# Z4-11Al mediates olfactory attraction and courtship in *Drosophila melanogaster*

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## Summary

[27 words]

The female-produced, species-specific volatile pheromone of *D. melanogaster* attracts both sexes from a distance, alone and in concert with food odorants, and elicits courtship in experienced males.

## Abstract

[250 words]

Specific mate communication and recognition underlies reproduction and hence speciation. Mate communication evolves during adaptation to ecological niches and makes use of social signals and habitat cues.

Our study provides new insights in *Drosophila melanogaster* premating olfactory communication, showing that female pheromone *Z*4-11Al and male pheromone cVA interact with food odour in a sex-specific manner. Furthermore, *Z*4-11Al, which mediates upwind flight attraction in both sexes, also elicits courtship in experienced males.

Twin variants of the olfactory receptor Or69a are co-expressed in the same olfactory sensory neurons, and feed into the same glomerulus in the antennal lobe. Female pheromone *Z*4-11Al is perceived via Or69aB, while the food odorant (*R*)-linalool is a main ligand for the other variant, Or69aA. That *Z*4-11Al mediates courtship in experienced males, not (*R*)-linalool, is probably due to courtship learning. Behavioural discrimination is reflected by calcium imaging of the antennal lobe, showing distinct glomerular activation patterns by these two compounds.

Male sex pheromone cVA is known to affect male and female courtship at close range, but does not elicit upwind flight attraction as a single compound, in to contrast to *Z*4-11Al. A blend of cVA and the food odour vinegar attracted females, while a blend of female pheromone *Z*4-11Al and vinegar attracted males instead.

Sex-specific upwind flight attraction to blends of food volatiles and male and female pheromone, respectively, adds a new element to *Drosophila* olfactory premating communication and is an unambiguous paradigm for identifying the behaviourally active components, towards a more complete concept of food-pheromone odour objects.



## Keywords

Specific Mate Recognition • Olfaction • Neuroethology • (*Z*)-11-octadecenyl acetate • cis-vaccenyl acetate • (*Z*)-4-undecenal


## Introduction

Structure and function of the olfactory system have been studied in *Drosophila* like in no other insect, from peripheral odorant perception to central pathways generating

behavioural output. And, a particular emphasis of this work has been placed on courtship, inspired by a characteristic behavioural response (Depetris-Chauvin et al. 2015, Kohl et al. 2015, Auer and Benton 2016, Grabe et al. 2016, Bates et al. 2020). The combined molecular and physiological know-how enables even investigations of the evolutionary development of olfactory channels in response to pheromones (Khallaf et al. 2020 a,b) and host odorants (Dekker et al. 2006, Markow 2019, Auer et al. 2020), across *Drosophila* phylogenies.

What is still unclear is whether olfactory tuning and response to pheromones and host or food odours evolve independently. And, although odorants enable evaluation and recognition of the emitter or source from a distance, little attention has been paid to the role of species-specific sex pheromones in distant mate recognition and flight attraction, prior to courtship enactment.

Drosophila cuticular hydrocarbon (CHC) profiles are sexually dimorphic, they vary and rapidly diverge between species and therefore contribute to sexual, reproductive isolation (Howard et al. 2003, Legendre et al. 2008, Alves et al. 2010, De Oliveira et al. 2011, Davis et al. 2020). Naturally, this congruently applies to their volatile oxidation products, particularly monounsaturated aldehydes, which derive from diunsaturated CHCs.

A CHC produced by female flies, (*Z*,*Z*)-7,11-heptacosadiene (7,11-HD), has been shown to afford reproductive isolation between *D*. *melanogaster* and its sibling species *D*. *simulans*. It is perceived by gustatory receptors owing to its low volatility (Billeter et al. 2009, Thistle et al. 2012, Toda et al. 2012, Billeter and Wolfner 2018, Seeholzer et al. 2018, Sato and Yamamoto 2020). Oxidation of 7,11-HD gives rise to the volatile pheromone (*Z*)-4-undecenal (*Z*4-11Al) which is perceived by one of the two variants of the olfactory receptor DmelOr69a (Or69a) and elicits flight attraction in males and females (Lebreton et al. 2017). The Or69a gene encodes two or more proteins, in a range of *Drosophila* species (Robertson et al. 2003, Conceicao and Aguade 2008). In *D*. *melanogaster*, the two isoforms of Or69a, Or69aA and Or69aB, are tuned to food odorants, including (R)-linalool and to the female pheromone *Z*4-11Al , respectively. They are expressed in the same olfactory sensory neurons (OSNs), and afford combined input of sex and food stimuli (Lebreton et al. 2017).

In comparison, *cis*-vaccenyl acetate (cVA) is a pheromone produced by males that increases female receptivity and reduces male attraction to recently mated females (Bartelt et al. 1985, Ejima et al. 2007, Kurtovic et al. 2007, Keleman et al. 2012, Lebreton et al. 2014). However, cVA cannot account for specific mate communication since it is shared by many other *Drosophila* species (El-Sayed 2020). The processing of cVA stimuli in sexually dimorphic neural pathways has been mapped from antennal input to third-order neurons in the lateral horn, where gender-specific courtship behaviour is generated (Kohl et al. 2013, Clowney et al. 2015).

97 cVA activates the sexually dimorphic fruitless (*Fru*) circuitry that controls male
98 courtship (Manoli et al. 2005, Stockinger et al. 2005, Billeter et al. 2006, Cachero et al.
99 2010), whereas *Z*4-11Al is not part of this circuit. Since *Z*4-11Al is produced by
100 females, we investigated its effect on flight attraction and courtship, which are
101 successive steps in male reproductive behaviour. An upwind flight assay confirms that
102 *Z*4-11Al elicits attraction in naive males (Lebreton et al. 2017), while it elicits courtship
103 only in experienced, previously mated males.

104 Remarkably, males distinguished between *Z*4-11Al and the food odorant (*R*)-linalool,
105 which did not elicit courtship. Both compounds are perceived via the two isoforms of
106 Or69a, that are co-expressed in the same OSNs, while functional imaging of the
107 antennal lobe (AL) showed nonetheless different activation patterns for *Z*4-11Al and
108 (*R*)-linalool.

109 Food odours act as aphrodisiacs and promote courtship in *Drosophila* (Grosjean et al.
110 2011, Gorter et al. 2016, Ando et al. 2020). Vinegar has traditionally been used as a
111 source of food odorants in fruit fly research, and has a prominent synergistic effect on
112 cVA perception and attraction (Lebreton et al. 2015, Das et al. 2017, Cazale-Debat et
113 al. 2019). Vinegar headspace contains several active compounds (Becher et al. 2010)
114 but greatly varies in composition between makes and types (Callejón et al. 2009,
115 Chinnici et al. 2009). Over-ripe fermenting fruit, on the other hand, is a feeding,
116 mating, and oviposition site for fruit flies. Yeasts growing on fruit release a very rich
117 volatome, that accounts for strong fly attraction (Becher et al. 2012, Buser et al. 2014,
118 Christiaens et al. 2014, Ljunggren et al. 2019).

119 We compared the effect of vinegar and yeast headspace on sex attraction to
120 pheromone. Blends of vinegar odour and male or female pheromone produced sex-
121 specific flight attraction of females and males, respectively. In comparison, both sexes
122 respond to blends of yeast and cVA or *Z*4-11Al.

## 123 Materials and methods

### 124 Insects and chemicals

125 Flies were reared on a standard sugar-yeast-cornmeal diet at room temperature (19 to
126 22°C) under a 16:8-h L:D photoperiod. Newly eclosing flies were anesthetized with $CO_2$
127 and sexed under a microscope. Virgin flies were identified by the presence of
128 meconium, and were kept together with flies of the same sex. Mated males were kept
129 together with females, and were separated 2 d before experiments. Flies were kept in
130 30-mL Plexiglas vials with fresh food, and were six d old when used for experiments.

131 Isomeric purity of (*Z*)-4-undecenal (Z4-11Al) and (*E*)-4-undecenal (E4-11Al) was
132 98.6% and 97.8%, repectively, according to gas chromatography coupled to mass

133 spectrometry (6890 GC and 5975 MS, Agilent Technologies, Santa Clara, CA, USA).
134 Chemical purity of the synthetic aldehydes was >99.9%, and of the other test
135 chemicals >97%. Heptane (redistilled; Merck, Darmstadt, Germany) was used as
136 solvent.

