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Nectar tyramine decreases the duration of bumblebee visits on flowers

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1 **Title: Nectar tyramine decreases the duration of bumblebee visits on flowers**

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32

33 **Abstract:** Several studies on floral nectar demonstrated that the behaviour of visit performed by  
34 pollinators is influenced by nectar chemistry. Biogenic amines act as neurotransmitters in  
35 invertebrates and recently have been reported in the floral nectar of 15 plant species for the first  
36 time. However, both their occurrence in floral nectar and the effects of their nectar-like  
37 concentrations on bee behaviour remains largely unsubstantiated.. To increase knowledge on the  
38 role of biogenic amines on plant-pollinator interactions, here we i) investigated the biogenic amine  
39 composition of *Echium vulgare* nectar in relation to its floral sexual phases, and ii) studied how an  
40 artificial solution enriched with nectar-like concentrations of tyramine affects the visit on flowers of  
41 bumblebees under semi-controlled conditions. The chemical analysis reported the presence of  
42 tyramine in *E. vulgare* nectar and no difference in concentration between the two sexual phases. To  
43 explore potential effects of tyramine on bee behaviour, we designed a new method consisting in  
44 zucchini flowers emptied of their natural nectar and refilled with artificial tyramine-enriched nectar,  
45 and we used bumblebee workers as pollinator model. We found that bees fed tyramine-enriched  
46 solution spent less time foraging on a single flower than those fed control solution , suggesting that  
47 their behaviour of visit was overall more dynamic. Our results highlight the importance of  
48 addressing further investigations on this emerging class of nectarcompounds on insect cognition and  
49 behaviour, other than on its occurrence and distribution in nectar of other species.

50

51 **Keywords:** biogenic amines, *Bombus terrestris*, *Echium vulgare*, flight enhancer, pollinator  
52 behaviour

53

54 **Conflict of interest**

55 All authors declare that they have no conflict of interest.

56

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## 61 **1. Introduction**

62 An increasing number of studies on the chemistry of floral nectar shows that the frequency of  
63 pollinator visits (Pleasants 1981; Real and Rathcke 1991; Shykoff and Bucheli 1995), the duration  
64 of flower visits (Galen and Plowright 1984; Cresswell 1999) and the overall pattern of visit within a  
65 plant population (Fisogni et al. 2011; Barberis et al. 2021) are all variables influenced by both  
66 nectar volume and chemistry. In the past decades, hundreds of secondary metabolites have been  
67 found in nectar other than sugars (e.g. Baker and Baker 1977, 1986) and for some of them a direct  
68 influence on behaviour has been demonstrated (e.g. Wright et al. 2013; Barlow et al. 2017; Bogo et  
69 al. 2019; Barberis et al. 2023).

70 Among the chemicals most recently discovered in floral nectar, biogenic amines are nitrogenous  
71 compounds which are known to act as neurotransmitters in invertebrates (Blenau and Baumann  
72 2001). Their presence in floral nectar has been reported for the first time by Muth et al. (2022) in 15  
73 different plant species belonging to 6 different orders, where they were represented either by  
74 tyramine, octopamine or a combination of the two, with a maximum concentration averaging  
75 around 0.07 mM in the species *Cytrus x meyeri*. Both compounds work through their binding to G  
76 protein-coupled receptors (Roeder 2005), whose activation leads to the interaction with other  
77 proteins regulating enzymatic activity leading to changes in the levels of intracellular signaling  
78 molecules such as cAMP and Ca<sup>2+</sup>. These signals can, in turn, regulate the expression of genes, the  
79 activity of ion channels, and the functioning of further proteins (Mustard 2020). As compounds that  
80 can activate or inhibit G proteins, their consumption can potentially affect pollinator behavior both  
81 in a short- and long-term way (Mustard 2020).

