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Gender-biased nectar targets different behavioural traits of flower visitors

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1 **Title: Gender-biased nectar targets different behavioural traits of flower visitors**

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20 **Author contributions**

21 MB and GB share the first authorship. MG, MN, LC and LB conceived and designed the

22 experiments. GB, MA and MG performed the experiments. HPLC analyses were executed by

23 MN. MB and GB analysed data and wrote the paper. All authors read, provided editorial

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Abstract

Floral nectar is a chemically complex aqueous solution within which several secondary metabolites have been identified and that affect attractiveness for pollinators. Understanding preferences and aversions to nectar quality in flower visitors is crucial since this may influence the patterns of insect floral visitation with consequences on the plant fitness. We hypothesise that nectar chemical variation through different floral sexual phases may affect the number of insect visits that each phase receives. The study was realized on a population of *Echium vulgare* L. growing in a natural area close to Bologna. Nectar was collected from functionally male and female flowers to investigate its chemical composition through the HPLC technique. A total of 200 mins of behavioural observations on foraging insects were also carried out. Variation in nectar traits has been detected for the amino acid spectrum. The proportion of protein amino acids appeared to be significantly higher in male-phase flowers. This may explain the significantly higher number of visits on male flowers than expected observed for all bee taxa (except *Hoplitis adunca* females). Functionally male flowers presented higher concentrations of phenylalanine, whilst proline was highly represented in

2

51 functionally female flowers. Since a recent study demonstrated that hymenopterans can

52 oxidize proline at a high rate for ATP production, we can hypothesise that the quality of

53 nectar offered by the two sexually distinct floral phases targets different insect behavioural

54 traits and likely ensures an optimal pattern of visit among flower sexes, which are unequally

55 distributed within and among individuals in the population.

56

57 **Keywords**: *Echium vulgare*, flower visitors, inbreeding avoidance, nectar chemistry, plant-

58 pollinator interactions

59

3

Introduction

Floral nectar is a chemically complex aqueous solution in which the main components comprise sugars, followed by amino acids (Nicolson and Thornburg 2007). In recent decades considerable progress has been made in providing evidence that points to the involvement of nectar chemistry in the interactions between plants and a variety of organisms (Nepi 2014; Stevenson et al. 2017). Although there is wide variability in nectar traits (Pacini et al. 2003; Nocentini et al. 2013; Irwin et al. 2014), a general paradigm shared by plants is balancing nectar chemical composition in order to not deter specific pollinators exceeding their tolerance thresholds (Baker and Baker 1975; Adler 2000; Nicolson and Thornburg 2007; Wright et al. 2013; Stevenson et al. 2017). For example, a small increase in nectar sugar concentration can increase its viscosity (Harder 1986; Nicolson and Thornburg 2007), which is strongly related to the energy required by nectar consumers to visit flowers (Corbet 1978; Josens and Farina 2001; Borrell and Krenn 2006; Nepi and Stpiczyńska 2006; Kim et al. 2011).

After sugars the most abundant nectar solutes are the amino acids (Baker and Baker 1982; Nepi et al. 2012; Bogo et al. 2019). A study conducted by Inouye and Waller (1984) showed a general decline in nectar consumption in honeybees as amino acid concentrations increased, despite evidence supporting the preference for amino acid enriched sugar solutions in insects (Alm et al. 1990; Bertazzini et al. 2010; Bogo et al. 2019). Amino acids also contribute to the taste of nectar, stimulating specific insects' labellar chemoreceptors (Gardener and Gillman 2002). Among protein amino acids, Inouye and Waller (1984) found that phenylalanine and leucine were phagostimulant for honeybees at all concentrations tested, even at those that in the case of other amino acids resulted in deterrence. In the same way, a preference in honeybees for proline enriched artificial nectar was reported (Carter et al. 2006; Bertazzini et

84   al. 2010), as well as a strong phagostimulatory activity (Nicolson and Thornburg 2007;

85   Petanidou 2007).

86   Beside primary metabolites (such as sugars and amino acids) an array of secondary

87   metabolites with different chemical natures have been identified in nectar and all of them

88   positively or negatively affect attractiveness to pollinators, showing effects which depend on

89   metabolite concentration and pollinators' sensitivity (Baker and Baker 1977; Faegri and van

90   der Pijl 1979; Baker and Baker 1982; Adler 2000; Stevenson et al. 2017). Among them non-

91   protein amino acids (NPAAs) have been detected in nectar (Nicolson and Thornburg 2007;

92   Petanidou 2007; Nepi et al. 2012). Despite that they can constitute a large portion of the

93   amino acidic content of floral nectar, little is known about their role in determining

94   pollinators' preferences and feeding behaviour. For some of those, such as γ-aminobutyric

95   acid, a phagostimulant function has been reported in some caterpillars and adult beetles

96   (Mitchell and Harrison 1984; Shoonhoven et al. 2005), whilst Bogo et al. (2019) found that

97   both bumblebees and honeybees showed higher consumption of sucrose solution enriched

98   with ß-alanine, but exhibited the effect at different concentrations.

