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

Marta Barberis[1,†], Gherardo Bogo[2,*,†], Laura Bortolotti[3], Lucia Conte[1], Mattia Alessandrini[1,4], Massimo Nepi[5], Marta Galloni[1]

[1]Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Bologna, Italy.

[2]Grupo de Ecología de la Polinización, INIBIOMA, Universidad Nacional del Comahue-CONICET, Bariloche, Argentina.

[3]Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Centro di ricerca agricoltura e ambiente (CREA-AA), Bologna, Italy.

[4]Department of Biochemistry of Plant Secondary Metabolism, University of Hohenheim Garbenstr. 30, D-70599, Stuttgart

[5]Dipartimento di Scienze della Vita, Università di Siena, Siena, Italy.

[†]These authors contributed equally to this paper

*** Corresponding author:** Gherardo Bogo

Grupo de Ecología de la Polinización, INIBIOMA, Universidad Nacional del Comahue-CONICET, Bariloche, Argentina.

E-mail address: gherardo1985@hotmail.com

Author contributions

MB and GB share the first authorship. MG, MN, LC and LB conceived and designed the experiments. GB, MA and MG performed the experiments. HPLC analyses were executed by MN. MB and GB analysed data and wrote the paper. All authors read, provided editorial advice and approved the final manuscript.

ORCiD IDs

M. Barberis: 0000-0002-1062-4760

G. Bogo: 0000-0001-7415-2224

L. Bortolotti: 0000-0001-7372-1579

L. Conte: 0000-0003-0513-0616

M. Alessandrini: ND

M. Nepi: 0000-0002-6765-897X

M. Galloni: 0000-0001-5304-7820

Abstract

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

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.

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

Introduction

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

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

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 metabolites with different chemical natures have been identified in nectar and all of them positively or negatively affect attractiveness to pollinators, showing effects which depend on 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-protein amino acids (NPAAs) have been detected in nectar (Nicolson and Thornburg 2007; 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 pollinators' preferences and feeding behaviour. For some of those, such as γ-aminobutyric acid, a phagostimulant function has been reported in some caterpillars and adult beetles (Mitchell and Harrison 1984; Shoonhoven et al. 2005), whilst Bogo et al. (2019) found that both bumblebees and honeybees showed higher consumption of sucrose solution enriched with ß-alanine, but exhibited the effect at different concentrations.

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 outbreeding rate within a population. Minimal inbreeding is predicted when pollinators visit a 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 showing gender-biased nectar production (Feinsinger 1978; Pike 1978; Rathcke 1992). Despite many studies having already addressed the subject of gender-biased nectar composition, most of them investigated the existence of bias in relation to nectar volume or sugar content only (Langenberger and Davis 2002; Canto et al. 2011; Fisogni et al. 2011; Stpiczyńska et al. 2015; Antoń et al. 2017; Jacquemart et al. 2019; Konarska and

109 Masierowska 2020) and few reported the observation of insect visit bias (Carlson and Harms
110 2006 and references therein).
111 In this study we focused on the many-flowered hermaphrodite species *Echium vulgare* L., a
112 self-compatible plant which shows both herkogamy and incomplete protandry, that avoids
113 self-pollination within the same flower, but within which geitonogamy can still occur
114 (Rademaker et al. 1999). Melser et al. (1999) reported evidences of inbreeding depression in
115 *E. vulgare*, finding a significant decline in siring success when selfing occurs. A study on
116 geitonogamy conducted by Rademaker et al. (1999), though, found a consistently lower
117 percentage of selfing rate than expected. Also, they reported that bumblebees visited only a
118 small fraction of the flowers on *E. vulgare* as a result of the presence of different flower
119 stages simultaneously occurring on a single individual plant.
120 *E. vulgare* represents an important food resource for many insect visitors, despite containing
121 toxic pyrrolizidine alkaloids in both nectar and pollen (Lucchetti 2017). The pollen contains
122 high concentrations of pyrrolizidines, whilst more than 500 times lower concentrations are
123 found in nectar (Lucchetti et al. 2016). For this reason, only a few taxa show oligolecty or
124 floral constancy on *E. vulgare* by actively collecting pollen for larval nourishment (Cane and
125 Sipes 2006; Burger et al. 2010; Filella et al. 2011), even if its flowers are visited by a wide
126 spectrum of insect taxa among which bumblebees have often been reported as main
127 pollinators (Corbet 1978; Klinkhamer and de Jong 1990; Pappers et al. 1999; Rademaker et
128 al. 1999).
129 Here, we examined if floral visitation pattern may be influenced by variations in the chemical
130 composition of nectar through different floral stages, and thus we investigated (i) whether *E.*
131 *vulgare* produces a gender-biased nectar for volume, sugar and amino acid composition and
132 (ii) if flower visitation rates of insects looking for nectar varied among different floral stages.
133