82 For this reason, far before their discovery in floral nectar, a number of studies has been performed  
83 to investigate their functioning in insects, demonstrating how their consumption modulates several  
84 behavioural traits such as locomotion (e.g. Fussnecker et al. 2006, Hardie et al. 2007), reward-  
85 seeking (e.g. Schulz and Robinson 2001, Peng et al. 2020), learning (e.g. Mercer and Menzel 1982,  
86 Hammer and Menzel 1998) and social communication (e.g. Barron et al. 2007, Peng et al. 2020).

87 However, most of such studies tested concentrations much greater than those found in natural nectar  
88 (Barberis et al. 2023, and reference therein).

89 In this exploratory study, we studied the effect of the biogenic amine tyramine on the behaviour of  
90 flower visitors, using bumblebees and the plant *Echium vulgare* as models. In particular, we  
91 investigated i) the biogenic amine composition of the floral nectar of a natural population of *E.*  
92 *vulgare* in relation to its floral sexual phases and, on the basis of the former result, ii) how an  
93 artificial solution enriched with nectar-like concentrations of biogenic amines affects the visits on  
94 flowers performed by bumblebees under the semi-controlled conditions of an insect net greenhouse.  
95 Though the use of artificial flowers is common in nectar experiments (e.g. Thomson et al. 2015;  
96 Felicioli et al. 2018) it is not excluded that they may potentially affect animal behaviour. For this  
97 reason, we opted for a novel experimental design under conditions as much natural as possible,  
98 adopting real flowers emptied of their natural nectar and refilled with the treatment diets to test.

99

## 100 **2. Methods**

### 101 **2.1. Biogenic amine composition of *Echium vulgare* floral nectar**

102 The activity in the field was carried out in June 2018 and took place in the Parco Belpoggio, a  
103 natural park managed since 2010 by the WWF, in San Lazzaro di Savena (Bologna, Italy). The area  
104 is close to the protected area Parco dei Gessi Bolognesi e Calanchi dell'Abbadessa (44°27'14.5"N  
105 11°22'58.3"E) and the studied *Echium vulgare* population was detected on an open prairie along the  
106 public pathway and exposed to full sunlight.

107 *Echium vulgare* L. is a perennial hemicryptophyte belonging to the family Boraginaceae. It is  
108 distributed in Europe, Asia and North America and it shows a long flowering period, ranging, at our  
109 latitudes, between June and October (Barberis et al. 2021). Anthesis lasts 3-4 days and flowers  
110 show incomplete protandry (Melser et al. 1997): the anthers often start to dehiscence already at the bud  
111 stage, while the stigma becomes receptive only hours after the flower opening. In this study, we  
112 considered two phases of floral development: functionally male (M) and functionally female (F)

113 flowers. The male phase was represented by an open flower presenting pollen with non-receptive  
114 stigma, whilst the female phase was recognized as soon as the stigma became bifid and receptive  
115 (Corbet 1978; Barberis et al. 2021).

116 In order to let the nectar accumulate, flowers were bagged in the morning with 1 mm mesh size  
117 tulle fabric for 2 hours prior to sampling. Due to the small volume of nectar produced per single  
118 flower (less than 0.5  $\mu\text{L}$  and 0.7  $\mu\text{L}$  in functionally male and female flowers, respectively) (Barberis  
119 et al. 2021), nectar was gathered from multiple flowers to reach a minimum volume of 15  $\mu\text{L}$   
120 needed for the chemical analyses. We obtained a total of 9 samples: 5 samples from functionally  
121 female flowers (pooled from 5-14 flowers, each sample collected from a single individual plant),  
122 and 4 samples from functionally male flowers (pooled from 30-63 flowers, each sample from one or  
123 two individual plants). We collected nectar samples by means of Drummond Microcaps (1-3  $\mu\text{L}$ ;  
124 Drummond Scientific Co., Broomall, PA), then we transferred the samples in Eppendorf tubes filled  
125 with 100  $\mu\text{L}$  of pure ethanol and took them to the laboratory on the same day of field sampling with  
126 the help of thermal insulated ice containers. Samples were stored at 5°C until analyses.

