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Article *in* Acta Oecologica · July 2023

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Acta Oecologica 120 (2023) 103937

Contents lists available at ScienceDirect

Acta Oecologica

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Floral nectar and insect flower handling time change over the flowering season: Results from an exploratory study

M. Barberis [a,*,1], G. Bogo [b,c,1], L. Bortolotti [c], S. Flaminio [c,d], E. Giordano [e], M. Nepi [e,f], M. Galloni [a]

[a] Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Bologna, Italy
[b] Grupo de Ecología de la Polinización, INIBIOMA, Universidad Nacional del Comahue-CONICET, Bariloche, Argentina
[c] Consiglio per la ricerca in agricoltura e l'analisi dell'economia agraria, Centro di ricerca agricoltura e ambiente (CREA-AA), Bologna, Italy
[d] Laboratory of Zoology, Research Institute for Biosciences, University of Mons, Place du parc 20, 7000, Mons, Belgium
[e] Dipartimento di Scienze della Vita, Università di Siena, Siena, Italy
[f] National Biodiversity Future Center (NBFC), Palermo, Italy

ARTICLE INFO

Keywords:
Octopamine
Biogenic amines
Neuroactive nectar
Amino acids
Pollinators
Echium vulgare

ABSTRACT

In recent decades, hundreds of secondary metabolites have been found in floral nectar and many studies have demonstrated that they can play various roles in modulating the behaviour of floral visitors. However, temporal variations in nectar chemistry over extended flowering seasons have never been substantiated. Moreover, the effects of nectar chemicals on insect behaviour are often studied under laboratory conditions, focusing on few insect species under artificial conditions which may influence insect responses. The aim of this exploratory study was to compare nectar chemistry and the durations of pollinator visits in the early and late summer periods of the long-flowering species *Echium vulgare* L. in natural populations. Nectar samples were collected in the early and late summer periods and insects were observed for a total of 480 min. The biogenic amine octopamine, sugars and the protein to non-protein amino acid ratio increased as the season proceeded. It remains to clarify whether these changes are determined by biotic and abiotic factors or whether the plant expresses some chemical constraint, however it seems likely that changes in nectar chemistry may be the cause of the longer visits by bumblebees to single flowers at the end of the flowering season. Though not conclusive, these results set a baseline for future research and highlight an interesting question. Since long-flowering plants see changing contexts during their bloom period, do they express chemical constraints to regulate their attractiveness?

Author contributions

Marta Barberis: investigation, formal analysis, writing and original draft preparation. **Gherardo Bogo**: conceptualization, formal analysis, supervision. **Laura Bortolotti**: conceptualization, supervision. **Simone Flaminio**: data curation, validation. **Emanuele Giordano**: investigation. **Massimo Nepi**: investigation, reviewing. **Marta Galloni**: conceptualization, investigation, supervision.

1. Introduction

The chemistry of nectar is central to ecology, since it mediates interactions with pollinators, flower-visiting antagonists and microbes (Pyke, 2016; Nepi, 2017). Besides sugars and amino acids, the first and second most abundant nectar solutes, respectively (Baker and Baker 1986; Nepi et al., 2012; Bogo et al., 2019), hundreds of secondary metabolites have also been found in nectar since the 1970s (e.g. Baker and Baker, 1986). All nectar components may affect pollinator attractiveness, and differences in composition have been demonstrated to be related to specific pollinator types (Faegri and van der Pijl 1979; Baker and Baker 1983).

It is now well established that nectar chemistry modulates several behavioural traits of floral visitors, such as their motor learning skills and their flower handling time (e.g. Harder 1986; Arnold et al., 2021). Among the multiple biologically active compounds found in floral nectar, two classes of chemicals are particularly intriguing for the plethora of effects

* Corresponding author. Dipartimento di Scienze Biologiche, Geologiche e Ambientali, Università di Bologna, Italy.
E-mail address: marta.barberis2@unibo.it (M. Barberis).
[1] These authors contributed equally to the study.

https://doi.org/10.1016/j.actao.2023.103937
Received 15 January 2023; Received in revised form 27 June 2023; Accepted 21 July 2023
Available online 27 July 2023
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that they may impart on floral visitors. The first is represented by non-protein amino acids which can be a large portion of the amino acid content of floral nectar and are considered crucial in shaping interactions between organisms through insect preferences (Bogo et al., 2019), feeding (e.g. Schoonhoven et al., 2005; Petanidou et al., 2006), locomotion (Bogo et al., 2019; Felicioli et al., 2018), learning, memory (Carlesso et al., 2021) and flight muscle performance (Whitton et al., 1987; Felicioli et al., 2018). The second is represented by a class of nectar compounds – that of biogenic amines – only recently reported in floral nectar for the first time, yet its discovery raises a series of interesting questions as such molecules are known to act as neurotransmitters in invertebrates (Roeder 1999; Blenau and Baumann 2001). As compounds that work by activating or inhibiting G proteins, their consumption can potentially affect pollinator behaviour both in a short- and long-term way (Roeder 2005; Mustard 2020). Several studies have shown that nectar chemistry often differs between populations (Lanza et al., 1995), individuals of the same species (e.g. Carlson and Harms, 2006 and reference therein) and even in relation to aging of single flowers on the same plant (e.g. Petanidou et al., 1996; Bogo et al., 2021). Surprisingly, though, few studies (e.g. Torres and Galetto, 1998) have tackled the subject of how nectar chemistry changes in long-flowering species as the season progresses, despite the strong seasonality recorded at certain latitudes and the changing environment of long-flowering species during their bloom period.