Material and Methods

Study site

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

Study species

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

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

Plant phenology

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

159 patches were alternatively considered: the first one was a single plant carrying 6
160 inflorescences while the second one was made up of 6 plants carrying one or two
161 inflorescences each.
162
163 **Nectar quality**
164 *Sampling*
165 We collected nectar samples by means of Drummond Microcaps (3-5 µL; Drummond
166 Scientific Co., Broomall, PA), we transferred samples to Eppendorf tubes filled with 100 µL
167 of pure ethanol, and then we took them to the laboratory in thermal bags where they were kept
168 at 5°C until analyses. We collected each sample from multiple flowers at the same floral stage
169 in order to reach a minimum volume of 2 µL needed for the sugar and amino acid analyses. In
170 order to let the nectar accumulate, flowers were bagged in the morning for 2 hours prior to
171 sampling; all nectar present in the selected flowers was collected.
172 We collected a total of 8 nectar samples each one from 3-13 male flowers belonging to 1-7
173 plants, and a total of 8 samples from 2-9 female flowers belonging to 1-3 plants. Both sugar
174 and amino acid compositions were investigated on these samples. We then collected 14
175 additional samples from 1-22 buds belonging to 1-10 plants. Since the amount of nectar
176 presents in the buds was very low, the minimum volume of 2 µL needed for amino acid
177 analysis could not be reached and thus these samples were tested for sugar composition only.
178
179 *Sugar analysis*
180 Sugar content was analysed by HPLC technique through a Waters LC1 with refractive index
181 detector (Waters 2410) connected to the output of a REZEX RCM Monosaccharide column
182 (Phenomenex, 300 mmx7.8 mm, grain 8 µm) maintained at 85°C. Water (MilliQ, pH 7) was

used as mobile phase at a flow rate of 0.6 mL min^{-1}; 20 µL of sample and standard solutions of sucrose, glucose and fructose were also injected (Nocentini et al. 2012).

Amino acids analysis

Amino acid analysis was performed by gradient HPLC with an ion exchange Novapack C18 (15 mm x 4.6 mm) cartridge with guard column maintained at 37°C and a Waters 470 scanning fluorescence detector (excitation at 295 nm, detection at 350 nm). A solvent composed of TEA-phosphate buffer (pH 5.0) mixed with a 6:4 acetonitrile-water solution was used as mobile phase at a flow rate of 1.0 mL min^{-1}. According to AccQtag protocol (Waters Corp.), the selected volume of each reconstituted sample was amino acid derivatized (Cohen and Micheaud 1993) with AQC fluorescent reagent and 0.02 M borate buffer (pH 8.6). In addition to all the protein amino acids, standard solutions of β-alanine, citrulline, L-homoserine, α-aminobutyric acid (AABA), γ-aminobutyric acid (GABA), hydroxyproline, ornithine and taurine were also used (Nocentini et al. 2012).

Flower visitors' observations

We carried out observations on flower visitors on the two fixed patches described previously, on 7 non-sequential days. Every survey consisted of two 15-mins periods separated by 10 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 of 200 mins of observation. Once a visitor left the patch, we counted the following approaching insect belonging to the same taxon as a different individual. Recorded data concerned the food resource collected (nectar or pollen, observing if the insect inserted its 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.

After each observation period, we performed a 15-mins period of net sampling throughout the 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 entomological boxes and inspected under a dissecting microscope for taxonomic identification.

Data analysis

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 glucose concentrations.

Data on nectar composition were grouped by floral stage and tested to assess homogeneity of variances and normality of distribution (Bartlett test and Shapiro Wilk test).

Data on sugars per flower, total sugar concentration and sucrose per flower were square root transformed to achieve normality. When the transformed data failed to match normality, we applied the corresponding non-parametric analyses.

