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1 **Antennae of psychodid and sphaerocerid flies respond to a high variety of**
2 **floral scent compounds of deceptive *Arum maculatum* L.**

3
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23 **Abstract**

24 Insect-pollinated plants often release complex mixtures of floral scents to attract their
25 pollinators. Yet scent compounds eliciting physiological or behavioural responses in
26 pollinators have only been identified in few plant species. The sapromyiophilous aroid *Arum*
27 *maculatum* releases a highly diverse dung-like scent with overall more than 300 different
28 compounds recorded so far to attract its psychodid and other fly pollinators. The volatiles'
29 role in pollinator attraction is mostly unknown. To identify potential behaviourally active
30 compounds, we recorded electroantennographic responses of four Psychodidae and one
31 Sphaeroceridae species to (1) inflorescence scents of *A. maculatum* and (2) the scents released
32 by cow dung, likely imitated by the plant species. Here we show that these flies are sensitive
33 to 78 floral volatiles of various chemical classes, 18 of which were also found in cow dung.
34 Our study, which for the first time determined physiologically active compounds in the
35 antennae of *Psychoda* spp. and Sphaeroceridae, identified various volatiles not known to be
36 biologically active in any floral visitors so far. The obtained results help to better understand
37 the chemical basis of how *A. maculatum* and other plants, pollinated by psychodids and
38 sphaerocerids, attract and deceive their pollinators.

39 **Key words:** *Arum maculatum*, brood-site mimicry, *Coproica ferruginata*, cow dung, GC–EAD,
40 GC–MS, *Psychoda* spp., physiologically active volatiles

41

42 **Introduction**

43 Floral scents are important mediators of plant–animal interactions¹. Typically, a floral scent
44 bouquet is composed of 20–60 volatiles per plant species², but some species emit highly
45 complex bouquets consisting of more than 100 volatiles^{3–5}. In generalist pollination systems,
46 widespread compounds (e.g., 2-phenylethanol, phenylacetaldehyde, benzaldehyde^{6–8}) are
47 typically involved in pollinator attractions, whereas in specialised systems^{reviewed by 9}, the
48 specificity in pollinator attraction is often reached by the emission of either specific blends
49 composed of common compounds^{e.g. 10} or highly specific compounds. Examples of such
50 particular compounds are diacetin, which attracts highly specialised oil-collecting bees^{11,12}, 4-
51 methyl-5-vinylthiazole, which attracts cyclocephaline beetles^{13–15}, or *p*-cresol as well as
52 dimethyl trisulphide, which attract flies seeking oviposition sites^{e.g., 16,17}. Although the
53 number of compounds known to be involved in pollinator attraction is increasing, the
54 biological role of the more than 2,000 floral volatile organic compounds (VOCs) that have
55 already been described^{2,18} is largely unknown.

56 In plant species with highly complex bouquets, it is often not possible to obtain all floral
57 compounds as pure substances and to disentangle their individual behavioural effects.
58 Additionally, many scent compounds are often not identifiable, because characterised
59 references are missing, and are then listed as unknowns, which complicates investigations of
60 their biological role^{4,5}. In consequence, there are large gaps in our understanding of the
61 chemical communication, especially between plants with complex scents and their pollinators.

62 The fly-pollinated and brood-site deceptive *Arum maculatum* L. (Araceae, Fig. 1), a
63 wide-spread European perennial herb, emits a remarkable diverse scent. A single individual
64 releases up to 150 different scent compounds, and overall more than 300 compounds have
65 been recorded for this plant species so far^{5,19–23}. However, most of these compounds have not
66 yet been identified⁵. Among the identified compounds, some are widespread floral scents
67 (e.g., methyl salicylate, germacrene D) and/or well-known from brood-site deceptive plants
68 (e.g., indole, *p*-cresol, 1-octen-3-ol¹⁶), while others are rarely found as floral scents (e.g., β -
69 lutidine, *p*-cresyl butyrate^{2,18}).

70 In Central and much of Western Europe, *A. maculatum* is predominantly visited and
71 pollinated by female *Psychoda phalaenoides* L. (Psychodidae, Diptera)^{24,25}. Yet, in Western
72 France and the Mediterranean region, the most abundant visitors are often other psychodid
73 species (e.g., *P. grisescens* TONN., *P. trinodulosa* TONN., *P. zetterstedti* JEŽEK) as well as non-
74 psychodid Diptera, such as Sphaeroceridae^{24,26,27}. Psychodidae and Sphaeroceridae are rare
75 pollinators of angiosperms, but apart from *A. maculatum* also pollinate other species of *Arum*

76 (*e.g.*, *A. concinatum*, *A. cylindraceum*, *A. cyrenaicum*, *A. italicum*,) and other species/genera
77 of Araceae (*e.g.*, *Typhonium eliosurum*, *Arisaema heterocephalum*)^{28–32}. In *A. maculatum*,
78 insects are attracted on the first day of anthesis (female stage) to the pitfall-trap inflorescence
79 (Fig. 1). They slip and fall into the floral chamber (*i.e.*, the lower part of the inflorescence
80 where the fertile flowers are situated), where they are trapped overnight and are only released
81 on the second and final day of flowering, after being dusted with fresh pollen^{19,32,33}.

82 The strong, dung-like inflorescence scent of *A. maculatum* attracts its fly-
83 pollinators^{21,34} which breed in (and mate on) a range of different decaying organic matter,
84 such as moist leaf litter, mushrooms, cow or horse dung^{35–38}. Although the pollination biology
85 of *A. maculatum* is well-studied^{31–33}, the role of the individual floral volatiles for pollinator
86 attraction remains largely unknown. To date, only five compounds of *A. maculatum* (*i.e.*,
87 indole, *p*-cresol, 2-heptanone, α -humulene, and skatole), all also released by cow dung^{39,40},
88 have been tested for attractiveness to potential pollinators, and their attractive function in
89 (female) Psychodidae has been proven^{28,34,41}. However, preliminary bioassays at two natural
90 sites of *A. maculatum* (Salzburg/Austria; Daone/North Italy) could not verify that these
91 compounds, alone or as a blend, are attractive to psychodid (and sphaerocerid) flies,
92 suggesting that other compounds are more likely to be responsible for the main attractiveness
93 of the inflorescence ^{Gfrerer, E. *et al.*, unpubl.}

