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Abstract	In insects such as *Drosophila melanogaster*, flight guidance is based on converging sensory information provided by several modalities, including chemoperception. Drosophila flies are particularly attracted by complex odors constituting volatile molecules from yeast, pheromones and microbe-metabolized food. Based on a recent study revealing that adult male courtship behavior can be affected by early preimaginal exposure to maternally transmitted egg factors, we wondered whether a similar exposure could affect free-flight odor tracking in flies of both sexes. Our main experiment consisted of testing flies differently conditioned during preimaginal development in a wind tunnel. Each fly was presented with a dual choice of food labeled by groups of each sex of *D. melanogaster* or *D. simulans* flies. The combined effect of food with the *cis*-vaccenyl acetate pheromone (*c*VA), which is involved in aggregation behavior, was also measured. Moreover, we used the headspace method to determine the "odorant" identity of the different labeled foods tested. We also measured the antennal electrophysiological response to *c*VA in females and males resulting from the different preimaginal conditioning procedures. Our data indicate that flies differentially modulated their flight response (take off, flight duration, food landing and preference) according to sex, conditioning and food choice. Our headspace analysis revealed that many food-derived volatile molecules diverged between sexes and species. Antennal responses to *c*VA showed clear sex-specific variation for conditioned flies but not for control flies. In summary, our study indicates that preimaginal conditioning can affect Drosophila free flight behavior in a sex-specific manner.

Keywords (separated by '-')	*Cis*-Vaccenyl acetate - Microbiota - Preimaginal conditioning
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Abstract

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Keywords *Cis*-Vaccenyl acetate · Microbiota · Preimaginal conditioning

Introduction

Flying allows insects to escape from predators, to predate on other animals (Baines et al. 2014; Dickinson 2014; Misof et al. 2014) and to disperse and find new food sources and/or potential mates. In *Drosophila melanogaster*, flight guidance is based upon converging information from several sensory modalities (proprioception, vision, mechanoperception, hygroperception and chemoperception (Bhandawat et al. 2010; Budick and Dickinson 2006; Budick et al. 2007; Duistermars et al. 2009). When they are at a relatively long distance from an odor source, flying Drosophila flies use the mechanosensory system to estimate wind velocity and olfaction to orient through the odor gradient (Budick et al. 2007; Dahake et al. 2018; Duistermars et al. 2009; Krishnan and Sane 2014). When they arrive near the odor source, they use visual and chemical signals to land on this source (Bhandawat et al. 2007; Budick and Dickinson 2006; Saxena et al. 2018). To detect volatile chemical cues, *D. melanogaster* flies use sensory hairs (sensilla) covering the antennae, the maxillary palps (Stocker 1994)) and the wings (Houot et al. 2017; Raad et al. 2016), whose signal influx is sent to specific (and/or sex-specific) brain centers, which in turn trigger adapted behaviors according to sex and mating status (Couto et al. 2005; Das et al. 2017; Datta et al. 2008; Fishilevich et al. 2005; Ruta et al. 2010).

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D. melanogaster adults mainly use three pheromone classes. First, sex-specific cuticular hydrocarbons (CHCs), mostly detected by gustation but also by close range olfaction, can either stimulate or inhibit courtship behavior (Everaerts et al. 2010a, b; Farine et al. 2012; Ferveur and Sureau 1996; Jallon 1984). Second, several volatile compounds derived either from 7,11-heptacosadiene, the principal female CHC (Z4-11Al aldehyde; Lebreton, 2017 #3307) or from male 7-tricosene CHC (methyl-laurate, methyl-myristate and methyl-palmitate; Dweck et al. 2015) can change the behavior of males and females at some distance. Third, 11-*cis*-vaccenyl acetate (*c*VA) (Butterworth 1969; Guiraudie-Capraz et al. 2007), a volatile lipid-derived substance produced in the ejaculatory bulb of several *Drosophila* species, can be detected at a relatively long distance (Bartelt et al. 1985b; Hedlund et al. 1996; Jaenike et al. 1992; Schaner et al. 1987, 1989a, b; Symonds and Wertheim 2005). cVA is transferred from the male into the female genital apparatus during copulation and subsequently deposited on eggs laid a few days after copulation (Everaerts et al. 2018). When combined with other infochemicals, *c*VA can modulate several Drosophila subsocial behaviors. At a close distance, *c*VA combined with male-specific CHCs inhibits male−male courtship, stimulates female sexual receptivity and induces male−male aggression (Bartelt et al. 1985a; Butterworth 1969; Das et al. 2017; Ejima 2015; Fernandez and Kravitz 2013; Guiraudie-Capraz et al. 2007; Jallon et al. 1981; Kurtovic et al. 2007; Laturney and Billeter 2016; Lebreton et al. 2015; Schaner et al. 1987; Wang et al. 2011; Wertheim et al. 2005; Zawistowski and Richmond 1986). At a longer distance, *c*VA associated with food volatile metabolites resulting from the activity of gut-associated bacteria (Keesey et al. 2016) is often deposited in frass and can enhance fly aggregation on food sources (Bartelt et al. 1985b; Das et al. 2017; Duménil et al. 2016; Lebreton et al. 2012). Recently, Cazalé-Débat et al. (2019) described the long-range effect on *D. melanogaster* free flight of *c*VA combined with CHCs and food-derived chemicals. This study (performed in a wind tunnel) showed that *c*VA and sex-specific CHCs interact with food volatile chemicals to induce sex-specific flight responses.

For a long time, responses to *c*VA were considered to be stereotypic and unconditional. Recently, some of us discovered that early preimaginal exposure to maternally transmitted substances—cVA likely associated with microbes—induced partial suppression of male courtship inhibition to *c*VA (Everaerts et al. 2018). Here, we tested Drosophila female and male free flight responses to a dual choice of food labeled by flies of various genotypes with or without *c*VA. Focal flies were differently exposed during their early preimaginal development by maternally transmitted substances. Using headspace, we determined the identity of the volatile substances emitted by the various fly-labeled food types. Moreover, we measured the antennal electrophysiological response to *c*VA of flies resulting from different preimaginal conditionings.

Materials and Methods

Drosophila Strains and Rearing

We used a *D. melanogaster* wild-type strain, Canton-S (CS), and a *Drosophila simulans* wild-type strain (line #K509, a gift from Prof. Daisuke Yamamoto). Flies were raised on yeast/cornmeal/agar medium [for 1 L of food: 50 g of yeast, 66 g of maize flour, 9 g of agar and 30 ml of Tegosept (®Apex) completed with distilled water] and kept under a 12:12 h light/dark cycle (artificial day from 8:00 am to 8:00 pm) at 24 ± 0.5 °C with 65 ± 5% humidity. All flies resulted from mass-rearing stocks transferred every 2–3 days to avoid competition and regularly provide progeny. Flies were screened 2 to 6 h after emergence under light CO_2 anesthesia. and kept at 24 ± 0.5 °C The flies were kept in same-sex groups (20 flies) for food labeling. Focal female flies tested in the wind tunnel experiment or used for chemical and electrophysiological analysis were also kept in groups (20 flies), whereas focal males were isolated to prevent social interactions potentially affecting behavior (Svetec and Ferveur 2005).

Egg Collection and Treatment (Fig. 1): Focal flies resulted from eggs laid by Cs females (i) less than 24 h after mating (D1) or at least 5 days after mating (D5). More precisely, one hour after artificial dawn, 30 males and 10 females, all 4-day-old Cs flies, were placed in a 30 ml glass vial containing 4 ml fresh plain food. After 3 h, they were cold-anesthetized (15 min at 4 °C). Then, males were discarded, and females were transferred into egg-laying devices (50 mm Petri dish filled with 1 ml 3% agar striped with fresh yeast to stimulate egg laying). Females were removed after three hours, and their eggs were collected (D1). To obtain D5 eggs, mated females (without males) were placed in rearing tubes for 4 days and then transferred into egg-laying devices before being discarded three hours later. We also tested flies resulting from D5 eggs deposited on food enriched with synthetic *c*VA (15 ng/mm^3 according to Everaerts et al. 2018; D5 + *c*VA).

As a reference for comparison and to check our device, we used, as focal flies, virgin Cs males and females randomly sampled from mass-rearing stocks 2 to 6 h after emergence screened and kept in similar conditions as those described for D1- and D5-derived flies.

Food Labeling

To investigate the effect of the molecules potentially involved in free flight odor tracking and landing preference,

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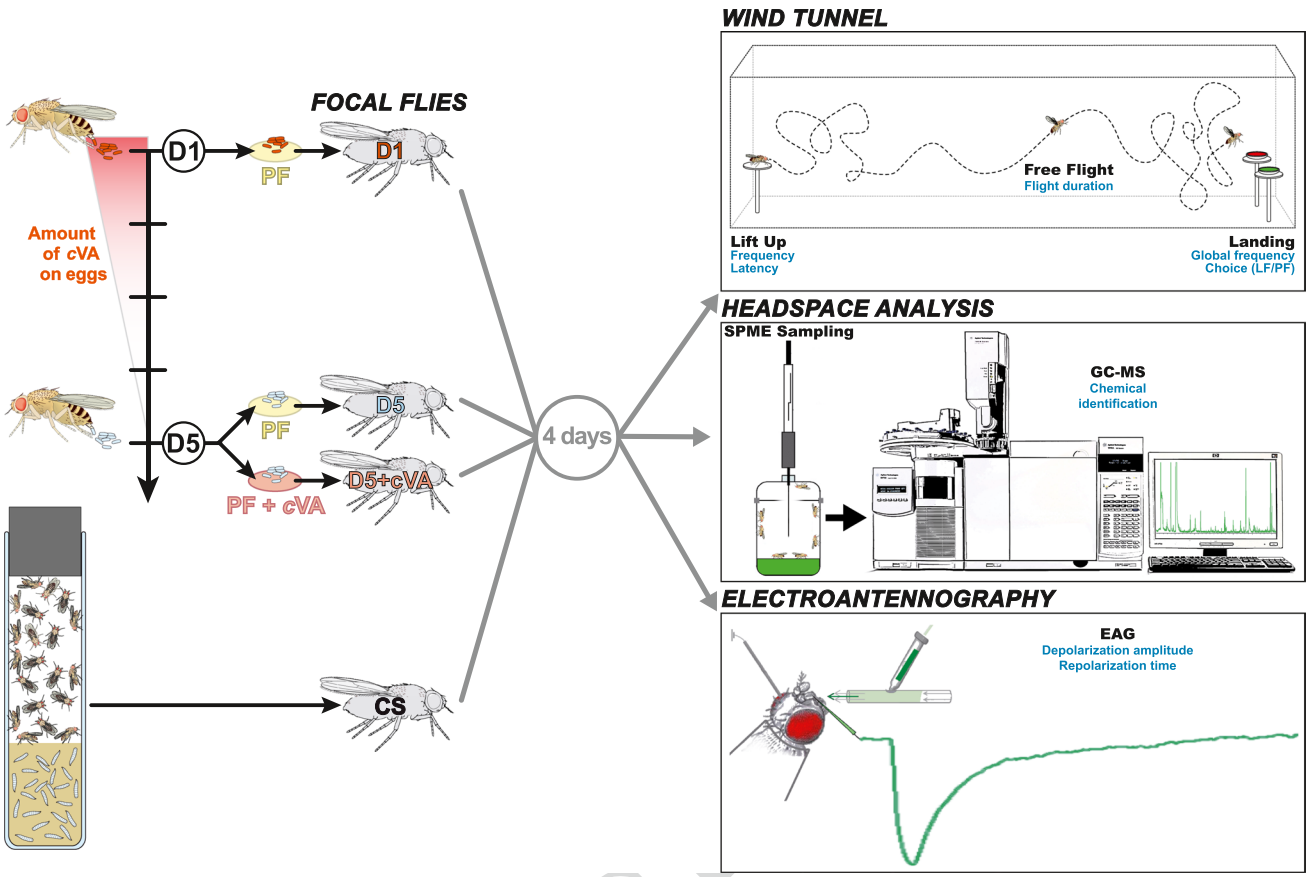

Fig. 1 Egg collection and treatment to obtain focal flies and experiments conducted to evaluate the effect of precocious *c*VA exposure on free-flight odor tracking in Drosophila

we labeled fresh plain laboratory food with live flies. To label food, 100 "labeling" flies were kept for 15 h in a petri dish (∅ = 5.4 cm) filled with 20 g fresh food and covered by a plastic lid (h = 6.4 cm) under similar experimental conditions as described above. "Labeling flies" were removed 24 h before the flight experiment. The food was either labeled by (*i and ii*) *D. melanogaster* virgin females or males, (*iii and iv*) *D. simulans* virgin females or males, or (*v*) *D. melanogaster* virgin females and enriched with synthetic *c*VA. For *c*VA labeling, 100 ng *c*VA (® Cayman Chemical, Ann Arbor, MI, USA; 50 mg/ml solution in ethanol; purity > 98%) diluted in 5 µl hexane was added to a Whatman filter paper patch (∅ = 1 cm, ® GE Healthcare Life Sciences), which was deposited on fly-labeled food a few minutes prior to each test (according to Cazalé-Débat et al. 2019). We used plain laboratory food as control food.

