



# OPEN Phenological stage dependent sensory and behavioral responses of *Zeugodacus cucurbitae* (Coquillett) to cucurbit volatiles

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*Zeugodacus cucurbitae* (Coquillett) flies significantly impact vegetable production in many tropical regions. This study aimed to identify physiologically and behaviorally relevant volatiles from host plants that could potentially be used for future monitoring and control of female *Z. cucurbitae* flies. Volatile organic compounds were collected from flower, immature fruit, and mature fruit stages of *Cucumis sativus* L., *Cucurbita pepo* L., and *Cucurbita mixta* L. in field conditions, and were analyzed using gas chromatography/mass spectrometry (GC–MS). A total of 81 compounds were identified from across the three species and their phenological stages. Volatilome diversity was higher within phenological stages of each species than between species. Electrophysiological responses of sexually mature *Z. cucurbitae* females to host volatiles were recorded using gas chromatography coupled electroantennogram detection (GC–EAD). Active compounds were then formulated into blends for behavioral assays conducted in a six-choice olfactometer. Synthetic blends based on physiologically active compounds from flower and immature fruit headspace attracted more females than blends derived from mature fruit and the paraffin oil control ( $P < 0.001$ ). Some of the physiologically active compounds were found to be behaviorally redundant. The performance of these blends needs to be assessed under field conditions.

**Keywords** Behavioral bioassay, Cucurbitaceae, Electroantennogram, Female attractants

The melon fly, *Zeugodacus cucurbitae* (Coquillett), is a serious pest of fruits and vegetables worldwide<sup>1</sup>. It is native to Asia and widely distributed in tropical, and subtropical regions<sup>2–4</sup>. While it shows a strong preference for plant species in the Cucurbitaceae family<sup>3</sup>, *Z. cucurbitae* has been recorded on 136 plant species across 30 families, with the highest infestation rates observed in Cucurbitaceae, followed by Solanaceae<sup>5</sup>.

Female melon flies lay their eggs in young fruits, flowers, and stems of host plants. Besides direct damage caused by larval feeding<sup>1,6</sup>, indirect damage results from pathogens that infest fruits through oviposition punctures and feeding galleries created by the larvae, ultimately reducing yield. Infestations are particularly severe in cucurbit fields, where unmanaged outbreaks can lead to yield losses of up to 100%<sup>1,3,7</sup>.

Growers commonly use cover sprays against fruit flies during the fruit's susceptible stage<sup>8</sup>. However, synthetic insecticides pose significant risks to human health and the environment, highlighting the urgent need for sustainable alternatives<sup>9,10</sup>. In response, Integrated pest management (IPM) is a strategy through which the use of insecticides can be reduced. This requires careful monitoring of the pests and use of sustainable alternatives when needed. IPM has been established in selected areas, using a range of tools to target various developmental stages of tephritid flies worldwide<sup>11–15</sup>. Among the tools deployed in IPM of tephritid fruit flies are semiochemicals, which manipulate the behavior of insect pests<sup>16</sup>. Semiochemicals play a crucial role in IPM systems for many tephritid pests, often including male attractants used for male annihilation<sup>17</sup>, and food-based attractants that show a female-biased response<sup>18</sup>. Food-based attractants, such as fermenting sugars, hydrolyzed proteins, and yeast, tend to broadly attract non-target insects, have a limited field life, and can be challenging to handle<sup>18,19</sup>. Additionally, gravid *Z. cucurbitae* females that feed on natural protein sources may bypass borders treated with proteinaceous bait and infest cucurbit fields<sup>20</sup>. This creates a clear need for the development of

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specific gravid female attractants, which could be deployed in individual orchards or, particularly in smaller fields, as part of area-wide applications in an integrated manner.

Female tephritid fruit flies rely on host plant volatiles for host location and oviposition<sup>21</sup>. The preference of females for odors emanating from food sources versus host plants is influenced by their age and physiological state<sup>20,22–25</sup>. As female flies undergo ovarian development and mating, their search focus shifts from food to host plants. Developing lures based on volatiles from host plants that specifically attract gravid females could provide an effective strategy to reduce infestation.

Research on fruit flies with narrow host ranges, such as the apple maggot fly, *Rhagoletis pomonella* (Walsh)<sup>26</sup> and the cucumber fly, *Bactrocera cucumis* (French), has led to the successful development of host-based female lures<sup>27</sup>. Similar efforts have been made to develop host volatile-based female lure for *Z. cucurbitae*. Since *Z. cucurbitae* females are attracted to cucumber (*Cucumis sativus* L.)<sup>20,28</sup>, physiologically and behaviorally active compounds have been identified from sources including: pureed cucumbers (Sidurhurst and Jang,<sup>19</sup>), three-month old cucumber and tomato plants (*Solanum lycopersicum* cultivar “cal- J”, Njuguna et al.<sup>29</sup>), and mature fruits of ridge gourd (*Luffa acutangula* L. cv. Mallika, Shivaramu et al.<sup>30</sup>). These studies, however, primarily focused on mature fruits as the source of host volatiles. Melon flies, in contrast, lay eggs on flowers and young fruits of cucurbit plants<sup>6</sup>. To address this, the present study collected and analysed headspace volatiles emitted by cucurbit host plants at different phenological stages, highlighting similarities and variations. Moreover, this study compared physiologically and behaviorally relevant compounds from intact flowers, immature and mature fruits of cucurbitaceous plants. The attractiveness of volatile blends from flowers and fruits, differing in composition and component ratios, was tested using a six-choice olfactometer.

## Results

### Organic volatile compounds of cucurbitaceous plants at different developmental stages

A total of 81 compounds were identified across the flowering, immature, and mature stages of *C. sativus*, *C. pepo* and *C. mixta*. At the species level, *C. sativus*, *C. pepo*, and *C. mixta* shared 64.19%, 62.96%, and 51.85% of the total volatiles, respectively (Fig. 1). Among these, 22.22%, 15.51%, and 12.34% were unique to *C. sativus*, *C. pepo*, and *C. mixta* respectively. Immature fruits contributed the largest proportion of headspace volatiles (60.49%), followed by flowers (53.08%) and mature fruits (46.91%). Overall, 32.5% of the headspace volatiles were shared by all three cucurbitaceous species (Fig. 1, Supplementary Table S1).

