The *Drosophila* pheromone Z4-11Al is encoded together with habitat olfactory cues and mediates species-specific communication

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Abstract

Mate recognition in animals evolves during niche adaptation and involves habitat and social olfactory signals. *Drosophila melanogaster* is attracted to fermenting fruit for feeding and egg-laying. We show that, in addition, female flies release a pheromone (*Z*)-4-undecenal (*Z*4-11Al), that elicits flight attraction in both sexes. The biosynthetic precursor of *Z*4-11Al is the cuticular hydrocarbon (*Z,Z*)-7,11-heptacosadiene (7,11-HD), which is known to afford reproductive isolation between *D. melanogaster* and *D. simulans*. A pair of alternatively spliced receptors, Or69aB and Or69aA, is tuned to *Z*4-11Al and to food olfactory cues, respectively. These receptors are co-expressed in the same olfactory sensory neurons, and feed into a neural circuit mediating species-specific, long-range communication: the close relative *D*. *simulans* does not respond. That Or69aA and Or69aB have adopted dual olfactory traits highlights the interplay of habitat and social signals in mate finding. These olfactory receptor genes afford a collaboration between natural and sexual selection, which has the potential to drive phylogenetic divergence.

Keywords

pheromone, kairomone, olfaction, reproductive isolation

Introduction

Volatile insect pheromones transmit species-specific messages over a distance. Premating communication with pheromones facilitates and accelerates mate-finding, and reduces predation risk and energy expenditure, which is particularly adapative in short-lived insects (1,2).

Sexual communication subserves mate-finding and reproduction. The sensory drive hypothesis predicts that mate recognition in animals evolves during niche adaptation and that premating sexual communication involves olfactory specialization to both social signals and habitat cues (3). Pheromones are released into an atmosphere that is filled with environmental, habitat-related olfactory cues, some of which signal mating sites and food sources. The response to pheromones and habitat odourants (kairomones) is under sexual and natural selection, respectively. Pheromones and kairomones are always perceived as an ensemble in a natural context and this leads to interaction of sexual and

natural selection during adaptive divergence of sexual signaling, which is thought to facilitate premating reproductive isolation (3-6).

Olfactory sexual communication is studied at cellular and molecular resolution in the fruit fly *Drosophila melanogaster*, but volatile pheromones encoding species-specific, long-range mate recognition have not yet been found. *Drosophila* is attracted to yeast and fruit odorants for feeding, mating and oviposition (7-9) and the interconnection between perception of pheromones and food semiochemicals is a current research theme (10, 11). For example, the male-produced sex pheromone cVA and food stimuli are integrated to coordinate feeding, courtship behavior and oviposition site selection (12-15). Perception of cVA is a current and outstanding paradigm for studying the molecular and neuronal logic of innate, olfactory-mediated reproductive behavior (14, 16, 17). cVA and other olfactory pheromones are active during courtship. Since they are shared with other *Drosophila* species, they cannot account for species-specific communication (18-20).

Interspecific matings of *D. melanogaster* with sibling species are inhibited by the female-produced cuticular hydrocarbon (*Z,Z*)-7,11-heptacosadiene (7,11-HD), which is not volatile and perceived through gustatory receptors only at close range (21-23). This raises the question whether *Drosophila* uses, in addition, volatile pheromone signals that mediate specific mate recognition at a distance.

We have identified the first long-range, species-specific pheromone in *D. melanogaster*. A pair of spliced olfactory receptors, feeding into the same neural circuit, has developed a dual affinity to this pheromone and to environmental semiochemicals, encoding adult and larval food. A blend of this pheromone and a food odourant specifically attracts *D. melanogaster*, but not the close relative *D. simulans*. This becomes an excellent paradigm to study the interaction of social signals and habitat olfactory cues in premating reproductive isolation and phylogenetic divergence.

Results

Drosophila melanogaster females produce a suite of volatile aldehydes.

We analysed volatile compounds released by *D. melanogaster* flies, by gas chromatography-mass spectrometry (GC-MS), and found 16 aliphatic aldehydes. Males and females shared saturated aldehydes with a carbon chain length of C7 to C18, but mono-unsaturated aldehydes were released by females only (Fig. 1a; Table 1). The most abundant compound was identified as (*Z*)-4-undecenal (*Z*4-11Al) and synthesized.

GC-MS analysis showed that *Z*4-11Al was present also in cuticular extracts of females, although in lower amounts (0.27 ± 0.12 ng/female, n = 5) than in headspace collections

84 (3.0 ± 0.81 ng/female, n = 10; P<0.01 Mann-Whitney test). Cuticular profiles of
85 *Drosophila* flies have been investigated, but *Z*4-11Al or other aldehydes have not been
86 reported (18, 24, 25).

87 The closely related *D. simulans* did not release *Z*4-11Al, or other monounsaturated
88 aldehydes (Fig. 1a). Unlike *D. melanogaster*, *D. simulans* does not produce (*Z,Z*)-7,11-
89 heptacosadiene (7,11-HD) (21). This led us to hypothesize that the production of mono-
90 unsaturated aldehydes with a double bond in position 4 was linked to oxidation of di-
91 unsaturated cuticular hydrocarbons. Oxidation of 7,11-HD is expected to generate two
92 saturated aldehydes, heptanal and hexadecanal, and two unsaturated aldehydes, *Z*4-11Al
93 and (*Z*)-4-eicosenal (Fig. 1b). This was experimentally verified by applying 100 ng
94 synthetic 7,11-HD to a glass vial. After 60 min, 1.92 ± 0.42 ng *Z*4-11Al were retrieved (n
95 = 3). Based on the cuticular hydrocarbon profile of *D. melanogaster* (24), 26 aldehydes
96 are expected to be formed by oxidation, 16 of which were found in our headspace
97 analysis (Table 1), others may have been below detection level.

98 Next, single sensillum electrophysiological recordings (SSR) from all basiconic, trichoid,
99 coeloconic, and intermediate olfactory sensilla in *D. melanogaster* (Fig. 1c) and GC-
100 coupled SSR recordings (GC-SSR) from ab9 sensilla (Fig. 1d) showed that *Z*4-11Al
101 strongly activates ab9A olfactory sensory neurons (OSNs).

102 The olfactory receptor Or69aB responds to *Z*4-11Al.

103 ab9A OSNs express the olfactory receptor (Or) Or69a (26). We therefore screened ab9A
104 OSNs with known ligands of Or69a (27) and *Z*4-11Al. In the *D. melanogaster* strains
105 Canton-S and Zimbabwe, the monoterpene (*R*)-carvone elicited the strongest response
106 from ab9A, although the response to *Z*4-11Al was not significantly different. In *D.*
107 *simulans*, Z4-11Al elicited a significantly lower response than (*R*)-carvone (Fig. 3a).