The main aim of this exploratory study was to pave the way for future research by exploring whether nectar chemistry changes over a season in the long-flowering species *Echium vulgare* L. In Northern Italy, in fact, this species blooms from early June to October (Barberis et al., 2021). A second aim was to make preliminary observations on the durations of flower visits by insect pollinators in natural plant populations. The length of flower visits may be influenced by nectar chemistry. Most studies investigating the effects of nectar chemistry on pollinator behaviour have been laboratory assessments, which are obviously simpler than field studies (Muth et al., 2020), but have their limitations. Controlled settings may not always yield a realistic picture of how a certain behaviour is affected (e.g. Mujagic and Erber, 2009; Ayestaran et al., 2010), and often concern a limited number of insect species and single compounds.

Besides sugars and amino acids, we also analysed biogenic amines, hypothesized in nectar but only recently reported for the first time (Nepi, 2017; Muth et al., 2022).

2. Methods

2.1. Study sites

Field work was conducted in summer 2020 in two periods: the second half of June (henceforth "early period"), when *E. vulgare* populations were already in full bloom, and late August-first half of September 2020 ("late period"). It concerned two natural populations in rural areas of northern Italy: one in the municipality of Lama Mocogno, province of Modena, named MO (44°18′52″N, 10°43′42″E), and the other in the municipality of Chiaverano, province of Torino, named TO (45°29′30″N, 7°53′22″E) (Fig. S1). The former population is located nearby the Parco Nazionale Appennino Tosco-Emiliano, in an area mainly dedicated to both pastoralism and agriculture. The latter population falls within the boundaries of the Natura 2000 site IT1110021 – Laghi di Ivrea, a smaller area characterized by several residual lakes of glacial origin and surrounded by areas dedicated to both pastoralism and agriculture. Both populations counted a number of individuals ranging between 50 and 100 and covered an area of about 20 m^2 on open meadows along public countryside roads in full sunlight.

2.2. Study species

Echium vulgare L. is a self-compatible biennial hemicryptophyte of the Boraginaceae family, native to Europe, Asia and North America. In Northern Italy, its flowering period ranges from June to October (Barberis et al., 2021). Cymes diverge from the main flowering stem, carrying flowers that develop sequentially (Nicholls, 1987). Flower anthesis lasts 3–4 days, and autogamy is limited by incomplete protandry: if the anthers may already be dehiscent at bud stage, the stigma elongates and its two lobes diverge, whilst becoming fully receptive only hours after bud opening (Melser et al., 1997). Along with this mechanism, which nevertheless does not limit geitonogamy (i.e. self-pollination among flowers of the same individual plant), intra-flower autogamy is also prevented by herkogamy (Rademaker et al., 1999). The petals of the corolla are fused at their bases to form a bell-shaped flower tube presenting floral nectar at its bottom (Rademaker et al., 1999).

Despite containing toxic pyrrolizidine alkaloids in both nectar and pollen (Lucchetti 2017), *Echium vulgare* represents an important food source for many insect visitors. Both pollen and nectar contain such alkaloids, though by far more concentrated in the former than in the latter (Lucchetti et al., 2016). Nectar is sucrose dominant and is secreted at concentrations ranging between 20 and 35% (Corbet 1978). Even if its flowers are visited by a wide spectrum of insect taxa (Barberis et al., 2021), bumblebees have more often been reported as the main pollinators of the species (Corbet 1978; Klinkhamer and de Jong 1990; Pappers et al., 1999; Rademaker et al., 1999).

2.3. Nectar sampling

Flowers were bagged before 8:00 a.m. with 1 mm mesh tulle fabric, 2 h prior to sampling to avoid nectar depletion, as nectar volumes in *Echium vulgare* result otherwise extremely low and challenging to extract (e.g. Corbet, 1978; Klinkhamer and de Jong, 1990; Barberis et al., 2021).

Due to the small volume of nectar produced per flower and to reduce the possible influence of individual flower phenology on nectar chemistry, samples were collected from multiple functionally female flowers up to the minimum volume of 5 μL needed for analysis of sugars, amino acids and biogenic amines. We collected a total of 21 nectar samples, each obtained by pooling the nectar collected from 4 to 25 flowers from 1 to 6 plants (Table 1). Nectar was collected by means of Drummond Microcaps (1–3 μL; Drummond Scientific Co., Broomall, PA), between 8:30am and 12:30pm on at least two non-consecutive sunny days per period. We also recorded temperature and relative humidity at the beginning and end of each sampling session (Table 1).

Table 1
Behavioural surveys on floral visitors and nectar sampling by period (early and late summer) and by population (MO and TO). Values are expressed as mean ± SE.

