyl acetone*	+	+
10	1497	2-Tridecanone*	+	+
11	1500	Pentadecane*	+	+
12	1521	Unknown	+	+
13	1527	Methyl laurate*	+	+
14	1548	Unknown	+	+
15	1593	Hexadecene*	+	+
16	1600	Hexadecane*	+	+
17	1665	Octylether*	+	+
18	1693	Monoene*	+	+
19	1700	Heptadecane*	+	+
20	1726	Methyl myristate*	+	+
21	1731	Ethanolamide ester*	+	+
22	1800	Octadecane*	+	+
23	1900	Nonadecane*	+	+
24	1924	Terpene*	+	+
25	1931	Methyl palmitate*	+	+
26	1941	Ethanolamide ester*	+	+
27	1975	Unknown	+	+
28	2000	Eicosane	+	+
29	2029	Unknown	+	+
30	2037	Heneicosene	+	+
31	2067	2-Methyleicosane	+	+
32	2082	(*Z*)-7-Heneicosene	+	+
33	2086	(*Z*)-5-Heneicosene	+	+
34	2100	Heneicosane	+	+
35	2123	Unknown	+	+
36	2145	Unknown	+	+
37	2182	7-Docosene	+	+
38	2194	(*Z*)-11-Vaccenyl acetate	+	—
39	2200	Docosane	+	+
40	2262	Tricosadiene	—	+
41	2263	2-Methyldocosane	+	—
42	2269	Tricosadiene (conjugated)*	+	—
43	2274	(*Z*)-9-Tricosene	+	+
44	2281	(*Z*)-7-Tricosene	+	+
45	2291	(*Z*)-5-Tricosene	+	+
46	2300	Tricosane	+	+
47	2317	Unknown	+	+
48	2322	Unknown	+	+
49	2335	Unknown	+	+
50	2341	7-Methyltricosane	+	—
51	2350	5-Methyltricosane	+	—
52	2358	Tetracosadiene	—	+
53	2362	2-Methyltricosane	+	+
54	2378	(*Z*)-7-Tetracosene	+	+
55	2382	Tetracosene	+	—
56	2387	(*Z*)-5-Tetracosene	+	+
57	2400	Tetracosane	+	+
58	2456	(*Z*,*Z*)-9,13-Pentacosadiene	—	+
59	2462	2-Methyltetracosane	+	—
60	2463	(*Z*,*Z*)-7,11-Pentacosadiene	—	+
61	2475	Unknown	+	+
62	2482	(*Z*)-7-Pentacosene	+	+
63	2484	Unknown	+	—

Table S1. Cont.

Peak no.	Kovats index	Compound name	Male	Female
64	2493	4-Pentacosene	+	+
65	2500	Pentacosane	+	+
66	2533	Unknown	+	+
67	2548	5-Methylpentacosane	+	+
68	2561	2-Methylpentacosane	+	—
69	2564	(*Z*,*Z*)-7,11-Hexacosadiene	—	+
70	2571	Hexacosene	+	+
71	2579	Hexacosene	+	+
72	2600	Hexacosane	+	+
73	2655	(*Z*,*Z*)-9,13-Heptacosadiene	—	+
74	2661	2-Methylhexacosane	+	+
75	2665	(*Z*,*Z*)-7,11-heptacosadiene	—	+
76	2677	(*Z*)-9-Heptacosene	+	+
77	2684	(*Z*)-7-Heptacosene	+	+
78	2700	Heptacosane	+	+
79	2760	Octacosene	—	+
80	2765	(*Z*,*Z*)-7,11-Octacosadiene	—	+
81	2772	Octacosene	—	+
82	2800	Octacosane	+	+
83	2861	2-Methyloctacosane	+	+
84	2866	(*Z*,*Z*)-7,11-Nonacosadiene	—	+
85	2900	Nonacosane	+	+

—, Compound absent; +, compound present.
*Compounds newly identified by TD-GC-MS.

MANUSCRIPT V

Olfactory Channels Associated with the Drosophila Maxillary Palp Mediate Short- and Long-range attraction

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Olfactory Channels Associated with the *Drosophila* Maxillary Palp Mediate Short- and Long-range Attraction

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Abstract: The vinegar fly *Drosophila melanogaster* is equipped with two peripheral olfactory organs, antenna and maxillary palp. The antenna is involved in finding food, oviposition sites and mates. However, the functional significance of the maxillary palp remained unknown. Here, we screened the olfactory sensory neurons of the maxillary palp (MP-OSNs) using a large number of natural odor extracts to identify novel ligands for each MP-OSN type. We found that each type is the sole or the primary detector for a specific compound, and detects these compounds with high sensitivity. We next dissected the contribution of MP-OSNs to behaviors evoked by their key ligands and found that MP-OSNs mediate short- and long-range attraction. Furthermore, the organization, detection and olfactory receptor (Or) genes of MP-OSNs are conserved in the agricultural pest *D. suzukii*. The novel short and long-range attractants could potentially be used in integrated pest management (IPM) programs of this pest species.

Introduction

Like all insects, the vinegar fly, *D. melanogaster*, is equipped with two peripheral olfactory organs, the antenna and maxillary palp. The antenna, the main olfactory organ, is covered with four types of sensilla: basiconic, trichoid, intermediate and coeloconic. These four sensillum types house olfactory sensory neuron (OSN) types responding to different kinds of chemical stimuli and thus serve distinct chemosensory functions. In contrast, the palp has only three different subtypes of basiconic sensilla, each housing two MP-OSNs. Because of the overlapping response spectra between MP- and antennal OSNs (Ant-OSNs) (de Bruyne et al., 1999; de Bruyne et al., 2001) as well as the location of the maxillary palp in close vicinity to the labellum, the main taste organ in flies, a function connected to taste enhancement has been

suggested for the MP-OSNs (Shiraiwa et al., 2008). However, taste enhancement would be a very general function for six types of MP-OSNs expressing seven different odorant receptors (Ors). In our previous work we presented data on the importance of Or71a, which is expressed in the maxillary palp sensillum pb1B, in proxy detection of dietary antioxidants (Dweck et al., 2015). It is not yet known whether the other MP-OSNs are also dedicated to detect specific ecologically relevant chemical compounds, and if so, what the ecological importance of these compounds is.

In several other insects, MP-OSNs are involved in detection of specific chemical compounds that are not covered within the receptive range of Ant-OSNs. For example, in both the hawk moth *Manduca sexta* and the African malaria mosquito *Anopheles gambiae*, CO_2 detection is primarily mediated via maxillary and/or labial palp OSNs (Thom et al., 2004; Lu et al., 2007). Mammals are also known to possess several peripheral olfactory organs. In mouse, e.g., the main olfactory epithelium is complemented with the vomeronasal organ, the septal organ and the Grueneberg ganglion, each having distinct functions (reviewed in Knaden and Hansson, 2014). The presence of specific functions in different olfactory organs in other insects and in mammals suggests that the maxillary palp may also be involved in the detection of specific chemicals in *Drosophila*.

In the present study, we present a systematic electrophysiological examination of MP-OSNs of *D. melanogaster* using 52 different complex odor sources containing more than 11300 chemical compounds. We find that each MP-OSN is either the sole or the primary detector of a specific chemical compound and that the maxillary palp contains independent and important olfactory channels that mediate both short- and long-range attraction. Finally, we find that the organization, detection and Or genes of MP-OSNs are conserved in the agricultural pest *D. suzukii*, and identify novel short and long-range attractants that could potentially be used in IPM programs of this pest species.

Results and Discussion

Screen for novel natural ligands for MP-OSNs

Although extensive work has been done on the olfactory sense of the vinegar fly, *D. melanogaster*, none (e.g. for pb3A-OR59c, Pb2A-Or33c) or very few ligands have been identified for the different MP-OSNs (deBruyne et al., 1999, Goldman et al., 2005; Marshall et al., 2010). In addition, the previously identified ligands activate Ant-OSNs as well as MP-OSNs and have been shown to be much better ligands for Ant-OSNs (i.e. require high concentrations to activate MP-OSNs) (deBruyne et al., 1999; Hallem and Carlson, 2006). This suggests that the best ligands of the different MP-OSNs have not yet been identified. Towards this end, we screened each of the six MP-OSNs with headspace collections from 52 different complex, ecologically relevant odor sources using GC-SSR (**Figure 1A,B** and **Figure 1B-figure supplement 1, Figure 1B-figure supplement 2, Figure 1B-figure supplement 3, Figure 1B-figure supplement 4, Figure 1B-figure supplement 5, and Figure 1B-figure supplement 6**). These odor sources included 34 fruits, seven microbes, and eleven types of mammal feces. Our GC-SSR measurements revealed that each of the tested headspace collections triggered a response from at least one palp OSN type. Fecal, fruit and microbial volatiles elicited responses from six, five and two palp OSN types, respectively. The pb2B MP-OSN was activated exclusively by fecal volatiles, whereas the other five types were activated by fruit, microbial, and fecal volatiles.

These large-scale GC-SSR experiments allowed us to test whether the 52 headspace collections of fruits, microbes and mammal feces are separated in the neural space of the maxillary palp. We performed a nonmetric multidimensional scaling (NMDS) based on a presence/absence matrix for the SSR active peaks across the tested samples using Bray-Courtis dissimilarity. This analysis indicated that the 52 headspace collections were separated

into three distinct groups; one group for fruit samples, another group for microbial samples and the last group for fecal samples (**Figure 1C, Figure 1-source data 1**). The significance of the differences between these three groups was assessed by the analysis of similarity (ANOSIM) score ($R = 0.61$, $P < 0.0001$). These results suggest that the information provided by the different MP-OSNs is sufficient to categorize fruits, microbes, and feces.

The physiologically active peaks from each extract were then identified via GC-mass spectroscopy (GC-MS) and co-injection with synthetic standards, which were purchased except for 5-hexen-3-one and buty-3-hydroxy butyrate, which were synthesized in house (see Materials and methods). The total number of distinguishable flame ionization detection (FID) peaks in the samples was 11326, of which only 328 FID peaks elicited responses (**Figure 1B, Figure 1B-figure supplement 7, and Figure 1B-figure supplement 8**). 225 of these peaks corresponded to 20 different compounds (table 1). The remaining peaks corresponded to eight different compounds, which remain unidentified because their mass spectra did not match that of any reference compound. The identified compounds belonged to four different chemical classes: alcohols, esters, phenols and ketones. Six of the physiologically active compounds occurred in most extracts, whereas the other 22 compounds were extract specific. Phenol and 4-methylphenol occurred exclusively in fecal extracts (**Figure 1B, Figure 1B-figure supplement 4**).

Figure 1 (new)

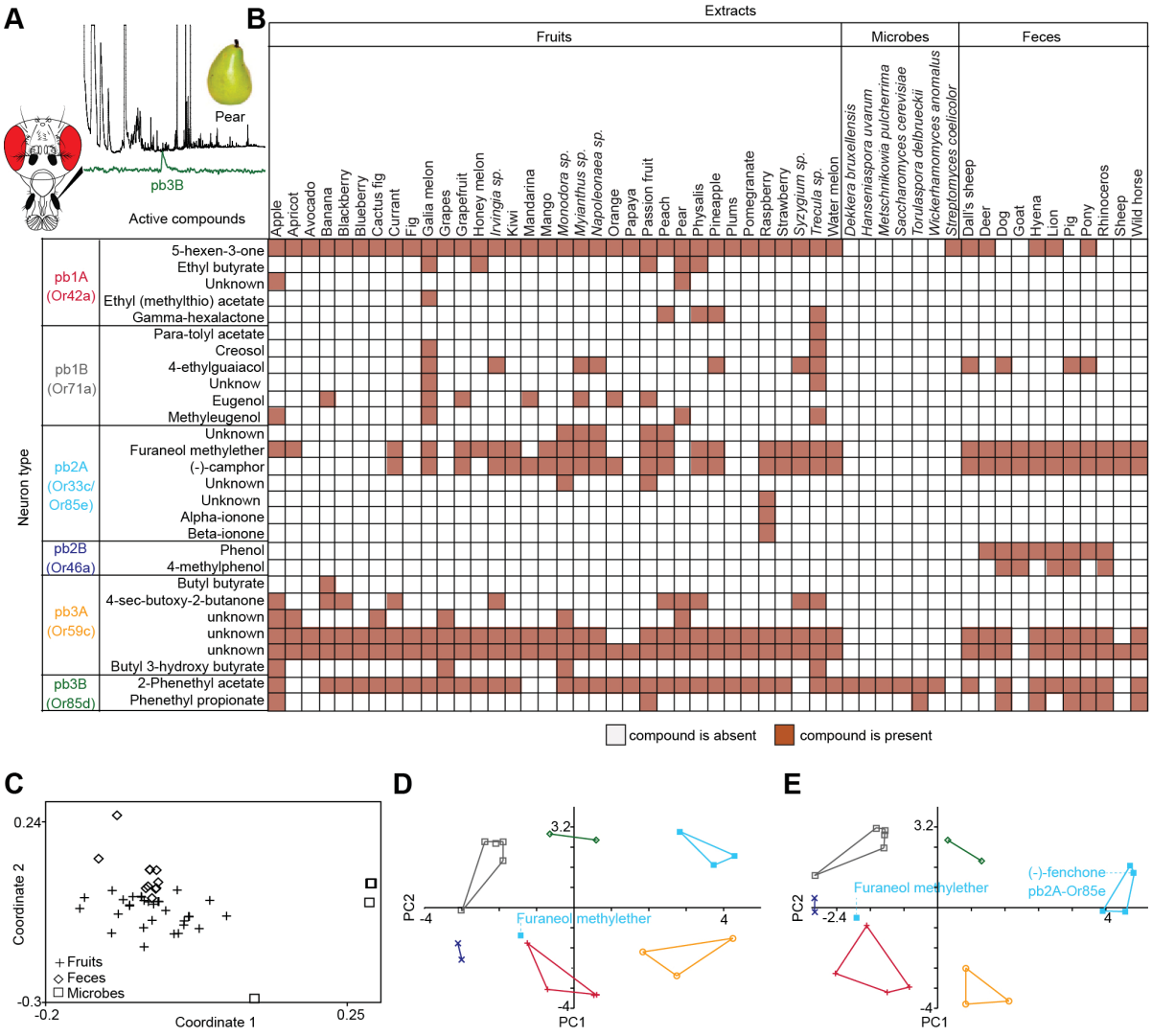


Figure 1. Screen for novel natural ligands for MP-OSNs. (**A**) Representative gas chromatography-linked single sensillum measurement (GC-SSR) from pb3B (green trace) stimulated with headspace extract of pear (black trace). (**B**) Heatmap of the physiologically active compounds identified from the different headspace extracts for each MP-OSN in the GC-SSR experiments. (**C**) NMDS plot based on a presence/absence matrix for the active peaks across the tested samples. (**D**) PCA plot showing the distribution of the ligands recognized by MP-OSNs in a 32-dimensional odor space. PC1 and PC2 explain 23 % and 22 % of the variance, respectively. (**E**) PCA plot showing the distribution of the ligands recognized by MP-OSNs and (-)-fenchone (the main ligand of Or85e-expressing OSNs) in a 32-dimensional odor space. PC1 and PC2 explain 24 % and 21 % of the variance, respectively.

Compound	CAS no.
5-hexen-3-one	24253-30-3
Ethyl butyrate	105-54-4
Ethyl (methylthio) acetate	4455-13-3
Gamma-hexalactone	695-06-7
Para-tolyl acetate	140-39-6
Creosol	93-51-6
4-ethylguaiacol	2785-89-9
Eugenol	97-53-0
Methyleugenol	93-15-2
Furaneol methylether	4077-47-8
(-)-camphor	464-48-2
Alpha-ionone	127-41-3
Beta-ionone	14901-07-6
Phenol	108-95-2
4-methylphenol	106-44-5
Butyl butyrate	109-21-7
4-sec-butoxy-2-butanone	57545-63-8
Butyl 3-hydroxy butyrate	53605-94-0
2-Phenethyl acetate	103-45-7
Phenethyl propionate	122-70-3

Table 1. List of physiologically active compounds identified for MP-OSNs including their Chemical Abstract Service numbers (CAS no.).

We next compared the distribution of the ligands recognized by different MP-OSNs in an odor space of 32 DRAGON descriptors (i.e. physicochemical properties such as number of benzene-like rings and number of double bonds), which were previously selected by Haddad et al. (2008). The 32 descriptors were then normalized using *z*-scrores and visualized in a two-dimensional principal component analysis (PCA) plot using variance-covariance matrix (**Figure 1D, Figure 1-source data 1**). In this odor space, odors with similar descriptors mapped close to each other, whereas odors with diverse descriptors distributed widely. Indeed, compounds that activated different MP-OSNs differed in their descriptors and, hence, distributed widely in the two-dimensional odor space. Compounds, however, that activated the same MP-OSN clustered together except for the ligands recognized by pb2A (**Figure 1D**).

