Food odors trigger *Drosophila* males to deposit a pheromone that guides aggregation and female oviposition decisions

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Running title: Food odors trigger pheromone signaling.

ABSTRACT:
Animals use olfactory cues for navigating complex environments. Food odors in particular provide crucial information regarding potential foraging sites. Many behaviors occur at food sites, yet how food odors regulate such behaviors at these sites is unclear. Using *Drosophila melanogaster* as an animal model, we found that males deposit the pheromone 9-tricosene upon stimulation with the food-odor apple cider vinegar. This pheromone acts as a potent aggregation pheromone and as an oviposition guidance cue for females. We use genetic, molecular, electrophysiological, and behavioral approaches to show that 9-tricosene activates antennal basiconic Or7a receptors, a receptor activated by many alcohols and aldehydes such as the green leaf volatile E2-hexenal. We demonstrate that loss of Or7a+ neurons or the Or7a receptor abolishes aggregation behavior and oviposition site-selection towards 9-tricosene and E2-hexenal. 9-Tricosene thus functions via Or7a to link food-odor perception with aggregation and egg-laying decisions.

INTRODUCTION:

Animals must navigate a complex and changing environment for survival and reproduction. Odorants function as molecular cues for objects in the environment, and the olfactory system translates these cues into appropriate behaviors [1-4]. Living organisms are also a source of odorants, broadly termed pheromones, which play important roles in olfactory communications between different organisms of the same species [5]. Despite a wealth of knowledge of pheromone identities and their physiological functions, how environmental cues interact with pheromone signaling is not well understood.

A behavior largely mediated through pheromone signaling is population aggregation, which is hypothesized to ensure efficient use of resources [6]. Aggregation behavior may reduce interspecific competition and also be important for finding mates [7]. However, how aggregation pheromones are induced or deposited to mark certain geographical location and modulate animal behaviors remains largely unknown. In *Drosophila melanogaster*, aggregation behavior has been observed at locations that contain male flies and food substrates. The male specific pheromone cis-vaccenyl acetate (cVA) has been implicated as the key aggregation pheromone that attracts both males and females [8, 9]. cVA is manufactured and stored in an internal male organ (ejaculatory bulb) and transferred to females during copulation [10, 11], where it plays a role in inhibiting male courtship of previously mated females [12]. Low levels of cVA may be present on males prior to mating [8, 13]. Interestingly, flies defective in sensing cVA exhibit residual aggregation behavior, suggesting the existence of an aggregation compound besides cVA from male flies [9].

Drosophila pheromones are typically cuticular hydrocarbons that are produced by specialized cells (oenocytes) in the fly abdomen and form a waxy layer on the body surface [6, 14, 15]. Given the chemical nature of long chain hydrocarbons, most cuticular hydrocarbons are not volatile and are instead detected by gustatory contact [16]. For instance, 7-tricosene, an abundant male cuticular hydrocarbon, functions as an aphrodisiac for females and anti-aphrodisiac for males and is sensed via the gustatory system [17, 18]. Nonetheless, recent solid-phase micro-extraction gas chromatography experiments indicated the presence of volatile cuticular hydrocarbon pheromones, suggesting pheromone detection might be medicated through the olfactory system [13, 16]. All together, these studies suggest an uncharacterized cuticular hydrocarbon might function as an aggregation pheromone and possibly signaling via the olfactory system.

Understanding how volatile pheromones may affect animal behavior is aided by identifying the odorant receptors activated by that pheromone. Studies using cuticular extracts or flies as odor sources identified neurons within trichoid sensilla as responding most robustly to *Drosophila* pheromones [19]. The pheromones activating each of the four pheromone receptors (Or67d, Or65a, Or88a, Or47b) have now been identified: cVA robustly activates Or67d [12, 20-22] and, to a lesser degree, Or65a [12, 23]; and fatty acid methyl ethers methyl laurate, methyl myristate, and methyl palmitate activate Or88a [24], whereas only methyl laurate activates Or47b [24]. In each case, the pheromone receptor is specifically tuned to respond only to the identified pheromones, and exhibit little response to a large panel of other odorants [24, 25]. In addition, the pheromones activate only the identified pheromone receptors, and show little effect on other olfactory receptors.

Here, we report the finding that male flies deposit an aggregation pheromone onto their surroundings upon apple cider vinegar odor and food odor stimulation. The pheromone, 9-tricosene, is a volatile male-specific cuticular hydrocarbon and requires the olfactory, but not gustatory, system for detection. By electrophysiological and behavior studies, we identify the Or7a olfactory neurons housed in basiconic sensilla as being necessary and sufficient for 9-tricosene pheromone detection. This is surprising as Or7a, in contrast to previously characterized pheromone receptors, can be classified as a 'generalist' odorant receptor as it can respond to many aldehydes and alcohols [25]. Behaviorally, 9-tricosene promotes aggregation and modulates female oviposition site selection- a behavior that was previously considered a female exclusive decision [26, 27]. The green leaf volatile E2-hexenal, a previously characterized robust Or7a agonist [25, 28], can also guide Or7a-dependent attraction and egg-laying decisions. Three additional Or7a odor agonists could also guide positive oviposition site selection, suggesting that Or7a neuron activation might directly influence this behavior. Indeed, selective optogenetic stimulation of Or7a neurons was also sufficient to guide egg-laying site selection. Our study provides important insights into biological communication by identifying an olfactory mechanism that links together food-odor perception, male pheromone deposition, species aggregation, and female oviposition decision-making.

RESULTS

A Novel Chemosensory Assay Identifies a Post-Stimulus Aggregation Behavior

Traditional olfactory assays monitor either single flies [1] or multiple flies in small spaces [29] and might overlook important aggregation behaviors. We modified a four-

104 field olfactory arena and fly tracking system [1, 30] to monitor large fly populations
105 responding to odors over a large arena space. Flies are contained in a star-shaped arena
106 between two glass plates (See Experimental Procedures for details), tracked in a dark
107 chamber using infrared illumination (which is invisible to flies), and detected by an
108 infrared camera (Figure 1A). We validated our experimental design by monitoring
109 attraction to apple cider vinegar, repulsion to citronellal, and neutral responses to clean
110 air (Figure 1—figure supplements 1-3). Flies mutant for *orco*, a necessary co-receptor for
111 most olfactory receptor neurons [31], showed reduced responses to control odorants,
112 confirming the experimental design accurately assesses olfactory behaviors (Figure 1—
113 figure supplement 4).

114 During investigations with the food-odor apple cider vinegar, we identified a
115 novel olfactory behavior in which flies showed robust aggregation to the original odor
116 quadrant for substantial time periods subsequent to odor application (Figures 1B, 1C, and
117 1G). In these experiments, flies were stimulated with apple cider vinegar for 5 minutes,
118 apple cider vinegar odor was switched to clean air for 10 minutes, and flies tracked in an
119 arena that had been rotated 90 degrees to rule out contamination in the odor delivery
120 system (Figure 1B, Videos 1 and 2). Interestingly, this aggregation behavior in the
121 absence of exogenous odor-stimulation persisted for >25 minutes (Figure 1—figure
122 supplement 5). We called this behavior “post-stimulus aggregation”. Perfusion of apple
123 cider vinegar into the empty arena for 5 minutes in the absence of flies, and introduction
124 of naïve flies into the arena, did not produce post-stimulus aggregation to the original
125 quadrant (Figure 1D), suggesting the post-stimulus aggregation behavior is not due to
126 residual apple cider vinegar on the glass plates.

Post-Stimulus Aggregation Involves Pheromone Deposition

Fly bodies are coated with cuticular hydrocarbons that can function as chemosensory pheromones [14, 16, 19, 32]. To rule out the possibility of passive pheromone deposition onto the glass plates due to crowding of many flies into a small space, flies were corralled into the odor quadrant by attraction to humidified air and monitored for post-stimulus aggregation behavior. Under these conditions, there was no detectable post-stimulus aggregation (Figures 1E and 1F). All together, these data suggest that flies may deposit pheromone(s) in a specific response to the food-odor apple cider vinegar. Different concentrations of apple cider vinegar generated post-stimulus aggregation responses of different potencies (Figure 1—figure supplement 6).

Post-Stimulus Aggregation Behaviors are Stimulated by Food-Odors

To determine if post-stimulus aggregation could be induced by other food odors, we tested the food odors banana and yeast paste (Figure 1—figure supplement 7). These stimuli were highly attractive to flies and also induced post-stimulus aggregation. As a further test, we examined stimulus attraction by a prominent attractive monomolecular odorant of apple cider vinegar: ethyl acetate. Although this odorant was highly attractive, it failed to generate post-stimulus aggregation behaviors (Figure 1—figure supplement 7). Together, these data suggest that post-stimulus aggregation behaviors may be guided specifically by food odors, and possibly by food odor perceptions. Future studies will be aimed at characterizing how food-odor perceptions might be encoded by a fly’s olfactory system to direct pheromone deposition.

Males are the Source of the Aggregation Pheromone

Intra-species communications via pheromones are often sex-specific. We used different combinations of flies (mixed genders, virgin females, or males) as potential pheromone depositors upon apple cider vinegar stimulation, and new naïve mixed genders as detectors for the presence of the pheromone (Figure 1H). Only in the presence of male depositor flies did detector flies show post-stimulus aggregation (Figure 1I). This indicates that male flies are the source of the pheromone. The aggregation pheromone was equally attractive to both virgin and mated males and females (Figure 1—figure supplement 8).

Detection of the Aggregation Pheromone Requires *Orco*

Pheromones are detected by the olfactory and gustatory systems [6, 17, 18, 32]. Since the pheromone is deposited onto the glass plates, it might be detected by either chemosensory system. We utilized genetic mutants that are defective in specific modes of chemosensory signaling as detectors (Figure 2A). *Poxn* mutants, which exhibit no functional gustatory receptor neurons [33], were as attracted as wild-type animals (Figure 2B), suggesting that the gustatory system is not necessary for post-stimulus aggregation. Pickpocket channel 23 (ppk23), a Degenerin/epithelial sodium channel, is necessary for the detection of a male-predominant cuticular hydrocarbon, 7-tricosene [17, 18]. *ppk23* mutants exhibited similar post-stimulus aggregation compared to wild-type animals (Figure 2B), suggesting Ppk23 function is not necessary for aggregation and that 7-tricosene is unlikely to be the aggregation pheromone. Most insect olfactory receptors

require a coreceptor(s) for normal olfactory responses: *Orco* for Odorant Receptors [31] and *Ir8a* or *Ir25a* for ionotropic receptors [34, 35]. The *Ir8a* and *Ir25a* double mutant flies exhibited normal post-stimulus aggregation, showing that most ionotropic receptors are not required for the pheromone attraction (Figure 2C). Interestingly, in *orco* mutant flies, attraction behavior to the pheromone was completely abolished and instead repelled by the apple cider vinegar quadrant (Figure 2C). This repulsion is likely due to acid sensing of minimal residual acetic acid on the quadrant mediated by the *Ir8a*/*Ir64a* complex [36] (Figure 2—figure supplement 1). Indeed, the *orco*, *Ir8a* double mutant was no longer repelled by the odor quadrant (Figure 2C), and the use of neutralized apple cider vinegar as the stimulus eliminated the post-stimulus repulsion demonstrated by *orco* mutants (Figure 2—figure supplement 2). We further tested if acidity of the apple cider vinegar was necessary for triggering wild-type post-stimulation behavior by neutralizing apple cider vinegar to pH=7.0. No phenotypic difference was found compared to non-neutralized apple cider vinegar (pH=3.2)(Figure 2—figure supplement 3). In sum, these data suggest that detection of the food-odor-induced pheromone is mediated through the *orco*-dependent olfactory system.

cVA has been suggested to be a male-derived aggregation pheromone in *Drosophila melanogaster* [8]. cVA induces conformational changes in the odorant binding protein LUSH, which enhances activation of Or67d/Orco complexes [9, 22]. Furthermore, the *Drosophila* CD36 homologue, sensory neuron member protein (Snmp), is essential for optimal Or67d neuronal activation [20]. cVA can also activate Or67d/Orco complexes directly [37]. Mutations of the key components in the signaling pathway (*Or67d*, *lush*, *snmp*) do not alter the post-stimulus aggregation behavior (Figure

196 2—figure supplement 4), suggesting that cVA is not the food-odor induced aggregation
197 pheromone.
198
199 **9-Tricosene is a Food-Odor Induced Aggregation Pheromone**
200 Since most insect pheromones are lipophilic carbohydrates dissolvable in hexane
201 [19], we reasoned that hexane might extract the active pheromone off the glass surface in
202 the food-odor-induced quadrant. We induced wild-type flies to deposit the food-odor
203 induced pheromone onto the glass plate and dissolved the deposited molecules into
204 hexane. We then painted the hexane extract onto a new glass plate in a letter 'E' pattern.
205 Naïve new flies were able to trace and follow the E pattern but do not follow control
206 hexane extracts of flies stimulated by humidified air painted in the same pattern (Figures
207 3A, 3B, Figure 3—figure supplement 1 and Video 3). These experiments demonstrated
208 that an active pheromone component(s) was successfully preserved during the pheromone
209 extraction. Behavioral results suggested the pheromone was volatile because: 1) it
210 required the olfactory system for detection (Figure 2C); 2) constant air flushing reduced
211 behavioral attraction after ~25 minutes (Figure 1—figure supplement 5); and 3) heating
212 the pheromone-containing arena to 32˚C to increase odor volatility eliminated post-
213 stimulus behaviors (Figure 3—figure supplement 2). Recently, four cuticular
214 hydrocarbons were identified as male-specific volatile pheromones: 7-docosene, 5-
215 tricosene, 23-methyldocosane, and 9-tricosene [13]. To identify the nature of the
216 pheromone(s), we performed gas chromatography-mass spectrometry (GC-MS) analyses
217 of hexane extracts from quadrants stimulated with apple cider vinegar alone, with
218 humidified air+flies, and with apple cider vinegar+flies. Consistent with behavioral