	Early		Late	
	MO	TO	MO	TO
No. floral visitor observations (30 min each)	4	4	4	4
Days of floral visitor observations	2	2	2	2
No. nectar samples	7	5	3	6
Days of nectar sampling	2 (1 + 1, non-consecutive)		3 (1 + 2[a], non-consecutive)	
No. flowers sampled per sample mean ± SE (min – max)	7.3 ± 0.9 (4–10)	8.2 ± 1.5 (5–13)	18.3 ± 3.5 (13–25)	17.7 ± 1.5 (14–24)
No. plants sampled per sample mean ± SE (min – max)	1.3 ± 0.2 (1–2)	3.4 ± 0.8 (2–6)	3.3 ± 1.5 (1–6)	4.0 ± 0.6 (2–6)
Mean temperature (°C)	18.9 ± 0.6	22.1 ± 0.5	22.1 ± 0.8	25.7 ± 0.1
Mean RH (%)	56.0 ± 0.0	59.0 ± 3.0	56.0 ± 1.0	57.0 ± 1.0

[a] Nectar sampling was performed on a single day (MO) and two consecutive days (TO).

We transferred samples to Eppendorf tubes containing 100 μL pure ethanol, took them to the laboratory in thermally insulated ice containers on the day of field sampling, and then stored them at 5 °C until analysis. Mean volume per flower was calculated by proportions, using the length of the microcapillary tube occupied by the nectar, measured with a calliper, tube capacity and the total number of flowers individual samples were collected from.

2.4. Nectar analysis

We analysed the sugar, amino acid and biogenic amine compositions of all samples. Sugar content was analysed by HPLC with a Waters LC1 equipped with refractive index detector (Waters 2410) connected to the output of a Water Sugar-Pak column (6.5 × 300 mm) maintained at 90 °C. Water (MilliQ) was used as mobile phase at a flow rate of 0.5 mL/min; 20 μL of sample and standard solutions of sucrose, glucose and fructose were also injected (Nocentini et al., 2012).

Amino acid and biogenic amine analysis was performed by gradient HPLC with a Supelco Ascentis C18 column (250 mm × 4.6 mm × 5 μm). The amino acid analysis was thermostated at 46 °C and a Waters 470 scanning fluorescence detector (excitation wavelength 295 nm, detection 350 nm) was used. 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. In line with the 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 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).

We analysed the content of biogenic amines by HPLC with diode array detector (HPLC-DAD) using a PerkinElmer series 200 chromatographic system with auto-sampler. Detection and quantification were based on UV absorption at 230 nm. The bandwidth was set at 6 nm. The injection volume was 50 μL and column temperature was set at 25 °C. The flow rate was 1.0 mL/min. A binary gradient system was used: eluent A consisted of 0.02 M potassium phosphate buffer (KH_2PO_4) adjusted to pH 2.5 with ortho phosphoric acid; eluent B was methanol. The composition of the mobile phase was modified according to the following time program: 0–10 min 97% A and 3% B; 10–14 min 80% A and 20% B; 22–23 min 97% A and 3% B; end run at 30 min. We calculated the concentration of each analyte by calibration curves obtained with external standard. Analyte identification was achieved by comparison with the UV spectrum of the pure standards of eight biogenic amines: dopamine (Dop), octopamine (Oct), serotonin (Ser), tyramine (Tyr), tryptamine (Tryp), epinephrine (Epi), norepinephrine (Nor), histamine (His). The retention time (RT) and the limit of detection (LOD) are reported in Supplementary Materials (Table S1). LOD for each amine was calculated by adding three times the standard deviation to the mean of 10 blank samples.

All standards (purity ≥98%) and solvents were from Sigma-Aldrich.

2.5. Flower visitor observations

We conducted behavioural surveys on floral visitors in the early and late periods in selected patches of the two *E. vulgare* populations. Each patch contained three flowering stems. Each survey consisted of two 15-min periods separated by 10 min rest, adapting the protocol of Fisogni et al. (2016). Behavioural surveys on floral visitors were performed twice a day on two consecutive days between 10:30am and 14:30pm for each population, both in the early and late periods (for a total of 16 censuses; Table 1). All observations were conducted in favourable weather conditions.

We recorded visitor taxa in as much detail as possible, the number of flowers visited in a single trip to the patch and the total duration spent in the patch. From this data we calculated the mean duration of visits to a single flower. Since the second objective of the study was to investigate possible effects of nectar consumption on wild pollinators, insects observed collecting pollen on the observed flowers were excluded from our analysis. When it was not possible to visually distinguish two closely related species, we combined them in higher categories (family, genus or species group). Since it was impossible to visually distinguish certain species, two artificial species groups were created: a *Bombus pascuorum* species group (consisting of *B. pascuorum* (Scopoli, 1763), *B. humilis* Illiger, 1806 and *B. muscorum* (Fabricius, 1793)) and a *Bombus hortorum* species group (consisting of *B. hortorum* (L., 1761), *B. ruderatus* (Fabricius, 1775) and *B. argillaceus* (Scopoli, 1763)).