Three out of four identified ligands for pb2A grouped close to each other, whereas the fourth ligand (furaneol methylether) spaced very widely. This could be explained by the fact that pb2A is the only MP-OSN that expresses two olfactory receptors, Or33c and Or85e (Couto et

al., 2005; Goldmann et al., 2005). In order to predict which of these two receptors could detect which of the pb2A ligands, we included (-)-fenchone, a previously identified best ligand for Or85e (Goldman et al., 2005), in our PCA. (-)-fenchone distributed widely from furaneol methylether and instead clustered with the other three ligands of pb2A (**Figure 1E, Figure 1-source data 1**). This result suggests that the responsiveness of pb2A to furaneol methylether was due to the expression of Or33c, while the responsiveness of pb2A to (-)-camphor, alpha- and beta-ionone was due to the expression of Or85e.

Each MP-OSN is either the sole or the primary detector for specific chemical compounds

To determine which of the identified ligands is the best activator for each MP-OSN, we examined the dose-response relationships in SSR (**Figure 2, Figure 2-source data 1**) and GC-SSR (**Figure 3, Figure 3-source data 1**) experiments. Only one best ligand was identified for most MP-OSNs except for pb1B and pb3B, where two best ligands for each were identified (**Figure 2**). The detection threshold of pb1A, pb1B, pb2B and pb3B for their best activators was 10^{-7} dilution, whereas the detection threshold of pb2A and pb3A was 10^{-6} and 10^{-4} dilution, respectively (**Figure 2**). This high sensitivity suggests that the maxillary palps could be involved in evaluating odor sources over long distance similar to the antennae.

Figure 2 (New)

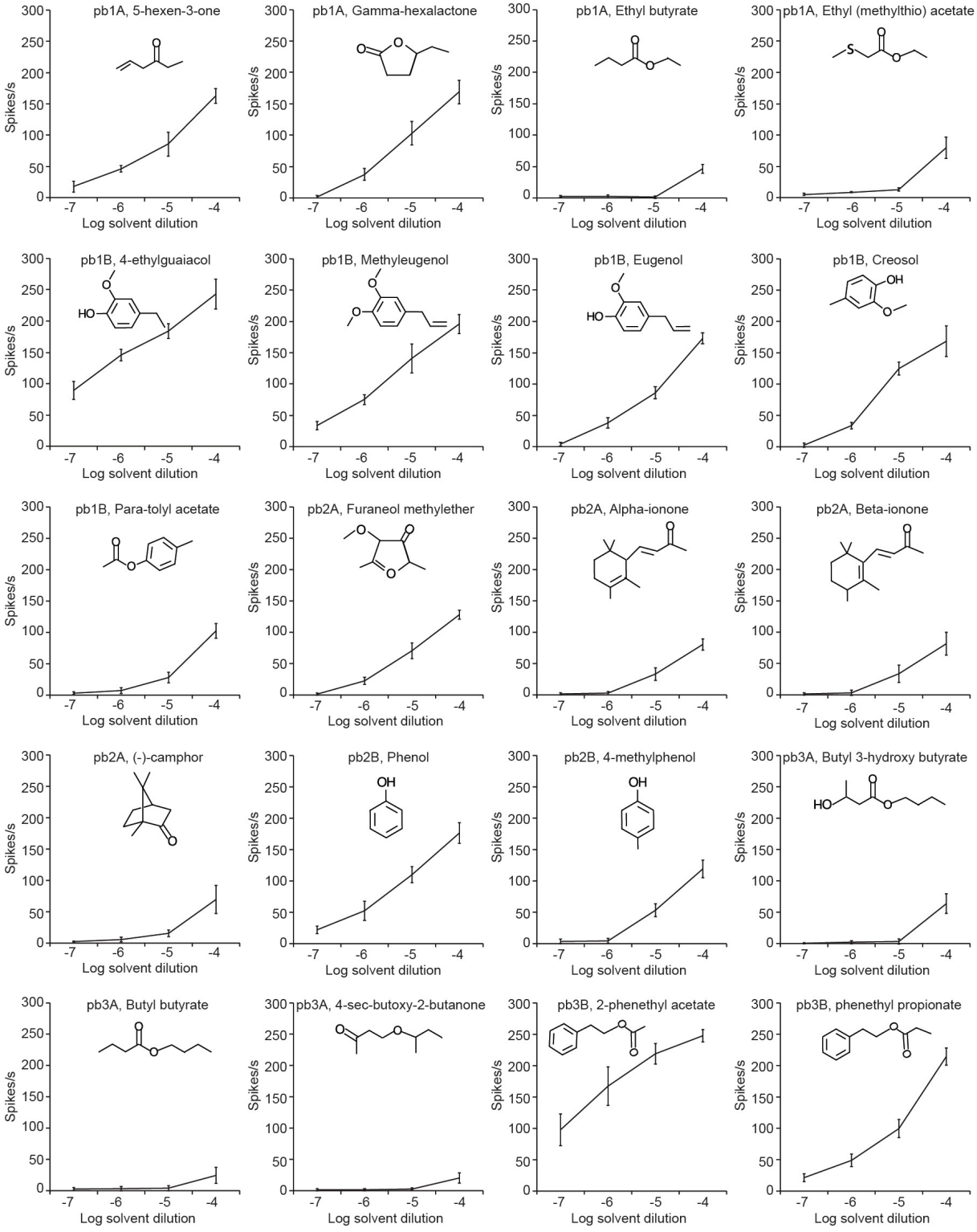


Figure 2. SSR dose-response curves for each MP-OSN stimulated with its physiologically active compounds (n = 5). Error bars represent SEM.

Figure 3 (New)

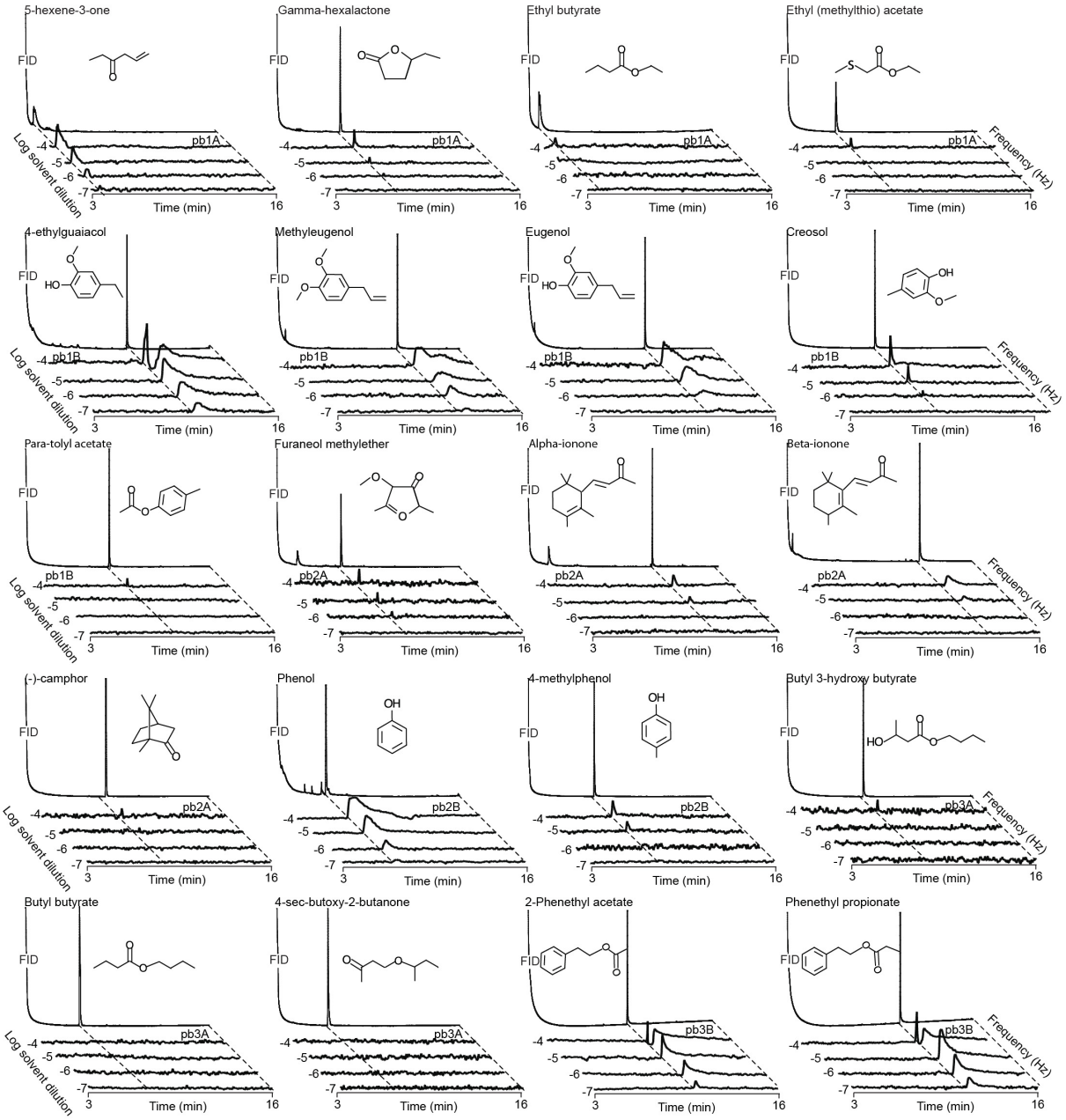


Figure 3. Representative GC-SSR dose-response traces for each MP-OSN stimulated with its physiologically active compounds (n = 3).

Several studies have suggested the existence of a labeled-line mode of odor coding in the olfactory and gustatory systems that signifies the presence of ecologically relevant signals of high biological importance (reviewed in Depetris-Chauvin et al., 2015). We next tested whether the best activators of MP-OSNs are detected via a single information channel. We screened all OSN types present on the antenna with these activators at 10^{-4} dilution using GC-SSR (**Figure 4A, Figure 4A-figure supplement 1**). We used this dosage because it is the

maximum concentration that we can inject in the GC. Four of these best activators (5-hexen-3-one, methyleugenol, furaneol methylether, and phenol) elicited no response from any of the Ant-OSNs, while the other four triggered responses from four additional Ant-OSNs (**Figure 4A, Figure 4A-figure supplement 1**). Interestingly, when odors activated both Ant-OSNs and MP-OSNs (4-ethylguaiacol: Or69a and Or71a; 2-phenethyl acetate and phenethyl propionate: Or67a and Or85d; butyl 3-hydroxy butyrate: Or59c and Or85a) in the latter two cases the receptor pairs cluster on a phylogenetic tree, suggesting a shared ancestor (Robertson et al., 2003).

Figure 4 (new)

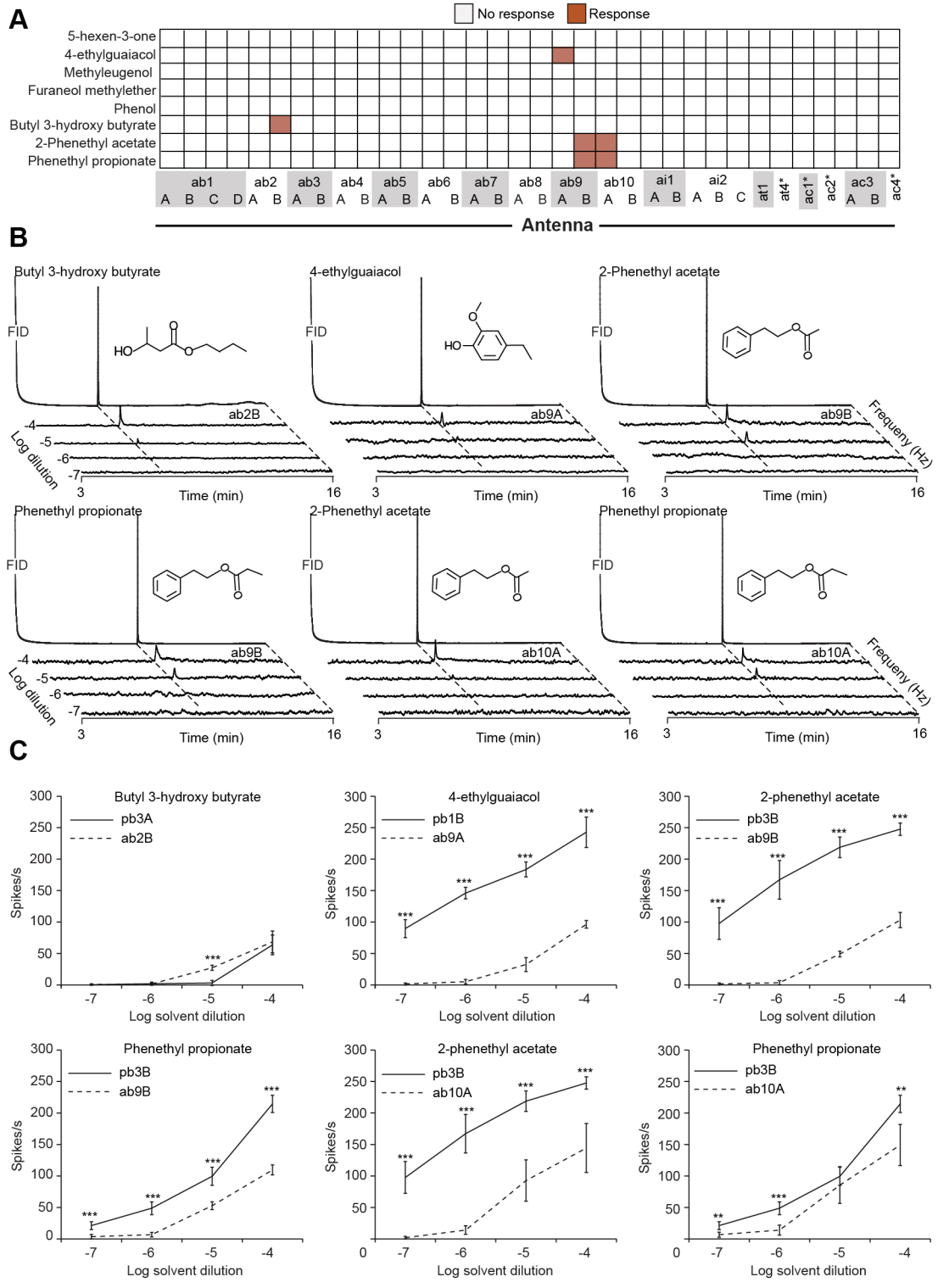


Figure 4. MP-OSNs are dedicated to detect specific chemical compounds. (**A**) Heatmap of the GC-SSR responses of the MP-OSNs best activators across the Ant-OSNs (n = 3). Asterisks denote the total response of a sensillum type when spike sorting of OSNs failed. (**B**) Representative traces of GC-SSR dose response relationship from ab2A, ab9A, ab9B and ab10A OSNs (n = 3). (**C**) SSR dose-response

curves. Error bars represent SEM. The symbols ** and *** indicate statistically significant differences between OSN types with $p < 0.001$, and $p < 0.0001$, respectively (two-tailed Independent Samples T Test, n = 5).

To know the primary olfactory detector of the four activators that activate both MP- and Ant-OSNs, we performed dose-response relationships, but this time from the activated Ant-OSNs (**Figure 3B,C**). Three of the compounds were primarily detected by the MP-OSNs as the 10^{-5} detection threshold for these Ant-OSNs was two orders of magnitude higher than the 10^{-7} detection threshold for MP-OSNs. In addition, the number of spikes elicited by these three compounds at any tested concentration from MP-OSNs is significantly higher from Ant-OSNs except for phenethyl propionate at 10^{-5} concentration from pb3B and ab10A. The fourth activator, butyl 3-hydroxy butyrate, was primarily detected by the Ant-OSN ab2B. The detection threshold of ab2B to this compound was one order of magnitude (10^{-5} dilution) lower than that of the corresponding MP-OSN pb3A (10^{-4} dilution). Together, this data suggests that the MP-OSNs are either the sole or the primary detectors of ecologically relevant concentrations of 5-hexen-3-one, 4-ethylguaiacol, methyleugenol, furaneol methylether, phenol, 2-phenethyl acetate, and phenethyl propionate (**Table 2**).

