The Interplay of Olfaction and Vision in Host Plant Selection by Anthrenus verbasci

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Article

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Abstract

Anthrenus verbasci (Coleoptera: Dermestidae) is a notorious pest of museums, where the infamous larvae can destroy valuable collections and displays. In households they are a nuisance; carpets, fabrics, food-items, or any proteinaceous material can be damaged and even in agriculture their presence is an unwelcome one, as stored grains, spices, etc. can be contaminated by larvae. We know very little about the host preference of adults, they are frequently seen on plants of the Apiaceae and Asteraceae family, yet we do not know why they prefer these species. The main concept is that visual contrast of white flowers attract these insects primarily, yet we know little about how floral volatiles mediate host recognition. We observed a high abundance of *A. verbasci* adults on the umbels of ground elder (*Aegopodium podagraria*) and sought to determine whether this mass attraction is mediated by its floral volatiles. With gas chromatography coupled electroantennography (GC-EAD/FID), we have detected 8 antenally active compounds from the headspace volatilome of *A. podagraria*. Y-tube behavioral assays revealed that the odor of intact ground elder (*Aegopodium podagraria*) umbels attracted *A. verbasci* adults significantly; however, this attraction was reduced when damaged umbels were presented. In Petri dish experiments, germacrene-D induced an aversional effect from adults, highlighting a possible repellency. Beetles seemed to prefer discs baited with *A. podagraria* headspace volatiles at first, yet choice changed and shifted towards the visual stimulus of dried ground elder umbellets at the end of the experiments highlighting the importance of vision and olfaction. From our studies we have concluded that olfaction could play an important role in host recognition and could be implemented in integrated pest management of *A. verbasci.*

INTRODUCTION

The varied carpet beetle (*Anthrenus verbasci*) is a cosmopolitan Coleoptera found worldwide, the presence of the insect is common in the palearctic, oriental, saharo-arabian and sino-japanese zoogeographical regions [1]. Generally speaking, the larvae feed on dried protein material, thus its diet range can be quite extensive: from household items such as clothing, fabrics, carpet, food items, etc. [2,3], to more *"gourmet"* like preserved insect or other animal specimens displayed in collections or in museums [4]. The occurrence of *Anthrenus* beetles is an unwelcome one in the human environment. In seven zoological museums in India, *Anthrenus coloratus* caused extremely severe damage to the collections of 379 stuffed specimens; 195 birds and 27 mammals were damaged. Mainly the feather, fur, skin and patagium were destroyed [5]. Among the *Anthrenus* species causing damage to museum materials, *Anthrenus verbasci* is the most frequent, since larvae can penetrate storage boxes through the smallest gaps and crevices [6]. According to a pest faunistic survey of 10 museum collections in Berlin, *Anthrenus verbasci* was the third most common species, while out of 9 museums in Vienna, it was the fourth most abundant among the museum pests [7]). In addition to museum collections, the damage of *A. verbasci* on musical instruments have also been described. On a certain clarinet, the larvae caused such damage that the instrument was rendered unusable [8], Furthermore, *Anthrenus* species can be a nuisance for violinists as well, truly leaving them to "*end on a down note"*. Its presence can also cause

human health problems. A 23-year-old woman living in Germany suffered from an allergic skin reaction caused by the larvae of *Anthrenus verbasci*[9]. In its natural habitat, the larvae are associated with bird and insect nests [10], spider webs [11], even with bat guano in caves [12,13]. Interestingly, the predatory behavior has also been documented; the larvae feed on the eggs of the moth species *Lymantria dispar* and *Orgyia detrita*[14,15]. The species can be significant in agriculture, as larvae can damage stored cereals and spices, reducing its marketability [16]. In early spring from May, the emerging adults opportunistically feed on the pollen and nectar of almost any available flower [10], but the adults are frequently seen on the florets of umbelliferae [17,18]. Due to their damage, chemical ecological studies of *Anthrenus* species have revealed the pheromones of several species. The sex pheromone of *Anthrenus flavipes*, (*Z*)-3-decenoic acid was determined as the first in the genus *Anthrenus*[19]. The decyl-butyrate has proved to be the sex pheromone of *Anthrenus sarnicus*, besides decanol [20]. The sex pheromone (*Z*)-5- and (*E*)-5-undecenoic acid released by females of *Anthrenus verbasci*[21] has now also been identified. In addition to the chemicals released by conspecifics, plant odorants have also proven to be attractive to the varied carpet beetle. *p*-anisaldehyde, a component of the odorant often found in flowers, had an attractive effect on both sexes. In addition to *p*-anisaldehyde, *p*-methoxyphenylacetone and *p*-ethoxybenzaldehyde were also similarly effective [22]). It is known that *Anthrenus* spp. adults prefer *Apiaceae* species as a food source where they forage for nectar and pollen. It is known that the white florets contrasting to the background serves as a visual stimuli that helps the carpet beetles find a suitable host [17]. Visual signals are particularly relevant to *A. verbasci*, Goulson et al. demonstrated that the darker florets of the central umbellets of *Daucus carota* attracted more adults, compared to inflorescence where central florets were removed. Furthermore, replacing the darker florets with dead *A. verbasci* adults resulted in even greater attraction, whereas placing larger insects on the flower diminished this effect. It was proposed that the contrast between the darker central florets and the white outer florets serves as a crucial visual signal, darker florets potentially mimic *A. verbasci* adults [17]. Yet studies revealed that compounds like *p*-anisaldehyde induce attraction, meaning that olfaction too could be relevant. Overall knowledge of *Anthrenus* spp. chemical ecology is limited, furthermore how olfaction and vision can interact with each other.

In the spring of 2023 and 2024 large numbers of *Anthrenus verbasci* adults were detected on *Aegopodium podagraria* umbels. Although the presence of varied carpet beetles has been documented on ground elder before, the olfactory background as a possible answer to the high attraction of *A. verbasci* to *A. podagraria* has not been experimented with. The aim of this study was to identify certain compounds found in the headspace of *A. podagraria* that elicit antennal response from varied carpet beetles. To uncover the physiologically active volatiles, gas chromatography coupled electroantennography (GC-EAD/FID) was used. Furthermore, Y-tube experiments were performed with the antennally active components and the evoked behavior elicited by compounds on adults was documented. While we focused on insect olfaction, we have tested visual cues and paired it against odour cues to see the possible interaction between them in Petri dish experiments.

MATERIAL AND METHODS

Insect collection and identification

For electroantennography and behavioural experiments, *A. verbasci* adults were collected during May from a uniform patch of blooming *A. podagraria* plants located at N 47.54768°, E 18.93468°, Pest County, Hungary (Fig. 1.a). The taxonomic identification of insect adults (1.b / 1.c) was done according to the description in the manual: Taxonomy and Biology of Nearctic Species of *Anthrenus* (Coleoptera: Dermestidae) [10]. Botanic identification of *A. podagraria* was concluded by the description in the manual: The Identification Guide to the Vascular Flora of Hungary – Ferns – Flowering Plants [23]. Voucher specimens of *A. verbasci* were collected and stored in ~ 70% EtOH, while *A. podagraria* specimen was placed in a herbarium. The voucher specimens are stored at Plant Protection Institute, HUN-REN ATK, Hungary.

Volatile collection and mass spectrometry

(1) Headspace volatiles were collected from the whole inflorescence of *A. podagraria*. Pre-blooming ground elder plants were dug up and potted individually in containers. Any dirt or external material was removed with water. Until blooming, plants were isolated with fine mesh nets to avoid unwanted insect colonisation. When blooming, the compound umbels were covered with cooking bags (Hewa roasting bags, Germany). To avoid any damage to the plants, and to form an airtight seal, cotton pads mixed with dental wax were wrapped around the flower stem. The cooking bags were fastened to the cotton wrapped stem with zip-ties. Adsorbent filters were inserted inside the cooking bags from the top and connected with PTFE tubings (id.: 5 mm) to a mobile volatile collection system, which provided necessary airflow and air filtering (Volatile Assay Systems,USA). The adsorbent was 50 mg of HaySep Q (80 mesh), and the airflow was set to 0.5 l min^{-1}. The headspace was saturated for 30 minutes prior sampling and the total sampling time lasted for 1.5 h. Headspace sampling was repeated on three individually bagged compound umbels from separate ground elder plants. From here forward, we will refer to the undamaged inflorescence of *A. podagraria* as '*intact umbels*'.

(2) Furthermore, individual blooming umbellets were cut from the peduncle of *A. podagraria* and headspace volatiles were sampled immediately. We used plant materials described above. We only sampled as many cut umbellets that would be originally on a whole compound umbel. Cut umbellets were placed in droplet shaped glass containers. The headspace was saturated for 30 minutes prior sampling. The glass container was equipped with ground joints, so a charcoal air filter (30 g) could be connected to it airtight, which supplied filtered air to the system. The adsorbent used for volatile trapping was 50 mg of HaySep Q (80 mesh) and was connected to the funnel end of the container with PTFE (ID.: 5 mm) tubings. A constant air flow of 0.5 l min^{-1} was supplied by air pumps. The total time per sampling lasted for 1.5 h under laboratory conditions, and headspace was collected in three repetitions. We will refer to samples where we used cut umbellets as '*cut umbels*'.

Headspace sampling of isolated blooming *A. podagraria* plants were only initiated, when we verified the presence of *A. verbasci* adults on ground elder flowers in natural conditions. The compounds captured by the adsorbents in both volatile collection method were eluted with 300 µl n-Hexane (purity 99.9%, VWR Chemicals) and stored at -40°C until electrophysiological experiments (GC-EAD/FID) and compound identification (GC-MS).

The methodology of chemical analyses was based on the work of Kecskeméti et al. [24] Plant volatiles were analyzed using gas chromatography coupled mass spectrometry (GC-MS) (HP Agilent 5890 GC and 5975 MS, Agilent Technologies). Samples were analyzed on standard non-polar capillary columns, HP-5 UI (30 m × 0.25 mm × 0.25 µm, J&W). The injector temperature was set to 250°C and operated in splitless mode for 0.5 min, the oven temperature was maintained at 50°C for 1 min, then increased at 10°C min^{-1} to 270°C and held for 4 min. The flow rate of the helium was 1.0 ml min^{-1}. Positive electron ionisation (EI+) was used, with an electron energy level of 70 eV, 2 scans s^{-1} were recorded in the range of 35–400 m/z. Compounds were tentatively identified by matching their mass spectra with those in the MS Libraries (NIST 23 and Wiley) using MassHunter (B.10.00, Agilent USA). Kováts retention indices were calculated for all compounds using C8-C20 alkanes calibration standards on HP-5 column. Calculated RIs were compared to RI values available in the NIST database. Furthermore, antennally active compounds' fragmentation pattern, retention time and retention indices were also compared with those of synthetic standards previously used on the GC-MS system to verify identification. Identical volatile collection setups, comprising empty cooking bags and adsorbent filters, were additionally prepared as control systems. The volatile compounds detected in these control samples were subsequently subtracted from those identified in the plant headspace samples to ensure accuracy in the analysis.

