

## Manuscript Details

<b>Manuscript number</b>	PHYTOCHEM_2018_187_R2
<b>Title</b>	Floral scent in <i>Iris planifolia</i> (Iridaceae) suggests food reward
<b>Article type</b>	Full Length Article

### Abstract

*Iris* species can adopt different pollination strategies to attract their pollinators, generalized shelter-mimicking, specialized deceptive sexual-mimicking or food-rewarding. As attractive stimuli, *Iris* flowers may use their colours, large-size, symmetry, and volatile organic compounds (VOCs). However, relatively few studies investigated *Iris* floral olfactory cues in the context of plant-visitor/pollinator interactions. In the present study we combined the identification of the floral volatiles of the nectariferous *I. planifolia* with insects visiting its flowers to gather data on its biology. Floral volatiles were collected in the natural environment by dynamic headspace and analysed by gas chromatography-mass spectrometry (GC-MS). Insect visitors/pollinators were also recorded. The volatile bouquet was aromatic-dominated with 1,4 dimethoxybenzene as major compound. Among the insects visiting its flowers, bumble and honey bees were the most abundant followed by hover flies. Overall, our results suggest that *I. planifolia* advertises its food reward by an aromatic dominated volatile composition.

<b>Keywords</b>	<i>Iris planifolia</i> ; Iridaceae; Aromatic compounds; Dynamic headspace; GC-MS; Bumble bees; Honey bees; Hover flies; Pollination
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## Submission Files Included in this PDF

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Ref: PHYTOCHEM\_2018\_187\_R1

Title: Floral scent in *Iris planifolia* (Iridaceae) suggests food reward

Journal: Phytochemistry

Dear Editor,

Thank you very much for giving us the opportunity to review our manuscript, for the very useful comments and for accepting our manuscript.

As requested we modified the manuscript (MS Word revision mode) according to the suggestions/requests.

Please note that we accepted all the revisions done in the version PHYTOCHEM\_2018\_187\_R1 and highlighted only the new changes in the text.

The revised files have been uploaded in the system together with the replies to editor and reviewer remarks.

Best regards,

Pietro Zito on behalf of co-authors.

Ref: PHYTOCHEM\_2018\_187\_R1

Title: Floral scent in *Iris planifolia* (Iridaceae) suggests food reward

Journal: Phytochemistry

Dear Dr. Zito,

I am pleased to inform you that the overall recommendation of the reviewers and the specialist subject Editor is that your paper should be accepted for publication once you have attended to a few final corrections. These are detailed below.

Please check again for any further typos.

Please indicate in a point-by-point 'reply to reviewers' the modifications made in response to the comments, or explain why you have not done so.

*Dear Editor,*

*Thank you very much for giving us the opportunity to review our manuscript, for the very useful comments and for accepting our manuscript.*

*Please find below our replies with the final corrections done according to the suggestions of the Reviewer.*

*Please note that we accepted all the revisions done in the version PHYTOCHEM\_2018\_187\_R1 and highlighted the new alterations in the text by using Microsoft Word revision mode.*

Comments from the editors and reviewers:

-Editor

-

-Reviewer 1

- The authors have responded with care to the reviewer remarks and the paper now feels less speculative, its arguments less forced.

Additional edits for fine tuning:

Discussion

Line 139: hydrocarbons

*Reply: Done, line 131 in the new revised version*

Line 149: benzyl tiglate also was detected

*Reply: Done, line 141 in the new revised version*

Line 172: it is not likely to have detrimental effects

*Reply: Done, line 162 in the new revised version*

Line 183: data from the literature

*Reply: Done, line 173 in the new revised version*

Line 199: blue-violet

*Reply: Done, line 183 in the new revised version*

Line 227: Although more data are needed...

*Reply: Done, line 205 in the new revised version*

*Additional changes:*

*Please note that we added the asterisk to phenylacetaldehyde in the table 1 since in the previous file it was missing.*

*We added in the acknowledgements section Dr. Roman Fuchs*

Best regards

Pietro Zito

# 1 **Floral scent in *Iris planifolia* (Iridaceae) suggests food reward**

2  
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11 Declarations of interest: none.

## 12 13 **Abstract**

14 *Iris* species can adopt different pollination strategies to attract their pollinators, generalized shelter-  
15 mimicking, specialized deceptive sexual-mimicking or food-rewarding. As attractive stimuli, *Iris*  
16 flowers may use their colours, large-size, symmetry, and volatile organic compounds (VOCs).  
17 However, relatively few studies investigated *Iris* floral olfactory cues in the context of plant-  
18 visitor/pollinator interactions. In the present study we combined the identification of the floral  
19 volatiles of the nectariferous *I. planifolia* with insects visiting its flowers to gather data on its biology.  
20 Floral volatiles were collected in the natural environment by dynamic headspace and analysed by gas  
21 chromatography-mass spectrometry (GC-MS). Insect visitors/pollinators were also recorded. The  
22 volatile bouquet was aromatic-dominated with 1,4 dimethoxybenzene as major compound. Among  
23 the insects visiting its flowers, bumble and honey bees were the most abundant followed by hover  
24 flies. Overall, our results suggest that *I. planifolia* advertises its food reward by an aromatic  
25 dominated volatile composition.

26 **Keywords:** *Iris planifolia*; Iridaceae; Aromatic compounds; Dynamic headspace; GC-MS; Bumble  
27 bees; Honey bees; Hover flies; Pollination

## 28 **1. Introduction**

29 *Iris* L., with about 250-300 species distributed in Eurasia, North America and North Africa, is the  
30 largest genus in the family Iridaceae (Goldblatt and Manning, 2008; Mavrodiev et al., 2014).  
31 Although some species are found in mesic or wet environments, most occur in desert, semi-desert, or  
32 dry, rocky and montane habitats (Wilson et al., 2016). *Iris* spp. were well known and cultivated, for  
33 their use as a perfume, throughout ancient Greece and Roman Empire (Crişan and Cantor, 2016).  
34 Cherokee Indians or other Native Americans used *Iris* plants for medicinal purposes (e.g. gastric  
35 problems, kidney and bladder disorders, among others) (Crişan and Cantor, 2016). Indeed, *Iris* plants  
36 have been extensively studied because the species are a rich source of specialised metabolites, such  
37 as flavonoids, isoflavonoids, benzoquinones, terpenoids and glycosides which have a wide range of  
38 biological activities, including anti-inflammatory, antioxidant, antimicrobial and anticancer with  
39 chemo-preventive properties (Al-Jaber, 2016; Crişan and Cantor, 2016). To date, *Iris* plants have a  
40 large economic value as flavoring in food products and soft drinks, ornamental purposes and uses in  
41 the cosmetic industry (Crişan and Cantor, 2016).

42 *Iris* flower is considered an inflorescence with three functionally separate pollination units  
43 (meranthia), acting as a single bilabiate gullet/tunnel flower (Goldblatt and Bernhardt, 1999). In each  
44 unit (meranthium), the petaloid-style branch and its associated stamen form the upper lip (roof), while  
45 the closely opposed large tepal, that works like a landing platform for insect visitors, forms the lower  
46 lip (perigon lobe/floor) (Goldblatt and Bernhardt, 1999; Westerkamp and Claßen-Bockhoff, 2007).  
47 Interestingly, in *Iris* spp. the lower lips are often embellished with various colour patches, lines,  
48 hairs/beards and ridges, among others, that may play an attractive role for the insects visiting the  
49 flowers (Guo, 2015; Morinaga and Sakai, 2006). Studies on *Iris* pollination showed that most species  
50 are pollinated by social and/or solitary bees belonging to Apidae, Andrenidae, Halictidae and  
51 Colletidae (e.g., *I. atropurpurea* Baker, Sapir et al., 2005; Vereecken et al., 2013; Watts et al., 2013;  
52 *I. bulleyana* Dykes, Ye et al., 2017; *I. lutescens* Lam., Imbert et al., 2014a, Radović et al., 2017; *I.*  
53 *paradoxa* Steven, Vereecken et al., 2012; *I. pumila* L., Radović et al., 2017; *I. tuberosa* L., Pellegrino

54 et al., 2016). In *Iris* species the rewards may consist of pollen, nectar or oils (Pellegrino et al., 2015);  
55 although, such as in the nectarless *Oncocyclus* (Siems.) Baker section (*I. atrofusca* Baker, *I.*  
56 *atropurpurea*, *I. bismarckiana* Damman & Sprenger, among others) the flowers can provide  
57 protective shelters (non-nutritive form of reward) that are used by visitors/pollinators (Sapir et al.,  
58 2005; Vereecken et al., 2012, 2013; Watts et al., 2013).

59 As attractive stimuli, *Iris* flowers may use their colours (Imbert et al., 2014a, 2014b; Vereecken et  
60 al., 2012, 2013; Wang et al., 2013; Wang et al., 2016), large-sized flowers (Lavi and Sapir, 2015;  
61 Radović et al., 2017), floral symmetry (Radović et al., 2017), and a range of volatile organic  
62 compounds (VOCs) (Ohler et al., 2016; Pellegrino et al., 2015, 2016; Vereecken et al., 2012, 2013;  
63 Wang et al., 2013). However, while floral visual cues *i.e.* floral size, symmetry, colour and/or its  
64 polymorphism have been extensively studied in *Iris* spp. (Imbert et al., 2014a, 2014b; Lavi and Sapir,  
65 2015; Pellegrino et al., 2015, 2016; Radović et al., 2017; Souto-Vilarós, 2018; Vereecken et al., 2012,  
66 2013; Wang et al., 2013, 2016), relatively few studies (additionally or exclusively) investigated *Iris*  
67 floral olfactory cues *i.e.* floral VOCs in the context of plant-visitor/pollinator interactions (*I.*  
68 *atropurpurea* and *I. paradoxa*, Vereecken et al., 2012, 2013; *I. lutescens*, Wang et al., 2013, *I. pallida*  
69 Lam., Ohler et al., 2016; *I. tuberosa*, Pellegrino et al., 2016).