Table 1. Best activators of MP-OSNs

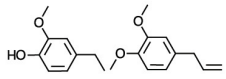
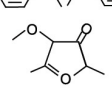
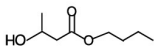
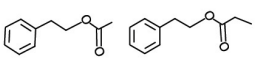
Palp OSN	Odorant	Chemical structure	Detection threshold	Source/remarks	Reference
pb1A	5-hexen-3-one		10^{-7}	Fruits	Present study
pb1B	4-ethylguaiacol/Methyleugenol ether		10^{-7}	Fruits, Proxy od dietary antioxidants	Dweck et al., 2015
pb2A	2,5-Dimethyl-4-methoxy-3(2H)-furanone		10^{-6}	Strawberries and a variety of other fruits, antioxidant activity	Ulrich et al., 1997 Slauchter, 1999
pb2B	Phenol		10^{-7}	Mammal feces, Toxic to invertebrate	Walker, 2006
pb3A	Butyl 3-hydroxy butyrate		10^{-4}	Fruits	Present study
pb3B	Phenylethyl acetate/Phenylethyl propionate		10^{-7}	Common yeast metabolites	Christiaens et al., 2014

Table 2. Best activators of MP-OSNs.

208

209 ***Contribution of MP-OSNs to short-range and long-range attraction***

210 We next screened innate behavioral responses of flies to the best activators of MP-OSNs. We
211 used trap and T-maze assays to measure short-range attraction, and wind tunnel assays to
212 measure long-range attraction. In trap and T-maze experiments, we used 10^{-4} concentration,
213 which is similar to the concentration used to measure the specificity of these ligands to
214 different OSN types. In wind tunnel experiments, we used 10^{-2} concentration because the
215 wind tunnel is supplied with a continuous airstream (0.3 m/s), which further dilutes this
216 concentration. Six out of the eight tested compounds were behaviorally active; two
217 compounds, 5-hexen-3-one and furaneol methylether, in T-maze assays, four compounds, 4-
218 ethylguaiacol, methyleugenol, 2-phenethyl acetate and phenethyl propionate, in trap assays,
219 and one compound, furaneol methylether, in wind tunnel assays (**Figure 5A, Figure 5-source**
220 **data 1**). The finding that odors are differentially attractive in the trap and the T-maze assays is
221 not new. E.g, the well-known *Drosophila* attractant, ethyl acetate, is attractive in T-maze
222 assays (Farhan et al., 2013) and neutral in trap assays (Knaden et al., 2012). Part of the
223 explanation of this variation might be due to flies flying in traps assays for 24 h, while
224 walking in T-maze assays for only 40 min. However, as so far never any odor was observed to
225 be attractive in one and repellent in the other assay, we regard each odor that elicited at least
226 attraction in one assay as attractive.

227 We next tested the behavioral responses of anosmic *Orco[2]* mutant flies, lacking the co-
228 receptor necessary for the function of canonical Or receptors (Larsson et al., 2004), to the
229 behaviorally active compounds. In contrast to WT flies, *Orco[2]* mutant flies were not
230 attracted by these compounds (**Figure 5B, Figure 5-source data 1**), suggesting that the flies'
231 behavior displayed to these compounds requires Or genes.

232 In the t-maze many pure chemicals become repellent at higher concentrations (Strutz et al.
233 2014). We, thus, measured innate responses of flies to 10^{-2} concentration of these ligands. We
234 found, indeed, that flies are strongly repelled by 5-hexen-3-one, 4-ethylgauaicol, and phenol
235 (Figure 5-figure supplement 1 and Figure 5-source data 1). Interestingly, the aversion elicited
236 by this concentration was independent of functioning Ors (Figure 5-figure supplement 1 and
237 Figure 5-source data 1). We, therefore, excluded this concentration in our further experiments.
238 From our results we conclude that the palp best ligands represent positive cues at lower
239 concentration, and that the processing of this information requires Or genes, while the
240 processing of higher concentrations seems to be independent of functioning Ors.

Figure 5 (new)

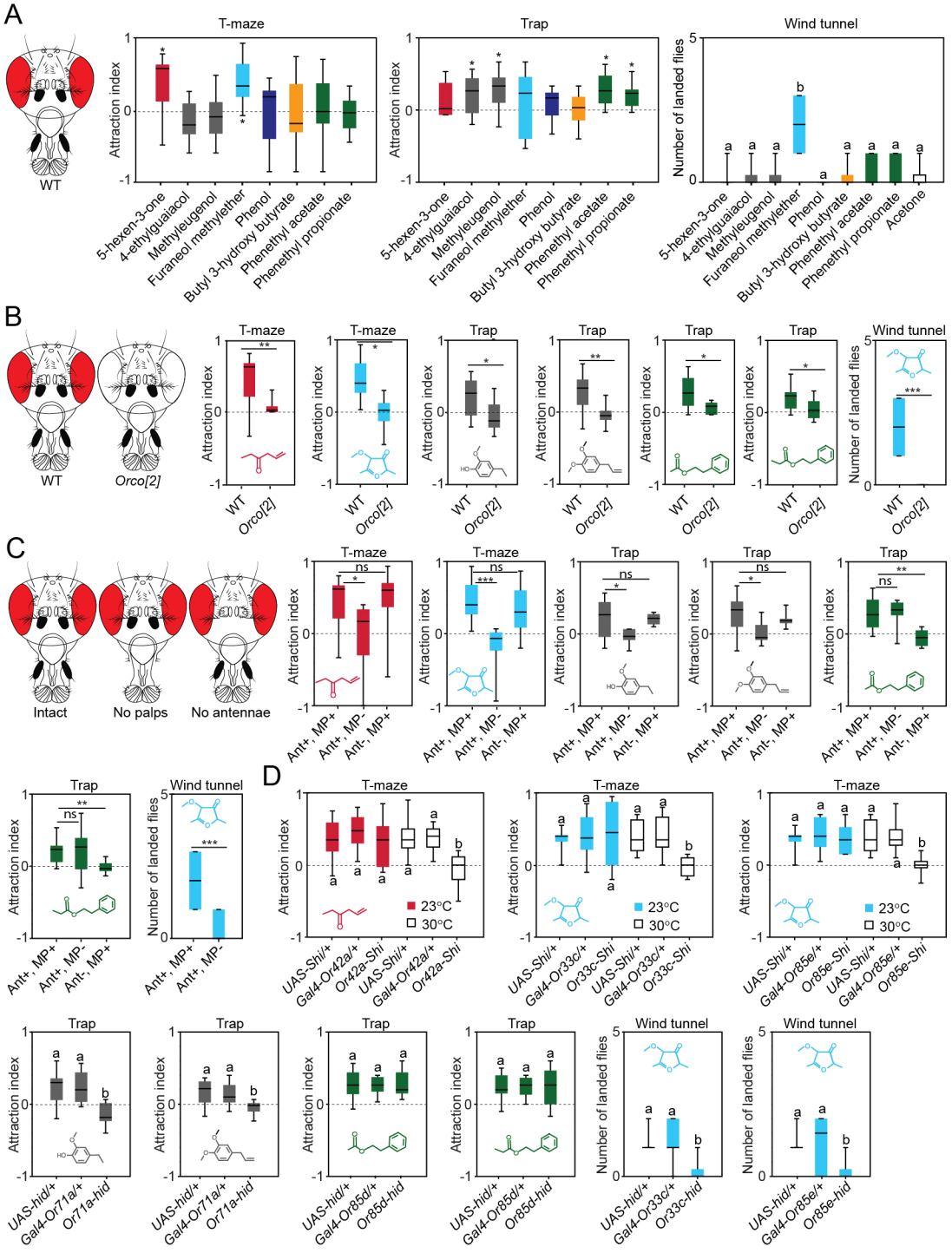


Figure 5. Contribution of the maxillary palp to the behaviors evoked by the palp best activators. (**A**) Behavioral responses of WT flies to the palp best activators (10^{-4} dilution used for trap and T-maze experiments, and 10^{-2} dilution used for wind tunnel experiments). For T-maze and trap assays, the symbol * indicates significant differences from a neutral attraction index of 0 ($p < 0.05$, Wilcoxon signed rank test, n = 10). For wind tunnel assays, different letters indicate significant differences

between groups ($p < 0.05$, Kruskal Wallis test with Dunn's multiple comparison, n = 10). (**B**) Behavioral responses of WT and *Orco[2]* flies to the behaviorally active compounds (10^{-4} dilution used for trap and T-maze experiments and 10^{-2} dilution used for wind tunnel experiments). The symbols *, ** and *** indicate statistically significant differences between the attraction indices of the genotypes with $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively (two-tailed Mann-Whitney U test, n = 10). (**C**) Behavioral responses of WT (Ant+, MP+), palp-amputated flies (Ant+, MP-) and antenna-amputated flies (Ant-, MP+) to the behaviorally active compounds (10^{-4} dilution used for trap and T-maze experiments and 10^{-2} dilution used for wind tunnel experiments). The symbols *, ** and *** indicate statistically significant differences between groups with $p < 0.01$, $p < 0.001$, and $p < 0.0001$, respectively; 'ns' indicates no significant differences between groups (Kruskal Wallis test with Dunn's multiple comparison for selected groups, n = 10). (**D**) Behavioral responses of flies with a killed or silenced specific MP-OSN population, the corresponding parental lines, and WT flies. Different letters indicate significant differences between groups (Kruskal Wallis test with Dunn's multiple comparison). Black line: median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.

To ensure that the behaviors evoked by the active compounds were mediated through the maxillary palps, we surgically removed either maxillary palps or antennae (we excluded wind tunnel experiments here, as the antenna has been shown to be involved in flight control (Budick et al. 2007)), and then tested behavioral responses of the manipulated flies. For 4-ethylguaiacol, 5-hexen-3-one, methyleugenol and furaneol methylether, amputation of the palps resulted in loss of attraction, while amputation of the antennae did not affect the behavior (**Figure 4C, Figure 5-source data 1**). Interestingly for 2-phenethyl acetate and phenethyl propionate amputation of the palps had no effect on the behavior, while amputation of the antennae abolished attraction elicited by these two compounds (**Figure 4C, Figure 5-source data 1**). We next killed or silenced a specific MP-OSN population using the

temperature-sensitive mutant dynamin shibire^{ts} (*UAS-shi^{ts}*) or head involution defective (*UAS-hid*). We avoided using the *shibire^{ts}* effector in trap assays and wind tunnel experiments to avoid any temperature effect on flying flies. In T-maze and trap assays, the behavioral response to the corresponding ligand was abolished except for 2-phenethyl acetate and phenethyl propionate (**Figure 5D, Figure 5-source data 1**). In wind tunnel experiments, killing pb2A OSNs via expression of *hid* from Or33c- or Or85e-promoter significantly reduced attraction towards furaneol methylether compared to both parental lines (**Figure 5D, Figure 5-source data 1**). Taken all together, we conclude that the maxillary palp contains olfactory channels that mediate both short- and long-range attraction to specific chemical compounds.

Organization, detection, and Or genes of MP-OSNs are conserved in D. suzukii

We selected *D. suzukii* to test whether the organization, detection and Or genes of MP-OSNs are conserved in another species. *D. suzukii* has recently invaded North America and Europe from Asia (Rota-Stabelli et al., 2013) and has become a serious agricultural pest for soft fruits causing devastating economic cost for farmers each year. Unlike most drosophilid flies including *D. melanogaster* that feed and oviposit on damaged and overripe fruits, *D. suzukii* feed and oviposit on undamaged, ripening fruits. Thus, *D. suzukii* represents an interesting neuroethological model to study olfactory changes that parallel the evolutionary shift in the preference towards ripening over fermenting fruits in *D. suzukii*.

Figure 6 (New)

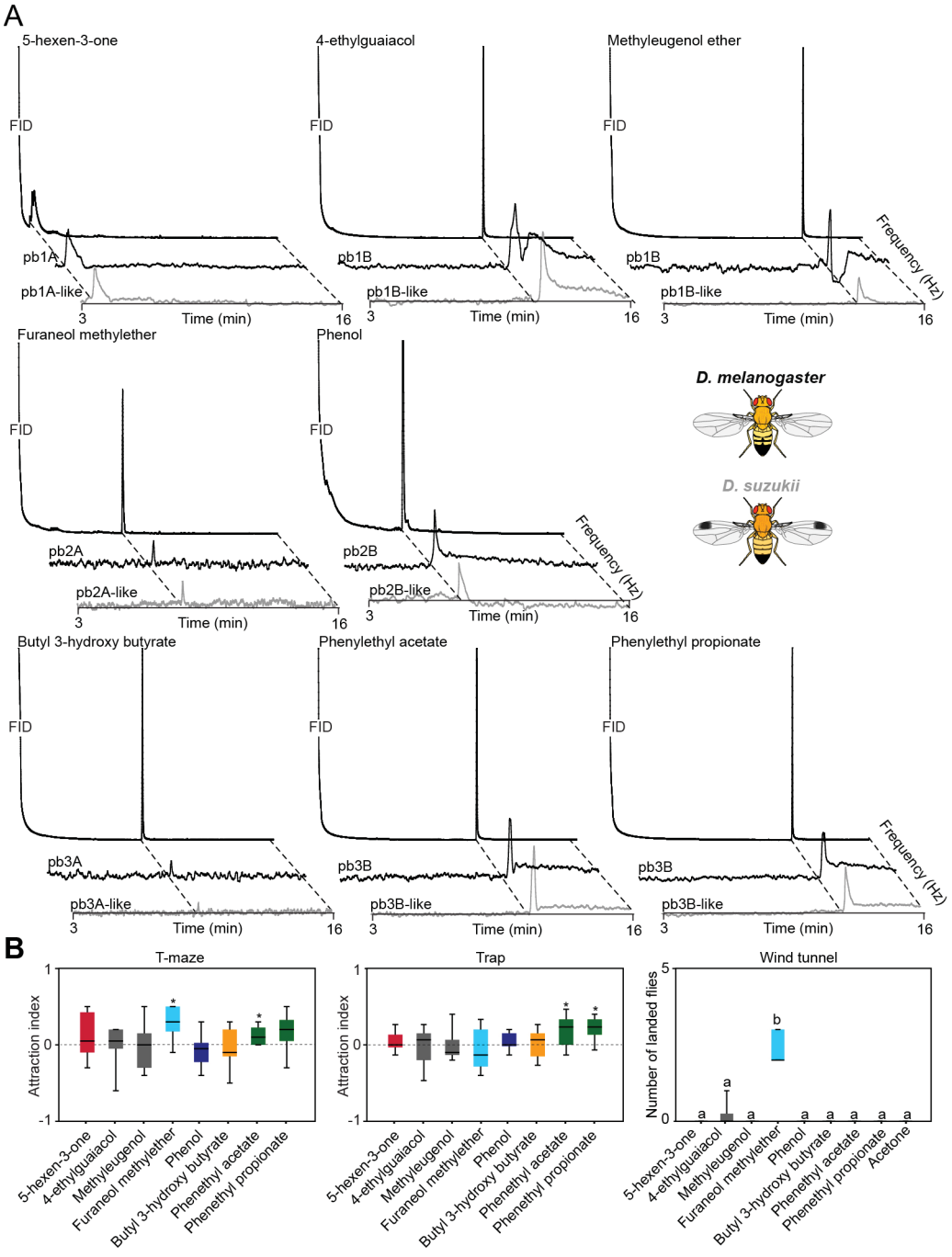


Figure 6. Organization, Detection, and Or genes of MP-OSNs are Conserved in *D. suzukii*. (**A**) Representative GC-SSR traces from palp OSNs in *D. suzukii* and *D. melanogaster*, stimulated with the palp best ligands (dilution 10^{-4}) (n = 3). (**B**) Behavioral responses of *D. suzukii* to *D. melanogaster* palp best activators (10^{-4} dilution used for trap and T-maze assays, and 10^{-2} dilution used for wind tunnel experiments). For T-maze and trap assays, the symbol * indicates significant differences from a neutral attraction index of 0 ($p < 0.05$, Wilcoxon signed rank test, n = 10). For wind tunnel assays, different letters indicate statistically significant differences between groups ($p < 0.05$, Kruskal Wallis

with Dunn's multiple comparison). Black line: median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.

We hence screened the MP-OSNs of *D. suzukii* with the best activators of *D. melanogaster* using GC-SSR. We found that the maxillary palp of *D. suzukii* contains three sensillum types as found in *D. melanogaster*, and that OSN types and their pairing within a particular sensillum type in *D. suzukii* are the same as in *D.* melanogaster. In addition, our screen revealed that the detection of these compounds is also conserved in *D. suzukii* (**Figure 5A**). Whether MP-OSNs of *D. suzukii* are the sole or the primary detectors of these ligands as found in *D. melanogaster* remains subject for future investigation.