127 We characterized the content of biogenic amines in nectar samples by high performance liquid  
128 chromatography coupled with Diode Array Detector (HPLC-DAD), A Perkin Elmer series 200  
129 chromatographic system equipped with DAD detector and auto-sampler was used for the  
130 determination. Detection and quantification were based on UV absorption at 230 nm. The  
131 bandwidth has been set to 6 nm. The injection volume was 50  $\mu\text{L}$ , and column temperature was set  
132 at 25°C. The flow rate was 1.0 mL/min. A binary gradient system was used. The eluent (A)  
133 consisted of 0.02 M potassium phosphate buffer ( $\text{KH}_2\text{PO}_4$ ) adjusted at pH 2.5 with ortho  
134 phosphoric acid, the eluent (B) was methanol. The composition of the mobile phase was changed  
135 according to the following time program: 0-10 min 97% (A) and 3% (B); 10-14 min 80% (A) and  
136 20% (B); 22-23 min 97% (A) and 3% (B); end run at 30 min. We calculated the concentration of  
137 each individual analyte by calibration curve obtained with external standard. Analyte identification  
138 was achieved by comparison with the UV spectrum of the pure standards of 8 biogenic amines:

139 dopamine (Dop), octopamine (Oct), serotonin (Ser), tyramine (Tyr), tryptamine (Tryp), epinephrine  
140 (Epi), norepinephrine (Nor), histamine (His). The retention time (RT) and the limit of detection  
141 (LOD) were reported in Supplementary Material (Table S1). LOD for each amine was calculated by  
142 adding 3 times the standard deviation to the mean of 10 blank samples.

143 All used standards (purity  $\geq$  98%) and solvents were purchased by Sigma-Aldrich.

144

## 145 **2.2 Effect of biogenic amines on bumblebee duration of visit**

146 The behaviour of visit on flowers of bumblebee workers was analysed in a greenhouse using  
147 queenless micro-colonies and *Cucurbita pepo* L. flowers previously emptied of their natural nectar  
148 and refilled with artificial solutions mimicking the biogenic amine composition of the floral nectar  
149 of *Echium vulgare* sampled in the field. Since flowers of *C. pepo* are large and nectar is easily  
150 accessible, they are particularly suitable for nectar experiments (Nepi et al. 2011).

151

### 152 **2.2.1 Plants and bumblebees**

153 Plants of *Cucurbita pepo* L. cv. Genovese were grown at CREA-AA in Bologna during April-July  
154 2021. They were first planted in a seedbed and kept indoor at  $20 \pm 2$  °C, then transplanted outdoor  
155 in a greenhouse where they were watered daily in the evening. Six plants were transplanted in each  
156 compartment. During the experiment, we kept an equal number of open flowers in each  
157 compartment cutting off potential supernumerary flowers, and male flowers were emasculated to  
158 avoid pollen contamination of the artificial nectar replaced. Some extra plants were kept in mobile  
159 pots in order to move them daily to one compartment or the other according to the need. Bees of the  
160 species *Bombus terrestris* (Linnaeus, 1758) were obtained from a commercial colony (Bioplanet  
161 S.r.l., Cesena, Italy). We set up two queenless micro-colonies capturing 30 workers (15 worker  
162 each, marked with a different colour code to be individually recognizable) from the mother colony.  
163 Very small ( $< 0.10$  g) and very large ( $> 0.35$  g) individuals, and newly emerged and old bumblebees  
164 (visually discriminated on the basis of whitish colour and lack of hairs, respectively) were avoided

165 (Sgolastra et al. 2017). The micro-colonies were acclimatised at  $25 \pm 1^\circ\text{C}$  and  $40 \pm 5\%$  relative  
166 humidity (RH) in continuous darkness, fed *ad libitum* sucrose syrup (20% w/v) for three days  
167 before the experiment. After the acclimatisation, each micro-colony was relocated in the greenhouse  
168 the day before the beginning of the experiment (in two different compartments). Between  
169 observations, a feeder containing the same artificial nectar offered during observations was  
170 collocated nearby the entrance of both nest boxes.