To investigate whether the floral stage affected sugar content and volume a one-way ANOVA followed by Tukey's HSD post hoc test with Benjamini-Hochberg correction for 'false discovery rate' (Verhoeven et al. 2005) were performed. When distribution was not normal a Kruskal Wallis H-test followed by a Mann Whitney pairwise comparison with Benjamini-Hochberg correction were carried out instead.

Data on single amino acid concentrations were ln transformed to achieve normality when needed and a Student t-test was applied in all analyses.

For both phenological stages (functionally male and functionally female flowers), three 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 calculated as $D = \sum_{i=1}^{n} \left(\frac{ni}{n}\right)^2$, where *n*i is the abundance of the *i*th amino acid and *n* is the total mean concentration (Ranjbar et al. 2017). This index ranges from 0 (one amino acid dominates the spectrum) to 1 (all amino acids equally represented) (Harper 1999).

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 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 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 represented by relatively low concentrations (Harper 1999; Hubálek 2000; Fattorini et al. 2016).

The third one was the Buzas and Gibson's evenness index, a measure of the relative abundance of the different amino acids within the floral stage. The index is calculated as the proportion of equally dominant amino acid in the phenological stage $E = e^H/S$, where H is Shannon's H index and S is the number of amino acids within the floral stage. This index ranges from 0 (highest dominance by a single amino acidic species) to 1 (all amino acids have the same abundance) (Buzas and Hayek 2010; Fattorini et al. 2016).

Insect visit data were first analysed by comparing the observed number of male and female flowers visited to the expected ones by χ^2 test. The expected number of visits was calculated on the basis of the ratio between the functionally male and the functionally female flowers occurring in the population.

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

All data are presented as mean ± SE and all statistics were performed using R software (version 3.6.1) with the significance level set at 0.05.

Results

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.

Nectar analyses

Sugars and volume

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 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 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 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 ± 63.2 nmol mL^{-1} and F = 143.6 ± 32.6 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 ± 243.5 nmol mL^{-1}), whilst the latter in the functionally male stage (352.7 ± 63.2 nmol mL^{-1}).

Among non protein amino acids, in both male and female stages GABA showed the highest concentration (51.4 ± 12.2 nmol mL^{-1} and 202.0 ± 73.4 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

353 sugar concentration to ensure pollinator visits has been previously reported in other species
354 (Nepi and Stpiczyńska 2008; Nepi et al. 2011).
355 When we compared the number of insect visits on male and female flowers observed to the
356 expected ones, all bee taxa except female *Hoplitis adunca* showed a higher number of visits to
357 male flowers than expected. This result could be explained by the higher proportion of protein
358 amino acids found in the male stage: preferences have often been reported in bees for protein
359 amino acid enriched solutions (Inouye and Waller 1984; Bertazzini et al. 2010; Hendriksma et
360 al. 2014), suggesting that flower visitors may actively choose to visit functionally male
361 flowers. Comparable results have been reported by Klinkhamer and de Jong (1990) and by
362 Rademaker et al. (1999) on bumblebees: when calculating the probabilities of visits on
363 different floral stages, the oldest female stage was less likely to be visited than a male-phase
364 flower. Females of *Hoplitis adunca* are the only bees collecting both pollen and nectar on *E.*
365 *vulgare*: this different foraging behaviour might explain the difference from the other bee
366 species.
367 Individuals of *Lasioglossum* sp. were observed visiting the flower and collecting pollen only.
368 A tendency for afternoon trips for nectar only have been reported for the subfamily Halictinae
369 by Michener (2003) so we cannot conclude that *Lasioglossum* sp. does not exploit *E. vulgare*
370 nectar since the species may simply collect the resource at different time of the day.
371 Despite Lepidoptera having been reported to prefer nectar rich in PAAs (Baker and Baker
372 1986; Erhardt and Rusterholz 1998), our study reports that Pieridae butterflies visited as many
373 male flowers as expected, indicating that these insects did not actively look for functionally
374 male flowers (containing a higher proportion of protein amino acids). A study conducted by
375 Alm et al. (1990) showed that male individuals of the species *Pieris rapae* do not discriminate
376 between artificial nectars containing sugar only or sugar solution enriched with protein amino
377 acids, and Romeis and Wäckers (2000) reported that feeding and source-selection in *Pieris*

brassicae is elicited by sucrose more than protein amino acids. We report a similar result for the species *Macroglossum stellatarum*, but to date no study has been done in order to assess amino acid preferences in the species and whether taste receptors on the proboscis can sense their presence in nectar remains unsubstantiated (Stöckl and Kelber 2019).