108 The Or69a gene encodes two proteins, Or69aA and Or69aB, as a result of alternative
109 splicing (Fig. 3d), prior to the split of the *D. obscura* and *D. melanogaster* groups (28,
110 29). Heterologous co-expression of both Or69a splice variants in ab3A (*Δhalo*) empty
111 neurons (30) produced a response similar to native ab9A OSNs; whereas individual
112 expression revealed distinct response profiles for Or69aA and Or69aB (Fig. 3a,b). Or69aB
113 responds best to both isomers of carvone, followed by *Z*4-11Al. These compounds share
114 a structural motif, a carbonyl functional group with an equidistant double bond in position
115 4 (Fig. 3b,c). Upon binding to the same Or, ligands adopt complementary bioactive
116 conformations; the strain energy required to achieve the bioactive conformation should
117 typically not exceed 5 kcal/mol (31). Conformational analysis showed that *Z*4-11Al aligns
118 with (*R*)-carvone at a strain energy cost of only 1.5 kcal/mol. Or69aA, on the other hand,
119 is tuned to terpenoid alcohols and responded significantly less to *Z*4-11Al. The most
120 active ligands (*S*)-terpineol, (*S*)- and (*R*)-linalool, which again share the functional group
121 and a double bond in position 4, align at 3.0 kcal/mol (Fig. 3b,c).

The Or69a splice event enabled adaptive changes in ligand tuning in the newly emerged twin receptor. Functional divergence has apparently been biased towards structurally related ligands (Fig. 3b,c; 27) and ecologically relevant odorant signals. Citrus peel, a preferred oviposition substrate (8), is a common source of all main ligands of Or69aA and Or69aB (32) and possibly a driver of Or69a ligand evolution. Diversification of the splice variants is, however, constrained to a behavioral theme, since both Ors feed into one OSN.

Z4-11Al elicits upwind flight attraction in *D. melanogaster*, but not in *D. simulans*.

*Z*4-11Al elicited upwind flight and landing at the source, in cosmopolitan Dalby and Canton-S strain *D. melanogaster* males and females. In contrast, males of the Zimbabwe strain and the sister species *D. simulans* were not attracted (Fig. 4a,b). This shows that *Z*4-11Al, in addition to its precursor 7,11-HD (Fig. 1) participates in sexual isolation between *D. melanogaster* and *D. simulans* (21), and between cosmopolitan and African *D. melanogaster* strains (33-35). Moreover, admixture of *Z*4-11Al eliminated *D. simulans* attraction to the yeast volatile (*R*)-linalool (31) (Fig. 4a). Finally, we used tetanus toxin mutants to verify that Or69a encodes *Z*4-11Al. Upwind flight attraction was significantly reduced when Or69a OSNs were disrupted (Fig. 4b).

The response rate to Z4-11Al released at a rate of 10 ng/min was similar to the upwind flight response to vinegar, when acetic acid, the main compound of vinegar headspace, was released at ca. 17 µg/min (Fig. 4a; 36). In summary, *Z*4-11Al is a powerful attractant that enables specific mate recognition at a distance and its interspecific role was enhanced through interaction with the food odour linalool.

Discussion

*Z*4-11Al is the first species-specific, long-range sex pheromone of *D. melanogaster*. It is produced by females and perceived by Or69aB in both sexes. We further show that Or69aB and its twin receptor Or69aA bind kairomonal terpenoids, such as linalool, found in fruit and yeast headspace (32, 37). Combined social and food odour tuning in the two Or69a splice variants underscores the tie between sexual and natural selection during the evolution of specific mate communication (3, 4). Tinting food odorants with sex pheromone foregrounds communication channels and facilitates mate finding. This is particularly adaptive in *Drosophila* when mating sites, fruit and berries, are abundant and widely spread.

155 Olfactory representations of other *Drosophila* Ors involved in food and pheromone
156 perception project through separate channels to the LH, where they partially overlap and
157 integrate (14). In stark contrast, Or69a is the first olfactory gene known to encode dual
158 olfactory traits. Or69aA and Or69aB co-express in the same ab9A OSNs (26) and thus
159 achieve a coordination of mating and food stimuli already in first order neurons, at the
160 antennal periphery. This makes Or69a a target for selection during phylogenetic
161 divergence. The tuning range of Ors evolves more rapidly than hardwired neural circuits
162 in higher brain centres (38) and selection pressure is further relaxed following a splice
163 event. Differential tuning of Or69a in closely related cosmopolitan and African strains of
164 *D. melanogaster* corroborates this idea.

165 Tuning changes in the two splice forms of Or69a are constrained with respect to the
166 behavioral and ecological role of their ligands, since they both feed into a neural circuit
167 mediating sexual and habitat attraction, and courtship. The two splice forms provide, on
168 the other hand, degrees of freedom during adaptive divergence, since they allow fly
169 populations to adopt new kairomone and pheromone signals; alteration of either one
170 produces a new communication channel. Reproductive isolation may arise as a byproduct
171 and the Or69a gene therefore has the potential to drive speciation (39, 40).

172 # Methods

173 ## Insects

174 Canton-S, Zimbabwe (S-29; Bloomington #60741) and Dalby-HL (Dalby, Sweden) (41)
175 strains of *D. melanogaster* were used as wild type flies for behavioral experiments.
176 Canton-S was used for comparison with knockouts of the same background. Further tests
177 were done with the sister species *D. simulans*.

178 We used the Or69a-Gal4/UAS TeTx, tetanus toxin knockout line to verify the role of
179 Or69a in flight attraction to *Z*4-11Al. Canton-S/UAS TeTx (Bloomington #28838 and
180 28997) and Canton-S/Or69a-Gal4 (Bloomington #10000) were used as parental controls.

181 Flies were reared on a standard sugar-yeast-cornmeal diet at room temperature (19 to
182 22°C) under a 16:8-h L:D photoperiod. Newly emerged flies were anesthetized under CO_2
183 and sexed under a dissecting microscope. Virgin flies were identified by the presence of
184 meconium, and were kept together with flies of the same sex. Flies were kept in 30-ml
185 Plexiglas vials with fresh food. Experiments were done with 3- to 5-d-old flies.

186 ## Chemicals

187 (*Z*)-4-undecenal (*Z*4-11Al) and (*E*)-4-undecenal (*E*4-11Al) were synthesized. A short
188 description follows below, for a complete account of the chemical synthesis, see
189 Supplemental Information.

190 (*Z*)-4-Undecenoic acid was synthesized via a modified version of Wube et al. (42) in 80%
191 stereoisomeric purity. Esterification under acidic conditions with sulfuric acid in methanol
192 resulted in 80% *Z*-isomer and a 93% yield over two steps. Stereoisomeric purity was
193 controlled with NMR and GC-FID by comparing analysis for acid and ester, the
194 appearance of a small quartet, in the NMR spectra, at 1.96 indicates the presence of *E*-
195 isomer. Gas chromatographic separation on a polar Varian factorFOUR vf-23ms of *Z*- and
196 *E*-ester proved that the stereochemistry was not affected by the acidic conditions during
197 esterification. Methyl (*Z*)-4-undecenoate was purified on regular silica gel and on silver
198 nitrate impregnated silica gel to obtain a stereoisomeric purity of 98.6%. Methyl (*Z*)-4-
199 undecenoate was reduced to (*Z*)-4-undecenol with lithium aluminum hydride in
200 diethylether and oxidized to *Z*4-11Al with Dess-Martin periodinane in dichloromethane.