Once a visitor left the patch, we counted the next approaching insect of the same taxon as a new visit, irrespective of whether or not it was the same individual. After each observation session, individuals that could not be visually identified, even at family, genus or species group level, during the session, were caught outside the patch. Captured individuals were put in separate vials with ethyl acetate to kill them, then transferred to clean empty vials to be brought to the laboratory for taxonomic determination under a stereo microscope, and subsequent sample preparation. All captured specimens, except those belonging to the dipteran families Syrphidae and Culicidae, and the lepidopteran genus *Pieris* sp., were identified at species level (Table S2). The captured specimens are conserved at the Laboratory of Plant Reproductive Ecology, Department of Biological, Geological and Environmental Sciences, University of Bologna.

2.6. Data analysis

Since the focus of this paper is not to describe geographical patterns of nectar changes and because we did not find significant differences in nectar chemistry between populations in a preliminary analysis (MANOVA: $F_{7,13} = 2.676$, $p = 0.060$), we pooled the data from TO and MO, setting "population" as random factor and "period" as fixed factor in all models.

We performed principal component analysis (PCA) to explore similarities in nectar composition. The data was scaled and centred around the mean, and analyses were performed using the function *dudi. pca* in the R-package *ade4* (Venables and Ripley, 2002). We considered volume per flower, total sugar concentration, sucrose:hexose ratio, and concentrations of total amino acids, non-protein amino acids (NPAAs) and biogenic amines. The data was then tested for homogeneity of variance and normal distribution (Bartlett test and Shapiro Wilk test).

We built a series of linear mixed models (LMMs) using the *lme* function of R package *nlme* (Pinheiro et al., 2020) to examine the effects of the flowering period (early or late) on nectar characteristics. In each model, nectar parameters such as volume, sugar concentration, total amino acid concentration, sucrose:hexose ratio and protein:non-protein amino acid (PAA:NPAA) ratio were set as dependent variables. Data on nectar volume, total amino acid concentration and PAA:NPAA ratio were log-transformed to meet model assumptions.

A second investigation was performed specifically on the amino-acidic composition of nectar chemistry. To do so, we performed a second PCA to explore similarities in amino acid spectra, based on the concentrations of each amino acid species. Then, single amino acid concentrations were tested to assess homogeneity of variance and normal distribution, and a second series of LMMs was built by means of the *lme* function of R package *nlme* (Pinheiro et al., 2020). The concentration of each amino acid was thus set as dependent variables.

Descriptive statistical analysis was performed on insect visits and insect diversity data.

Finally, we built a generalized linear mixed model (GLMM) with a Gamma error structure-log-link function, using the *glmer* function of R package *lmer4* (Bates and Machler, 2015) to examine the effects of the flowering period (early or late) on the duration of visits to single flowers by pollinators of the artificial *Bombus pascuorum* species group. All data

is presented as mean ± SE. All statistics were performed using RStudio software (version 4.0.2) with α-error set at 0.05.

3. Results

3.1. Nectar composition

The PCA on nectar parameters showed a clear separation between early and late periods, with the first two components explaining 69.1% of the variance (Fig. 1). The first component was positively correlated with volume per flower (PC1 loading = 0.54), while the second was positively correlated with total amino acid concentration (PC2 loadings = 0.71, Table S3).

Regarding single nectar parameters, nectar volume was lower in the late than the early period (PeriodLate: $t_{18} = -5.431$, $p < 0.001$, Fig. 2a), nectar in the late period showed a significantly higher concentration of total sugars (PeriodLate: $t_{18} = 4.581$, $p < 0.001$), a lower sucrose:hexose ratio (PeriodLate: $t_{18} = -3.369$, $p = 0.003$) and a higher PAA:NPAA ratio (PeriodLate: $t_{18} = 4.562$, $p < 0.001$), while no difference was found in total amino acid concentration (PeriodLate: $t_{18} = 1.276$, $p = 0.297$) (Fig. 2b–e). Octopamine was the only biogenic amine found. Although it was detected in the early and late periods, it was significantly more concentrated in the late period (PeriodLate: $t_{18} = 5.164$, $p < 0.001$) (early period: 0.314 ± 0.058 μmol/mL; late period: 1.328 ± 0.212 μmol/mL; Fig. 2f; Table S4).

The PCA on the amino acid spectrum showed partial separation of the two periods, the first two components explaining 67.4% of the variance (Fig. 3). The first component was correlated with phenylalanine and isoleucine concentrations (PC1 loadings = 0.71 and −0.56, respectively). The second component was correlated with ornithine and isoleucine (PC2 loadings = −0.82 and 0.51, respectively; Table S5).

Modelling the concentrations of the amino acid species showed that tyrosine, valine, alanine and phenylalanine were significantly higher in the late period (PeriodLate: $t_{18} = 6.103$, $p < 0.001$; $t_{18} = 2.580$, $p = 0.019$; $t_{18} = 2.139$, $p = 0.046$ and $t_{18} = 4.914$, $p < 0.001$, respectively), while proline was significantly lower (PeriodLate: $t_{18} = -2.319$, $p = 0.032$; Table S5 and Fig. S2).

