The *Drosophila melanogaster* 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 the sibling species *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*, which shares food resources and co-occurs with *D. melanogaster*, 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

Significance Statement

Volatile insect sex pheromones carry a message over a distance, they are perceived by dedicated olfactory receptors, and elicit a sequence of innate behaviours. Pheromones mediate specific mate recognition, but are embedded in and perceived together with environmental olfactory cues. We have identified the first long-range, species-specific pheromone in *Drosophila melanogaster*. A pair of spliced olfactory receptors, feeding into the same neural circuit, has developed a dual affinity to this pheromone and kairomones, encoding adult and larval food. Blends of this pheromone and kairomone specifically attract *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.

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Introduction

Volatile insect pheromones transmit species-specific messages and elicit long-range flight attraction. Premating communication with pheromones facilitates and accelerates mate-finding, and reduces predation risk and energy expenditure, which is particularly adaptive in short-lived insects (1,2).

Sexual communication subserves mate-finding and ultimately reproduction. Female insects search for larval food and oviposition sites soon after mating, and both sexes

58 forage to offset the nutritional cost of reproduction. The search for mates and food is
59 accordingly interconnected, and so is the response to sex and habitat olfactory signals.
60 The sensory drive hypothesis predicts that mate recognition in animals evolves during
61 niche adaptation and that premating sexual communication involves olfactory
62 specialization to both social signals and habitat cues (3,4).

63 Pheromones are released into an atmosphere that is filled with environmental, habitat-
64 related olfactory cues, some of which signal mating sites and food sources. The response
65 to sex pheromones and food or habitat odourants (kairomones) is under sexual and
66 natural selection, respectively. Pheromones and kairomones are always perceived as an
67 ensemble in a natural context and this leads to an interaction of sexual and natural
68 selection during adaptive divergence of sexual signaling, which is thought to facilitate
69 premating reproductive isolation (3-7).

70 Olfactory sexual communication is studied at cellular and molecular resolution in the fruit
71 fly *Drosophila melanogaster*, but volatile pheromones encoding species-specific, long-
72 range mate recognition have not yet been found. *Drosophila* is attracted to yeast and
73 fruit odorants for feeding, mating and oviposition (8-10) and the interconnection between
74 perception of pheromones and food semiochemicals is a current research theme (11, 12).
75 For example, the male-produced sex pheromone cVA and food stimuli are integrated to
76 coordinate feeding, courtship behavior and oviposition site selection (13-16). Perception
77 of cVA is a current and outstanding paradigm for studying the molecular and neuronal
78 logic of innate, olfactory-mediated reproductive behavior (15, 17, 18). cVA and other
79 known olfactory pheromones are active during courtship, and since they are all shared
80 with other *Drosophila* species, they cannot account for species-specific communication
81 (19, 20, 21).

82 *D. melanogaster* and *D. simulans* are sibling species with no gene flow between them
83 (22). Interspecific matings of *D. melanogaster* with *D. simulans*, or other closely related
84 species are inhibited by the female-produced cuticular hydrocarbon (*Z,Z*)-7,11-
85 heptacosadiene (7,11-HD), which is perceived through gustatory receptors at close range
86 (23-25). 7,11-HD or other non-volatile cuticular hydrocarbons convey species-specificity,
87 but they are not volatile and cannot account for long-range communication and flight
88 attraction. This raises the question whether *Drosophila* uses, in addition, volatile
89 pheromone signals that mediate specific mate recognition at a distance.

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

97 # Results

98 ## *Drosophila melanogaster* females produce a suite of volatile aldehydes.

99 A focus in *Drosophila* pheromone research has been on cuticular hydrocarbons, which are
100 active during close-range courtship. Our scope was to investigate volatile compounds
101 encoding long-range communication. We therefore collected volatile compounds released
102 by *D. melanogaster* flies in a glass aeration apparatus and found 16 aliphatic aldehydes,
103 according to chemical analysis by gas chromatography-mass spectrometry (GC-MS).
104 Males and females shared saturated aldehydes with a carbon chain length of C7 to C18,
105 but mono-unsaturated aldehydes were released by females only (Fig. 1*A*; Table 1). The
106 most abundant compound was identified as (*Z*)-4-undecenal (*Z*4-11Al) and synthesized.

107 GC-MS analysis showed that *Z*4-11Al was present also in cuticular extracts of females,
108 although in lower amounts (0.27 ± 0.12 ng/female, n = 5) than in headspace collections
109 (3.0 ± 0.81 ng/female, n = 10; P<0.01 Mann-Whitney test). Cuticular profiles of
110 *Drosophila* flies have been investigated, but *Z*4-11Al or other aldehydes have not been
111 reported (19, 26, 27).

112 The sister species *D. simulans* did not release *Z*4-11Al, nor other monounsaturated
113 aldehydes (Fig. 1*A*). Unlike *D. melanogaster*, *D. simulans* does not produce (*Z,Z*)-7,11-
114 heptacosadiene (7,11-HD) (23). This led us to hypothesize that the production of mono-
115 unsaturated aldehydes with a double bond in position 4 was linked to oxidation of di-
116 unsaturated cuticular hydrocarbons. Oxidation of 7,11-HD is expected to generate two
117 saturated aldehydes, heptanal and hexadecanal, and two unsaturated aldehydes, *Z*4-11Al
118 and (*Z*)-4-eicosenal (Fig. 1*B*). This was experimentally verified by applying 100 ng
119 synthetic 7,11-HD to a glass vial. After 60 min, 1.92 ± 0.42 ng *Z*4-11Al were retrieved (n
120 = 3). Based on the cuticular hydrocarbon profile of *D. melanogaster* (24), 26 aldehydes
121 are expected to be formed by oxidation, 16 of which were found in our headspace
122 analysis (Table 1), others may have been below detection level.

123 Next, single sensillum electrophysiological recordings (SSR) from all basiconic, trichoid,
124 coeloconic, and intermediate olfactory sensilla in *D. melanogaster* (Fig. 1*C*) and GC-
125 coupled SSR recordings (GC-SSR) from ab9 sensilla (Fig. 1*D*) showed that *Z*4-11Al
126 strongly activates ab9A olfactory sensory neurons (OSNs). A weaker response from ab4A
127 neurons to *Z*4-11Al probably reflects the sensitivity of Or7a (expressed in ab4A) to
128 aldehydes, such as the leaf volatile (*E*)-2-hexenal or bombykal, a lepidopteran
129 pheromone compound (28-30).