We next aimed to know whether Or genes underlying these responses are also conserved in MP-OSNs of *D. suzukii*. We extracted the ortholog sequences of the genes expressed in the MP-OSNs in *D. melanogaster* from the public database of the *D. suzukii* genome (http://spottedwingflybase.oregonstate.edu/). These genes were then amplified from cDNA of our lab strain of *D. suzukii* (stock no. 14023–0311.01), cloned and sequenced. Five gene sequences were identified in full length, while the other two gene sequences (Or85d and Or85e) were partial. The gene sequences generated from the cDNA of our lab strain were submitted to the European Nucleotide Archive under the accession numbers LT555550-LT555555. We next aligned the amino acid sequences of the genes generated from the cDNA of our lab strain with those of *D. melanogaster* to compare their similarities. This comparison revealed that the amino acid sequences of these genes are well conserved in the *D. suzukii* genome: Or33c (82.2 %), Or42a (90 %), Or46a (83.5 %), Or59c (78.4 %), Or71a (81.4 %), Or85d (86.5%) and Or85e (85%). We thus demonstrate that the organization, the detection of *D. melanogaster* palp best activators and the *D. melanogaster* Or genes of MP-OSNs are conserved in *D. suzukii*.

Like *D. suzukii* and *D. melanogaster*, also other closely related species share the same set of orthologs of olfactory genes expressed in MP-OSNs (Guo and Kim, 2007; de Bruyne et al., 2010), which might be either due to phylogenetic constraints or to their similar feeding habits (all examined species in this group feed on fruit-associated yeast). However, differences have been described for more distantly related species, such as *D. mojavensis* (cactus feeder), *D. virilis* (sap feeder), *D. grimshawi* (tree feeder) and *Scaptomyza flava* (leaf feeder). *D. mojavensis*, *D. virilis* and *D. grimshawi* have lost orthologs of Or59c, which is expressed in the *D. melanogaster* pb3A (Guo and Kim, 2007; de Bruyne et al., 2010), whereas *S. flava* has lost the ortholog of Or85d, which is expressed in the *D. melanogaster* pb3B (Goldman-Huertas et al., 2015). In line with these notions pb3A was not found during electrophysiological recordings from palp sensilla of *D. virilis* (de Bruyne et al., 2010).

While the MP-OSNs of all close relatives of *D. melanogaster* express the same set of olfactory receptors, the similar tuning of these MP-OSNs that we found in *D. suzukii* and *D. melanogaster* cannot necessarily be assumed for all species of the *D. melanogaster* species group. On the antenna e.g. the change of only few amino acids in a *D. sechellia* ortholog of the *D. melanogaster* Or22a gene has been shown to result in changed tuning curves of the corresponding OSN (Dekker et al., 2005). Hence, future studies will reveal whether the tuning of MP-OSNs is generally more conserved than the one of Ant-OSNs

Conservation of the behavioral readouts to palp best activators in D. suzukii

To investigate whether the behavioral readouts of the olfactory inputs to the palp best activators are conserved in *D. suzukii*, we next examined innate responses of *D. suzukii* to these activators in trap, T-maze and wind tunnel assays. The yeast producing volatile 2-phenethyl acetate and phenethyl propionate (Dweck et al., 2015; Christiaens et al., 2014), elicited positive chemotaxis in trap and/or T-maze two-choice assays, while the ripening signal, furaneol methylether ((Ulrich et al., 1997), induced positive chemotaxis and upwind

attraction. The fermentation signals produced by the metabolism of hydrocinnamic acids in fruits by yeasts, 4-ethylguaiacol and methyleugenol (Dweck et al., 2015), did not induce any behavioral response in *D.suzukii* in contrast to *D. melanogater*. Pham and Ray (2014) reported a similar case, where they found that the avoidance behavior of *D. melanogaster* to CO_2, which is highly emitted by ripe fruits, is not conserved any more in *D. suzukuii*, although the CO_2 detection and the genes responsible for this detection are conserved.

Conclusions

In summary, we demonstrate that the maxillary palp in the vinegar fly, *D. melanogaster* contains OSNs that mediate both short-and long-range attraction evoked by specific chemical compounds in the flies' ecological niche. Interestingly, although the sensitivity of MP-OSNs was described to be rather low (deBruyne et al., 1999; Hallem and Carlson, 2006), which led to the assumption that MP-OSNs are basically involved in taste enhancement (Shiraiwa et al., 2008), we show that their sensitivity to some compounds can be as high as in Ant-OSNs. Furthermore, MP-OSN specific ligands did not only attract walking flies over short distance, but in one case (furaneol methylether, Or33c) even motivated flies to fly towards the source.

Finally we found that although the detection of *D. melanogaster* palp best activators and Or genes of MP-OSNs are conserved in the agricultural pest *D. suzukii*. However, only behavioral readouts to 2-phenethyl acetate and phenethyl propionate produced by yeast volatiles, and furaneol methylether that represent ripening signal in strawberries are conserved. Contrary, behavioral readouts to the yeast metabolites 4-ethylguaiacol and methyleugenol that represent fermentation signals, are not conserved in this pest species These behavioral changes might represent a taxon-specific adaptation to the newly emerging ecological niche of this pest species.

Materials and methods

Fly stocks

All experiments with wild type (WT) *D. melanogaster* were carried out with the Canton-S strain. *D. suzukii* (stock no. 14023–0311.01) was obtained from the UCSD *Drosophila* Stock Center (www.stockcenter.ucsd.edu). Transgenic lines were obtained from the Bloomington *Drosophila* stock center (http://flystocks.bio.indiana.edu/), except for *UAS-Shibire^{ts}*, which was a kind gift from G.M. Rubin (Janelia Farm Research Campus, USA).

Complete genotypes of all strains used in this study

Shi^{ts}-Or42a

Females

w^{118}/w^{118}; +/+; UAS-Shi^{ts}/+

w^{118}/w^{118}; +/+; +/Or42a-Gal4

w^{118}/w^{118}; +/+; UAS-Shi^{ts}/Or42a-Gal4

Shi^{ts}-Or33c

Females

w^{118}/w^{118}; +/+; UAS-Shi^{ts}/+

w^{118}/w^{118}; +/+; +/Or33c-Gal4

w^{118}/w^{118}; +/+; UAS-Shi^{ts}/Or33c-Gal4

Shi^{ts}-Or85e

Females

w^{118}/w^{118}; +/+; UAS-Shi^{ts}/+

w^{118}/w^{118}; +/+; +/Or85e-Gal4

w^{118}/w^{118}; +/+; UAS-Shi^{ts}/Or85e-Gal4

hid-Or71a

Females

w^{118}/w^{118}; UAS-hid/+; +/+

w^{118}/w^{118}; +/+; +/Or71a-Gal4

w^{118}/w^{118}; UAS-hid/+; +/Or71a-Gal4

Males

w^{118}/Y; UAS-hid /+; +/+

w^{118}/Y; +/+; +/Or71a-Gal4

w^{118}/Y; UAS-hid/+; +/Or71a-Gal4

hid-Or85d

Females

w^{118}/w^{118}; UAS-hid/+; +/+

w^{118}/w^{118}; +/+; +/Or85d-Gal4

w^{118}/w^{118}; UAS-hid/+; +/Or85d-Gal4

Males

w^{118}/Y; UAS-hid /+; +/+

w^{118}/Y; +/+; +/Or85d-Gal4

w^{118}/Y; UAS-hid/+; +/Or85d-Gal4

Odor samples

Fruit samples were either ripe or in early fermentation stage. Microorganisms were kept on strain-specific media (HiMedia, http://www.himedialabs.com), following standard protocols. Mammalian fecal samples were provided by the Leipzig Zoo.

Headspace collections

The headspaces of the different samples were collected for 24 h on a Super-Q filter (50mg, Analytical Research Systems, Inc., www.ars-fla.com). The samples were placed individually in an l liter laboratory glass bottle that was halfway filled with samples and closed with a custom-made polyether ether ketone (PEEK) stopper. Airflow at 0.5 L/min was drawn through the flask by a pressure pump. Filters were eluted with 300 µl hexane and samples stored at -20°C until analysis.

SSR/GC-SSR/GC-MS

Adult flies were immobilized in pipette tips, and the palps or antennae were placed in a stable position onto a glass coverslip. Sensilla were localized under a binocular at 1,000× magnification, and the extracellular signals originating from the OSNs were measured by inserting a tungsten wire electrode into the base of a sensillum. The reference electrode was inserted into the eye. Signals were amplified (10×; Syntech Universal AC/DC Probe; www.syntech.nl), sampled (10,667 samples/s), and filtered (100–3,000 Hz with 50/60-Hz suppression) via a USBIDAC connection to a computer (Syntech). Action potentials were extracted using Syntech Auto Spike 32 software. For SSR, neuron activities were recorded for 10 s, starting 2 s before a stimulation period of 0.5 s. Responses from individual neurons were calculated as the increase (decrease) in the action potential frequency (spikes/s) relative to the prestimulus frequency. For GC-SSR, neuron activities were recorded for 1220 s, the time of a single GC run. For GC stimulation, 1 µl of the odor sample was injected into a GC (Agilent 6890, column: DB5, 30 m long, 0.32 mm id, 0.25 µm film thickness; inlet at 250°C, oven: 50°C for 2 min, then 15°C min^{-1} up to 250°C, held for 5 min; carrier gas: helium, 2.0 ml min^{-1} constant flow). The GC was equipped with a 4-arm effluent splitter (Gerstel, www.gerstel.com), with split ratio 1:1 and N2(30.3 kPa) as makeup gas. One arm was connected with the flame ionization detector (FID) of the GC and the other arm introduced

into a humidified air stream (200 ml min^{-1}). GC-separated components were directed toward the palps of the mounted fly. Signals from OSNs and FID were recorded simultaneously. Headspace samples were analyzed by GC-MS (Agilent 6890GC & 5975bMS, Agilent Technologies, www.agilent.com).

Chemicals

All odorants tested were purchased from commercial sources (Sigma, http://www.sigma-aldrich.com and TCI America, http://www.tcichemicals.com/en/us/) except for 5-hexen-3-one and butyl 3-hydroxy butyrate, which were synthesized in house from propionitrile and allyl bromide using the protocol of Rousseau *et al*.(1981), and from ethyl-3-oxobutanoate using the protocol of Padhi *et al*. (2003), respectively.

Trap experiments

In this paradigm thirty 4-5 day-old mated female flies that were starved with free access to water for 24 hrs were introduced into a small box (length, 10 cm; width, 8 cm; height, 10 cm) that contained two smaller containers (height, 4.5 cm; diameter, 3 cm). The reason for performing these experiments with only females is that hungry females live longer than hungry males. For 24 hr, flies could enter (but not leave) these containers through a pipette tip (tip opening, 2 mm). Containers were equipped with the lid of an Eppendorff cap that was loaded with either an odorant or solvent. The attraction index (AI) was calculated as AI = (O−C) / 30, where O is the number of flies entered the odorant containing trap and C is the number of flies entered the solvent containing trap. The index could range from -1 (complete avoidance) to 1 (complete attraction). A value of 0 characterizes no response, i.e. the odor is not detected or is neutral. Experiments were carried out in a climate chamber at 20, 25 or 30°C and 70% humidity. Experiments were started in the morning with 12 hrs of white light, followed by 12 hrs of no light.

T-maze experiments

T-maze experiments were carried out as described in Stensmyr et al. 2012. In brief, thirty 4-5 days old starved and mated female and male flies were introduced into the bottom part of a t-shaped tube (length of each arm, 4 cm; diameter, 1 cm) and during 40min were allowed to enter (but not to leave) via pipette tips (tip opening, 2mm) eppendorff caps attached to the two upper arms of the t-shaped tube. The lids of the Eppendorff caps contained 0.5ml agar (1%) that was loaded either with 50 µl of the odorant or with solvent only. The positions of odorant and solvent within the t-mazes were changed repeatedly. The attraction index (AI) was calculated as AI = (O-C)/30, where O is the number of flies entered the odorant containing trap and C is the number of flies entered the solvent containing trap. Experiments were carried out in a climate chamber at 20, 25 or 30°C and 70% humidity. For shibire experiments, flies were warmed at 30°C for 30 min prior to behavioral assays. All t-maze assays were performed under white light.

Wind tunnel experiments

Free-flight experiments were performed in a wind tunnel that was built as described previously (Becher et al., 2010), with the airstream in the tunnel (0.3 m/s) produced by a fan and filtered through activated charcoal. The wind tunnel was maintained within a climate chamber set to 27 °C and 50-55 % humidity. Five flies (following suggestions from Becher et al. (2010) for highest responses rates in wind tunnel assays, we used 2 days old flies that were mated and starved for 24 hours) were together released at the center position of the downwind side of the tunnel. No differences between sexes were noted, and thus the data were pooled (Figure 5-figure supplement 2, Figure 5-figure supplement 2-source data 1). 50 µl of a 10^{-2} dilution of the odorant in acetone (solvent) was delivered onto a filter paper, which was placed in a plastic tube (diameter, 3 cm). The tube was horizontally suspended within the airstream in the center position of the upwind side of the tunnel. Flies landing at the tube were

counted for the first 10 min after release. All wind tunnel experiments were performed under white light.

Gene annotation

Annotated genomic sequences of *Drosophila suzukii* were obtained from SpottedWingFlyBase (http://spottedwingflybase.oregonstate.edu). *Drosophila melanogaster* Or sequences were downloaded from flybase.org. Using the BLAST algorithm we identified gene models of the *Drosophila melanogaster* orthologs of Or33c, Or42a, Or46a, Or59c, Or71a, Or85d and Or85e in the *Drosophila suzukii* genome. The gene models were curated manually by comparison with the sequences of *Drosophila melanogaster*. For gene sequences see supplementary document.

RT-PCR and cDNA cloning

The third antennal segment and palps of ~100 *D. suzukii* flies were collected and transferred to Eppendorf cups chilled on dry ice. Subsequently they were homogenized with ceramic beads for 15 min at 50 Hz in a TissueLyser LT (Qiagen, Hilden Germany). Total RNA was isolated using TRizol isolation following the manufacture´s protocol. The extracted total RNA was dissolved in RNAse free water. The quality was checked by gel electrophoresis and the concentration was measured photometrically. cDNA synthesis for RT-PCR was done by using SuperScript III First-Strand Synthesis Kit (Invitrogen, Life Technology, Grand Island, USA). RT-PCR was performed according to standard protocols, using primers of the table below with an annealing temperature of 57°C. PCR products were cloned into pCR®2.1 vector (Invitrogen, Life Technology, Grand Island, USA). Sequencing was performed by Eurofins Genomics.

Gene	Forward primer	Reverse primer
DsuzOr33c	5′ACC ATG GTC ATC ATC GAT AGT GTT CAT 3′	5′CTA TAT ACC TTT CAC CCG CAC CAC 3′
DsuzOr42a	5′ATG GAG CTG CAA AGA ATC ATT CCG 3′	5`TCA ATC GTC TTC ATC AGA TTT GGC TAA 3′
DsuzOr46a	5′ACC ATG AGC AAC AGA GTG GAA ATC 3′	5′CTA ACT GTT GAC CCG CTT TAG CAA 3′
DsuzOr59c	5′ACC ATG AAG AAG CCG CTC TTT GAA CGT 3′	5′TTA GGG CTC TAC TTC CCC TGC ATT 3′
DsuzOr71a	5′ACC ATG GAT TAC GAC CGA ATT CGA CCA 3′	5′CTA TTG GTT CAT GTT GAG CAG CAA G 3′
DsuzOr85d	5′ATG GCA GCG AAG AAG CAA ACT CAA 3′	5′ TCA GGT ACT ATA CAT TGT GCG CAG 3′
DsuzOr85e	5′ATG GCC AGT CTT CAG TTC CAC GG 3′	5′GGG CGT GTT TCC ACCATG AGC 3′

522

523 *Data analysis*

524 Chemometric analysis was performed as outlined in Haddad *et al*.2008, and was used as basis
525 for a Principal Component Analysis (PCA) performed in PAST (folk.uio.no/ohammer/past/).
526 Normality and homogeneity of variances were tested in SPSS (www.spss.com) to select
527 appprociate statistical tests. All statistical tests were performed with SPSS or Graphpad
528 Instat.