219 results that cVA is unlikely to be the food-odor induced pheromone (Figure 2—figure
220 supplement 4), cVA was not detected in pheromone extracts from the glass plates
221 (Supplementary File 1). 7-docosene and 23-methyldocosane were also not detected, while
222 5-tricosene was detected at trace amounts (Figure 3C and Supplementary File 1). The
223 levels of 7-tricosene were increased in the experimental conditions. However, our
224 behavioral results excluded 7-tricosene as the food-odor-induced pheromone (Figure 2B;
225 also see below and Figure 5—figure supplement 3). Interestingly, only one other peak
226 was significantly enriched in the experimental but not humidified air+flies control group:
227 (Z) 9-tricosene (9-T) (Figure 3C, Supplementary File 1). Little is known regarding the
228 function of 9-tricosene in *Drosophila melanogaster* besides its presence as a male-
229 specific volatile pheromone [11, 13]. To determine if 9-tricosene was attractive to
230 *Drosophila*, as would be predicted for the food-odor induced aggregation pheromone, we
231 used 9-tricosene as the stimulus in the 4-field olfactory assay. Indeed, 9-tricosene elicits
232 attraction behaviors (Figures 3D-3E). To determine if 9-tricosene could direct
233 aggregation behaviors, we repeated the 'E' experiment using 9-tricosene alone (Figure
234 3F). Naïve flies did trace and follow the 9-tricosene pattern, paying particular attention to
235 the odor border (Figure 3—figure supplement 3). Nonetheless, the aggregation response
236 was not identical to the full hexane extract. In particular, flies appeared to be repelled by
237 the center of a concentrated 9-tricosene odor trail. The sensory cause of this repulsion
238 remains to be identified, but may arise from high concentrations of 9-tricosene mediating
239 olfactory or gustatory contact repulsion. Such highly concentrated 9-tricosene deposits
240 are unlikely to be found after food-odor-induced pheromone deposition in the four-field
241 olfactory assay. Overall, these data suggest that 9-tricosene under certain conditions can

function as an aggregation pheromone, although additional pheromone components may contribute to the full aggregation phenotype.

9-Tricosene is a member of cuticular hydrocarbons produced by oenocytes on the fly abdominal wall [11, 15]. 9-Tricosene and other cuticular hydrocarbon components, but not cVA or methyl ethers pheromones, can be genetically eliminated by specifically ablating oenocytes [15, 24]. Oenocyte-less males mixed with wild-type females no longer produced a post-stimulus aggregation behavior, suggesting that an oenocyte-derived cuticular hydrocarbon is essential for post-stimulus aggregation (Figure 3—figure supplement 4). Although other oenocyte-derived cuticular hydrocarbons may contribute to the aggregation phenotype, this is consistent with the identification of 9-tricosene as a food-odor induced pheromone.

The Or7a Receptor is Necessary and Sufficient for 9-tricosene Activation

The olfactory sensory neurons in *Drosophila* can be classified as those that require either *orco* (odorant receptors expressed in basiconic, intermediate, trichoid and ac3 sensilla) [31, 38] or those that are *orco*-independent (ionotropic receptors expressed in coeloconic sensilla and gustatory receptors expressed in the ab1C neuron) [2, 34, 35]. To determine which odorant receptors are required for 9-tricosene responses, we performed electroantennogram (EAG) recordings, which measure global detection of odor-induced antennal responses, in *WT* and *orco* mutants (Figure 4A). The *orco* mutants completely lacked responses to 9-tricosene (Figures 4A and 4B). This suggests that 9-tricosene activates an *orco*-dependent odorant receptor, and does not require signaling from *Ir* or *Gr* receptors.

We next identified the *orco*-positive olfactory receptor neurons (ORNs) that respond to 9-tricosene, by using Fluorescence-guided Single Sensillum Recording (FgSSR) [39], which detects the activity of olfactory neurons within single sensory sensilla. Previously characterized volatile pheromones typically activate trichoid sensillar neurons [19], and kairomones (odorants released by other animals or plants) typically activate intermediate sensillar neurons [3, 30, 39-41]. Surprisingly, 9-tricosene did not stimulate these sensillar neurons (Figure 4C). We found that 9-tricosene elicits rapid and robust firing patterns in the antennal basiconic ab4 sensillum, which houses two neurons (ab4A and ab4B) that express Or7a (ab4A) or Or56a receptors (ab4B) (Figure 4C) [3, 38, 42]. 9-Tricosene stimulates spiking of the larger amplitude neuron indicating the 9-tricosene-responsive ORNs are ab4A, which express Or7a receptors. Stimulation of ab4 sensilla by flies housed in a glass vial pre-stimulated with apple cider vinegar, compared to flies pre-stimulated with dry air alone, also led to significant increases in ab4A (Or7a) activation (Figure 4—figure supplement 1). The identification of Or7a as a 9-tricosene pheromone receptor was surprising as, unlike previously identified pheromone receptors, it had been shown to respond to a broad range of odors, including many aldehydes and alcohols [25, 28]. To determine if the Or7a receptor is sufficient for 9-tricosene responses, we misexpressed Or7a in an olfactory neuron that lacks an odorant receptor in ab3A sensillar neurons [43]. Expression of Or7a endowed ab3A neurons the ability to respond to 9-tricosene comparable to the 9-tricosene activation pattern detected in endogenous Or7a-positive ab4 sensillum (Figures 4D and 4E). These data indicate that the Or7a receptor responds to 9-tricosene. This is unexpected since basiconic sensilla were traditionally considered food odor detectors [2, 4, 31, 42, 44]. Both male and female

ab4A/Or7a neurons responded equally to 9-tricosene (Figure 4E), consistent with 9-tricosene being attractive to both males and females (Figure 1—figure supplement 8). Or7a neurons did not respond to cVA (Figure 4—figure supplement 2).

To further verify that Or7a was responsible for the 9-tricosene responses of ab4A neurons, we generated *Or7a* mutants using homologous recombination (Figure 4—figure supplement 3). Ab4 sensilla in *Or7a* mutants were identified based on their shape and the specific response of the ab4B neuron to geosmin [3]. No spontaneous or 9-tricosene stimulated ab4A spiking activity was observed in *Or7a* mutant flies (Figures 4F and 4G), indicating that Or7a is necessary for 9-tricosene activation in ab4 sensilla. All together, these data suggest that 9-tricosene specifically activates the 'generalist' Or7a receptor.

The Or7a Receptor is Necessary for Pheromone and 9-tricosene Induced Aggregation

To identify the full expression pattern of Or7a, we genetically converted our *Or7a* mutant to an *Or7a-GAL4* knock-in, in which *GAL4* is under control of the endogenous *Or7a* promoter [45]. *Or7a-GAL4* specifically drives effector expression only in olfactory neurons that target the DL5 antennal lobe glomerulus (Figure 5A) [38]. We did not detect expression outside the antennae (Figure 5—figure supplement 1). We thus could use the *Or7a-GAL4* line to specifically ablate $Or7a^{+}$ antennal neurons and test for changes in behavior. Aggregation responses to both the naturally deposited food-odor induced pheromone (Figures 5B and 5C) and to 9-tricosene (Figures 5D and 5E) were completely abolished in Or7a neuron-ablated flies obtained using *Or7a-Gal4+UAS-hid* (Figure 5) or *Or7a-Gal4+UAS-DTI* (Figure 5—figure supplement 2). Ablation of other odorant

311 receptors (*OrX-Gal4+UAS-hid*) did not affect aggregation (Figure 5C). Similarly, *Or7a*
312 mutants completely lacked attraction to the naturally deposited food-odor-induced
313 pheromone and to 9-tricosene (Figures 5B-5E). To verify that aggregation behavior is
314 specific to 9-tricosene, we repeated the experiments using the 9-tricosene pheromone
315 isomer 7-tricosene (7-T), which contains an identical carbon chain length to 9-tricosene
316 but a double bond at an alternate location. Interestingly, 7-tricosene induced a neutral to
317 slightly repulsive behavior in WT flies (AI=-0.07 ± 0.026, Figure 5—figure supplement
318 3). These data suggest that aggregation behavior to the naturally deposited food-odor-
319 induced pheromone depends on 9-tricosene and proper function of the *Or7a* receptor.
320
321 **9-Tricosene Guides Oviposition Site Selection via Or7a Neurons**
322 Many behaviors occur at food sources, including courtship and egg-laying, but the
323 molecular signals that help guide these behaviors remain poorly characterized [6]. Since
324 9-tricosene may aggregate flies to sites of food-odor perception, we asked whether 9-
325 tricosene could be an olfactory mechanism for catalyzing such behaviors.
326 *Drosophila* preferentially lay their eggs in food sources so as to increase survival
327 of their progeny [27, 41, 46]. Since 9-tricosene acts as a geographical marker for food, it
328 could function as a male-generated guide for female egg-laying decisions. We modified
329 our 4-field arena by spreading a thin layer of 1% agarose onto one of the glass plates to
330 serve as a substrate for the deposited pheromone and an appropriate medium for female
331 egg-laying (Figure 6A). In order to rule out potentially confounding roles of males in this
332 behavior, only previously mated females were assayed. Under conditions in which the
333 food-odor-stimulated pheromone was deposited onto the agarose (Figure 6A’), females

laid five-fold more eggs in the pheromone quadrant. This suggests that a deposited pheromone could guide female egg-laying site selection decisions. We next generated an arena in which one quadrant contained 9-tricosene (Figure 6—figure supplement 1). Female flies also laid significantly more eggs in locations containing only 9-tricosene (Figure 6B'). The 9-tricosene egg-laying preference was abolished when Or7a neurons were ablated (*Or7a-Gal4/UAS-hid* or *Or7a-Gal4/UAS-DTI*) (Figures 6C' and Figure 6—figure supplement 2). The 9-tricosene guided egg laying preference was also abolished in *Or7a* mutant flies (Figure 6D). The oviposition preference for the 9-tricosene quadrant was not due to the innate attraction to 9-tricosene because females spent similar time in the four quadrants over the course of the 23 hour egg-laying assay (Figure 6E).

Hydrocarbons could be potential food sources for larvae and female flies might thus preferentially lay eggs in locations containing cuticular hydrocarbons. To verify that oviposition guidance is specific to 9-tricosene, we repeated the oviposition experiments using the 9-tricosene pheromone isomer 7-tricosene (7-T), the most abundant cuticular hydrocarbon in male flies [11]. Female flies did not preferentially oviposit in the 7-T quadrant (Figure 6F). Interestingly, total egg numbers laid were significant higher in *Or7a* mutant and Or7a neuron ablated flies, implying a potential connection of oviposition site selection and egg deposition number (Figure 6G). The female ovipositor can be involved in the detection and guidance to egg-laying cues [26]. However, since Or7a is expressed only in the antennae and not in the female ovipositor (Figure 5—figure supplement 1), this implicates Or7a signaling in the antennae as the main driver of Or7a-directed oviposition guidance. All together, these data suggest that male derived 9-

tricosene can guide female egg-laying preferences, and this decision-making process requires proper antennal Or7a neuronal function.

E2-Hexenal Activation of Or7a Mimics 9-Tricosene Guided Behaviors

Comprehensive SSR surveys of odorant-receptor activities identified multiple ligands for the Or7a receptor [25]. E2-hexenal, a leafy green volatile released upon fruit or leaf damage [47], was identified as the most potent ligand for Or7a [25]. E2-hexenal could thus be an abundant odorant for Or7a at damaged or rotting fruits and might contribute towards Or7a-mediated behaviors. We thus examined if E2-hexenal could direct similar behaviors as those triggered by 9-tricosene (Figure 7). Indeed, we found that E2-hexenal, like 9-tricosene, directed Or7a-dependent egg-laying site selection (Figures 7A-7D) and attraction (Figure 7E).

The presence of E2-hexenal, as a potentially abundant Or7a activator, might confound localized 9-tricosene guided behaviors. The concentration of E2-hexenal at a foraging site will vary depending on the fruit source, the stage of ripening [48], and the extent of fruit or leaf damage. Most undamaged plants emit undetectable levels of E2-hexenal [47, 49-51]. However, a single cut on an *Arabidopsis* leaf can produce ~ 28 parts per trillion of E2-hexenal (~$3x10^{-11}$ E2-hexenal) [52]. As an upper limit of E2-hexenal concentrations at a foraging site, a fully disrupted source (*e.g*, a blended apple, tomato, or *Arabidopsis* leaf) can produce approximately 5-10 parts per million of E2-hexenal (~10^{-5} -10^{-6} E2-hexenal) [47, 48, 51-53]. To determine if 9-tricosene pheromone cues remained recognizable in a surrounding of E2-hexenal, we repeated the 9-tricosene (10^{-4} concentration) 3-choice oviposition-selection assays in the presence of high (10^{-6}, 10^{-7}),

middle (10^{-8}) or low (10^{-9},10^{-10}) E2-hexenal concentrations (Figure 7—figure supplement 1). 9-tricosene functioned as an oviposition guidance cue in the presence of low and middle, but not high, E2-hexenal concentrations. These results suggest that 9-tricosene can effectively guide oviposition in the surrounding presence of E2-hexenal.