171

### 172 **2.2.1 Artificial nectars**

173 Since tyramine resulted to be the only biogenic amine found in the floral nectar of *E. vulgare*, in the  
174 behavioural essay we tested this compound only. A 20% sucrose solution (w/v) was used as control  
175 (named C), whilst an identical solution containing tyramine (Sigma-Aldrich, Italy) in the mean  
176 concentration found in the natural population (0.29 mM) was used as experimental solution (named  
177 T). Prior to each observation, nectar was removed from flowers by means of glass disposable 20  $\mu\text{L}$   
178 microcapillary tubes and a strip of absorbent paper was introduced through the nectary pores to  
179 remove the remaining natural nectar. Nectar was then replaced by 60  $\mu\text{L}$ /flower of artificial solution  
180 using a micropipette. A strip of absorbent paper was introduced through the nectary pores to remove  
181 as much natural nectar as possible. After half an hour, 40  $\mu\text{L}$  of solution were added into the  
182 emptied flowers so that the bees never found unrewarding flowers.

183

### 184 **2.2.2 Behavioural observations**

185 Due to the very warm weather, zucchini flowers always closed early in the morning, so that we had  
186 to perform the experiment, every day for six days, between 7:00 and 9:00 am at the latest.  
187 During the observation periods, for every bumblebee that came out of the micro-colony to forage  
188 we recorded: the colour code of the bee, the number of flowers visited for each plant, the number of  
189 successive approaches to the nectar source on the same flower, the duration of movements between

190 two successive flower visits, the time spent feeding or walking and the total time of the visit. We  
191 included data only for those bees that fed on nectar during flower visit.

192

### 193 **2.3 Data analysis**

194 We performed a preliminary exploration of our dataset through a first principal component analysis  
195 (PCA) to assess possible difference in the behaviour of visit of bees fed Control (C) vs Tyramine  
196 (T) artificial solutions. The behavioural parameters considered were the number of flowers  
197 approached during each visit, the time spent feeding and walking during the entire visit, the total  
198 duration of visit and the number of consecutive approaches to nectar performed by a worker bee on  
199 the same flower. Data were scaled and centred around the mean, and analyses were performed using  
200 the function ‘*dudi.pca*’ in the R-packages *ade4* (Venables and Ripley 2002). Subsequently, we run a  
201 one-way PERMANOVA on the same parameters.

202 To conclude, we focused on the behaviour exhibited by bees on single flowers. To evaluate  
203 differences between treatments on each behavioural parameter, we fitted a generalized linear mixed-  
204 effect model (GLMM) with a Poisson error structure-log-link function. We set each behaviour  
205 (feeding, walking, total permanence on flower and no. of distinct approaches to nectar on a single  
206 flower) as response variable, whilst treatment as explanatory variable. We included as random factors:  
207 i) the bee ID, to account for individual autocorrelation and variability, and ii) the progressive number  
208 of the flower visited, as previous studies demonstrated a correlation between the handling time and  
209 the increasing number of flowers visited. The nature of such correlation may depend on various  
210 variables such as, for instance, the complexity of the flower, the reachability of the nectar, or the  
211 visitor’s degree of specialization (e.g. Harder 1983; Lavery 1994). All GLMMs were built through  
212 the *glmmPQL* function of the R package *nlme* (Pinheiro et al. 2022).

213 All data are presented as mean  $\pm$  SE and all statistics were performed using RStudio software  
214 (version 4.0.2) with the  $\alpha$ -error set at 0.05.

215

216 **3. Results**

217 **3.1 Biogenic amine composition of *E. vulgare* floral nectar**

218 In nectar samples we found only the biogenic amines tyramine, in a mean concentration of  $0.286 \pm$   
219  $0.034 \mu\text{mol/mL}$ , with no statistical difference between functionally male and functionally female  
220 flowers ( $t_{6.86} = -1.845$ ,  $p = 0.108$ ;  $0.238 \pm 0.047$  and  $0.347 \pm 0.035 \mu\text{mol/mL}$ , respectively).