94 Compounds potentially behaviourally active in plants with complex scents, such as *A.*
95 *maculatum*, can be pre-selected by measuring the insects' peripheral olfactory detection of
96 floral scents, using gas chromatography coupled to electroantennographic detection (GC–
97 EAD^{42,43}). In previous studies testing scent of various plant species (*e.g.*, *Ceropegia*
98 *sandersonii*⁴⁴, *Encephalartos villosus*⁴⁵), such measurements frequently pointed to candidate
99 attractants and subsequently allowed the identification of behaviourally active compounds of
100 (complex) scent blends^{44–46}. A comparison of the physiologically active floral compounds
101 with the physiologically active compounds of the imitated substrate (or element) also helped
102 to identify the key compounds of the interaction^{11,47,48}. Here, we investigated the antennal
103 responses of four psychodid and one sphaerocerid species to the inflorescence scents of *A.*
104 *maculatum* and to scent released by cow dung. Specifically, we asked (1) which of the many
105 compounds recorded from this plant species elicit antennal responses in inflorescence visitors,
106 (2) whether antennal responses differ among insect species, and between sexes within species,
107 and (3) how many of the EAD-active scent compounds are shared between *A. maculatum* and
108 cow dung. The study overall aimed to identify potential scent candidates responsible for
109 attracting and deceiving different pollinators of *A. maculatum*.

110

111 **Material & Methods**

112 **Insect sampling**

113 During 2017–2020, we obtained insects at two natural sites of *A. maculatum*, one located in
114 Salzburg (Austria, 47°46'59"N 13°04'30"E) and the other in Marktschellenberg (Southern
115 Germany, 47°41'05"N 13°03'30"E). In 2017 and 2018, containers (5 L microboxes, Model:
116 TP5000 + TPD5000–18.5 cm × 18.5 cm × 19.1 cm; Combiness nv, Nevele, Belgium) filled
117 with fresh cow dung (c. 2 L) were offered to insects for three consecutive days as oviposition
118 substrate at the Salzburg site (April–October). Afterwards, the containers were brought to the
119 lab. Once psychodids and sphaerocerids started to hatch in the boxes, the flies were
120 transferred to a small outdoor flight cage (60 cm × 60 cm × 60 cm; BugDorm, Talchung,
121 Taiwan), in which they were offered different breeding substrates (soil, leaf litter,
122 mushrooms, cow or horse dung). Insects reproduced in the cage and were available for
123 electrophysiological measurements for four to six weeks, depending on the species. In spring
124 2019 and 2020, all insects used for measurements were obtained by bagging *A. maculatum*
125 individuals with mesh bags at both sites in the morning of the second day of anthesis, prior to
126 the release of the trapped insect visitors. Once released by the plant and trapped in the bag, the
127 flies were transferred to a flight cage and bred as described above.

128

129 **Floral scent collection, electrophysiological analyses (GC-EAD), and identification of** 130 **EAD-active compounds**

131 To acquire solvent scent samples for electroantennographic analyses, we collected
132 inflorescence scent of *A. maculatum* and volatiles released by cow dung using dynamic
133 headspace methods, following^{44,49}. Plant volatile samples were collected from a total of eight
134 populations (see Supplementary information Table S1), covering most of the observed scent
135 diversity⁵, whereas dung volatiles were obtained from dung samples (fresh or 1-day old) used
136 for the rearing of flies (see above). Each inflorescence was enclosed in a plastic oven bag (c.
137 30 cm × 12 cm; Toppits, Melitta, Germany) on the first day of anthesis between 17:30 and
138 20:00, when scent emission is strongest^{5,22}. C. 60 ml dung was placed into 250 ml glass jars,
139 covered with a plastic oven bag (see above). Volatiles were collected on adsorbent tubes
140 (length: 8 cm, diameter: 2 mm), filled with a mixture of Tenax-TA (mesh 60–80) and
141 Carbotrap B (mesh 20–40; 10 mg each; both Supelco, Germany), that were inserted through
142 small holes into the headspace of the inflorescence and dung each. Samples were collected for
143 0.5–1.5 h with a flow of 100 mL min⁻¹, generated by a battery-operated vacuum pump (rotary

144 vane pump G12/01 EB, Gardner Denver Austria GmbH, Vienna, Austria). Due to the
145 thermogenic activity of the inflorescence^{22,50}, we partly opened the plastic oven bag at the top
146 to avoid strong condensation of water inside the bag. Samples were eluted from each
147 adsorbent tube using 80–100 μL acetone (SupraSolv, Merck KGaA, Germany; following^{49,51}).
148 Then, samples were pooled per population (plant) or per age (dung, *i.e.*, fresh or 1-day-old) to
149 be used for the physiological measurements. To confirm physiological responses to specific
150 compounds, we recorded antennal responses to mixtures of synthetic compounds for a subset
151 of compounds found in *A. maculatum* (Table 1, Supplementary information Table S1).

152 Electrophysiological measurements were performed with a gas chromatograph
153 (Agilent 7890A, Santa Clara, California, USA) equipped with a flame ionization detector
154 (FID) and an electroantennographic detection system (GC–EAD)^{44,49}. One microliter of a
155 solvent scent sample was injected in splitless mode (250 °C), with hydrogen as the carrier gas
156 (column flow: 3 mL min⁻¹). During the period of testing (2017–2020), the GC was equipped
157 with three different columns. In 2017, it was a ZB-5 fused silica column (5% phenyl
158 polysiloxane; 30 \times 0.32 mm, 0.25 μm film thickness; Phenomenex, Torrance, CA, USA),
159 which was replaced in 2018 by a chiral fused silica capillary (30 m \times 0.23 mm I.D.), coated
160 with a 0.23 μm film of 0.4% heptakis (2,3-di-O-methyl-6-O-tert-butyl-dimethylsilyl)- β -
161 cyclodextrin (DIME- β -CD) (30%) in SE-52 (70%), the same as described in⁵² and⁵³. From
162 mid-2019 onwards, the GC was equipped with another DIME- β -CD chiral column (MEGA-
163 DEX DMT Beta SE, 30 m \times 0.25 mm ID, 0.23 μm film thickness, MEGA S.r.l., Legnano,
164 Italy). The end of each column was split into two capillaries by a μFlow splitter (Gerstel,
165 Mühlheim, Germany), with nitrogen (N₂) as make-up gas (flow rate 25 mL min⁻¹). One of the
166 capillaries (2 m \times 0.15 μm inner diameter) led to the FID and the other (1 m \times 0.2 μm inner
167 diameter) to the EAD setup. The EAD was set up by a transfer line, heated at 220 °C, and a 2-
168 channel USB acquisition controller (Syntech, Kirchzarten, Germany). The outlet of the EAD
169 was placed in a cleaned, humidified airflow, directed onto the mounted antenna. Prior to
170 measurements, each fly was anaesthetised with CO₂, and the head and last antennomere
171 (apical-tip) were removed. Subsequently, the head and one randomly selected antenna were
172 each connected to a glass micropipette electrode, filled with 95% insect Ringer’s solution (8.0
173 g L⁻¹ NaCl, 0.4 g L⁻¹ KCl, 4.0 g L⁻¹ CaCl₂) and 5% Tween 20 (Sigma Aldrich, Vienna,
174 Austria), and connected to silver wires. The recording electrode was attached to the tip of the
175 antenna, while the reference electrode was connected to the caudal side of the head^{44,54}.

