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

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18

19

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
24 advice and approved the final manuscript.

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26

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35

36 **Abstract**

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

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

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 Stpiczynska 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 β -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

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.

152

153 *Plant phenology*

154 On the first day of the study we counted all plants and inflorescences per plant constituting
155 the population (approximately 600 m² of extension) and we observed all open flowers to
156 assess whether the phenomenon of gynodioecy, firstly described in *E. vulgare* populations by
157 Darwin (1877), occurred in our study population. Each day, prior to visitor observations, on
158 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 μ L; Drummond
166 Scientific Co., Broomall, PA), we transferred samples to Eppendorf tubes filled with 100 μ 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
169 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
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 μ L 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 μ m) 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 $D = \sum_{i=1}^n \left(\frac{n_i}{n}\right)^2$, 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 $H = -\sum_i \frac{n_i}{N} \ln \frac{n_i}{N}$, where n_i 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 \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**

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 HSD:
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 HSD: $p =$

0.021; Table 1). Mean percentage of sucrose per flower did not appear to be significantly different among floral stages (Table 1).

Amino acids

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

The only amino acid with a statistically significant difference was phenylalanine ($t_{14} = 2.94$, $p = 0.011$), showing a higher concentration in male floral phase ($M = 352.7 \pm 63.2 \text{ nmol mL}^{-1}$ and $F = 143.6 \pm 32.6 \text{ nmol mL}^{-1}$; Fig. 1).

Among all protein amino acids, proline and phenylalanine showed the highest concentrations: the former appeared to reach higher concentrations in the functionally female stage ($674.8 \pm 243.5 \text{ nmol mL}^{-1}$), whilst the latter in the functionally male stage ($352.7 \pm 63.2 \text{ nmol mL}^{-1}$).

Among non protein amino acids, in both male and female stages GABA showed the highest concentration ($51.4 \pm 12.2 \text{ nmol mL}^{-1}$ and $202.0 \pm 73.4 \text{ nmol mL}^{-1}$, respectively).

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 19.0 ± 0.3 , respectively), while no differences were found in Simpson, Shannon and Evenness indices between male and female stages (Table 2).

Insect visit analyses

Flower visitors' abundance

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

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 expected ones, all bee taxa except female *Hoplitis adunca* showed a higher number of visits to male flowers than expected. This result could be explained by the higher proportion of protein 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 al. 2014), suggesting that flower visitors may actively choose to visit functionally male 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 different floral stages, the oldest female stage was less likely to be visited than a male-phase flower. Females of *Hoplitis adunca* are the only bees collecting both pollen and nectar on *E. vulgare*: this different foraging behaviour might explain the difference from the other bee species.

Individuals of *Lasioglossum* sp. were observed visiting the flower and collecting pollen only. 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* nectar since the species may simply collect the resource at different time of the day.

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

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 Martin 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

(Darwin 1876, 1877; Charlesworth and Charlesworth 1987). In dichogamous species, gender-biased nectar often occurs (Carlson and Harms 2006; Stpiczyńska et al. 2015; Konarska and Masierowska 2020), and this, according to the mentioned above hypothesis, may contribute to decrease geitonogamous selfing through its effects on a pollinator's behaviour (Carlson and Harms 2006). Our results suggest that the quality of nectar offered by the two sexually distinct floral phases may target different insect needs, thus affecting simultaneously different behavioural traits and ensuring an optimal pattern of visit among functionally different floral stages, unequally present in the population throughout the anthesis period. The more nutritional nectar found in the less frequent sexual phase occurring in the population (male flowers) may enhance movements among plants by encouraging "better-resource hunt", whilst the flight efforts accomplished for doing so may be sustained by a rapidly oxidable fuel such proline offered in female-phase flowers. In the light of this hypothesis, it appears clear that gender-biased nectar studies in dichogamous, many-flowered species should be undertaken in relation to the occurrence of floral sexual phases in the population (when a bias in the frequency of sex occurrence exists). Despite no study yet providing strong scientific evidence that gender-biased nectar in fact reduces inbreeding (Carlson and Harms 2006), it is reasonable to assume that by offering variable quality nectar through sexually different floral phases the plant may produce a mosaic of food targeting different pollinator behavioural traits aiming to promote cross-pollination.