Wind Tunnel

The design of the wind tunnel was previously described in detail (Cazalé-Débat et al. 2019; Fry et al. 2008; Houot et al. 2017, 2018). The tunnel was made of clear acrylic (length = 155 cm; width and height = 30.5 cm) and was illuminated by four band strips of white LEDs (BDL- F300 W-05–3528, Boulevard des LEDs, France; length = 1 m) located below the tunnel base and separated with a red screen. Tracing paper was placed over the tunnel to homogenize the light intensity inside the flying section, and the two lateral panels of the tunnel were covered with a randomized pattern consisting of black and white squares (side = 3 cm). A "departure/starting" platform (height = 16 cm) was placed in the downwind section at 90 cm from the two landing platforms (height = 16 cm, ∅ = 1.7 cm) located in the upwind section. The two landing platforms — with a food source on top of each — were placed 10 cm from each lateral panel and were separated from each other by 7.5 cm. For each behavioral test, approximately 1 cm^3 of food was deposited on a microscope slide at the top of each platform. A humidifier (® OKOIA, AH400; Tianjin, China) was placed at the entrance of the airflow to maintain a constant humidity (65–75%) in the flying section. A laminar airflow (0.4 ms^{-1}) was running through the section. After each session of tests (performed

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between 9:00 am and 3:00 pm), the wind tunnel was washed with a 70% ethanol solution, and the room was ventilated until the next day. The temperature and relative humidity of the room were 25 ± 1 °C and 60 ± 5%, respectively.

We measured several flight parameters and landing preference in binary food choice assays. Four-day-old subject flies were individually introduced with a mouth aspirator into an acclimation chamber (consisting of an acrylic tube; ∅ = 5 mm) separated by a gate from the inside of the wind tunnel. After 3 min of acclimation, subjects were allowed to reach the part of the tube opening inside the wind tunnel. Once the fly reached the lift off platform, we successively noted (*i*) its latency (and frequency) for taking upwind flight; (*ii*) its "time duration to reach food" (between upwind flight latency and landing latency); and, (*iii*) in case of landing, the food source chosen (food choice preference). "Landing on food frequency" corresponds to the sum of landing frequencies on the two food sources. Each experiment lasted a maximum of 10 min (or less if the fly landed on a food source before 10 min).

We tested several dual food choice combinations consisting of (*i*) two plain food sources as a double control (PF/PF), (*ii* and *iii*) PF combined with food labeled either by Cs female (PF/FCs) or by Cs male flies (PF/MCs), (*iv*) FCs enriched or not with synthetic *c*VA (FCs/FCs + cVA), (*v*) FCs/MCs, and (*vi*) food labeled by *D. simulans* females and by *D. simulans* males (FSim/MSim). In these experiments, we tested 31–130 individual flies.

Tunnel experiments were conducted with starving flies to stimulate upwind flight attraction (Lebreton et al. 2012). Briefly, the night before the test, flies were individually kept at 25 °C in a glass vial containing only a piece of cotton wool moistened with 90 µL of distilled water.

Identification of Volatile Compounds by HS–SPME–GC−MS

To analyze volatile chemicals produced by the different food sources tested in the wind tunnel, we used headspace-solid phase microextraction-GC-mass spectrometry (HS–SPME–GC−MS).

Samplings were performed with 9 different odor sources: plain food, *D. melanogaster* females and males (without or with food), and *D. simulans* females and males (without or with food; for each sampling type: $3 \leq n \leq 4$).

The media to be analyzed were prepared 15 h before sampling. Depending on the case, 5 g of plain food (cooked 3 days before and stored at 4 °C as regular laboratory medium) was kept plain or was labeled by 20 four-day-old flies. Vials covered with a cotton mesh (to avoid excessive humidity) were maintained at room temperature. Before sampling, flies were discarded, and the mesh cap was replaced by a Teflon septum. These vials were placed at 26 °C for 1 h. Then, a triphasic SPME fiber (30 µm layers CAR-PDMS—50 µm layer DVB; SUPELCO), previously conditioned for 15 min in a GC injector set at 240 °C, was introduced into the vial through the septum and exposed for 10 min to the vapor phase inside the headspace.

To identify chemicals present in the headspace after odorant uptake analysis, we used an HP6890 GC coupled to an MSD 5973 N selective detector (Agilent Technologies operated in electron ionization mode at 70 eV). The HP6890 GC was fitted with an SPME injection port (splitless mode) set at 240 °C and with a DB-Wax capillary column (length 30 m; ID 0.25 mm; film thickness 0.050 µm; Agilent® J&W). The GC oven temperature was maintained at 40 °C for 5 min, raised to 240 °C at 3 °C/min and maintained for 10 min at this temperature. Helium was used as the carrier gas at a linear velocity of 44 cm/s. The SPME fiber was introduced into the injector of the GC and desorbed for a 15 min period. The MSD 5973 N mass spectrometer scanned the ion mass fragments *(m/z)* from 29 to 350. The ion source was set at 230 °C, and the transfer line was set at 250 °C.

Chromatograms were analyzed with MSD-ChemStation software (Agilent Technologies).

Identification of the volatile compounds was carried out by comparison of their mass spectra with those of Wiley (Wiley Registry 2020) and Inramass libraries (personal database). We did not take into account chemicals with m/z features distinctive of polydimethylsiloxane (PDMS; m/z = 73, 147, 207, 221, 281), which are contaminants derived from the silica column.

EAG Assays

Electrophysiological antennal responses of Cs, D1, D5 and D5 + *c*VA four-day-old virgin females and males stimulated by various *c*VA doses were measured using electroantennography (EAG).

Living 3- to 7-day-old flies were secured in an Eppendorf 200 µl cone, leaving the eyes and antennae exposed. EAGs were recorded with two glass capillary electrodes (tip diameter 2.8 µm, filled with 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$, and 10 mM HEPES buffer). The reference electrode was inserted in the left eye, and the recording electrode was leaned against the distal part of the right third antennal segment without being inserted. The signal was amplified (total gain × 5), low-pass filtered (0.5 kHz) with an AxoPatch 2008 (Molecular Devices, Union City, CA, USA) and digitized at 1 kHz (Digidata 1440A; Molecular devices) with Axoscope® (Axon™pCLAMP™ 11.1, Molecular devices) and Clampfit® (Molecular devices) software.

Odor Delivery System A 5-mm Teflon tube held 10 mm from the insect antenna continuously delivered a humidified air

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stream (Pump Wisa; 1 L/min; using a bubbler with reverse osmosis water and fitted with a charcoal filter). Stimuli were applied by inserting a Pasteur pipette 15 cm containing a small piece of filter paper (Whatman; 20×3 mm^3) loaded with 10 µl of the odorant diluted in paraffin oil into the Teflon tube. An air puff (200 ms, 1 PSI) was delivered through the pipette with an electrovalve (Kendrion Kuhnke Micro solenoid valve, 64.060) controlled by a digital output module (PDES-02DX, NPI Electronics).

Odorants were presented every min in a fixed sequential pattern: (*i*) hexan-1-ol (Sigma−Aldrich, 10^{-1} M) and heptan-2-one (Sigma−Aldrich, 10^{-1} M) diluted in paraffin oil, (*ii*) pure paraffin oil, (*iii*) pure hexane (99%, Sigma−Aldrich), (*iv*) increasing *c*VA dose (1, 100, 300 and 500 µg in hexane), and (*v*) hexan-1-ol. Initial stimulations with hexan-1-ol and heptan-2-one allowed us to check the electrical connection to obtain an obvious antennal signal response (Chertemps et al. 2012), while the final hexan-1-ol stimulation allowed us to check the stability of the fly physiological state. Liquid paraffin and pure hexane were set up as blank controls. Each compound series was tested in 15 flies.

Both the maximum depolarization amplitude (DA) elicited by a volatile stimulus and the repolarization time (RT) duration were measured and compared between groups of flies. Although depolarization and repolarization times were shown to vary between species, depolarization amplitude and repolarization time showed a strong intraspecific correlation (Bau et al. 2002). According to this study, faster recovery rates allowed for a better resolution of odor mixtures. As it was shown that in the fall armyworm, *Spodoptera frugiperda,* amplitude and repolarization to its pheromone can be unlinked by inhibitors of antennal serine esterases (Luis et al. 2010), we tested whether such an effect could occur in unconditioned flies.

All electrophysiological recordings were performed from 9 am to 1 PM at 24 ± 0.5 °C with 65 ± 5% humidity.

Statistics

Behavioral frequencies (upwind flight and landing) were compared using the Wilks G^2 likelihood ratio test completed with a computation of significance by cell (Fisher's exact test). While the choice between the two food sources was tested using the z test, these choices were compared using the Wilks G^2 test as described above.

Headspace results were analyzed using principal component analysis (PCA; Pearson's correlation matrix type; with standardized values) with the amount of chemical used as variables and the type of fly (sex and species) used as individuals. PCA and ANOVA were used to analyze EAG results with amplitude of depolarization and time of repolarization as qualitative variables and the treatment (CS, D1, D5 and D5 + *c*VA) as quantitative variables.

All statistical analyses were performed using XLSTAT Premium 2021.5.1.1220 (Addinsoft 2021).

Results

Free Flight in a Wind Tunnel

To determine the effect of early exposure to *c*VA and other maternally transmitted factors, we measured free flight orientation in individual female and male flies tested for a dual food choice in a wind tunnel. In addition to control Cs flies, we tested flies resulting from (*i*) eggs laid less than 24 h after copulation (D1), (*ii*) eggs laid 5 days after copulation (D5) and (*iii*) D5 eggs raised in food enriched with *c*VA (D5 + cVA).

We measured the frequencies of flies (Figs. 2A and 3A) taking upwind flight, (Figs. 2B and 3B) landing on food, and (Figs. 2 and 3C) landing on each food type (food choice) in females and males. We also measured the latency to take upwind flight and the flight duration between the starting platform and landing on the food (Suppl. Figure 1). The two latter parameters are either shown for all individuals (A and C for females; E and G for males) or according to their choice to land on each food type (B and D for females; F and H for males). All parameters were determined relative to the total number of flies tested. In addition to plain food (PF), the different types of food consisted of food labeled by Cs males (MCs), Cs females (FCs), Cs females and enriched with *c*VA (FCs + *c*VA), *D. simulans* females (FSim) or *D. simulans* males (MSim).