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

131 ab9A OSNs express the olfactory receptor (Or) Or69a (31). We therefore screened ab9A
132 OSNs with known ligands of Or69a (30) and *Z*4-11Al. In the *D. melanogaster* strains
133 Canton-S and Zimbabwe, the monoterpene (*R*)-carvone elicited the strongest response
134 from ab9A, although the response to *Z*4-11Al was not significantly different. In *D.*
135 *simulans*, Z4-11Al elicited a significantly lower response than (*R*)-carvone (Fig. 2*A*).

136 The Or69a gene encodes two proteins, Or69aA and Or69aB, as a result of alternative
137 splicing (Fig. 2*D*), which occurred prior to the split of the *D. obscura* and *D. melanogaster*
138 groups (32, 33). Heterologous co-expression of both Or69a splice variants in ab3A
139 (*Δhalo*) empty neurons (34) produced a response similar to native ab9A OSNs; whereas
140 individual expression revealed distinct response profiles for Or69aA and Or69aB (Fig. 2 *A*
141 and *B*). Or69aB responds best to both isomers of carvone, followed by *Z*4-11Al.

142 Carvone and *Z*4-11Al, which seem unalike at first glance, share a structural motif, a
143 carbonyl functional group with an equidistant double bond in position 4 (Fig. 2 *B* and *C*).
144 Different ligands, upon binding to the same Or, are thought to adopt a complementary
145 bioactive conformation. The strain energy required for any compound to assume a steric
146 conformation that aligns with an active ligand should typically not exceed 5 kcal/mol
147 (35). Conformational analysis showed that *Z*4-11Al aligns with (*R*)-carvone, which elicited
148 the strongest Or69aB response, at a strain energy cost of only 1.5 kcal/mol. Or69aA, on
149 the other hand, is tuned to terpenoid alcohols and responded significantly less to *Z*4-
150 11Al. The most active ligands (*S*)-terpineol, (*S*)- and (*R*)-linalool, which again share the
151 functional group and a double bond in position 4, align at 3.0 kcal/mol (Fig. 2 *B* and *C*).
152 Conformational analysis confirms that the most active ligands for Or69aA and Or69aB,
153 respectively, are structurally related.

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

156 *Z*4-11Al elicited upwind flight and landing at the source, in cosmopolitan Dalby and
157 Canton-S strain *D*. *melanogaster* males and females. In contrast, males of the Zimbabwe
158 strain and *D*. *simulans* were not attracted (Fig. 3 *A* and *B*). This shows that *Z*4-11Al, in
159 addition to its precursor 7,11-HD (Fig. 1) participates in sexual isolation between *D*.
160 *melanogaster* and its sister species *D*. *simulans* (23), and between cosmopolitan and
161 African *D*. *melanogaster* strains (36-38).

162 Moreover, admixture of *Z*4-11Al eliminated *D*. *simulans* attraction to the yeast volatile
163 (*R*)-linalool (39) (Fig. 3*A*). *D*. *melanogaster* and its sister species *D*. *simulans* co-occur in
164 the same habitat and use partially overlapping food resources. The very rarely occurring
165 hybrid matings are sterile; the antagonistic interaction between pheromone and food
166 stimulus is therefore adaptive and substantiates a contributing role of Or69a in

167 reproductive isolation. A tentative explanation for the significantly reduced attraction
168 response of *D. melanogaster* females to the blend of *Z*4-11Al and linalool, compared to
169 males (Fig. 3*A*) is that females would avoid food resources and rendezvous sites
170 overcrowded with other females.

171 The response magnitude of wild-type flies to Z4-11Al released at a rate of 10 ng/min was
172 similar to the upwind flight response to vinegar headspace, when the main compound
173 acetic acid was released at a 170-fold amount (Fig. 3*A*; 36). This illustrates the high
174 responsiveness and sensitivity of *D. melanogaster* males and females to Z4-11Al.

175 Finally, we used tetanus toxin mutants to verify that Or69a encodes *Z*4-11Al. Upwind
176 flight attraction to *Z*4-11Al or linalool was significantly reduced when Or69a OSNs were
177 disrupted (Fig. 3*B*). In summary, *Z*4-11Al is a powerful attractant that enables specific
178 mate recognition at a distance. Its heterospecific role was conserved even in blends with
179 the food attractant linalool.

180 Conclusions

181 *Z*4-11Al is the first species-specific, long-range sex pheromone of *D. melanogaster*. It is
182 produced by females and perceived by Or69aB in both sexes. The precursor of *Z*4-11Al is
183 the cuticular hydrocarbon 7,11-HD, which mediates isolation between *D. melanogaster*
184 and its sister species *D. simulans* during courtship (23-25).

185 In addition to pheromone, Or69aB and its twin receptor Or69aA bind kairomonal
186 terpenoids, such as linalool or terpineol, which are found in both fruit and yeast
187 headspace. Citrus peel, a preferred oviposition substrate (8), and baker's yeast which
188 grows on ripe fruit and elicits fly attraction and oviposition (9) are sources of all main
189 ligands of Or69aA and Or69aB (39, 41).

190 Combined pheromone and food odour tuning in the two Or69a splice variants underscores
191 the tie between sexual and natural selection during the evolution of specific mate
192 communication (3, 4). Flies releasing pheromone tint pervading habitat and food
193 odorants and thus shape and foreground a communication channel that facilitates mate
194 finding. This is particularly adaptive in *Drosophila* when mating sites, fruit and berries,
195 are abundant and widely spread.

196 A twin receptor, emerging from the Or69a splice event (32,33), facilitated adaptive
197 changes in ligand tuning, without compromising the established functional role of the
198 Or69a channel. Functional divergence has apparently been biased towards structurally
199 related ligands (Fig. 2 *B* and *C*; 30) and ecologically relevant odorant signals. This is

expected; conservative diversification of the splice variants is constrained to a behavioral theme, since both Ors feed into one OSN.