448

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453

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/xxxxxxx> (Barberis,
459 Bogo et al. 2020)

460

461 **References**

- 462 Adler LS (2000) The ecological significance of toxic nectar. *Oikos* 91:409-420 doi:
463 10.1034/j.1600-0706.2000.910301.x
- 464 Aguado Martin LO, Fereres Castiel A, Viñuela Sandoval E (2017) Guía de campo de los
465 polinizadores de España, 2nd edn. Mundi-Prensa Eds, Madrid.
- 466 Alm J, Ohnmeiss TE, Lanza J, Vriesenga L (1990). Preference of cabbage white butterflies
467 and honeybees for nectar that contains amino acids. *Oecologia* 84:53-57 doi:
468 10.1007/BF00665594
- 469 Antón S, Denisow B, Komoń-Janczara E, Targoński Z (2017) Nectary and gender-biased
470 nectar production in dichogamous *Chamaenerion angustifolium* (L.) Scop. *Plant Spec.*
471 *Biol.* 32:380-391 doi: 10.1111/1442-1984.12169
- 472 Armbruster WS (1985) Patterns of character divergence and the evolution of reproductive
473 ecotypes of *Dalechampia scandens* (Euphorbiaceae). *Evolution* 39:733-752 doi:
474 10.1111/j.1558-5646.1985.tb00416.x
- 475 Baker HG, Baker I (1975) The study of nectar-constitution and pollinator plant coevolution.
476 In: Gilbert LE, Raven PH (eds), *Coevolution of plants and animals*. Univ. of Texas Press,
477 Austin, pp 100-140

478 Baker HG, Baker I (1977) Intraspecific constancy of floral nectar amino acid complements.
 479 Bot. Gaz. 138:183-191 doi: 10.1086/336914
 480 Baker HG, Baker I (1982) Chemical constituents of nectar in relation to pollination
 481 mechanisms and phylogeny. In: Nitecki MH (ed), Biochemical Aspects of Evolutionary
 482 Biology. Univ. of Chicago Press, Chicago, pp 131-171
 483 Baker HG, Baker I (1986) The occurrence and significance of amino acids in floral nectars.
 484 Plant Syst. and Evol. 151:175-186
 485 Bertazzini M, Medrzycki P, Bortolotti L, Maistrello L, Forlani G (2010) Amino acid content
 486 and nectar choice by forager honeybees (*Apis mellifera* L.). Amino Acids 39:315-318 doi:
 487 10.1007/s00726-010-0474-x
 488 Bogo G, Bortolotti L, Sagona S, Felicioli A, Galloni M, Barberis M, Nepi M (2019) Effects of
 489 non protein amino acids in nectar on bee survival and behaviour. J. Chem. Ecol. 45:278-
 490 285 doi: 10.1007/s10886-018-01044-2
 491 Borrell BJ, Krenn HW (2006) Nectar feeding in long-proboscid insects. In: Herrel A, Speck
 492 T, Rowe N (eds), Ecology and biomechanics: a mechanical approach to the ecology of
 493 animals and plants. CRC Press, Boca Raton, pp 185-211
 494 Burger H, Ayasse M, Häberlein, Schulz S, Dötterl S (2010) *Echium* and *Pontechium* specific
 495 floral cues for host-plant recognition by the oligolectic bee *Hoplitis adunca*. S. Afr. J. Bot.
 496 76:788-795 doi: 10.1016/j.sajb.2010.08.003
 497 Buzas MA, Hayek LAC (2010) Surveying natural populations. Quantitative tools for
 498 assessing biodiversity. 2nd edn. Columbia Univ. Press, New York
 499 Cane JH, Sipes S (2006) Floral specialization by bees: analytical methodologies and a revised
 500 lexicon for oligolecty. In: Waser N, Ollerton J (eds), Plant-Pollinator Interactions: From
 501 Specialization to Generalization. Univ. Chicago Press, Chicago, pp 99-122