In the PF/PF control choice assay (consisting of two similar PF sources), 56–75% of females and 53–69% of males took upwind flight, while 32–47% and 39–51%, respectively, landed on food without showing preference. Their median upwind flight latencies were 80–150 s and 91–163 s, while their median flight durations lasted 14–42 s and 32–56 s, respectively. Cs females showed a shorter flight duration than D5 + *c*VA females.

In the MCs/PF choice assay, 68–84% of flies took upwind flight, while 30–76% landed on food. However, D1 females and males landed on food with a significantly lower frequency (30%) compared to flies of the three other treatments. D1 flies, Cs males and D5 males clearly preferred landing on MCs food than on PF. Both the latency of upwind flight and the flight duration of these flies were generally similar to those found in the PF/PF assay. Only Cs males showed a delayed upwind flight latency compared to the three other treatments.

In the FCs/PF assay, 63–80% of flies took upwind flight with a median latency of 146–173 s in females, while this

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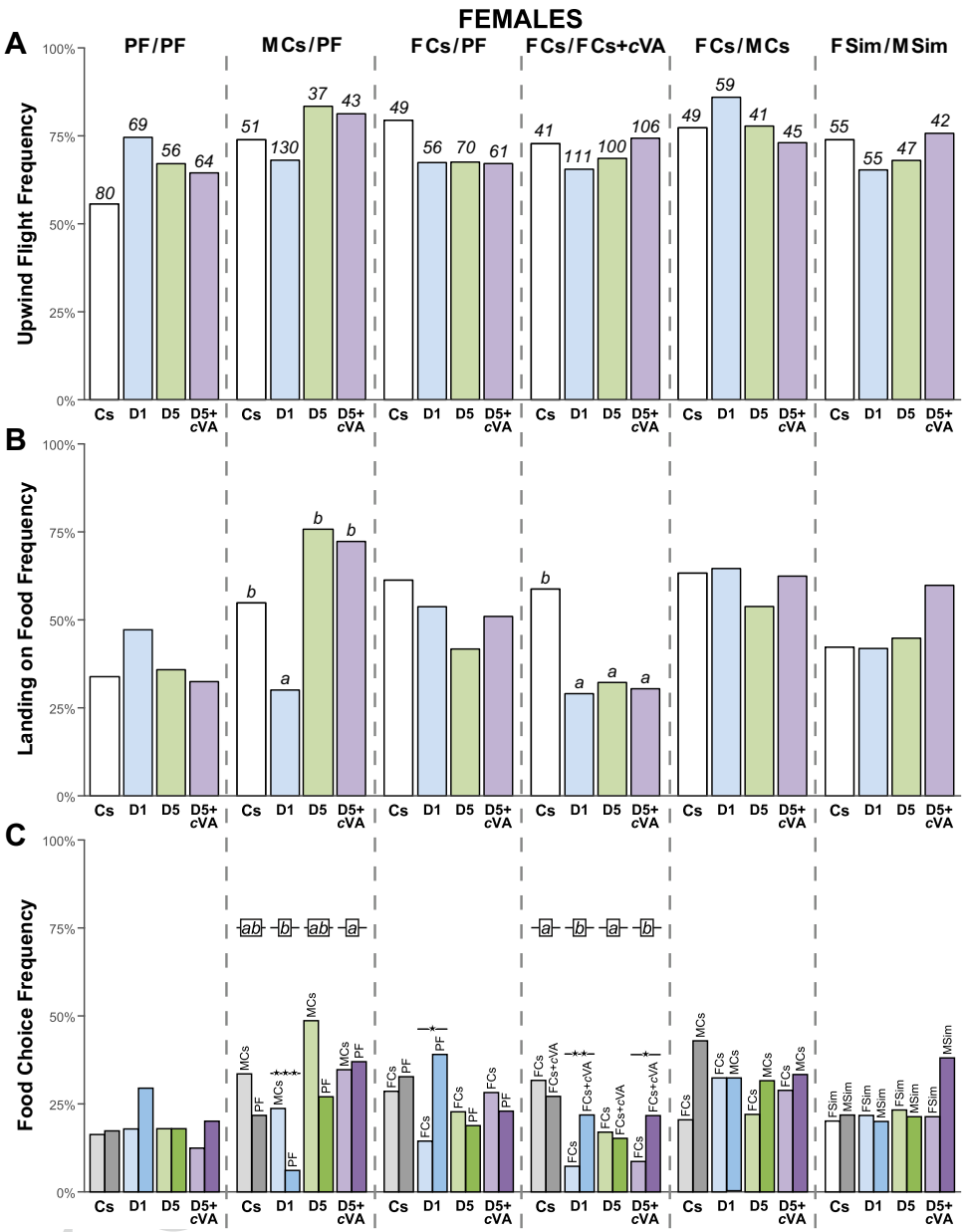

was more variable in males (79–187 s), with a significant difference between Cs and D5 males. A slight difference in flight duration was found between D1 and D5 + *c*VA females. No difference in landing frequency on food was noted. No food preference was noted except in D1 females, which landed more frequently on PF than on FCs food.

In the choice assay involving food labeled by Cs females without or with *c*VA (FCs/FCs + *c*VA), 61–75% flies took upwind flight. Upwind flight latency was either similar between males (29–64 s) or longer in Cs females (198 s) compared to the three other females (12–30 s). D1, D5 and D5 + *c*VA female and male flies showed a strongly decreased landing frequency (28–34%) compared to Cs flies (59%). Additionally, D1 and D5 + *c*VA females preferred landing on FCs + *c*VA food than on FCs food, whereas males showed no preference.

In the FCs/MCs assay, flies showed relatively high upwind flight (73–86%) and landing frequencies (54–69%). While their upwind flight latency was approximately 100 s, their flight duration was often very brief (10–60 s). The flies showed no food preference except D5 + *c*VA males, which preferred landing on MCs food over FCs food.

In the choice assay performed with *D. simulans*-labeled food (FSim/MSim), 66–77% flies took upwind flight, while 42–60% females and 41–69% males landed on food. D1 males landed significantly less often on food than D5 and D5 + *c*VA males. Female and male flies showed a very brief flight duration (12–35 s and 24–32 s, respectively).

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◂**Fig. 2** Flight and landing preference in single female flies tested for food labeled by flies of various genotypes. The histograms represent (top row) the frequency of female flies taking upwind flight (calculated from the total number of flies tested: see top of each histogram bar), (medium row) the overall landing frequency (calculated from all individuals), and (bottom row) the landing preferences on a dual food choice. At the top of each histogram group (*delineated by dashed lines*), the dual food choices tested are indicated (*from left to right*): "plain food/plain food" (PF/PF), "food labeled by Cs males/plain food" (MCs/PF), "food labeled by Cs females/plain food" (FCs/PF), "food labeled by Cs females/food labeled by Cs females and enriched with synthetic *c*VA" (FCs/FCs + *c*VA), "food labeled by Cs females/food labeled by Cs males" (FCs/MCs) and "food labeled by *D. simulans* females/food labeled *D. simulans* males" (FSim/MSim). For each dual food choice, we compared Cs flies resulting from different preimaginal conditioning conditions. We tested (*from left to right*) (*i*) Cs control flies (empty bars or gray bars) to flies resulting from (*ii*) eggs laid less than 24 h after copulation (D1; blue bars), (*iii*) eggs laid at least 5 days after copulation (D5; green bars) and (*iv*) D5 eggs raised in *c*VA-rich food (D5 + *c*VA; purple bars). For food preference, the frequency of flies landing on each food source is represented by twin bars; the bar with lighter color density depicts the food shown on the left side of the dual choice, and the bar with darker color density depicts the food on the right side. For each dual food choice, the differences between upwind flight and between landing frequencies were tested with the Wilks G^2 likelihood ratio test completed with a computation of significance by cell (Fisher's exact test), whereas landing preference was tested with the z test, and the corresponding frequencies were compared between the different LFs using the Wilks G^2 likelihood ratio test. For the two frequency parameters, significant differences (at $\alpha = 0.05$) are indicated by different letters, while the level of significance for food preference is represented (or not) by asterisks (*: $\alpha < 0.05$; **: $\alpha < 0.01$; ***: $\alpha < 0.001$; no star: not significant). (*Upwind flight frequency*: Wilks G^2 likelihood ratio test, PF/PF: $G^2_{(3df)} = 5.95$, $p = 0.114$, MCs/PF: $G^2_{(3df)} = 5.41$, $p = 0.148$, FCs/PF: $G^2_{(3df)} = 2.79$, $p = 0.425$, FCs/FCs + *c*VA: $G^2_{(3df)} = 2.28$, $p = 0.524$, FCs/MCs: $G^2_{(3df)} = 0.07$, $p = 0.811$, Fsim/MSim: $G^2_{(3df)} = 1.90$, $p = 0.598$; *Landing on food frequency:* PF/PF: $G^2_{(3df)} = 0.90$, $p = 0.273$, MCs/PF: $G^2_{(3df)} = 40.51$, $p < 10^{-4}$ FCs/PF: $G^2_{(3df)} = 5.02$, $p = 0.170$, FCs/FCs + *c*VA: $G^2_{(3df)} = 12.47$, $p = 0.006$, FCs/MCs: $G^2_{(3df)} = 1.29$, $p = 0.722$, Fsim/MSim: $G^2_{(3df)} = 0.89$, $p = 0.272$; *Food Choice Frequency*: —for sake of clarity, only the significant values are provided—: MCs/PF- D1: $z = 4.56$, $p = 0.0002$, FCs/PF—D1: $z = 2.89$, $p = 0.004$, FCs/FCs + cVA—D1: $z = 3.27$, $p = 0.0012$, FCs/FCs + cVA—D5 + cVA: $z = 3.71$, $p = 0.0002$, MSim/FSim—Cs: $z = 3.39$, $p = 0.0008$, Sim/FSim—D1: $z = 3.67$, $p = 0.0004$, MSim/FSim—D5: $z = 2.98$, $p = 0.003$; *Food Choice Frequency Differences:* PF/PF: $G^2_{(3df)} = 1.26$, $p = 0.731$, MCs/PF: $G^2_{(3df)} = 7.66$, $p = 0.049$, FCs/PF: $G^2_{(3df)} = 6.69$, $p = 0.083$, FCs/FCs + *c*VA: $G^2_{(3df)} = 9.32$, $p = 0.025$, FCs/MCs: $G^2_{(3df)} = 2.43$, $p = 0.489$, Fsim/MSim: $G^2_{(3df)} = 1.73$, $p = 0.630$). Two other flight parameters (upwind flight latency and time to reach food) are shown in Supplemental Fig. 1

Moreover, Cs females showed a shorter flight than D1 females. Males showed a slight preference (or a tendency) to land on MSim (than on FSim), while focal females showed no food preference.

Headspace Analysis of Compounds Present in Food Sources

To determine the identity of food compounds potentially involved in various aspects of free flight behavior in female and male flies, we performed headspace analysis of most food sources tested in the tunnel (Fig. 4A, B). In particular, we compared the volatile compounds produced by PF, FCs, MCs, FSim and MSim types of food.