Decanal, 1H-indole, nona-2,6-dien-1-ol and (*E*)-non-2-enal were the most abundant compounds in the flower headspace. In contrast, benzyl alcohol, linalool, and benzaldehyde were abundant in the headspace of both flowers and immature fruits. The amount of these compounds in the headspace decreased from the flower to the immature fruit stage. Compounds such as 2-cyclopentylcyclopentan-1-one, 1-(4-ethylphenyl) ethenone, 3-ethylbenzaldehyde and cyclopentanone were abundant across all growth stages (Fig. 1). Overall, the volatilome of *C. sativus*, *C. pepo*, and *C. mixta* exhibited more diversity within the developmental stages than between species (Fig. 2).

### Chemical class of cucurbitaceous plants

The flower headspace of both *C. sativus* and *C. pepo* was quantitatively dominated by ketones, followed by terpenoids, whereas *C. mixta* was dominated by terpenoids, followed by aldehydes. In all three species, the headspace of immature and mature fruits was dominated by ketones (Fig. 3).

### Physiologically active compounds

Fourteen GC-EAD active compounds were tentatively identified, of which 10 were confirmed with synthetic standards. Decanal, benzaldehyde, linalool, benzyl alcohol, (*E*)-non-2-enal, 1,4-dimethoxybenzene, 4-ethylbenzaldehyde, nona-2,6-dien-1-ol, nonanal and propanoic acid were confirmed with synthetics (Figs. 4, 5, and Supplementary Fig. S1–6). Across developmental stages, flowers had the highest number of EAD active compounds (9/10), while immature fruits had six. Five EAD active compounds of immature fruits were also present in the flower headspace, except for 1,4-dimethoxybenzene. Fifty % of the antenna-active compounds were shared by all three cucurbit species. The antenna-active compounds in the headspace of flowers and immature fruits were more similar to each other than to those found in mature fruits (Figs. 5, 6). Regression analysis showed no correlation between the amount of a given volatile compound and the strength of the EAD response (Supplementary Fig. S7).

### Flower and immature fruit volatiles attract females

In six-choice assays, both the flower blend (FB) and immature fruit blend (IFB) were more attractive than the mature fruit blend (MFB) and the control ( $P < 0.001$ ). The highest mean fly catch was recorded for FB (2.29) and IFB (2.14), with no significant difference between them (Fig. 7).

### Fly catch comparison among different blends

The All\_active, Tephri\_active, and Zeugo\_active blends attracted significantly higher number of flies than the controls ( $P < 0.001$ ). Moreover, there was no significant difference in mean fly catch among Zeugo\_active, Tephri\_active and All\_active blends. The highest mean fly catch was recorded from the All\_active blend (3.27). However, it was not significantly different from Zeugo\_active and Tephri\_active blends (Fig. 8).

The two-component blend caught significantly fewer flies than the All\_active ( $P = 0.007$ ), but did not differ from the control (Fig. 9).



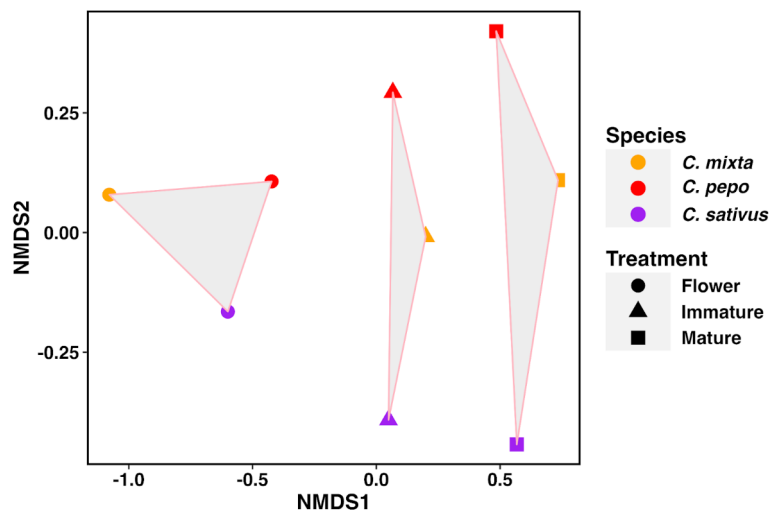
**Fig. 1.** From left to right, the heatmap shows (1) Name of the compounds, (2) chemical classes of the compounds, and (3) amount of the compounds in headspaces at different phenological stages (flower, immature fruit, and mature fruit) of *C. mixta*, *C. pepo* and *C. sativus* respectively. Volatiles are arranged in descending order based on sharedness. The relative abundance (surface area of GCMS peak) of the compounds represented by a gradient from light blue (lowest) to red (highest). Cas numbers of compounds shown in red text were confirmed via synthetic injection.