502 Cane JH, Minckley R, Kervin L, Roulston T (2005) Temporally persistent patterns of
 503 incidence and abundance in a pollinator guild at annual and decadal scales: the bees of
 504 *Larrea tridentata*. Biol. J. Linn. Soc. 85:319-329 doi: 10.1111/j.1095-8312.2005.00502.x
 505 Canto A, Herrera CM, Garcia IM, Pérez R, Vaz M (2011) Intraplant variation in nectar traits
 506 in *Helleborus foetidus* (Ranunculaceae) as related to floral phase, environmental
 507 conditions and pollinator exposure. Flora 206:668-675 doi: 10.1016/j.flora.2011.02.003
 508 Carlson J, Harms KE (2006) The evolution of gender-biased nectar production in
 509 hermaphrodite plants. Bot. Rev. 72:179-205 doi: 10.1663/0006-
 510 8101(2006)72[179:TEOGNP]2.0.CO;2
 511 Carter C, Sharoni S, Yehonatan L, Palmer, RG, Thornburg R (2006) A novel role for proline in
 512 plant floral nectars. Sci. Nat. 93:72-79 doi: 10.1007/s00114-005-0062-1
 513 Charlesworth D, Charlesworth B (1987) Inbreeding depression and its evolutionary
 514 consequences. Annu. Rev. Ecol. Systemat. 18:237-268 doi:
 515 10.1146/annurev.es.18.110187.001321
 516 Chwil M, Weryszko-Chmielewska E (2011) Nectar production and pollen yield of *Echium*
 517 *vulgare* L. in the climatic conditions of Lublin. Acta Sci. Pol., Hortorum Cultus 10:187-
 518 196
 519 Cohen SA, Micheaud DP (1993) Synthesis of a fluorescent derivatizing reagent, 6-
 520 aminoquinolyl-N-hydroxysuccinimidyl carbamate, and its application for the analysis of
 521 hydrolysate amino acids via High Performance Liquid Chromatography. Anal. Biochem.
 522 211:279-287 doi: 10.1006/abio.1993.1270
 523 Corbet SA (1978) Bee visits and the nectar of *Echium vulgare* L. and *Sinapis alba* L. Ecol.
 524 Entomol. 3:25-37 doi: 10.1111/j.1365-2311.1978.tb00900.x
 525 Darwin CR (1876) The effects of cross and self fertilization in the vegetable kingdom.
 526 Murray, London

527 Darwin, C. R. 1877. The different forms of flowers on plants of the same species. Murray,
 528 London
 529 De Groot AP (1953) Protein and amino acid requirements of the honeybee (*Apis mellifera* L.).
 530 Physiol. Comp. Oecol. 3:1-83 doi: 10.1371/journal.pone.0034137
 531 Dupont YL, Padrón B, Olesen JM, Petanidou T (2009). Spatio-temporal variation in the
 532 structure of pollination networks. Oikos 118:1261-1269 doi: 10.1111/j.1600-
 533 0706.2009.17594.x
 534 Erhardt A, Rusterholz HP (1998) Do peacock butterflies (*Inachis io* L.) detect and prefer
 535 amino acids and other nitrogenous compounds? Oecologia 117:536-542 doi:
 536 10.1007/s004420050690
 537 Faegri K, van der Pijl L (1979) The principles of pollination ecology, 3rd edn. Pergamon
 538 Press, doi: 10.1016/C2009-0-00736-3
 539 Fattorini S, Rigal F, Cardoso P, Borges PAV (2016) Using species abundance distribution
 540 models and diversity indices for biogeographical analyses. Acta Oecol. 70:21-28 doi:
 541 10.1016/j.actao.2015.11.003
 542 Feinsinger P (1978) Ecological interactions between plants and hummingbirds in a
 543 successional tropical community. Ecol. Monogr. 48:269-287 doi: 10.2307/2937231
 544 Felicioli A, Sagona S, Galloni M, Bortolotti L, Bogo G, Guarnieri M, Nepi N (2018) Effects
 545 of non-protein amino acids on survival and locomotion of *Osmia bicornis*. Insect Mol.
 546 Biol. 27: 556–563 doi: 10.1111/imb.12496
 547 Filella I, Bosch J, Llusà J, Peñuelas J (2011) Chemical cues involved in the attraction of the
 548 oligolectic bee *Hoplitis adunca* to its host plant *Echium vulgare*. Biochem. Syst. Ecol.
 549 39:498-508 doi: 10.1016/j.bse.2011.07.008