PCA revealed a clear separation between the sexes for each species (*D. melanogaster* = Cs; *D. simulans*; Fig. 4C). While FCs completely overlapped with PF and partly overlapped with MSim, the two other food types (MCs, FSim) showed clear segregation. Each MCs and FSim food type was "correlated" with a large number of compounds (Fig. 4D and Table 1). Specifically, MCs-specific compounds correspond to acids (acetichydrazide, acetic, isovaleric, hexanoic, and isobutyric acids), ethanal, acetoin, 6-methyl, 5-hepten-2-one, 2-propanol, pentanol, ethyl-acetate, ethyl-butyrate, ethyl-caprate, ethyl-9-decenoate, ethyl-hexanoate and ethyl-octanoate and to 3 other diverse compounds (5,5-dimethyl-2(5H)-furanone, methoxy-phenyl-oxime and α-caryophyllene).

FSim-specific volatile molecules are heptanoic and propionic acids, aldehydes (butanal, decanal, dodecanal, nonanal, octanal, undecanal), 4-methyl-2-pentanone, alcohols (2-methyl-butanol, 2-pentanol, 2-hexanol, 4-methyl-pentanol, butanol, pentanol and nonanol), and diverse compounds such as dimethyl disulfide, 2-butoxyethanol, ethoxy-ethene, humulen, phenylmethane, 2-butamine and N-methyl-methanamine.

We performed a more extensive PCA to compare the compounds produced by flies on food with those produced by flies without food (Figure Supp 2; Table 1). The results indicated that compounds emitted by flies of the four genotypes (without food) largely overlapped and showed a large divergence with the chemical profiles corresponding to PF and fly-labeled food (FCs, MCs, FSim and MSim).

Electrophysiological Antennal Response to cVA

To determine the involvement of the peripheral olfactory system in the perception of fly-labeled food and, more particularly, of *c*VA, we measured the electrophysiological response of female and male antennae stimulated with a range of *c*VA doses (1–500 µg). We took into account two parameters: the depolarization amplitude (DA) and the repolarization time (RT) duration (Fig. 5). The analysis of variance (one-way ANOVA) revealed that DA showed a dose-dependent response similar in both sexes, with a clear increase induced by 300 µg and 500 µg *c*VA (Fig. 5A and B). DA variation was continuous in D1 males, while it was discontinuous in other conditions. RT showed a very different variability range between the sexes (Fig. 5C and D).

Based on these observations, we plotted the DA and RT data obtained in individual Cs, D1, D5 and D5 + *c*VA females and males. These data reveal several differences according

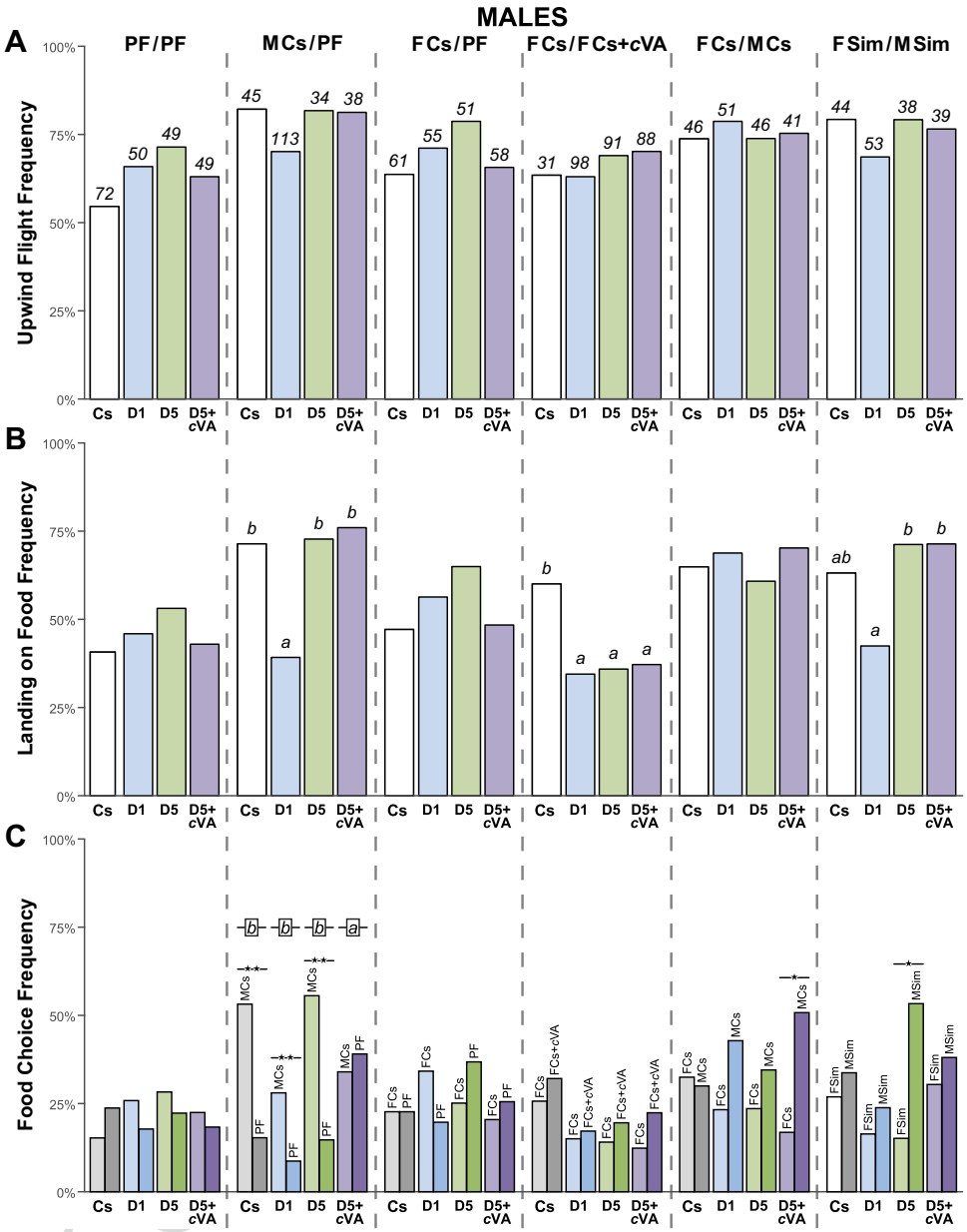

Fig. 3 Flight and landing preference in single male flies tested for food labeled by flies of various genotypes. The histograms represent (**top row**) the frequency of male flies taking upwind flight (calculated from the total number of flies tested: see top of each histogram bar), (**medium row**) the overall landing frequency (calculated from all individuals), and (**bottom row**) the landing preferences on a dual food choice. For parameters and statistics, please refer to the legend of Fig. 1. Two other flight parameters (upwind flight latency and time to reach food) are shown in Supplemental Fig. 1. (*Upwind flight frequency*: Wilks G^2 likelihood ratio test, PF/PF: $G^2_{(3df)}=0.69$, $p=0.297$, MCs/PF: $G^2_{(3df)}=0.87$, $p=0.276$, FCs/PF: $G^2_{(3df)}=0.16$, $p=0.711$, FCs/FCs+cVA: $G^2_{(3df)}=1.52$, $p=0.72$, FCs/MCs: $G^2_{(3df)}=0.82$, $p=0.949$, Fsim/MSim: $G^2_{(3df)}=1.82$, $p=0.611$; *Landing on food frequency:* PF/PF: $G^2_{(3df)}=1.89$, $p=0.596$, MCs/PF: $G^2_{(3df)}=27.19$, $p<10^{-4}$ FCs/PF: $G^2_{(3df)}=4.12$, $p=0.248$, FCs/FCs+cVA: $G^2_{(3df)}=7.96$, $p=0.047$, FCs/Mcs: $G^2_{(3df)}=1.06$, $p=0.787$, Fsim/MSim: $G^2_{(3df)}=10.86$, $p=0.013$; *Food Choice Frequency*: MCs/PF- Cs: $z=3.65$, $p=0.0002$, MCs/PF- D1: $z=3.99$, $p<10^{-4}$, MCs/PF- D5: $z=3.52$, $p=0.0002$, FCs/FCs+cVA- D5: $z=8.73$, $p<0.\ 10^{-4}$, MSim/FSim- D1: $z=3.13$, $p=0.002$; *Food Choice Frequency Differences:* PF/PF: $G^2_{(3df)}=2.49$, $p=0.476$, MCs/PF: $G^2_{(3df)}=9.406$, $p=0.024$, FCs/PF: $G^2_{(3df)}=3.62$, $p=0.306$, FCs/FCs+cVA: $G^2_{(3df)}=0.90$, $p=0.825$, FCs/MCs: $G^2_{(3df)}=4.57$, $p=0.206$, Fsim/MSim: $G^2_{(3df)}=4.10$, $p=0.251$). Two other flight parameters (upwind flight latency and time to reach food) are shown in Supplemental Fig. 1

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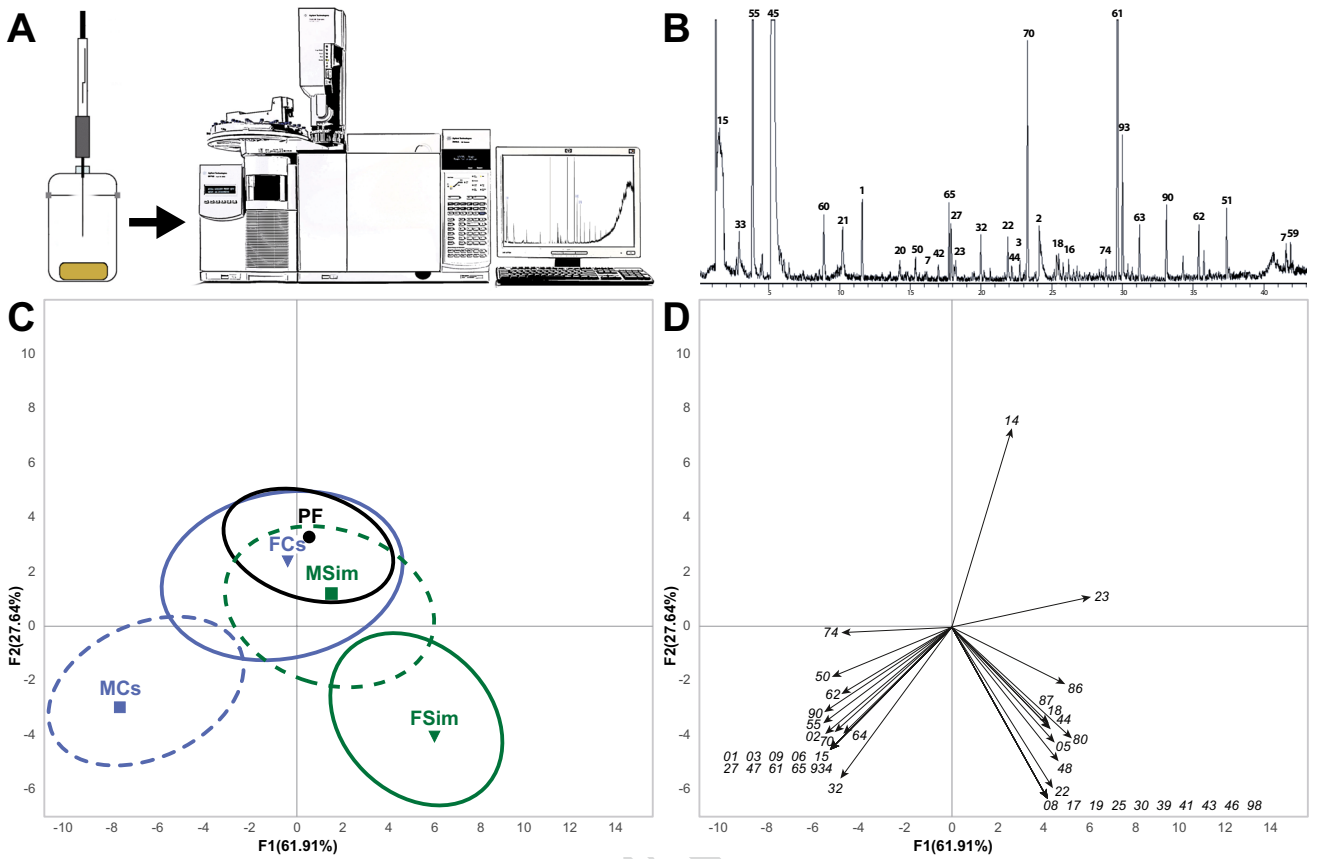

