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The female sex pheromone (*Z*)-4-undecenal mediates flight attraction and courtship in *Drosophila melanogaster*

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ABSTRACT

Specific mate communication and recognition underlies reproduction and hence speciation. Our study provides new insights in *Drosophila melanogaster* premating olfactory communication. Mate communication evolves during adaptation to ecological niches and makes use of social signals and habitat cues. Female-produced, species-specific volatile pheromone (*Z*)-4-undecenal (*Z*4-11Al) and male pheromone (*Z*)-11-octadecenyl acetate (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. Two isoforms of the olfactory receptor Or69a are co-expressed in the same olfactory sensory neurons. *Z*4-11Al is perceived via Or69aB, while the food odorant (*R*)-linalool is a main ligand for the other variant, Or69aA. However, only *Z*4-11Al mediates courtship in experienced males, not (*R*)-linalool. 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 contrast to *Z*4-11Al. A blend of the food odour vinegar and cVA attracted females, while a blend of vinegar and female pheromone *Z*4-11Al 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.

1. 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 sequence of behavioural responses (Depetris-Chauvin et al., 2015; Kohl et al., 2015; Auer and Benton, 2016; Bates et al., 2020; Chakraborty and Sachse, 2021). 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, 2021) and host odorants (Dekker et al., 2006; Markow 2019; Auer et al., 2020), across *Drosophila* phylogenies.

What remains unclear is whether olfactory tuning and response to pheromones and food odours evolve independently. And, although odorants enable evaluation and recognition of other flies or a food 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., 2021). Naturally, this congruently applies to their volatile oxidation products, particularly monounsaturated aldehydes, which derive from diunsaturated CHCs. The *D. melanogaster* female pheromone (*Z*,*Z*)-7,11-heptacosadiene (Z7,Z11-27Hy; 7,11-HD) has been shown to afford reproductive isolation between *D. melanogaster* and its sibling species *D.*

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simulans, which does not produce 7,11-HD. Owing to its low volatility, 7,11-HD is perceived by gustatory receptors, at close range (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 (Or) DmelOr69a (Or69a) and elicits flight attraction in conspecific males and females, but not in *D. simulans* (Lebreton et al., 2017). This lends support to the idea that 7,11-HD and *Z*4-11Al are species-specific. The closely related *D. mauritiana* and *D. sechellia* also produce 7,11-HD, together with other unsaturated hydrocabons (Khallaf et al., 2021), but it is yet unclear whether the behavioural activity of these blends differs from the single compounds, as this is the case in lepidopteran pheromones (El-Sayed, 2021). Females of the Zimbabwe strain of *D. melanogaster* produce (Z,Z)-5,9-heptacosadiene (Z5,Z9-27Hy; 5,9-HD) in addition to 7,11-HD (Dallerac et al., 2000) and release accordingly a blend of (*Z*)-4-nonenal (Z4-9Al) and *Z*4-11Al (Frey et al., 2021).

Singular expression of Ors in olfactory sensory neurons (OSNs) and convergence of OSNs for first-order processing, is fundamental to olfaction in insects and vertebrates alike (Monahan and Lomvardas, 2015; Mika and Benton, 2021). The Or69a gene, which encodes two isoforms throughout the *Drosophila* lineage, is a rare exception to this rule (Robertson et al., 2003; Conceicao and Aguade, 2008). In *D. melanogaster*, the Or69aA and Or69aB isoforms are tuned to food odorants, such as (*R*)-linalool, and the female pheromone *Z*4-11Al, respectively (Lebreton et al., 2017). Co-expression of Ors can be viewed as a transient state, following Or duplication and prior to the evolution of separate processing in the antennal lobe (AL) (Ramdya and Benton, 2010; Mika and Benton, 2021). Co-expression of the Or69a variants may, however, also be adaptive, since combined coding of sex and food stimuli (Lebreton et al., 2017) affords a trait that integrates natural and sexual selection (Maan and Seehausen, 2011; Servedio et al. 2011).

In comparison, (*Z*)-11-octadecenyl acetate (Z11-18Ac) or *cis*-vaccenyl acetate (cVA), is a male pheromone that mediates attraction in conjunction with food odour, 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, 2021). 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).

cVA activates the sexually dimorphic fruitless (*fru*) neuronal circuitry that controls male courtship (Manoli et al., 2005; Stockinger et al., 2005; Billeter et al., 2006; Cachero et al., 2010; Peng et al., 2021). Curiously, the *fru* circuit does not comprise olfactory neurons responsive to the female pheromone *Z*4-11Al.

We therefore investigated the effect of *Z*4-11Al on flight attraction and courtship, which are successive steps in male reproductive behaviour. An upwind flight assay confirms that *Z*4-11Al, unlike cVA, elicits attraction in naive males (Lebreton et al., 2017), but enhances courtship only in experienced, previously mated males.