3.2. Flower visitors

A total of 319 insect visits to *Echium vulgare* were recorded during 480 min of field surveys in the two populations. The total numbers of insect visits recorded were 233 and 86 in the early and late periods, respectively (Table 2a and Table S2); the exact number of visits by each pollinator taxon per population and period is reported in Table S2. Taxonomic richness was much higher in population TO than in MO in the early period, and nearly equal in the two populations in the late period (Table 2b). The genus *Bombus* Latreille, 1802 was the most frequent visitor taxon overall (40.8% of visits), though the different species of the genus were distributed differently in the two populations in line with the period. The second most abundant taxon overall was *Apis mellifera* L., 1758, however the two populations showed opposite abundance of visit trends for this species. In June, honeybees were the most abundant taxon in TO, while no honeybee was observed in MO at all, whereas at the end of the flowering season, the opposite situation was recorded for the two sites.

The taxon *Hoplitis* Klug, 1807 was the third most frequent visitor in the population MO in the early period (17.9%). The genera *Ceratina* Latreille, 1802 and *Anthophora* Latreille, 1803 and the family Culicidae were the next most frequent taxa, all recorded with more than 5% of visits (Fig. 4). Since the *Bombus pascuorum* species group proved to be the only one omnipresent in both periods and populations, behavioural analysis was conducted exclusively on it. Bumblebees visited flowers for significantly longer time in the late than the early period (PeriodLate: $t_{78} = 3.257$, $p = 0.002$; Table S7) (Late: 4.257 ± 0.574 s (N = 41) and Early: 2.392 ± 0.178 s (N = 38), respectively, Fig. 5).

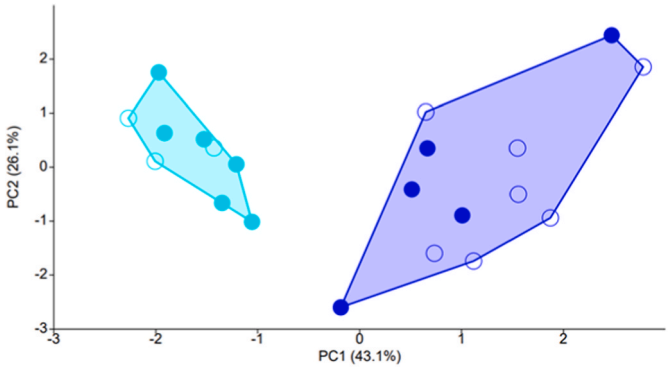

Fig. 1. Principal components analysis (PCA) on nectar parameters. Early and late periods are indicated in dark blue (right) and light blue (left), respectively; the MO and TO populations are indicated by empty and solid circles, respectively.

4. Discussion

4.1. Nectar composition

Our results showed that the nectar volume and composition of *E. vulgare* changed as the flowering season proceeded, decreasing in volume per flower, with a concomitant increase in total sugar concentration in relation to season, and a decrease in the sucrose:hexose ratio. These results are in line with those obtained by Torres and Galetto (1998). In their study conducted on *Mandevilla pentlandiana*, in fact, they recorded a general decrease in nectar availability as the season advanced, coupled with an increase in sugar concentration.

Another interesting change over the flowering season that we observed was an increase in the PAA:NPAA ratio.

Although we did not delve into the possible causes of nectar changes in time, several hypotheses are suggested. The most parsimonious one is that the fluctuations recorded in the nectar constituent concentrations may be due to environmental and ecological factors. In the late flowering period, we observed fewer flower visitors, which suggests that the nectar is less frequently collected and sugars are likely to concentrate due to evaporation of water. However, this explanation alone does not justify the differences in sucrose:hexose ratio and PAA:NPAA ratio observed.

Another possible hypothesis is that nectar chemistry changes in relation to plant phenology. On one hand, as the flowering season advances, plants enter demanding new phases, such as seed production. Since nectar production represents a considerable investment for the plant (e.g. Pyke 1991), the possibility that nectar volumes decrease in relation to the increase of seed production could be explained as a trade-off between resources allocated to reproduction: the flowers investment in attracting pollinators, such as nectar secretion, may be subsequently saved for the maturation of fruits and seeds (Obeso 2002; Ornelas and Lara 2009; Galetto et al., 2018). It is reasonable that this trade-off is driven also by resources availability implying that stressed plants invest the low resources more in seeds development than in nectar production. Our study seems to fit in this frame: plant experiencing higher water stress in full summer (August–September) reduce nectar production to ensure higher seed production. On the other hand, as the flowering season advances, the insect community becomes depleted, so that long-flowering plants may need to tackle the problem of how to optimize visitor attraction. It is now well established that as plants develop from seedlings to mature stages, their ontogeny can constrain the expression of chemical resistance to herbivory (Boege and Marquis, 2005), but little or nothing has been done to investigate whether similar chemical constraints exist in relation to pollination in long-flowering species which experience dramatic changes in the surrounding context