## 137 Functional imaging

138 The head capsule was opened by incising the cuticle between the antennae and the
139 eyes. With the brain immersed in Ringer's saline, the ALs were exposed by removing
140 muscle tissue, glands and trachea. In vivo recordings of illuminated preparations were
141 processed using custom software (Strutz et al. 2014).

142 We used a Till Photonic imaging system with an upright Olympus microscope (BX51WI)
143 and a 20x Olympus objective (XLUM Plan FL 20x/0.95W). A Polychrome V provided light
144 excitation (475 nm), which was then filtered (excitation: SP500, dicroic: DCLP490,
145 emission LP515) and captured by a CCD camera (Sensicam QE, PCO AG) with
146 symmetrical binning. For each measurement, a series of 300 frames was taken (1 Hz).
147 Data were analyzed using IDL (Research Systems Inc., Boulder, CO, USA).

148 A 3-D map of the fruit fly AL (Grabe et al. 2015) served to link the active area to
149 individual glomeruli. All experimental flies contained the calcium dependent fluorescent
150 sensor G-CaMP 3.0 (Nakai et al. 2001) together with a promoter Gal4 insertion to direct
151 expression of the calcium sensor to specific neuron populations. Stimulus-evoked
152 fluorescence in these flies arises from the population of labeled neurons that are
153 sensitive to the specific odour.

154 We tested the physiological responses in input neurons, i.e. the axonal terminals of
155 OSNs in the AL. Mass labelling of olfactory sensory neurons (OSNs) was achieved using
156 the transgenic line Orco-GAL4 that drives expression in at least 60% of all OSNs
157 (Larsson et al. 2004). Additionally, for mass labelling of PNs, the transgenic line
158 GH146-GAL4, expressing G-CaMP in 83 out of the 150 PNs in each AL (Vosshall and
159 Stocker 2007) has been used. Fly lines were obtained from the Bloomington *Drosophila*
160 Stock Center (Indiana University, Bloomington, IN, USA). Transgenic flies have been
161 generated using standard procedures.

## 162 Courtship assay

163 Wild-type flies for courtship experiments were the *D. melanogaster* strains Dalby-HL
164 (Ruebenbauer et al. 2008) and Canton-S, and and the sister species *D. simulans*.

165 Orco-Gal4/uas-Or69aRNAi flies, and the Orco-Gal4 and uas-Or69aRNAi (VDCR, Vienna)
166 parental lines were used to confirm the effect of *Z*4-11Al on courtship behavior.
167 Canton-S served as comparison for knockouts of the same background. We resorted to
168 the Orco-Gal4 line, since males of the Or69a-Gal4 line did not court. Because of the

169 broad expression of the Or69aRNAi, we cannot exclude that other Ors involved in
170 courtship in experienced males are also affected.

171 Courtship experiments were done on a light box (37x28x2.5 cm; color temperature
172 5.000 ± 5% Kelvin; Kaiser Slimlite 2422, Kaiser Fototechnik GmbH & Co. KG, Buchen,
173 Germany). The courtship arena consisted of three glass plates (17x13x0.5 cm) placed
174 on top of each other. Twelve circular holes (ø 3 cm) were cut in the middle plate to
175 form 12 circular single-pair mating chambers. All glassware was heated to 350°C for 8
176 h before use. Tests were done between 2 and 5 h after onset of scotophase (16:8 L:D
177 photoperiod). Target flies, unmated males or females, were anesthetized on ice and
178 decapitated before experiments. Test flies were either mated or unmated males. A
179 single target fly was added to each mating chamber, and 1 µl heptane (control) or 1 µl
180 heptane containing 1 ng *Z*4-11Al was applied onto its abdomen. After solvent
181 evaporation (2 min), a single live test fly was introduced into each mating chamber and
182 observed during 20 min. Males were scored when showing vigorous courtship
183 behaviour, including wing vibration, licking, and attempted copulation. Treatments ($n$ =
184 80) and controls ($n$ = 80) were conducted simultaneously, and each fly was tested
185 once.

186 **Food odour collection**

187 Brewer's yeast, *Saccharomyces cerevisiae* (strain S288C), was grown in minimal media
188 (Merico et al. 2007) during 20 h in a shaking incubator at 25°C and 260 RPM. 50 mL
189 yeast broth or white vine vinegar (7.1%, Zeta, Sweden) were filled into a wash bottle
190 and charcoal filtered air was bubbled through the bottle at a rate of 200 mL/min.
191 Headspace was trapped in 4x40-mm glass tubes holding 300 mg of Porapak Q 50/30
192 (Waters Corporation, Milford, Ma, USA) during 2 h (Becher et al. 2012). These air filters
193 were eluted with 1.2 mL redistilled ethanol (Labscan), the eluate was sprayed during 2
194 h of wind tunnel experiments.

195 **Wind tunnel assay**

196 Upwind flight attraction was observed in a glass wind tunnel (30x30x100 cm). The wind
197 tunnel was lit diffusely from above, at 13 lux, temperature ranged from 20°C to 24°C,
198 relative humidity from 38% to 48% and charcoal filtered air (Camfil AB, Malmö,
199 Sweden), at a velocity of 0.25 m/s, was produced by a fan (Fischbach GmbH,
200 Neunkirchen, Germany). Yeast headspace and odour blends were delivered from the
201 centre of the upwind end of the wind tunnel via a piezo-electric microsprayer (Becher et
202 al. 2010).

203 Forty flies were flown individually to each treatment. Flies were scored when flying
204 upwind over 80 cm in the wind tunnel centre, from the release cage towards the odour
205 source. Unmated, fed, 3-d-old males and females were flown towards yeast and vinegar
206 headspace alone and in blends with *Z*4-11Al sprayed at 10 ng/min (Lebreton et al.

207 2017) and 300 ng/min *cis*-vaccenyl acetate (cVA) (Lebreton et al. 2015). Isomeric
208 purity of the synthetic compounds, according to gas chromatography, was 98.6%. and
209 97.3%, respectively.

### 210 Statistical analysis

211 Generalized linear models (GLM) with a Bernoulli binomial distribution were used to
212 analyse wind tunnel data. Landing at source and sex were used as the target effects.
213 *Post-hoc* Wald pairwise comparison tests were used to identify differences between
214 treatments. Courtship assays were analysed using chi-squared tests to compare
215 between treatments and their respective control. Statistical analysis was calculated with
216 R (R Core Team 2013) and SPSS Version 22 (IBM Corp.).

## 217 Results

### 218 Flight attraction to blends of pheromone and food odour

219 The male-produced pheromone cVA (Bartelt et al. 1985) is a core paradigm for
220 behavioural and neurophysiological studies in *Drosophila*. The volatile, female-produced
221 and species-specific pheromone *Z*4-11Al has been discovered more recently (Lebreton
222 et al. 2017). We therefore compared flight attraction to male pheromone cVA and
223 female pheromone *Z*4-11Al, in *D. melanogaster* males and females. Pheromones were
224 tested alone and in blends with odorants from vinegar and yeast, signalling food
225 sources (Figure 1).

226 Male pheromone cVA alone attracted only few flies, while a blend of cVA and vinegar
227 was highly attractive to females, not to males (Figure 1b). In comparison, both sexes
228 responded to a blend of cVA and yeast headspace.

229 Female pheromone *Z*4-11Al alone attracted males and females. Blending vinegar with
230 *Z*4-11Al reduced attraction in both sexes, but significantly more males than females
231 responded. And, a blend of yeast headspace and *Z*4-11Al attracted as many flies as *Z*4-
232 11Al alone (Figure 1c).

233 Yeast headspace by itself attracted more males and females than vinegar headspace
234 (Figure 1a). Females attraction to yeast headspace (Figure 1a) and to the blends of
235 yeast headspace and cVA and *Z*4-11Al, respectively, was not significantly different
236 (Figures 1b,c).