Olfactory representations of other *Drosophila* Ors involved in food and pheromone perception project through separate channels to the LH, where third-order neurons partially overlap and integrate (15). In stark contrast, Or69a is the first olfactory gene known to encode dual olfactory traits. Or69aA and Or69aB co-express in the same ab9A OSNs (31) and thus achieve a coordination of mating and food stimuli already in first order neurons, at the antennal periphery. This makes Or69a a target for selection during phylogenetic divergence. The tuning range of Ors evolves more rapidly than hardwired neural circuits in higher brain centres (42) and selection pressure is further relaxed following a splice event. Differential tuning of Or69a in closely related cosmopolitan and African strains of *D. melanogaster* (Fig. 2 *A*) corroborates this idea.

Tuning changes in the two splice forms of Or69a are restricted with respect to the behavioral and ecological role of their ligands, since they both feed into a neural circuit mediating sexual and habitat attraction. The two splice forms provide, on the other hand, degrees of freedom during adaptive divergence, since they allow fly populations to adopt new kairomone or pheromone signals; alteration of either one produces a new communication channel. Reproductive isolation may arise as a byproduct (43-45) and the Or69a gene therefore has the potential to drive speciation (46, 47). Species in the *D. melanogaster* and *D. obscura* groups provide a rich substrate for studying Or ligand evolution and its consequences on disruptive selection on ecological interactions and mate choice.

Methods

Insects

Canton-S, Zimbabwe (S-29; Bloomington #60741) and Dalby-HL (Dalby, Sweden) (48) strains of *D. melanogaster* were used as wild type flies for behavioral experiments. Canton-S was used for comparison with knockouts of the same background. Further tests were done with the sister species *D. simulans*.

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

Flies were reared on a standard sugar-yeast-cornmeal diet at room temperature (19 to 22°C) under a 16:8-h L:D photoperiod. Newly emerged flies were anesthetized under CO_2 and sexed under a dissecting microscope. Virgin flies were identified by the presence of

234 meconium, and were kept together with flies of the same sex. Flies were kept in 30-ml
235 Plexiglas vials with fresh food. Experiments were done with 3- to 5-d-old flies.

236 Chemicals

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

240 (*Z*)-4-Undecenoic acid was synthesized via a modified version of Wube et al. (49) in 80%
241 stereoisomeric purity. Esterification under acidic conditions with sulfuric acid in methanol
242 resulted in 80% *Z*-isomer and a 93% yield over two steps. Stereoisomeric purity was
243 controlled with NMR and GC-FID by comparing analysis for acid and ester, the
244 appearance of a small quartet, in the NMR spectra, at 1.96 indicates the presence of *E*-
245 isomer. Gas chromatographic separation on a polar Varian factorFOUR vf-23ms of *Z*- and
246 *E*-ester proved that the stereochemistry was not affected by the acidic conditions during
247 esterification. Methyl (*Z*)-4-undecenoate was purified on regular silica gel and on silver
248 nitrate impregnated silica gel to obtain a stereoisomeric purity of 98.6%. Methyl (*Z*)-4-
249 undecenoate was reduced to (*Z*)-4-undecenol with lithium aluminum hydride in
250 diethylether and oxidized to *Z*4-11Al with Dess-Martin periodinane in dichloromethane.

251 A modified version of Virolleaud's (50) metathesis was used to produce (*E*)-4-undecenoic
252 acid in a 56% yield (87.5% of the *E*-isomer). (*E*)-4-undecenoic acid was esterified under
253 the same conditions as the (*Z*)-acid, without isomerisation of the double bond (according
254 to GC-FID and ^{1}H-NMR). The methyl-(*E*)-4-undecenoate was reduced to the alcohol with
255 lithium aluminum hydride in diethylether and purified on silver nitrate impregnated silica
256 gel to obtain a purity of 99.8% of the (*E*)-isomer, which was oxidized with Dess-Martin
257 periodinane in dichloromethane to obtain *E*4-11Al.

258 Commercially available compounds were: (*R*)-carvone (97% chemical purity, CAS #6485-
259 40-1, Firmenich), (*S*)-carvone (98%, CAS #2244-16-8, Firmenich), (*S*)-terpineol (97%,
260 CAS #10482-56-1, Aldrich), (*S*)-linalool (97%, CAS #126–91–0, Firmenich), (*R*)-linalool
261 (97%, CAS #126-90-9, Firmenich), citronellol (99%, CAS #106-22-9, Aldrich), geraniol
262 (98%, CAS #106-24-1, Aldrich), 3-octanol (99%, CAS #589-98-0, Aldrich), decanol
263 (99%, CAS #112-30-1, Fluka), 11-Al (99%, CAS #112-44-7, Aldrich).

264 Odor collection and chemical analysis

265 Twenty *D. melanogaster* (Dalby), *D. melanogaster* (Canton) (*n* = 10) or 20 *D. simulans*
266 (*n* = 10) unmated female or unmated male flies were placed in a glass aeration
267 apparatus designed for collection of airborne pheromone (effluvia) (51). The flies were
268 held in a glass bulb with a narrow open outlet (ø 1 mm), which prevented them from
269 escaping. A charcoal-filtered air flow (100 mL/min) passed over the flies during 75 min.

270 Fly effluvia were collected on the glass surface, breakthrough was monitored by attaching
271 a 10-cm glass capillary (ø 1 mm) attached to the outlet. After 75 min, flies were
272 removed, 100 ng of heptadecyl acetate (internal standard) was deposited in the glass
273 bulb, which was then rinsed with 50 µl hexane, the solvent was concentrated to 10 µl in
274 Francke vials.

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

279 Oxidation of (*Z,Z*)-7,11-heptacosadiene (7,11-HD) was analysed by dropping 100 ng
280 synthetic 7,11-HD into a 1.5-mL glass vial, at 19°C. Vials were rinsed with 10 µl of
281 hexane, which contained 100 ng heptadecyl acetate as an internal standard, after 15, 30,
282 45, 60 and 75 min (*n* = 3).

