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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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- 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
- 24 advice and approved the final manuscript.
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26

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36 Abstract

Floral nectar is a chemically complex aqueous solution within which several secondary 37 38 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 39 influence the patterns of insect floral visitation with consequences on the plant fitness. We 40 hypothesise that nectar chemical variation through different floral sexual phases may affect 41 the number of insect visits that each phase receives. The study was realized on a population of 42 Echium vulgare L. growing in a natural area close to Bologna. Nectar was collected from 43 44 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 45 also carried out. Variation in nectar traits has been detected for the amino acid spectrum. The 46 proportion of protein amino acids appeared to be significantly higher in male-phase flowers. 47 48 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 49 presented higher concentrations of phenylalanine, whilst proline was highly represented in 50

- functionally female flowers. Since a recent study demonstrated that hymenopterans can
 oxidize proline at a high rate for ATP production, we can hypothesise that the quality of
 nectar offered by the two sexually distinct floral phases targets different insect behavioural
 traits and likely ensures an optimal pattern of visit among flower sexes, which are unequally
 distributed within and among individuals in the population.
- 57 Keywords: Echium vulgare, flower visitors, inbreeding avoidance, nectar chemistry, plant-
- 58 pollinator interactions
- 59

60 Introduction

61	Floral nectar is a chemically complex aqueous solution in which the main components
62	comprise sugars, followed by amino acids (Nicolson and Thornburg 2007). In recent decades
63	considerable progress has been made in providing evidence that points to the involvement of
64	nectar chemistry in the interactions between plants and a variety of organisms (Nepi 2014;
65	Stevenson et al. 2017). Although there is wide variability in nectar traits (Pacini et al. 2003;
66	Nocentini et al. 2013; Irwin et al. 2014), a general paradigm shared by plants is balancing
67	nectar chemical composition in order to not deter specific pollinators exceeding their
68	tolerance thresholds (Baker and Baker 1975; Adler 2000; Nicolson and Thornburg 2007;
69	Wright et al. 2013; Stevenson et al. 2017). For example, a small increase in nectar sugar
70	concentration can increase its viscosity (Harder 1986; Nicolson and Thornburg 2007), which
71	is strongly related to the energy required by nectar consumers to visit flowers (Corbet 1978;
72	Josens and Farina 2001; Borrell and Krenn 2006; Nepi and Stpiczyńska 2006; Kim et al.
73	2011).
74	After sugars the most abundant nectar solutes are the amino acids (Baker and Baker 1982;
75	Nepi et al. 2012; Bogo et al. 2019). A study conducted by Inouye and Waller (1984) showed a
76	general decline in nectar consumption in honeybees as amino acid concentrations increased,
77	despite evidence supporting the preference for amino acid enriched sugar solutions in insects
78	(Alm et al. 1990; Bertazzini et al. 2010; Bogo et al. 2019). Amino acids also contribute to the
79	taste of nectar, stimulating specific insects' labellar chemoreceptors (Gardener and Gillman
80	2002). Among protein amino acids, Inouye and Waller (1984) found that phenylalanine and
81	leucine were phagostimulant for honeybees at all concentrations tested, even at those that in
82	the case of other amino acids resulted in deterrence. In the same way, a preference in
83	honeybees for proline enriched artificial nectar was reported (Carter et al. 2006; Bertazzini et

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

Beside primary metabolites (such as sugars and amino acids) an array of secondary 86 metabolites with different chemical natures have been identified in nectar and all of them 87 positively or negatively affect attractiveness to pollinators, showing effects which depend on 88 89 metabolite concentration and pollinators' sensitivity (Baker and Baker 1977; Faegri and van der Pijl 1979; Baker and Baker 1982; Adler 2000; Stevenson et al. 2017). Among them non-90 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 amino acidic content of floral nectar, little is known about their role in determining 93 pollinators' preferences and feeding behaviour. For some of those, such as y-aminobutyric 94 acid, a phagostimulant function has been reported in some caterpillars and adult beetles 95 (Mitchell and Harrison 1984; Shoonhoven et al. 2005), whilst Bogo et al. (2019) found that 96 both bumblebees and honeybees showed higher consumption of sucrose solution enriched 97 with ß-alanine, but exhibited the effect at different concentrations. 98 99 Understanding preferences and aversions to nectar traits is crucial since they likely influence the patterns of floral visitation by nectar consumers and thus the plant inbreeding and 100 outbreeding rate within a population. Minimal inbreeding is predicted when pollinators visit a 101 102 small fraction of the open flowers on a plant (Iwasa et al. 1995; Ohashi and Yahara 2001): this behaviour may be enhanced by within-plant variation in nectar, as occurs in plants 103 showing gender-biased nectar production (Feinsinger 1978; Pike 1978; Rathcke 1992). 104 Despite many studies having already addressed the subject of gender-biased nectar 105 106 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

Masierowska 2020) and few reported the observation of insect visit bias (Carlson and Harms2006 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

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

Here, we examined if floral visitation pattern may be influenced by variations in the chemical
composition of nectar through different floral stages, and thus we investigated (i) whether *E. vulgare* produces a gender-biased nectar for volume, sugar and amino acid composition and
(ii) if flower visitation rates of insects looking for nectar varied among different floral stages.