Electrophysiological reading of A. verbasci (GC-FID/EAD)

To identify the electrophysiologically active compounds in the headspace of *A. podagraria* samples, gas chromatography coupled electroantennographic detections were carried out (GC-EAD/FID). The setup of instruments were based on the work of Kecskeméti et al. [24] An Agilent 6890 N gas chromatograph (Agilent Technologies Inc., Santa Clara, CA, USA), equipped with an HP-5 capillary column (30 m × 0.32 mm × 0.25 µm, J&W Scientific, Folsom, CA, USA) and a flame ionization detector (FID) was used for separations. From the volatile samples, 2 µl was injected to the heated (220°C) injector port in splitless mode. The starting oven temperature was initiated from 50°C, held for 1 minute, and constantly increased to 230°C at a steady rate of 10°C min^{-1}. The Helium carrier gas was set to a constant flow rate of 2 ml min^{-1}. The GC effluent was split equally in a low dead volume glass four-way splitter. Two pieces of deactivated fused silica capillary columns (100 cm × 0.32 mm x 0.25 µm) were connected to the four-way splitter; one led to the flame ionization detector (FID) (280°C) and the other led to a heated (240°C) EAD transfer line (Syntech, Kirchzarten, Germany) and into a glass tube (10 mm I.D.) with a charcoal-filtered and humidified airflow of 1 l min^{-1} that was led over to the antennal preparation.

During the electrophysiological readings, *A. verbasci* adults were prepared with the following method: The insect was held with soft grip-strength entomological tweezers with its ventral side facing upwards. This way the head was accessible, since *Anthrenus* sp. contract their body parts tightly when threatened. The head was slightly pierced in the middle with the apex of a no. 11 surgical scalpel-blade (Swann-Morton) and the caput was pulled from the thorax. From the head, one the antennae was cut off with a surgical scalpel-blade (no. 11). For the recordings the removed antenna was held between two silica glass capillaries filled with Ringer's solution [25]. The glass capillaries were fixed on silver electrodes that connected to a pre-amplifier (EAG Combi Probe, Ockenfels Syntech GmbH, Germany) where the recorded signals were converted in to digital (IDAC-2, Ockenfels Syntech) (Fig. 2). The recording software used was GcEAD (version 1.2.5 by Syntech), during recordings, external amplification was set to 9, and lower threshold (low cutoff) was set to 5 Hz. Experiments were conducted in five repetitions. Only responses consistent across all replicates were analysed further. The fundamentals of data preparation and analysis was based on Biasazin et al. 's publication. Based on morphological keys, it is not possible to identify sexes of *A. verbasci* adults [10]. However the electrophysiological data did not indicate any discrepancies between individual's volatile perception, therefore the data were pooled together.

Behaviour experiments

I. Y-tube behaviour experiments

Experimental design

Bioassay experiments were performed in order to verify whether the attraction of *A. verbasci* towards *A. podagraria* is mediated by olfaction. The overall experimental setup was as follows: Experiments were carried out in a Y-tube glass system. The constant airflow was supported via an air pump with PTFE inner coating. The effluent air was filtered with activated charcoal (50 g) and connected through a gas-bubbler filled with sterilized distilled water for proper humidification. The filtered and humidified air was split into two lines, and each splitted line connected to a sample-container that held the test materials. The air flowing through the sampling-containers – carrying the volatilome of samples – was connected to the two diverging ends of the Y-tube with a threaded glass fitting. To ensure equal flow through the two diverging branches of the Y-tube, adjustable flow meters were also implemented (the air-flow through each line was set to 0.5 l min^{-1}). Every tubing used in the setup was made from PTFE and glass components had Duran ground joints, ensuring airtight connection. Behind the Y-tube setup, an LED light source was placed (V-TAC G-series, 36 W, 4320 lm, 4500 K) approximately 1 meter behind the experimental setup). Light stimulus was used during each experiment while no other luminous source was present. Experiments were carried out at 23°C (± 1°C) with 50–55% relative humidity (Fig. 3). In every experimental setup a single adult *A. verbasci* per repetition was placed at the end of the Y-tube and were given 5 minutes to choose. Prior to the experiments, insects were collected from nearby flora and were deprived from any food source for 48 hours. Only responding specimens were taken into statistical analyses, but the number of non-respondents are presented in figures and referred to in the results section.The responsiveness of individuals was determined based on their movement in the Y-tube assay.

An individual was considered responsive if it covered at least 80% of the distance from the bifurcation point, toward the volatile source. Since the sex of adults were not known, the Y-shaped glass where individuals traversed was washed with ≥ 99.9% acetone (Roth, Germany), after every measurement, and oven baked at 250°C for 0,5 h after the 5th repetition of an experimental trial.

Experiments

I/(1) To ensure that there is no bias towards either branch in our Y-tube setup, a control experiment was carried out, where there were no volatile stimuli placed inside in either treatment holding container. A total of 20 insects were tested individually.

I/(2) In this set of experiments, *A. verbasci* adults were tasked to find the blooming umbels of *A. podagraria* only via olfactory cues in a Y-tube olfactory system. Before the experiments took place, ground elder plants with ~ 10 pre-blooming umbels were placed in plastic pots individually and transferred to an experimental glass house. If any animals were present on the plants, then they were removed, the total plant surfaces was rinsed with 25°C water, to eliminate dirt, possible animal excrement, honeydew residue, etc. Test plants were wrapped in fine plastic mesh as isolation for about a week, and monitored daily until full blooming. Before experiments, the florets of the ground elder plant was covered in a cooking bag. The floral stems were covered in a thick layer of dental wax and sterilized cotton-pads; the cooking bag was tightened with zip-ties (paying attention to not damage the plant) to ensure airtight sealing. PTFE tube from the air pump connected into the bottom side of the cooking bag, and a separate PTFE tube connected to the top side of the bag, which led to one of the branches of the Y-tube (with an adjustable flow meter in between). To the other branch of the Y-tube an identical setup was connected, but instead of plant material, autoclaved and moist cotton balls were placed as control stimulus inside the cooking bag (Fig. 3). The two stimuli were not visible to the insects and were randomly assigned in every repetition. A total of 100 adults were tested in these experiments one by one. The aim of this behavioral experiment was to see whether the attraction is reproducible if only olfactory cues are presented for insects.

I/(3) The third set of experiments were identical in design with the previously described setup, but instead of using undamaged plants of *A. podagraria*, freshly cut umbels were placed in the sample container used as olfactory stimuli. Every other parameter was the same as I/(2). In total 50 adults were tested in this experimental trial individually.

I/(4) Based on the GC-EAD/FID experiments, 8 compounds elicited consistent and strong antennal responses. The synthetic equivalent of these active volatiles were tested in behavioral experiments. The synthetic volatiles were mixed with each other in a 1:1 ratio and diluted in mineral oil to give a final concentration of 10 ug/µl per compound. During the experiments 10 µl of synthetic mixture was pipetted onto a filter paper (1 cm diameter) as chemical stimulus for the carpet beetle adults. The filter paper was placed into a silica glass tube (15 cm long, 2 cm diameter) with ground olive fittings placed at both ends. For control treatment, 10 µl of mineral oil was dispensed on filter paper and placed into an identical container. The synthetic compounds were supplied by Sigma-Aldrich (*α*-pinene, *β*-pinene, *β*-myrcene, *α*-

phellandrene, limonene, (*Z*)-*β*-ocimene, (*E*)-*β*-ocimene, germacrene-D). In total the choice of 50 individual insects were recorded.

II. Petri dish experiments

Experimental setup

Multiple trials were conducted to observe the behavioural effects of various experiments on varied carpet beetles in a free moving environment. A silica glass Petri dish (D: 150 mm) was used as an experimental arena where insects were placed. The collected insects were deprived of any food source for 48 hours and were kept at 23 ± 1°C 60% RH and 16:8 L:D period. Only one beetle was placed in the arena per trial, and every experiment was repeated 20 times. Each trial involved placing a filter paper disk (D: 10 mm) inside the arena, treated with either a volatile sample, visual cue, both volatile and visual cues simultaneously, or left untreated to serve as a control (Fig. 4). A 10 minute adaptation time was given to insects after placing them inside the arena. Each experiment lasted a maximum of 25 minutes, and every trial was recorded at a 1920 x 1080 resolution, 30 frames per second in .mp4 format. Experiments were conducted from 10.00 a.m. at an average temperature of 24 ± 1°C; air pressure around 766 ± 2 mmHg, with a relative humidity of 54 ± 2%. Two identical lightsources (V-TAC G-series, 36 W, 4320 lm, 4500 K) were placed directly above (3 m high) the experimental arenas and diffusers ensured that light was dispersed in the room equally. After every experiment the glass arenas were oven baked at 150°C for 4 hours, and beetles were discarded, using only naive specimens in further trials. In experiments we recorded these behavior incidents: Initial reaction – meaning the exact time when beetles changed its trajectory after introducing stimulus. Directional change – the possible direction a beetle could move relatively to the stimulus: away, towards or no directional change. Stay duration of stimulus – time spent by beetles on stimulus. First stimulus found and difference between final decision – Only recorded when odour and visual stimulus was compared simultaneously. Duration of 3 cm locomotion – the time needed to move 3 cm away in any direction compared to germacrene-D baited discs. The possible differences in experimental setups were as follows:

II/(1) To test the possible aversional effect of germacrene-D, we dispensed 10 µl of this compound (10 µg/µl dilution of n-Hexane > 99.9%) on a filter paper disk and placed it in the ~ 2 cm vicinity of the carpet beetle (but never directly in front of its path) and observed behavioural changes. Furthermore we recorded the time needed for the beetle to move 3 cm away from the filter paper. For comparison, we placed 20 other beetles in empty arenas separately and timed the period when insects moved 3 cm without changing any direction serving as control movement duration data.

II/(2) To test the effect of intact *A. podagraria* inflorescence headspace volatiles, 10 µl of intact ground elder flower volatile collection was pipetted on a filter paper and placed in the ~ 2 cm vicinity of the carpet beetle (but never directly in front of its path).

II/(3) Visual attractivity of *A. podagraria* umbellets. The inflorescence of ground elder was separated to the individual umbellets and dried out at 40°C for one week. This ensured that no plant odour remained

and floretts presented only visual stimuli. These dried out umbellets were adhered with the mixture of cornstarch and water to the filter papers used in previous experiments. Only one dried out flower stimulus was placed inside the arena with one beetle. Like before, the visual stimulus was also placed in the ~ 2 cm vicinity of the carpet beetle (but never directly in front of its path).

II/(4) Choice comparison of beetles between *A. podagraria* headspace and visual stimuli. In this set of experiments two stimuli were present in the arena at the same time: One was the volatile collection of ground elder described in II/(2) and one was a visual stimulus of an umbellet same as used in experiment II/(3) while one beetle was introduced in the arena. These two stimuli and beetle were positioned inside the arena so that the distances between them were equal, forming an equilateral triangle.

II/(5) Control filter paper experiment. In the control experiment we used the same filter paper discs as mentioned before, but without applying any volatile or visual treatment. The filter paper was placed in the center of the arena.

The results will not be presented sequentially by experiment, as similar types of data were collected across different experiments. Findings will be grouped by data type to allow for a comparison and discussion of differences between experiments. Collected data types according to experiments are shown in the table below (Table 1):

Table 1
Data collection types in Petri dish experiments

	II/(1)	II/(2)	II/(3)	II/(4)	II/(5)
Initial reaction after stimulus introduction	x	x	x		x
Directional change compared to stimulus	x	x	x		x
Stay duration on stimulus		x	x	x	x
First stimulus found and difference between final decision				x	
Duration of 3 cm locomotion	x				

Data analysis

Y-tube behavioural experiments

To see whether the distribution of frequencies of *A. verbasci* adults differs significantly in Y-tube behavioural experiments, we conducted multiple *Chi-square goodness of fit* tests with expected frequencies of 1:1 distribution.