The ability of both 9-tricosene and E2-hexenal to guide oviposition-site selection suggested that Or7a activation might be a key signal for this behavior. We therefore tested the oviposition-site selection guidance to 3 additional Or7a-agonists (benzaldeyde, 1-butanol, 1-propanol) as well as 3 odorants that do not activate Or7a (hexyl butyrate, pentanoic acid, ethyl lactate) [25]. Interestingly, all odorants that activated Or7a directed positive oviposition-site selection to some degree, whereas all odorants not activating Or7a showed mainly neutral or negative oviposition-site selection effects (Figure 8). The exception is pentanoic acid at high concentrations. This high concentration may recruit additional odorant receptors to positively influence site selection. Egg-laying site selection was associated with the specificity of the odorant for Or7a: those odorants (*e.g.*, benzaldehyde or 1-butanol) that activated many additional odorant receptors exhibited decreased oviposition towards that odorant whereas more specific Or7a agonists (*e.g.*, 9-tricosene and 1-propanol) demonstrated the highest positive oviposition site selection (Figure 8—figure supplement 1).

To further examine the sufficiency of Or7a neuron activation in guiding oviposition-site preference behaviors we utilized an optogenetics approach to specifically activate Or7a neurons with red light confined to one of the egg-laying wells (Figure 8—figure supplement 2). Since flies require feeding of all-trans-retinal for efficient light-induced activation of Channel Rhodopsin, we compared the behavioral responses elicited

by red light to the same genotype of flies that were, or were not, fed all-trans-retinal (genotype: *Or7a-GAL4, UAS-CsChrimson*) [54]. Flies fed all-trans-retinal exhibited significantly increased egg-laying preference for the red light quadrant (Figure 8—figure supplement 2). These results support the findings that Or7a neuron activity can influence egg-laying decisions.

DISCUSSION

We have identified a phenomenon in which *Drosophila* males deposit the pheromone 9-tricosene in response to apple cider vinegar food-odor stimulation. This male-predominant cuticular hydrocarbon acts as an aggregation pheromone to attract both males and females and as a chemosensory cue to influence female oviposition site selection (Figure 9). The behavioral effects of 9-tricosene are mediated via specific activation of a broadly tuned odorant receptor. To females, activation of this odorant receptor imparts biologically relevant information regarding potentially beneficial egg-laying sites. This behavioral choice is likely modulated by the ensemble activity of other odorant receptors stimulated at the egg-laying site.

Aggregation Behavior Guided by Food-Odor Perception

Our results suggest that aggregation at a food-site might be strongly influenced by the olfactory perception of an optimal food source. We found that an aggregation pheromone is only deposited upon food-odor stimulations, such as apple cider vinegar, ripe banana and yeast. An attractive odorant component (ethyl acetate) found in apple cider vinegar

424 was not sufficient for pheromone deposition, suggesting that attraction *per se* to an odor
425 is not sufficient for aggregation pheromone deposition.

426 cVA has been suggested as the aggregation pheromone in *Drosophila*
427 *melanogaster*. However, in contrast to aggregation pheromones identified in other
428 *Drosophila* species [7, 8], cVA is only weakly attractive on its own [9], although does
429 enhance aggregation when coupled with food or food-derived odors [8, 11]. However,
430 mutating key components in the cVA signaling pathway did not affect the food-odor
431 induced aggregation behavior. Moreover, GC-MS analysis of the active aggregation
432 pheromone extract found no evidence that cVA was deposited. Therefore, cVA is likely
433 not responsible for the aggregation behaviors triggered by food odors in these
434 experiments. Nonetheless, it remains possible that under natural conditions, cVA is used
435 as a long distance co-attractant with food odors at mating sites while 9-tricosene is used
436 as a short-range aggregation pheromone at food sites.

437 For many flying and non-flying insects, pheromone trails play an important role
438 in organizing social foraging behaviors [6, 55]. The first animals to find a suitable food
439 source use a pheromone trail to recruit even more animals to the food source, which in
440 turn deposit additional pheromone trails, and thereby establish a positive feedback of
441 pheromone signaling towards the foraging site [6, 55]. Our experiments identified
442 pheromone tags deposited when *Drosophila melanogaster* males perceived food odors,
443 implicating the existence of such a behavioral occurrence in this insect. We found that 9-
444 tricosene, and Or7a neuron activities, are likely key mediators of this particular
445 pheromone response. Interestingly, the “E” experiments with 9-tricosene suggests that 9-
446 tricosene may cause repulsion at high concentrations, although it remains to be

447 determined if these high concentrations represent natural conditions. Future experiments
448 will investigate how 9-tricosene responses are modulated by other chemicals or
449 pheromones in guiding pheromone trailing. It also remains to be explored if different
450 pheromone trails are deposited under different environmental conditions.
451
452 **9-Tricosene Is an Important Close-Range Social Pheromone**
453 Food searching behaviors rely on volatile chemosensory cues, which create long-distance
454 odor plumes or homogeneous odor clouds to guide foraging behaviors [56]. E2-hexenal,
455 because of its high volatility, might represent such a long-distance cue for recently fallen
456 fruit or plants. Since E2-hexenal is generated by a plant-dependent enzymatic reaction, its
457 levels will diminish over extended time periods [47, 51]. In contrast, 9-tricosene, as a
458 low volatility olfactory pheromone, might be ideally suited as a stable close-range
459 olfactory marker and utilized to convey environmental conditions. This is supported by
460 the variety of functions 9-tricosene exhibits in other species. For instance, 9-tricosene is a
461 sex attractant for female houseflies (*Musca domestica*) [57] and honey bee waggle
462 dancers (*Apis mellifera*) use a pheromone cocktail that includes 9-tricosene to
463 communicate with nest mates about the locations of food sources [58]. Future
464 experiments will be required to determine the ecological niche whereby 9-tricosene
465 signaling in *Drosophila* is most prevalent.
466
467 **Pheromones Can Activate Basiconic Sensillar Neurons**
468 Three basic-types of olfactory sensilla have been classified based on their size, shape, and
469 predicted functions [38]. ORNs housed in basiconic sensilla tend to be strongly activated

by food odors; ORNs in intermediate/trichoid sensilla tend to be activated by kairomones and pheromones; and ORNs in coeloconic sensilla are activated by amines, ammonia, water vapor and putrescine [25, 34]. Only two exceptions are known—phenylacetic acid is a food odor that activates coeloconic Ir84a+ neurons [59]; and carbon dioxide is detected by basiconic neurons expressing Gr21a/Gr63a [44]. 9-tricosene represents the first *Drosophila* cuticular hydrocarbon pheromone that, to our knowledge, is demonstrated to activate a *Drosophila* basiconic sensilla. Intriguingly, the same sensilla and Or7a olfactory receptor responds to the silk moth pheromone bombykol [60]. It is interesting to speculate that this *Drosophila* neuron's response to bombykol might represent off-target ligand specificity for 9-tricosene. Our data further suggests that activation of odorant receptors by both pheromone and plant volatiles might be more widespread than previously anticipated. Indeed, an olfactory receptor in the moth *Agrotis ipsilon* that was previously considered to be pheromone specific was recently found to also respond to the plant volatile heptanal, despite the plant volatile showing no structural similarity to the moth pheromone [61].

Male Pheromones Influence a Female Oviposition Decision

Oviposition site selection is a model system to study simple decision-making in *Drosophila* [26, 27]. The behavior comprises three steps—an ovipositor motor program, a clean/rest period and a search-like behavior. The ovipositor motor program that leads to egg deposition is relatively short (6-7 seconds) as compared to clean/rest and search-like behaviors (100-130 seconds) [26]. Rapid egg-laying associated with an extended positional search is consistent with our observations that over long time periods there

were no detectable positional preferences in the 9-tricosene pheromone quadrant even though female flies preferentially laid eggs in this quadrant (Figure 6). These findings also suggest that 9-tricosene might mediate two temporally distinct responses in our experimental design. A short-term aggregation behavior that lasts ~25 minutes and a long-term oviposition site selection behavior that lasts for hours. A possible mechanism underlying these different behaviors could be that detection thresholds for the two behaviors are different, *i.e.* higher concentrations of 9-tricosene triggers aggregation whereas low 9-tricosene concentrations affect oviposition.

In many insects, eggs are vulnerable and larvae have restricted motility, thus oviposition site selection is a crucial decision for progeny survival. The hypothesis of "mother-knows-best" stipulates that female egg-laying decisions evolved to oviposit in places offering the best survival of offspring [62]. As expected, oviposition decisions require multiple sensory modalities, such as visual, olfactory, gustatory and proprioception [26, 27, 46]. Our study shows that a previously considered female-only decision can in fact be modulated by a male-deposited pheromone. Since 9-tricosene is enriched only upon food-odor stimulation, and acts to aggregate animals and increase courtship (Lin and Potter, unpublished observations), it could be a mechanism used by *Drosophila* males to increase the likelihood that their progeny will be laid in an optimal location. Thus, in addition to "mother-knows-best", this suggest that "father" may have co-opted a female's olfactory system in order to influence an egg-laying decision for maximizing progeny survival.

The identification of Or7a as a pheromone receptor represents an intriguing puzzle. 9-Tricosene is a specific activator of Or7a, but Or7a is not activated only by 9-

516 tricosene. How can a specific pheromone response be mediated by a ‘generalist’ odorant
517 receptor? Our behavioral experiments suggest a strong positive correlation with the
518 specificity of Or7a activation and oviposition-guidance. This suggests that activation of
519 the Or7a olfactory receptor neuron can strongly influence egg-laying decisions. 9-
520 Tricosense may therefore have a specific influence on oviposition decisions due to its
521 specific activation of Or7a. Or7a olfactory neurons join a growing list of olfactory
522 neurons found to mediate specific olfactory behaviors like oviposition-site selection [40,
523 41], aversion [2, 3, 36], courtship [21], and attraction [1]. Odorants besides 9-tricosene
524 that stimulate Or7a neurons will activate additional olfactory receptor neurons that may
525 function to mask or modulate Or7a-mediated egg-laying behaviors. This likely reflects
526 how olfactory systems function to make sense of a complex environment, by assigning
527 biological weights and values to different olfactory neuron activity patterns that together
528 influence a behavioral choice.

529 Food odors and pheromone signals have been shown to project to non-
530 overlapping divisions in higher brain regions in *Drosophila*, suggesting that distinct brain
531 divisions may be involved in mediating different biological functions [63]. Might these
532 disparate olfactory signals for oviposition decision converge in the female brain? ORNs
533 expressing the same ORs converge onto the same glomeruli and synapse with second
534 order projection neurons, which relay the olfactory information to higher brain regions
535 (mushroom body calyx and lateral horn) [63]. We demonstrate that basiconic Or7a+
536 neurons are responsible for 9-tricosene and E2-hexenal guided oviposition decisions
537 (Figures 6C, 6D, 7C and 7D). Recently, it was found that flies preferred citrus fruit as
538 oviposition substrates as detected by Or19a+ olfactory neurons [41], and also preferred to

oviposit on substrates containing ethylphenols as detected by Or71a+ olfactory neurons [40]. Interestingly, the Or7a DL5, Or19a DC1, and Or71a VC2 projection neurons share highly similar axonal projection patterns in the lateral horn that are distinct from previously described food and pheromone regions [30, 63]. This suggests that oviposition site-selection might be strongly guided by a dedicated olfactory processing brain center.

MATERIALS AND METHODS:

EXPERIMENTAL PROCEDURES:

Fly stocks:

Wildtype flies are isogenized w^{1118} (*IsoD1* w^{1118}) and *IsoD1* w^{+}. Sources of the lines used in the study: *poxn* mutant, *ppk23*$^{-}$ [17], *Ir8a*$^{-}$ [35], I*r25a*$^{-}$ [34], *Ir64a-Gal4* [36], *snmp1* mutant [20], *lush* mutant, constitutive active *lush* [22], *Or67d-Gal4* [21], Δ*halo/CyO; UAS-Or7a,* Δ*halo; Or22a-Gal4* [25], *PromE(800)-Gal4, Tub-Gal80ts/TM6B* [15], *Or7a-Gal4* (BS#23907 [38]), *orco* mutant (BS# 23130 [31]), *Or83c-Gal4* (BS#23910), *Or43a-Gal4* (BS#9974), *Or88a-Gal4* (BS#23138). Before any behavioral analyses were performed, mutant or transgenic stocks were backcrossed at least 5 generations to *IsoDI* w^{1118}.

Imaging and Immunohistochemistry:

Confocal images were taken on a LSM 700 Confocal Microscope (Zeiss). The procedures for fixation, immunochemistry and imaging were as described previously [64]. Primary antibodies used were Rat anti-CD8 (Caltag Laboratories, 1:200) and Mouse anti-nc82 (DSHB, 1:25).