Fig. 4 Headspace analysis of volatile chemicals released by food labeled by flies of various genotypes. To analyze the volatile chemicals from several food sources labeled by flies and tested in the wind tunnel, we used (**A**) headspace-solid phase microextraction-GC-mass spectrometry. (**B**) We obtained chromatograms with many peaks, each corresponding to an identified volatile compound (labeled by a number; please refer to the nomenclature shown in Table 1). (**C**) We used principal component analysis (PCA) to compare all the compounds released by plain food (PF) to food sources labeled by (*i*) Cs females (FCs), (*ii*) Cs males (MCs), (*iii*) *D. simulans* females (FSim), and (*iv*) *D. simulans* males (MSim). (**D**) Each ellipse representing each food source corresponds to the compounds (identified with their numbers) located at a similar place on the PCA shown in C. For each sampling type, $3 \leq n \leq 4$. We also tested volatile compounds emitted by flies of similar genotypes but without food (see Supplemental Fig. 2)

to sex, *c*VA dose and treatment (Fig. 6). In females, *c*VA doses ≥ 100 µg induced similar electrophysiological responses in all treatments. The empty (0) and 1 µg stimulations induced noncoherent responses. In males, stimulations with 1–500 µg *c*VA induced similar slope responses in Cs males regardless of the dose tested. Cs male responses clearly diverged from those shown by D1, D5 and D5 + *c*VA males. In particular, the latter males showed less increased DA and almost no RT variation. This was particularly clear with the 1 µg *c*VA dose.

Control solutions were either tested before the *c*VA stimulation (hexanol [1], heptanone and paraffin oil) or after the *c*VA stimulation test (hexanol [2]). The two hexanol stimulations and the paraffin oil stimulation induced slight differences that mostly remained within the error variation range, while heptanone induced a divergent response (mostly due to increased RT) in D1 males and in D5 females compared to the other same-sex treatments (Suppl Fig. 3).

The PCA performed with all parameters extracted from these data revealed more subtle effects (Fig. 6C). Both sexes showed a substantial overlap for the response of D5 and D5 + *c*VA flies and a clear segregation of Cs and D1 flies. The segregation of the "D5/D5 + *c*VA" group was mostly linked with DA (red arrows) induced by higher *c*VA doses (300–500 µg) in both sexes. D1 female segregation was related to RT (dashed blue arrows) induced by low (1 µg) or 0 *c*VA doses, while Cs female segregation was related to the RT induced by 100 µg *c*VA. In contrast, D1 males segregated with the DA induced by 1 and 100 µg *c*VA, while the segregation of Cs males was linked to the RT induced by the higher *c*VA doses (100–500 µg).

Discussion

The present study aimed to test whether and to what extent early preimaginal exposure to maternally transmitted factors (*c*VA, microbes, etc.) could affect free flight olfactory tracking behavior in Drosophila flies. Specifically, we

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Table 1 Volatile chemicals from the different flies or labelled food sources tested in the wind tunnel, identified using Headspace-Solid Phase Micro-Extraction-GC-Mass-Spectrometry (HS-SPME-GC-MS). The X indicates the occurrence of the compound in SPME sampling if 25 D. melanogaster (or D. simulans) females and males without food or on 5 g of plain food

		Melano.				Simulans			
		No Food		Food		No Food		Food	
	#	F	M	F	M	F	M	F	M
ACIDS									
Acethydrazide	1			X					
Acetic acid	2			X					
Isovaleric acid	3			X					
Decanoic acid	4					X			
Heptanoic acid	5							X	
Hexanoic acid	6			X					
Octanoic acid	7								
Propanoic acid	8					X	X	X	
Isobutyric acid	9			X					
Pyruvic acid	10								
ALDEHYDS									
3-Methyl-2-butenal	11								
2-Hexenal	12								
2-Meethyl butanal	13								
3-Methyl butanal	14								
Ethanal	15			X					
Benzaldehyde	16								
Butanal	17						X	X	
Decanal	18					X	X	X	
Dodecanal	19					X		X	
Heptanal	20						X		
Hexanal	21								
Nonanal	22							X	
Octanal	23							X	
Pentanal	24					X		X	
Undecanal	25							X	
CETONES									
Butanone	26								
Hydroxy-3-butanone-2	27	X	X	X					
3-Methyl-2-butanone	28								
3-Methyl-2-pentanone	29								
4-Methyl-2-pentanone	30							X	
3-Methyl-3-buten-2-one	31					X			
6-Methyl-5-hepten-2-one	32			X		X	X		
Acetone	33	X	X						
ALCOOLS									
2-Methyl-1-butanol	34					X		X	
1,3-Butanediol	35								
2,3-Butanediol	36					X			
3-Methyl-2-butanol	37								
2-Methyl-3-buten-2-ol	38								
2-Pentanol	39						X	X	
Hexanol	40								
2-Hexanol	41							X	
3-Methyl-butanol	42						X		
4-Methyl-pentanol	43							X	
Butanol	44							X	
Ethanol	45	X	X						
Heptanol	46						X	X	
2-Propanol	47			X					
Nonanol	48							X	

		Melano.				Simulans			
		No Food		Food		No Food		Food	
	#	F	M	F	M	F	M	F	M
Octanol	49					X	X		
Pentanol	50			X					
2-Phenyl-ethanol	51								
Phenylmethanol	52								
ESTERS									
Isoamyl acetate	53								
1,2,4-Benzenetricarboxylic acid, 1,2-dimethyl ester	54								
Ethyl acetate	55	X	X	X					
Benzoic acid, 2-methoxy-,methyl ester	56								
Benzoic acid, 3-hydroxy-,methyl ester	57								
Benzoic acid, 2-hydroxy-,methyl ester	58								
Methyl anisate	59							X	
Butanoic acid, 3-hydroxy-,ethyl ester	60							X	
Ethyl butyrate	61			X					
Ethyl caprate	62			X					
Ethyl laurate	63								
Ethyl 9-decenoate	64			X					
Ethyl hexanoate	65			X					
Isoamyl butyrate	66								
Isobutyl acetate	67								
Isobutyl butanoate	68								
Isoamyl decanoate	69								
Ethyl octanoate	70			X					
Ethyl pentanoate	71								
Various									
1-Aziridineethanamine	72								
4-Nonyne	73								
5,5-Dimethyl-2(5H)-furanone	74			X					
2-Pentylfuran	75								
5-Ethylcyclopent-1-enecarboxaldehyde	76								
Acetamide	77							X	
4-Methoxybenzaldoxime	78							X	
Benzoyl bromide	79	X	X						
Dimethyl disulfide	80								X
2-Butoxyethanol	81							X	X
Ethoxyethene	82							X	X
sec-Butylisopropyl ether	83							X	
Geranylacetone	84								
3-Methoxy-hexane	85							X	
Humulene	86								X
Phenylmethane	87							X	X
Methyl-D3 1,1-dideutero-2-propenyl ether	88							X	X
Methyl-paraben	89	X	X						
Methoxy-phenyl-oxime	90	X	X	X					
Styrene	91								
Triacetine	92							X	
α-Caryophyllene	93			X					
ß-Caryophyllene	94								
11-Tetradecen-1-ol acetate	95								
3-Methyl-1-butanamine	96							X	
2-Butanamine	97							X	X
N-Methyl-methanamine	98								X
Octane	99							X	

compared individual flies resulting from eggs laid less than 24 h (D1) or more than 5 days (D5) after the copulation of their progenitors. Since D5 eggs are devoid of *c*VA, we also attempted to rescue the cVA exposure effect in flies resulting from D5 eggs exposed to cVA-rich food (D5 + *c*VA). Flies resulting from these three "conditioning" experiences were compared to control Cs flies randomly sampled from culture vials. The pivotal experiment of our study was performed in a wind tunnel to measure fly ability to take upwind flight and to land on food with regard to food preference in a dual food choice. Since most food sources tested in the wind tunnel were "contaminated" by flies of various genotypes, likely disseminating different microbes on the food (Wong et al. 2013; Farine et al. 2017), we hypothesized that these fly-labeled food sources could emit different volatile food-derived metabolites that we identified using headspace analysis. Moreover, to partly determine the involvement of the peripheral nervous system in the different flight responses shown by Cs, D1, D5 and D5 + cVA males and females, we measured the electrophysiological response of their antennae to *c*VA.

Our free flight experiment revealed that preimaginal conditioning differentially affected some behavioral aspects between the sexes. Most female and male groups showed very similar upwind flight frequencies to the PF/PF control choice (61–80%), except for Cs flies (53–56%). As this tendency occurred in Cs flies of both sexes, plain food elicited upwind flight less often than fly-contaminated food. We observed a similar tendency with Cs male-processed food (MCs in MCs/PF; FCs/MCs), which elicited increased flight frequency in both sexes. Similarly, *D. simulans*-labeled food (MSim) elicited very frequent upwind male flights. Together, these data suggest that flies can detect food volatile odors before initiating flight. In other words, their ability to discriminate odors determines the behavioral decision preceding their upwind flight.

Females and males showed a relatively similar variation in their "Landing on food" frequencies. Relatively low landing responses were induced in all fly groups by the PF/PF control and by the "FCs/FCs + *c*VA" choice except in Cs flies. The "FSim/MSim choice" induced low responses in most females but only in D1 males. The "MCs/PF choice induced significantly less responses in D1 flies compared to the three other conditions. How can we interpret the decreased "landing on food" frequency shown by these groups of flies? Four remarks may help to understand such variation. (1) D5 and D5 + cVA flies showed similar responses. (2) D1 flies landed less often in the "MCs/PF" choice than in the "FCs/MCs"