550 Fisogni A, Cristofolini G, Rossi M, Galloni M (2011) Pollinator directionality as a response
 551 to nectar gradient: promoting outcrossing while avoiding geitonogamy. Plant Biol.
 552 13:848-856 doi: 10.1111/j.1438-8677.2011.00453.x
 553 Fisogni A, Rossi M, Sgolastra F, Bortolotti L, Gogo G, de Manincor N, Quaranta M, Galloni
 554 M (2016) Seasonal and annual variations in the pollination efficiency of a pollinator
 555 community of *Dictamnus albus* L. Plant Biol. 18:445-454 doi: 10.1111/plb.12417
 556 Gade G, Auerswald L (2002) Beetles' choice proline for energy output: control by AKHs.
 557 Comp. Biochem. Phys. B 132:117-129 doi: 10.1016/S1096-4959(01)00541-3
 558 Gardener MC, Gillman MP (2002) The taste of nectar - a neglected area of pollination
 559 ecology. Oikos 98:552-557 doi: 10.1034/j.1600-0706.2002.980322.x
 560 Hansen K, Wacht S, Seebauer H, Schnuch M (1998) New aspects of chemoreception in flies.
 561 Ann. NY Acad. Sci. 855:143-147 doi: 10.1111/j.1749-6632.1998.tb10556.x
 562 Harder L (1986) Effects of nectar concentration and flower depth on flower handling
 563 efficiency of bumble bees. Oecologia 69:309-315 doi: 10.1007/BF00377639
 564 Harper DAT (1999) Numerical palaeobiology: computer-based modelling and analysis of
 565 fossils and their distributions. John Wiley and Sons, New York
 566 Hendriksma HP, Oxman KL, Shafir S (2014) Amino acids and carbohydrate tradeoffs by
 567 honeybee nectar foragers and their implications for plant-pollinator interactions. J. Insect
 568 Physiol. 69:56-64 doi: 10.1016/j.jinsphys.2014.05.025
 569 Hubálek Z (2000) Measures of species diversity in ecology: an evaluation. Folia Zool.
 570 49:241-260
 571 Inouye DW, Waller GD (1984) Responses of honeybees (*Apis mellifera*) to amino acid
 572 solutions mimicking floral nectars. Ecology 65:618-625 doi: 10.2307/1941424

573 Irwin RE, Cook D, Richardson LL, Manson JS, Gardner DR (2014) Secondary compounds in
 574 floral rewards of toxic rangeland plants: impacts on pollinators. J. Agric. Food Chem.
 575 62:7335-7344 doi: 10.1021/jf500521w
 576 Iwasa Y, de Jong TJ, Klinkhamer PGL (1995) Why pollinators visit only a fraction of the
 577 open flowers on a plant-the plants point-of-view. J. Evol. Biol. 8:439 doi: 10.1046/j.1420-
 578 9101.1995.8040439.x
 579 Kim W, Gilet T, Bush JW (2011) Optimal concentrations in nectar feeding. PNAS
 580 108:16618-16621 doi: 10.1073/pnas.1108642108
 581 Langenberger MW, Davis AR (2002) Temporal changes in floral nectar production,
 582 reabsorption, and composition associated with dichogamy in annual caraway (*Carum*
 583 *carvi*; Apiaceae). Am. J. Bot. 89:1588-1598 doi: 10.3732/ajb.89.10.1588
 584 Lázaro A, Nielsen A, Totland Ø (2010) Factors related to the inter-annual variation in plants'
 585 pollination generalization levels within a community. Oikos 119:825834 doi:
 586 10.1111/j.1600-0706.2009.18017.x
 587 Lucchetti MA (2017) Pyrrolizidine alkaloids: occurrence in bee products and impact on
 588 honeybees (*Apis mellifera* L.). PhD Dissertation, Faculty of Science, Institute of Biology,
 589 University of Neuchâtel, Switzerland
 590 Lucchetti MA, Glauser G, Kilchenmann V, Dübecke A, Beckh G, Praz C, Kast C (2016)
 591 Pyrrolizidine alkaloids from *Echium vulgare* in honey originate primarily from floral
 592 nectar. J. Agric. Food Chem. 64:5267-5273 doi: 10.1021/acs.jafc.6b02320
 593 Jacquemart AL, Buyens C, Hérent MF, Quetin-Leclercq J, Lognay G, Hance T, Quinet M
 594 (2019) Male flowers of *Aconitum* compensate for toxic pollen with increased floral signals
 595 and rewards for pollinators. Sci. Rep. 9: 16498 doi: 10.1038/s41598-019-53355-3

