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ORIGINAL PAPER

Female melon fruit flies, *Zeugodacus cucurbitae*, are attracted to a synthetic chemical blend based on male epicuticular components

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Abstract

The melon fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae), is considered to be the most destructive pest of melons and other related cucurbit crops worldwide. Despite the potential of behaviour-based control strategies, little is known about the mechanisms involved in female mate choice. Herein, we investigated the production and chemoreception of cuticular hydrocarbons in both sexes of *Z. cucurbitae,* and the behavioural responses they induce. We studied the epicuticular composition of virgin males and females, using two-dimensional gas chromatography coupled with mass spectrometric detection. Data were interpreted using multivariate factorial analysis. The differentiation of chemical profiles was consistently observed over time. In young individuals, the chemical profiles did not differ between sexes, while sex-specific differences were noted in mature flies. The fly olfactory sensitivity to these compounds was explored using gas chromatography combined with chopped triple electroantennography and electropalpography detectors. This extensive exploration of the pest olfactory sensitivity highlighted three compounds produced by the male. When blended, they induced a robust positive response in unmated naive females in a six-choice olfactometer. The responsiveness of other Tephritidae species (a polyphagous species *Bactrocera dorsalis* (Hendel) and the cucurbit specialist *Dacus demmerezi* (Bezzi)) to whole body extracts of *Z. cucurbitae* was also investigated. Our findings showed that *Z. cucurbitae* uses species-specific olfactory receptors to detect male produced compounds. In addition, the palps were sensitive to a female-specific component, 1,7-dioxaspiro[5.5]undecane, which the males produce in minute quantities. Overall, this study provides a starting point for a pheromone-based tephritid lure that targets unmated females. The potential implications for pest management are discussed.

Keywords Behaviour-based control · Cuticular hydrocarbons · Pheromones · Age-dependent production · GC × GC-TOFMS · GC-EAD · GC-EPD · Tephritidae

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Introduction

The melon fruit fly, *Zeugodacus cucurbitae* (Coquillett) (Diptera: Tephritidae), is one of the most widespread and destructive fruit fly pests in the world. It infests over 45 plant species, most of which belong to the Cucurbitaceae family, including several commercially grown crops (White and Elson-Harris 1992; Dhillon et al. 2005; De Meyer et al. 2015). According to a recent study of macrogeographic population structure (Virgilio et al. 2010), the melon fly originated in Central Asia and expanded its range to East Asia, Hawaii, Africa, and the islands in the western Indian Ocean. Since 1972, *Z. cucurbitae* has been the subject of eradication programmes in the southernmost part of Japan (Koyama et al. 2004). It has been detected repeatedly in other parts of the world, including California (USA) (Papadopoulos et al. 2013). Male and female attractants are key tools for tephritid population management (Giunti et al. 2023). Tephritid monitoring and control techniques can rely on male attractants, which are either compounds closely interconnected with the pheromonal system (e.g., cue lure or methyl eugenol) (Howlett 1912, 1915; Beroza et al. 1979; Tan et al. 2014), or directly involve a female pheromone constituent, as documented for the olive fruit fly, *Bactrocera oleae* (Rossi) (Haniotakis et al. 1977; Baker et al. 1980; Daane and Johnson 2010). Female attraction to male pheromones could lead to the development of new control tools (Tan et al. 2014). A more general understanding of sexual communication is a prerequisite for improving the fitness of mass-reared individuals for autocidal control strategies (Benelli et al. 2014a, b).

In the melon fly, it has been reported that both sexes produce volatile compounds eliciting specific behavioural response in the opposite sex (Kobayashi et al. 1978; Baker et al. 1982; Ohinata et al. 1982; Baker and Bacon 1985; Khoo and Tan 2000; Benelli et al. 2014a). *Zeugodacus cucurbitae* males produce courtship calls using wing stridulation. They also emit sex pheromones by wind fanning at dusk (Kuba and Sokei 1988). Adults form leks, where each male fights to defend a small territory for mating (Kuba et al. 1984; Kuba and Koyama 1985; Koyama et al. 2004). This is a typical feature of many tephritid species (Benelli 2015a). The involvement of an aggregation pheromone in lek formation has not been confirmed in this species. Male attraction to other males was observed in some cases (Khoo and Tan 2000) but not in others (Kobayashi et al. 1978). The sex pheromone emitted by males attracts conspecific virgin females (Sugimoto 1979; Suzuki and Koyama 1980, 1981; Kuba and Sokei 1988), but we do not know if this attraction occurs over long distances. Male emissions consist of chemically diverse compounds, such as linear hydrocarbons, amides, pyrazines, esters, alcohols and acids (Sugimoto 1979; Suzuki and Koyama 1980, 1981; Baker et al. 1982; Ohinata et al. 1982; Kuba and Sokei 1988; Nishida et al. 1990). Pheromone-like volatiles extracted from the rectal glands of male *Z. cucurbitae* and their possible role in courtship behaviour have been studied previously (Baker et al. 1982; Perkins et al. 1990; Nishida et al. 1993; Tan 2000; Koyama et al. 2004). Volatiles produced by female *Z. cucurbitae* contain a variety of 1,7-dioxaspiro[5.5]undecanes (DSU) and *N*-(3-methylbutyl)acetamide (3MA). However, the biological role of spiroacetals remains unclear (Baker and Bacon 1985). The maturation kinetics of sex pheromone production have not been studied in this species. It is still unclear whether young male *Z. cucurbitae* can produce the female compounds (DSU and/or 3MA), as reported for other tephritid species, e.g., *B. oleae* (Benelli et al. 2013, 2014a). Overall, current knowledge of the epicuticular composition in *Z. cucurbitae* (Carlson and Yocom 1986) is limited. Research on the variation of epicuticular chemistry in relation to age and sex is particularly important for a better understanding of chemical communication in insects (Vaníčková et al. 2012).

Cuticular hydrocarbons (CHs) are important components of the insect surface lipids and often account for the majority (up to 90%) of epicuticular lipids (Goh et al. 1993). These non-polar compounds serve primarily to protect insects from desiccation (Blomquist and Bagnères 2010). They can also play a major role in inter- and intra-specific recognition, especially mate recognition and territorial displays (Blomquist and Bagnères 2010; Everaerts et al. 2010; Benelli 2015b; Vaníčková et al. 2017). In tephritid flies CHs have been used for species differentiation in cryptic species complexes, such as the *Anastrepha fraterculus* complex or the so-called *Ceratitis* FAR complex (Vaníčková 2012; Vaníčková et al. 2014, 2015a, b, c). Within the *Bactrocera* genus, clear species segregation has been observed in terms of the complex cuticular profiles of *Bactrocera carambolae* Drew & Hancock and *Bactrocera dorsalis* (Hendel). Results supported both taxonomic synonymization of *Bactrocera invadens* Drew, Tsura & White, *Bactrocera papayae* Drew & Hancock, and *Bactrocera philippinensis* Drew & Hancock with *B. dorsalis*, as well as the exclusion of *B. carambolae* from *B. dorsalis* (Vaníčková et al. 2017). In addition to CHs, abundant complex mixtures of sex-specific oxygenated lipids (3 fatty acids and 22 fatty acid esters) with unknown functions have been identified in epicuticular extracts from *B. dorsalis* and *B. carambolae* females.

The present study investigated the chemical composition of the epicuticle of young and sexually mature *Z. cucurbitae* males and females. To explore the role of these compounds in intraspecific chemical communication, the fly olfactory sensitivity was investigated. Responses to

synthetic compounds were tested with triple electroantennography (EAG_3) and electropalpography (EPG). Whole body extracts of mature virgin females and males were analysed using chopped gas chromatography coupled with EAG_3 (GC-EAD_3) and EPG (GC-EPD) detectors. The responsiveness of young and mature virgin males and mature virgin females was compared. To gain insights into interspecific interaction, we explored the antennal responsiveness of females from two other tephritid species, i.e., *Dacus demmerezi* (Bezzi) and *B. dorsalis*, to the whole body extract of *Z. cucurbitae* males. Lastly, using a six-choice olfactometer, we tested the attraction of mature unmated females of *Z. cucurbitae* to a synthetic blend that included electrophysiologically active male compounds.

Materials and methods

Insects

Zeugodacus cucurbitae was used for chemical analyses, electrophysiological experiments and behavioural assays. *Dacus demmerezi* and *B. dorsalis* females were used only in electrophysiological experiments to test their antennal responsiveness to body extracts of *Z. cucurbitae* males. Larvae of *Z. cucurbitae* and *D. demmerezi* were reared on zucchini (*Cucurbita pepo* L.). *Bactrocera dorsalis* larvae were reared on an artificial diet following Duyck and Quilici (2002). The diet was composed of dehydrated carrot powder, brewer's yeast, sugar, dehydrated potato, water, nipagin/sodium benzoate, HCl, agar and wheat germ. All flies were reared in the CIRAD, UMR PVBMT laboratories (La Réunion, France) at 25 ± 1 °C, R.H. 65 ± 10% and 12:12 h (L:D) photoperiod. *Zeugodacus cucurbitae* males and females were separated 1–2 days after emergence, fed with sugar, water, and proteins, and tested in electrophysiological experiments 15–25 days after emergence, unless specified otherwise. For chemical analysis, 100 individuals from the same *Z. cucurbitae* laboratory population were transported, at the pupal stage, to the Institute of Chemistry and Biochemistry CAS. Immediately after emergence, males and females were sexed and stored in different glass chambers (30 × 20.5 × 16 cm). The rearing conditions were identical to those stated above for *Z. cucurbitae*. Throughout the study, the term mature individuals refers to flies that are 15 to 25 days old after emergence.

Extraction of epicuticular compounds

At five days intervals, starting at the 5th day after emergence till day 30 (i.e., at days 5, 15, 25, 30), five virgin males and females of *Z. cucurbitae* were immobilized (− 18 °C) and placed in a desiccator for 15 min to remove the surface moisture. Compounds from the whole body surface were extracted individually with 0.5 mL hexane (Fluka, Germany) for 5 min in small glass vials. An internal standard (1-bromodecane, 5 ng per 1 mL of extract) was used for quantification. Each extract was concentrated to approximately 100 µL by a constant flow of nitrogen and stored in a freezer (− 18 °C) until GC × GC-TOFMS analysis. We performed an electrophysiological analysis on a pooled sample of 40 and 120 virgin males and females, respectively, aged 15 to 20 days after emergence. Flies were anaesthetized with CO_2. Males were placed together in 6 mL of hexane (Sigma-Aldrich), and females were placed in 12 mL of hexane for 5 min. Each extract was concentrated, first under reduced pressure at room temperature using a rotavapor (Hei-VAP, Heidolph, Schwabach, Germany), then with a constant flow of nitrogen to reach a final volume of 200 µL. Extractions were performed in the afternoon (3 h before the end of the photophase, which corresponds to peak pheromone emissions in other Dacinae species, Levi-Zada et al. 2020). For behavioural assays, we performed another test on a pooled sample of 250 males, 15 to 20 days old. They were placed in 25 mL of hexane for 5 min, the solution was then concentrated, as with the electrophysiology samples up to about 500 µL. This was further diluted at 0.1 or 0.033 v/v in mineral oil. Data for both dilutions were combined.