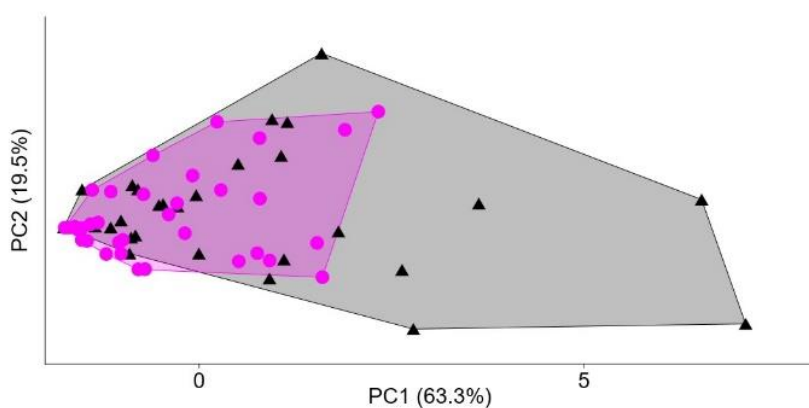
221

222 **3.2 Effect of biogenic amines on bumblebees' flower visit pattern**

223 A total of 70 visits on flowers were performed by 16 individual bees during the 6 days of running  
224 experiment (Table S2). Of these visits, 4 were excluded from the dataset because one of the  
225 behavioural categories appeared to exceed 70% of the time of visit.

226 The PCA on the parameters selected as descriptors of the pattern of visit showed a partial separation  
227 between control and tyramine solutions, with the first two components explaining 82.8% of the  
228 variance (Figure 1). The first component was positively correlated with the total time of visit and  
229 with the time spent feeding (PC1 loadings = 0.55 and 0.49, respectively). The second component  
230 was positively correlated with the number of consecutive approaches to nectar showed on the same  
231 flower and negatively correlated with the number of visited flowers (PC2 loadings = 0.84 and -0.50,  
232 respectively; Table S3).

233



234

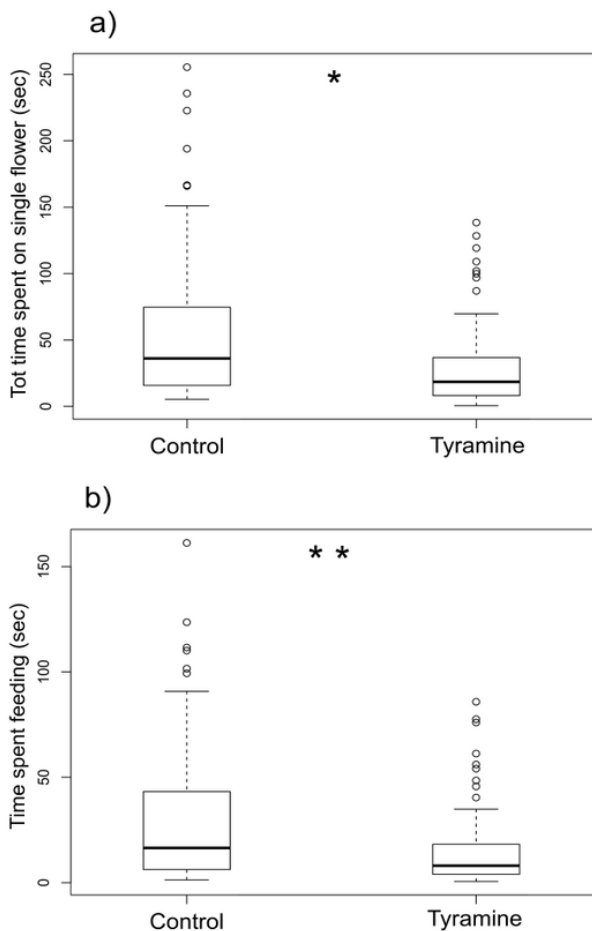
235 **Fig. 1** Principal components analysis (PCA) on the behavioural parameters describing visits  
236 performed by free-flying bumblebees. Black triangles represent visits recorded for the treatment C,  
237 whilst fuchsia circles represent visits recorded for the treatment T.