70 *Iris planifolia* (Mill.) T. Durand & Schinz (Iridaceae), occurring mainly in the western Mediterranean  
71 and North Africa, is the only member of the subgenus *Scorpiris* Spach (section *Juno* Dykes) native  
72 to Europe (Boltenkov and Govaerts, 2017). Since this nectariferous species occurs at low/middle  
73 altitude in the Mediterranean region, where the winters are not severe, it may bloom at any time from  
74 November to February (Mathew, 1986). This species has been studied from a taxonomical (Boltenkov  
75 and Govaerts, 2017), molecular (Ikinici et al., 2011) and morphological (Guo, 2015; Ikinici et al., 2011)  
76 points of view. The single chemical study on *I. planifolia* was performed by Chikhi et al., (2012).  
77 The authors used the essential oil to study some biological roles (*i.e.* antimicrobial and antioxidant  
78 activity) of whole *I. planifolia* plant volatiles, but there are no studies investigating the inflorescence-  
79 specific volatile emissions of *I. planifolia* by using more biologically appropriate and non-invasive

80 methods, such as headspace. In the present study we combined the identification of the floral volatiles  
81 of the blue-violet *I. planifolia* with insects visiting its flowers to gather data on its biology. In detail,  
82 we (i) collected the inflorescences scent by dynamic headspace, (ii) analysed the samples by gas  
83 chromatography and mass spectrometry (GC-MS) and (iii) recorded first data on its insect  
84 visitors/pollinators.

85

## 86 **2. Results**

### 87 *2.1. Floral scent*

88 Twelve VOCs were identified in *I. planifolia* inflorescences: eleven aromatic compounds and one  
89 monoterpene (Table 1). The total absolute amount of VOCs emitted by *I. planifolia*, estimated by  
90 using anisole as internal standard (see section 5.2 for details), was about 2.0 µg/inflorescence/hour.  
91 The relative amount was reported as mean percentage of the integrated chromatogram peak areas.  
92 The aromatic-dominated volatile bouquet was composed of four aromatic aldehydes (12 %), three  
93 aromatic alcohols (10 %), two aromatic esters (5 %) and two aromatic ethers (72 %). The single  
94 exception was represented by the monoterpene alcohol linalool, which contributed to less than 0.5 %.  
95 Overall, the floral scent of *I. planifolia* was strongly dominated by 1,4-dimethoxybenzene (also  
96 known as hydroquinone dimethyl ether) with about 71 %. Two compounds: 2-phenylethanol and 2-  
97 methoxybenzaldehyde were respectively found in relative amounts of 8 % and 6 %. Finally, five  
98 compounds ranged between 4 and 1% whereas four contributed with less than 1% (Table 1).

### 99 *2.2. Flower-visiting insects*

100 Overall, in 8 hours of observation (8:00 - 16:00) we recorded 42 insects on *I. planifolia* inflorescences,  
101 with bees and flies landing on all the observed plants. Insect visitation mostly occurred between 11:00  
102 - 14:00. Bumble bees and honey bees (Apidae) were the most abundant visitors (32 registered visits)  
103 followed by hover flies (10 registered visits). *Bombus ruderatus* (Fabricius) (Apidae) (Fig. 1A, B)  
104 with 18 visits was the main insect visitor followed by *Apis mellifera* Linnaeus (Apidae) (Fig. 1C)



105 with 14 visits and by *Episyrphus balteatus* (De Geer) (Syrphidae) (Fig. 1D) with 10 visits. Since only  
106 bumble bees and honey bees entered inside the flower tunnels and carried the pollen on their body  
107 (mainly on their head and torax), they can be considered as potential pollinators. On the contrary, the  
108 syrphid fly *E. balteatus* never entered inside the flowers and thus was considered as visitor.  
109 Interestingly, *E. balteatus* spent most of its time eating the pollen grains left on the flowers by  
110 pollinators when they crawled out of the flower tunnels.

111

### 112 **3. Discussion**

113 The floral scent of *I. planifolia* is characterized by VOCs that are reported (1) as floral volatiles of  
114 different angiosperm families (Knudsen et al., 2006) and (2) as semiochemicals mediating several  
115 plant-animal interactions (El Sayed, 2018).

116 With the single exception represented by the monoterpene alcohol linalool (less than 0.5 %), the floral  
117 scent bouquet of *I. planifolia* has a strong aromatic chemical composition (Tab. 1) dominated with  
118 more than 70 % by the aromatic ether 1,4-dimethoxybenzene (hereafter 1,4-DMB). This VOC was  
119 also found as major compound in the floral scents of *Salix* L. spp. (Salicaceae) pollinated during the  
120 daytime by mining bees and honey bees (Dötterl et al., 2005, 2014; Jürgens et al., 2014), and of  
121 *Anacamptis coriophora* (L.) R.M. Bateman, Pridgeon & M.W. Chase (Orchidaceae) pollinated by  
122 bumble and honey bees (Salzmann et al., 2007).

123 Despite the similarity with the floral scents of plant species belonging to different families (Knudsen  
124 et al., 2006), the floral scent composition of *I. planifolia* differs from those found within the genus by  
125 other authors. For example, while 1,4-DMB was the main VOC found in our dynamic headspace  
126 samples, it was detected in minor amounts in the essential oil of *I. planifolia* (0.5 %, Chikhi et al.,  
127 2012) and in the static headspace samples of *I. lutescens* (less than 3.0 %, Wang et al., 2013).  
128 Furthermore, it was not found in solvent extracts of *I. pallida* (toluene samples by Ohler et al., 2016),  
129 *I. atropurpurea*, *I. paradoxa* and *I. tuberosa* (hexane samples by Pellegrino et al., 2016 and Vereecken  
130 et al., 2012, 2013). In contrast, *I. planifolia* by Chikhi et al. (2012) was dominated by aliphatic

131 hydrocarbons and fatty acids (e.g. *n*-alkanes and hexadecanoic acid); *I. lutescens* by monoterpenes  
132 (myrcene, (*E*)- $\beta$ -ocimene and limonene) (Wang et al., 2013); *I. pallida* by methyl anthranilate (Ohler  
133 et al., 2016) and *I. atropurpurea*, *I. paradoxa* and *I. tuberosa* by aliphatic hydrocarbons (*n*-alkanes  
134 and *n*-alkenes) (Pellegrino et al., 2016; Vereecken et al., 2012, 2013). As reported by other authors  
135 (e.g. El-Sajed et al., 2018; Jürgens et al., 2009; Knudsen and Gershenzon, 2006; Tholl et al., 2006),  
136 it is not surprising that the limited overlap in floral VOCs between *Iris* species could be due to the  
137 different volatile sampling methods (e.g., headspace vs hydrodistillation or solvent extractions), to  
138 the polarity and affinities of VOCs to the adsorbent fibers used (headspace methods), to the  
139 interspecific variation, and/or to the insect pollinators/pollination strategies of the investigated *Iris*,  
140 among others. Indeed, excluding 1,4-DMB, we found only a few co-occurrences between our floral  
141 volatiles and those of other *Iris* species. Among these, benzyl tiglate also was detected ~~also~~ in *I.*  
142 *planifolia* by Chikhi et al. (2012); benzaldehyde, 2-(4-methoxyphenyl)ethanol and 4-  
143 methoxybenzaldehyde in *I. lutescens* (Wang et al., 2013); linalool in *I. planifolia* (Chikhi et al., 2012)  
144 and in *I. lutescens* (Wang et al., 2013).

145 Autonomous self-pollination has been described in some *Iris* species (e.g. *Iris versicolor* L.) (Kron  
146 et al., 1993) but not in others (e.g. *Oncocyclus* irises) (Watts et al., 2013; Sapir et al., 2005). To date,  
147 information on pollination biology of *I. planifolia* has been lacking; however, irises pollination data  
148 from the literature show that insects play an important role for the reproductive success in both self-  
149 incompatible and self-compatible *Iris* species (Watts et al., 2013; Kron et al., 1993). Among *I.*  
150 *planifolia* visitors, *Bombus ruderatus* and *Apis mellifera* (Fig. 1A, B, C) were found as candidates  
151 that, forcing the tepal (lower lip) and the opposite style branch of the meranthium, entered inside the  
152 tunnel and passively collected pollen. We did not find any insects inside the flowers (sheltering) as  
153 in *Iris atropurpurea* and *Iris paradoxa* (Vereecken et al., 2012, 2013; Watts et al., 2013). Although  
154 more extensive field observations (e.g. data collection and night-sheltering) and experiments (e.g.  
155 seed- fruit-set) are needed to confirm the exclusive role of *B. ruderatus* and *A. mellifera* as pollinators  
156 of *I. planifolia*, our findings are consistent with previous studies of other *Iris* species pollinated by

157 bumble bees and/or honey bees, among others (Goldblatt and Manning, 2006; Imbert et al., 2014a,  
158 2014b; Segal et al. 2006; Souto-Vilarós et al., 2018; Watts et al., 2013; Ye et al., 2017). In addition  
159 to the legitimate bee pollinators, the syrphid fly *E. balteatus* was observed eating the pollen grains  
160 lost by *B. ruderatus* and *A. mellifera* on the lower lip of the *I. planifolia* flowers. Since *E. balteatus*  
161 was not observed as pollen robber from the anthers of *I. planifolia* (it never entered into the  
162 gullet/tunnel flower) it is not likely to have ~~has not~~ detrimental effects for *I. planifolia*. Conversely,  
163 we speculate that its “cleaning service” could have a beneficial effect by restoring the original visual  
164 displays.