Chemical analyses

The analyses to identify and quantify compounds from the hexane body washes of males and females were performed using a LECO Pegasus 4D instrument (LECO Corp., St. Joseph, MI, USA), equipped with a non-moving quad-jet cryomodulator. A DB-5 column (J&W Scientific, Folsom, CA, USA; 30 m × 250 µm i.d. × 0.25 µm film) was used for GC in the first dimension. The second dimension analysis was performed on a polar BPX-50 column (SGE Inc., Austin, TX, USA; 2 m × 100 µm i.d. × 0.1 µm film). Helium was used as a carrier gas at a constant flow of 1 mL min^{-1}. The temperature programme for the primary GC oven was as follows: 80 °C for 2 min, then 80–300 °C at 10 °C min^{-1} and a 10 min hold at 320 °C. The programme in the secondary oven was 10 °C higher than in the primary oven and was operated in an iso-ramping mode. The modulation period, the hot-pulse duration and the cool time between the stages were set to 3.0, 0.4 and 1.1 s, respectively. The transfer line to the TOFMS was operated at 260 °C. The source temperature was 250 °C with a filament bias voltage of − 70 eV. The data acquisition rate was 100 Hz (scans s^{-1}) for the mass range of 29–400 amu. The detector voltage was 1750 V. For each sample, 1µL was injected in the splitless mode. The inlet temperature was 200 °C. The purge time was 60 s at a flow of 60 mL min^{-1}. The data was processed and visualized consecutively on 2D and 3D chromatograms using LECO

ChromaTOF™ software. First, we analysed a mixture of *n*-alkanes C_8-C_{32} (1×10^{-3} µg µL^{-1}, Sigma-Aldrich) under the given conditions, followed by the samples. LECO ChromaTOF™ is equipped with a retention index (RI) calculation function. The compounds were identified by comparing their MS fragmentation patterns, retention times and RI (Baker et al. 1982; Carlson and Yocom 1986; Nishida et al. 1990; Perkins et al. 1990; Goh et al. 1993; Vaníčková 2012; Vaníčková et al. 2014).

Synthesis of 2-methoxy-N-(3-methylbutyl) acetamide

All the synthetic compounds used in this study were purchased from Sigma-Aldrich, except 2-methoxy-N-(3-methylbutyl)acetamide, which is not commercially available. This compound was synthetized as follows. We prepared a solution of isoamylamine (0.096 g; 1.11 mmol), methoxyacetic acid (0.10 g; 1.11 mmol) and triethylamine (0.20 g; 2.0 mmol) in dry dichloromethane (5 mL), to which we added *N*,*N*,*N*′,*N*′-tetramethyl-*O*-(1*H*-benzotriazol-1-yl) uronium hexafluorophosphate (0.46 g; 1.22 mmol). The reaction mixture was stirred overnight and then quenched with the addition of a 5% aqueous solution of citric acid (5 mL). The organic layer was separated, washed with water (2 mL), brine (2 mL) and evaporated under reduced pressure. Standard column chromatography (silica gel, eluent ethyl acetate) of the residue yielded 0.16 g (90%) of the title compound; spectral data agreed with values found in the literature (Baker et al. 1982). ^{1}H NMR (401 MHz, $CDCl_3$) δ 3.90 (s, 2H), 3.43 (s, 3H), 3.36 – 3.23 (m, 2H), 1.81 – 1.57 (m, 1H), 1.49 – 1.38 (m, 2H), 0.94 (d, J = 6.6 Hz, 6H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.4, 72.0, 59.2, 38.4, 37.1, 25.8, 22.4. HRMS (ESI+): m/z calculated for $C_8H_{18}O_2N$ = 160.1332; found = 160.1331 $[M+H]^+$.

Odour delivery system

For electrophysiological experiments, a charcoal-filtered and humidified air stream (23 mL s^{-1}, air speed 60 cm s^{-1}) was continuously delivered to the fly antenna through a 7-mm glass tube held at 4 mm. Stimuli were applied by inserting a Pasteur pipette containing a small piece of filter paper, loaded with 1 µL of an odorant solution, 15 cm upstream. Odorant solutions consisted of compounds diluted in hexane or pure hexane for control stimulations. A puff of air (200 ms duration, 5 mL s^{-1}) was delivered through the pipette with a solenoid valve (LHDA-1233215-H, Lee Company, France), controlled by a digital output module (NI 9472, National Instr., Nanterre, France) and the software Labview (National Instr., France). For each experiment, the compounds were applied in random order. For each compound, a control stimulus (0 ng) was followed by three consecutive stimuli with an increasing quantity of the compound (1, 100 or 10,000 ng). Time intervals of 1 min were applied between consecutive puffs, except after the stimuli with 10,000 ng of a compound, where 2 min intervals were applied. We tested 2-methoxy-*N*-(3-methylbutyl)acetamide (2M3MA) in an independent set of experiments. This was systematically followed by stimulation with ethyl decanoate at a dose rate of 10^2 ng. The response was similar between the two sets of experiments (Wilcoxon's rank sum test, $p = 0.27$). The compounds used in this study were commercially available chemicals (chemical purity > 98%), purchased from Sigma-Aldrich.

For the GC-EAD_3 and GC-EPD experiments, tephritid antennae and palps were stimulated through a GC (Clarus 580, Perkin Elmer), equipped with a Rxi-5 ms column (Restek, 30 m × 0.32 mm i.d., 0.25 µm film) and injected with the hexane body washes. The temperature programme for the GC was as follows: 1 min at 70 °C, ramped up by 8 °C min^{-1} to 270 °C, hold 10 min. The compound identity was confirmed with a GC–MS injection with a similar phase column. The column was branched to a D-Swafer Dean's Switch (Perkin Elmer) to modulate the output between the flame ionization detector (FID) and the EAD_3 at 0.5 Hz, copying the chopped GC-EAD setup suggested by (Myrick and Baker 2018). The three solenoid valves used for modulation were controlled by the NI 9472 digital output module and the software LabView. The gauge pressure was set at 15 psi with nitrogen (as a makeup gas) in one branch of the D-swafer and measured at 12 psi in the other branch. The helium flow in the column was set at 2 mL min^{-1}, whereas the total flow in the odour delivery system was around 25 mL min^{-1} (N_2 + He).

Electrophysiology assays

EAG_3 and GC-EAD_3 were performed as in Ramiaranjatovo et al. (2023). Adult flies were secured in a plastic tube and their protruding head was fixed with dental wax, leaving the antennae and maxillary palp exposed. For EAG_3 and GC-EAD_3, the scapes (1st antennal segments, no olfactory functions) were also fixed with silicon adhesive (Kwik-Sil, WPI, Hitchin, UK). Glass capillary electrodes (tip diameter 1–2 µm) were filled with 120 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 4 mM $MgCl_2$ and 10 mM HEPES. The reference electrode was inserted in the right eye. For EAG_3 and GC-EAD_3, three recording electrodes, simultaneously placed against the left funiculus, were positioned latero-proximally, latero-distally and at the centre of the medial face. For EPG and GC-EPD, one single electrode was placed against the left maxillary palp. The signal was amplified (× 1000), band-pass filtered (0.1 Hz to 1 kHz) with a 4-channel differential amplifier (Model 1700, AM-Systems, Sequim, USA), and digitized at 500 Hz (NI 9215, National instr.), with the

Labview software. In all recordings, a control stimulus with 1-octen-3-ol (chemical purity > 98%, Sigma-Aldrich) was applied before and after the runs to ensure responsiveness.

Behavioural assays

The orientation preferences of mature naive virgin females (11 to 25 days after emergence, with no previous exposure to male odours), were tested in a six-choice olfactometer, replicated from Biasazin et al. (2019). Experiments were performed in the morning (3–4 h after the beginning of the photophase) or in the afternoon (2–3 h before the end of the photophase, shortly before the period of sexual activity in this species, Suzuki and Koyama 1980). Females tested in the afternoon were starved for 6 h, females tested in the morning were starved for 16 h. A schematic representation of the six-choice olfactometer is shown in Fig. 8A. Thirty females were released into an arena consisting of a glass cage (420 × 420 × 420 mm), with six circularly arranged traps at the top and ventilation holes on the five remaining sides (diameter 120 mm, covered with an insect proof mesh). The number of flies in each trap was counted 30 min after insertion. Each trap had a circular opening (diameter 50 mm), covered with a glass funnel (height 45 mm, basal diameter 60 mm) inside a trapping chamber, which consisted of a closed glass cylinder (diameter 70 mm, height 50 mm). The trapping chamber was connected through a small hole (diameter 5 mm) to an odorized chamber consisting of another glass cylinder (diameter 70 mm, height 50 mm). The setup was lit using six lightbulbs positioned above each trap and light was diffracted using an opaque white plexiglass panel. A charcoal-filtered and humidified air flow (0.5 L min^{-1}) was injected through each trap via the odorized chamber. An open Eppendorf containing a piece of filter paper, loaded with 10 µL of an odorant solution, was put inside the odorized chambers. The odorant solutions consisted of paraffin oil (control), a male whole body extract or a synthetic blend of TMP, 3MA, 2M3MA, ethyl decanoate, ethyl myristate and ethyl palmitate, in ratios of 15:100:25:2:2:5, respectively. Each part of the six-choice olfactometer was thoroughly cleaned between experiments, and the trap parts were deodorized in an oven (at 300 °C for 2 to 6 h). We performed two sets of experiments: first, we tested the male whole body extract vs. control, then we tested the synthetic blend vs. control. In each experiment, every other trap was assigned to each treatment, and we alternated the positions for each treatment between trials. The first fly entering a trap should make a choice solely based on the volatile samples tested, while subsequent insects could also use aggregation cues that might be multimodal. Thus, even though the assay tests an olfactory preference, the percent of trapped individuals cannot be interpreted as a percent of individuals attracted to the tested odour.

Data analysis

Relationships between the epicuticular composition of *Z. cucurbitae* and fly age or sex were analysed using multiple factorial analysis (MFA) (Escofier and Pagès 2008). Prior to the MFA, the peaks of the 45 compounds were integrated in LECO ChromaTOF™ software and exported. The relative peak areas, as well as absolute amounts of all the substances, were calculated. MFA analyses a set of observations, which are described by several groups of variables within the same framework, and normalizes individual data sets. The analysis generates an integrated picture of the observations and the relationships between the groups of variables. MFA was performed in two steps. First, a principal component analysis (PCA) was computed for each data set, which was then "normalized" by dividing all its elements by the square root of the first eigenvalue obtained from its PCA. Then, the normalized data sets were merged to form a single matrix and a global PCA was performed on the matrix. The individual data sets were then projected onto the global analysis to identify shared features and discrepancies. The coordinates of each variable are the correlation coefficients with the first two principal components. Compounds contributing significantly to MFA dimensions were used to explain differences between sexes and age groups (normal law adjustment test on compound correlation coefficients, $\alpha = 0.05$).