Petri dish behavioural experiments

In Petri dish experiments, to evaluate if there is a significant difference between the initial reaction time between trials ANOVA was performed (α = 0.05). The raw data was transformed with log(x) function, and the normality of residuals were verified by *Shapiro-Wilk's* test (p = 0,068). Since the homogeneity of variances failed, post hoc test was run by *Games-Howell's* method (α = 0.05).

To compare the time spent by beetles on filter papers in every experimental trial (except germacrene-D baited papers) *Kruskal-Wallis* test was performed (α = 0.05) (since normality of residuals were not proven according to *Shapiro-Wilk's* test p < 0,001), and differences in each subset was determined by *Dunn's Post Hoc* test with *Bonferroni correction* (α = 0.05).

To statistically evaluate directional change of insects based on treatment effect (except simultaneous comparison of *A.podagraria* volatilome and visual stimulus of dried *A. podagraria* florets) *Chi-square* tests were performed since the distribution of data was not normal. Pairwise comparisons of possible significance between trials were also done by *Chi-square* tests. Distribution within an experiment was analysed by *Chi-square goodness of fit* test expected frequencies of 1:1:1 or *One-Sample Binomial* test with hypothesised distribution of 1:1.

To highlight differences between the first choice of beetles during the comparison of *A.podagraria* volatilome and dried *A. podagraria* florets, *One-Sample Binomial* test was performed with hypothesised distribution of 1:1. To see how the final choice changed from the first one until the end of the experiment, *Chi-square* test was done.

To compare the locomotion time needed to reach 3 cm in germacrene-D aversion experiment *Mann-Whitney U* test was performed.

Statistical analyses were performed via IBM SPSS Statistics ver. 22 (IBM Corporation, Armonk, New York, USA). In Y-tube experiments only actively responding adults were taken into statistical analyses. Figures and results also report the number of non-responding specimens however. In Petri dish experiments the data of perished individuals were discarded.

Heatmap visualization was created with R Studio ver. 2022.07.1 Build 554 (R Core Team, 2023) using the package “ggplot2”. Additional image editing was done using Adobe Illustrator (Adobe Systems, Mountain View, California, USA), InkScape vector graphics program 1.4 ((86a8ad7, 2024-10-11) and GNU Image Manipulation Program (GIMP 2.10.18). Transformation of raw data (if needed) was done in Microsoft Office Excel 2016 (Microsoft Corporation, Redmond Washington, USA).

Video recordings were rendered with Camtasia software (TechSmith LLC., Michigan, USA) and behaviour of insects and insidents were analysed using Behavioral Observation Research Interactive Software 9.4.1 [26].

RESULTS

Volatile collection and mass spectrometry (GC-MS)

We have identified 69 volatile compounds from the headspace of cut and intact *Aegopodium podagraria* umbels. We detected qualitative and relative areal differences between cut and intact umbels. The majority of identified compounds were terpenoids; from the 20 monoterpenoids the first five most abundant volatiles were: limonene (cut: 25.92%, intact: 17.81%); *β*-pinene (cut: 15.27%, intact: 16.28%); *β*-myrcene (cut: 8.93%, intact: 7.15%); *α*-pinene (cut: 8.27%, intact: 9.29%); (*Z*)-*β*-ocimene (cut: 2.65%, intact: 1.72%). We have identified 18 sesquiterpenoids, where the five most abundant compounds were: germacrene-D (cut: 14.94%, intact: 2.91%); *α*-farnesene (cut: 7.14%, intact: 1.35%); (*Z*)-*β*-farnesene (cut: 2.4%, intact: 0.78%); *α*-caryophyllene (cut: 0.63%, intact: 0.2%); *β*-elemen (cut: 0.49%, intact: 0.12%). We also identified 9 hydrocarbons, 8 aldehydes, 5 alcohols and 3 ketones. We have detected 12 compounds that were unique to cut umbel volatilome and were not detected in intact florets. The full list of detected peaks and identified compounds are listed in Supplementary Table 1.

Electrophysiological readings of A. verbasci (GC-FID/EAD)

Regardless of using headspace samples from intact or cut ground elder florets, the antennae of *A. verbasci* gave robust signals to eight compounds consistently from the 135–144 detected compounds (Fig. 5). These antennally active compounds were *α*-pinene (80-56-8), *β*-pinene (127-91-3), *β*-myrcene (123-35-3), *α*-phellandrene (99-83-2), limonene (138-86-3), (*Z*)-*β*-ocimene (3338-55-4), (*E*)-*β*-ocimene (3779-61-1) and germacrene-D (23986-74-5). All of these compounds were terpenoids, germacrene-D being sesquiterpenoid while other active compounds were monoterpenoids. Independently from the quantity of each compound, largest signals were given to *α*-pinene and limonene (Fig. 6.a). However if responses are adjusted accordingly to a single percent of a compound area, then the strongest antennal responses were given to *α*-phellandrene, (*E*)-ocimene (Fig. 6.b). All antennally active compounds were tested with its synthetic equivalents (*α*-pinene ~ 70% mixture of isomers (PhytoLab); (-)-*β*-pinene ≥ 95.0%; (+)-*β*-pinene ≥ 95.0%; *β*-myrcene ≥ 90%; *α*-phellandrene ≥ 75% (stabilised); (R)-(+)-limonene ≥ 99%; *β*-ocimene ≥ 90% mixture of isomers (Sigma-Aldrich/Merck); Germacrene-D ≥ 90.0% (MedChemExpress) (Fig. 7).

Behavior experiments

I. Y-Tube Behaviour experiments

I/(1) During the testing of the experimental Y-tube setup, we did not indicate a difference in choice the *A. verbasci adults* made between the two blank arms of the olfactometer setup. Based on the Chi-square test of goodness of fit, the expected distribution did not differ significantly from the observed distribution [$X^2_{(1)}$ = 0.0 p = 1, N = 14]. This indicated that there wasn't any external factor in the experimental setup that could have influenced the choice of *A. verbasci* adults, ensuring that the setup was reliable for use in other experiments. From the 20 tested specimens, 6 adults (30%) did not make a choice and remained at the starting position (Fig. 8/1).

I/(2) During the second set of experiments, adult *A. verbasci* beetles significantly chose intact umbels of ground elder [$X^2_{(1)}$ = 8.562 p = 0.003, N = 73]. From the 73 responding specimens, 49 (~ 67%) adults selected ground elder florets, and 24 (~ 33%) wet cotton. From the total 100 tested insects, only 27 did not respond at all (Fig. 8/2).

I/(3) In contrast, the preference of adults shifted when umbels with freshly cut stems were presented. Although not significant, adults chose wet cotton more (~ 62%), and only a smaller percent preferred cut umbels (~ 38%) [$X^2_{(1)}$ = 1.190 p = 0.275, N = 21]. As compared to the blank - blank (1) and intact umbel - wet cotton (2) experimental settings, the number of non-responding specimens grew notably. The proportion of non responders were around 58%, almost double what we have previously detected (27–30%), meaning a possible unidentified factor had a negative effect on the insects' tendency to start (Fig. 8/3).

I/(4) In this set of experiments, the synthetic mixture of antennally active compounds detected in GC-EAD recordings were tested. Statistically not significant, yet adults chose wet cotton in greater numbers (22) as opposed to the synthetic mixture (17) [$X^2_{(1)}$ = 0.641 p = 0.423, N = 39]. From the 50 tested specimens, 11 did not move from the starting position (Fig. 8/4).

II. Petri dish experiments

Initial reaction after stimulus introduction

In these sets of experiments we measured how much time passed until the beetle gave a first reaction to the stimulus placed inside the arena. A significant difference was detected between the experiments (F(3.74) = 21.418 (p < 0.001)). The shortest time for beetles to give a reaction was in the experiment where germacrene-D or the headspace volatile of intact *A. podagraria* inflorescence (odour stimulus) was added on the filter paper. Both of these trials significantly differed from the control paper and visual stimulus trials (germacrene-D – control paper: p < 0.001; germacrene-D – visual stimulus: p < 0.001; odour stimulus – control paper: p < 0.001; odour – visual stimulus: p = 0.003), however did not differ from each other (germacrene-D – odour stimulus: p = 0.935). The slowest initial reaction was induced when only control filter paper was introduced to the insects but this result was not significantly longer than visual stimulus (p = 0.147) (Fig. 9).

Directional change compared to stimulus

In these sets of experiments we recorded how certain stimuli affect the locomotion trajectory of carpet beetles, either beetles moved towards, away or did not change its path from the stimulus when it was introduced. Based on the *Chi-square* test, there were significant differences in directional distribution between experiments with a very strong association [$X^2_{(6)}$ = 103.74 p < 0.001, N = 78; Cramer's V = 0.815]. When germacrene-D was placed near the insects, a significant number of individuals changed their direction and went away from the stimulus [$X^2_{(2)}$ = 19.6 p < 0.001, N = 20]. When *A. podagraria* headspace

sample was dispensed on filter papers, every beetle (N = 19) chose to go towards the odour stimuli. A significant number of insects similarly changed their direction towards the flower stimulus ($p < 0.001$, N = 20). When untreated filter paper was placed in, most beetles did not change their path and continued in the direction where they were facing ($p = 0.19$, N = 19). Multiple Chi-square tests were performed to distinguish the distributional differences between pairwise cases (Supplementary Table 3) (Fig. 10):

Stay duration on stimulus

There was a significant difference in time spent on filter papers depending on the experimental trial indicated by *Kruskal-Wallis* test [$H(4) = 57.85$, $p < 0.001$, N = 98]. The longest periods were spent by insects on visual stimuli, almost reaching 1200 seconds on average. This was also true, when visual stimuli was compared with odour stimuli simultaneously (visual x odour stimulus). It is important to note, that if beetles found the visual stimulus, then many participants did not leave it even after experimental time ended. By its own, beetles stayed an average of ~ 230 seconds on odour stimulus and this duration was not significantly different, when odour was paired against visual stimuli simultaneously. The shortest time was spent on control paper discs, on average the insects stayed on it ~ 10 seconds before leaving it (Fig. 11). By *Dunn's Post Hoc* test, significant differences were revealed between groups (Supplementary Table 2):

First stimulus found and difference between final decision

During the simultaneous comparison of odour and visual stimulus more insects chose the odour stimulus first (70%), however this was not significantly greater than the expected distribution of 50% indicated by the *One-Sample Binomial* test ($p = 0.115$, N = 20) (Fig. 12.a).

When comparing the distribution of the first (14:6) and final choice (2:18) of insects, Chi-square test revealed a significant difference [$X^2_{(1)} = 15$ $p < 0.001$, N = 40; Cramer's V = 0.612]. At the end of the experiment the initial odour stimulus choice shifted towards the visual one (Fig. 12.b).

Duration of 3 cm locomotion

The duration at which beetles move 3 cm from the germacrene-D treated papers (~ 20,3 s) did not differ significantly than how insect move in an empty arena (~ 18,7 s), revealed by *Mann-Whitney U* test ($U = 165$, $z = -0.44$, $p = .675$, N = 38).