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

292 Behavioural assays

293 Upwind flight behavior was observed in a glass wind tunnel (30 x 30 x 100 cm) equipped
294 with a piezo sprayer (40). The flight tunnel was lit diffusely from above, at 13 lux,
295 temperature ranged from 22 to 24°C, relative humidity from 38% to 48% and charcoal
296 filtered air, at a velocity of 0.25 m/s, was produced by a fan (Fischbach GmbH,
297 Neunkirchen, Germany). Compounds were delivered from the centre of the upwind end of
298 the wind tunnel via a piezo-electric micro-sprayer (52). Forty flies were flown individually
299 to each treatment. "Attraction" was defined as upwind flight, directly from a release tube
300 at the end of the tunnel over 80 cm towards the odor source, followed by landing.
301 Unmated fed, 3-d-old Dalby wild-type males and females, *D. melanogaster* Zimbabwe
302 strain males and *D. simulans* males were flown towards (*Z*)-4-undecenal (released at 10
303 ng/min), (*R*)-linalool (10 ng/min) and the blend of (*Z*)-4-undecenal and (*R*)-linalool (10
304 ng/min, each).

305 **Heterologous expression of Or69aA and Or69aB**

306 Or69aA and Or69aB receptors were cloned from antennae of *D. melanogaster*, Dalby line
307 (53). Briefly, cDNA was generated from RNA extracts of antennae of 100 males and
308 females using standard procedures. Or69a variants were PCR amplified with the following
309 primers: Or69aA_5': GTCATAGTTGAAACCAGGATGCAGTTGC, Or69aB_5':
310 ATAATTCAGGACTAGATGCAGTTGGAGG, Or69aAB_3':
311 TGCACTTTTGCCCTTTTATTTAAGGGAC.

312 The splice variants were amplified with unique 5' primers and a common 3' primer,
313 reflective of genomic structure at this locus. These primers encompass the entire open
314 reading frame of the receptor variants, and are located partially upstream and
315 downstream of the start and stop codons. PCR amplicons were gel-purified and cloned
316 into the pCR8/GW/Topo-TA Gateway entry vector (Thermo-Fisher Scientific, Waltham,
317 MA, USA) according to standard procedure, with vector inserts sequenced to confirm
318 fidelity of Or sequence. Or inserts were subsequently transferred to pUAS.g-HA.attB (54)
319 with LR Clonase II enzyme (Thermo-Fisher Scientific), according to manufacturers
320 protocol; vector inserts were sequenced to confirm fidelity of Or sequence.

321 Mini-prep purified pUAS.g-HA.attB plasmid with Or69aA or Or69aB insert were delivered
322 to Best Gene Inc. (Chino Hills, CA USA) for generation of transgenic *D. melanogaster*
323 flies. Using the PhiC31 targeted genomic-integration system (54) vectors with Or69aA or
324 Or69aB were injected into the following fly strain, for integration on the 3rd chromosome:
325 M{3xP3-RFP.attP}ZH-86Fb (with M{vas-int.Dm}ZH-2A) (Bloomington *Drosophila* Stock
326 Number: 24749). For expression of single receptor variants in the empty neuron system,
327 Or69a transgenes were crossed into the *Δhalo* background to give genotype: w;
328 *Δhalo*/Cyo; UAS-DmelOr69a(A or B), and these flies were crossed to flies with genotype:
329 w; *Δhalo*/Cyo; DmelOr22a-Gal4, as described previously (53). Experimental
330 electrophysiology assays were performed on flies with genotype: w; *Δhalo*; UAS-
331 DmelOr69a(A or B)/DmelOr22a-Gal4.

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

341 ## Conformational analysis

342 MacroModel version 11.0 (Schrodinger LLC, New York, NY, USA) in the Maestro Version
343 10.4.017 were used to build, minimize and to perform conformational analysis of *Z*4-
344 11Al, (*R*)-carvone, (*S*)-terpineol and (*R*)-linalool, using default settings (OPLS3 as force
345 field, water as the solvent and mixed torsional/low-mode sampling method). The
346 assumed bioactive conformations of the conformationally more flexible compounds, *Z*4-
347 11Al and (*R*)-linalool, were based on the positon of the shared functional groups in the
348 conformationally more restricted compounds, (*R*)-carvone and (*S*)-terpineol. The
349 carbonyl and the double bond atoms were kept fixed during minimization of the proposed
350 bioactive conformation of Z4-11Al; the alcohol functional group and the double bond were
351 kept fixed in (*R*)-linalool. The strain energies, the energy cost for adopting proposed
352 bioactive conformations, were then calculated as the difference between the lowest
353 energy conformations and the assumed bioactive conformation.

354 ## Electrophysiological recordings

355 Single sensillum recordings (SSR) were done as described earlier (19). Unmated males
356 were restrained in 100-µl pipette tips, with half of the head protruding, the third antennal
357 segment or palps were placed on a glass microscope slide and held by dental wax. For
358 the initial screening, all basiconic, trichoid, coeloconic, and intermediate sensilla (31)
359 were localized in *D. melanogaster* (Canton-S strain) males, under a binocular at 1000x
360 magnification. Further recordings were made from small basiconic ab9 sensilla, in *D.*
361 *melanogaster* (Canton-S and Zimbabwe strains) and in *D. simulans* males, and from large
362 basiconic ab3 sensilla in mutant *D. melanogaster*, where Or69aA and Or69aB were
363 heterologously expressed (see above).

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

374 Odorants were diluted in redistilled hexane, 10 µg of test compounds in 10 µl hexane
375 were applied to filter paper (1 cm^2), kept in Pasteur pipettes. The test panel contained
376 the most active ligands known for Or69a (30) and several aldehydes. Diagnostic
377 compounds for confirmation of sensillum identity were 2-phenyl ethanol (ab9) and 2-
378 heptanone (ab3). Control pipettes contained solvent only. Puffs (2.5 ml, duration 0.5 s)

from these pipettes, produced by a stimulus controller (Syntech GmbH, Kirchzarten, Germany), were injected into a charcoal-filtered and humidified airstream (0.65 m/s), which was delivered through a glass tube to the antenna.

For GC–SSR recordings, GC columns and the temperature programmes were the same as 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.