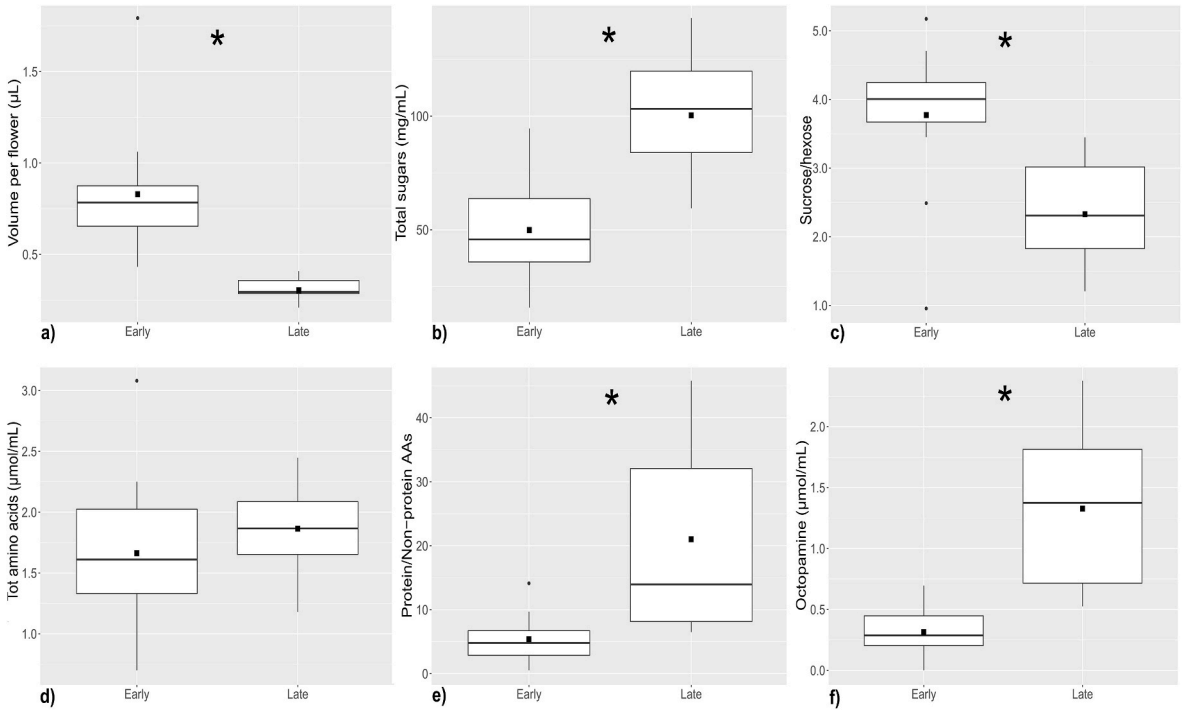

Fig. 2. Nectar chemistry of the 21 nectar samples collected from two populations (TO and MO) in the two periods (early and late summer): volume (a), total sugar concentrations (b), sucrose:hexose ratio (c), total amino acid concentrations (d), PAA:NPAA ratio (e) and octopamine concentration (f). Asterisks indicate a significant difference ($p < 0.001$) according to a linear mixed model with "period" as fixed factor and "population" as random factor. Solid black squares inside the box indicate the mean.

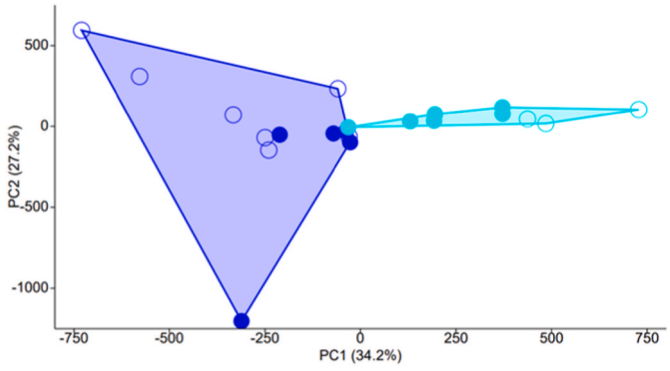

Fig. 3. Principal components analysis (PCA) of amino acid spectrum. Early and late periods are indicated in dark blue (left) and light blue (right), respectively; the MO and TO populations are represented by empty and solid circles, respectively.

Table 2
Number of visits (a) and number of insect taxa recorded (b) in the two populations (TO and MO) and periods (early and late summer).

Number of visits					Number of insect taxa recorded				
	TO	MO	Tot			TO	MO	Tot	
early	91	142	233		early	12	6	18	
late	29	57	86		late	3	4	7	
Tot	120	199	**319**	a)	Tot	15	10	**25**	b)

as the blooming season unfolds.

Finally, since the flower visitor guild changes over the season, so must microbial communities, which are mainly dispersed among flowers by insect visitors (e.g. Adler et al., 2021; Bogo et al., 2021; Pozo et al., 2014). Since we sampled nectar from functionally female flowers on the second-third day after bud opening, the changes in composition observed over the season could be at least partly due to shifts in the community of nectar microbes and their activity. Indeed, an increasing number of recent studies have linked microbial abundance to a variety of nectar traits such as sugar composition (de Vega and Herrera, 2012) and amino acid concentrations (e.g. Vannette and Fukami, 2018), often reporting that bacteria and yeasts can have contrasting effects on nectar chemistry (Vannette et al., 2013; Good et al., 2014).