596 Josens R, Farina W (2001) Nectar feeding by the hovering hawk moth *Macroglossum*
 597 *stellatarum*: intake rate as a function of viscosity and concentration of sucrose solutions. J.
 598 Comp. Physiol. 187:661-665 doi: 10.1007/s00359-001-0238-x
 599 Klinkhamer PGL, de Jong TJ (1990) Effects of plant size, plant density and sex differential
 600 nectar reward on pollinator visitation in the protandrous *Echium vulgare*. Oikos 57:399-
 601 405 doi: 10.2307/3565970
 602 Konarska A, Masierowska M (2020) Structure of floral nectaries and female-biased nectar
 603 production in protandrous species *Geranium macrorrhizum* and *Geranium phaeum*.
 604 Protoplasma 257:501-523 doi: 10.1007/s00709-019-01454-3
 605 Magurran A (2004) Measuring Biological Diversity. Blackwell Science Ltd, Oxford
 606 Melser C, Rademaker M, Klinkhamer PGL (1997) Selection on pollen donors by *Echium*
 607 *vulgare* (Boraginaceae). Sex. Plant Reprod. 10:305-312 doi: 10.1007/s004970050103
 608 Melser C, Bijleveld A, Klinkhamer PGL (1999) Late-acting inbreeding depression in both
 609 male and female function of *Echium vulgare* (Boraginaceae). Heredity 83:162-170 doi:
 610 10.1046/j.1365-2540.1999.00568.x
 611 Michener CD (2003) The social behaviour of the bees: a comparative study. Annu. Rev.
 612 Entomol. 14:299-342 doi: 10.1007/BF02223852
 613 Micheu S, Crailsheim K, Leonhard B (2000) Importance of proline and other amino acids
 614 during honeybee flight (*Apis mellifera carnica* POLLMANN). Amino Acids 18:157-175
 615 doi: 10.1007/s007260050014
 616 Mitchell BK Harrison GD (1984) Characterization of galeal chemosensilla in the adult
 617 Colorado beetle, *Leptinotarsa decemlineata*. Physiol. Entomol. 9:49-56 doi:
 618 10.1111/j.1365-3032.1984.tb00680.x
 619 Nepi M (2014) Beyond nectar sweetness: the hidden ecological role of non protein amino
 620 acids in nectar. J. Ecol. 102:108-115 doi: 10.1111/1365-2745.12170

621 Nepi M, Stpiczyńska M (2006) Nectar resorption and trans location in *Cucurbita pepo* L. and
 622 *Platanthera chlorantha* Custer (Rchb.). Plant Biol. 9:93-100 doi: 10.1055/s-2006-924287
 623 Nepi M, Stpiczyńska M (2008) Do plants dynamically regulate nectar features through sugar
 624 sensing? Plant Signal. Behav. 3(10):874-876 doi: 10.4161/psb.3.10.6228
 625 Nepi M, Cresti L, Guarnieri M, Pacini E (2011) Dynamics of nectar production and nectar
 626 homeostasis in male flowers of *Cucurbita pepo* L. Int. J. Plant Sci. 172:183-190 doi:
 627 10.1086/657648
 628 Nepi M, Soligo C, Nocentini D, Abate M, Guarnieri M, Cai G, Bini L, Puglia M, Bianchi L,
 629 Pacini E (2012) Amino acids and protein profile in floral nectar: much more than a simple
 630 reward. Flora 207:475-481 doi: 10.1016/j.flora.2012.06.002
 631 Nicolson SW (2007) Nectar consumers. In: Nicolson SW, Nepi M, Pacini E (eds), Nectaries
 632 and nectar. Springer, Dordrecht, pp 289-342
 633 Nicolson SW, Thornburg RW (2007) Nectar chemistry. In: Nicolson SW, Nepi M, Pacini E
 634 (eds), Nectaries and nectar. Springer, Dordrecht, pp 215-264
 635 Nocentini D, Pacini E, Guarnieri M, Nepi M (2012) Flower morphology, nectar traits and
 636 pollinators of *Cerithe major* (Boraginaceae- Lithospermeae). Flora 207:186-196 doi:
 637 10.1016/j.flora.2012.01.004
 638 Nocentini D, Pacini E, Guarnieri M, Martelli D, Nepi M (2013) Intrapopulation heterogeneity
 639 in floral nectar attributes and foraging insects of an ecotonal Mediterranean species. Plant
 640 Ecol. 214:799-809 doi: 10.1007/s11258-013-0204-z
 641 Ohashi K, Yahara T (2001) Behavioral responses of pollinators to variation in floral display
 642 size and their influence on the evolution of floral traits. In: Chittka L, Thomson JD (eds),
 643 Cognitive ecology of pollination. Cambridge Univ. Press, Cambridge, pp 274-296
 644 Pacini E, Nepi M, Vesprini JL (2003) Nectar biodiversity: a short review. Plant Syst. Evol.
 645 238:7-21 doi: 10.1007/s00606-002-0277-y