Food is an aphrodisiac and promotes courtship in *Drosophila* (Grosjean et al. 2011, Gorter et al., 2016; Ando et al., 2020). Vinegar has been widely used as a convenient proxy for food odour in fruit fly research, and has a prominent synergistic effect on cVA perception and attraction (Lebreton et al., 2015; Das et al., 2017; Cazale-Debat et al., 2019). Vinegar headspace contains several active odorants that enhance flight attraction to acetic acid (Becher et al., 2010) and cancel out avoidance of acetic acid at close range (Ai et al., 2010). Naturally, vinegar flavour widely varies in composition between makes and types (Callejón et al., 2009; Chinnici et al., 2009). Vinegar is the end point of fruit fermentation, whereas live yeast colonies on overripe fruit stimulate feeding, mating, and oviposition. Fermenting yeasts release a very rich volatome, that accounts for strong fly attraction (Becher et al., 2012, 2018; Buser et al., 2014; Christiaens et al., 2014; Ljunggren et al., 2019).

We therefore compared the effect of vinegar and yeast volatiles on flight attraction to pheromone. Blends of male or female pheromone with vinegar produced a sex-specific, converse response in females and males, respectively. In comparison, both sexes responded to blends of yeast and cVA or *Z*4-11Al.

2. Materials and methods

2.1. Insects and chemicals

Flies (*D. melanogaster* strains Dalby-HL, Canton-S and *D. simulans*) were reared on a BDSC standard cornmeal diet (Bloomington, IN, USA) at room temperature (19–22 °C) under a 16:8-h L:D photoperiod. Newly eclosing flies were anesthetized with CO_2 and sexed under a microscope. Virgin flies were identified by the presence of meconium, and were kept together with flies of the same sex. Flies were kept in 30-mL Plexiglas vials with fresh food.

Z4-11Al and E4-11Al were synthesized (Lebreton et al., 2017), isomeric purity was 98.6% and 97.8%, respectively, according to gas chromatography coupled to mass spectrometry (6890 GC and 5975 MS, Agilent Technologies, Santa Clara, CA, USA). Isomeric purity of cVA (Pherobank, Wageningen, The Netherlands) was 97.3%. Chemical purity of the aldehydes was >99.9%, and of cVA, (*R*)-linalool and ethyl butyrate >97%. Heptane (redistilled; Merck, Darmstadt, Germany) was used as solvent.

2.2. 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 20×/0.95 W). 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, 2016) served to link the active area to individual glomeruli. All experimental flies (3 d old) 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). For mass labelling of PNs, the transgenic line GH146-GAL4, expressing G-CaMP in 83 out of the 150 PNs in each AL (Heimbeck et al., 2001) was used. Fly lines, including OR43b-Gal4, were obtained from the Bloomington *Drosophila* Stock Center (Indiana University, Bloomington, IN, USA). In addition, OR22a-Gal4 (Leslie Vosshall, The Rockefeller University, NY, USA) and OR43b-Gal4 (Bloomington) served to image specific AL glomeruli. Transgenic flies have been generated using standard procedures.

2.3. Courtship assay

Wild-type flies for courtship experiments were the *D. melanogaster* strains Dalby-HL (Ruebenbauer et al., 2008) and Canton-S, 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 courtship behaviour in males of two Or69a-Gal4 lines (lines #9999 and #10000, Bloomington Drosophila Stock Center) was not consistent.

Courtship experiments were done on a light box (37 × 28 × 2.5 cm; color temperature 5.000 ± 5% Kelvin; Kaiser Slimlite 2422, Kaiser Fototechnik GmbH & Co. KG, Buchen, Germany). The courtship arena consisted of three glass plates (17 × 13 × 0.5 cm) placed on top of each other. Twelve circular holes (ø 3 cm) were cut in the middle plate to form 12 circular single-pair mating chambers. All glassware was heated to 350 °C for 8 h before use. Tests were done between 2 and 5 h after onset of scotophase (16:8 L:D photoperiod). Target flies, unmated males or females, were anesthetized on ice and decapitated before experiments. Test flies were either mated or unmated 6-d-old males. Mated males were kept together with females, and were separated 2 d before experiments.

A single target fly was added to each mating chamber, and 1 μL heptane (control) or 1 μL heptane containing 1 ng *Z*4-11Al was applied onto its abdomen. After solvent evaporation (2 min), a single live test fly was introduced into each mating chamber and observed during 20 min. Males either responded or did not respond at all to decapitated target males or females. Since we tested different fly lines and chemical treatments, naive and experienced flies, female and male target flies, it was parsimonious to compare the number of courting males. Test males were scored when they initiated the courtship sequence, including orientation, tapping and wing vibration (Greenspan and Ferveur, 2000; Ellendersen and von Philipsborn, 2017). Treatments ($n = 80$) and controls ($n = 80$) were conducted simultaneously, and each fly was tested once.

2.4. Food odour collection

Brewer's yeast, *Saccharomyces cerevisiae* (strain S288C), was grown in minimal media (Merico et al., 2007) during 20 h in a shaking incubator at 25 °C and 260 RPM. 50 mL yeast broth or white wine vinegar (7.1%, Zeta, Sweden) were filled into a wash bottle and charcoal filtered air was led through the bottle at a rate of 200 mL/min. Headspace was trapped in 4 × 40-mm glass tubes holding 300 mg of Porapak Q 50/30 (Waters Corporation, Milford, Ma, USA) during 2 h (Becher et al., 2012). These air filters were eluted with redistilled ethanol (Labscan), the eluate was sprayed during 2 h of wind tunnel experiments.