99   Understanding preferences and aversions to nectar traits is crucial since they likely influence

100   the patterns of floral visitation by nectar consumers and thus the plant inbreeding and

101   outbreeding rate within a population. Minimal inbreeding is predicted when pollinators visit a

102   small fraction of the open flowers on a plant (Iwasa et al. 1995; Ohashi and Yahara 2001):

103   this behaviour may be enhanced by within-plant variation in nectar, as occurs in plants

104   showing gender-biased nectar production (Feinsinger 1978; Pike 1978; Rathcke 1992).

105   Despite many studies having already addressed the subject of gender-biased nectar

106   composition, most of them investigated the existence of bias in relation to nectar volume or

107   sugar content only (Langenberger and Davis 2002; Canto et al. 2011; Fisogni et al. 2011;

108   Stpiczyńska et al. 2015; Antoń et al. 2017; Jacquemart et al. 2019; Konarska and

109 Masierowska 2020) and few reported the observation of insect visit bias (Carlson and Harms

110 2006 and references therein).

111 In this study we focused on the many-flowered hermaphrodite species *Echium vulgare* L., a

112 self-compatible plant which shows both herkogamy and incomplete protandry, that avoids

113 self-pollination within the same flower, but within which geitonogamy can still occur

114 (Rademaker et al. 1999). Melser et al. (1999) reported evidences of inbreeding depression in

115 *E. vulgare*, finding a significant decline in siring success when selfing occurs. A study on

116 geitonogamy conducted by Rademaker et al. (1999), though, found a consistently lower

117 percentage of selfing rate than expected. Also, they reported that bumblebees visited only a

118 small fraction of the flowers on *E. vulgare* as a result of the presence of different flower

119 stages simultaneously occurring on a single individual plant.

120 *E. vulgare* represents an important food resource for many insect visitors, despite containing

121 toxic pyrrolizidine alkaloids in both nectar and pollen (Lucchetti 2017). The pollen contains

122 high concentrations of pyrrolizidines, whilst more than 500 times lower concentrations are

123 found in nectar (Lucchetti et al. 2016). For this reason, only a few taxa show oligolecty or

124 floral constancy on *E. vulgare* by actively collecting pollen for larval nourishment (Cane and

125 Sipes 2006; Burger et al. 2010; Filella et al. 2011), even if its flowers are visited by a wide

126 spectrum of insect taxa among which bumblebees have often been reported as main

127 pollinators (Corbet 1978; Klinkhamer and de Jong 1990; Pappers et al. 1999; Rademaker et

128 al. 1999).

129 Here, we examined if floral visitation pattern may be influenced by variations in the chemical

130 composition of nectar through different floral stages, and thus we investigated (i) whether *E.*

131 *vulgare* produces a gender-biased nectar for volume, sugar and amino acid composition and

132 (ii) if flower visitation rates of insects looking for nectar varied among different floral stages.

133

Material and Methods

Study site

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

Study species

Echium vulgare L. is a perennial hemicryptophyte belonging to the family Boraginaceae. It is distributed in Europe, Asia and North America and it shows a long flowering period, ranging between June and October. Flower anthesis lasts 3-4 days and flowers show an incomplete protandry (Melser et al. 1997): the anthers are often dehiscent already at the bud stage, while the stigma becomes receptive only hours after the flower opening.

In this study we considered three phases of floral development: closed flower (Bud), functionally male (M) and functionally female (F) flowers. The male phase was represented by an open flower presenting pollen with non receptive stigma, whilst the female phase was recognised as soon as the stigma became bifid and receptive.

Plant phenology

On the first day of the study we counted all plants and inflorescences per plant constituting the population (approximately 600 m^2 of extension) and we observed all open flowers to assess whether the phenomenon of gynodioecy, firstly described in *E. vulgare* populations by Darwin (1877), occurred in our study population. Each day, prior to visitor observations, on the same patch we recorded the number of flowers per developmental stage. Two fixed

159 patches were alternatively considered: the first one was a single plant carrying 6

160 inflorescences while the second one was made up of 6 plants carrying one or two

161 inflorescences each.

162

163 **Nectar quality**

164 *Sampling*

165 We collected nectar samples by means of Drummond Microcaps (3-5 μL; Drummond

166 Scientific Co., Broomall, PA), we transferred samples to Eppendorf tubes filled with 100 μL

167 of pure ethanol, and then we took them to the laboratory in thermal bags where they were kept

168 at 5°C until analyses. We collected each sample from multiple flowers at the same floral stage

169 in order to reach a minimum volume of 2 μL needed for the sugar and amino acid analyses. In

170 order to let the nectar accumulate, flowers were bagged in the morning for 2 hours prior to

171 sampling; all nectar present in the selected flowers was collected.