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Author contributions

Conception and design: HKMD, MK, BSH

Acquisition of data: HKMD, SAME, MAK, CK, AF, RS, EGW

Analysis and interpretation of data HKMD, MK, BSH, EGW

Drafting or revising the article HKMD, MK, BSH

Contributed unpublished, essential data, or reagents JW, AS

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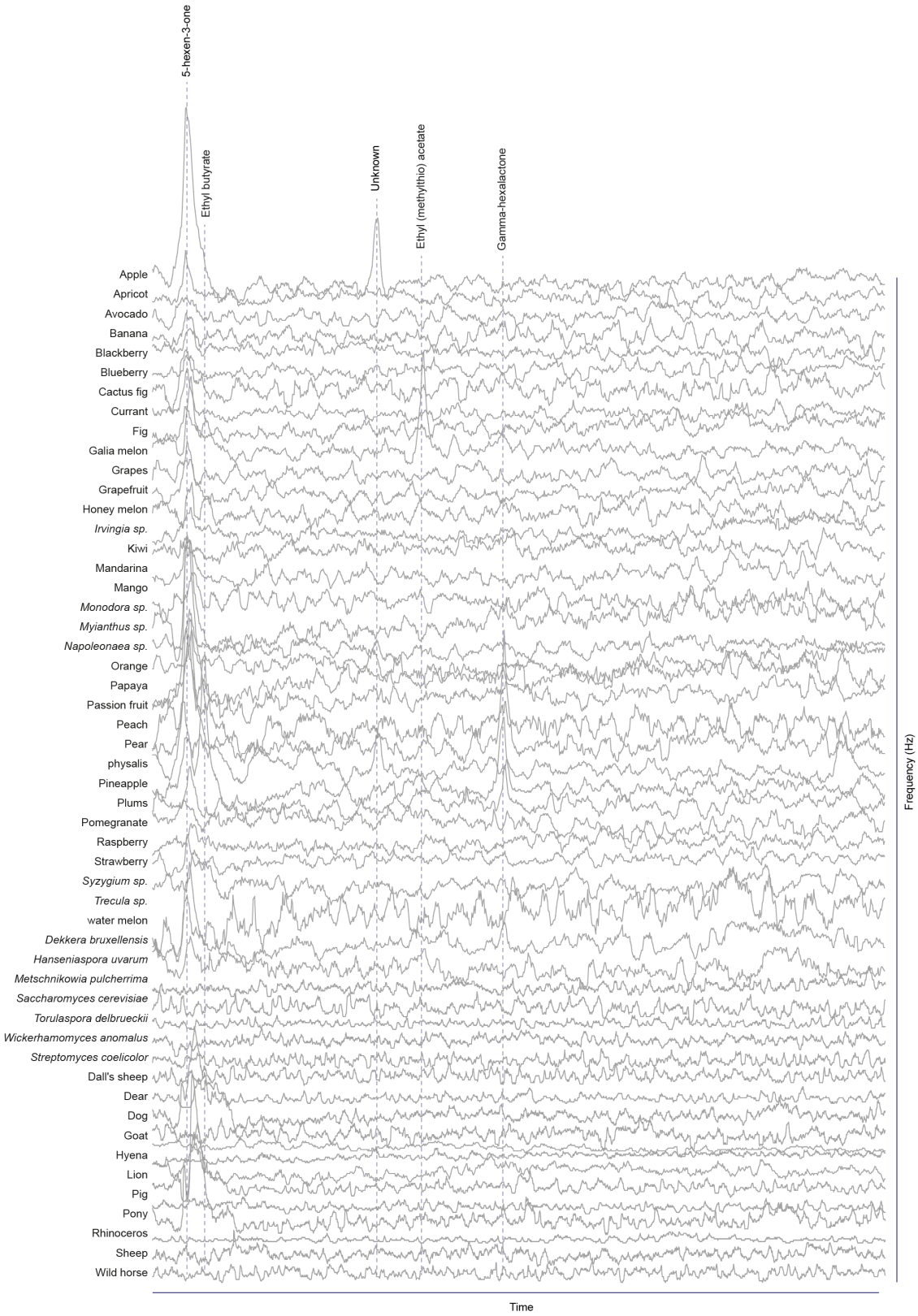
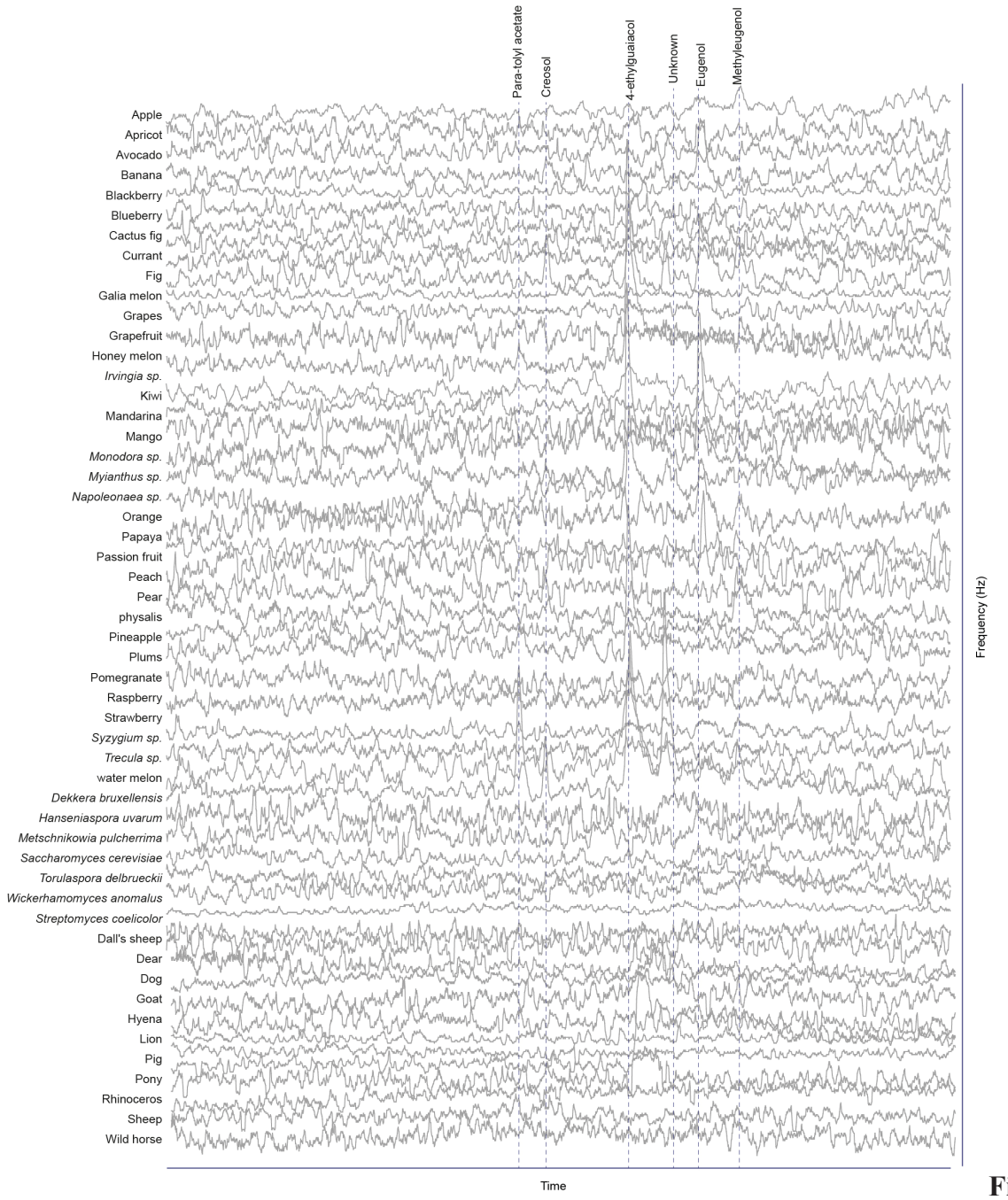


Figure 1B-figure supplement 1. Responses of pb1A OSNs type to physiologically active compounds in different extracts.



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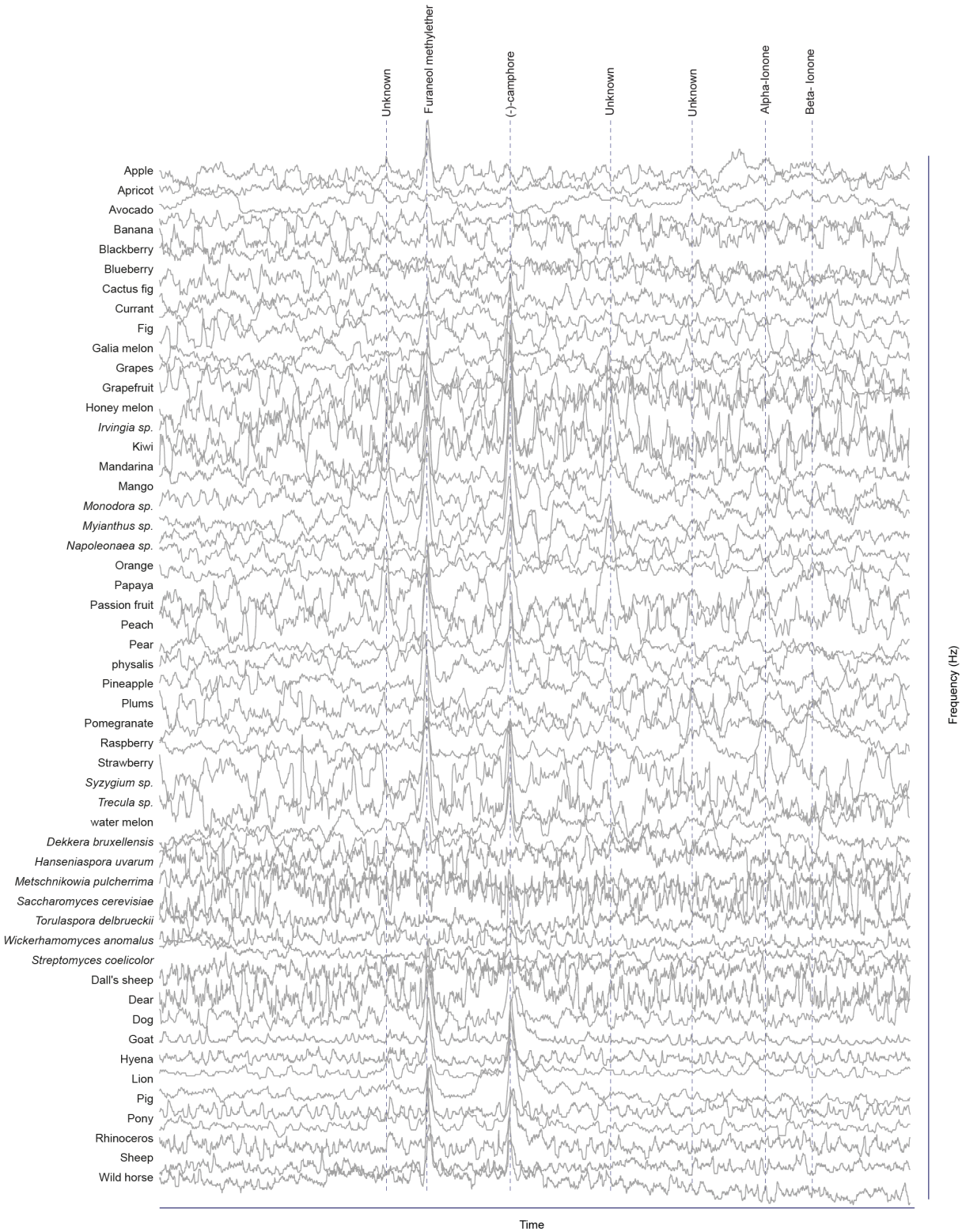
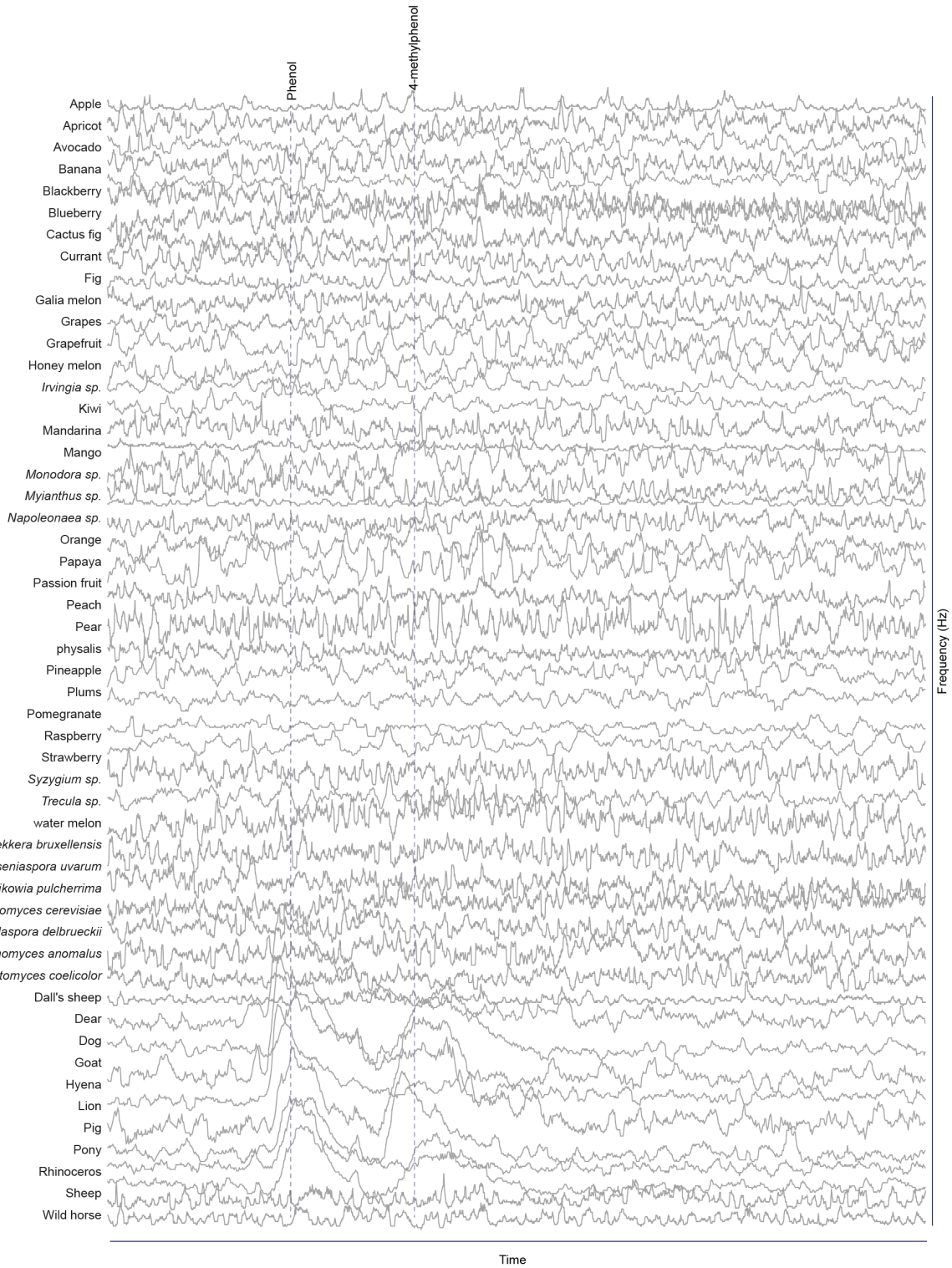


Figure 1B-figure supplement 3. Responses of pb2A OSNs type to physiologically active compounds in different extracts.