2.5. Wind tunnel assay

Upwind flight attraction was observed in a glass wind tunnel (30 × 30 × 100 cm). The wind tunnel was lit diffusely from above, at 13 lx, temperature ranged from 20 °C to 24 °C, relative humidity from 38% to 48% and charcoal filtered air (Camfil AB, Malmö, Sweden), at a velocity of 0.25 m/s, was produced by a fan (Fischbach GmbH, Neunkirchen, Germany). Yeast headspace and odour blends were delivered from the centre of the upwind end of the wind tunnel via a piezo-electric microsprayer (Becher et al., 2010).

The microsprayer (El-Sayed et al., 1999) consists of a motor-driven syringe (CMA Microdialysis AB, Solna, Sweden) that delivers solutions of test chemicals through teflon tubing at a constant rate (10 μL/min) to a vibrating glass capillary with an elongated tip. A piezo-ceramic disc (Valvo, Hamburg, Germany) vibrates the glass capillary at ca. 200 kHz, and thus produces an aerosol that evaporates from the tip of the capillary. Unlike passive dispenser materials, such as filter paper, the sprayer ensures delivery of test chemicals in constant and known rates and blend ratios, independent of vapour pressure. Another advantage is that very small amounts of chemicals can be applied, including headspace collected from odour sources during short intervals or extracts of single insect glands. The sprayer has been successfully used with flies and moths. For example, optimum release of codling moth *Cydia pomonella* sex pheromone is 10 ng/min, which rather precisely matches the female release rate of ca. 6 ng/h (Bäckman et al., 1997, Witzgall et al. 2001)

Unmated, fed, 3-d-old males and females (n = 40) were flown individually to each treatment. *Z*4-11Al and cVA were sprayed at 10 ng/min and 300 ng/min, respectively (Lebreton et al., 2015, 2017), alone and in blends with yeast and vinegar headspace. Flies were scored when they approached the odour source (<10 cm), after showing the typical odour-mediated, undulating upwind flight in the wind tunnel centre over 80 cm, from the release cage at the downwind end of the wind tunnel, to the source. Flies landed at the glass cylinder (6 × 9.5 cm) holding the microsprayer, or on the metal mesh (2 mm) covering the open end of the cylinder to protect the vibrating capillary. Glass cylinder and metal mesh were heated to 350 °C during 6 h between experiments using different test chemicals, and the sprayer was flushed with solvent during at least 1 h.

2.6. Statistical analysis

Generalized linear models (GLM) with a Bernoulli binomial distribution were used to analyse wind tunnel data and courtship assays. Upwind flight and sex were used as the target effects for the wind tunnel tests. *Post-hoc* Wald pairwise comparison tests were used to identify differences between treatments for wind tunnel assays, and differences between the treatments and their respective control for the courtship assays. Statistical analysis was calculated with R (R Core Team, 2013) and SPSS Version 22 (IBM Corp.).

3. Results

3.1. Flight attraction to blends of pheromone and food odour

We oftentimes observed that groups of feeding and courting *D. melanogaster* females and males attract other flies. This observation inspired an investigation of flight attraction to known volatile pheromones and food odorants.

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 compared flight attraction of males and females to cVA and *Z*4-11Al, alone and in blends with vinegar and yeast volatiles (Fig. 1). Use of the microsprayer (El-Sayed et al., 1999) enables the release of synthetic chemicals and headspace collections at known and constant release rates. This is particularly important when comparing chemicals that greatly differ with respect to vapour pressure, such as *Z*4-11Al and cVA.

Male pheromone cVA alone attracted only few flies, while a blend of cVA and vinegar was highly, and significantly more attractive to females than to males ($z = 4.22$, $df = 79$, $p < 0.0001$, -4.51 ± 1.07 (estimate ± SE); Fig. 1b. In comparison, no significant differences were found in the response of both sexes towards a blend of cVA and yeast headspace.

Female pheromone *Z*4-11Al alone attracted unmated males and females. Blending vinegar with *Z*4-11Al reduced attraction in both sexes, but significantly more males than females responded ($z = 3.37$, $df = 79$, $p < 0.0001$, 1.84 ± 0.54). And, a blend of yeast headspace and *Z*4-11Al attracted as many flies as *Z*4-11Al alone (Fig. 1c).

Yeast headspace by itself attracted more males ($z = 3.22$, $df = 79$, $p < 0.001$, 1.85 ± 0.57) and more females ($z = 2.25$, $df = 79$, $p < 0.02$, 1.07 ± 0.48) than vinegar headspace (Fig. 1a). Female and male attraction to the blends of yeast headspace and cVA and *Z*4-11Al, respectively, was not significantly different (Fig. 1b,c).