176 Solvent scent samples of *A. maculatum* were tested on the antennae of five Diptera
177 species: the Sphaeroceridae *Coproica ferruginata* STENH. (one female) and four Psychodidae

178 species, *i.e.*, *Psychoda phalaenoides* (12 females and eight males), *P. zetterstedti* (two
179 females and two males), *P. trinodulosa* (one male), and *P. cinerea* BANKS (two females, all
180 Psychodidae) (Table 1, Supplementary information Table S1). All these fly species are
181 visitors of *A. maculatum*^{24,25;27}, except for *P. cinerea*, which is a pollinator of *A. hygrophilum*
182 and *A. italicum*²⁹, with the latter sharing several floral compounds with *A. maculatum*^{21,32}.
183 Two additional female *Psychoda* individuals (collected directly from *A. maculatum*) could not
184 be determined to species level, as their abdomen were too damaged, and might belong to the
185 four above mentioned species or (an)other species.

186 For identification of EAD-active compounds, scent samples were run on a gas
187 chromatograph/mass spectrometer (GC/MS, model QP2010 Ultra EI, Shimadzu, Tokyo,
188 Japan), equipped with either a non-chiral ZB-5 column (in 2017; see above) or a chiral
189 column (2018–2020; MEGA-DEX DMT Beta SE, see above). Helium was used as carrier gas
190 (flow: 3 mL min⁻¹) and samples (injection volume: 1 µL) were run with a split ratio of 1:1^{44,49}.
191 Obtained data were handled using *GCMSolution* v.4.41 (Shimadzu Corporation, Kyoto,
192 Japan). We tentatively identified components by comparison of Kováts' retention indices⁵⁵
193 (KRIs; based on commercially available *n*-alkanes C₇–C₂₀) and mass spectra available in the
194 libraries of Adams⁵⁶, FFNSC 2, Wiley9, NIST11, and ESSENTIAL OILS (available in
195 *MassFinder 3*, Hochmuth Scientific Consulting, Hamburg, Germany). The identity of some of
196 the components was verified by authentic reference standards, available in the collection of
197 the Plant Ecology Lab of Salzburg University (see Table S1). Compounds were classified as
198 inflorescence-specific or as vegetative compounds, according to Gfrerer *et al.*⁵. Absolute
199 amounts of compounds tested in the GC/EAD measurements (Table 1) were quantified by
200 injecting known amounts of various aliphatics and terpenoids and the resulting mean peak
201 areas were used for quantification⁵.

202 **Results**

203 Across all tested dipterans, we found a total of 78 volatile organic compounds (VOCs) from
204 *A. maculatum* (together c. 88% of the relative inflorescence scent emission of this species⁵)
205 that were electroantennographically active. The majority of these compounds were
206 inflorescence-specific, but five of them were vegetative compounds [*i.e.*, (*Z*)-3-hexenyl
207 acetate, nonanal, limonene, linalool, α -pinene; Table 1]. Overall, 55 of the EAD-active VOCs
208 could be (tentatively) identified (Table 1). They represented several chemical classes,
209 including monoterpenoids (*n*=25 VOCs), aliphatic compounds (15), sesquiterpenoids (eight),
210 irregular terpenoids (two), nitrogen-bearing compounds (three), and aromatic compounds

211 (two). Seven of these VOCs elicited antennal responses in all tested insect species: the
212 monoterpene 2,6-dimethylocta-2,6-diene (isomer 2), the aliphatic compound 1-octen-3-ol,
213 the sesquiterpene (*E*)- β -caryophyllene, the nitrogen-containing component indole, the
214 aromatic components methyl salicylate and *p*-cresol, and the unknown UNK1415 (Table 1,
215 Fig. 2). All other VOCs elicited responses only in a subset of insect species.

216 On average, 38 scent compounds yielded a response per species (and sex), with a
217 minimum of 13 volatiles in male *P. trinodulosa* (Psychodidae; one individual tested on one
218 scent sample), and a maximum of 60 VOCs in female *P. phalaenoides* (12 individuals tested
219 on nine scent samples). When considering both sexes of *P. phalaenoides* (total of 20
220 individuals tested on 16 scent samples) 61 VOCs were EAD-active, and in both sexes of *P.*
221 *zetterstedti* 49 VOCs (four individuals on eight scent samples; Table 1, Supplementary
222 information Table S1). For the female *C. ferruginata* (Sphaeroceridae; one individual tested
223 on three samples) 36 VOCs resulted in an antennal response. Notably, the aliphatic compound
224 2-nonanone was perceived by all psychodid species but did not elicit signals in *C. ferruginata*,
225 even though this compound was present in all three scent samples tested on the latter species.
226 In contrast, the monoterpene γ -terpinene was EAD-active only in *C. ferruginata*, but not in
227 the two psychodid species tested on this compound, *i.e.*, *P. phalaenoides* and *P. sp.*

228 Some compounds elicited specific responses in certain *Psychoda* taxa. For instance,
229 the aliphatic compound nonanal induced responses in *P. phalaenoides* and *P. zetterstedti*, but
230 not in *P. cinerea* and *P. trinodulosa*. The nitrogen-bearing compound skatole was EAD-active
231 in *P. phalaenoides* and *P. cinerea*, but not in *P. zetterstedti* and *P. sp.* (Table 1). The
232 sesquiterpene α -humulene resulted in responses in most individuals of *P. zetterstedti* and in a
233 few individuals of *P. phalaenoides*, but not in *P. cinerea* and *P. sp.* A number of the unknown
234 volatiles (*e.g.*, UNK883, UNK1394, UNK1492) elicited responses in *P. phalaenoides* and *P.*
235 *zetterstedti*, but not in *P. cinerea*.