4.2. Biogenic amines

Of all the biogenic amines tested, we only found octopamine, which proved to be approximately four times more concentrated in the nectar collected in the late than in the early period. Besides the increase in concentration, octopamine showed higher variation in samples collected later in the season, possibly due to the greater number of flowers and plants sampled. Octopamine was found in all samples except one from population TO in the early period. The mean overall concentration of this biogenic amine was 0.70 mM, one order of magnitude higher than the maximum mean concentration reported in the study conducted by Muth et al. (2022), specifically found in the species *Citrus x meyeri* (mean approximately 0.07 mM).

Biogenic amines are nitrogenous compounds known to act as neurotransmitters, neurohormones and neuromodulators in invertebrates (Roeder, 1999; Blenau and Baumann, 2001). Since several studies have focused on the effects of biogenic amines on insects, demonstrating that their consumption modulates a plethora of behavioural traits, such as reward-seeking, learning, memory acquisition and social communication of food sources (e.g. Barron et al., 2007; Peng et al., 2020; Finetti et al., 2021; Barberis et al., 2023), the urgency of further studies on nectar-like concentrations of such compounds appears clear. Since their presence in nectar was predicted to be a possible product of microbial decarboxylation of free amino acids (Nepi, 2017; Nepi et al., 2018), future research into their origin is warranted.

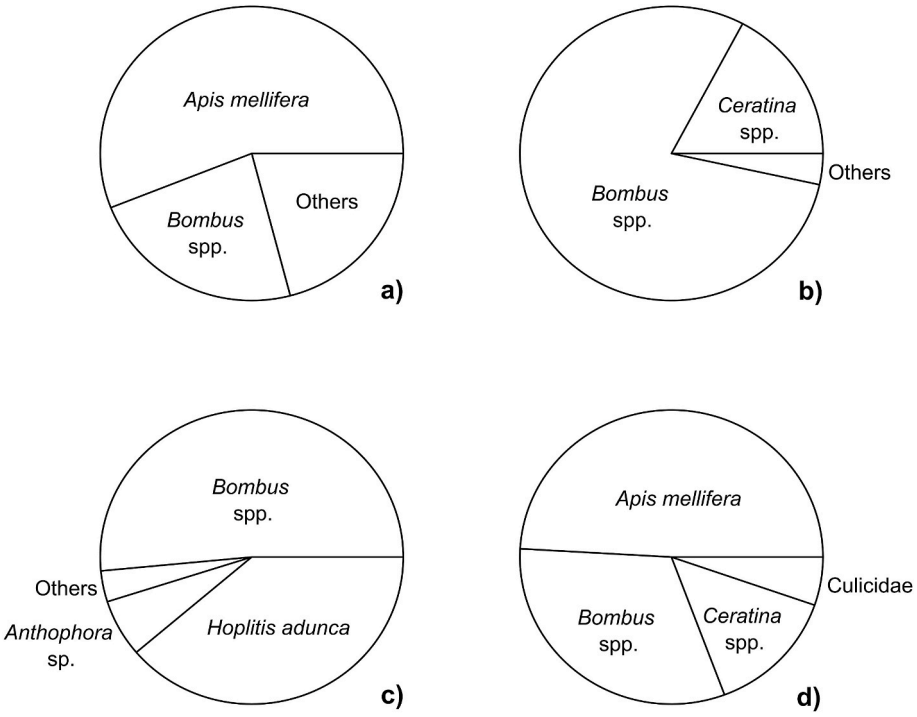

Fig. 4. Abundance of visits by the main insect taxa recorded in population TO in the early (a) and late (b) periods, and in population MO in the early (c) and late (d) periods of the study.

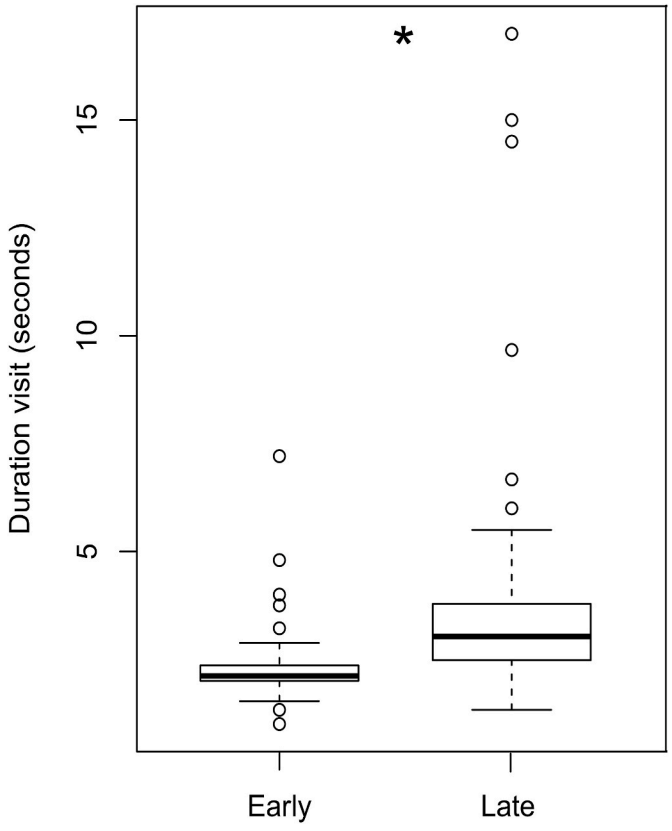

Fig. 5. Duration of visits to single flowers recorded for 79 visits by individuals of the *Bombus pascuorum* group in the early and late periods of observation. The asterisk indicates a significant difference between periods according to a GLMM with a Gamma error structure-log-link function.