237 Taken together, only few flies responded to the male pheromone cVA, while the female
238 pheromone *Z*4-11Al strongly attracted both sexes. Blending vinegar with cVA and *Z*4-
239 11Al, respectively, produced an inverse response pattern in male and female flies. Our

240 results show further that vinegar, a frequently used food odorant source for *Drosophila*,
241 is an inferior attractant in comparison with yeast aroma (Figure 1).

## 242 Male courtship in response to *Z*4-11Al

243 Male-produced cVA mediates aggregation, suppresses male-to-male courtship, and
244 males learn to avoid mated females tainted with cVA (Ejima et al. 2007, Kurtovic et al.
245 2007, Keleman et al. 2012). Since female-produced *Z*4-11Al elicits male flight
246 attraction (Figure 1c), we asked whether *Z*4-11Al also has an effect on courtship,
247 following landing at females. Males were tested with decapitated target flies, laced with
248 blank solvent or synthetic test chemicals.

249 Compared with solvent control, *Z*4-11Al elicited a significant response in experienced
250 males that had mated earlier, and not in naive, unmated males. Males scored showed
251 vigorous courtship including attempted copulation (Figure 2a). More experienced males
252 courted decapitated females than males, plausibly because cVA, present on the cuticula
253 of male flies, has an antagonistic effect on experienced males. That a few males
254 nonetheless courted decapitated males further confirms a role of *Z*4-11Al in male
255 courtship (Figure 2a). Tests with Canton-S test males corroborated results obtained
256 with males of the Dalby strain (Figure 2b).

257 Importantly, males also courted decapitated *D*. *simulans* target females, laced with
258 synthetic *Z*4-11Al (Figure 2b). This experiment rules out a contributing role of the
259 cuticular hydrocarbon pheromone 7,11-HD, which is specific for *D*. *melanogaster* and
260 not found in *D*. *simulans* females (Billeter et al. 2009), and underlines that *Z*4-11Al
261 elicits male courtship, in the absence of 7,11-HD.

262 On the other hand, *D*. *simulans* males did not respond in a significant manner to Dalby
263 females painted with *Z*4-11Al (Figure 2b). This was expected, since *D*. *simulans* males
264 are not attracted to *Z*4-11Al (Lebreton et al. 2017) and since *D*. *simulans* females do
265 not produce 7,11HD, which is the precursor for *Z*4-11Al (Billeter et al. 2009, Lebreton
266 et al. 2017).

267 Moreover, we used a RNAi fly line to corroborate that Or69a encodes *Z*4-11Al-mediated
268 courtship. Significantly fewer males responded when olfactory sensory neurons (OSNs)
269 expressing Or69a were disrupted (Figure 2c).

270 Finally, we tested three non-pheromonal chemicals: *E*4-11Al, the geometric isomer of
271 *Z*4-11Al, which is perceived via the Or69aB variant; (*R*)-linalool, a food compound,
272 which is the main ligand of the other variant, Or69aA; and ethyl butyrate, which is a
273 ligand for Or22a (DM2 glomerulus in the AL), for Or43b (VM2 glomerulus) and also for
274 Or85a (DM5 glomerulus; see also Figure 3). None of these compounds elicited a
275 significant courtship response in experienced males (Figure 2d).

### Activation of antennal lobe glomeruli in response to *Z*4-11Al

The D glomerulus in the antennal lobe (AL) collects input from ab9A olfactory sensory neurons (OSNs) that co-express Or69aA and Or69aB, the two isoforms of Or69a (Couto et al. 2005). Single sensilllum recordings (SSR) have further shown that the food odour (*R*)-linalool and female pheromone *Z*4-11Al are the respective key ligands for Or69aA and Or69aB (Lebreton et al. 2017).

We used calcium imaging of the AL to first confirm the role of the Or69a channel including the D glomerulus in the perception of *Z*4-11Al. Moreover, we compared the AL response to the key ligands (*R*)-linalool and *Z*4-11Al. Both compounds elicit upwind flight (Lebreton et al. 2017), but only *Z*4-11Al participates in male courtship, which goes to show that the flies discriminate between these two compounds (Figure 2).

In males and females, *Z*4-11Al activated the D glomerulus, as expected, but also DM2 and VM2 (Figure 3a). These broadly-tuned glomeruli have also been shown to respond to a mix of cVA and vinegar (Lebreton et al. 2015). However, neither ab3A OSNs (expressing Or22a, connecting to DM2), nor ab8A (expressing Or43b, connecting to VM2; Grabe et al. 2015, Münch and Galizia 2016) responded to *Z*4-11Al, according to SSR (Lebreton et al. 2017). Simultaneous activation of DM2 and VM2 glomeruli by *Z*4-11Al might accordingly reflect interglomerular communication in the AL, via local neurons (Wilson 2013).

(*R*)-linalool, on the other hand, is also a ligand for ab7A OSNs expressing Or98a, which accounts for additional activation of the VM5v glomerulus (Couto et al. 2005, Münch and Galizia 2016). In comparison, activation of DM2 and VM2 glomeruli by (*R*)-linalool was much lower compared to *Z*4-11Al (Figure 3a).

Activation of the DM2 and VM2 glomeruli by *Z*4-11Al was substantiated by imaging Or22a-Gal4 males and Or43b-Gal4 males, respectively. Ethyl butyrate is a diagnostic stimulus for Or22a, Or43b and Or85a (DM2, VM2 and DM5 glomeruli) (Figure 3b; Grabe et al. 2015, Münch and Galizia 2016).

Imaging of the glomerular activation pattern of projection neurons (PNs), connecting the AL to the lateral horn (LH), where behavioural responses are generated, corroborated activity of *Z*4-11Al in the D and DM2 glomeruli (Figure 3c). A possible response of VM5v to linalool does not show, since GH146-Gal4 does not label this glomerulus (Grabe et al. 2015).

## Discussion

Volatile chemicals that are cognate ligands for odorant receptors (ORs) are selectively perceived against noisy backgrounds and they capacitate behavioural decisions at a

311 distance, since they deliver reliable information about the source, including identity and
312 physiological state of the emitter, in the case of pheromones.

313 The female pheromone *Z*4-11Al attracts naive males and females by flight (Figure 1).
314 At close range, *Z*4-11Al also elicits courtship, but only in experienced, previously mated
315 males (Figure 2). A response in naive males is not expected, since neurons mediating
316 perception of *Z*4-11Al are not part of the sexually dimorphic fruitless circuitry that
317 elicits innate male courtship (Manoli et al. 2005, Stockinger et al. 2005).

318 Importantly, males respond also to decapitated *D. simulans* females laced with *Z*4-
319 11Al. This shows that a response to *Z*4-11Al does not require presence of the close-
320 range CHC pheromone 7,11-HD, since 7,11-HD is not produced by *D. simulans* (Billeter
321 et al. 2009, Lebreton et al. 2017, Billeter and Wolfner 2018, Sato and Yamamoto
322 2020).

## 323 Courtship discrimination between Or69a ligands

324 Female pheromone Z4-11Al and the food odorant (*R*)-linalool are main ligands for
325 Or69aB and Or69aA, which are co-expressed in ab9A OSNs, branching to the D
326 glomerulus in the AL (Couto et al. 2005, Lebreton et al. 2017).

327 Whereas both compounds elicit innate flight attraction (Lebreton et al. 2017), males
328 distinguish between Z4-11Al and (*R*)-linalool during courtship (Figure 2a,d), probably
329 because males learn to associate Z4-11Al with females during mating. Courtship
330 learning confirms production of Z4-11Al by females. The question arises whether males
331 would even learn to associate (*R*)-linalool or other food-related compounds with
332 females.

333 A differential response to Z4-11Al and (*R*)-linalool is reflected by their respective
334 activation patterns in the AL (Figure 3a). (*R*)-linalool is also a ligand for Or98a, which
335 accounts for stimulation of the VM5v glomerulus (Couto et al. 2005, Münch and Galizia
336 2016)

337 In contrast, direct activation of DM2 and VM2 by Z4-11Al is not supported by SSR data
338 (Lebreton et al. 2017) and lateral activation by local AL neurons is a possible explanation,
339 instead (Wilson 2013). DM2 and VM2 are respond broadly, also to blends of vinegar and cVA
340 (Lebreton et al. 2015).