165 Generalist bees, such as *Bombus* spp. and *Apis mellifera*, have a broad pollen/nectar diet and collect  
166 pollen from different species with variable floral cues (Dobson, 2006; Dötterl and Vereecken, 2010).  
167 Consequently, generalist bees might benefit by relying on floral VOCs related to a broad spectrum of  
168 potential host flowers (Dötterl and Vereecken, 2010). In food-rewarding plants, the aromatic  
169 compounds as alcohols, aldehydes, esters, and ethers alone or in combination with some monoterpene  
170 alcohols (e.g. linalool) are often reported as floral VOCs that attract nectar/pollen feeders, such as  
171 bees, butterflies, moths and flies (Dobson 2006; Dötterl and Vereecken 2010; Jürgens et al., 2009;  
172 Primante and Dötterl, 2010). Although in our study we did not perform electroantennographic or  
173 behavioral experiments, data ~~form~~ from the literature provide evidence that 1,4-DMB (Jürgens et al.,  
174 2014; Salzman et al., 2007), benzaldehyde (Theis, 2006), 4-methoxybenzaldehyde (= *p*-  
175 anisaldehyde), phenylacetaldehyde (Dötterl and Vereecken 2010; Knauer and Schiestl, 2015; Theis,  
176 2006), 2-phenylethanol, benzyl alcohol and linalool (Dötterl and Vereecken 2010; Theis, 2006) are  
177 VOCs perceived and attractive for *Bombus* spp. and/or *Apis mellifera*. In addition, 2-phenylethanol  
178 is known as attractant for syrphid flies (Zhu and Park, 2005) and potentially also for *E. balteatus*  
179 (Primante and Dötterl, 2010); whereas linalool was recently reported as electrophysiologically-active  
180 for Syrphidae (Braunschmid et al., 2017).

181 *Iris* spp. use floral visual and olfactory cues for attracting their pollinators (e.g. Pellegrino et al., 2016;  
182 Vereecken et al., 2012, 2013; Wang et al., 2013). It is interesting to note that bumble bees’ UV-blue

183 photoreceptors can perceive the predominant blue-violet *I. planifolia* floral colour (human  
184 perception) (Arnold et al., 2010) as attractive and innate signals of rewarding flowers (Knauer and  
185 Schiestl, 2015; Kunze and Gumbert, 2001; Raine and Chittka, 2007). Furthermore, since syrphid flies  
186 prefer yellow over other colours, the yellow or orange pubescent median ridge in the lower lips of *I.*  
187 *planifolia* meranthia can be attractive for *E. balteatus*, despite it mainly uses olfactory cues to find a  
188 pollen/nectar host-plants (Primante and Dötterl, 2010).

189

#### 190 **4. Conclusions**

191 In agreement with Vereecken et al. (2015), in this study we adopted an accepted and modern  
192 methodology (dynamic headspace) that allows the collection and analysis of floral scents from the  
193 prospective of plant-visitor/pollinator interactions (El-Sajed et al., 2018; Zito et al., 2015). To our  
194 surprise, this is the first study that investigated the floral volatiles in the genus *Iris* by using a dynamic  
195 headspace method. Furthermore, it is the first report on visitor/pollinators of *I. planifolia*. Our results  
196 suggest that the floral volatiles of the nectariferous *I. planifolia* may play a synergistic role when also  
197 visual cues occur. This hypothesis is supported by other studies that reported how social bees (e.g.  
198 bumble bees and honey bees) elicited the strongest behavioural responses when floral visual and  
199 olfactory cues co-occur (Chittka and Raine, 2006; Dötterl et al., 2014; Junker and Parachnowitsch,  
200 2015).

201 Interestingly, sexual mimicry has evolved from generalized food deception, shelter pollination, or  
202 food reward systems (Johnson and Schiestl, 2016). By floral visual and olfactory cues *Iris* spp. can  
203 adopt different pollination strategies to attract their pollinators *i.e.* from generalized shelter-  
204 mimicking species or specialized sexual-mimicking deceptive species (Pellegrino et al., 2015, 2016;  
205 Vereecken et al., 2012, 2013) to food-rewarding species (present study). ~~Although more~~ ~~Despite~~  
206 ~~further~~-data are needed to confirm the pollination strategies adopted in other un-investigated *Iris*  
207 species among the sections, our results suggest that *I. planifolia* advertises the food reward by its  
208 aromatic dominated volatile composition probably in synergy with its visual cues. Further studies are

209 needed to better understand the interplay between floral olfactory and visual cues for attracting *I.*  
210 *planifolia* visitors and pollinators.

211

## 212 **5. Experimental**

### 213 *5.1. Study species*

214 *Iris planifolia* (Mill.) T. Durand & Schinz (Iridaceae) [subg. *Scorpiris* Spach; section *Juno* Dykes],  
215 described as *Xiphium planifolium* in 1768 by Philip Miller, is a perennial bulbous herb about 10-25  
216 cm high (Mathew, 1986). It is naturally occurring in Spain, Portugal, Sardinia, Sicily, Crete, Greece,  
217 Libya, Tunisia, Algeria and Morocco (Barker and Govaerts, 2018). As described by Mathew (1986),  
218 the stems are very short, more or less subterranean and hidden by the leaf bases. The lanceolate and  
219 often undulate-falcate leaves (4-7) are bright shiny green above and glaucous beneath. Each plant,  
220 blooming from November to February, has 1-3 inflorescences (6-12 cm across) pale to deep blue or  
221 violet-blue and rarely white. The lower lips of the three meranthia of its inflorescence are widely  
222 reflexed, blue, veined and dotted in dark violet with a yellow or orange pubescent median ridge. The  
223 anthers are whitish or purplish whereas style branches flattened and petaloid are bilobed at the apex.  
224 To date, no information is available on the pollination ecology of *I. planifolia*; the plant produces  
225 nectar (supplementary material in Herrera et al., 2012) as pollinators reward. The seeds, ovoid and  
226 rugose are dark reddish-brown. In its natural habitat, the species usually grows on sloping rocky  
227 ground on limestone formations where the soil is the typical Mediterranean reddish clay (Mathew,  
228 1986).

229 In the present study, all the field activities were performed in November 2017, wet season in Sicily,  
230 in a natural site located in San Martino delle Scale - Sicily (38°5'18.29"N, 13°15'23.48"E).

### 231 *5.2. Floral scent sampling*

232 Floral scent was collected, on a sunny day in November 2017, from plants growing in their natural  
233 habitat (38°5'18.29"N, 13°15'23.48"E - San Martino delle Scale - Sicily). All samples from

234 inflorescences ( $N= 7$ ) were collected using a dynamic headspace method (pull system) (Tholl et al.,  
235 2006). For each sampling, one inflorescence per plant was enclosed in an oven bag (size:  $15 \times 12$  cm,  
236 CUKI® Cofresco S.p.A.) and the emitted volatiles were collected for 6 hours (9:00 - 15:00) by an  
237 adsorbent tube using a vacuum pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany), with a  
238 flow rate of 200 ml/min. The adsorbent tube (shortened glass Pasteur pipette) was filled with 20 mg  
239 Tenax-TA (mesh 60-80) and 20 mg Carbotrap B (mesh 20-40) both Supelco (Bellefonte, PA, USA).  
240 The adsorbent materials were fixed in the tubes using glass wool (Sigma Aldrich). At the same time,  
241 by using the same approach described before, headspace samples from leaf ( $N= 3$ ) and an empty oven  
242 bag ( $N= 1$ ), were used as negative controls. Volatiles trapped in the tubes were eluted within a few  
243 minutes after collection with 200  $\mu$ l of acetone (Sigma Aldrich, > 99 %). All samples were stored in  
244 screw cap vials at  $-20$  °C until chemical analyses. The peak areas on the chromatograms were  
245 integrated to obtain the total ion current, which was used to determine the relative amount (%) of each  
246 peak. -To quantify the absolute amount of scent emitted from an inflorescence, one  $\mu$ g of anisole  
247 (Sigma-Aldrich, Anisole ReagentPlus®, 99 %) was added to each sample as internal standard.  
248 Subsequently to calculate the absolute amounts of the VOCs in each sample, peak areas on the  
249 chromatograms were integrated to obtain the total ion current signal and compared to that of the  
250 internal standard.

### 251 5.3. Chemical analysis

252 All samples were analyzed by GC-MS on a single quadrupole Shimadzu GC-MS-QP2010 Plus  
253 equipped with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) and a Supelcowax 10 capillary  
254 column (30 m long, 0.25 mm i.d., 0.25  $\mu$ m film thickness). One  $\mu$ l of each sample was injected at 250  
255 °C in the splitless mode, and the column flow (carrier gas: helium) was set at 1 ml/min. The GC oven  
256 temperature was held for 5 min at 40 °C, then increased by 2°C/min to 250 °C, held for 15 min and  
257 finally raised to 270 °C at 10 °C/min. The MS interface worked at 280 °C, and the ion source at 250

258 °C. Mass spectra were taken at 70 eV (in EI mode) from m/z 30 to 500. The GC/MS data were  
259 analyzed using the GCMSolution package, Version 2.72.

260 Identification of compounds was carried out using the mass spectral libraries FFNSC 2, W9N11,  
261 ESSENTIAL OILS (available in MassFinder 3), and Adams (2007). These identifications were also  
262 compared by mass spectra and Kovats retention indices found in NIST11, SciFinder and Pherobase  
263 (El-Sayed, 2018) database. Many of the compounds were confirmed by comparison of mass spectra  
264 and retention times with authentic standard components (Sigma Aldrich) provided by Prof. Dr. Stefan  
265 Dötterl (Plant Ecology Lab - University of Salzburg). Kovats retention indices were calculated using  
266 a series of n-alkanes (C<sub>8</sub> - C<sub>30</sub>). Compounds found in the flowers were compared with those found in  
267 the negative controls (empty oven bag and green leaves) to discriminate contaminations from air  
268 ambient and to identify those exclusively emitted by flowers.

