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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 Ca²⁺. 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 μ L and 0.7 μ 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 μ 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 μ L;
124 Drummond Scientific Co., Broomall, PA), then we transferred the samples in Eppendorf tubes filled
125 with 100 μ 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 μ 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 (KH_2PO_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 ± 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 assay 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 μL
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 μL /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 μL 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; Laverly 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 α -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 ± 0.047 and $0.347 \pm 0.035 \mu\text{mol/mL}$, respectively).

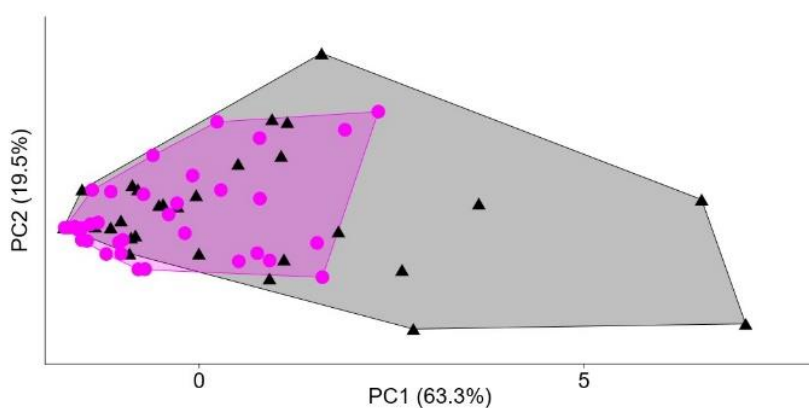
221

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

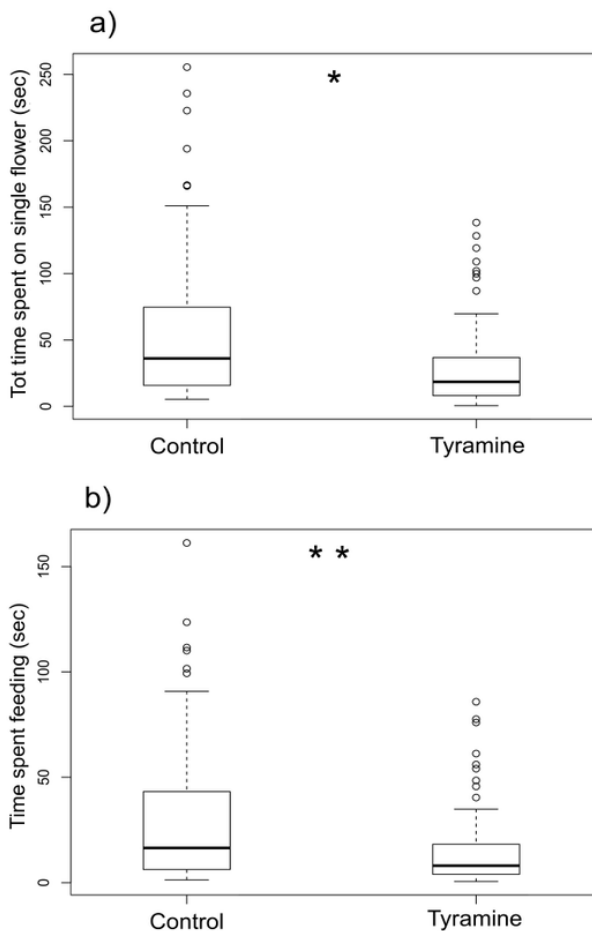
235 **Fig. 1** Principal components analysis (PCA) on the behavioural parameters describing visits
236 performed by free-flying bumblebees. Black triangles represent visits recorded for the treatment C,
237 whilst fuchsia circles represent visits recorded for the treatment T.