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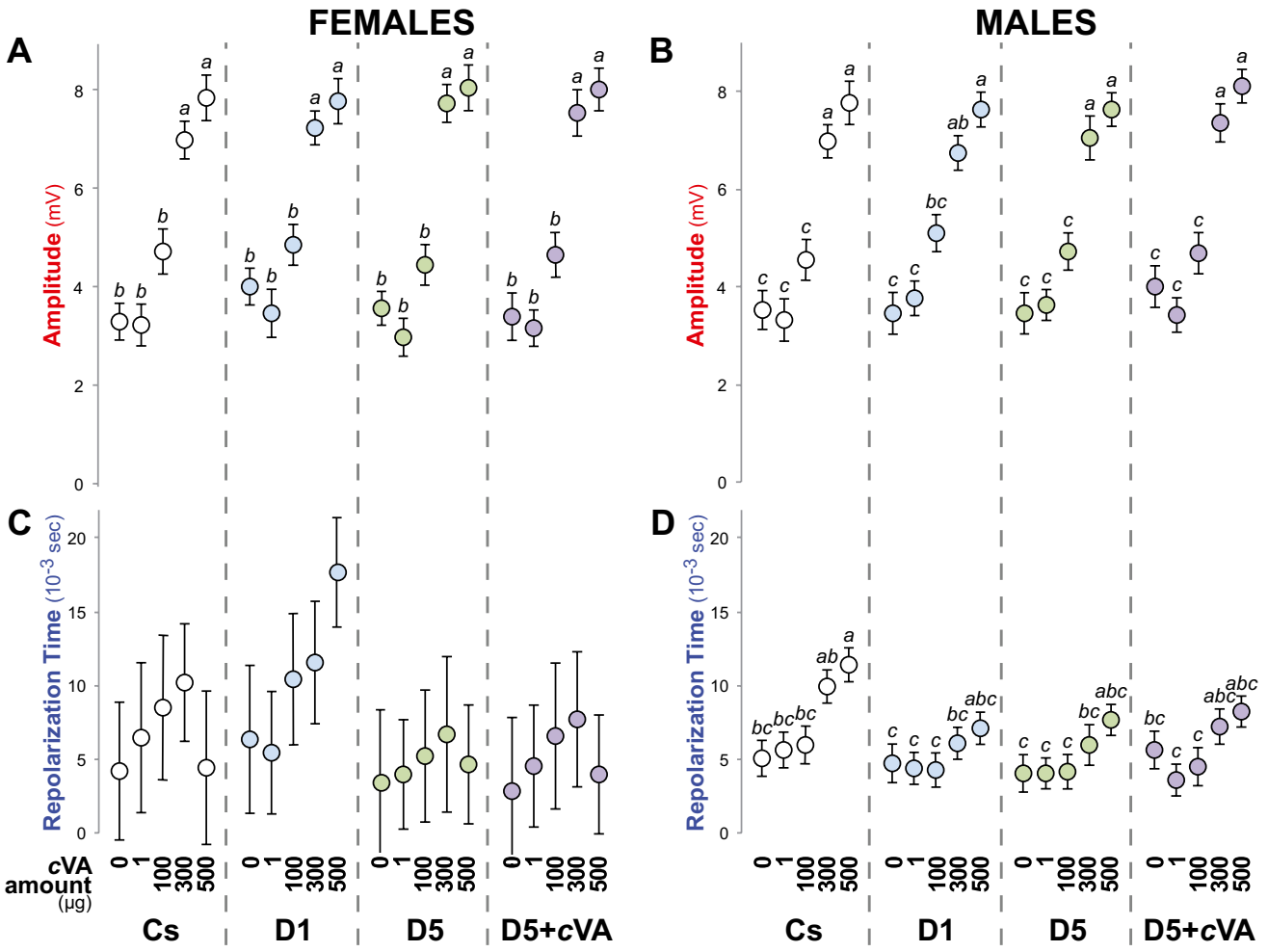

Fig. 5 Electrophysiological antennal response in variously conditioned flies stimulated by *cis*-vaccenyl acetate (*c*VA). We used one-way ANOVA to analyze the possible effect of conditioning on the amplitude of depolarization (Females: A, $F_{(19,\ 280)}=28.4$—$p<10^{-4}$; Males: B, $F_{(19,\ 275)}=26.6$—$p<10^{-4}$) and repolarization time (Females: C, $F_{(19,\ 280)}=1.2$—$p=0.243$; Males: D, $F_{(19,\ 275)}=4.8$—$p<10^{-4}$). Significant differences (at $\alpha=0.05$) are indicated by different letters

choice, indicating that unlike PF, FCs masked the repulsive (or nonattractive) effect induced by MCs on D1 flies. (3) Flies resulting from the three conditioning groups—but not Cs—were repulsed by *c*VA added to FCs. (4) The "FSim/MSim" choice induced a clear sex difference: most females, but only D1 males, showed low landing frequencies.

The examination of the "food choice" preference can shed some light on the analysis of the previous parameter. In the "MCs/PF" choice, all but D5 + *c*VA flies preferred—significantly or not—MCs over PF. Moreover, D5 males landed preferentially on MSim (more than on FSim), while D5 + *c*VA males preferred MCs (over FCs). These data indicate that *c*VA added to the preimaginal diet affected some—but not all—male behavioral responses. However, the fact that all males were indifferent to *c*VA-rich food in the "FCs/FCs + cVA" choice test suggests that in the "MCs/PF" choice test, male preference was not driven by *c*VA but by other factors specifically provided by *D. melanogaster,* such as cuticular hydrocarbons (CHCs) and/or microbes.

In addition to the parallel effect described for the "MCs/PF" choice in both sexes, several sex differences were noted: D1 females avoided FCs (in the "FCs/PF" choice), while D1 and D5 + *c*VA females preferred *c*VA-rich food (in the "FCs/FCs + cVA" choice). If D1 and D5 + *c*VA females are attracted by *c*VA-rich food, such preference could allow them to find—in nature—a food source labeled by recently mated females and by males. In contrast, non *c*VA-conditioned D5 females were not attracted to *c*VA-rich food, indicating that, in nature, flies prefer to visit food sources with no or fewer males and mated females. Consequently, (*i*) D5 females would be subjected to less sexual harassment (Makowicz and Schlupp 2013), and (*ii*) their larvae would be exposed to reduced competition for food (Wertheim et al. 2005). In turn, a low adult male

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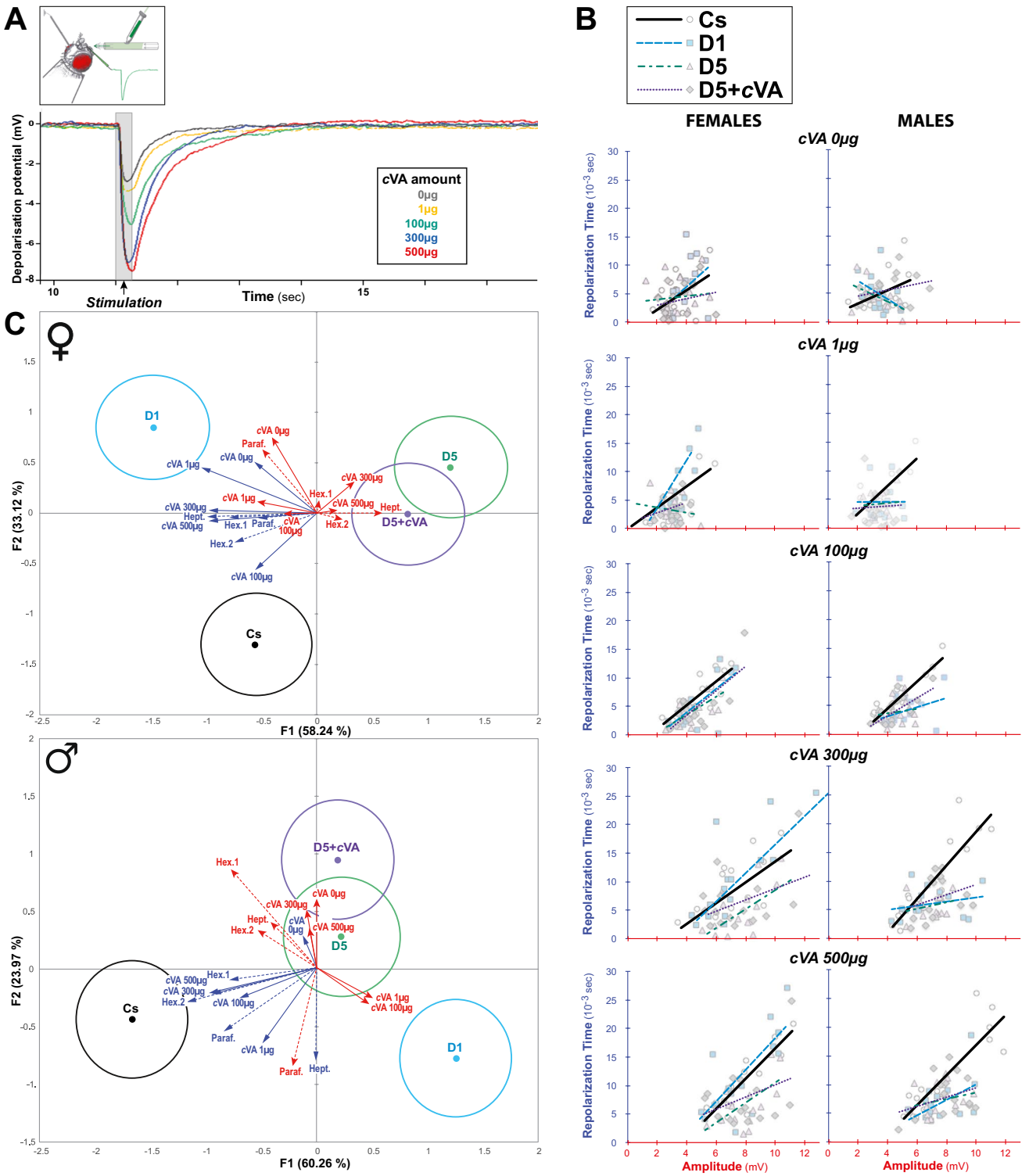

density could reduce the probability for a female to choose the most appropriate male, with possible negative effects on offspring fitness (Kohlmeier et al. 2021; Wertheim et al. 2002) and an increased risk of being parasitized at a lower population density (Hamilton 1971).

The difference between D5 + *c*VA and D1 flies indicates that *c*VA addition to the preimaginal diet did not mimic its maternal transmission during egg laying. As previously discussed (Everaerts et al. 2018), the difference between D1 and D5 + *c*VA is related not only to *c*VA concentration and

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◂**Fig. 6** Electrophysiological antennal response in variously conditioned flies stimulated by *cis*-vaccenyl acetate (*c*VA) and other control chemicals. (**A**) Each live fly was maintained with its head protruding at the tip of a pipette cone. A puff of air with various *c*VA doses was sent onto the whole antenna, whose electrical response was recorded according to the *c*VA dose. The time at which the stimulation took place is indicated with an arrow below the electroantennograms with its duration shown as a gray bar. For each electrical response, we measured both its amplitude corresponding to the depolarization potential (measured in mV) and the duration of repolarization (return until the baseline; measured in seconds). (**B**) We determined the relationship between the amplitude of depolarization (x-axis; red color) and repolarization time (y-axis; blue color) in females (left) and in males (right) for each *c*VA dose. In each frame, we compared the response of Cs flies (plain dark lines) to the response of conditioned flies resulting from D1 eggs (D1; long blue dashed lines), D5 eggs (D5; medium green dashed lines) and D5 eggs raised in *c*VA (D5 + *c*VA; dark dotted lines). (**C**) The global response of each type of fly was compared using PCA taking into account both the depolarization amplitude (red plain arrows) and repolarization time (blue plain arrows) induced by all *c*VA doses. The PCA also takes into account the depolarization amplitude (red dashed arrows) and repolarization time (blue dashed arrows) induced by all control substances (see Supplemental Fig. 3). Females are shown on the top PCA; males are shown on the bottom PCA. Each compound was tested in 15 flies of each sex

nature (biological vs. synthetic) but also to its dispersion pattern—discontinuous vs. homogenous—in food and the simultaneous presence/absence of microbes on the embryonic chorion (Bakula 1969). Other factors could also be involved, such as accessory gland proteins (Herndon and Wolfner 1995), antibiotic peptides produced by the ejaculatory bulb (Wolfner 2002) and male CHCs (Duménil et al. 2016; Laturney and Billeter 2016). Moreover, we do not know whether similar microbes are present on D1 and D5 eggs. In nature, *c*VA is superficially deposited on food by females laying their first postmating eggs followed by mating plug ejection (Laturney and Billeter 2016; Lung and Wolfner 2001). *c*VA is also deposited by males either by passive transfer (Farine et al. 2012) or in their feces and fecal droplets (Keesey et al. 2016; Mercier et al. 2018). All these sources produce a discontinuous and superficial distribution of *c*VA onto the substrate, contrasting with the homogeneous presence of synthetic *c*VA added in *c*VA-rich food. In the first medium, first and second instar larvae crawling into the food intermittently encountered *c*VA, while homogeneous *c*VA food induced permanent exposure. These two exposure patterns could differentially affect the early preimaginal conditioning process (Durisko et al. 2014).