DISCUSSION

Anthrenus spp., commonly known as carpet beetles, is one of the most speciose genus of Dermestidae; with approximately 250 species described worldwide [27]. The varied carpet beetle, *Anthrenus verbasci* is known for its synanthropic behaviour, often inhabiting human dwellings as well as agricultural establishments. They are notorious for damaging stored products, spices, textiles, or museum collections. Their larvae can cause serious damage by feeding on wool, feathers, dried plant matter,

leather, fur, etc. [28,29]. Since the economic damage is attributed to the larvae of carpet beetles, little attention is paid to the host breadth of adult insects. In nature, *A. verbasci* adults can be found on the flowers of several plant species, where they opportunistically feed on pollen and nectar [30]. Most commonly, *A. verbasci* adults appear on the umbels of plants belonging to the Apiaceae family [17,31]. The presence of *A. verbasci* on ground elder have been described before [32], however the possible cause for this interaction between these beetles and this plant species have not been well studied. Furthermore, the overall knowledge of the chemical ecology of varied carpet beetles is scarce and little information was known about plant species that prove to be attractive. The aim of this study was to provide information regarding chemical ecology and highlight the possible importance of olfactory cues in host selection in this species. Furthermore we marginally experimented with visual cues on how they interact with odour sources.

Y-tube experiments revealed that olfactory cues alone were sufficient enough to elicit a positive response from adult varied carpet beetles toward ground elder flower odour. However this attraction was only observable, when uncut inflorescence was used in experiments, suggesting that due to damage, additional volatiles were released which caused a possible aversion behaviour in insects. Similarly in Petri dish experiments, discs containing the headspace volatile of undamaged *A. podagraria* flowers initiated a quick response when presenting the stimuli. This response was significantly shorter than the visual stimulus of dried out flowers and the empty control disc. Furthermore, every participant directed its trajectory towards the odour stimulus, reinforcing the fact that headspace volatiles alone were enough to induce a positive response from beetles. Our findings suggest that antennal receptors may influence behavior and could play a significant role in host selection, since every participant found the ground elder scented discs, crawling and residing on them.

Speaking about host selection – for herbivorous insects – generally the primary determinants are visual and olfactory cues emitted by the hostplant; vision playing a more significant role at greater distances, whereas olfaction becomes more influential at closer proximities [33]. The prevailing view suggests that floral visual cues play the dominant role for *A. verbasci* [34], with Imamura even suggesting that floral scent is likely irrelevant [35]. Petri dish experiments revealed that initial response time to visual stimuli was not significantly shorter than control filter paper disc treatments. In the case of control, this could suggest that the white coloration of the control disc may have mimicked some floral cues, despite disparities in shape and detailed features, and the presence of yellow and green hues within the actual flowers. It is possible that from the distance experienced by the beetles, they could not distinguish the background contrast well enough from the control paper and the flower stimulus. The vibrant colour of flowers were greatly diminished due to the drying process, a necessary step to ensure that visual stimuli did not emit any odour at all. Furthermore, adult insects, particularly those in flight, primarily perceive visual stimuli from various angles in their natural environment. Consequently, the perception of visual stimuli on the largely two-dimensional arena surface may have been compromised, leading to slower processing than for olfactory stimuli. This discrepancy in perception conditions may account for the

statistically significant difference observed between response times to olfactory and visual stimuli, the speed of response to olfactory stimuli potentially surpasses that of visual stimuli.

A significant number of adults shifted their direction towards the dried flower stimuli. Overall, in the context of the experimental design, both olfactory and visual stimuli (excluding the repellent germacrene-D) elicited an approach behavior. The animals consistently moved towards both types of stimuli, with no significant difference observed in the direction of movement between the two. This indicates that, based on these findings, both visual and olfactory cues are individually attractive and effective for the adult insects. This phenomenon is not unique; for example, the Asian longhorn beetle (*Anoplophora glabripennis*) demonstrates host plant recognition (specifically *Acer negundo*) through both visual and olfactory stimuli, whether presented separately or in combination [36]. However, it's worth noting that in some species, like the Asian citrus psyllid (*Diaphorina citri*), the combined effect of host plant odorants and visual signals can enhance responses in mated individuals, whereas odorants alone may not always be sufficient to trigger a response [37]. Collectively, our results suggest that both the odorants emitted by ground elder volatile stimuli and the visual signals of flowers play a crucial role in foraging behavior. Importantly, these findings indicate that a synergistic or sequential presentation of these two stimuli is not necessarily required to elicit a behavioral response in the animals.

The aforementioned results depicted the choice of beetles if stimuli were tested separately. When presented with a simultaneous choice between visual and olfactory stimuli, the insects initially selected the disc with headspace volatiles of ground elder in 70% of trials, though a large portion, this preference was not statistically significant from the hypothesised 50–50% distribution. This initial choice suggests a predominant role for olfactory cues in host plant location, particularly when compared to visual stimuli. However, it's crucial to acknowledge the potential limitations in visual stimulus perception within the experimental setup, as discussed previously. Insects that initially chose the scented disc exhibited characteristic searching behavior upon arriving. Interestingly, the duration of residence on discs was not significantly shorter than when the scented disc was presented in isolation. A notable observation was that a significant proportion of animals subsequently departed the scented disc and migrated to the flower disc. Upon reaching the flower disc, searching behavior recommenced. Following this, the insects concealed themselves among the flowers and remained there until the end of the experiment. This sequence of behaviors — initial olfactory attraction, followed by visual confirmation and subsequent concealment — suggests that the animals visited the visual stimulus source for potential feeding. Thus, both visual and olfactory stimuli contribute to host plant interaction, with the olfactory cue appearing to exert a stronger initial influence in this experimental setup. The retreat and prolonged presence within the flowers after initial searching on the flower disc imply that the insects may have been seeking refuge after unsuccessful feeding attempts, indicating that the time spent on the flower disc was not exclusively dedicated to foraging. It is worth noting – although not significant – that there was an observably prolonged presence of insects on scented paper discs when they were presented by itself, and beetles departed from these baited discs earlier, when visual stimulus was also available. Hypothetical, yet this suggests that it was worth abandoning the host plant scented discs and approach

the visual stimulus which had no odour, but only a physically perceivable dimension. The behavior implies that beetles were possibly evaluating stimulus quality and making strategic decisions. In the absence of a physical reward on the scented disc, almost all individuals appeared to abandon the olfactory cue and explore the visual stimulus instead. This implies a degree of behavioral flexibility or risk assessment [38], where beetles weighed the potential benefit of remaining with the initial stimulus against the possibility of finding a more promising resource. In this particular case it seemed that olfactory cues gave a stronger stimulus at first, but after realising that there is no resource to feed on, it was a better opportunity to approach the other stimulus. Stenberg and Ericson found that visual cues (like colored targets) can override olfactory cues when locating hosts in the beetle *Altica engstroemi*[39]. Furthermore, when speaking about behaviour of insects on dried flower stimulus, we noted that beetles crawled on the flower, displaying foraging, initiating tactile behaviour at first, yet this searching behaviour quickly diminished and instead concealed themselves within the flower and stayed there stationary until the end of the experiment.This means that even if the searching, foraging time of insects did not differ between odour and visual stimuli, the ability to hide at least somewhere made the dried out flowers more attractive. It is possible, that in nature, the visual stimuli created by the large patches of white umbels contrasting to the background are more perceivable from a distance for *A. verbasci*, yet adults rely primarily on olfactory cues to assess and select the most suitable flower at a close range, as chemical signals provide critical information about nectar availability, floral quality, and host suitability. It is important to note that in certain species, such as *Rhagoletis pomonella*, both visual and olfactory cues can induce approach behaviors, with the stronger stimulus dictating the response [40].

We analyzed the duration time of insects on the disc containing their chosen stimulus. The shortest time was consistently observed on the control paper, a duration significantly different from that spent on all other stimuli, with the exception when odour stimulus was compared with visual one together. Beyond mere duration, distinct behavioral patterns were noted. On the control paper, insects exhibited continuous movement, frequently attempting to leave the disc upon reaching its edge. Conversely, on discs containing experimental stimuli, insects often paused. While not definitively confirmed due to observational distance, this behavior suggests exploratory "tasting" or sampling of the disc surface. This exploratory behavior was also observed on discs presenting ground elder flowers, after which the insects typically hid under the inflorescences. In the vast majority of cases, they remained within the inflorescences, regardless of whether the ground elder visual stimulus was presented alone or with the odorant stimulus. When both stimuli were present, this specific behavior was independent of which stimulus the animals encountered first. Crucially, no significant difference was found in residence times on discs containing the same stimuli, irrespective of whether those stimuli were presented in isolation or in combination with other stimuli in the arena.

In addition to visual cues, chemical signals play a crucial role in the ecological interactions of *Anthrenus verbasci*. There is scarce information regarding the chemical ecology of varied carpet beetles, however it is known that females emit sex pheromones during mate calling [41]. The emitted compound was identified as a mixture of (*Z*)-5-undecanoic acid and (*E*)-5-undecanoic acid [42]. Apart from sex

pheromone, there is only one notable compound that elicited mass attraction behaviour in *A. verbasci*. Originally implemented in a Chrysanthemum experiment for trapping flower thrips [43], it was observed that *p*-anisaldehyde baited traps caught adults of varied carpet beetles; moreover it lured both sexes. In the case of males, 1 g dosage of *p*-anisaldehyde caught as many individuals as the female sex pheromone [22].

In accordance with the literature, we did not identify *p*-anisaldehyde in the headspace of *A. podagraria* [44–46]. Yet in behavioural experiments, adults significantly chose that particular branch in Y-tube experiments where the volatile plume of intact umbels came from. Volatile components in flowers play a crucial role in attracting insects and influencing plant interactions with their environment. The composition of these volatile compounds can vary significantly among different plant families, including the *Apiaceae* family, which is known for its aromatic species. Common volatile compounds found in various flowers include terpenes (linalool, *β*-ocimene, *α*-pinene, myrcene, limonene, caryophyllene), alcohols (2-phenylethanol, benzyl alcohol), aldehydes (hexanal, benzaldehyde), and esters (methyl salicylate, ethyl butyrate, methyl butyrate, ethyl acetate) [47–52]. In the Apiaceae family, specific volatile compounds such as estragole, carvone, sabinene and γ-terpinene, linalool, caryophyllene, benzaldehyde, are frequently reported [53,54].

In the headspace volatile of *A. podagraria* we have detected compounds that are common floral volatiles and which are characteristic for the Apiaceae family (Table S1). If we examine the antennally active volatiles of *A. verbasci*, the components seem more common flower volatiles than specific Apiaceae ones. In general, adult *A. verbasci* and other carpet beetle species prefer umbelliferous plants, yet can be observed on other flower families like Asteraceae [32]. Furthermore it must be underscored that in the context of host plant selection of herbivorous insects, that the ratio of behaviourally active volatiles appears to play a more critical role than the existence of behaviorally active compounds in a host plant. Herbivorous insects are adept at detecting and responding to a complex blend of plant volatiles, which provide essential information regarding plant identity and quality. Insects analyse these complex mixtures for host recognition, suggesting that the ability to discern ratios of volatiles is crucial for effective host selection [55]. All plants emit volatiles, the challenge lies in detecting reliable cues that indicate plant quality, further underscoring the importance of the ratio of volatiles over mere composition [56]. Even small modulations in ratio can be detected by insects, which can be a determining factor in evaluating host plant condition [57] and distinguishing between non-host and host plants based on ratios [58]. Even though the EAD active components were more common floral volatiles, the specific components' ratio can be a determining factor when it comes to selecting a concrete host for *A. verbasci*. The active volatiles detected during electrophysiology were all mono- and sesquiterpenes, which in itself does not exclude the possibility of detecting other chemical groups and volatiles. Yet based on the host plants of *A. verbasci* – which are mainly umbelliferous plants that contain an abundant part of terpenes in their chemical composition – it is possible that *A. verbasci* has a terpene-tuned olfactory receptor array.