341 Finally, we do not know whether the olfactory input of Or69aA and Or69aB through ab9A is
342 entirely equivalent. OSN spiking trains generated by Or69aA and Or69aB may deliver non-
343 congruent messages that may be distinguishable at the AL level. This is reminiscent of Or85e
344 and Or33c which are coexpressed in ab3A OSNs, where it is yet unclear whether different
345 response profiles and spiking patterns enable behavioural discrimination between the
346 respective Or ligands (Goldman et al. 2005).

347 ## Roles of cVA and Z4-11Al in upwind flight and courtship

348 Male courtship in *D. melanogaster* has been anatomically and physiologically dissected
349 at a neuronal circuit level, with a particular emphasis on the sex-dimorphic behavioural
350 effect of cVA. A central node is the sexually dimorphic P1 interneuron cluster that
351 integrates input from all sensory modalities to activate male courtship, and that is
352 regulated by social experience. Male-produced pheromone cVA provides antagonistic
353 olfactory input to the P1 node, preventing courtship of recently mated females
354 perfumed with male cVA (Manoli et al. 2005, Stockinger et al. 2005, Kimura et al.
355 2008, Ruta et al. 2010, Kohl et al. 2013, Yamamoto and Koganezawa 2013, Clowney et
356 al. 2015, Kohl et al. 2015, Auer & Benton 2016).

357 cVA does not elicit long-range flight by itself, only in combination with fly food (Figure
358 1; Bartelt et al. 1985, Lebreton et al. 2015) and is shared by many other *Drosophila*,
359 including the sibling species *D. simulans* (Schaner et al. 1987, El-Sayed 2020).
360 Females, on the other hand, produce the species-specific CHC 7,11-HD that feeds
361 gustatory input into P1 to activate male courtship and to achieve reproductive isolation
362 towards the sibling species *D. simulans* (Billeter et al. 2009, Thistle et al. 2012, Toda et
363 al. 2012, Seeholzer et al. 2018). What is yet unclear is whether P1 also receives
364 excitatory olfactory input.

365 Does Z4-11Al feed into P1? Z4-11Al encodes species-specificity, mediates long-range
366 attraction (Figure 1; Lebreton et al. 2017) and contributes to courtship, independently
367 of 7,11-HD (Figure 2a,b). Z4-11Al is perceived prior to and during upwind flight, and
368 since females release Z4-11Al, it follows that stimulation is sustained at close-range.
369 Courtship and upwind flight are not isolated episodes, they are inextricably
370 interconnected parts of a behavioural sequence that culminates in copulation.

371 ## A behavioural paradigm for bioactive odorant identification

372 An unambigious, stereotypical behavioural response is substrate for investigations of
373 the genetic basis, neural circuitry and physiology of mate communication in *D.*
374 *melanogaster*. We here show that upwind flight attraction is a behavioural paradigm
375 that is suitable to extend investigations of reproductive behaviour to include long-range
376 communication, and even decision-making upon odorant stimulation.

377 Comparison of pheromone blend attraction with vinegar and yeast shows that yeast
378 overrides differences between pheromones (Figure 1). Vinegar, derived from acetic acid
379 bacteria fermentation (Lynch et al. 2019), is a widely used standard food attractant for
380 *D. melanogaster*, although live yeast aroma is much richer in composition (Callejón et
381 al. 2009, Chinnici et al. 2009, Becher et al. 2010, Ljunggren et al. 2019). Yeast growing
382 on ripe fruit is also a biologically more relevant attractant, since it attracts flies for
383 oviposition and since yeast is a sufficient substrate for larval development (Becher et al.
384 2012, Grangeteau et al. 2018, Quan and Eisen 2018, Murgier et al. 2019).

Reproductive behaviour in *D. melanogaster* is mediated by an ensemble of pheromones and food odorants, emanating from food substrates and from aggregating or mating flies. Our knowledge of *D. melanogaster* pheromone chemistry is fairly comprehensive, compared to behaviourally salient food odorants, owing to the extraordinary complexity of microbial headspace.

Flies recognize food sources despite very considerable, inherent variation in the bouquets emanating from fermenting fruit, according to fruit substrates, microbial community composition and fermentation state. Towards an understanding of how neural representations of such complex and variable odours are generated (Endo et al. 2020) and how habitat cues are integrated with pheromonal signals we next need to identify the key odorants in microbial food headspace. An upwind flight bioassay is suitable to describe the key bioactive chemical constitutents that encode reliable recognition and evaluation of food odour objects and trigger the decision to engage in orientation flights.


## Acknowledgements

We thank two anonymous reviewers for constructive criticism.


## Competing interests

No competing interests declared.


## Funding

This study was supported by the Colombian Corporation for Agricultural Research (Corpoica), the Colombian Administrative Department of Science, Technology, and Innovation (Colciencias), the Linnaeus environment "Insect Chemical Ecology, Ethology and Evolution" IC-E3 (Formas, SLU, Sweden), and the Faculty of Landscape Architecture, Horticulture, and Crop Production Science (SLU, Alnarp, Sweden).


## Data availability

Data will be made avaliable at Dryad.

## References

[69]


Alves, H., Rouault, J. D., Kondoh, Y., Nakano, Y., Yamamoto, D., Kim, Y. K., & Jallon, J. M. (2010). Evolution of cuticular hydrocarbons of Hawaiian Drosophilidae. Behavior Genetics, 40(5), 694-705. (doi:10.1007/s10519-010-9364-y)

Ando, Y., Yoshimizu, T., & Matsuo, T. (2020). Food availability reverses the effect of hunger state on copulation rate in Drosophila prolongata females. Animal Behaviour, 166, 51-59. (doi:10.1016/j.anbehav.2020.06.003)

Auer, T. O., & Benton, R. (2016). Sexual circuitry in Drosophila. Current Opinion in Neurobiology 38:18-26. (doi:10.1016/j.conb.2016.01.004)

Auer TO, Khallaf MA, Silbering AF, Zappia G, Ellis K, Alvarez-Ocana R, Arguello JR, Hansson BS, Jefferis GS, Caron SJ, Knaden M, Benton R. 2020. Olfactory receptor and circuit evolution promote host specialization. Nature 579:402-408. (doi:10.1038/s41586-020-2073-7)

Bartelt, R.J., Schaner, A.M. & Jackson, L.L. 1985 cis-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol.* 11, 1747-1756. (doi:10.1007/BF01012124)

Bates AS, Schlegel P, Roberts RJV, Drummond N, Tamimi IFM, Turnbull R, Zhao X, Marin EC, Popovici PD, Dhawan S, Jamasb A, Javier A, Capdevila LS, Li F, Rubin GM, Waddell S, Bock DD, Costa M, Jefferis GSXE. 2020. Complete connectomic reconstruction of olfactory projection neurons in the fly brain. Current Biology 30, 3183-3199. (doi.org/10.1016/j.cub.2020.06.042)

Becher PG, Bengtsson M, Hansson BS, Witzgall P. 2010. Flying the fly: long-range flight behavior of *Drosophila melanogaster* to attractive odors. J chem Ecol 36:599-607 (doi: 10.1007/s10886-010-9794-2)

Becher, P.G., Flick, G., Rozpędowska, E., Schmidt, A., Hagman, A., Lebreton, S., Larsson, M.C., Hansson, B.S., Piškur, J., Witzgall, P., et al. 2012 Yeast, not fruit volatiles mediate Drosophila melanogaster attraction, oviposition and development. Funct. Ecol. 26, 822-828. (doi:10.1111/j.1365-2435.2012.02006.x).

Billeter, J. C., Rideout, E. J., Dornan, A. J, Goodwin SF. Control of male sexual behavior in Drosophila by the sex determination pathway. Curr. Biol. 16, R766–R776 (2006).