A heat map was used to visualize the complex data sets, that were organized as matrices. Thus, it was possible to identify differences in the relative amounts of CHs between males and females and age groups. Different compounds tend to form small clusters depending on quantity. The dendrograms were created using correlation-based distances and the Ward method of agglomeration was applied (Key 2012). To improve our understanding of the differences and similarities between individual age groups, we focused on the compounds used to construct factor maps. Compounds that were significantly correlated ($\alpha = 0.05$) to the first two dimensions were assigned in Table 1, by the corresponding correlation and *P*-values. In detail, 37 compounds were selected for their statistical relevance in the first dimension of MFA, while two compounds were chosen from the second dimension of MFA. All computations were performed with R 3.0.3 (R Core TEAM 2014); R packages *FactoMineR* (Le et al. 2008, 2013), and *gplots* (Warnes and et al. 2013) were used.

For quantifying the EAG_3 and EPG response amplitudes to synthetic compounds, the recordings were filtered with a Gaussian convolution with a width of 20 ms. The amplitude was defined as the maximum negative peak in the 0.5 s following stimulation, minus the average value in the 0.5 s preceding stimulation. The response to the first control stimulus was subtracted. The amplitudes were averaged for the three recording positions for EAG_3. The GC-EAD_3 and GC-EPD

Table 1 Compounds identified by GC×GC-TOFMS in the hexane body washes of virgin *Zeugodacus cucurbitae* males and females of four different age groups

No	Group[a]	Compound	RI[b]	Correlation[c]	*P*-value	Male				Female			
						day 5	day 15	day 25	day 30	day 5	day 15	day 25	day 30
1	a1	Decane[d]	1001	0.88	0.004	+	+	+	+	+	+	++	+
2	b1	δ-3-Carene[d]	1025	0.88	0.004	+	+	+	+	+	+	+	+
3	a2	4-Methyldecane[d]	1055	0.88	0.004	++	++	+	+	++	++	+++	++
4	b2	Tetramethylpyrazine[d]	1095	−0.96	0.0002	+	++++	++++	+++	−	−	−	−
5	a3	Undecane[d]	1102	0.88	0.004	++	+	+	+	++	+	++	++
6	b3	*N*-(3-Methylbutyl) acetamide[d]	1136	−0.96	0.0001	−	++++	++++	++++	−	++	−	−
7	a4	1,7-Dioxaspiro[5.5]undecane[d]	1152	0.73	0.039	−	+	+	−	++	++	++	++
8	a5	Dodecane[d]	1209	0.91	0.002	++	++	++	++	++	++	+++	++
9	b4	2-Methoxy-*N*-(3-methylbutyl)acetamide[d]	1271	−0.96	0.0002	−	++	+++	+++	−	−	−	−
10	a6	3-Methyldodecane[d]	1279	0.86	0.006	++	+	+	+	++	+	++	++
11	a7	Tridecane[d]	1302	0.84	0.009	+	+	+	+	+	+	+	+
12	a8	10-Methyltridecane[d]	1321	0.79	0.019	+	+	+	+	+	+	++	+
13	a9	11-/13-/15-Methyltridecane[d]	1339	0.84	0.009	+	+	+	+	+	+	+	+
14	c1	Ethyl decanoate[d]	1394	−0.96	0.0002	−	+	+	+	−	−	−	−
15	a10	Tetradecane[d]	1402	0.91	0.001	++	+	+	+	++	+	++	+
16	a11	Methyltetradecane[d]	1484	0.90	0.002	+	+	+	+	+	+	+	+
17	a12	Pentadecane[d]	1502	0.88	0.004	+	+	+	+	+	+	+	+
18	a13	11-/13-/15-Methylpentadecane[d]	1536	0.92	0.001	+	+	+	+	+	+	+	+
19	c2	Ethyl 4-hydroxybenzoate[d]	1693	−0.96	0.0001	−	+	+	+	−	−	−	−
20	c3	Ethyl tetradecanoate[d]	1816	−0.75	0.033	−	+	+	+	−	−	−	−
21	c4	Ethyl hexadecanoate[d]	2003	−0.95	0.0004	−	+	+	+	−	−	−	−
22	c5	Ethyl-9,12,15-octadecatrienoate[d]	2176	−0.92	0.001	+	+	+	+	−	+	−	−
23	c6	Ethyl octadecanoate[d]	2202	−0.98	0.00001	+	+	+	+	−	+	−	−
24	c7	Ethyl icosenoate[d]	2280	−0.95	0.0003	−	+	+	+	−	+	−	−
25	a14	Tricosane	2300	−	−	+	+	+	+	+	+	+	−
26	c8	Vinyl 2-ethylhexanoate	2411	−	−	+	+	+	+	+	+	+	+
27	a15	7,11-Dimethyltricosane[d]	2472	0.89	0.003	++	+	+	+	++	+	+	++
28	a16	4,6-/4,10-Dimethyltricosane[d]	2503	0.88	0.004	++	+	+	+	++	++	++	++
29	a17	10-/12-Methylpentacosane[d]	2523	0.88	0.004	++	+	+	+	++	++	++	++
30	a18	9,13-Dimethyltetracosane[d]	2543	0.90	0.002	++	++	+	+	++	++	++	++
31	b5	Squalene[e]	2554	−0.97	0.0001	+	+	+	+	+	+	+	+
32	a19	2-Methylpentacosane[d]	2564	0.88	0.004	++	++	+	++	+++	++	++	+++
33	a20	Methylpentacosane	2585	−	−	++	++	++	++	++	++	++	++
34	a21	Hexacosane[d]	2605	0.90	0.002	+++	++	++	++	+++	+++	+++	++++

Table 1 (continued)

No	Group[a]	Compound	RI[b]	Correlation[c]	*P*-value	Male				Female			
						day 5	day 15	day 25	day 30	day 5	day 15	day 25	day 30
35	a22	11-/13-/15-Methylhexacosane[d]	2625	0.93	0.001	++++	++++	++++	++++	++++	++++	++++	++++
36	a23	Unknown[d]	2656	0.80	0.016	+	+	+	+	+	+	+	+
37	a24	12-/14-Methylhexacosane[d]	2677	0.94	0.0004	++++	++++	+++	+++	++++	++++	++++	++++
38	a25	Heptacosane	2708	−	−	+	+	+	+	+	+	+	+
39	a26	11-/13-/15-Methylheptacosane	2728	−	−	+	+	+	+	+	+	+	−
40	a27	Methylheptacosane[d]	2748	0.91	0.002	+	+	+	+	+	+	+	+
41	a28	Methylheptacosane[d]	2789	0.92	0.001	+++	++	++	++	++	+++	++	+++
42	a29	10-/12-/14-Methyloctacosane	2830	−	−	+	+	+	+	+	+	++	+
43	a30	12,14-Dimethylheptacosane[d]	2860	0.91	0.002	++	+	+	+	++	++	++	++
44	a31	Nonacosane[d]	2900	0.91	0.002	++	+	+	+	++	++	++	++
45	a32	Methylnonacosane[e]	2993	−0.76	0.0001	−	+	+	+	−	+	+	+

[a]Group in which were the compounds separated acording to their chemical character, a – cuticular hydrocarbons, b – non-hydrocarbon compounds, c – esters

[b]Retention index on DB-5 column

[c]Correlation coefficient to the corresponding axis

[d]Compound significantly correlated ($\alpha = 0.05$) to the first dimension of the individual factor map (Fig. 3)

[e]Compound significantly correlated ($\alpha = 0.05$) to the second dimension of the individual factor map (Fig. 3).+ ≤ 1%;++ < 5%;+++ < 10%;++++ ≥ 10%

recordings stimulated with GC were downsampled at 10 Hz. The EAD_3 and EPD recordings were demodulated at 0.5 Hz (frequency of modulation of the GC output) through a Morlet's wavelet transform (cran R package "*Rwave*", function vwt). The demodulated EAD_3 was then averaged for the three positional recordings. The amplitude of responses was defined as the maximum value of the demodulated EAD_3 or EPD in 5 s time windows, manually time-locked on the FID peaks and subtracted with a reference level. The reference level was defined as the median of maximum value of demodulated EAD_3 or EPD in 5 s moving windows encompassing the entire GC run. Signal to noise ratio was estimated with z statistics, z being the amplitude of responses divided by the standard deviation of the maximum values in 5 s moving windows encompassing the entire GC run.

In all the electrophysiological and behavioural data, bootstrap statistics on median values were used, including the 95% confidence interval (c.i.), unless stated otherwise (number of repetitions above 1000). For GC-EPD response to female whole body extract, males (n = 3) and females (n = 5) were combined for bootstrapping. In this case, sexual difference was not assessed statistically. Dose-sensitivity of EAG_3 antennal responses was tested with an ANOVA and the different doses were considered as factors. The inter-species comparison of GC-EAD_3 responses to male *Z. cucurbitae* extract was qualitative, since the number of repetitions is too low for statistical analysis. For behavioural data, we tested if the number of trapped females depended on treatment using a generalized mixed linear model (glmm, Poisson family) with trap position, experimental replicate (each replicate is a set of catches from six traps sampled simultaneously) and timing (morning or afternoon) as random factors. Independently, the percentage of trapped females per replicate was tested with bootstrap statistics.

Results

Cuticular hydrocarbons of Z. cucurbitae *vary with sex and age*

The chemical analyses of whole body extracts of virgin males and females of *Z. cucurbitae* identified 45 compounds, consisting of a chemically diverse mixture of 10 linear and 22 methyl branched hydrocarbons, seven esters, a pyrazine, two amides, two terpenes, and one unidentified compound (Table 1). The chain-length of the carbon backbones ranged from C_{10} to C_{30}. The most prominent peaks identified in all chromatograms were hexacosane (34 in Table 1 and Fig. 1, a21 in Fig. 2), a mixture of 11-/13-/15-methylhexacosanes (35, a22), and mixture of 12-/14-methylhexacosanes (37, a24) (Figs. 1, 2).

A comparison of the chemical profiles of virgin males and females revealed qualitative and quantitative differences in the composition of the hexane body washes. Male compounds were tetramethylpyrazine (TMP) (4, b2), 2-methoxy-*N*-(3-methylbutyl)acetamide (2M3MA) (9, b4), ethyl decanoate (14, c1), ethyl 4-hydroxybenzoate (19, c2), ethyl tetradecanoate (20, c3), and ethyl hexadecanoate (21, c4). We constructed a heat map to visualize quantitative patterns across all identified compounds and age groups (Fig. 2). Compounds from the same chemical class formed small clusters (Fig. 2). TMP (b2), 3MA (b3), and 2M3MA (b4) were present in higher concentrations in older males (15, 20, 30 days old) compared to young ones (5 days old) (Fig. 2).

In females, older flies (15 and 25 days old) had higher amounts of 10-/12-/14-methyloctacosanes (a29) compared to younger females or males (5 days old) (Fig. 2). Interestingly, minute amounts of the female pheromone constituent DSU (a4) were identified in sexually mature males (15 and 25 days old) (Table 1, Fig. 2).

Significantly different chemical patterns were confirmed in both sexes when running multiple factorial analysis (MFA), based on 45 compounds classified in three chemically distinct groups (Fig. 3). Factorial axis 1 (72.68% of variance) separated the samples according to sex, whereas the second axis (11.63% of variance) separated female samples according to age (Fig. 3 A). CHs were generally positively correlated to the first dimension, whereas the esters and three non-hydrocarbon compounds (b2, b3, b4) were negatively correlated. However, squalene (b5) was the main compound responsible for the separation in the second axis (Fig. 3 B).