201 A modified version of Virolleaud's (43) metathesis was used to produce (*E*)-4-undecenoic
202 acid in a 56% yield (87.5% of the *E*-isomer). (*E*)-4-undecenoic acid was esterified under
203 the same conditions as the (*Z*)-acid, without isomerisation of the double bond (according
204 to GC-FID and ^{1}H-NMR). The methyl-(*E*)-4-undecenoate was reduced to the alcohol with
205 lithium aluminum hydride in diethylether and purified on silver nitrate impregnated silica
206 gel to obtain a purity of 99.8% of the (*E*)-isomer, which was oxidized with Dess-Martin
207 periodinane in dichloromethane to obtain *E*4-11Al.

208 Commercially available compounds were: (*R*)-carvone (97% chemical purity, CAS #6485-
209 40-1, Firmenich), (*S*)-carvone (98%, CAS #2244-16-8, Firmenich), (*S*)-terpineol (97%,
210 CAS #10482-56-1, Aldrich), (*S*)-linalool (97%, CAS #126–91–0, Firmenich), (*R*)-linalool
211 (97%, CAS #126-90-9, Firmenich), citronellol (99%, CAS #106-22-9, Aldrich), geraniol
212 (98%, CAS #106-24-1, Aldrich), 3-octanol (99%, CAS #589-98-0, Aldrich), decanol
213 (99%, CAS #112-30-1, Fluka), 11-Al (99%, CAS #112-44-7, Aldrich).

214 ## Odor collection and chemical analysis

215 Twenty *D. melanogaster* (Dalby), *D. melanogaster* (Canton) (*n* = 10) or 20 *D. simulans*
216 (*n* = 10) virgin female or virgin male flies were placed in a glass aeration apparatus
217 designed for collection of airborne pheromone (effluvia) (44). The flies were held in a
218 glass bulb with a narrow open outlet (ø 1 mm), which prevented them from escaping. A
219 charcoal-filtered air flow (100 mL/min) passed over the flies during 75 min. Fly effluvia
220 were collected on the glass surface, breakthrough was monitored by attaching a 10-cm
221 glass capillary (ø 1 mm). After 75 min, flies were removed, 100 ng of heptadecenyl

222 acetate (internal standard) was deposited in the glass bulb, which was then rinsed with
223 50 µl hexane, the solvent was concentrated to 10 µl in Francke vials.

224 Cuticular extracts (*n* = 5) were obtained by dropping 20 *D. melanogaster* females for 5
225 min in 400 µl hexane containing 100 ng heptadecenyl acetate. After 5 min, the extracts
226 were transferred to Francke vials and concentrated to 10 µl before analysis. Fly extracts
227 and volatile collections were stored at -20°C.

228 Oxidation of (*Z,Z*)-7,11-heptacosadiene (7,11-HD) was analysed by depositing 100 ng
229 synthetic 7,11-HD in a 1.5-mL glass vial, at 19°C. Vials were rinsed with 10 µl of hexane,
230 which contained 100 ng heptadecenyl acetate as an internal standard, after 15, 30, 45,
231 60 and 75 min (*n* = 3).

232 Samples were analysed by combined gas chromatography and mass spectrometry (GC-
233 MS; 6890 GC and 5975 MS, Agilent technologies Inc., Santa Clara, CA, USA). Two µl
234 were injected (injector temperature 225°C) splitless (30 s) into fused silica capillary
235 columns (60 m x 0.25 mm), coated with HP-5MS UI (Agilent Technologies Inc., d_f = 0.25
236 µm) or DB-wax (J&W Scientific, Folsom, CA, USA, d_f = 0.25 µm), that were temperature-
237 programmed from 30 to 225°C at 8°C/min. Helium was used as mobile phase at 35 cm/s.
238 The MS operated in scanning mode over m/z 29-400. Compounds were tentatively
239 identified based on their mass spectra and Kovats retention indices, using custom and
240 NIST (Agilent) libraries, as well as authentic standards.

241 Behavioural assays

242 Upwind flight behavior was observed in a glass wind tunnel (30 x 30 x 100 cm) equipped
243 with a piezo sprayer (36). The flight tunnel was lit diffusely from above, at 13 lux,
244 temperature ranged from 22 to 24°C, relative humidity from 38% to 48% and charcoal
245 filtered air, at a velocity of 0.25 m/s, was produced by a fan (Fischbach GmbH,
246 Neunkirchen, Germany). Compounds were delivered from the centre of the upwind end of
247 the wind tunnel via a piezo-electric micro-sprayer (45). Forty flies were flown individually
248 to each treatment. "Attraction" was defined as upwind flight, directly from a release tube
249 at the end of the tunnel over 80 cm towards the odor source, followed by landing.
250 Unmated fed, 3-d-old Dalby wild-type males and females, *D. melanogaster* Zimbabwe
251 strain males and *D. simulans* males were flown towards (*Z*)-4-undecenal (released at 10
252 ng/min), (*R*)-linalool (10 ng/min) and the blend of (*Z*)-4-undecenal and (*R*)-linalool (10
253 ng/min, each).

254 Heterologous expression of Or69aA and Or69aB

255 Or69aA and Or69aB receptors were cloned from antennae of *D. melanogaster*, Dalby line
256 (46). Briefly, cDNA was generated from RNA extracts of antennae of 100 males and
257 females using standard procedures. Or69a variants were PCR amplified with the following

258 primers: Or69aA_5′: GTCATAGTTGAAACCAGGATGCAGTTGC, Or69aB_5′:
259 ATAATTCAGGACTAGATGCAGTTGGAGG, Or69aAB_3′:
260 TGCACTTTTGCCCTTTTATTTAAGGGAC.

261 The splice variants were amplified with unique 5′ primers and a common 3′ primer,
262 reflective of genomic structure at this locus. These primers encompass the entire open
263 reading frame of the receptor variants, and are located partially upstream and
264 downstream of the start and stop codons. PCR amplicons were gel-purified and cloned
265 into the pCR8/GW/Topo-TA Gateway entry vector (Thermo-Fisher Scientific, Waltham,
266 MA, USA) according to standard procedure, with vector inserts sequenced to confirm
267 fidelity of Or sequence. Or inserts were subsequently transferred to pUAS.g-HA.attB (47)
268 with LR Clonase II enzyme (Thermo-Fisher Scientific), according to manufacturers
269 protocol; vector inserts were sequenced to confirm fidelity of Or sequence.

270 Mini-prep purified pUAS.g-HA.attB plasmid with Or69aA or Or69aB insert were delivered
271 to Best Gene Inc. (Chino Hills, CA USA) for generation of transgenic *D. melanogaster*
272 flies. Using the PhiC31 targeted genomic-integration system (47) vectors with Or69aA or
273 Or69aB were injected into the following fly strain, for integration on the 3rd chromosome:
274 M{3xP3-RFP.attP}ZH-86Fb (with M{vas-int.Dm}ZH-2A) (Bloomington *Drosophila* Stock
275 Number: 24749). For expression of single receptor variants in the empty neuron system,
276 Or69a transgenes were crossed into the *Δhalo* background to give genotype: w;
277 *Δhalo*/Cyo; UAS-DmelOr69a(A or B), and these flies were crossed to flies with genotype:
278 w; *Δhalo*/Cyo; DmelOr22a-Gal4, as described previously (46). Experimental
279 electrophysiology assays were performed on flies with genotype: w; *Δhalo*; UAS-
280 DmelOr69a(A or B)/DmelOr22a-Gal4.