Four-quadrant behavioral assay: A four-quadrant olfactometer [1, 65] was used to track the olfactory responses of multiple flies at 30 frames/second [66]. Central air passed through a carbon filter before being split into multiple channels each regulated by a high-resolution flowmeter (Cole-Parmer). Electronically controlled 3-way solenoid valves (Automate Scientific, Berkeley, CA) regulated if clean air leaving the flowmeters expelled into the room or entered into custom made odor chambers [67]. Teflon tubing was used for odor delivery. The Teflon fly arena is 19.5 cm by 19.5 cm, with a thickness of 0.7 cm. Glass plates were secured onto the arena using clamps. The airflow of each quadrant was maintained at a rate of 100 ml min^{-1} and verified by an electronic flowmeter before each experiment. Apple cider vinegar was diluted in H_2O to make the final concentrations of 6.25% (1/16), 1.56% (1/64) and 0.39% (1/256) and acetic acid in water to make final concentration of 0.33%. Ethyl acetate and 9-tricosene (Sigma #859885) were diluted in paraffin oil for final concentration of 0.001% and 0.1%. When paraffin oil was used as solvent in the odor chamber, paraffin oil alone used in the three non-odor control chambers. 40-50 flies with an isogenized genetic background (*IsoD1* w^{1118}) were used. At the time of the assay, flies were 4-6 days old and had been starved in vials containing 1% agarose for 40-42 hours to increase locomotor activity. The dark arena was illuminated by 2 infrared LED arrays (AL4554-880; Advanced Illumination, Rochester, VT), monitored by an infrared camera (Sony XC-EI50), and flies tracked by previously described software [66]. Data was analyzed by custom Matlab scripts. On average, each fly generates approximately 1800 tracked positional data points per min. If two flies intersect, their respective previously continuous tracks are considered

completed, and new independent tracks begun once they move apart. This assures continuously labeled tracks originated from the same fly. An Attraction Index (AI) is defined as $(Ot_5\text{-}Cavgt_5)/ (Ot_5+Cavgt_5)$, in which Ot_5 is the number of tracked positional data points in the odor quadrant and $Cavgt_5$ is average number of tracked positional data points in non-odor control quadrants over a 5 min testing period. An AI = 1 indicates all flies were tracked to the odor quadrant, and an AI = 0 indicates flies were equally distributed to all four quadrants.

Pheromone extraction: A 1:1 mixture of 40-44-hour starved 50 male:female flies were stimulated with humidified air or apple cider vinegar for 5 minutes to deposit substrates onto cleaned glass plates. Apple cider vinegar stimulation alone (without flies) was used as a negative control. Odors were followed by clean air perfusion for another 5 minutes. To generate a hexane pheromone extract, the glass plates were treated 3× with 500 µl hexane solvent. The apple cider vinegar-stimulated pheromone extract was used to pipette a pattern onto a new clean glass plate or stored at -20 ºC for GC-MS analysis. The humidified air-stimulated hexane extract was used as a negative control in the hexane painting. In GC-MS experiments, to monitor extraction efficiency, 750 ng of internal standard controls (hexacosane (Sigma #241687) and triacontane (Sigma #263842) as dissolved in hexane) were added on to the glass plates immediately before pheromone extraction procedures. In the 9-tricosene "E" paint experiment, 9-tricosene was dissolved in hexane solvent (1:25,000 dilution) and 150 µl was evenly pipetted onto the clean glass plate to form an "E" pattern.

610 **Gas Chromatography/Mass Spectrometry (GC/MS)**. A sample volume of 2 µL of the
611 hexane extract was injected in splitless mode into a Thermo Scientific ISQ single
612 quadrupole GC/MS (Waltham, MA) with Xcalibur software (ThermoElectron Corp.) for
613 separation and analysis of the deposited hydrocarbons. The GC/MS was equipped with a
614 Stabilwax column, 30 m × 0.32 mm with 1.0 µm film thickness (Restek Corp.,
615 Bellefonte, PA). The injection port was set at 230°C. The oven temperature was set to
616 60°C, raised to 180°C at 6°C min^{-1}, held at 180°C for 20 min, and then raised to 220°C at
617 6°C min^{-1} where it was maintained for an additional 20 min. Helium carrier gas
618 constantly flowed at 2.5 mL min^{-1}. The mass spectrometer was operated at an ionizing
619 energy of 70 eV with a 2 scan/sec rate over a scan range of *m/z* 40–400 and an ion source
620 temperature of 200°C. Identification of structures/compounds was performed using the
621 National Institute of Standards and Technology library, as well as comparisons with
622 known literature compounds and commercially available standards. Relative retention
623 times were obtained by comparison of sample hydrocarbons to authentic standards. All
624 standards were purchased from Sigma or Cayman Chemical Company at the highest
625 available purity.
626
627 **Electroantennography (EAG):** Electroantennograms were recorded with capillary glass
628 electrodes (1.5mm outer diameter) containing *Drosophila* saline plus Triton X-100
629 (188mM NaCl, 5mM KCl, 2mM $CaCl_2 \cdot 2H_2O$, 0.02% Triton X-100). The reference
630 electrode was placed in the head capsule close to the base of the antenna. A polished
631 large diameter (~40-50 µm) recording electrode was capped onto the anterior distal
632 region of the *Drosophila* third antennal segment. Control odorant stimulations (1% and

633 10% cVA; data not shown) were used to verify that the recording electrode was properly
634 sealed onto the distal antenna, 30 µl of different dilutions of 9-tricosene in mineral oil on
635 filter paper was used as the pheromone stimulus. Electrical signals were acquired with a
636 Syntech Intelligent Data Acquisition Controller IDAC-4-USB and quantified by
637 measuring the mV value at the greatest deflection in the EAG trace.
638
639 **Fluorescence Guided Single Sensillum Recording (SSR):** Recordings were performed
640 as previously described [39]**.** Sensillum of targeted ORNs was identified using green
641 fluorescence signals by crossing *OrX-Gal4* to *15xUAS-IVS-mCD8GFP* (Bloomington
642 Stock #32193) [68]. Extracelluar activity was recorded by inserting a glass electrode to
643 the base of the sensillum of 4-10 day-old flies. Signals were amplified 100X (USB-IDAC
644 System; Syntech, Hilversum, The Netherlands) and inputted into a computer via a 16-bit
645 analog-digital converter and analyzed off-line with AUTOSPIKE software (USB-IDAC
646 System; Syntech). The low cutoff filter setting was 50Hz, and the high cutoff was 5kHz.
647 Stimuli consisted of 1000 ms air pulses passed over odorant sources [43]. The
648 Δspikes/second is obtained by counting the spikes in a 1000ms window from 500 ms
649 after odor stimuli were triggered, subtracting the spikes in a 1000ms window prior to
650 stimulation. Furthermore, the response generated by control solvent was further
651 subtracted. The formula is as below:
652 (odor response-spontaneous response)-(solvent response-spontaneous response).
653 9-Tricosene from three different sources (Sigma Cat#859885, TCI America
654 Cat#T1242, AK Scientific Cat#M691) robustly activated ab4A neurons. 9-tricosene from
655 Sigma was used in all reported experiments. For SSR experiments using fly body odors,

50 male and 50 female flies were starved in vials containing 1% agarose gel for 40 hours as described above and then transferred to a 40-ml glass vial (Thermo scientific B7999-6). Dry air or apple cider vinegar (6.25%) was perfused through the vial for 30 minutes (flowrate = 100 ml/min). The vials were then used as odor sources to stimulate the ab4 sensilla in the standard SSR setup as described above. Each odor vial was used less than 3 times to avoid depletion of deposited odors.

Generation of Or7a transgene:

5' and 3' homology arms of the *Or7a* gene were generated by PCR amplifying from bacterial artificial chromosome (C.H.O.R.I, RP98-39F18) and *WT* genomic DNA, respectively and subcloned into the pTV^{Cherry} vector [45]. 5' homologous sequence immediately 5' to the ATG start site of Or7a (A of ATG is included) (4199 b.p.) was subcloned between NheI and KpnI restriction sites. A 4304 b.p sequence starting from 1368 base pairs downstream to the ATG start codon of Or7a was cloned between SpeI and BglII sites. In-Fusion cloning was used for subcloning into the pTV vector (Clontech Laboratories, Inc.) (Figure 4—figure supplement 3).

Primers used for PCR (Vector specific sequence in red, Or7a specific sequence in blue; lowercase letters indicate designed b.p. to preserve restriction sites):

5' homology arm: 5'Or7a_FOR, GCT ACC GCG GGC TAG cCA ACA TGC CGA TTA TGT CG; 5'Or7a_REV, AGT TGG GGC ACT ACG gta ccT GGC TGA TGG ACT TTT GAC G

3’ homology arm: 3’Or7a_FOR, CGA AGT TAT CAC TAG tAG CCA AGT TCT CGT TTT CGC; 3’Or7a_REV, TTA TGC ATG GAG ATC tTT TGG CAT TGT GTG TTG CAC

Generation of Or7a deletion mutant and GAL4 knockin:

Accelerated homologous recombination was performed according to Baena-Lopez LA et al. [45]. Briefly, P-element insertion lines containing the Or7a knockout construct were crossed to *hs-Flp, hs-SceI* (BS#25679) and heat-shocked at 48 and 72 hours after egg-laying (1 hour duration each time). Female progeny with mottled eyes were crossed to *ubi-Gal4[pax-GFP]* [45] in order to select against flies containing non-homologous recombination events. Stocks were generated from candidate flies that contained both w^+ and GFP markers. *Or7a* mutants were verified by single sensillum recordings and PCR (Figures 4F, 4G, Figure 4—figure supplement 3). In order to identify the ab4 sensillum, 30 μl of geosmin (Sigma #16423-19-1), an odor that specifically activates only ab4B (Or56a) [3], was used (Figures 4F and 4G).

Primers used for verification: G4polyA_FOR: TCG ATA CCG TCG ACT AAA GCC; gOr7a_REV:TCG CCG TTG AGT TTT CAG AG

The Or7a-Gal4 knockin was generated by co-injection of the pRIV-Gal4 donor plasmid [45] with PhiC31 integrase to target GAL4 to the attP site within the knockout locus, as described in [45].

Four-quadrant egg laying behavior assay and positional recording: The schematic of the hybrid 9-tricosene or 7-tricosene gel is as shown in Figure 6—figure supplement 1. Control agarose gel was made by pouring 70 ml of a 1% agarose gel onto a glass plate assembled onto the 4-field arena. 9-tricosene or 7-tricosene gel was made by mixing 0.8 mg pure 9-tricosene (Sigma #859885) or 7-tricosene (Cayman Chemical Company #9000313) into 70 ml of 1% agarose gel (temperature = ~50 ºC). One quadrant of the control gel or 9-tricosene or 7-tricosene gel was cut out and transferred to a Petri dish. In order to increase egg production, mixed population of male and female flies were pre-induced in vials with wet yeast paste (yeast + 0.5% propionic acid) overnight. Mated females from premixed population were rapidly separated by cooling on ice and transferred to the arena gel. The egg laying behavior was performed in a dark enclosure at room temperature for 22-23 hours. Simultaneous recordings of the fly positions were performed using the same setup for tracking of the four-quadrant behavior assay described above except that the frame rate was set at 1 frame/5 sec due to the large file size generated over the extended time period. On average, each fly generated approximately 16560 tracked positions per experiment. The recorded data was analyzed with custom Matlab programs and analyzed for the AI as defined above. Analyses scripts are available as associated source code or upon request.

3-well egg-laying behavior assay: A schematic of the 3-well (34x85 mm) egg-laying assay is shown in Figure 7. The 9-tricosene well was made by mixing 1 µl 9-tricosene into a 70 mL 1% agarose gel solution. 1.2-1.3 mL of this solution was allowed to set in a well for 10 mins before the experiment. Control wells contained only 1% agarose. Odorant wells (E2-hexenal (Sigma W256005), benzaldeyde (Sigma

418099), 1-butanol (Sigma 281549), 1-propanol (Sigma 279544), hexyl butyrate (Sigma W256803), pentanoic acid (Sigma 240370), and ethyl lactate (Sigma W244007)) were made by mixing 1 µl of 10^{-1} to 10^{-6} odorant concentrations into a 70 ml 1% agarose solution. Odor concentrations listed in Figures or Figure legends are the approximated final diluted concentrations in the agarose gel. Females were pre-stimulated as described above, placed in the 3-well agarose spots, covered with Petri dish lids (Diameter=90mm, Fischer Scientific FB0875712), and allowed to oviposit in a dark temperature controlled chamber for 23 hours. The oviposition preference index (OPI) is defined as $(E_o-E_{avg})/ (E_o+E_{avg})$, in which E_o is the number of eggs in the odor well and E_{avg} is average number of egg numbers in non-odor wells. An OPI = 1 indicates all eggs were laid in the odor well, an OPI = 0 indicates eggs were equally distributed to all 3 wells, and an OPI= -1 indicates all eggs were laid in the non-odor wells.

Channel Rhodopsin Activation: Newly eclosed flies were transferred to fly vials containing 0.4 mM of all-trans-retinal (Sigma-Aldrich # R2500, dissolved in pure ethanol) in fly food. The flies were fed with all-trans-retinal food in the dark for 3 days and used for the 3-well egg-laying assay as described above. A 627nm LED light source (1-up LED Lighting Kit, PART #: ALK-1UP-EH-KIT; LEDSupply.Com) was placed directly beneath the glass 3-well egg-laying chamber with a white acrylic diffuser in between (Cat# 8505K11, McMaster-Carr). The LED was powered and controlled by an Arduino Uno to activate *UAS-CsChrimson* [54] (Bloomington Stock # 55135). The power supplied to the LED by the Arduino was set to 2 V with light on for 8 ms and off for 17 ms by using a custom Arduino program. The red light stimulus was supplied continuously throughout the 23 hour egg-laying assay. The Arduino program is available as associated source code.

AUTHOR CONTRIBUTIONS:

C.-C.L. performed the experiments and co-wrote the manuscript. K.A.P.-P. and G.P. performed the GC-MS analyses. C.J.P. co-wrote the manuscript and supervised the project.

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COMPETING INTERESTS:

The authors declare no competing interests.

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FIGURE LEGENDS:

Figure 1. Identification of an apple cider vinegar odor induced post-stimulus aggregation behavior mediated by males.