#### 269 5.4. Flower-visiting insects

270 Insects visiting *I. planifolia* inflorescences were recorded during the day and in the same site of the  
271 headspace collections. The sampling of flower-visiting insects was limited to ten inflorescences in  
272 order to minimize the potential negative impact on the local entomofauna and on pollination  
273 processes. Observations were performed simultaneously by two researchers staying near the plants  
274 for 8 hours (08:00 - 16:00). The limited number of hours of observation was linked to good weather  
275 condition (sunny day) occurring in the flowering period of *I. planifolia* in Sicily (rainy season).  
276 Insects visiting *I. planifolia* were recorded when they landed on a meranthium. Only a few insect  
277 visitors belonging to different morphospecies were captured by entomological hand nets or plastic  
278 bags (10 x 7 cm), otherwise they were annotated and photographed with a digital camera (NIKON  
279 D3100). Since not all insect individuals were captured, we cannot exclude that the same specimen  
280 after leaving, came back to revisit the inflorescence again. The data of insects visiting the ten different  
281 *I. planifolia* inflorescences were pooled together. The flower visitors that landed, entered inside the  
282 meranthium and came out it with pollen on their body were recorded as potential pollinators. On the

283 contrary, the insects that after their landing on the inflorescences never entered inside the meranthium  
284 were recorded as visitors. Insect specimens, identified at specific taxonomic level, are stored in an  
285 entomological box and kept in the Entomological collection of the Department of Biological,  
286 Chemical and Pharmaceutical Sciences and Technologies of the University of Palermo.

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288 us the authentic standards necessary to verify the identification of the compounds. We would like to  
289 thank Ms. Francesca Tavella for her support during the headspace samplings and for the photos of  
290 the pollinating bumble bee. This work was supported by grant from MIUR-ITALY PRIN2015  
291 (Project N. 2015MSCCKCE\_003).

292

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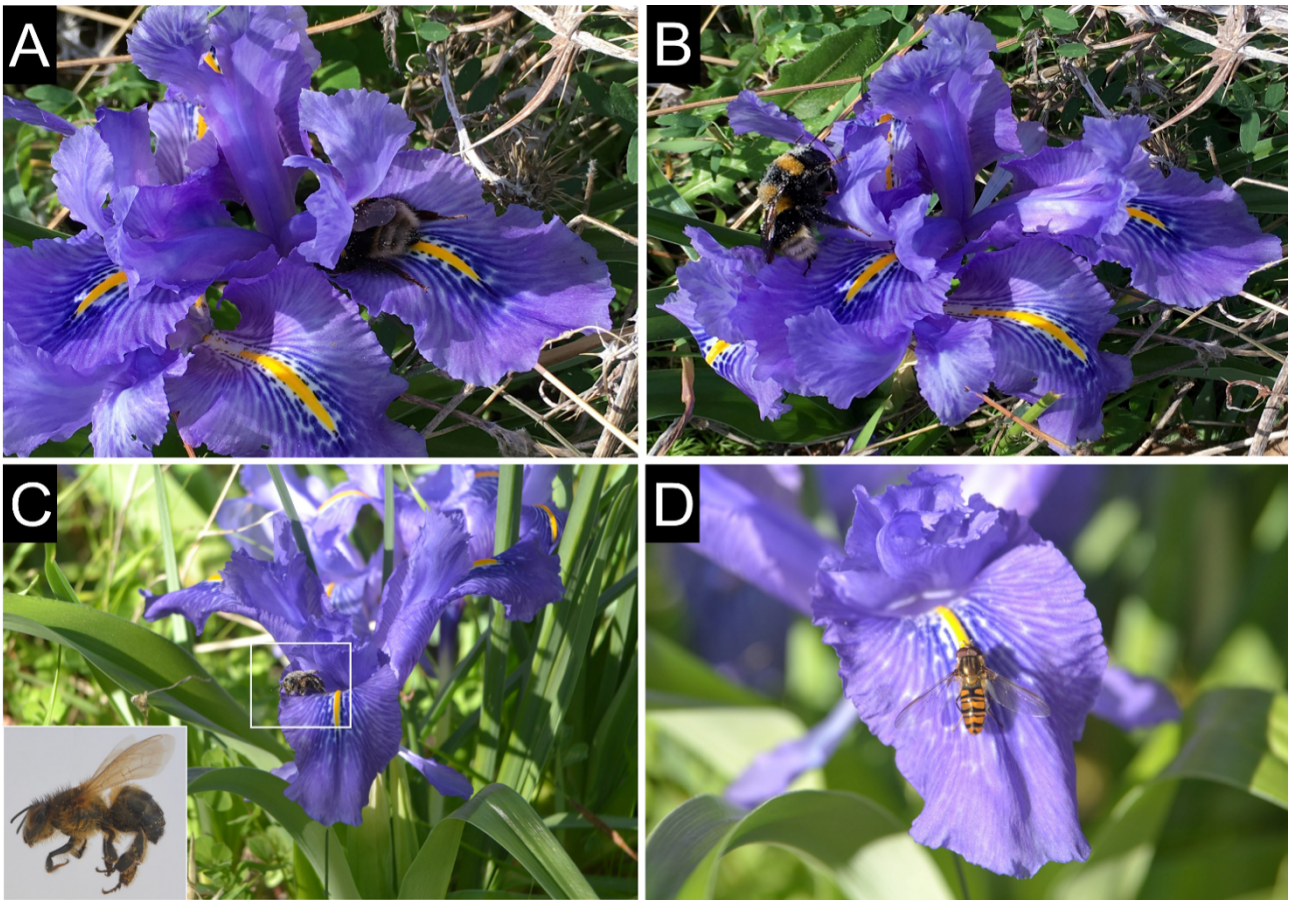
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442

443 **Table 1.** Relative and total absolute amounts of floral scent detected in *Iris planifolia*. Compounds  
 444 belonging to the same chemical class and functional group (Knudsen et al., 2006) are arranged  
 445 according to Kovats retention indices (KRI) of the Supelcowax 10 column. Odour characteristic:  
 446 olfactory descriptions according to Acree and Arn (2004) and El-Sayed (2018). \* = identification  
 447 verified by authentic standard.

Compound	KRI	Relative amount (%) Mean $\pm$ SE (N= 7)	Occurrence of samples (%)	Odour characteristic
<b>Aromatic alcohols</b>				
Benzyl alcohol*	1856	0.3 $\pm$ 0.1	100	Sweet, floral, fruity
2-Phenylethanol*	1886	8.4 $\pm$ 3.6	100	Honey, spicy, rose, lilac, sweet, yeast, floral, herbal
2-(4-Methoxyphenyl)ethanol	2303	1.4 $\pm$ 0.2	100	-
<b>Aromatic aldehydes</b>				
Benzaldehyde*	1503	3.7 $\pm$ 1.3	100	Almond, burnt sugar, woody
Phenylacetaldehyde*	1620	2.0 $\pm$ 1.6	57	Honey, sweet, rose, green, grassy, floral
2-Methoxybenzaldehyde	1936	6.2 $\pm$ 1.4	100	-
4-Methoxybenzaldehyde*	2004	0.4 $\pm$ 0.2	71	Mint, sweet
<b>Aromatic esters</b>				
Methyl benzoate*	1599	1.9 $\pm$ 0.5	86	Prune, lettuce, herbal, sweet, floral, watermelon
Benzyl tiglate*	2080	3.1 $\pm$ 1.0	86	Earthy, mushroom
<b>Aromatic ethers</b>				
1,4-Dimethoxybenzene*	1716	71.5 $\pm$ 4.9	100	Sweet, floral, herbal
1,2,4-Trimethoxybenzene*	2068	0.8 $\pm$ 0.3	86	-
<b>Monoterpene alcohols</b>				
Linalool*	1536	0.4 $\pm$ 0.1	100	Muscat, sweet, green, floral, lemon, parsley, lavender, fruity
<b>Total absolute amount: 2.0 <math>\pm</math> 0.6 <math>\mu</math>g/inflorescence/hour</b>				
<b>Total number of compounds: 12</b>				

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451

452 **Figure legend.**

453 **Fig. 1.** *Bombus ruderatus* inside (A) and on (B) the meranthium of *Iris planifolia* (photos by  
454 Francesca Tavella). *Apis mellifera* outgoing from the tunnel of the meranthium (C) and *Episyrphus*  
455 *balteatus* eating the pollen grains left by pollinators (D) (photos by Pietro Zito).

456

457 **Graphical abstract Legend.**

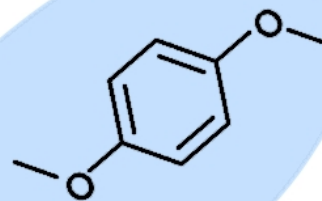
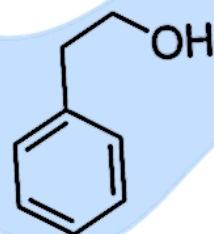
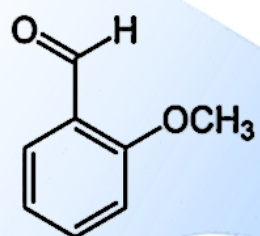
458 *Iris planifolia* (Iridaceae) advertises its food reward by an aromatic dominated floral volatile  
459 composition.

460



## Highlights

- Aromatic compounds dominate the floral scent bouquet.
- 1,4-dimethoxybenzene is the major compound.
- The floral scent advertises a food-rewarding species.
- Bumble and honey bees are pollinators whereas a syrphid fly is visitor.
- Floral olfactory cues probably act in synergy with visual ones to attract bees and flies.