134 Material and Methods

135 Study site

- 136 The activity in the field was carried out in June 2018 and took place in the Parco Belpoggio, a
- 137 public park managed since 2010 by the WWF, in San Lazzaro di Savena (Bologna, Italy). The
- 138 area is situated close to the protected area Parco dei Gessi Bolognesi e Calanchi
- 139 dell'Abbadessa (44°27'14.5"N 11°22'58.3"E). The studied population was located on an open
- 140 prairie along the public pathway.
- 141

142 Study species

- 143 Echium vulgare L. is a perennial hemicryptophyte belonging to the family Boraginaceae. It is
- 144 distributed in Europe, Asia and North America and it shows a long flowering period, ranging
- 145 between June and October. Flower anthesis lasts 3-4 days and flowers show an incomplete
- 146 protandry (Melser et al. 1997): the anthers are often dehiscent already at the bud stage, while
- 147 the stigma becomes receptive only hours after the flower opening.
- 148 In this study we considered three phases of floral development: closed flower (Bud),
- 149 functionally male (M) and functionally female (F) flowers. The male phase was represented
- 150 by an open flower presenting pollen with non receptive stigma, whilst the female phase was
- 151 recognised as soon as the stigma became bifid and receptive.

- 153 *Plant phenology*
- On the first day of the study we counted all plants and inflorescences per plant constituting the population (approximately 600 m² 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 $100 \,\mu L$

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

in order to reach a minimum volume of 2 μ L 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

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

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

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

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 μ L needed for amino acid

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

178

179 Sugar analysis

Sugar content was analysed by HPLC technique through a Waters LC1 with refractive index
detector (Waters 2410) connected to the output of a REZEX RCM Monosaccharide column
(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
- 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

- and female flowers approached per visit. We also recorded the visitor's taxon, indicating the
 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
- 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
- flower. Total sugar concentration was calculated as the sum of sucrose, fructose and glucoseconcentrations.
- 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.

For both phenological stages (functionally male and functionally female flowers), three 231 232 diversity indices were calculated on the nectar amino acid composition. The first index was the reciprocal Simpson's diversity index 1-D of the nectar amino acidic spectrum. D was 233 calculated as $D = \sum_{i=1}^{n} \left(\frac{ni}{n}\right)^2$, where *n* is the abundance of the *i*th amino acid and *n* is the 234 total mean concentration (Ranjbar et al. 2017). This index ranges from 0 (one amino acid 235 236 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 concentrations as well as the total mean concentration of amino acids. The index is calculated 238 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 239 240 total number of amino acids (Magurran 2004). This index varies from 0 for a spectrum with only a single amino acid to high values for a spectrum with many amino acids, each 241 represented by relatively low concentrations (Harper 1999; Hubálek 2000; Fattorini et al. 242 2016). 243 The third one was the Buzas and Gibson's evenness index, a measure of the relative 244 abundance of the different amino acids within the floral stage. The index is calculated as the 245 proportion of equally dominant amino acid in the phenological stage $E = e^{H}/S$, where H is 246 Shannon's H index and S is the number of amino acids within the floral stage. This index 247 ranges from 0 (highest dominance by a single amino acidic species) to 1 (all amino acids have 248 the same abundance) (Buzas and Hayek 2010; Fattorini et al. 2016). 249 Insect visit data were first analysed by comparing the observed number of male and female 250 flowers visited to the expected ones by χ^2 test. The expected number of visits was calculated 251 252 on the basis of the ratio between the functionally male and the functionally female flowers

253 occurring in the population.

Frequencies of male flowers visited by each taxon were compared by a Kruskal Wallis H-testfollowed by a Mann-Whitney pairwise comparison with Benjamini-Hochberg correction.

All data are presented as mean \pm 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

In June 2018, the studied population contained 47 flowering individuals, all hermaphrodites. The mean number of inflorescences per plant was 3.17 ± 0.44 , while the mean number of cymes per inflorescence was 14.30 ± 0.81 . Moreover, the mean number of male flowers per inflorescence was 2.69 ± 0.171 , while the mean number of female flowers per inflorescence was 21.07 ± 0.858 . On the basis of the data collected on the population structure the ratio of 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,
- with volume in buds statistically lower than in both male- and female-phase flowers (U = 15,
- 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:

- p = 0.028), whilst sugar concentration did not differ significantly among floral stages (Table
- 275 1).
- A more in depth analysis on sugars reported that hexose sugar quantity per flower in the bud stage differed significantly from both male- and female-phase flowers (U = 12, p = 0.008 and U = 19, p = 0.018, respectively), whilst sucrose quantity per flower found in bud differed statistically only from the average amount found in the female stage (Tukey's HDS: p =

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

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

and $F = 143.6 \pm 32.6 \text{ nmol mL}^{-1}$; 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 \pm

292 243.5 nmol mL⁻¹), whilst the latter in the functionally male stage ($352.7 \pm 63.2 \text{ nmol mL}^{-1}$).