Zeugodacus cucurbitae *antennae are sensitive to male compounds*

A pooled whole body extract of 40 mature virgin males of *Z. cucurbitae* was analysed using chopped GC-EAD_3 (Fig. 4). For eight mature female and seven mature male *Z. cucurbitae*, electroantennograms were simultaneously recorded and averaged for three positions scanning the funiculus surface. Both female and male EAD_3 responded significantly to 2M3MA (females: z = 2.18, c.i. 1.57—3.02, $p < 0.001$ bootstrap; males: z = 1.47, c.i. 0.03—1.97, $p = 0.03$ bootstrap). Response to ethyl decanoate was significant in males (z = 0.57, c.i. − 0.3, − 0.96, $p = 0.03$ bootstrap), but not in females (z = 0.41, c.i. − 0.17 − 0.94, $p = 0.27$ bootstrap). However, there was no significant difference between sexes ($p = 0.77$ bilateral bootstrapped difference in median). Females responded significantly to TMP (z = 0.4, c.i. 0.24—0.56, $p = 0.008$ bootstrap), but males did not (z = 0.01, c.i. − 0.12 − 0.16, $p = 0.33$ bootstrap); the difference between sexes was significant ($p = 0.023$ bilateral

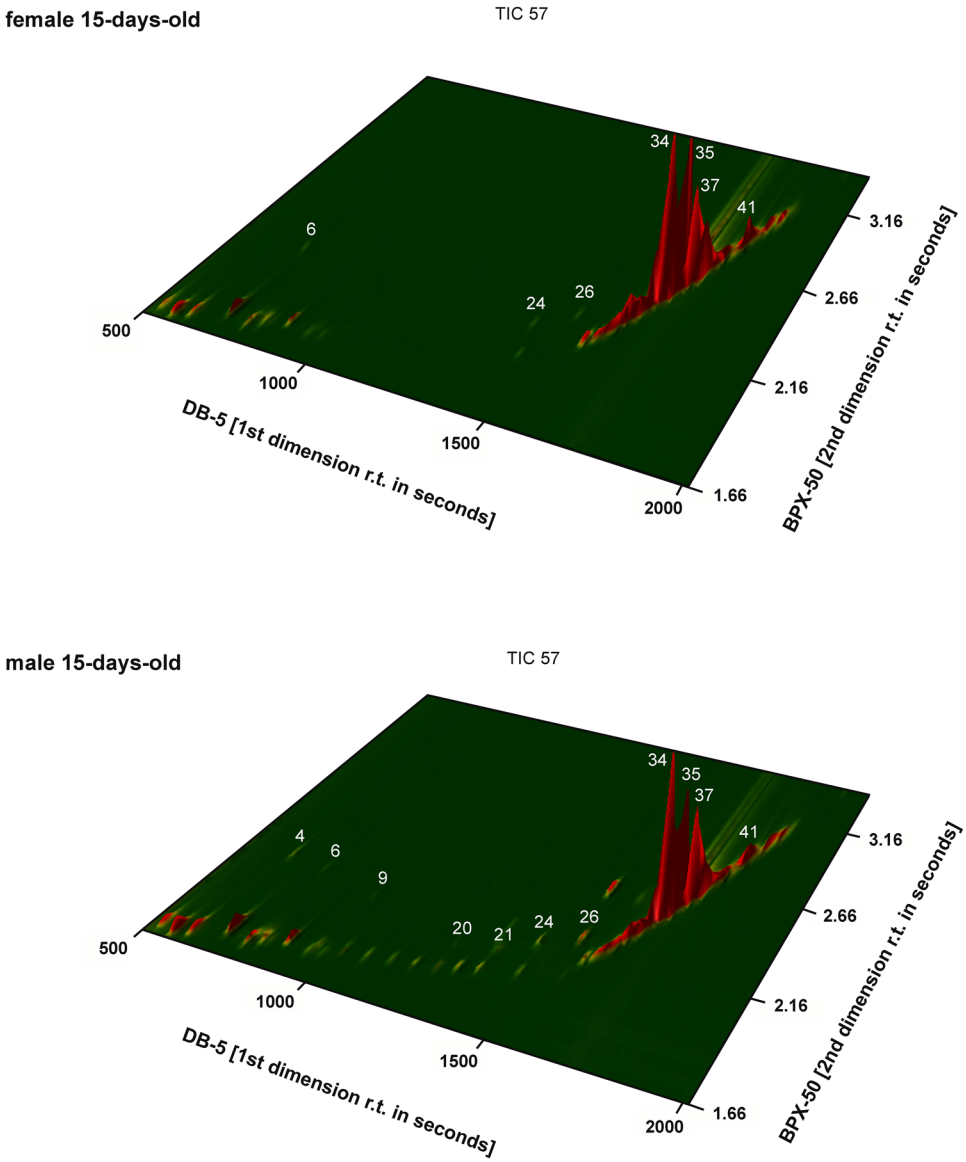


Fig. 1 GC × GC-TOFMS analysis of the cuticle body washes from 15-day-old *Zeugodacus cucurbitae* females and males. The intensity of the signals is colour-coded from blue (zero) to red (maximum). The numbers correspond to the compounds listed in Table 1

bootstrapped difference in median). A small response to ethyl paraben (z = 0.38, c.i. – 0.06 –0.71, $p = 0.025$ bootstrap) was observed in males and a small response to ethyl laurate (z = 0.18, c.i. 0.04 – 0.24, $p = 0.022$ bootstrap) was noted in females.

The whole body extract of males was then analysed using chopped GC-EAD$_3$ performed in nine 7-day-old males, which were not sexually mature. These males responded to 2M3MA (z = 1.33, c.i. 1.12—3.08, $p < 0.001$ bootstrap), ethyl decanoate (z = 0.88, c.i. 0.21—1.29, $p < 0.001$ bootstrap), ethyl paraben (z = 0.30, c.i. 0.12—0.35, $p < 0.001$ bootstrap) and ethyl laurate (z = 0.14, c.i. 0.07—0.28, $p = 0.01$ bootstrap). The response patterns in these males were similar to mature males, except their response to 2M3MA, which was greater ($p = 0.042$ bilateral bootstrapped difference in median).

Zeugodacus cucurbitae *maxillary palps, but not antennae are sensitive to DSU produced by females*

A pooled whole body extract of mature *Z. cucurbitae* females was analysed using chopped GC-EAD$_3$ (Fig. 5). Electroantennograms were recorded simultaneously and averaged for the three antennal positions of six mature males and four mature females. A significant response to 2M3MA was observed in both females and males (z = 1.25, c.i. 0.53—1.42, $p < 0.001$ bootstrap, female and males combined n = 10).

GC-EPD recordings were then carried out in mature individuals to evaluate the responses evoked by whole body extracts (Fig. 6). The analysis of the mature female body extract revealed a strong palp response to the female

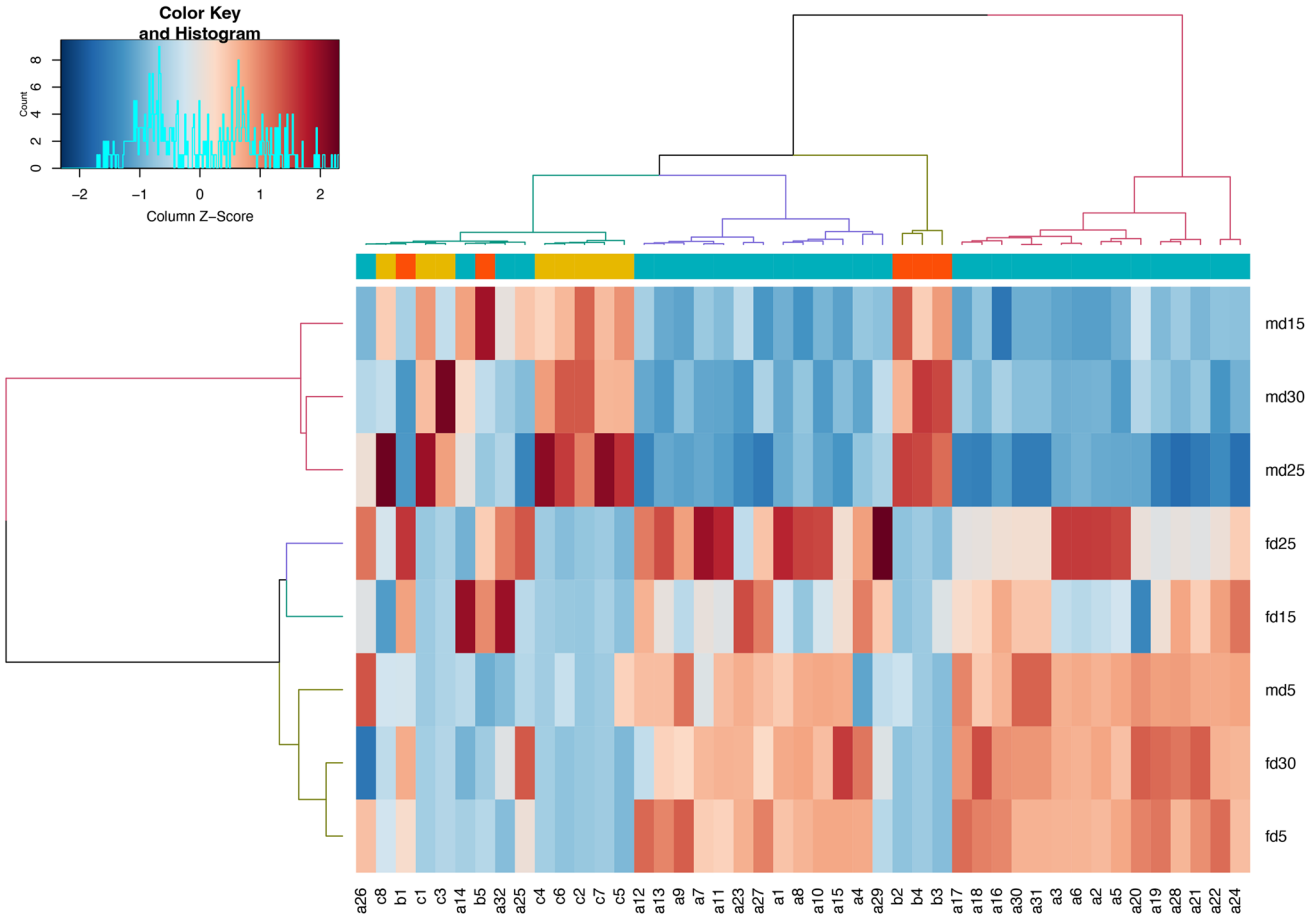


Fig. 2 Heat map of the 45 compounds (columns) and the four age groups (5, 15, 25, 30 days old) of *Zeugodacus cucurbitae* males (m) and females (f) (rows) from GC × GC-TOFMS dataset. Colour patterns of the columns differentiate chemical classes of compounds (turquoise = cuticular hydrocarbons a1-32, orange = nonhydrocarbon compounds b1-5, yellow = esters c1-c8). The dendrograms were created using correlation-based distances and the Ward method of hierarchical clustering ($p < 0.05$)

compound DSU (z = 6.77, c.i. 4.51—10.00, $p < 0.001$ bootstrap, both sexes combined, n = 8), with no obvious difference between sexes (Fig. 6). While analysing the mature male body extract with GC-EPD, a small but significant response was observed in males (z = 1.39, c.i. 0.15—2.56, $p < 0.001$ bootstrap, n = 8), but not in females (z = 0.27, c.i. − 0.05 − 2.5, $p = 0.31$, bootstrap test, n = 9). The response levels of EPD to male body extracts did not differ significantly between males and females at the timing of DSU release ($p = 0.32$, bilateral bootstrapped difference in median).