# 1 2 3 **Floral scent in *Iris planifolia* (Iridaceae) suggests food reward** 4

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7 3 Pietro Zito<sup>a,\*</sup>, Sergio Rosselli<sup>b</sup>, Maurizio Bruno<sup>a</sup>, Antonella Maggio<sup>a</sup>, Maurizio Sajeve<sup>a</sup>,

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19  
20 11 Declarations of interest: none.  
21

## 22 23 24 13 **Abstract**

25  
26 14 *Iris* species can adopt different pollination strategies to attract their pollinators, generalized shelter-  
27  
28 15 mimicking, specialized deceptive sexual-mimicking or food-rewarding. As attractive stimuli, *Iris*  
29  
30 16 flowers may use their colours, large-size, symmetry, and volatile organic compounds (VOCs).  
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32 17 However, relatively few studies investigated *Iris* floral olfactory cues in the context of plant-  
33  
34 18 visitor/pollinator interactions. In the present study we combined the identification of the floral  
35  
36 19 volatiles of the nectariferous *I. planifolia* with insects visiting its flowers to gather data on its biology.  
37  
38 20 Floral volatiles were collected in the natural environment by dynamic headspace and analysed by gas  
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40 21 chromatography-mass spectrometry (GC-MS). Insect visitors/pollinators were also recorded. The  
41  
42 22 volatile bouquet was aromatic-dominated with 1,4 dimethoxybenzene as major compound. Among  
43  
44 23 the insects visiting its flowers, bumble and honey bees were the most abundant followed by hover  
45  
46 24 flies. Overall, our results suggest that *I. planifolia* advertises its food reward by an aromatic  
47  
48 25 dominated volatile composition.  
49

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51  
52 26 **Keywords:** *Iris planifolia*; Iridaceae; Aromatic compounds; Dynamic headspace; GC-MS; Bumble  
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54 27 bees; Honey bees; Hover flies; Pollination  
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62 **1. Introduction**  
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64 29 *Iris* L., with about 250-300 species distributed in Eurasia, North America and North Africa, is the  
65  
66 30 largest genus in the family Iridaceae (Goldblatt and Manning, 2008; Mavrodiev et al., 2014).  
67  
68 31 Although some species are found in mesic or wet environments, most occur in desert, semi-desert, or  
69  
70 32 dry, rocky and montane habitats (Wilson et al., 2016). *Iris* spp. were well known and cultivated, for  
71  
72 33 their use as a perfume, throughout ancient Greece and Roman Empire (Crişan and Cantor, 2016).  
73  
74 34 Cherokee Indians or other Native Americans used *Iris* plants for medicinal purposes (e.g. gastric  
75  
76 35 problems, kidney and bladder disorders, among others) (Crişan and Cantor, 2016). Indeed, *Iris* plants  
77  
78 36 have been extensively studied because the species are a rich source of specialised metabolites, such  
79  
80 37 as flavonoids, isoflavonoids, benzoquinones, terpenoids and glycosides which have a wide range of  
81  
82 38 biological activities, including anti-inflammatory, antioxidant, antimicrobial and anticancer with  
83  
84 39 chemo-preventive properties (Al-Jaber, 2016; Crişan and Cantor, 2016). To date, *Iris* plants have a  
85  
86 40 large economic value as flavoring in food products and soft drinks, ornamental purposes and uses in  
87  
88 41 the cosmetic industry (Crişan and Cantor, 2016).  
89

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92 42 *Iris* flower is considered an inflorescence with three functionally separate pollination units  
93  
94 43 (meranthia), acting as a single bilabiate gullet/tunnel flower (Goldblatt and Bernhardt, 1999). In each  
95  
96 44 unit (meranthium), the petaloid-style branch and its associated stamen form the upper lip (roof), while  
97  
98 45 the closely opposed large tepal, that works like a landing platform for insect visitors, forms the lower  
99  
100 46 lip (perigon lobe/floor) (Goldblatt and Bernhardt, 1999; Westerkamp and Claßen-Bockhoff, 2007).  
101  
102 47 Interestingly, in *Iris* spp. the lower lips are often embellished with various colour patches, lines,  
103  
104 48 hairs/beards and ridges, among others, that may play an attractive role for the insects visiting the  
105  
106 49 flowers (Guo, 2015; Morinaga and Sakai, 2006). Studies on *Iris* pollination showed that most species  
107  
108 50 are pollinated by social and/or solitary bees belonging to Apidae, Andrenidae, Halictidae and  
109  
110 51 Colletidae (e.g., *I. atropurpurea* Baker, Sapir et al., 2005; Vereecken et al., 2013; Watts et al., 2013;  
111  
112 52 *I. bulleyana* Dykes, Ye et al., 2017; *I. lutescens* Lam., Imbert et al., 2014a, Radović et al., 2017; *I.*  
113  
114 53 *paradoxa* Steven, Vereecken et al., 2012; *I. pumila* L., Radović et al., 2017; *I. tuberosa* L., Pellegrino  
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119  
120  
121 54 et al., 2016). In *Iris* species the rewards may consist of pollen, nectar or oils (Pellegrino et al., 2015);  
122  
123 55 although, such as in the nectarless *Oncocyclus* (Siems.) Baker section (*I. atrofusca* Baker, *I.*  
124  
125 56 *atropurpurea*, *I. bismarckiana* Damman & Sprenger, among others) the flowers can provide  
126  
127  
128 57 protective shelters (non-nutritive form of reward) that are used by visitors/pollinators (Sapir et al.,  
129  
130 58 2005; Vereecken et al., 2012, 2013; Watts et al., 2013).

131  
132 59 As attractive stimuli, *Iris* flowers may use their colours (Imbert et al., 2014a, 2014b; Vereecken et  
133  
134 60 al., 2012, 2013; Wang et al., 2013; Wang et al., 2016), large-sized flowers (Lavi and Sapir, 2015;  
135  
136 61 Radović et al., 2017), floral symmetry (Radović et al., 2017), and a range of volatile organic  
137  
138 62 compounds (VOCs) (Ohler et al., 2016; Pellegrino et al., 2015, 2016; Vereecken et al., 2012, 2013;  
139  
140 63 Wang et al., 2013). However, while floral visual cues *i.e.* floral size, symmetry, colour and/or its  
141  
142 64 polymorphism have been extensively studied in *Iris* spp. (Imbert et al., 2014a, 2014b; Lavi and Sapir,  
143  
144 65 2015; Pellegrino et al., 2015, 2016; Radović et al., 2017; Souto-Vilarós, 2018; Vereecken et al., 2012,  
145  
146 66 2013; Wang et al., 2013, 2016), relatively few studies (additionally or exclusively) investigated *Iris*  
147  
148 67 floral olfactory cues *i.e.* floral VOCs in the context of plant-visitor/pollinator interactions (*I.*  
149  
150 68 *atropurpurea* and *I. paradoxa*, Vereecken et al., 2012, 2013; *I. lutescens*, Wang et al., 2013, *I. pallida*  
151  
152 69 Lam., Ohler et al., 2016; *I. tuberosa*, Pellegrino et al., 2016).

153  
154 70 *Iris planifolia* (Mill.) T. Durand & Schinz (Iridaceae), occurring mainly in the western Mediterranean  
155  
156 71 and North Africa, is the only member of the subgenus *Scorpiris* Spach (section *Juno* Dykes) native  
157  
158 72 to Europe (Boltenkov and Govaerts, 2017). Since this nectariferous species occurs at low/middle  
159  
160 73 altitude in the Mediterranean region, where the winters are not severe, it may bloom at any time from  
161  
162 74 November to February (Mathew, 1986). This species has been studied from a taxonomical (Boltenkov  
163  
164 75 and Govaerts, 2017), molecular (Ikinci et al., 2011) and morphological (Guo, 2015; Ikinci et al., 2011)  
165  
166 76 points of view. The single chemical study on *I. planifolia* was performed by Chikhi et al., (2012).  
167  
168 77 The authors used the essential oil to study some biological roles (*i.e.* antimicrobial and antioxidant  
169  
170 78 activity) of whole *I. planifolia* plant volatiles, but there are no studies investigating the inflorescence-  
171  
172 79 specific volatile emissions of *I. planifolia* by using more biologically appropriate and non-invasive  
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178  
179  
180 80 methods, such as headspace. In the present study we combined the identification of the floral volatiles  
181  
182 81 of the blue-violet *I. planifolia* with insects visiting its flowers to gather data on its biology. In detail,  
183  
184 82 we (i) collected the inflorescences scent by dynamic headspace, (ii) analysed the samples by gas  
185  
186 83 chromatography and mass spectrometry (GC-MS) and (iii) recorded first data on its insect  
187  
188  
189 84 visitors/pollinators.

## 193 86 **2. Results**

### 195 87 *2.1. Floral scent*

197 88 Twelve VOCs were identified in *I. planifolia* inflorescences: eleven aromatic compounds and one  
198  
199 89 monoterpene (Table 1). The total absolute amount of VOCs emitted by *I. planifolia*, estimated by  
200  
201 90 using anisole as internal standard (see section 5.2 for details), was about 2.0 µg/inflorescence/hour.  
202  
203 91 The relative amount was reported as mean percentage of the integrated chromatogram peak areas.  
204  
205 92 The aromatic-dominated volatile bouquet was composed of four aromatic aldehydes (12 %), three  
206  
207 93 aromatic alcohols (10 %), two aromatic esters (5 %) and two aromatic ethers (72 %). The single  
208  
209 94 exception was represented by the monoterpene alcohol linalool, which contributed to less than 0.5 %.  
210  
211 95 Overall, the floral scent of *I. planifolia* was strongly dominated by 1,4-dimethoxybenzene (also  
212  
213 96 known as hydroquinone dimethyl ether) with about 71 %. Two compounds: 2-phenylethanol and 2-  
214  
215 97 methoxybenzaldehyde were respectively found in relative amounts of 8 % and 6 %. Finally, five  
216  
217 98 compounds ranged between 4 and 1% whereas four contributed with less than 1% (Table 1).  
218  
219  
220

### 221 99 *2.2. Flower-visiting insects*

223 100 Overall, in 8 hours of observation (8:00 - 16:00) we recorded 42 insects on *I. planifolia* inflorescences,  
224  
225 101 with bees and flies landing on all the observed plants. Insect visitation mostly occurred between 11:00  
226  
227 102 - 14:00. Bumble bees and honey bees (Apidae) were the most abundant visitors (32 registered visits)  
228  
229 103 followed by hover flies (10 registered visits). *Bombus ruderatus* (Fabricius) (Apidae) (Fig. 1A, B)  
230  
231 104 with 18 visits was the main insect visitor followed by *Apis mellifera* Linnaeus (Apidae) (Fig. 1C)  
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105 with 14 visits and by *Episyrphus balteatus* (De Geer) (Syrphidae) (Fig. 1D) with 10 visits. Since only  
106 bumble bees and honey bees entered inside the flower tunnels and carried the pollen on their body  
107 (mainly on their head and torax), they can be considered as potential pollinators. On the contrary, the  
108 syrphid fly *E. balteatus* never entered inside the flowers and thus was considered as visitor.  
109 Interestingly, *E. balteatus* spent most of its time eating the pollen grains left on the flowers by  
110 pollinators when they crawled out of the flower tunnels.