A crucial point in our study was the fact that positive behaviour towards ground elder volatiles was only observable if intact umbels were used in bioassays. This positive attitude of adults was diminished if the volatiles came from cut umbels. Although not significant, yet more adults chose wet cotton pads over cut umbel volatiles. It is further worth noticing that the number of non responding specimens took a sharp rise compared to what we recorded during other experimental trials. The percentage of non responding specimens in the blank vs. blank scenario was 30%; which could be interpreted as such, that the likelihood of non respondent behaviour in this specific *A. verbasci* population is around 30%. If we compare this non respondent percentage to the other experimental trials we get a similar result. Intact umbel vs. wet cotton: 27%; Synthetic Mix vs. wet cotton: 22%. Yet the non responding percentage rose drastically when cut umbels were used: 58%. This means that there was an extra factor that withheld insects from leaving the starting position, which resulted in an almost double the observed non respondent percentage. If we compare the ratio of EAD active compounds in the volatilome of intact and cut umbels we can see that most of the compounds have increased slightly. The only compound which drastically increased was germacrene-D. In the volatilome of cut umbels the relative area of germacrene-D was almost 15%, yet in the intact umbels it was around 3%; a five times difference in relative area. Germacrene-D is commonly found in various plant species, and has significant effects on insect behavior and physiology. This compound is primarily recognized for its role in plant defense mechanisms against herbivores, acting as a repellent or deterrent to various insect species. Germacrene-D exhibits repellent effect on ticks and mites [59,60], while it can attract beneficial insects such as pollinators, while simultaneously deterring herbivorous pests [61,62]. Furthermore, germacrene-D may serve as a cue for predators, thus affecting the dynamics of plant-insect interactions [63]. The elevated amount of germacrene-D could have been an important cue for carpet beetle adults, that the offered umbel sample suffered significant damage, meaning either herbivore competition is high or the possibility of predator presence is likely. The behaviour importance of germacrene-D is further supported by the observations made in Petri dish experiments: Initial reaction time to germacrene stimulus was the shortest, meaning the insect perceived a change in its environment that forced out a rapid action. Although it is important to state, that no significant difference was detected in the reaction times to the putatively repellent germacrene-D and ground elder odorant stimuli. Behavioral assays clearly demonstrated that germacrene-D elicits an aversion effect, characterized by a distinct "moving away" response. This behavior sharply contrasted with responses to both the floral visual and headspace stimuli, as well as the control. The combination of this unique behavioral pattern and the rapid response time strongly supports the conclusion that germacrene-D acts as a repellent. In nearly all instances, the beetles actively moved away from the stimulus source. This movement was linear walking and sharp directional change. Such directed avoidance behavior is consistent with observations in other insect species reacting to repellent substances [64]. Given that the adult insects have an approximate body length of 3 mm [65], the 3 cm distance represents a significant displacement, roughly ten times their body size. The time required to travel the avoidance distance did not differ significantly from locomotion duration in an empty arena. This finding could suggest that the insects are moving at their maximal walking speed. In a true emergency, one might expect the beetles to attempt to fly from the source

instantaneously. It is noteworthy, however, that even when moving towards attractive stimuli, locomotion was primarily achieved by walking rather than flying. Furthermore, it is plausible that the inherent biological significance of these olfactory cues elicits a more pronounced and rapid behavioral response in the animals compared to visual signals.

According to the heatmaps, the strongest antennal responses were elicited by *α*-pinene, *β*-pinene, *β*-myrcene and limonene. However if we scale EAD signals to a single percent of the corresponding compound's relative area, the strongest responses seem to come from the detection of *α*-phellandrene and (*E*)-*β*-ocimene, both of which play an important role in insect herbivory. On one hand, both of these monoterpenes have an attractive effect on certain insect species and has an important role when insects seek suitable hosts [66], while in some cases the increased levels of this compound – which can be the result of herbivory – have been linked to the defensive responses of plants, which subsequently reduces the palatability, or results in the higher toxicity to herbivores [67], furthermore these compounds are an important volatile cue for predatory insects and parasitoids [68]. While there are parasitoids that are associated with Dermestidae, the parasitoid wasp *Laelius pedatus* and the predatory stink bug *Xylocoris flavipes* only prey on eggs and larvae of *A. verbasci*. The antennal sensitivity to these compounds could also be important for *A. verbasci* adults, when it comes to selecting the most suitable host, since these compounds – and other terpenes – elevated levels are key indicators of herbivory. The high amount of herbivore induced plant volatiles (HIPVs) serves to alert potential herbivores about their competitors' presence on a host plant. This is crucial, as herbivores may choose to avoid plants emitting high levels of HIPVs, signaling that they have been previously infested. It has been noted that some plants with altered volatile emissions following herbivore damage actually attract fewer herbivores in subsequent encounters, supporting the notion that high HIPV levels can indicate an occupied resource [69]. It is possible that the sensitivity to these compounds help varied carpet beetle adults determine if a host plant has already been colonised by other herbivores.

Although in trace amounts, 4,8-Dimethyl-1,3,7-nonatriene – or DMNT – has also been identified, which is an important compound regarding plant insect interaction. This acyclic monoterpenoid functions as a defensive compound that enhances the protective responses of plants. DMNT emission is strongly associated with insect herbivory, the accumulation of this compound can further induce plant defence by the production of protease inhibitors that render the plant less palatable to herbivores, while promoting jasmonate-independent defenses [70,71]. Additionally, DMNT plays a crucial role in the chemical communication between plants and insects, natural enemies can be attracted to herbivore-infested plants. The presence of DMNT has been shown to attract parasitoids and predators that prey on herbivores, acting as an indirect defense for plants [72]. DMNT can influence the host plant selection and foraging behaviour; as it can suppress the olfactory responses of herbivores, acting as a repellent in some species [73]. However, during GC-EAD the antennae did not respond to DMNT when testing cut umbel volatiles. It must be underscored that the relative area of DMNT in the collected headspace was around 0.01%, which may be under the detectable threshold of the antennae. Another answer could be that during antennal preparation for EAD experiments, the DMNT sensitive sensilla were covered by the

capillary during EAD thus no response was given to this compound. Furthermore it is important to note that different adsorbents have varying adsorbing quality, which is further affected by other factors such as temperature, humidity, pump speed, etc. Meaning that the DMNT emanating from freshly cut ground elder umbels could have been higher which was relevant during behavioural assays, however during volatile collection, the adsorbent filter could only absorb a trace amount; which subsequently was under detection rate of *A. verbasci* antennae during electrophysiology.

In summary, apart from visual signals, volatile compounds alone can play a determining factor when it comes to host recognition in *A. verbasci.* We first report the physiologically active volatiles to *A. verbasci* from the headspace of *A. podagraria*, yet the specific compounds which elucidates positive behaviour was not specified, the 1:1 ratio of synthetic blend did not attract adults. It was proven via Y-tube and Petri dish bioassays that olfactory cues alone are enough to elicit a positive attraction and searching behaviour from varied carpet beetle adults. This attraction was negated if odour was originating from a damaged floral umbel, suggesting that there are certain volatiles that increase avoidance in adults. Furthermore it was determined that adults initially located flower scented odour sources, but later almost all insects migrated to visual stimuli highlighting the importance of olfaction and vision in searching behaviour. Experiments showed that adults avoided germacrene-D and quickly moved to distant positions if germacrene-D was in close vicinity suggesting that this compound is repellent for *A. verbasci*. We hypothesise that elevated compound levels, probably germacrene-D can hinder the explorative behaviour of adults.

Declarations

AUTHOR CONTRIBUTIONS

Conceptualisation and investigation of behaviour phenomenon: DF, SK. Formal analysis, validation, visualization: DF, SK. Conceived and designed the experiments: DF, SK. Experiment performed by DF, SK. Structure elucidation: DF, SK, Analysed the data: DF, SK. Preparation of the manuscript: DF, SK. All authors read and approved the manuscript.

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The authors received no financial support for the research.

DATA AVAILABILITY

Raw data of experiments and recordings have been uploaded and publicly available at figshare: (https://figshare.com/s/b983bf3b153a371f783d).

COMPETING INTERESTS

The authors declare no competing interests.

RESEARCH INVOLVING PLANT AND ANIMAL COLLECTION

The plant species *Aegopodium podagraria* and the insect species *Anthrenus verbasci* used in the

present study are commonly found and are not protected in Hungary. Therefore, they can be freely collected and used without permit or approval from national authorities under Hungarian law 13/2001. (V. 9.).

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Figures

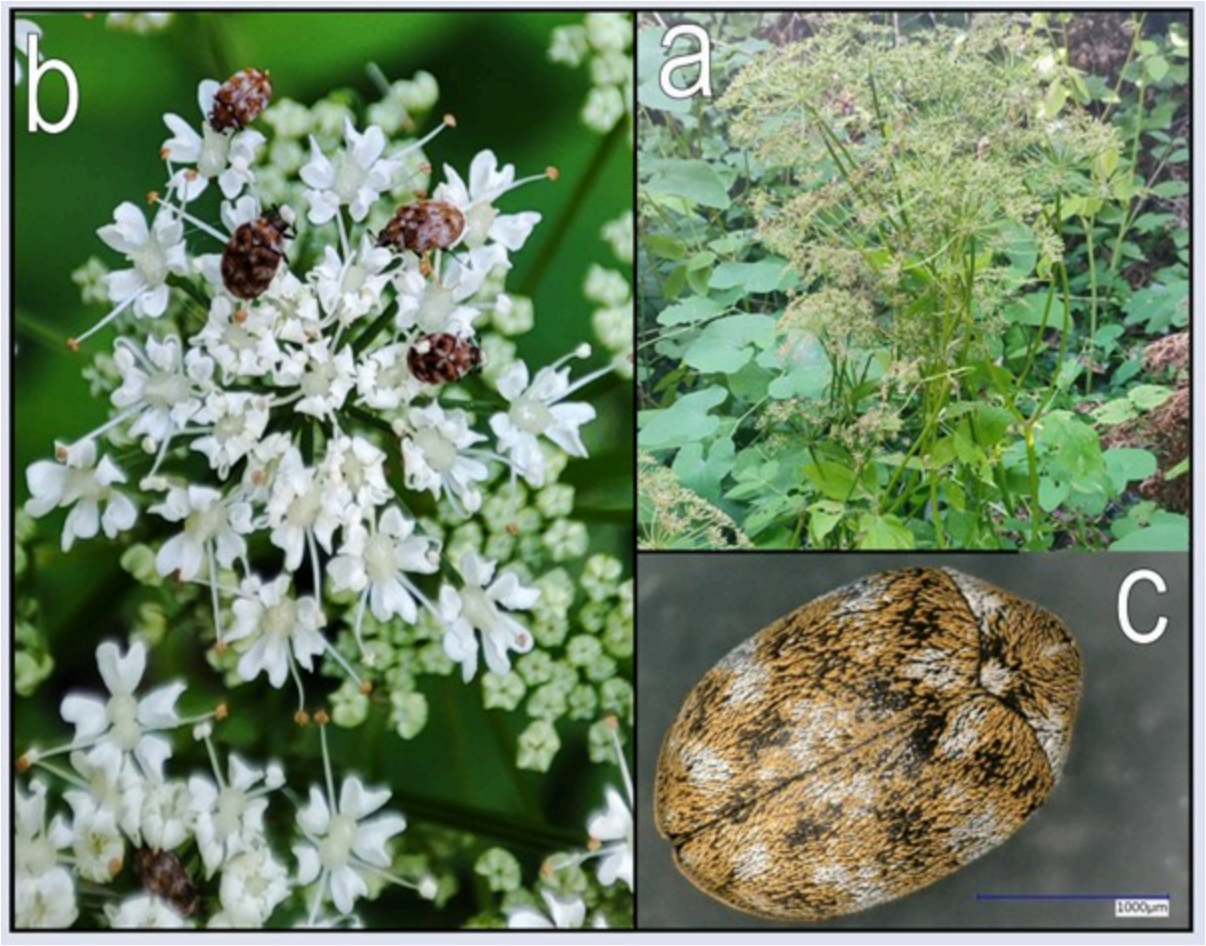

Figure 1

Showcasing ground elder **(a)** umbellets with foraging carpet beetles **(b)**; the collected specimens **(c)** were identified as *A. verbasci.*