Billeter JC, Atallah J, Krupp JJ, Millar JG, Levine JD. 2009. Specialized cells tag sexual and species identity in Drosophila melanogaster. Nature 461:987-U250 (doi:10.1038/nature08495)

Billeter, J. C., & Wolfner, M. F. (2018). Chemical cues that guide female reproduction in Drosophila melanogaster. Journal of chemical Ecology 44:750-769. (doi:10.1007/s10886-018-0947-z)

Buser, C.C., Newcomb, R.D., Gaskett, A.C. & Goddard, M.R. 2014 Niche construction initiates the evolution of mutualistic interactions. Ecol. Lett. 17, 1257-1264. (doi:10.1111/ele.12331).

Cachero, S., Ostrovsky, A. D., Yu, J. Y., Dickson, B. J. & Jefferis, G. S. X. E. Sexual dimorphism in the fly brain. Curr. Biol. 20, 1589–1601 (2010).

453 Callejón, R.M., Tesfaye, W., Torija, M.J., Mas, A., Troncoso, A.M. and Morales, M.L.,
454 2009. Volatile compounds in red wine vinegars obtained by submerged and surface
455 acetification in different woods. Food Chemistry, 113(4), 1252-1259.
456 (doi:10.1016/j.foodchem.2008.08.027)

457 Cazale-Debat, L., Houot, B., Farine, J. P., Everaerts, C., & Ferveur, J. F. (2019). Flying
458 Drosophila show sex-specific attraction to fly-labelled food. Scientific reports, 9(1),
459 1-13. (doi:10.1038/s41598-019-51351-1)

460 Chinnici, F., Duran Guerrero, E., Sonni, F., Natali, N., Natera Marin, R. and Riponi, C.,
461 2009. Gas Chromatography− Mass Spectrometry (GC− MS) characterization of
462 volatile compounds in quality vinegars with protected European geographical
463 indication. Journal of agricultural and food chemistry, 57(11), 4784-4792.
464 (doi:10.1021/jf804005w)

465 Christiaens, J.F., Franco, L.M., Cools, T.L., De Meester, L., Michiels, J., Wenseleers, T.,
466 Hassan, B.A., Yaksi, E. & Verstrepen, K.J. 2014 The fungal aroma gene ATF1
467 promotes dispersal of yeast cells through insect vectors. Cell Reports 9, 425-432.
468 (doi:10.1016/j.celrep.2014.09.009).

469 Clowney, E.J., Iguchi, S., Bussell, J.J., Scheer, E. & Ruta, V. 2015 Multimodal
470 Chemosensory Circuits Controlling Male Courtship in Drosophila. *Neuron* 87, 1036-
471 1049. (doi:10.1016/j.neuron.2015.07.025).

472 Conceicao IC, Aguade M. 2008. High incidence of interchromosomal transpositions in
473 the evolutionary history of a subset of Or genes in Drosophila. J Molec Evol.
474 66:325-32. (doi:10.1007/s00239-008-9071-y)

475 Couto A, Alenius M, Dickson BJ. 2005. Molecular, anatomical, and functional
476 organization of the *Drosophila* olfactory system. Current Biology 15:1535-1547
477 (doi:10.1016/j.cub.2005.07.034)

478 Davis, J. S., Pearcy, M. A., Yew, J. Y., & Moyle, L. C. (2020). A shift to shorter cuticular
479 hydrocarbons causes sexual isolation in the Drosophila americana species group.
480 bioRxiv (doi:10.1101/2020.09.07.286294)

481 De Oliveira, C. C., Manfrin, M. H., de M Sene, F., Jackson, L. L., & Etges, W. J. 2011.
482 Variations on a theme: diversification of cuticular hydrocarbons in a clade of
483 cactophilic Drosophila. BMC Evolutionary Biology 11:179. (doi:10.1186/1471-2148-
484 11-179)

485 Dekker, T., Ibba, I., Siju, K. P., Stensmyr, M. C., & Hansson, B. S. (2006). Olfactory
486 shifts parallel superspecialism for toxic fruit in Drosophila melanogaster sibling, D.
487 sechellia. Current Biology 16:101-109. (doi:10.1016/j.cub.2005.11.075)

488 Depetris-Chauvin, A., Galagovsky, D. & Grosjean, Y. 2015 Chemicals and
489 chemoreceptors: ecologically relevant signals driving behavior in Drosophila. *Front*
490 *Ecol Evol* **3**. (doi:10.3389/fevo.2015.00041).

491 Ejima A, Smith BPC, Lucas C, Van Naters WV, Miller CJ, Carlson JR, Levine JD, Griffith
492 LC. 2007. Generalization of courtship learning in Drosophila is mediated by cis-
493 vaccenyl acetate. Curr Biol 17:599-605. (doi:10.1016/j.cub.2007.01.053)

494 El-Sayed, A. M. (2020). The pherobase: database of pheromones and semiochemicals.
495 www.phero base.com

496 Endo K, Tsuchimoto Y, Kazama H. 2020. Synthesis of conserved odor object
497 representations in a random, divergent-convergent network. Neuron 108, 367–
498 381. (doi:10.1016/j.neuron.2020.07.029)

499 Goldman, A. L., van Naters, W. V. D. G., Lessing, D., Warr, C. G., & Carlson, J. R.
500 (2005). Coexpression of two functional odor receptors in one neuron. Neuron
501 45:661-666. (doi:10.1016/j.neuron.2005.01.025)

502 Gorter, J.A., Jagadeesh, S., Gahr, C., Boonekamp, J.J., Levine, J.D. and Billeter, J.C.,
503 2016. The nutritional and hedonic value of food modulate sexual receptivity in
504 Drosophila melanogaster females. Scientific reports, 6, 19441
505 (doi:10.1038/srep19441)

506 Grabe, V., Strutz, A., Baschwitz, A., Hansson, B.S. and Sachse, S., 2015. Digital in vivo
507 3D atlas of the antennal lobe of *Drosophila melanogaster*. Journal of Comparative
508 Neurology, 523(3), pp.530-544 (doi:10.1002/cne.23697).

509 Grabe, V., Baschwitz, A., Dweck, H.K., Lavista-Llanos, S., Hansson, B.S. and Sachse,
510 S., 2016. Elucidating the neuronal architecture of olfactory glomeruli in the
511 Drosophila antennal lobe. Cell Reports, 16(12), pp.3401-3413.
512 (doi:10.1016/j.celrep.2016.08.063)

513 Grangeteau, C., Yahou, F., Everaerts, C., Dupont, S., Farine, J.P., Beney, L. and
514 Ferveur, J.F. 2018. Yeast quality in juvenile diet affects Drosophila melanogaster
515 adult life traits. Scientific Reports 8, 1-9. (doi:10.1038/s41598-018-31561-9)

516 Grosjean Y, Rytz R, Farine JP, Abuin L, Cortot J, Jefferis GSXE, Benton R. 2011. An
517 olfactory receptor for food-derived odours promotes male courtship in Drosophila.
518 Nature 478:236-U123. (doi: 10.1038/nature10428)

519 Howard, R.W., Jackson, L.L., Banse, H. and Blows, M.W., 2003. Cuticular hydrocarbons
520 of Drosophila birchii and D. serrata: identification and role in mate choice in D.
521 serrata. Journal of Chemical Ecology 29(4):961-976.
522 (doi:10.1023/A:1022992002239)

523 Keleman, K., Vrontou, E., Krüttner, S., Jai, Y. Y., Kurtovic-Kozaric, A., & Dickson, B. J.
524 (2012). Dopamine neurons modulate pheromone responses in Drosophila courtship
525 learning. Nature 489:145-149. (doi:10.1038/nature11345)

526 Khallaf MA, Auer TO, Grabe V, Depetris-Chauvin A, Ammagarahalli B, Zhang D-D,
527 Lavista-Llanos S, Kaftan F, Weißflog J, Matzkin LM, Rollmann SM, Löfstedt C,
528 Svatos A, Dweck HKM, Sachse S, Benton R, Hansson BS, Knaden M. 2020a. Mate
529 discrimination among subspecies through a conserved olfactory pathway. Sci. Adv.
530 6: eaba5279. (doi:10.1126/sciadv.aba5279)

531 Khallaf, M.A., Cui, R., Weißflog, J., Svatoš, A., Dweck, H., Valenzano, D.R., Hansson,
532 B.S. and Knaden, M., 2020b. Large-scale characterization of sex pheromone
533 communication systems in Drosophila. bioRxiv, September 22
534 (doi:10.1101/2020.09.21.305854)

535 Kimura, K., Hachiya, T., Koganezawa, M., Tazawa, T., Yamamoto, D. 2008. Fruitless
536 and Doublesex coordinate to generate male-specific neurons that can courtship.
537 Neuron 59, 759-769. (doi:10.1038/nrn3567)

538 Kohl, J., Ostrovsky, Aaron D., Frechter, S. & Jefferis, Gregory S. 2013 A Bidirectional
539 Circuit Switch Reroutes Pheromone Signals in Male and Female Brains. *Cell* 155,
540 1610-1623. (doi:10.1016/j.cell.2013.11.025).