238

239 The one-way PERMANOVA showed that the behaviour of visit significantly differed between bees  
240 visiting flowers containing C artificial nectar and those visiting flowers containing T artificial  
241 nectar ( $F_{1,64} = 5.756$ ,  $p = 0.013$ ).

242 When considering the single behavioural parameters, the total time spent on a single flower by  
243 bumblebee workers resulted higher in bees fed C than in those fed T ( $t_{14} = -2.308$ ,  $p = 0.036$ , Figure  
244 2a), as did the total time spent feeding ( $t_{14} = -3.456$ ,  $p = 0.004$ , Figure 2b). All the other behavioural  
245 parameters did not show any significant difference (Table S4).

246



247

248 **Fig. 2** Total time spent on a single flower (a) and time spent feeding on a single flower (b) out of  
249 the 161 flower approaches recorded. Asterisks indicates a significant difference (\* =  $p < 0.05$  and  
250 \*\* =  $p < 0.01$ ) between treatments according to Generalized Linear Mixed Model where Control  
251 treatment C was set as intercept.

252

#### 253 **4. Discussion**

254 Our finding of the biogenic amine tyramine in the floral nectar of *Echium vulgare* represents the  
255 first report of this compound in the plant order Boraginales, in concentrations which appear dozens  
256 of times greater than those reported in the only study published to date highlighting the presence of  
257 this class of compounds in floral nectar (Muth et al. 2022). Despite *E. vulgare* presents a gender-  
258 biased chemistry of its floral nectar ( Barberis et al. 2021), no difference between the functionally  
259 male and female flowers were found in the current study for what concerns the concentration of  
260 tyramine. However, the lack of difference may be also imputable to the small sample size.

261 Given that our current knowledge on the occurrence and distribution of biogenic amines in floral  
262 nectar is still extremely limited, we consider this finding as a nonetheless valuable data for a class  
263 of nectar compounds which represents a breaking-through finding for the field.

264 Since tyramine is synthesized from the amino acid tyrosine through the action of the enzyme  
265 tyrosine decarboxylase and then converted into octopamine by the enzyme tyramine b-hydroxylase,  
266 for years it has been considered as the simple precursor of the better-known octopamine. For this  
267 reason, its influence on insect behaviour remains, nowadays, largely unstudied, though in recent  
268 years tyramine has been proved to function as an independent neurotransmitter (Kutsukake et al.  
269 2000; Nagaya et al. 2002; Roeder 2004; Alkema et al. 2005; Fussnecker et al. 2006; Lange 2009).

270 In this sense, the current work provides preliminary results on the effects of nectar-like  
271 concentrations of tyramine on bumblebee behaviour, reporting that bees fed tyramine-enriched  
272 solution spent significantly less time foraging on a single flower compared to those fed control. This  
273 aspect needs further investigations, since tyramine may act in different ways, for example affecting

274 palatability (acting as deterrent), or instead imparting the sensation of satiety, by interfering with the  
275 nervous system functioning. However, the presence of tyramine in nectar seems to enhance bee  
276 dynamic behaviour, as they appear more prone to leave the flower sooner and reach out for the next.  
277 This, seen from a plant's perspective, may encourage pollen transfer and thus potentially promote  
278 cross-pollination. In addition, less time spent in foraging should result in lower volumes of nectar  
279 consumed, reducing nectar depletion and increasing the number of possible insect visits.

280 Tyramine and octopamine represent the invertebrate counterparts of the vertebrate adrenergic  
281 transmitters (Roeder 2005), thus ruling the so-called fight or flight response, which is to say the  
282 quick adaptation to energy-demanding situations (Roeder 2005). They have physiological roles  
283 similar to adrenaline and noradrenaline, with whom they share a similar chemical structure,  
284 suggesting an early evolutionary origin of the adrenergic/octopaminergic/tyraminergetic system,  
285 which points to an ancient origin of complex behavioural traits (Roeder 2005).