Nectar of male-phase flowers in *E. vulgare* presented, among all the amino acids, the highest concentration of phenylalanine, representing an average of 35% of total amino acid content. Phenylalanine is an essential protein amino acid (de Groot 1953) and several studies proved that it exerts a phagostimulatory effect on several insects, especially on honey bees, and it is strongly correlated with pollinator preferences (Inouye and Waller 1984; Hendriksma et al. 2014; Tiedge and Lohaus 2017; Seo et al. 2019). Consequently, this could explain the higher frequency of visit on male flowers than expected. A correlation between phenylalanine concentration and nectar feeding by Megachilids, that were the more numerous pollinators in our study, was demonstrated in a phriganic community, a plant association typical of the East Mediterranean (Petanidou et al. 2006).

Proline, instead, represented the most concentrated amino acid in functionally female flowers, and the second in the early-stage functionally male flowers (representing more than 30% and almost 20% of the total amino acid content, respectively). This non-essential amino acid, commonly found in nectar (Nicolson and Thornburg 2007), can stimulate the insect salt cell increasing intensity of feeding behaviour (Hansen et al. 1998; Wacht et al. 2000). Proline also represents an energy substrate to fuel the earliest or most expensive stages of insect flight (Micheu et al. 2000; Gade and Auerswald 2002), resulting in short-term bursts of energy production (Teulier et al. 2016).

Finally, in both male- and female-phase flower nectar GABA showed the highest concentration among the non-protein amino acids representing more than 5% and 9% of total 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 Martìn et al. 2017), bees, bee flies (Proctor et al. 1996) and syrphids (Willmer and Finlayson
412 2014). Also, even though the species has often been reported as mainly pollinated by
413 bumblebees (Corbet 1978; Klinkhamer and de Jong 1990; Pappers et al. 1999; Rademaker et
414 al. 1999), we observed only one individual of *Bombus pascuorum* visiting the
415 flowers. Pollinators of wide spread plant species can vary in relation to their geographical
416 distribution (Armbruster 1985; Thompson 2006; Pérez-Barrales et al. 2007) and, moreover, as
417 reported by Lázaro et al. (2010), the plant and pollinators assemblages of an entire community
418 may also influence the composition of visitors of a particular species by determining, for
419 instance, the strength of competition or the intensity of attraction to that species rather than
420 another. Thus, the scarcity of bumblebees observed on *Echium vulgare* in 2018 may either
421 depend on several factors and/or reflect a temporal fluctuation in the species composition of
422 the pollinator community, as previously reported by many studies (Cane et al. 2005;
423 Petanidou et al. 2008; Dupont et al. 2009).
424

425 ## Conclusions

426 The inbreeding avoidance hypothesis states that some mechanisms develop within a species in
427 order to prevent breeding among related individuals and its damaging effects on fitness

(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 anthesic 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.

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Conflict of interest

The authors declare that they have no conflict of interest.

Data availability

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

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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

726 **Table 1.** Comparison of nectar volume, sugar and amino acid (AA: amino acids; PAA: protein
727 amino acids; NPAA: non-protein amino acids) compositions among the three phenological
728 stages (bud, male and female flowers). Values (expressed by mean ± SE) marked with different
729 letters were significantly different according to one-way ANOVA or Kruskal-Wallis test
730 followed by the respective post hoc test with Benjamini-Hochberg correction.

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

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733 **Table 2.** Comparison of diversity indices calculated on nectar amino acid concentration
734 between male and female phases (8 samples for both floral phases).

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

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737 **Table 3.** *Echium vulgare* visitors recorded in June 2018 (215 visits in total), their abundance
738 and the percentage of them looking for nectar as reward.

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

739 [a]value calculated only on individuals with fully recorded data (n = 21)
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741 **Table 4.** Male (a) and female (b) flowers visited by each taxon (mean ± SE). Chi-square test is
742 calculated on the basis of the ratio 1:9 between male and female flowers occurred in the studied
743 population.

a)

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

b)

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

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