541 Kohl J, Huoviala P, Jefferis GSXE. 2015. Pheromone processing in Drosophila. Curr Op
542 Neurobiol 34:149-157. (doi:10.1016/j.conb.2015.06.009)

543 Kurtovic A, Widmer A, Dickson BJ. 2007. A single class of olfactory neurons mediates
544 behavioural responses to a Drosophila sex pheromone. Nature 446:542-546.
545 (doi:10.1038/nature05672).

546 Larsson, M. C., Domingos, A. I., Jones, W. D., Chiappe, M. E., Amrein, H., & Vosshall,
547 L. B. (2004). Or83b encodes a broadly expressed odorant receptor essential for
548 *Drosophila* olfaction. Neuron 43:703-714.(doi:10.1016/j.neuron.2004.08.019)

549 Lebreton S, Grabe V, Omondi AB, Ignell R, Becher PG, Hansson BS, Sachse S, Witzgall
550 P. 2014. Love makes smell blind: mating suppresses pheromone attraction in
551 *Drosophila* females via OR65a olfactory neurons. Sci Rep 4:7119
552 (doi:10.1038/srep07119)

553 Lebreton S, Trona S, Borrero-Echeverry F, Bilz F, Grabe V, Becher PG, Carlsson MA,
554 Nässel DR, Hansson BS, Sachse S, Witzgall P. 2015. Feeding regulates sex
555 pheromone attraction and courtship in *Drosophila* females. Sci Rep 5:13132
556 (doi:10.1038/srep13132)

557 Lebreton, S., Borrero-Echeverry, F., Gonzalez, F., Solum, M., Wallin, E.A., Hedenström,
558 E., Hansson, B.S., Gustavsson, A.-L., Bengtsson, M., Birgersson, G., et al. 2017 A
559 Drosophila female pheromone elicits species-specific long-range attraction via an
560 olfactory channel with dual specificity for sex and food. BMC Biol. 15, 88.
561 (doi:10.1186/s12915-017-0427-x).

562 Legendre, A., Miao, X. X., Da Lage, J. L., & Wicker-Thomas, C. (2008). Evolution of a
563 desaturase involved in female pheromonal cuticular hydrocarbon biosynthesis and
564 courtship behavior in *Drosophila*. Insect biochemistry and molecular biology, 38(2),
565 244-255. (doi:10.1016/j.ibmb.2007.11.005)

566 Ljunggren J, Borrero-Echeverry F, Chakraborty A, Lindblom TU, Hedenström E, Karlsson
567 M, Witzgall P, Bengtsson M. 2019. Yeast volatomes differentially effect larval
568 feeding in an insect herbivore. Appl Environm Microbiol 85: e01761-19
569 (doi:10.1128/AEM.01761-19)

570 Lynch, K.M., Zannini, E., Wilkinson, S., Daenen, L. and Arendt, E.K., 2019. Physiology
571 of acetic acid bacteria and their role in vinegar and fermented beverages.
572 Comprehensive Reviews in Food Science and Food Safety, 18, 587-625.
573 (doi:10.1111/1541-4337.12440)

574 Manoli, D. S., Foss, M., Villella, A., Taylor, B. J., Hall, J. C., & Baker, B. S. 2005. Male-
575 specific fruitless specifies the neural substrates of Drosophila courtship behaviour.
576 Nature 436:395-400. (doi:10.1038/nature03859)

577 Markow, T. A. (2019). Host use and host shifts in *Drosophila*. Current Opinion in Insect
578 Science 31:139-145.(doi:10.1016/j.cois.2019.01.006)

579 Merico, A., Sulo, P., Piskur, J. & Compagno, C. (2007) Fermentative lifestyle in yeasts
580 belonging to the Saccharomyces complex. FEBS Journal, 274, 976–989.
581 (doi:10.1111/j.1742-4658.2007.05645.x)

582 Münch D, Galizia CG. 2016. DoOR 2.0 - Comprehensive mapping of *Drosophila*
583 *melanogaster* odorant responses. Scientific Reports 6:21841
584 (doi:10.1038/srep21841)

585 Murgier, J., Everaerts, C., Farine, J.P. and Ferveur, J.F., 2019. Live yeast in juvenile
586 diet induces species-specific effects on Drosophila adult behaviour and fitness.
587 Scientific Reports 9,1-12. (doi:10.1038/s41598-019-45140-z)

588 Nakai, J., Ohkura, M., & Imoto, K. (2001). A high signal-to-noise Ca2+ probe composed
589 of a single green fluorescent protein. Nature Biotechnology 19:137-
590 141.(doi:10.1038/84397)

591 Quan AS, Eisen MB (2018) The ecology of the *Drosophila*-yeast mutualism in wineries.
592 PLoS One 13(5): e0196440. (doi:10.1371/journal.pone.0196440)

593 R Core Team. 2013 R: A Language and Environment for Statistical Computing. Vienna,
594 Austria, R Foundation for Statistical Computing.

595 Robertson HM, Warr CG, Carlson JR. 2003. Molecular evolution of the insect
596 chemoreceptor gene superfamily in Drosophila melanogaster. Proc Natl Acad Sci
597 USA 100:14537-42. (doi:10.1073/pnas.233584710)

598 Ruebenbauer, A., Schlyter, F., Hansson, B.S., Löfstedt, C., Larsson, M.C. 2008. Genetic
599 variability and robustness of host odor preference in *Drosophila melanogaster*.
600 Curr. Biol. 18: 1438-1443. (doi:10.1016/j.cub.2008.08.062)

601 Ruta V, Datta SR, Vasconcelos ML, Freeland J, Looger LL, Axel R. 2010. A dimorphic
602 pheromone circuit in Drosophila from sensory input to descending output. Nature
603 2010, 468:686-690. (doi:10.1038/nature09554)

604 Sato K and Yamamoto D (2020) Contact-chemosensory evolution underlying
605 reproductive isolation in Drosophila species. Front. Behav. Neurosci. 14:597428.
606 (10.3389/fnbeh.2020.597428)

607 Schaner, A.M., Bartelt, R.J. & Jackson, L.L. 1987. (Z)-11-octadecenyl acetate, an
608 aggregation pheromone inDrosophila simulans . J Chem Ecol 13, 1777–1786
609 (doi:10.1007/BF00980218)

610 Seeholzer, L. F., Seppo, M., Stern, D. L., & Ruta, V. (2018). Evolution of a central
611 neural circuit underlies Drosophila mate preferences. Nature, 559(7715), 564-569.
612 (doi:10.1038/s41586-018-0322-9)

613 Stockinger P, Kvitsiani D, Rotkopf S, Tirian L, Dickson BJ. 2005. Neural circuitry that
614 governs Drosophila male courtship behavior. Cell 121:795-807
615 (doi:10.1016/j.cell.2005.04.026).