172 We collected a total of 8 nectar samples each one from 3-13 male flowers belonging to 1-7

173 plants, and a total of 8 samples from 2-9 female flowers belonging to 1-3 plants. Both sugar

174 and amino acid compositions were investigated on these samples. We then collected 14

175 additional samples from 1-22 buds belonging to 1-10 plants. Since the amount of nectar

176 presents in the buds was very low, the minimum volume of 2 μL needed for amino acid

177 analysis could not be reached and thus these samples were tested for sugar composition only.

178

179 *Sugar analysis*

180 Sugar content was analysed by HPLC technique through a Waters LC1 with refractive index

181 detector (Waters 2410) connected to the output of a REZEX RCM Monosaccharide column

182 (Phenomenex, 300 mmx7.8 mm, grain 8 μm) maintained at 85°C. Water (MilliQ, pH 7) was

183   used as mobile phase at a flow rate of 0.6 mL min^{-1}; 20 µL of sample and standard solutions

184   of sucrose, glucose and fructose were also injected (Nocentini et al. 2012).

185

186   *Amino acids analysis*

187   Amino acid analysis was performed by gradient HPLC with an ion exchange Novapack C18

188   (15 mm x 4.6 mm) cartridge with guard column maintained at 37°C and a Waters 470

189   scanning fluorescence detector (excitation at 295 nm, detection at 350 nm). A solvent

190   composed of TEA-phosphate buffer (pH 5.0) mixed with a 6:4 acetonitrile-water solution was

191   used as mobile phase at a flow rate of 1.0 mL min^{-1}. According to AccQtag protocol (Waters

192   Corp.), the selected volume of each reconstituted sample was amino acid derivatized (Cohen

193   and Micheaud 1993) with AQC fluorescent reagent and 0.02 M borate buffer (pH 8.6). In

194   addition to all the protein amino acids, standard solutions of β-alanine, citrulline, L-

195   homoserine, α-aminobutyric acid (AABA), γ-aminobutyric acid (GABA), hydroxyproline,

196   ornithine and taurine were also used (Nocentini et al. 2012).

197

198   **Flower visitors' observations**

199   We carried out observations on flower visitors on the two fixed patches described previously,

200   on 7 non-sequential days. Every survey consisted of two 15-mins periods separated by 10

201   mins of rest, adapting the protocol of Fisogni et al. (2016). Every day we performed 1 to 3

202   surveys, between 10:30 am and 3:00 pm and under favourable weather conditions, for a total

203   of 200 mins of observation. Once a visitor left the patch, we counted the following

204   approaching insect belonging to the same taxon as a different individual. Recorded data

205   concerned the food resource collected (nectar or pollen, observing if the insect inserted its

206   mouth-parts deeply inside the corolla or if it manipulated the anthers) and the number of male

207 and female flowers approached per visit. We also recorded the visitor's taxon, indicating the

208 taxonomic level in as much detailed as possible, and its sex.

209 After each observation period, we performed a 15-mins period of net sampling throughout the

210 area, collecting insects that alighted on flowers of *E. vulgare*. Captured individuals were put

211 in separate vials with ethyl acetate and brought to the laboratory where they were pinned in

212 entomological boxes and inspected under a dissecting microscope for taxonomic

213 identification.

214

215 **Data analysis**

216 Sugar and amino acid quantities and the mean nectar volume were calculated per single

217 flower. Total sugar concentration was calculated as the sum of sucrose, fructose and glucose

218 concentrations.

219 Data on nectar composition were grouped by floral stage and tested to assess homogeneity of

220 variances and normality of distribution (Bartlett test and Shapiro Wilk test).

221 Data on sugars per flower, total sugar concentration and sucrose per flower were square root

222 transformed to achieve normality. When the transformed data failed to match normality, we

223 applied the corresponding non-parametric analyses.

224 To investigate whether the floral stage affected sugar content and volume a one-way ANOVA

225 followed by Tukey's HSD post hoc test with Benjamini-Hochberg correction for 'false

226 discovery rate' (Verhoeven et al. 2005) were performed. When distribution was not normal a

227 Kruskal Wallis H-test followed by a Mann Whitney pairwise comparison with Benjamini-

228 Hochberg correction were carried out instead.

229 Data on single amino acid concentrations were ln transformed to achieve normality when

230 needed and a Student t-test was applied in all analyses.