236 For *P. phalaenoides* and *P. zetterstedti*, the two species for which we tested male and
237 female individuals, the analyses revealed some sex-specific responses. In *P. phalaenoides*, 24
238 compounds were EAD-active in both sexes, but one compound elicited responses only in
239 females (unknown UNK1030; $n=12$ individuals). In *P. zetterstedti*, 21 compounds were EAD-
240 active in both sexes, but four [2,6-dimethylocta-2,6-diene isomer 1, (*Z*)- β -ocimene, α -
241 copaene, 6-methyl-5-hepten-2-one] and two [(+)- α -citronellene, unknown UNK1378/81]
242 compounds elicited responses only in females ($n=2$) and males ($n=2$), respectively.

243 Of the 78 EAD-active floral compounds, 18 were also physiologically active in cow
244 dung samples (Fig. 2, Table 1), representing all different chemical classes recorded in *A.*

245 *maculatum*. More specifically, six of the seven VOCs that elicited antennal responses in all
246 tested insect species were among those 18 compounds [*i.e.*, 2,6-dimethylocta-2,6-diene
247 isomer 1, 1-octen-3-ol, (*E*)- β -caryophyllene, indole, *p*-cresol, and UNK1415; Fig. 2]. Among
248 those 18 were also VOCs that elicited different responses among fly families (2-nonanone),
249 fly species (nonanal, skatole), and within species between sexes [*i.e.*, 2,6-dimethylocta-2,6-
250 diene isomer 1, 6-methyl-5-hepten-2-one, (*E*)- β -caryophyllene].

251 **Discussion**

252 Our study is the first to identify electroantennographically active compounds in *Psychoda*
253 spp. (Psychodidae) and a Sphaeroceridae (*Coproica ferruginata*). It shows that these insect
254 visitors of deceptive *Arum maculatum* are sensitive to a high number of the plants'
255 inflorescence scent compounds. The EAD-active compounds identified represent various
256 chemical classes, including mono- and sesquiterpenoids, aliphatic, aromatic, nitrogen-bearing,
257 and unknown compounds. Antennal responses differed among insect species and between
258 sexes within species. More than a fifth of the physiologically active scent compounds were
259 also released by cow dung, linking insect breeding/mating sites, floral VOCs of *A.*
260 *maculatum*, and its floral visitors.

261 A few of the compounds recorded as physiologically active in the antennae of
262 *Psychoda* spp. (1-octen-3-ol, butanoic acid, α -pinene, and α -terpinene) were also found to
263 elicit electrophysiological responses in females of the phlebotomine sandfly *Lutzomyia*
264 *longipalpis* (Psychodidae, Diptera)^{56,57}, the only other psychodid used so far for physiological
265 measurements in the olfactory circuitry. The sandfly, which was tested on faeces from
266 vertebrates and canid host odours, additionally responded to several other volatiles that do not
267 occur in *A. maculatum* (including different isomers of monoterpenoids), but we provide the
268 first evidence that psychodids are able to perceive sesquiterpenoids. Physiological
269 measurements on antennae of Sphaeroceridae were not available before our measurements,
270 and thus, our study overall increases the knowledge about the peripheral olfactory circuitry of
271 psychodids and Sphaeroceridae.

272 Among the EAD-active volatiles recorded in the present study are the most abundant
273 inflorescence scents of *A. maculatum* included (*i.e.*, indole, *p*-cresol, (+)- β -citronellene, 2-
274 heptanone, 3,7-dimethyloct-2-ene, UNK1415,^{5,19,21–23,32}), but also numerous compounds
275 emitted only in small relative amounts by the plant (*e.g.*, 3-octanone, 2,6-dimethyl-7-octen-2-
276 ol; Fig. 2). Several of these abundant and less abundant compounds are also released from
277 cow dung (Table 1, Fig. 2) or other breeding/mating sites of the tested insect species. These

278 are, for instance, *p*-cresol, 2-heptanone, terpinen-4-ol, α -citronellene, and 2,6-dimethylocta-
279 2,6-diene (cow dung; Table 1,⁴⁰), 2-octanone (horse dung³⁹), β -citronellene and α -humulene
280 (both cow and horse dung^{39,40,59}), or 3-octanone and (*E*)-2-octen-1-ol (fungi e.g.,⁶⁰). Some of
281 the EAD-active compounds have not been detected in ours or others' scent samples from
282 (cow) dung and have not been described elsewhere. We speculate that those volatiles are
283 released from various other, likely differently scented breeding and/or mating substrates of *A.*
284 *maculatum* pollinators, such as mud-flats or leaf litter. It has been suggested that the
285 hyperdiverse floral scent of *A. maculatum* might result from the imitation of various
286 differently scented breeding substrates⁵. The sensitivity of the tested flies to a variety of
287 different compounds would support this idea, although other possible roles for those volatiles
288 cannot be excluded (e.g., repellence of florivores⁶¹, defence against pathogens⁶²).

289 Altogether five inflorescence scent compounds of *A. maculatum* have previously been
290 reported as attractive to psychodids. In detail, indole and *p*-cresol, together with α -humulene
291 or 2-heptanone, were found to attract female *P. phalaenoides*⁴¹ and *Psychoda* spp³⁴. In Kite *et*
292 *al.*³⁴, the attracted psychodids have not been identified to the species level; hence, it is
293 unknown whether they are flower visitors of *A. maculatum* or not. A synthetic mixture of
294 skatole, indole, and *p*-cresol, together with VOCs not occurring in *A. maculatum* (geranyl
295 acetone, dihydro-, and β -ionone), was shown to attract psychodid and sphaerocerid pollinators
296 of *Typhonium eliosurum*, a dung-mimicking aroid endemic to Australia²⁸. In preliminary
297 bioassays in the field, we tested the above five compounds, using the same composition and
298 concentration as released by the inflorescences of *A. maculatum*. Yet those volatiles did not
299 attract psychodid or sphaerocerid flies. This suggests that other, not yet tested scent
300 compounds (additionally) contribute to pollinator attraction in *A. maculatum*. Potential
301 candidates are other odours known also from cow or horse dung (e.g., 2,6-dimethylocta-2,6-
302 diene, unknown UNK1415; Table 1) or compounds known from other breeding substrates
303 (e.g., fungi: 3-octanone e.g.,⁶⁰). The unknown UNK1415, one of the main scent compounds of
304 *A. maculatum*⁵, yielded antennal responses in all insect taxa and in nearly all individuals
305 tested in the present study. Interestingly, this unknown volatile is possibly identical to
306 unknown "RI 1531" found in *T. eliosurum*²⁸, as both volatiles have the same mass spectra
307 (Supplementary information Fig. S1).