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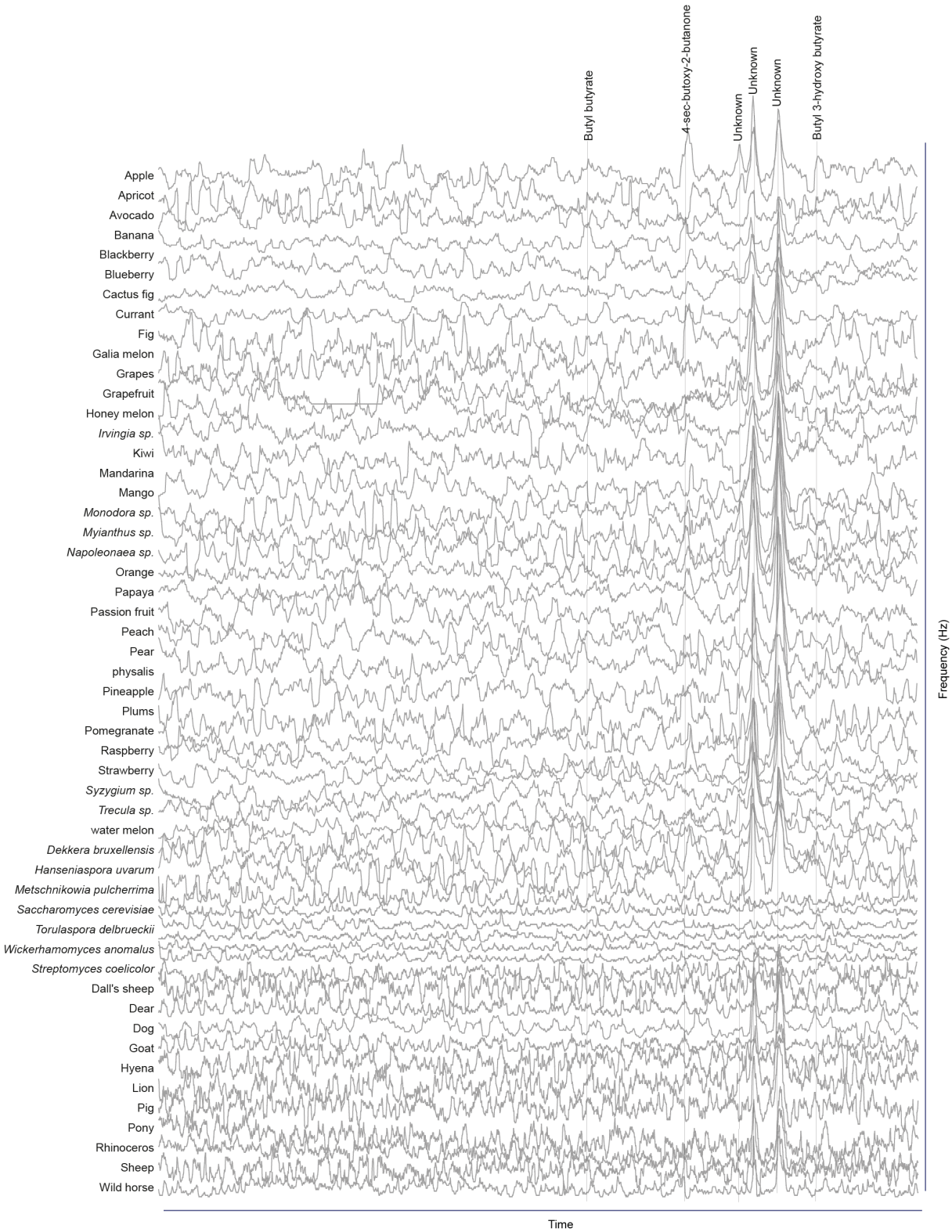


Figure 1B-figure supplement 5. Responses of pb3A OSNs type to physiologically active compounds in different extracts.

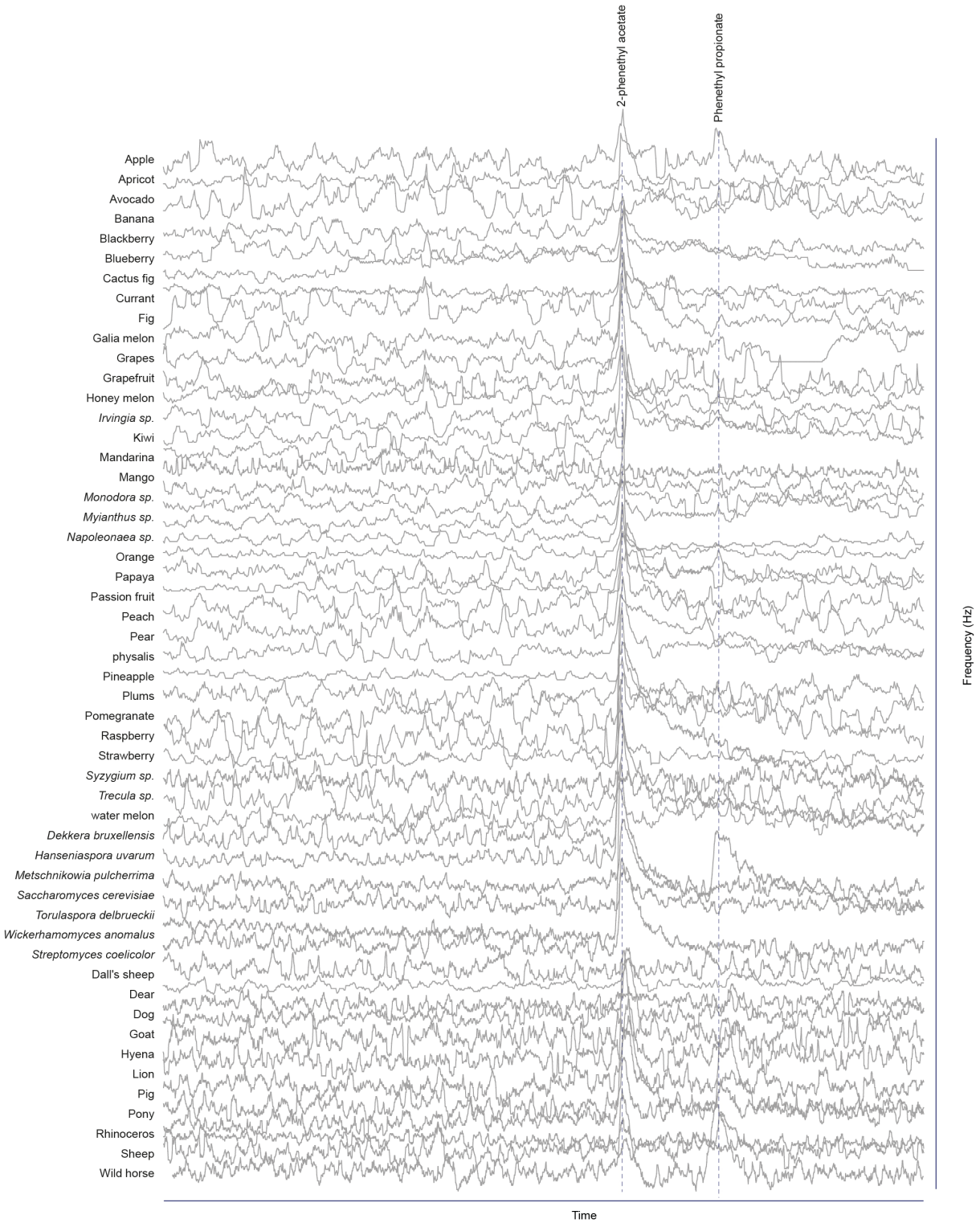


Figure 1B-figure supplement 6. Responses of pb3B OSNs type to physiologically active compounds in different extracts.

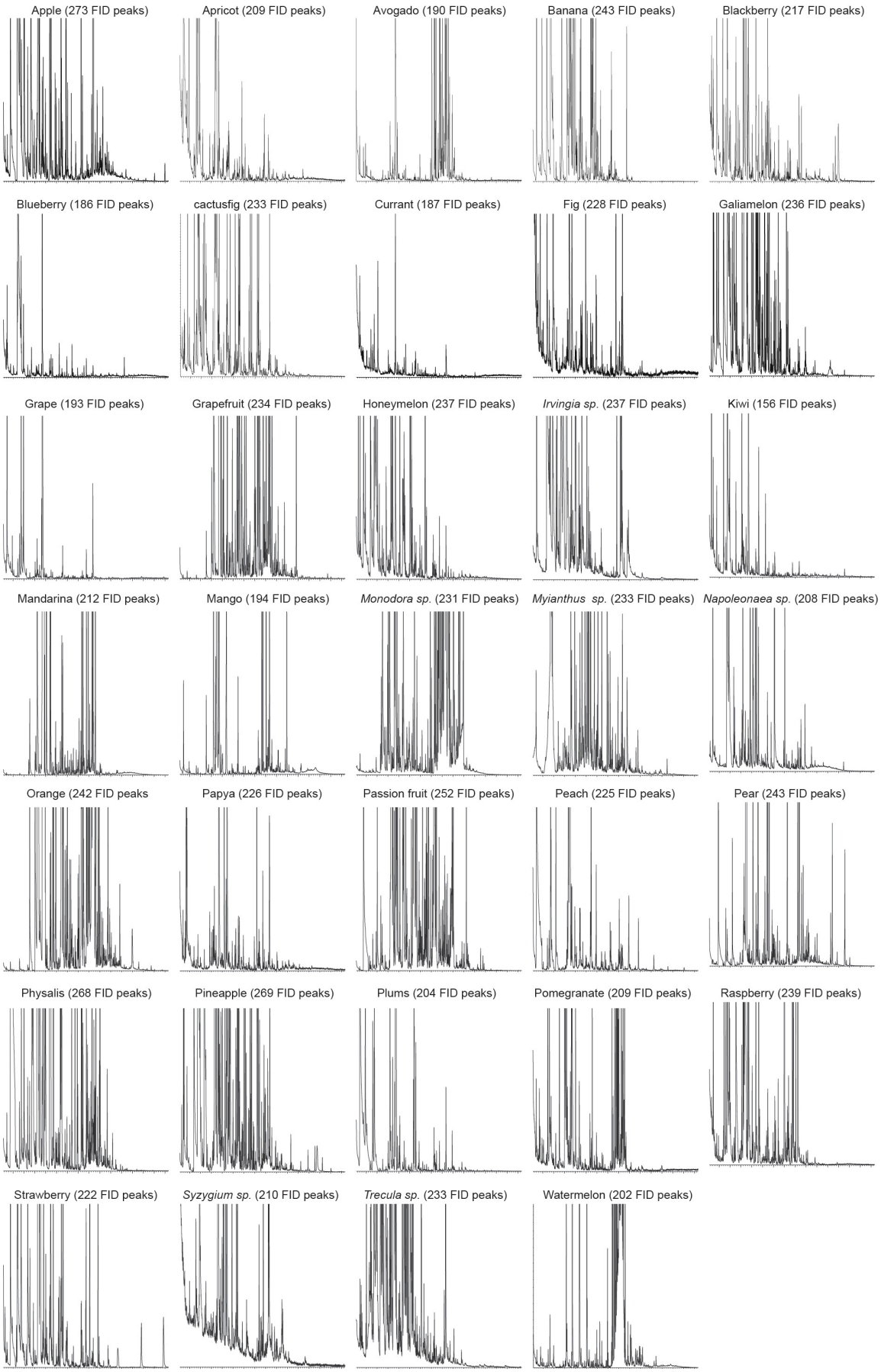
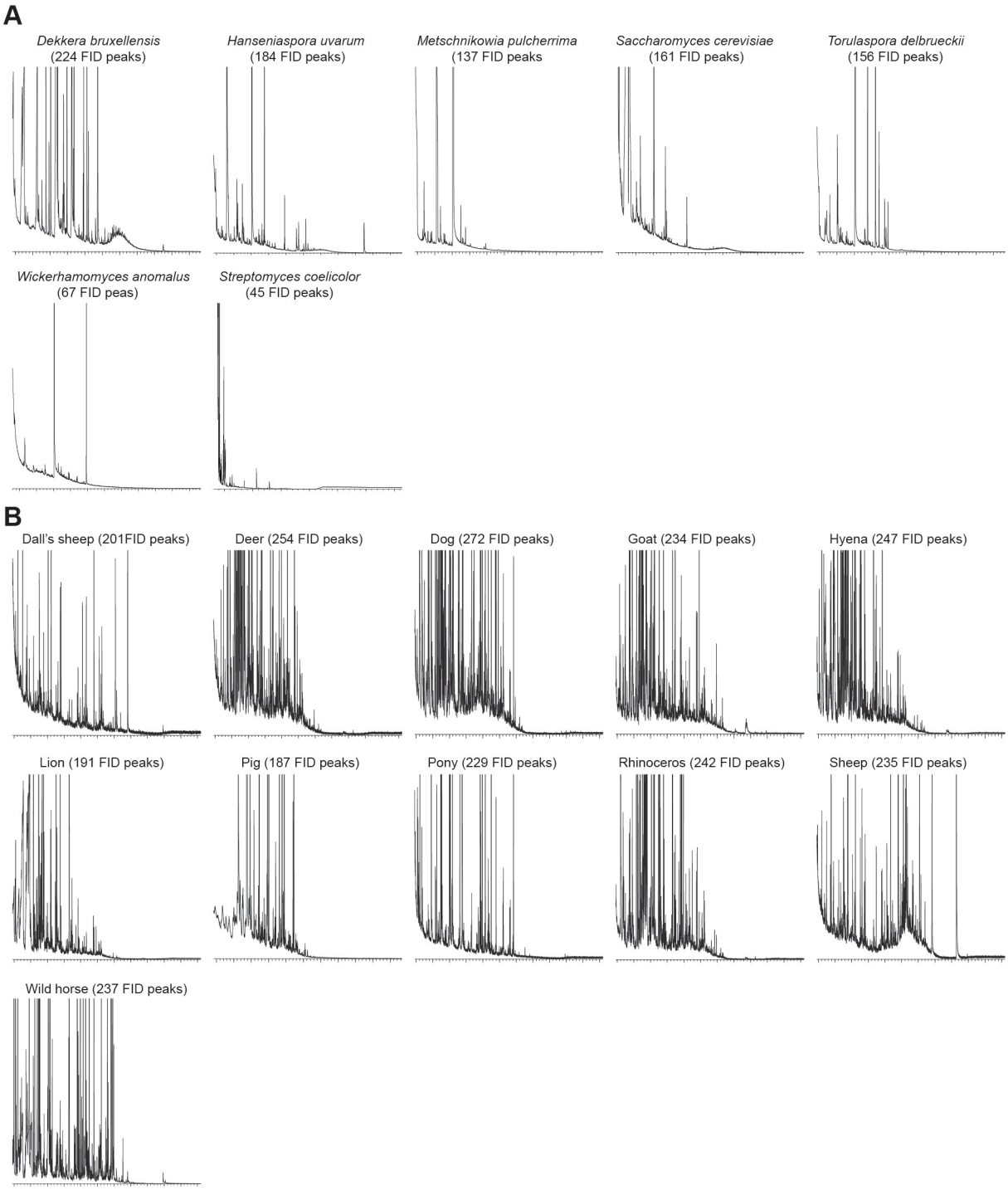
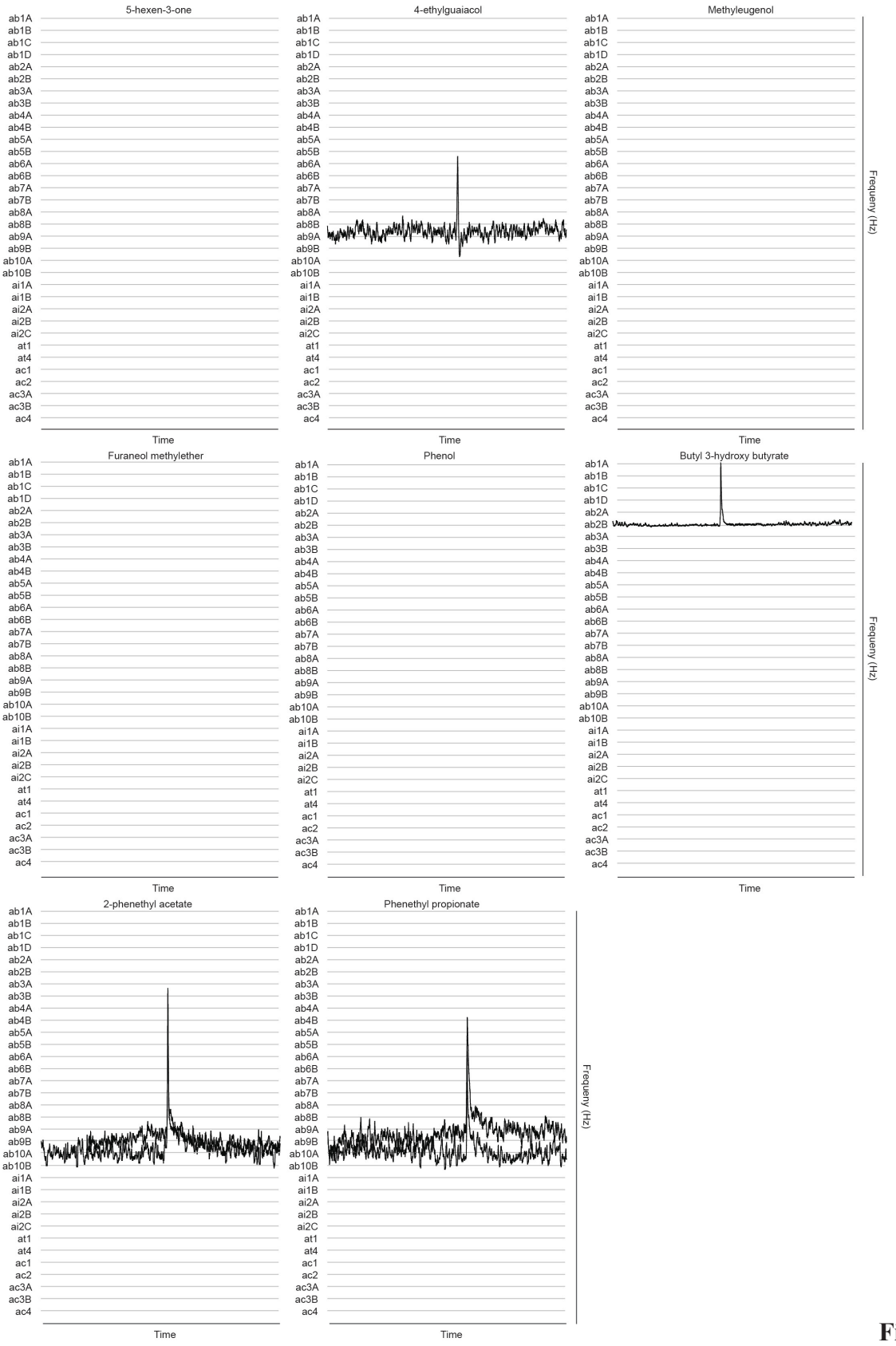


Figure 1B-figure supplement 7. GC-MS chromatographs showing number of FID peaks in each fruit sample.