231   For both phenological stages (functionally male and functionally female flowers), three

232   diversity indices were calculated on the nectar amino acid composition. The first index was

233   the reciprocal Simpson's diversity index 1-*D* of the nectar amino acidic spectrum. *D* was

234   calculated as $D = \sum_{i=1}^{n} \left(\frac{ni}{n}\right)^2$, where *n*i is the abundance of the *i*th amino acid and *n* is the

235   total mean concentration (Ranjbar et al. 2017). This index ranges from 0 (one amino acid

236   dominates the spectrum) to 1 (all amino acids equally represented) (Harper 1999).

237   The second was the Shannon's *H-* index, by taking into account mean amino acid

238   concentrations as well as the total mean concentration of amino acids. The index is calculated

239   as $H = -\sum_{i} \frac{ni}{N} \ln \frac{ni}{N}$, where *ni* is the mean concentration for the *i*th amino acid and N is the

240   total number of amino acids (Magurran 2004). This index varies from 0 for a spectrum with

241   only a single amino acid to high values for a spectrum with many amino acids, each

242   represented by relatively low concentrations (Harper 1999; Hubálek 2000; Fattorini et al.

243   2016).

244   The third one was the Buzas and Gibson's evenness index, a measure of the relative

245   abundance of the different amino acids within the floral stage. The index is calculated as the

246   proportion of equally dominant amino acid in the phenological stage $E = e^H/S$, where H is

247   Shannon's H index and S is the number of amino acids within the floral stage. This index

248   ranges from 0 (highest dominance by a single amino acidic species) to 1 (all amino acids have

249   the same abundance) (Buzas and Hayek 2010; Fattorini et al. 2016).

250   Insect visit data were first analysed by comparing the observed number of male and female

251   flowers visited to the expected ones by χ^2 test. The expected number of visits was calculated

252   on the basis of the ratio between the functionally male and the functionally female flowers

253   occurring in the population.

254   Frequencies of male flowers visited by each taxon were compared by a Kruskal Wallis H-test

255   followed by a Mann-Whitney pairwise comparison with Benjamini-Hochberg correction.

256   All data are presented as mean ± SE and all statistics were performed using R software

257   (version 3.6.1) with the significance level set at 0.05.

258

259   **Results**

260   **Plant phenology**

261   In June 2018, the studied population contained 47 flowering individuals, all hermaphrodites.

262   The mean number of inflorescences per plant was 3.17 ± 0.44, while the mean number of

263   cymes per inflorescence was 14.30 ± 0.81. Moreover, the mean number of male flowers per

264   inflorescence was 2.69 ± 0.171, while the mean number of female flowers per inflorescence

265   was 21.07 ± 0.858. On the basis of the data collected on the population structure the ratio of

266   male and female floral stages in the observation patches was determined at 1:9.

267

268   **Nectar analyses**

269   *Sugars and volume*

270   Mean nectar volume per flower showed a clear trend of increasing in relation to floral age,

271   with volume in buds statistically lower than in both male- and female-phase flowers ($U = 15$,

272     $p = 0.009$ and $U = 2$, $p = 0.001$, respectively). A significant difference for mean sugar

273   quantity per flower was also reported between buds and female-phase flowers (Tukey's HDS:

274     $p = 0.028$), whilst sugar concentration did not differ significantly among floral stages (Table

275   1).

276   A more in depth analysis on sugars reported that hexose sugar quantity per flower in the bud

277   stage differed significantly from both male- and female-phase flowers ($U = 12$, $p = 0.008$ and

278     $U = 19$, $p = 0.018$, respectively), whilst sucrose quantity per flower found in bud differed

279   statistically only from the average amount found in the female stage (Tukey's HDS: $p =$

280   0.021; Table 1). Mean percentage of sucrose per flower did not appear to be significantly

281   different among floral stages (Table 1).

282

283   *Amino acids*

284   There was no significant difference for total, protein, and non-protein amino acid quantity per

285   flower between male and female flowers, while the ratio between protein and non protein

286   amino acid concentrations was significantly higher for male-phase flowers (Table 1).

287   The only amino acid with a statistically significant difference was phenylalanine (t_{14} = 2.94, p

288   = 0.011), showing a higher concentration in male floral phase (M = 352.7 ± 63.2 nmol mL^{-1}

289   and F = 143.6 ± 32.6 nmol mL^{-1}; Fig. 1).

290   Among all protein amino acids, proline and phenylalanine showed the highest concentrations:

291   the former appeared to reach higher concentrations in the functionally female stage (674.8 ±

292   243.5 nmol mL^{-1}), whilst the latter in the functionally male stage (352.7 ± 63.2 nmol mL^{-1}).

293   Among non protein amino acids, in both male and female stages GABA showed the highest

294   concentration (51.4 ± 12.2 nmol mL^{-1} and 202.0 ± 73.4 nmol mL^{-1}, respectively).