Taken together, only few flies responded to the male pheromone cVA, while the female pheromone *Z*4-11Al strongly attracted both sexes. Our results show further that vinegar, a frequently used food odorant

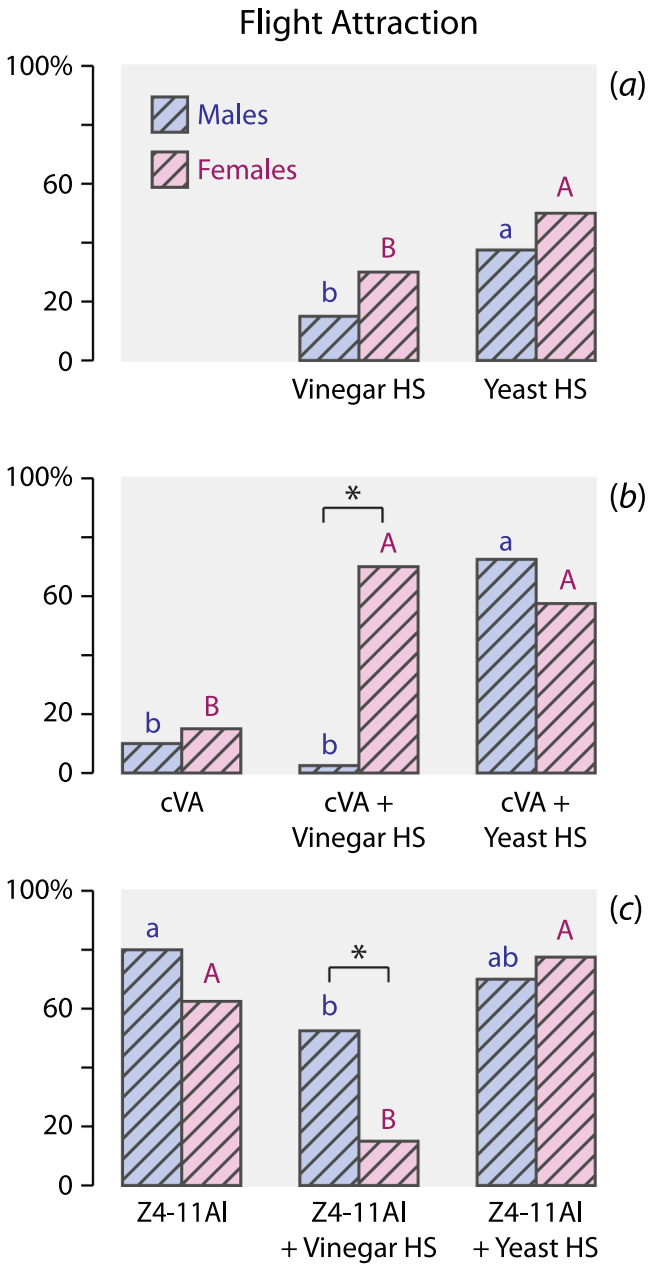

Fig. 1. Odour-mediated upwind flight attraction of fruit fly *Drosophila melanogaster* males (blue bars) and females (pink bars) to (a) vinegar and yeast headspace, (b) 10 ng/min male sex pheromone cVA, (c) 10 ng/min female sex pheromone *Z*4-11Al, alone and in blends with vinegar or yeast headspace, respectively (n = 40 for each sex, in each treatment). Capital and lower-case letters show differences between treatments, for females and males, respectively, using Wald pairwise comparisons. Asterisks show treatments that differ significantly between sexes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

source for *Drosophila*, is a weaker attractant than yeast aroma (Fig. 1a). Blends with the suboptimal food stimulus vinegar accentuate sex-specific differences in the behavioural response to male and female pheromone. Blends of vinegar with cVA and *Z*4-11Al, respectively, produced an inverse response pattern in male and female flies (Fig. 1b, c).

3.2. Male courtship in response to *Z*4-11Al

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

Compared to solvent control, *Z*4-11Al elicited a significant response in experienced males that had mated earlier, but not in naive, unmated males ($z = -2.86$, $df = 319$, $p < 0.0001$, -0.95 ± 0.33 (estimate $\pm$ SE); Fig. 2a. Experienced males courted decapitated females rather than males, possibly because cVA, present on the cuticula of male flies, has an antagonistic effect on experienced males. That a few males nonetheless courted decapitated males underlines a role of *Z*4-11Al in male courtship ($z = -6.61$, $df = 319$, $p < 0.0001$, -2.68 ± 0.41; Fig. 2a). A test with Canton-S test males corroborated the result obtained with males of the Dalby strain ($z = 2.72$, $df = 319$, $p < 0.007$, 0.91 ± 0.33; Fig. 2b).

Importantly, males also courted decapitated *D. simulans* target females, laced with synthetic *Z*4-11Al ($z = 2.21$, $df = 319$, $p < 0.03$, 0.7148 ± 0.32; Fig. 2b). This experiment rules out a contributing role of the cuticular hydrocarbon pheromone 7,11-HD, which is specific for *D. melanogaster* and not found in *D. simulans* females (Jallon, 1984; Billeter et al., 2009), and further corroborates that *Z*4-11Al elicits male courtship, in the absence of 7,11-HD.

On the other hand, *D. simulans* males did not respond in a significant manner to Dalby females painted with *Z*4-11Al ($z = 1.82$, $df = 319$, $p < 0.07$, 0.62 ± 0.34; Fig. 2b). This was expected, since *D. simulans* males are not attracted to *Z*4-11Al and since *Z*4-11Al has even an antagonistic effect on D. simulans attraction to (*R*)-linalool. *D. simulans* females do not produce 7,11HD, which is the precursor for *Z*4-11Al (Billeter et al., 2009; Lebreton et al., 2017).