### 112 3. Discussion

113 The floral scent of *I. planifolia* is characterized by VOCs that are reported (1) as floral volatiles of  
114 different angiosperm families (Knudsen et al., 2006) and (2) as semiochemicals mediating several  
115 plant-animal interactions (El Sayed, 2018).

116 With the single exception represented by the monoterpene alcohol linalool (less than 0.5 %), the floral  
117 scent bouquet of *I. planifolia* has a strong aromatic chemical composition (Tab. 1) dominated with  
118 more than 70 % by the aromatic ether 1,4-dimethoxybenzene (hereafter 1,4-DMB). This VOC was  
119 also found as major compound in the floral scents of *Salix* L. spp. (Salicaceae) pollinated during the  
120 daytime by mining bees and honey bees (Dötterl et al., 2005, 2014; Jürgens et al., 2014), and of  
121 *Anacamptis coriophora* (L.) R.M. Bateman, Pridgeon & M.W. Chase (Orchidaceae) pollinated by  
122 bumble and honey bees (Salzmann et al., 2007).

123 Despite the similarity with the floral scents of plant species belonging to different families (Knudsen  
124 et al., 2006), the floral scent composition of *I. planifolia* differs from those found within the genus by  
125 other authors. For example, while 1,4-DMB was the main VOC found in our dynamic headspace  
126 samples, it was detected in minor amounts in the essential oil of *I. planifolia* (0.5 %, Chikhi et al.,  
127 2012) and in the static headspace samples of *I. lutescens* (less than 3.0 %, Wang et al., 2013).  
128 Furthermore, it was not found in solvent extracts of *I. pallida* (toluene samples by Ohler et al., 2016),  
129 *I. atropurpurea*, *I. paradoxa* and *I. tuberosa* (hexane samples by Pellegrino et al., 2016 and Vereecken  
130 et al., 2012, 2013). In contrast, *I. planifolia* by Chikhi et al. (2012) was dominated by aliphatic

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297  
298 131 hydrocarbons and fatty acids (e.g. *n*-alkanes and hexadecanoic acid); *I. lutescens* by monoterpenes  
299  
300 132 (myrcene, (*E*)- $\beta$ -ocimene and limonene) (Wang et al., 2013); *I. pallida* by methyl anthranilate (Ohler  
301  
302 et al., 2016) and *I. atropurpurea*, *I. paradoxa* and *I. tuberosa* by aliphatic hydrocarbons (*n*-alkanes  
303 133 and *n*-alkenes) (Pellegrino et al., 2016; Vereecken et al., 2012, 2013). As reported by other authors  
304  
305 134 (e.g. El-Sajed et al., 2018; Jürgens et al., 2009; Knudsen and Gershenson, 2006; Tholl et al., 2006),  
306  
307 135 (e.g. El-Sajed et al., 2018; Jürgens et al., 2009; Knudsen and Gershenson, 2006; Tholl et al., 2006),  
308  
309 136 it is not surprising that the limited overlap in floral VOCs between *Iris* species could be due to the  
310  
311 137 different volatile sampling methods (e.g., headspace vs hydrodistillation or solvent extractions), to  
312  
313 138 the polarity and affinities of VOCs to the adsorbent fibers used (headspace methods), to the  
314  
315 139 interspecific variation, and/or to the insect pollinators/pollination strategies of the investigated *Iris*,  
316  
317 140 among others. Indeed, excluding 1,4-DMB, we found only a few co-occurrences between our floral  
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319  
320 141 volatiles and those of other *Iris* species. Among these, benzyl tiglate also was detected in *I. planifolia*  
321  
322 142 by Chikhi et al. (2012); benzaldehyde, 2-(4-methoxyphenyl)ethanol and 4-methoxybenzaldehyde in  
323  
324 143 *I. lutescens* (Wang et al., 2013); linalool in *I. planifolia* (Chikhi et al., 2012) and in *I. lutescens* (Wang  
325  
326 144 et al., 2013).  
327  
328 145 Autonomous self-pollination has been described in some *Iris* species (e.g. *Iris versicolor* L.) (Kron  
329  
330 146 et al., 1993) but not in others (e.g. *Oncocyclus* irises) (Watts et al., 2013; Sapir et al., 2005). To date,  
331  
332 147 information on pollination biology of *I. planifolia* has been lacking; however, irises pollination data  
333  
334 148 from the literature show that insects play an important role for the reproductive success in both self-  
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336  
337 149 incompatible and self-compatible *Iris* species (Watts et al., 2013; Kron et al., 1993). Among *I.*  
338  
339 150 *planifolia* visitors, *Bombus ruderatus* and *Apis mellifera* (Fig. 1A, B, C) were found as candidates  
340  
341 151 that, forcing the tepal (lower lip) and the opposite style branch of the meranthium, entered inside the  
342  
343 152 tunnel and passively collected pollen. We did not find any insects inside the flowers (sheltering) as  
344  
345 153 in *Iris atropurpurea* and *Iris paradoxa* (Vereecken et al., 2012, 2013; Watts et al., 2013). Although  
346  
347 154 more extensive field observations (e.g. data collection and night-sheltering) and experiments (e.g.  
348  
349 155 seed- fruit-set) are needed to confirm the exclusive role of *B. ruderatus* and *A. mellifera* as pollinators  
350  
351 156 of *I. planifolia*, our findings are consistent with previous studies of other *Iris* species pollinated by  
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355  
356  
357 157 bumble bees and/or honey bees, among others (Goldblatt and Manning, 2006; Imbert et al., 2014a,  
358 2014b; Segal et al. 2006; Souto-Vilarós et al., 2018; Watts et al., 2013; Ye et al., 2017). In addition  
359 158 to the legitimate bee pollinators, the syrphid fly *E. balteatus* was observed eating the pollen grains  
360 158 lost by *B. ruderatus* and *A. mellifera* on the lower lip of the *I. planifolia* flowers. Since *E. balteatus*  
361 159 to the legitimate bee pollinators, the syrphid fly *E. balteatus* was observed eating the pollen grains  
362 159 lost by *B. ruderatus* and *A. mellifera* on the lower lip of the *I. planifolia* flowers. Since *E. balteatus*  
363 160 was not observed as pollen robber from the anthers of *I. planifolia* (it never entered into the  
364 160 gullet/tunnel flower) it is not likely to have detrimental effects for *I. planifolia*. Conversely, we  
365 161 speculate that its “cleaning service” could have a beneficial effect by restoring the original visual  
366 161 displays.  
367 162  
368 162 Generalist bees, such as *Bombus* spp. and *Apis mellifera*, have a broad pollen/nectar diet and collect  
369 163 pollen from different species with variable floral cues (Dobson, 2006; Dötterl and Vereecken, 2010).  
370 163 Consequently, generalist bees might benefit by relying on floral VOCs related to a broad spectrum of  
371 164 potential host flowers (Dötterl and Vereecken, 2010). In food-rewarding plants, the aromatic  
372 164 compounds as alcohols, aldehydes, esters, and ethers alone or in combination with some monoterpene  
373 165 alcohols (e.g. linalool) are often reported as floral VOCs that attract nectar/pollen feeders, such as  
374 165 bees, butterflies, moths and flies (Dobson 2006; Dötterl and Vereecken 2010; Jürgens et al., 2009;  
375 166 Primante and Dötterl, 2010). Although in our study we did not perform electroantennographic or  
376 166 behavioral experiments, data from the literature provide evidence that 1,4-DMB (Jürgens et al., 2014;  
377 167 Salzman et al., 2007), benzaldehyde (Theis, 2006), 4-methoxybenzaldehyde (= *p*-anisaldehyde),  
378 167 phenylacetaldehyde (Dötterl and Vereecken 2010; Knauer and Schiestl, 2015; Theis, 2006), 2-  
379 167 phenylethanol, benzyl alcohol and linalool (Dötterl and Vereecken 2010; Theis, 2006) are VOCs  
380 168 perceived and attractive for *Bombus* spp. and/or *Apis mellifera*. In addition, 2-phenylethanol is known  
381 168 as attractant for syrphid flies (Zhu and Park, 2005) and potentially also for *E. balteatus* (Primante and  
382 169 Dötterl, 2010); whereas linalool was recently reported as electrophysiologically-active for Syrphidae  
383 169 (Braunschmid et al., 2017).  
384 170  
385 170 *Iris* spp. use floral visual and olfactory cues for attracting their pollinators (e.g. Pellegrino et al., 2016;  
386 170 Vereecken et al., 2012, 2013; Wang et al., 2013). It is interesting to note that bumble bees’ UV-blue  
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416 183 photoreceptors can perceive the predominant blue-violet *I. planifolia* floral colour (human  
417  
418 184 perception) (Arnold et al., 2010) as attractive and innate signals of rewarding flowers (Knauer and  
419  
420  
421 185 Schiestl, 2015; Kunze and Gumbert, 2001; Raine and Chittka, 2007). Furthermore, since syrphid flies  
422  
423 186 prefer yellow over other colours, the yellow or orange pubescent median ridge in the lower lips of *I.*  
424  
425 187 *planifolia* meranthia can be attractive for *E. balteatus*, despite it mainly uses olfactory cues to find a  
426  
427 188 pollen/nectar host-plants (Primante and Dötterl, 2010).  
428