## Discussion

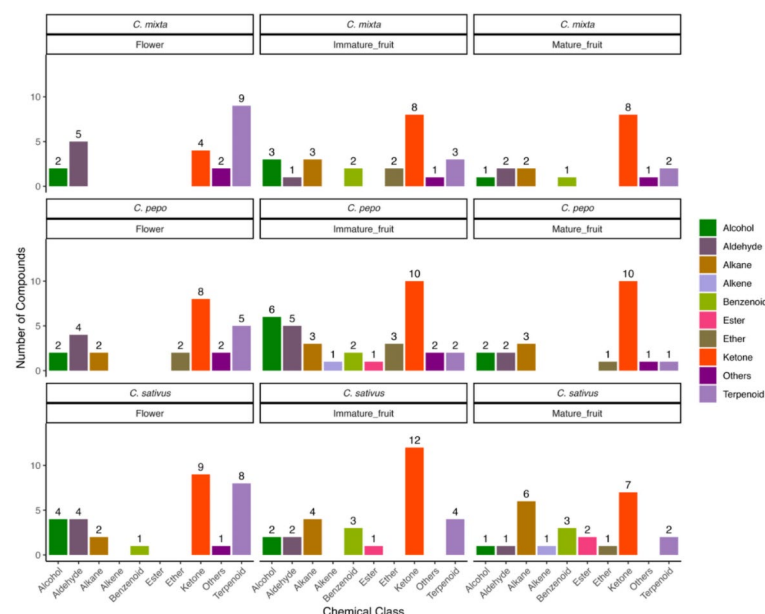
The melon fly *Z. cucurbitae*, is among the most destructive horticultural pests worldwide<sup>4</sup>. Current control strategies that target females mainly depend on food baits, which are broadly attractive to insects and thus affect non-target species, including beneficials<sup>18</sup>. A species-selective tool that targets the damaging sex is sorely needed<sup>19</sup>. This study identified antennally active and behaviorally attractive volatiles from different phenological stages of cucurbitaceous host plants of *Z. cucurbitae* that could be of use in novel control strategies.

Most of the antenna-active compounds identified in this study, including decanal, benzaldehyde, linalool, nonanal, benzyl alcohol, (*E*)-non-2-enal, and nona-2,6-dien-1-ol, have been previously reported to elicit physiological responses in *Z. cucurbitae*<sup>19,30</sup>, and attracted both females and males<sup>19</sup>. Three of these antenna-active compounds, 1,4-dimethoxybenzene, 4-ethylbenzaldehyde, and propanoic acid, are reported for the first time. Out of the 81 compounds that were individually identifiable, 10 were antenna-active, and 50% of these were shared among *C. sativus*, *C. pepo* and *C. mixta*. This supports the notion of Biasazin et al.<sup>31</sup> that the probability of antennal detection by tephritid fruit flies increased for shared volatiles of ripe fruits, even for volatiles from closely related hosts.

Among the antenna-active compounds, linalool, benzaldehyde, and benzyl alcohol have been reported in the floral headspace of more than half of the seed plant species sampled<sup>32</sup>, while decanal and nonanal are ubiquitous and shared among different plant families<sup>32,33</sup>. The presence of typical floral volatiles like linalool and



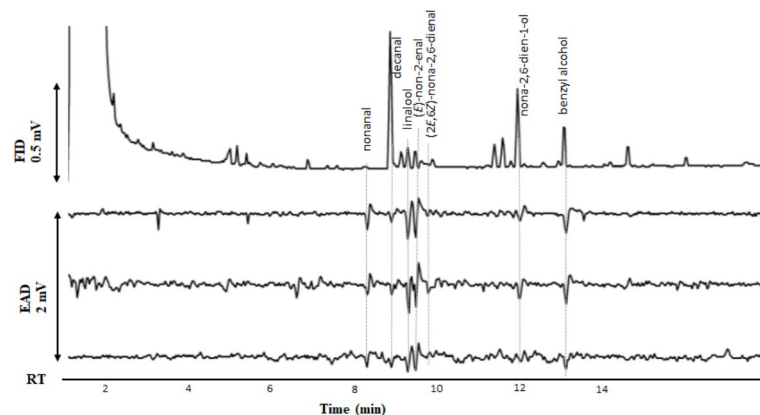
**Fig. 2.** Nonmetric multidimensional scaling (NMDS) analysis of *C. sativus*, *C. pepo* and *C. mixta* headspaces at different developmental stages (flower, immature, and mature). The diversity within the volatiles of different phenological stages was relatively higher than the diversity among species; the stress value for the analysis was 0.081.



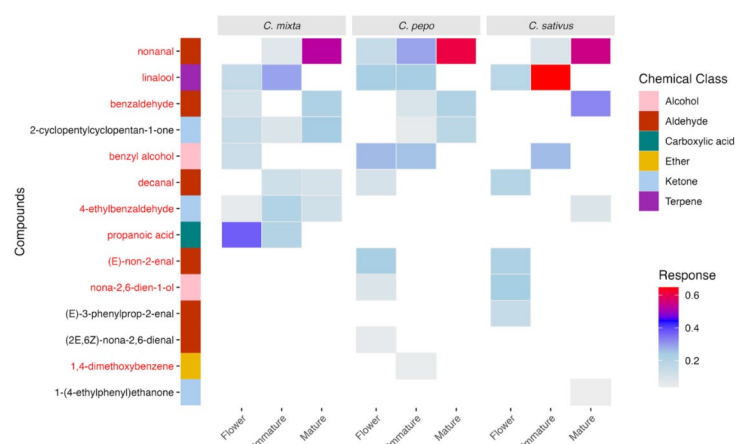
**Fig. 3.** Chemical class of headspace volatiles of three cucurbitaceous plants at different phenological stages. The graph shows that ketones are the dominant chemical class in the sampled cucurbit plants headspace across all developmental stages. The numbers at the top of each bar indicate the number of compounds in each chemical class. Carboxylic acid, amine, amide and unknown chemical classes are grouped under the "others" category.

methoxybenzene in immature fruit headspace might indicate that decaying flowers carry these volatiles in the course of fruit development. The characteristic cucumber-like odor in cucumber fruits is associated with the aroma of nona-2,6-dien-1-ol and (*E*)-non-2-enal<sup>34</sup>. However, these two compounds were only present in flower headspace and absent from both immature and mature fruit headspace, possibly due to their very low release in the latter. Atiama-Nurbel et al.<sup>35</sup> reported that both nona-2,6-dien-1-ol and (*E*)-non-2-enal were present in low amounts in the headspace of mature cucumber pieces. These two compounds increase when the fruit is mechanically ruptured in the presence of oxygen<sup>36</sup>.