(**A** and **B**) Schematic of behavior setup and experimental design. (**C**) Fly tracking for 5-minutes of 25 male and 25 female wild-type flies. Flies are highly attracted to apple cider vinegar food odor, which gives rise to a post-stimulus aggregation behavior in the absence of exogenous odorants (right). (**D**) The lack of flies with apple cider vinegar stimulation (left) led to a lack of a post-stimulus aggregation (right). (**E**) Humidified air versus dry air is attractive (left), but does not lead to a post-stimulus aggregation (right). (**F**) Post-stimulus response summary (p=0.0002 and 0.00001 for apple only and humidified air + WT, respectively; t-test, n=3-6 per trial) (**G**) Definition of attraction index, A.I. Error bars indicate ± s.e.m. throughout. (**H**) Schematic of 4-quadrant arena using different populations of depositor and detector flies. (**I**) Different depositor fly populations (females+males, females only, males only) were used as pheromone sources and assayed for post-stimulation aggregation by female+male detector flies (p=0.003; t-test, n=3-5 per combination).

Figure 1—figure supplement 1. Basic characterization of the four-field olfactometer.

Fly tracking for 5-minutes of 25 male and 25 female wild-type flies to dry air (left) and insect repellent citronellal (right). Example single fly tracks within the dataset are shown color-coded from start (blue) to end (red) of a continuous track.

Figure 1—figure supplement 2. Colormap of all fly trajectories from 0 minute to 7 minutes.

Apple cider vinegar was applied at 0.5 minute. Right, attraction index over time is shown.

Figure 1—figure supplement 3. Four-field behavioral control experiments.

(**A**) Wild-type flies stimulated with apple cider vinegar, humidified air, and apple cider vinegar with humidified air perfusion in the other 3 quadrants (apple cider vinegar + 3 humidified air). (**B**) *Orco* mutant flies responding to apple cider vinegar, humidified air and apple cider vinegar + 3 humidified air.

Figure 1—figure supplement 4. Summary of four-field olfactometer controls.
Response summary of wild-type and *orco* mutant flies to apple cider vinegar, humidified air and apple cider vinegar + 3 humidified air. n=4-6 for each condition ±s.e.m.

Figure 1—figure supplement 5. Time-course of aggregation pheromone responses.
Recordings of post-stimulus aggregation in 5-minute intervals up to 25 minutes post clean air perfusion.

Figure 1—figure supplement 6. Post-stimulus aggregation induced by various concentrations of apple cider vinegar.
Post-stimulus aggregation was assayed using different dilutions of apple cider vinegar.

Figure 1—figure supplement 7. Post-stimulus aggregation induced by additional food odors, but not by an attractive odorant.
Quantification of post-stimulus aggregation by food-odors (apple cider vinegar, banana and yeast), and by ethyl acetate, an attractive component of apple cider vinegar ($p<0.001$ for post-stimulus phase ethyl acetate; t-test, n=4-5 per odor stimulation).

Figure 1—figure supplement 8. The aggregation pheromone is similarly attractive to virgin or mated males and females.
Post-stimulus aggregation to natural pheromone is similar among virgin males, virgin females, virgin male + virgin females (1:1) and premixed males+females (1:1).

Video 1. Tracking flies stimulated with apple cider vinegar in four-quadrant behavior assay.

Odor is supplied to the bottom right quadrant at time 30 seconds. The graph at the bottom reflects the attraction index over time. Related to Figure 1.

Video 2. Tracking flies responding to post-stimulus aggregation pheromone in four-quadrant behavior assay.

The arena has been rotated 90 degrees counter-clockwise with the aggregation pheromone deposits located at the top right quadrant. The graph at the bottom reflects the attraction index over time. Related to Figure 1.

Figure 2. Post-stimulus aggregation requires Orco-dependent olfactory signaling.

(**A**) Diagram indicating the different genetic components required for gustatory or olfactory-based pheromone detection. (**B**) Post-stimulus aggregation responses by gustatory receptor (*Poxn;* p=0.2258; t-test) and *ppk-23* mutants (p=0.0951; t-test, n=4-5 per trail). (**C**) Post-stimulus aggregation responses by olfactory receptor (*orco*), and Ionotropic receptor (*Ir8*, *Ir25a*) mutants ($Ir8a^{-/-};Ir25a^{-/-}$: p=0.1524; $orco^{-/-}$: p<0.001; $Ir8a^{-/-};;orco^{-/-}$: p=0.004; t-test, n=4-6 per genotype). Wild-type flies were used as pheromone depositors.

Figure 2—figure supplement 1. Ablation of the Ir64a+ acid-sensing neurons increases post-stimulus attraction.

Post-stimulus attraction responses of flies are increased when acid-sensing olfactory neurons are removed (*Ir64a-Gal4/UAS-hid*)(p=0.021; t-test, n=3-4 per trial ±s.e.m.).

Figure 2—figure supplement 2. Repulsion of *orco* mutant flies to the aggregation pheromone is likely mediated by acid sensing of residual apple cider vinegar.

Post-stimulus repulsive behavior is abolished in *orco* mutant flies assayed with neutralized apple cider vinegar (pH=7.0).

Figure 2—figure supplement 3. Acidity of apple cider vinegar is not required for post-stimulus responses.

Post-stimulus responses using neutralized apple cider vinegar (pH=7).

Figure 2—figure supplement 4. Mutating components of the cis-vaccenyl acetate pheromone pathway does not disrupt post-stimulus responses.

Quantification of post-stimulus aggregation in mutants of key components in the cVA signaling pathway (p=0.4405, one-way ANOVA test, n=3 for each trial). Error bars indicate ± s.e.m. throughout.

Figure 3. 9-Tricosene is a food-odor induced pheromone.

(**A**) Schematic of pheromone extract paint experiment. (**B**) Hexane extracts of the pheromone quadrant were used to paint the letter "E" onto the glass plate. Shown are traces of naïve new flies in the painted arenas by deposited pheromone extract (apple cider vinegar+flies) or control (humidified air+flies). The blue to red color trace indicates a single fly track from start to end of tracking. (**C**) GC-MS results of hexane extracts from quadrants stimulated by apple cider vinegar-only, humidified air and flies, and apple cider vinegar with flies. Peak #2 is (Z)9-tricosene. 9-Tricosene exhibited a 2.8 fold enrichment on the glass plates upon food-odor stimulation. (**D**) Olfactory behavioral response of flies to 0.1% 9-tricosene. (**E**) Dose-response curve of 9-tricosene for mediating attraction. (**F**) Traces of flies in response to 9-tricosene deposited in an "E" pattern on the glass plate. In this context, flies appear to be repelled by a concentrated 9-tricosene pattern and prefer to trail the 9-tricosene pheromone border. The behavioral differences between (B) and (F) maybe modulated by additional pheromone components present in the hexane extracts, or reflect that 9-tricosene trailing occurs only over a narrow odor range.

Figure 3—figure supplement 1. Single fly trajectories of painted "E" experiment.

The blue to red color trace indicates a single fly track from the start to end of one continuous track. See also Video 2

Figure 3—figure supplement 2. The aggregation pheromone is heat-sensitive.

To determine effects of heat on pheromone stability, the arena was maintained at 32 ºC for the entire testing period (red). The wild-type response at 25 ºC is shown for comparison.

Figure 3—figure supplement 3. Additional examples of flies responding to a 9-tricosene "E" pattern.

Traces of flies in response to 9-tricosene deposited in an "E" pattern on the glass plate.

Figure 3—figure supplement 4. Apple cider vinegar stimulation of oenocyte-less males leads to a reduction in post-stimulus aggregation responses.
Post-stimulus behavior is reduced when using mixtures of oenocyte-negative males (*UAS-hid; PromE(800)-Gal4,Tub-Gal80ts*) with wild-type females as the source of the aggregation pheromone (p=0.0021; t-test, n=4 per trial ±s.e.m.).

Video 3. Tracking flies responding to hexane extract of the post-stimulus aggregation pheromone painted as an "E" pattern. Related to Figure 3.

Figure 4. Electrophysiological results identify Or7a as the receptor for 9-tricosene.

(**A**) Electroantennography (EAG) traces of wild-type and *orco*$^{-/-}$ flies stimulated with 100% 9-tricosene. (**B**) EAG response summaries of different 9-tricosene concentrations in different sexes of wild-type and *orco*$^{-/-}$ flies (n=5-7 per stimulation). (**C**) Single sensillum recording (SSR) in all *orco*-positive antennal and maxillary palp sensilla. n=3-6 per sensillum. (**D**) SSR traces showing responses to 9-tricosene stimulation in ab4 (9-tricosene responsive), ab3 empty neuron (*halo/halo;Or22a-Gal4*), and ab3 rescue (*halo/halo;Or22a-Gal4/UAS-Or7a*) sensilla. (**E**) SSR response summary to 9-tricosene of native ab4 and rescued ab3 sensilla in different sexes. n=7-8 per sensillum. (**F** and **G**) SSR trace responses and quantitative summary of ab4 sensillum of *Or7a*$^{-/-}$ mutant flies stimulated with 100% 9-tricosene and geosmin (n=4). Error bars indicate ± s.e.m. throughout.

Figure 4—figure supplement 1. Fly odors can stimulate ab4A neurons.

(**A**) SSR trace of ab4 responding to fly odors after apple cider vinegar stimulation. Flies were housed in a glass vial and stimulated with apple cider vinegar to induce pheromone deposition. (**B**) Box plot of changes in ab4 spike frequency to vials containing flies stimulated with dry air, or vials containing flies that had been stimulated with apple cider vinegar (p=0.03; t-test, n=5 per condition).

Figure 4—figure supplement 2. Response of ab4 sensillum to cVA.

(A) SSR response traces and (**B**) activity summary of WT ab4 sensillum to different dilutions of cVA (n=4 per concentration ±s.e.m.).

Figure 4—figure supplement 3. Generation of *Or7a* mutant.

(A) Schematic of constructs and crosses utilized for accelerated homologous recombination of the *Or7a* locus (See Experimental Procedures for details). (**B**) PCR verification of *Or7a* mutant. Primer locations are diagramed in (**A**).

Figure 5. Or7a neurons are necessary for the behavioral response to naturally deposited aggregation pheromone and 9-tricosene.

(**A**) Immunostaining of Or7a-expressing neurons innervating the DL5 glomerulus in the antennal lobe (*Or7a-Gal4/UAS-mCD8GFP*). (**B-C**) Four-field behavior responses of WT, *Or7a* mutant, Or7a-neuron ablated, and control OrX-neuron ablated flies (*Or83c, Or43a* and *Or88a-Gal4 x UAS-hid*) to naturally deposited pheromone (p=0.0012 and 0.006 comparing WT to Or7a neurons ablated and $Or7a^{-/-}$ flies; t-test, n=4-6 per experiment). (**D-E**) Behavioral response of WT, *Or7a* mutant, and Or7a neuron ablated flies to 9-tricosene (0.1%) (p<0.001; t-test; n=4-5 per trial). Error bars indicate ± s.e.m. throughout.

Figure 5—figure supplement 1. Whole-animal Or7a expression pattern.

Shown is the expression profile of *Or7a-GAL4* in whole animals (males and females), and in dissected brain and ventral nerve cord. *Or7a-GAL4* expression was determined by using a strong *20xUAS-mCD8GFP* reporter. GFP expression was not detected in tissues outside of the antennal olfactory neurons.

Figure 5—figure supplement 2. Or7a-ablation experiments for the deposited pheromone and 9-tricosene.

Post-stimulus aggregation response of wild-type or Or7a neuron ablated flies (*Or7a-Gal4/UAS-DTI)* to natural pheromone (p=0.0014; t-test, n=4-5 per stimulation) and 0.1% 9-tricosene (p=0.0049; t-test, n=4-5 per stimulation). Error bars indicate ± s.e.m.

Figure 5—figure supplement 3. Olfactory assays for 7-tricosene.

Four-field behavior response (left) and summary (right) of wild-type flies to 0.1% 7-tricosene (n=4 per odor ±s.e.m.).

Figure 6. 9-Tricosene modulates female oviposition site selection.

(**A**) Quantification and positions of eggs laid over ~23 hours in the 1% agarose arena with apple cider vinegar-only control or naturally deposited aggregation pheromone (A: p=0.4836; n=10; A': p<0.001; n=9; one-way ANOVA test) (**B**) Quantification and positions of eggs laid over ~23 hours in the agarose arena in blank control or with 9-tricosene (yellow, 0.001%) (B: p=0.9499; n=11; p<0.001; t-test, n=9 per trial, One-way ANOVA test). (**C**) The effect of 9-tricosene on female oviposition site selection was assayed in Or7a neuron ablated flies (C: *UAS-hid/+*, p<0.001, n=9 per trial; C': *Or7a-Gal4/UAS-hid*, p=0.384; n=9, One-way ANOVA test). (**D**) 9-Tricosene guided oviposition site selection assayed in $Or7a^{-/-}$ mutant flies (p=0.69; n=9, One-way ANOVA test). (**E**) Positional recording throughout the 23 hr course of female oviposition behavior with a 9-tricosene hybrid gel (p=0.1; n=6, One-way ANOVA test). (**F**) Oviposition site selection using a 7-tricosene hybrid gel. (p=0.28; n=8, One-way ANOVA test). (**G**) Box plots indicating the total number of eggs laid in **A-D** (p=0.0021 comparing WT and *Or7a>hid*, p=0.0001 comparing WT and $Or7a^{-/-}$, p=0.68 comparing *Or7a>hid* and $Or7a^{-/-}$; t-test ; n=9-11). In all panels, colored dots indicate actual egg locations. Different colors represent different experiment trials. Error bars indicate ± 2.5 s.e.m. throughout. Data points not within this range are plotted as circles.

Figure 6—figure supplement 1. Schematic of hybrid 9-tricosene gel construction.