281 For co-expression of Or69aA and Or69aB in the same empty neurons, a second fly-line
282 with Or69aB was generated with Or69aB present on the X-chromosome. The same UASg-
283 HA.attB:Or69aB plasmid generated previously was injected into the following fly strain:
284 y,w, P{CaryIP}su(Hw)attP8 (Bloomington *Drosophila* Stock Number: 32233). The Or69aB
285 transgene was crossed into the DmelOr22a-Gal4 line in *Δhalo* background to give
286 genotype: UAS-DmelOr69aB; *Δhalo*/Cyo; DmelOr22a-Gal4; these flies were crossed to
287 flies with genotype: w; *Δhalo*/Cyo; UAS-DmelOr69aA. Experimental electrophysiology
288 assays were performed on flies with genotype: UAS-DmelOr69aB/w; *Δhalo*; UAS-
289 DmelOr69aA/DmelOr22a-Gal4.

290 Conformational analysis

291 MacroModel version 11.0 (Schrodinger LLC, New York, NY, USA) in the Maestro Version
292 10.4.017 were used to build, minimize and to perform conformational analysis of *Z*4-
293 11Al, (*R*)-carvone, (*S*)-terpineol and (*R*)-linalool, using default settings (OPLS3 as force
294 field, water as the solvent and mixed torsional/low-mode sampling method). The

295 assumed bioactive conformations of the conformationally more flexible compounds, *Z*4-
296 11Al and (*R*)-linalool, were based on the positon of the shared functional groups in the
297 conformationally more restricted compounds, (*R*)-carvone and (*S*)-terpineol. The
298 carbonyl and the double bond atoms were kept fixed during minimization of the proposed
299 bioactive conformation of Z4-11Al; the alcohol functional group and the double bond were
300 kept fixed in (*R*)-linalool. The strain energies, the energy cost for adopting proposed
301 bioactive conformations, were then calculated as the difference between the lowest
302 energy conformations and the assumed bioactive conformation.

303 Electrophysiological recordings

304 Single sensillum recordings (SSR) were done as described earlier (18). Unmated males
305 were restrained in 100-µl pipette tips, with half of the head protruding, the third antennal
306 segment or palps were placed on a glass microscope slide and held by dental wax. For
307 the initial screening, all basiconic, trichoid, coeloconic, and intermediate sensilla (26)
308 were localized in *D. melanogaster* (Canton-S strain) males, under a binocular at 1000x
309 magnification. Further recordings were made from small basiconic ab9 sensilla, in *D.*
310 *melanogaster* (Canton-S and Zimbabwe strains) and in *D. simulans* males, and from large
311 basiconic ab3 sensilla in mutant *D. melanogaster*, where Or69aA and Or69aB were
312 heterologously expressed (see above).

313 Tungsten electrodes (diameter 0.12 mm, Harvard Apparatus Ltd, Edenbridge, United
314 Kingdom) were electrolytically sharpened with a saturated KNO_3 solution. The recording
315 electrode was introduced with a DC-3K micromanipulator equipped with a PM-10 piezo
316 translator (Märzhäuser Wetzler GmbH, Germany) at the base of the sensilla. The
317 reference electrode was inserted into the eye. The signal from olfactory sensory neurons
318 (OSNs) was amplified with a probe (INR-02; Syntech), digitally converted by an IDAC-4-
319 USB (Syntech) interface, and analyzed with Autospike software v. 3.4 (Syntech). Neuron
320 activities were recorded during 10 s, starting 2 s before odor stimulation. Neuron
321 responses were calculated from changes in spike frequency, during 500 ms before and
322 after odor stimulation.

323 Odorants were diluted in redistilled hexane, 10 µg of test compounds in 10 µl hexane
324 were applied to filter paper (1 cm^2), kept in Pasteur pipettes. The test panel contained
325 the most active ligands known for Or69a (27) and several aldehydes. Diagnostic
326 compounds for confirmation of sensillum identity were 2-phenyl ethanol (ab9) and 2-
327 heptanone (ab3). Control pipettes contained solvent only. Puffs (2.5 ml, duration 0.5 s)
328 from these pipettes, produced by a stimulus controller (Syntech GmbH, Kirchzarten,
329 Germany), were injected into a charcoal-filtered and humidified airstream (0.65 m/s),
330 which was delivered through a glass tube to the antenna.

331 For GC–SSR recordings, GC columns and the temperature programmes were the same as
332 for the GC–MS analysis. At the GC effluent, 4 psi of nitrogen was added and split 1:1 in a

3D/2 low dead volume fourway-cross (Gerstel, Mühlheim, Germany) between the flame ionization detector and the antenna. Towards the antenna, the GC effluent capillary passed through a Gerstel ODP-2 transfer line, that tracked GC oven temperature, into a glass tube (30 cm x 8 mm ID), where it was mixed with charcoal-filtered, humidified air (20°C, 50 cm/s).

Statistical analysis

Generalized linear models (GLM) with a Bernoulli binomial distribution were used to analyse wind tunnel data. Landing at source and sex were used as the target effects. *Post-hoc* Wald pairwise comparison tests were used to identify differences between treatments. For all the electrophysiological tests, differences in spike activity derived from SSRs were analyzed with Kruskall Wallis H test followed by pairwise comparisons with Mann Whitney U *post hoc* test. All statistical analysis were carried out using R (R Core Team 2013) and SPSS Version 22 (IBM Corp.).

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Author Contributions

S.L., P.G.B. and P.W. conceived the study. F.B.-E. and M.S. carried out behavioural studies, F.G. and W.B.W produced empty neuron flies, F.G. and H.D. did single sensillum recordings, supervised by B.S.H, E.W. and E.H. synthesized the compound, A.-L.G. calculated the confirmational analysis, M.B., G.B. and S.L. performed chemical analysis, P.W. wrote the paper with input from all co-authors.

481 ## Legends

482 **Figure 1. Headspace analysis of *Drosophila* females and males by GC-MS**
483 **and electrophysiological screening of the candidate pheromone**
484 **compound *Z*4-11Al on male antennae.**

485 (**a**) Chromatograms of headspace collections from *D. melanogaster* females (lilac traces;
486 upper trace: amplified signal; lower trace: entire chromatogram), males (blue trace), and
487 *D. simulans* females (green trace). The headspace of *D. melanogaster* females contains
488 16 yet undescribed compounds: heptanal (1), octanal (2), (*Z*)4-nonenal (3), nonanal (4),
489 (*Z*)4-undecenal (*Z*4-11Al) (5), undecanal (6), dodecanal (7), (*Z*)4-tridecenal (8),
490 tridecanal (9), tetradecanal (10), pentadecanal (11), (*Z*)4-hexadecenal (12), hexadecanal
491 (13), (*Z*)4-octadecenal (14), octadecanal (15) and (*Z*)4-eicosenal (16) (see Table 1).
492 Female-specific compounds are coloured, the most abundant cuticular hydrocarbon, 7-
493 tricosene (17) is shown for reference, the internal standard (IS) was heptadecenyl
494 acetate. Inset: mass spectrum of the most abundant female-specific compound *Z*4-11Al.

495 (**b**) Oxidation of the most abundant female cuticular hydrocarbon (*Z*,*Z*)-7,11-
496 heptacosadiene (7,11-HD), affording two saturated and two unsaturated aldehydes,
497 heptanal, hexadecanal, *Z*4-11Al and (*Z*)4-eicosenal.