566 ## Legends

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

570 (**a**) Chromatograms of headspace collections from *D. melanogaster* females (lilac traces;
571 upper trace: amplified signal; lower trace: entire chromatogram), males (blue trace), and
572 *D. simulans* females (green trace). The headspace of *D. melanogaster* females contains
573 16 yet undescribed compounds: heptanal (*1*), octanal (*2*), (*Z*)4-nonenal (*3*), nonanal (*4*),
574 (*Z*)4-undecenal (*Z*4-11Al) (*5*), undecanal (*6*), dodecanal (*7*), (*Z*)4-tridecenal (*8*),
575 tridecanal (*9*), tetradecanal (*10*), pentadecanal (*11*), (*Z*)4-hexadecenal (*12*), hexadecanal
576 (*13*), (*Z*)4-octadecenal (*14*), octadecanal (*15*) and (*Z*)4-eicosenal (*16*) (see Table 1).
577 Female-specific compounds are coloured, the most abundant cuticular hydrocarbon, 7-
578 tricosene (*17*) is shown for reference, the internal standard (IS) was heptadecyl acetate.
579 Inset: mass spectrum of the most abundant female-specific compound *Z*4-11Al.

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

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

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

587 **Figure 2. SSR-Response of Or69a splice variants to ten odorants, in**
588 **native ab9A OSNs and in ab3A OSNs, following heterologous expression.**

589 (**a**) SSR from ab9A OSNs, in *D. melanogaster* (Canton-S, Zimbabwe) and *D. simulans*
590 males, which natively express both splice variants Or69aA and Or69aB. Responses were
591 normalized to the most active odorant.

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

597 (**c**) Key ligands for Or69aA, (*S*)-terpineol (*1*) and (*R*)-linalool (*4*), and for or Or69aB, (*R*)-
598 carvone (*8*) and Z4-11Al (*10*). Alignment of these ligands illustrates shared structural
599 motifs.

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

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

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

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

614

615 **Table 1. Saturated and unsaturated aldehydes found in headspace**
616 **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	-

617
618 ***a*** - traces
619 ***b*** - not found

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 ^1H, 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). ^1H-NMR: 5.52–5.30 (m, 2H), 2.35 (m, 4H), 2.04 (q, *J* = 6.5 Hz, 1.6H,

659 *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)
660 ppm. The NMR data is in accordance with data previously reported (46, 52). The
661 relationship by integration between protons at 2.04 and 1.95 indicates approximately a
662 *Z*:*E*-ratio of 80:20 which is supported by GC-MS analysis on a Varian factorFOUR vf-23ms
663 column. The obtained crude product was used in the next step without further
664 purification.

665 **Methyl (*Z*)-4-undecenoate**. (*Z*)-4-Undecenoic acid (0.547 g, 2.97 mmol) from above
666 was dissolved in methanol (15 mL) and 7 drops of concentrated H_2SO_4 were added
667 followed by heating at 70°C over night. The mixture was allowed to reach room
668 temperature and the methanol was evaporated and the remaining crude product was
669 dissolved in Et_2O (15 mL). The organic phase was washed with H_2O (3 x 10 mL) and brine
670 (2 x 10 mL), dried over Na_2SO_4 (anhydr.) and solvent evaporated resulting in 0.547 g of
671 a yellow oil (92.8% yield). GC-MS (FactorFour vf-23ms) shows a *Z*:*E*-ratio of 80:20. ^{1}H-
672 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*-
673 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)
674 ppm (no data found in the literature). ^{13}C-NMR($CDCl_3$): 134.2, 119.9, 32.3, 31.9, 29.5,
675 29.3, 27.43, 22.7, 14.1 ppm. ^{13}C-NMR data similar to reported (53). Proton NMR shows a
676 80:20 *Z*:*E*-ratio between the diastereomers. Enrichment of the *Z*-isomer on $AgNO_3$ (10%)
677 impregnated silica resulted in 63 mg of 98.6:1.4 *Z*:*E*-ratio according to GC-FID analysis
678 on the vf-5 column as the diastereoisomeric purity was not possible to measure when
679 using ^{1}H-NMR.

680 **(*Z*)-4-Undecenol**. Methyl (*Z*)-4-undecenoate (63 mg, 0.32 mmol) was dissolved in Et_2O
681 (5 mL) and $LiAlH_4$ (2 spatel tips) was added followed by stirring at room temperature for
682 30 min. HCl (2 M, 2 mL) was added to quench the reaction and the mixture was extracted
683 with Et_2O (2 x 3 mL), the combined organic layer was dried over $MgSO_4$ (anhydr.) and
684 solvent was evaporated. Purification with flash chromatography on SiO_2 resulted in 37
685 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
686 (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
687 were similar to Kim and Hong (54) and Davis and Carlsson (55). Diastereomeric purity
688 was checked with GC-FID before next step.

689 **(*Z*)-4-Undecenal**. (*Z*)-4-Undecenol (37 mg, 0.22 mmol) in DCM (3 mL) was added to
690 Dess-Martin periodinane (0.140 g, 0.33 mmol) in DCM (0.5 mL). After 50 min, NaOH
691 (2M, 10 mL) was added to quench the reaction. The two layers were separated and the
692 aqueous phase was extracted with Et_2O (3 x 10 mL), the combined organic layers were
693 washed with NaOH (2M, 10 mL), dried over $MgSO_4$ (anhydr.) and solvent was evaporated
694 resulting in 30 mg of a yellow oil (81% yield). The crude product was purified with
695 Kugelrohr distillation at bp 65–70 °C (1.6 mbar), resulted in 17 mg. ^{1}H-NMR($CDCl_3$): 9.77
696 (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* =
697 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,

698 127.0, 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
699 were in accordance with published results (56, 57). Analysis on GC-MS (FactorFour vf-
700 23ms) resulted in a 98.6:1.4 *Z*:*E*-ratio, the *E*-isomer could not be detected by ^{1}H-NMR.