The chemical class of volatiles showed slight differences among the headspaces of flowers. Ketones and terpenoids dominated the flower headspaces of *C. sativus* and *C. pepo*, whereas terpenoids and aldehydes predominated in the flower headspace of *C. mixta*. In all three species studied, ketones dominated the headspace



**Fig. 4.** Representative GC-EAD traces of *Z. cucurbitae* females in response to *C. pepo* flower headspace volatiles. The top trace represents the FID chromatogram, while the three lower traces show antennal responses. Gray vertical lines connect antennal responses to their corresponding FID peaks. The time base was 2.00 min, scale: FID = 0.5 mV, EAD = 2 mV. RT refers to the retention time of the compounds.

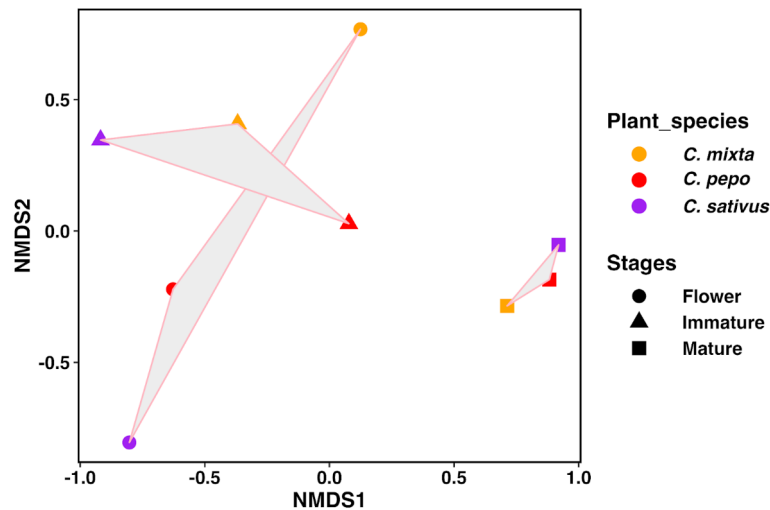


**Fig. 5.** *Z. cucurbitae* antennal sensitivity to headspace volatiles of *C. mixta*, *C. pepo*, and *C. sativus* at flowering, immature, and mature fruit stages. The heatmap shows the normalized strength of antennal responses, calculated using the overall antennal response to each plant species and developmental stage (flower, immature fruit, and mature fruit) as the denominator. Compounds are arranged in descending order based on their sharedness and the strength of the antennal response. Compounds labelled in red text were confirmed with synthetic standards.

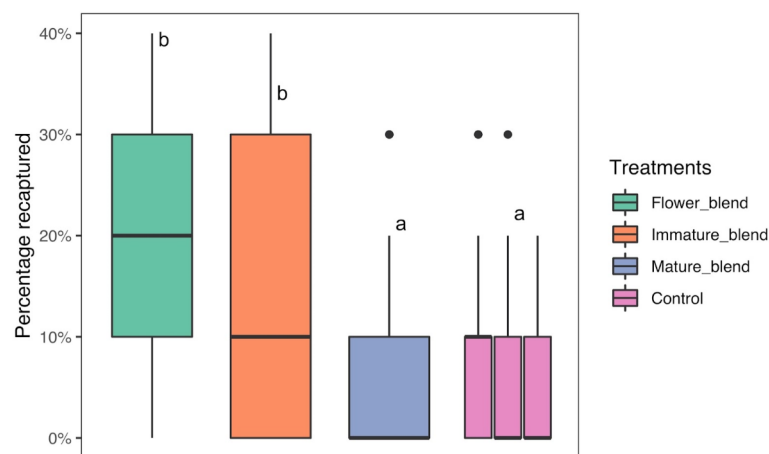
of immature and mature fruit. Previous studies have found that aldehydes and alcohols dominated the headspace of mature cucumbers<sup>19,35</sup>. The variation in chemical classes might be due to differences in sampling. Damaged and macerated mature cucumber fruits<sup>36</sup> release many oxygenated volatiles that are only present in very low amounts in intact fruits (this study). Such variations due to level of damage of the plant material has been reported previously for, for instance, *Brassica* and *Sinapis* species<sup>37</sup>.

In agreement with Siderhurst and Jang<sup>19</sup>, the physiologically active compounds in this study were dominated by aldehydes, while the number and quantity of esters were very low and responses to these were absent. The headspaces of mini-watermelons *Citrullus lanatus* and *Cucumis melo* L. fruits also had low abundances of esters<sup>38,39</sup>. Esters are typical of mature fruits, and dominate the olfactome of four tephritid fruit fly species<sup>31</sup>. The amount of ester volatiles increases throughout the ripening of sweet fruits, and synthetic blends of these volatiles were attractive to tephritid females<sup>31,40,41</sup>. Interestingly, *Z. cucurbitae* showed lower overall sensitivity to esters compared to other fruit fly species<sup>31</sup>, indicating that their olfactory receptor repertoire has shifted to other compounds to accommodate the Cucurbitaceae niche, which typically has very low levels of esters (with perhaps the exception of ripe sweet melons which may be abundant in esters)<sup>42,43</sup>.

Unlike *B. dorsalis* and *C. capitata*, which prefer ripe fruits for oviposition<sup>44</sup>, *Z. cucurbitae* females preferentially infest flowers, immature, while mature fruits are less preferred<sup>1,6,45</sup>. This study investigated whether this phenomenon is due to the overlap of odor profiles and antennal sensitivity to those of flowers and immature fruits and mature fruits, while the plants were intact in the field. Indeed, the antenna active compounds from



**Fig. 6.** Nonmetric multidimensional scaling (NMDS) analysis of *Z. cucurbitae* antenna-active compounds to headspace volatiles from *C. sativus*, *C. pepo* and *C. mixta* at different developmental stages (flower, immature fruits, and mature fruits). Responses to flower and immature fruit stages are more similar to each other (closer in ordination space) compared to those from the mature fruit stage; the stress value for the analysis was 0.079.

