The female pheromone (*Z*)-4-undecenal mediates flight 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

[249 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-undecenal (*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. *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).

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

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

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

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

124 Materials and methods

125 Insects and chemicals

126 Flies were reared on a standard sugar-yeast-cornmeal diet at room temperature (19 to
127 22°C) under a 16:8-h L:D photoperiod. Newly eclosing flies were anesthetized with CO_2
128 and sexed under a microscope. Virgin flies were identified by the presence of
129 meconium, and were kept together with flies of the same sex. Mated males were kept
130 together with females, and were separated 2 d before experiments. Flies were kept in
131 30-mL Plexiglas vials with fresh food, and were six d old when used for experiments.

132 Isomeric purity of (*Z*)-4-undecenal (Z4-11Al) and (*E*)-4-undecenal (E4-11Al) was
133 98.6% and 97.8%, repectively, according to gas chromatography coupled to mass

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

Functional imaging

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

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

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

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

Courtship assay

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

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

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

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

187 **Food odour collection**

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

196 **Wind tunnel assay**

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

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

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

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. Courtship assays were analysed using chi-squared tests to compare between treatments and their respective control. Statistical analysis was calculated with R (R Core Team 2013) and SPSS Version 22 (IBM Corp.).

Results

Flight attraction to blends of pheromone and food odour

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

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

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

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

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

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

243 Male courtship in response to *Z*4-11Al

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

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

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

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

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

271 Finally, we tested three non-pheromonal chemicals: *E*4-11Al, the geometric isomer of
272 *Z*4-11Al, which is perceived via the Or69aB variant; (*R*)-linalool, a food compound,
273 which is the main ligand of the other variant, Or69aA; and ethyl butyrate, which is a
274 ligand for Or22a (DM2 glomerulus in the AL), for Or43b (VM2 glomerulus) and also for
275 Or85a (DM5 glomerulus; see also Figure 3). None of these compounds elicited a
276 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

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

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

319 Importantly, males respond also to decapitated *D. simulans* females laced with *Z*4-
320 11Al. This shows that a response to *Z*4-11Al does not require presence of the close-
321 range CHC pheromone 7,11-HD, since 7,11-HD is not produced by *D. simulans* (Billeter
322 et al. 2009, Lebreton et al. 2017, Billeter and Wolfner 2018, Sato and Yamamoto
323 2020).

324 Courtship discrimination between Or69a ligands

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

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

334 A differential response to Z4-11Al and (*R*)-linalool is reflected by their respective
335 activation patterns in the AL (Figure 3a). (*R*)-linalool is also a ligand for Or98a, which
336 accounts for stimulation of the VM5v glomerulus (Couto et al. 2005, Münch and Galizia
337 2016)

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

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

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

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

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

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

372 ## A behavioural paradigm for bioactive odorant identification

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

378 Comparison of pheromone blend attraction with vinegar and yeast shows that yeast
379 overrides differences between pheromones (Figure 1). Vinegar, derived from acetic acid
380 bacteria fermentation (Lynch et al. 2019), is a widely used standard food attractant for
381 *D. melanogaster*, although live yeast aroma is much richer in composition (Callejón et
382 al. 2009, Chinnici et al. 2009, Becher et al. 2010, Ljunggren et al. 2019). Yeast growing
383 on ripe fruit is also a biologically more relevant attractant, since it attracts flies for
384 oviposition and since yeast is a sufficient substrate for larval development (Becher et al.
385 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.

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638 ## Figure legends

639 ### Figure 1

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

647 ### Figure 2

648
649 Effect of *Z*4-11Al on courtship in *D. melanogaster* males. Decapitated target flies were
650 painted with 1 ng test compound or heptane (solvent control). The number of courting
651 and non-courting males in each test was compared using a chi-square test, asterisks
652 show significant differences.