616 Strutz, A., Soelter, J., Baschwitz, A., Farhan, A., Grabe, V., Rybak, J., Knaden M,
617 Schmuker M, Hansson BS, Sachse, S. (2014). Decoding odor quality and intensity
618 in the Drosophila brain. Elife, 3, e04147. (doi:10.7554/eLife.04147)

619 Thistle, R., Cameron, P., Ghorayshi, A., Dennison, L., & Scott, K. (2012). Contact
620 chemoreceptors mediate male-male repulsion and male-female attraction during
621 Drosophila courtship. Cell, 149(5), 1140-1151. (doi:10.1016/j.cell.2012.03.045)

622 Toda, H., Zhao, X., & Dickson, B. J. (2012). The Drosophila female aphrodisiac
623 pheromone activates ppk23+ sensory neurons to elicit male courtship behavior.
624 Cell Reports 1:599-607. (doi:10.1016/j.celrep.2012.05.007)

625 Vosshall, L. B., & Stocker, R. F. (2007). Molecular architecture of smell and taste in
626 Drosophila. Annu. Rev. Neurosci. 30:505-533.
627 (doi:10.1146/annurev.neuro.30.051606.094306)

628 Wilson RI. 2013. Early olfactory processing in *Drosophila*: mechanisms and principles.
629 Annu Rev Neurosci 36:217-241 (doi:10.1146/annurev-neuro-062111-150533)

630 Yamamoto, D., & Koganezawa, M. (2013). Genes and circuits of courtship behaviour in
631 Drosophila males. Nature Reviews Neuroscience 14:681-692.
632 (doi:10.1038/nrn3567)

633 ## Figure legends

634 ### Figure 1

635 Odour-mediated upwind flight attraction of fruit fly *Drosophila melanogaster* males
636 (blue bars) and females (ochre bars) to (a) vinegar and yeast headspace, (b) 10
637 ng/min male sex pheromone cVA, (c) 10 ng/min female sex pheromone *Z*4-11Al, alone
638 and in blends with vinegar or yeast headspace, respectively. Letters of the
639 corresponding colours show differences between treatments using Wald pairwise
640 comparisons. Asterisks show treatments with significant differences between sexes
641 ($n$=40).

642 ### Figure 2

643
644 Effect of *Z*4-11Al on courtship in *D. melanogaster* males. Decapitated target flies were
645 painted with 1 ng test compound or heptane (solvent control). The number of courting
646 and non-courting males in each test was compared using a chi-square test, asterisks
647 show significant differences.

648 (a) Proportion of unmated or mated *D. melanogaster* (Dalby) test males courting
649 unmated decapitated female or male target flies treated with 1 ng *Z*4-11Al or heptane
650 (solvent control) ($n$=80, $P$=0.006 and $P$=0.007).

651 (b) Proportion of mated Canton-S and Dalby strain *D. melanogaster* males and *D*.
652 *simulans* males, courting decapitated Dalby or *D*. *simulans* females, treated with 1 ng
653 *Z*4-11Al or heptane ($n$=80, $P$=0.009 and $P$=0.039).

654 (c) Effect of RNA interference, in Orco-Gal4/uas-Or69aRNAi mated males, courting
655 Dalby target females, treated with 1 ng *Z*4-11Al or heptane. Parental lines, Orco-Gal4
656 and uas-Or69aRNAi, show significant courtship behaviour ($n$=80, $P$=0.036 and
657 $P$=0.032).

658 (d) Proportion of mated Dalby males courting decapitated Dalby females, treated with
659 *E*4-11Al, (*R*)-linalool or ethyl butyrate ($n$=80).

660 **Figure 3**

661 Calcium imaging responses in the antennal lobe (AL). Colours show the median
662 normalized calcium activity (Δ F/F [%]) in response to controls and odor applications,
663 according to the colour bar on the left (*n*=10; mean±SD).

664 (a) Calcium activity in Orco-Gal4 males (top) and females (below) in response to
665 control (air, mineral oil), to 60 and 600 ng *Z*4-11Al, and to 600 ng (*R*)-linalool. *Z*4-11Al
666 and (*R*)-linalool are key ligands for the D glomerulus (Lebreton et al. 2017).

667 (b) Responses of Or22a-Gal4 males to *Z*4-11Al in the DM2 glomerulus and of Or43b-
668 Gal4 males in the DM5 and VM2 glomeruli; ethyl butyrate is a diagnostic stimulus for
669 these glomeruli.

670 (c) Projection neuron (PN) responses in GH146-Gal4 males to *Z*4-11Al and (*R*)-linalool
671 in D and DM2 glomeruli.

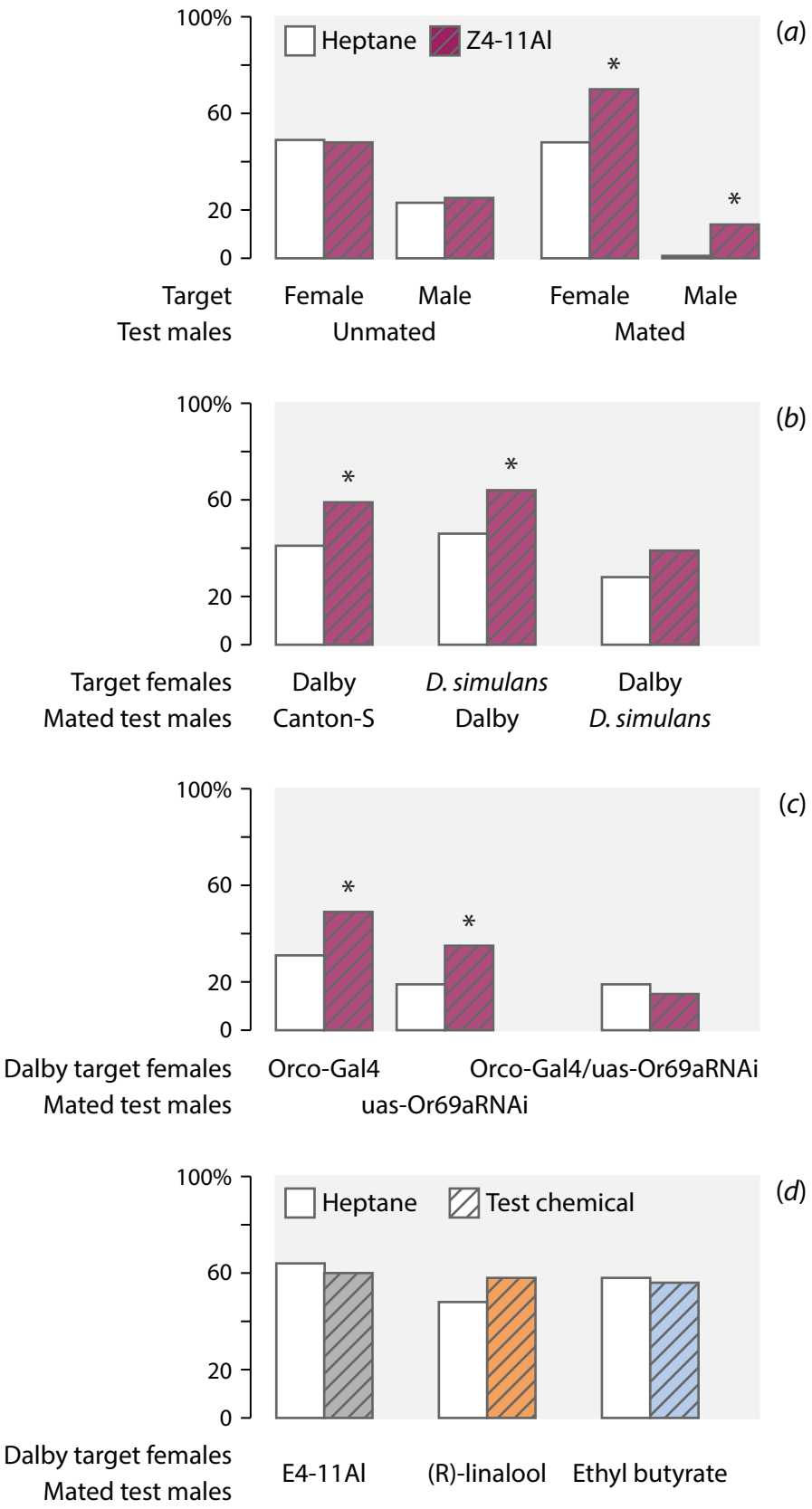


**Figure 2**. Effect of Z4-11Al on courtship in D. melanogaster males. Decapitated target flies were painted with 1 ng test compound or heptane (solvent control). The number of courting and non-courting males in each test was compared using a chi-square test, asterisks show significant differences.
(a) Proportion of unmated or mated D. melanogaster (Dalby) test males courting unmated decapitated female or male target flies treated with 1 ng Z4-11Al or heptane (solvent control) (n=80, P=0.006 and P=0.007).
(b) Proportion of mated Canton-S and Dalby strain D. melanogaster males and D. simulans males, courting decapitated Dalby or D. simulans females, treated with 1 ng Z4-11Al or heptane (n=80, P=0.009 and P=0.039).
(c) Effect of RNA interference, in Orco-Gal4/uas-Or69aRNAi mated males, courting Dalby target females, treated with 1 ng Z4-11Al or heptane. Parental lines, Orco-Gal4 and uas-Or69aRNAi, show significant courtship behaviour (n=80, P=0.036 and P=0.032).
(d) Proportion of mated Dalby males courting decapitated Dalby females, treated with E4-11Al, (R)-linalool or ethyl butyrate (n=80).