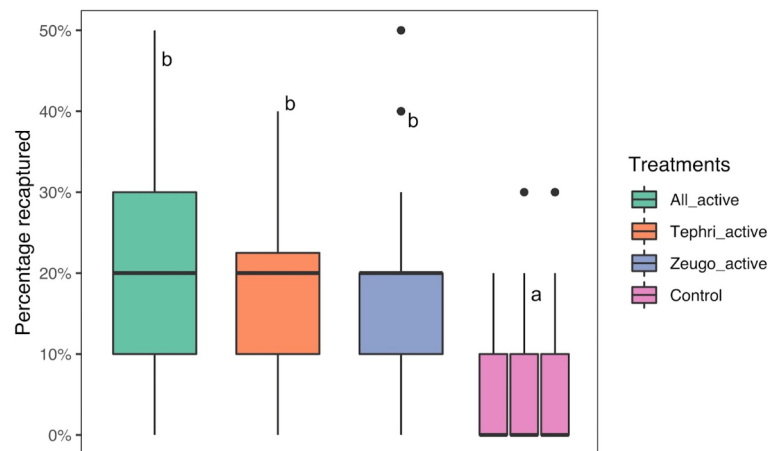


**Fig. 7.** Behavioral preference of sexually mature *Z. cucurbitae* females for blends of antenna-active compounds in ratios corresponding to each phenological stage of cucurbit hosts. Three paraffin oil controls were placed in between each treatment. The box plots display the median, interquartile ranges, and outliers for fly catches across the different blends and the control. The experiment included 14 replicates, each with 30 sexually mature female flies. Different letters indicate significant differences between means at  $p < 0.05$  means-Kramer, followed by Tukey post hoc tests.

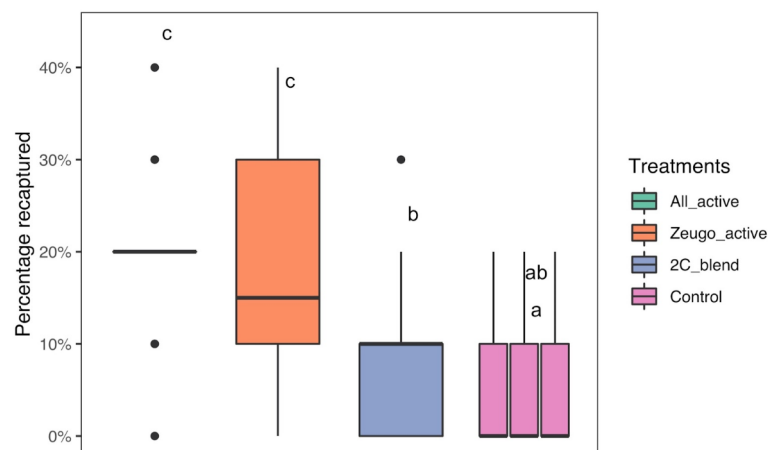
flowers and immature fruits headspace did not differ (Fig. 6), and the flower blend (FB) and immature fruit blend (IFB) attracted a significantly higher number of females than mature fruit blend (MFB). The preference for younger fruit might be due to the hard epidermis of mature fruits<sup>6</sup>. According to reports, *B. dorsalis* also showed a low preference for infestation of fruits with a tough pericarp<sup>46,47</sup>. The difficulty to penetrate the pericarp of ripe fruit may have led to an increased preference for volatiles in earlier development stages, such as aldehydes, to which the melon fly responds. Most aldehydes were high at the early stages of cantaloupe fruits (*Cucumis melo* var. *reticulatus* cv. Sol Real) and decreased towards the harvesting stage<sup>48</sup>. However, mechanically damaging mature fruit has been shown to restore the attraction of gravid *Z. cucurbitae* females<sup>20</sup>, which makes sense, as this damage causes the release of large amounts of volatiles typical of early developmental stages, while also offering oviposition sites through bypassing the pericarp.

Insects display defined host preferences, and even within a host, insects are selective in their niche. The olfactory correlate of this behavior is differential attraction to host volatiles that emanate from different niches or phenological stages of the host plant<sup>49</sup>. For instance, the pea moth *Cydia nigricana* (Fabricius) can distinguish different phenological stages of its host *Pisum sativum* L. by headspace extract volatiles in a wind tunnel bioassay, showing a preference for the flowering stage<sup>50</sup>. Späthe et al.<sup>51</sup> also reported that *Manduca sexta* L. females can





**Fig. 8.** Behavioral preference of sexually mature *Z. cucurbitae* females to All\_active, Tephri\_active and Zeugo\_active blends. Three paraffin oil controls were placed between each treatment. Box plots show the median, interquartile ranges, and outliers of fly catches for the different blends and control. Number of replicates = 11, with 30 sexually mature female flies per replicate. Different letters indicate significant differences between means at  $p < 0.05$  means-Kramer, followed by Tukey post hoc tests.



**Fig. 9.** Behavioral response of sexually mature *Z. cucurbitae* females to 2- component, Zeugo\_active and All\_active blends. Three paraffin oil controls were placed between each treatment. Box plots show the median, interquartile ranges, and outliers of fly catches for the different blends and controls. Number of replicates = 8, with 30 sexually mature female flies per replicate. Different letters indicate significant differences between means at  $p < 0.05$  means-Kramer, followed by Tukey post hoc tests.

rank different host plants and even choose quality plants from individuals of the same species using olfactory cues. Similarly, the west Indian fruit fly *Anastrepha obliqua* (Macquart) differentiated mango cultivars and ripeness stages using synthetic blends in multilure traps in a semi-natural conditions<sup>40</sup>. Considering that females are under strong selection to maximize the fitness of their offspring<sup>52</sup>, immature fruits should be preferable over flowers due to their size and ability to support many larvae to reach adulthood. However, in this study, there was no significant difference between FB and IFB in attracting *Z. cucurbitae* females. There are several possible explanations for the equal attractiveness of FB and IFB blends in the olfactometer. 1) Flowering and fruiting seasons in cucurbit plants do not show temporal differences; even flowers and harvestable fruits are available on a single plant. Thus, flower volatiles might indicate the presence of a suitable oviposition site for foraging females. In a wind tunnel bioassay, mature females of the tomato fruit fly *Neoceratitis cyanescens* (Bezzi), which doesn't oviposit on flowers and infests unripe fruits of Solanaceae, were attracted to flower odor<sup>53</sup>. Flower odor might thus be used as a cue for the presence of a suitable host at a relatively long distance. Once in close contact with the host, tephritid females use additional sensory inputs to olfaction before accepting the plant part for oviposition<sup>50,51,54–56</sup>. Piñero et al.<sup>55</sup> reported that *Z. cucurbitae* females synergistically use olfaction and vision to locate host plants. Therefore, a bioassay that includes olfaction and vision might reveal whether flowers are equally attractive as immature fruits. 2) *Z. cucurbitae* might not discriminate between the two blends (FB and IFB) since the physiologically active compounds of flower and immature fruit stages overlapped. This may be

ecologically relevant as senescing flowers remain attached to developing immature cucurbits for many days and thus may indicate a suitable oviposition site.