308 Our study shows that some of the antennal responses to scent differ among insect
309 species, and some also between males and females within species. This finding is in
310 agreement with results obtained by physiological measurements in other insects^{43,63}. Some of
311 the species-level effects described in the present study might have been influenced by sex-

312 specific effects, because for some species (*P. cinerea* and *P. trinodulosa*) we only tested
313 males or females. Hence, differences in antennal responses among these species need to be
314 interpreted with caution. Nonetheless, species- and sex-specific differences in the peripheral
315 olfactory circuitry of insects can result in different behaviours e.g.,^{64,65}. Interestingly, antennae
316 of *P. cinerea*, the only non-pollinating species of *A. maculatum* (but of other *Arum* spp.) we
317 tested in this study, did not respond to some abundant compounds emitted by *A. maculatum*
318 (e.g., UNK1394, UNK1492), which otherwise elicited responses in the other pollinating
319 species tested (e.g., *P. phalaenoides* and *P. zetterstedti*). The lack of antennal sensitivity to
320 (some of) those compounds might explain why *P. cinerea* does not visit *A. maculatum*, while
321 close relatives including *P. phalaenoides* and *P. zetterstedti* are important pollinators^{24,25}.

322

323 **Conclusions**

324 Until now, it has been not known which (and how many) volatile compounds of the complex
325 floral scent of *Arum maculatum* can be perceived by its floral visitors. Our study identified 78
326 physiologically active compounds from hundreds of potentially behaviourally active VOCs,
327 which is still a rather high number the psychodid and sphaerocerid flies are sensitive to. Our
328 results thus provide a basis for future studies that aim to understand the floral volatiles of *A.*
329 *maculatum* involved in the chemical attraction and deception of its pollinators, and which
330 VOCs guide the flies to their breeding/mating substrates. Some of the EAD-active VOCs (4-
331 terpinenol, α -terpinene, 2-heptanol, 2-nonanol, UNK1503) have recently been shown to be
332 under phenotypic selection in *A. maculatum*⁵. These compounds and those EAD-active ones
333 shared with the pollinators' breeding substrates (e.g., UNK1415, 3-octanone) are the most
334 promising candidates for future behavioural assays. As the tested Diptera species
335 (Psychodidae, Sphaeroceridae) are also known pollinators of other (similarly-scented) species
336 of *Arum* (e.g., *A. italicum*, *A. concinatum*) as well as other species/genera of Araceae (e.g.,
337 *Typhonium eliosurum*,^{28,31,66}), our study should also help to elucidate the chemical
338 interactions between these plants and their fly pollinators. Future research is now needed to
339 test the behavioural function of physiologically active floral volatiles, which is crucial for a
340 better understanding of olfactory cues mediating plant–animal interactions in general, and in
341 sapromyophilous species, in particular.

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352 **Author contributions**

353 MG, ACH, HPC and SD designed the study. EG and DL collected the scent samples. DL and
354 RW identified the Psychodidae to the species level. EG reared the insects, performed all
355 measurements, analysed all data, and wrote the first draft of the manuscript. All authors
356 contributed to the final manuscript and approved the submitted version.

357 **Additional Information**

358 Data availability

359 All data that support the findings of this study are included in this published article (and its
360 supplementary information files).

361 Competing interests

362 The authors declare no competing interests.

363 Experimental research and field studies on plants

364 All samplings were carried out in compliance with the current laws of the respective countries

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527 **Figure legends**

528

529 **Fig. 1.** A flowering individual of *Arum maculatum* in a natural habitat. (Photo E. Gfrerer;
530 Salzburg, Austria).

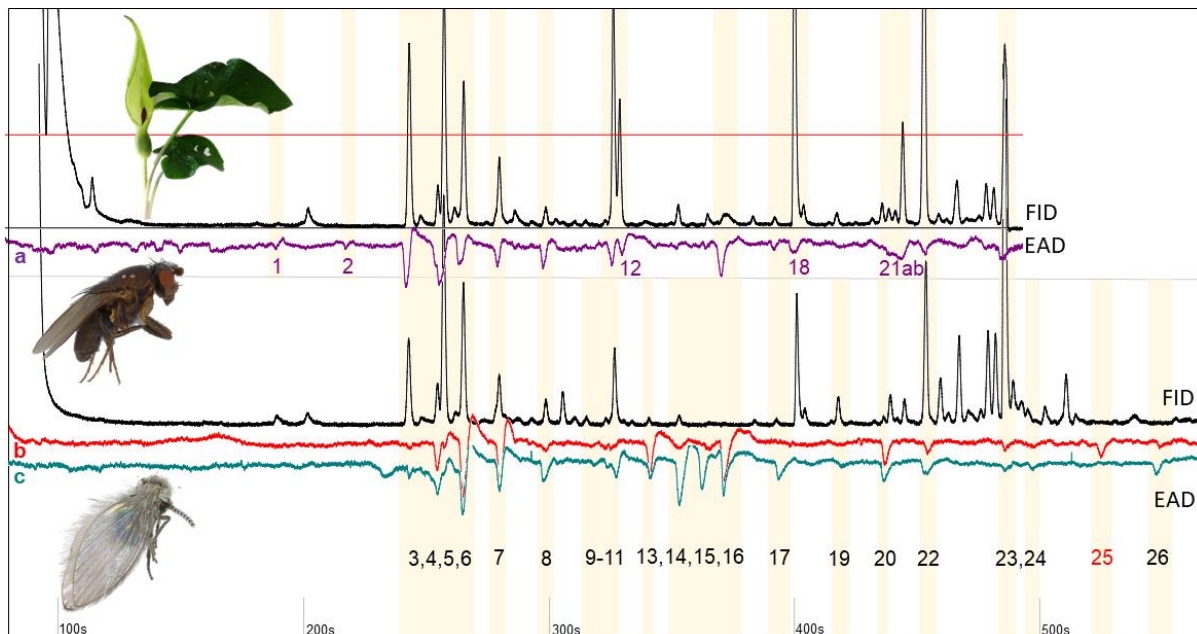
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534 **Fig. 2.** Representative physiological responses (gas chromatography coupled to
535 electroantennographic detection) of a female *Psychoda* sp. (Psychodidae; red) and a female
536 *Coproica ferruginata* (Sphaeroceridae; violet) to scent samples of **A**) inflorescences of *Arum*
537 *maculatum* and **B**) cow dung (fresh and 1-day old). EAD-active compounds (see also Table
538 1): (1) UNK883; (2) hexanal; (3) 1-hexanol; (4) 3,7-dimethyloct-1-ene; (5) (+)- α -citronellene;
539 (6) (+)- β -citronellene; (7) 3,7-dimethyl-2-octene isomer 1; (8) 3,7-dimethyl-2-octene isomer
540 2; (9) 2,6-dimethyl-2,6-octadiene isomer 2; (10) 6-methyl-5-hepten-2-one; (11) UNK1030;
541 (12) octanal; (13) *p*-cymene; (14) 6-methyl-5-heptene-2-ol; (15) 1-octen-3-ol; (16) 2-
542 methylbutanoic acid; (17) 2-nonanone; (18) nonanal; (19) 2,6-dimethyl-7-octen-2-ol; (20)
543 (*E*)-2-octen-1-ol; (21) (*S*)-linalool; (22) 2-decanone; (23) UNK1135; (24) decanal; (25) *p*-
544 cresol; (26) 3,7-dimethyl-1-octanol; (27) α -copaene; (28) UNK1378/81; (29) UNK1394; (30)
545 UNK1415; (31) indole; (32) UNK1503/UNK1492; (33) UNK 1658. Numbers in bold (top
546 line) are VOCs eliciting signals in A) and B); numbers in italics (bottom line) are VOCs that
547 do not occur in *A. maculatum*. FID1 *A. maculatum*, population Josefiau; FID2 *A. maculatum*,
548 population Murnau; FID3 cow dung fresh; FID4 cow dung 1-day-old. See Supplementary
549 Table S1 for detailed population information. All samples shown were run on a chiral fused
550 silica capillary column (30% DIME- β -CD in 70% SE-52, see methods) and each
551 measurement lasted either 8 (*Coproica ferruginata*) or 10 min (*Psychoda* sp.).
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556 **Table 1.** Inflorescence scents of *Arum maculatum* that elicited electroantennographic responses in *Psychoda phalaenoides*, *P. zetterstedti*, *P. cinerea*, *P.*
557 *trinodulosa*, *P. sp.* (Psychodidae) and *Coproica ferruginata* (Sphaeroceridae). Compounds are sorted according to chemical classes, and within those
558 alphabetically. The volatiles printed in bold were also detected in cow dung. In parentheses, the numbers of individuals tested (*n*) and runs performed per fly
559 species and sex are denoted. Superscript values indicate the amounts (mean±SD) of volatiles tested in the GC/EAD measurements on different fly species and
560 sexes.