498 (**c**) Single sensillum recordings (SSR) from all *D. melanogaster* olfactory sensory neurons
499 (OSNs) with *Z*4-11Al (error bars show SEM; n = 5).

500 (**d**) SSR coupled to GC (GC-SSR), showing a response of ab9A to three different amounts
501 of *Z*4-11Al.

502 **Figure 2. SSR-Response of Or69a splice variants in native ab9A OSNs**
503 **and in ab3A OSNs, following heterologous expression, to ten odourants.**

504 (**a**) SSR from ab9A OSNs, in *D. melanogaster* males (Canton-S, Zimbabwe) and *D.*
505 *simulans* males, which natively express both splice variants Or69aA and Or69aB.

506 (**b**) SSR from ab3A OSNs in *D. melanogaster,* heterologously expressing Or69aA and
507 Or69aB, together and singly. Test panel includes the known most active ligands for Or69a
508 (27) and three aldehydes. Cross-hatched bars indicate behaviorally active compounds
509 (Fig. 3). Bars followed by different letters indicate statistically significant differences for
510 each fly type (P<0.05; Mann-Whitney test, n = 5 for ab9A, n = 10 for ab3A).

511 (**c**) Key ligands for Or69aA, (*S*)-terpineol (*1*) and (*R*)-linalool (*4*), and for or Or69aB, (*R*)-
512 carvone (*8*) and Z4-11Al (*10*).

513 (**d**) Alternative splicing of Or69a, where coloured boxes A and B show unique exons
514 encoding the splice products; dark boxes show shared exons, generating co-expression of
515 Or69aA and Or69aB in the same neurons in ab9 sensilla in *D*. *melanogaster*.

516 **Figure 3. Z4-11Al mediates long range attraction in *D. melanogaster*.**

517 (**a**) Upwind flights to 10 ng/min of *Z*4-11Al and (*R*)-linalool, followed by landing at the
518 source, in *D*. *melanogaster* (Dalby) males and females, in *D*. *melanogaster* (Zimbabwe)
519 males and *D*. *simulans* males. Lower case letters indicate statistical differences between
520 test insect strains and species, for each treatment. Asterisks indicate significant
521 differences between treatments (n = 40, P<0.001; binomial GLMs followed by post-hoc
522 Wald pairwise comparison tests).

523 (**b**) Upwind flights to 10 ng/min of *Z*4-11Al in *D. melanogaster* (Canton-S) males
524 expressing a tetanus toxin in OSNs expressing Or69a, and in the parental lines. Letters
525 indicate statistical differences within treatments (n = 40, P<0.001; binomial GLM,
526 followed by *Post-hoc* Wald pairwise comparison tests).

527

528 **Table 1. Saturated and unsaturated aldehydes found in headspace**
529 **collections of *D. melanogaster* (Dalby) females and males.**

Compound	Females (%±SD, *n*=10)	Males (%±SD, *n*=10)
heptanal	3.1 ± 0.8	9.9 ± 4.8
octanal	tr ***a***	0.2 ± 0.5
nonanal	4.1 ± 0.9	8.7 ± 7.9
(*Z*)-4-nonenal	tr	- ***b***
undecanal	tr	-
(*Z*)-4-undecenal	23.3 ± 1.8	-
dodecanal	7.9 ± 1.3	-
tridecanal	tr	-
(*Z*)-4-tridecenal	0.4 ± 0.9	-
tetradecanal	11.2 ± 0.8	4.9 ± 4.1
pentadecanal	3.2 ± 0.9	2.4 ± 2.1
hexadecanal	27.8 ± 3.3	69.1 ± 9.7
(*Z*)-4-hexadecenal	2.9 ± 0.3	-
octadecanal	4.7 ± 1.0	4.4 ± 3.9
(*Z*)-4-octadecenal	3.0 ± 0.6	-
(*Z*)-4-eicosenal	5.6 ± 1.7	-

530
531 ***a*** - traces
532 ***b*** - not found
533

Supplementary Information: Chemical Synthesis

Dry THF and dry Et_2O was obtained from a solvent purification system (Activated alumina columns, Pure Solv PS-MD-5, Innovative technology, Newburyport, USA) and used in the reactions when dry conditions were needed. All other chemicals were used without purification. Reactions were performed under Argon atmosphere unless otherwise stated. Flash chromatography was performed on straight-phase silica gel (Merck 60, 230–400 mesh, 0.040–0.063 mm, 10–50 g/g of product mixture) employing a gradient technique with an increasing concentration (0–100 %) of distilled ethyl acetate in distilled cyclohexane. In cases of very polar products chromatography was continued with ethanol in ethyl acetate (0-20 %). Thin-layer chromatography (TLC) was performed to monitor the progress of the reaction on silica gel plates (Merck 60, precoated aluminium foil), using ethyl acetate (40 %) in cyclohexane as an eluent, and plates were developed by means of spraying with vanillin in sulfuric acid and heating at 120°C. Purity of the product was checked with gas chromatography (GC) analysis on a Varian 3300 GC instrument equipped with a flame ionization detector (FID) using a capillary column Equity-5 (30 m x 0.25 mm i.d, d_f = 0.25 µm, with nitrogen (15 psi) as carrier gas and a split ratio of 1:20). The oven temperature was programmed at 50°C for 5 min followed by a gradual increase of 10°C min^{-1} to reach a final temperature of 300°C. An Agilent 7890 GC equipped with a polar capillary column FactorFOUR vf-23ms (30 m x 0.25 mm i.d., d_f = 0.25 µm) coupled to an Agilent 240 ion-trap MS detector for separation of some isomeric intermediates. The injector was operated in split mode (1:20) at 275°C, and a helium flow rate of 1 ml min^{-1} and a transfer line temperature of 280 °C. The analyses were performed in the external ionisation configuration. EI spectra were recorded with a mass range of m/z 50-300 at fast scan rate. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance 500 (500 MHz ^{1}H, 125.8 MHz ^{13}C) spectrometer using $CDCl_3$ as solvent and internal standard.

(*Z*)-4-Undecenoic acid. NaHMDS (6.78 mmol, 1 M in hexane) was added dropwise, during 30 min, to a suspension of (3-carboxypropyl)triphenylphosphonium bromide (1.45 g, 3.39 mmol) in THF (25 mL). The mixture was stirred for 2 h then cooled to 0°C on ice/water bath, and heptanal (0.387g, 3.39 mmol) in THF (2.5 mL) was added slowly during 15 min. The mixture was stirred for 5 h at 0°C then allowed to reach room temperature over night. The reaction was quenched with H_2O (20 mL) and the organic solvent was evaporated. The remaining water phase was extracted with Et_2O (3 x 20 mL), the obtained organic phases discarded and the basic aqueous phase was acidified with HCl (2M) until pH 1 and extracted with Et_2O (3 x 20 mL). The combined organic phases were dried over $MgSO_4$ (anhydr.) and the solvent evaporated off. The obtained crude product was dissolved in pentane, cooled at -18°C and filtered to remove the precipitated $OPPh_3$ followed by evaporation of the solvent to result in 0.547 g of a yellow oil (87.5% yield). ^{1}H-NMR: 5.52–5.30 (m, 2H), 2.35 (m, 4H), 2.04 (q, *J* = 6.5 Hz, 1.6H,

573 *Z*-isomer), 1.96 (q, *J*=6.5 Hz, 0.4H, *E*-isomer), 1.37–1.19 (m, 8H), 0.89 (t, *J*=7 Hz, 3H)
574 ppm. The NMR data is in accordance with data previously reported (42, 48). The
575 relationship by integration between protons at 2.04 and 1.95 indicates approximately a
576 *Z*:*E*-ratio of 80:20 which is supported by GC-MS analysis on a Varian factorFOUR vf-23ms
577 column. The obtained crude product was used in the next step without further
578 purification.