The attraction to different blends, with varying numbers of components according to the natural ratio at different phenological stages of host plants, shows the redundancy of some components in the blends and the behavioral plasticity of the females. Studies on Tephritidae and other insects have reported some physiologically active compounds that were behaviorally redundant<sup>41,57,58</sup>. The concept that herbivores depend on a ratio of ubiquitous plant compounds to locate hosts or oviposition sites is well documented<sup>33,59,60</sup>. At the same time, herbivore insects can also rank their hosts and use taxa-specific odors<sup>51,61,62</sup>. In this study, the IFB that contains ubiquitous volatiles was as attractive as the FB, which contain both ubiquitous volatiles and compounds that are more characteristic of 'cucumber-like' aroma in cucurbitaceous fruits (nona-2,6-dien-1-ol and (*E*)-non-2-enal). Since none of the components of IFB were taxa-specific (to cucurbitaceous plants), our results suggest that *Z. cucurbitae* females can locate their host plants based on volatiles shared by different plant families at a particular ratio. However, more specific volatiles may play a role in further defining the attractiveness. In a wind tunnel assay, *Lobesia botrana* (Denis and Shiffermüller) females were attracted to synthetic blends shared by two host plants and blends specific to each host plant. At the same time, higher attraction was recorded when the specific volatiles were added to the shared synthetic blends<sup>63</sup>.

A controlled and charcoal-filtered laboratory bioassay environment is different from the field, where the insect is exposed to a complex mixture of odors. This may give rise to conflicting results in the performance of blends as observed for the apple fruit moth, *Argyresthia conjugella* (Zeller)<sup>64,65</sup>. The interaction of behaviorally relevant blends with background odor can have synergistic or antagonistic effects, and there are examples for both scenarios. Knudsen and Tasin<sup>66</sup> reported a difference in the attraction of apple fruit moth *A. conjugella*, to two-component blends and seven-component blends in the presence of background odor. In the absence of background odor, the two-component blend is equally attractive as the seven-component blend. However, in the presence of background odor at the field, seven-component blend traps attracted three-fold of the two-component blend. On the other hand, background odor enhanced the attraction of moths to a synthetic blend by forming synergy<sup>65</sup>. Therefore, the performance of the cucurbit flower and fruit-based synthetic blends in attracting females should be evaluated in the field. This study identified behaviorally relevant volatiles from different phenological stages of cucurbitaceous hosts. These findings will contribute to the ongoing efforts to develop female-biased lures for *Z. cucurbitae*. It will be fascinating to further investigate how generalist tephritids such as *B. dorsalis* and *C. capiata* respond physiologically and behaviorally to cucurbit host odor that are low in esters.

## Materials and methods

### Experimental plants

*Cucumis sativus* L., *Cucurbita pepo* L., and *Cucurbita mixta* L. grown in Alnarp were used for odor sampling. Sampling was performed at the field while the plants were intact. Volatiles were collected from three developmental stages of each species. Stage 1: at a flowering stage when the corollas were still intact; stage 2: when the fruits were young and tender; and stage 3: when the fruits were mature and ready for harvesting.

### Experimental insects

The International Atomic Energy Agency (IAEA) provided startup material to establish colonies in Sweden and Ethiopia. Flies were held in bugdorm cages (325 × 325 × 325) at 24–28 °C, 60–65% RH, and a 12:12 L:D photoperiod. Honeydew melon (Sweden) or cucumber (Ethiopia) served as the oviposition medium. Adult flies were fed on a standard 1:3 yeast-sugar ratio and provided cotton balls soaked with water.

### Odor collection

A 6 cm Teflon tube was filled with 35 mg of Porapak TM type Q 50–80 mesh, with two stoppers and polypropylene wool at both ends. Before collection, the adsorption columns were rinsed with 1 ml of distilled n-hexane and 1 ml of methanol. The column washes were saved in vials and used to assess the cleanliness of the columns. Host flowers and fruits were enclosed in polyamide bags (Toppits Stekpåsar, Mingen, Germany, 35 × 43 cm), and charcoal-filtered air was pumped through the system. The column was placed in the bag and connected to a pump through Teflon tubing. Aerations were run for four hours, and adsorbed compounds were extracted with 0.5 ml of n-hexane into glass vials. The samples were stored at -20 °C until analysis. Each stage of the three cucurbit species was sampled five times.

### Gas chromatography coupled electroantennogram (GC-EAD)

The antennal response of sexually mature *Z. cucurbitae* females to host volatiles was recorded using gas chromatography coupled electroantennogram (GC-EAD) software GC-EAD 2011 (V.1.2.3, Syntech, Kirchzarten, Germany). This software received input from a high impedance GC amplifier interface box (IDAC-2; Syntech, Kirchzarten, Germany), which synced incoming antennal (OpAmp preamplifier probe, Syntech, Kirchzarten, Germany) and GC-Flame Ionization detector (FID) (Agilent Technologies 6890 GC (Santa Clara, CA, USA) signals. The GC was equipped with a DB-Wax column (30 m 0.32 mm 0.25 µm, Agilent Technologies). Hydrogen was used as the carrier gas for the mobile phase. The GC oven temperature was programmed to start at 40 °C (held for 3 min), increase by 10 °C/min<sup>-1</sup> to 240 °C, and hold at 240 °C for 5 min. The effluent was split 1:1 between the GC flame ionization detector (FID, at 250 °C) and the EAD. A humidified airstream (1500 ml/min) delivered the effluent to the mounted antenna. Amplified signals from both the FID and EAD were converted to digital signals and displayed on a computer.