646 Pappas SM, de Jong TJ, Klinkhamer PGL, Meelis E (1999) Effects of nectar content on the
 647 number of bumblebee approaches and the length of visitation sequences in *Echium*
 648 *vulgare* (Boraginaceae). *Oikos* 87:580-586 doi: 10.2307/3546822
 649 Pérez-Barralés R, Arroyo J, Armbruster WS (2007) Differences in pollinator faunas may
 650 generate geographic differences in floral morphology and integration of *Narcissus*
 651 *papyraceus* (Amaryllidaceae). *Oikos* 116:1904-1918 doi: 10.1111/j.0030-
 652 1299.2007.15994.x
 653 Petanidou T (2007) Ecological and evolutionary aspects of floral nectars in Mediterranean
 654 habitats. In: Nicolson SW, Nepi M, Pacini E (eds), *Nectaries and nectar*. Springer,
 655 Dordrecht, pp 343-375
 656 Petanidou T, Van Laere A, Ellis WN, Smets EF (2006) What shapes amino acid and sugar
 657 composition in Mediterranean floral nectars? *Oikos* 115:155-169 doi:
 658 10.1111/j.2006.0030-1299.14487.x
 659 Petanidou T, Kallimanis AS, Tzanopoulos J, Sgardelis SP, Pantis JD (2008) Long-term
 660 observation of a pollination network: fluctuation in species and interactions, relative
 661 invariance of network structure and implications for estimates of specialization. *Ecol.*
 662 *Lett.* 11:564-575 doi: 10.1111/j.1461-0248.2008.01170.x
 663 Proctor M, Yeo P, Lack A (1996) *The natural history of pollination*. Timber Press, Portland,
 664 Oregon
 665 Pyke GH (1978) Optimal foraging in bumblebees and coevolution with their plants.
 666 *Oecologia* 36:281-293 doi: 10.1007/BF00348054
 667 Rademaker MCJ, de Jong TJ, Van der Meijden E (1999) Selfing rates in natural populations
 668 of *Echium vulgare*: a combined empirical and model approach. *Funct. Ecol.* 13:828-837
 669 doi: 10.1046/j.1365-2435.1999.00384.x

670 Ranjbar MH, Gharekhloo J, Soltani A (2017) Diversity and evenness of weeds in forage corn
 671 field under different tillage systems. J. Plant Prot. 31:213-222 doi:
 672 10.22067/jpp.v0i0.45478
 673 Rathcke BJ (1992) Nectar distributions, pollinator behaviour and plant reproductive success.
 674 In: Hunter MD, Ohgushi T, Price PW (eds), Effects of resource distribution on animal-
 675 plant interactions. Academic Press, pp 113-138
 676 Romeis J, Wackers FL (2000) Feeding responses by female *Pieris brassicae* butterflies to
 677 carbohydrates and amino acids. Physiol. Entomol. 25:247-253 doi: 10.1046/j.1365-
 678 3032.2000.00188.x
 679 Schoonhoven LM, van Loon JJA, Dicke M (2005) Insect-Plant Biology. 2nd edn. Oxford
 680 Univ. Press, Oxford
 681 Seo HJ, Song J, Yoon HJ, Lee KY (2019) Effects of nectar contents on the foraging activity
 682 of honeybee (*Apis mellifera*) on Asian pear (*Pyrus pyrifolia* Nakai). Sci. Hortic. 245:185-
 683 192 doi: 10.1016/j.scienta.2018.10.009
 684 Shelp BJ, Bown AW, Zarei A (2017) 4-aminobutyrate (GABA): a metabolite and signal with
 685 practical significance. Botany 95:1015-1032 doi: 10.1139/cjb-2017-0135
 686 Stevenson PC, Nicolson SW, Wright GA (2017). Plant secondary metabolites in nectar:
 687 impacts on pollinators and ecological functions. Funct. Ecol. 31:65-75 doi: 10.1111/1365-
 688 2435.12761
 689 Stöckl AL, Kelber A (2019) Fuelling on the wing: sensory ecology of hawkmoth foraging. J.
 690 Comp. Physiol. 205:399-413 doi: 10.1007/s00359-019-01328-2
 691 Stpiczyńska M, Nepi M, Zych M (2015) Nectaries and male-biased nectar production in
 692 protandrous flowers of *Angelica sylvestris* L. (Apiaceae). Plant Syst. Evol. 301:1099-1113
 693 doi: 10.1007/s00606-014-1152-3