579 **Methyl (*Z*)-4-undecenoate**. (*Z*)-4-Undecenoic acid (0.547 g, 2.97 mmol) from above
580 was dissolved in methanol (15 mL) and 7 drops of concentrated H_2SO_4 were added
581 followed by heating at 70°C over night. The mixture was allowed to reach room
582 temperature and the methanol was evaporated and the remaining crude product was
583 dissolved in Et_2O (15 mL). The organic phase was washed with H_2O (3 x 10 mL) and brine
584 (2 x 10 mL), dried over Na_2SO_4 (anhydr.) and solvent evaporated resulting in 0.547 g of
585 a yellow oil (92.8% yield). GC-MS (FactorFour vf-23ms) shows a *Z*:*E*-ratio of 80:20. ^{1}H-
586 NMR($CDCl_3$): 5.4 (m, 2H), 3.67 (s, 3H), 2.3 (m, 4H), 2.03 (q, *J* = 6.5 Hz, 1.6H, *Z*-
587 isomer), 1.96 (q, *J*=6.5 Hz, 0.4H, *E*-isomer), 1.33–1.21 (m, 8H), 0.89 (t, *J*=6.5 Hz, 3H)
588 ppm (no data found in the literature). ^{13}C-NMR($CDCl_3$): 134.2, 119.9, 32.3, 31.9, 29.5,
589 29.3, 27.43, 22.7, 14.1 ppm. ^{13}C-NMR data similar to reported (49). Proton NMR shows a
590 80:20 *Z*:*E*-ratio between the diastereomers. Enrichment of the *Z*-isomer on $AgNO_3$ (10%)
591 impregnated silica resulted in 63 mg of 98.6:1.4 *Z*:*E*-ratio according to GC-FID analysis
592 on the vf-5 column as the diastereoisomeric purity was not possible to measure when
593 using ^{1}H-NMR.

594 **(*Z*)-4-Undecenol**. Methyl (*Z*)-4-undecenoate (63 mg, 0.32 mmol) was dissolved in Et_2O
595 (5 mL) and $LiAlH_4$ (2 spatel tips) was added followed by stirring at room temperature for
596 30 min. HCl (2 M, 2 mL) was added to quench the reaction and the mixture was extracted
597 with Et_2O (2 x 3 mL), the combined organic layer was dried over $MgSO_4$ (anhydr.) and
598 solvent was evaporated. Purification with flash chromatography on SiO_2 resulted in 37
599 mg. ^{1}H-NMR($CDCl_3$): 5.43-5.32 (m, 2H), 3.67 (m, 2H), 2.16–2.10 (m, 2H), 2.08–2.02
600 (m, 2H), 1.69–1.60 (m, 2H), 1.39–1.22 (m, 8H), 0.89 (t, *J*=6.5 Hz, 3H) ppm. NMR-data
601 were similar to Kim and Hong (50) and Davis and Carlsson (51). Diastereomeric purity
602 was checked with GC-FID before next step.

603 **(*Z*)-4-Undecenal**. (*Z*)-4-Undecenol (37 mg, 0.22 mmol) in DCM (3 mL) was added to
604 Dess-Martin periodinane (0.140 g, 0.33 mmol) in DCM (0.5 mL). After 50 min, NaOH
605 (2M, 10 mL) was added to quench the reaction. The two layers were separated and the
606 aqueous phase was extracted with Et_2O (3 x 10 mL), the combined organic layers were
607 washed with NaOH (2M, 10 mL), dried over $MgSO_4$ (anhydr.) and solvent was evaporated
608 resulting in 30 mg of a yellow oil (81% yield). The crude product was purified with
609 Kugelrohr distillation at bp 65–70 °C (1.6 mbar), resulted in 17 mg. ^{1}H-NMR($CDCl_3$): 9.77
610 (s, 1H), 5.48–5.22 (m, 2H), 2.47 (t, *J* = 7 Hz, 2H), 2.37 (q, *J* = 7 Hz, 2H), 2.04 (q, *J* =
611 7Hz, 2H), 1.37–1.23 (m, 8H), 0.88 (t, *J* = 7 Hz, 3H) ppm. ^{13}C-NMR($CDCl_3$): 202.1, 131.8,

127.0, 43.9, 31.8, 29.5, 29.0, 27.2, 22.6, 20.1, 14.1 ppm. Both ^{1}H- and ^{13}C-NMR data were in accordance with published results (52, 53). Analysis on GC-MS (FactorFour vf-23ms) resulted in a 98.6:1.4 *Z*:*E*-ratio, the *E*-isomer could not be detected by ^{1}H-NMR.

(*E*)-4-Undecenoic acid. 4-Pentenoic acid (0.5 g, 5 mmol) and 1-octene (2.8 g, 25 mmol) was dissolved in DCM (50 mL) and Grubbs II catalyst (85 mg, 0.1 mmol) was added and the reaction was refluxed. After 7 h was a second portion of Grubbs II catalyst (85 mg, 0.1 mmol) added and the reaction refluxed for 16 h. Reaction was allowed to reach room temperature and the solvent was evaporated. The obtained crude product was dissolved in Et_2O (50 mL) and filtered through a short pad of silica gel. The product was purified with flash chromatography by gradient elution (0–100% EtOAc in c-hexane followed by 0–10% EtOH in EtOAC) resulting in 0.52 g oil (56% yield). ^{1}H-NMR($CDCl_3$): 5.51–5.33 (m, 2H), 2.41 (q, *J*=7 Hz, 2H), 2.32 (q, *J*=7 Hz, 2H), 2.04 (q, *J*=6.5 Hz, 0.25 H, *Z*-isomer), 1.97 (q, *J*=6.5 Hz, 1.75H, *E*-isomer), 1.37–1.22 (m, 9H), 0.88 (t, *J*=7.5 Hz, 3H) ppm. The relation between proton at 2.04 and 1.97 reveals a 87.5:12.5 *E*:*Z*-ratio. The isolated product was used in the next step without further purification.

Methyl (*E*)-4-Undecenoate. (*E*)-4-Undecenoic acid (0.52 g, 2.82 mmol) was dissolved in methanol (25 mL) and a catalytic amount H_2SO_4 was added and the mixture was refluxed over night. After evaporation of the solvent the crude product was dissolved in Et_2O (10 mL) and washed with H_2O (20 mL). The aqueous phase was extracted with Et_2O (2 × 25 mL), the combined organic layer was washed with H_2O (20 mL) and brine (20 mL), dried over $MgSO_4$ (anhydr.) and evaporation of solvent resulted in 0.439 g (78% yield). ^{1}H-NMR($CDCl_3$): 5.51–5.33 (m, 2H), 3.67 (s,3H), 2.40–2.27 (m, 4H), 1.96 (q, *J*=6.5 Hz, 2H), 1.38–1.21 (m, 8H), 0.88 (t, *J*=6.5 Hz,3H) ppm. Purification with flash chromatography resulted in 0.401g (71.7% yield). GC-FID showed the same stereoisomeric ratio as for the acid above.