Concentration of 9-tricosene in the agarose gel is ~0.001%.

Figure 6—figure supplement 2. Oviposition guidance of Or7a-neuron ablated flies to 9-tricosene.

(**A**) Egg laying preference summary to the 9-tricosene quadrant in control (*UAS-DTI/+*, p= 0.0028, 0.0028 and 0.0353 to 9-tricosene quadrant; t-test, n=8) and (**B**) Or7a neuron ablated flies (*Or7a-Gal4/UAS-DTI*, p=0.1349; one-way ANOVA test, n=11).

Figure 7. E2-hexenal modulates oviposition site selection.

(**A**) The effects of 9-tricosene guidance on egg-laying using a 3-well spot plate (34x85 mm) containing 9-tricosene (10^{-4} dilution) in a 1% agarose gel (yellow, 0.001%; $p<0.001$; t-test, n=12) or control 1% agarose gel (blue) ($p=0.53$, One-way ANOVA test, n=17). (**B**) Egg laying preference of *Or7a*$^{-/-}$ mutant flies in the 3-well spot 9-tricosene egg laying assay ($p=0.69$, One-way ANOVA test, n=15). (**C**) Egg laying preference of *w*^{1118} flies in a 3-well spot egg laying assay (10^{-6} dilution) (p=1.73x10-4, t-test, n=10). (**D**) Egg laying preference of *Or7a*$^{-/-}$ mutant flies in a 3-well spot E2-hexenal egg laying assay ($p=0.68$, One-way ANOVA test, n=10). For box plots (**A**-**D**), error bars indicate ± 2.5 s.e.m. Data points not within this range are plotted as circles. (**E**) Attraction of wild-type *w*^{1118} or *Or7a* mutant flies to E2-hexenal as determined in the four-field olfactory assay ($p=2.4\text{x}10^{-4}$ for comparing 10^{-6} and 10^{-5} E2-hexenal dilutions; $p=0.0149$ for comparing *WT* and *Or7a*$^{-/-}$ at 10^{-5} E2-hexenal dilution; t-test,. n=4-6 for each condition).

Figure 7—figure supplement 1. Oviposition selection to 9-tricosene in a surrounding presence of E2-hexenal odors.

(**A**) Schematic of the 3-choice spot plate oviposition assay in which the center well contains 9-tricosene (yellow) and the surrounding wells contain differing concentrations of E2-hexenal (green). (**B**) Box plots of the number of eggs laid per well per day in the center 9-tricosene well, or in surrounding wells that contain high (10^{-6}, 10^{-7}), medium (10^{-8}) or low (10^{-9}, 10^{-10}) concentrations of E2-hexenal. (**C**) The potency of 9-tricosene relative to differing E2-hexenal concentrations in guiding egg-laying decisions. Values >1 (dotted-line) highlight concentrations of E2-hexenal that are more potent as oviposition cues than 9-tricosene.

Figure 8. Odorants that activate Or7a guide oviposition site selection.

(A) Summary graph of Or activities induced by 7 different odorants as detected by single sensillum recordings. All odorant responses are from [25]. ++++, spikes ≥ 200; +++, spikes ≥ 150; ++, spikes ≥ 100; +, spikes ≥ 50; 0, spikes ≥0; -, spikes ≤ 0. (**B**) Oviposition-guidance preference for each odorant as assayed in the 3-choice assay. An oviposition preference index (OPI) was calculated as: (# of eggs laid in odor well – average # of eggs laid in control wells) / (# of eggs laid in odor well + average # of eggs laid in control wells). ++++, OPI ≥ 0.8; +++, OPI ≥ 0.5; ++, OPI ≥ 0.2; +, OPI =0∼0.2; -. OPI =0∼-0.2; --, OPI≤-0.2; ---, OPI≤-0.5. n=5-9 for each odor concentration. n.d., not determined.

Figure 8—figure supplement 1. Oviposition preferences by different Or7a agonists and control odorants in a 3-well egg-laying assay.

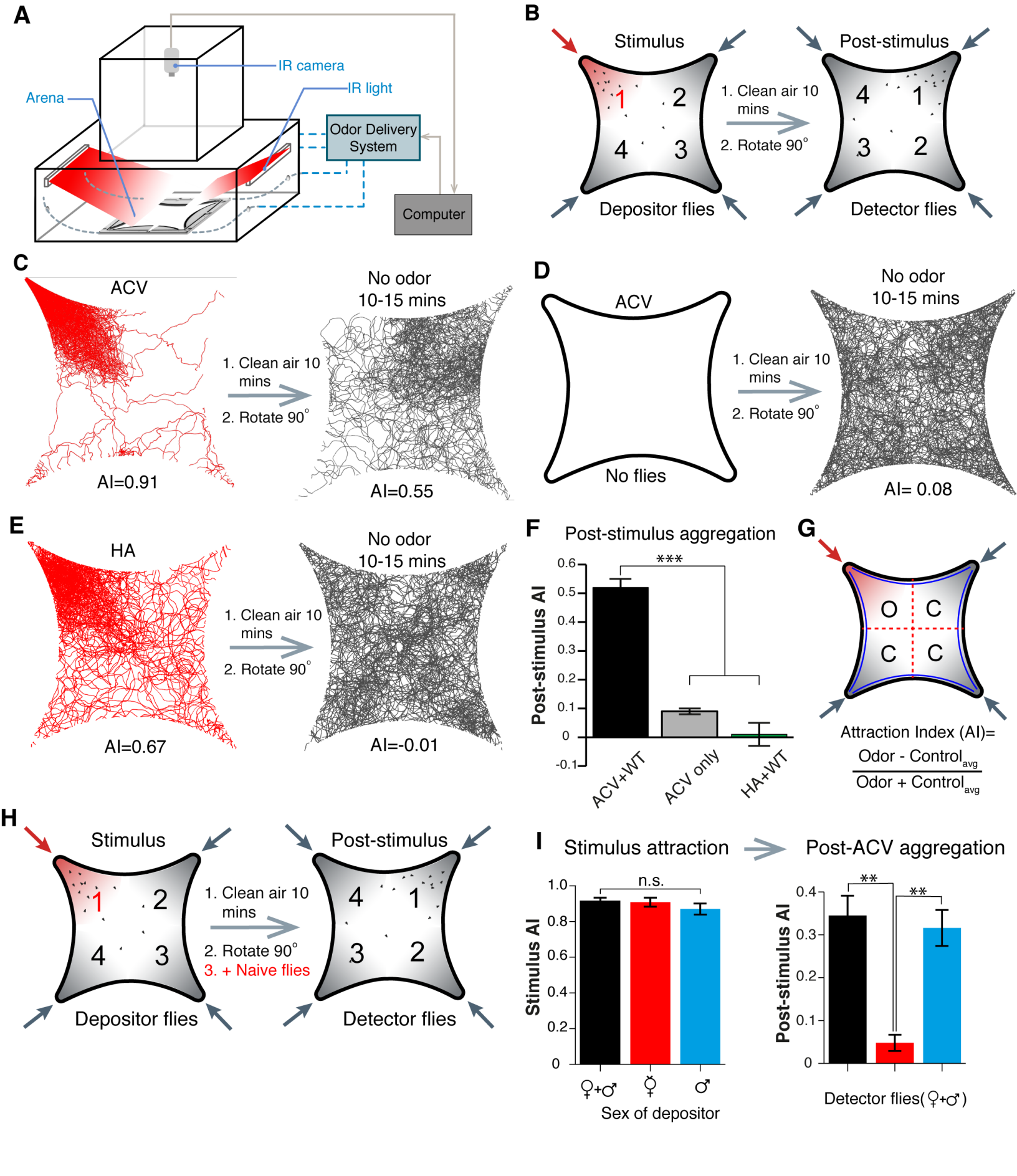
The final odorant concentrations in the 1% agarose gel are listed. *p*-values are calculated by using one-way ANOVA test.

Figure 8—figure supplement 2. Optogenetic activation of Or7a neurons in egg-laying assay.

Oviposition preference was determined in a 3-well egg-laying assay in which the center well was illuminated by red (627nm wavelength) light. The red light activated the Or7a neurons of flies fed the co-factor all-trans-retinal (ATR), but not the same genotype of flies not fed all-trans-retinal. Genotype: *Or7a-GAL4*; *UAS-CsChrimson*. $p = 0.022$ by t-test. n=8 for (–) all-trans-retinal and 10 for (+) all-trans-retinal condition.

Figure 9. Model of food-odor induced pheromonal behavioral responses. Upon exposure to food odors, male *Drosophila melanogaster* deposit the pheromone 9-tricosene. 9-Tricosene functions via Or7a odorant receptors to guide aggregation and oviposition site-selection decisions. Activation of Or7a by other odorants may also guide similar behaviors.

Supplementary File 1. Volatile male-specific and male-enriched pheromones detected by GC-MS under control and experimental conditions.