Moreover, we used a RNAi fly line to corroborate that Or69a encodes *Z*4-11Al-mediated courtship (Fig. 3c). Significantly fewer males responded when expression of Or69a was disrupted in olfactory sensory neurons (OSNs). The response by Orco-Gal4/uas-Or69aRNAi males was significantly different from males of the Orco-Gal4 ($z = -4.38$, $df = 319$, $p < 0.0001$, -1.69 ± 0.39) and uas-Or69aRNAi lines ($z = -2.85$, $df = 319$, $p < 0.004$, -1.12 ± 0.39). That flies with disrupted neurons courted flies coated with Z4-11Al as well as those washed in heptane, further corroborates the role of Or69a in the detection of Z4-11Al ($z = -0.63$, $df = 319$, $p < 0.53$, -0.268 ± 0.424; Fig. 2c).

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

3.3. 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 (Fig. 2).

In G-CaMP males and females, *Z*4-11Al activated the D glomerulus, as expected, but also DM2 and VM2 (Fig. 3a). These broadly-tuned glomeruli have also been shown to respond to a mix of cVA and

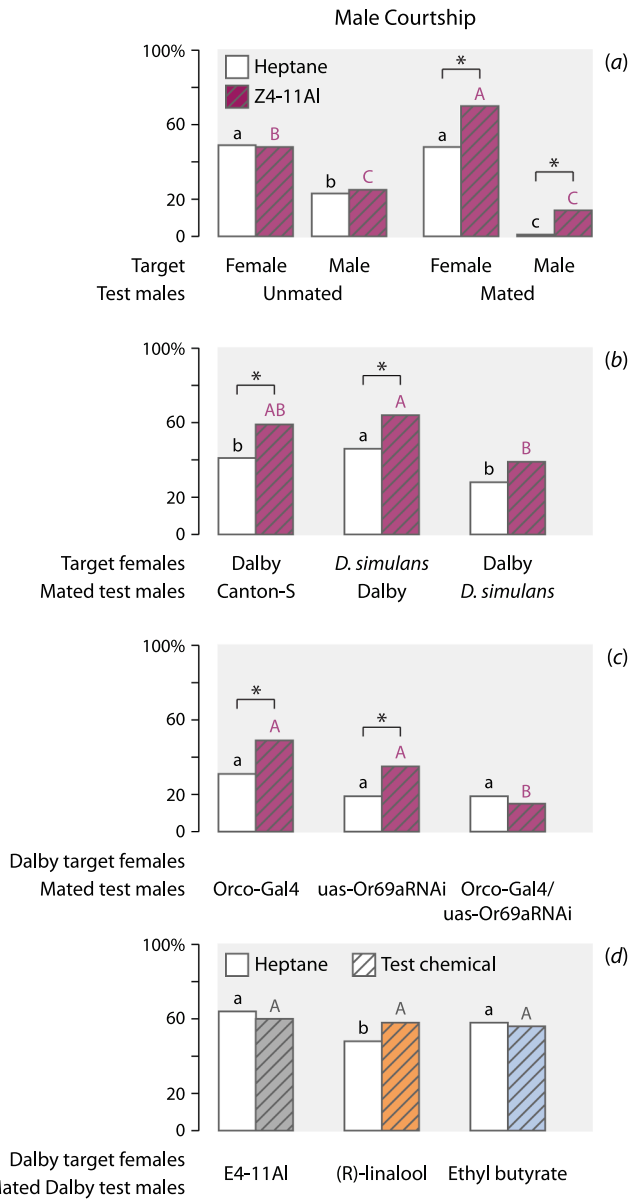

Fig. 2. Effect of *Z*4-11Al on male courtship (n = 80, for each treatment). Decapitated target flies were painted with 1 ng test compound (filled bars) or heptane (solvent control, empty bars). (a) Proportion of unmated or mated *D. melanogaster* (Dalby) test males courting unmated decapitated female or male target flies treated with 1 ng *Z*4-11Al or heptane. (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 *Z*4-11Al or heptane. (c) Effect of RNA interference, in Orco-Gal4/uas-Or69aRNAi mated males, and males of both parental lines (Orco-Gal4 and uas-Or69aRNAi) courting Dalby target females, treated with 1 ng *Z*4-11Al or heptane. (d) Proportion of mated Dalby males courting decapitated Dalby females, treated with *E*4-11Al, (*R*)-linalool or ethyl butyrate. Capital and lower-case letters show differences between treatments (treated flies and control flies, respectively) using Wald pairwise comparisons. Asterisks show differences within treatments, between treated flies and their respective control ($p < 0.05$).

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, 2016; 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 (Fig. 3a).

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

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 (Fig. 3c). A possible response of VM5v to linalool does not show, since GH146-Gal4 does not label this glomerulus (Grabe et al., 2015, 2016).

4. Discussion

4.1. Female pheromone Z4-11Al evokes attraction and courtship in D. melanogaster

Volatile chemicals that are cognate ligands for odorant receptors (ORs) are selectively perceived against noisy odorant backgrounds and they capacitate behavioural decisions at a distance, since they deliver reliable information about the source, including identity and physiological state of the emitter, in the case of pheromones.

The female *D. melanogaster* pheromone *Z*4-11Al attracts naive conspecific males and females from a distance (Fig. 1) and also enhances courtship in experienced males (Fig. 2). Fig. 4 summarizes the current knowledge of the sensory and behavioral physiology of the Or69a channel underlying communication with Z4-11Al.