The headspace experiment focused on the quality of food sources tested in the wind tunnel. This highlighted the existence of a strong sexual dimorphism within each species (*D. melanogaster* and *D. simulans*). The intersex difference for volatile chemicals produced by flies interacting on the food may — at least partly — explain divergent food preference between our tests. The high number of compounds diverging between genotypes makes it currently difficult to identify the molecule(s) potentially involved in multiple flight decisions (upwind flight, landing, choice). However, the clear intersex difference together with the "FCs – MSim" overlap (both genotypes strongly diverging for their CHCs) suggests that the difference in volatile chemicals is linked not only to CHC identity but also to other divergent factors, very likely microbes involved in food and CHC degradation. Indeed, some volatile compounds detected here are related to bacterial activity (isovaleric, hexanoic, and isobutyric acids, ethanal, acetoine and ethyl butyrate) and/or to yeast activity (hexanoic acid, acetoin, ethyl butyrate, hexanoate and 9-decenoate) (Becher et al. 2012; Beck et al. 2000; Farine et al. 2014; Palanca et al. 2013; Ryu et al. 2004). The hypothesis of a "food-microbe-CHC" interaction is reinforced by the PCA comparison between flies without food (showing an important overlap without regard to sex and species) segregating far from fly-labeled food types (Fig. Suppl 2).

The electrophysiological experiment was a preliminary attempt to explore, in the peripheral olfactory system, the influence of preimaginal conditioning on olfactory-driven free flight in adults. This experiment was designed to compare the antennal response to *c*VA of Cs, D1, D5 and D5 + cVA females and males. We chose *c*VA since it is a compound potentially involved both in some of the behavioral responses observed in the wind tunnel (present study) and in preimaginal conditioning (Everaerts et al. 2018). We also chose *c*VA by default: the identity of food-derived compounds potentially involved in food preference remained unknown (see above). The antennal response shown by both Cs sexes (control flies) was similar with a proportional relationship between the depolarization amplitude (DA) and the repolarization time (RT), with DA increasing with the *c*VA dose. This observation is supported by a report showing a similar antennal response to *c*VA in Cs females and males (Kurtovic et al. 2007). Here, we observed marked sexual differences in differently conditioned flies. Within the 100–500 µg *c*VA range, conditioned females showed DA/RT "regression slopes" relatively well aligned with those of Cs control females. In contrast, within the 1–500 µg *c*VA range, conditioned males showed a relatively flat DA/RT "correlation slope due to repolarization times shorter than in Cs males. The sexually dimorphic response of antennae stimulated by *c*VA, especially the highly different RT variability range, may partly explain sex-specific variations in dual food choice. These differences could be caused by the alteration of sex-specific features of the *D. melanogaster* antennae: (*i*) the male funiculus harbors more trichoid sensilla than the female funiculus (Xu et al. 2005); (*ii*) the esterase-6 enzyme, involved in *c*VA degradation, shows higher expression in males than in females (Chertemps

et al. 2012); and (*iii*) the odorant-binding protein OBP69a, required for the activity of *c*VA-responsive neurons, is reciprocally regulated by *c*VA between the sexes (*c*VA stimulation decreases the OBP level in males, whereas it increases the OBP level in females (Bentzur et al. 2018)).

Moreover, flight differences observed between conditioned females and males could also be related to a different integration in olfactory signals in their brain. Indeed, since EAG was performed on immobilized flies, many sensory aspects shown by free flying Drosophila were not taken into account, such as vision and mechanosensation (Bentzur et al. 2018; Dahake et al. 2018; Saxena et al. 2018). Drosophila uses information from mechanoreceptors on wings and halters and in campaniform sensillae to control flight (Deora et al. 2021). Chemosensory receptors on the anterior wing margin can also change some aspects of free flight (Houot et al. 2017; Raad et al. 2016).

In summary, our study reveals that preimaginal exposure to *c*VA and/or to unidentified maternally transmitted factors can affect several aspects of free flight olfactory tracking behavior in Drosophila females and males. Such a plasticity effect could underlie the natural variation in behavioral dispersion in Drosophila populations, allowing differently conditioned flies to explore a higher diversity of food patches. We do not know whether it is possible for a female that mated more than 5 days earlier to lay eggs on a food patch devoid of conspecifics. In nature, this may happen considering that (1) mated and virgin females show similar flight ability (Becher et al. 2010) and (2) Drosophila flies can show a very long range flight capacity (~12 km in a single flight (Leitch et al. 2021)), which increases the probability for a AQ5 fly to land on a food spot devoid of conspecifics.

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Data Availability An xlsx file containing all raw data is available as supplemental material.

Declarations

Competing Interests The authors declare no competing interests.

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Suppl. Fig. 1

FEMALE

MALE

A — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Upwind Flight Latency (sec)

p=0.022

B — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Upwind Flight Latency (sec)

p=0.022 p=0.046

C — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Time to Reach Food (sec)

p=0.016 p=0.048 p=0.048 p=0.048 p=0.044

D — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Time to Reach Food (sec)

p=0.029

E — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Upwind Flight Latency (sec)

F — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Upwind Flight Latency (sec)

G — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

Time to Reach Food (sec)

Cs D1 D5 D5+cVA

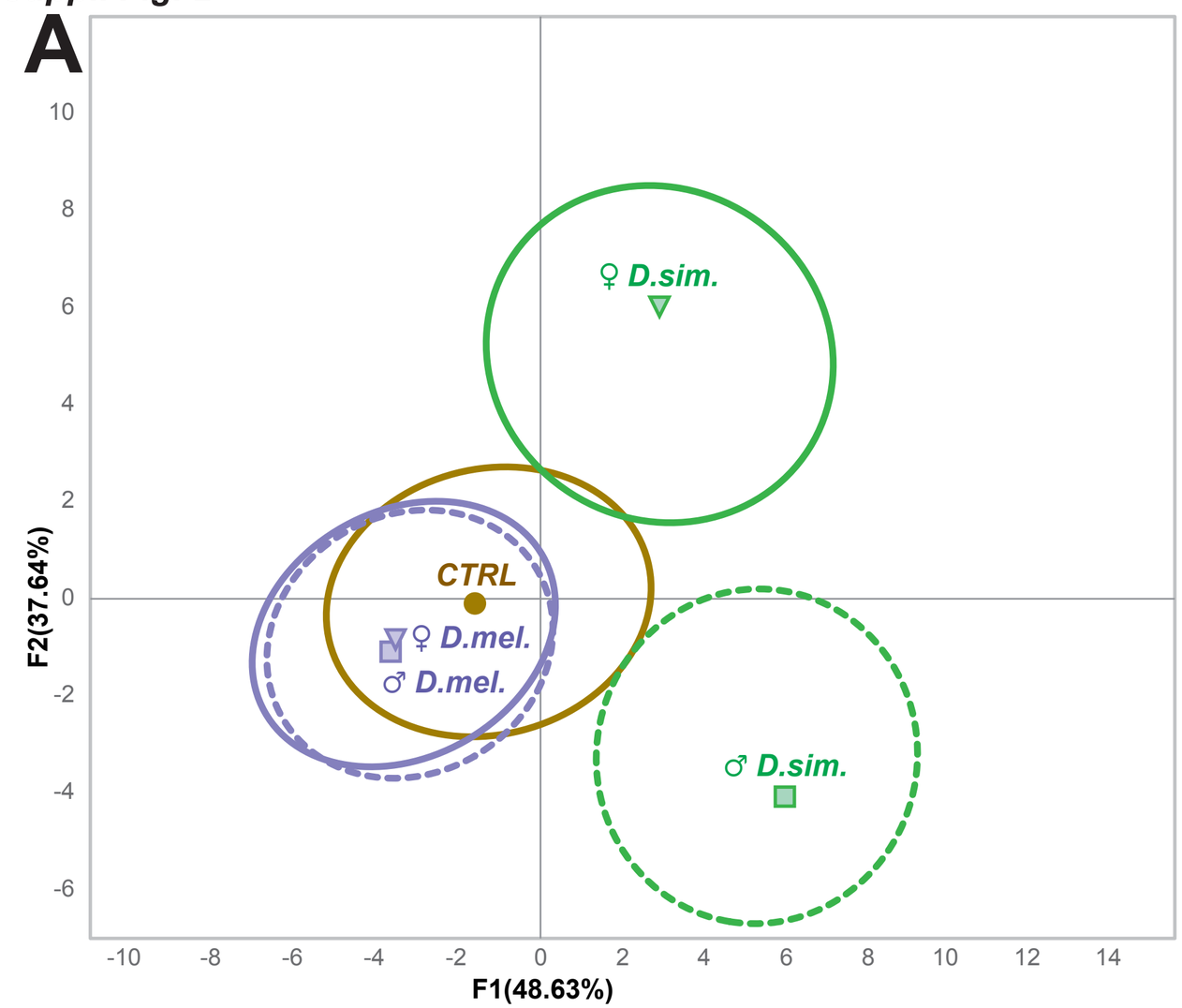
H — PF/PF | MCs/PF | FCs/PF | FCs/FCs+*c*VA | FCs/MCs | FSim/MSim

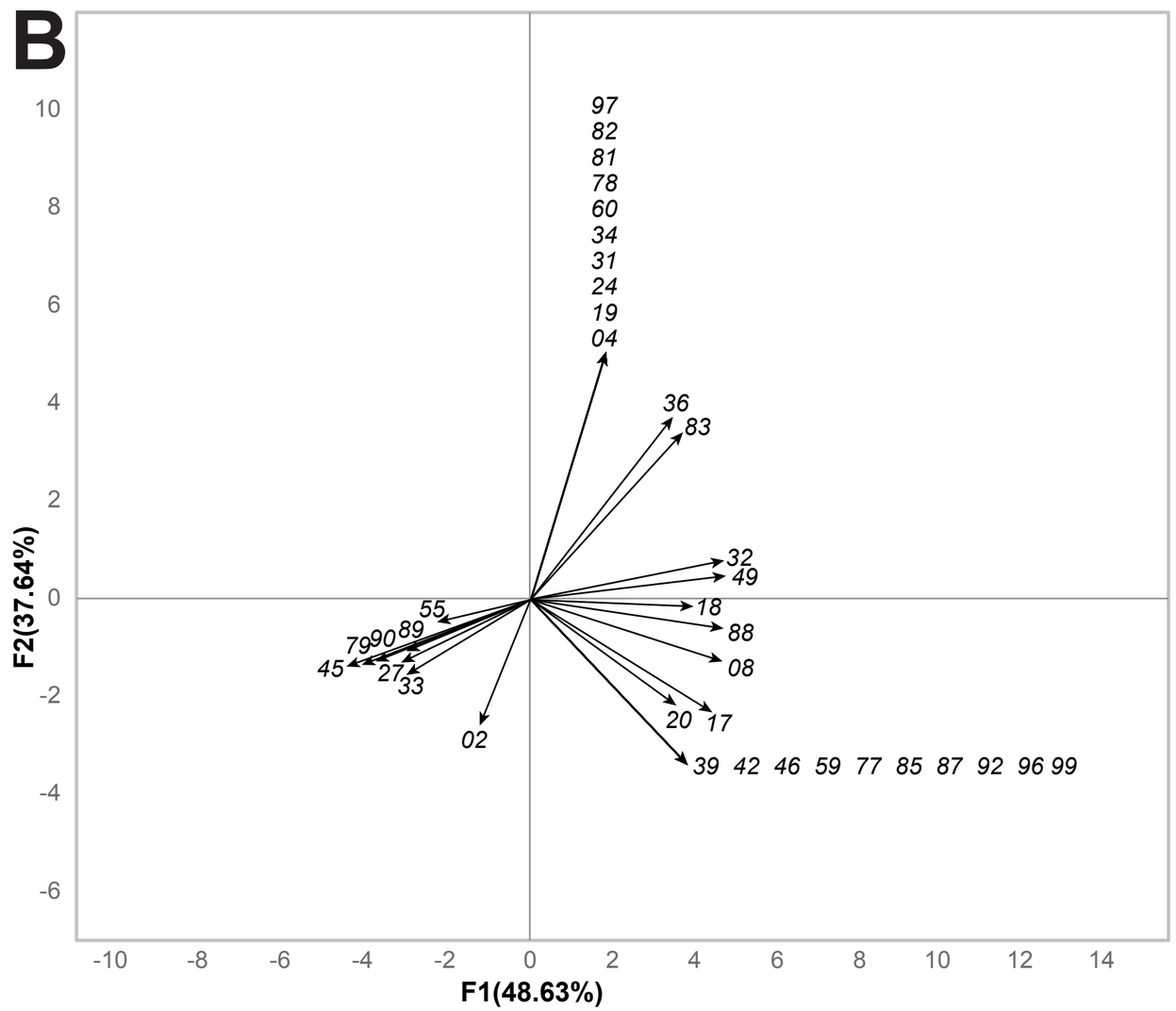
Time to Reach Food (sec)