694 Teulier L, Weber JM, Crevier J, Darveau CA (2016) Proline as a fuel for insect flight:
 695 enhancing carbohydrate oxidation in hymenopterans. P. Roy. Soc B 283: 20160333 doi:
 696 10.1098/rspb.2016.0333
 697 Thompson JN (2006) The geographic mosaic of coevolution. 2nd edn. Univ. of Chicago
 698 Press, Chicago
 699 Tiedge K, Lohaus G (2017) Nectar sugars and amino acids in day- and night-flowering
 700 Nicotiana species are strongly shaped by pollinators' preferences than organic acids and
 701 inorganic ions. PLoS One 12:1-25 doi: 10.1371/journal.pone.0176865
 702 Verhoeven KJF, Simonsen KL, McIntyre LM (2005) Implementing false discovery rate
 703 control: increasing your power. Oikos 108:643-647 doi: 10.1111/j.0030-
 704 1299.2005.13727.x
 705 Wacht S, Lunau K, Hansen K (2000) Chemosensory control of pollen ingestion in the
 706 hoverfly *Eristalis tenax* by labellar taste hairs. J. Comp. Physiol. A 186:193-203 doi:
 707 10.1007/s003590050019
 708 Willmer P, Finlayson K (2014) Big bees do a better job: intraspecific size variation influences
 709 pollination effectiveness. J. Pollinat. Ecol. 14:244-254 doi: 10.26786/7603(2014)22
 710 Wright GA, Baker DD, Palmer MJ, Stabler D, Mustard A, Power EF, Borland AM, Stevenson
 711 PC (2013) Caffeine in floral nectar enhances a pollinator's memory of reward. Science
 712 339:1202-1204 doi: 10.1126/science.1228806
 713

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 ± 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
722 **Figure 2.** Frequency of male flowers visited by each taxon. Different letters denote statistical
723 differences according to Kruskal Wallis H-test followed by Mann-Withney pairwise
724 comparison with Benjamini-Hochberg correction ($p < 0.05$).

725

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

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 <i>H</i>	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

735

736

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

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

741 **Table 4.** Male (a) and female (b) flowers visited by each taxon (mean \pm 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
<i>Anthidium florentinum</i>	0.96 ± 0.192	37.80	21	0.014
<i>Apis mellifera</i>	1.59 ± 0.384	39.39	16	<0.001
<i>Hoplitis adunca</i> male	0.51 ± 0.100	70.51	40	0.002
<i>Hoplitis adunca</i> female	0.14 ± 0.143	8.50	13	0.810
<i>Macroglossum stellatarum</i>	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
<i>Anthidium florentinum</i>	3.95 ± 0.826	4.20	21	1.000
<i>Apis mellifera</i>	7.47 ± 1.652	4.38	16	0.998
<i>Hoplitis adunca</i> male	2.37 ± 0.312	7.84	40	1.000
<i>Hoplitis adunca</i> female	1.64 ± 0.199	0.94	13	1.000
<i>Macroglossum stellatarum</i>	15.67 ± 14.696	0.50	8	1.000
Pieridae	4.22 ± 1.656	0.58	8	1.000

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