4.2. Evidence for perception of Z4-11Al via the Or69a channel

Evidence for perception of female pheromone Z4-11Al via OSNs expressing Or69a is derived from electrophysiological recordings (Lebreton et al., 2017), functional imaging (Fig. 3) and behavioural responses of transgenic flies in which OSNs expressing Or69a have been disrupted, during upwind flight (Lebreton et al., 2017) and courtship (Fig. 2).

An electrophysiological screening of single sensilla on the *Drosophila* antenna renders ab9A sensilla as the most responsive to Z4-11Al and points towards a role of Or69a (Couto et al., 2005; Lebreton et al., 2017). This was then corroborated by comparing single sensillum responses from native OSNs contained in ab9 with OSNs that heterologously expressed the two isoforms Or69aA and Or69aB, singly and together (Lebreton et al., 2017).

OSNs expressing Or69aB and Or69aA branch to the D glomerulus in the AL (Couto et al., 2005). Functional imaging of the antennal lobe including projection neurons confirms that Z4-11Al stimulates the D glomerulus (Fig. 3).

Courtship is strongly reduced after disrupting Or69a (Fig. 2), even though we cannot exclude that other Ors might have been affected by the broad expression of Or69aRNAi in Orco-Gal4/uas-Or69aRNAi flies. However, the combined experimental evidence strongly suggests that Z4-11Al mediates both upwind attraction and courtship via Or69a.

4.3. Courtship discrimination between Or69a ligands

Female pheromone Z4-11Al and the food odorant (*R*)-linalool are main ligands for Or69aB and Or69aA, the two isoforms of Or69a, which are coexpressed in the same OSNs (Fig. 4; Lebreton et al., 2017). Remarkably, males distinguish between Z4-11Al and (*R*)-linalool during courtship, while both compounds elicit innate flight attraction (Fig. 2a, d, 4; Lebreton et al., 2017).

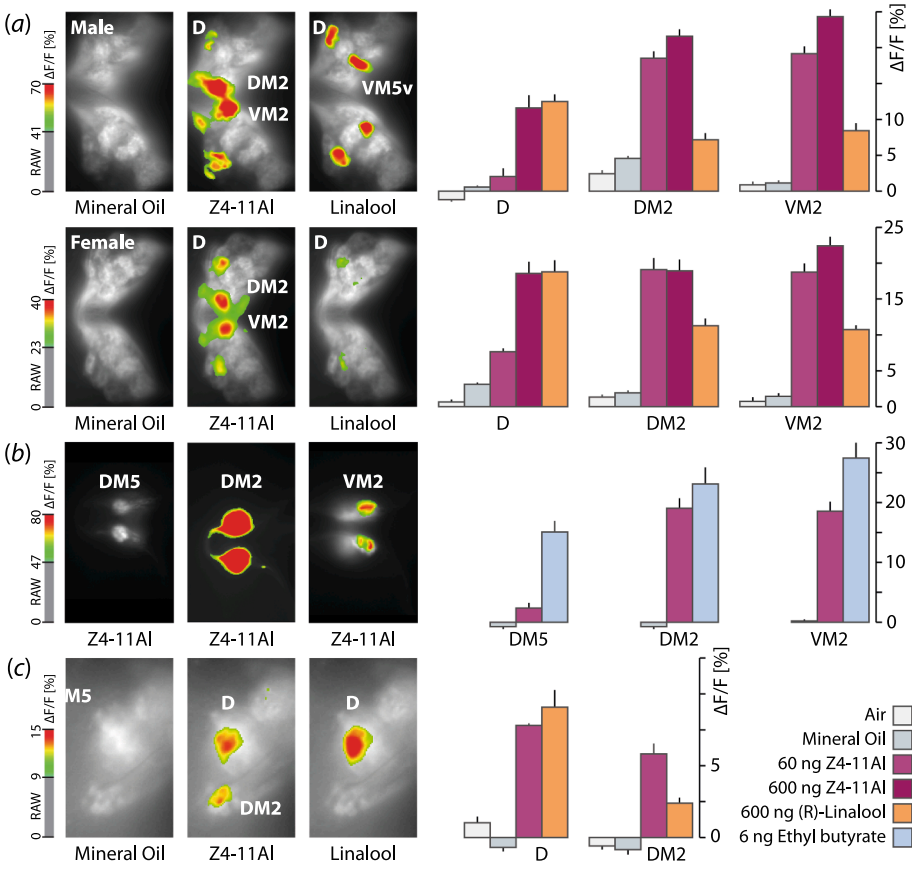

Fig. 3. Calcium imaging responses in the antennal lobe (AL). Colours show the median normalized calcium activity (Δ F/F [%]) in response to controls and odor applications, according to the colour bar on the left ($n = 10$; mean ± SD). (a) Calcium activity in Orco-Gal4 males (top) and females (below) in response to control (air, mineral oil), to 60 and 600 ng *Z*4-11Al, and to 600 ng (*R*)-linalool. *Z*4-11Al and (*R*)-linalool are key ligands for the D glomerulus (Lebreton et al. 2017). (b) Responses of Or22a-Gal4 males to *Z*4-11Al 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 *Z*4-11Al and (*R*)-linalool in D and DM2 glomeruli.