295   The number of different amino acids (richness) detectable in the male stage was significantly

296   lower than number of amino acids in the female stage (t_{15} = 3.54, p = 0.003; 16.5 ± 0.6 and

297   19.0 ± 0.3, respectively), while no differences were found in Simpson, Shannon and Evenness

298   indices between male and female stages (Table 2).

299

300   **Insect visit analyses**

301   *Flower visitors' abundance*

302   A total of 215 insect visits were recorded on *Echium vulgare* during 200 minutes of field

303   surveys (Table 3).

304   Visitors belonged to three order: Hymenoptera (87.4%), Lepidoptera (9.8%) and Diptera

305   (2.8%). The order Hymenoptera was mainly represented by individuals belonging to the

306   family Megachilidae (59%), followed by the family Halictidae (26.5%) and Apidae (14%).

307   The order Lepidoptera was represented mainly by individuals belonging to the species

308   *Macroglossum stellatarum* (43%) and the family Pieridae (43%). The order Diptera was

309   represented only by 6 individuals belonging to the families Bombyliidae and Syrphidae. The

310   most frequent visitors were solitary bees of the species *Hoplitis adunca* (42%).

311

312   *Flower visitor observations*

313   Among the 215 insects visiting the plant, we fully recorded data for 189 individuals.

314   Statistical analyses were carried out only on the 112 individuals which were looking for

315   nectar and for which the number of total visits exceeded 5 (*Macroglossum stellatarum*,

316   Pieridae, *Anthidium florentinum*, *Apis mellifera* and *Hoplitis adunca*). The family Pieridae

317   was analysed as a single taxon in order to reach a total number of visits above 5. Since

318   *Hoplitis adunca* was the most abundant taxon and the only species strongly oligolectic on

319   *Echium*, we therefore decided to analyse the sexes separately.

320   Although nectar is produced before flower opening and insects can force the bud searching

321   for nectar (personal observation), this event occurred very rarely. Consequently, we did not

322   consider the phenological stage bud in these analyses.

323   For each insect taxon, we compared the number of visits to male and female flowers with the

324   expected ones, calculated according to the ratio 1:9 between male and female flowers

325   registered in the studied population.

326   Regarding the number of male flowers visited, no significant difference was reported for

327   lepidopterans (Pieridae spp., *Macroglossum stellatarum*) and for females *Hoplitis adunca*,

328   while *Anthidium florentinum*, *Apis mellifera* and *Hoplitis adunca* males visited more male

329 flowers than expected (Table 4). The number of female flowers visited was never statistically

330 different from that expected.

331 The frequency of male flowers visited in relation to the total number of flowers visited among

332 taxa was statistically different ($H_4 = 14.01$, p = 0.016). Statistical analyses confirmed that the

333 female *Hoplitis adunca* visited fewer male flowers than did *Anthidium florentinum* (U = 65, p

334 = 0.002), *Apis mellifera* (U = 48, p = 0.002) and *Macroglossum stellatarum* (U = 28.5, p =

335 0.043; Fig. 2).

336

337 **Discussion**

338 Our studied population did not show the phenomenon of gynodioecism, as all flowers were

339 hermaphrodite, and our data confirmed the ratio of 1:9 found by Rademaker et al. (1999)

340 between functionally male and functionally female flowers.

341 Our analyses confirmed that nectar is secreted in the bud, as reported by Chwil and

342 Weryszko-Chmielewska (2011). Contrary to Klinkhamer and de Jong (1990), we found that

343 nectar volume, as well as sugar quantity per flower, increased with the age of the flower (from

344 bud to female phase), although the positive trend between male and female phases was not

345 statistically significant. Both quantity of hexose sugars and sucrose per flower increased with

346 the age of the flower, the latter reaching a mean almost 7 fold higher in functionally female

347 flowers than the mean amount found in the bud stage and almost twice the amount found in

348 functionally male flowers. At the same time, the mean percentage of sucrose per flower

349 appeared to be lower in male-phase flowers, even though not significantly, meaning that the

350 total sugar increase in relation to floral age is due to the rise of nectar volume, since total

351 sugar concentration and composition remained constant during the entire flower phenology.

352 The existence of nectar homeostasis mechanisms which actively maintain a constant nectar

15

353 sugar concentration to ensure pollinator visits has been previously reported in other species

354 (Nepi and Stpiczyńska 2008; Nepi et al. 2011).