Females aged 14–21 days were mounted by immobilizing the fly's body in a 200 µl micropipette tip. Part of the head and antennae were exposed outside the pipette tip for recording. To create conduction between



the silver electrodes and the insect antennae, glass capillaries filled with Beadle-Ephrussi ringer solution were used. The ringer solution had salts dissolved with different concentrations (7.5 g NaCl, 0.35 g KCl, and 0.29 g CaCl dissolved in 1 L of distilled water). The recording electrode was connected to the medio-central part of the antenna, while the reference electrode was inserted into the head of the fly. Antennal depolarizations were analyzed using Syntech data acquisition software. A minimum of three repeatable recordings were conducted for each of the three cucurbit plant species and the three developmental stages.

### Gas chromatography-mass spectrometry (GC–MS)

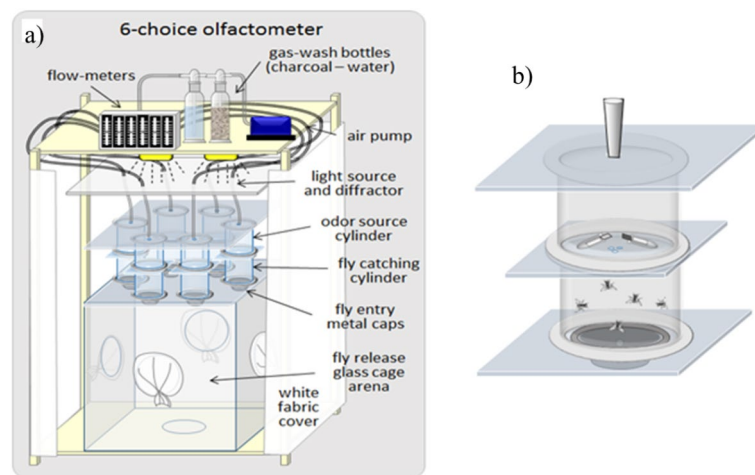
Samples were analyzed individually by GC/mass spectrometry (MS) using an Agilent 6890/ 5975 GC–MS system with two different columns: a polar DB-Wax and a non-polar HP-5 (both Agilent), each 30 m × 0.25 mm id × 0.25 µm film thickness. Then, samples were pooled after confirming their similarity. The use of both columns enabled complementary separation of compounds, as some compounds that co-elute on one column could be resolved on the other. This approach ensured a more comprehensive identification of volatile compounds. Kovats alkanes (c8–c20) were injected under the same conditions to calculate Kovats retention indices (KI) for the detected compounds. Helium was used as the carrier gas, and the GC temperature was set the same as in the GC-EAD. The peaks were identified by comparing their mass spectra and Kovats retention indices (KI) with the NIST14 database and published values. For estimating the relative amount of compounds, 10 ng of heptyl acetate (Cas # 112-06-1) was added as an internal standard to the pooled headspace extracts. The relative amount of each analyte in the pooled samples was determined by comparing the peak areas of the analyte of interest to the peak area of the internal standard. Then, amount of analytes in the non-polar column was used for further analysis, including comparisons across phenological stages, as most of the analytes were well separated. Further, the pooled samples were used for electrophysiological assay.

### Description of six choice olfactometer

The six-choice olfactometer was made up of a reinforced glass cage (420 mm × 420 mm × 420 mm<sup>3</sup>) and was developed by Tephri Group at SLU, Alnarp. It was described and used by Biasazin et al.<sup>31</sup> to evaluate the attraction of tephritid fruit flies to fruit volatiles and different synthetic blends. Later, Figueroa<sup>67</sup> used this olfactometer to evaluate the attraction of fermentation products and repellence of plant essential oils against *C. capitata*.

The olfactometer setup had six circular openings on its top surface, each 70 mm in diameter. Fitted onto these openings are conical glasses with 10 mm holes at the aperture, providing entry points for the flies. At the top of the six circular openings, two cylindrical glasses were positioned, separated by a glass plate with a 2 mm opening to facilitate air passage. The upper cylinder housed the odor source, while the lower cylinder functioned as a collection chamber for flies that had made a choice (Fig. 10b). A single glass plate encompassing six 5-mm holes served both as a conduit for Teflon tubing and a cover for all six chambers. A pump-generated air stream, charcoal-filtered and humidified, was directed to reach each of the chambers at a 0.5 l/min flow rate. Positioned centrally at the top was the light source, strategically placed to minimize bias. Teflon tubing was used throughout the system, and the setup contained five large holes with a 120 mm diameter on the sides and bottom part of the cube, used for releasing and collecting flies (Fig. 10a).

Electrophysiologically active synthetic compounds were diluted to a 10<sup>−4</sup> concentration and prepared according to their respective ratios in the headspace. Paraffin oil (prepared by Fine Chemical G. T. PLC, Addis Ababa, Ethiopia) was used to formulate the blends (Table 1). Prior to the experiments, flies underwent an 18 h starvation period with access to water only. Thirty sexually mature females were released for each experiment, with the positions of the treatments rotated to mitigate any potential biases. A volume of 10 µl from each treatment was placed in a plastic bottle cap with a diameter of 30 mm and a height of 10.2 mm.