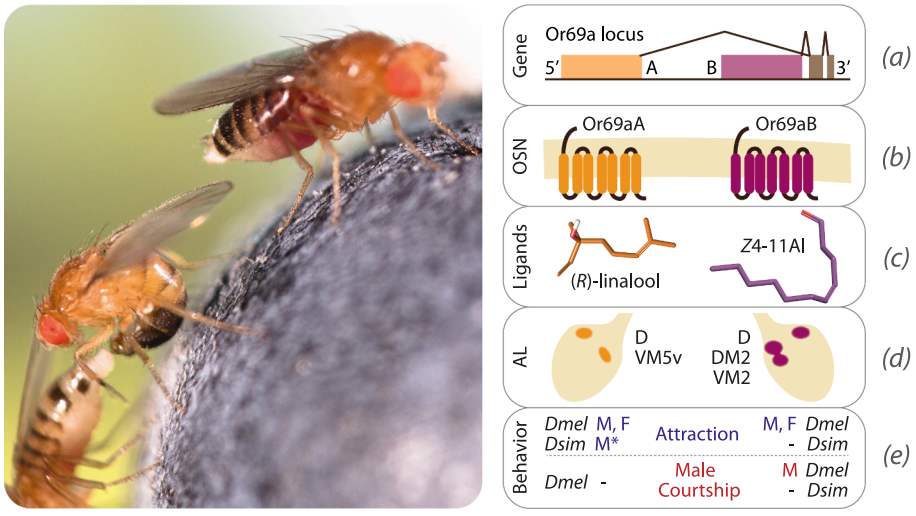

Fig. 4. Schematic of the sensory and behavioral physiology of the Or69a olfactory channel in *D. melanogaster*. (a) Alternative splicing of Or69a generates (b) the transcript variants Or69aA and Or69aB (Robertson et al., 2003), which are co-expressed in OSNs in ab9A antennal sensilla (Couto et al., 2005; Lebreton et al., 2017). (c) (*R*)-linalool and Z4-11Al are main ligands of Or69aA and Or69aB, respectively (Lebreton et al., 2017), (d) and elicit distinct response patterns in the AL (Fig. 3). (e) (*R*)-linalool elicits upwind flight attraction in *D. melanogaster* (*Dmel*) males and females, as well as the sibling species *D. simulans* (*Dsim*; only males have been tested). In comparison, Z4-11Al attracts only conspecific males and females (Lebreton et al., 2017) and stimulates courtship in experienced *D. melanogaster* males (Fig. 2).

A differential courtship response to Z4-11Al and (*R*)-linalool is reflected by their respective activation patterns in the AL (Fig. 3a). (*R*)-linalool, which is also found in yeast headspace (Ljunggren et al., 2019) is a ligand for Or98a, which accounts for stimulation of the VM5v glomerulus (Couto et al., 2005; Münch and Galizia, 2016). In contrast, direct activation of DM2 and VM2 by Z4-11Al is not supported by SSR data (Lebreton et al., 2017) and lateral activation by local AL neurons is a possible explanation, instead (Wilson, 2013). DM2 and VM2 respond broadly, also to blends of vinegar and cVA (Lebreton et al., 2015).

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

4.4. *Or69a is outside the fruitless neuronal circuitry*

Male sexual behaviour in *D. melanogaster* is regulated by the *fruitless* (fru) gene, that encodes male-specific proteins (FruM), in sensory

neurons dedicated to signals from conspecific females, as well as in motor neurons generating courtship (Manoli et al., 2005; Stockinger et al., 2005; Peng et al. 2021). Peripheral and first-order neurons that respond to *Z*4-11Al do not express FruM, and are accordingly not part of the sexually dimorphic fruitless circuitry that elicits innate male courtship (Manoli et al., 2005; Stockinger et al., 2005). This aligns with the observation that naive males do not respond significantly to Z4-11Al in the courtship assay and that both sexes are attracted by flight (Fig. 1c, 2a, 4).

Mutant males that lack FruM nonetheless learn to court females. This capacity to acquire courtship through adult experience is determined by male-specific proteins encoded by the *doublesex* (dsx) gene (Pan and Baker, 2014). However, dsx-expressing neurons are absent from brain regions involved in olfactory processing, namely the AL, LH and mushroom body (Robinett et al., 2010)

An apparent role of Or69a in mate communication, and a differential behavioural response to Z4-11Al and (*R*)-linalool during courtship invites the question whether olfactory input via Or69a is indeed entirely disconnected from fru- or dsx-positive neuronal circuitry, downstream from the AL. In this context, several other, fru-negative glomeruli in the AL show sex-specific size differences, while it is yet unclear whether they interconnect with sex-specific circuitry (Grabe et al. 2016).