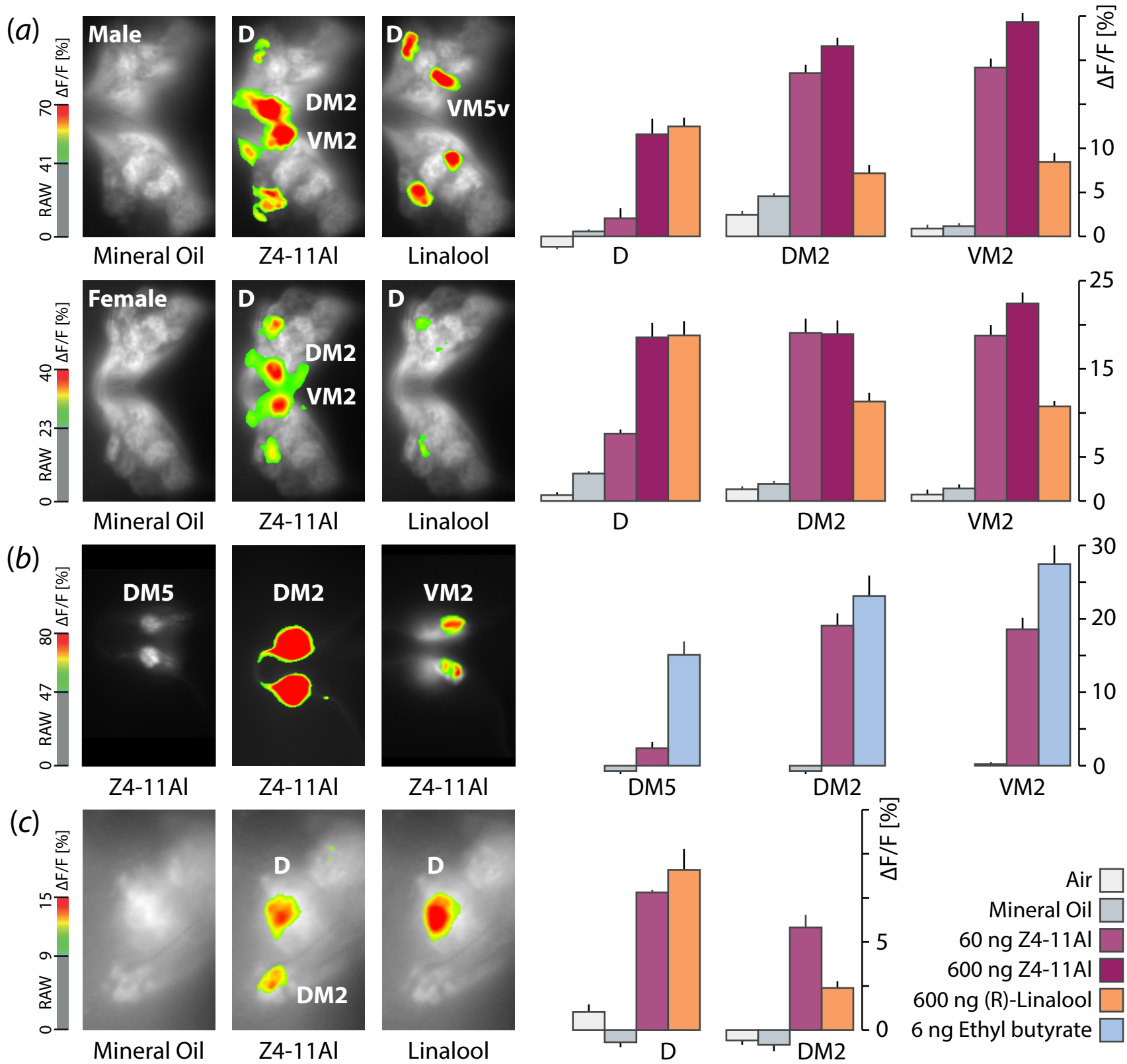


**Figure 3**. Calcium imaging responses in the antennal lobe (AL). Colours show the median normalized calcium activity (Δ F/F [%]) in response to controls and odor applications, according to the colour bar on the left (n=10; mean±SD).
(a) Calcium activity in Orco-Gal4 males (top) and females (below) in response to control (air, mineral oil), to 60 and 600 ng Z4-11Al, and to 600 ng (R)-linalool. Z4-11Al and (R)-linalool are key ligands for the D glomerulus (Lebreton et al. 2017).
(b) Responses of Or22a-Gal4 males to Z4-11Al in the DM2 glomerulus and of Or43b-Gal4 males in the DM5 and VM2 glomeruli; ethyl butyrate is a diagnostic stimulus for these glomeruli.
(c) Projection neuron (PN) responses in GH146-Gal4 males to Z4-11Al and (R)-linalool in D and DM2 glomeruli.

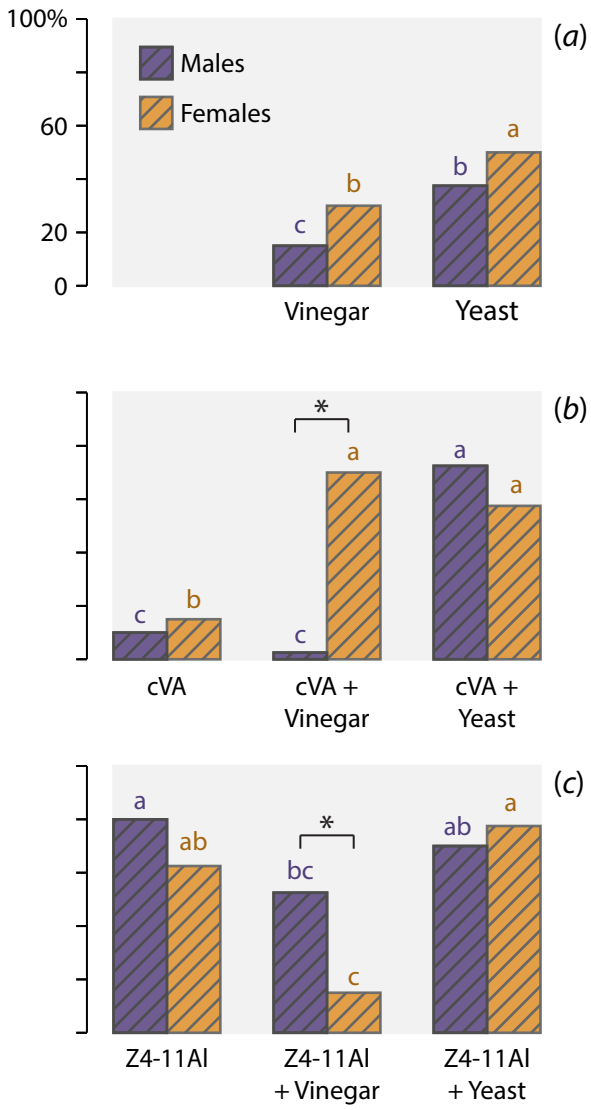


**Figure 1**. Odour-mediated upwind flight attraction of fruit fly Drosophila melanogaster males (blue bars) and females (ochre bars) to
(a) vinegar and yeast headspace,
(b) 10 ng/min male sex pheromone cVA,
(c) 10 ng/min female sex pheromone Z4-11Al, alone and in blends with vinegar or yeast headspace, respectively.
Letters of the corresponding colours show differences between treatments using Wald pairwise comparisons. Asterisks show treatments with significant differences between sexes (n=40).