(*E*)-4-Undecen-1-ol. $LiAlH_4$ (0.055 g, 1.46 mmol) was added to methyl (*E*)-4-undecenoate (0.145 g, 0.73 mmol) dissolved in Et_2O (5 mL). After 30 minutes was HCl (2 M, 5 mL) added to quench the reaction. The acidic water phase was extracted with Et_2O (3 × 10 mL) and the combined organic layers were dried over $MgSO_4$ (anhydr.) and evaporation of solvent resulted in 0.104 g (99% yield). Enrichment of the *E*-isomer with medium pressure liquid chromatography (MPLC) on $AgNO_3$ (10% impregnated) silica resulted in 30 mg of a clear oil (>99.8 % *E*). ^{1}H-NMR($CDCl_3$): 5.43 (m, 2H), 3.65 (m, 2H), 2.08 (q, *J*=7 Hz, 2H), 1.97 (q, *J*=7 Hz, 2H), 1.63 (pent, 2H), 1.35–1.21 (m, 9H), 0.88 (t, *J*=6.5 Hz, 3H) ppm. ^{13}C-NMR($CDCl_3$): 134.4, 131.3, 129.4, 62.6, 32.6, 32.5, 31.7, 29.6, 29.5, 28.9, 28.8, 22.6, 14.1 ppm. All NMR-data were in accordance with previous published data (54).

(*E*)-4-Undecenal. Dess-Martin Periodinane (0.110 g, 0.26 mmol) was added to (*E*)-4-undecen-1-ol (0.030 g, 0.22 mmol) in DCM (4 mL). NaOH (2 M, 10 mL) was added after 1 h to quench reaction. The aqueous phase was extracted with Et_2O (3 × 10 mL) and the

651 combined organic layers were dried over $MgSO_4$ (anhydr.) and evaporation of the solvent
652 resulted in 30 mg (98% yield). Purification of the crude with Kugelrohr distillation at 65°C
653 (2 mbar) resulted in 10 mg of product (33% yield, 97% chemical purity, 3% undecenal).
654 ^{1}H-NMR($CDCl_3$): 9.76 (t, *J*=1.5 Hz, 1H), 5.50–5.36 (m, 2H), 2.48 (d of t, *J*=7.5, 1.5 Hz,
655 2H), 2.33 (q, *J*=7 Hz, 2H), 1.97 (q, *J*=6.5 Hz, 2H), 1.32–1.19 (m, 8H), 0.87 (t, *J*=6.5 Hz,
656 3H). ^{13}C-NMR ($CDCl_3$): 202.5, 132.2, 127.6, 43.6, 32.5, 31.7, 29.4, 28.8, 25.2, 22.6,
657 14.1. The NMR-data were in accordance with published data (53, 54).

658 (*Z*)-4-Undecenoic acid was synthesized via a modified version of Wube and Hüfner (42) in
659 80% stereoisomeric purity. Esterification under acidic conditions with sulfuric acid in
660 methanol resulted in 80% *Z*-isomer and a 93% yield over two steps. Stereoisomeric
661 purity was controlled with NMR and GC-FID by comparing analysis for acid and ester, the
662 appearance of a small quartet, in the NMR spectra, at 1.96 indicates the presence of *E*-
663 isomer. Gas chromatographic separation on a polar Varian factorFOUR vf-23ms of *Z*- and
664 *E*-ester proved that the stereochemistry was not affected by the acidic conditions during
665 esterification. Methyl-(*Z*)-4-undecenoate was purified on regular silica gel and on silver
666 nitrate impregnated silica gel to obtain a stereoisomeric purity of 98.6 %. Methyl-(*Z*)-4-
667 undecenoate was reduced to (*Z*)-4-undecenol with lithium aluminum hydride in
668 diethylether and oxidized to (*Z*)-4-undecenal with Dess-Martin periodinane in
669 dichloromethane.