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.
- 328 - This work was partly supported by the project BeeNet (Italian National Fund under FEASR
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- 331 - All data generated or analysed during this study are included in this published article [and its
332 supplementary information files]

333

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338

339 **References**

- 340 Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR (2005) Tyramine functions independently of
341 octopamine in the *Caenorhabditis elegans* nervous system. *Neurons* 46:247-260.
342 <https://doi.org/10.1016/j.neuron.2005.02.024>
- 343
- 344 Baker HG, Baker I (1977) Intraspecific constancy of floral nectar amino acid complements. *Bot*
345 *Gaz* 138:183-191. <https://doi.org/10.1086/336914>
- 346 Baker HG, Baker I (1986) The occurrence and significance of amino acids in floral nectars. *Plant*
347 *Syst Evol* 151:175-186. <https://doi.org/10.1007/BF02430273>
- 348 Barberis M, Bogo G, Bortolotti L, Conte L, Alessandrini M, Nepi M, Galloni M (2021) Gender-
349 biased nectar targets different behavioural traits of flower visitors. *Plant Ecol* 222:233-246.
350 <https://doi.org/10.1007/s11258-020-01101-5>

351 Barberis M, Calabrese D, Galloni M, Nepi M (2023) Secondary metabolites in nectar-mediated
352 plant-pollinator relationships. *Plants* 12(3):550. <https://doi.org/10.3390/plants12030550>

353 Barlow SE, Wright GA, Ma C, Barberis M, Farrell IW, Marr EC, Brankin A, Pavlik BM, Stevenson
354 PC (2017) Distasteful nectar deters floral robbery. *Curr Biol* 27:2552-2558.
355 <https://doi.org/10.1016/j.cub.2017.07.012>

356 Barron AB, Maleszka R, Vander Meer RK, Robinson GE (2007) Octopamine modulates honey bee
357 dance behavior. *PNAS* 104(5):1703-1707. <https://doi.org/10.1073/pnas.0610506104>.

358 Blenau W, Baumann A (2001) Molecular and pharmacological properties of insect biogenic amine
359 receptors: lessons from *Drosophila melanogaster* and *Apis mellifera*. *Arch Insect Biochem*
360 *Physiol* 48:13-38. <https://doi.org/10.1002/arch.1055>

361 Bogo G, Bortolotti L, Sagona S, Felicioli A, Galloni M, Barberis M, Nepi, M (2019) Effects of non
362 protein amino acids in nectar on bee survival and behavior. *J Chem Ecol* 45:278-285.
363 <https://doi.org/10.1007/s10886-018-01044-2>

364 Bogo G, Fisogni A, Rabassa-Juvanteny J, Bortolotti L, Nepi M, Guarnieri M, Conte L, Galloni M,
365 (2021) Nectar chemistry is not only a plant's affair: floral visitors affect nectar sugar and
366 amino acid composition. *Oikos* 103:1180-1192. <https://doi.org/10.1111/oik.08176>

367 Boppré M (2011) The ecological context of pyrrolizidine alkaloids in food, feed and forage: an
368 Overview. *Food Addit Contam Part A* 28 (3):260-281.
369 <https://doi.org/10.1080/19440049.2011.555085>

370 Carlson J, Harms KE (2006) The evolution of gender-biased nectar production in hermaphrodite
371 plants. *Bot Rev* 72:179-205. [https://doi.org/10.1663/0006-](https://doi.org/10.1663/0006-8101(2006)72[179:TEOGNP]2.0.CO;2)
372 [8101\(2006\)72\[179:TEOGNP\]2.0.CO;2](https://doi.org/10.1663/0006-8101(2006)72[179:TEOGNP]2.0.CO;2)

373 Corbet SA (1978) Bee visits and the nectar of *Echium vulgare* L. and *Sinapis alba* L. *Ecol Entomol*
374 3:25-37. <https://doi.org/10.1111/j.1365-2311.1978.tb00900.x>

375 Cresswell JE (1999) The influence of nectar and pollen availability on pollen transfer by individual
376 flowers of oil-seed rape (*Brassica napus*) when pollinated by bumblebees (*Bombus*
377 *lapidarius*). J Ecol 87:670-677. <https://doi.org/10.1046/j.1365-2745.1999.00385.x>

378 Felicioli A, Sagona S, Galloni M, Bortolotti L, Bogo G, Guarnieri M, Nepi M (2018) Effects of
379 nonprotein amino acids on survival and locomotion of *Osmia bicornis*. Insect Mol Biol
380 27(5):556-563. <https://doi.org/10.1111/imb.12496>

381 Fisogni A, Cristofolini G, Rossi M, Galloni M (2011) Pollinator directionality as a response to
382 nectar gradient: promoting outcrossing while avoiding geitonogamy. Plant Biol 13:848-856
383 <https://doi.org/10.1111/j.1438-8677.2011.00453.x>