4.5. Does *Z*4-11Al deliver excitatory olfactory input to the male P1 node?

Male courtship in *D. melanogaster* has been anatomically and physiologically dissected at a neuronal circuit level, with a particular emphasis on the sex-specific behavioural effect of cVA. A central node is the sexually dimorphic P1 interneuron cluster that integrates input from all sensory modalities to activate male courtship, and that is regulated by social experience. Male-produced pheromone cVA delivers antagonistic olfactory input to the male fru-positive P1 node, and prevents courtship with freshly mated females that are still perfumed with male cVA (Manoli et al., 2005; Stockinger et al., 2005; Kimura et al., 2008; Ruta et al., 2010; Kohl et al., 2013, Yamamoto and Koganezawa, 2013; Clowney et al., 2015; Kohl et al., 2015; Auer and Benton, 2016).

cVA does not elicit long-range flight by itself, only in combination with fly food (Fig. 2; Bartelt et al., 1985; Lebreton et al., 2015) and is shared by many other *Drosophila*, including the sibling species *D. simulans* (Schaner et al., 1987; El-Sayed, 2021). *D. melanogaster* females, on the other hand, produce the species-specific CHC 7,11-HD that feeds gustatory input into P1 to activate male courtship and to achieve reproductive isolation towards the sibling species *D. simulans* (Billeter et al., 2009; Thistle et al., 2012; Toda et al., 2012; Seeholzer et al., 2018). What is yet unclear is whether P1 also receives excitatory olfactory input.

Does Z4-11Al feed into P1? Z4-11Al encodes species-specificity, mediates long-range attraction (Fig. 1; Lebreton et al., 2017) and contributes to courtship, independently of its biosynthetic precursor 7,11-HD. Males responded to decapitated *D. simulans* females laced with *Z*4-11Al (Fig. 2a,b), although *D. simulans* does not produce 7,11-HD (Jallon 1984; Billeter et al., 2009; Lebreton et al., 2017; Billeter and Wolfner, 2018; Sato and Yamamoto, 2020). Mated *D. melanogaster* males have been shown to outcompete naive males during courtship (Saleem et al., 2014), and a participation of Or69a in courtship competition is a testable hypothesis.

Z4-11Al is perceived during upwind flight, and since females release Z4-11Al, it follows that stimulation is sustained at close-range. Courtship and upwind flight are not isolated episodes, they are part of a behavioural sequence that culminates in copulation.

4.6. A behavioural paradigm for bioactive odorant identification

An unambigious behavioural response is substrate for investigations of the genetics, neural circuitry and physiology of mate communication in *D. melanogaster*. We here show that upwind flight attraction is a behavioural paradigm that is suitable to extend investigations of reproductive behaviour to include long-range communication, which includes decision-making upon odorant stimulation prior to flight activation.

We used fed flies for the pheromone attraction assay to exclude a mere food attraction response to vinegar or yeast (Becher et al., 2010; Lebreton et al., 2012). Feeding and courtship promoting neurons, TyR and P1, are antagonistically modulated according to starvation state (Cheriyamkunnel et al., 2021).

Females and males are attracted, in an inverse ratio, to blends of vinegar with male or female pheromone, cVA and Z4-11Al, while equally many males and females respond to blends of these pheromones with yeast (Fig. 1).

Vinegar is a widely used standard food attractant for *D. melanogaster*, although live yeast aroma is much richer in composition (Callejón et al., 2009; Chinnici et al., 2009; Becher et al., 2010; Ljunggren et al., 2019). Fermenting yeast signals suitable substrates for larval development and attracts flies for oviposition (Becher et al., 2012; Grangeteau et al., 2018; Quan and Eisen, 2018; Murgier et al., 2019). Vinegar, derived from acetic acid bacterial fermentation (Lynch et al., 2019) is the end point of fruit fermentation, but contains attractant volatiles that synergize flight attraction to acetic acid (Becher et al., 2010) and override acetic acid avoidance at close range (Ai et al., 2010). Since vinegar is a suboptimal attractant, compared with yeast, it is more suitable to accentuate behavioural differences between male and female pheromone.

Taken together, reproductive behaviour in *D. melanogaster* is mediated by an ensemble of food odorants and pheromones emitted by aggregating or mating flies. Our knowledge of *D. melanogaster* pheromone chemistry is fairly comprehensive, in contrast to the extraordinary complexity of food headspace. Differential interaction of male and female pheromones with food odorants accentuates the question which food volatiles mediate specific attraction responses.

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.

Another open question is the species-specificity of sex attraction via Or69a, and which volatile female pheromones are used by closely related species. For example, Females of an African strain of *D. melanogaster* release a blend of Z4-9Al and Z4-11Al (Frey et al., 2021), where it is yet unclear whether this blend is behaviourally different from Z4-11Al alone. In *D. simulans*, Z4-11Al has an antagonistic effect (Lebreton et al., 2017). An upwind flight bioassay is suitable to describe the key bioactive chemicals that encode reliable recognition and evaluation of conspecific mates and food odour objects and trigger the decision to engage in orientation flights.

5. Data availability

https://datadryad.org/stash/dataset/doi:10.5061/dryad.5mkkwh752.

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Competing interests

No competing interests declared.

CRediT authorship contribution statement

Felipe Borrero-Echeverry: Methodology, Software, Formal analysis, Investigation, Writing – review & editing. **Marit Solum:** Methodology, Formal analysis, Investigation. **Federica Trona:** Methodology, Formal analysis, Investigation. **Paul G. Becher:** Methodology. **Erika A. Wallin:** Methodology, Investigation. **Marie Bengtsson:** Methodology, Resources. **Peter Witzgall:** Methodology, Conceptualization, Validation, Writing – original draft, Visualization, Project administration, Funding acquisition. **Sebastien Lebreton:** Methodology, Conceptualization, Formal analysis, Writing – review & editing.

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