355 When we compared the number of insect visits on male and female flowers observed to the

356 expected ones, all bee taxa except female *Hoplitis adunca* showed a higher number of visits to

357 male flowers than expected. This result could be explained by the higher proportion of protein

358 amino acids found in the male stage: preferences have often been reported in bees for protein

359 amino acid enriched solutions (Inouye and Waller 1984; Bertazzini et al. 2010; Hendriksma et

360 al. 2014), suggesting that flower visitors may actively choose to visit functionally male

361 flowers. Comparable results have been reported by Klinkhamer and de Jong (1990) and by

362 Rademaker et al. (1999) on bumblebees: when calculating the probabilities of visits on

363 different floral stages, the oldest female stage was less likely to be visited than a male-phase

364 flower. Females of *Hoplitis adunca* are the only bees collecting both pollen and nectar on *E.*

365 *vulgare*: this different foraging behaviour might explain the difference from the other bee

366 species.

367 Individuals of *Lasioglossum* sp. were observed visiting the flower and collecting pollen only.

368 A tendency for afternoon trips for nectar only have been reported for the subfamily Halictinae

369 by Michener (2003) so we cannot conclude that *Lasioglossum* sp. does not exploit *E. vulgare*

370 nectar since the species may simply collect the resource at different time of the day.

371 Despite Lepidoptera having been reported to prefer nectar rich in PAAs (Baker and Baker

372 1986; Erhardt and Rusterholz 1998), our study reports that Pieridae butterflies visited as many

373 male flowers as expected, indicating that these insects did not actively look for functionally

374 male flowers (containing a higher proportion of protein amino acids). A study conducted by

375 Alm et al. (1990) showed that male individuals of the species *Pieris rapae* do not discriminate

376 between artificial nectars containing sugar only or sugar solution enriched with protein amino

377 acids, and Romeis and Wäckers (2000) reported that feeding and source-selection in *Pieris*

16

378   *brassicae* is elicited by sucrose more than protein amino acids. We report a similar result for

379   the species *Macroglossum stellatarum*, but to date no study has been done in order to assess

380   amino acid preferences in the species and whether taste receptors on the proboscis can sense

381   their presence in nectar remains unsubstantiated (Stöckl and Kelber 2019).

382   Nectar of male-phase flowers in *E. vulgare* presented, among all the amino acids, the highest

383   concentration of phenylalanine, representing an average of 35% of total amino acid content.

384   Phenylalanine is an essential protein amino acid (de Groot 1953) and several studies proved

385   that it exerts a phagostimulatory effect on several insects, especially on honey bees, and it is

386   strongly correlated with pollinator preferences (Inouye and Waller 1984; Hendriksma et al.

387   2014; Tiedge and Lohaus 2017; Seo et al. 2019). Consequently, this could explain the higher

388   frequency of visit on male flowers than expected. A correlation between phenylalanine

389   concentration and nectar feeding by Megachilids, that were the more numerous pollinators in

390   our study, was demonstrated in a phriganic community, a plant association typical of the East

391   Mediterranean (Petanidou et al. 2006).

392   Proline, instead, represented the most concentrated amino acid in functionally female flowers,

393   and the second in the early-stage functionally male flowers (representing more than 30% and

394   almost 20% of the total amino acid content, respectively). This non-essential amino acid,

395   commonly found in nectar (Nicolson and Thornburg 2007), can stimulate the insect salt cell

396   increasing intensity of feeding behaviour (Hansen et al. 1998; Wacht et al. 2000). Proline also

397   represents an energy substrate to fuel the earliest or most expensive stages of insect flight

398   (Micheu et al. 2000; Gade and Auerswald 2002), resulting in short-term bursts of energy

399   production (Teulier et al. 2016).

400   Finally, in both male- and female-phase flower nectar GABA showed the highest

401   concentration among the non-protein amino acids representing more than 5% and 9% of total

402   amino acid content, respectively. Recent studies indicated that GABA could affect both

17

403 insects' physiology and behaviour, feeding rate and flight muscles performances (Shelp et al.

404 2017; Felicioli et al. 2018; Bogo et al. 2019). Besides GABA, or possibly the combination of

405 GABA and NaCl, can constitute an important nectar phagostimulant and its presence

406 correlates with visits by an array of pollinators such as long tongued bees, ex-anthophorid and

407 andrenid bees, as well as anthomyiid and syrphid flies (Petanidou 2007 and reference

408 therein).