384 Fox LE, Soll DR, Wu CF (2006) Coordination and modulation of locomotion pattern generators in
385 *Drosophila* larvae: effects of altered biogenic amine levels by the tyramine beta hydroxlyase
386 mutation. J Neurosci 26:1486-1498. <https://doi.org/10.1523/JNEUROSCI.4749-05.2006>

387 Fussnecker BL, Smith BH, Mustard JA (2006) Octopamine and tyramine influence the behavioral
388 profile of locomotor activity in the honey bee (*Apis mellifera*). J Insect Physiol 52:1083-1092.
389 <https://doi.org/10.1016/j.jimsphys.2006.07.008>

390 Galen C, Plowright RC (1984) The effects of nectar level and flower development on pollen carry-
391 over in inflorescences of fireweed (*Epilobium angustifolium*) (Onagraceae). Canadian J Bot
392 63:488-481. <https://doi.org/10.1139/b85-060>

393 Hammer M, Menzel R (1998) Multiple sites of associative odor learning as revealed by local brain
394 microinjections of octopamine in honeybees. Learn Mem 5:146–156.
395 <https://doi.org/10.1101/lm.5.1.146>

396 Harder LD (1983) Flower handling efficiency of bumble bees: morphological aspects of probing
397 time. Oecologia 57:274-280. <https://doi.org/10.1007/BF00379591>

398 Hardie SL, Zhang JX, Hirsh J (2007) Trace amines differentially regulate adult locomotor activity,
399 cocaine sensitivity, and female fertility in *Drosophila melanogaster*. Dev Neurobiol
400 67(10):1396-1405. <https://doi.org/10.1002/dneu.20459>

401 Hartmann T, Witte L (1995) Chemistry, biology and chemoecology of the pyrrolizidine alkaloids.
402 In: Pelletier SW (ed) Alkaloids: Chemical and Biological Perspectives. Pergamon Press,
403 Oxford, United Kingdom, Vol. 9, pp 155-233.

404 Kessler D, Bhattacharya S, Diezel C, Rothe E, Gase K, Schöttner M, Baldwin IT (2012)
405 Unpredictability of nectar nicotine promotes outcrossing by hummingbirds in *Nicotiana*
406 *attenuata*. Plant J 71(4):529-538. <https://doi.org/10.1111/j.1365-313X.2012.05008.x>

407 Kutsukake M, Komatsu A, Yamamoto D, Ishiwa-Chigusa S (2000) A tyramine receptor gene
408 mutation causes a defective olfactory behavior in *Drosophila melanogaster*. Gene 245:31-42.

409 Lange AB (2009) Tyramine: from octopamine precursor to neuroactive chemical in insects. Gen
410 Comp Endocrinol 162(1):18-26. <https://doi.org/10.1016/j.ygcen.2008.05.021>

411 Lavery TM (1994) Bumble bee learning and flower morphology. Anim Behav 47:531-545.
412 <https://doi.org/10.1006/anbe.1994.1077>

413 Lucchetti MA, Glauser G, Kilchenmann V, Dübecke A, Beckh G, Praz C, Kast C (2016)
414 Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral nectar. J
415 Agric Food Chem 64:5267-5273. <https://doi.org/10.1021/acs.jafc.6b02320>

416 Melser C, Rademaker MCJ, Klinkhamer PGL (1997) Selection on pollen donors by *Echium vulgare*
417 (Boraginaceae). Sex Plant Reprod 10:305-312. <https://doi.org/10.1007/s004970050103>.

418 Mercer AR, Menzel R (1982) The effect of biogenic amines on conditioned and unconditioned
419 responses to olfactory stimuli in the honeybee *Apis mellifera*. J Comp Physiol A 145:363-368.
420 <https://doi.org/10.1007/BF00619340>

421 Mustard JA (2020) Neuroactive nectar: compounds in nectar that interact with neurons. Arthropod
422 Plant Interact 14:151-159. <https://doi.org/10.1007/s11829-020-09743-y>

423 Muth F, Philbin CS, Jeffrey CS, Leonard AS (2022) Discovery of octopamine and tyramine in
424 nectar and their effects on bumblebee behavior. Iscience 25(8):104765.
425 <https://doi.org/10.1016/j.isci.2022.104765>