701 **(*E*)-4-Undecenoic acid**. 4-Pentenoic acid (0.5 g, 5 mmol) and 1-octene (2.8 g, 25
702 mmol) was dissolved in DCM (50 mL) and Grubbs II catalyst (85 mg, 0.1 mmol) was
703 added and the reaction was refluxed. After 7 h was a second portion of Grubbs II catalyst
704 (85 mg, 0.1 mmol) added and the reaction refluxed for 16 h. Reaction was allowed to
705 reach room temperature and the solvent was evaporated. The obtained crude product
706 was dissolved in Et_2O (50 mL) and filtered through a short pad of silica gel. The product
707 was purified with flash chromatography by gradient elution (0–100% EtOAc in c-hexane
708 followed by 0–10% EtOH in EtOAC) resulting in 0.52 g oil (56% yield). ^{1}H-NMR($CDCl_3$):
709 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
710 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
711 Hz, 3H) ppm. The relation between proton at 2.04 and 1.97 reveals a 87.5:12.5 *E*:*Z*-
712 ratio. The isolated product was used in the next step without further purification.

713 **Methyl (*E*)-4-Undecenoate**. (*E*)-4-Undecenoic acid (0.52 g, 2.82 mmol) was dissolved
714 in methanol (25 mL) and a catalytic amount H_2SO_4 was added and the mixture was
715 refluxed over night. After evaporation of the solvent the crude product was dissolved in
716 Et_2O (10 mL) and washed with H_2O (20 mL). The aqueous phase was extracted with Et_2O
717 (2 × 25 mL), the combined organic layer was washed with H_2O (20 mL) and brine (20
718 mL), dried over $MgSO_4$ (anhydr.) and evaporation of solvent resulted in 0.439 g (78%
719 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,
720 *J*=6.5 Hz, 2H), 1.38–1.21 (m, 8H), 0.88 (t, *J*=6.5 Hz,3H) ppm. Purification with flash
721 chromatography resulted in 0.401g (71.7% yield). GC-FID showed the same
722 stereoisomeric ratio as for the acid above.

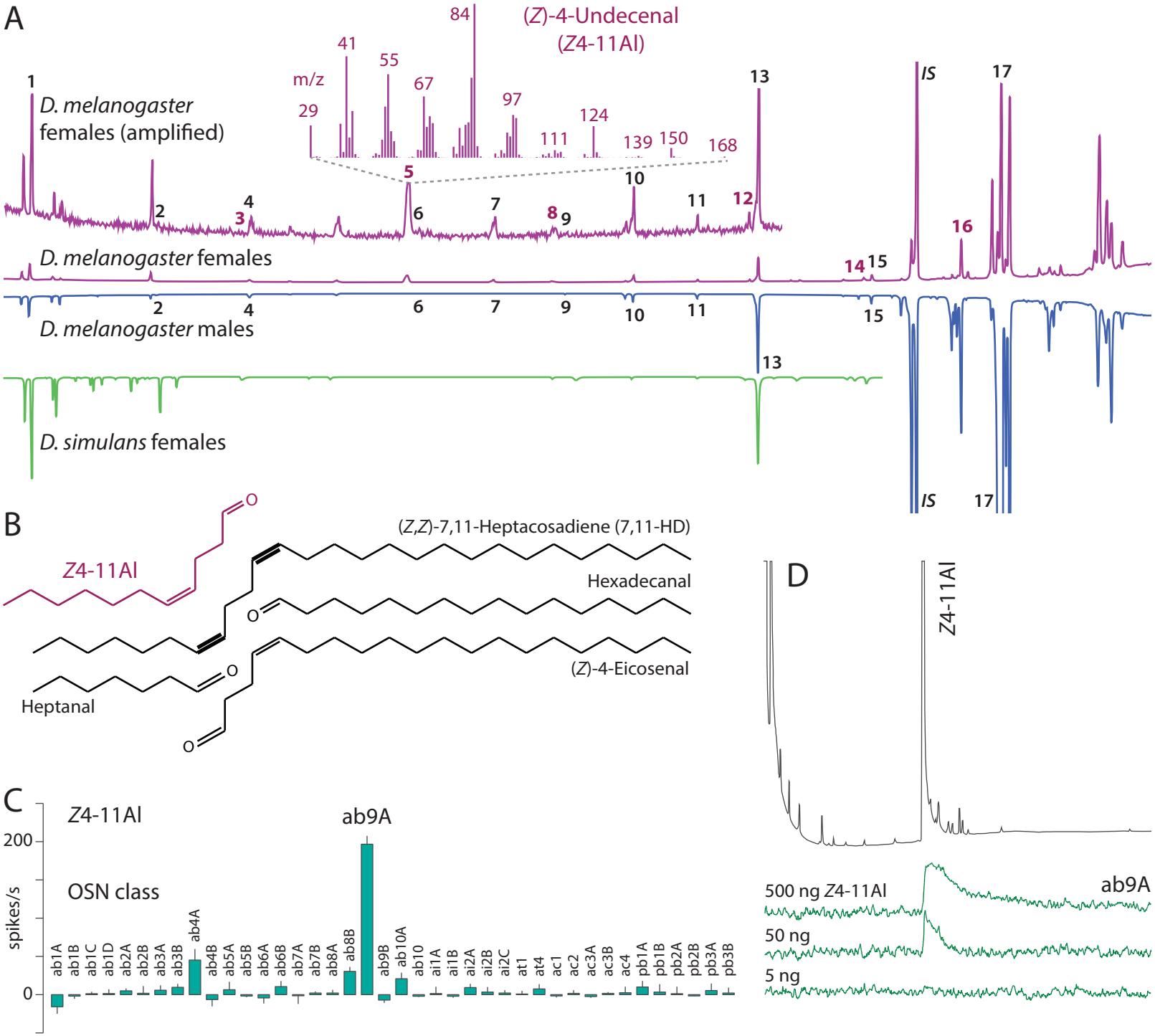
723 **(*E*)-4-Undecen-1-ol**. $LiAlH_4$ (0.055 g, 1.46 mmol) was added to methyl (*E*)-4-
724 undecenoate (0.145 g, 0.73 mmol) dissolved in Et_2O (5 mL). After 30 minutes was HCl (2
725 M, 5 mL) added to quench the reaction. The acidic water phase was extracted with Et_2O
726 (3 × 10 mL) and the combined organic layers were dried over $MgSO_4$ (anhydr.) and
727 evaporation of solvent resulted in 0.104 g (99% yield). Enrichment of the *E*-isomer with
728 medium pressure liquid chromatography (MPLC) on $AgNO_3$ (10% impregnated) silica
729 resulted in 30 mg of a clear oil (>99.8 % *E*). ^{1}H-NMR($CDCl_3$): 5.43 (m, 2H), 3.65 (m,
730 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),
731 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,
732 31.7, 29.6, 29.5, 28.9, 28.8, 22.6, 14.1 ppm. All NMR-data were in accordance with
733 previous published data (58).