#### 430 189 431 190 **4. Conclusions**

432  
433 191 In agreement with Vereecken et al. (2015), in this study we adopted an accepted and modern  
434  
435 192 methodology (dynamic headspace) that allows the collection and analysis of floral scents from the  
436  
437  
438 193 prospective of plant-visitor/pollinator interactions (El-Sajed et al., 2018; Zito et al., 2015). To our  
439  
440 194 surprise, this is the first study that investigated the floral volatiles in the genus *Iris* by using a dynamic  
441  
442 195 headspace method. Furthermore, it is the first report on visitor/pollinators of *I. planifolia*. Our results  
443  
444 196 suggest that the floral volatiles of the nectariferous *I. planifolia* may play a synergistic role when also  
445  
446 197 visual cues occur. This hypothesis is supported by other studies that reported how social bees (e.g.  
447  
448 198 bumble bees and honey bees) elicited the strongest behavioural responses when floral visual and  
449  
450 199 olfactory cues co-occur (Chittka and Raine, 2006; Dötterl et al., 2014; Junker and Parachnowitsch,  
451  
452 200 2015).  
453

454 201 Interestingly, sexual mimicry has evolved from generalized food deception, shelter pollination, or  
455  
456  
457 202 food reward systems (Johnson and Schiestl, 2016). By floral visual and olfactory cues *Iris* spp. can  
458  
459 203 adopt different pollination strategies to attract their pollinators *i.e.* from generalized shelter-  
460  
461 204 mimicking species or specialized sexual-mimicking deceptive species (Pellegrino et al., 2015, 2016;  
462  
463 205 Vereecken et al., 2012, 2013) to food-rewarding species (present study). Although more data are  
464  
465 206 needed to confirm the pollination strategies adopted in other un-investigated *Iris* species among the  
466  
467 207 sections, our results suggest that *I. planifolia* advertises the food reward by its aromatic dominated  
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469 208 volatile composition probably in synergy with its visual cues. Further studies are needed to better  
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475 209 understand the interplay between floral olfactory and visual cues for attracting *I. planifolia* visitors  
476  
477 210 and pollinators.  
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480 211

## 481 482 212 **5. Experimental**

### 483 484 213 *5.1. Study species*

485  
486 214 *Iris planifolia* (Mill.) T. Durand & Schinz (Iridaceae) [subg. *Scorpiris* Spach; section *Juno* Dykes],  
487  
488 215 described as *Xiphium planifolium* in 1768 by Philip Miller, is a perennial bulbous herb about 10-25  
489  
490 216 cm high (Mathew, 1986). It is naturally occurring in Spain, Portugal, Sardinia, Sicily, Crete, Greece,  
491  
492 217 Libya, Tunisia, Algeria and Morocco (Barker and Govaerts, 2018). As described by Mathew (1986),  
493  
494 218 the stems are very short, more or less subterranean and hidden by the leaf bases. The lanceolate and  
495  
496 219 often undulate-falcate leaves (4-7) are bright shiny green above and glaucous beneath. Each plant,  
497  
498 220 blooming from November to February, has 1-3 inflorescences (6-12 cm across) pale to deep blue or  
499  
500  
501 221 violet-blue and rarely white. The lower lips of the three meranthia of its inflorescence are widely  
502  
503 222 reflexed, blue, veined and dotted in dark violet with a yellow or orange pubescent median ridge. The  
504  
505 223 anthers are whitish or purplish whereas style branches flattened and petaloid are bilobed at the apex.  
506  
507 224 To date, no information is available on the pollination ecology of *I. planifolia*; the plant produces  
508  
509 225 nectar (supplementary material in Herrera et al., 2012) as pollinators reward. The seeds, ovoid and  
510  
511 226 rugose are dark reddish-brown. In its natural habitat, the species usually grows on sloping rocky  
512  
513 227 ground on limestone formations where the soil is the typical Mediterranean reddish clay (Mathew,  
514  
515 228 1986).

516  
517  
518 229 In the present study, all the field activities were performed in November 2017, wet season in Sicily,  
519  
520 230 in a natural site located in San Martino delle Scale - Sicily (38°5'18.29"N, 13°15'23.48"E).

### 521 522 523 231 *5.2. Floral scent sampling*

524  
525 232 Floral scent was collected, on a sunny day in November 2017, from plants growing in their natural  
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527 233 habitat (38°5'18.29"N, 13°15'23.48"E - San Martino delle Scale - Sicily). All samples from  
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534 234 inflorescences ( $N= 7$ ) were collected using a dynamic headspace method (pull system) (Tholl et al.,  
535  
536 235 2006). For each sampling, one inflorescence per plant was enclosed in an oven bag (size: 15 × 12 cm,  
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538  
539 236 CUKI® Cofresco S.p.A.) and the emitted volatiles were collected for 6 hours (9:00 - 15:00) by an  
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541 237 adsorbent tube using a vacuum pump (G12/01 EB, Rietschle Thomas, Puchheim, Germany), with a  
542  
543 238 flow rate of 200 ml/min. The adsorbent tube (shortened glass Pasteur pipette) was filled with 20 mg  
544  
545 239 Tenax-TA (mesh 60-80) and 20 mg Carbotrap B (mesh 20-40) both Supelco (Bellefonte, PA, USA).  
546  
547 240 The adsorbent materials were fixed in the tubes using glass wool (Sigma Aldrich). At the same time,  
548  
549 241 by using the same approach described before, headspace samples from leaf ( $N= 3$ ) and an empty oven  
550  
551 242 bag ( $N= 1$ ), were used as negative controls. Volatiles trapped in the tubes were eluted within a few  
552  
553 243 minutes after collection with 200 µl of acetone (Sigma Aldrich, > 99 %). All samples were stored in  
554  
555 244 screw cap vials at -20 °C until chemical analyses. The peak areas on the chromatograms were  
556  
557  
558 245 integrated to obtain the total ion current, which was used to determine the relative amount (%) of each  
559  
560 246 peak. To quantify the absolute amount of scent emitted from an inflorescence, one µg of anisole  
561  
562 247 (Sigma-Aldrich, Anisole ReagentPlus®, 99 %) was added to each sample as internal standard.  
563  
564 248 Subsequently to calculate the absolute amounts of the VOCs in each sample, peak areas on the  
565  
566 249 chromatograms were integrated to obtain the total ion current signal and compared to that of the  
567  
568 250 internal standard.

### 571 251 *5.3. Chemical analysis*

572  
573 252 All samples were analyzed by GC-MS on a single quadrupole Shimadzu GC-MS-QP2010 Plus  
574  
575  
576 253 equipped with an AOC-20i autoinjector (Shimadzu, Kyoto, Japan) and a Supelcowax 10 capillary  
577  
578 254 column (30 m long, 0.25 mm i.d., 0.25 µm film thickness). One µl of each sample was injected at 250  
579  
580 255 °C in the splitless mode, and the column flow (carrier gas: helium) was set at 1 ml/min. The GC oven  
581  
582 256 temperature was held for 5 min at 40 °C, then increased by 2°C/min to 250 °C, held for 15 min and  
583  
584 257 finally raised to 270 °C at 10 °C/min. The MS interface worked at 280 °C, and the ion source at 250  
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592  
593 258 °C. Mass spectra were taken at 70 eV (in EI mode) from m/z 30 to 500. The GC/MS data were  
594  
595 259 analyzed using the GCMSolution package, Version 2.72.  
596  
597  
598 260 Identification of compounds was carried out using the mass spectral libraries FFNSC 2, W9N11,  
599  
600 261 ESSENTIAL OILS (available in MassFinder 3), and Adams (2007). These identifications were also  
601  
602 262 compared by mass spectra and Kovats retention indices found in NIST11, SciFinder and Pherobase  
603  
604 263 (El-Sayed, 2018) database. Many of the compounds were confirmed by comparison of mass spectra  
605  
606 264 and retention times with authentic standard components (Sigma Aldrich) provided by Prof. Dr. Stefan  
607  
608 265 Dötterl (Plant Ecology Lab - University of Salzburg). Kovats retention indices were calculated using  
609  
610 266 a series of n-alkanes (C<sub>8</sub> - C<sub>30</sub>). Compounds found in the flowers were compared with those found in  
611  
612 267 the negative controls (empty oven bag and green leaves) to discriminate contaminations from air  
613  
614 268 ambient and to identify those exclusively emitted by flowers.  
615  
616

#### 617 269 5.4. Flower-visiting insects

619 270 Insects visiting *I. planifolia* inflorescences were recorded during the day and in the same site of the  
620  
621 271 headspace collections. The sampling of flower-visiting insects was limited to ten inflorescences in  
622  
623  
624 272 order to minimize the potential negative impact on the local entomofauna and on pollination  
625  
626 273 processes. Observations were performed simultaneously by two researchers staying near the plants  
627  
628 274 for 8 hours (08:00 - 16:00). The limited number of hours of observation was linked to good weather  
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630 275 condition (sunny day) occurring in the flowering period of *I. planifolia* in Sicily (rainy season).  
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632 276 Insects visiting *I. planifolia* were recorded when they landed on a meranthium. Only a few insect  
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634 277 visitors belonging to different morphospecies were captured by entomological hand nets or plastic  
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636 278 bags (10 x 7 cm), otherwise they were annotated and photographed with a digital camera (NIKON  
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638 279 D3100). Since not all insect individuals were captured, we cannot exclude that the same specimen  
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641 280 after leaving, came back to revisit the inflorescence again. The data of insects visiting the ten different  
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643 281 *I. planifolia* inflorescences were pooled together. The flower visitors that landed, entered inside the  
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645 282 meranthium and came out it with pollen on their body were recorded as potential pollinators. On the  
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652 283 contrary, the insects that after their landing on the inflorescences never entered inside the meranthium  
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654 284 were recorded as visitors. Insect specimens, identified at specific taxonomic level, are stored in an  
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657 285 entomological box and kept in the Entomological collection of the Department of Biological,  
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659 286 Chemical and Pharmaceutical Sciences and Technologies of the University of Palermo.