A
Arena
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Stimulus
1
2
4
3
Depositor flies
1. Clean air 10 mins
2. Rotate 90°
Post-stimulus
4
1
3
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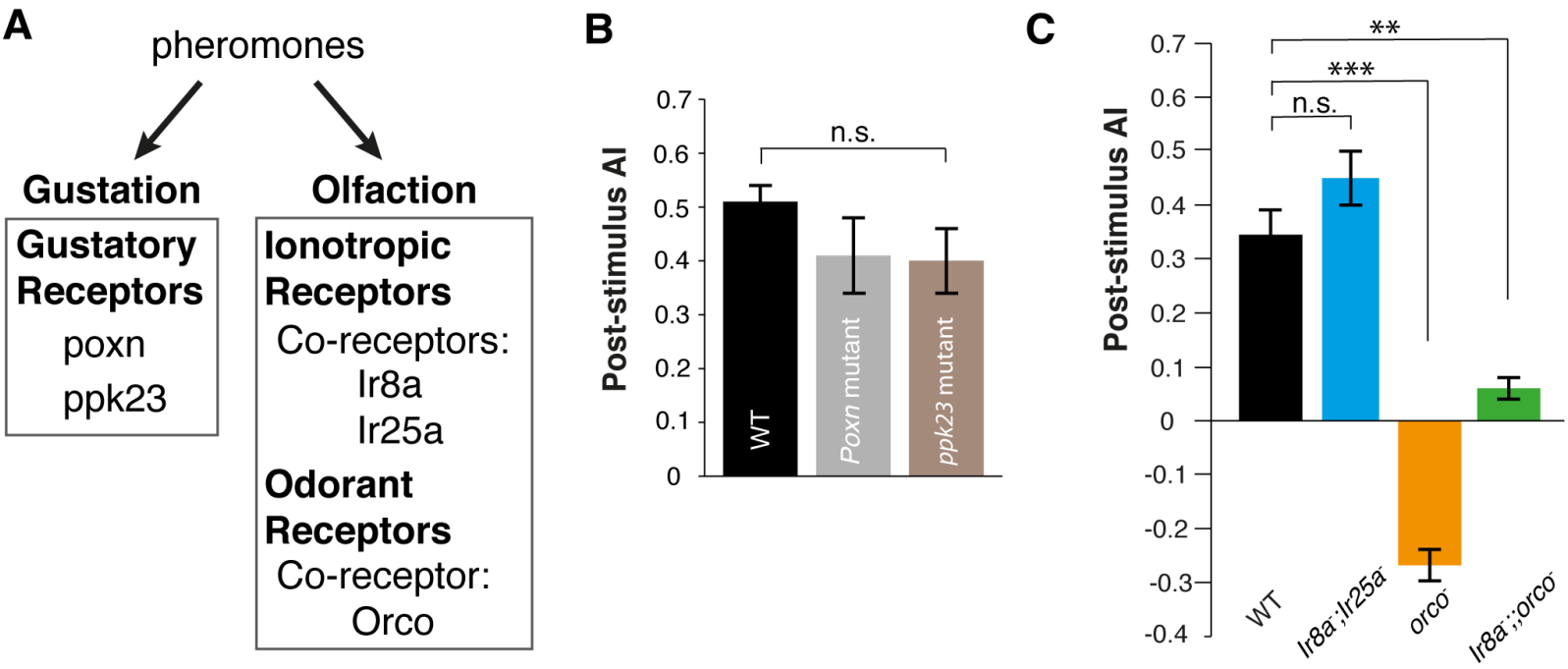
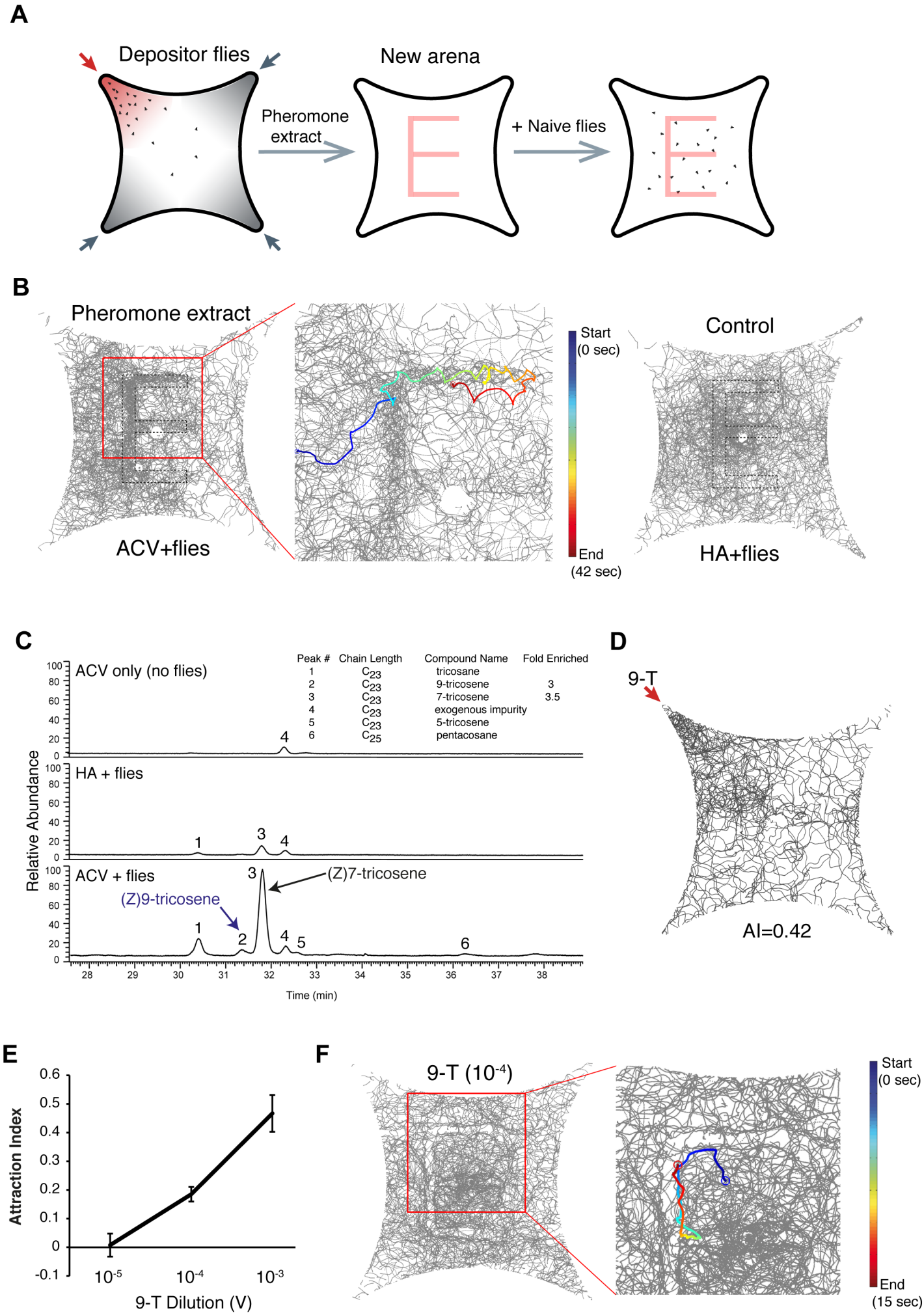
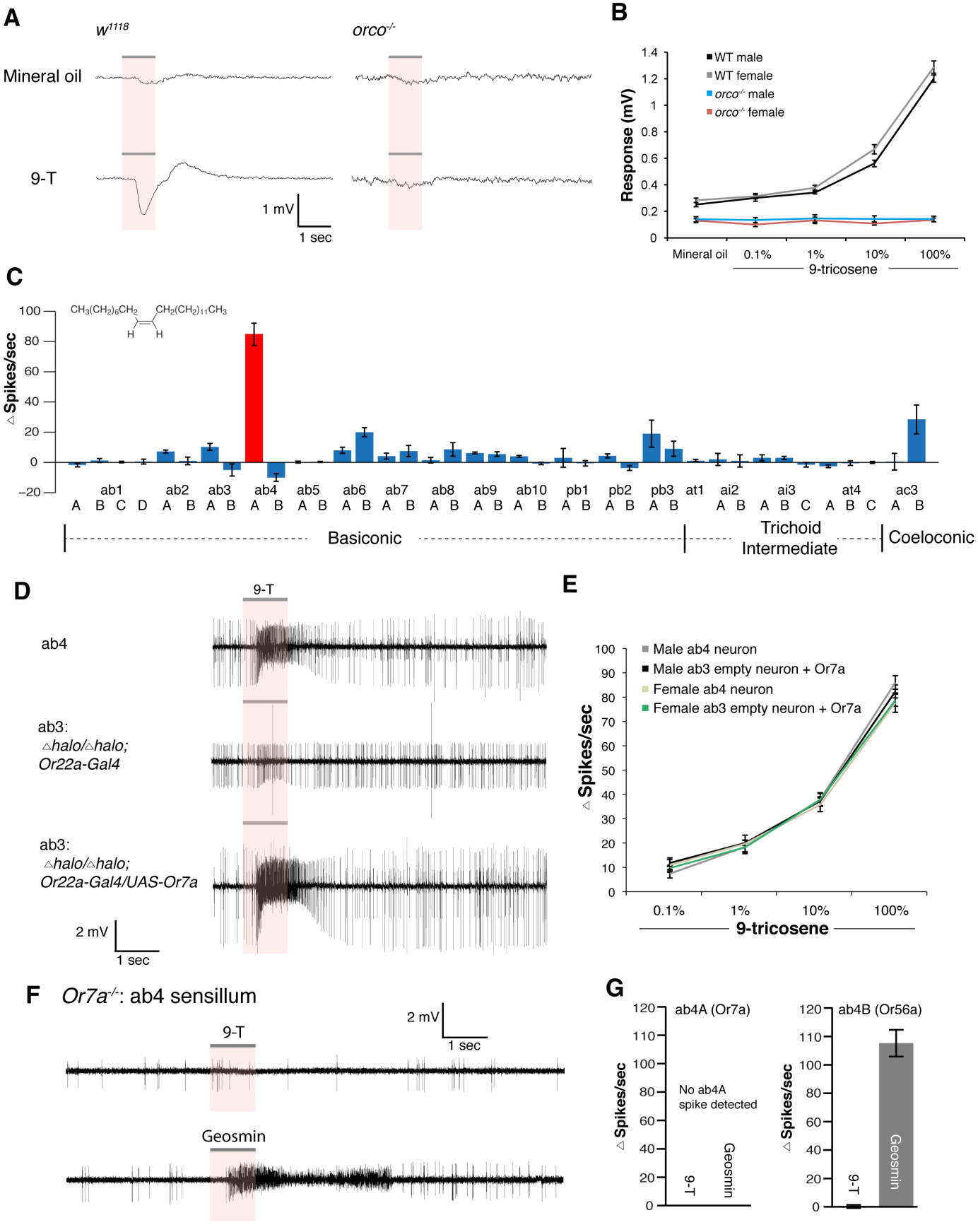
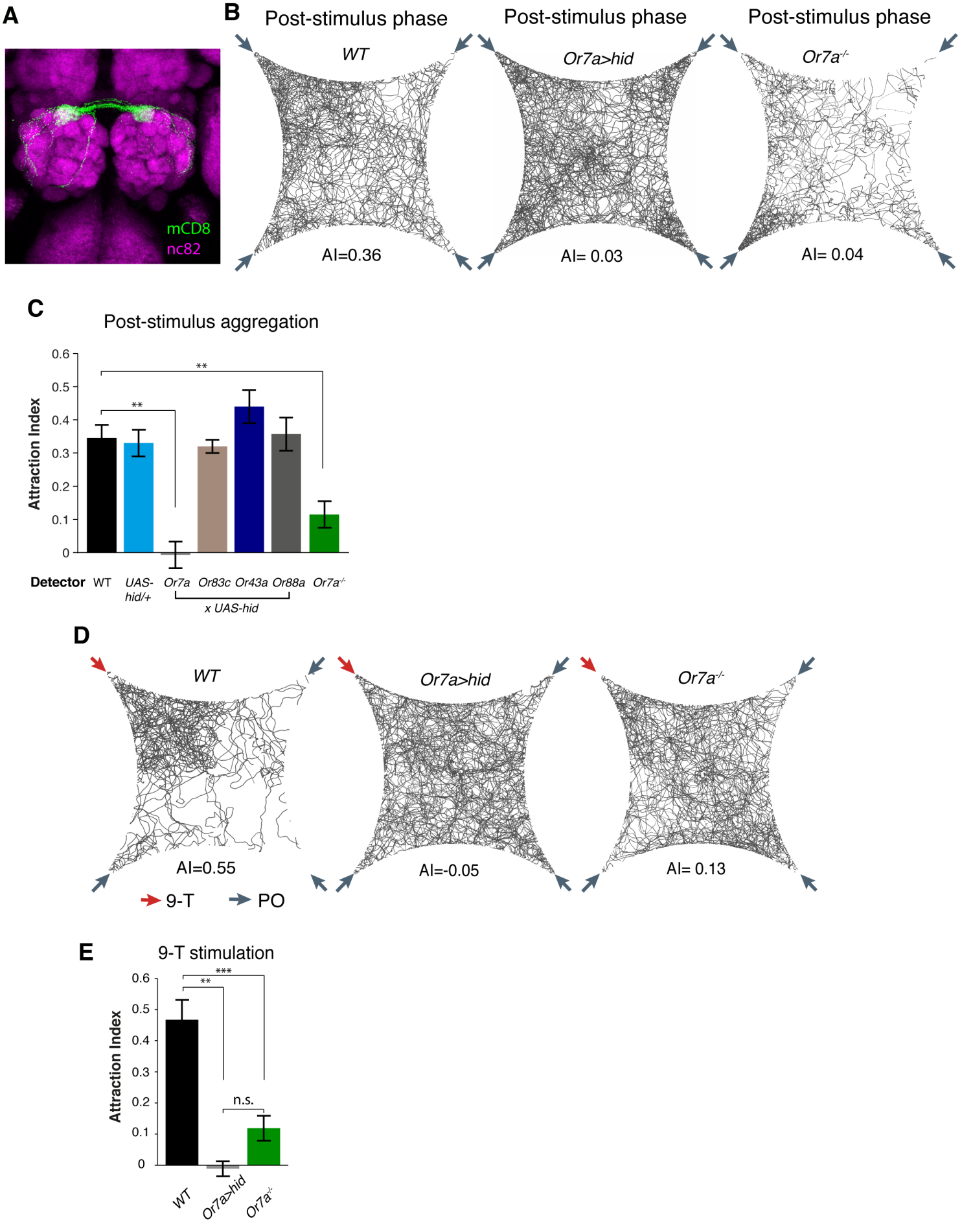
Detector flies
C
ACV
AI=0.91
1. Clean air 10 mins
2. Rotate 90°
No odor 10-15 mins
AI=0.55
D
ACV
No flies
1. Clean air 10 mins
2. Rotate 90°
No odor 10-15 mins
AI= 0.08
E
HA
AI=0.67
1. Clean air 10 mins
2. Rotate 90°
No odor 10-15 mins
AI=-0.01
F
Post-stimulus aggregation
***
Post-stimulus AI
0.6
0.5
0.4
0.3
0.2
0.1
0
-0.1
ACV+WT
ACV only
HA+WT
G
O
C
C
C
Attraction Index (AI)=
Odor - Control avg
Odor + Control avg
H
Stimulus
1
2
4
3
Depositor flies
1. Clean air 10 mins
2. Rotate 90°
3. + Naive flies
Post-stimulus
4
1
3
2
Detector flies
I
Stimulus attraction
Post-ACV aggregation
n.s.
Stimulus AI
1.0
0.8
0.6
0.4
0.2
0
♀+♂
♀
♂
Sex of depositor
**
**
Post-stimulus AI
0.4
0.3
0.2
0.1
0
Detector flies(♀+♂)