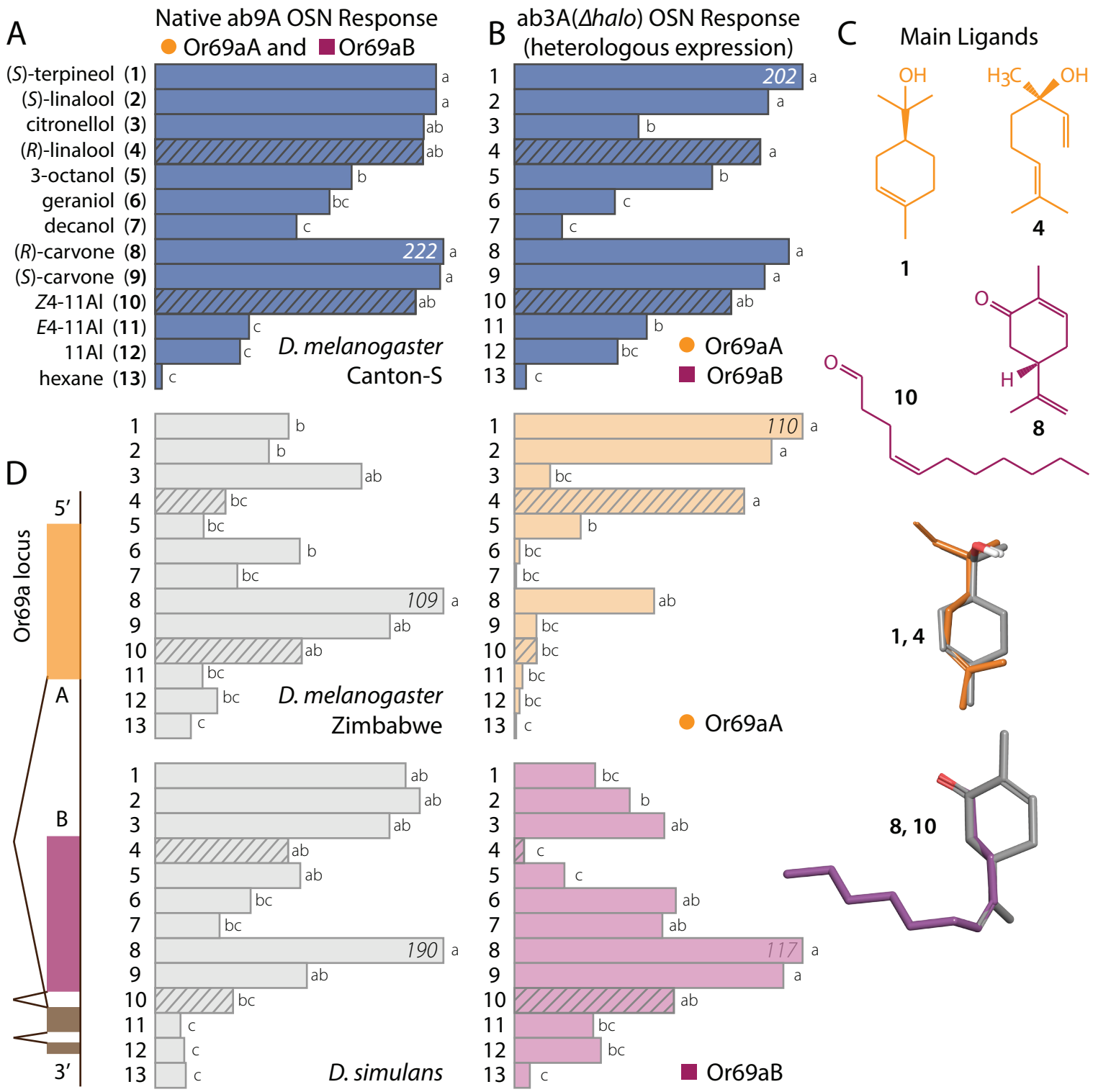
734 **(*E*)-4-Undecenal**. Dess-Martin Periodinane (0.110 g, 0.26 mmol) was added to (*E*)-4-
735 undecen-1-ol (0.030 g, 0.22 mmol) in DCM (4 mL). NaOH (2 M, 10 mL) was added after
736 1 h to quench reaction. The aqueous phase was extracted with Et_2O (3 × 10 mL) and the

737 combined organic layers were dried over $MgSO_4$ (anhydr.) and evaporation of the solvent
738 resulted in 30 mg (98% yield). Purification of the crude with Kugelrohr distillation at 65°C
739 (2 mbar) resulted in 10 mg of product (33% yield, 97% chemical purity, 3% undecenal).
740 ^{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,
741 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,
742 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,
743 14.1. The NMR-data were in accordance with published data (57, 58).

744 (*Z*)-4-Undecenoic acid was synthesized via a modified version of Wube and Hüfner (46) in
745 80% stereoisomeric purity. Esterification under acidic conditions with sulfuric acid in
746 methanol resulted in 80% *Z*-isomer and a 93% yield over two steps. Stereoisomeric
747 purity was controlled with NMR and GC-FID by comparing analysis for acid and ester, the
748 appearance of a small quartet, in the NMR spectra, at 1.96 indicates the presence of *E*-
749 isomer. Gas chromatographic separation on a polar Varian factorFOUR vf-23ms of *Z*- and
750 *E*-ester proved that the stereochemistry was not affected by the acidic conditions during
751 esterification. Methyl-(*Z*)-4-undecenoate was purified on regular silica gel and on silver
752 nitrate impregnated silica gel to obtain a stereoisomeric purity of 98.6 %. Methyl-(*Z*)-4-
753 undecenoate was reduced to (*Z*)-4-undecenol with lithium aluminum hydride in
754 diethylether and oxidized to (*Z*)-4-undecenal with Dess-Martin periodinane in
755 dichloromethane.