Stimulations with synthetic compounds suggest that Z. cucurbitae *lacks sensitivity to 3MA*

The antennae and palps of mature females and males of *Z. cucurbitae* were stimulated with synthetic compounds at different doses while recording EAG_3 and EPG (Fig. 7). Electroantennogram response patterns did not vary with the position of recording, thus recordings at the three positions were averaged for each fly. Dose-sensitive antennal responses were found to TMP (females: $F_{3,24} = 10.03$, $p < 0.001$; males $F_{3,20} = 7.17$, $p = 0.002$), DSU (females: $F_{3,24} = 5.31$, $p < 0.01$; males $F_{3,20} = 5.46$, $p < 0.01$), 2M3MA (females: $F_{3,20} = 13.1$, $p < 10^{-4}$; males $F_{3,20} = 25.9$, $p < 10^{-6}$) and to ethyl decanoate (females: $F_{3,24} = 17$, $p < 10^{-5}$; males $F_{3,20} = 20.06$, $p < 10^{-5}$). A small dose-sensitive response to ethyl myristate was observed in males ($F_{3,20} = 4.91$, $p = 0.01$), but not in females ($F_{3,24} = 1.16$, $p = 0.35$). No response was observed to 3MA (females: $F_{3,24} = 0.61$, p = 0.61; males $F_{3,20} = 0.15$, $p = 0.93$)

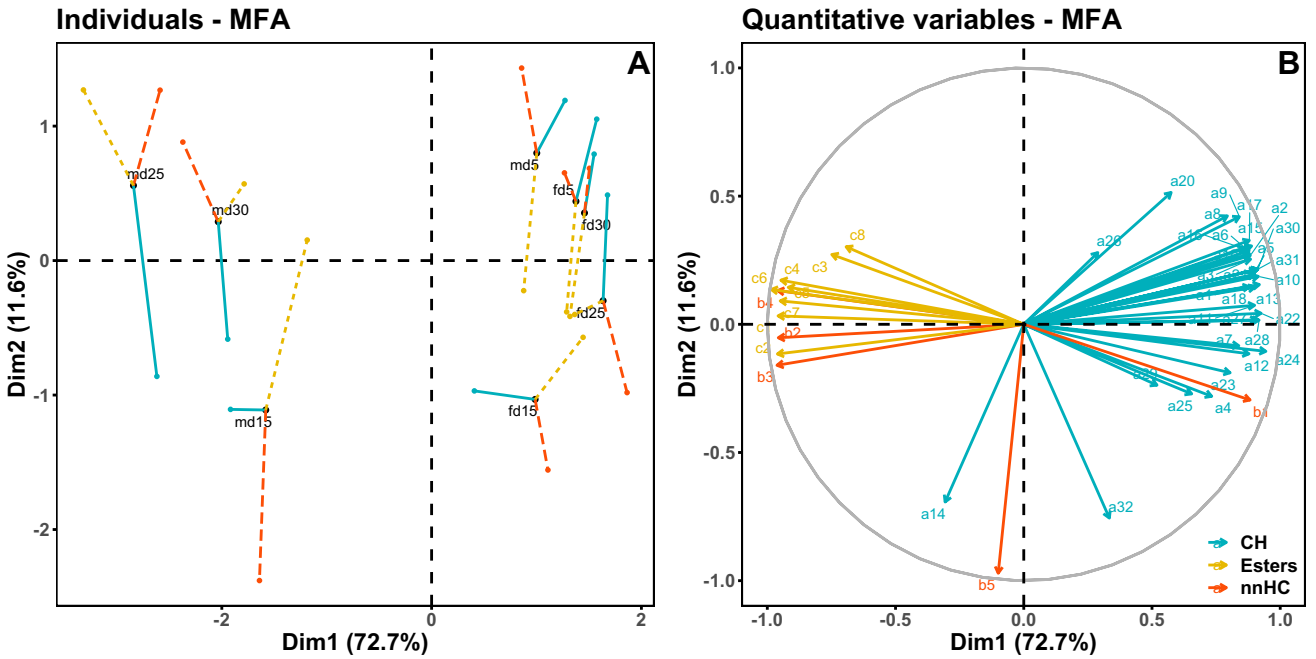


Fig. 3 Multiple factor analyses (MFA) of transformed GC×GC-TOFMS data of 45 compounds identified in *Zeugodacus cucurbitae* males and females from 4 age groups. **A** Score plot describing the age groups and variables (chemical classes). **B** Vector representation of the contribution of each compound to the distinction of age groups and sexes. The coordinates of each variable are the correlation coefficients with the two first principal components. Key: md5-30—male 5 to 30 days old, fd5-30—female 5 to 30 days old, turquoise CH a1-32—cuticular hydrocarbons, orange nnHC b1-5—nonhydrocarbon compounds, yellow esters c1-8—esters, respectively

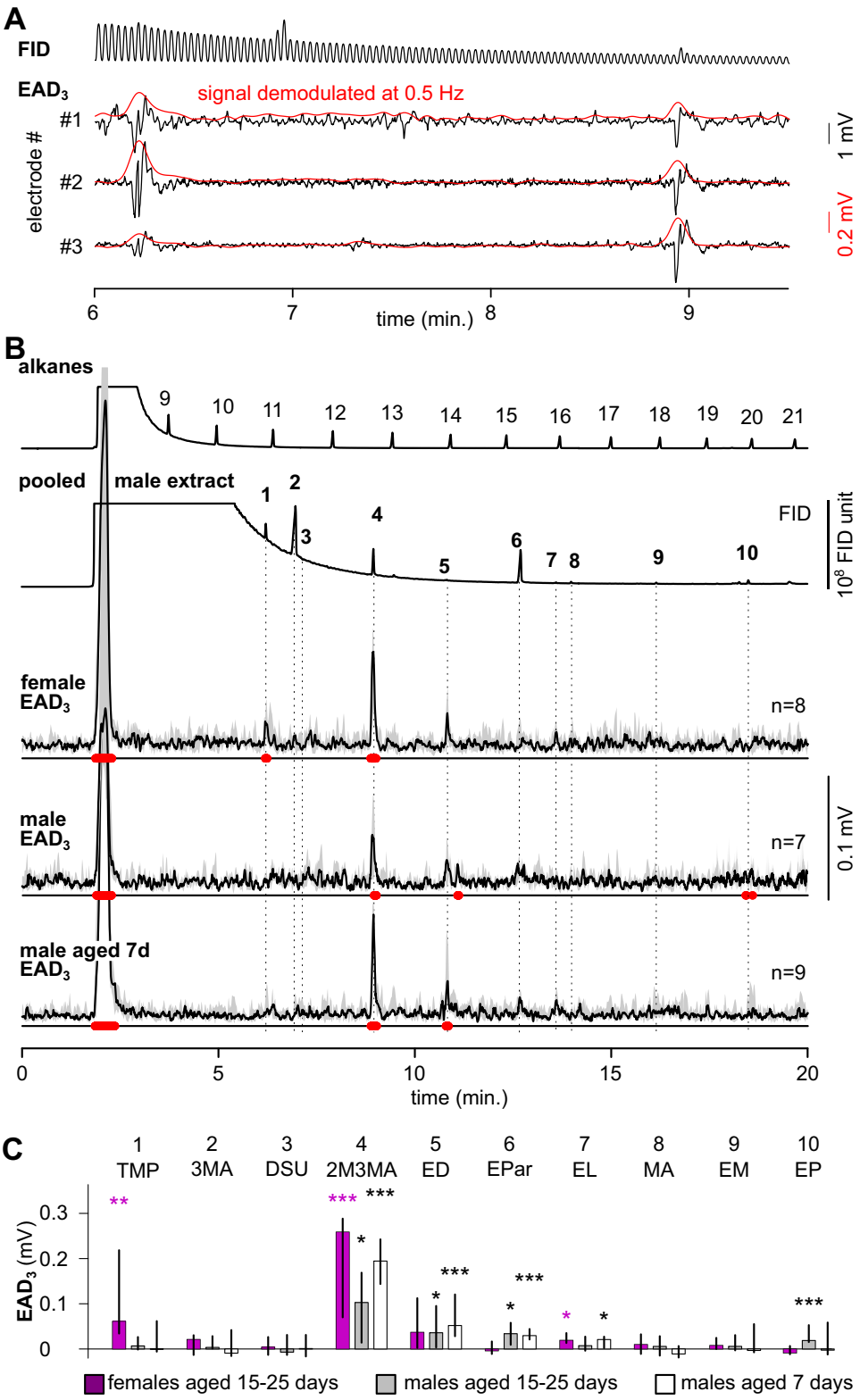
or ethyl palmitate (females: $F_{3,24}=1.01$, $p=0.41$; males $F_{3,20}=0.15$, $p=0.93$). For all compounds and doses tested, no significant difference was observed between sexes (bilateral bootstrapped difference in median, $p>0.1$ for all cases). To ensure that the responses were not due to impurities, we performed a GC-EAD$_3$ analysis of an iso-massic solution of synthetic compounds with a female *Z. cucurbitae* and confirmed a qualitative response to TMP, 2M3MA, ethyl decanoate and ethyl myristate. The palps of both females and males responded to DSU and appear to be dose sensitive (females: $F_{3,20}=10.45$, $p<0.001$; males $F_{3,20}=6.20$, $p<0.01$). The other compounds tested did not elicit palp responses.

Natural and synthetic male blends attract virgin females of Z. cucurbitae

The mature virgin females' attraction to the male extract was tested in a six-choice olfactometer in morning and afternoon experiments (Fig. 8). After 30 min, 25% (c.i. 11.7—33.3%, n = 20) of the females were trapped, of which 60% (c.i. 52—77.5%) were trapped using the natural extract. More females were trapped using the natural extract than with the control solution (glmm Poisson family, $p<0.05$). The time of day had no clear effect (random effect, p = 0.2; 57% and 67% of trapped females were attracted to the natural extract in the morning and afternoon, respectively). We then tested the attractiveness of a synthetic blend of TMP, 3MA, 2M3MA, ethyl decanoate, ethyl myristate and ethyl palmitate in respective ratios of 15:100:25:2:2:5 (FID peak amplitude), diluted at 100 ng/µL in paraffin oil. The ratios were chosen to mimic the natural extract. We found that 36.7% (c.i. 23.3 – 50%, n = 21) of the females were trapped, of which 62.5% (c.i. 53.9 – 73.3%) were attracted to the synthetic blend. More females were trapped using the synthetic blend than the control solution (glmm Poisson family, $p<0.01$) and the time of day had no effect (random effect, $p=0.18$; in the morning and afternoon, respectively, 64.6% and 62.5% of females were trapped using the synthetic blend).