409 The spectrum of visitors recorded through our observations confirm that reported by previous

410 studies stating that flowers of *E. vulgare* are visited by hummingbird hawkmoths (Aguado

411 Martìn et al. 2017), bees, bee flies (Proctor et al. 1996) and syrphids (Willmer and Finlayson

412 2014). Also, even though the species has often been reported as mainly pollinated by

413 bumblebees (Corbet 1978; Klinkhamer and de Jong 1990; Pappers et al. 1999; Rademaker et

414 al. 1999), we observed only one individual of *Bombus pascuorum* visiting the

415 flowers. Pollinators of wide spread plant species can vary in relation to their geographical

416 distribution (Armbruster 1985; Thompson 2006; Pérez-Barrales et al. 2007) and, moreover, as

417 reported by Lázaro et al. (2010), the plant and pollinators assemblages of an entire community

418 may also influence the composition of visitors of a particular species by determining, for

419 instance, the strength of competition or the intensity of attraction to that species rather than

420 another. Thus, the scarcity of bumblebees observed on *Echium vulgare* in 2018 may either

421 depend on several factors and/or reflect a temporal fluctuation in the species composition of

422 the pollinator community, as previously reported by many studies (Cane et al. 2005;

423 Petanidou et al. 2008; Dupont et al. 2009).

424

425 **Conclusions**

426 The inbreeding avoidance hypothesis states that some mechanisms develop within a species in

427 order to prevent breeding among related individuals and its damaging effects on fitness

428 (Darwin 1876, 1877; Charlesworth and Charlesworth 1987). In dichogamous species, gender-

429 biased nectar often occurs (Carlson and Harms 2006; Stpiczyńska et al. 2015; Konarska and

430 Masierowska 2020), and this, according to the mentioned above hypothesis, may contribute to

431 decrease geitonogamous selfing through its effects on a pollinator's behaviour (Carlson and

432 Harms 2006). Our results suggest that the quality of nectar offered by the two sexually

433 distinct floral phases may target different insect needs, thus affecting simultaneously different

434 behavioural traits and ensuring an optimal pattern of visit among functionally different floral

435 stages, unequally present in the population throughout the anthesic period. The more

436 nutritional nectar found in the less frequent sexual phase occurring in the population (male

437 flowers) may enhance movements among plants by encouraging "better-resource hunt",

438 whilst the flight efforts accomplished for doing so may be sustained by a rapidly oxidable fuel

439 such proline offered in female-phase flowers. In the light of this hypothesis, it appears clear

440 that gender-biased nectar studies in dichogamous, many-flowered species should be

441 undertaken in relation to the occurrence of floral sexual phases in the population (when a bias

442 in the frequency of sex occurrence exists).

443 Despite no study yet providing strong scientific evidence that gender-biased nectar in fact

444 reduces inbreeding (Carlson and Harms 2006), it is reasonable to assume that by offering

445 variable quality nectar through sexually different floral phases the plant may produce a

446 mosaic of food targeting different pollinator behavioural traits aiming to promote cross-

447 pollination.

448

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

Data available from the Zenodo Digital Repository: http://doi.org/xxxxxxxxxx (Barberis, Bogo et al. 2020)

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713

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Figure captions

Figure 1. Amino acid concentrations (nmol mL^{-1}) detected in functionally male (dark bars)

and in functionally female (light bars) flowers (mean ± SE). Amino acids hydroxyproline,

homoserine, citrulline, cysteine, histidine, glutamine, asparagine and L-thyronine were not

detected in either floral stages and thus not shown in the graph. The asterisk denotes a

statistically significant difference according to Student t-test. NPAA = non-protein amino

acids; PAA = protein amino acids.

Figure 2. Frequency of male flowers visited by each taxon. Different letters denote statistical

differences according to Kruskal Wallis H-test followed by Mann-Withney pairwise

comparison with Benjamini-Hochberg correction (p < 0.05).

726 **Table 1.** Comparison of nectar volume, sugar and amino acid (AA: amino acids; PAA: protein

727 amino acids; NPAA: non-protein amino acids) compositions among the three phenological

728 stages (bud, male and female flowers). Values (expressed by mean ± SE) marked with different

729 letters were significantly different according to one-way ANOVA or Kruskal-Wallis test

730 followed by the respective post hoc test with Benjamini-Hochberg correction.

Nectar parameters	Bud	Male flower	Female flower	Test value	p-value
Volume (μL flower^{-1})	0.159 ± 0.019 a	0.427 ± 0.080 b	0.669 ± 0.135 b	H_2 = 16.83	< 0.001
Total sugar (μg flower^{-1})	0.013 ± 0.006 a	0.040 ± 0.013 ab	0.070 ± 0.026 b	$F_{2,27}$ = 5.78	< 0.001
Total sugar concentration (μg μL^{-1})	0.089 ± 0.033	0.094 ± 0.022	0.090 ± 0.020	$F_{2,27}$ = 0.45	0.642
Hexose sugars (μg flower^{-1})	0.005 ± 0.004 a	0.007 ± 0.001 b	0.008 ± 0.002 b	H_2 = 11.43	0.003
Sucrose (μg flower^{-1})	0.009 ± 0.003 a	0.033 ± 0.012 ab	0.061 ± 0.024 b	$F_{2,27}$ = 5.63	0.007
Sucrose (% per flower)	82.278 ± 7.824	72.896 ± 5.776	81.900 ± 3.817	H_2 = 4.10	0.129
Total AA (nmol flower^{-1})	-	0.367 ± 0.061	1.349 ± 0.611	U = 21	0.270
PAA (nmol flower^{-1})	-	0.321 ± 0.054	1.058 ± 0.467	U = 23	0.372
NPAA (nmol flower^{-1})	-	0.045 ± 0.007	0.290 ± 0.145	U = 15	0.083
PAA:NPAA ratio	-	7.31 ± 0.670	4.65 ± 0.437	t_{14} = -3.34	0.005