756 A modified version of Virolleaud´s (47) metathesis was used to produce the (*E*)-4-
757 undecenoic acid and in a 56 % yield (87.5% of the *E*-isomer). (*E*)-4-undecenoic was
758 esterified under the same conditions as the (*Z*)-acid and once again there was no
759 isomerisation of the double bond (according to GC-FID and ^{1}H-NMR. The methyl-(*E*)-4-
760 undecenoate was reduced to alcohol with $LiAlH_4$ in diethylether and purified on silver
761 nitrate impregnated silica gel to obtain a purity of 99.8 % of the (*E*)-isomer, which was
762 oxidized with Dess-Martin periodinane in dichloromethane to obtain the wanted (*E*)-4-
763 undecenal.


A
(Z)-4-Undecenal
(Z4-11Al)
m/z
29
41
55
67
84
97
111
124
139
150
168
D. melanogaster
females (amplified)
D. melanogaster females
D. melanogaster males
D. simulans females
IS
B
Z4-11Al
(Z,Z)-7,11-Heptacosadiene (7,11-HD)
Hexadecanal
(Z)-4-Eicosenal
Heptanal
C
Z4-11Al
ab9A
OSN class
spikes/s
200
0
ab1A ab1B ab1C ab1D ab2A ab2B ab3A ab3B ab4A ab4B ab5A ab5B ab6A ab6B ab7A ab7B ab8A ab8B ab9B ab10A ab10 ai1A ai1B ai2A ai2B ai2C at1 at4 ac1 ac2 ac3A ac3B ac4 pb1A pb1B pb2A pb2B pb3A pb3B
D
Z4-11Al
500 ng Z4-11Al
50 ng
5 ng
ab9A


A
Native ab9A OSN Response
Or69aA and Or69aB
(S)-terpineol (1)
(S)-linalool (2)
citronellol (3)
(R)-linalool (4)
3-octanol (5)
geraniol (6)
decanol (7)
(R)-carvone (8)
(S)-carvone (9)
Z4-11Al (10)
E4-11Al (11)
11Al (12)
hexane (13)
222
D. melanogaster
Canton-S
B
ab3A(Δhalo) OSN Response
(heterologous expression)
202
Or69aA
Or69aB
C
Main Ligands
OH
H3C
OH
1
4
O
H
10
8
D
Or69a locus
5′
A
B
3′
D. melanogaster
Zimbabwe
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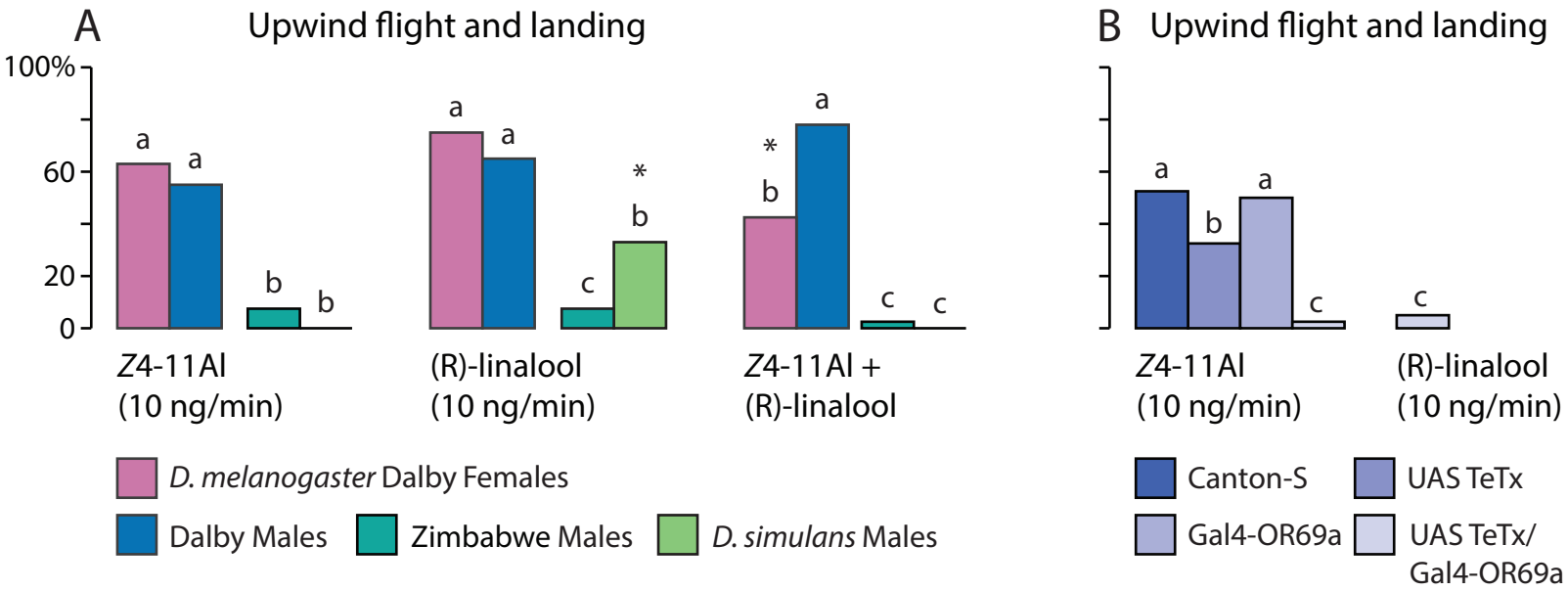
110
Or69aA
D. simulans
190
117
Or69aB
1, 4
8, 10


A
Upwind flight and landing
100%
60
20
0
a
a
b
b
a
a
c
*
b
*
b
a
c
c
Z4-11Al
(10 ng/min)
(R)-linalool
(10 ng/min)
Z4-11Al +
(R)-linalool
D. melanogaster Dalby Females
Dalby Males
Zimbabwe Males
D. simulans Males
B
Upwind flight and landing
a
b
a
c
c
Z4-11Al
(10 ng/min)
(R)-linalool
(10 ng/min)
Canton-S
UAS TeTx
Gal4-OR69a
UAS TeTx/
Gal4-OR69a