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

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

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

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

665 **Figure 3**

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

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

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

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

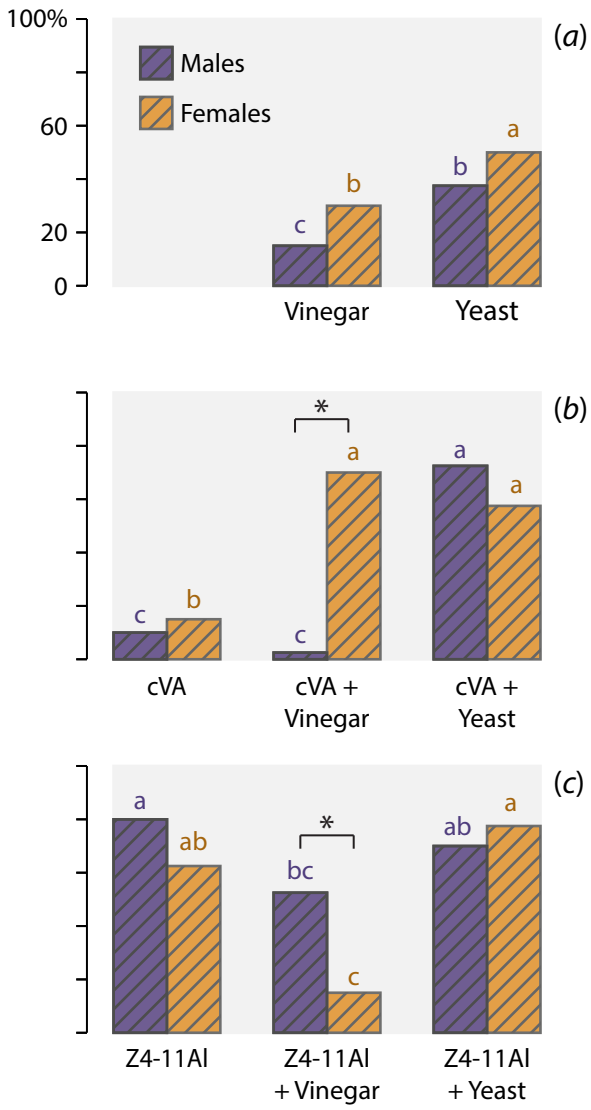

Figure 1. Odour-mediated upwind flight attraction of fruit fly Drosophila melanogaster males (blue bars) and females (ochre bars) to
(a) vinegar and yeast headspace,
(b) 10 ng/min male sex pheromone cVA,
(c) 10 ng/min female sex pheromone Z4-11Al, alone and in blends with vinegar or yeast headspace,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

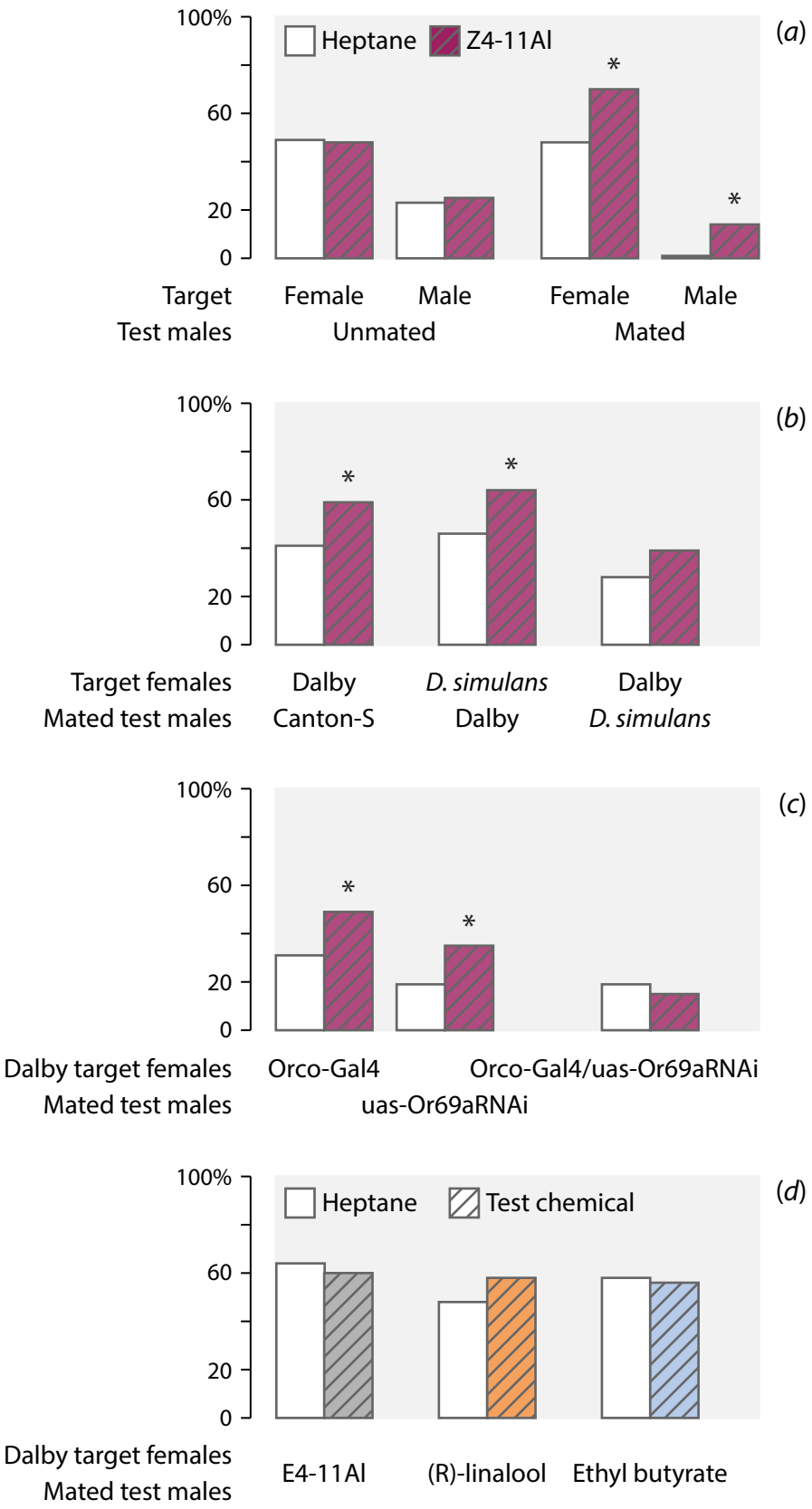

Figure 2. Effect of Z4-11Al on courtship in D. melanogaster males. Decapitated target flies were painted with 1 ng test compound or heptane (solvent control). The number of courting and non-courting males in each test was compared using a chi-square test, asterisks show significant differences.
(a) Proportion 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).