Species	<i>P. phalaenoides</i>		<i>P. zetterstedti</i>		<i>P. cinerea</i>	<i>P. trinodulosa</i>	<i>P. sp.</i>	<i>C. ferruginata</i>
Sex	♀	♂	♀	♂	♀	♂	♀	♀
# individuals, # runs	(n=12, 33)	(n=8, 14)	(n=2, 6)	(n=2, 4)	(n=2, 6)	(n=1, 1)	(n=2, 8)	(n=1, 3)
Chemical class / compound								
Aliphatic compounds								
Butanoic acid ^S	++++ ^{0.6±1.2}	NP	NP	NP	NP	NP	++++ ^{0.7±1.6}	NP
Decanal ^S	++ ^{1.9±1.1}	+ ^{1.3±0.9}	++++ ^{0.5±0.8}	NP	+++ ^{1.0±1.4}	+++ ^{3.4}	++++ ^{1.3±1.2}	++++ ^{1.4±1.6}
2-Decanone	++++ ^{0.4±0.5}	NP	++++	NP	+++ ^{0.3±0.5}	NP	++++ ^{0.3±0.3}	++++ ^{0.4±0.7}
2-Heptanol* ^S	++ ^{5.8±10}	+++ ^{8.9±11}	++ ^{2.1±3.6}	++ ^{6.6±9.0}	+++ ^{20.5±27}	NP	++ ^{4.4±7.1}	+++ ^{18.7±27}
2-Heptanone* ^S	++ ^{14.8±17}	+ ^{22.2±22}	+++ ^{24.5±27}	++++ ^{17.6±18}	+++ ^{28.7±35}	NP	- ^{15±13}	+ ^{28.8±33}
1-Hexanol ^S	NP	NP	NP	NP	NP	NP	NP	+++ ^{0.2±0.2}
(Z)-3-Hexenyl acetate ^{S,L}	+++ ^{0.5±0.9}	NP	NP	NP	++ ^{0.5±1.1}	NP	NP	NP
Nonanal ^{S,L}	++ ^{1.3±1.6}	NP	++ ^{0.8±0.7}	NP	- ^{0.6±0.9}	- ^{2.6}	++ ^{0.5±1.0}	++ ^{0.3±0.5}
2-Nonanol ^S	++ ^{0.4±0.7}	NP	+++ ^{1.5±2.6}	NP	++ ^{3.5±4.7}	NP	+ ^{0.3±0.5}	+++ ^{3.1±4.9}
2-Nonanone * ^S	++ ^{2.1±1.6}	++ ^{3.0±2.7}	+++ ^{3.9±3.7}	++ ^{2.7±1.9}	+++ ^{5.4±6.7}	NP	+++ ^{1.9±1.7}	- ^{5.16±6.5}
2-Octanone ^S	+ ^{0.6±0.6}	NP	- ^{0.5±0.3}	NP	NP	NP	+ ^{0.5±0.7}	NP
3-Octanone ^S	++ ^{2.4±2.0}	NP	++++ ^{0.3±0.4}	++++ ^{4.0±1.6}	+++ ^{3.7±4.8}	NP	++ ^{3.1±4.4}	++++ ^{5.7±5.5}
1-Octen-3-ol * ^S	+++ ^{41.9±31}	+++ ^{32.9±18}	+++ ^{1.71±2.0}	+++ ^{54.1±29}	+++ ^{119±130}	+++	+++ ^{27.5±28}	++++ ^{111±132}
(E)-2-Octen-1-ol ^S	+ ^{0.3±0.5}	NP	NP	++++ ^{0.4±0.6}	+++ ^{4.0±8.4}	NP	NP	++++ ^{6.7±10}
2-Undecene ^S	++ ^{5.2±15}	NP	NP	NP	NP	NP	NP	NP
Aromatic compounds								
p-Cresol * ^S	+++ ^{56.4±45}	+++ ^{39.6±12}	++ ^{13.4±9.7}	++ ^{92.0±53}	+++ ^{41.9±49}	+++ ^{0.2}	++ ^{41.6±38}	++++ ^{52.2±67}
Methyl salicylate ^S	++ ^{1.9±1.4}	+ ^{1.1±1.1}	++++ ^{2.1±1.2}	++++ ^{2.2±1.9}	+++ ^{1.6±2.1}	+++ ^{0.8}	++++ ^{1.3±1.7}	++++ ^{1.4±2.4}
Irregular terpene								
6-Methylhept-5-ene-2-ol	++ ^{2.3±1.2}	NP	NP	NP	NP	NP	+++ ^{1.5±1.7}	NP
6-Methylhept-5-ene-2-one ^S	++ ^{2.8±1.4}	NP	- ^{1.8±1.5}	++++ ^{2.5±1.6}	- ^{1.0±1.3}	NP	++ ^{1.9±2.2}	++++ ^{1.3±1.4}
Monoterpenoids								
(+)- α -Citronellene* ^S	+++ ^{9.2±6.2}	NP	++ ^{2.4±3.5}	- ^{14.6±1.5}	+++ ^{6.9±5.1}	NP	+++ ^{6.3±4.2}	++ ^{9.6±3.9}