426 Nagaya Y, Kutsukake M, Chigusa SI, Komatsu A (2002) A trace amine, tyramine, functions as a
427 neuromodulator in *Drosophila melanogaster*. *Neurosci Lett* 329:324-328.
428 [https://doi.org/10.1016/s0304-3940\(02\)00596-7](https://doi.org/10.1016/s0304-3940(02)00596-7)

429 Nepi M, Cresti L, Guarnieri M, Pacini E (2011) Dynamics of nectar production and nectar
430 homeostasis in male flowers of *Cucurbita pepo* L. *Int J Plant Sci* 172(2):183-190.
431 <https://doi.org/10.1086/657648>

432 Nepi M (2017) New perspectives in nectar evolution and ecology: simple alimentary reward or a
433 complex multiorganism interaction? *Acta Agrobot* 70(1):1704.
434 <https://doi.org/10.5586/aa.1704>.

435 Nepi M, Grasso DA, Mancuso S (2018) Nectar in plant–insect mutualistic relationships: from food
436 reward to partner manipulation. *Front Plant Sci* 9:1063.
437 <https://doi.org/10.3389/fpls.2018.01063>.

438 Peng T, Schroeder M, Grüter C (2020) Octopamine increases individual and collective foraging in a
439 neotropical stingless bee. *Biol Lett* 16:20200238. <https://dx.doi.org/10.1098/rsbl.2020.0238>

440 Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team (2020) Linear and Non-Linear Mixed
441 Effects Models. <https://CRAN.R-project.org/package=nlme>

442 Pleasants JM (1981) Bumblebee response to variation in nectar availability. *Ecology* 62:1648-1661.
443 <https://doi.org/10.2307/1941519>

444 Pyke GH (2016) Floral nectar: pollinator attraction or manipulation? *Trends Ecol Evol* 31(5):339-
445 341. <https://doi.org/10.1016/j.tree.2016.02.013>

446 Real RA, Rathcke BJ (1991) Individual variation in nectar production and its effects on fitness in
447 *Kalmia latifolia*. *Ecology* 72:149-155. <https://doi.org/10.2307/1938910>

448 Rhoades DF, Bergdahl JC (1981) Adaptive significance of toxic nectar. *Am Nat.* 117:798-803.

449 Roeder T (2005) Tyramine and octopamine: ruling behavior and metabolism. *Annu Rev Entomol*
450 50:447-77. <https://doi.org/10.1146/annurev.ento.50.071803.130404>

451 Saraswati S, Fox LE, Soll DR, Wu CF (2004) Tyramine and octopamine have opposite effects on
452 the locomotion of *Drosophila* larvae. J Neurobiol 58(4):425-441.
453 <https://doi.org/10.1002/neu.10298>

454 Schulz DJ, Robinson GE (2001) Octopamine influences division of labor in honey bee colonies. J
455 Comp Physiol A 187:53-61. <https://doi.org/10.1007/s003590000177>

456 Sgolastra F, Medrzycki P, Bortolotti L, Renzi MT, Tosi S, Bogo G, Teper D, Porrini C, Molowny-
457 Horas R, Bosch J (2017) Synergistic mortality between a neonicotinoid insecticide and an
458 ergosterol biosynthesis-inhibiting fungicide in three bee species. Pest Manag Sci 73:1236–
459 1243. <https://doi.org/10.1002/ps.4449>

460 Shykoff JA, Bucheli E (1995) Pollinator visitation patterns, floral rewards and the probability of
461 transmission of *Microbotryum violaceum*, a venereal disease plant. J Ecol 83:189-198.
462 <https://doi.org/10.2307/2261557>

463 Thomson JD, Draguleasa MA, Guorui Tan M (2015) Flowers with caffeinated nectar receive more
464 pollination. Arthropod-Plant Interact 9:1-7. <https://doi.org/10.1007/s11829-014-9350-z>

465 Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th edn. Springer.

466 Wright GA, Baker DD, Palmer MJ, Stabler D, Mustard A, Power EF, Borland AM, Stevenson PC
467 (2013) Caffeine in floral nectar enhances a pollinator's memory of reward. Science 339:1202-
468 1204. <https://doi.org/10.1126/science.1228806>

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.