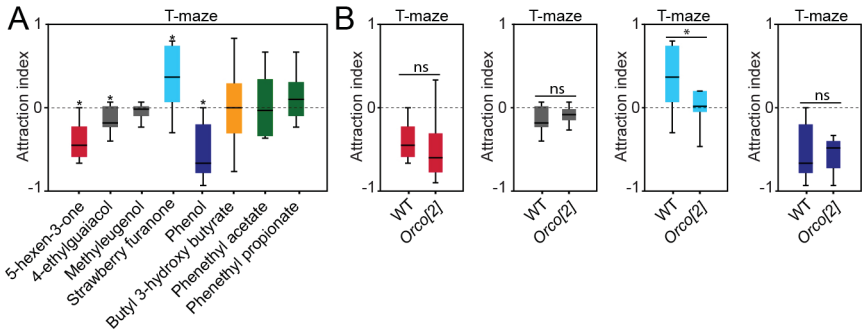


672
673 **Figure 1B-figure supplement 8.** GC-MS chromatographs showing number of FID peaks in
674 each microbial (A) and fecal (B) sample.



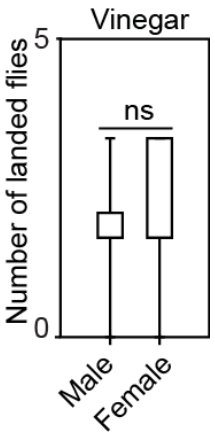
675 **Figure**

676 **4A-figure supplement 1.** Responses of Ant-OSNs to palp best activators.



677

678 **Figure 5A-figure supplement 1. (A).** Behavioral responses of WT flies to the palp best
679 activators at 10^{-2} dilution in T-maze experiments. The symbol * indicates that data are
680 significantly different from zero ($p < 0.05$, One-Sample T test, n = 10). Whiskers represent
681 the minimum and maximum values. **(B).** Behavioral responses of WT and *Orco[2]* flies to the
682 the behaviorally active compounds at 10^{-2} dilution in T-maze two-choice experiments. The
683 symbol * indicates significant differences between groups with $p < 0.01$ (two-tailed
684 Independent Samples T Test, n = 10). 'ns' indicates no statistically significant differences
685 between groups ($p > 0.05$, two-tailed Independent Samples T Test, n = 10). Black line:
686 median; boxes: upper and lower quartiles; whiskers: minimum and maximum values.



687

688 **Figure 5-Figure supplement 2.** Behavioral responses of male and female WT flies to 10%
689 vinegar in wind tunnel experiments. 'ns' indicates no statistically significant differences
690 between groups ($p > 0.05$, two-tailed Mann-Whitney U Test, n = 10). Black line: median;
691 boxes: upper and lower quartiles; whiskers: minimum and maximum values.

GENERAL DISCCUSION

The olfactory system is tuned to detect and interpret cues from an animal's ecological niche important for survival and reproduction. The recognition of nutrients and mates, as well as the avoidance of predators, toxins, and other noxious stimuli relies on sensory systems that receive and process signals from the environment. In this thesis I contributed to the identification of olfactory circuits underlying the avoidance of parasitoids (Ebrahim et al., 2015, Manuscript I), egg-laying preference to citrus fruits (Dweck et al., 2013, Manuscript II), preference towards dietary antioxidants (Dweck et al., 2015a, Manuscript III) and pheromones mediating copulation and aggregation (Dweck et al., 2015b, Manuscript IV). I also contributed to the dissection of the functional significance of the maxillary palp, the second olfactory organ, in *Drosophila* (Manuscript V).

Protection against parasitism in *Drosophila*

Endoparasitoid wasps are a common predator of *Drosophila* larvae, and can infect up to 80% of larvae in the wild (Fleury et al., 2004). These wasps pierce the *Drosophila* larva's thick cuticle by their needle-like ovipositor to inject an egg inside (Carton et al., 1986). After hatching, the developing wasp larva feeds on the *Drosophila* larva alive from the inside and eventually a new wasp emerges rather than a fly (Silvers and Nappi, 1986).

Beside physiological defenses, *Drosophila* has evolved several defense mechanisms to avoid being parasitized. Upon parasitoid wasp attack, *Drosophila* larvae respond with a specific rolling behavior that occasionally flips the attacker to the back (Hwang et al., 2007). This rolling behavior is mediated by a multimodal circuit that includes mechanosensory chordotonal neurons as well as nociceptive multidendritic class IV (MD IV) neurons (Hwang et al., 2007; Robertson et al., 2013; Zhang et al., 2013; Ohyama et al., 2015). Mechanosensory and nociceptive sensory neurons converge on specific types of first-order Basin interneurons that integrate their inputs. Artificial activation of a single type of first-order multisensory interneurons triggers rolling behavior. Co-activation of first-order interneurons that receive distinct combinations of mechanosensory and nociceptive inputs increases rolling behavior. Then, interneurons that receive distinct combinations of mechanosensory and nociceptive inputs converge again at multiple levels downstream, all the way to command-like neurons, called Goro, in the nerve cord. Although the Goro neurons clearly mediate larval rolling behavior, their silencing does not abolish the rolling behavior completely, raising the

possibility that other neurons and presynaptic neural networks are involved in the rolling behavior as well.

In manuscript I (Ebrahim et al., 2015) of this thesis I examined the larval behavioral responses of several *Drosophilid* species to the smell of the larval endparasitoid *Leptopilina boulardi*. The behavioral experiments revealed that the larvae of these species avoid the smell of *Leptopilina boulardi*, and that this avoidance behavior in *D. melanogaster* is Orco dependent. Orco is the co-receptor that is necessary for the function of canonical Or receptors. This means that the observed larval avoidance behavior requires Or genes. *Drosophila* larvae smell the surrounding environment through a pair of dorsal organs located at both sides of the head. Each dorsal organ houses 21 OSNs, which express 23 Or genes. Two of these Or genes, Or2a and Or49a, remained so far orphan. Interestingly, these two Or genes are also expressed in adult *Drosophila* in the ai2B neuron (previously named at3B/Or2a) and the ab10B neuron (Or49a). Electrophysiological experiments showed that the *Leptopilina*'s sex pheromone, iridomyrmecin, exclusively elicited strong responses from the dorsal organs of the examined species. Furthermore, ectopic expression of Or49a revealed that Or49a respond exclusively to this compound from the smell of the *Leptopilina* wasps. This is consistent with the notion that the larval avoidance behavior of the *Leptopilina*'s smell in *D. melanoster* is olfactaory guided. Thermo- and optogentic experiments revealed that Or49a-expressing neurons are necessary and sufficient to govern the avoidance behavior of the *Leptopilina*'s smell.

It will be interesting in future to known whether the first-order Basin interneurons that integrate mechanosensory and nociceptive inputs caused by the wasp ovipositor, also integrate the olfactory information generated from the *Leptopilina*'s smell via Or49a, and whether the command Goro neurons receive also input from the first-order interneurons of this olfactory information.

Drosophila adults have also evolved complex behavioral mechanisms to protect their offspring from the attack of the parasitoid wasps. An example of such a mechanism is that *Drosophila* adults self-mediate their offspring by laying eggs in ethanol-rich medium, which protect larvae from parasitoid wasps (Kacsoh et al., 2013). This is because *Drosophila* larvae have higher ethanol tolerance than their parasitoids. Flies rely on their visual system to initiate this oviposition preference switch. In addition, exposure to the parasitoid wasp *Leptopilina heterotoma* results in reduced egg-laying behavior (Kacsoh et al., 2015).

However, apart from the visually mediated avoidance, one could imagine that Or49a detecting the wasp pheromone also contributes to wasp avoidance in adult flies as this receptor is also expressed in adult flies. I found that female *Drosophila* avoid to lay eggs on oviposition plates

spiked with the smell of *Leptopilina* wasps, and that *Orco* mutant flies lacked this behavior. Electrophysiological studies revealed that adult *Drosophila* detect three compounds from the *Leptoplina*'s body wash, iridomyrmecin, actinidine and nepetalactol, via ab10B OSNs. These OSNs express Or49a and Or85f. Ectopic expression experiments revealed that the responsiveness to iridomyrmecin is due to the expression of Or49a, while the responsiveness to actinidine and nepetalactol is due to the expression of Or85f. Thermo- and optogenetic experiments revealed that ab10B neurons are necessary and sufficient to trigger oviposition avoidance behavior. Optical-imaging and immunostaining experiments revealed that this information is transferred to a specific region in the antennal lobe as well as in the lateral horn known to be involved in coding aversive stimuli (Knaden et al., 2012 and Stensmyr et al., 2012). Thus, this manuscript is the first that dissects the olfactory circuit underlying avoidance of natural enemies in insects.

I also contributed to the demonstration that *Drosophila* adults prefer *Citrus* fruits as oviposition substrates (Dweck et al., 2013, Manuscript II). This preference is due to the high content of terpenes in the flavedo (i.e. colored rind found in *Citrus*). Behavioral experiments revealed that chemicals present in the flavedo are important as flies clearly preferred intact oranges over peeled oranges and as flies no longer preferred oranges when the terpene content was genetically decreased. Finally, flies strongly preferred to oviposit on food plates spiked with synthetic terpene volatiles. Electrophysiological experiments showed that only the antennal intermediate sensillum type 2A (ai2A) neurons responded strongly to a number of terpene compounds in the headspace of *Citrus* fruits. The ai2A OSNs express *Or19a* as revealed from the calcium-imaging experiments and ectopic expression of *Or19a*. Interestingly, all of terpene compounds activating ai2A triggered oviposition, but not chemotaxis (i.e. movement of an organism to a chemical stimulus). Thus, the ai2A-*Or19a* OSNs are specifically tuned to detect terpenes. Furthermore, thermogenetic experiments revealed that the function of ai2A is necessary and sufficient to mediate oviposition preference for *Citrus* fruits. Behavioral experiments also revealed that the same terpene ligands that mediate fly oviposition are also potent repellents for parasitic wasps that prey on fly larvae. Thus, the same cues that indicate suitable oviposition substrates for flies also indicate host unsuitability for parasitoid wasps.

Protection against oxidative stress in *Drosophila*

Flies not only have to find suitable oviposition sites and avoid natural enemies but also need to identify healthy food sources. Healthy food plays a fundamental role in preventing oxidative stress by regulating levels of free radicals and other reactive oxygen species (Vertuani et al., 2004; Dai and Mumper, 2010; Soto-Vaca et al., 2012). In flies, oxidative stress can be induced by immune defense responses and detoxification processes upon consumption or infection by entomopathogenic microorganisms (Limmer et al., 2011; Wu et al., 2012; Panayidou and Apidianakis, 2013). In fruits – the primary breeding substrate of flies (Lachaise and Tsacas, 1983) – antioxidants are abundant (Soto-Vaca et al., 2012). Manuscript III of this thesis describes that flies are attracted to dietary antioxidants. Behavioral experiments revealed that flies are more attracted towards, feed, and lay eggs on yeast-inoculated media containing the common hydroxycinnamic acids (HCAs) p-coumaric acid and ferrulic acid- potent dietary antioxidants abundant in fruits. Fly larvae, too, were attracted to media containing these HCAs. Flies were neither repelled nor attracted to HCAs in food in the absence of yeast, but they were attracted to the ethylphenols- 4-ethylguaiacol and 4-ethylphenol- produced by the metabolism of HCAs by yeasts as revealed by analytical and behavioral experiments. Adult flies found both of these compounds highly attractive and were more likely to feed and lay eggs on standard food media containing them. Fly larvae, too, were more attracted to the ethylphenols. Comprehensive series of electrophysiological experiments revealed that flies use specific olfactory receptors to detect the ethylphenols produced by yeast metabolism of HCAs. Adult flies use a different receptor (Or71a) than larvae do (Or94b) to detect these compounds. Thus, these results provide the first indication that animals are able to use olfactory cues to judge content of dietary antioxidants. The ethylphenol pathway described in manuscript III of this thesis adds another layer to the fly's defensive arsenal against toxic microbes. The importance played by toxic microbes in the fly's ecology is also demonstrated by the remarkably sensitive and selective detection system for geosmin- a volatile indicating the presence of harmful microorganisms (Stensmyr et al., 2012).

Olfactory pheromones in *Drosophila*

So far I have shown olfactory circuits underlying avoidance of parasitoids (Ebrahim et al., 2015, Manuscript I), egg-laying preference to citrus fruits (Dweck et al., 2013, Manuscript II) and preference towards dietary antioxidants (Dweck et al., 2015a, Manuscript III). However apart from that flies need to evaluate mating partners. Many organisms use chemical signals called pheromones to indicate species and sex (Wyatt, 2003). Pheromones are used by insects to distinguish conspecifics from others (Jallon, 1984; Wyatt, 2003; Ferveur, 2005), and also contribute to intraspecific courtship and mating interactions (Jallon, 1984; Wyatt, 2003; Ferveur, 2005). In *Drosophila*, the only identified olfactory pheromone is the male-specific cis-Vaccenyl Acetate (cVA). cVA is produced in the male ejaculatory bulb and transferred to virgin females during copulation (Butterworth, 1969; Brieger and Butterworth, 1970). cVA reduces courtship towards mated females or other males, modulates male-male aggression, and increases receptivity in females (Jallon, 1984; Ejima et al., 2007; Kurtovic et al., 2007; Wang and Anderson, 2010; Liu et al., 2011). At long ranges, cVA acts as an aggregation factor for males and females (Bartelt et al., 1985). cVA is detected by two olfactory receptors in both sexes, Or67d and Or65a (van der Goes van Naters and Carlson, 2007; Kurtovic et al., 2007). Or67d is one of three olfactory receptors that express the male- specific isoforms of the transcription factor *fruitless* (FruM) (Manoli et al., 2005; Stockinger et al., 2005). The role of cVA on sexual behavior seems to be mediated by OR67d activation (Ejima et al., 2007; Kurtovic et al., 2007). Acute promotion of aggression depends on OR67d, while chronic exposure to cVA reduces aggression through OR65a activation (Wang and Anderson, 2010; Liu et al., 2011).

In addition to the fly produced olfactory pheromone cVA, Grosjean et al. (2011) identified other compounds, phenylacetaldehyde (PA) and phenylacetic acid (PAA), that fulfill pheromone-like functions but are present in various fly food sources. These two aromatic compounds activate the second olfactory receptor that express the male-specific isoforms of the transcription factor *fruitless* (FruM), Ir84a (Benton et al., 2009; Grosjean et al., 2011; Silbering et al., 2011). PAA/Ir84a enhances male courtship behavior towards other males and females (Grosjean et al., 2011).

Apart from these two olfactory pheromonal systems, it was several times suggested that two other receptors might be involved in pheromone detection in *Drosophila* (Shorey and Bartell, 1970; Averhoff and Richardson, 1974; Tompkins,1984; Ferveur and Sureau 1996; Savarit, 1999). Or47b-and Or88a-expressing OSNs that house in trichoid sensilla respond to

unidentified odors in male and female body wash extracts (Van der Goes van Naters and Carlson, 2007), suggesting the presence of other volatile pheromones. In manuscript IV (Dweck et al., 2015b) of this thesis, I contributed to the identification and behavioral characterization of three novel volatile pheromones. One of these fly produced odorant, methyl laurate, activates Or47b-expressing OSNs that express the male-specific isoforms of the transcription factor *fruitless* (FruM). Single pair and competition mating experiments revealed that Or47b and Or47b-expressing OSNs are required for optimal male copulation, and that activation of Or47b-expressing OSNs in the male is sufficient to provide a competitive mating advantage. Methyl laurate in addition to the other two fly produced odorants methyl myristate and methyl palmitate activate Or88a-expressing OSNs. Behavioral experiments revealed that flies which lack the olfactory receptor Or88a are no longer attracted to the three fly-produced odorants; however, their mating behavior remains intact. Both receptors and their ligands are conserved over a number of drosophilid species. These results do not only close a significant gap in the understanding of how olfaction mediates mating and aggregation in *Drosophila* but also reveal that while reproductive isolation barriers between species are created mainly by species-specific signals, the mating enhancing signal in several *Drosophila* species is conserved. With the findings of manuscript IV of this thesis, the understanding of male olfactory-based sexual arousal is becoming more complete, with all FruM-positive OSNs now having known ligands.