The olfactory sensitivity to Z. cucurbitae *hydrocarbons is species-specific*

To evaluate the species-specificity of the epicuticular compound detection, chopped GC-EAD$_3$ assays were conducted to compare the antennal sensitivity of *Z. cucurbitae*, *D. demmerezi* and *B. dorsalis* females to a whole body extract of *Z. cucurbitae* males (Fig. 9). The responses to TMP and ethyl decanoate noted in *Z. cucurbitae* were not observed in the other species tested. *Bactrocera dorsalis* antennae responded



to 2M3MA (z = 0.96, 1.05, 0.94 and 0.96). This was the case for *Z. cucurbitae* (z = 2.18, c.i. 1.57—3.02), but not for *D. demmerezi* (z = 0.66, 0.63 and − 0.71). A response to 3MA was observed in *B. dorsalis* (z = 0.58, 0.72, 0.29 and 0.23), but not in *Z. cucurbitae* (z = 0.08, c.i. − 0.23 − 0.26), while a response to ethyl paraben was observed in *D. demmerezi* (1.21, 1.52 and 1.14), but not in *Z. cucurbitae* (z = − 0.12, c.i. − 0.33 − 0.25).

◂**Fig. 4** GC-EAD_3 analysis of the whole body extract of *Zeugodacus cucurbitae* males. **A** Typical recording in a *Z. cucurbitae* female. The modulation at 0.5 Hz of the FID recording includes the tail of the solvent peak (hexane), superimposed with three FID peaks. Below, electroantennograms are simultaneously recorded at three antennal positions (black). The demodulated signal at 0.5 Hz is also included (red). The three signals are averaged for subsequent analysis. **B** Population analysis. A mixture of alkanes was injected in the GC to be used at reference times and the number of carbons corresponding to each peak is indicated. The FID signal corresponds to an independent injection of the whole body extract without modulating the GC output. Below, the EAD_3 time plots show the median (black line) and 95% bootstrap c.i. (grey area) of demodulated EAD_3, averaged for the three recording positions. The three-time plots correspond to the mature female, mature male and immature male, respectively. The number of flies is indicated for each plot. Thick red lines below the time plots show the timing when a significant EAD_3 signal was observed ($p < 0.05$, bootstrap comparison with a threshold defined as mean + 3 SE of the mean EAD_3 signal during the first 90 s of the runs). The significant peaks at 2 min are a response to the solvent hexane. Each peak is identified with a number and the timing of the analysed response is indicated with dashed lines. **C** The bar plot shows median and 95% bootstrap c.i. of EAD_3 quantified at the timings corresponding to the 10 identified compounds. TMP: tetramethylpyrazine; 3MA: *N*-(3-methylbutyl)acetamide; DSU: 1,7-dioxaspiro[5.5]undecane; 2M3MA: 2-methoxy-*N*-(3-methylbutyl) acetamide; ED: ethyl decanoate; EPar: ethyl paraben; EL: ethyl laurate; MA: myristyl aldehyde; EM: ethyl myristate; EP: ethyl palmitate. * $p < 0.05$, Response significance is indicated: ** $p < 0.01$, *** $p < 0.001$, bootstrap

Discussion

Our investigation of the epicuticular chemical composition of *Z. cucurbitae* revealed variability depending on age and sex. We found six epicuticular compounds that elicit neuronal responses on the antenna or maxillary palp, the fly two olfactory organs. This suggests they play a role in intra-specific communication. A blend including synthetic standards of these compounds with a natural ratio attracted *Z. cucurbitae* females in a six-choice olfactometer. Thus, the melon fly can be added to the list of Tephritidae species in which females have been observed to be attracted to a synthetic pheromone blend. These species include *Anastrepha fraterculus* (Milet-Pinheiro et al. 2015), *Anastrepha ludens* (Loew) (Robacker and Hart 1985), *Anastrepha suspensa* (Loew) (Nation 1975), *Anastrepha obliqua* (Macquart) (De Aquino et al. 2021), *Ceratitis capitata* (Wiedemann) (Heath et al. 1991; Jang et al. 1994; Light et al. 1999), *Bactrocera carambolae* Drew & Hancock (Wee and Tan 2005), *B. oleae* (Carpita et al. 2012; Canale et al. 2013, 2015), and *B. dorsalis* (Hee and Tan 1998; Khoo et al. 2000).

Here, we demonstrated that the chemical composition of circular profiles of virgin males and females of *Z. cucurbitae* differs qualitatively and quantitatively. These findings are in partial agreement with earlier research by Goh et al. (1993) and Carlson and Yocom (1986), who identified CHs up to C_{36} in males and females of two populations of *B. dorsalis* complex (from Malaysia) and one population of *Z. cucurbitae* (from Hawaii). *Zeugodacus cucurbitae* from Hawaii were characterized by the presence of 11-/13-/15-methylnonacosanes, 3-methylnonacosane and triacontane on their cuticle (Carlson and Yocom 1986), whereas the prominent chromatographic peak identified here was composed by a mixture of methylhexacosanes. Variations in the cuticle chemical profiles of the same insect species among geographically distinct populations have been documented for several groups, including termites (Haverty et al. 1997, 2000), ants (Blight et al. 2012; Fox et al. 2012; Buellesbach et al. 2018), wasps (Bonelli et al. 2015), and drosophilid and tephritid flies (Alves et al. 2010; Bontonou et al. 2013; Jennings et al. 2014; Vanickova et al. 2015a; 2015c). Geographic variability is thought to be driven by an adaptation of desiccation resistance to climatic conditions. Recent studies of the populations belonging to the same tephritid species pointed out that the cuticle chemical profiles are also influenced by geoclimatic conditions, such as relative humidity, altitude and relative temperature (Vaníčková et al. 2015c, b, a).

In addition to the differences identified in linear and methyl branched alkanes, the content of amides and fatty acid esters in the cuticle washes of *Z. cucurbitae* varied depending on age and sex. *Zeugodacus cucurbitae* male flies produce relatively small quantities of 3MA, 2M3MA, methyl, ethyl, and propyl 4-hydroxybenzoate, and a large quantity of 1,3-nonanediol endogenously in their rectal glands (Ohinata et al. 1982; Baker and Bacon 1985; Nishida et al. 1990; Perkins et al. 1990; Fletcher and Kitching 1995; Tan 2000). The quantities stored in the rectal glands start to increase with age from two weeks after emergence (Nishida et al. 1993). Our study revealed that the production of male pheromone components on the cuticle increased with age, and that young 5-day-old males did not produce these compounds, i.e., 3MA and ethyl esters of decanoic, tetradecanoic and hexadecanoic acids. However, we did not observe any differences in the antennal sensitivity of 7-day-old and mature males, suggesting that the chemoreception for these compounds already functions 7 days after emergence.

Several components identified here, namely TMP, 3MA and series of ethyl esters, are putative sex attractants for *Z. cucurbitae* females (Baker et al. 1982; Fletcher 1989; Nishida et al. 1990; Perkins et al. 1990; Fletcher and Kitching 1995). 3MA has been suggested to elicit activation and increase flight activity in *Z. cucurbitae* females (Baker et al. 1982), but the exact role of the other components has not been identified (Fletcher and Kitching 1995). The electrophysiological data included here highlight their relative importance for the chemical communication of *Z. cucurbitae*. In decreasing order, we observed an electrophysiological response to 2M3MA, ethyl decanoate and TMP, with no clear difference between sexes. Furthermore, we

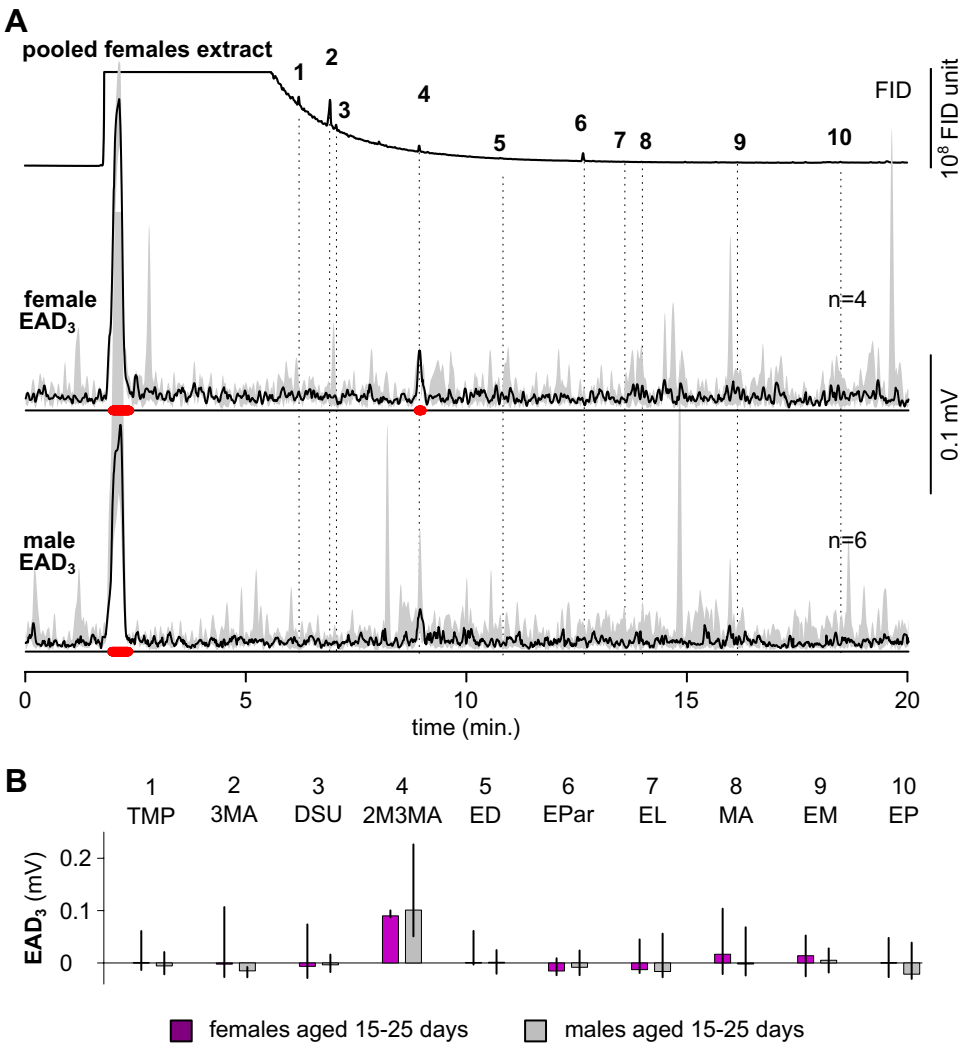


Fig. 5 GC-EAD_3 analysis of the whole body extract of *Zeugodacus cucurbitae* females. **A** Population analysis. The FID signal corresponds to an independent injection of the whole body extract without modulating the GC output. Below, the EAD_3 time plots show the median (black line) and 95% bootstrap c.i. (grey area) of demodulated EAD_3 averaged for the three recording positions. The two-time plots correspond to the mature female and mature male, respectively. The number of flies is indicated for each plot. Thick red lines below the time plots show the timing when a significant EAD_3 signal was observed ($p < 0.05$, bootstrap comparison with a threshold defined as mean + 3 SE of the mean EAD_3 signal during the first 90 s of the runs). The significant peaks at 2 min were a response to the solvent hexane. Each peak is identified with a number and the timing of the analysed response is indicated with dashed lines. **B** The bar plot shows median and 95% bootstrap c.i. of EAD_3 quantified at the timings corresponding to the 10 identified compounds. TMP: tetramethylpyrazine; 3MA: *N*-(3-methylbutyl)acetamide; DSU: 1,7-dioxaspiro[5.5]undecane; 2M3MA: 2-methoxy-*N*-(3-methylbutyl) acetamide; ED: ethyl decanoate; EPar: ethyl paraben; EL: ethyl laurate; MA: myristyl aldehyde; EM: ethyl myristate; EP: ethyl palmitate. * $p < 0.05$, Response significance is indicated: ** $p < 0.01$, *** $p < 0.001$, bootstrap

detected a weak EAG_3 response to ethyl myristate and no response to ethyl palmitate, which could be due to their lower volatility. On the other hand, 3MA did not elicit antennal or palp responses. Nonetheless, this compound might have a pheromonal role, given that some activation of the olfactory sensory neurons can occur even in the absence of an EAG response (Blight et al. 1995). However, the use of three points for electroantennography and of EPG allowed us to scan the activity of most of the insect olfactory receptor neurons (Jacob 2018; Ramiaranjatovo et al. 2023), and reduces the probability of missing an olfactory response.