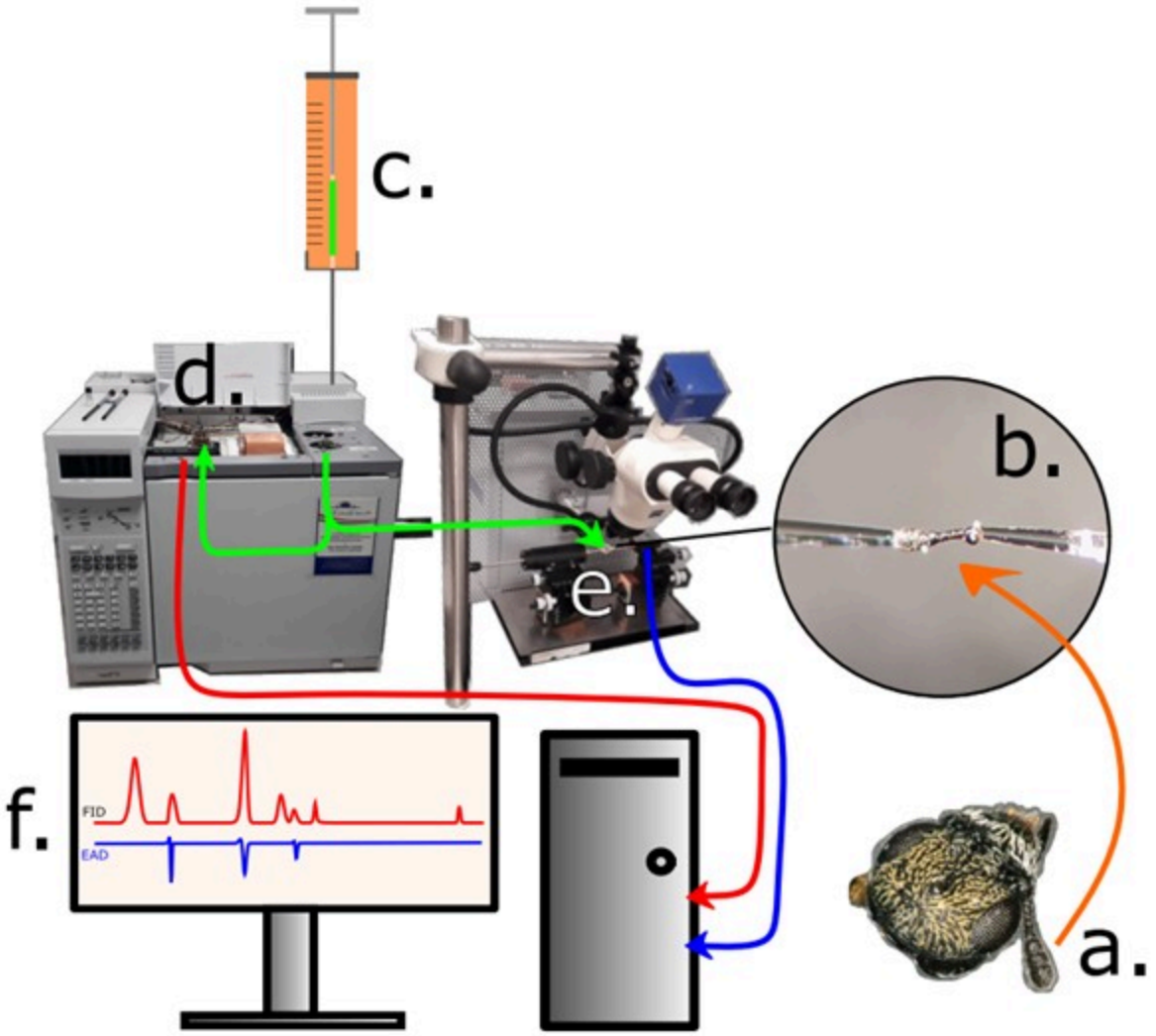

Figure 2

Schematic representation of gas chromatography-coupled electroantennographic detection (GC-FID/EAD). The antenna of *Anthrenus verbasci* **(a)** is excised and positioned between two glass capillaries **(b)**to establish an electrical connection. A volatile sample **(c)** is injected into the gas chromatograph, where individual compounds are separated based on their volatility. The effluent is split in a 1:1 ratio, directing one portion to a flame ionization detector **(d)**, which generates an electric signal proportional to the quantity of ionized compounds **(f)**. The other portion is delivered to the excised antenna via a humidified airstream **(e)**. If a volatile compound interacts with an olfactory receptor, an electric potential is induced, which is subsequently amplified and converted into detectable signals **(f)**.

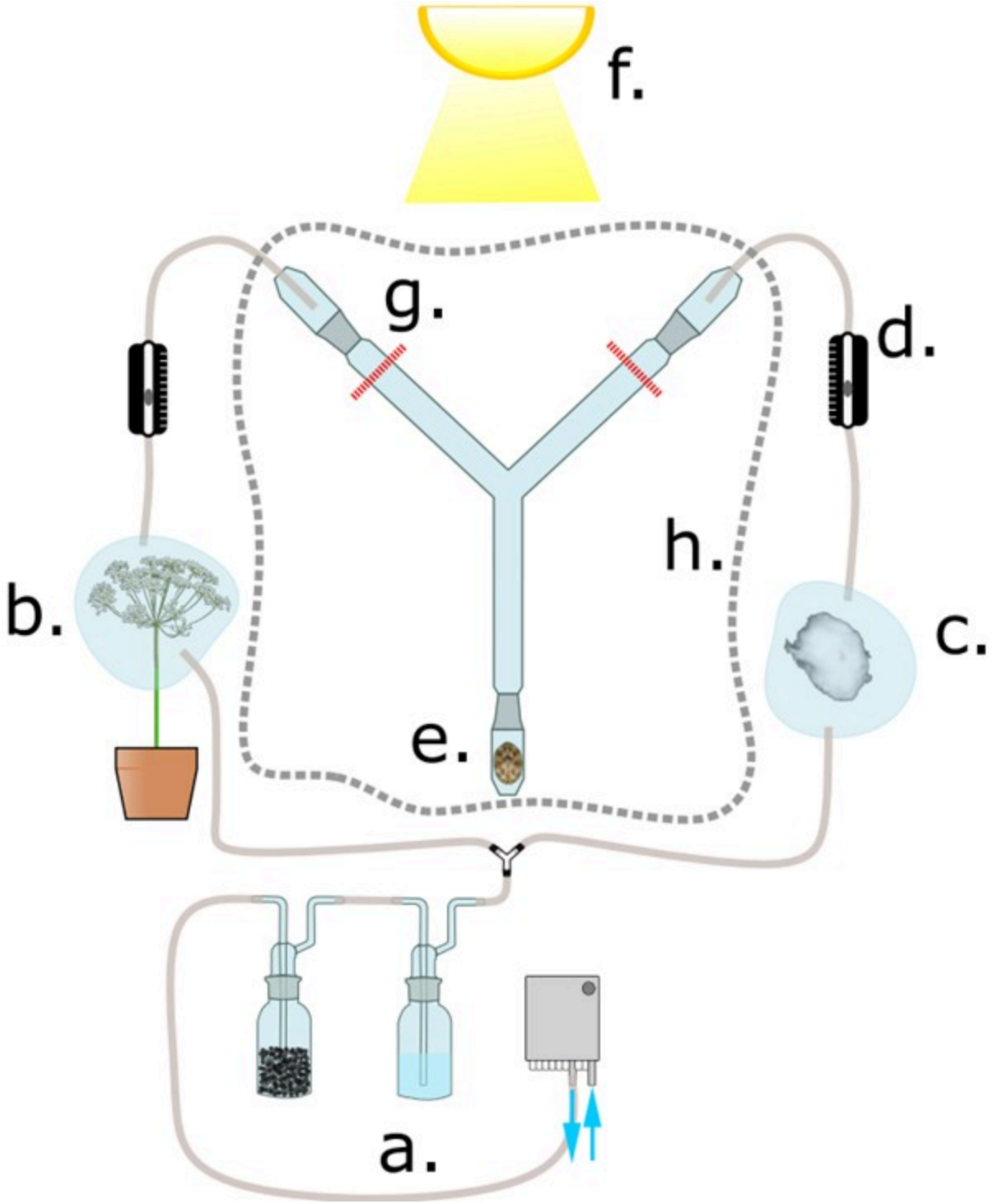

Figure 3

Schematic representation of the Y-tube olfactometer bioassay. Filtered and humidified air, supplied by an air pump **(a)**, is divided into two streams directed toward the sample container **(b)** and the control container **(c)**. Headspace volatiles from both chambers are conveyed through PTFE tubing into the olfactometer. Airflow balance between the two arms is maintained using adjustable flowmeters **(d)**. The test insect is introduced at the starting position **(e)**, where movement is stimulated by an LED light source **(f)**. A choice is recorded if the insect traverses at least 80% of the total distance from the Y-tube

bifurcation point **(g)** toward either arm. The Y-tube olfactometer was enclosed with a fleece barrier **(h)** to minimize external disturbances.

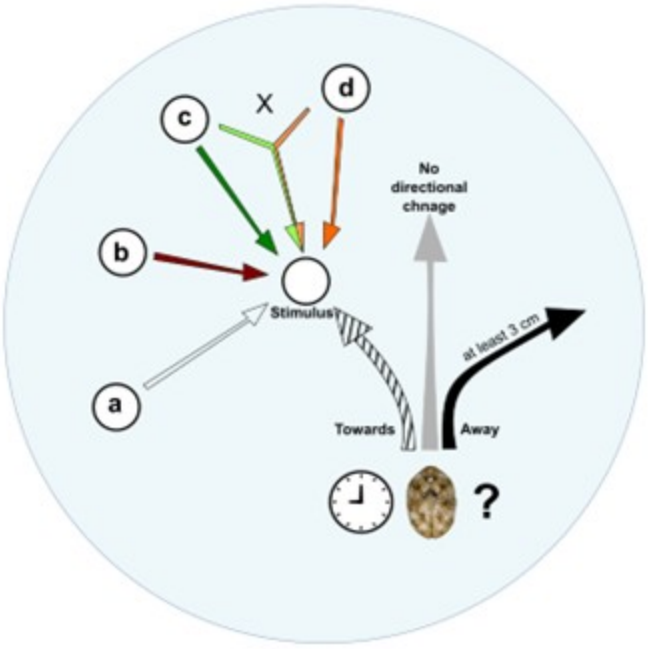

Figure 4

Concept of Petri dish behaviour experiments. Depending on the experimental trial, the initial reaction movement direction, stay duration on stimulus, first – last choice, or travel distance of *A. verbasci*adults was recorded. Stimulus was filter paper which either contained nothing (serving as the control) **(a)**, infused with germacrene-D **(b)**, baited with the headspace volatile of *A. podagraria* inflorescence **(c)** or held dried *A. podagraria* umbellet on it **(d)**. The “X” means that **(c)** and **(d)** treatment was introduced to the beetle simultaneously.