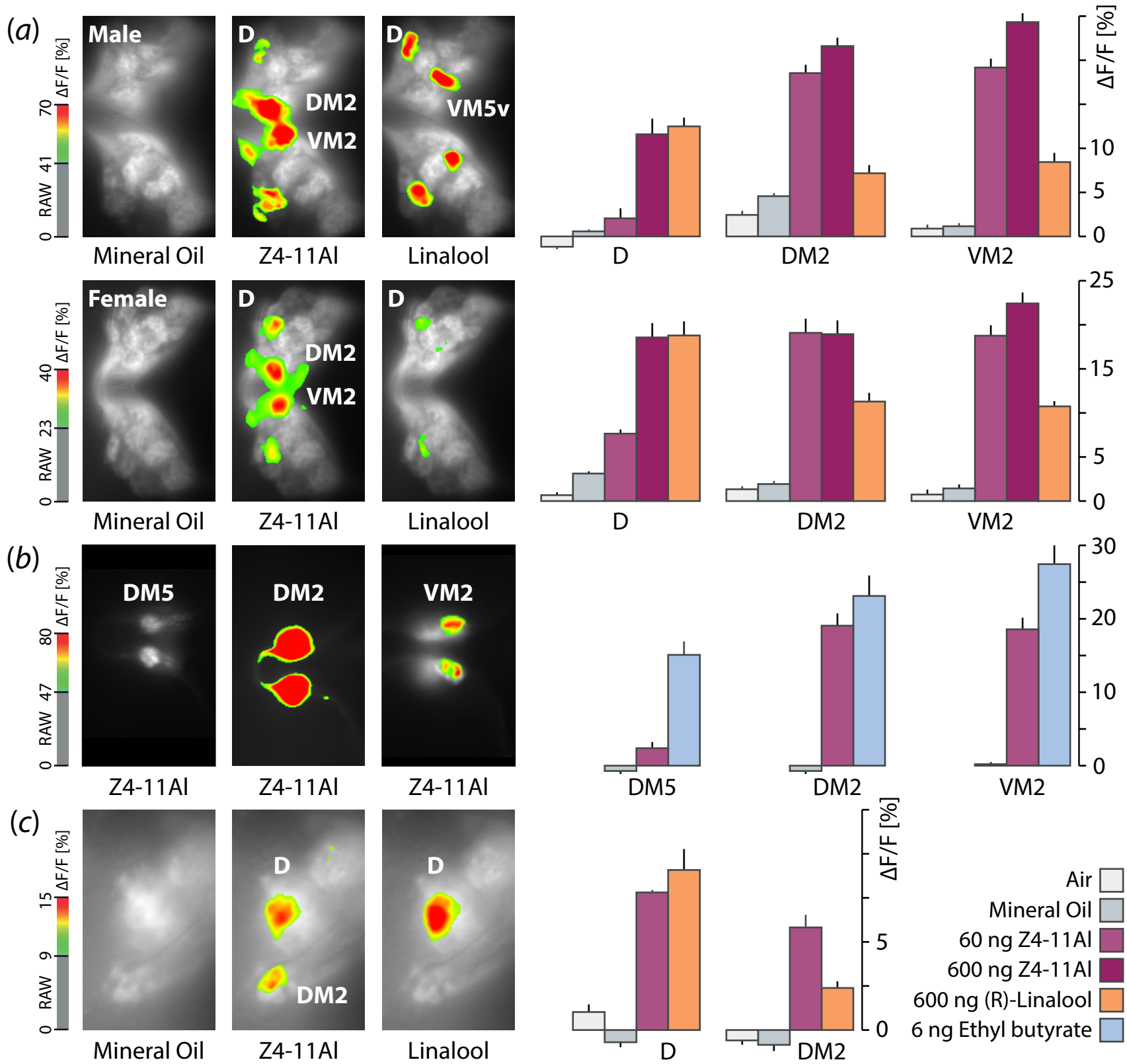

Figure 3. Calcium imaging responses in the antennal lobe (AL). Colours show the median normalized calcium activity (Δ F/F [%]) in response to controls and odor applications, according to the colour bar on the left (n=10; mean±SD).
(a) Calcium activity in Orco-Gal4 males (top) and females (below) in response 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.