DSU was first reported by (Baker and Bacon 1985) in the aeration extracts from melon fly females. Our study confirmed DSU as the main female compound. We observed that both a synthetic spiroacetal and fly-borne DSU are detected predominantly by olfactory receptor neurons located in the maxillary palps, notwithstanding a small antennal response. The involvement of palps was also reported for the detection of the male attractants methyl eugenol (Chieng et al. 2018)

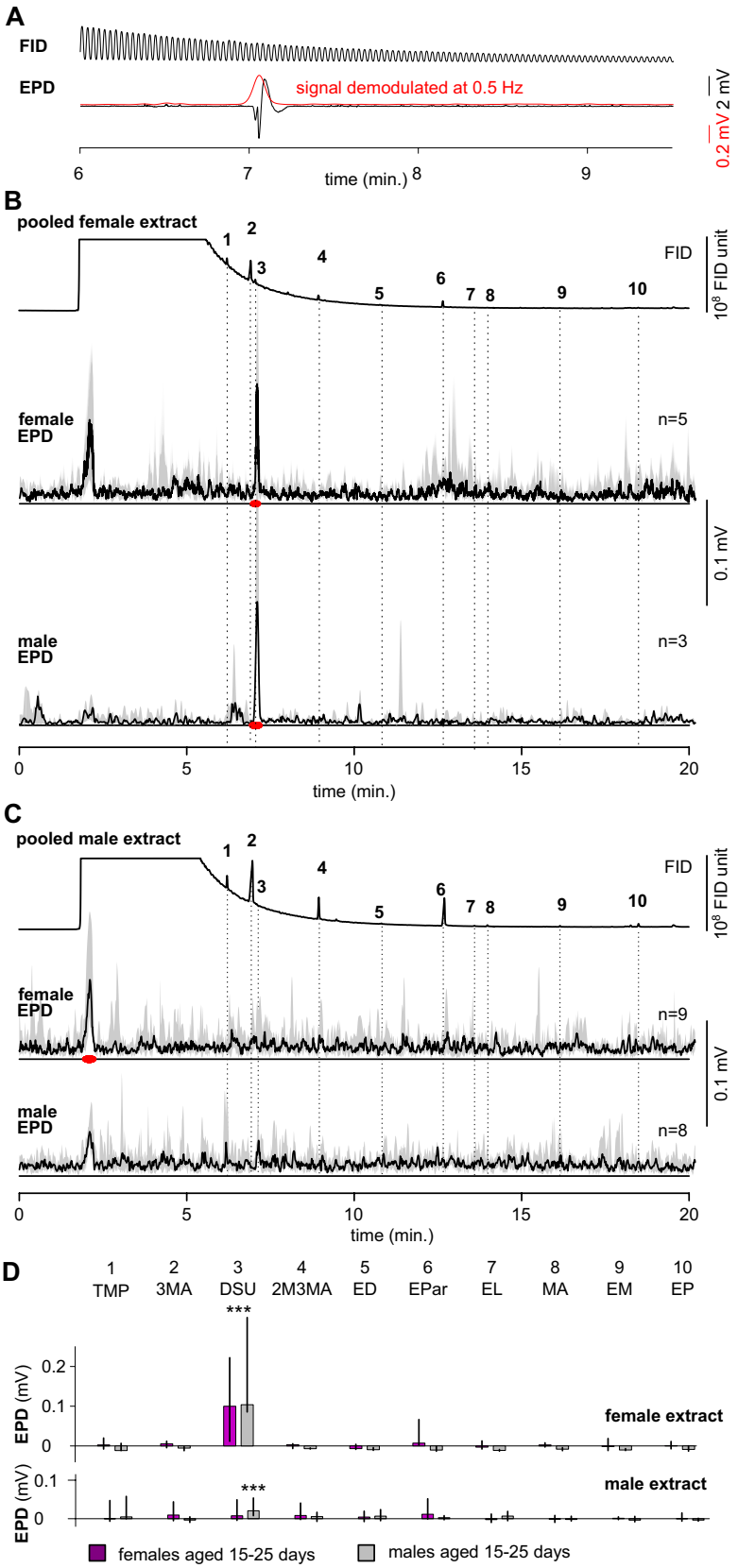


Fig. 6 GC-EPD analysis of the whole body extract of *Zeugodacus cucurbitae* females and males. **A** Typical recording in a male. FID peaks are barely visible due to the superimposition with the tail of the solvent peak (hexane). Same convention as in Fig. 4A. **B** GC-EPD of *Z. cucurbitae* females and males in response to the whole body extract of a female. Same conventions as in Fig. 4. **C** GC-EPD *Z. cucurbitae* females and males in response to whole body extract of a male. Same conventions as in Fig. 4. **D** The bar plot shows median and 95% bootstrap c.i. of EPD quantified at the timings corresponding to the 10 identified compounds. TMP: tetramethylpyrazine; 3MA: *N*-(3-methylbutyl) acetamide; DSU: 1,7-dioxaspiro[5.5]undecane; 2M3MA: 2-methoxy-*N*-(3-methylbutyl) acetamide; ED: ethyl decanoate; EPar: ethyl paraben; EL: ethyl laurate; MA: myristyl aldehyde; EM: ethyl myristate; EP: ethyl palmitate. * $p < 0.05$, Response significance is indicated: ** $p < 0.01$, *** $p < 0.001$, bootstrap. For female whole body extract (top), female and male responses were combined to calculate *p*-value (***) due to low number of tested flies

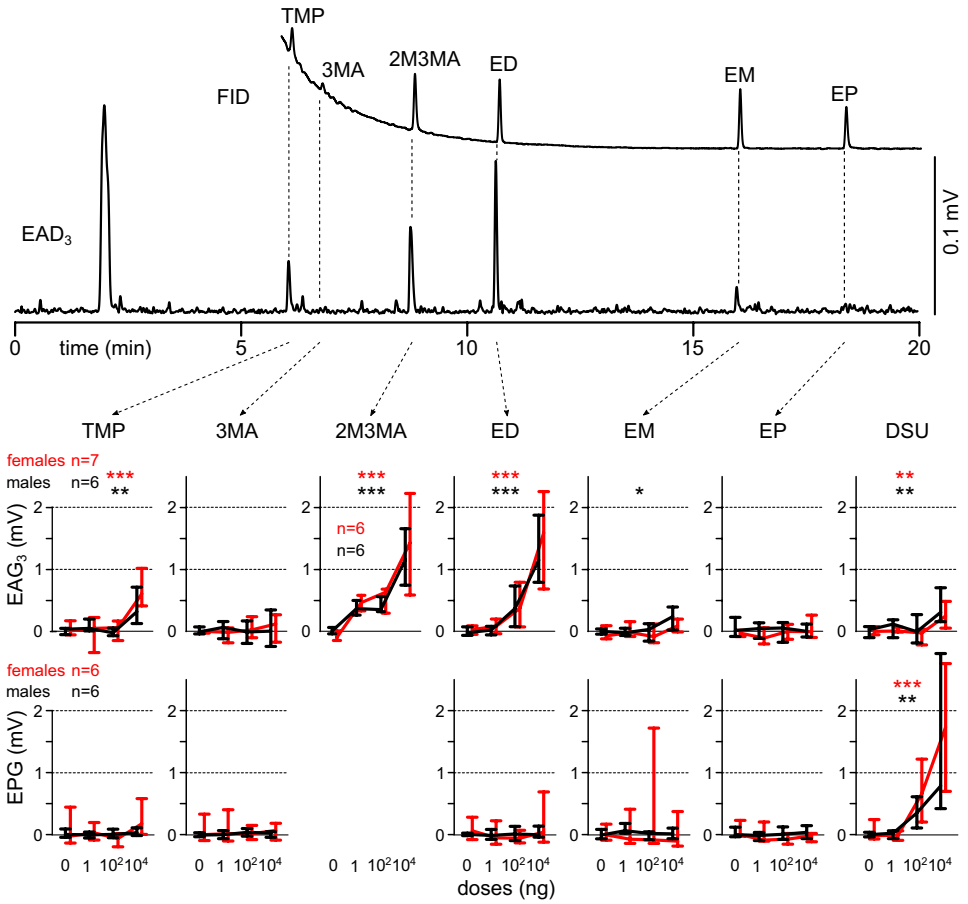


Fig. 7 Dose–response curves of EAG_3 and EPG in mature *Zeugodacus cucurbitae* to synthetic compounds. **A** For each sex, each tested compound and dose, the graph shows the median and 95% bootstrap confidence interval of EAG_3 response levels. The response levels correspond to the average response at the three recording electrodes. Significant dose-sensitivity are indicated (ANOVA, * $p<0.05$, ** $p<0.01$ and *** $p<0.001$). Red: females, black: males. TMP: tetramethylpyrazine; 3MA: *N*-(3-methylbutyl) acetamide; 2M3MA: 2-methoxy-*N*-(3-methylbutyl)acetamide; ED: ethyl decanoate; EM: ethyl myristate; EP: ethyl palmitate; DSU: 1,7-dioxaspiro[5.5] undecane. **B** median and 95% bootstrap confidence interval for EPG response levels. Same convention as in **A**

and cue lure (Verschut et al. 2018) in other tephritid species. These compounds may have an optional pheromonal role. *Zeugodacus cucurbitae* males feed on cue lure, raspberry ketone or zingerone that accumulate in their rectal glands (Nishida et al. 1990, 1993) and enhance the attraction of females (Khoo and Tan 2000). Further research is required to determine whether cue lure and DSU activate the same olfactory pathway or different olfactory sensory neurones located within the maxillary palps.