670 A modified version of Virolleaud´s (43) metathesis was used to produce the (*E*)-4-
671 undecenoic acid and in a 56 % yield (87.5% of the *E*-isomer). (*E*)-4-undecenoic was
672 esterified under the same conditions as the (*Z*)-acid and once again there was no
673 isomerisation of the double bond (according to GC-FID and ^{1}H-NMR. The methyl-(*E*)-4-
674 undecenoate was reduced to alcohol with $LiAlH_4$ in diethylether and purified on silver
675 nitrate impregnated silica gel to obtain a purity of 99.8 % of the (*E*)-isomer, which was
676 oxidized with Dess-Martin periodinane in dichloromethane to obtain the wanted (*E*)-4-
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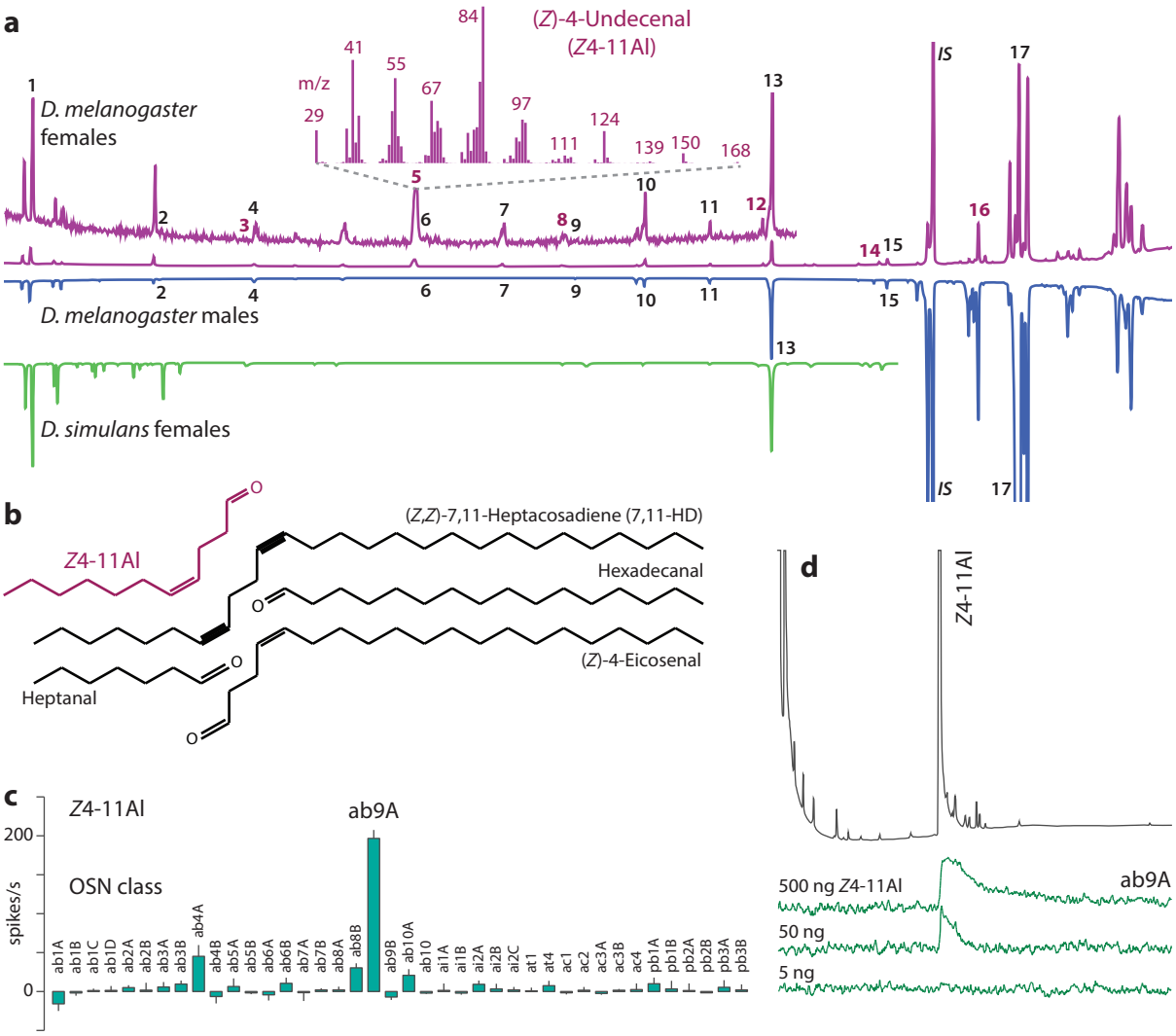
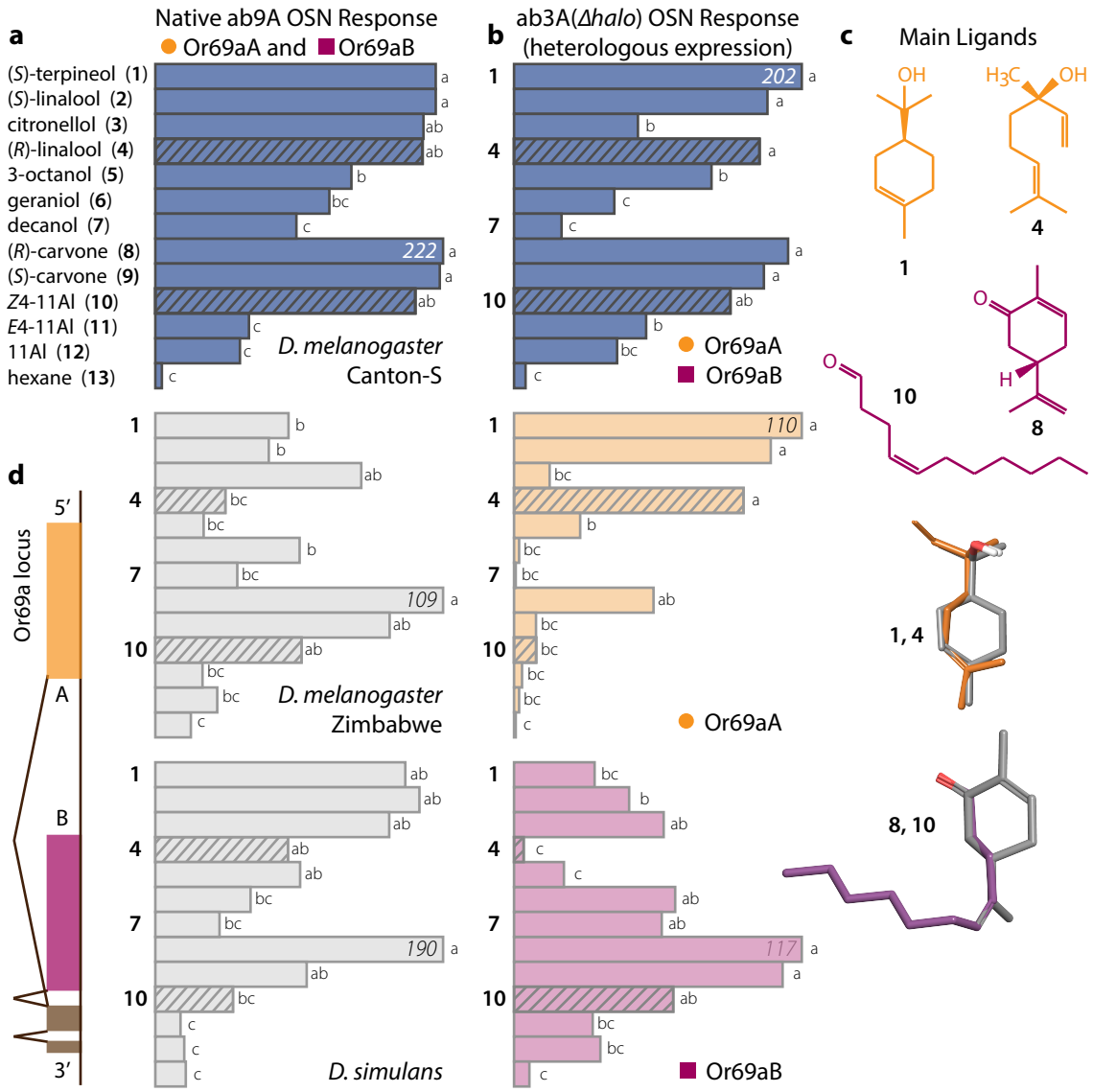
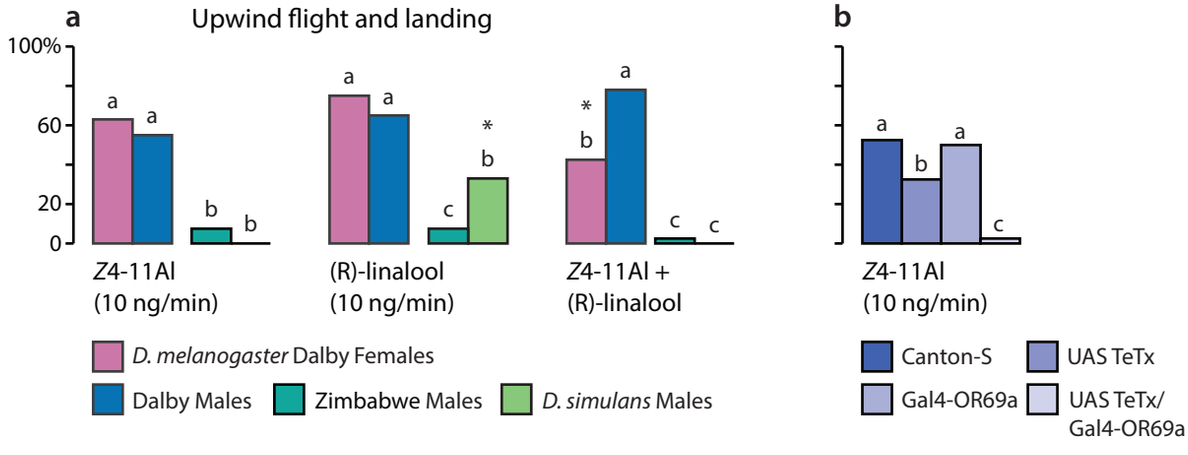


Lebreton Figure 1



Lebreton Figure 2



Lebreton Figure 3