4.3. Flower visitors

Along with changes in nectar chemistry, we found that both the number and the taxonomic richness of floral visitors were lower in the late period than in the early period. This observation is due to two main causes: on one hand, most insect species are strongly seasonal and the adult activity of most solitary bees found in June has a narrow temporal window (Danforth et al., 2019). Moreover, in the late period most of the individuals recorded were social bee species. On the other hand, as the overall number of flowering scapes lowered as the season advanced (personal observation), this may have contributed to the decrease of the overall number of visits, since less conspicuous flowering masses may reduce attractiveness (Ohashi and Yahara 2001 and reference therein).

4.4. General conclusions on flower handling time

Our results showed that the nectar chemistry of the long-flowering species *Echium vulgare* changes as the blooming season unfolds, as does the duration of visit to single flowers exhibited by bumblebees.

We observed a decrease in nectar volume in relation to season and a significant increase in sugar concentrations. Since more concentrated nectars are more rewarding, but also more time-consuming to imbibe due to their greater viscosity (Borrell, 1986; Pattrick et al., 2020), the influence of nectar viscosity on the duration of visits to single flowers may be the simplest explanation of why bumblebees spent longer on flowers in the late than the early period. Nevertheless, in their recent study, Muth et al. (2022) reported that the bumblebee visitation rate was lowest when bees were fed a combination of tyramine and octopamine instead of control solutions, while Farooqui (2012) describes the latter as a regulator of bee motivation (Farooqui, 2012).

Concentrations of NPAAs were also higher in the early period. Taurine, β-alanine and ornithine were the most abundant amino acid species found. Some NPAAs have been suggested to have various effects on plant visitors, such as reducing fatigue and sustaining muscle performance (Nepi, 2014). Taurine, for example, concentrates in the thoracic region of many adult insects, where it is associated with fully

functional flight muscles (Whitton et al., 1987), while β-alanine is the precursor of the dipeptide carnosine, found in the skeletal muscle of invertebrates, and appears to be a limiting factor for carnosine synthesis (Harris et al., 2006). In the study of Bogo et al. (2019), bumblebees fed solutions enriched in β-alanine at natural concentrations showed a higher flying-index.

Among PAAs, phenylalanine, isoleucine and tyrosine were the most abundant. Phenylalanine, known to act as a strong phagostimulant in several insects and to be correlated with pollinator preferences (Petanidou et al., 2006; Tiedge and Lohaus, 2017; Seo et al., 2019), was abundant in the early and late periods, showing an increasing trend as the season proceeded. It is still unclear whether solely increased concentrations of phenylalanine may be responsible for the longer durations of visits to single flowers by bumblebees. Likewise, tyrosine was more concentrated in the late period. Tyrosine is the precursor of the biogenic amine tyramine, which is in turn decarboxylated by enzymes to the biogenic amine octopamine (Finetti et al., 2021). By contrast, isoleucine was highly concentrated only in the early period. Interestingly, Simcock et al. (2014) found that worker honeybees fed with sucrose solutions enriched with isoleucine ate more isoleucine-laced solutions the following day.

In conclusion, the significant variations in specific nectar molecules during the flowering season of *E. vulgare* appear related to the longer insect visits to flowers in the late period.

4.5. Limitations of the study

Far from exhausting the topic of how floral nectar changes during the blooming season in long-flowering plants, we believe that the current study has the merit of highlighting a gap in our knowledge and bringing interesting new questions to the field. However, these preliminary results present their limitations. A first critical aspect concerns the moderate number of nectar samples due to the small volumes available per each individual flower. For the same reason, we also pool nectar from different flowers, which represents a second aspect of potential concern. A third point regards the fact that we did not account for individual variability in the model because we did not mark individual bees in order to not interfere with the system. And last, a potential fourth worrying aspect is represented by the total amount of time spent performing behavioural surveys on floral visitors, which appears relatively restricted. Altogether, these points make our study and results somehow exploratory, suggesting the need for further investigations.

4.6. Future perspectives

At our latitudes, long-flowering plants face changing contexts during their blooming season, characterized by a decrease in flower abundance and in the number of floral visitors. The nectar changes observed in this exploratory study may be imputed to various factors, but the main question arising from our findings is whether nectar changes passively under the influence of external biotic and abiotic factors (i.e. insect and microbial communities, environmental variables), or rather whether the plant somehow expresses chemical constraints aimed at modifying insect handling time. This first exploratory data sets a baseline for future research into the question.

Declaration of competing interest

All authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest or non-financial interest in the subject matter or materials discussed in this manuscript.

Data availability

Data will be made available on request.

Acknowledgements

We thank the anonymous reviewers for their useful suggestions, Fedele Greco, Nicolò Brunelli, Massimo Guarnieri and Michela Boi for their precious help. We are grateful to Lucia Conte for her support during late flowering season field surveys. Marta Barberis was supported by a PhD grant from the University of Bologna.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.actao.2023.103937.

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