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

concentration (51.4 \pm 12.2 nmol mL⁻¹ and 202.0 \pm 73.4 nmol mL⁻¹, respectively).

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

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

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

- 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
- 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

flowers than expected (Table 4). The number of female flowers visited was never statistically
different from that expected.
The frequency of male flowers visited in relation to the total number of flowers visited among

- 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
- 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
- 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
- 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
- total sugar increase in relation to floral age is due to the rise of nectar volume, since total
- sugar concentration and composition remained constant during the entire flower phenology.
- 352 The existence of nectar homeostasis mechanisms which actively maintain a constant nectar

sugar concentration to ensure pollinator visits has been previously reported in other species(Nepi and Stpiczyńska 2008; Nepi et al. 2011).

When we compared the number of insect visits on male and female flowers observed to the 355 expected ones, all bee taxa except female Hoplitis adunca showed a higher number of visits to 356 male flowers than expected. This result could be explained by the higher proportion of protein 357 358 amino acids found in the male stage: preferences have often been reported in bees for protein amino acid enriched solutions (Inouye and Waller 1984; Bertazzini et al. 2010; Hendriksma et 359 360 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 Rademaker et al. (1999) on bumblebees: when calculating the probabilities of visits on 362 different floral stages, the oldest female stage was less likely to be visited than a male-phase 363 flower. Females of Hoplitis adunca are the only bees collecting both pollen and nectar on E. 364 vulgare: this different foraging behaviour might explain the difference from the other bee 365 species. 366 Individuals of Lasioglossum sp. were observed visiting the flower and collecting pollen only. 367 368 A tendency for afternoon trips for nectar only have been reported for the subfamily Halictinae by Michener (2003) so we cannot conclude that Lasioglossum sp. does not exploit E. vulgare 369 nectar since the species may simply collect the resource at different time of the day. 370 371 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 male flowers as expected, indicating that these insects did not actively look for functionally 373

male flowers (containing a higher proportion of protein amino acids). A study conducted by
Alm et al. (1990) showed that male individuals of the species *Pieris rapae* do not discriminate
between artificial nectars containing sugar only or sugar solution enriched with protein amino
acids, and Romeis and Wäckers (2000) reported that feeding and source-selection in *Pieris*

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 <i>E. vulgare</i> 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

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 <i>E. vulgare</i> 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 <i>Echium vulgare</i> 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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454	Conflict of interest
455	The authors declare that they have no conflict of interest.
456	
457	Data availability
458	Data available from the Zenodo Digital Repository: http://doi.org/xxxxxxxxx (Barberis,
459	Bogo et al. 2020)
460	
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Codice campo modificato

714 Figure captions

715	Figure 1. Amino acid concentrations (nmol mL ⁻¹) detected in functionally male (dark bars)
716	and in functionally female (light bars) flowers (mean \pm SE). Amino acids hydroxyproline,
717	homoserine, citrulline, cysteine, histidine, glutamine, asparagine and L-thyronine were not
718	detected in either floral stages and thus not shown in the graph. The asterisk denotes a
719	statistically significant difference according to Student t-test. NPAA = non-protein amino
720	acids; PAA = protein amino acids.
721	

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).

Table 1. Comparison of nectar volume, sugar and amino acid (AA: amino acids; PAA: protein amino acids; NPAA: non-protein amino acids) compositions among the three phenological stages (bud, male and female flowers). Values (expressed by mean ± SE) marked with different letters were significantly different according to one-way ANOVA or Kruskal-Wallis test 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)	$0.159 \pm 0.019 \ a$	$0.427\pm0.080\ b$	$0.669\pm0.135\text{ b}$	$H_2 = 16.83$	< 0.001
Total sugar (µg flower ⁻¹)	$0.013 \pm 0.006 \; a$	$0.040\pm0.013\ ab$	$0.070\pm0.026~b$	$F_{2,27} = 5.78$	< 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)	$0.005 \pm 0.004 \; a$	$0.007\pm0.001\ b$	$0.008\pm0.002~b$	$H_2 = 11.43$	0.003
Sucrose (µg flower ⁻¹)	$0.009 \pm 0.003 \ a$	$0.033\pm0.012\ ab$	$0.061\pm0.024~b$	$F_{2,27} = 5.63$	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

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

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

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

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

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
		Lasioglossum interruptum (Panzer, 1798)		
Hymenoptera	Halictidae	Lasioglossum laticeps (Schenck, 1869)	0.233	0
		Lasioglossum corvinum (Morawitz, 1878)		
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: 66.6ª
Diptera	Bombyliidae	Bombylius (Linnaeus, 1758) sp.	0.009	100
Diptera	Syrphidae	Syrphidae (Latreille, 1802) sp.	0.019	0
Lenidontera	Hesperiidae	Hesperia comma (Linnaeus, 1758)	0.019	100
Lepidoptera	nespendae	<i>1 hymelicus acteon</i> (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

740

^avalue calculated only on individuals with fully recorded data (n = 21)

741	Table 4. Male (a	a) and female (b)	flowers visited l	by each taxon	(mean \pm SE).	. Chi-square test is
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calculated on the basis of the ratio 1:9 between male and female flowers occurred in the studied

743	population.	
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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