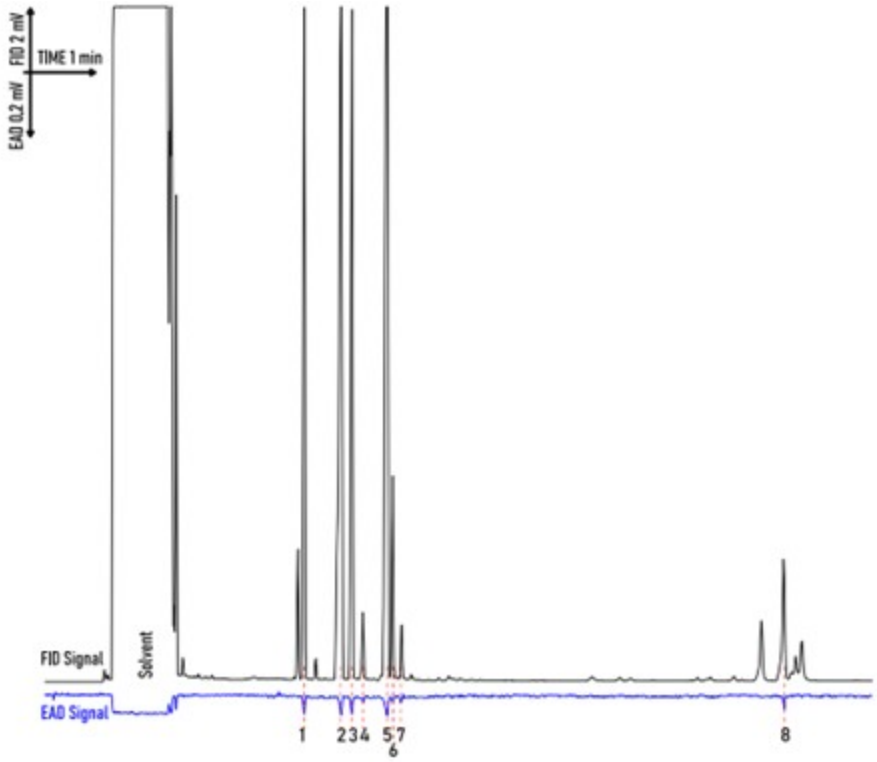

Figure 5

Representative Headspace volatiles compounds of *A. podagraria* umbels, detected by the flame ionisation detector (FID) (black line) and chromatogram (blue line) of potential changes in the antennae of *A. verbasc*i during gas chromatography coupled electroantennography (GC-EAD) recordings. Antennal responses are marked with arabic numerals (1-8) that align (dashed line) with a compound peak. The number of EAD peak was the same in the case of cut and intact umbel volatilome. (1: *α*-pinene; 2: *β*-pinene; 3: *β*-myrcene; 4: *α*-phellandrene; 5: limonene; 6: (*Z*)-*β*-ocimene; 7: (*E*)-*β*-ocimene; 8: germacrene D).

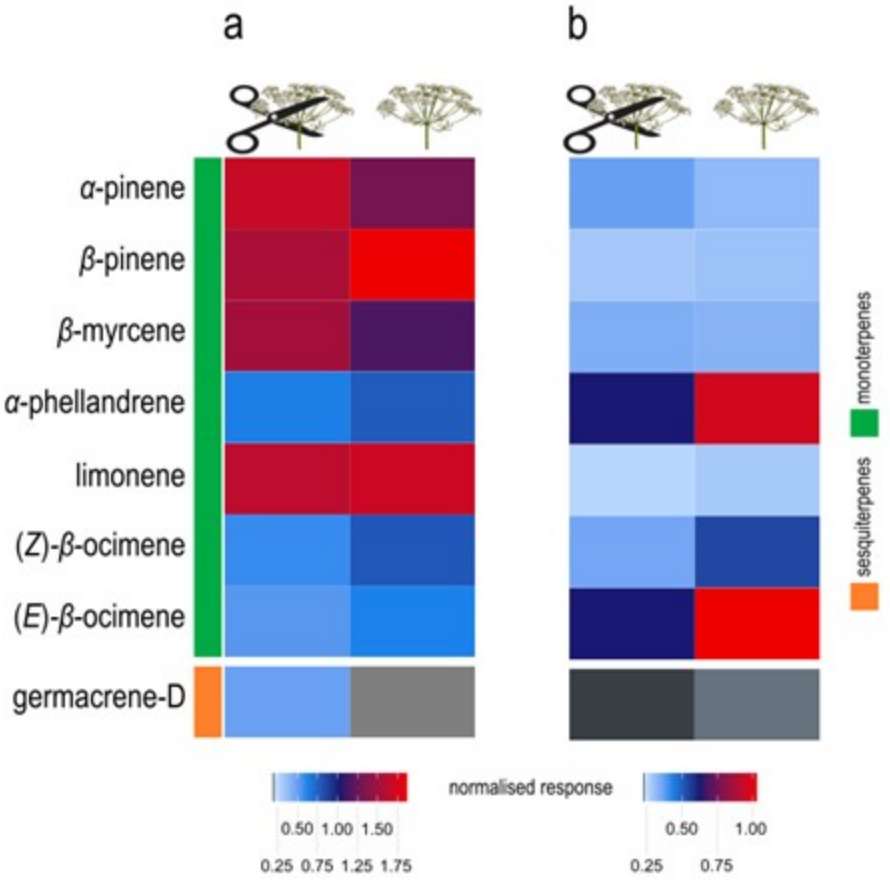

Figure 6

Heatmap illustrating antennal responses measured by GC-EAD using cut and intact headspace collection of *A. podagraria*. From left to right: columns show names of the identified volatile constituents, colour code for chemical classes, heatmaps of *A. verbasci* antennal responses to *A. podagraria* volatiles. Heatmap (**a**) represents the normalised antennal responses to physiologically active volatiles, whereas heatmap (**b**) depicts the normalized antennal activity adjusted to a standardised 1% relative area for each active compound. Compounds are organized according to chemical classes and respective retention time.

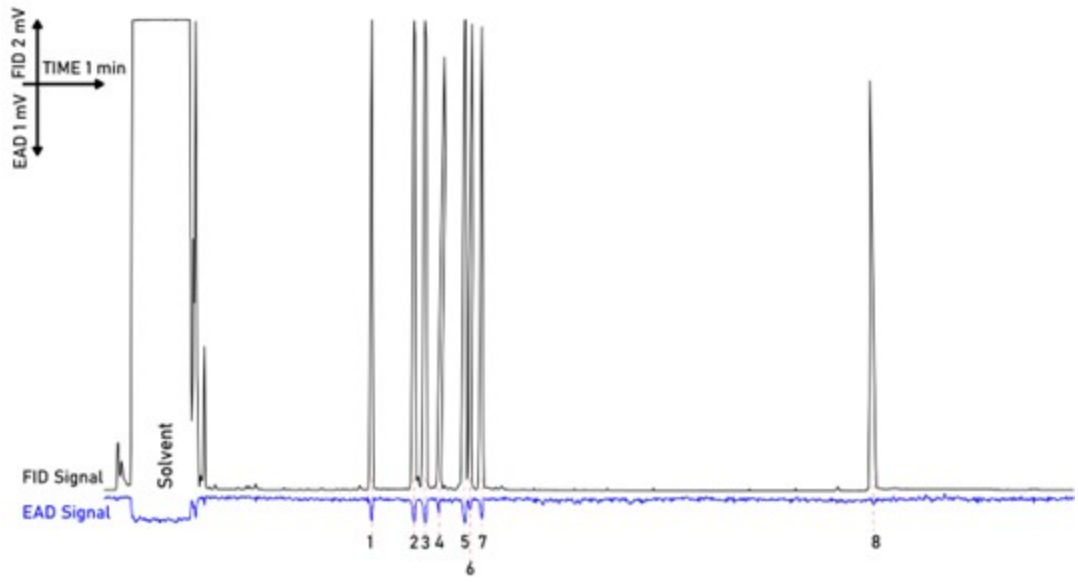

Figure 7

The recording of antennal response of *A. verbasci* adults to the synthetic equivalent of the 8 antennally active compounds detected from the headspace of *A. podagraria* umbels during gas chromatography coupled electroantennography (GC-EAD/FID). EAD signal (blue line) contains the antennal responses marked with arabic numbers from 1-8. (1: *α*-pinene; 2: *β*-pinene; 3: *β*-myrcene; 4: *α*-phellandrene; 5: limonene; 6: (*Z*)-*β*-ocimene; 7: (*E*)-*β*-ocimene; 8: germacrene-D).

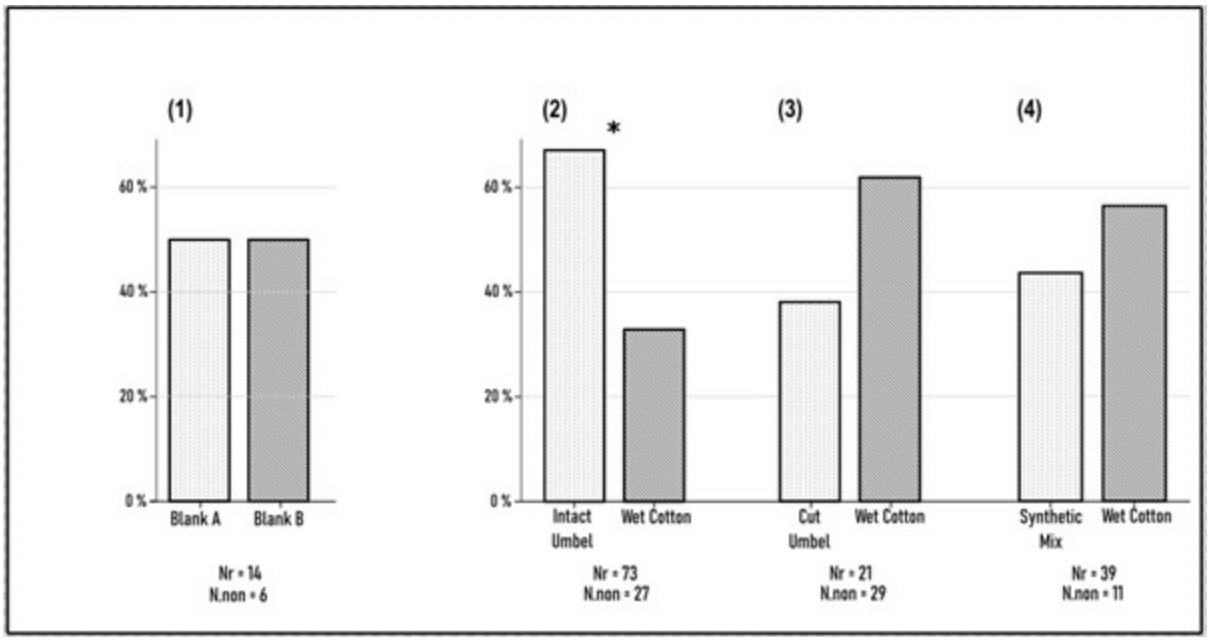

Figure 8

Observed distribution of responding (Nr) *A. verbasci* adults during Y-tube behavioural assays. (**1):** The observed distribution of adults in a blank vs. blank scenario to verify the unbiasedness of the experimental setup. The number of non responding specimens (N.non) are shown under each

experimental trial. Asterisk show significant difference between expected distribution (50-50%) and observed distribution based on *Chi-square goodness of fit* test. (**2)-(4):**Experiments involving volatile compounds emanating from intact and cut umbels of *A. podagraria*, and the 1:1 synthetic mixture of EAD active compounds. Asterisk indicate statistical significance (*p<0.05)