The present study is the first to identify small amounts of DSU in sexually mature males of *Z. cucurbitae*. The EPG of *Z. cucurbitae*, which can be considered as a specific detector of DSU, confirmed this observation. Sexually mature flies of intermediate age (15, 25 days old) produce small quantities of this compound, whereas it was not observed in immature (5-day-old) or older mature 30-day-old male melon flies. In the olive fruit fly, *B. oleae*, young mature males, but not older ones, also release a spiroacetal female sex pheromone, which attracts rivals and induces courtship behaviour (Benelli et al. 2013). Male-male mating attempts have also been reported in the melon fly, but the age-dependence of this interaction has not been studied (Kuba and Koyama 1985). It has been hypothesised that male-male mating attempts may distract older males from courting females, however no female chemical mimicry linked to DSU production has been reported (Ruther and Steiner 2008; Benelli et al. 2013).

Sex pheromones contribute to the reproductive isolation between species (Bontonou and Wicker-Thomas 2014). Within the *Drosophila* genus, a congruence between cuticular composition and species phylogeny suggests that pheromone composition has gradually evolved (Symonds and Wertheim 2005). However, the reproductive isolation between species may be linked to rapid changes in the olfactory pathways involved in pheromone detection (Dekker et al. 2015). Among Dacinae species, many cuticular compounds are shared or derived (Baker and Bacon 1985; Carlson and Yocom 1986; Goh et al. 1993; Fletcher and Kitching 1995; Benelli et al. 2014a; Vaníčková et al. 2017; Noushini et al. 2020), and the olfactory system is largely conserved at the molecular and functional levels (Jacob et al. 2017). This also suggests that pheromone emission and detection may evolve gradually. Moreover, interspecific attraction demonstrated by *B. dorsalis* females for *Z. cucurbitae* males has been reported (Kobayashi et al. 1978), suggesting similarities between the pheromone compounds of the two species. To challenge this hypothesis, we recorded the antennal activity of *B. dorsalis* and *D. demmerezi* females induced

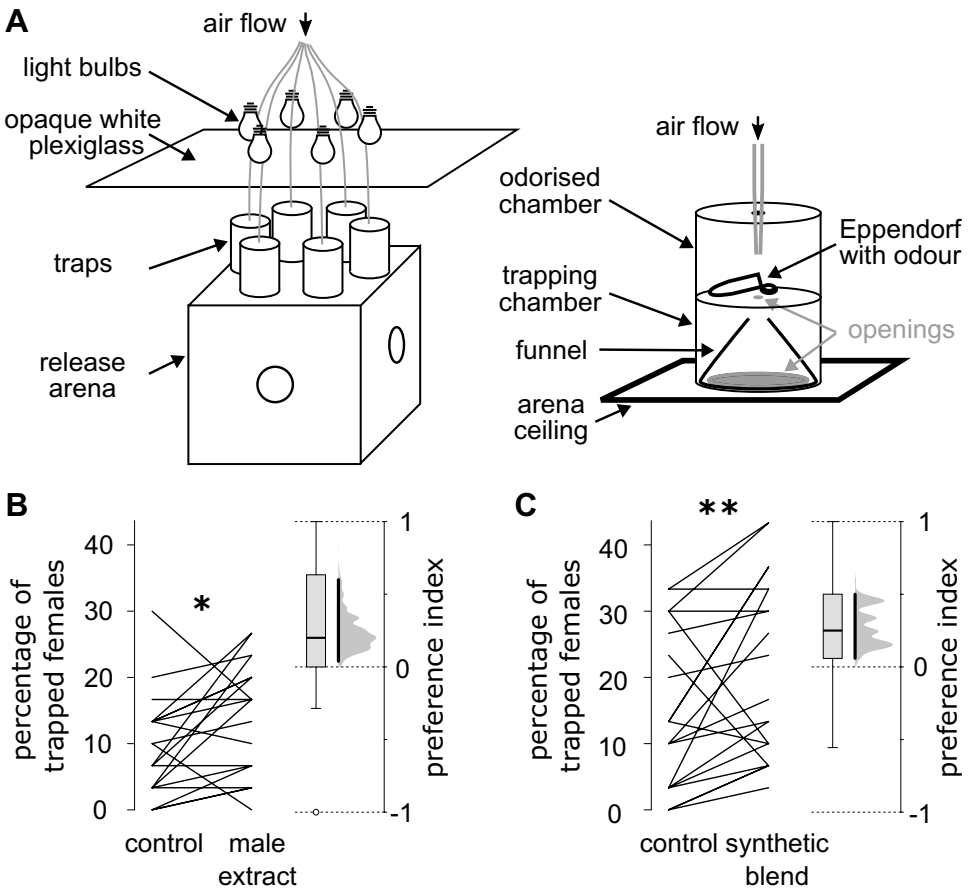


Fig. 8 Attractiveness of mature virgin female of *Zeugodacus cucurbitae* in a six-choice olfactometer. **A** Diagram of the six-choice olfactometer, with the structure of a trap detailed on the right. **B** Gardner-Altman estimation plot comparing the paired percentage of females trapped with a natural male extract and a control solution. Data are plotted on the left axes as a slope graph: each paired set of observations is connected by a line, * $p < 0.05$; ** $p < 0.01$ (generalized linear mixed model, Poisson family). A preference index (difference divided by the sum) is plotted on the right with a boxplot illustrating the quartile distribution and a bootstrap sampling distribution of the median value superimposed with its 95% confidence interval (thick vertical line). **C** Gardner-Altman estimation plot comparing the paired percentage of females trapped with the synthetic blend and a control solution. Same conventions as in panel **A**

by the epicuticular compounds of *Z. cucurbitae* males. We observed similar EAD responses to 2M3MA in *Z. cucurbitae* and *B. dorsalis*, thus we argue that this compound might have a specific ecological role. The antennae of *Z. cucurbitae* and possibly *B. dorsalis* responded to TMP, as also reported for *Bactrocera zonata* (Saunders) (Levi-Zada et al. 2020). Inversely, only *Z. cucurbitae* antennae responded to ethyl decanoate. Surprisingly, *D. demmerezi* antennae, but not *Z. cucurbitae* ones, responded to ethyl paraben. *Bactrocera dorsalis* antennae and not *Z. cucurbitae* ones responded to 3MA, which elicited anemotaxis behaviour in the sibling species *B. carambolae* (Wee and Tan 2005). We also compared our results with the few studies on the electrophysiological response of Dacinae species to cuticular compounds (Canale et al. 2013, 2015; Levi-Zada et al. 2020; Noushini et al. 2020). In agreement with our observation, an EPD response to DSU was detected in *Bactrocera frauenfeldi* (Schiner) (Noushini et al. 2020), suggesting that palp sensitivity to spiroacetals is a widespread trait among Dacinae species. While we observed no EAG response to C_{14}-C_{16} ethyl esters in *Z. cucurbitae*, an EAG response was reported in *B. oleae* and *B. zonata* (Canale et al. 2015; Levi-Zada et al. 2020). To conclude on the evolution of pheromonal systems, our data supports the hypothesis that both the emission and detection of epicuticular compounds are partially conserved in Dacinae, with qualitative differences linked to just a few compounds in the pheromonal blend.

Overall, our detailed analysis and identification of cuticular components add fundamental knowledge to the chemical ecology of *Z. cucurbitae*, which could have implications for Integrated Pest Management programs. The synthetic blend formulated here attracted *Z. cucurbitae* females in an olfactometer, but its potential for trapping in the field was not assessed. A low, but significant, percentage of females placed close to the source were captured. Female attraction to males peaks during the last hour of the photophase (Kobayashi et al. 1978; Khoo and Tan 2000), thus female attraction to the blend might be higher during this time window. Female attraction could be enhanced by combining the blend with cue lure or its derivatives (Khoo and Tan 2000), and with a more finely tuned compound ratio and concentrations. To optimize the blend, future studies are required with further laboratory, semi-field, and field behavioural assays, which may lead to the development of a novel pest management tool.

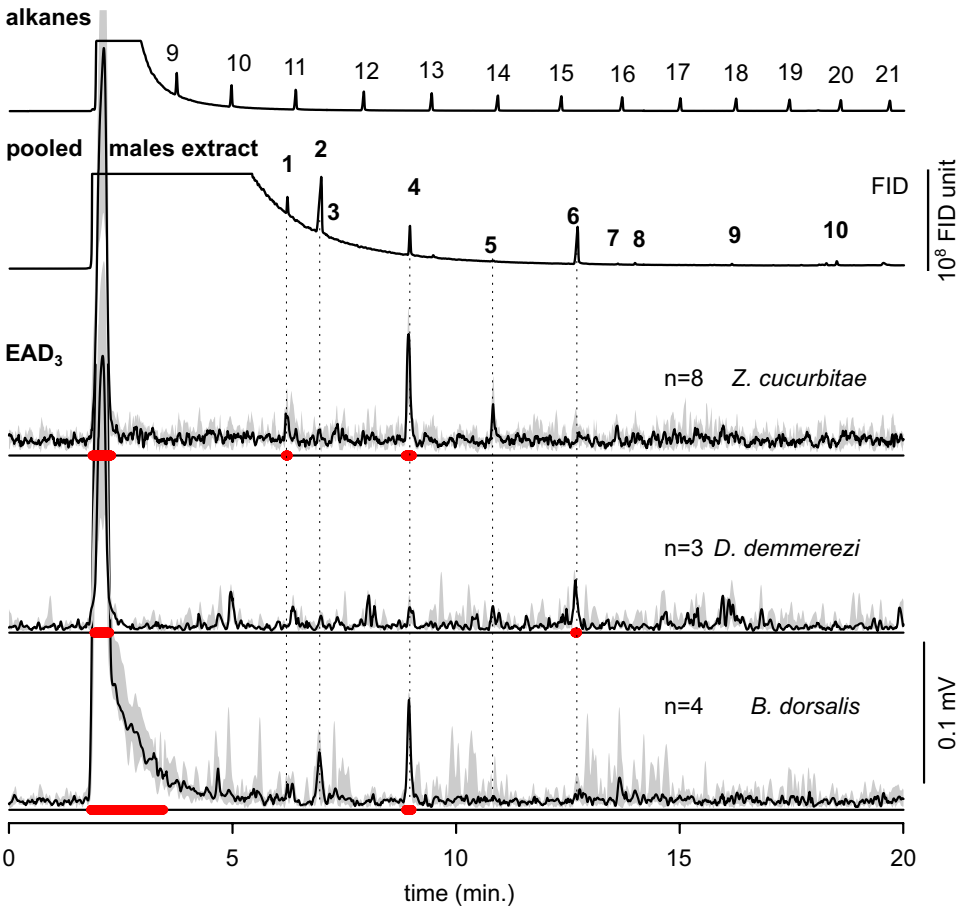


Fig. 9 Cross-specific comparison of GC-EAD_3 analysis of the whole body extract of *Zeugodacus cucurbitae* males. Same conventions as in Fig. 4

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Author contributions LV, TAN, VJ, GB, GR, EP, and MM conceived and designed the research project. LV, TAN, AM, PK, VJ, GR and EP conducted experiments. LV, VJ, AP analysed data. LV, VJ, AP, GB, and MM drafted the manuscript. All authors read and approved the manuscript.

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Declarations

Conflict of interest The authors have not disclosed any competing interests.

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