**Fig. 10.** (a) The six-choice olfactometer setup was used to compare the attractiveness of different cucurbitaceous host odor-based blends to *Z. cucurbitae* females. (b) Close-up view of the fly-catching and odor-source glass cylinders. Source<sup>67</sup>

Compound name	Cas- number	Purity (%)	Blend Ratio						
			All_active	Tephri_active	Zeugo_active	2-C	FB	IFB	MFB
decanal	112-31-2	97	152	152	152		55		
1,4-dimethoxybenzene	150-78-7	99	90	90			32		
nona-2,6-dien-1-ol	7786-44-9	99	76	76	76	76	27		
benzyl alcohol	100-51-6	99.8	32	32	32		14	114	
linalool	78-70-6	97	30	30			18	108	
(E)-non-2-enal	18829-56-6	95	16	16	16	16	6		
4-ethylbenzaldehyde	4748-78-1	97	2					4	
benzaldehyde	100-52-7	99	2	2			6	8	1
nonanal	124-19-6	98	1	1	1		0.5	5	29
propanoic acid	79-09-4	90	1				0.1	1	

**Table 1.** Purity and ratio of antennae-active compounds used in behavioral assays. 2-C, FB, IFB and MFB represent two components: fruit blend, immature fruit blend, and mature fruit blend, respectively.

Bioassay experiments

Experiment one

A blend of antenna-active volatile compounds was prepared for each stage based on the ratio of the components found in the headspace. Although most of the antenna-active components of flowers and immature fruits overlap in presence, the ratio of the components in the headspace varied. Therefore, the blends were prepared to reflect the specific ratio characteristics of each stage. The flower blend (FB) comprised nine compounds including decanal, 1,4-dimethoxybenzene, nona-2,6-dien-1-ol, linalool, benzyl alcohol, benzaldehyde, (E)-non-2-enal, nonanal and propanoic acid, in the following ratio of 55:32:27:18:14:6: 6:0.5:0.1. The immature fruit blend (IFB) contained six components, including linalool, benzyl alcohol, benzaldehyde, nonanal, 4-ethylbenzaldehyde, and propanoic acid in the ratio of 108:114:8:5:4:1. The mature fruit blend (MFB) contained only two components: nonanal and benzaldehyde in a ratio of 29:1, respectively (Table 1). This experiment compared the response of females *Z. cucurbitae* to the three blends (FB, IFB and MFB) in a 6-choice olfactometer assay.

Experiment two

In the second experiment, three additional blends were formulated. The first blend, termed “All\_active,” encompassed all antenna-active compounds. The second blend, termed “Tephri\_active,” consisted of compounds identified as bioactive in our studies and previously reported as attractive in other Tephritidae fruit flies. The third blend, termed “Zeugo\_active,” consisted of compounds that are antenna-active in our assays and have been reported as attractants only in *Z. cucurbitae*. These compounds contribute to a distinctive cucumber-like aroma<sup>34,68</sup>. For each blend, the compounds were prepared in a ratio similar to those found in the headspace of immature fruits. Additionally, compounds exclusively present in the headspace of flowers were incorporated at the ratio observed in flower headspace.

The All\_active blend contained all the synthetically confirmed antenna-active compounds from the three cucurbit species. Decanal, 1,4-dimethoxybenzene, nona-2,6-dien-1-ol, benzyl alcohol, linalool, (E)-non-2-enal, 4-ethylbenzaldehyde, benzaldehyde, nonanal and propanoic acid at the ratio of 152:90:76:32:30:16:2:2:1:1, respectively. The Tephri\_active blend contained antenna-active components that elicited response from flowers and fruits, excluding two compounds (propanoic acid and 4-ethylbenzaldehyde) from the All\_active blend. These exclusions were made as these two compounds were not previously reported in the attraction of tephritid fruit flies, either individually or synergistically with other compounds. The components of the Tephri\_active blend include decanal, 1,4-dimethoxybenzene, nona-2,6-dien-1-ol, benzyl alcohol, linalool, (E)-non-2-enal, benzaldehyde, and nonanal at the ratio of 152: 90: 76: 32: 30: 16: 2: 1, respectively. The Zeugo\_active blend contains decanal, nona-2,6-dien-1-ol, benzyl alcohol, (E)-non-2-enal and nonanal are mixed at the ratio of 152: 76: 32: 16: 1, respectively (Table 1).

Experiment three

The two-component blend was compared with the Zeugo\_active and All\_active blends. The two-component blend is composed of nona-2,6-dien-1-ol and (E)-non-2-enal, which are known to be released upon mechanical damage of cucurbit fruits, and are among those compounds responsible for the distinctive cucumber-like aroma<sup>34,68</sup>. The ratio of the blends was similar to those in experiments two and three (Table 1).

Data analysis

Generalized linear model (GLM)-ANOVA fitted with Poisson distribution (for experiments 1 and 2) and gaussian distribution (for experiment 3) were carried out to compare the mean response of females to different synthetic blends in the six-choice olfactometer. Emmeans package was used for pair-wise mean comparison and mean separation (Tukey Test). Nonmetric multidimensional scaling (NMDS) analysis using Jaccard dissimilarity index in vegan package was used to compare similarity in the headspaces of the three species (*C. sativus*, *C. pepo* and *C. mixta*) and the three developmental stages (flower, immature fruit and mature fruit). The average relative response of EAD amplitudes was computed using three replicates of antennal responses of female *Z. cucurbitae*. To normalize the responses, each response in each EAD trace was divided by the weighted mean of all the

responses in that trace. The weighted mean was obtained by using the back transformed (exp) average of the ln transformed depolarization values of each response in that trace. Then, normalized responses were averaged across traces by dividing them by the total sum of average normalized responses, in order to scale the responses from 0 to 1 as seen in the heatmap. Moreover, regression analysis was conducted to determine whether the amount of volatile had an impact on response strength. To produce the heatmap, bar chart, box plots, and NMDS plots; ggplot2 package in R software version 4.1.2 was used.

## Data availability

**The datasets used and/or analysed during the current study available from the corresponding author on reasonable request.**

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### Author contributions

Y.B., Y.W., T.D.B. and T.D. conceptualization. Y.B. and T.D.B. data Collection. Y.B. and T.D.B. data Analysis. Y.B. Writing the draft, and all authors, reviewed the manuscript.

### Declarations

### Competing interests

The authors declare no competing interests.

### Additional information

**Supplementary Information** The online version contains supplementary material available at <https://doi.org/10.1038/s41598-025-94928-9>.

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