The second olfactory organ in *Drosophila*

Most of the findings of this thesis (parasitoid avoidance, oviposition preference towards *Citrus* fruits, olfactory detection of dietary antioxidants and pheromones mediating aggregation and mating) and most of the work published on *Drosophila* olfaction focuses on cues that are detected by the antennae. However the vinegar fly, *D. melanogaster*, is equipped with two peripheral olfactory organs, the antenna and the maxillary palp. The antenna, the main olfactory organ, is covered with four types of sensilla: basiconic, trichoid, intermediate and coeloconic. These four sensillar types house OSN types responding to compounds of special biological importance. These include detection of pheromones that promote sexual, aggression and social aggregation behaviors via Or67d (Kurtovic et al., 2010), Or47b, Or88a (Dweck et al., 2015, Manuscript IV), CO2 avoidance via Gr21a and Gr63a (Suh et al., 2004; Jones et al., 2007; Kwon et al., 2007), attraction toward vinegar via Or42b and Or92a (Semmelhack et al., 2009), aversion toward select acids via Ir64a (Ai et al., 2010), preference

for the yeast metabolites phenylacetic acid and phenylacetaldehyde via Ir84a (Grosjean et al., 2011), avoidance of geosmin producing harmful microbes via Or56a (Stensmyr et al., 2012), avoidance of DEET via Ir40a (Kain et al., 2013), oviposition preference for *Citrus*-like fruits via Or19a (Dweck et al., 2013, Manuscript II), attraction to ammonia and select amines through Ir92a (Min et al., 2013), attraction toward farnesol (exact ecological function unclear) via Or83c (Ronderos et al., 2014), and avoidance of parasitoids via Or49a and Or85f in adult and only Or49a in larvae (Ebrahim et al., 2015, Manuscript I).

In contrast, the maxillary palp has only three different types of basiconic sensilla, each housing two different OSNs. It has been shown that the maxillary palp is involved in detecting compounds from mated females (Stocker and Gendre, 1989). Detailed electrophysiological analysis of the maxillary palp olfactory neurons did not reveal such function (deBruyne et al., 1999), and recent studies have localized olfactory receptor neurons responsible for this type of inhibitory behavior toward mated females to the antennal trichoid sensilla (van der Goes van Naters and Carlson, 2007; Kurtovic et al., 2007). Another suggested function of the maxillary palp is avoidance of very high odor concentrations (Charro and Alcorta, 1994). Finally, it was suggested that the maxillary palp contribute to taste enhancement (Shiraiwa et al., 2008). This is because of the overlapping response spectra between maxillary palp and antennal OSNs (deBruyne et al., 1999; deBruyne et al., 2001) as well as the location of the maxillary palp in close vicinity to the labellum, the main taste organ in flies. However, taste enhancement would be a very general function for six types of OSNs expressing seven different odorant receptors (Ors). In manuscript III of this thesis we presented data on the importance of Or71a, which is expressed in pb1B, in proxy detection of dietary antioxidants (Dweck et al., 2015a). It is not yet known whether the other MP-OSNs are also dedicated to detect specific ecologically relevant chemical compounds, and if so, what the ecological importance of these compounds is.

In several other insects, maxillary palp OSNs (MP-OSNs) are involved in the detection of specific chemical compounds that are not covered within the receptive range of antennal OSNs (Ant-OSNs). For example, in both the hawk moth *Manduca sexta* and the African malaria mosquito *Anopheles gambiae*, CO2 detection is primarily mediated via maxillary and/or labial palp OSNs (Thom et al., 2004; Lu et al., 2007), while *Drosophila* detects CO_2 with specific neurons housed in sensilla on the antenna (deBruyne et al., 2001). Mammals are also known to possess several peripheral olfactory organs. In mouse, e.g., the main olfactory epithelium is complemented with the vomeronasal organ, the septal organ and the Grueneberg ganglion, each having distinct functions (reviewed in Knaden and Hansson, 2014). The

presence of specific functions for olfactory organs in other insects and in mammals suggests that the maxillary palp may also be involved in the detection of specific chemicals in *Drosophila*.

In manuscript V of thesis I contributed to the demonstration that the maxillary palp in the vinegar fly, *D. melanogaster*, contains OSNs that mediate short-and long-range attraction evoked by specific chemical compounds. These compounds are either primarily or exclusively detected by MP-OSNs as revealed from electrophysiological experiments using 52 different complex odor sources containing more than 12700 chemical compounds as well as from the dose-response curve experiments. Behavioral experiments in three different paradigms measuring short- and long-range attraction revealed that the maxillary palp contains OSNs that mediate short-and long-range attraction, and that most of the MP-OSNs seem to be tuned to positive cues emanating from food sources and potential feeding and oviposition sites. Especially, 4-ethylguaiacol and methyleugenol that are produced by yeast metabolism of hydroxycinnamic acids, which are important antioxidants in fruits (Tan and Nishida, 2012; Dweck et al., 2015a), strawberry furanone that is found in strawberries and a variety of other fruits (Ulrich et al., 1997), and has antioxidant activity (Slauchter, 1999), and phenyethyl acetate and phenylethyl propionate that are common yeast metabolites (Christiaens et al., 2014). The only exception is the MP-OSN, pb2B that responds to phenol, which is found only in mammal's feces (i.e. sources not suitable for feeding or oviposition in vinegar flies) and has been reported to be toxic (Walker, 2006).

Furthermore, I contributed in comparing the organization, detection and molecular machinery of the maxillary palp in the invasive agricultural pest *D. suzukii*. *D. suzukii* is an international threat that has been spreading rapidly in North America and Europe (Rota-Stabelli et al., 2013). Unlike *D. melanogaster*, *D. suzukii* has a serrated ovipositor that allows penetration of the skin of healthy ripening fruits (Sasaki and Sato, 1995) causing devastating economic cost for farmers each year. Thus, *D. melanogaster* and *D. suzukii* provide an excellent opportunity to understand the olfactory changes that could have occurred in MP-OSNs due to the shift in the feeding and oviposition preference in *D. suzukii*. This comparison revealed that MP-OSN types and their pairing within a particular sensillar type, the responses to the palp specific compounds, and the receptors expressed in MP-OSNs are conserved in *D. suzukii*. Olfactory changes associated with the maxillary palp have been reported for distantly related species of *D. melanogaster*, such as *D. mojavensis* (cactus feeder), *D. virilis* (sap feeder), *D. grimshawi* (tree feeder) and *Scaptomyza flava* (leaf feeder) (Guo and Kim, 2007; deBruyne et al., 2010; Goldman-Huertas et al., 2015). *D. mojavensis*, *D. virilis* and *D. grimshawi* have lost orthologs

of Or59c, which is expressed in pb3A (Guo and Kim, 2007; deBruyne et al., 2010), whereas *S. flava* has lost ortholog of Or85d, which is expressed in ab3B (Goldman-Huertas et al., 2015). In line with these notions pb3A was not found in *D. virilis* (deBruyne et al., 2010). On the other hand, for closely related species of *D. melanogater*, no olfactory changes associated with the maxillary palp have been reported (Guo and Kim, 2007; deBruyne et al., 2010), which is consistent with our results from *D. suzukii*.

GENERAL SUMMARY

Because of its genetic tractability and the anatomical simplicity of the olfactory system, the vinegar fly *Drosophila melanogater* has become the leading model for the discovery of olfactory neuronal pathways underlying the behavioral responses to compounds of specific biological importance.

In this thesis I describe the identification of olfactory circuits underlying avoidance of *Leptopilina* larval endoparasitoid wasps in *Drosophila* adults and larvae. This avoidance is mediated by a highly specific olfactory sensory neuron (OSN) type that is tuned to detect odors of one of the main parasitoids, wasps of the genus *Leptopilina*. The larval OSN expresses the olfactory receptor Or49a and is tuned to the waps' sex pheromone iridomyrmecin exclusively, while the adult OSN expressing both Or49a and Or85f detects in addition to iridomyrmecin, the wasp odors actinidine and nepetalactol. This information is transferred via the primary olfactory processing center, the antennal lobe to a specific region in the lateral horn that is known to be involved in coding aversive stimuli. The OSN type expressing Or49a and Or85f is both necessary and sufficient to govern the parasitoid avoidance behavior, and is conserved across several *Drosophila* species.

I also contributed to the demonstration that *Drosophila* adults prefer *Citrus* fruits as oviposition substrates. This preference is due to the high content of terpenes in the flavedo (i.e. the colored rind found in *Citrus* fruits). Flies detect these terpenes via only a single class of olfactory sensory neurons, which in this case express the odorant receptor Or19a. These neurons are both necessary and sufficient for this oviposition preference. The preference has likely been driven by the need to avoid parasitism from endoparasitoid wasps, since the same terpene ligands that mediate fly oviposition are also potent repellents for parasitic wasps that oviposit in fly larvae.

Furthermore, this thesis describes that flies are attracted to dietary antioxidants. Adult flies are more attracted towards, feed and lay eggs on yeast-inoculated media containing the common hydroxycinnamic acids (HCAs) p-coumaric acid and ferrulic acid – potent dietary antioxidants abundant in fruit. Fly larvae, too, are attracted to media containing these acids. Flies detect the presence of HCAs via ethylphenols, which are exclusively derived from the yeast metabolism of these acids. Adult flies use a different olfactory receptor (Or71a) that is expressed in olfactory sensory neurons on the maxillary palps than larval flies (Or94b) to detect these compounds. These neurons in both adult and larval flies are necessary and

sufficient for the proxy detection of dietary antioxidants. These results provide the first indication that animals are able to use olfactory cues to judge content of dietary antioxidants.

Moreover, I contributed to the identification and behavioral characterization of the three novel volatile pheromones methyl laurate, methyl myristate and methyl palmitate. One of these fly produced odorants, methyl laurate, activates Or47b-expressing OSNs that express the male-specific isoforms of the transcription factor *fruitless* (FruM). Or47b-expressing OSNs are required for optimal male copulation, and activation of Or47b-expressing OSNs in the male is sufficient to provide a competitive mating advantage. In addition all three volatiles activate Or88a-expressing OSNs. Or88a-expressing OSNs are necessary and sufficient for aggregation behavior in both males and females. Both receptors and their ligands are conserved over a number of drosophilid species. These results close a significant gap in the understanding of how olfaction mediates mating and aggregation in *Drosophila*.

Finally, I contributed to the dissection of the functional significance of the maxillary palp in *Drosophila*. The maxillary palp of *D. melanogaster* contains OSNs that mediate short-and long-range attraction evoked by specific chemical compounds. These compounds are either primarily or exclusively detected by maxillary palp OSNs, which detect these compounds with remarkably high sensitivity.

In conclusion, the findings of this thesis clarify how the antennae and the palps as well as specific olfactory circuit contribute to the decision of a fly, whether or not to feed, oviposit or mate.

ZUSAMMENFASSUNG

Aufgrund der umfangreichen molekular genetischen Werkzeuge, die für *Drosophila melanogaster* etabliert wurden und ihrer relativen anatomischen Einfachheit eignet sich die Essigfliege für die besonders für die Erforschung von duftgesteuertem Verhalten und die zugrunde liegenden neuronalen Netzwerke.

In meiner Arbeit beschreibe ich, wie sich Essigfliegen und ihre Larven durch die Detektion und Vermeidung des Sexuallockstoffes eines ihrer Hauptfeinde vor Parasitierung schützen. Parasitoide Wespen der Gattung *Leptopilina* legen ihre Eier in die Larven von *Drosophila.* Um sich dagegen zu schützen, haben die Fliegen ein sensorisches Neuron entwickelt, das spezifisch auf das Sexualpheromon der Wespe (und bei adulten Fliegen zwei weitere Wespendüfte) reagiert. Während dieses Neuron in der Larve nur den olfaktorischen Rezeptor Or49a exprimiert, der hochspezifisch ist für das Wespenpheromon Iridomyrmecin, exprimiert das gleiche Neuron in den adulten Fliegen zusätzlich Or85f, was die zusätzliche Detektion von zwei weiteren Wespendüften (Actinidin und Nepatalactol) ermöglicht. Durch künstliche Aktivierung bzw. Inaktivierung dieses Neurons, konnten wir zeigen, dass es notwendig und hinreichend ist, um das duftgesteuerte Vermeidungsverhalten von Larven und adulten Fliegen zu erklären. Interessanterweise wird die Information, die dieses Neuron liefert, in einem höheren Hirnzentrum, dem lateralen Horn, an einer Stelle verarbeitet, die auch für die Verarbeitung eines schon früher beschriebenen abschreckenden Duftes verantwortlich ist.

Ich nahm zusätzlich an einer Untersuchung teil, die ergab, dass Essigfliegen durch den Duft von Zitrusfrüchten zur Eiablage motiviert werden. Als ausschlaggebende Düfte konnten wir Terpene in der Schale der Früchte identifizieren, die von dem Duftrezeptor Or19a detektiert werden. Die Vorliebe für Zitrusfrüchte scheint für die Fliegen von Vorteil zu sein, da die gleichen Früchte aus bisher unbekannten Gründen von parasitoiden Wespen vermieden werden.

Weiterhin beschreibe ich in meiner Arbeit, dass Fliegen in der Lage sind, Nahrung mit einem hohen Anteil an gesunden Antioxidantien zu erkennen. Sie steuern diese Nahrung bevorzugt an und legen dort auch mehr Nahrung als auf Kontrollmedium. Die Fliegen detektieren die Antioxidantien jedoch nicht direkt sondern detektieren stattdessen Ethylphenole, deren Vorkommen jedoch an das Vorhandensein von Antioxidantien streng gekoppelt ist. Interessanterweise detektieren adulte Fliegen diesen Duft mit einem anderen Rezeptor (Or71a) als die Larven (Or94b). Wie schon bei dem Parasitoiden-spezifischen Neuron,

konnten wir zeigen, dass die Neurone, welche entweder Or71a oder Or94b exprimieren, für das beobachtete Verhalten notwendig und hinreichend sind.

Fliegen müssen jedoch nicht nur Feinde vermeiden und Nahrungsquellen evaluieren. Sie müssen auch Paarungspartner finden und deren Qualität einschätzen. Ein weiterer Teil meiner Arbeit beschreibt, dass sie dieses anhand der drei neu entdeckten Pheromone Methyllaurat, Methylmyristat und Methylpalmitat tun. Während alle drei Düfte von einem sensorischen Neuron, das den Rezeptor Or88a exprimiert wahrgenommen werden, wird Methyllaurat zusätzlich von einem Neuron, das Or47b exprimiert, detektiert. Wir konnten zeigen, dass über das Or47b-Neuron das Paarungsverhalten der Fliegen beeinflusst wird (erhöhte Konzentrationen von Methyllaurat auf Weibchen führen zu mehr erfolgreichen Paarungen), während das Or88a-Neuron nicht direkt in die Paarung eingreift, sondern eine generelle Attraktivität der drei Düfte garantiert. Sowohl die Rezeptoren als auch die drei Düfte fanden wir in vielen unterschiedlich nah verwandten Fruchtfliegenarten. Sie dienen daher nicht der Artentrennung sondern ausschliesslich der Kommunikation innerhalb der Arten.

Abschliessend konnte ich noch zeigen, inwieweit das zweite olfaktorische Organ der Fliege, die Maxillarpalpen, duftgesteuertes Verhalten beeinflussen. Die Neurone in den Sensillen der Palpen detektieren zum Teil Düfte, die von der Antenne nicht wahrgenommen werden. Somit ergänzen sich beide Organe in ihrer Funktion. Ein Grossteil der von den Palpen wahrgenommenen Düfte ist attraktiv und lockt Fliegen sowohl über kurze Distanzen (in kleinräumigen Arenaexperimenten) als auch über größere Distanzen (im Windkanal) an.

Insgesamt zeigt meine Arbeit, dass es im olfaktorischen System der Fliege mehr spezifische Informationskanäle gibt, als bisher angenommen wurde. Gerade lebenswichtige Entscheidungen, was gefressen werden soll, mit wem man sich paaren sollte, und wo die Eier hingelegt werden sollten, scheinen oft durch spezifische Neuronen gesteuert zu werden.

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DECLARATION OF INDEPENDENT ASSIGNMENT

I declare in accordance with the conferral of the degree of doctor from the School of Biology and Pharmacy of Friedrich-Schiller University, Jena that the submitted thesis was written only with the assistance and literature cited in the text.

People who assisted in experiments, data analysis and writing of the manuscripts are listed as co-authors of the respective manuscript. I was not assisted by a consultant for doctorate theses.
The thesis has not been previously submitted whether to the Friedrich-Schiller University Jena or to any other university.

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