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661  
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665  
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668 290 the pollinating bumble bee. This work was supported by grant from MIUR-ITALY PRIN2015  
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670 291 (Project N. 2015MSCCKCE\_003).

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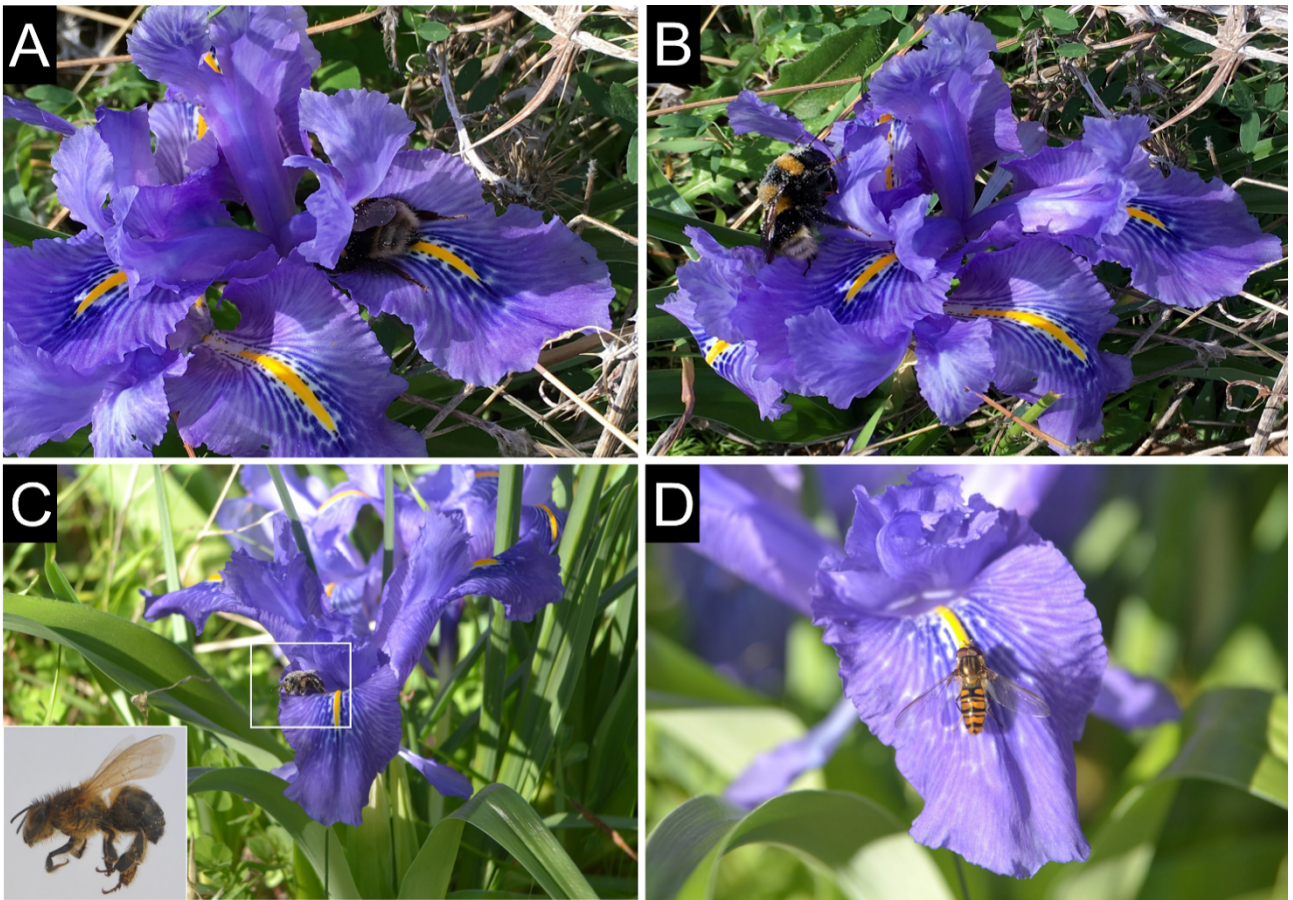
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**Table 1.** Relative and total absolute amounts of floral scent detected in *Iris planifolia*. Compounds belonging to the same chemical class and functional group (Knudsen et al., 2006) are arranged according to Kovats retention indices (KRI) of the Supelcowax 10 column. Odour characteristic: olfactory descriptions according to Acree and Arn (2004) and El-Sayed (2018). \* = identification verified by authentic standard.

Compound	KRI	Relative amount (%) Mean $\pm$ SE (N= 7)	Occurrence of samples (%)	Odour characteristic
<b>Aromatic alcohols</b>				
Benzyl alcohol*	1856	0.3 $\pm$ 0.1	100	Sweet, floral, fruity
2-Phenylethanol*	1886	8.4 $\pm$ 3.6	100	Honey, spicy, rose, lilac, sweet, yeast, floral, herbal
2-(4-Methoxyphenyl)ethanol	2303	1.4 $\pm$ 0.2	100	-
<b>Aromatic aldehydes</b>				
Benzaldehyde*	1503	3.7 $\pm$ 1.3	100	Almond, burnt sugar, woody
Phenylacetaldehyde*	1620	2.0 $\pm$ 1.6	57	Honey, sweet, rose, green, grassy, floral
2-Methoxybenzaldehyde	1936	6.2 $\pm$ 1.4	100	-
4-Methoxybenzaldehyde*	2004	0.4 $\pm$ 0.2	71	Mint, sweet
<b>Aromatic esters</b>				
Methyl benzoate*	1599	1.9 $\pm$ 0.5	86	Prune, lettuce, herbal, sweet, floral, watermelon
Benzyl tiglate*	2080	3.1 $\pm$ 1.0	86	Earthy, mushroom
<b>Aromatic ethers</b>				
1,4-Dimethoxybenzene*	1716	71.5 $\pm$ 4.9	100	Sweet, floral, herbal
1,2,4-Trimethoxybenzene*	2068	0.8 $\pm$ 0.3	86	-
<b>Monoterpene alcohols</b>				
Linalool*	1536	0.4 $\pm$ 0.1	100	Muscat, sweet, green, floral, lemon, parsley, lavender, fruity
<b>Total absolute amount: 2.0 <math>\pm</math> 0.6 <math>\mu</math>g/inflorescence/hour</b>				
<b>Total number of compounds: 12</b>				

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**Figure 1**



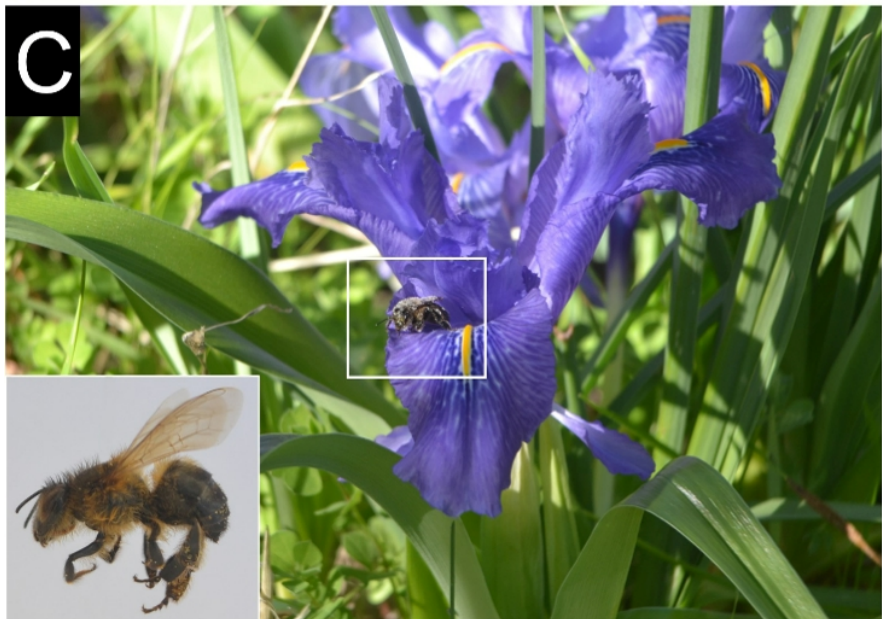
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452 **Figure legend.**

453 **Fig. 1.** *Bombus ruderatus* inside (A) and on (B) the meranthium of *Iris planifolia* (photos by  
454 Francesca Tavella). *Apis mellifera* outgoing from the tunnel of the meranthium (C) and *Episyrphus*  
455 *balteatus* eating the pollen grains left by pollinators (D) (photos by Pietro Zito).

456  
457 **Graphical abstract Legend.**

458 *Iris planifolia* (Iridaceae) advertises its food reward by an aromatic dominated floral volatile  
459 composition.



**Table 1.** Relative and total absolute amounts of floral scent detected in *Iris planifolia*. Compounds belonging to the same chemical class and functional group (Knudsen et al., 2006) are arranged according to Kovats retention indices (KRI) of the Supelcowax 10 column. Odour characteristic: olfactory descriptions according to Acree and Arn (2004) and El-Sayed (2018). \* = identification verified by authentic standard.

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