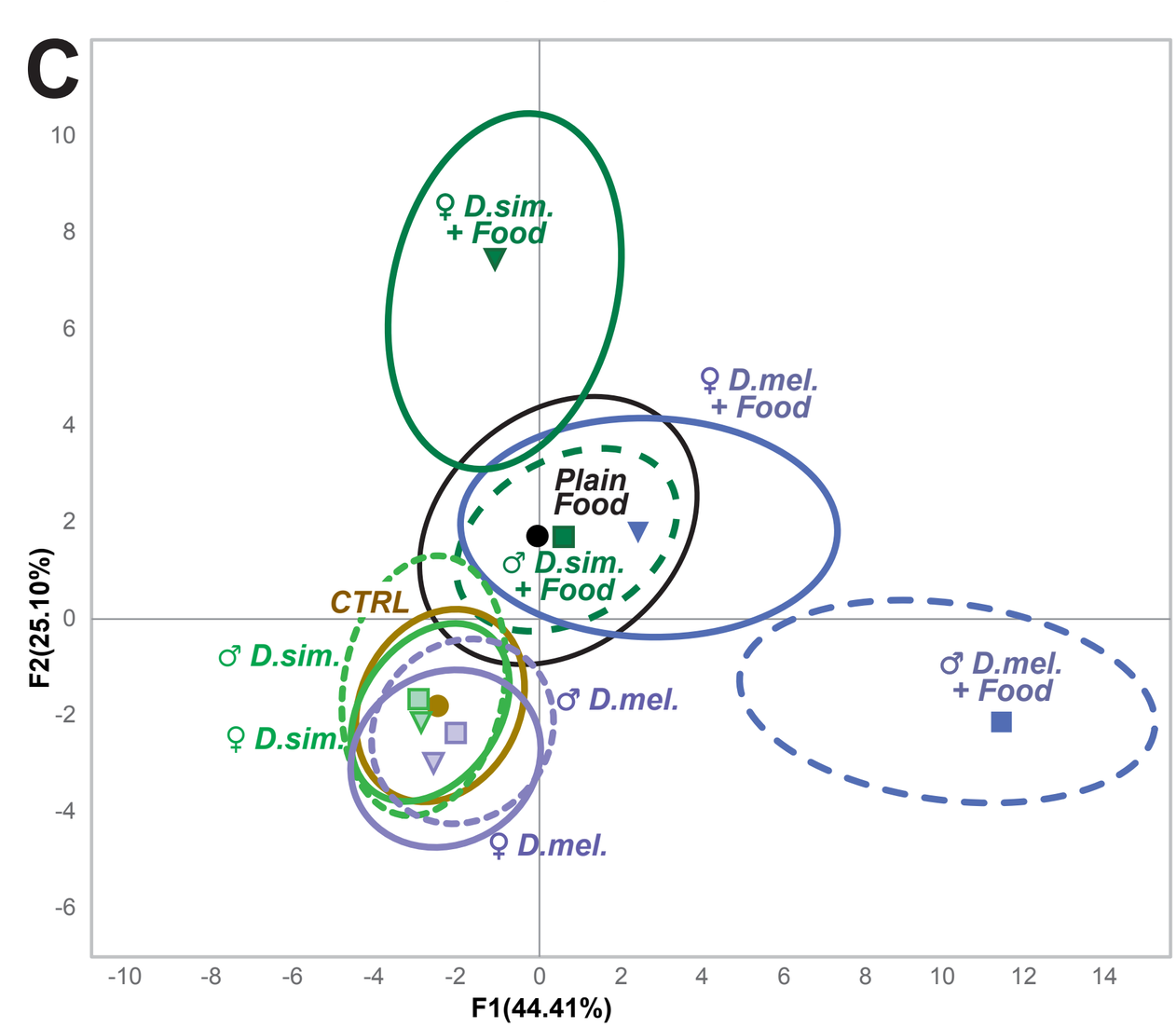
p=0.026

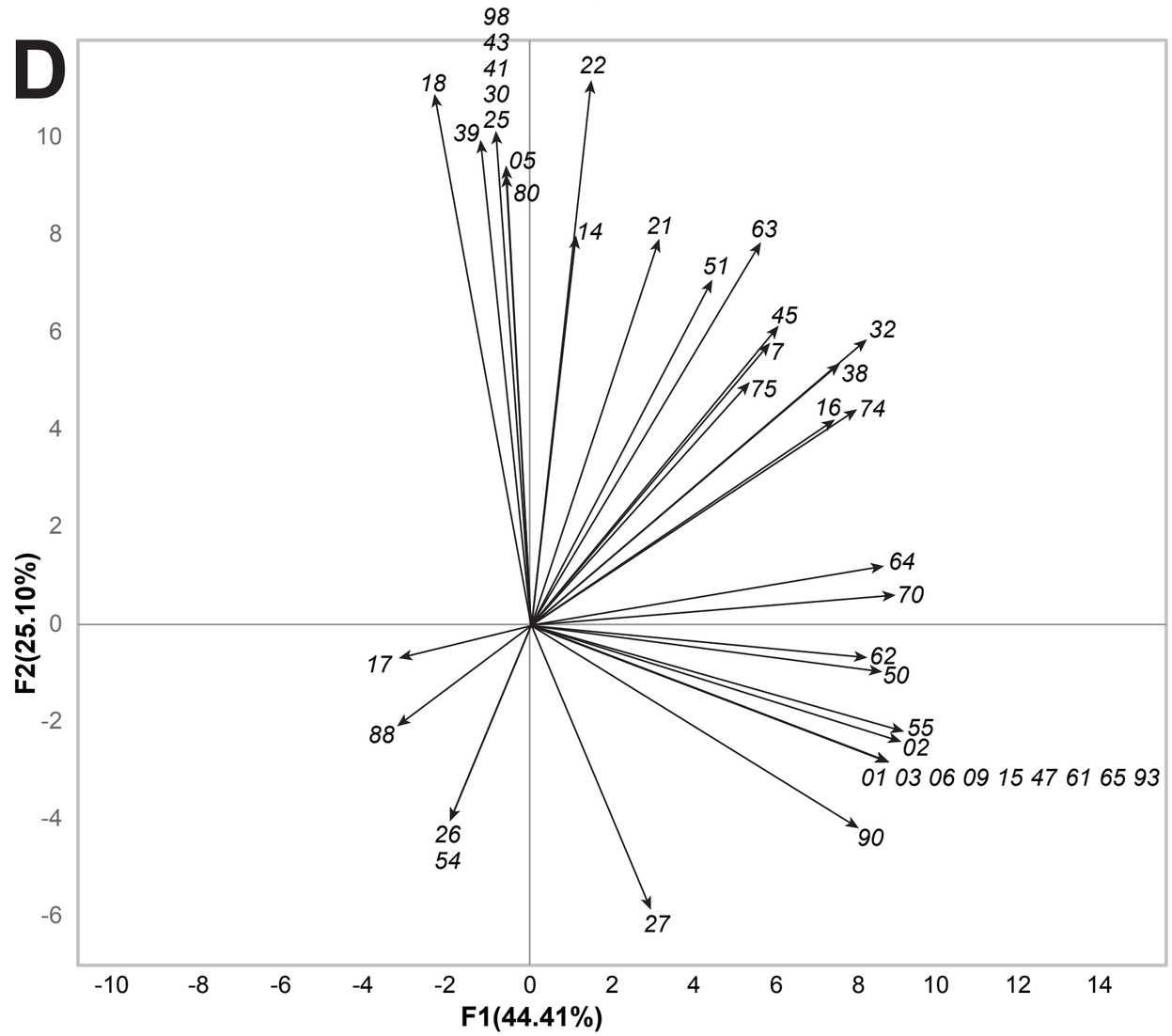
Cs D1 D5 D5+cVA

Suppl. Fig. 2


A
♀ D.sim.
CTRL
♀ D.mel.
♂ D.mel.
♂ D.sim.
F2(37.64%)
F1(48.63%)


B
97 82 81 78 60 34 31 24 19 04
36 83
32 49
18
88
08
20 17
39 42 46 59 77 85 87 92 96 99
02
55
79 90 89
45 27 33
F2(37.64%)
F1(48.63%)


C
♀ D.sim. + Food
♀ D.mel. + Food
Plain Food
♂ D.sim. + Food
CTRL
♂ D.sim.
♀ D.sim.
♂ D.mel.
♀ D.mel.
♂ D.mel. + Food
F2(25.10%)
F1(44.41%)


D
98 43 41 30 25
18
39
05 80
22
14
21
63
51
45 7
75
32 38
16 74
64 70
17
62 50
88
55 02
01 03 06 09 15 47 61 65 93
90
26 54
27
F2(25.10%)
F1(44.41%)

Suppl. Fig. 3

CONTROLS

FEMALES **MALES**

Hexanol (1)

Heptanone

Paraffin Oil

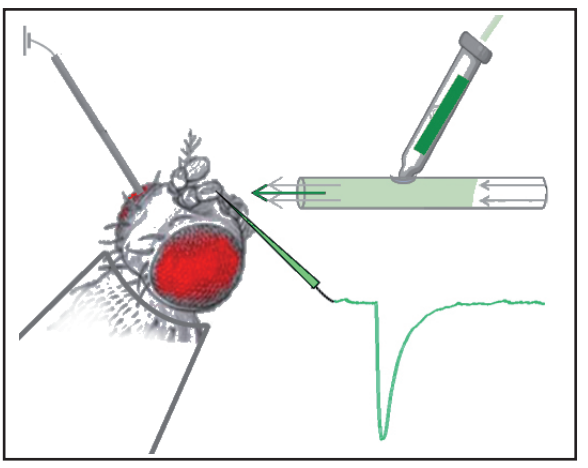
Hexanol (2)

Repolarization Time (10^{-3} sec)

0 10 20 30 40 50

0 5 10 15

Amplitude (mV)



Cs
D1
D5
D5+cVA