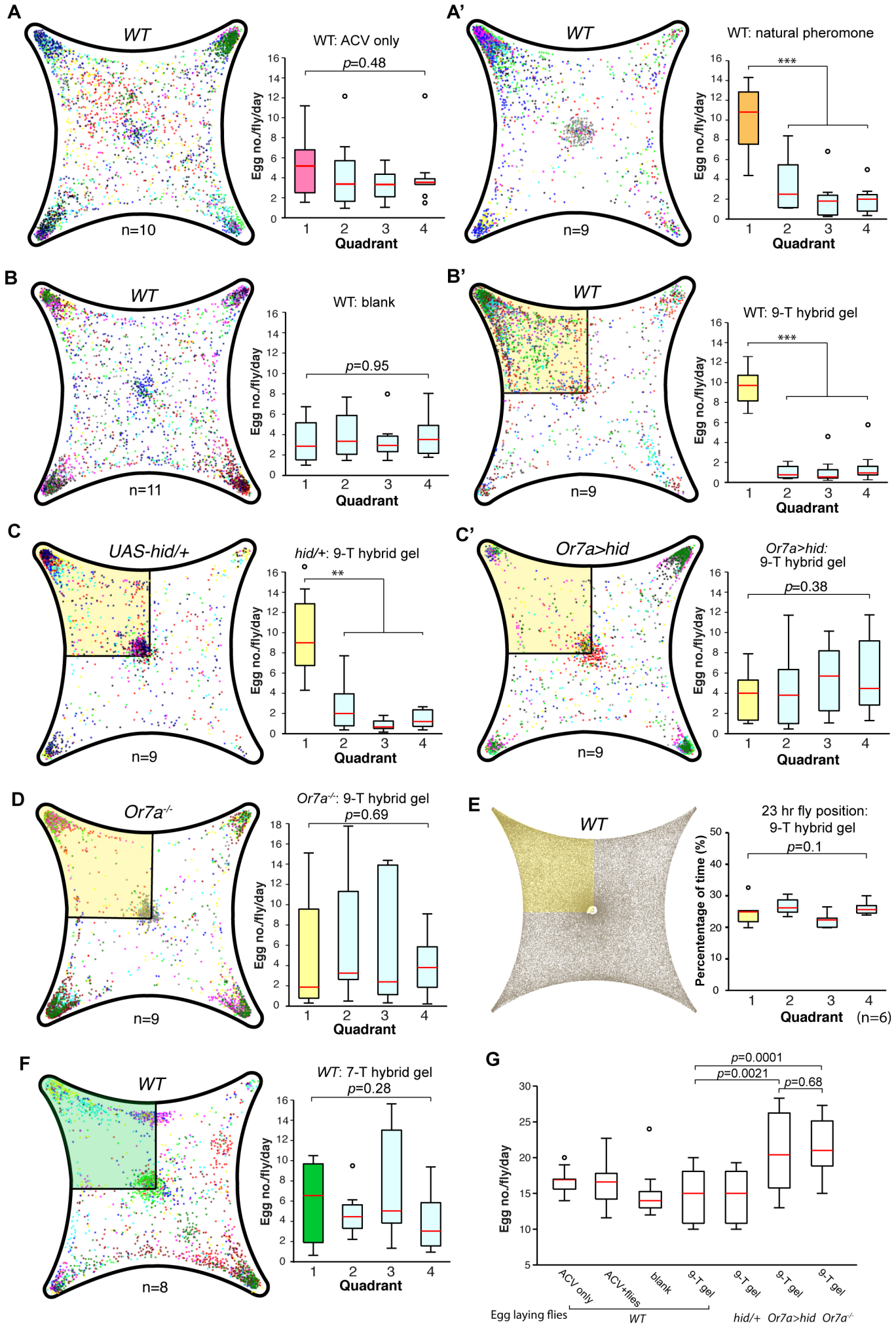
Lin et. al. Figure 2



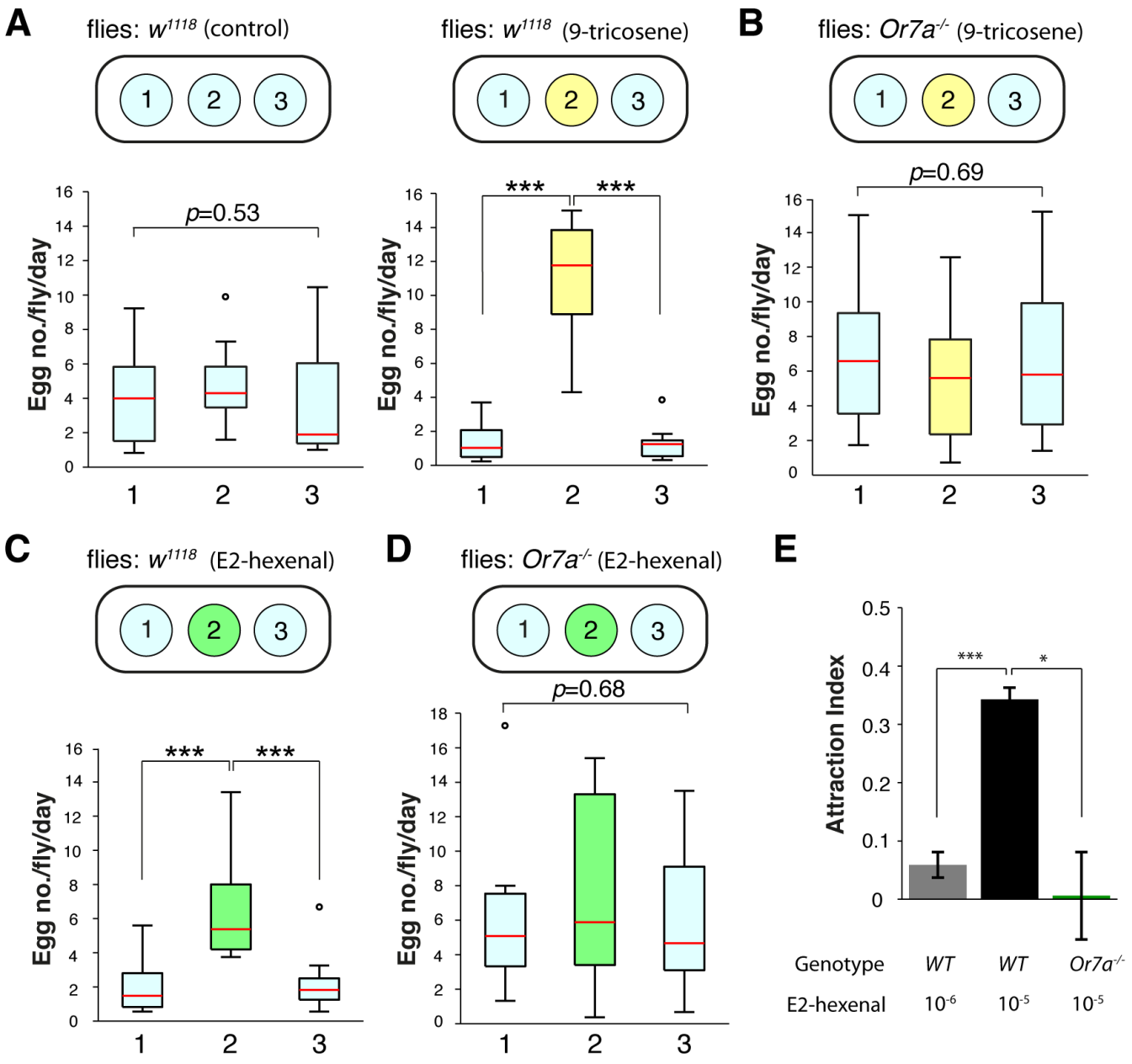

A
Depositor flies
Pheromone extract
New arena
+ Naive flies
B
Pheromone extract
ACV+flies
Start (0 sec)
End (42 sec)
Control
HA+flies
C
ACV only (no flies)
HA + flies
ACV + flies
Relative Abundance
Time (min)
Peak # Chain Length Compound Name Fold Enriched
1 C23 tricosane
2 C23 9-tricosene 3
3 C23 7-tricosene 3.5
4 C23 exogenous impurity
5 C23 5-tricosene
6 C25 pentacosane
(Z)9-tricosene
(Z)7-tricosene
D
9-T
AI=0.42
E
Attraction Index
9-T Dilution (V)
F
9-T (10-4)
Start (0 sec)
End (15 sec)


A
w1118
orco-/-
Mineral oil
9-T
1 mV
1 sec
B
WT male
WT female
orco-/- male
orco-/- female
Response (mV)
Mineral oil
0.1%
1%
10%
100%
9-tricosene
C
CH3(CH2)6CH2
CH2(CH2)11CH3
△Spikes/sec
ab1 ab2 ab3 ab4 ab5 ab6 ab7 ab8 ab9 ab10 pb1 pb2 pb3 at1 ai2 ai3 at4 ac3
Basiconic
Trichoid
Intermediate
Coeloconic
D
9-T
ab4
ab3:
△halo/△halo;
Or22a-Gal4
ab3:
△halo/△halo;
Or22a-Gal4/UAS-Or7a
2 mV
1 sec
E
Male ab4 neuron
Male ab3 empty neuron + Or7a
Female ab4 neuron
Female ab3 empty neuron + Or7a
△Spikes/sec
0.1%
1%
10%
100%
9-tricosene
F
Or7a-/-: ab4 sensillum
9-T
2 mV
1 sec
Geosmin
G
ab4A (Or7a)
No ab4A
spike detected
△Spikes/sec
9-T
Geosmin
ab4B (Or56a)
9-T
Geosmin


A
mCD8
nc82
B
Post-stimulus phase
WT
AI=0.36
Post-stimulus phase
Or7a>hid
AI= 0.03
Post-stimulus phase
Or7a-/-
AI= 0.04
C
Post-stimulus aggregation
Attraction Index
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
Detector
WT
UAS-hid/+
Or7a
Or83c
Or43a
Or88a
Or7a-/-
x UAS-hid
D
WT
AI=0.55
Or7a>hid
AI=-0.05
Or7a-/-
AI= 0.13
9-T
PO
E
9-T stimulation
Attraction Index
0.6
0.5
0.4
0.3
0.2
0.1
0
***
**
n.s.
WT
Or7a>hid
Or7a-/-


A
WT
n=10
WT: ACV only
p=0.48
Egg no./fly/day
Quadrant
A'
WT
n=9
WT: natural pheromone
***
B
WT
n=11
WT: blank
p=0.95
B'
WT
n=9
WT: 9-T hybrid gel
***
C
UAS-hid/+
n=9
hid/+: 9-T hybrid gel
**
C'
Or7a>hid
n=9
Or7a>hid:
9-T hybrid gel
p=0.38
D
Or7a-/-
n=9
Or7a-/-: 9-T hybrid gel
p=0.69
E
WT
23 hr fly position:
9-T hybrid gel
p=0.1
Percentage of time (%)
Quadrant (n=6)
F
WT
n=8
WT: 7-T hybrid gel
p=0.28
G
p=0.0001
p=0.0021
p=0.68
ACV only
ACV+flies
blank
9-T gel
9-T gel
9-T gel
9-T gel
Egg laying flies
WT
hid/+ Or7a>hid Or7a-/-

Lin et. al. Figure 7



Lin et. al. Figure 8

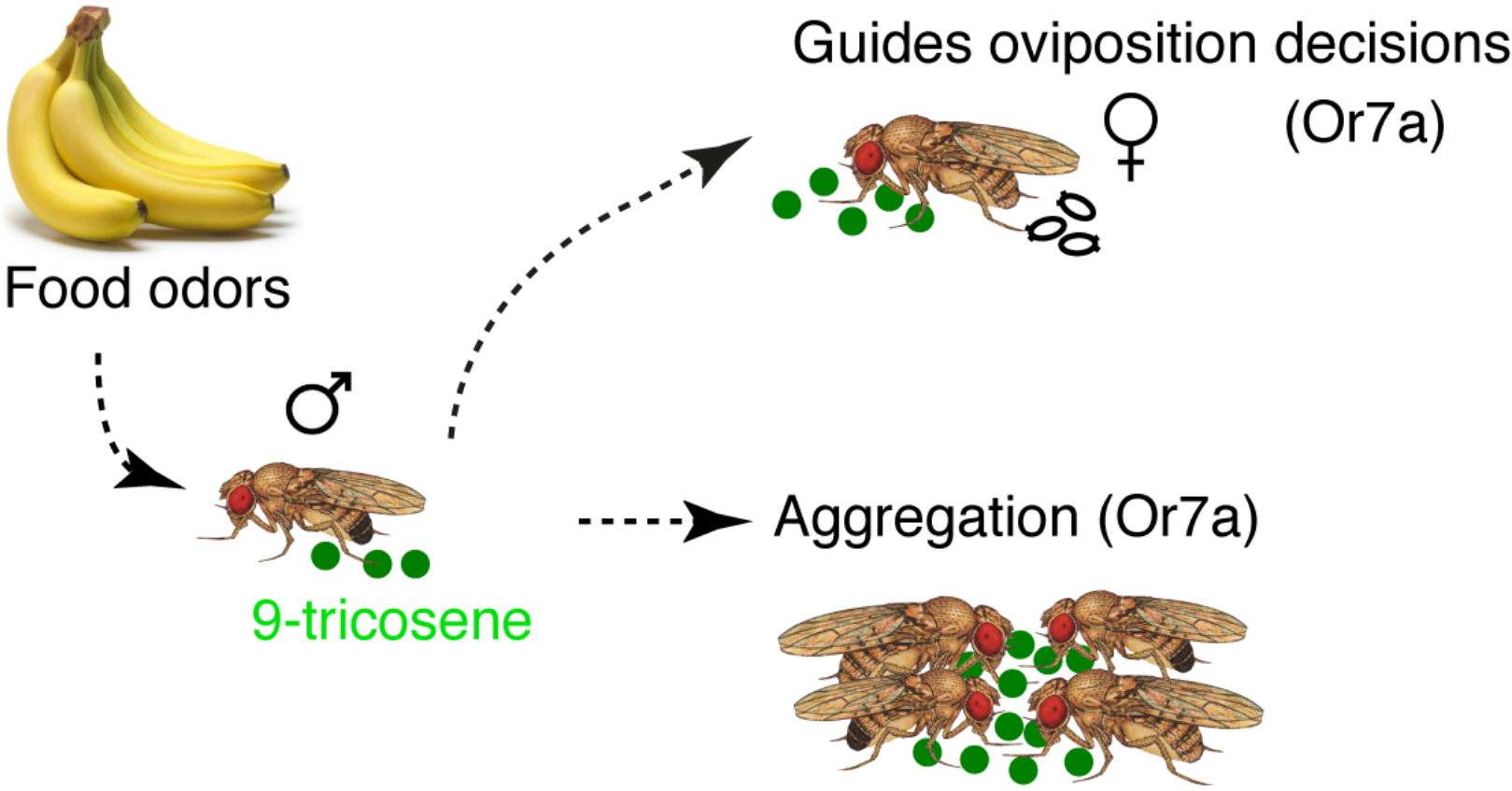
A

Odorant to OR activites

	Or7a	Or35a	Or67a	Or67c
E2-hexenal	++++	++++	++	0
Benzaldehyde	++++	+	++++	0
1-butanol	+++	+++	+	+
1-propanol	+++	+	0	0
Hexyl butyrate	-	++++	+	0
Pentanoic acid	0	0	++++	0
Ethyl lactate	0	0	0	++++

B

Oviposition preference

	10^{-4}	10^{-3}	10^{-2}	10^{-1}
E2-hexenal	--	++	+++	n.d.
Benzaldehyde	-	++	+++	--
1-butanol	n.d.	+	++	++
1-propanol	n.d.	-	+	++++
Hexyl butyrate	n.d.	-	--	-
Pentanoic acid	--	---	++	n.d.
Ethyl lactate	--	-	-	n.d.

Lin et. al. Figure 9