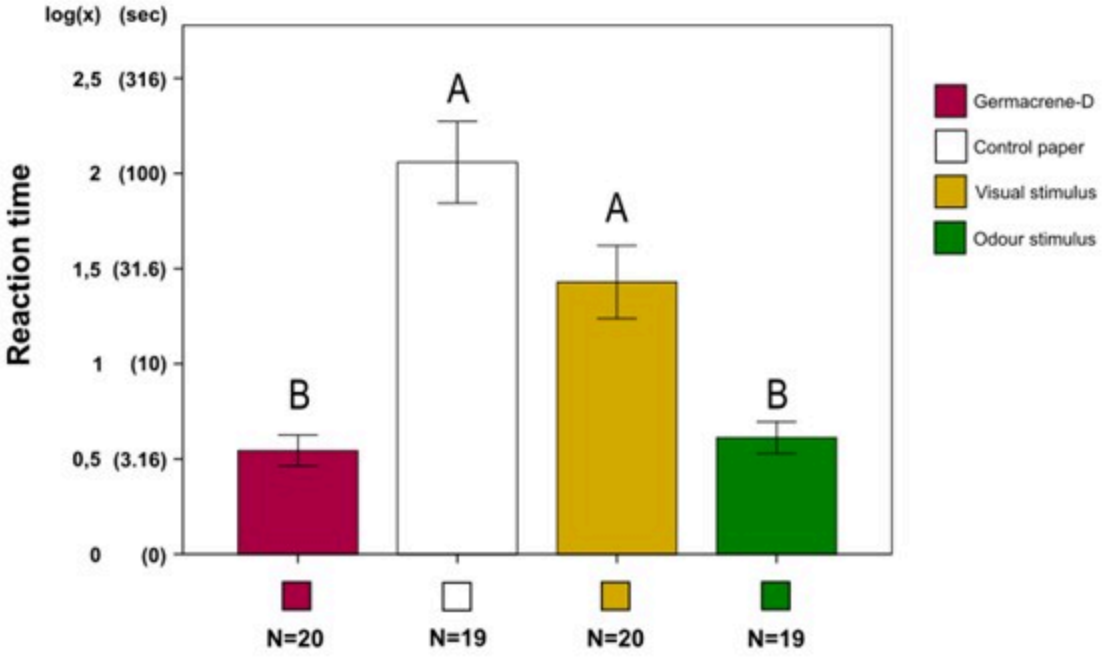

Figure 9

The average time it took for *A. verbasci* adults to give any form of behavioural response to the introduced stimuli (± SE). The results are presented in a logarithmic scale. Statistically different groups are indicated with capital letters revealed by *Games-Howell's* post hoc test (α = 0.05)

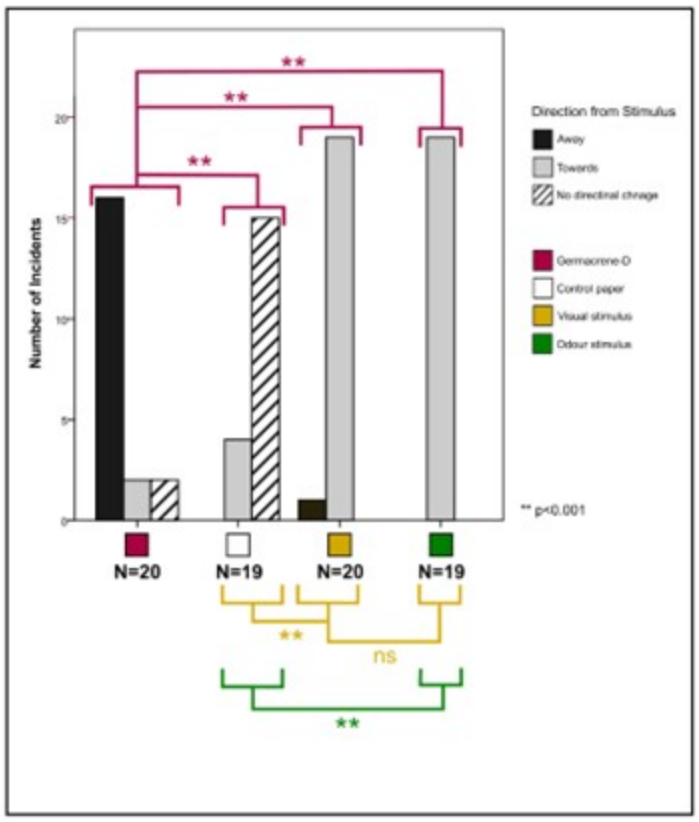

Figure 10

Distribution of incidents within and between experiments given by *A. podagraria* adults. The possible reaction of adults could vary between moving towards or away from the introduced stimulus or not changing direction at all. Significant differences between distribution of trials were marked with asterisks and were revealed by *Chi-square*test (α = 0.05).

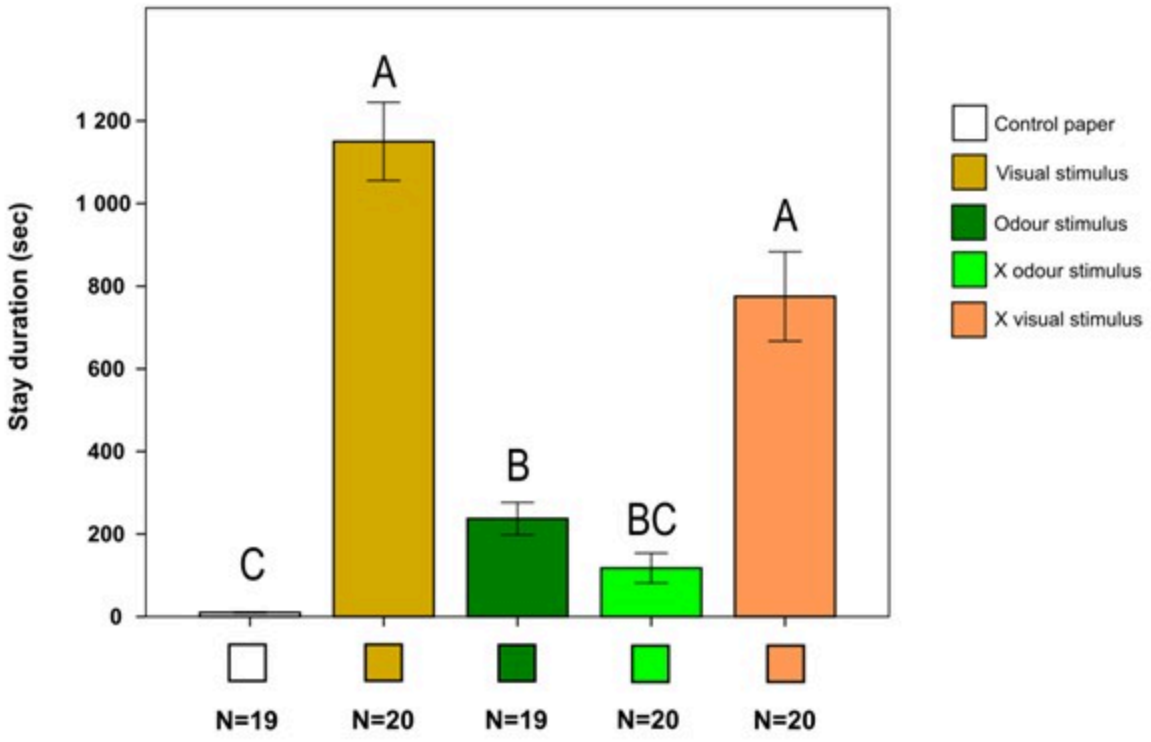

Figure 11

Stay duration of *A. verbasci* adults on different stimuli (± SE). Capital letters indicate statistically significant groups according to *Dunn's post hoc* test (α = 0.05). In the legend "x odor stimulus" and "x visual stimulus" indicates when these two stimuli were presented to the beetles at the same time.

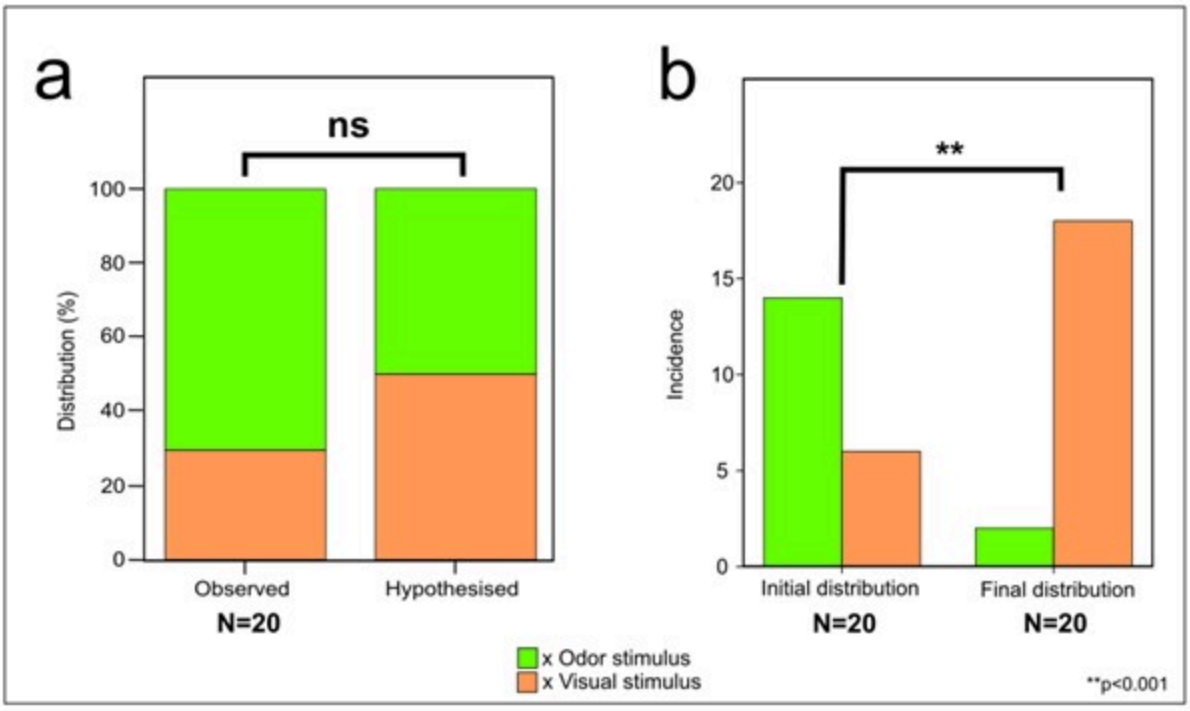

Figure 12

Distribution of insects according to their first choice (**a**) and the distributional difference at the end of the experiment (**b**). Statistical differences were revealed by *One-Sample Binomial* test (**a**) and *Chi-square* test (**b**) (α = 0.05). In the legend “x odor stimulus” and “x visual stimulus” indicates when these two stimuli were presented to the beetles at the same time.

Supplementary Files

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