(+)- β -Citronellene* ^S	+++ 93.8±69	+++ 93.8±106	+++ 28.7±31	++++ 160±55	++ 105±91	NP	+++ 90.4±55	++++ 142±50
β -Citronellol ^S	++ 2.4±2.5	NP	++ 0.6±0.9	NP	+++ 4.6±5.9	NP	++++ 0.8±1.2	NP
<i>p</i> -Cymene ^S	- 1.6±0.9	NP	NP	NP	- 0.3±0.7	NP	NP	++ 0.9±0.6
2,6-Dimethyloct-7-en-2-ol	+++ 4.8±2.9	NP	++++ 6.8±3.7	NP	+++ 2.7±4.6	NP	+++ 3.2±0.2	++++ 4.6±5.4
2,6-Dimethyloct-7-en-4-one	++ 0.5±0.6	NP	NP	NP	NP	NP	++++ 0.5±0.3	++++ 0.3±0.3
2,6-Dimethylocta-2,6-diene isomer 1 * ^S	++ 0.4±0.6	++ 0.3±0.5	- 0.1±0.2	++ 1.0±0.8	++ 0.4±0.6	NP	+ 0.2±0.2	- 0.6±0.7
2,6-Dimethylocta-2,6-diene isomer 2 * ^S	+++ 19.1±15	+++ 14.5±2.2	+++ 4.1±5.3	++++ 32.6±20	+++ 17.9±9.1	+++ 1.4	++++ 8.8±5.1	++++ 19.0±10
2,7-Dimethylocta-4,6-dien-2-ol	NP	NP	++++ 0.1±0.2	++ 0.7±1.2	NP	NP	NP	NP
3,7-Dimethyloct-1-ene* ^S	+++ 30.8±27	++ 16.5±11	++ 3.7±3.0	+++ 58.7±31	++ 31.4±26	NP	++ 17.5±6.5	++++ 44.1±20
3,7-Dimethyloct-2-ene isomer 2 * ^S	+++ 35.9±28	++ 24.3±1.3	++++ 9.6±5.5	++++ 58.3±35	++ 26.8±17	NP	++++ 21.4±20	++++ 45.4±14
3,7-Dimethyloctan-1-ol	++ 3.9±3.7	NP	++ 7.0±12	+++ 6.1±3.8	++ 6.1±10	NP	+++ 3.6±5.0	++ 1.9±2.5
Limonene ^{S,L}	+ 2.6±2.4	NP	- 0.2±0.3	NP	+ 3.2±4.9	NP	++++ 2.1±1.3	++ 2.9±4.7
(S)-Linalool ^{S,L}	++ 3.3±5.5	NP	NP	NP	+++ 3.7±7.9	NP	+++ 0.5±0.5	++++ 6.0±10
β -Myrcene ^S	++ 3.5±1.9	NP	++ 2.4±2.5	NP	+++ 7.6±13	NP	+ 3.1±1.4	NP
Nerol ^S	- 3.1±5.4	NP	NP	NP	++ 3.8±5.1	NP	NP	NP
(<i>E</i>)- β -Ocimene ^S	NP	NP	++ 1.1±1.8	NP	NP	NP	NP	NP
(<i>Z</i>)- β -Ocimene ^S	NP	NP	- 2.2±3.8	++ 0.5±0.8	NP	NP	NP	++ 2.0±3.3
<i>neoallo</i> -Ocimene ^S	NP	NP	- 0.1±0.1	NP	+++ 0.04±0.1	NP	NP	NP
α -Pinene ^L	++ 4.4±10	NP	- # 0.1±0.1	NP	- # 0.7±0.8	NP	NP	NP
Sabinene ^S	NP	NP	NP	NP	NP	NP	+ 1.2±1.0	NP
α -Terpinene ^S	NP	NP	- 1.1±0.9	NP	+++ 0.1±0.3	NP	NP	NP
α -Terpinolene ^S	+ 2.1±2.8	+ 1.7±0.1	NP	- 1.4±0.9	++ 0.9±0.8	NP	++ 1.7±1.3	NP
4-Terpinenol ^S	+ 0.4±0.8	NP	NP	NP	++ 0.5±1.0	NP	NP	NP
γ -Terpinene ^S	- 0.9±1.0	NP	NP	NP	NP	NP	- 0.5±0.6	++++ 0.3±0.5
Nitrogen-bearing compounds								
Indole * ^S	++ 220±117	++ 273±203	+++ 2.4±4.0	++ 208±174	++ 136±72	+++ 79.2	+ 150±84	++++ 100±76
β -Lutidine	++ 4.0±4.6	NP	NP	NP	+++ 1.7±2.4	+++ 2.1	++ 2.1±2.5	NP
Skatole ^S	++ 6.0±5.1	++ 7.1±4.3	- 5.2±5.2	NP	+ 2.8±2.2	NP	- 2.4±3.4	NP
Sesquiterpenoids								
Bicyclogermacrene	++ 53.4±67	NP	++++ 20.3±13	++++ 91.5±117	+++ 30.7±34	+++ 25.9	+++ 26.9±25	++ 10.4±10
α -Cadinene	NP	NP	- 9.7±4.9	NP	+++ 1.2±1.6	NP	NP	NP