286 This, contrarily to our finding, suggested how tyramine is expected to reduce the overall bee  
287 dynamism. However, tyramine works by binding to G protein-coupled receptors (Roeder 2005),  
288 whose activation leads to a plethora of possible metabolic responses involving enzymatic activity,  
289 intracellular signalling, and gene expression (Roeder 2005, Mustard 2020).

290 Finally, it is well known that the nectar of *Echium vulgare* contains pyrrolizidine alkaloids  
291 (Lucchetti et al. 2016), whose toxicity has been assessed (Hartmann and Witte 1995; Boppré 2011),  
292 contrarily to their potential role in shaping animal behavior through neuroactive action. Muth et al.  
293 (2022) demonstrated that nectar biogenic amines can modulate a bee's perception of other  
294 compounds, as caffeine. They found that biogenic amines neutralized the effects of caffeine in  
295 enhancing sucrose responsiveness and that, apparently, erased the aversion of bees towards the  
296 presence of caffeine. Moreover, the combination of the biogenic amines with caffeine decreased the  
297 time of visit on a single flower compared to that recorded for bees feeding on the biogenic amines  
298 only. Therefore, we can't exclude that the presence and maintenance of biogenic amines in the

299 floral nectar of *Echium vulgare* may have been driven by its regulation of the tolerance threshold of  
300 nectar feeding insects for pyrrolizidine alkaloids.

301

#### 302 **4.1. Knowledge gaps, conclusive remarks and future research**

303 To date, most of our knowledge on the effects of nectar biogenic amines on floral visitors involves  
304 studies where these compounds have been tested alone and at much higher concentrations than  
305 those found in natural nectar (Barberis et al. 2023 and reference therein). Given that different  
306 compounds may exert different actions when coupled together, and can show diverse, dose-  
307 dependent effects, this lack of investigations leads to the evidence that future studies should address  
308 these aspects. Moreover, a growing number of studies has been demonstrating how microorganisms  
309 occurring in flowers can impart significant modifications in the chemistry of floral nectar (e.g. Bogo  
310 et al. 2021). As some authors have suggested that nectar biogenic amines may be by-products of the  
311 activity of nectar microbes rather than being plant exudates (Nepi 2017, Nepi et al. 2018, Barberis  
312 et al. 2023), the assessment of their origin would be of great interest for the advancement of the  
313 field. If the potential aminogenic activity of nectar microbes resulted beneficial for the plant  
314 reproductive success, then we could even hypothesize that plants do not simply passively undergo  
315 microbial-induced changes of their nectar, but rather modulate or even facilitate the settlement of  
316 specific microorganisms whose by-produced metabolites exert positive effects on pollinator visits.  
317 Last, we want to highlight how little scientific evidence has so far proved the direct influence  
318 exerted by specific nectar secondary metabolites on the plant reproductive outputs (e.g. Kessler et  
319 al. 2012), as predicted by the “pollinator manipulation” hypothesis (Rhoades and Bergdahl 1981;  
320 Pyke 2016). In this regard, the implementation of experimental designs making use of real flowers  
321 instead of artificial feeders may allow future studies to explore further this aspect, for instance by  
322 examining difference between the ratio of self- and cross-obtained progeny in the presence and  
323 absence of specific nectar secondary metabolites.

324

325

## 326 **Declarations**

- 327 - All authors declare that they have no conflict of interest.
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- 331 - All data generated or analysed during this study are included in this published article [and its  
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333

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469 **Fig. 1** Principal components analysis (PCA) on the behavioural parameters describing visits  
470 performed by free-flying bumblebees. Black triangles represent visits recorded for the treatment C,  
471 whilst fuchsia circles represent visits recorded for the treatment T.

472

473 **Fig. 2** Total time spent on a single flower (a) and time spent foraging on a single flower (b) out of  
474 the 161 flower approaches recorded. The asterisk indicates a significant difference between  
475 treatments according to Generalized Linear Mixed Model where Control treatment C was set as  
476 intercept.