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733   **Table 2.** Comparison of diversity indices calculated on nectar amino acid concentration

734   between male and female phases (8 samples for both floral phases).

Diversity indices	Male flower	Female flower	t	p-value
Amino acids richness	16.50 ± 0.627	19.00 ± 0.327	3.54	0.003
Simpson	0.793 ± 0.035	0.822 ± 0.024	0.68	0.506
Shannon H	2.109 ± 0.103	2.233 ± 0.111	0.82	0.428
Evenness	0.527 ± 0.059	0.511 ± 0.050	-0.20	0.842

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737 **Table 3.** *Echium vulgare* visitors recorded in June 2018 (215 visits in total), their abundance

738 and the percentage of them looking for nectar as reward.

Order	Family	Species	Relative frequency	Looking for nectar (%)
Hymenoptera	Apidae	*Apis mellifera* Linnaeus, 1758	0.079	100
Hymenoptera	Apidae	*Bombus pascuorum* (Scopoli, 1763)	0.005	100
Hymenoptera	Apidae	*Ceratina* (Latreille, 1802) sp.	0.023	100
Hymenoptera	Apidae	*Eucera* (Scopoli, 1770) sp.	0.018	100
Hymenoptera	Halictidae	*Lasioglossum interruptum* (Panzer, 1798) *Lasioglossum laticeps* (Schenck, 1869) *Lasioglossum corvinum* (Morawitz, 1878)	0.233	0
Hymenoptera	Halictidae	*Halictus subauratus* (Rossi, 1792)	0.005	100
Hymenoptera	Colletidae	*Hylaeus* cfr. *angustatus* (Schenck, 1859)	0.005	100
Hymenoptera	Megachilidae	*Anthidium florentinum* (Fabricius, 1775)	0.102	100
Hymenoptera	Megachilidae	*Hoplitis adunca* (Panzer, 1798)	Male: 0.191 Female: 0.219	Male: 100 Female: 66.6[a]
Diptera	Bombyliidae	*Bombylius* (Linnaeus, 1758) sp.	0.009	100
Diptera	Syrphidae	Syrphidae (Latreille, 1802) sp.	0.019	0
Lepidoptera	Hesperiidae	*Hesperia comma* (Linnaeus, 1758) *Thymelicus acteon* (Rottemburg, 1775)	0.019	100
Lepidoptera	Papilionidae	*Iphiclides podalirius* (Linnaeus, 1758)	0.005	100
Lepidoptera	Pieridae	*Pieris brassicae* (Linnaeus, 1758) *Pieris mannii* Mayer, 1851 *Colias croceus* (Fourcroy, 1785) *Pontia edusa* (Fabricius, 1777)	0.042	100
Lepidoptera	Sphingidae	*Macroglossum stellatarum* (Linnaeus, 1758)	0.042	100

739 [a]value calculated only on individuals with fully recorded data (n = 21)
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741 **Table 4.** Male (a) and female (b) flowers visited by each taxon (mean ± SE). Chi-square test is

742 calculated on the basis of the ratio 1:9 between male and female flowers occurred in the studied

743 population.

a)

Taxon	Male flowers visited	χ^2	d.f.	p-value
Anthidium florentinum	0.96 ± 0.192	37.80	21	0.014
Apis mellifera	1.59 ± 0.384	39.39	16	<0.001
Hoplitis adunca male	0.51 ± 0.100	70.51	40	0.002
Hoplitis adunca female	0.14 ± 0.143	8.50	13	0.810
Macroglossum stellatarum	2.33 ± 0.799	4.54	8	0.806
Pieridae	0.33 ± 0.236	5.21	8	0.735

b)

Taxon	Female flowers visited	χ^2	d.f.	p-value
Anthidium florentinum	3.95 ± 0.826	4.20	21	1.000
Apis mellifera	7.47 ± 1.652	4.38	16	0.998
Hoplitis adunca male	2.37 ± 0.312	7.84	40	1.000
Hoplitis adunca female	1.64 ± 0.199	0.94	13	1.000
Macroglossum stellatarum	15.67 ± 14.696	0.50	8	1.000
Pieridae	4.22 ± 1.656	0.58	8	1.000

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