δ-Cadinene		++ 35.0±27	NP		++ 5.46±6.2	NP	+++ 30.9±37	+++ 2.6	+++ 20.6±6.5	NP
(E)-β-Caryophyllene*^S		+++ 46.4±32	+++ 48.5±1.6		++ 1.71±1.4	++++ 64.8±54	+++ 77.4±68	+++ 1.6	++ 28.1±12	++ 68.4±71
α-Copaene^S		++ 15.6±12	++ 16.2±7.5		- 32.5±21	+++ 19.8±21	+ 20.3±16	+++ 0.9	++ 8.60±3.2	++ 18.1±17
Germacrene D*^S		++++ 48.2±50	NP		NP	NP	++ 7.2±8.1	NP	NP	NP
α-Humulene*^S		+ 64.8±50	+ 70.5±4.2		+++ 27.7±13	++ 91.7±85	- 88.6±75	NP	- 41.0±17	- 75.7±78
Isocaryophyllene		++ 20.5±15	++ 22.4±5.4		++ 0.5±0.8	++ 26.9±26	+ 37.1±40	NP	- 13.3±5.6	NP
Unknowns										
UNK 807	<i>m/z</i> : 70,98,42,71	+ 3.5±1.5	NP		NP	++ 4.01±1.0	NP	NP	NP	NP
UNK 829	<i>m/z</i> : 84,67,110,41	+ 3.3±3.0	NP		+++ 43.3±28	++++ 3.7±0.5	- 5.1±8.2	NP	NP	++ 1.4±2.3
UNK 883	<i>m/z</i> : 79,77,108,91	+ 0.3±0.6	++++ 0.5±0.8		- 0.5±0.4	NP	NP	NP	NP	NP
UNK 962	<i>m/z</i> : 68,67,69,41	+ 0.8±0.5	NP		NP	NP	+++ 0.9±1.2	NP	NP	NP
UNK 1030	<i>m/z</i> : 95,67,138,82	+++ 4.9±5.8	- 1.6±1.3		+ 0.4±0.6	++ 8.3±9.5	+++ 2.2±2.0	NP	++++ 1.2±0.9	++ 2.3±2.6
UNK 1135	<i>m/z</i> : 82,67,59,121	NP	NP		NP	++++ 0.9±0.5	+++ 0.6±0.4	NP	NP	++ 0.5±0.5
UNK 1279	<i>m/z</i> : 43,68,81,54	NP	NP		++++ 0.1±0.1	NP	+++ 1.0±1.3	NP	NP	NP
UNK 1349	<i>m/z</i> : 69,70,55,57	NP	NP		++++ 1.9±1.4	NP	+++ 0.03±0.0	NP	NP	NP
UNK 1367_1	<i>m/z</i> : 81,95,55,93	+++ 5.1±3.8	NP		++ 8.5±3.7	NP	+++ 6.3±4.7	NP	++++ 5.2±3.7	NP
UNK 1378/ 1381	<i>m/z</i> : 95,147,96,79/ <i>m/z</i> : 55,69,95,81	++ 3.9±3.8	+ 3.4±2.3		++ 0.5±0.5	- 8.0±4.0	+ 2.1±2.5	+++ 0.5	NP	NP
UNK 1394	<i>m/z</i> : 69,55,41,82	++ 13.1±15	NP		NP	++++ 23.2±23	- 11.1±21	NP	NP	++ 22.1±24
UNK 1409	<i>m/z</i> : 69,55,41,82	++ 0.8±1.4	NP		++ 0.7±0.8	NP	+++ 4.8±6.3	NP	NP	NP
UNK 1415	<i>m/z</i> : 69, 81,41,95	+++ 119±80	+++ 128±81		+++ 0.6±0.7	++ 209±51	+++ 105±57	+++ 6.2	++++ 102±63	++++ 118±63
UNK 1466_1	<i>m/z</i> : 161,107,122	++ 2.7±2.0	NP		++ 0.9±0.9	NP	++ 6.2±6.8	NP	NP	NP
UNK 1492	<i>m/z</i> : 105,161,91,41	NP	NP		++ 22.9±15	NP	- 41.6±52	NP	NP	NP
UNK 1492 ⁺⁺ & UNK 1503 ⁺⁺		+++ 14.1±17	+++ 56.7±20		NP	++++ 17.1±22	++ 56.7±20	NP	+++ 32.9±6.9	++++ 33.0±57
UNK 1503 ⁺ & Germacrene D ^{S,+}	<i>m/z</i> : 81,107,163	NP	NP		+++ 78.4±77	NP	+ 48.3±29	NP	NP	NP
UNK 1518	<i>m/z</i> : 105,93,107,79	NP	NP		- 0.4±0.5	NP	+++ 2.6±4.8	NP	NP	NP
UNK 1521_2	<i>m/z</i> : 191,81,163,109	++ 8.5±9.9	NP		NP	NP	NP	NP	++ 3.6±3.8	NP
UNK 1541/ 1507_1	<i>m/z</i> : 161,105,119,163	NP	+ 19.1±2.2		- 18.5±32	NP	+++ 6.1±11	NP	+++ 10.4±4.2	NP
UNK 1584	<i>m/z</i> : 85,57,41,53	++ 4.8±9.7	NP		NP	NP	NP	NP	+++ 1.9±1.9	NP
UNK 1607	<i>m/z</i> : 43,119,159,91	NP	NP		NP	NP	++ 0.5±1.0	NP	NP	NP
UNK 1658	<i>m/z</i> : 109,161,81,204	++++ 5.8±3.8	NP		NP	++++ 6.0±1.6	+++ 7.8±5.0	NP	NP	NP

UNK 1699	<i>m/z</i> : 81,163,191,95	++ ^{112±90}	+ ^{107±109}	<i>NP</i>	<i>NP</i>	++ ^{78.3±90}	<i>NP</i>	<i>NP</i>	<i>NP</i>
561	Levels of EAD-activity: ++++ (EAD-active in >80% of runs with samples that contained this compound), +++ (80–50%), ++ (50–25%), + (>25%), – (VOC did not elicit a signal,								
562	even though the same or a lower amount elicited a signal in other sexes or species), <i>NP</i> (VOC not present in scent samples tested). * The physiological activity was confirmed by								
563	a synthetic standard (see Supplementary information Table S1); ^S identity was confirmed by synthetic standard; ^L in leaf blanks ^{see 5} ; <i>m/z</i> = mass-to-charge ratio in decreasing								
564	order of abundance; † coeluted on non-chiral ZB-5 column; ‡ coeluted on chiral columns.								